Investigation of the role of epigenetic silencing mechanisms in neural stem cells

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DECLARATION

I declare that this thesis was composed by myself and the research presented is my own except where otherwise stated

Isabel Martín Caballero

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To my sister, Ana,
the begining of my questions,
the motor of my efforts,
my other self.

To know the brain is equivalent to acertaining the material course of thought and will, to discovering the intimate history of life in its perpetual duel with external forces, a history sumarized, and in a way engraved in the defensive neuronal coordinations of the reflex of instinct, and of the association of ideas. [...] What a delight it was when by dint of much patience, we succeded in isolating a neuroglia element with its typical spido-like form, what a triumph to capture a cortical pyramid, that is the noble and enigmatic cell of thought !

Santiago Ramón y Cajal

Recollections of my life

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ABSTRACT

Neural stem cells (NSCs) are multipotent cells capable of differentiating into neurons, astrocytes and oligodendroctyes in response to developmental signals. Although they do not have the required gene expression patterns to identify a differentiated stage, their chromatin will be epigenetically modified to achieve distinct stages of differentiation. The most abundant epigenetic modification in vertebrate DNA is methylation, which is normally associated with transcriptional silencing. This occurs primarily through recruitment of methyl-CpG binding proteins (MeCPs) to methylated DNA, which further recruits chromatin-modifying activity, resulting in the formation of repressive chromatin.

Additionally, another protein from the MeCP family, Mbd3 does not itself bind methylated DNA but acts as a scaffold protein of the Nucleosome Remodelling and Deacetylation complex, NuRD. Previous evidence has outlined a central nervous system function for the MeCPs, but MeCPs mutant mice are viable and fertile, with the exception of MeCP2 deficient mice. However, Mbd3 has a role in early cell fate decisions during development, and Mbd3 mutant mice do not survive post-implantation stage.

In my thesis I have investigated the role of the two different mechanisms of epigenetic silencing: Methyl-CpG binding protein-dependent repression and chromatin remodelling activity in the neural stem cell system. I hypothesised firstly, that MeCPs may have a redundant role in neural stem cell function. Secondly, that the effect of lacking Mbd3 in neural stem cells may be different and more severe than the absence of the methyl-CpG binding proteins. The approach to investigate these hypotheses consisted of a first stage in the study of the neuroectoderm differentiation capacity of ES cells lacking one MeCP, Kaiso, followed by studying the function of neural stem cell (NS) lines derived from the cortex of mice lacking the MeCPs Kaiso, MeCP2 and Mbd2. I have found no defects in proliferation or self-renewal of triple null NS cells. Additionally, although triple null NS cells present a normal astrocyte differentiation, they present a delay in neuronal differentiation, a defect that is only visible at early differentiation stages. Therefore,

these proteins are dispensable for viability and differentiation of NS cells *ex vivo*. In contrast, I have found that Mbd3 is important for differentiation of neuroectoderm in culture, as Mbd3 null ES cells differentiate into neurons at very low frequency but do not differentiate into astrocytes. Moreover, Mbd3 is essential for establishment and/or maintenance of ES cell-derived NS cell lines. Thus while there is no evidence for a role of three MeCPs in NS cell maintenance or differentiation, the activity of the NuRD co-repressor complex is important for both properties.

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COMMON ABBREVIATIONS

ANC	Adult neural stem cell
ATP	Adenosine-tri-phosphate
Bdnf	Brain derived neurotrophic factor
bFGF or FGF2	Basic fibroblast growth factor or fibroblast growth factor 2
bHLH	Basic helix-loop-helix
BMP4	Bone morphogenic protein 4
bp	base pair
CNS	Central nervous system
Blbp	Brain lipid-binding protein
CAG	Chicken β-actin Actin Globin promoter
Cre	Causes recombination
D	Day
d.p.c	Days post coitum
DAPI	4', 6'-diamindino-2-phenylindole
DNA	Deoxyribonucleic acid
EB	Embryoid bodies
EGF	Epidermal growth factor
ER	Estrogen receptor
ES	Embryonic stem
Ex	Exon
FACS	Fluorescence activated cell sorting
FCS	Fetal calf serum
Gfap	Glial fibrillary astrocytic factor
GFP	Green fluorescent protein
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
ICM	Inner cell mass
IP	IRES-*puromycin-N-acetyltransferase*
IRES	Internal ribosome entry site
Kb	Kilobase
KO	Knock out
LIF	Leukaemia inhibitory factor
Lox	Locus of crossing over (x)
M	Molar
MBD	Methyl-binding domain
MeCP	Methyl-CpG binding protein
Mg	Milligram
Min	Minutes
ml	Mililiter
mol	Mole
MTA	Metastasis associated
N-CoR	Nuclear Corepressor
ng	Nanogram
NLS	Nuclear localisation signal
NS	Neural stem
NSC	Neural stem cell
NuRD	Nucleosome Remodeling and Histone Deacetylation
^{0}C	degree centigrade

Pax6	Paired box homeotic gene 6
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PDL	Poly-D-lysine
POZ	Pox virus and zinc fingers
PURO	Puromycin
RA	Retinoic acid
Rpm	Revolutions per minute
RNA	Ribonucleic acid
RG	Radial glia
RT-PCR	Reverse transcription polymerase chain reaction
RTT	Rett Syndrome
SDIA	Stromal cell-derived inducing activity
SAM	S-adenosyl-L-methionine
SDS	Sodium dodecyl sulphate
SOX	SRY-like HMG box
SVZ	Subventricular zone
T	Tamoxifen
TE	Trophectoderm
TK	Hygromycin thymidine kinase
WT	Wild type
μg	Microgram

CHAPTER 1

INTRODUCTION

1. 1 Chromatin compaction

Chromatin structure in eukaryotic nuclei is highly ordered. DNA is wrapped around histone proteins, forming the nucleosome. The nucleosome consists of 146 base pairs of DNA wrapped around a core histone octamer which consists of two molecules each of histones H2A, H2B, H3 and H4 (Kornberg and Lorch, 1999; Luger et al., 1997). Additionally, the linker histone H1 binds to nucleosome core particles and organizes the linker DNA on either side of the nucleosome to facilitate the folding of chromatin into a ~30 nm fiber *in vitro* (Bednar et al., 1998; Thoma et al., 1979).

To gain access to the highly compacted structure of the chromatin, there are in the cells chromatin remodelling activities that can be divided in two major mechanisms: The first one relies on the use of the energy derived from ATP hydrolysis to remodel nucleosomes (Kadonaga, 1998; Travers, 1999; Tsukiyama and Wu, 1997; Varga-Weisz and Becker, 1998). The other mechanism involves covalent modification of the histone tails of the nucleosome. These modifications include methylation, acetylation, phosphorylation, ubiquitylation, sumoylation and ADP-ribosylation. The acetylation of lysine residues on histone N-terminal tails is catalyzed by histone acetyltransferases (HATs) (Wolffe and Pruss, 1996) and is generally associated with transcriptional activation. In contrast, deacetylation carried out by histone deacetylases (HDACs). (Pazin and Kadonaga, 1997) is generally associated with transcriptional repression. Both groups of proteins function in different large multiprotein complexes, where sequence-specific DNA-binding proteins target them to specific genes, leading to chromatin modification.

1. 2 NuRD

The NuRD (Nucleosome Remodeling and Histone Deacetylation) co-repressor complex (Wade et al., 1998; Xue et al., 1998b; Zhang et al., 1998a) is a co-repressor implicated in silencing in mammals, flies, nematodes, and plants (Ahringer, 2000; Bowen et al., 2004). In vertebrates, NuRD is a multi-subunit protein complex

containing both histone deacetylase and nucleosome-dependent ATPase subunits. In mammals NuRD plays a role in cell fate decisions by transcriptional silencing mechanisms in B-cell (Fujita et al. 2004), thymocyte (Hutchins et al., 2002) and erythroid development (Hong et al., 2005; Rodriguez et al., 2005).

The largest subunit in the NuRD complex is Mi-2, a member of the SWI2/SNF2 helicase/ATPase family (Eisen et al., 1995; Tong et al., ; Zhang et al.) that also associates with histone deacetylases. In mammals there are two highly similar proteins Mi-2α and Mi-2β transcribed from two different genes *Mi-2α* and *Mi-2β* (Seelig et al., 1996). In the NuRD complex, the predominant form is Mi-2β (Feng and Zhang, 2003; Zhang et al., 1998a). Mi-2β contains two PHD (plant homeo domain)-zinc finger domains, two chromodomains and a SWI2/SNF2-type ATPase/helicase domain.

Another subunit consists of a four-unit complex formed by two histone binding proteins, RbAp46 and RbAp48, and two histone deacetylases, HDAC1 and HDAC2. This complex is also present in the co-repressor complex Sin3. Hdac1-deficient ES cells have slow growth, and Hdac1-deficient mice die by 9.0 dpc (Lagger et al., 2002). A compensation effect of Hdac2 and Hdac3, which are upregulated in Hdac1 deficient mice, could explain this late embryonic lethality.

There is a major polypeptide of approximately 70-80kDa called Mta2, that is thought to have a role in modulating histone deacetylase activity in NuRD (Zhang et al., 1999b). MTA2 is 65% similar to the metastasis associated protein MTA1, which has also been reported to associate with NuRD (Xue et al., 1998a);(Mazumdar et al., 2001). Further, MTA3, the shorter isoform translated from the *MTA3* gene in humans, does also form part of NuRD (Fujita et al., 2003a). It is currently believed that individual members of MTA family associate with NuRD depending of the location and function (Bowen et al., 2004).

The smallest component of the NuRD complex is the MBD3 protein (Wade et al., 1999; Xue et al., ; Zhang et al., 1999a). MBD3 was originally identified in mice and humans as a protein containing a region with high homology to the methyl-CpG binding domain (MBD) of MeCP2 that, unexpectedly, did not specifically bind methylated DNA (Hendrich and Bird, 1998). Mammalian MBD3 does not bind methylated DNA because it has two amino acid residues different than the rest of

MBD containing proteins that eliminate the methyl-CpG binding activity (Ohki et al., 2001; Saito and Ishikawa, 2002).

Nevertheless, the NuRD complex can interact with methylated DNA by association with another methyl-CpG binding protein, MBD2 (Feng and Zhang, 2001; Ng et al., 1999). The transcriptional repressor complex formed by Mbd2 and NuRD complex was originally named MeCP1 complex (Meehan et al., 1989).

Mbd3 is ubiquitously expressed from morula to the egg cylinder stage (6.5 dpc) and is localised into the nucleus. Maternal Mbd3 protein is expressed until the morula stage. The development of *Mbd3* null embryos is morphologically normal until 5.5 dpc, when they display a runted morphology with a reduced number of pluripotent Oct4 positive cells. This population will be gradually lost in time synchrony with the lost expression of maternal Mbd3, since a majority of diapaused $Mbd3^{(-/-)}$ blastocysts fail to express Oct4. By 5.5 dpc, Mbd3 null embryos fail to initiate rapid proliferation, cavitation and distal displacement of ICM population, together with a failure of extraembryonic development. Thus, Mbd3 is required for transition of pluripotent cells in peri-implantation embryos. Hence Mbd3 is necessary for maintenance of pluripotent population (Kaji and Hendrich, unpublished).

$Mbd3^{(-/-)}$ ICMs fail to downregulate expression of a number of preimplantation specific genes, possibly contributing to the failure of embryonic development beyond 5.5 dpc. This gene missexpression correlates with inappropriate expression of those genes in $Mbd3^{(-/-)}$ ES cells (Kaji et al., 2006; Kaji et al., unpublished). This aberrant expression is accompanied by increased levels of histone acetylation, providing evidence of a failure of histone deacetylation. On the other hand, expression of *Dppa3*, an early marker of primordial germ cells (also known as *Pgc7* or *Stella*) (Saitou et al., 2002; Sato et al., 2002) is inappropriately silenced in $Mbd3^{(-/-)}$ ES cells (Kaji et al., 2006).

While $Mbd3^{(-/-)}$ ICM cells fail to mantain expression of Oct4, Mbd3-deficient ES cells are viable, and show persistent Oct4 expression when induced to differentiate *in vivo* and *in vitro*. $Mbd3^{(-/-)}$ ES cells show no difference in gene expression of NuRD subunits compared with wild type ES cells but their protein levels are reduced for Mta1, Mta2 and RbAp48, although the levels of Hdac1 are not changed.

Additionally, Mta1, Mta2, Hdac1 and Mbd3 can not be coimmunoprecipitated in *Mbd3*$^{(-/-)}$ ES cells. These facts indicate that Mbd3 is necessary for the formation of NuRD complex.

There are three independent Mbd3 isoforms in the NuRD complex differing in their N-termini: Mbd3a, b, and c (Kaji et al., 2006; Zhang et al., 1999b). Mbd3b is the major isoform in ES cells, but Mbd3a and Mbd3c are also detectable (Kaji et al., 2006). Mbd3b is also the prevalent isoform in NuRD of proliferating HeLa cells (Zhang et al., 1999b) and in embryonic neural cells (Jung et al.,2003). Interestingly, the ratio between Mbd3a and b will vary during brain development and hence it may indicate a role for Mbd3 in development. Mbd3 is expressed in the embryonic forebrain, including cortical neuroepithelia, striatum and hippocampus. These regions show expression of Mbd3 during adulthood though at decreased levels. Meanwhile, olfactory bulb and cerebellum will show an increase in levels of Mbd3 during adulthood (Jung et al., 2003).

Mbd3 in *Xenopus* binds to methylated DNA and is essential for embryonic development (Wade et al., 1999). xMbd3 is highly expressed during embryogenesis, specifically in the developing eye, brain and bronchial arches. Concordantly, partial inhibition of xMBD3 affects the eye formation and brain development. This eye phenotype occurs also with the over expression of xMbd3. When xMbd3 is almost completely depleted, the embryos are arrested at the late gastrula or early neurula stages, in concordance with Mbd3 deficient mice (Iwano et al., 2004).

1.3 DNA methylation

DNA methylation in vertebrates is achieved by the transfer of a methyl group to cytosine by a DNA methyltransferase to the 5'position of the pyrimidine ring. This cytosine is followed by a guanine forming a dinucleotide CpG (Johnson, 1925); (Hotchkiss, 1948).

DNA methylation is found in most organisms, from bacteria to mammals, but the distribution of methylation in animals varies between species. *Caenorhabditis elegans* does not have methylated cytosines in its genome and does not encode a DNA methyl-transferase. *Drosophila melanogaster* has a DNA methyltransferase-like gene (Hung et al., 1999; Tweedie et al., 1999) and contains very low levels of

methylcytosine, mostly in the CpT dinucleotide (Gowher et al., 2000; Lyko et al., 2000). In contrast, another invertebrate, *Ciona intestinalis,* has blocks of methylated DNA separated by equivalent domains of unmethylated DNA (Bird et al., 1979; Tweedie et al., 1997). In vertebrates, DNA methylation is present throughout the genome at 60-90% of all CpGs. The majority of the unmethylated CpG dinucleotides occurs in CpG islands. CpG islands are found in short regions of 1-2kb containing unmethylated cytosine, GC-rich in ~60-70%, meanwhile the rest of the genome has a GC content of 40%. These regions are usually functioning as strong promoters, being found in all the house keeping genes and majority of genes with a tissue-restrictive pattern of expression (Delgado et al., 1998; Gardiner-Garden and Frommer, 1987). The majority of CpG islands are unmethylated in the germline, at all stages of development and in all somatic tissue types. Thus, CpG islands can remain methylation-free even when their associated gene is silent.

1.4 The roles of DNA methylation

Methylation interferes with protein-DNA interactions, leading to changes in chromatin structure. This interference can cause transcriptional repression. Methylation of CpG rich promoters is used by mammals to prevent transcriptional intitiation and to ensure the silencing of genes on the inactive X chromosome, imprinted genes and parasitic DNAs (Colot and Rossignol, 1999; Yoder et al., 1997).

The most direct mechanism of repression is to interfere with the binding of basal transcriptional machinery or transcription factors that require contact with cytosine in the major groove of the double helix (Watt and Molloy, 1988). However, the majority of the repression of methylated genes occurs by the binding to methyl CpG to methyl-CpG binding proteins (Boyes and Bird, 1991). Transcription can then be inhibited by steric impediment, where the MeCP bound to the DNA blocks the access of the transcription factor, by impediment of RNA polymerase activity or by chromatin changes directed by Methyl-CpG binding proteins (MeCPs).

1.4.1 X chromosome inactivation

During early development in mammals, one X chromosome is inactivated in females. This inactivation is random in embryonic cells, and inactivation status will

be propagated to their daughter cells creating a mosaic of X chromosome inactivation. The extraembryonic lineages placenta and primitive endoderm in mice are the exception since it is the paternal X chromosome that remains silenced (Huynh and Lee, 2003; Mak et al., 2004; Okamoto et al., 2004). X chromosome inactivation is controlled by the X chromosome inactivation centre, from where are expressed two non coding RNAs: Xist and its antisense Tsix. The initiation of X inactivation by Xist starts very early in development (2-4 cell stage). Xist will coat the entire X chromosome initiating the inactivation process (Huynh and Lee, 2003; Okamoto et al., 2004). The maintenance of the silencing state is achieved by chromosome wide inactivation, where the combination of methylation of CpG islands of most of the genes, recruiting of polycomb group proteins Ezh2/Eed and histone modification leads to a heterochromatin status and stable silencing (de Napoles et al., 2004; Fang et al., 2004) (Brockdorff, 2002; Plath et al., 2003).

1.4.2 Imprinting

Genomic imprinting results in the differential expression of the two alleles of a gene in somatic cells due to epigenetic modification of the parental chromosome in the gamete or the zygote (Efstratiadis, 1994). This differential expression is due to the specific heritable parental methylation patterns, termed differentially methylated regions (DMRs). DMRs include imprinting control regions (ICRs), which control gene expression within imprinted domains, often over large distances. These ICRs carry allele-specific methylation patterns established in the germ line and retained thereafter (Reik and Walter, 2001). ICRs subsequently will influence epigenetically the modulation of allele-specific, tissue-specific or temporal-specific regulation of imprinting genes (Ferguson-Smith and Surani, 2001).

A number of *in vivo* and *in vitro* studies of ICRs have revealed that the chromatin insulator protein CTCf binds only to the unmethylated parental allele, and then regulates the ability of distant enhancers to access promoters in an allele-specific manner (Kurukuti et al., 2006; Ling et al., 2006; Schoenherr et al., 2003). Currently, there are 80 genes known to be imprinted, being this number in continuous increase.

1.4.3 DNA methylation and disease

Loss of imprinting (LOI) is the disruption of imprinted epigenetic marks through gain or loss of DNA methylation, losing normal allele-specific gene expression (Robertson, 2005). This aberrant imprinting leads to human diseases normally as a result of activation of a normally silent allele. Hence, it is present in many tumour types as a consequence of LOI of growth inhibitory imprinting genes. Examples are Wilms' tumour and some types of breast and ovarian cancer, lung and colon.

In general, loss of genomic methylation is a frequent event in cancer that correlates with disease severity and metastasic potential in many tumor types (Widschwendter et al., 2004). The abnormal methylation of CpG islands in tumor suppressor genes is frequent in tumorigenic cells, although it is currently unknown whether this silencing is a cause or consequence of the onset of tumorigenesis (Prokhortchouk and Hendrich, 2002). Early and general demethylation in tumorigenesis may predispose cells to genomic instability meanwhile a gene-specific demethylation might occur later as a adaptative mechanism of the tumorigenic cell to the local environment and promote metastasis (Robertson, 2005).

Apart from cancer, there are an extensive number of diseases known to be related with an aberrant imprinting such as Beckwith-Wiedeman, Prader-Willi and Angelman syndrome.

1.4.4 Silencing of parasitic DNA elements

A high fraction of methylated CpG dinucleotides are located within parasitic DNA elements or retrotransposons, which are inactivated copies of repetitive DNA in the mammalian genome. These elements are classified in SINE or short interpersed DNA elements, LINE or long interpersed DNA elements and LTR containing elements (Smit and Riggs, 1996), and can be activated or inserted in novel locations causing chromosome rearrangements or translocations with devastating effects (Kazazian and Moran, 1998; Montagna et al., 1999). SINES and LINEs are very CpG rich, heavily methylated and may recruit chromatin modifiying repression (Yoder et al., 1997). Intracisternal A particules or IAPs, are a class of

LTR that tend to decrease its methylation levels during aging in mouse with the consequence of increase in transcriptional activation of these elements (Barbot et al., 2002).

1. 5 DNA cytosine methyl transferases:

1.5.1 Dnmt1

Maintenance methylation describes the process that reproduces DNA methylation patterns between cell generations (Riggs, 1975). During DNA replication, both new double-stranded DNA molecules are hemimethylated, being only methylated on the parental strand. Dnmt1, which has a strong preference (15 fold) for hemimethylated DNA (Goyal et al., 2006), will methylate the cytosine on the unmethylated strand restoring the symmetry. With this mechanism, methylation patterns are inherited to the next cell generation (Bird, 2002). The recruitment of Dnmt1 to the replication fork is targeted by the proliferating cell nuclear antigen, PCNA (Chuang et al., 1997). PCNA has a role in DNA replication and repair. This interaction can be disrupted by the cell cycle regulator, $p21^{WAF/CIP}$, which can bind PCNA in an exclusive manner in relation with Dnmt1. Therefore, $p21^{WAF/CIP}$, whose function is to mediates the ability of p53 to arrest cell division in response to DNA damage (Li et al., 1996), prevents Dnmt1 access to damaged DNA and consequently can lead to hypomethylation (Chuang et al., 1997).

The catalytic domain of Dnmt1, which is common to the Dnmt family, consists on a set of six highly conserved motifs located in the C-terminal domain (Kumar et al., 1994). Additionally, Dnmt1 has a region of alternating glycine and lysine residues and in its N-terminal region has several domains attributed with a number of functions. Firstly, there is a nuclear localisation signal (NLS) that is required for import Dnmt1 into nuclei. Secondly, the N-terminal region is believed to be necessary to target the association with replication foci in S phase (Leonhardt et al., 1992). Furthermore, the N-terminal region is believed to confer specificity for hemimethylated DNA (Bestor et al., 1992) and also play a role in coupling stabilization of DNA to the growth state of cells (Ding and Chaillet, 2002). Dnmt1 has a diffuse nucleoplasmic distribution in G1 but associates with replication foci

during S phase, being expressed at very low levels in non-cycling cells (Fan et al., 2001).

Role of Dnmt1 through the study of the *Dnmt1* deletion

There is a severe demethylation in mice and ES cells homozygous for null alleles of Dnmt1, although methylation levels persist in 5% (Lei et al., 1996). As a result, multiple consequences are observed in null mice and ES cells. Firstly, mutant mice lacking a functional *Dnmt1* gene are normal during pre-implantation development but they start to show developmental delay at around 9.5 dpc, failing to develop past 12.5 dpc (Li et al., 1992). Secondly, deletion of *Dnmt1* causes the biallelic expression of most imprinted genes in homozygous embryos; some, such as H19 are expressed from both alleles, whereas others such as Igf2 are not expressed (Li et al., 1993). Thirdly, there is an inactivation of all X chromosomes in homozygous mutant embryos due to the demethylation and activation of *Xist* (Panning and Jaenisch, 1996). Lastly, deletion of *Dnmt1* causes demethylation and expression of very high levels of transposons of the intracisternal A particle (IAP) class, (Walsh et al., 1998). The Dnmt1 is the only gene known to be required for the repression of transposons in mammalian somatic cells, protecting these elements from demethylation during cleavage. The study of Dnmt1 deficient ES cells revealed an enhaced mutation rate at several endogenous loci (Hprt and tk) caused by chromosomal rearragements, namely loss of heterozygosity because of increased mitotic recombination (Chen et al., 1998).

Interestingly, ES cells that lack Dnmt1 grow normally in the undifferentiated state and retain low but detectable levels of DNA methylation through more than 20 cell divisions (Lei et al., 1996). However, *Dnmt1* null ES die by apoptosis when induced to differentiate *in vivo* or *in vitro*. These effects are paralleled by the apoptosis observed in Dnmt1 deficient embryos (Li et al., 1992). The mechanisms that lead to apoptosis are not known.

Dnmt1 is highly expressed in the cytoplasm of postmitotic neurons. The cytoplasmic localisation of Dnmt1 could be explained by a regulatory mechanism of the neuron to ensure the optimal protein in a post-mitotic state, or by an additional function in the cytoplasm. Conditional mutant transgenic studies where Dnmt1 is specifically deleted in the central nervous system have been used to show that Dnmt1

is necessary for the regulation of the respiratory function and the survival of post-mitotic neurons (Fan et al., 2001).

Dnmt1 deletion has been shown to affect the differentiation of $CD8^+$ T cells in the presence of a viral infection (Chappell et al., 2006). Additionally, when *Dnmt1* is knocked out with a mutation that leads to 10% expression levels of Dnmt1, this mutation causes a very high rate of lethal T-cell lymphomas (Gaudet et al., 2003). Demethylation and mobilization of endogenous retrovirus cause the majority of leukemias and lymphomas in most mouse strains. And ultimately, studies of overexpression of Dnmt1 reveal both that Dnmt1 has *de novo* methylase activity and that high levels of Dnmt1 are lethal because it causes *de novo* methylation of normally unmethylated allele at imprinted loci (Biniszkiewicz et al., 2002).

1.5.2 Dnmt2

Dnmt2 has sequential and structural high affinity with the other DNA methyltransferases Dnmt1 and Dnmt3. However, Dnmt2 lacks biochemically detectable methyltransferase activity (Okano et al., 1998b; Yoder and Bestor, 1998) and *Dnmt2* deficient ES cells (Okano et al., 1998b) *D.melanogaster* and *A. thaliana* are viable, fertile and morphogically indistinguishable from wild type (Goll et al., 2006). The role of Dnmt2 is to methylate the small RNA aspartic acid transfer $tRNA^{Asp}$, function that is highly conserved in among mammals, flies and plants. Dnmt2 shares with the rest of Dnmts an indirect sequence recognition requirement in eukaryotes, giving the hypothesis of an ancestral Dnmt2 as origin of eukaryote Dnmts (Goll et al., 2006).

1.5.3 Dnmt3

There are two known *de novo* methyltransferases, Dnmt3A and Dnmt3B (Okano et al., 1999; Okano et al., 1998a) which primarily methylate CpG dinucleotides. A third homologue, Dnmt3L lacks cytosine methyltransferase activity, but interacts with *de novo* methyltransferases targeting methylation to the dispersed retrotransposon elements (Bourc'his and Bestor, 2004; Bourc'his et al., 2001; Hata et al., 2002). Dnmt3L functions as well as a regulatory factor in male and female germ cells, playing an important role in genomic imprinting (Bourc'his and Bestor, 2004;

Hata et al., 2002). Dnmt3a and Dnmt3b are highly similar proteins. Both have C-terminal catalytic domains, and in their N-terminal domain they have a PWWP domain and a cysteine rich zinc-binding region related to the one contained in the chromatin remodelling protein ATRX (Xie et al., 1999). The PWWP domain is a highly conserved aminoacid sequence found in many chromatin associated proteins (Qiu et al., 2002; Slater et al., 2003; Stec et al., 2000).

The question of how the methyltransferases are targeted to unmethylated DNA has been extensively studied with *in vitro* cell culture systems. There are three working possibilities. Firstly, in mouse cells Dnmt3 enzymes might be targeted to the pericentromeric heterochromatin regions by its PWWP domain (Chen et al., 2004). An alternative targeting mechanism might be by transcription factors that interact with the Dnmt to recruit methyltransferase activity as part of a molecular silencing signal to shutdown gene expression (Brenner et al., 2005; Di Croce et al., 2002). A third way of targeting could be via an RNA-mediated interference system targeting *de novo* methylation to specific DNA sequences (Santoro and Grummt, 2005). A combination of the three mechanisms should be also considered.

Dnmt3a and b are highly expressed in early embryonic cells, where most programmed *de novo* methylation events occur, and to much lesser extent in differentiated cells and adult tissues (Okano et al., 1998a). Because DNA methylation is believed to play an important role in the central nervous system, extensive studies of Dntm3a and Dnm3b in the mouse CNS have been made. Both proteins are expressed throughout the developing cortex in neural precursors. While Dnmt3b is preferentially expressed between E10.5 and E13.5, Dnmt3a remains expressed through E17.5 and switches its expression from neural precursors to post-mitotic neurons with an increased expression through the first 3 weeks of postnatal development followed by a decreased expression into adulthood (Feng et al., 2005). The same pattern of expression is followed in other sensory systems, as in the olfactory system Dnmt3b is expressed in mitotic olfactory precursors, while Dnmt3a is expressed only in post-mitotic immature neurons. Furthermore, in the vomeronasal organ, the retina and taste buds, Dnmt3b is found in more primitive mitotic progenitors, whereas Dnmt3a is expressed in more highly differentiated post-mitotic neurons (MacDonald et al., 2005).

Dnmt3b is thought to be involved in methylation of specific genomic regions: including pericentromeric repetitive DNA sequences and CpG islands on the inactive X chromosome. This is known because *Dnmt3b* null mice and also human patients with DNMT3B mutations are deficient in methylation of pericentromeric repetitive DNA sequences and CpG islands on the inactive X (Okano et al., 1999). Point mutations in human *DNMT3B* are responsible for the rare autosomal recessive human disorder immunodeficiency, centromere instability, and facial anomalies syndrome (ICF) (Hansen et al., 1999; Xu et al., 1999). ICF is characterised by developmental defects, mental retardation and distinct facial features. Patients have a specific loss of methylation of classical satellite DNA, also known as satellites 2 and 3 at the pericentromeric regions of chromosomes 1, 9, and 16 in their lymphocytes.

Dnmt3a$^{(-/-)}$ mice are born but die after 4 weeks, while *Dnmt3b*$^{(-/-)}$ mice are not viable and have multiple developmental defects (Okano et al., 1999). Both present global demethylation of their genomes, although in lesser degree than *Dnmt1*$^{(-/-)}$ mutant embryos. *Dnmt3b*$^{(-/-)}$/Dmt3a$^{(-/-)}$ double mutant ES cells are unable to methylate newly integrated retroviral DNA. Conditional deletion of *Dnmt3a* in germ cells results in sex-specific phenotypes. Female Dmt3a$^{(-/-)}$ germ cells die *in utero* lacking methylation and allele-specific expression of maternally imprinted genes. Meanwhile conditional Dmt3a$^{(-/-)}$ null male germ cells have impaired spermatogenesis and misregulation of paternally imprinted genes. On the other hand, germ cells are not affected by deletion of Dnmt3b (Kaneda et al., 2004a; Kaneda et al., 2004b).

Lsh forms part of the chromatin remodelling SNF family of proteins. Lsh control normal heterchromatin sturucture and function in mice, contributing to the silencing of repetitive elements (Huang et al., 2004; Yan et al., 2003a; Yan et al., 2003b). Concordantly, *Lsh*$^{(-/-)}$ mutant mice have developmental defects and early lethality (Dennis et al., 2001; Geiman et al., 2001). Recently it has been discovered that Lsh interacts with Dnmt3a and Dnmt3b and it is required for their function in *de novo* methylation of endogenous genes (Zhu et al., 2006).

1.6 Epigenetic reprogramming cycle

Epigenetic modification undergoes reprogramming during the life cycle in two phases: during gametogenesis and preimplantation development.

In the zygote, after fertilization, the paternal genome has been carried by the mature sperm and is single copy packaged densely with protamines. The maternal genome is arrested at metaphase II and is packaged with histones. Upon fertilization, protamines in the sperm chromatin are rapidly replaced with highly acetylated histones (Adenot et al., 1997; Santos et al., 2002) although histone methylation is also detectable (Erhardt et al., 2003; Lepikhov and Walter, 2004; Santos et al., 2005). Meanwhile, the maternal genome completes meiosis. Following this process begins the genome wide demethylation: The paternal genome undergoes active demethylation and is completed by one cell stage before DNA replication starts in the paternal pronucleus. On the contrary, the maternal genome undergoes passive demethylation, and is completed by the morula stage (Santos et al., 2002). There are some regions of the genome that are designed to not become demethylated; These are heterochromatin in the centromeres and close regions, probably to maintain chromatin stability (Rougier et al., 1998; Santos et al., 2002), IAP retrotansposons to avoid transpositions (Lane et al., 2003) and paternally methylated imprinted genes for imprinting maintenance (Olek and Walter, 1997). These processes have been observed in the mouse, rat, pig, bovine and human zygotes. However, DNA methylation dynamics of sheep zygotes are slightly different because the paternal pronucleus retains more DNA methylation.

At the blastocyst stage in the mouse there are two cell lineages, the inner cell mass (ICM) and the trophectoderm (TE). DNA methylation is still decreased through the cleavage divisions (Monk et al., 1987) because of the exclusion of oocyte Dnmt1 (Dnmt1o) from the nucleus during the first three cleavage divisions (Carlson et al., 1992). This progressive demethylation that depends on DNA replication (Howlett and Reik, 1991) is known as passive demethylation. As a result, there will be a low level of methylation in the TE meanwhile the ICM will go through an extensive *de novo* methylation starting from morula stage (Santos et al., 2002). This difference may be the cause of the fact that Dnmt3b is detectable in blastocyst in the ICM but not in the TE (Watanabe et al., 2002). The difference in DNA methylation changes

between ICM, and TE could be that the differentiation potential and longevity of extraembryonic tissues are much more limited than the embryonic tissues (Morgan et al., 2005).

Primordial germ cells (PGC) are derived from epiblast cells in the posterior primitive streak at embryonic day E7.5, from where they start to migrate to the genital ridge and arrive at E11.5 (McLaren, 2003). Female PGCs enter meiotic arrest in prophase of meiosis I, while male PGCs enter mitotic arrest about birth when mitosis of spermatogonial stem cells is resumed. The epigenetic marks that the PGCs are carrying are similar to other epiblast cells, such as random X chromosome inactivation, imprinted gene expression and DNA methylation. However, these marks need to be erased, and indeed this happens when the PGCs reach the genital ridges with a wave of demethylation that occurs between E11.5 and E12.5 (Hajkova et al., 2002). This demethylation is believed to be active because it happens in very short period of time and in presence of Dnmt1 in the nucleus. Following demethylation, the genomes of the gametes are *de novo* methylated and acquire parent-specific imprints. This process continues until E18.5 in males and in maturing oocytes before ovulation in females (Morgan et al., 2005) (figure 1.1).

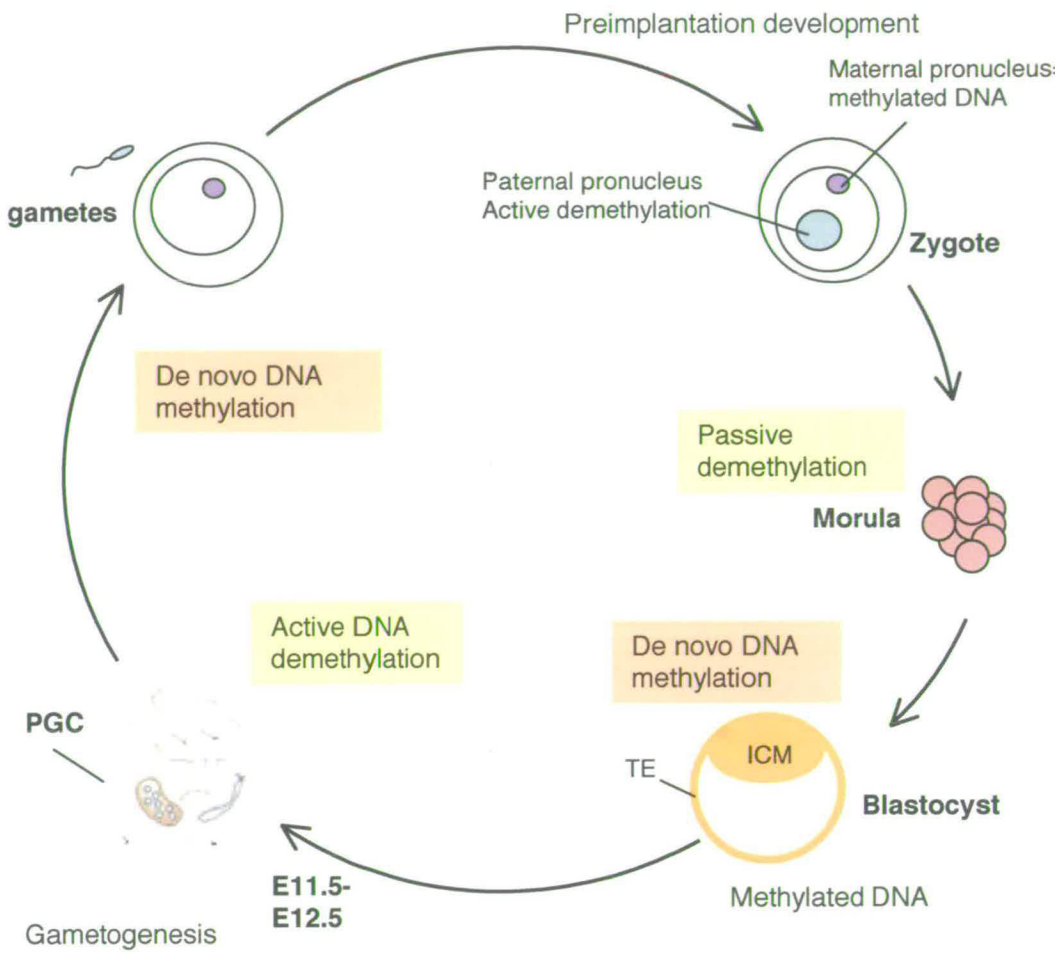


Figure 1.1 Epigenetic reprogramming cycle. After fertilisation, the paternal DNA undergoes active demethylation, while maternal DNA undergoes passive demethylation that is completed at morula stage. At blastocyst stage, the inner cell mass undergoes extensive *de novo* methylation, while the trophectoderm chromatin remain demethylated. Epigenetic marks at primordial germ cells (PGCs) are erased by another wave of demethylation between embryonic age E11.5 and E12.5. Subsequently, the genomes of the gametes are *de novo* methylated and acquire parent-specific imprints. This process continues until E18.5 in males and in maturing oocytes before ovulation in females. *Adapted from Morgan et al., 2005.*

1.7 Methyl-CpG binding proteins

The first entity found to bind methylated DNA and mediate transcriptional repression was MeCP1 (Meehan et al., 1989). This factor later was revealed as a multi-component complex. Subsequently, another was identified capable to bind methylated DNA, MeCP2 (Lewis et al., 1992) that can bind a single symmetrically methylated CpG pair. MeCP2 contains both a methyl-CpG binding domain (MBD) and a transcriptional repression domain (TRD) (Nan et al., 1997; Nan et al., 1993). By searching for sequences homologous of the MBD from the MeCP2, the MBD-containing protein Mbd1 was identified (Cross et al., 1997), and subsequently Mbd2, Mbd3 and Mbd4 (Hendrich and Bird, 1998) (figure 1.2 and figure 1.3).

The multi-component complex MeCP1 was shown to contain a methyl-CpG binding protein able to repress transcription in a methyl-CpG binding dependent fashion. This protein was initially believed to be Mbd1 (Cross et al., 1997), but subsequent studies revealed that Mbd2 was the methyl-CpG binding activity in the complex (Ng et al., 1999). Additionally, MeCP1 was later on shown to be a complex formed by the Nucleosome Remodelling and Deacetylation Repressor NuRD Complex, and the Mbd2 protein (Feng and Zhang, 2001).

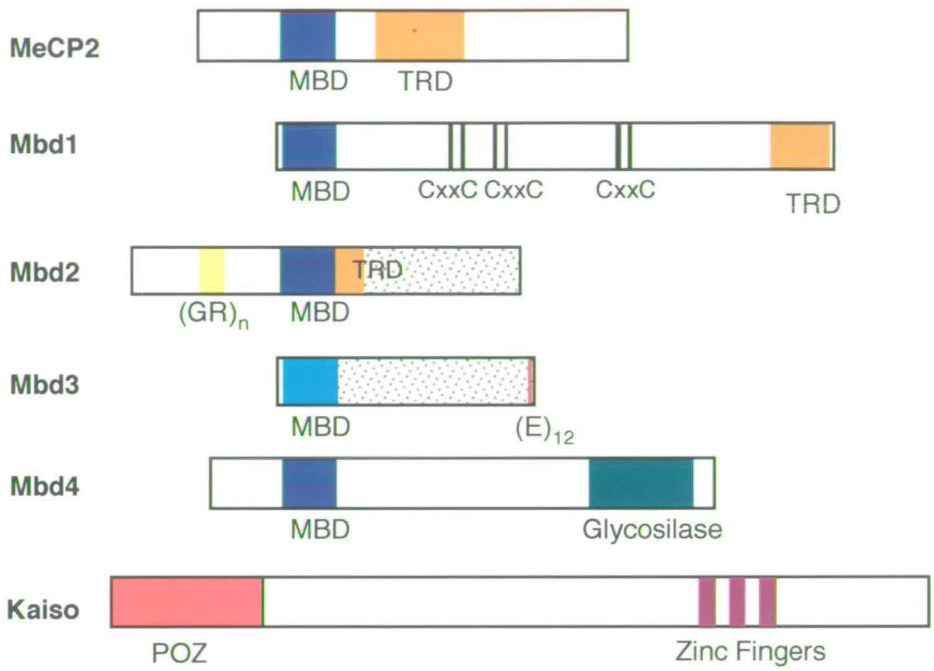


Figure 1.2 Methyl-CpG binding proteins. MePC2, Mbd1-4 share MBD domain which allows the proteins to bind methylated DNA, while Kaiso bind methylated DNA through its zinc fingers. Mbd1 also contains three zinc finger domains (CxxC), one of them allows Mbd1 to bind non-methylated CpG dinulceotides. MeCP2 and Mbd1 have a transcriptional repression domain TRD. Kaiso repress transcription in a methylation dependent manner with involvement of both zinc fingers and POZ domain. Mbd2 and Mbd3 are 70% homologous, but Mbd3 MBD does not bind methylated DNA in mammals due to two amino acid residues different that eliminate the methyl-CpG binding activity. Mbd4 can bind methylated DNA through its MBD domain and repair mismatched base pairs through its glycosilase domain.

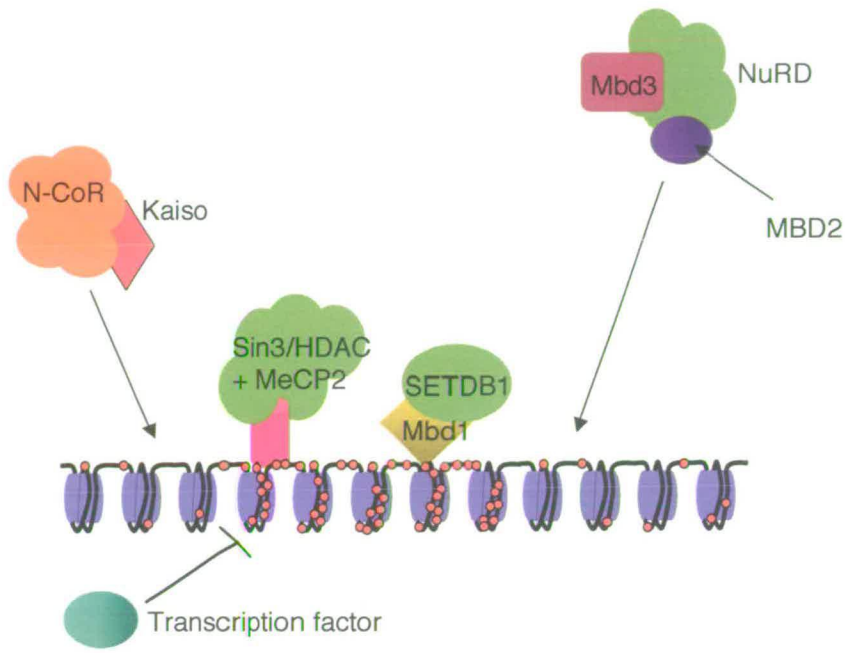


Figure 1.3 Transduction of the methylated signal. MeCPs bind methylated DNA (red circles) targeting the chromatin remodeling complexes where they are included, which leads to silencing of gene expression. In HeLa cells Kaiso forms part of the NCoR corepressor complex and Mbd1 associates with the H3-lysine9-specific methylase SETDB1. Mbd3, which does not bind methylated DNA in mammals, forms part of the nuclear corepressor NuRD, which associates with Mbd2 that bind methylated DNA. MeCP2 may associate with several co-repressor complexes including Dnmt1, CoREST, Suv39H1, and Sin3a. *Adapted from Bird., 2002.*

1.8 MeCP2

MeCP2 is a 80kDa protein that binds a single symetrically methylated CpG (Lewis et al., 1992). Its MBD, as in the rest of MBD proteins, is located in the N-terminus of the protein. In the middle of the protein is located its transcription repression domain (TRD) of 100 amino acids (Nan et al., 1997) that can repress transcription up to 2kb from the transcription initiation site. The transcriptional repression activity is linked to histone deacetylation (Jones et al., 1998; Nan et al., 1998) and histone methylation (Fuks et al., 2003b). There are several co-repressor complexes that may associate with MeCP2 in mammals including Dnmt1, CoREST, Suv39H1, c-SKI and Sin3a (Kimura and Shiota, 2003; Kokura et al., 2001; Lunyak et al., 2002; Nan et al., 1998). This last complex, once believed to be the corepressor partner of MeCP2, has been demonstrated to intereact with MeCP2 in low proportion with a non stable interaction (Klose and Bird, 2004).

Recently it has been demonstrated that MeCP2 requires an A/T run of four or more base pairs adjacent to the methyl-CpG for efficient binding (Klose et al., 2005). In the absence of another MeCP, Mbd2, MeCP2 can not bind to the sites normally bound by Mbd2 in the majority of the cases. On the other hand, in the absence of MeCP2, Mbd2 can bind to most of the normal MeCP2 binding sites (Klose et al., 2005). Therefore, Mbd2 can functionally compensate for MeCP2 but not vice versa.

There are two MeCP2 isoforms as a result of alternate splicing of the four exon MeCP2 gene. These are termed Mecp2α/β in mice, and MECP2A/B in humans, and they differ in the aminoacid composition of the N-terminus (Kriaucionis and Bird, 2004; Mnatzakanian et al., 2004). *MECP2B* in humans or *Mecp2α* in mouse are more highly expressed than *MECP2A/Mecp2β* transcripts in most tissues. Although MeCP2 is ubiquitously expressed through the mouse and human tissues, is the brain where the expression is remarkably high.

Within the brain, MeCP2 is predominantly expressed in neurons, with strongest expression in the more mature neurons (Kishi and Macklis, 2004; Mullaney et al., ; Shahbazian et al.). The extensive studies of Mecp2 expression in rat (Mullaney et al., 2004) and mouse (Braunschweig et al., ; Shahbazian et al., 2002b)Shahbazian et al., 2002) have revealed a pattern of expression that correlates

with the maturation of the different brain regions, and indicates a function for MeCP2 in mature neurons. Moreover, neuronal precursor differentiation assays reveal no evidence for defects in the proliferation or fate decisions of neuronal precursors in the absence of MeCP2 (Kishi and Macklis, 2004). However, increasing evidence suggests a role for MeCP2 in maturation and maintenance of postmitotic neurons in murine brains (Kishi and Macklis, 2004; Matarazzo et al., 2004).

1.8.1 Rett syndrome (RTT)

Rett syndrome is caused by mutations in the *MECP2* gene (Amir et al., 1999). RTT is a neurological disorder that affects primarily girls, with an incidence of 1/10000 to 1/15000 live births (Hagberg, ; Kerr and Stephenson, 1985; Rett, 1966). The affected individuals are born asymptomatic and develop normally during the first few months of life, achieving the expected milestones in motor language and social areas. After 6-18 months of age, however, acquired speech and motor skills begin to be lost (Hagberg). Purposeful hand movements are replaced by characteristic hand-wringing motions and gait apraxia, followed by growth retardation and deceleration of head growth. In addition, half of affected individuals develop seizures and autistic behaviour, and by 4-7 years display gross cognitive and motor impairment together with profound hypoactivity which remains throughout their lives (Hagberg and Witt-Engerstrom).

Histopathological studies on post-mortem RTT brains have revealed that the observed microcephaly correlates with reduced brain size, particularly of the prefrontal, posterior frontal, and anterior temporal regions (Subramaniam et al.). This characteristic reduction in size roughly correlates with both a decrease in the size of individual neurons and increased neuronal packing densitiy in these regions (Bauman et al., 1995a; Bauman et al., 1995b). Additionally a reduction of dendritic arborization in cerebral cortical layers II and IV in the frontal, motor and inferior temporal regions has been observed (Armstrong et al., 1995).

1.8.2 MeCP2 mutant mice models

Once MeCP2 was linked to be the cause of Rett syndrome, many laboratories started the generation of mouse models to try to understand the function of MeCP2. *Mecp2* heterozygous female mice are viable, fertile, and appear normal well into

adulthood (Chen et al., 2001; Guy et al., 2001) even though they have the same genotype as RTT girls. At around six months of age, however, these females begin to show neurological symptoms reminiscent of RTT (Guy et al., 2001), indicating that the onset of Rett Syndrome is unlikely to depend upon the developmental stage of affected individuals, as humans and mice develop a similar disease after the same amount of time, despite being at completely different developmental stages. Hemizygous null male or homozygous null female mice appear healthy at birth, but began to display RTT-like phenotypes after about six weeks and die at an average age of approximately 8 weeks (Chen et al., 2001; Guy et al., 2001). Brain architecture in null mice is grossly normal, although a slight decrease in the size of neurons and an increased packing density in the hippocampus, cerebral cortex and cerebellum could be identified (Chen et al., 2001; Kishi and Macklis, 2004). This finding correlates with disease pathology in humans where a decrease in the size of individual neurons and increased packing density are likely contributory factors to the smaller brain size often observed in RTT patients (Armstrong et al., 1995; Bauman et al., 1995a; Bauman et al., 1995b).

Another mouse model was produced that expresses a truncated form of MeCP2 often seen in RTT patients (Shahbazian et al., 2002a). These *Mecp2* mutant males ($MeCP2^{308/Y}$) were aphenotypic until 4- 5 months of age, after which time they began to display tremors, kyphosis and motor dysfunction. Heterozygous females ($MeCP2^{308/X}$) have impaired motor features at 35-39 weeks and show phenotypic variability ascribed to differences in patterns of X chromosome inactivation. In early studies, results of learning and memory tests were reported to be normal (Shahbazian et al., 2002a). However, recent thorough studies of behaviour, synaptic function, electrophysiological and ultrastructural experiments revealed compound memory and learning deficits associated with synaptic function (Moretti et al., 2006).

Mice in which MeCP2 was deleted in Nestin-positive neural precursors (and their progeny, i.e. all neurons and glia) displayed a phenotype indistinguishable from that seen in MeCP2-null mice (Chen et al., 2001; Guy et al., 2001), indicating that the primary site of action for MeCP2 is in the brain. Furthermore, Chen et al. produced mice in which the *Mecp2* gene was only deleted in cells expressing αCamKII, a kinase specifically present in postmitotic neurons (Chen et al., 2001).

The αCamKII promoter is normally active in postnatal excitatory neurons of the forebrain, hippocampus and brainstem, and to a much lesser degree in the cerebellum (Silva, 2003), although the exact distribution and strength of its activity within the forebrain can vary from one transgenic line to another (Chen et al., 2001; Tsien et al., 1996). These mice appeared normal until about 3 months of age, at which time they began to display ataxic gait and reduced nocturnal activity. Histological analysis revealed reduced brain weights and smaller neuronal cell bodies in cortex and hippocampus (Chen et al., 2001). Interestingly, these phenotypes were not seen in the cerebellum where αCamKII is not expressed (Caballero et al., 2005).

1.8.3 MeCP2 in *Xenopus*

MeCP2 is also expressed in *Xenopus* ubiquitously, and concordantly with mice and human, it is also highly expressed in all neural tissues. However, the depletion of xMeCP2 results in arrest of development at the neurula stage, which results in significant developmental defects such as reduced dorsal axis and abnormal head structures (Stancheva et al., 2003). Hence, xMeCP2 play an essential role in early development of *Xenopus* in contrast with the situation in mammals where MeCP2 is not essential for embryogenesis. *Xenopus* Hairy2a was identified as a target gene for repression by xMeCP2. xHairy2a is a downstream gene of the Notch/Delta signalling pathway. When *Xenopus* embryos express a truncated form of MECP2, (a frequent mutation in Rett syndrome), xHairy2a is inefficiently repressed which causes an increase in neuronal precursors with consequent disruption in neuronal patterning (Stancheva et al., 2003). Hence, identified target genes in *Xenopus* are related with neuronal precursor functionality, but given the differences on the onset of the phenotype as a consequence of depletion of MECP2 in human and mice versus *Xenopus*, its target genes may differ between species.

1.8.4 MeCP2 target genes

Since MeCP2 is involved in transcriptional repression when bound to the widely distributed methyl-CpGs of the genome, it was predicted that loss of MECP2 in a cell would result in inappropriate expression of a large number of genes (Willard and Hendrich, 1999). However, microarray studies with cell lines and tissues of mice

or mutant human patients found only very subtle gene expression changes (Colantuoni et al., 2001; Matarazzo and Ronnett, 2004; Tudor et al., 2002) or protein expression changes (Matarazzo and Ronnett, 2004). Some of the genes found to be discretly upregulated are involved with glucocorticoid signaling pathway like *Sgk* and *Fkbp5* (Nuber et al., 2005). Interestingly, the changes appear before the onset of Rett-like symptoms in the mice which indicates that the upregulation of these genes may not be consequence of the Rett-like phenotype (which includes misregulation in metabolism). Additionally, both genes have been linked to neuronal function and therefore is plausible that misregulation of these genes would be involved in the Rett syndrome phenotype.

On the other hand, candidate gene approaches have lead to the identification of additional MeCP2 *bona fide* target genes. The first example was the brain-derived neurotrophic factor (BDNF), which was found to be misexpressed in *Mecp2*-null brains. This missexpression occurs only in certain cell types, and only to a level of about two-fold (Chen et al., 2003; Martinowich et al., 2003). BDNF is a neurotrophin required for survival, growth, and maintenance of neurons during development (Barde, 1994). BDNF is known to be involved in learning and memory and has the ability to modulate synaptic plasticity by regulating axonal and dendritic branching and remodeling (Alsina et al., 2001; Lo, 1995; Lom and Cohen-Cory, 1999; Shimada et al., 1998; Yacoubian and Lo, 2000). MeCP2 was found to associate with and maintain repression of *Bdnf* in resting neuronal cultures. Following neuronal depolarization, MeCP2 becomes phosphorylated and disassociates from the *Bdnf* promoter, allowing for full transcription of the gene. In the absence of MeCP2, *Bdnf* repression becomes leaky in unstimulated neurons, resulting in a two-fold increase of *Bdnf* mRNA levels (Chen et al., 2003). This increase in mRNA levels does not constitute 'activation' of the *Bdnf* gene, as transcript levels in resting *Mecp2*-deficient neurons are still approximately 100-fold lower than those found in activated neurons (Chen et al., 2003). Rather, the absence of MeCP2 results in incomplete repression of *Bdnf*. No difference in *Bdnf* expression was detectable in depolarised neuronal cultures derived from *Mecp2*-deficient or wild-type mice (figure 1.4).

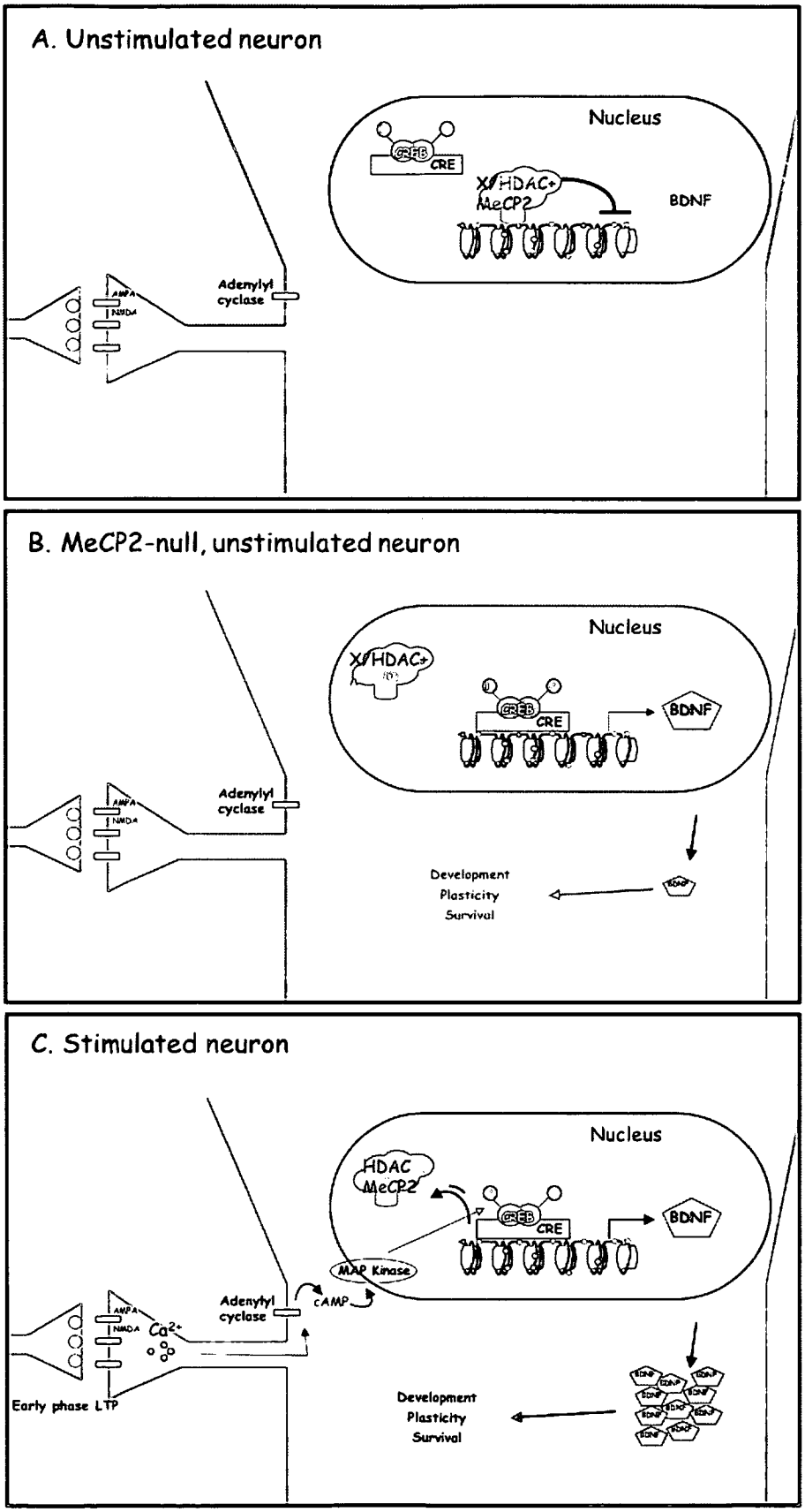

A. Unstimulated neuron
Nucleus
CREB
CRE
HDAC
MeCP2
BDNF
Adenylyl cyclase
AMPA
NMDA
B. MeCP2-null, unstimulated neuron
Nucleus
HDAC
CREB
CRE
BDNF
Adenylyl cyclase
AMPA
NMDA
Development
Plasticity
Survival
C. Stimulated neuron
Nucleus
HDAC
MeCP2
CREB
CRE
BDNF
MAP Kinase
Adenylyl cyclase
cAMP
AMPA
NMDA
Ca2+
Early phase LTP
Development
Plasticity
Survival

Figure 1.4. MeCP2 regulation of the neurotrophic factor Bdnf. A. In resting neurons, MECP2 (orange) and its associated co-repressor (HDAC, in green) represses transcription of the *Bdnf* promoter III. B. In the absence of MeCP2, repression of *Bdnf* is leaky, resulting in low-level transcription and, presumably, Bdnf protein production that may cause a variety of downstream effects leading to dysfunction at different levels such as reduced long term potentiation (LTP), altered learning and memory and decreased dendritic arborization. C. Upon membrane depolarisation (i.e. neuronal stimulation), MeCP2 is displaced from promoter III and *Bdnf* transcription is activated, resulting in Bdnf protein production. *Figure adapted from Kandel, Schwartz, Jessell, Principles of Neural Science. 4th Edition 2000.*

Although MeCP2 has been shown to physically associate with the silent alleles of various imprinted genes (Drewell et al., 2002; Fournier et al., 2002; Gregory et al., 2001), no loss of imprinting could be found at these loci in *Mecp2*-deficient mice (Chen et al., 2001; Guy et al., 2001). However, the imprinted *Dlx5* gene was recently found as a target for MeCP2 repression (Horike et al., 2005). *Dlx5* and *Dlx6* encode homeobox proteins known to regulate the expression of genes involved in neurotransmitter production (Stuhmer et al., 2002) and are important in various aspects of embryonic development, including brain patterning (McLarren et al., 2003).

Moreover, another imprinting gene, *Ube3a* has been found to be misregulated in *Mecp2*-deficient animals (Samaco et al., 2004). The deficiency or deletion of this gene is the cause of Angelman Syndrome (Kishino et al., 1997; Matsuura et al., 1997). Again, a small but significant decrease in expression of *Ube3a/UBE3A* was found in brains of *Mecp2*-deficient mice and RTT patients (Samaco et al., 2004). Further, expression of a nearby non-imprinted gene implicated in autism, *Gabrb3/GABRB3,* was reduced in the same samples. A similar reduction in *UBE3A* and *GABRB3* expression was also found in brain samples from autism and Angelman Syndrome patients, indicating that the similarities in phenotype between these syndromes may have a common molecular etiology (Samaco et al., 2004). In this case no evidence for loss of imprinting or for direct MeCP2 binding to either gene was found.

Recently MECP2 has been reported to have a direct role in splicing regulation through its association with YB-1 (Young et al., 2005), which controls multiple steps of mRNA processing (Raffetseder et al., 2003), giving a new vision to MeCP2 as a multifunctional protein.

1.9 MBD2

Mbd2 has two isoforms, Mbd2a, which is the full-length protein and Mbd2b that has an N-terminal truncation expressed by an alternative start codon. Both isoforms can bind methylated DNA (Hendrich and Bird, 1998).

Mbd2 shares an identical genomic structure with *Mbd3* in mammals. Methyl-CpG binding protein Mbd3, forms part of the methyl binding domain (MBD) family of proteins, but does not bind methylated DNA in mammals in contrast with the rest of the family. *Mbd2* and *Mbd3* encode proteins that are 70% identical. These facts support the theory that Mbd2 and Mbd3, could have been separated in vertebrates by a gene duplication event from the ancestral Mbd2/Mbd3 gene in invertebrates (Hendrich and Tweedie, 2003).

1.9.1 Mbd2 knock out mice and Mbd2 function

The function of Mbd2 has been investigated through the study of the knock out mice (Hendrich et al., 2001). *Mbd2*$^{(-/-)}$ mice are viable and fertile, and their DNA methylation and imprinting levels are normal. Mbd2 is a transcriptional repressor and therefore, when *Mbd2* is deleted from fibroblast cell lines there is a decrease in repression of methylated reporter genes. However, the level of expression is only 25% of the expression level with unmethylated promoters. This data indicates a possible redundancy between MeCPs in the repression of methylated promoters (Hendrich et al., 2001).

Abnormal maternal behaviour.

The *Mbd2*$^{(-/-)}$ pups were observed to be smaller than normal because a defect in nurturing behaviour of the *Mbd2*$^{(-/-)}$ mothers. This observation was concluded after cross-fostering experiments showed that pups from *Mbd2*$^{(-/-)}$ mothers developed normally when nurtured by *wild type* mothers, whereas when nurtured by *Mbd2*$^{(-/-)}$ mothers the pups showed low weight. This low weight was proven to be independent of the maternal milk composition (Hendrich et al., 2001).

Normal postpartum nurturing behavior in rodents is quite stereotyped and includes creating a nest, cleaning the pups, retrieving them to the nest and crouching over them for warmth and nursing (Barnett and Burn, 1967). The ability of the

postpartum mother to retrieve the newborn pups to their nest is the definitive diagnosis of a possible abnormal nurturing behaviour (Li et al., 1999). *Mbd2*$^{(-/-)}$ mothers were significantly slower than the wild type in this task. The postpartum nurturing response has two components, one experiential and one hormonal (Rosenblatt, 1994). Both require specific hippocampal circuitry involving the main olfactory bulb that receives the olfactory stimulus from the newborn pup. This signal is transmitted to the amygdala and preoptic area (POA) of the hypothalamus. Therefore, it is possible that *Mbd2* null mice suffer from an unidentified neurological defect related with neuronal circuitry.

A mutation in the *fosB* gene, which is involved in the POA circuitry, causes a nurturing behavior phenotype while other sensory and cognitive functions are normal (Brown et al., 1996). By a targeted disruption of the *dopamine-β-hydroxylase* gene, mice are unable to synthesise norepinephrine (NE) and epinephrine (Thomas and Palmiter, 1997). With the absence of the ligand NE mice present nurturing abnormalities, which have been specifically associated with a disruption of noradrenergic input in the olfactory bulb and other primary olfactory regions of the CNS. Both gene disruptions, *foxB* and *Dbh* results in a dysfunction of the induction of early genes in response to pup exposure.

Other proteins involved with maternal nurturing behavior are Peg3 and Mest, which are imprinting genes. No misexpression of imprinted genes has been shown in *Mbd2* mutant animals, including *Peg3* or *Mest*. Therefore it is more likely that Mbd2 is required as a transcriptional repressor in the olfactory circuitry of the central nervous system.

T cell differentiation.

Looking closely to possible gene misregulations in the absence of Mbd2, investigators studied organization of T cell development in absence of Mbd2. Helper T cell differentiation comprises a complex cascade of gene expression that is known to involve transcriptional reprogramming and developmental effects (Bird et al., 1998). The progenitor naïve helper T cell starts a differentiation program under microbial threat and differentiates into Th1 or Th2 lineage. Th1 is characterised by the expression of the transcription factor T-bet and the secretion of IFN-γ.

Meanwhile, Th2 is characterised by the expression of the transcriptional activator Gata-3 and secretion of IL-4 (Mullen et al., 2001; Ouyang et al., 1998; Szabo et al., 2000; Szabo et al., 2002; Zheng and Flavell, 1997). However, when Mbd2 is not present, both Th1 and Th2 cells, and also the progenitor helper T, express IL4, bypassing the transcriptional differentiation effect of Gata-3. In normal conditions, Gata-3 is believed to compete with the binding of Mbd2 to the *IL4* enhancers, so in the absence of Mbd2 IL4 is expressed regardless the cell type (Hutchins et al., 2002). Additionally, it was found that Mbd2 also competes with T-bet in the expression of IFN-γ, since Mbd2 repress and T-bet activates expression of IFN-γ. Consequently, the number of IFN-γ expressing cells is higher in *Mbd2* null mice. These *in vitro* observations have recently been assayed one step further when Mbd2 deficient mice were challenged with parasitic pathogens. The interesting outcome from these experiments is that Mbd2 deficiency confers enhanced resistance to *Leishmania major* because of the enhanced population of IFN-γ expressing cells, but impaired immunity to *Trichuris murs*. This intestinal helminth activates the immune response of Th2 cells that secrete IL-4, and the resistance to the infection is antagonized by IFN-γ (Hutchins et al., 2005).

Tumorigenesis.

Another characteristic of Mbd2 null mice is that they have a decreased incidence of tumour formation. Mice that are heterozygous for the tumour suppressor *Apc* gene are very susceptible to develop intestinal tumours (Su et al., 1992). *Mbd2* homozygous null mice, on an *Apc* $^{Min/+}$ heterozygous background present less number of tumours with less size, and consequently they live longer. The absence of Dnmt1 has been also shown to reduce the intestinal tumours in Apc$^{min/+}$ mice (Laird et al., 1995). It is believed to be related with a decrease in the aberrant methylation of tumour suppressor genes (Campbell and Szyf, 2003). However, the hypomethylation of the genome caused by Dnmt1 deficiency can also result in increased or accelerated tumorigenesis. The type of tumours seen with Dnmt1 defficiency appears to be linked with chromosomal instability, in particular sarcomas and aggressive T cell lymphomas (Eden et al., 2003; Gaudet et al., 2003). In contrast, Mbd2 null mice do not develop lymphomas. This conclusion was derived from the study of *Mbd2-*

null mice crossed with lymphomagenesis-prone p53-deficient mice (Sansom et al., 2005). p53 misregulation is the most established model of lymphomagenesis, and does not induce intestinal tumorigenesis.

It is an open question what methylated sequences Mbd2 binds (Klose et al., 2005). There is no sequence specificity known for this protein, and therefore it could be targeted to any selected sequence. This idea has been reinforced with the discovery of a protein named MIZF (Mbd2-interacting zinc finger), that can repress transcription in an HDAC-dependent manner (Sekimata et al., 2001). This protein has been shown to enhance the repression function of Mbd2 in co-transfection experiments. Since this protein can bind to a specific 5bp recognition sequence with a subsequent repression of transcription of the genes that have that consensus sequence within its promoter (Sekimata and Homma, 2004), it is plausible that MIZF targets Mbd2 and the NuRD complex to specific genomic regions.

1.10 Mbd1

Ectopically expressed of Mbd1, 2, and 4 can bind to methylated CpG sequences that in mouse cells is concentrated in the major satellite. These heterochromatic foci can be visualized as DAPI bright spots (Miller et al., 1974). However, this localisation is lost for Mbd2 and Mbd4 in methylation deficient mutant cells (Hendrich and Bird, 1998). In contrast, Mbd1 can still be visualized at DAPI bright spots in deficient cells because it can also bind unmethylated CpGs. This particular binding capability will be described below (Jorgensen et al., 2004).

Mbd1 is the largest protein in the MBD family. In the C-terminus, Mbd1 has a specific transcriptional repression domain TRD that is capable of strong methyl-dependent repression from binding sites far upstream of its promoter (Ng et al., 2000) (Fujita et al., 2003b). Since TSA treatment variably relieves the repression by Mbd1, histone deacetylation may not be consistently involved in the repression (Ng et al., 2000).

Both human and mouse cells have several isoforms of MBD1 protein, derived from alternative splicing of *MBD1* mRNA and differing in the number and spacing of cysteine rich motifs (CxxC) (Fujita et al., 1999; Hendrich and Bird, 1998). These CxxC motifs are located in the N-terminal domain, which also contains the MBD

that can bind a single symmetrically methylated mCpG pair. In mice there are three known Mbd1 transcripts, Mbd1a, b and c. The three of them have two CxxC, and Mbd1a has additionally a third CxxC. Mbd1a can bind to unmethylated CpGs and repress transcription through its CxxC (Jorgensen et al., 2004). This special capability confers Mbd1 the ability to bind to CpG sequences regardless their methylated status. Similar CxxC domains can be found in Dnmt1, CpG binding protein (CGBP), and the mixed lineage leukemia protein (MLL) (Ng et al., 2000), all of which have been demonstrated to bind to nonmethylated CpG sites *in vitro* (Birke et al., 2002; Lee et al., 2001; Voo et al., 2000).

In HeLa cells MBD1 associates with the histone H3-lysine9-specific methylase SETDB1 via its second CxxC motif and to a chromatin assembly factor, CAF-1 by the first CxxC motif (Sarraf and Stancheva, 2004). CAF-1 is a complex of three subunits, p150, p60, and p48, and is responsible for the assembly of nucleosomes onto newly replicated DNA (Kaufman et al., 1995). The p150 subunit is known to interact with the proliferating cell nuclear antigen PCNA, a protein implicated in a wide range of DNA replication and repair processes (Moggs et al., 2000; Reese et al., 2003) that functions in S phase. This complex meets a neat process of transmission of transcriptional silencing through the cell cycle. MBD1 and SETDB1 form a stable pair in all the cell cycle stages. At G1, MBD1/SETDB1 complex is bound to methylated DNA to repress transcription. When the cell enter S phase to start the DNA replication, MBD1 recruits CAF-1, and the CAF-1/MBD1/SETDB1 complex is formed. CAF-1 then recruits PCNA. This complex is transient because it may prevent spreading of H3-K9 methylation beyond the methylated DNA region. During S phase, this complex may associate with Dnmt1, which also binds to PCNA, to copy the parental methylation pattern to the daughter DNA strand, and together with the histone methylation activity by the SETDB1, the epigenetic status of the silenced chromatin is maintained at the replicated DNA.

1.10.1 Mbd1 expression

Mbd1 is expressed in the majority of somatic tissues, being expressed highly in brain and only at very low levels in ES cells. In the mouse brain Mbd1 is expressed everywhere and the highest expression is in the hippocampus, being particularly high

the *Cornu Ammoni* 1 (CA1) and dentate gyrus (DG) regions. The cells that express Mbd1 most abundantly are Neuronal Nuclei (NeuN)-positive neurons, but it is also expressed in Nestin-positive precursor cells. Mbd1 is not expressed in oligodendroctyes neither in astrocytes. The CA1 and DG are the neuronal layers with higher expression of Mbd1.

1.10.2 Mbd1 deficient mice and Mbd1 function

Mbd1$^{(-/-)}$ mice are healthy and fertile with a normal life span (Zhao et al., 2003). They display no defects in routine neurological tests and the cellular and structure brain arrangements are normal. *In vitro* studies showed normal proliferation rates in hippocampal adult neural stem cells but revealed a decrease in neuronal differentiation while astrocytic differentiation was not affected. Given this finding, *in vivo* studies followed the neuronal differentiation phenotype to find that cell proliferation as measured by BrdU incorporation was not affected in the null hippocampus. However 4 weeks after injection there were less 50 percent of proliferating cells in the (DG) of *Mbd1* null than wild type, and from this percentage around 50 percent were newborn neurons, giving it a total of approximately 75% less newborn neurons in total in null adult mice. Ultimately, the neuronal differentiation and survival impairment in null Mbd1 hippocampus was conclusively linked with specific DG hippocampal learning and memory tests studies, since this test showed impaired spatial learning ability and a three-fold decrease in LTP in the DG-specific region, which has been associated with decreased neurogenesis (van Praag et al., 1999).

Adult hippocampal neural stem cells were used in microarray studies in the search for genes misregulated in Mbd1-null cells. From these experiments intracisternal A particles (IAP) were found to be expressed at higher level in null adult neural stem cells (ANCs) than in wild type. As described before, the increased expression in IAPs has also been observed in the absence of Dnmt1 (Walsh et al., 1998) since IAPs are heavily methylated (Yoder et al., 1997). In contrast, in Mbd1$^{(-/-)}$ ANCs there is no evidence of demethylation. Interestingly, upon TSA treatment the IAP expression increases three times more in null cells than in wild type cells, giving evidence of a mechanism of repression that involves deacetylation machinery. IAP

expression is a signal for chromosomal aberrations, and in concordance, Mbd1 null ANC have double aneuploidy rates than in wild type cells, namely gaining of chromosomes. Given that aneuploidy is a cause of cell death, it is possible that the decreased cell survival in the DG is the cause of the increased expression of IAP found in their precursors.

Another piece of evidence that corroborate a function for Mbd1 in regulation of precursor cell fate is the recent discovery that Mbd1 is required in hematopoietic precursors to fully prevent cell differentiation. This function requires Mbd1 association with methylated PML-RARα target promoters, namely RARβ2 via HDAC3 repression (Villa et al., 2006).

1.11 Mbd4

Mbd4 binds symmetrically methylated CpG sites through its MBD (Hendrich and Bird, 1998) although it has a higher affinity for 5mCpG/TpG mistmatches (Hendrich et al., 1999), which are common mutation products of the deamination of methylcytosine at mCpGs and account to more than 20% of all base substitutions in human genetic diseases (Krawczak et al., 1998). Mbd4 also contains a glycosylase domain (Hendrich and Bird, 1998; Petronzelli et al., 2000) which can remove T or U in a mismatched base pair *in vitro* with G without cleaving the DNA strand (Hendrich et al., 1999). Both domains confer upon Mbd4 the appropriate characteristics to function as a mutability repair enzyme to reduce mutation at methylated CpG sites *in vivo*, and indeed mice lacking Mbd4 have an increase in the frequency of mutation at m5CpG pairs, and are prone to intestinal tumorigesis on an Apc^{Min} background (Millar et al., 2002; Wong et al., 2002). Moreover, loss of Mbd4 alters wild type Apc allele due to CpG to TpG mutations that causes APC protein truncation (Millar et al., 2002). Deficiency of Mbd4 also causes reduction of the apoptosis response in DNA damage (Sansom et al., 2003). Additionally, the MBD has recently been found to function not only to direct the enzyme to possible mismaches, but also as part of the transcriptional repressor machinery (Kondo et al., 2005). Mbd4 is involved in transcriptional repression at hypermethylated promoters, which are also H3/H4 deacetylated and H3K9 methylated. Repression of

transcription probably occurs through recruitment of HDAC1/Sin3A (Kondo et al., 2005).

1.12 Kaiso

Kaiso protein is a member of the BTB/POZ (broad complex, Tramtrack, Bric à brac/pox viruses and zinc fingers) family of proteins. The BTB/POZ domain consists of a highly conserved hydrophobic domain of approximately 120 amino acids located at the extreme N terminus. This POZ/BTB domain enables Kaiso to homodimerize or heterodimerize and interact with other proteins, recruiting corepressor complexes such as N-CoR, Sin3a and possibly Groucho or TLE (van Roy and McCrea, 2005). However, in the Kaiso-containing methyl-CpG specific complex, mSin3a, HDAC1 or SMRT where not isolated (Prokhortchouk et al., 2001).

In the carboxy-terminal there are three krupple-like C_2H_2 zinc fingers that have the ability to bind from two to three consecutive methyl-CpG base pairs with the highest reported affinity of any protein for DNA methylated at CpG (Prokhortchouk et al., 2001). Kaiso binds to DNA through its zinc fingers 2 and 3 and localised, as the rest of the MeCPs with the major satellite repeats in the chromosome, although mouse major satellite repeats do not contain the sequence CGCG and therefore Kaiso may not be targeted by methylation binding (Filion et al., 2006).

p120 catenin ($p120^{ctn}$) is a member of the armadillo family of proteins, characterised by the presence of an Armadillo domain which consist of series of ten or more tandem copies of a 42 amino acid repeats in helical conformation (Huber et al., 1997; Kobe, 1999). The function of the armadillo domain is protein-protein interaction (Peifer et al., 1994). As other catenins of this family, like β and γ catenins, $p120^{ctn}$ is involved in cell-cell adhesion, and hence related to metastasic events, but also is directly involved in regulation of gene expression by regulating the transcriptional repression activity of Kaiso (Daniel et al., 2002). Through its NLS, $p120^{ctn}$ is translocated to the nucleus and associates through its armadillo domain with Kaiso. Steric hindrance of Kaiso by $p120^{ctn}$ may inhibit Kaiso transcriptional repression activity (Kelly et al., 2004b).

Intracellular localisation of Kaiso is still controversial. There have been reported two different nuclear localisations: both a bright dot spots and diffuse localisation, with a variation between cell lines but also among same cell lines (Daniel and Reynolds, 1999; Filion et al., 2006). Apart from nuclear localisation, some cases of cytosolic localisation have also been reported for Kaiso. Although a role for p120 in sequestering Kaiso to the cytosol to modulate its transcriptional repression activity was initially postulated (Daniel and Reynolds, 1999), it has been recently shown that a NLS mutant form of p120 unable to translocate to the nucleus and fails to inhibit Kaiso-mediated transcriptional repression (Kelly et al., 2004b). Additionally, Kaiso itself has a highly basic ten amino acid NLS, upstream of the Kaiso zinc-finger domain that target Kaiso to the nucleus and that is involved in the transcriptional repression activity of the protein (Kelly et al., 2004a). One report assigned Kaiso a cellular localisation that is environment-dependent. For example, *in vitro* cultured cells were shown to mainly have a nuclear Kaiso localisation, while three dimensional cultures, normal tissues and tumor growths seemed to show predominantly cytoplasmic localisation, perhaps being cell density dependent (Soubry et al., 2005). However, other immunocytochemistry studies have pointed out a predominantly nuclear localisation *in vivo* in mice colon tumors (Prokhortchouk et al., 2006).

Kaiso can repress transcription in a methylation dependent manner and both POZ and zinc finger domains are necessary. Transcriptional silencing may not depend upon histone deacetylase activity since addition of TSA does not disturb the repression activity (Prokhortchouk et al., 2001). Additionally, through its zinc fingers Kaiso can bind the consensus Kaiso binding site (KBS) TCCTGCNA. Through this consensus Kaiso has been shown to repress the transcription of the genes *MATRYSILIN* (Spring et al., 2005) in humans and *Wnt-11* in *Xenopus* (Kim et al., 2004), but also is able to activate the transcription of rapsyn (Rodova et al., 2004). Additionally, Kaiso interacts with the DNA binding protein CTC-binding factor (CTCF) through its Kaiso binding site (KBS). The enhancer blocking activity of CTCF has been shown to be negatively regulated by Kaiso (Defossez et al., 2005). Through recognition of mCGmCG Kaiso can repress transcription of MTA2 (Yoon et al., 2003b), and can bind to the human tyrosine hydroxylase *TH* gene (Aranyi et

al., 2005). TH is essential for catecholaminergic neuronal specification and display different tissue specific methylation status, which determines KAISO binding. Therefore, it would be interesting to check whether the absence of Kaiso in neural stem cells increases their TH specification. However, these characterised target genes were not found to be upregulated in Kaiso-null tissues (Prokhortchouk et al., 2006).

1.12.1 Kaiso in Xenopus

In *Xenopus,* xKaiso is essential for development. Depletion of Kaiso in *Xenopus* embryos causes a striking phenotype consisting of developmental arrest and cellular apoptosis, characteristics that mimic the phenotype of Dnmt1 depletion in *Xenopus* (Stancheva et al., 2001) and mouse embryos (Jackson-Grusby et al., 2001). These severe effects are consequence of the premature activation of genes expressed in the mid blastula stage that are normally silenced by DNA methylation. Mid blastula transition is the time when a dependence upon maternal transcripts gives way to the start of zygotic transcription, and precedes a period of transcriptional silencing lasting 12 cell divisions. Thus, loss of Kaiso results in up-regulation of more than 10% of genes in early development.

xKaiso cooperates with the T cell factor/lymphocyte enhancer factor (TCF/LEF) family to repress transcription of targets from the canonical Wnt signalling pathway with the recruitment of the corepressor N-CoR (Kim et al., 2004). Particularly, xKaiso interacts with xTCF-3, and exogenous xTCF-3 can rescue some defects resulting from xKaiso depletion (Park et al., 2005). xKaiso represses transcription of Siamois, Xnr3 (Park et al., 2005) and xWnt-11(Kim et al., 2004), and other β-catenin gene targets such as PPAR-δ , c-Myc, Cyclin D1 and Matrilysin, which has a Kaiso consensus site adjacent to TCF binding site. From these targets, Cyclin D1 and c-myc have been shown to be upregulated after xKaiso depletion *in vitro* and further confirmed *in vivo* for cyclin D1 (Park et al., 2005). To circle the pathway, it is interesting to note that Xp120-catenin can relieve xKaiso repression of Siamois and xWnt-11.

1.12.2 Kaiso and the family of MeCP Zinc finger proteins

Recently two human proteins were identified containing Kaiso-like zinc fingers, ZBTB4 and ZBTB38 (Filion et al., 2006). These two proteins can bind sequences containing a single methylated CpG and have been shown to be methyl-dependent transcriptional repressors. The homolog of ZBTB38 in rat is called Zenon (Kiefer et al., 2005), and in mouse is called CIBZ (Sasai et al., 2005). Kaiso protein is common to all vertebrates and it might have duplicated in evolution from an ancestor that originated Kaiso and another protein that probably duplicated itself in ZBT38 and ZBTB4 because they are 75% similar within each other and around 40% with Kaiso. ZBT38 is expressed in brain and in neuroendocrine tissues, while ZBTB4 is expressed in most tissues, being the highest in brain, lung, kidney, muscle and heart, intermediate level in placenta, liver, spleen and thymus, and lowest expression in the testis (Filion et al., 2006).

1.13 Nuclear CoRepressor Complex

N-CoR is a class I HDAC-containing corepressor, primarily associated with HDAC3 (Guenther et al., ; Li et al., 2000; Wen et al.). N-CoR purified from HeLa nuclear extract contains 10-12 proteins (Yoon et al.). Among these are: TBL1 and TBLR1, two highly related WD-40 repeats histone-binding proteins; GPS2, an intracellular signalling protein and IR10 a coronin-like actin binding protein. TBL1/TBLR1 bind to histones H2B and H4 preferentially through their N-terminal region (Yoon et al., 2003a). Additionally, Kaiso has been identified as a component of the N-CoR complex in HeLa cells. N-CoR has been shown to repress transcription in a methylation-dependent manner *in vivo* (Yoon et al., 2003b).

Neuron-specific genes have a conserved 21-23 base pair DNA response element, known as RE-1 or NRSE (repressor element1/neuron restrictive silencer element) which is bound by the RE-1 silencing transcription factor/neuronal restricted silencing factor (REST-NRSF) (Chong et al., 1995; Schoenherr and Anderson, 1995). N-CoR associates with the REST/NRSF transcriptional repressor and brings together histone deacetylase activity (HDAC3) to the promoter of neuronal genes, which is essential for the repression of these genes in non-neuronal cells (Jepsen et al., 2000).

1.13.1 N-CoR function through N-CoR null mice

N-CoR-deficient embryos exhibit defects in the developmental progression of specific erythroctye, thymocyte and neural events (Jepsen et al., 2000). MAP2 expression, a marker for late neuronal differentiation, was reproducibly enhanced in the outer cortical layers in *N-CoR*$^{(-/-)}$ embryos (Jepsen et al., 2000). MAP2 can be induced by retinoic acid and harbours Retinoic Acid Response Elements (RAREs) in its promoter, and its upregulation in the cortex at the onset of neurogenesis identifies it as a putative target gene for N-CoR mediated repression through unliganded RAR (Neuman et al.). Decreased levels of Map2 have been suggested to inhibit neuronal differentiation and neurite formation (Dinsmore and Solomon, 1991). Concordantly, E12.5 N-CoR-deficient embryos also express decreased levels of Nestin (Jepsen et al.). Additionally, Gfap expression, indicative of astrocytic differentiation, is increased and precocious in N-CoR deficient embryos, starting the expression as early as E14.5 (Jepsen et al., 2000). These results indicate an early fate specification of nestin-expressing neural stem cells differentiated cells.

In addition to the *in vivo* studies, detailed *in vitro* experiments have been used to dissect the specific role of N-CoR in neural stem cell function. Neural stem cells are able to be maintained in an undifferentiated state and retain the capacity to self-renew in response to fibroblast growth factor-2 (FGF2) (Temple). Cultured *in vitro* cortical progenitors derived from E13 *N-CoR*$^{(-/-)}$ mice fail to proliferate and self-renew, and undergo spontaneous differentiation into astrocytes even in the presence of high concentrations of FGF2 (Hermanson et al.). Therefore, the phenotype of *N-CoR*$^{(-/-)}$ neural stem cells may be due to an intrinsic inability to maintain the FGF2-mediated undifferentiated and proliferative state.

Treatment with ciliary neurotrophic factor (CNTF), which promotes a rapid and efficient differentiation of neural stem cells into Gfap-expressing astrocytic cells (Johe et al., 1996), results in a translocation of N-CoR from the nucleus to the cytoplasm which may result in degradation (Hermanson et al., 2002). This mechanism occurs by enzymatic regulation: CNTF co-ordinately down-regulates protein phosphatase-1 (Aggen et al., 2000; Egloff et al., 1997) and activates Akt1-kinase (Bellacosa et al.), resulting in an increased phosphorylation and cytoplasmic localisation of N-CoR. Since Fgf2-treated neural stem cell cultures show endogenous

nuclear N-CoR localisation, and overexpression of N-CoR inhibits the astrocytic differentiation and Gfap expression that occurs in response to CNTF stimulation, it can be concluded that N-CoR is a principal component of cell type determination.

Platelet-derived growth factor (Pdgf) increases neuronal differentiation of neural stem cells after initial Fgf2-dependent expansion (Johe et al., 1996). Treatment with Pdgf in *N-CoR*$^{(-/-)}$ neural stem cell cultures did not increase the number of TuJ1-positive cells compared with wild-type cells. This indicates that in contrast to the spontaneous differentiation along the glial pathway, neuronal differentiation is not induced by the absence of N-CoR in cultured cells.

1.14 Self-reinforcing cycle of repressed epigenetic status

At the earliest developmental stages the embryo is formed by cells that have the potential to give rise to any cell of the adult organism. This pluripotency will gradually be restricted through multiple cell divisions, giving a range of differentiated cells with specialised protein expression patterns to function in the different tissues of the organism. Cell memory is the process by which the cell inherit its protein expression pattern and it is established early in development. There are two important epigenetic mechanisms that regulates cell memory: DNA methylation and histone modification. Considerable evidence indicates that these two mechanisms are interegulated in mammals.

Firstly, DNA methyltransferases and methyl-CpG binding proteins recruit histone deacetylase-containing corepressor complexes (Bird, 2002). Secondly, the MeCP Mbd1 binds to the histone H3-lysine9-specific methylase SETDB1 and together with the chromatin-assembly factor CAF-1 favours the stable transmission of silenced chromatin states at methylated DNA (Sarraf and Stancheva, 2004). Similar interaction with histone methylases may occur with the other MeCPs. In mammals, Dnmt transferases require interaction with the Suv39h H3K9 methyltransferase and heterochromatin protein 1 adaptor molecule (HP1). This has been concluded from the observation that Suv39h-knockout ES cells decrease Dnmt3b-dependent CpG methylation at major centromeric satellites (Fuks et al., 2003a; Lehnertz et al., 2003).

Another repression mechanism is methylation at histone H3 at lysine 27 (H3K27) by the Polycomb group protein EZH2. This enzyme form together with the EED and SUZ12 proteins the Polycomb repressive complexes 2 and 3 (PCRC2/3) (Kuzmichev et al., 2004). Whether EZH2 methylates H1 or H3 will depend upon which other proteins are in the complex (Kuzmichev et al., 2004). Recently, EZH2 has been shown to interact with the three DNA methyltransferases (DNMT1, DNMT3A and DNMT3B) *in vivo* and *in vitro* (Vire et al., 2006). Repression of EZH2 target genes require both EZH2 and DNMTs. Depletion of EZH2 disturbs recruitment of DNMTs to the regulatory regions of the EZH2 target genes which cause the expression of these genes.

1.15. Mouse Embryonic Stem Cells

The mouse 3.5 days post-coitum (d.p.c) embryo is called a blastocyst. It consists of around 60 cells organised in two different structures: the inner cell mass or ICM and the trophectoderm where the former is attached. The cells contained in the ICM are pluripotent and they will give rise to all the tissues of the organism with the exception of the trophectoderm.

Arresting the further development of the blastocyst and culturing the outgrowth of the ICM *in vitro* makes it possible to obtain a very special type of cells, embryonic stem cells, that are also pluripotent and undergo symmetrical self-renewal divisions so they can be maintained for indefinite time in *in vitro* cultures in defined conditions. (Evans and Kaufman, 1981; Martin, 1981). The confirmation of pluripotency is obtained by their capacity to be reintroduced in the mouse embryo and contribute to a variety of tissues of the resulting chimera and even to pass through the germline (Bradley et al., 1984).

There is an orchestrated signalling network involved in the maintenance of pluripotency of the embryonic stem cells that has been extensively studied and fairly well established. An embryonic stem cell maintains its pluripotency during its replication by its capacity to self renew by a symmetric cell division into two identical daughter embryonic stem cells. There are two cytokines indispensable for the self-renewal of ES cells, Leukaemia Inhibitory Factor, LIF (Smith et al., 1988; Williams et al., 1988) and Bone Morphogenetic Protein, Bmps (Ying et al., 2003a).

Each one stimulates a signalling pathway through the cell involving many downstream elements. Lif is a member of the IL6 family of cytokines and targets a heterodimeric cell surface receptor complex comprising the Lif receptor subunit (Gearing et al., 1991), and glycoprotein 130 (Davis et al., 1993). The downstream signalling is followed by JAK-kinase mediated recruitment, activation and nuclear translocation of the signal transducer and activator of transcription factor Stat3. There are other related cytokines, like CNTF, that interact with the Lif receptor LIFR/gp130 complex and can substitute LIF for ES cell self-renewal (Pennica et al., 1995). Gp130 cytokines can also activate the mitogen activated protein kinases (MAPK) Erk1 and Erk2 (Fukada et al., 1996) of the ERK pathway, which appears to be a pro-differentiative signal (Burdon et al., 1999b). Thus it has been found useful to reduce ERK signalling in order to facilitate ES cell derivation (Buehr and Smith, 2003) and to promote self-renewal (Burdon et al., 1999a). In the absence of LIF, ES cells lose their self-renewal capacity and differentiate into flattened epithelial-like cells. In the absence of BMP4, ES cells differentiate into the neural lineage (Ying et al., 2003a). Bmp family members known to contribute to pluripotency and self-renewal are Bmp2, Bmp4 and Gdf6 and they act by inducing expression of members of the Id family of negative transcriptional modulators (Ying et al., 2003a).

Additionally, there are two key transcription factors involved in self-renewal, Oct4 and Nanog, which together with Sox2 complete a transcription cascade, to active genes involved in self-renewal and pluripotency and to inactivate genes involved in differentiation (Boyer et al., 2005; Loh et al., 2006). Depletion of Oct4 in ES cells causes their differentiation into trophectodermal cells, meanwhile overexpression of Oct4 induces differentiation into primitive endoderm and mesoderm lineages (Niwa et al., 2000). Nanog confers cytokine independent self-renewal, acting synergistically with Stat3 signalling since overexpressing Nanog cells form pure stem cell colonies at clonal density but with significant increased efficiency when LIF is added (Chambers et al., 2003).

1.16. Neural Induction

ES cells have the capacity to differentiate into any cell of any tissue of the organism. Different cell types can be obtained under specific *in vitro* conditions,

despite the lack of germ line signals and normal developmental environment, or niche signals. There is a very important potential for ES cell differentiation as a tool for tissue regeneration since the amplification of ES cells is almost unlimited. Additionally, ES cells provide a practical system to study which signals govern cell specification. Neuronal differentiation from ES cells is a well-established differentiation system since there is a great interest in understanding the origins of neurodegenerative diseases in order to design therapeutic approaches.

A high percentage of ES cells growing in the presence of LIF, but in the absence of BMP ligands, will become specified towards neural lineages and consequently a high percentage will adopt a neuronal fate (Ying et al., 2003a). An established, though debated hypothesis is that of neural induction by default. This model considers that in the absence of repressive signals from BMP a mouse ES cell will adopt a neural fate (Tropepe et al., 1999). This theory origines from work in *Xenopus laevis* with the identification by Speman and Mangold of a region that could induce neural specification, which was called the organizer (known as Spemann-Mangold organizer (Spemann and Mangold, 2001) or, in the chick, as Hensen's node) (Hensen, 1876; Waddington, 1936). The signals released by the organizer that were needed for the neural induction were identified subsequently to be antagonists of BMP receptors, namely Noggin, Chordin, Cerberus, Xn3 and follistatin (Hemmati-Brivanlou et al., 1994; Hemmati-Brivanlou and Melton, 1994). However, later observations suggested that the default mechanism may not explain the complete picture of neural specification. For example, mice deficient for Noggin and Chordin can form neural tissue, although they have severe forebrain defects (Anderson et al., 2002). Additionally, ES cells require FGF/MAPK signalling in order to generate neural derivatives *in vitro* (Ying et al., 2003b)(and it will be explained in the next section). It is most likely that neural induction depends upon a variety of signals, including Fgf signalling and Wnt signalling which are both involved in repression of Bmps (Linker and Stern, 2004) but may also act independently of BMP repression.

1.17. ES neural specification *in vitro*

The so-called monolayer differentiation system permits us to dissect signals and conditions that may be important for ES cells to differentiate into neurons (Ying et al., 2003b). This adherent culture system is relatively homogenous and the components of media culture are defined. From this system we know, firstly the time course with which neural precursors start to appear in the cultures; and secondly, that cell density is critical for differentiation, since high density leads to no neuronal differentiation, and low density leads to poor cell survival. Hence, cell-cell communication is necessary for cell specification. In concordance, a culture system with a high percentage of dopaminergic neuronal specification from ES cells described the importance of cell contact of the stromal feeder cell layer from mouse skull bone marrow and the ES cells, stromal-cell-derived inducting activity or SDIA (Kawasaki et al., 2000).

These ideas correlate with what have been previously described from other less homogenous culture systems, such as neurospheres generated from low density cultures of ES cells in the presence of LIF, possibly as a cell survival cytokine (Tropepe et al., 1999). After 7 days, FGF signalling start to play an important role in neural specification, since FGF receptor 1 FGFR1 deficient ES have a decrease in the number of neural colonies (Ciruna et al., 1997; Tropepe et al., 2001).

1.18. Development of the mouse nervous system

The cells within the inner cell mass will generate two layers: the hypoblast or primitive endoderm and the epiblast. The primitive endoderm will contribute to extraembryonic tissues, and the epiblast will give rise to the three main germ layers in the embryo. The innermost layer is the endoderm, which gives rise to the gut, lungs, liver and pancreas. The middle layer or mesoderm, gives rise to connective tissues, muscle and vascular system, and the ectoderm, which is the outermost layer, gives rise to the central and peripheral nervous system and epidermis. All layers have a common origin from the primitive streak formed at 6.5 dpc. At the gastrula stage, around 7, or 7.5 dpc the ectoderm forms a sheet called neural plate that, receiving the joint influence of signals from the node and the axial mesoderm, acquires neural properties. At 9.5 dpc, the neural plate will fold into the neural tube. The caudal

region of the tube will give rise to the spinal cord, and the rostral region will form the prosencephalon. Because of a difference in the proliferation rate among the cells forming the neural tube, it will develop in different 'bulbs'. Thus, the prosencephalon will form three brain vesicles: the forebrain, the midbrain and the hindbrain. The forebrain will give rise to the telencephalon and diencephalons, and the hindbrain vesicle will give rise to the metencephalon and myelencephalon. The dorsal part of the telencephalon or Pallium, is the origin of the cerebral cortex, that itself is divided in three structures: neocortex, paleocortex or piriform cortex and hippocampus or archicortex.

Cerebral cortex development follows an inside-out model, so the newly generated neurons migrate towards the more apical brain areas through the older neuronal layers already formed (Angevine and Sidman, 1961). The cerebral cortex will be populated with a majority of excitatory glutamatergic projecting neurons that form layers by radial migration from the germinal zone of the dorsal telencephalon. The ventral part of the telencephalon will generate the basal ganglia or subpallium, primarily arising from the medial and lateral ganglionic eminences, or primordial globus pallius and primordial striatum, respectively, at the wall of the ventricle. Gabaergic interneurons are formed in the basal ganglia, and basal ganglia neurons migrate tangentially to the cerebral cortex, where they become cortical interneurons (Anderson et al., 1997; Nery et al., 2002; Wichterle et al., 2001). The spatial and temporal origin of the eminence progenitors is correlated with the type of mature cortical interneuron that will originate from them (Butt et al., 2005). A third eminence, the caudal ganglionic eminence or CGE contributes to layer-5 cortical neurons, the striatum and the limbic system. Interestingly, the migratory routes taken appear to be cell intrinsic to the CGE-derived neurons (Nery et al., 2002) (figure 1.5).

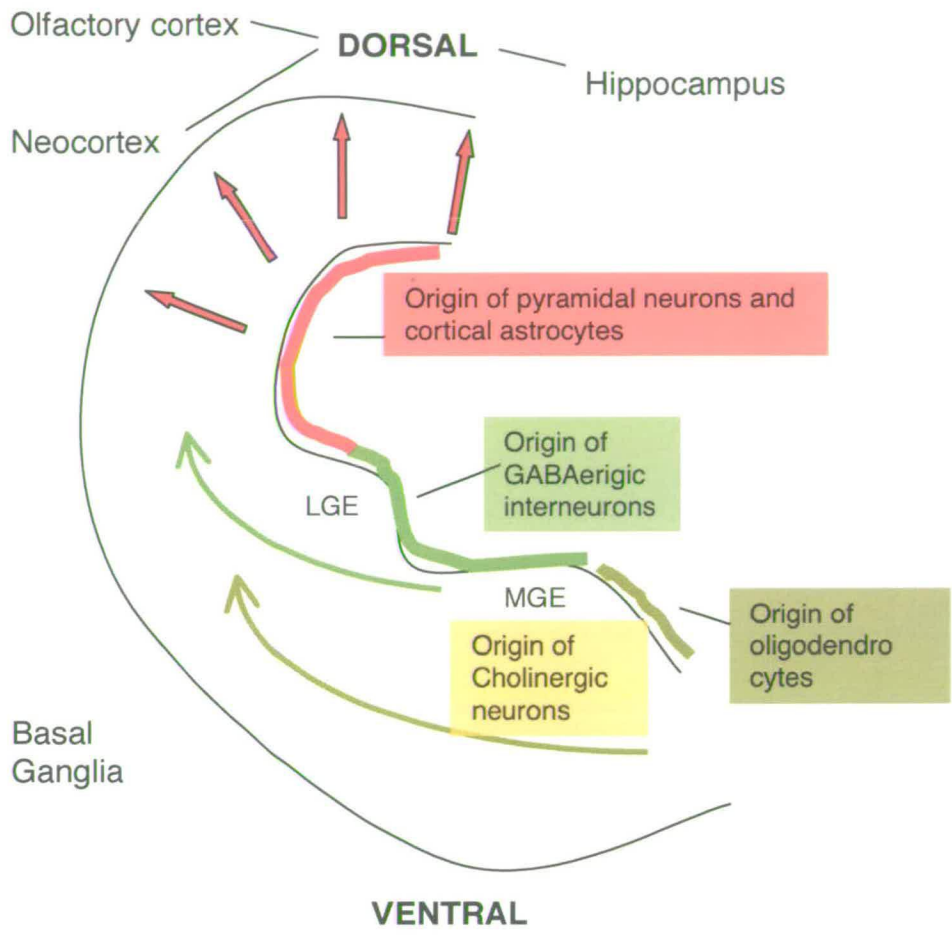


Figure 1.5. Developmental telencephalon representation.

Pyramidal neurons arise from the dorsal proliferative zone or ventricular zone (red), and migrate outward in an inside-out manner giving rise to the six layers of the neocortex. GABAergic interneurons arise in the proliferative zone of the ganglionic eminences (green) and will migrate tangentially towards the neocortex. Oligodendrocytes are born from the most ventral region of the telencephalon (dark green) and will also migrate dorsally. Cholinergic neurons, and striatal astrocytes are born in the ventral telencephalon and will remain in the basal ganglia. LGE: lateral ganglionic eminence, MGE: medial ganglionic eminence. *Adapted from Ross et al., 2003 and Guillemot, 2005.*

1.19 Progenitor cells in the Telencephalon

1.19.1 History of developmental neurobiology

Golgi was the first to describe epithelial cells in the developing neural tube that extended radial fibres from the ventricular surface to the pial surface (Golgi et al., 2001). Golgi preparations were invaluable to neurobiologists to study the morphology of cells in the developing cortex and study their temporal sequence. He defined them as spongioblasts, but designated the neural source to the rounded germinal cells visible at the ventricular surface. In contrast, Magini proposed that the spongioblasts where immature nerve cell, and called them radial neuroglial cells after the observed varicositites along their filaments (Magini, 1888) Morest presented evidence to show that neurons grow radial processes and subsequently translocate to the cortex (Morest, 1970). In the 70's Rakik defined the epithelial or spongioblasts as radial glial cells, being glial supportive cells with the role to guide neuronal migration (Rakic, 1971; Rakic, 1972). Thus radial glial cells were considered as a type of glial support cell. Surprisingly, in 2000 was discovered that radial glial cells not only could generate cortical neurons, but that they are the major source of cortical pyramidal neurons, which was originally proposed by Magini in 1888 (Malatesta et al., 2000; Miyata et al., 2001; Noctor et al., 2001).

1.19.2 Neuroepithelial cells

Neuroepithelial cells are the first progenitor cells to appear during the formation of the neural plate and neural tube. They have epithelial characteristics and are polarized, with an apical distribution towards the inner or ventricular lumen, and basal distribution forming the basal lamina at the outer or pial surface. The apical membrane presents tight junctions and adherens junctions which are important for the apical-basal polarity of the cell, and the basal membrane presents receptors for basal lamina (Huttner and Brand, 1997). The neuroepithelium appears layered because of the interkinetic nuclear migration of the neuroepithelial cells, or the nuclear migration during mitosis from the apical surface at G1 and G2 and to basal lamina at S phase and M phase (Sauer, 1935).

During forebrain development, neuroepithelial cells give rise to the earliest born neurons of the cortex by asymmetric cell division at E10, and by the onset of neurogenesis, neuroepithelial cells lose some epithelial features such as the tight junctions, and the apical-versus-basal polarity of delivery of certain plasma-membrane proteins (Langman et al., 1966; Martin, 1967; Reid et al., 1995). Neuroepithelial cells express Rc1 and Rc2 markers (Misson et al., 1988) together with intermediate filament protein Nestin (Lendahl et al., 1990) and can be detected at E9/E10.

1.19.3 Radial glia

With the onset of neurogenesis between E12 and E14, neuroepithelial cells become a more fate-restricted population called radial glia. This population constitutes the most common neuronal precursor in the central nervous system (CNS). Characteristic radial glia markers are glycogen granules (Gadisseux and Evrard, 1985), astrocyte-specific glutamate transporter Glast, TN-C, vimentin, and subsets have Blbp, s100β, and glutamine synthase GS and Rc2 in some species (Malatesta et al., 2003; Malatesta et al., 2000; Mori et al., 2005). Human, primates and adult mouse radial glia also express GFAP. Radial glial cells have neuroepithelial characteristics, such as the expression of intermediate filament protein Nestin (Hartfuss et al., 2001), apical-basal polarity, adherent junctions and interkinetic nuclear migration (Chenn and McConnell, 1995; Gotz and Huttner, 2005). This last feature differs with neuroepithelial cells being more restricted nucleus migration through the cytoplasm.

Radial glia cells act both as neuronal precursors boundary and patterning and guide structures for migrating neurons. They primarily divide asymmetrically, generating a radial glial cell and a postmitotic neuron, or a radial glial cell and a basal progenitor (Noctor et al., 2001). They generate neurons in many distinct areas of the CNS. In most brain regions, radial glia cells constitute the majority of progenitors at E13/E14, and they persist until the end of neurogenesis, when the remainder transforms into astrocytes (Noctor et al., 2004). Radial glia generate neurons in many distinct areas of the CNS, but the numbers are highest in dorsal

telencephalon, while the majority of ventral telencephalon neurons are most likely derived from basal progenitors.

Pax6 is a neurogenic fate determinant factor for radial glial cells. Pax6 also inhibits the generation of basal progenitors, since *Pax6* mutant mice have an increased population of basal progenitors. In this mutant neurogenesis still happens by differentiation from basal progenitors and migration from ventral sources (Hartfuss et al., 2001; Heins et al., 2002). Pax6 can induce neurogenesis in non neurogenic postnatal astrocytes (Heins et al., 2002) and is sufficient to stimulate adult neurogenesis (Hack et al., 2005). Additionally, Pax6 is expressed specifically by dopaminergic interneurons in the glomerunal layer during adult neurogenesis (Hack et al., 2005).

1.19.4 Basal progenitors

Basal progenitors originate from the asymmetric division of neuroepithelial and radial glial cells at the apical surface of the neuroepithelium and ventricular zone respectively. They are characterised by the absence of ventricular or apical contact, they have a long G2 phase and undergo mitosis at the basal side of the ventricular zone (Haubensak et al., 2004; Miyata et al., 2004; Noctor et al., 2004). Their molecular characterisation includes Tbr2 (Englund et al., 2005), Ngn2 (Miyata et al., 2004) and the absence of Glast or Pax6 (Haubst et al., 2004; Malatesta et al., 2003). Additionally basal progenitors express neuronal markers (Englund et al., 2005).

At E13 in the ventral telencephalon and E15 in the dorsal telencephalon, basal progenitors become abundant enough to form a secondary progenitor layer called the subventricular zone (SVZ), located on top of the precursors lining the ventricle in the ventricular zone. The basal progenitors usually divide symmetrically and generally produce two neurons (Haubensak et al., 2004; Miyata et al., 2001; Noctor et al., 2002) (Haubensak et al., 2004). However, after E15, they will acquire EGF receptor expression, that will be sometimes distributed asymmetrically in correlation with the generation of astrocytes or oligodendroctyes (Sun et al., 2005). Therefore, early SVZ precursors are mostly neurogenic, and late SVZ precursors produce mainly glia.

1.20 Neural stem cell/ Neural progenitors signalling

During the central nervous system development, neural progenitor cells are immersed in a variety of signals that will direct the cell fate specification of these cells. In this section, some of the key factors involved in neural specification are described (figure 1.6)

1.20.1 Retinoic Acid

Retinoic acid (RA) has long been identified as a efficient neural differentiation signal for ES cells *in vitro* (Bain et al., 1995; Doetschman et al., 1985; Fraichard et al., 1995; Okabe et al., 1996; Strubing et al., 1995). RA treated embryoid bodies dissociated and replated originate high percentage of differentiated neurons (Li et al., 1998).

In addition to being able to induce neural and neuronal differentiation, RA has an effect on the regional identity and subtype of the neurons that are generated. Very similar protocols can generate either glutaminergic neurons when RA is added (Bibel et al., 2004) or GABAergic neurons when RA is not added (Conti et al., 2005). There is also some evidence that RA restricts progenitors to CNS rather than PNS identity (Plachta et al., 2004) and can posteriorise CNS identity (Blumberg et al., 1997). There is however, opposite evidence from studies where RA was used to promote neural crest derivatives, motoneurons and GABAergic interneurons (Barberi et al., 2003; Mizuseki et al., 2003; Renoncourt et al., 1998; Wichterle et al., 2002).

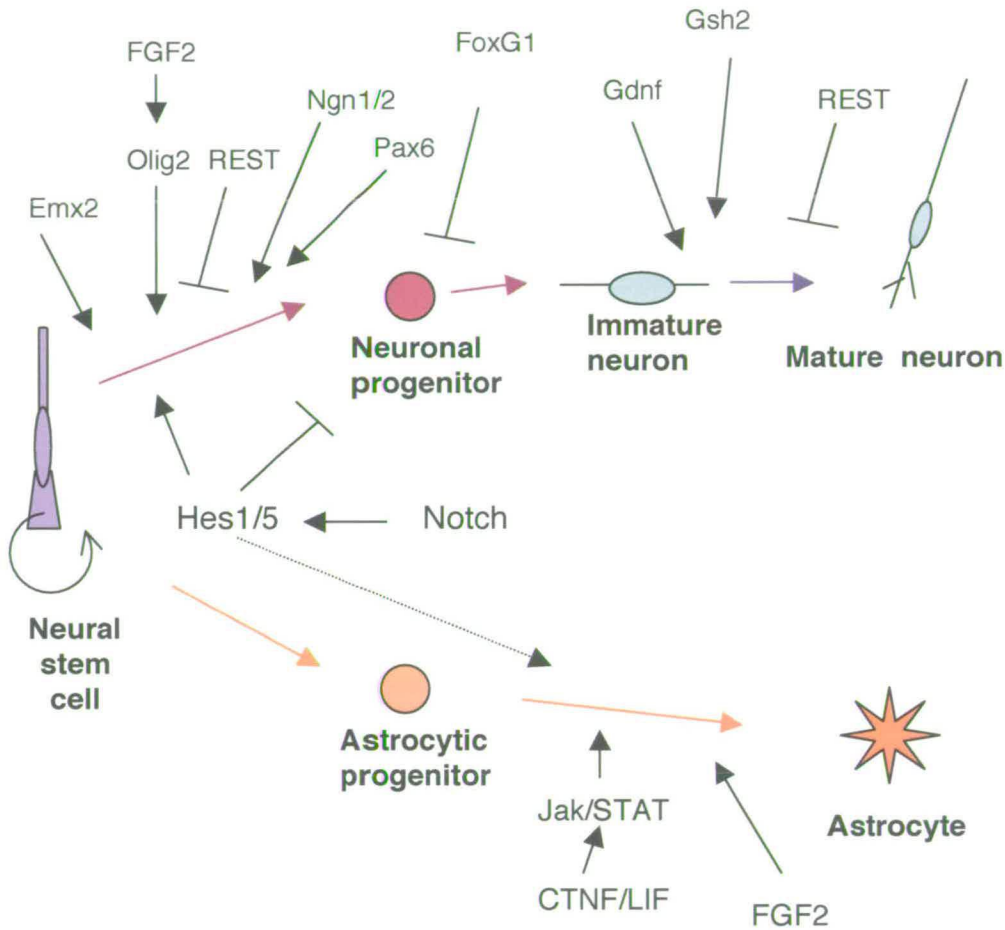


Figure 1.6 Diagram of some signaling pathways involved in cell fate specification of neural stem cells in the telencephalon. *Adapted from Gullemot, 2005.*

1.20.2 bHLH factors

Neurogenin 1, Neurogenin 2, Mash1 and NeuroD are basic helix-loop-helix (bHLH) genes which promote neurogenesis and are expressed in the developing cortex. These factors form heterodimers with the ubiquitously expressed bHLH protein E2A and activate neuronal gene expression by binding to the E box (CANNTG) (Guillemot and Joyner, 1993). Expression of neurogenic bHLH transcription factors are downregulated by another pair of bHLH transcription factors, Hes1 and Hes5. These factors can repress neurogenic effects not only by a direct binding to a N box (CACNAG), but as well through competitive binding to the heterodimeric binding of the E box (Akazawa et al., 1992; Sasai et al., 1992; Takebayashi et al., 1995). The proneural proteins bHLH Neurogenin 1 and Neurogenin 2 are responsible for both
neuronal cell migration and cell fate specification by separate mechanisms. These proteins are primarily expressed in the cortical ventricular zone, and Ngn2 is also expressed in the intermediate zone. Ngn1 and Ngn2 are implicated together with Pax6 in the acquisition of cortical neuron specification as well as to repress genetic programs that would regulate non cortical neuronal differentiation such as those operating in the ventral telencephalon. In the absence of these factors, cortical progenitors are misspecified, expressing genes typical of ventral telencephalic progenitors. The result seems to be a respecification toward ventral neuronal fates. Indeed, the cells generated appear molecularly more akin to the GABAergic interneurons produced in the ganglionic eminences (Fode et al., 2000; Schummers et al., 2004; Stoykova et al., 1997; Sun et al., 2001; Toresson et al., 2000).

1.20.3 Notch

The Notch signalling pathway is involved in cell-cell signalling. In vertebrates there are four Notch receptors, and Notch1-3 are expressed in developing brain and postnatal CNS germinal zones (Irvin et al., 2001). When the ligands, which are grouped into Delta and Jagged families, bind to their receptors, the intracellular domain of Notch (NICD) is released from the plasma membrane and translocates into the nucleus (Schroeter et al., 1998), where it converts the Cbf1 (or RBPJ-κ)

repressor complex into an activator complex (Weinmaster, 1997). The NICD/CDBF1 activator complex upregulates Hes, Hey and Herp genes, which are basic helix-loop-helix transcriptional regulators that antagonize proneural genes such as Mash1 and the neurogenins (Iso et al., 2001a; Iso et al., 2001b; Jarriault et al., 1995; Leimeister et al., 1999). This antagonism blocks early neuronal gene expression and is central to the inhibition of neuronal differentiation (Ohtsuka et al., 1999).

In mammals Notch activation is involved in maintaining the undifferentiated state by blocking neuronal gene expression, in a process called lateral inhibition (Lardelli et al., 1996). The disruption of Notch leads to precocious neuronal differentiation (de la Pompa et al., 1997) of mouse neural precursors. However, Notch can also function to promote neighbouring cells to adopt the same cell fate, in a process called lateral induction.

In the striatum, Notch governs the asymmetric division that leads to neuronal differentiation at different stages of neurogenesis (Mason et al., 2005). In the mouse forebrain, Notch signalling directly activates the radial glia marker brain lipid binding protein (Blbp) (Anthony et al., 2005). Moreover, Notch1 and Notch3 promotes radial glia identity during embryogenesis and to dispersed and periventricular astrocytes postnatally (Dang et al., 2006; Gaiano et al., 2000). It has been shown that activated Notch1 promotes a proliferative response to bFGF *in vitro* (Yoon et al., 2004).

1.20.4 bFGF

The FGF family of proteins consists of 22 members that can be grouped into seven subfamilies based on sequence similarities and functional properties (Popovici et al., 2005). FGFs bind four high-affinity lingand-dependent FGF receptor tyrosine kinase molecules (FGFR1-4) forming stable dimers in the presence of heparan sulfate glycosaminoglycans and in the presence of heparan sulfate HS glycosaminoglycans (Zhang et al., 2006). Basic fibroblast growth factor (bFGF, also denominated FGF2) is a single-chain polypeptide composed of 146 amino acids, which was first purified from the bovine pituitary by high affinity binding to heparin, and was named after its biological activity of promoting the growth of fibroblasts (Gospodarowicz et al., 1984; Gospodarowicz et al., 1986).

bFGF binds to the four cell surface receptors, binding with the highest affinity to FGFR1 (Stachowiak et al., 1997). bFGF signals through its receptor tyrosine kinase activity linked to the G-protein Ras, which activates ERK/MEK phosphorylation signaling. Additionally, heparin and cell surface heparan sulfate proteoglycan modulate FGF activity. Independently of the bFGF paracrine effect, another three bFGF isoforms with a higher molecular weight localize to the nucleus and exert activities through an intracrine pathway (Arese et al., 1999).

bFGF has been reported to have both mitogenic and neurotrophic actions. bFGF is a survival factor for cultured CNS neurons (Vescovi et al., 1993). It also has an effect in the promotion of axonal branch growth (Aoyagi et al., 1994). Additionally bFGF has been reported to regulate proliferation of purified populations of astrocytes (Eccleston and Silberberg, 1985; Pettmann et al., 1985; Sensenbrenner et al., 1987) and oligodendrocytes (Eccleston and Silberberg, 1985).

bFGF induces ventralisation of cortical neural precursors independently of their age. Cortical progenitor cultures in the presence of bFGF increase their expression of ventral markers like Mash1, and Olig2 (which is a marker of oligodendroctye precursors) and downregulate Emx1, Pax6, (Hack et al., 2004), Neurogenin1 and Neurogenin2 (Abematsu et al., 2006). Although GABA neurons are differentiated from ventral neural precursors, the ventralising effect of bFGF is not sufficient to induce GABAergic-specific differentiation of cortical progenitors (Abematsu et al., 2006). That effect is in concordance with the lack of any increase of Dlx2 in cortical progenitors, a transcription factor necessary for GABAergic specification. In the contrary, in neurosphere cultures the addition of bFGF appears to induce differentiation of a GABAergic neuronal phenotype (Hack et al., 2004). Further, neurosphere cultures appear to present an increase in GABAergic neurons independently of the brain regional origin (Ciccolini et al., 2003; Hitoshi et al., 2002; Parmar et al., 2002). Interestingly, neurosphere cultures appear to be close to ES cells in terms of transcriptional factors expression (Ramalho-Santos et al., 2002), which may explain the reason for the high percentage of GABAergic neuronal differentiation from ES cell derivation *in vitro* (Ying et al., 2003b).

1.20.5 EGF

Egf signals via the 170-kD tyrosine kinase Egf receptor (EgfR) (Pimentel 1994). EgfR can influence cortical progenitor fate choice, as its overexpression at midgestation causes astrocyte differentiation at the expense of neuronal lineages (Burrows et al., 1997). EgfR expression increases during cortical development at E13, which is the peak of neurogenesis, and the time when Egf-responsive stem cells arise (Kornblum et al., 1997; Tropepe et al., 1999). During cortical development, EgfR is asymmetrically distributed between two daughter cells, correlates distinct cell fates. The cell with higher EgfR expression will also express markers of radial glia such as Rc2, Glast, CD-15/Lewis X. In contrast, the cell expressing the lowest level of EgfR will preferentially become an oligodendrocyte precursor (Sun et al., 2005).

Egf responsive stem cells from the striatal subventricular zone are capable of differentiating into GABAergic neurons (Kornblum et al., 1995).

1.20.6 Neuregulin-ErbB signalling

Migrating neurons expressing the ErbB ligand Neuregulin signal through ErbB receptors to maintain radial glia (RG) characteristics. In the absence of NRG there is a reduction in Rc2 expression in cortical explants, and this can be rescued by addition of exogenous NRG. Furthermore a dominant form of erB2 can turn RG cells into astrocytes (Schmid et al., 2003).

1.20.7 SOX Proteins

The Sox proteins comprise a group of transcription factors with an SRY box, which is a 79 amino acid motif that encodes an high mobility group (HMG) DNA binding domain. There are seven groups within the Sox family, from which three groups have representation in the nervous system with a total of 12 proteins (Kamachi et al., 2000). These include the SOXB1 and SOXB2 subgroups in the CNS and SOXE subgroup in the PNS. The SOXB1 subgroup comprises SOX1, 2 and 3 which share more than 90% amino acid identity in the HMG-DNA binding domains (Bowles et al., 2000; Pevny and Lovell-Badge, 1997).

In mice, the initial phase of Sox2 and Sox3 expression is pan-ectodermal; Sox2 being expressed from pluripotent ES cells (Yuan et al., 1995) and Sox3 from very early stages of differentiation towards the neurectodermal fate (Collignon et al., 1996; Wood and Episkopou, 1999). Sox1 expression appears as the earliest transcription factor to be expressed in ectodermal cells committed to the neural fate (Pevny et al., 1998). Concomitanly, expression of Sox2 and Sox3 becomes confined to cells that are committed to a neural fate. Forced expression of *Sox1* or *Sox2* promotes the differentiation of mouse embryonic stem cells into neuroectoderm at the expense of mesoderm and endoderm (Zhao et al., 2004). SoxB1 proteins interact with POU domain factors to activate a *Nestin* neural enhancer directly (Tanaka et al., 2004).

Sox2 plays an essential role in early embryo precursor cells, as *Sox2*-null embryos can not give rise to embryonic or trophectoderm lineages. Additionally, Sox2 is also expressed in the adult subventricular zone of the lateral ventricles and in the subgranular layer of the hippocampal dentate gyrus, where stem cells are found in the adult brain (Ferri et al., 2004; Wegner and Stolt, 2005). Sox2 is also expressed in adult-brain derived neural stem cells grown in vitro (Conti et al., 2005; Ferri et al., 2004). High expression of Sox2 was found in subtypes of postmitotic neurons including pyramidal cells of the cerebral cortex, thalamic neurons, medial dorsal striatum and septum of the adult brain. It is possible that Sox2 has a role in neuronal function and maintenance, since mice *Sox2* deficient display neurological abnormalities that correlates with the neuronal disruption of the neuronal type where Sox2 is expressed (Fantes et al., 2003). Furthermore, *SOX2* mutation is linked to the neurological symptoms of anophtalmia in humans (Ragge et al., 2005). *Sox1*-null adult mice exhibit spontaneous epileptic seizures associated with the loss of Sox1 expressing neurons in the ventral striatum, and the phenotype of *Sox3*-null mice suggests a role for Sox3 in a subset of hypothalamic neurons that regulate the hormonal output of the anterior pituitary. Overall these phenotypes are relatively mild, and given the strong overlapping expression between them and their biochemical and functional similarities, there must be most likely functional redundancy between these proteins.

1.21 *In vitro* neural stem cells

Neural stem cells (NSCs) are undifferentiated cells with the ability to: (1) proliferate, (2) exhibit self-renewal, (3) generate a large number of differentiated progeny, (4) retain their multilineage potential over time, and (5) generate new cells in response to injury or disease (Reynolds and Weiss, 1996). NSCs appears in the CNS as early as E8.5 and are characterised by their proliferation, *in vitro*, in response to Fgf 2 (Tropepe et al., 1999). At E11-12 a second population of NSCs appears that divide in response to Egf or transforming growth factor (Tgf) (Tropepe et al., 1999).

1.21.1 Asymmetric versus symmetric cell division

Asymmetric cell division occurs when a precursor cell undergoes mitosis and divides into two different cell types. An asymmetric cell division usually produces another precursor cell, maintaining self-renewal, and a more restricted cell type.

Symmetric cell division is characterised by the origination of two cells of the same type after mitosis. These two daughter cells can be the same cell type as the

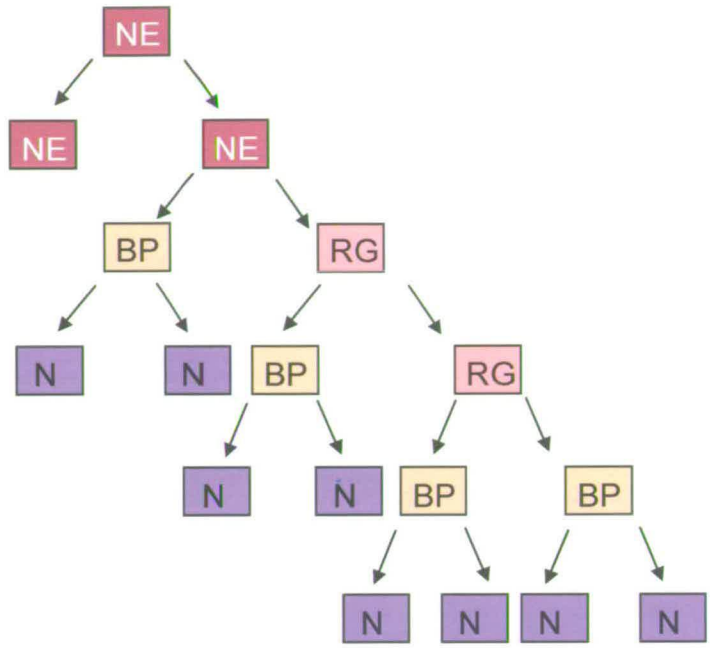


Figure 1.7. Lineage trees of neurogenesis. Asymmetric versus symmetric divisions in cell fate determination. NE: neuroepithelial cell. N: neuron. RG: radial glia cell. BP: basal progenitor. *Adapted from Gotz and Huttner, 2005.*

mother self-renewal, or two differentiated daughter cells. There are certain genes responsible for the intrinsic cell determination such as Notch (Conlon et al., 1995) and Numb (Zhong et al., 2000). These genes are dispensable before the onset of neurogenesis, meanwhile extrinsic signals such as Wnts, BMPs and Sonic Hedgehog Shh are all essential for neural patterning during this period.

1.21.2 NS cells

ES cells can differentiate *in vitro* into a pure population of neural stem (NS) cells niche independent cultured in monolayer. These NS cells can undergo symmetric cell divisions giving two same cell NS daughters at a rate of double every 24 h (Conti et al., 2005). They need Egf and bFgf signalling to maintain their characteristics. In the absence of Egf cells undergo apoptosis, and in the absence of bFGF differentiation occurs. The NS phenotype corresponds with radial glia cells because they are positive for Glast, Blbp, Nestin, Vimentin, and Pax6. However, in contrast with radial glia cells, NS cells also express markers of the entire telencephalon. Therefore, these cells may represent an isolated temporal neural stem cell that occurs *in vivo* at a specific time during radial glia formation, and being isolated *in vitro* and by continuous stimulation with Egf and bFgF they aquire the capacity for indefinite self-renewal capacity through symmetric cell division. The NS-derived neurons appear to be preferentially GABAergic as they show GAD67 and GABA expression.

Neural precursors obtained through different protocols, including embryoid body formation and addition of Retinoic Acid are also phenotypically defined as radial glia expressing RC2, Glast, Blbp, and Pax6. In contrast with NS-derived neurons, and as expected from this gene expression pattern, these cells will differentiate into predominantly of glutamatergic cells that express the vesicular glutamate transporter vGlut1, a membrane protein specific to pyramidal cortical neurons (Bibel et al., 2004).

The cause of the lack of consistency between the two protocols may reside in the two different signalling pathways to which both neural precursors are exposed: Retinoic Acid in one, and Egf and bFgF in the other. Additionally, NS cells can be

clonally isolated, proliferate and self-renew indefinitely, meanwhile RA-derived radial glia cells can not. Furthermore, NS cells can be isolated from different forebrain regions maintaining the same neural stem cell characteristics and apparently the same differentiation capabilities. Egf responsive stem cells from the striatal subventricular zone are capable of differentiating into GABAergic neurons (Kornblum et al., 1995). The presence of Fgf2 activates the expression of transcription factors ganglionic eminence. Overall, the preferential GABAergic specification of NS differentiation may be related with the continuous Egf and bFgf signalling.

1.22 Neuronal specification

Many genes are known to be involved in neuronal fate specification. The gene expression profile of a restricted neuronal precursor will determine their final neuronal phenotype. Restricted post-mitotic neuronal precursors that have been specified to a particular neuronal fate will migrate to their final destination and acquire dendritic morphology, generating an axon that will connect to the correct target. The proneural genes *Neurogenin1* and *Neurogenin2* promote dorsal telencephalic fate by repressing the ventral proneural gene *Mash1* (Fode et al., 2000) and by specifying cortical projection neuron characteristics like glutamatergic neurotransmission and dendritic morphology (Hand et al., 2005; Schuurmans et al., 2004). Striatal neuronal specification is led by Gsh2 expression with the involvement of retinoic acid (Waclaw et al., 2004). Cortical interneurons are directed towards tangential migration by Dlx1 and Dlx2 (Cobos et al., 2005), and they require of Gdnf (glial derived neurotrophic factor) to delimit their morphology and axonal growth. Apart from its role in dorsal radial glia specification, Pax6, together with the nuclear receptor Tlx are involved in cortical neuronal specification, particularly of superficial neurons, which are the last neurons generated during development (Hack et al., 2005; Roy et al., 2004).

1.23 Cell intrinsic mechanisms of cell fate determination

Forebrain neural progenitors can differentiate into neurons early in development, starting from E11.5 with a peak of neurogenesis around E14.5.

However, gliogenesis will not occur until after completion of neurogenesis, peaking at E16.5 (Levers et al., 2001; Qian et al., 2000). This time-directed cell fate specification can be also observed within isolated neural progenitors *in vitro* (Qian et al., 1998; Qian et al., 2000). Interestingly, neuroepithelial cells aquire gliogenic potential simultaneously with loss of their neurogenic potential. Time specification of gliogenesis is crucial in forebrain development to ensure a coordinated nervous tissue organization; glial cells are important in critical neuronal maturation processes such as axonal pathfinding, synapse formation, and myelination (Shu and Richards, 2001; Ullian et al., 2001). Since oligodendrocytes are generated from the ventral telencephalon, gliogenesis in the developing cortex is mostly directed towards astrocytic differentiation. There are a variety of mechanisms involved in astrocytogenesis, both intrinsic or extrinsic to the neuroepithelial cell. The main signalling astrocytic pathway is the JAK-STAT pathway, which is itself dynamically regulated through development (He et al., 2005). Several factors activate this pathway, including Cnft, Lif and IL-6 (Bonni et al., 1997). Bmp2, which belongs to the transforming growth factor β (TGF-β) super-family, and Lif share gp130 as a signal transducing receptor component (Taga and Kishimoto, 1997) and both signal through Stat3 activation (Nakashima et al., 1999a). Activated Stat3, in its phosphorylated state, associates with the transcriptional coactivator Creb binding protein (CBP/p300) to activate expression of astrocyte-specific genes (Nakashima et al., 1999b). On the other hand, neurogenic bHLH family members are shown to suppress gliogenesis by sequestering Smad1-CBP/p300 complex away from astrocyte-specific genes and inhibiting activation of Stat3 (Nieto et al., 2001; Sun et al., 2001).

Stat3 acts downstream of these cascade through the glial fibrillary acidic protein (*Gfap)* promoter. Gfap is the major intermediate filament protein in mature astrocytes (Eng et al., 2000) and is highly conserved throughout vertebrates (Messing and Brenner, 2003). The access of Stat3 to the *Gfap* promoter is strongly regulated as well by a number of mechanisms including histone methylation, DNA methylation, and transcriptional repressor complexes.

bFGF regulates Stat3 access to the *Gfap* promoter by inducing an increase in lysine-4 methylation, which facilitates *Gfap* transcription, and a decrease in lysine 9

methylation at the Stat-binding site of the *Gfap* promoter with the opposite effect (Song and Ghosh, 2004). This chromatin-regulated mechanism is similarly seen at another astrocyte- specific gene *s100β*. Equally, bFgf stimulation modifies histone methylation switch from repressive lysine 9 methylation to permissive lysine 4 methylation at the *s100β* promoter (Song and Ghosh, 2004).

A second mechanism of control over access to the *Gfap* promoter is DNA methylation-dependent repression. The *Stat3* binding element in the *Gfap* promoter is highly methylated in E11.5 neuroepithelial cells, post-mitotic neurons and non nervous system cells, but is demethylated in cells where Stat3 induces the expression of Gfap (Takizawa et al., 2001). In parallel, the *s100β* promoter sequence also contains a highly methylated CpG site at E11.5 neuroepithelial cells that is significantly demethylated in E14.5 neuroepithelial cells. Furthermore, Mecp2 binds to this methylated CpG dinucleotide at E11.5 stage which results in inactivation of the gene and does not bind it at E14.5 stage (Namihira et al., 2004). At this stage of high level methylation state, MeCP2 has been also shown to bind to the promoters of *Gfap* and *Stat1* (Fan et al., 2005). Moreover, these promoters have inactive histone marks during neurogenesis (dimethyl-lysine 9 of histone H3; H3dmK9) and active histone marks during astrogliogenesis (di- or tri-methyl-lysine4 of histone H3; H3d/tmK4). Therefore DNA methylation plays an important role in astrocytogenesis. Transgenic nestin-Cre Dnmt1 mice, which suffer from global hypomethylation during CNS development show precocious astrocytogenesis. This effect is caused by a precocious elevation of JAK-STAT signalling, which as discussed previously, controls the onset of astrogliogenesis. Consistenly, Dnmt1-deficient neural precursors display an increase in H3d/tmK4 (active chromatin status) and decrease in H3dmK9 (inactive chromatine status) (Fan et al., 2005).

In addition to DNA methylation, several transcription regulators are implicated in the control of astrocytogenesis, such as the nuclear corepressor complex N-CoR, and the orphan nuclear receptor TLX (Tailess homolog) (Shi et al., 2004). In parallel with precocious Gfap expression in *N-CoR* deficient mice, *TLX* deficient adult NSC show increased differentiation into Gfap-positive astrocytes together with upregulation of Gfap and s100β in the TLX deficient forebrain. As previously discussed, N-CoR is associated with histone deacetylation, and this is also

the case for the neuron-restrictive silencer factor/repressor element-1 silencing transcription factor (NRSF/REST).

Nrsf/Rest is a major controller of neuronal differentiation (Chong et al., 1995; Schoenherr and Anderson, 1995). Nrsf/Rest represses neuronal genes containing a region called repressor element 1 or Nrse/Rest. Rest mediates repression via recruitment of its corepressors mSin3a (Huang et al., 1999; Roopra et al., 2000) and CoRest (Andres et al., 1999). This in turn promotes additional transcriptional silencing by recruiting MeCP2, histone H3-lysine9 methyltransferase Suv39H1 and heterochromatin protein 1 (Hp1), which causes compactation of chromatin. The chromatin where this machinery acts will show histone deacetylation, the absence of H3-K4 methylation and the presence of H3-K9 methylation, creating a condensed-silenced state of neuronal genes in non-neuronal cells (Lunyak et al., 2002).

An alternative mechanism is necessary to maintain a semi-permissive chromatin state for embryonic stem cells that have the potential to differentiate into mature neurons. In ES cells Rest binds to *Re1* sites and repress gene expression, but when ES cells differentiate into mature neurons, the Rest corepressor complex dissociates from *Re1* sites allowing the expression of fundamental neuronal genes. However, evidence suggests that there are other neuronal genes that contain an extra repression mechanism, where in addition to the *Re1* site binding and repression, a methylated site is bound by MeCP2 which recruits mSin3a and HDAC and CoREST and is not released until an additional stimulus occurs, for example neuronal depolarization, which leads to MeCP2 phosphorilation and de-repression of the neuronal gene (Ballas et al., 2005), as investigated in the *Bdnf* promoter (Chen et al., 2003) (Martinowich et al., 2003).

In contrast with the transcriptional repression activity of REST, small noncoding double-stranded RNA (dsRNA) have been identified which have the ability to bind to REST sequences in neuronal progenitors of the adult hippocampus (Kuwabara et al., 2004). These dsRNAs have the ability to convert REST from a repressor to an activator complex, which leads to the activation of neuron-specific genes necessary for the induction of neurogenesis.

In adult neural precursors and neurons, global levels of H3 and H4 acetylation are increased as compared with oligodendrocytes and astrocytes (Hsieh et

al., 2004). Concordantly, an anti-epileptic drug, valproic acid, which has been shown to inhibit HDAC activity (Gottlicher et al., 2001; Phiel et al., 2001), have a interesting effect of increasing neuronal differentiation both *in vivo* and *in vitro* in adult neural precursors at the expense of oligodendrocyte and astrocyte differentiation (Hsieh et al., 2004).

Overall, it can be concluded that dynamic changes in chromatin status such as DNA methylation and histone modifications are crucial in cell fate determination (figure 1.8)

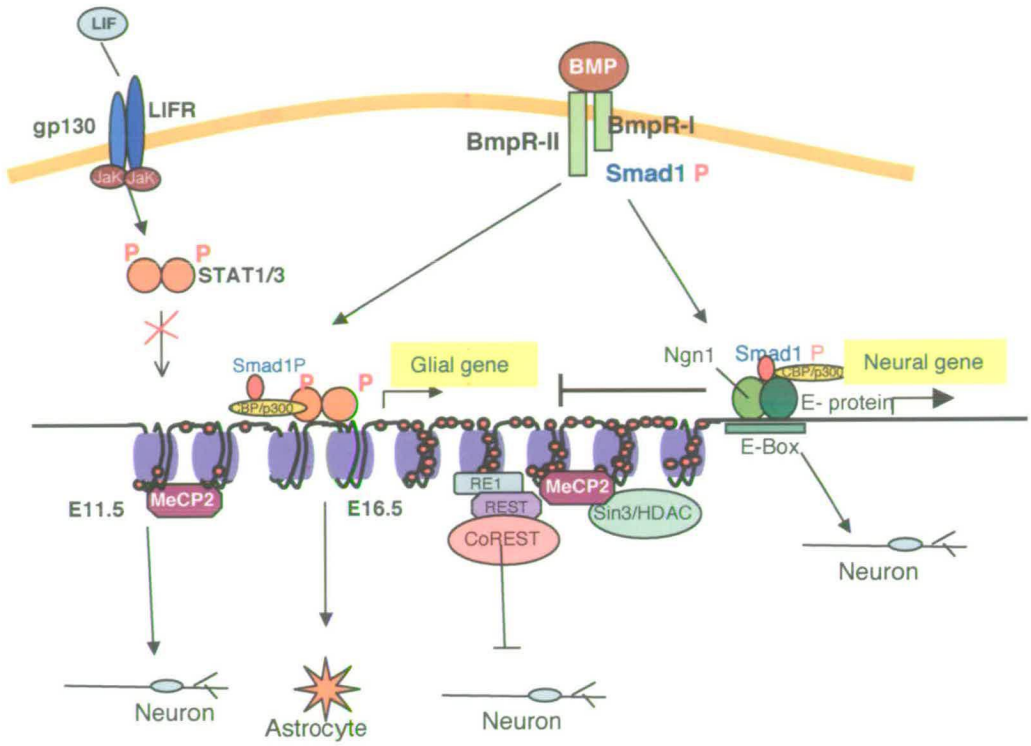


Figure 1.8 Diagram of epigenetic signaling pathways that control cell fate specification. At embryonic age E11.5 and in post mitotic neurons, STAT3 activation is blocked by DNA methylation. At embryonic age E14.5 onwards, Stat3 binding site is demethylated which leads to Gfap activation and astrocyte differentiation. bHLH neurogenic transcription factors as Neurogenin1 block Stat3 pathway and activates neuronal gene transcription. P=Phosphorylation. *Adapted from Sun et al., 2003, Fukuda et al., 2004 and Hsieh et al., 2004.*

1.24 Aim of the thesis

During brain development, an orchestra of signals are necessary to drive the correct patterning that lead pluripotent stem cells to give rise to all the cell types necessary for brain tissue function. Individual cell chromatin status will determine active or repressive transcriptional signals that will dictate its cell fate within its time and space environment.

The aim of this thesis is the study of epigenetic mechanisms involved in neural stem cell function. Here I will describe the functional analysis I carried out to understand the role of the methyl-CpG binding proteins in neural stem cell function. The chapters 3 and 4 are concentrated in answering the question: What are the consequences of the absence of methyl-CpG binding proteins MeCP2, Kaiso and Mbd2 in neural development. The chapter 5 is focused in the study of an epigenetic silencing mechanism independent of binding to methylated DNA. Mbd3 forms part of the Nucleosome remodelling and deacetylation complex (NuRD), and in contrast with the rest of MBD proteins do not bind methylated DNA in mammals. This last chapter will uncover the key role of Mbd3 in stem cell fate decisions.

Chapter 2

MATERIALS AND METHODS

2.1 Materials

Analytical grade chemicals were obtained from either Sigma or BDH Laboratory Supplies (unless otherwise specified). Stock solutions were prepared with reverse osmosis purified (ROP) water (MilliQ biocel, Millipore) and filtered or autoclaved as necessary. Synthetic oligonucleotides were synthesised by SIGMA. Agarose for electrophoresis was supplied by Invitrogen. Radioisotopes were supplied by Amersham Biosciences.

2.1.1 Solutions and reagents

DEPC treated water: 0.2% DEPC, mixed well and autoclaved.

SSC 20x: 3M NaCl, 0.3M tri-Na citrate.

TE: 10mM Tris-HCL, 1mM EDTA pH=8.

Orange G DNA loading buffer: 15% Ficol, 0.2 M EDTA, 0.33% Orange G.

TAE electrophoresis buffer (10x): 0.4 M Tris-acetate, 100mM EDTA, pH=8.5.

TBE electrophoresis buffer (10x) 890 mM Tris, 890 mM Boris acid, 20mM EDTA, ph=8.

TBS: 20mM Tris-HCL, 100mM NaCL pH=8.

Loading buffer (SDS-sample buffer) 2X: 100mM Tris-HCL pH=6.8, 4% SDS, 0.2% bromophenol blue, 20% glycerol, 200mM DTT.

5X tri-glycine electrophoresis buffer: 125mM Tris, 1.25 M glycine, 1% SDS.

Transfer buffer SDS-PAGE: 48mM Tris, 390 mM glycine, 0.1% SDS, 20% methanol (for high molecular weight proteins), 25mM Tris, 190mM glycine, 20% methanol (for low molecular weight proteins).

Phosphate buffered saline (PBS): 2.7 mM KCl, 137 mM NaCl, 4.3 mM $Na_2HPO_4.7H_2O$, 1.4 mM KH_2PO_4 (pH 7.4).

PBST: 1% Tween in PBS.

Blocking solution for protein blots: 5% skimmed milk powder, PBST.

Stripping buffer protein blot membranes: 20% SDS, 0.5 M Tris pH=6.8, 2.8% 2-mercaptoethanol.

Luria-Bertani (LB) broth: 1% (w/v) tryptone (Difco), 0.5% (w/v) yeast extract, 85mM NaCl.
LB agar: 1.5% (w/v) agar (Difco) in LB broth.

STE buffer: 10mM Tris-Cl (pH 8.0), 0.1M NaCl, 1mM EDTA pH 8.0.

PEG-Hybridisation buffer: 0.25 M NaCL, 1mM EDTA, 7% SDS, 10% PEG-6000, 50μg/ml salmon sperm DNA, 125 mM NaPi pH=7.2.

Lysis buffer for genomic PCR: 10% NP40, 10% Tween, 25 mg/ml Proteinase K (Roche) added just before use.

PCR buffer: 750mM Tris pH=8.8, 200mM $(NH_4)_2SO_4$, 0.1% Tween, 30mM $MgCL_2$.

Lysis buffer for DNA extraction: 20% SDS, 25 mg/ml Proteinase K, TE buffer.
$CaCl_2$ solution: 60mM $CaCl_2$, 10MM Pipes pH=7, 15% glycerol. (Filtered, and autoclaved).

EMSA binding buffer: 0.2 M Hepes, 10mM EDTA, 20mM $MgCl_2$, 100 μM 2-mercaptoethanol, 40% glycerol, 500mM NaCl.

Homogenization buffer: 2M sucrose, 10% glycerol, 25mM KCL, 20mM Hepes pH=7.8, 0.15 Spermine, 0.5mM Spermidine, 1mM EDTA, 0.5mM EGTA, 0.5mM PMSF and protease inhibitors cocktail (Sigma).

Resuspension buffer: 75mM KCL, 25mM $MgCl_2$, 1mM EDTA, 0.5mM EGTA, 0.5mM Spermidine, 0.5 mM Spermine, 10mM 2-mercaptoethanol, 20% glycerol, 0.23M sucrose, 20mM Hepes 7.9.

Buffer A for nuclei isolation: 0.5 mM EGTA, 20mM Hepes pH=7.9, 0.23 M sucrose, 60mM KCL, 15mM NaCL, 0.25 mM $MgCl_2$, 0.5mM Spermine, 0.15 mM spermidine, 14 mM 2-mercaptoethanol, 15 mM Tris-HCL, pH=7.4, 0.5mM PMSF and protease inhibitors cocktail (Sigma).

Buffer B for nuclear extraction: 5mM Hepes pH=7.9, 26% glycerol, 1.5mM $MgCl_2$, 0.2mM EDTA, 400mM NaCL, 0.5mM PMSF and protease inhibitors cocktail (Sigma).

Solution 1 for minipreps: 50mM Glucose, 10mM EDTA, 25mM Tris pH 8, 100μg/ml RNase.

Solution 2 for minipreps: 0.2N NaOH, 1% SDS.

2.1.2 Antibodies

Primary Antibody	Host/Type	Used dilution	Manufacturer or reference	Catalog number
anti-Oct 4	Goat	1:200	Santa Cruz	SC-8628
anti-Oct4	Mouse IgG2b	1:1000	Santa Cruz	SC-5279
anti-GFAP	Rabbit	1:200	Sigma	G9269
anti-GFAP(n-18)	Goat	1:200	Santa Cruz	SC-617
anti-Tuj1	Mouse IgG2a	1:1000	Covance	MMS435P
anti-Map2 (a+b)	Mouse IgG1	1:200	Sigma	M1406
anti-GABA	Rabbit	1:800	Sigma	A2052
anti-TH	Rabbit	1:200	PelFreeze	P40101-0
anti-RC2	Mouse IgM	1:20	DSHB	RC2
anti-Vimentin	Mouse IgM	1:20	DSHB	40E-C
anti-nestin	Mouse IgM	1:50	DSHB	Rat-401
anti-caspase-3	Rabbit	1:400	R&D	AF-835
anti-s100β	Mouse IgG1	1:500	Sigma	S-2532
anti-ER	Rabbit	1:50	Santa Cruz	EC-20
anti-MECP2	Rabbit	1;500 WB	(Nan et al., 1998)	674
anti-MECP2	Rabbit	1:200/1:500 WB	Upstate	07-013
anti-kaiso clone 6F	Goat	1:1000	Upstate	05-659
anti MBD2	Sheep	1:200/1:1000 WB	(Ng et al., 1999)	S923
anti Kaiso (1303)	Goat	1:500	Abcam	AB1303
Anti GFP polyclonal	Chicken	1:200	Chemicon	AB16901
anti flag M2	Mouse	1:1000	Sigma	F3165
anti-MBD3 c-18	Goat	1:1000	Santa Cruz	SC-9402

Anti MBD3 (N-20)	Goat	1:200	Santa Cruz	SC-9400
anti-αtubulin	Mouse	1:1000	Santa Cruz	SC-5286
anti-GAD65/67	Rabbit	1:200	Chemicon	AB1511
Anti-Gata4	Goat	1:200	Santa Cruz	SC-1237

Secondary antibodies:

All secondary antibodies used in immunocytochemistry were Alexa Fluor, Molecular Probes, used 1:1000 dilution.

Secondary antibodies used in western blot were ECL peroxidase labelled anti-mouse and anti-rabbit (Amershan) and anti-goat (Sigma).

2.2 Molecular biology methods

2.2.1. Plasmid isolation

Overnight cultures of single bacterial colonies in LB broth were used to isolate plasmid DNA using Quiagen's mini, midi and maxiprep kits for small, medium and large scale preparations respectively, according to manufacturers instructions.

2.2.2 Genomic DNA isolation

ES cells and NS cells:

Cells were grown in 96 well plate (or 4 well plate (Nunc) to confluence, harvested, centrifuged 5 minutes at 100g, washed in PBS, centrifuged again, and resuspended in 10-25μl of lysis buffer. This solution was incubated at 55°C for two hours and 94°C for 10 minutes. 2μl of lysis sample was used as template for genomic PCR.

2.2.3 RNA isolation

Total RNA was isolated from cells using Tri Reagent™ (Sigma), according to the manufacturer instructions.

2.2.3 DNA purification

Same volumes of DNA solution and phenol/chlorophorm are mixed and centrifuged at 10600g for 3 minutes. The aqueous phase is extracted and mixed with same volume of chlorophorm. The solution is centrifuged at 10600g for 3 min and the water phase is extracted to precipitate DNA by incubation with 2 volumes of 100% ethanol and 0.1 volumes of NaOAc at -80^{0}C for 30 minutes. The solution is then centrifuged at 20000g for 30 minutes at 4^{0}C, washed with 70% ethanol and resupended in TE buffer.

2.2.4 Polymerase chain reaction

PCR were carried out using Red Hot Taq (Abgene) following manufacturers instructions with 7.5pmol/μl of reverse and forward primers, 5-100ng DNA as template, Thermocycling incubations were done in DNA engine DYAD.

2.2.5 RT-PCR

First strand cDNA was synthesised from 1μg of total RNA using SuperScript^{TM} II reverse transcriptase or M-MLV RT (Invitrogen) according to manufacturers instructions.

2.2.6 qRT-PCR

Quantitative real time PCR was done using a Roche light cycler. The optimised PCR reaction contained: 2μl of LightCycler Master SYBR Green I mix (Tth DNA polymerase, reaction buffer, dNTP mix and SYBR Green I), 2.5μl of dH_2O, 0.25μl of reverse and forward primers (10pmol/μl). The following reaction conditions were used:

95°C -5 min

95°C -14 sec

X -5 sec

72°C -14 sec

Annealing temperatures (X):

Gapdh, Sox2 and Kaiso: 58°C

Mbd2, Mbd3a, Mbd3b: 64°C

Mbd1: 60°C

2.2.7 Methylation of DNA fragments

Methylation reactions using MSssI methyltransferase (NEB) were carried out according to manufacturers instructions with the exception that the methylation reactions were incubated for 2-3 hours at 37°C, followed by purification with Zymoclean Kit (Zymo Research) and re-methylated for another 2-3 hours at 37°C.

2.2.8 Radiolabelling

Probe radiolabelling was done using replacement synthesis method.

100ng of methylated (MeCG11) and unmethylated (CG11) DNA probes were incubated with T4 DNA polymerase, 100μM dATP, 1X buffer and BSA·(100μg/ml) for 15 min at 12°C to activate the 3'5' exonuclease activity of T4 DNA polymerse, eliminating G bases of the 3' end of the MeCG11 and CG11. After this time 100mM dTTP and dGTP were added as well as P^{32} -αdCTP (40μCi, ~3000 Ci/mmol, Amersham) and incubated for 15 min at 12°C. The enzyme was inactivated by addtion of 0.5M EDTA. Final radioactivity of the probe was read with a liquid scintillation analyzer (Tri-Carb, Packard).

2.2.9 Nuclei extraction from mouse liver

25ml Homogenization buffer was used to homogenise the liver using a tight fitting dounce. The solution was centrifuged through a 10ml cushion of homogenization buffer at 0°C 24000 rpm in a SW28 rotor for 40-50 minutes. The pellet was resuspended in 5 ml of resuspension buffer and stored at -80°C.

2.2.10 Nuclei extraction from ES and NS cells

Cells were washed with PBS and harvested with a cell scraper. Suspension was centrifuged at 1500g for 3 minutes and the pellet washed in PBS and resuspended in 10 volumes of buffer A and incubated on ice for 10 min. The suspension was centrifuged at 1500g for 5 min and the pellet resuspended in 3 volumes of buffer A. The suspension was homogenized using a tight fitting dounce and then diluted 3 fold in 2M sucrose in buffer A. The lysate was centrifuged at

1500g for 5 min, 20% glycerol was added and the sample was stored at -80°C until needed.

2.2.11 Salt extraction of nuclear protein

The frozen solution was thawed and resuspended in same volume of buffer C, homogenized with a loose fitting dounce, stirred for 1-2 hours on ice and centrifuged at 13000 rpm at 4°C for 30 min in a SS-34 rotor.

2.2.12 Measurement of protein concentration

Protein concentration was measured using Bradford reagent (Sigma) according to manufacturer instructions. The absorbance at 595nm was analysed and recorded using a precision microplate reader (Molecular Devices).

2.2.13 SDS-PAGE

Gels were prepared as follows: Separating gel (1.5 M Tris pH=8, 20% SDS, TEMED, acrylamide and APS concentration depending on percentage of separation desired) was casted on an assembly kit (Bio-Rad), and subsequently 5% stacking gel was casted on top and assembled in a Bio-Rad mini protean apparatus. Proteins were diluted in loading buffer, boiled for 5 min at 100°C and loaded on the gel. Gels were run in tri-glycine electrophoresis buffer at room temperature until the loading dye migrated off the bottom of the gel.

2.2.14 Transfer to nitrocellulose membrane from SDS-PAGE gel

Gel and nitrocellulose membrane (Protran, Whatman) were assembled in Bio Rad mini protean apparatus according to manufacturer instructions and run at 90V for 1-2 hours at 4°C in transfer buffer.

2.2.15 Western blot

Membrane was washed in PBST for 10 min, incubated in blocking solution for 30 min, and incubated with primary antibody in blocking solution overnight. Secondary antibody was added after washing with blocking solution for 2x10 min and incubated for 1 hour at room temperature. All washes and incubations were done

in a shaker. Subsequently the membrane was washed in PBST, air-dried, and incubated with freshly mixed ECL reagents (Amershan) for one min. Membrane was wrapped in cling film and exposed to Kodak film. Development of the film was done using SRX-101A developer.

2.2.16 Electrophoretic mobility shift assay:

5 μg of protein, 10^4cpm methylated (MeCG11) or unmethyated (CG11) probe (Meehan et al., 1989), and 2μg of *E.coli* DNA as competitor were incubated on ice with binding buffer for 30 min. The binding reaction was loaded in a 1.5% agarose gel in TBE buffer, and run at 4°C for 4-5 hours. Gel was dried on two layers of 0.2mm Whatman paper in a gel drier (Bio Rad 583) and exposed to a phosphor-imager screen overnight. Images were developed the next day using phosphoimager Fujifilm FLA 3000.

2.2.17 Cloning and subcloning

Vector construction involved restriction enzyme digestion, gel purification and ligation. Restriction enzyme digestions were performed according to NEB's instructions. Digested DNA was isolated by electrophoresis of (TAE) 0.8% -2% agarose gel for fragment resolution. DNA was then isolated from desired gel fragment using Zymoclean (Zymo Reearch). Vector and insert ligation was done using Rapid DNA ligation Kit (Roche) according to manufacturers instructions.

2.2.18 Transformation of competent cells.

100μl of competent cells and 10-100ng of DNA were incubated on ice for 30 minutes, heat shock treated for 45 seconds at 45°C and incubated on ice for 3 minutes. Solution was diluted with 400 μl of SOC media (Invitrogen) and incubated at 37°C for 60 minutes. Bacteria were spread evenly over the surface of LB agar with antibiotic plates using a sterile bent glass rod. Plates were inverted and incubated overnight at 37°C.

2.2.19 Analysis of transformants

Colonies grown in agar plates were screened to isolate the desired vector using the following protocol. Single colonies were inoculated in a 5ml LB broth containing the appropriate selection and incubated in a shaker at 37°C overnight. 3.5ml of overnight culture was centrifuged for 5 min at 107g. Pellet was resuspended in 100μl of Solution 1 and 200μl of Solution 2 (see pg 82), mixed gently and added 150μl of 3M NaOAc pH=4.8. The solution was mixed, centrifuged at 20000g for 10 min, and from the supernatant plasmid DNA was purified as described before for genomic DNA (2.2.3).

2.3 Cell culture methods

Cells were maintained at 7.5% CO_2 at 37°C in a humidified incubator. All solutions were tested for bacteria contamination before use. All tissue culture manipulations were undertaken inside a laminar flow sterile hood (Microflow) and surfaces and objects were sprayed with 70% industrial methylated spirits (IMS) before use. Cell suspension centrifugation was done at 200g for 3.5 min unless specified.

2.3.1 Cell culture media

ES cell medium: Glasgow modified Eagle's medium (GMEM), 10% fetal bovine serum (Gibco), 1mM sodium pyruvate (Gibco), 100X MEM non-essential amino acids, 2mM L-glutamine (Gibco), 0.1mM 2-mercaptoethanol (BDH).

N2B27 medium: DMEM/F12 and Neurobasal 1:1 mixture (both Gibco), N2 (25 g/ml insulin, 100 μg/ml apo-transferrin, 6 ng/ml progesterone, 16 μg/ml putrescine, 30 nM sodium selenite and 50 μg/ml bovine serum albumin fraction V (Gibco), 100x B27 supplement (Gibco), 1mM L-glutamine and 0.1mM 2-mercaptoethanol.

NS medium: NS-A (Euroclone), 1mM L-glutamine (Gibco), 10ng/μl bFGF and 10ng/μl EGF (Prepotech).

Trypsin solution: 0.025% trypsin (Gibco), 1.3mM EDTA, 0.1% chicken serum (Flow Labs) in PBS.

PA-6 co-culture differentiation medium: GMEM, 10% knock-out serum replacement (Gibco), 1mM sodium pyruvate, 100X MEM non-essential amino acids, 2mM L-glutamine and 0.1mM 2-mercaptoethanol.

PA-6 co-culture induction medium: N2B27 medium supplemented with 200μM ascorbic acid, 100X MEM non-essential amino acids, 2mM L-glutamine.

Neurosphere medium: DMEM/F12 medium supplemented with B27, 25μg/μl bFGF, 100x antibiotic-anti-mycotic solution (Invitrogen).

Neurosphere differentiation medium (NB+): Neurobasal medium supplemented with B27, 1mM L-glutamine, 0.1mM 2-mercaptoethanol and 100x antibiotic-anti-mycotic solution.

2.3.2 ES cell routine culture

ES cells were cultured on 0.1% gelatine-coated tissue culture flasks or plates in Glasgow modified Eagle's medium (GMEM) supplemented with 100units/ml leukaemia inhibitory factor (LIF). LIF was prepared in house by transfecting COS-7 cells with a human LIF expression plasmid and harvesting the medium. The concentration of LIF was assayed using CP1 indicator cells (LIF preparation was done by L. Taylor). All media reagents were pre-warmed at 37°C.

To passage the cells, media was removed and cells were washed twice with PBS. 1x (0.025%) trypsin (Invitrogen) was added at the minimum volume required to cover the cell monolayer and incubated at 37°C for 1 min. Subsequently the cell suspension was diluted with GMEM media (4 ml of media per every 1ml of trypsin). The cell suspension was centrifuged and the cell pellet was resuspended in media to achieve a cell concentration of 10^6 cells per 25cm^2 culture surface. ES cells were passaged every 2-3 days.

2.3.3 Freezing cells

Cells were harvested as described above, and the cell pellet was resuspended in the required media and 10% dimethylsulphoxide (DMSO, BDH) at concentration of 10^6 cells per cryotube (Nunc) vial. Cryotubes were immediately transferred at -80°C overnight and then transferred to a liquid nitrogen cell bank (Series 2300, Custom biogenic systems).

To freeze cells in 96 or 24 well plates, cells were harvested with trypsin solution and cell suspension was diluted with freezing mix (150μl for 96 well plate or 500μl for 24 well plate). Freezing mix for ES cells consisted of ES media, 10% DMSO and 20% FCS for ES cells; freezing mix for NS cells consisted of NS-A

media, 10% DMSO and 3% BSA (Sigma) for NS cells). Plates were placed in a polystryrene box and stored at -80^{0}C.

2.3.4 Thawing cells

Frozen cryotubes were thawed at 37^{0}C for 1-3 min and immediately transferred to a universal tube with 9.5ml of required media and centrifuged. Cell pellet was then resuspended in 10ml of required media and transferred to a 25cm^2 flask. An additional media change was done 8-10 hours later to remove dead cells and remaining DMSO.

To thaw cells in 96 or 24 well plates, pre-warmed media was added to cover the well, and cell suspension was transferred to a gelatin-coated parallel plate. Media was replaced with fresh media after 8-10 hours.

2.3.5 Monolayer differentiation (ES neuroectoderm differentiation)

ES cells were passaged the day before the experiment to 90% confluence. Next day ES cells were harvested, and resuspended in GMEM media with 10% FCS. Cells were centrifuged and the cell pellet resuspended in Neurobasal media and centrifuged again. Cell pellet was resuspended in N2B27 and counted using a haemocytometer. Culture wells were coated with 0.1% gelatin and incubated for 30 min. To aid even cell distribution within the well gelatin was then aspirated and N2B27 media was added to the well into half the volume required for the culture. Cells suspension was diluted in half the total required volume and plated at a density of 10^6 cells per 10cm^2 surface. N2B27 media was replaced with fresh N2B27 media every two days to remove dead cells and cell debris.

2.3.6 Replating monolayer cultures

Plates (usually 1.88 cm^2 growth area) were coated with 100x poly-D-lysine (Sigma) in filtered distilled water (MilliQ Biocel, Millipore) and incubated at room temperature for at least 30 minutes. Poly-D-lysine (PDL) was removed, wells were allowed to dry and washed twice with PBS. Then the wells were coated with 1000x

laminin (Sigma) for 3 hours at 37°C and subsequently removed and washed twice with PBS.

Monolayer differentiation cultures were harvested using pre-warmed PBS, incubating for 5 min at 37°C and gently pippetting up and down the cell culture to achieve cell suspension. Cell suspension was then diluted with N2B27 and centrifuged at 200g for 3.5 min. Cell pellet was resuspended in N2B27 media and plated in PDL/laminin pre-coated wells as described above at a density of 2-2.5 10^4 cells/cm^2 when replating 7 days old monolayer cultures or 3.5-4 10^4/cm^2 when replating 10 day old monolayer cultures. N2B27 media was replaced with fresh media every 3-4 days to remove dead cells and cell debris.

2.3.7 PA-6 co-culture differentiation protocol

PA-6 feeder cells were routinely cultured in the same conditions as ES cells (as described above) in ES media without addition of LIF. PA-6 cells were irradiated to arrest proliferation, harvested and plated at a cell density of $8x10^4$ cells/cm^2 one day before the differentiation experiment. ES cells cultured at 90% confluency which had been passaged previous day were harvested, centrifuged and resuspended in differentiation medium. ES cell suspension was counted and plated on the PA-6 feeder monolayer at a density of 25-30 cells/cm^2. Differentiation media was replaced from the wells after 4 days and on the 8^{th} day replaced with induction media. Media was then replaced every 2 days with fresh induction media for 8 days.

2.3.8 Neurosphere culture

Embryonic age E14.5 or E16.5 mouse cortices were dissected and dissociated by pipetting up and down using a fire polished Pasteur pipette to obtain single cell suspension in PBS supplemented with 1 unit (potency contained in 0.60μg of Penicillin G master standart) of penicillin, 100 μg streptomycin and 0.0025μg of ampothericin B per ml of media (antibiotic-anti-mycotic 100X solution, Invitrogen.). Cell suspension was passaged through a 40μm nylon mesh (BD Falcon), diluted in DMEM/F12 media and centrifuged. Cell pellet was resuspended in neurosphere medium and plated in single cell suspension in 25cm^2 flasks at density of 5000 cells/cm^2 for 7 days. After 7 days, primary neurospheres were

collected by centrifugation and the cell pellet resuspended in 1-2 ml neurosphere media. Suspension was pipetted up and down first with a fired polished Pasteur pipette followed by same procedure with a polished Pasteur pipette with narrower aperture. The cell suspension was passaged through a 40μm nylon mesh and diluted with neurosphere media. The single cell suspension was centrifuged, resuspended in neurosphere media, counted in a haemocytometer and plated at the same density as for primary neurospheres.

2.3.9 Neurosphere differentiation

Glass coverslips (13mm diameter) were sterilised by ultraviolet exposure for 30 min and allocated in 24 well plates. Subsequently the coverslips were coated with Poly-D-lysine and Laminin as described before. Primary and secondary neurospheres grown in 25cm^2 flasks were collected by centrifugation and resuspended in NB+ medium. 1:3 of the cell suspension was plated on the coated plates and cultured for 5 days.

2.3.10 Routine passage of NS cells

When they reached 30-60% confluent, NS were harvested and trypsined in 10x trypsin solution. Harvested cells were resuspended in NS medium and centrifuged at 300g for 4 min. Cell pellet was resuspended in NS medium to achieve a cell density of 8-16x10^4 cells/cm^2. Tissue culture plastic had been incubated with 0.1% gelatin for at least 30 min.

2.3.11 NS cell derivation from embryonic cortex

Embryonic age E14.5 or E16.5 cortices were dissected and dissociated as explained before. The entire cell suspension from each embryonic cortex was plated on 25cm^2 flasks in NS media. After 1-2 days cells were collected by centrifugation and replaced with fresh NS media. Cultures were allowed to form neurospheres for 7 days. After this time, neurospheres were collected by centrifugation at 100g for 1 min and plated on gelatinised 25cm^2 flasks. Flasks had been coated with gelatin at 37°C for at least 30 min. From this point cells were maintained in adherent

monolayer and passaged when 30-50% confluent aiming for homogeneous cell morphology.

2.3.12 NS cell derivation from ES neuroectoderm monolayer

10 cm^2 dish ES monolayer differentiation cultures were harvested after 7 days, centrifuged and resuspended in 20ml NS media. The cell suspension was plated in 75cm^2 flasks for 2-3 days in suspension. After this time, formed neurospheres were collected by centrifugation at 100g for 1 min, plated in gelatin-coated 75cm^2 flasks and cultured for 6-8 days. From this point cells were maintained in monolayer and passaged when 30-50% confluent as explained before.

2.4 Transfection of DNA into ES cells by electroporation

2.4.1 Stable transfection

200 -300μg of DNA was linearised by appropriate restriction enzyme digestion and checked for correct digestion by 1% agarose gel electrophoresis. DNA was purified by phenol/clorophorm and ethanol precipitation as described above. DNA was resuspended in 80μl of TE buffer inside the laminar flow sterile hood. 90-100% confluent culture of ES cells in T75cm^2 flask was harvested, centrifugated and resuspended in 800μl of PBS. Cell suspension was transferred to a sterile electroporation cuvette and mixed with the linearised DNA. Cells were then electroporated at 800V and 3μF for 0.1 seconds (BioRad Gene Pulser). Cells were left to rest in the cuvette for 10 min and then resuspended in ES media and plated on 10-15 pre-gelatinised 18cm^2 dishes. After 24 hours, drug selection was added (Hygromicin B 100μg/ml, puromycin 1μg/ml, Gancyclovir 25mM 0.1N HCL 1000x, Neomycin 166μg/ml) and media was changed every 2-3 days. 6-8 days later medium sized ES colonies were selected and transferred into 2-3 96 well plates. When colonies formed confluent cultures in 96 well plates, plates were replicated as explained above (2.5.6) for freezing stock and for screen the correct targeting by genomic PCR .

2.4.2 Transient transfection

$5x10^6$ ES cells were electroporated as described above with 50μg of non linearised (pCAGCreIP) plasmid to achieve transient Cre recombinase expression. After electroporation cells suspension was diluted with ES media and plated in a gelatinised $75cm^2$ flask and cultured for 24-35h. After this time, cells were harvested and plated at clonal density in gelatinised dishes. Cells were cultured until colony formation and then proceeded as described above for selection of the clones with the correct recombination by Cre recombinase.

2.5 Transfection of DNA into ES cells by lipofection

Lipofection was done using TransFast™ tranfection reagent (Promega). DNA plasmid was cut and purified as described before and resuspended in TE. 1μg of DNA was incubated with 6μl of TransFast reagent in 250μl pre-warmed media for 15 min at room temperature. ES cells were harvested, centrifuged and resuspended in ES media to a concentration of $2x10^6$ cells/ml. Cell suspension is prepared to a concentration of $5x10^5$ cells/250μl and mixed with DNA-TransFast solution. Subsequently the total 500μl are plated in 24 well plate and incubated for 1 hour. After this time, 1ml of media was added and culture overnight. Next day media was replaced with fresh media and 48 h later cells were harvested and plated in 10cm diameter dish at clonal density (1000 ES cells).

2.6 Transfection of DNA into NS cells by eletroporation

$10x10^6$ NS cells were mixed in 100μl PBS solution with 50μg of plasmid DNA transferred to a sterile electroporation cuvette and electroporated at 250mV and 9.6 capacitance. Immediately after cells were resuspended in 10 ml of NS media and plated at a density of 10^6cells per 10cm diameter dish. Media was changed every 4 days until colonies became large enough to be transferred to 96 well plate (about 10-15 days).

2.7 Transfection of DNA into NS cells by nucleofection

Nucleofection was done using Amaxa Biosystems nucleofector and solution V cell kit for NS cells. Nucleofector solution was pre-warmed at room temperature and NS media with 10% serum was pre-warmed at 37°C. NS cells were harvested,

counted and centrifuged at 300g for 4 minutes. Cell pellet was resuspended in 100µl of nucleofector solution B to a cell density of 2-5x10^6 cells and 2µg of plasmid DNA was mixed to the solution. Subsequently the cell suspension was transferred to a nucleofection cuvette and nucleofected using program T-030. Immediately after nucleofection, cells were transferred into an eppendorf with 1ml pre-warmed media and left to rest for 5 min. After this time cells were gently transferred to pre-gelatinised plates and media was changed to replace any trace of serum in the next two hours. 12 hours later drug selection was added to the media.

2.8 MTT assay

Cell proliferation quantification was done using the CellTiter^R 96 AQ_{ueous} one solution cell proliferation assay (Promega) according to manufacturer's instructions with the following modifications: 10x10^4cells were plated in 150µl of media and 30µl of CellTiter reagent was added to the culture. The absorbance was read after 4 hours using a precision microplate reader (Molecular devices) at 490nm wavelength.

2.9 Cell sorting

Cells were harvested and resuspended in 1ml of FACS sorting solution (10% FCS and 100 U/ml penicillin and100 µg/ml streptomycin in PBS for ES cells and or 3% BSA, penicillin/streptomycin in PBS for NS cells and monolayer differentiation cultures). Cells were sorted by J. Vrana using Cytomation MoFlo flow cytometer into tubes containing N2B27 medium. Immediately after cells were plated on pre-gelatinised plates at desired cell density with appropriate media or lysed for RNA extraction as described above.

2.10 Flow cytometry analysis.

Sox1-GFP knock-in ES (46C) cultured in 6 well plates in N2B27 serum-free monolayer system were harvested at days 3-8, centrifuged and resuspended in 1ml of FACS solution. Analysis was done using FACScalibur flow cytometer (Becton Dickinson) using CellQuest software. 10,000 events were scanned. Electronic gates were set by forward scatter (size) and side scatter (cell complexity) criteria to

eliminate cell debris from the analysis. ES with no GFP knock-in after monolayer differentiation were used as a control to eliminate autofluorescence events.

2.11 Immunocytochemistry

Cells cultured in tissue culture plates were fixed with 4% PFA (pre-warmed at 37°C) for 30 min, and washed three times in PBS. Cells were blocked in a 3% serum (goat or donkey if using goat or donkey host respectively as a secondary antibody) and 0.1% Triton-X for 60 min. Primary antibodies were diluted in 3% serum PBS solution and incubated overnight at 4°C. Cells were washed three times in PBS and incubated in secondary antibody solution (3% serum in PBS) for 1 hour at room temperature. After this incubation time, secondary antibody solution was removed and cells were washed three times in PBS. 4'-6-Diamidino-2-phenylindole (DAPI) solution of 1:5000 in PBS was incubated for 10 min and washed with PBS. Immunocytochemistry experiments were visualised using Olympus (1x50) microscope and images were captured with Olympus DP50 camera.

2.12 Quantification of immunolabelled cells

Velocity software was used for cell number (DAPI positive nuclei) and neuronal number (TuJ1 positive) quantification.

2.13 Statistical analysis

Data statistical analysis was performed using ANOVA test and Bonferroni method with XLSTAT software.

CHAPTER 3

INVESTIGATING THE ROLE OF KAISO IN NEURAL STEM CELL FUNCTION AND DIFFERENTIATION

3.1 Introduction

DNA methylation is a major epigenetic modification in mammalian genomes affecting gene expression. DNA methylation mediates transcriptional silencing, mainly functioning in tumour suppressor gene inactivation (Herman and Baylin, 2000), imprinting genes (Reik and Walter, 2001) and X chromosome inactivation (Robertson, 2005) by methylation of CpG islands, and to suppress repetitive DNA elements during murine embryogenesis. DNA methylation is also implicated in the regulation of cell- and tissue-specific gene expression (Bird and Wolffe, 1999; Jackson-Grusby et al., 2001).

Transduction of the DNA methylation signal is primarily induced by the methyl-CpG binding proteins (MeCPs) that selectively bind methylated CpG dinucleotides. The MeCPs can exert transcriptional repression to the methylated locus (even at long distances (Kass et al., 1993; Nan et al., 1997)) by recruiting chromatin remodelling corepressor complexes to the methylated site and changing the functional gene status (Jones et al., 1998; Nan et al., 1998; Ng et al., 1999; Sarraf and Stancheva, 2004; Wade et al., 1999; Yoon et al., 2003b).

MeCPs are ubiquitously expressed in somatic cells while are expressed at very low levels in ES cells, giving evidence to support a critical role in regulating tissue-specific gene expression. MeCP2, Mbd2 and Mbd1 are members of the MBD family of proteins, that share the methyl-CpG binding domain that has the ability to bind to methylated CpG dinucleotides (Nan et al., 1993). In contrast, Kaiso belongs to the zinc finger family of transcriptional repressors. Kaiso is the only MeCP that binds specifically to two to three consecutive methylated CpG dinucleotides by zinc fingers with the highest reported affinity of any protein of methylated DNA (Prokhortchouk et al., 2001). Two target genes have been found to be bound (Aranyi et al., 2005) and repressed (Yoon et al., 2003b) by Kaiso through recognition of its

specific binding site, human tyrosine hydroxylase *TH* and *MTA2*. In addition, Kaiso can bind through its zinc fingers unmethylated sequences that contain the consensus Kaiso binding site. xKaiso has been shown to repress transcription of the genes *MATRYSILIN* (Spring et al., 2005) and *Wnt-11*, *Siamois* and *Xnr3* (Kim et al., 2004; Park et al., 2005) in *Xenopus,* which are target genes of the canonical Wnt signalling pathway. Thus, xKaiso cooperates with TCF/LEF to repress transcription of canonical Wnt pathway target genes. The canonical wnt pathway is very important in cell fate specification and development. In the central nervous system, this signalling pathway is crucial in the decision of precursors to proliferate or differentiate during mammalian neural development (Hirabayashi et al., 2004) (Chenn and Walsh, 2002; Otero et al., 2004; Zechner et al., 2003).

Kaiso mediates transcriptional repression by association with the corepressor complex N-CoR in HeLa cells (Yoon et al., 2003b) and in *Xenopus* (Kim et al., 2004). The N-CoR complex is formed by more than ten proteins, containing histone binding proteins and histone deactylases. N-CoR associates with the Rest/Nrsf transcriptional repressor bringing histone deacetylase activity to the promoter of neuronal genes to drive their repression in non-neuronal cells (Jepsen et al., 2000).

N-CoR plays a crucial role in the regulation of early fate specification in neural stem cells (Jepsen et al., 2000). N-CoR deficient mice show during brain development early neuronal Map2 positive and astrocytic Gfap positive populations while their neural precursor population is decreased, indicating aberrant fate specification. Concordantly, *N-CoR* deficient neural stem cells undergo spontaneous astrocytic differentiation bypassing the bFGF-induced self-renewal signal. Moreover, N-CoR overexpression inhibits astrocytic differentiation (Hermanson et al., 2002). Hence, these detailed *in vitro* studies revealed that N-CoR regulates nestin positive bFGF dependent neural stem cell fate specification.

Since Kaiso is a key component of N-CoR repressor complex, I decided to study its function *in vitro* in neural stem cells. The hypothesis to be tested in this study is that Kaiso will play an important role in neural stem cell function via its role in the N-CoR complex.

3.2 Generation of Kaiso null ES cells expressing Sox1-GFP

Neural differentiation *in vitro* from ES cells has been demonstrated to correlate in time with neurogenesis *in vivo* (Qian et al., 2000; Shimozaki et al., 2003). Sox1 is a specific transcription factor of the earliest stages of neural development and it is present in the first neuroectoderm cells commited to the neural lineage (Pevny et al., 1998). Sox1 is largely coexpressed with Nestin in neuroectodermal cells and it can be used as a marker for neural progenitors.

To study the capacity of ES cells to differentiate into neural stem cells, previously generated *Sox1*-Gfp knock-in ES cells (46C) (Ying et al., 2003b), were chosen to be targeted with a Kaiso conditional deletion construct (a gift from Dr. E. Prokhortchouk) (figure 3.1.A). This construct consists of two fragments of mouse genomic DNA from the *Kaiso* locus (left arm and right arm) of 1925bp and 1282 bp respectively, and a *neo/tk* selectable marker cassette cloned into pBluescript II SK- (pBS KS- in Fig. 3.1.A) (Prokhortchouk et al., 2006). The *Kaiso* fragments right arm and left arm confer the homology necessary for homologous recombination to occur (Thomas and Capecchi, 1987). The neo/tk cassette contains the *neomycin-resistance-encoding* gene which confers neomycin resistance for positive selection and the herpes simplex virus type-1 *thymidine kinase (Tk)* gene, which confers ganciclovir sensitivity for negative selection (Horie et al., 1995). The *Kaiso* cDNA of the construct is tagged with C-terminal Flag, although this tag has not been used in this thesis. After Flag, the *Kaiso* cDNA is ended with a stop codon and 3' untranslated region (UTR) *Kaiso* sequence.

In the first targeting step, G418 resistant clones that had correctly replaced *Kaiso* with the *Kaiso* floxed construct were selected by genomic PCR (figure 3.1.A). Kaiso is a X-linked gene, allowing me to use PCR genotyping in our XY ES cells. One selected clone (46CT) was transfected with a Cre expression construct (PE-CRE) which expresses under a weak promoter (PE), the Cre recombinase protein that efficiently causes recombination between two loxP sites (Muller, 1999). Thus two different set of clones were selected: null clones with the selection cassette and Kaiso cassette deleted (figure 3.1B I), and floxed clones that have the selection cassette deletion only (figure 3.1.B II) . The PE-CRE plasmid was chosen because of the low activity of its promoter in order to obtain both floxed and null lines.

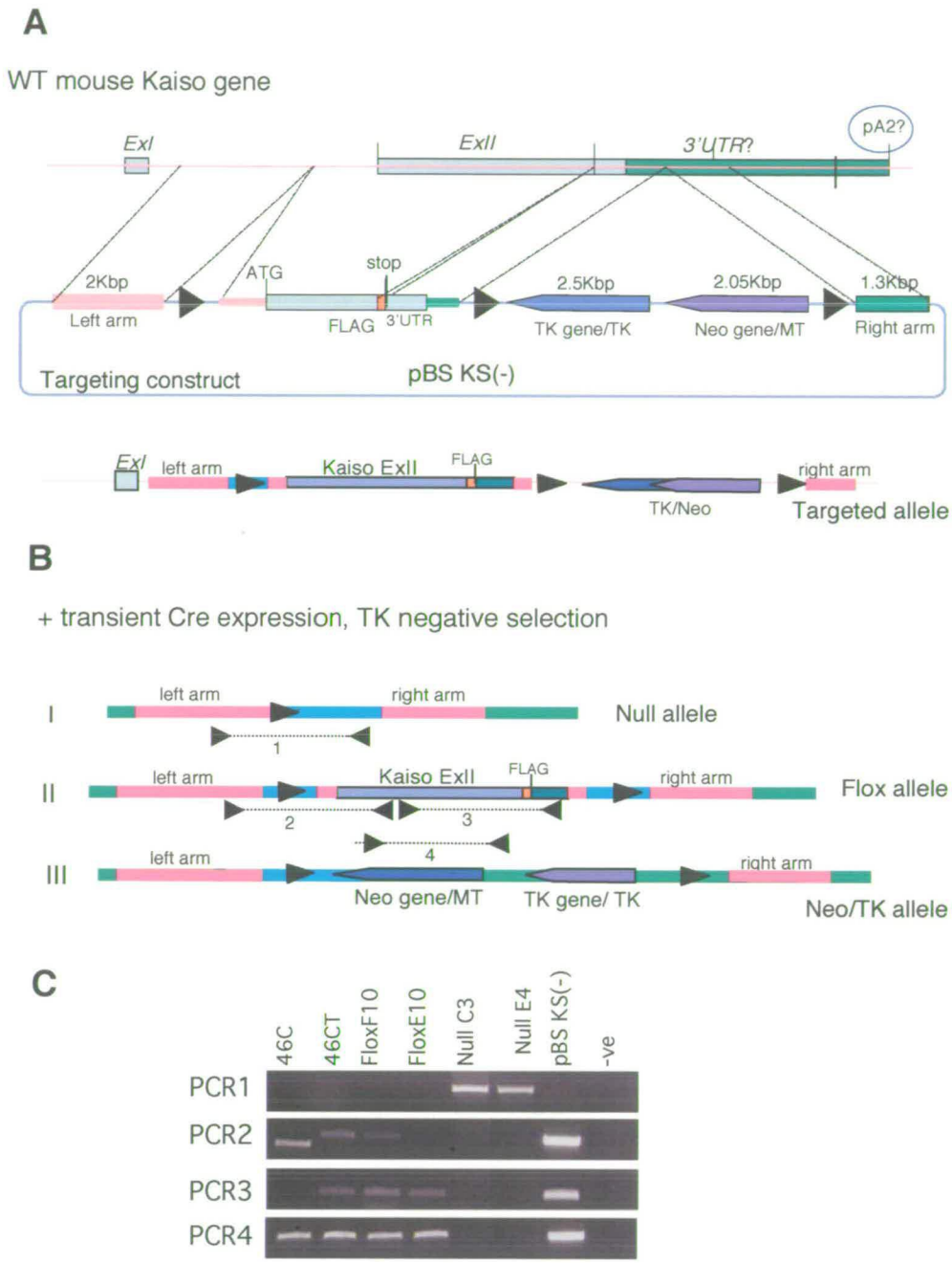


Figure 3.1 A) 46C Sox-GFP ES cells were targeted with the Kaiso deleting construct (Prokhortchouk et al., 2006). Correctly targeted cells (46CT) were identified by genomic PCR. **B)** Transient transfected with Cre expression vector results in three different recombinations I: clones with completed recombination giving a null allele, II: clones with Kaiso floxed allele and III: clones with Neo/TK cassette allele. After TK negative selection, 46C floxed (II) and 46C Kaiso$^{(-/y)}$ (I) ES cell clones were identified by genomic PCR. **C)** PCR with genomic DNA from parental line (46C), Targeted clone (46CT), 2 clones carrying floxed allele (F10, E10), 2 *Kaiso*$^{(-y)}$ clones (C3, E4) and Kaiso deleting plasmid as control (pBS KS(-). ▸:indicates LoxP site, ▸····▸: indicates primer positions for PCR results shown in C)

3.3 Quantification of neural precursors in the absence of Kaiso

ES cells require LIF and Bmp4 signalling to maintain an undifferentiated state (Ying et al., 2003a), and in the absence of both factors ES cells in monolayer cultures undergo differentiation into predominantly (70%) neuronal cells in about 7 days, with the first Sox1 positive neural precursors appearing at around 4 days in culture (Ying et al., 2003b). This well established system was chosen as a tool to study the neuroectoderm formation capacity of *Kaiso*$^{(-/y)}$ ES cells.

Two *Kaiso*$^{(-/y)}$ ES cell clones, E4 and C3, were challenged to differentiate using the monolayer differentiation protocol and Sox1-GFP expression was monitored from days 3 to 8 by flow-cytometry of GFP in comparison with the parental ES cell line, 46C. During this process of differentiation, GFP-positive 46C cells appear around D3, peak at day 6, and start to decrease after day 7. Days 3 to 8 were chosen to screen both possibilities of early or delayed differentiation compared with the control line. The results of this experiment demonstrate that there is no defect in differentiation of ES cells lacking Kaiso protein into Sox1-positive neural precursor cells (figure 3.2).

3.4 Study of differentiation potential of ES-cell derivated neuroectoderm

Monolayer differentiation cultures of the null cell lines were studied by immunocytochemistry to investigate the expression of markers of terminally differentiated cells such as astrocytes and neurons. The markers TuJ1 for post mitotic neurons and Gfap for astrocytes were assayed at different time points of differentiation to cover both possibilities of delay or early differentiation abnormalities, as described above. These experiments demonstrate that Kaiso is not necessary for the differentiation into mature neurons and astrocytes in this system (figure 3.3).

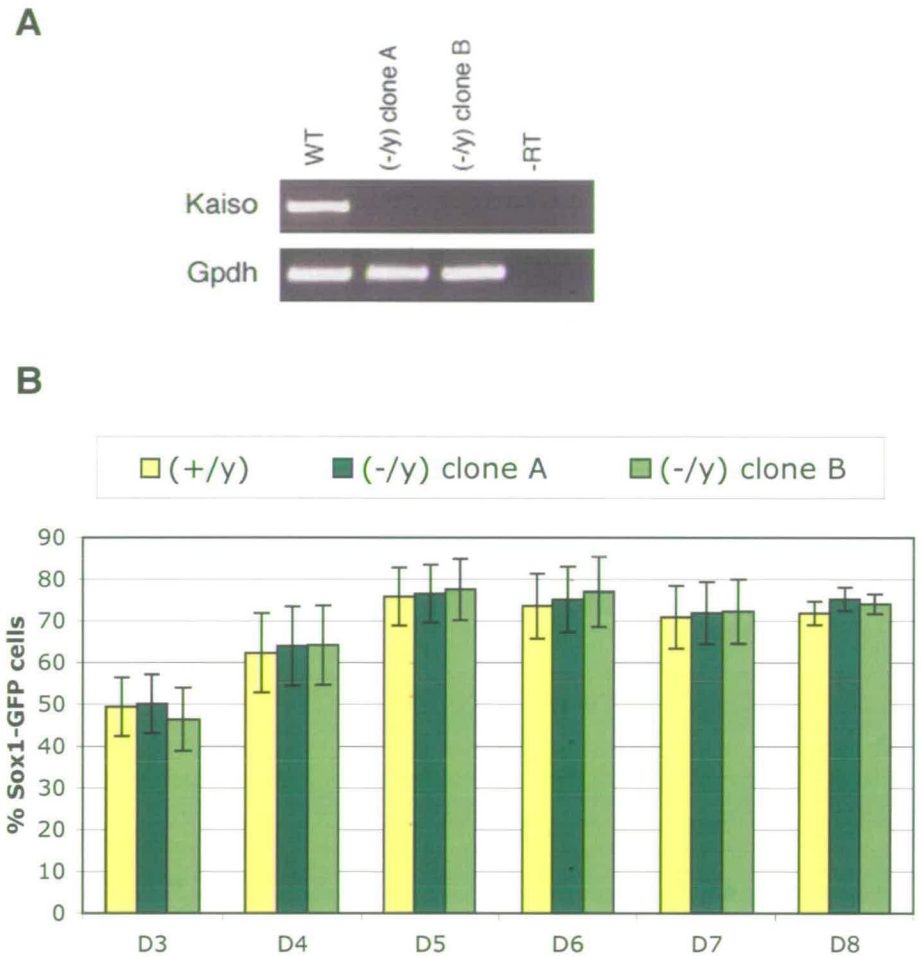
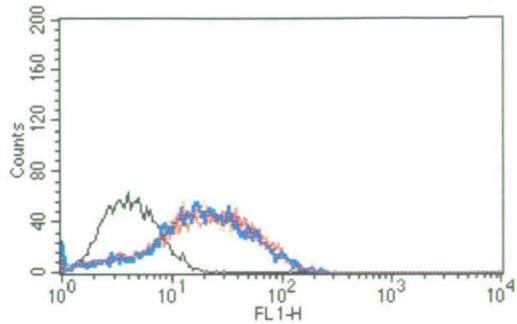
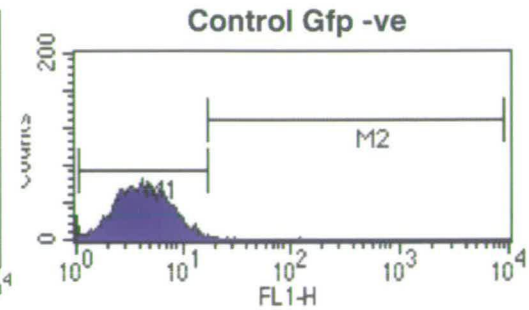
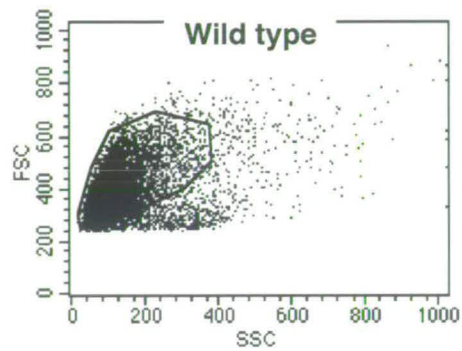


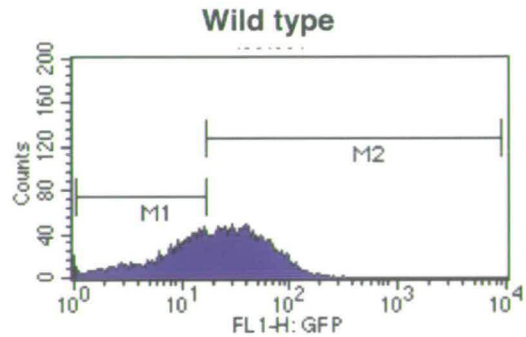
Figure 3.2 Kaiso is not required for neural precursor differentiation from ES cells. **A)** RT-PCR from two independent *Kaiso*$^{(-/y)}$ clones and wild type FACS-sorted Sox1-GFP cells. **B)** Quantification of Sox1-GFP positive neural progenitors generated from day 3 to day 8 during monolayer differentiation from two independent *Kaiso*$^{(-/y)}$ clones and its parental one wild type 46C cell line. Error bars indicate SEM of three independent experiments. Test Anova: p=0.980, hence samples are not significantly different. **C**) FACS analysis of typical neuroectoderm differentiation at D4. In the top left corner histogram of percentage of GFP populations for control cell line with non-modified cells (no Sox1-GFP knock-in), wild type cells (46C) and two independent null cell lines (A and B). Left below dot plots of cell granularity on the x-axis versus cell size. The gate represent all viable cells. On the right panel are histogram that represent fluorescence on the x-axis versus the number of cells on the y-axis. M1 is set to exclude 99% of control cells (non-modified) and M2 represent Sox1-GFP cell population.

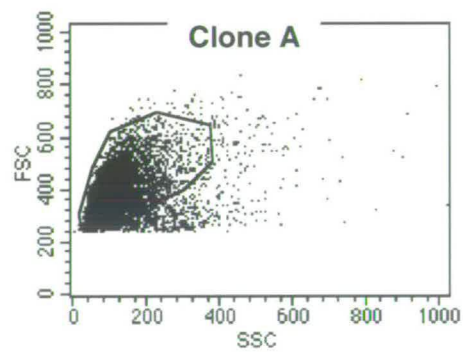
C

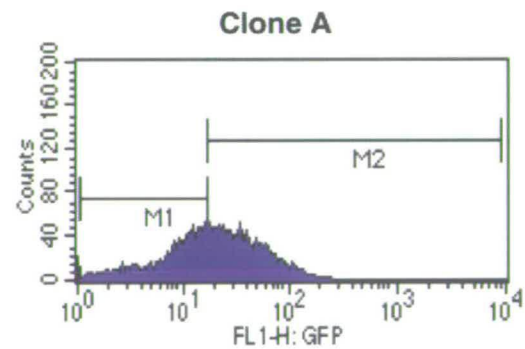

Counts
FL1-H

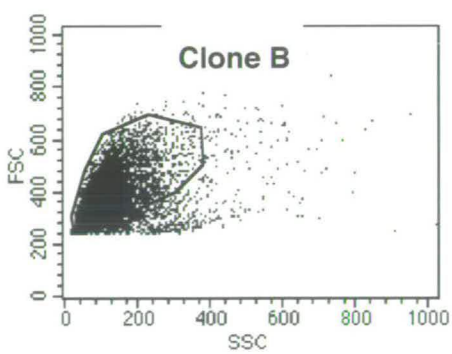

Control Gfp -ve
M1
M2
FL1-H

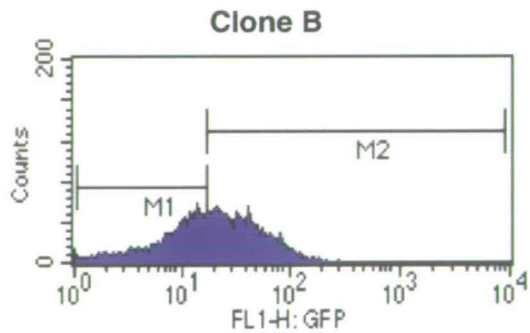

Wild type
FSC
SSC


Wild type
Counts
M1
M2
FL1-H: GFP


Clone A
FSC
SSC


Clone A
Counts
M1
M2
FL1-H: GFP


Clone B
FSC
SSC


Clone B
Counts
M1
M2
FL1-H: GFP

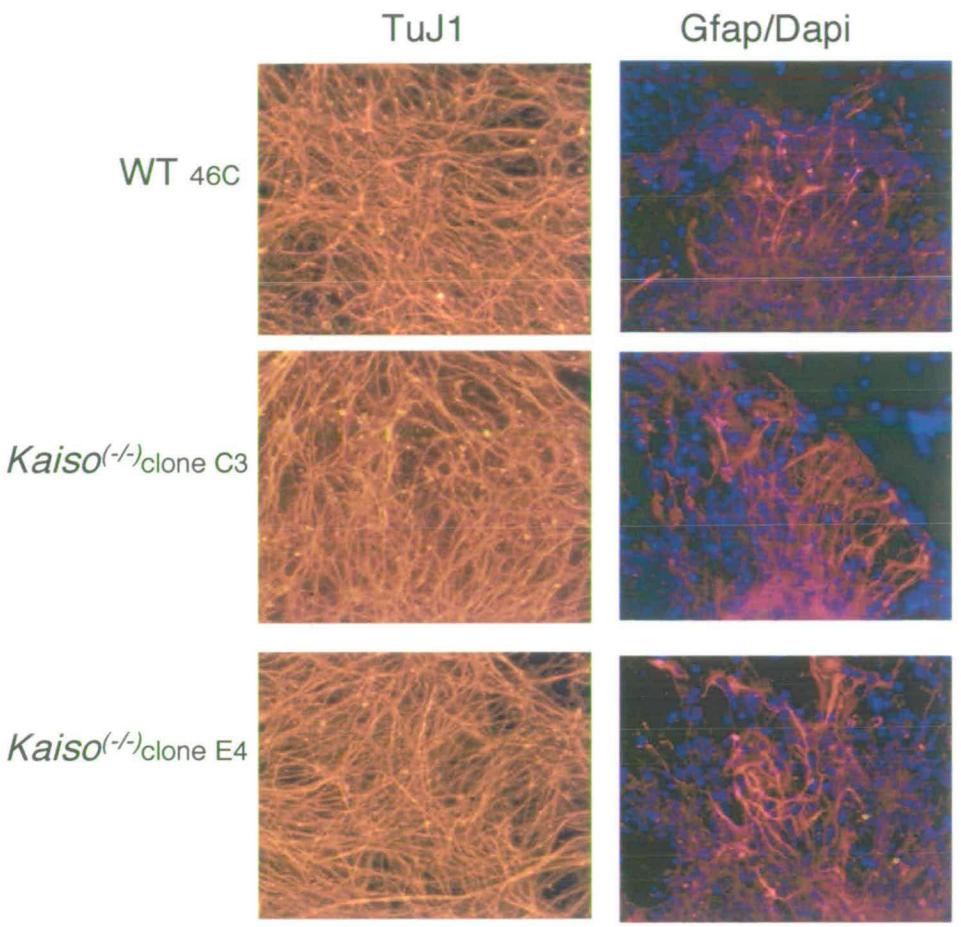


Figure 3.3 Kaiso is dispensable for neuron and astrocyte formation in the monolayer differentiation system. Left panels: wild type 46C cells, right panes: two independent *Kaiso*$^{(-/-)}$ differentiation cultures. Left panels: TuJ1; right panels: Gfap in red and Dapi in blue.

3.5 Study of neural stem cell function, self-renewal and differentiation.

Recently an *in vitro* system has been discovered to cultivate and maintain in monolayer culture a pure population of neural stem cells that undergo indefinite symmetric self-renewal divisions in the presence of the EGF and bFGF growth factors (Conti et al., 2005). This is an extraordinary useful tool to dissect neural stem cell function in the dish since analysis of pure clonigenic stem cells can be done. This is in remarkable contrast with neurosphere assays where cells undergo various differentiation stages within the neurosphere, or neuroepithelial primary cultures, where reproducibility is undermined by differences in dissection, various cell differentiation phases, and the inability to mantain long term cultures.

Since ES cell derived neuroectoderm differentiation is not affected by the lack of Kaiso, I decided to generate pure population of *Kaiso*$^{(-/y)}$ NS cells to specifically study the function of Kaiso from a cell status independent of ES cell differentiation. The previously studied *Kaiso*$^{(-/y)}$ C3 and E4 ES clones were chosen to derive NS cell lines. The derivation of such cell lines appeared normal compared with the control, as was their proliferation and self renewal. Immunostaining for key neural stem cell markers Rc2, Nestin and Vimentin revealed uniform *Kaiso*$^{(-/y)}$ NS cultures, where all the cells were positive for the three markers, and were indistinguishable from the 46C wild type NS line (figure 3.4).

Subsequently, *Kaiso*$^{(-/y)}$ NS cells were assayed for their differentiation potential into neurons and astrocytes. For differentiation into neurons, NS cells are plated on Poly-D-Lysine and Laminin coated wells for one week in media with N2 and bFGF only, and a second week with B27 only. After 15 days of differentiation, cells are fixed and immunostained for markers of neurons and astrocytes. The data processed revealed no observable impairment in differentiation into neurons and astrocytes (figure 3.5).

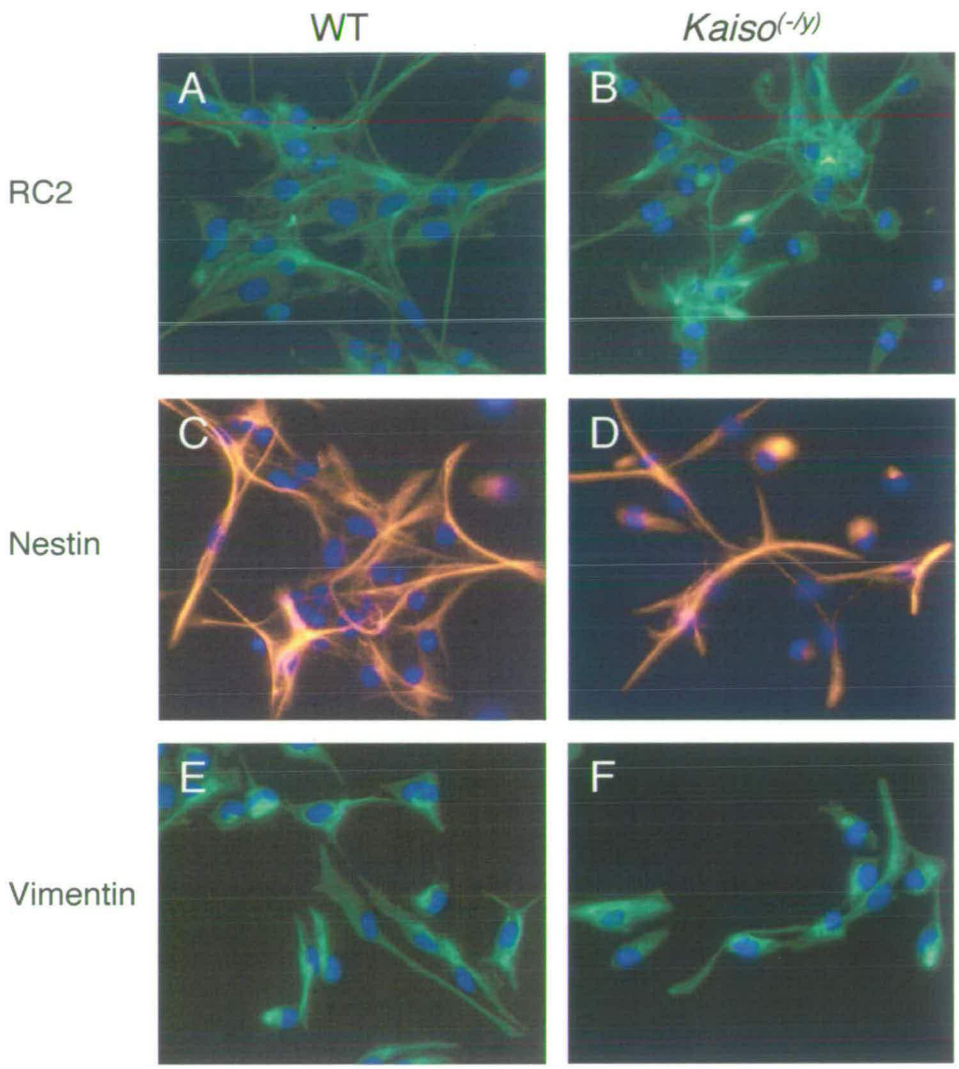


Figure 3.4 *Kaiso*$^{(-/y)}$ NS cells are indistinguishable from wild type 46C NS cells and do express neural stem cell markers Rc2 (top panels, green), Nestin (middle panels, red) and Vimentin (bottom panels, green). All cells are counterstained for Dapi (blue).

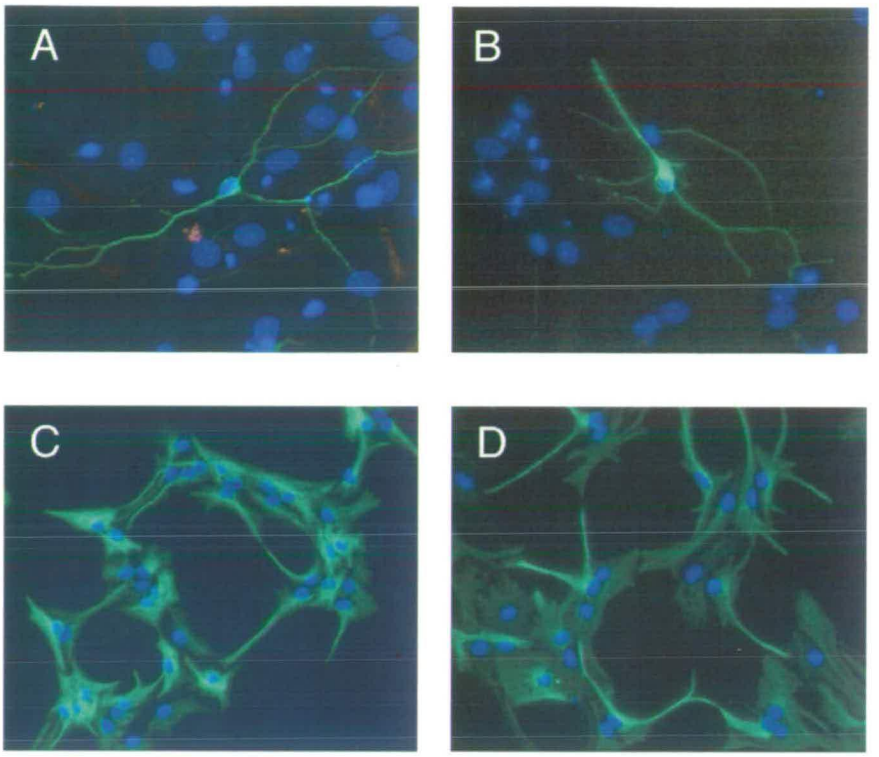


Figure 3.5 *Kaiso*$^{(-/-)}$ NS cells have normal differentiation potential. A and C wild type 46C, B and D *Kaiso*$^{(-/-)}$ NS cells. A and B TuJ1 in green, Dapi in blue. C and D Gfap in green, Dapi in blue.

3.6 Summary and discussion

Kaiso-null ES and NS cells were used to study the role of Kaiso in the proliferation, differentiation and self-renewal capacity of neural stem cells. *In vitro* neuroectoderm formation from *Kaiso*$^{(-/y)}$ was indistinguishable from wild type cultures and the frequency of neuronal and astrocytic differentiation was normal. Moreover, the capacity to form neural precursors, which was quantified by a clear-cut method, was remarkably identical between *Kaiso*$^{(-/y)}$ and wild type 46C parental lines. From these experiments I can conclude that Kaiso is not required for neural stem cell function. Depletion of xKaiso in *Xenopus* embryos causes an overall developmental arrest and cellular apoptosis (Ruzov et al., 2004). In contrast, my results presented here and parallel studies demonstrate that Kaiso is dispensable in mice (Prokhortchouk et al., 2006). This surprising dissimilarity between *Xenopus* and mice adds more evidence to a wide divergence between mice and *Xenopus* in DNA methylation-dependent function. This could be explained by species-specific developmental differences; in *Xenopus* zygotic transcription occurs later than mammalian embryos and the later may not be affected by the earlier transcriptional activation in the absence of Kaiso (Prokhortchouk et al., 2006).

In a recent study, *Kaiso* labelling by *in situ* hybridisation has been described to be uniform through the whole mouse brain, with a neuronal expression rather than glia (Della Ragione et al., 2006). Interestingly, my experiments with quantitative real time RT-PCR show astrocytes as the cell type with highest expression of *Kaiso* (see figure 4.1).

The localisation of Kaiso within the cell is still unknown, and the multiple Kaiso studies remain inconsistent whether localisation of Kaiso is predominantly nuclear or cytoplasmic. It will be interesting to study the Kaiso localisation further at the cell level, since it can bring insight into the function of this protein. I have tested several published Kaiso antibodies and none showed a Kaiso-specific band in western blot that was not present in null samples. Therefore, these antibodies probably also recognises Kaiso-like proteins.

It is rather surprising that ES and NS cells lacking a component of the N-CoR corepressor complex, which has been shown to be essential in neural stem cell

function, are indistinguishable from wild type cells in all the experiments described above. Kaiso is one of the four characterised methyl-CpG binding proteins. There is previous evidence of key genes involved in neurogenesis that can function redundantly such as the SoxB1 family (Wegner, 1999). Their overlapping roles have previously precluded the elucidation of the function of the individual proteins with the study of individual mice knock out models (Nishiguchi et al., 1998). It is plausible that functional redundancy occurs among this family of proteins and even in the absence of Kaiso, neural stem cells can function normally.

CHAPTER 4

INVESTIGATING THE ROLE OF THE METHYL-CPG BINDING PROTEINS IN NEURAL STEM CELLS.

4.1 Introduction

DNA methylation is essential for embryonic development since mice lacking the enzymes that establish and maintain methylated DNA cannot survive after day 9.5 dpc (Li et al., 1992; Okano et al., 1999). The cause of lethality is most likely due to an aberrant gene expression that causes disruption of developmental programs, while ES cells lacking methyltransferases remain immune to this disruption and can grow aphenotypically lacking normal DNA methylation levels (Li et al., 1992; Okano et al., 1998b). The maintenance methyltransferase Dnmt1 and *de novo* methyltransferases Dnmt3a and Dnmt3b are dynamically expressed in neurons throughout development and appear necessary for their function (Feng et al., 2005; Goto et al., 1994; Okano et al., 1999). Transgenic mice that specifically lack Dnmt1 in neural precursor cells from E9-E10 embryonic age have impaired neuronal function and die postnatally (Fan et al., 2001).

Mutations in the *MeCP2* gene cause the neurological disorder Rett syndrome in girls (Amir et al., 1999) and cause Rett-like phenotype in mice (Chen et al., 2001; Guy et al., 2001). Mice lacking Mbd2 are viable and fertile, but show gene missexpression in T cells (Hutchins et al., 2002) and mutant mothers show abnormal maternal behaviour with nurturing impairment that affect their pups development (Hendrich et al., 2001). Mice lacking Mbd1 are also viable and fertile, and no obvious abnormalities have been found in their brain structures. However, *Mbd1*$^{(-/-)}$ adult neural stem cells have a defect in neuronal differentiation which encompasses a defect in learning ability of the dentate gyrus region of the hippocampus (Zhao et al., 2003). This impairment was discovered with functional analysis *in vitro* of adult neural stem cells.

Therefore, disruption in the transduction of the methylation signal seems to trigger neural malfunction. While methyltransferase disruption has lethal consequences, the absence of methyl-CpG proteins leads to relatively mild

phenotypes with the exception of Rett-like phenotype in *MeCP2*$^{(-/y)}$ mice or no phenotype in the case of Kaiso$^{(-/y)}$ (previous chapter and Prokhourtchouk et al., 2006).

This leads to two interelated questions: why has evolution come up with four independent MeCPs in mammals to regulate transcription in methylated sites, and what are their functions? It is possible that methylated-dependent transcriptional repression occurs simultaneuously through multiple overlapping MeCPs to ensure gene silencing. Or it could be that their only function is fine silencing tuning and the only consequences of their disruption are visualised in complex neuronal function that we are barely beginning to understand.

In favour of the first possibility multiple methyl-CpG binding proteins have been shown to be associated with methylated genes *in vivo* (Fournier et al., 2002; (Ballestar et al., 2003; Fraga et al., 2003; Koizume et al., 2002), raising the possibility that these proteins may provide independent, but normally redundant, silencing activities at methylated genes. In favour of the second possibility is the slow but steady unveiling of specific and unique targets for the most extensively studied MeCP, MeCP2, involved in neuronal circuitry (Chen et al., 2003; Martinowich et al., 2003; Horike et al., 2005).

Hence, the question to be addressed in this chapter is: Do MeCPs display functional redundancy such that the absence of one has no overt consequence because there are still another three known proteins to apply methylated-dependent silencing, or is their function specialised in precise but distinct cell functions and therefore their absence has no major consequences?

To address this question, *Mbd2*$^{(-/-)}$*MeCP2*$^{(-/y)}$*Kaiso*$^{(-/y)}$ triple null mice (3KO) were engineered in our lab. 3KO mice are viable and fertile but develop same Rett-like phenotypes indistinguishable from those displayed by *MeCP2*$^{(-/y)}$ mice although they die earlier than do *MeCP2*$^{(-/y)}$ mice (Caballero et al., in preparation). This is evidence that the simultaneous lack of three MeCPs is more severe than MeCP2-deficiency, consistent with the hypothesis that the MeCPs are redundant. Therefore mice lacking three MeCPs have a stronger phenotype than single knock out.

Given this evidence and since mild phenotypes occurring in the absence of single MeCPs were only visualised through *ex vivo* analysis (Hutchins et al., 2002; Zhao et

al., 2003), I decided to investigate the function of MeCP2, Mbd2 and Kaiso simultaneously with the well characterised neural stem cell *in vitro* system.

4.2 Investigation of MeCP expression levels

Both Dnmt1 and MeCP2 are highly expressed in postmitotic neurons (Fan et al., 2001; Shahbazian et al., 2002b). This could be due to a DNA methylation independent role of these proteins, or as a strategy of the cell to ensure the necessary Dnmt1 and MeCP2 protein levels through life. The investigation of the gene expression levels on study is crucial to begin the understanding of their function. This was achieved with real time PCR on the cell types and tissues I have used through my experiments. A particularly important advantage in this experiment is the uniformity/purity that most of the cell types used in the extraction of RNA have.

The cell types chosen were:

- ES cells,
- Sox1 positive facs-sorted cells,
- Astrocytes: NS cells after 4 days of differentiation in the presence of 1-3% serum. In these conditions, 100% of the cells express the astrocytes marker Gfap.
- Neurons: 15 days NS differentiation cultures, where is believed that only 2 cell types are in majority: astrocytes and neurons, with a small population of undifferentiated Rc2 positive NS cells.
- Adult brain.

For the study of Mbd1 expression, triple null adult brain was also analyzed, in search of a possible upregulation of Mbd1 in the absence of other 3 Mbds, following the hypothesis of redundancy. I found an upregulation of Mbd1 of about 3 fold in the triple null brain sample, thus the upregulation of Mbd1 in the absence of MeCP2, Kaiso and Mbd2 could be caused by a compensation effect (figure 4.1).

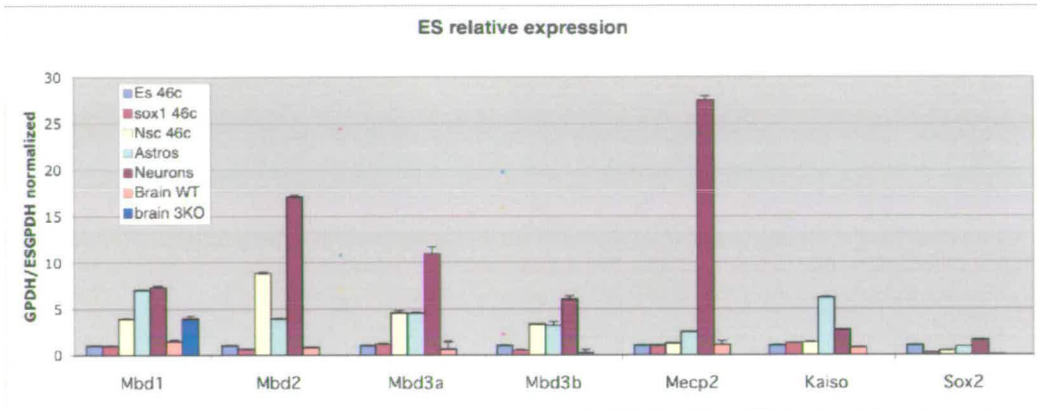
The expression of Mbd1, Mbd2, Mbd3a and Mbd3b, Kaiso and MeCP2 mRNA was analysed. Additionally, I included Sox2 as a reference gene for which expression levels are known. Sox2 is expressed in ES cells (Yuan et al., 1995); after neural induction, Sox2 is expressed in proliferating neural precursors along the entire antero-posterior axis of the developing embryo and is detected in neurogenic regions

in the postnatal and adult CNS (Ferri et al., 2004; Tanaka et al., 2004). Therefore, it is expressed in all the cell types chosen in the experiment.

As predicted, all the cell types express the four MeCPs studied throughout this thesis, plus Mbd1, and Sox2. The highest expression of MeCP2 is in neurons, being the highest expression among all the Mbds. This finding correlates with previous studies (Shahbazian et al., 2002) (figure 4.1).

To investigate whether MeCPs are expressed in NS cells, which have been used as a tool for this thesis, western blot and immunocytochemistry were performed for MeCP2 and western blot for Mbd2. From these experiments I can confirm the expression of both proteins in NS cells (figure 4.2).

A



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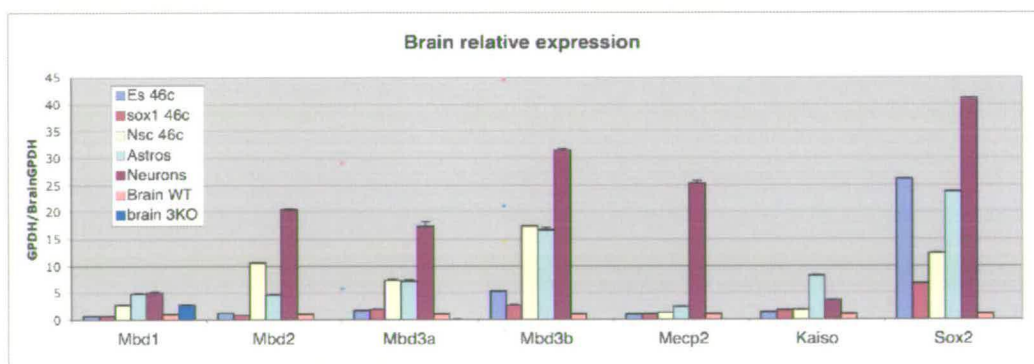


Figure 4.1.Real time RT-PCR expression with the cell types of study. Upper panel normalised against ES cell (46C ES cells) expression levels. Lower panel normalised against adult brain expression. All expression levels were measured in triplicate. Error bars indicate SEM.

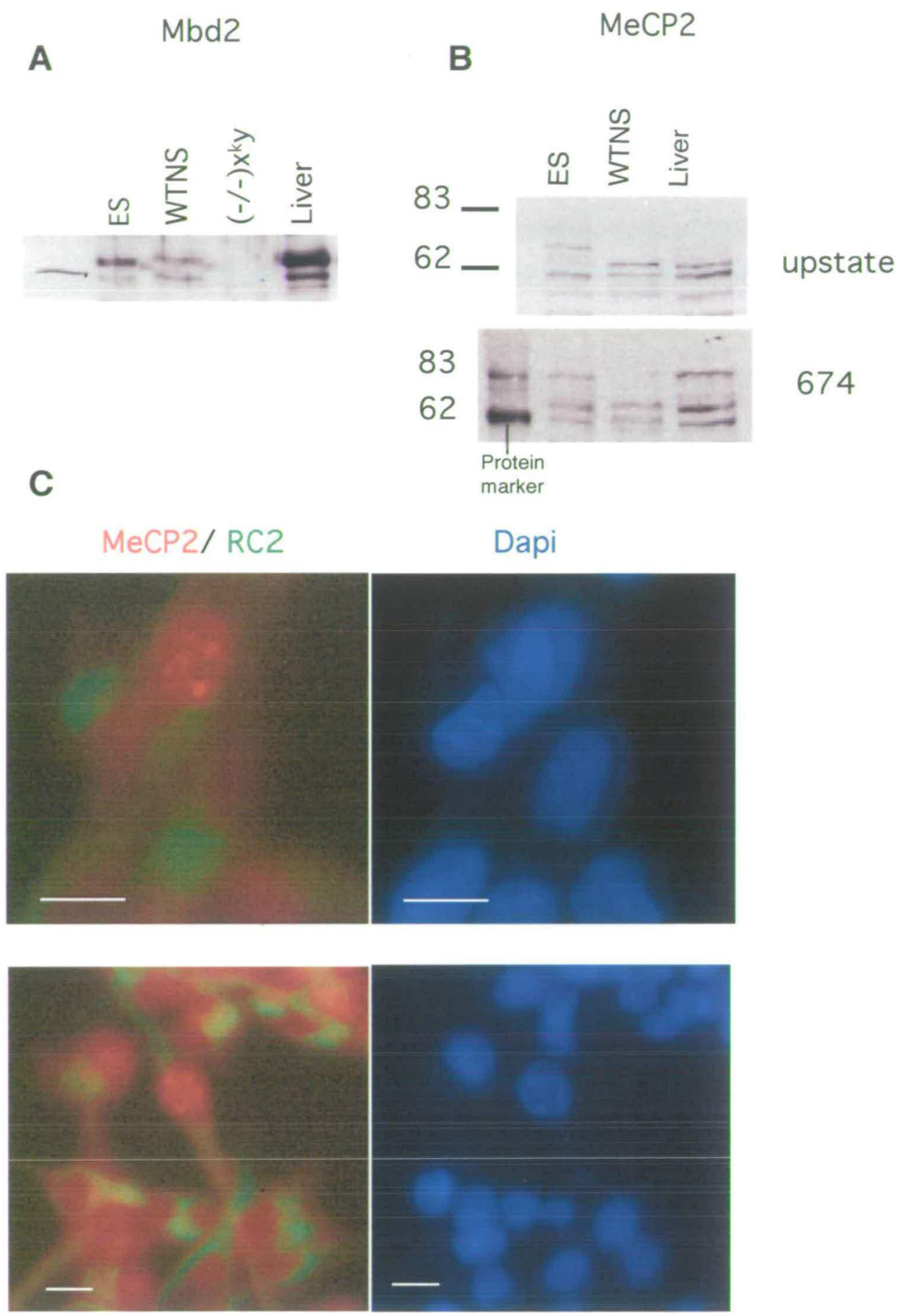


Figure 4.2. **A)** Western blot for Mbd2 in ES, wild type NS and *Kaiso/Mbd2* null NS cells, (-/-)$x^{K}y$, nuclear protein (from left to right). **B)** Western blot of MeCP2 with NS nuclear protein: upper, anti-MeCP2 (from Upstate); lower anti-MECP2 674 (Nan et al., 1998). **C)** Immunofluorescence for anti-MeCP2 in wild type NS cells. MeCP2 in red, Rc2 in green, Dapi in blue. Scale bars represent 20μm

4.3 Electrophoretic mobility assays

The defining characteristic of the MeCPs is their ability to bind methylated DNA; to a single mCpG dinucleotide in the case of MeCP2 and Mbd2 and to a double or triple mCpG dinucleotide in the case of Kaiso. Therefore, it is necessary to determine whether these proteins do bind to methylated DNA within NS cells and to determine whether the triple null NS cells contain the known MeCP activity encoded by Mbd2 and Kaiso (Prokhourtchouk et al., 2001; Hendrich et al., 2001).

The electrophoretic mobility shift assay (EMSA) is based on the observation that protein-DNA complexes migrate more slowly than free DNA molecules when subjected to non-denaturing polyacrylamide or agarose gel electrophoresis (Revzin, 1989). EMSA has been the key assay to investigate the properties of the family of methyl-CpG binding proteins, and its binding pattern is well characterised (Cross et al., 1997; Hendrich and Bird, 1998; Hendrich et al., 2001; Meehan et al., 1992). A methylated or unmethylated probe (CG11) containing four HhaI sites (GCGC) and two HpaII sites (CCGG) (Meehan et al., 1989) was incubated with NS cell nuclear extract in the presence of competing bacterial DNA. The mix was loaded on 1.5% agarose gel to resolve protein-DNA complexes from free DNA and subsequently an autoradiography revealed the complexes formed.

As expected, nuclear extract from wild type NS cells bound the methylated probe MeCG11 forming a shift band of similar size to the well-characterised doublet band in liver nuclear extract (Meehan et al., 1989). This doublet band is formed by two complexes, a slow migrating complex (complex I), and a faster migrating complex (complex II) which is not dependent upon Mbd2 for its formation (Hendrich et al., 2001) . On the other hand, both $Mbd2^{(-/-)}MeCP2^{(-/y)}Kaiso^{(-/y}$ NS extract and $Mbd2^{(-/-)}Kaiso^{(-/y)}$ NS cell nuclear extract produced very weak binding. From these experiments I can conclude that NS cells do have methyl-CpG binding activity (figure 4.3).

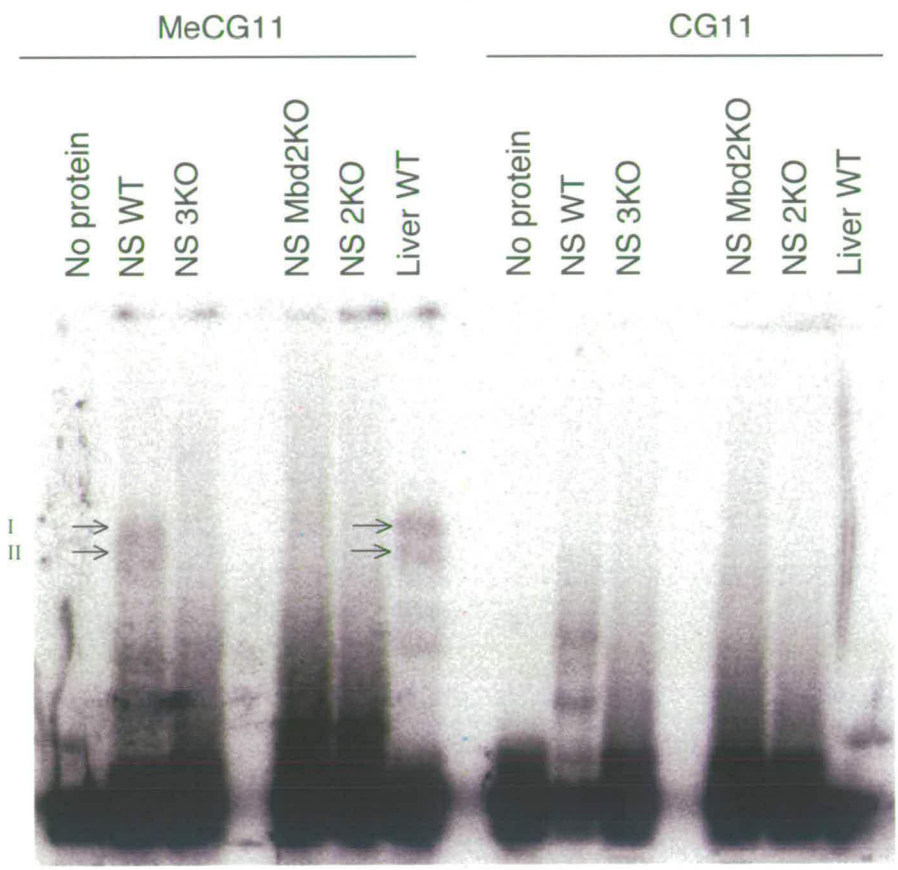


Figure 4.3. EMSA with methylated probe MeCG11 (left) in comparison with unmethylated probe CG11 (right). In the MeCG11 experiment, a doublet (indicated by two arrows (complex I and II)) is shifted in wild type NS cell nuclear extract as well as wild type liver, meanwhile triple *MeCP2/Mbd2/Kaiso* NS cell nuclear extract (NS 3KO) and double null *Mbd2/Kaiso* NS cell nuclear extract (NS 2KO) does not. *Mbd2* null NS (Mbd2KO) shift a faint (complex II) that is not present in the unmethylated panel.

4.4 Neurosphere assays from embryonic cortex

Embryonic age E14.5 is the peak of neurogenesis in mice, and E16 is the starting point of astrocytogenesis (Ghosh et al., 1995). Both stages were chosen to study neurosphere formation from mouse embryonic forebrain.

The neurosphere assay is a well-established method that has been extensively used in the field to study neural stem cell function. Hence, I used the neurosphere assay as a tool to investigate whether MeCP2, Kaiso and Mbd2 play redundant role in neural stem cells. First, I studied the proliferation capacity of single, double and triple null neurospheres (see table 4.1) by analysing the frequency that neurospheres were formed from single cells. Neurospheres were measured after a week of culture in size and number.

Single null	*Mbd2*$^{(-/-)}$	*MeCP2*$^{(-/y}$	*Kaiso*$^{(-/y)}$
Double null	*Mbd2*$^{(-/-)}$*MeCP2*$^{(-/y)}$	*MeCP2*$^{(-/y)}$*Kaiso*$^{(-/y)}$	*Mbd2*$^{(-/-)}$*Kaiso*$^{(-/y)}$
Triple null	*Mbd2*$^{(-/-)}$*MeCP2*$^{(-/y)}$*Kaiso*$^{(-/y)}$		

Table 4.1 Mouse genotypes analysed by neurosphere assay.

Secondly, to study the stem cell self-renewal ability in the absence of MeCPs, neurospheres that had been cultured for one week were dissociated to single cells in cloning conditions to study the ability of each single cell to form a new neurosphere. Again the frequency of neurosphere formation and the size were measured.

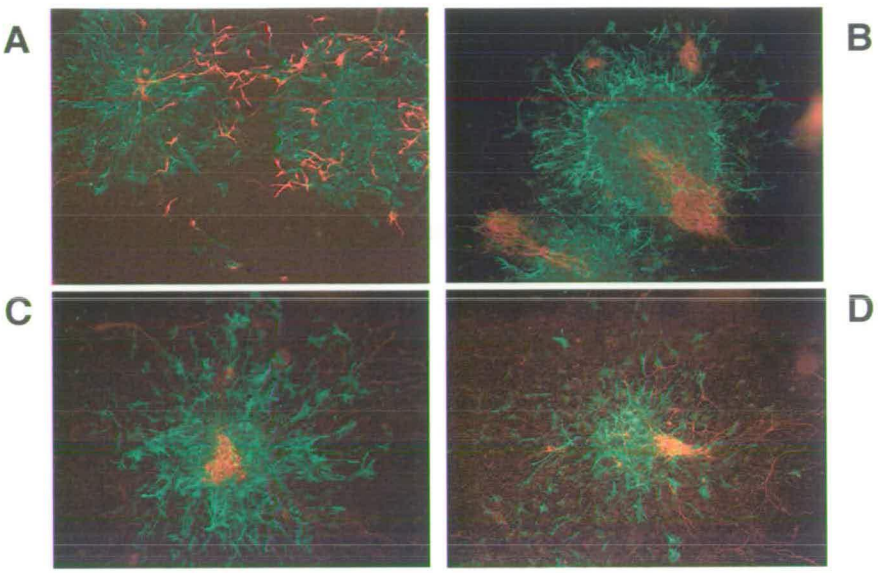
The data obtained from these assays was highly variable within the different clones obtained from different embryos, regardless their genotype. Therefore, no conclusions could be obtained and the data has been disregarded. This observation will be discussed at the end of the chapter.

Finally, to study the potential of neurospheres to differentiate into two different post-mitotic cell types, neurons and glia, neurospheres were cultivated for one week, plated on PDL/Laminin without growth factors and maintained in differentiation medium for 5 days. After this time, cells were immunostained to analyze the number of multipotent neurospheres giving rise to both neuronal and astrocytic cell types, or neurospheres with only astrocytes or only neurons.

Additionally, the same differentiation assay was performed from the second neurosphere generation obtained.

This assay proved to be the only informative neurosphere assay, and I could conclude that all the neurosphere populations derived from single, double and triple mutant and wild type brains had normal differentiation potential, being multipotent in 80% for first neurospheres and 60% for second neurospheres (figure 4.4).

1.



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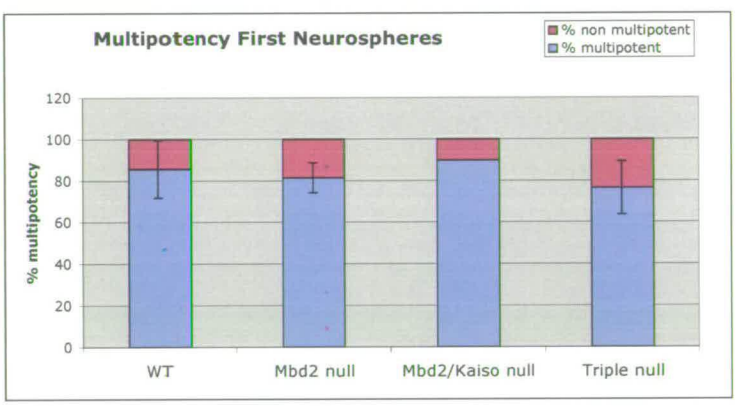


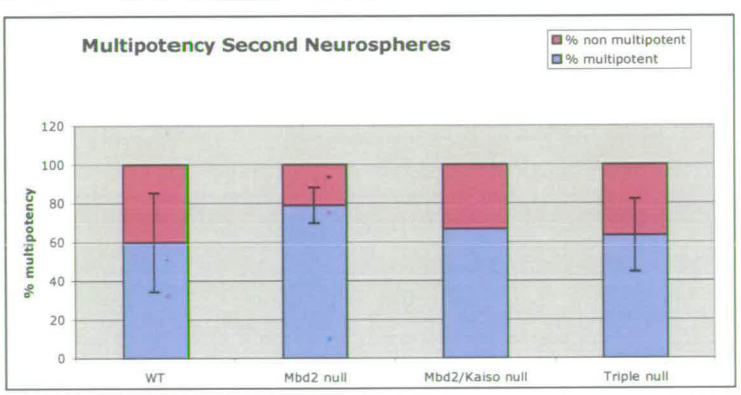


Figure 4.4 Neurosphere assay. 1. Neurosphere ICC after 5days in differentiation conditions. A: Wt, B: Mbd2 null, C: Kaiso null, D Kaiso/Mbd2/MeCP2 null. TuJ1 in red, GFAP in blue 2. Multipotency 1st and 2nd Neurospheres, % colonies Gfap positive/TuJ1 positive or only Gfap positive.

4.4 Derivation of neural stem cell lines from embryonic cortex.

As described before, a neural stem cell must be able to proliferate indefinitely, have unlimited self-renewal capacity and be clonogenic. Additionally, neural stem cells must be multipotent, giving rise to differentiated cell types by asymmetric division (Reynolds and Weiss, 1996). Since the neurosphere assay was not able to address the question of self-renewal and proliferation, I took the advantage of the recent discovery of NS cells, that as I described previously, are a pure population of neural stem cell that can be maintained in monolayer conditions (Conti et al., 2005), and are a very useful tool to address the questions I needed to study.

As in the neurosphere assays, embryonic stages E14.5 and E16.5, which are the beginning of neurogenesis and astrocytogenesis respectively, were chosen. Embryonic forebrain was extracted out from the embryonic brain, and the cortex was dissected. The tissue was dissociated and single cells were plated regardless of cell density in the presence of EGF and bFGF. After two or three days cells were forming neurospheres and these were collected and plated on gelatin. After several days, characteristic cells attached to the flask were dispersing out of the neurosphere. These are the derived NS cells that, after several passages, will form a pure population of neural stem cells. The cell lines that were derived are described in table 4.2.

Embryonic age		E14.5	E16.5		E14.5	E16.5		E14.5	E16.5
Wild type		√	√						
Single null	*Mbd2*$^{(-/-)}$	√	√	*MeCP2*$^{(-/y)}$	√	√	*Kaiso*$^{(-/y)}$	√	√
Double null	*Mbd2*$^{(-/-)}$ *MeCP2*$^{(-/y)}$	√					*Mbd2*$^{(-/-)}$ *Kaiso*$^{(-/y)}$	√	√
Triple null	*Mbd2*$^{(-/-)}$ *MeCP2*$^{(-/y)}$ *Kaiso*$^{(-/y)}$				√				

Table 4.2 Genotypes of NS cell lines derived from embryonic cortex.

However, because previous experiments with neurospheres showed a tendency for overlapping characteristics regardless of the genotype, I decided to concentrate on the investigation of triple null versus wild type genotypes.

4.5 Characterisation of NS lines

Three independent E14.5 triple null NS cell lines were selected to characterise their neural stem cell function.

4.5.1. Neural stem cell marker expression:

Cell lines were screened for the proper expression of neural stem cell markers Rc2, Nestin and Vimentin, and the absence of differentiated cell markers TuJ1 and Gfap. This was done at several points during more than 20 passages within these cell lines that have been studied (figure 4.5).

One working hypothesis was the possibility of spontaneous astrocytic differentiation during the culture of NS cells or, the possibility of early astrocytic differentiation. This hypothesis was driven by the observation that global hypomethylation, caused by deletion of *Dnmt1* in mice, causes precocious astrocytogenesis in the developing central nervous system, due to demethylation of a Stat binding element within the *Gfap* promoter (Takizawa et al., 2001) and the overall elevation in Jak-Stat signalling activity (Fan et al., 2005). *s100β, GFAP* and *Stat* promoters display highly methylated CpGs in neuroepithelial cells at early stages of development, and lose their methylation at later stages, in parallel with the onset of neurogenesis in the developmental brain. These three promoters are associated with MeCP2 during the highly methylated neurogenic stages of the neuroepithelial cells (Fan et al., 2005). As explained before, the absence of three methyl-CpG binding proteins could result in removal of compensation activities and result in a phentoype that resembled the *Dnmt1* knock out phenotype. However, I found no precocious or spontaneous astrocytic differentiation in the triple null NS cell cultures. Therfore, I can conclude that the absence of Kaiso, MeCP2 and Mbd2 does not cause early or spontaneous astrocytic differentiation in NS cells.

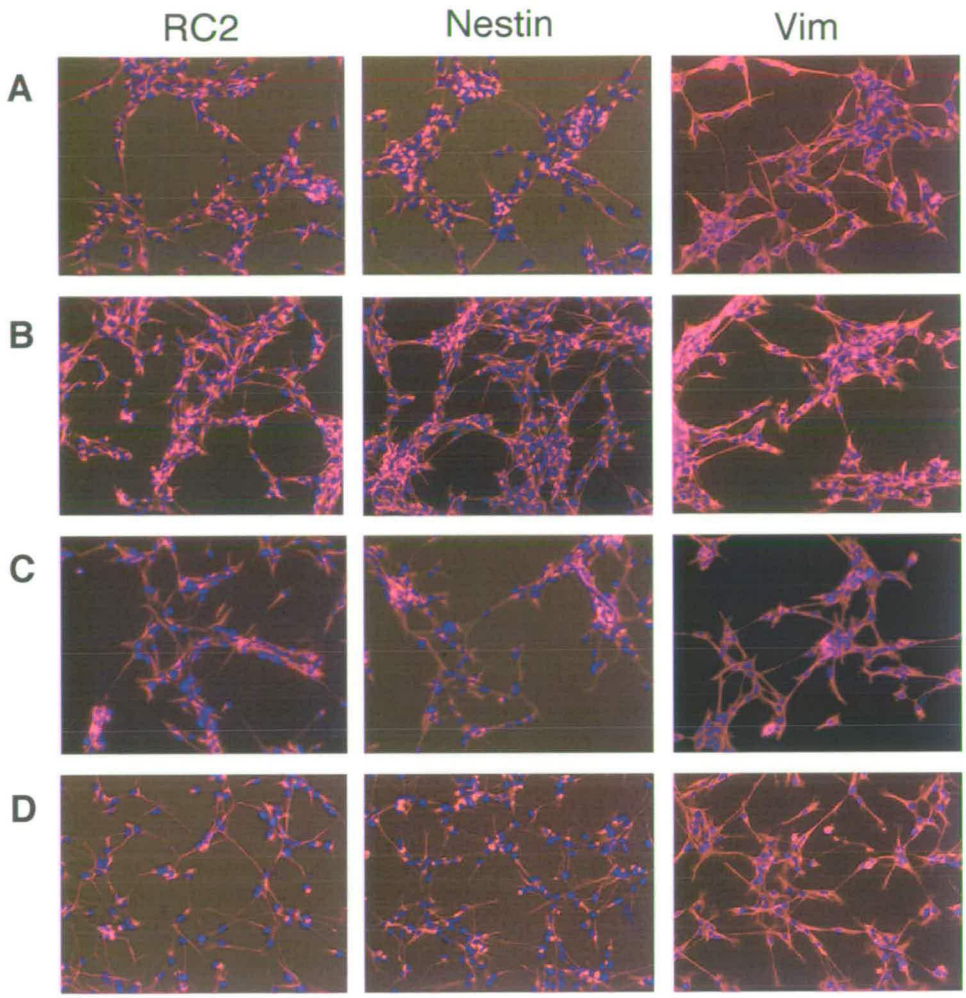


Figure 4.5. Characterisation of triple NS cell lines. Immunocytochemistry of neural stem cell markers Rc2, Nestin and Vimentin in red, Dapi in blue. A Wild type NS clone 4; B, C and D: 3 independent MeCP2/ Kaiso/Mbd2 triple null NS clones.

4.5.2 Proliferation

The MTT assay measures the bioreduction of the tetrazolium compound MTT to a coloured formazan product by the mitochondria of living cells, and therefore colorimetric analysis provides an accurate quantification of cell proliferation . To study the proliferation rate of the triple null NS cell lines, MTT assays were performed in 5 independent experiments for 2 different cell lines of each genotype, and 3 independent experiments for an extra cell line of each genotype. The conclusion of these experiments is that proliferation of NS cells is not affected by the simultaneous absence of MeCP2, Mbd2 and Kaiso as measured by the mitochondria respiration compared with wild type NS cells (figure 4.6).

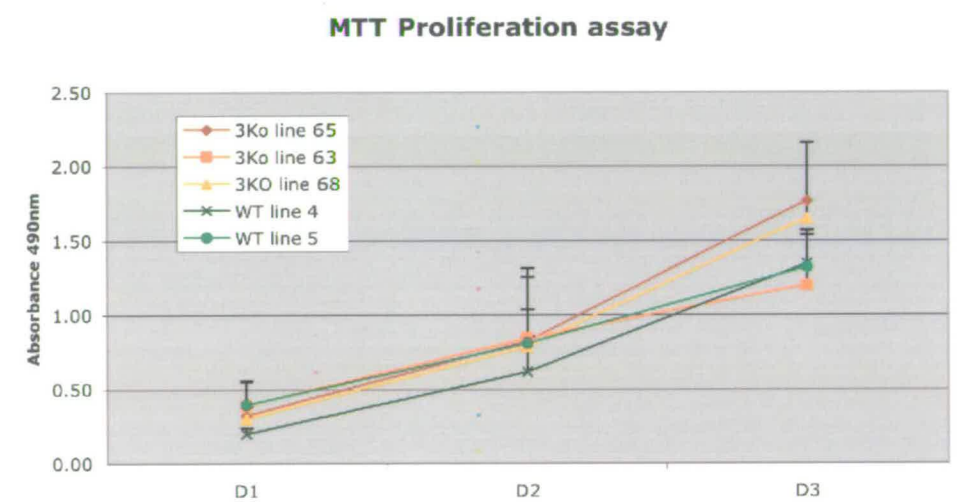


Figure 4.6 Triple null NS cells proliferate at a normal rate. NS cell proliferation rates were compared by MTT assay. Data for three independent MeCP2/Kaiso/Mbd2 triple null NS cell lines and two independent wild type NS cell lines are shown.

4.5.3 Self-renewal

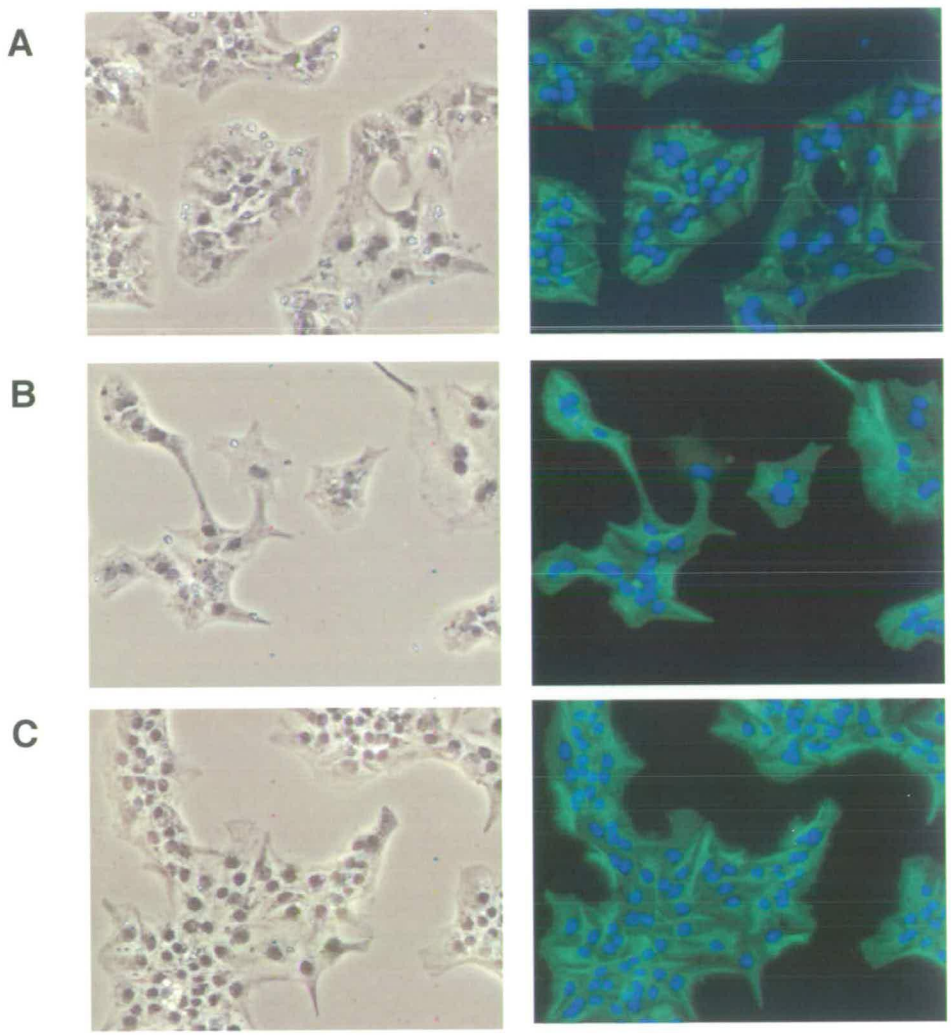
Triple null NS cells were maintained for between 20 and 60 passages without displaying differences in growth rate, maintaining the expression of neural stem cell markers and retaining the ability to give rise to neurons and astrocytes when plated on differentiation conditions. Triple null NS cells were able to form colonies from single cells at a normal rate compared with wild type.

4.6 Astrocytic differentiation potential

NS cells are able to differentiate to Gfap expressing astrocytes in less than 48 hours in the presence of serum or Bmp4 and in the absence of growth factors (Conti et al., 2005). Lif and Bmp signalling share gp130 as a signal transducing receptor component in the activation of the Jak/Stat pathway (Fan et al., 2005; Nakashima et al., 1999b). As mentioned above, one working hypothesis was the possibility of early astrocytic differentiation in 3KO NS cells. However monitoring of differentiation at 8h, 12h, 20h, 24h time points I did not observe any difference in astrocytic differentiation between triple null and wild type NS cells. Both wild type and triple null lines were able to differentiate to astrocytes normally in more than 5 independent experiments where cell density, serum concentration and presence/absence of LIF were tested along the ~40 passages of three independent 3KO NS cell lines.

Reverse transcription analysis revealed that both wild type and 3KO astrocytes express the expected genes of astrocytes: Gfap and s100β; as well as Blbp (Feng et al., 1994) (figure 4.7.D). Interestingly, neural stem cell markers such as Nestin and Pax6 were also expressed in some cell lines regardless of genotype. These results could suggest that not all the cells are fully differentiated into astrocytes .

Figure 4.7 Triple null astrocytes are indistinguishable from WT. Immunostaining after 4 days of NS cell culture in presence of serum and removal of growth factors. Left panels: bright field. Right panels: GFAP in green, Dapi in blue. A WT NS cells derived astrocytes. B and C two independent 3KO NS-derived astrocytes. **D** RT-PCR of astrocyte and NS cell markers. Cell types from left to right: 2 wild type NS-derived astrocytes (4 and Cor1) two triple null NS-derived astrocytes, one wild type NS cell line, one 3KO NS cell line, Adult brain and ES cells.


A
B
C
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WT4
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WT NSC
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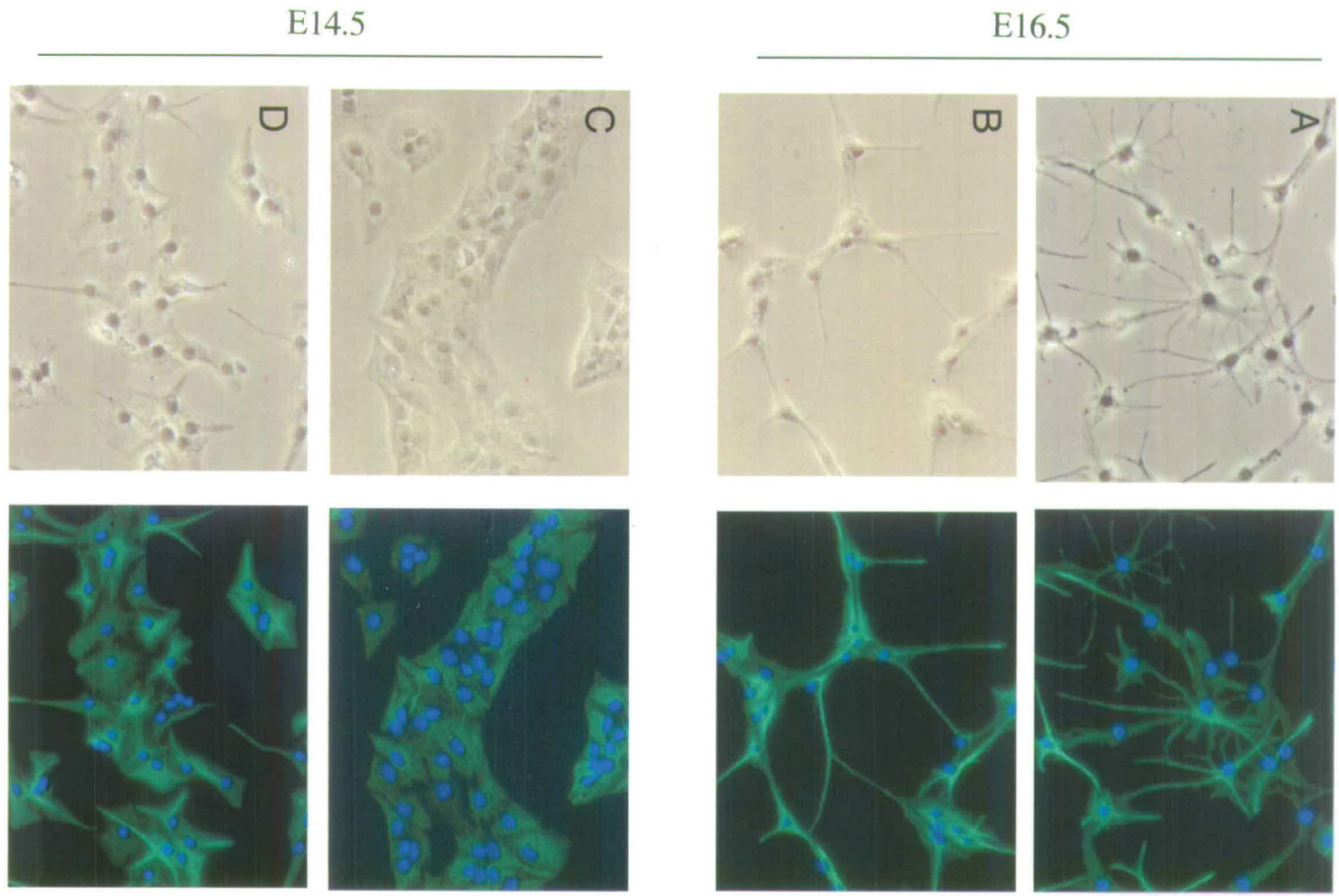
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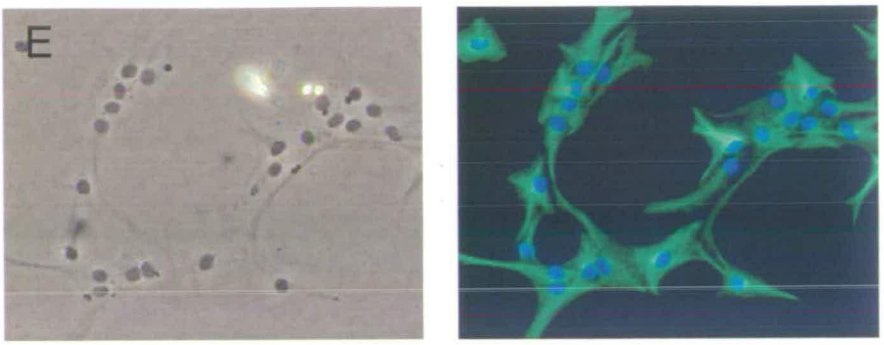
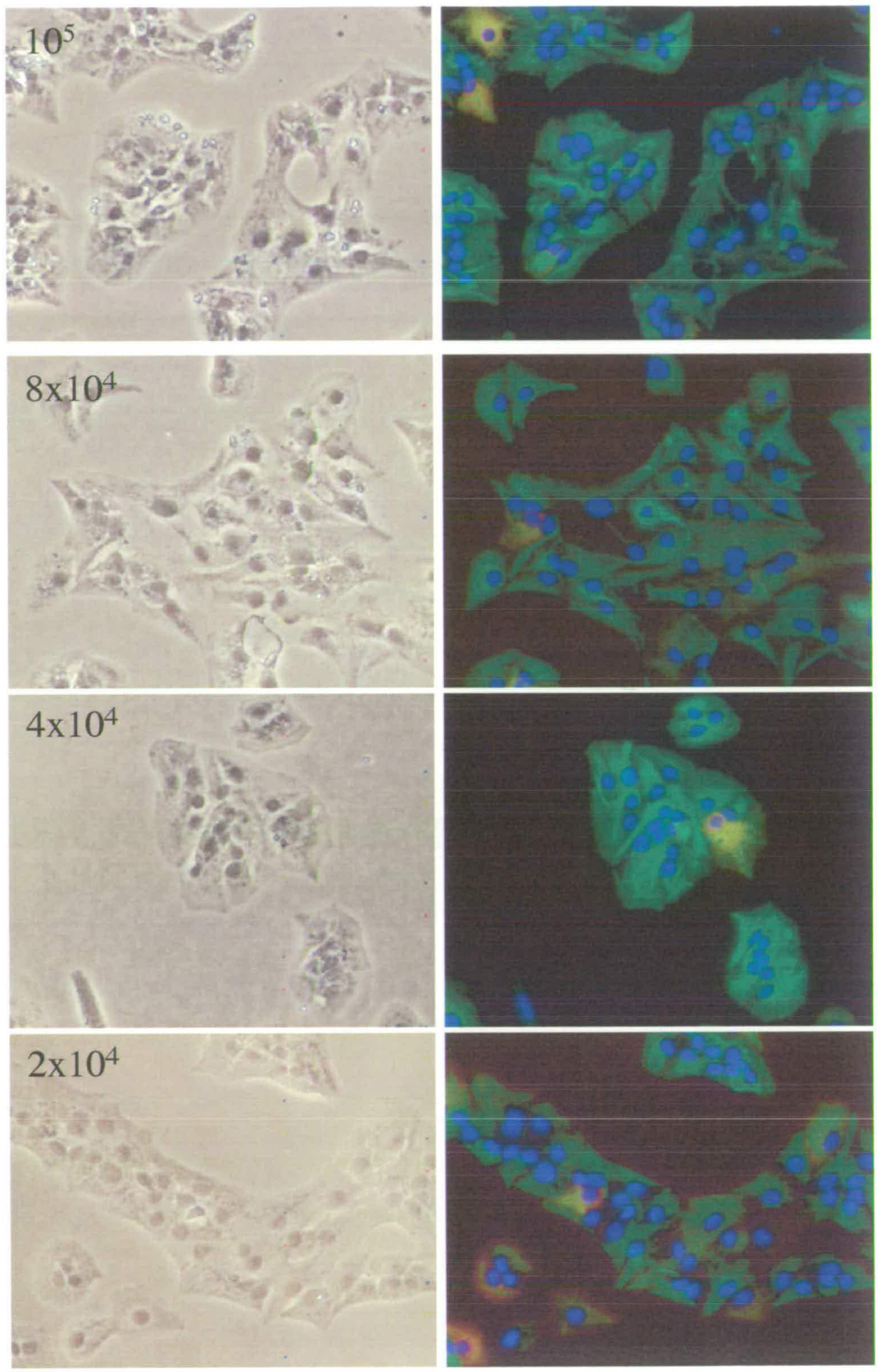


Figure 4.8 Astrocytes differentiated from E14-derived NS cells have different morphology than E16-derived NS cells. A and B: two independent E16.5 derived NS cell lines. C and D: two independent E14.5 derived NS cell lines. E: Astrocytes differentiated from ES-derived NS cells. Left panels: bright field; Right panels: Gfap in green, Dapi in blue.

However, during the process of characterization, I have observed a morphological divergence that make astrocytes differentiated from E14.5-derived NS cells (E14A) distinguishable from astrocytes differentiated from E16.5-derived NS cells (E16A) independently of their genotype. E16A were spiky, with long processes and connections to neighbour cells while E14A were short processed and fibrous with more cytoplasmic surface (figure 4.8). This different morphology could be a cell density effect, given that in a less dense environment cell-cell contact could be affected. I investigated further the morphology that E14.5 and E16.5 NS derived astrocytes were having at different densities. Wild type E14.5 derived NS cells were plated for astrocytic differentiation at densities of X, 2X, X/2, and 12.5X. In these experiments I observed no morphological difference between different densities and no similar morphology to E16.5 in the more dense cultures. Therefore I conclude that the morphological difference is independent of cell density at the time of differentiation (figure 4.9).

Figure 4.9 Astrocyte morphology is independent of cell density during differentiation from NS cells. E14.5 embryonic age wild type NS derived cells were plated at densities of 10^5, $8x10^4$, $4x10^4$ and $2x10^4$cells/well and cultured in astrocytic conditions without observing morphological differences. Left panel bright field. Right panel: Gfap in green, Rc2 in red and Dapi in blue.


10^5
$8x10^4$
$4x10^4$
$2x10^4$

The cause of a cell morphology difference is difficult to dissect; immunostaining markers were similar in both cases, with all cells being Gfap positive. Additionally, in both groups there was always present a small population of Rc2 positive cells that were simultaneously Gfap positive.

Radial glia and subventricular zone neural stem cells differentiate into primarily protoplasmic astrocytes. NS cells are believed to have characteristics of radial glia, and are indeed derived from cells residing where radial glia are located, lining the subventricular zone. However, protoplasmic astrocytes hardly express Gfap while NS derived astrocytes do express Gfap. Therefore, the astrocytic morphology expected to be predominant from NS cell differentiation would be closer to E14A morphology than E16A morphology.

Pure NS cell cultures are most likely to be independent of the embryonic age from which they were derived. This independency can be due to two explanations: first, neural stem cells *in vivo* at those stages are immersed in a different concentration of growth factors and other signalling molecules, while *in vitro* are exposed to the exactly same conditions and concentration of growth factors. Secondly, the effect of cultivating and passaging the cells is likely to unify the age-derived possible differences. However, morphology is indeed different in two E16.5 NS derived astrocyte cultures compared with two wild type E14.5 NS derived astrocyte cultures, and it is independent of the cell density. Furthermore, ES-derived NS cells differentiate into astrocytes resembling the type from E14.5 NS cell-derived. This difference is in relation with the findings that cultured radial cells produce different progeny depending on the age where they were isolated, being primarily neuronal colonies with few glial or mixed colonies when isolated from E14-E16 embryos and mostly glial colonies with few neuronal colonies when isolated from E18 (Qian et al., 2000; Takizawa et al., 2001; Malatesta et al., 2000). In conclusion, the different morphology of the astrocytes differentiated from E14.5-derived NS cells and E16.5-derived NS cells could be due to an epigenetic readiness after E16 to differentiate into astrocytes.

4.6 Neuronal Differentiation potential

To investigate the role of the simultaneous action of MeCP2, Mbd2 and Kaiso in neuronal differentiation, I next studied the capacity of 3KO NS cells to form post-mitotic neurons with wild type parallel experiments as comparison. NS cells were plated on PDL/Laminin for a period of 7 days in the presence of bFGF and further 7 days without growth factors in the presence of B27 for nutrition of the neurons. This protocol yields the highest NS neuronal differentiation (Conti et al., 2005). After 15 days in differentiated conditions three populations of cells could be identified: post-mitotic neurons, astrocytes and Rc2 positive NS cells. In these conditions, post-mitotic neurons marked by TuJ1 were in the 3KO NS cell cultures at the same frequency as wild type (figure 4.10). Hence, 3KO NS display normal span neuronal differentiation.

However, neuronal differentiation frequency achieved after only 7 days, where bFGF was still present, was remarkably lower than in wild type NS cells. Three NS lines derived from three independent E14.5 triple null embryos were investigated in comparison with four independent wild type cell lines, which one was derived from E16.5 wild type embryo and three from three independent E14.5 wild type embryos. The rate of Tuj1 positive neurons was dramatically reduced in the triple null cultures with statistical significance ($p<0.0024$) according to the Bonferroni method (Gordi and Khamis, 2004). In summary, while a normal differentiation span leads to indistinguishable neuronal differentiation capacity between wild type and triple null NS cells, 3KO NS cells show an initial delay in neurogenesis as compared to wild type NS cells (figure 4.11).

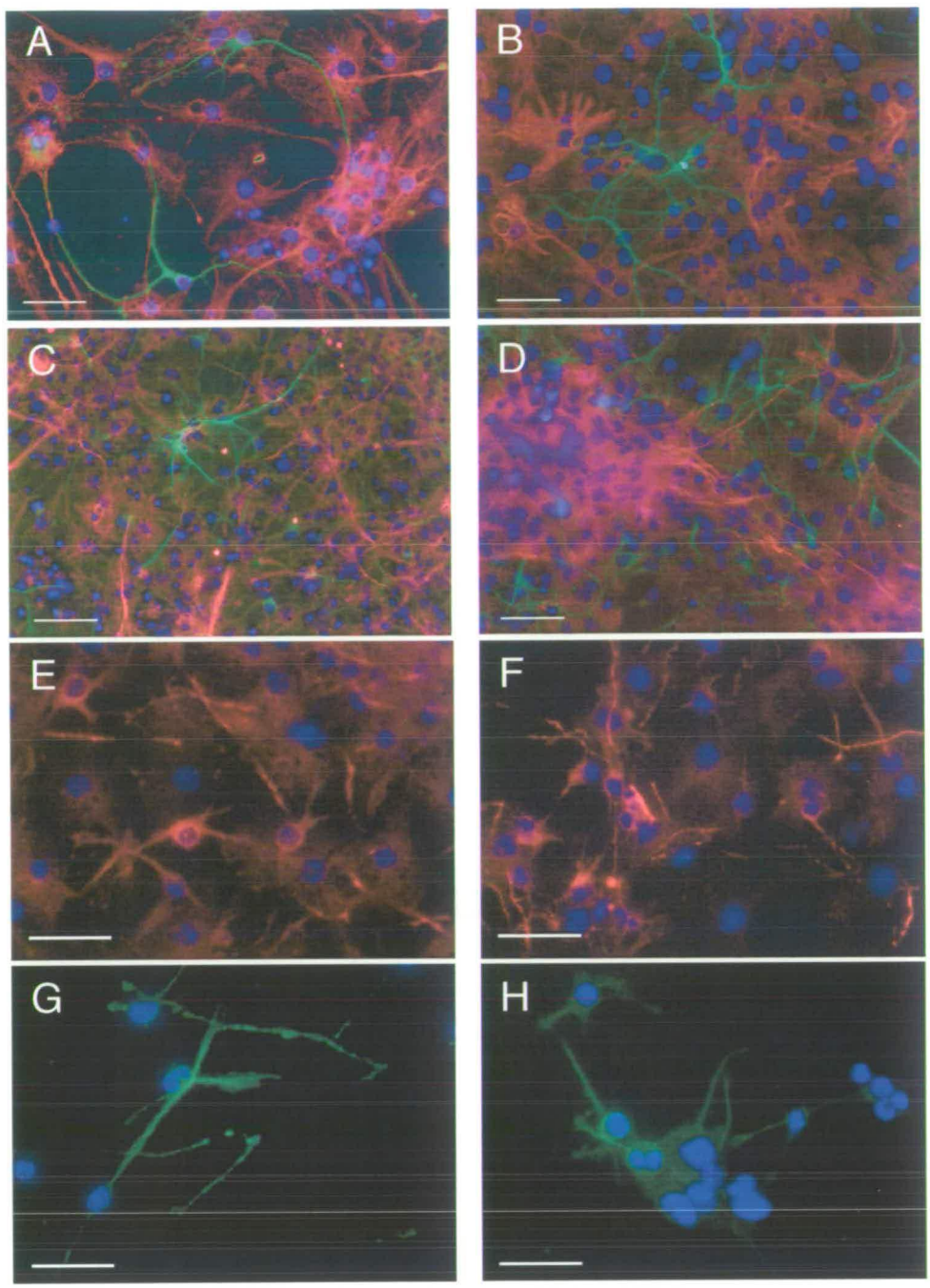
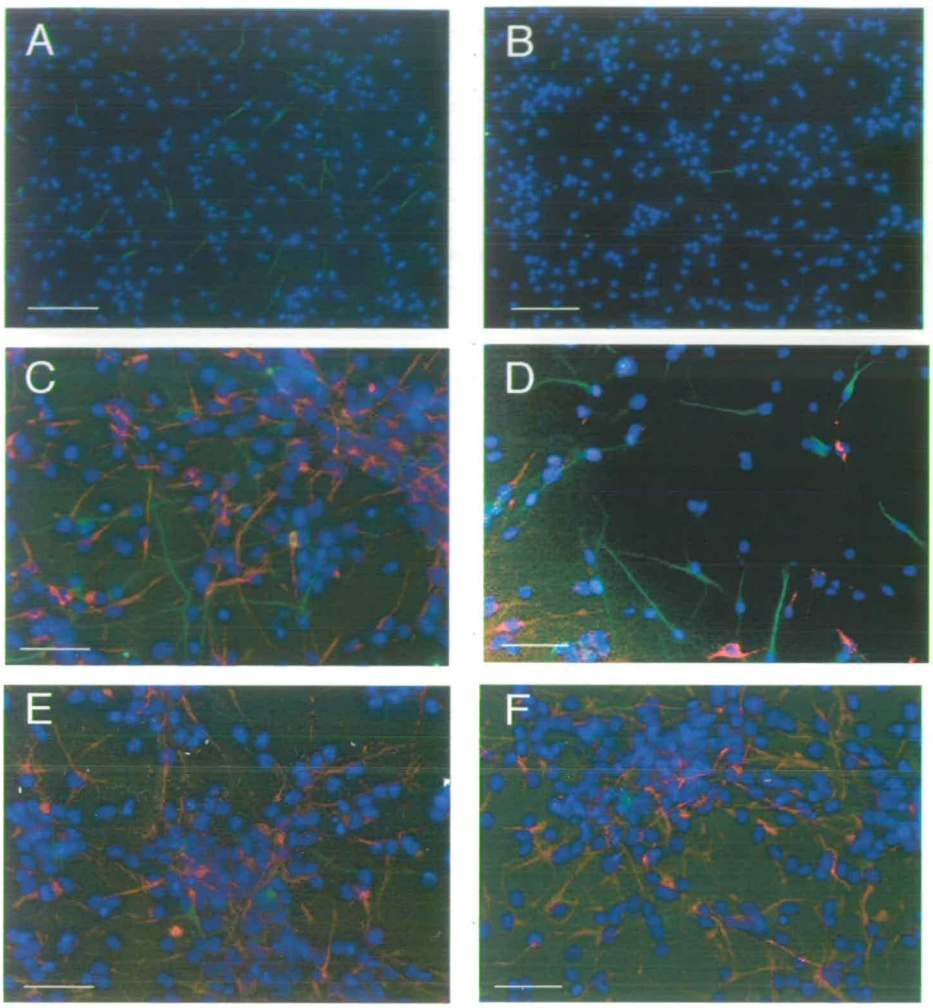


Figure 4.10 Neural differentiation of triple null NS cells is not impaired. NS cells are cultured for 15 days in neuronal differentiation conditions and subsequently immunostained. A and B, cultures derived from wild type NS cell clones: A wild type NS clone derived from embryonic age E16.5. B wild type clone derived from embryonic age E14.5. C and D cultures derived from two independent triple null NS cell clones TuJ1 in green, Gfap in red, Dapi in blue. E and F,MAP2 as a marker for mature neurons. G and H Gad67, GABAergic marker. E and G wild type. F and H triple null. Scale bars represent 5μm in A-D, and 3μm in E-H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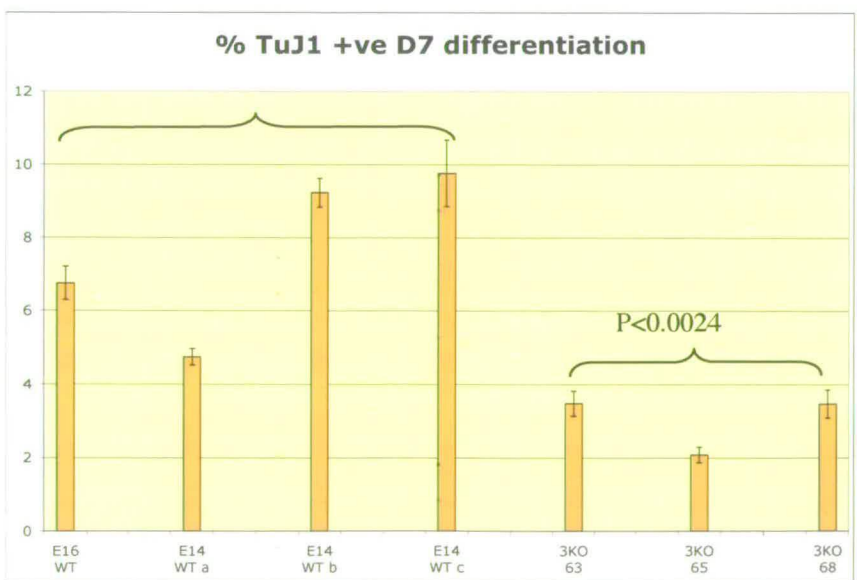

% TuJ1 +ve D7 differentiation
12
10
8
6
4
2
0
P<0.0024
E16 WT
E14 WT a
E14 WT b
E14 WT c
3KO 63
3KO 65
3KO 68

Figure 4.11 A) Triple null NSs have fewer differentiated TuJ1 positive neurons after 7 days of differentiation. A, Wild type E14.5 derived NS. B triple null E14.5 derived NS. TuJ1 in green, Dapi in blue.C, Wild type E16.5 derived NS. D, Wild type E14.5 derived NS. E and F, triple null E14.5 derived NS. TuJ1 in green, RC2 in red, Dapi in blue. A and B scale bars represent 10μm.C,D,E,F scale bars represent 5μm. B) Quantification of NS-derived TuJ1 positive neurons after 7 days of differentiation. On the left wild type NS cells form 2.5 fold more neurons than triple null NS cells on average. This phenotype is independent of embryonic age. All NS cell lines are E14.5 embryonic age-derived, with the exception of first left wild type NS cell line, which is E16.5 embryonic age-derived.

4.7 Summary and Discussion

4.7.1 MeCP2, Kaiso and Mbd2 are dispensable for neural stem cell function

Following the postulated hypothesis of functional redundancy among the methyl-CpG binding proteins, triple null neural stem cell lines were analysed in order to study their function. I can conclude that the triple null neural stem cell lines have no abnormal capability compared with the wild type, as both wild type and null NS cells showed comparable proliferation, self-renewal and differentiation capacities. Hence, my results do not support any role for MeCP2, Kaiso and Mbd2 in neural stem cell function.

4.7.2 Methyl-CpG binding protein and functional redundancy

This surprising finding raises the question of why there is no abnormal neural stem cell function in the absence of three out of four methyl-CpG binding proteins?

One possible explanation for these results is that there are more methyl-CpG binding proteins currently uncharacterised, and therefore the absence of three proteins is not sufficient to disrupt neural stem cell function. Indeed, two additional methyl-CpG binding proteins have been discovered. These proteins, called ZBTB4 and ZBTB38, are closely related to KAISO in that they can bind sequences containing a single methylated CpG and can function as methyl-dependent transcriptional repressors (Filion et al., 2006). Given their expression pattern and homology it is very plausible that they share functional redundancy with the first BTB/POZ methyl-CpG binding protein discovered. Hence, the lack of phenotype observed in my studies could be attributed to functional redundancy with other methyl-CpG binding proteins.

Another explanation could be that although all the evidence indicates a role of the methyl-CpG binding proteins in neural stem cell function, it could be that only Mbd1 is involved in the mechanisms of differentiation of neural precursors. In concordance with this possibility, a role for MeCP2 in neural stem cell function has been recently ruled out, being in contrast confirmed a neuronal role for this protein (Kishi et al., 2004).

4.7.3 A role for methyl-CpG binding proteins in neuronal function

My experiments demonstrate that despite the dispensability of Kaiso, MeCP2, and Mbd2 for neural stem cells, the number of TuJ1 positive neurons after seven days of differentiation *in vitro* is dramatically reduced in triple null cultures, while after a complete period of differentiation of 15 days there is no observable difference.

These results could be the consequence of a delay in neuronal maturation in 3KO lines, and therefore after an extended neuronal differentiation period would be indistinguishable from wild type. On the other hand, as only the expression of the postmitotic neuronal protein β tubulin III has been quantified, it is plausible that only the expression of this particular marker is delayed in 3KO lines. Therefore, it would be interesting to investigate the expression of additional neuronal genes that mark different neuronal differentiation stages, such as early maturation events (NeuN and Map2) and more advanced stages of differentiation (Neurofilament triplet proteins NF-L, NF-H, NF-M and synaptophysin) (Izant and McIntosh, 1980; Matus, 1990; Mullen et al., 1992; Przyborski and Cambray-Deakin, 1995; Steinschneider et al., 1996)

Consistenly with a delay in neuronal maturation phenotype in 3KO lines, several groups have suggested a role for MeCP2 in neuronal maturation (Shahbazian et al., 2002; Kishi et al., 2004; Matarazzo, 2004). Matarazzo et al, studying olfactory receptor neurons identified a delay in terminal differentiation of olfactory neurons that also leads to axonal disruption. On the other hand, Young and colleagues (Young et al., 2004) described a possible role of MeCP2 in neuronal survival, when plating 1:1 wild type and null neurons observed that after 7 days the majority of the cells were wild type, suggesting that the MeCP2 null neurons would die during

culture. If this were the case, neuronal survival should be affected both at day 7 and day 15, but I only see an observable difference after the first week of differentiation.

Additionally, Kishi et al described a reduced size and arborisation of *MeCP2*-null neurons in the neocortex that is linked to a defect in late stage post migratory maturation of the neurons. Moreover, MeCP2 is also believed to have a role in the establishment and maintenance of synapses (Johnston et al., 2001; Kaufmann et al., 2005; Moretti et al., 2006) (Fukuda et al., 2005). Therefore, MeCP2 is essential for proper regulation of neuronal gene expression that is fundamental in the complex balance of transcriptional factors involved in neuronal function (Chen et al., 2003; Horike et al., 2005; Martinowich et al., 2003; Nuber et al., 2005; Samaco et al., 2004).

Given this evidence it is not surprising to find delayed neuronal differentiation in my *in vitro* system. However, an additional role for Mbd2 or the combination of MeCP2, Kaiso and Mbd2 in controlling the specific transition between neural stem cells and neurons cannot be ruled out.

4.7.5 Astrocyte morphology

During my NS differentiation studies I observed an unequivocal morphological difference in astrocytes that was embryonic age-related instead of genotype dependent. I concluded that E14.5 embryo-derived NS cells differentiate into more flat and less spiked astrocytes with fewer connections than do E16.5 embryo-derived NS cells. As explained before, this is a surprising effect given that these cell lines are thought to be very homogenous since there are a pure population of cells that undergo continuous asymmetric divisions and are maintained in the same constant balance of growth factors.

This morphological difference is not observed in other differentiation assays when astrocytes are not induced by BMP4 present in the serum but other factors are involved in the process (PDL/Laminin, bFGF and b27)(figure 4.10). It would be very interesting to study what is the cause of the morphological difference. Which are the signals and factors that determine one shape or the other? Does different astrocytical morphologies correlates with different functions?

4.7.6 Heterogeneity of neurosphere assay

The neurosphere assay, although extensively used, has the drawback of heterogeneity. In the neurosphere, apart from the neural stem cell population that maintains the neurosphere status, there are other cells at various differentiation stages. Therefore, there are different cell growth rates and self-renewal capabilities within the same neurosphere. Hence, unless a study would involve a very dramatic phenotype it is very possible to dismiss it through this system, or alternatively, obtain results that are not easily reproduced in independent experiments involving different embryos.

The variability I found in the results of proliferation and self-renewal could be due to the combination of no differential phenotype and heterogeneity of neurospheres formed from individual embryos regardless their genotype.

CHAPTER 5

INVESTIGATION OF THE ROLE OF MBD3 IN NEURAL STEM CELLS

5. 1 Introduction

Mbd3 forms part of the NuRD complex and may function as a scaffold protein, since in the absence of Mbd3 the complex is not correctly formed. This is shown by the failure of components of the complex to coimmunoprecipitate in the absence of Mbd3 (Kaji et al., 2006). Mbd3 is required for the transition of pluripotent cells of the blastocyst to mature into epiblast upon implantation, resulting in early post implantation lethality. Accordingly, *Mbd3*$^{(-/-)}$ *ex vivo* inner cell mass culture fail to differentiate into epiblast (Kaji et al., 2006).

Mbd3 was also fished out in an mRNA substractive cDNA hippocampal library as a gene that was being expressed higher in the embryonic brain than in the adult brain (Jung et al., 2003). The consequent immunohistochemical and *in situ* analysis of *Mbd3* in mouse and rat brain delineated an early Mbd3 expression in embryonic cortex and hippocampus around the onset of neurogenesis that decrease toward adulthood. In contrast, striatum, olfactory bulb and cerebellum had a later expression during embryonic development that continued into adulthood. This differential expression parallels the presence of progenitor neural cells in these structures, and it could correlate with a role for Mbd3 in neural stem cell function.

5.1.1 Generation of *Mbd3*$^{(-/-)}$ ES cells

Although *Mbd3*$^{(-/-)}$ blastocysts fail to proliferate in culture making the derivation of *Mbd3*$^{(-/-)}$ ES cells from blastocysts impossible, *Mbd3*$^{(-/-)}$ ES cells have been made by selecting for loss of the wild type allele and duplication of the mutant allele by homologous recombination (Fix2 and Spl2 ES cell lines)(Kaji et al., 2006)(figure 5.1.A). A second null line was generated through the targeting of loxP sites flanking the entire Mbd3 gene (3Flox ES cell line) and consequent recombination of loxP sites by transient expression of Cre recombinase (3KO cell line)(Kaji et al., 2006).

There are three Mbd3 isoforms, Mbd3a, Mbd3b and Mbd3c (figure 5.1.B). Cre recombination of loxP sites in the 2lox ES cell line resulted in Mbd3c-expressing ES cell line (A7), as explained in figures 5.1.A and 5.1.C. To ensure that the defects explained below are a consequence of the absence of Mbd3 in the cells, rescued cell lines were generated by stable transfection of Mbd3a or Mbd3b isoforms in *Mbd3*$^{(-/-)}$ ES cells (Kaji et al., 2006).

Previous experiments have shown decreased proliferation of *Mbd3*$^{(-/-)}$ ES cells compared with wild type (Kaji et al., 2006). *Mbd3*$^{(-/-)}$ ES cells can grow at clonal density in the absence of LIF. Furthermore *Mbd3*$^{(-/-)}$ ES cells expressing Puromycin and GFP under the Oct4 promoter (*Oct4-GFP-ires-pac* (Ying et al., 2002)) could be cultured and passaged under puromycin selection and the absence of LIF for more than 2 weeks, with the aberrant expression of Fgf5, which is reversed after addition of LIF to the culture (Kaji et al., 2006). These experiments demonstrate that *Mbd3*$^{(-/-)}$ ES remain pluripotent (Oct4 positive) independently of LIF signalling.

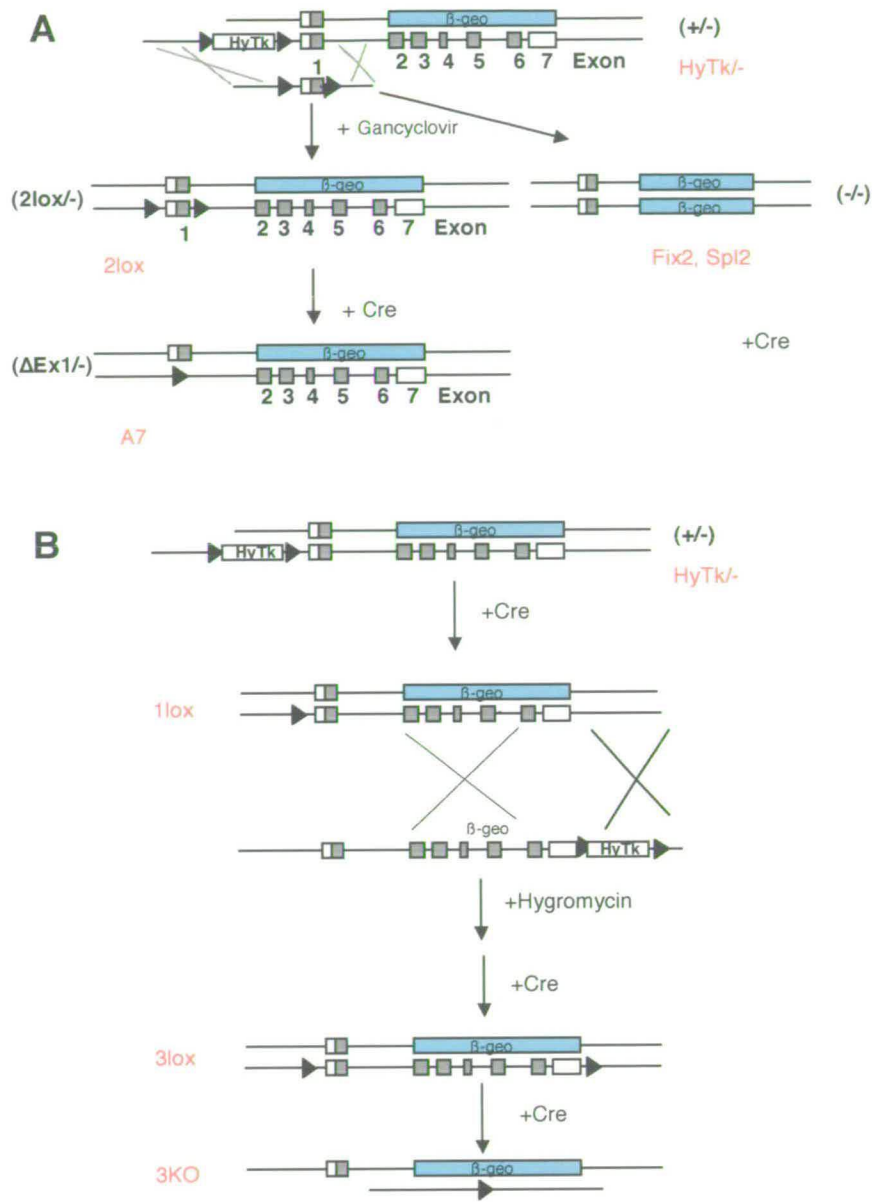


Figure 5.1 A) Schematic representation of the generation of *Mbd3*$^{(2lox-)}$, *Mbd3*$^{(-/-)}$ and *Mbd3* $^{(\Delta Ex1/-)}$ cell lines. *Mbd3*$^{(2lox-)}$ ES cell line which have exon 1 floxed between two loxP sites was generated from ES cells which have Hygromycin/Thymidine Kinase cassete upstream of Exon1 (HyTk/-), *Mbd3* $^{(\Delta Ex1/-)}$ ES cell line (A7) with Exon1 deletion in one allele was generated from *Mbd3*$^{(2lox-)}$ ES cell line by Cre recombination. *Mbd3*$^{(-/-)}$ ES cell lines (clones Spl2 and Fix2) were obtained by loss of the wild type allele and duplication of the mutant allele from HygTk/- cells by gancyclovir selection. Exons are indicated as boxes and non-coding sequences as unfilled boxes. Exon 1 contain both coding and non-coding sequences. Black triangles represent loxP sites. ES cell line names are indicated in red. These cell lines were generated by Dr. Hendrich and R. Macleod.

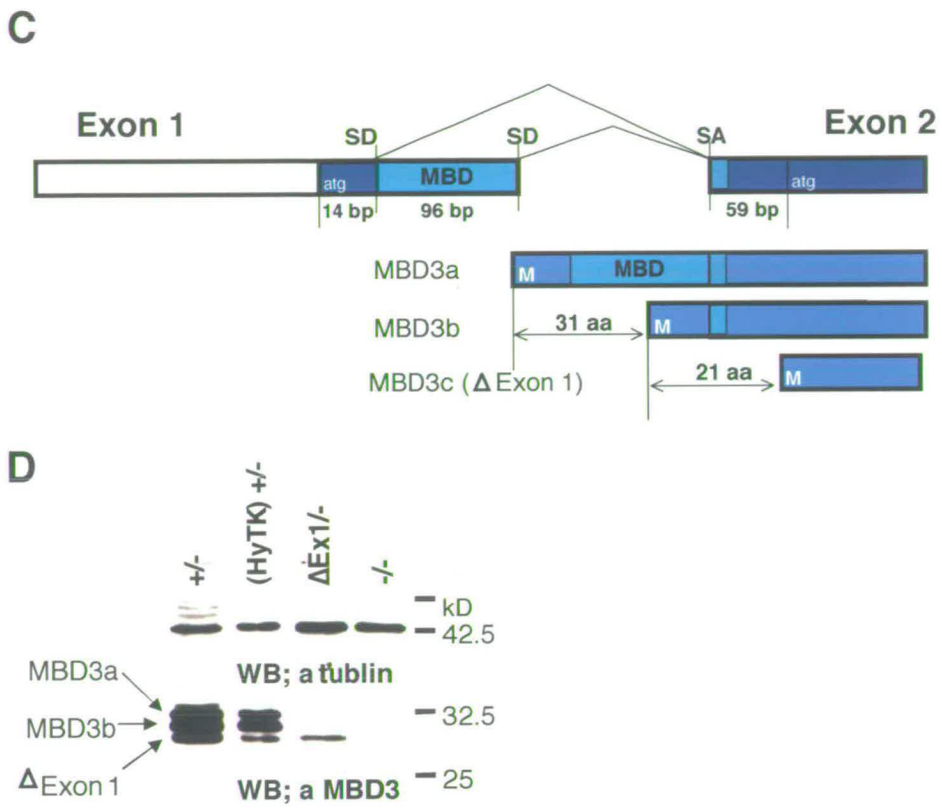


Figure 5.1 B) Schematic representation of the generation of *Mbd3*$^{(3Flox-)}$ and *Mbd3*$^{(-/-)}$ clone named 3KO. A cell line (1 lox) was generated from floxed ES cell HyTk/- by Cre recombination, then subsequently targeted with a construct designed to leave the entire *Mbd3* gene flanked by two loxP sites (*Mbd3*$^{(3Flox-)}$). After Cre recombination of *Mbd3*$^{(3Flox-)}$ cell line, *Mbd3*$^{(-/-)}$ 3K0 is generated. **C)** Schematic representation of Mbd3 isoforms: Mbd3b is a splice isoform, which lacks half of methyl CpG binding domain. Mbd3c might be translated from methionine in exon 2 as the transcripts are detectable in A7. SD=splice donor. SA=splice acceptor **D)** Western blot using *Mbd3*$^{(2lox-)}$, HyTK)/-, *Mbd3* $^{(\Delta Ex1/-)}$ (A7), and *Mbd3*$^{(-/-)}$ cell lines cell lines from left to right (Kaji et al., 2006).

5.1.2 Studies of differentiation capacity in *Mbd3*$^{(-/-)}$ ES cells

In the analysis of differentiation capacity, there were previously performed embryoid body (EB) differentiation experiments in the absence of LIF and gene expression analysis by RT-PCR at key time points of differentiation (Kaji et al., 2006). In embryoid bodies, the external endoderm signals, which correlates with the primitive endoderm in the embryo, differentiation and endoderm specific gene expression are necessary to form the columnar ectoderm layer (Li et al., 2004).

The results from these experiments (Kaji et al., 2006) revealed an unequivocal defect in differentiation of *Mbd3*$^{(-/-)}$ ES cells, denoted by two reasons. Firstly, *Mbd3*$^{(-/-)}$ embryoid bodies expressed pluripotent markers Oct4, Nanog and Rex1 after 10 days of differentiation, and secondly, *Mbd3*$^{(-/-)}$ EBs could be dissociated and cultured as ES cells, being positive for alkaline phosphatase. While *Mbd3*$^{(-/-)}$ ES cells seem to maintain undifferentiated conditions in absence of LIF, they do have activation of trophectoderm markers *Tbpa*, and *Pi-1* after 10 days, informing of an aberrant gene expression (Kaji et al., 2006).

Interestingly, Fgf5, which is normally activated in EBs after 3 days was also activated in *Mbd3*$^{(-/-)}$ EBs but failed to downregulate its expression after more than 10 days in culture. *Mbd3*$^{(-/-)}$ ES cells differentiate efficiently and lose Oct4 expression in presence of retinoic acid, giving evidence that differentiation is not fully blocked and Mbd3 is not required for survival of differentiated cells (Kaji et al., 2006).

Chimera analysis shows that *Mbd3*$^{(-/-)}$ ES cells can undergo some level of differentiation into primitive ectoderm and early mesoderm when placed in the appropriate signalling context. This differentiation is abnormal, and impedes the normal embryonic development of the host blastocyst, in a degree directly dependent on the level of *Mbd3*$^{(-/-)}$ contribution. Chimera analysis with Oct4-GFP labelled Mbd3$^{(-/-):GFP}$ ES cells shows continuous expression of Oct4 at 8.5 dpc, long after Oct4 should be switched off, reflecting that this defect is cell autonomous. These deficiencies could be restored upon addition of Mbd3b or partially restored upon addition of Mbd3a (Kaji et al., 2006).

These studies lead to an interesting question: What is the role of Mbd3 in neuroectoderm differentiation from ES cells? In contrast with embroid bodies, in the serum-free monolayer differentiation system the primitive endoderm and endoderm gene expression are absent, and therefore it makes an ideal system to investigate *Mbd3*$^{(-/-)}$ ES differentiation capacity in the absence of endodermal and mesodermal signalling. Additionally, I became interested to study the function of Mbd3 in cells independent of Oct4 signalling, and given the previous reports of a possible Mbd3 function in the developing nervous system (Jung et al., 2003), I aimed to isolate Mbd3 neural stem cells to study their function.

5.2 Neuroectodermal differentiation of Mbd3 null ES cells

The parental cell lines 3Flox (*Mbd3*$^{(3Flox/-)}$) and 2Lox (*Mbd3*$^{(2lox/-)}$); the 3KO, Spl2 and Fix2 (*Mbd3*$^{(-/-)}$) ES cell lines and the A7 (*Mbd3* $^{(\Delta Ex1/-)}$) (figure 5.1.A)ES cell line (Mbd3c expressing ES cell line) were challenged to differentiate in monolayer cultures in serum-free medium. These cultures were fixed and immunostained at different time points for the pluripotency marker Oct4, neural stem cell marker Nestin, the postmitotic neuron marker TuJ1, and the astrocytic marker Gfap.

5.2.1 Neural and neuronal differentiation

Very consistently during the 5 independent experiments performed, all three *Mbd3*$^{(-/-)}$ ES cell lines used presented a deficiency both in differentiation and in proliferation compared with wild type. These cells showed deficient differentiation into not only neurons but also Nestin positive neural stem cells. Both cell types were identified very rarely in the cultures after 10 days of differentiation in 12 well plates (figure 5.3). A7 cell line (*Mbd3* $^{(\Delta Ex1/-)}$) displayed different behaviour, since there was no observable proliferation defect, but the cells showed a differentiation defect. The cultures have few colonies positive for neural precursor and neuron markers although more than in null cell lines. In general, A7 cultures present a high number of cells with a characteristic flat, geometrically shape and an apparent normal proliferation. This is consistent with previous studies in the lab, were there was no proliferation defect identified in the A7 cell line (figure 5.2.A)

Fig. 5.2A

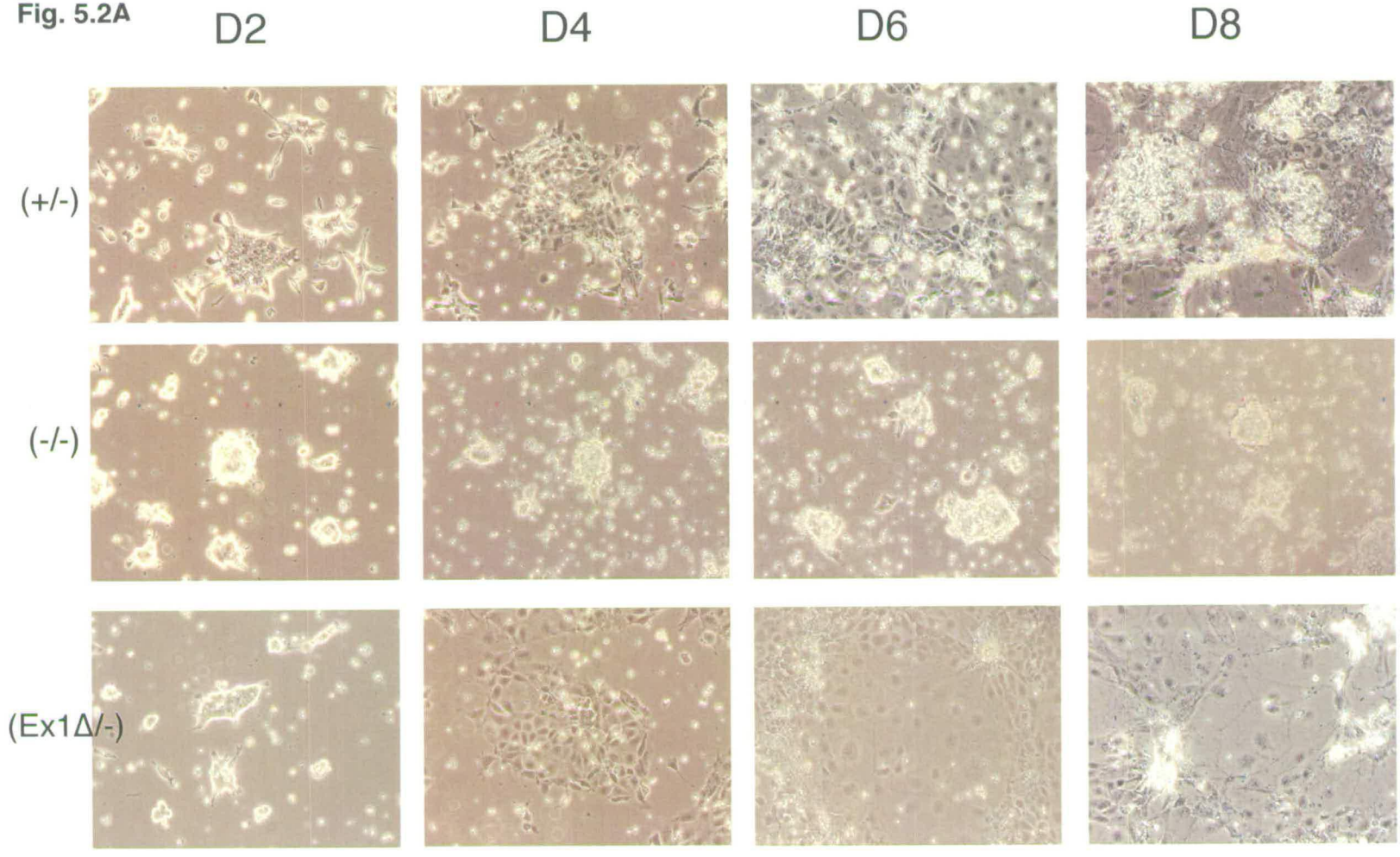


Fig. 5.2B

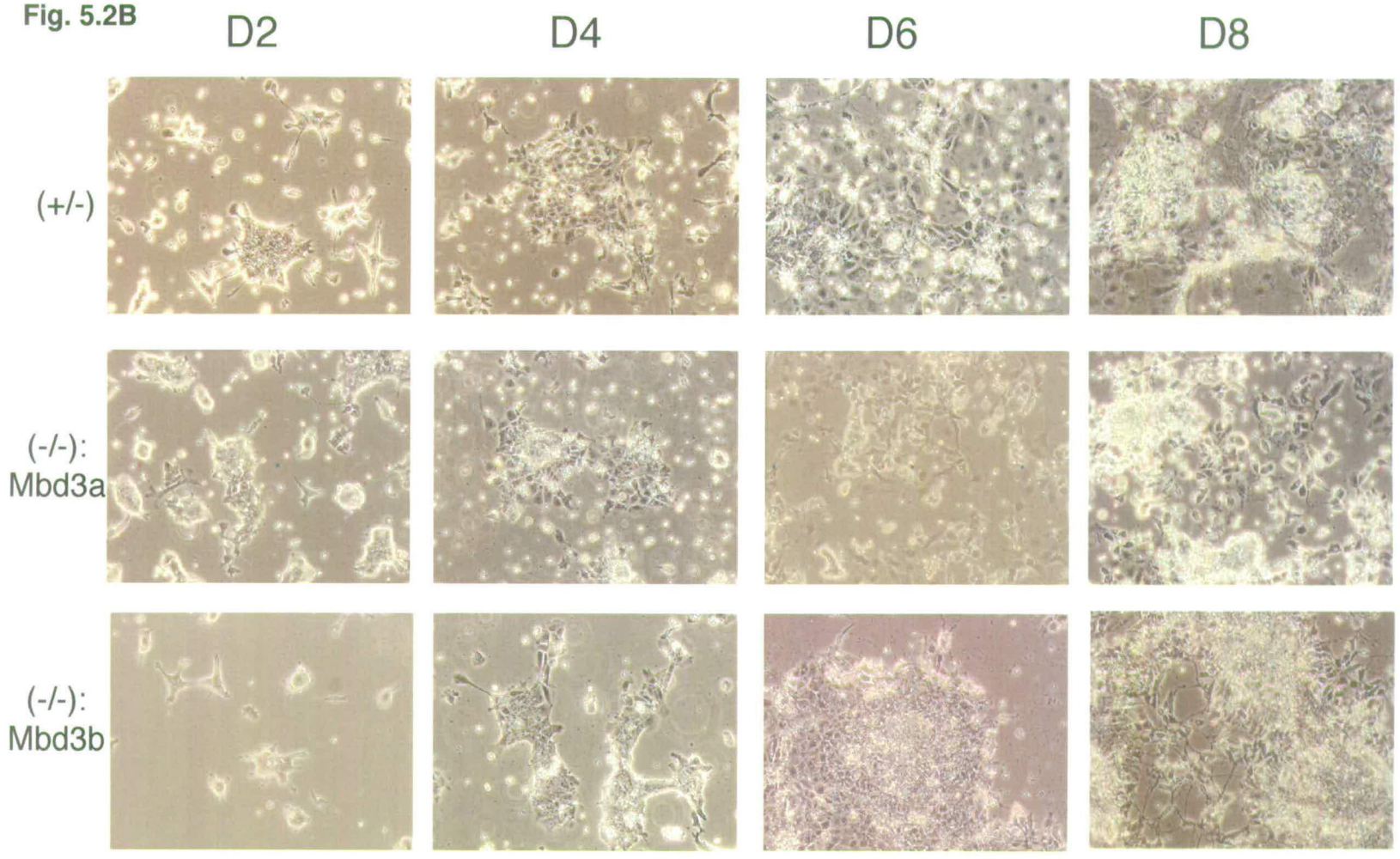


Figure. 5.2 Serum-free monolayer differentiation time course from day 2 to day 8. *Mbd3*$^{(-/-)}$ cultures remain as tight round ES colonies during the 8 days, and the proliferation is reduced; *Mbd3* $^{(\Delta Ex1/-)}$ do not display a proliferation defect but do display a differentiation defect with characteristic flat geometrically shaped cells in the cultures. **A)** Cell lines: *Mbd3*$^{(3flox/-)}$, *Mbd3*$^{(-/-)}$ and *Mbd3* $^{(\Delta Ex1/-)}$. **B)** Cell lines *Mbd3*$^{(2lox/-)}$, *Mbd3*$^{(-/-):Mbd3a}$, *Mbd3*$^{(-/-):Mbd3b}$.

To demonstrate that the above phenotype is caused by the lack of Mbd3, parallel experiments were performed with the two rescued cell lines previously described (5.1.1). Both rescued cell lines showed normal differentiation undistinguishable from wild type cultures (figure 5.3.B). The *Mbd3*$^{(-/-):Mbd3a}$ cell line showed an overall better degree of differentiation (even more than wild type) (figure 5.3.E, F). Although this observation can be merely due to intrinsic cell line differences, it could be also due to the presence of the Mbd3a isoform and absence of Mbd3b and Mbd3c isoforms. As mentioned before, in ES cells the more abundant Mbd3 isoform is Mbd3b, although Mbd3a and c can also be detected (Kaji et al., 2006). Mbd3b is also reported to be more prevalent in the embryonic brain, although Mbd3a increases its expression during differentiation (Jung et al., 2003). Additionally, the Mbd3b-rescued lines express near normal levels of Mbd3b, while Mbd3a lines express approximately two-fold higher levels of rescuing protein. Taking all together, the higher degree of differentiation observed in the Mbd3a-rescued cell line in monolayer differentiation conditions could be explained by 2 independent possibilities. First, Mbd3a could be involved in maintenance and/or differentiation of neural precursors into post-mitotic neural cells in a higher level than Mbd3b. This possibility would be backed by the increased presence of Mbd3a in the mature brain. Secondly, the increased levels of Mbd3 in the Mbd3a-rescued cell line could be responsible to push ES cell differentiation into neuroectoderm lineages to a higher degree than in wild type ES cells. This possibility will be discussed further below.

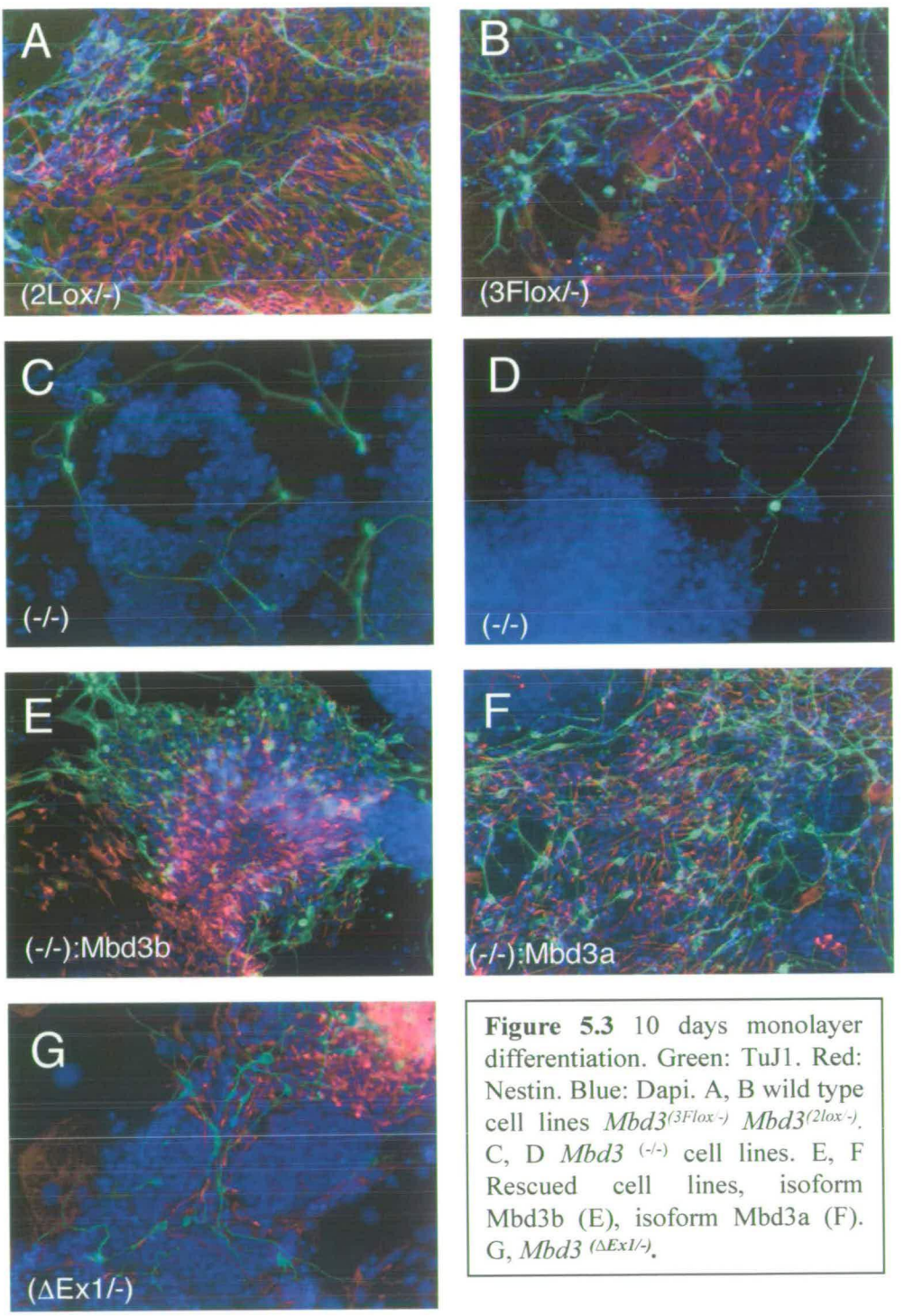


Figure 5.3 10 days monolayer differentiation. Green: TuJ1. Red: Nestin. Blue: Dapi. A, B wild type cell lines *Mbd3*$^{(3Flox/-)}$ *Mbd3*$^{(2lox/-)}$. C, D *Mbd3* $^{(-/-)}$ cell lines. E, F Rescued cell lines, isoform Mbd3b (E), isoform Mbd3a (F). G, *Mbd3* $^{(\Delta Ex1/-)}$.

5.4.2 Oct4 positive cells

Mbd3$^{(-/-)}$ ES cell lines differentiated for 10 days had a majority of Oct4 expressing cells in the culture. (figure 5.4 and figure 5.5). Although Oct4 positive cells can be observed in wild type cell lines after ten days of differentiation, there are very few compared with the null cell lines, as it is shown in figure 5.5. Further, colonies entirely positive for Oct4 are often observed in Mbd3 null cell lines. This is also the case for the *Mbd3* $^{(\Delta Ex1/-)}$ cell line, although still the phenotype is less severe than in the null cell lines. In general, Mbd3 null cells appear during the entire differentiation protocol as ES cell-like compact rounded colonies and they fail both to proliferate and to differentiate (figures 5.2.A, 5.3.C-D and 5.4).

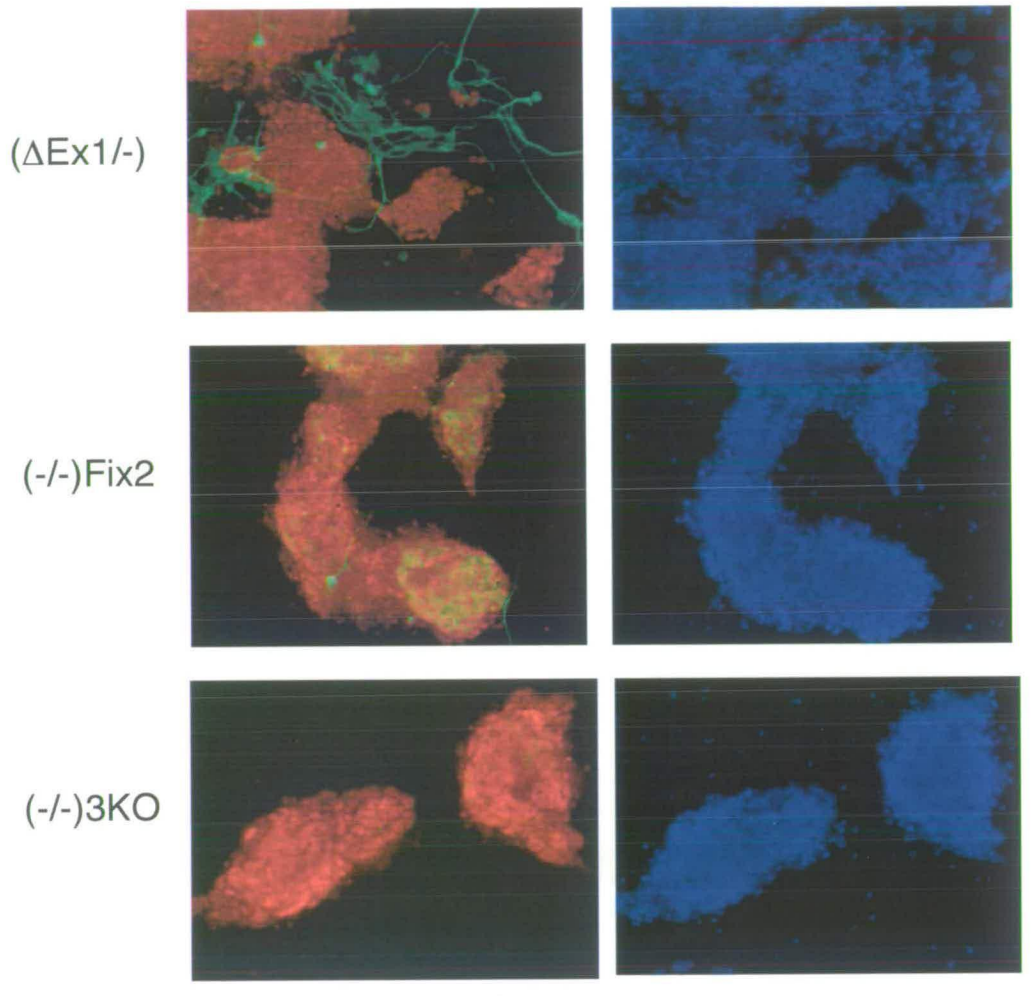


Figure 5.4 *Mbd3*$^{(-/-)}$ cells remain Oct4 positive after 10 days of differentiation conditions. Immunocytochemistry on monolayer culture system after 10 days of differentiation conditions. Oct4 red, TuJ1 green in left panels, DAPI blue in right panel.

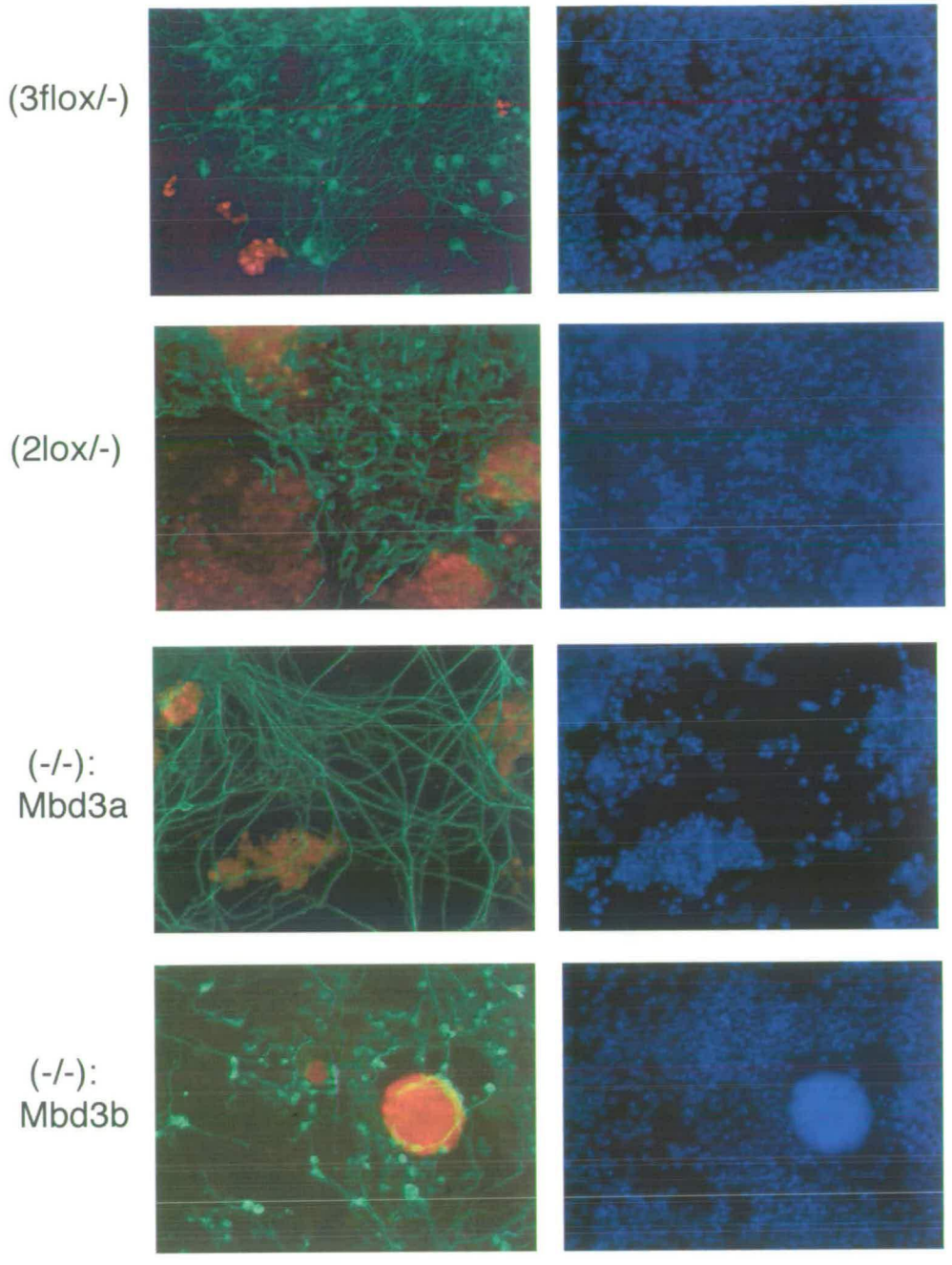


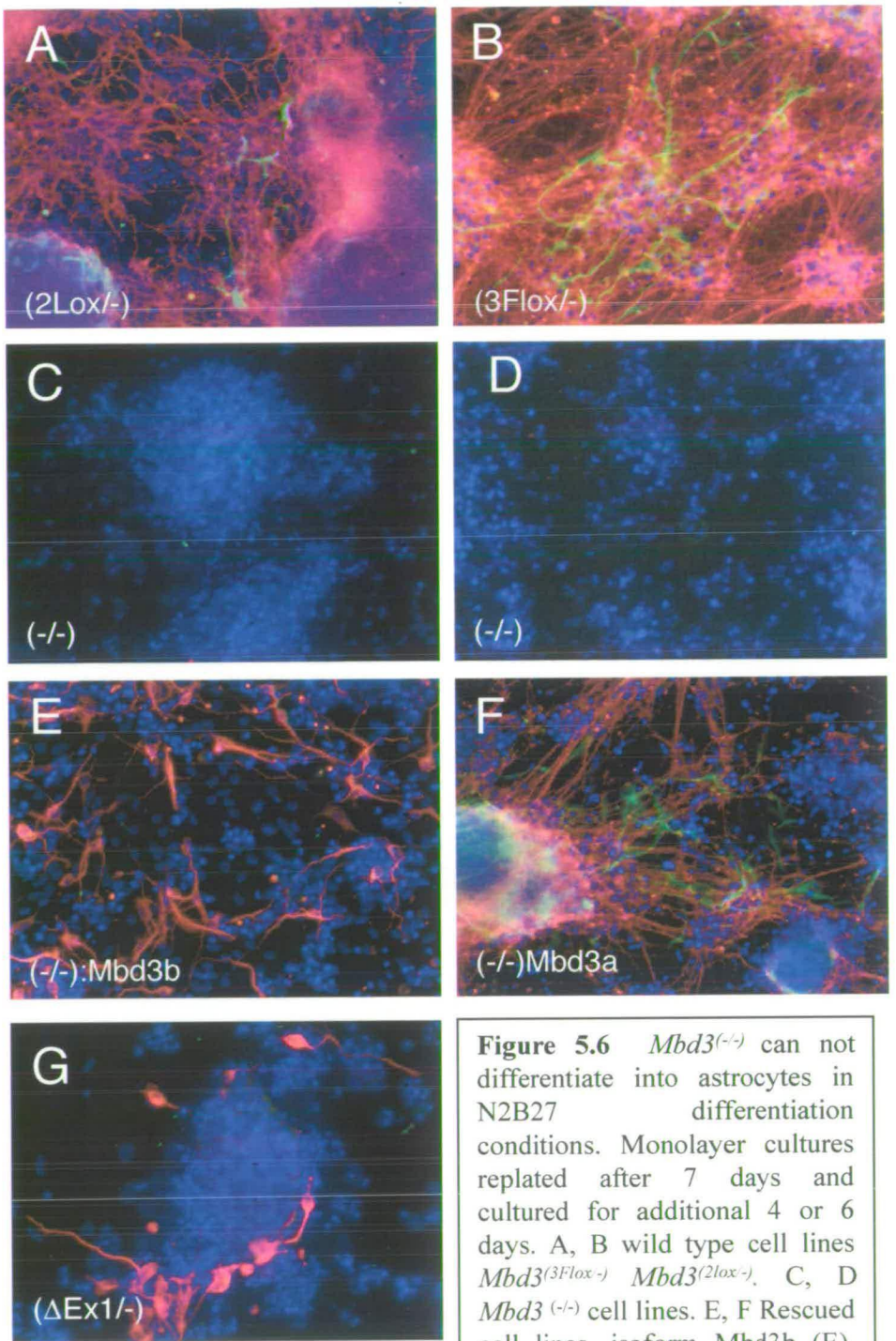
Figure 5.5 *Mbd3*$^{(-/-):Mbd3a}$ and *Mbd3*$^{(-/-):Mbd3b}$ rescued cell lines do differentiate in the same degree as floxed cell lines 3flox and 2Lox. Left panels: TuJ1 in green and Oct4 in red. Right panels: Dapi in blue.

5.4.3 Astrocyte differentiation

In order to obtain Gfap positive cells in the monolayer cultures, I replated seven day old monolayer cultures in Poly-D-Lysine and Laminin-coated 12 well plates. In general, with the serum-free monolayer system I have observed that the presence of Gfap positive cells is dependent upon time of differentiation requiring at least 15 days in culture. I have also observed that replating the cultures increases the proportion of Gfap positive cells. This is consistent with previous findings describing that *in vitro* cultures tend to mirror what happens *in vivo* in terms of timing in the appearance of neurons versus astrocytes differentiation (Fan et al., 2005; Qian et al., 2000; Takizawa et al., 2001). Replating may benefit from the elimination of both non neuroectodermal differentiated and dead cells three dimensionally clustered in the monolayer; but most importantly from the enrichment of neural precursors that adhere in PDL coated surfaces (figure 5.6).

Fetal calf serum contains Bmp4, which is a potent astrocytic induction signal (Gomes et al., 2003; Gross et al., 1996), therefore I studied whether addition of serum after the first days of neural induction in serum-free monolayer system would have an effect in astrocytic differentiation. I have found that addition of 10% serum in N2B72 culture media after 6-7 days does not improve astrocytic differentiation nor neuronal differentiation in this culture system. On the other hand, I have detected that addition of serum improves cell survival (figure 5.8), which is a positive factor given the long term culture necessary to obtain astrocytes in this system.

Neither *Mbd3*$^{(-/-)}$ ES cells, from two independent cell lines or *Mbd3* $^{(\Delta Ex1/-)}$ ES cells differentiated into astrocytes in any of the conditions described above (figures 5.6 and 5.7). These results suggest that Mbd3 is necessary for astrocytic differentiation, since no *Mbd3*$^{(-/-)}$ astrocytes can be generated from *Mbd3*$^{(-/-)}$ ES cells.



Legend: GFAP is in green. TuJ1 in red and DAPI in blue.

Figure 5.6 *Mbd3*$^{(-/-)}$ can not differentiate into astrocytes in N2B27 differentiation conditions. Monolayer cultures replated after 7 days and cultured for additional 4 or 6 days. A, B wild type cell lines *Mbd3*$^{(3Flox-)}$ *Mbd3*$^{(2lox-)}$. C, D *Mbd3* $^{(-/-)}$ cell lines. E, F Rescued cell lines, isoform Mbd3b (E), isoform Mbd3a (F). G, *Mbd3* $^{(\Delta Ex1/-)}$.

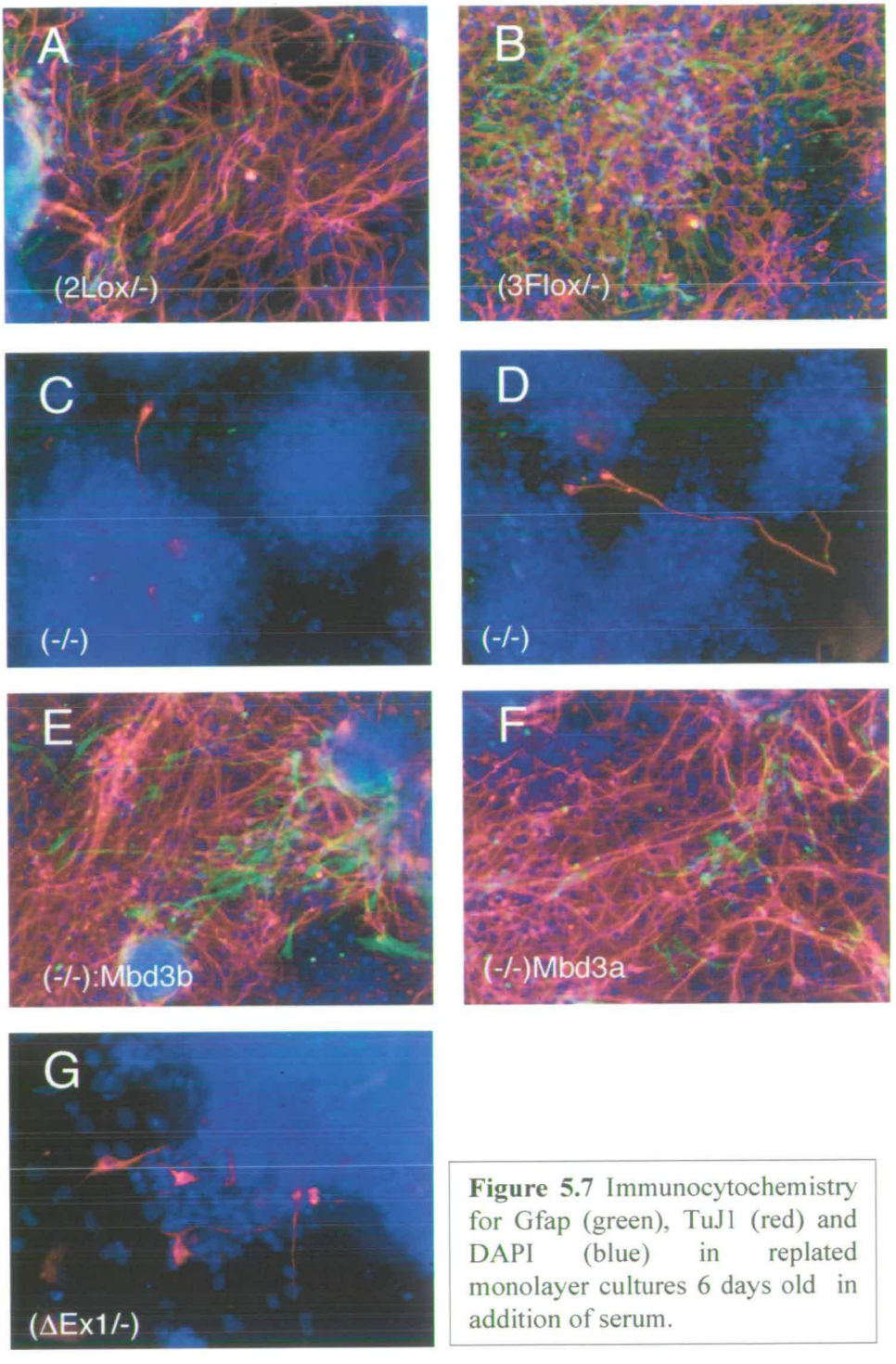


Figure 5.7 Immunocytochemistry for Gfap (green), TuJ1 (red) and DAPI (blue) in replated monolayer cultures 6 days old in addition of serum.

5.4.4 Long term cultures and apoptosis

Although my experiments this far show that Mbd3 is important for neuroectoderm differentiation after 10 days, it remain possible that this effect is due to a delay in differentiation more than a block in dfferentiation. To address the question of whether *Mbd3*$^{(-/-)}$ cells show a delay in differentiation defect instead of a lack of differentiation, non replated long term cultures of 17 days were stained for TuJ1, Gfap and activated Caspase-3 which is a marker of apoptosis. Cleavaged or activated Caspase-3 belongs to the protease subfamily of caspases that execute the apoptotic response during programmed cell death (Boatright and Salvesen, 2003). The 11 components of this subfamily are present in neural precurors, neurons and glia, since apoptosis plays an important role in brain development. Activated Caspase-3 filters the pro-apoptotic signals from both the mitochondria (intrinsic pathway) and the membrane (extrinsic pathway) to catalyse the specific cleavage of key cellular proteins (Troy and Salvesen, 2002).

My conclusion from these experiments is that *Mbd3*$^{(-/-)}$ cells do not present a delay in differentiation because there was no improvement in the phenotype in longer cultures (figure 5.9). In general, long term cultures show better survival in addition of serum, but the differentiation capacity is compromised (figure 5.8). Hence, these experiments demonstrate that the absence of Mbd3 prevents ES cells from undergoing neuroectoderm differentiation regardless the time in culture.

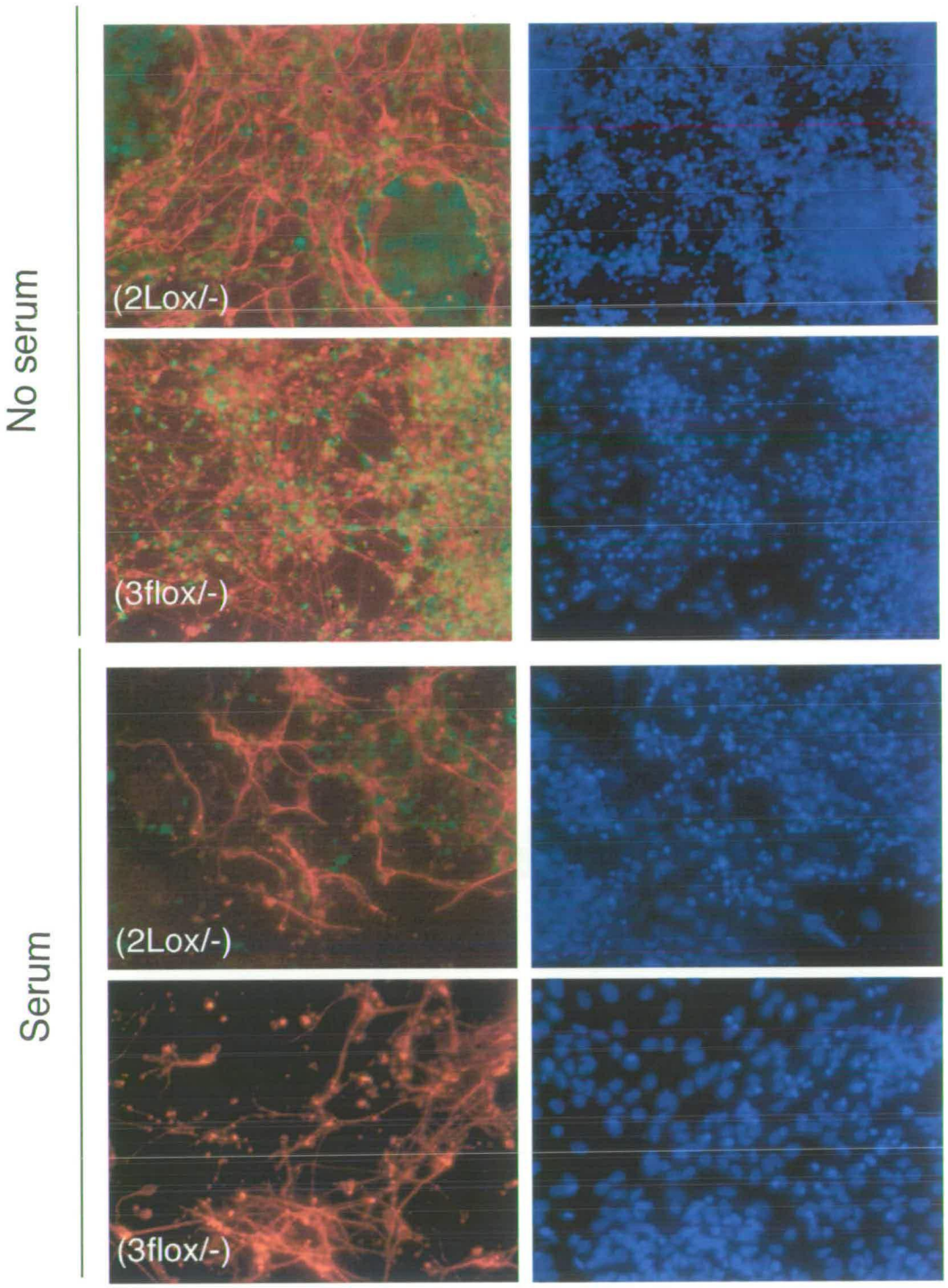


Figure 5.8. Immunocytochemistry for Caspase-3 (green), TuJ1 (red) and DAPI (blue) after monolayer differentiation for 17 days with two independent WT ES cell lines. Addition of serum improved cell viability but interferes with differentiation ratio.

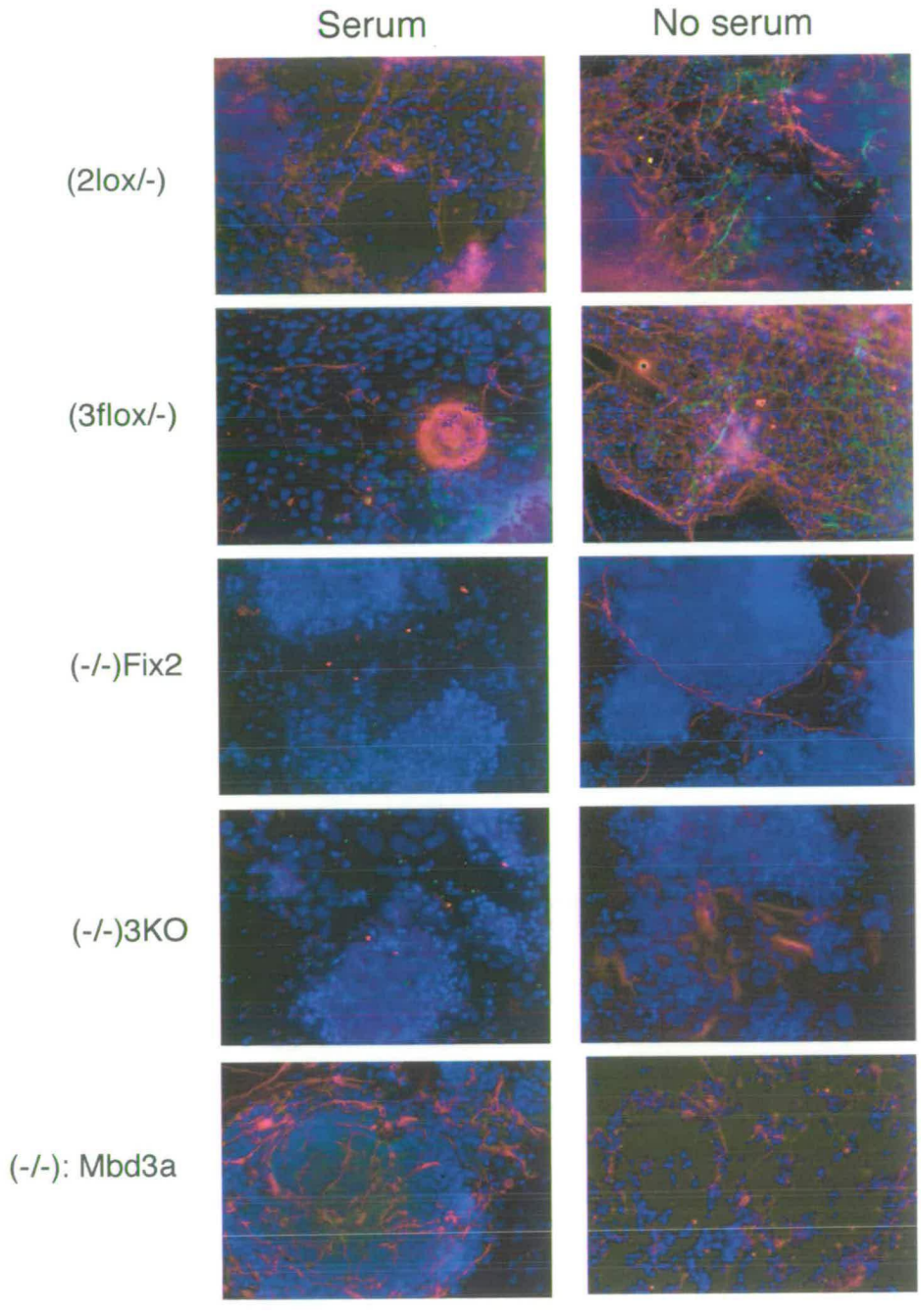


Figure. 5.9 *Mbd3*$^{(-/-)}$ cells do not show delayed neural differentiation. Immunocytochemistry on monolayer cultures after 17 days of differentiation conditions. TuJ1 red, Gfap green and DAPI blue. This assay confirms that addition of serum does not improve astrocytic differentiation but rather decrease differentiation capability.

5.4.5 Time points monolayer differentiation RT-PCR

To investigate the expression of genes known to be important in neural stem cell fate decisions, RNA was extracted from five time points of monolayer differentiation from day 0 to day 12 from the seven different cell lines, and subsequently RT-PCR was performed for the stem cell markers *Sox1, Sox2, Sox3, Pax6* and *Blbp*. In parallel, the expression of *Oct4, TuJ1* and *Gfap* was assayed. As can be observed in figure 5.10, $Mbd3^{(-/-)}$ cell lines show a complete lack of detectable expression for neural stem cell markers from day 2 to day 8. On the other hand, the exon one deleted cell line does express the studied neural stem cell markers although not as high as the wild type cultures. In contrast, the $Mbd3^{(-/-):Mbd3a}$ rescued cell line has recovered the expression of the studied genes in a parallel pattern to the wild type cell line. These experiments suggest that Mbd3 is involved in the differentiation from ES cells into neuroectoderm lineages, such that $Mbd3^{(-/-)}$ cells fail to express genes that are key of neural stem cell differentiation, at least at detectable levels by RT-PCR (figure 5.10).

In summary, although there are usually differences among the cell lines in terms of degree of differentiation capacity, there is always consistency among the wild type, null and rescued genotypes in the phenotypic overview.

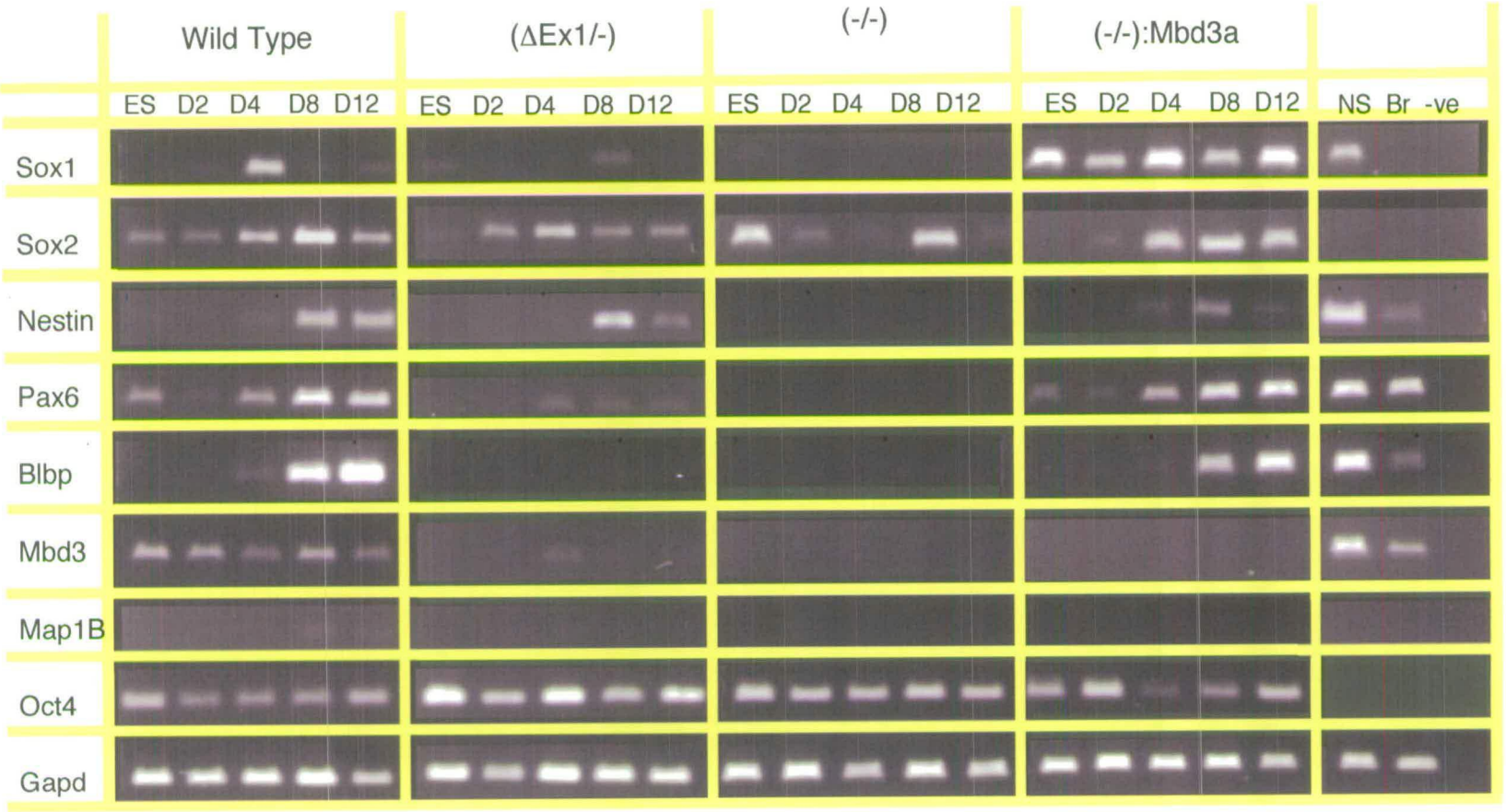


Figure 5.10 *Mbd3*$^{(-/-)}$ cells do not express NSC markers in differentiation conditions. RT-PCR panel with neural stem cell markers gene expression from ES cells to D12 of monolayer differentiation . NS= NS cells, Br= adult brain

5.5 PA-6 co-culture differentiation system.

My previous studies demonstrated that Mbd3 is necessary for the transition from Oct4-positive ES cells to differentiated neuroectoderm. Very few Nestin positive neural precursor cells and neurons and no astrocytes can be observed when *Mbd3*$^{(-/-)}$ ES are challenged to differentiate *in vitro*. These results generate a subsequent question: Can *Mbd3*$^{(-/-)}$ differentiated cells survive, or do *Mbd3*$^{(-/-)}$ ES cells fail to transit to a differentiated cell fate?

To address this question, the PA-6 co-culture differentiation system was used (Kawasaki et al., 2000). In this system, differentiation of ES cells proceeds in adherent co-cultures of ES cells with PA-6 stromal cells in serum free media. The main difference of this system is the decreased cell death compared with the monolayer differentiation system. Therefore, I could investigate whether *Mbd3*$^{(-/-)}$ cells die during the differentiation process by studying the apoptotic behaviour of *Mbd3*$^{(-/-)}$ cells in comparison with wild type ES cells. This protocol consists of plating a low number of ES cells in a layer of PA-6 cells in serum-free media containing knock out serum replacement KSR, known as 'differentiation medium' for a period of 7 days, followed by another 7 days in N2B27 medium containing ascorbic acid described as 'induction medium'.

PA-6 cells are derived from skull bone marrow and produce an inducing signal called stromal cell-derived inducing activity or SDIA (Kawasaki et al., 2000). Although other stromal cells promote neural differentiation when used as feeders, PA-6 cells have been found to produce higher induction. The SDIA effect remains through a filter (though results in weaker induction), does not condition the media and is not destroyed by fixation of stromal cells (Kawasaki et al., 2000). Therefore SDIA could be mediated by secreted factors that would anchor to the cell surface.

The experiment was designed to screen the presence of apoptosis at correlated time points during the process of PA-6 co-culture differentiation by immunocytochemistry for activated caspase-3. The time points selected were day 0, day 4, day 8, day 12 and day 16. It was observed in these experiments that cells lacking Mbd3 do not die by apoptosis in different fashion than wild type (figure 5.11).

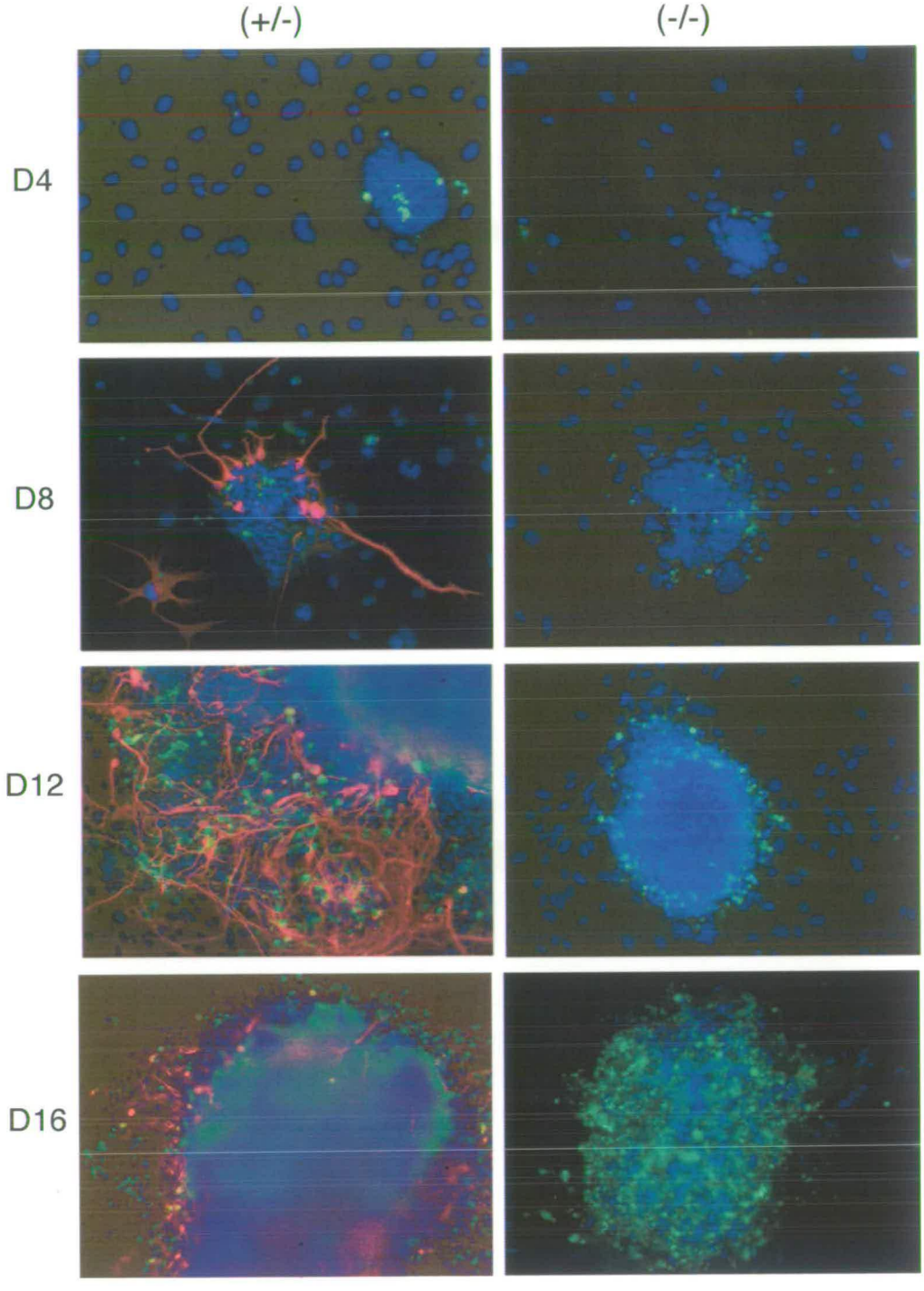


Figure 5.11 *Mbd3*$^{(-/-)}$ do not die by apoptosis upon differentiation conditions. Immunocytochemistry analysis after 4 time points in PA-6 co-culture differentiation system. Caspase-3 green, TuJ1 red. DAPI blue.

In summary, the previous experiments demonstrate that *Mbd3*$^{(-/-)}$ ES cells induced to undergo neural induction neither show a delay in differentiation nor die during the process. Rather, *Mbd3*$^{(-/-)}$ ES cells maintain their undifferentiated Oct4 positive state without expression of neural genes throughout the time in culture. In conclusion, these data suggest that Mbd3 is necessary to switch off pluripotency markers to transit ES cell to differentiated cell state.

5.6 Study of Sox1 overexpression in Mbd3 overexpressing cell lines

I have previously demonstrated that the lack of Mbd3 impairs ES cells to differentiate into neuronal lineages. A following question is whether overexpression of Mbd3 would lead to the opposite effect increasing the degree of differentiation in ES cells. During neuroectoderm differentiation experiments from ES cells I observed that the *Mbd3*$^{(-/-):Mbd3a}$ rescued cell line achieved a higher degree of neuronal differentiation than wild type or *Mbd3*$^{(-/-):Mbd3b}$ rescued cell lines. *Mbd3*$^{(-/-):Mbd3a}$ ES cells overexpress Mbd3 approximately two-fold (K.Kaji personal communication). Further, I also observed that *Mbd3*$^{(-/-):Mbd3a}$ ES were marked by Sox1 expression by RT-PCR (figure 5.10). Sox1 is a marker of neural precursors and is not observable in ES cells by RT-PCR in normal conditions. Sox1 overexpression has been shown to direct neuroectodermal differentiation in ES cells (Zhao et al., 2004). Given these previous observations, I decided to investigate whether an overexpression of Mbd3, which presumably leads to Sox1 overexpression, could cause an increased and/or premature neural differentiation.

Overexpressing ES cell lines 46C^{Mbd3a} and 46C^{Mbd3b} were created by inserting Mbd3a and Mbd3b expression construct respectively into *Sox1*-GFP knock-in ES cells (46C). Additionally, a mock construct with only puromycin selection was inserted in 46C ES cells as a control (46C^{Mock}). Colonies from these transfections were obtained at normal rates and the clones selected had no obvious abnormalities. However, RT-PCR experiments revealed no overexpression of Sox1 (figure 5.12).

To study whether Mbd3 overexpression could lead to premature and/or increased neuronal differentiation, two Mbd3a and two mock transfected 46C ES clones were selected to undergo neuroectoderm differentiation by serum-free monolayer system. The degree of differentiation was monitored from day 0 to day 10, and no obvious

neuronal differentiation was found in $46C^{Mbd3a}$ ES cell lines compared witth wild type (figure 5.12). To study a possible premature neural differentiation, 3 day and 4 day cultures were immunostained to check the expression of Nestin to quantify neural precursor differentiation. I observed same degree of Nestin positive staining in $Mbd3^{(-/-):Mbd3a}$, mock and wild type cultures (figure 5.13).

From these experiments I can conclude that the overexpression of Mbd3 in ES cells does not lead to precocious or increased neural differentiation.

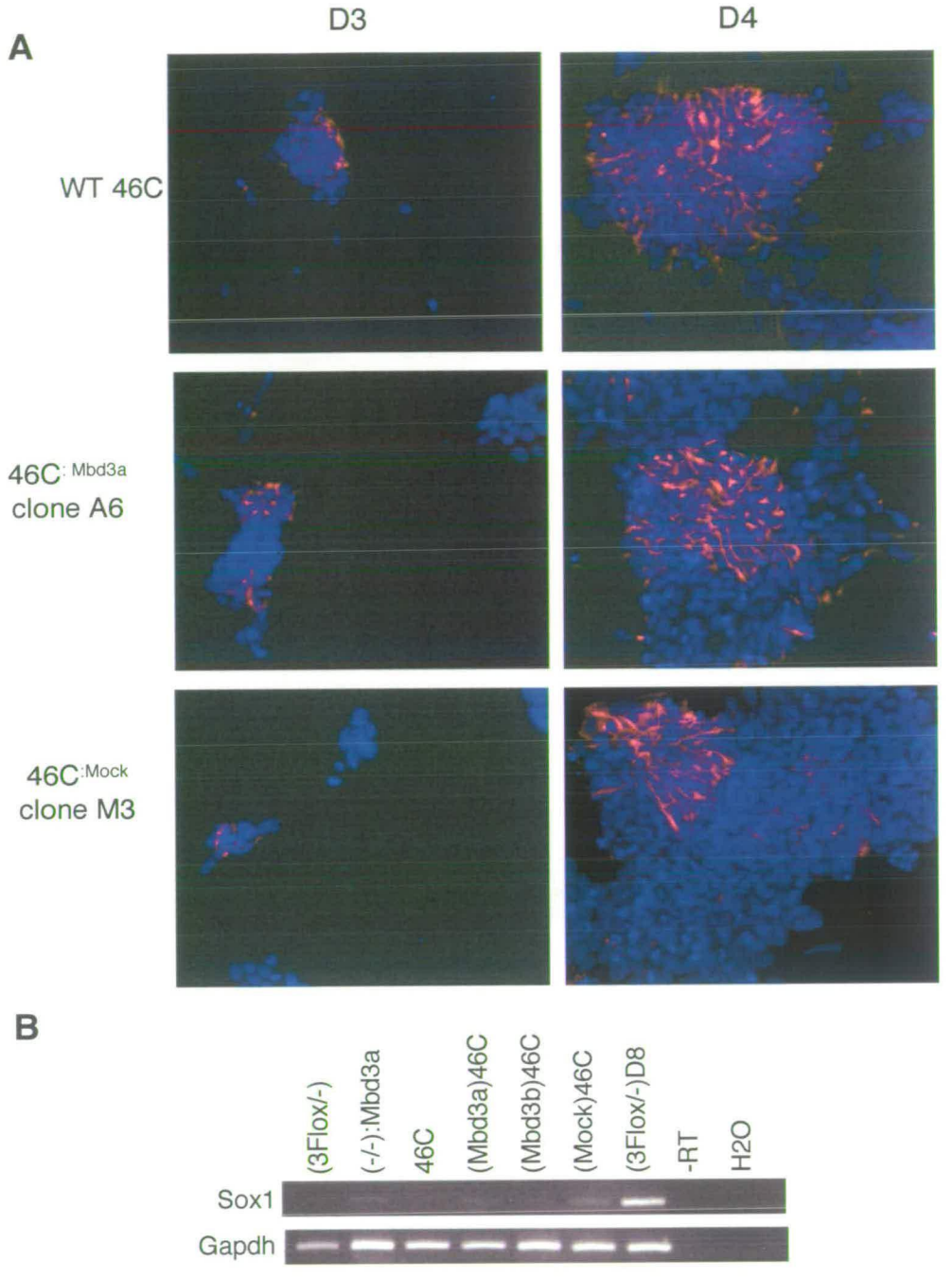


Figure 5.12 A) Mbd3 overexpressing ES cells were differentiated and immunostained for Nestin at day 3 and 4 of monolayer differentiation. **B)** RT-PCR reveal no overexpression of Sox1 in 46C ES cell lines overexpressing Mbd3a or Mbd3b compared with mock 46C lines. The highest Sox1 expression corresponds to *Mbd3* $^{(3Flox-)}$ ES cells after 8 days in serum-free monolayer differentiation conditions. From left to right: *Mbd3*$^{(3Flox-)}$ ES cells, *Mbd3*$^{(--)Mbd3a}$ *Mbd3a* overexpressing ES cells, 46C ES cells, 46C *Mbd3a* overexpressing ES cells, 46C *Mbd3b* overexpressing ES cells, 46C Mock ES cells, D8 monolayer differentiation sample from *Mbd3* $^{(3Flox-)}$ ES cells, minus reverse transcriptase and negative (water) sample.

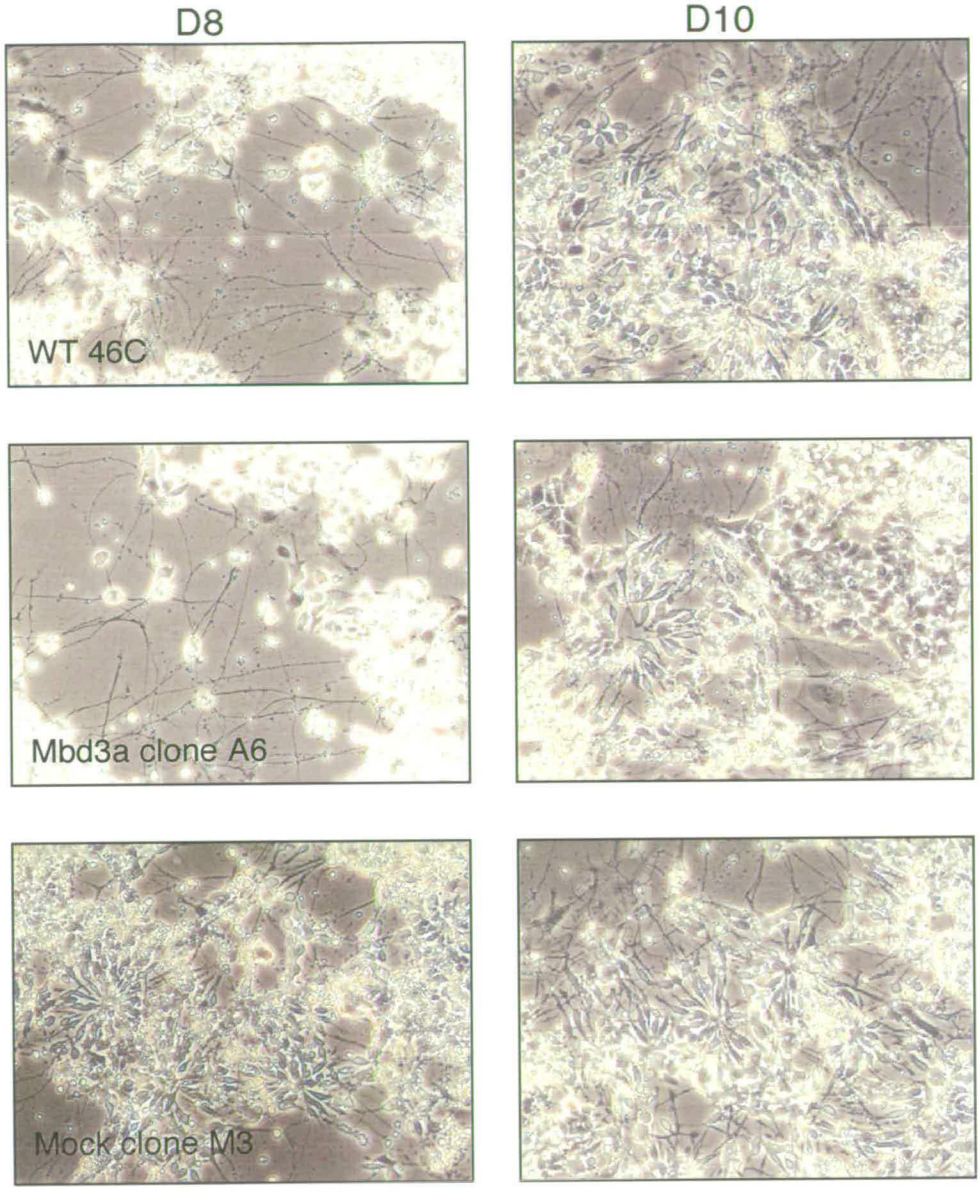


Figure 5.13 Monolayer differentiation at D8 and D10 live cultures shows no precocious or increased neuronal differentiation in overexpressing $46C^{Mbd3a}$ cell line, in comparison with both its parental line 46C and Mock cell line.

5.7 Study of neural stem cells lacking Mbd3

5.7.1 *Mbd3*$^{(-/-)}$ NS from *Mbd3*$^{(-/-)}$ ES

I observed a differentiation defect of *Mbd3*$^{(-/-)}$ ES cells into neural precursors and terminally differentiated neural cells. These observations raise the question of whether this deficiency is due to a defect in ES-specific signalling pathways, or whether Mbd3 is involved in other stages of differentiation. To answer that question, I aimed to obtain a pure population of *Mbd3*$^{(-/-)}$ neural stem cell lines (NS cells).

A first attempt to derive *Mbd3*$^{(-/-)}$ NS cells from *Mbd3*$^{(-/-)}$ ES cells was unsuccessful. This result was expected since the number of neural progenitors in the first step of neuroectoderm differentiation in monolayer is dramatically reduced in *Mbd3*$^{(-/-)}$ cultures. This was the case both for *Mbd3*$^{(-/-)}$ and *Mbd3*$^{(\Delta Ex1/-)}$ ES cells, although the later formed neurosphere-like colonies that failed to produce NS cells. These experiments may indicate that Mbd3 is necessary for the ES-NS cell transition.

5.7.2 *Mbd3*$^{(Flox/-)}$ NS Cre-deletion

Subsequently, a variety of strategies were tested to obtain *Mbd3*$^{(-/-)}$ NS cell lines. In first place, *Mbd3* floxed NS cells (*Mbd3* $^{(3Flox/-)}$), which have two loxP sites flanking the entire *Mbd3* gene, were electroporated with CAG-CRE-IP. This construct contains a CAG promoter that drives the expression of the *Cre recombinase* and *puromycin-N-acetyltransferase* (*pac*) genes simultaneously due to the encephalomyocarditis virus (EMCV) internal ribosome entry site (IRES) (Mountford et al., 1994). The CAG promoter, which includes chicken β-actin promoter and a CMV immediate-early enhancer element (Niwa et al., 1991), drives robust transgene expression in ES cells (Niwa *et al.*, 2000; Chambers *et al.*, 2003).

Additionally, the plasmid CAG-IP was electroporated in a control set of cells (mock experiment). I also co-transfected 1/10 amount of a CAG-GFP-IP construct, as an early marker for transfected cells. With this strategy, GFP positive NS cells were sorted out and replated at clonal density. However, no NS colonies could be obtained by this strategy in comparison with the mock transfected group where colonies were recovered at expected frequencies.

These results could be due to three reasons; first, *Mbd3*$^{(-/-)}$ NS cells may not be viable, and as soon as Mbd3 is not present in the cell, NS cells die. Second, *Mbd3*$^{(-/-)}$ NS cells may survive but can not proliferate and divide symmetrically in NS daughter cells to give rise a colony. Third, *Mbd3*$^{(-/-)}$ NS may be viable and can proliferate but the efficiency of Cre transfection is so low that colonies cannot be detected.

5.7.3 *Mbd3*$^{(-/-)}$ NS from *Mbd3*$^{(Flox/-)}$ Cre-ER^{T2} ES

To determine whether *Mbd3*$^{(-/-)}$ NS cells are viable, a second strategy was designed to obtain *Mbd3*$^{(Flox/-)}$ NS cells where *Mbd3* could be deleted at a determined time point. This strategy consisted in making conditional knock out *Mbd3* ES cells using the tamoxifen inducible form of the Cre recombinase, Cre-ER^{T2}, where the *Cre* recombinase is fused with the ligand binding domain of the estrogen receptor (ER) (Metzger et al., 1995). A mutation in the binding site of the receptor prevents the binding of cell endogenous estradiol, and is only activated by synthetic ligand 4-hidroxytamoxifen (OHT or tamoxifen) (Feil et al., 1996; Feil et al., 1997). This strategy would allow discerning in which different steps from ES cell to differentiated cell Mbd3 is necessary.

Mbd3$^{(Flox/-)}$ ES were stably transfected with the vector CAG-Cre-ER^{T2}-IP and CRE-ER^{T2}-ES clones were selected based on a correct response to tamoxifen. This selection screened those clones where *Mbd3* was not detected by genotyping PCR in addition of tamoxifen. However, PCR experiments denoted that some clones already contained cells where *Mbd3* had been deleted in absence of tamoxifen. This effect may be caused by the strong CAG promoter of the construct that would start the synthesis of Cre in the absence of tamoxifen. Nevertheless, I decided that it would be worth while to continue with the derivation of the NS cell lines (from the clones with less ''leaking''), since my previous data showed that *Mbd3*$^{(-/-)}$ ES cells are unable to differentiate into NS cells and so, those undifferentiated cells would be eliminated by the differentiation conditions.

Subsequently, four different clones of *Mbd3*$^{(Flox/-)}$ CRE-ER^{T2} ES cells were chosen to derive NS cell lines (A8, C4, F1 and F4), and once obtained, *Mbd3*$^{(Flox/-)}$ CRE-ER^{T2} NS cells were further characterised. To ensure that the tamoxifen

concentration necessary for the experiment is not toxic for the NS cells, I tritated the tamoxifen concentration that NS cells can tolerate and found that wild type cells can be maintained in double the working tamoxifen concentration without any observable effect. During derivation of NS cell lines, the cultures consist of a mixed population of cells, yet they become progressively more homogenous with the passages in the presence of EGF, bFGF and NS-A media. Hence, although, *Mbd3*$^{(Flox/-)}$ CRE-ER^{T2} NS cultures were expressing neural stem cell markers (Rc2, Nestin and Vimentin) (figure 5.14.2A-2E) in this non-pure first step, they had different morphology than *Mbd3*$^{(Flox/-)}$ NS cultures (figure 5.14.1A-1E), and other non NS cell types were found in the cultures. *Mbd3* deletion on addition of tamoxifen was confirmed by PCR genotyping. However, as in the case of the ES cells, a percentage of NS cells were already *Mbd3* null even without addition of tamoxifen (figure 5.14.3).

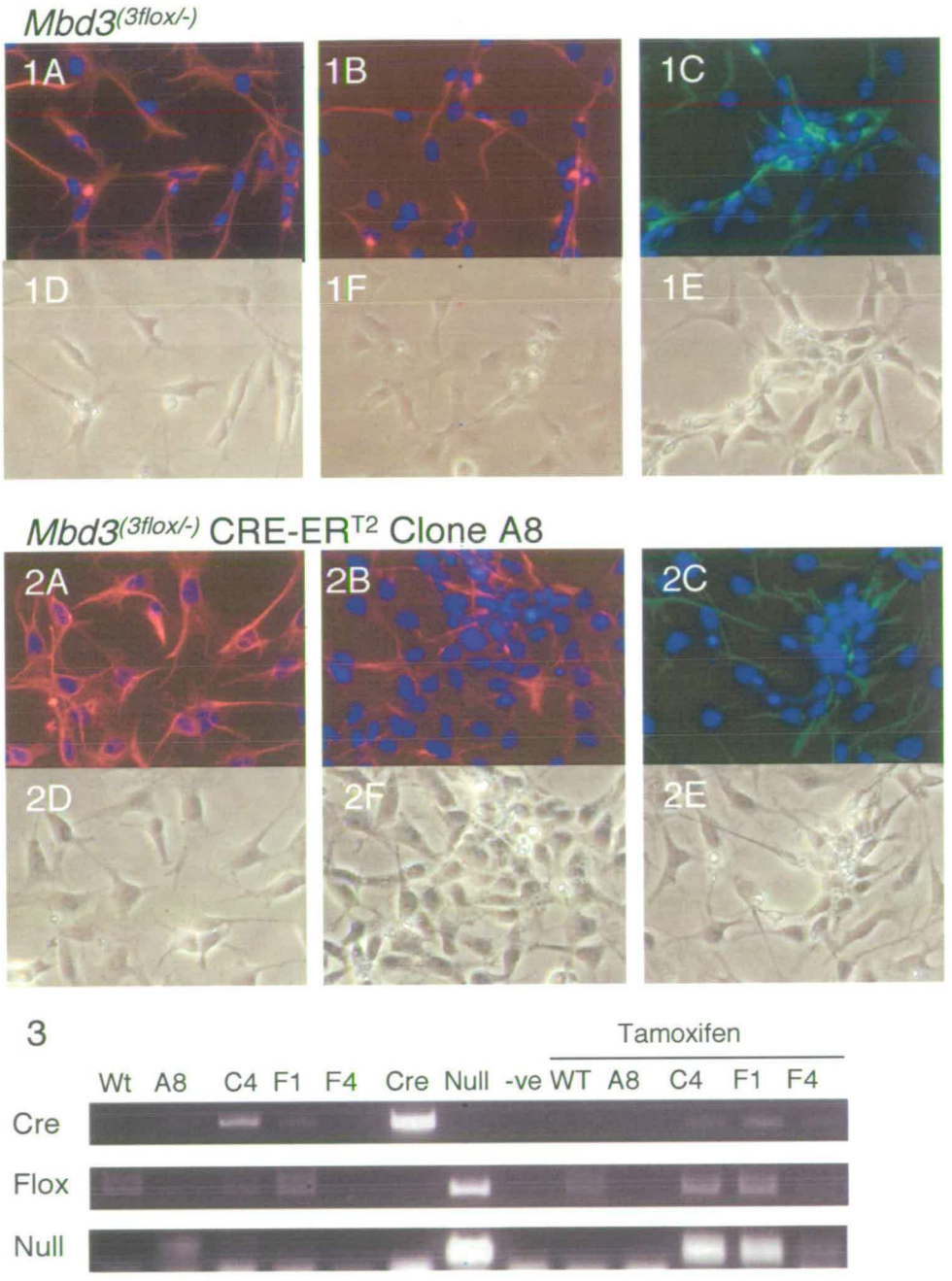


Figure 5.14. 1&2.Both *Mbd3*$^{(3flox-)}$ and *Mbd3*$^{(3flox-)}$CRE-ER^{T2} cell lines express neural stem cell markers after 3 passages from ES derivation. Panel 1. *Mbd3*$^{(3flox-)}$. Panel 2, *Mbd3*$^{(3flox-)}$CRE-ER^{T2}. A,Vimentin. B, RC2 and C, Nestin. D, E. F, bright field. Panel 3. Genotyping PCR, from left to right wild type NS cells, *Mbd3*$^{(3flox-)}$ floxed clones A8, C4, F1 and F4, Cre plasmid, *Mbd3*$^{(-/-)}$ ES cells and negative control (water sample) with and without tamoxifen. From up to down, set of primers to amplify Cre, floxed allele (Flox) and null allele. *Mbd3*$^{(-/-)}$ can be detected in absence of tamoxifen.

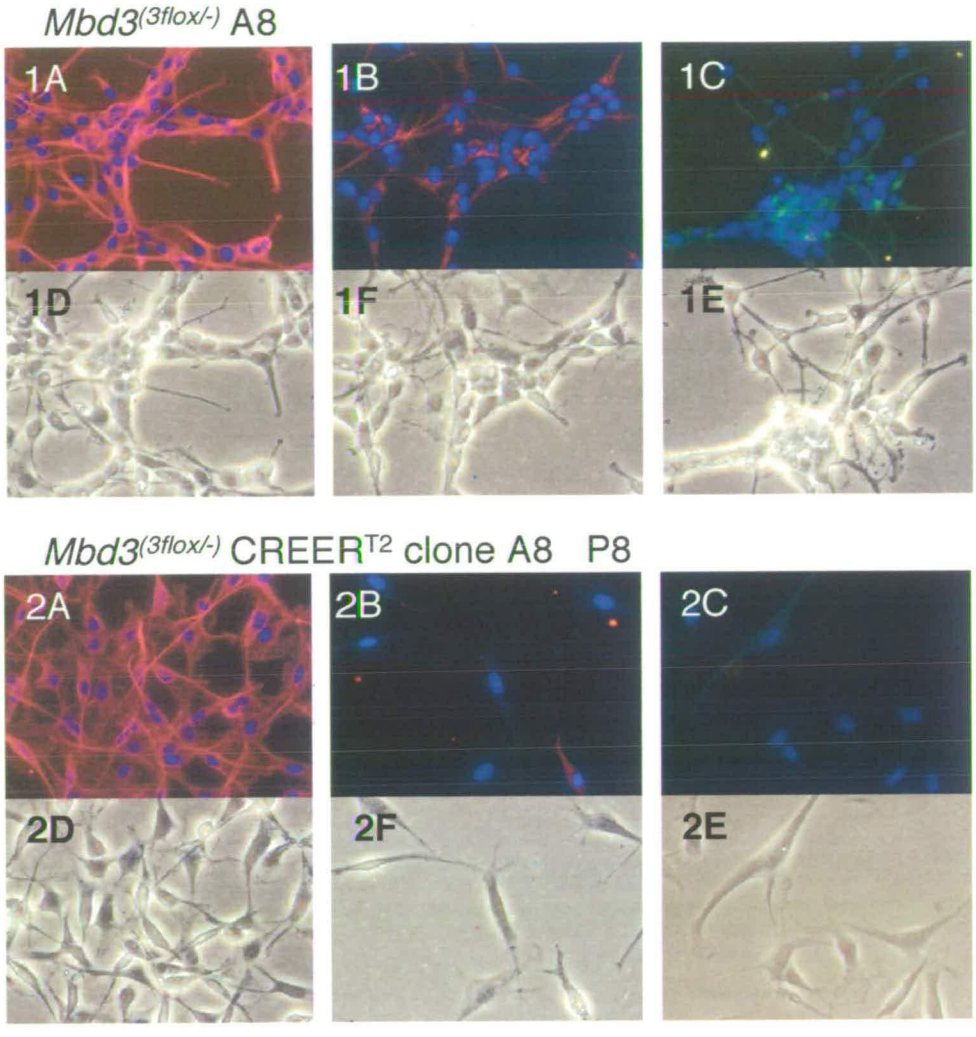


Figure 5.15 *Mbd3*$^{(3flox/-)}$ CRE-ERT^{2} stop proliferation and loses neural stem cell marker expression after 8 passages. Panel 1. *Mbd3*$^{(3flox/-)}$ Panel 2. *Mbd3*$^{(3flox/-)}$ CRE-ER^{T2} clone A8. A: Vimentin, B: Rc2 and C: Nestin. D, E F, bright field.

During subsequent passages the control wild type NS cell line was forming a uniform looking culture, consisting of a pure population of NS cells. In contrast the four CRE-ER^{T2} NS lines were losing their ability to proliferate and self renew until it was not possible to maintain the lines. Concordantly, these lines stopped expressing neural stem cell markers. This effect was observable both in the presence or absence of tamoxifen, although those not treated with tamoxifen showed a slightly higher proliferation rate (figure 5.15).

To study the effect of lack of Mbd3 in differentiated cells, Mbd3$^{(Flox/)}$ Cre-ER^{T2} NS lines were differentiated into astrocytes in the presence of serum and absence of growth factors. In these experiments I could observe that, although there were some Gfap positive astrocytes in the cultures (figure 5.17), they were dying by apoptosis within 6 days, in contrast with control experiments with *Mbd3*$^{(Flox/-)}$ Mock NS lines (figure 5.16). This effect was observable both in the presence and absence of tamoxifen, and it cannot be definitely concluded that Mbd3 is necessary for the maintenance of healthy astrocytes, because without tamoxifen, not all of the astrocytes might be *Mbd3* null.

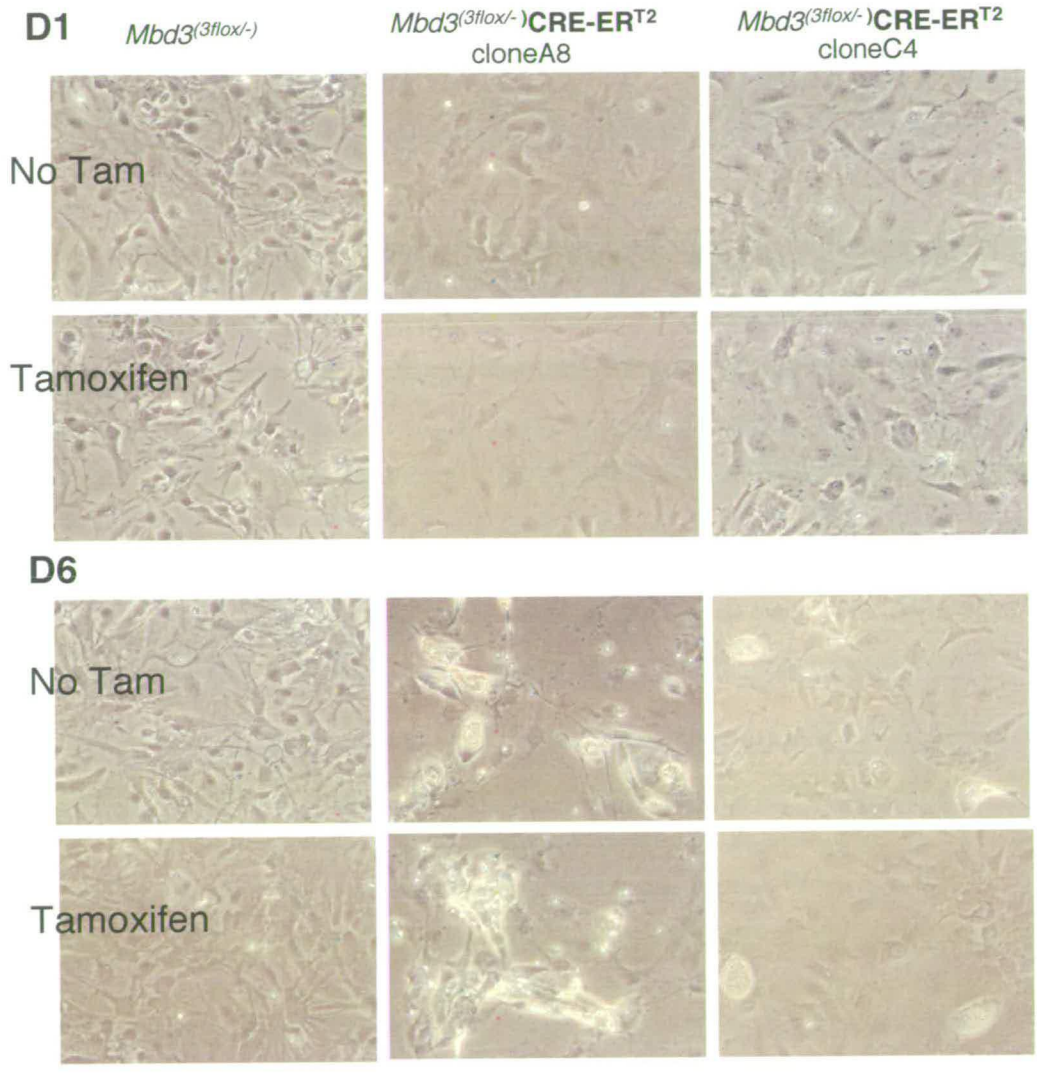


Figure 5.16 Upper panel. Day1 upon astrocytic differentiation conditions. Serum and growth factors withdrawal. Lower panel, Day6 upon astrocytic differentiation conditions. In contrast with *Mbd3*$^{(3flox/-}$. *Mbd3*$^{(3flox/-}$)CRE-ER^{T2} undergoes apoptosis after 5 days.

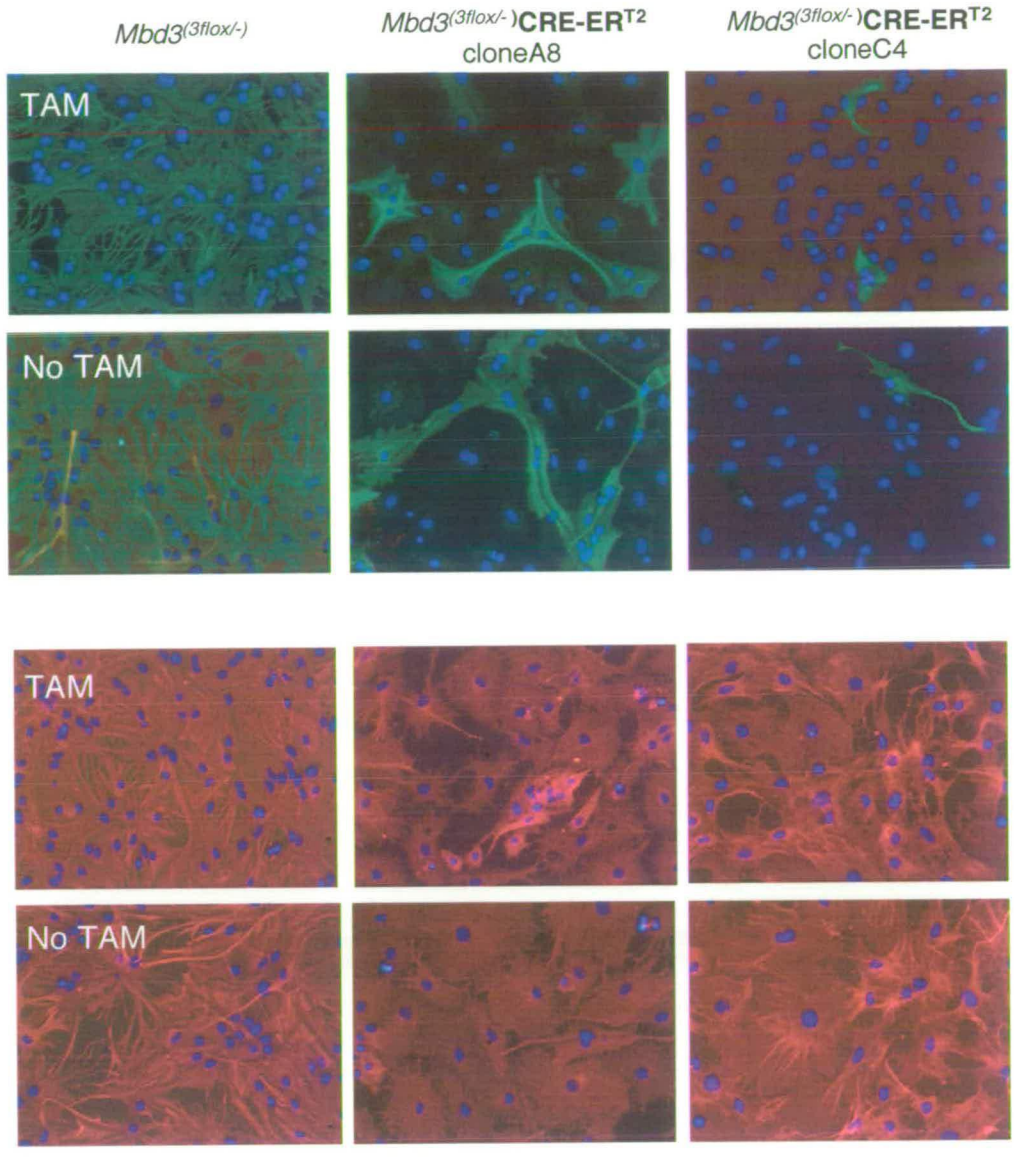


Figure 5.17 Immunocytochemistry of differentiated astrocytes from *Mbd3*$^{(3flox/-)}$ CRE-ER^{T2} cell lines in comparison with wild type. The frequency of astrocyte differentiation is dramatically lower, which correlates with a slower proliferation, loss of neural stem cell markers, and apoptotic process as seen in fig 5.17. Upper panel, immunostained for Gfap in green and Rc2 in red. Lower panel stained for Vimentin. The results are independent of tamoxifen addition. First and third row, Tamoxifen added. Second and fourth row, no tamoxifen added.

Given that the Cre-ER^{T2} system could not give a conclusive answer to whether Mbd3 is necessary for NS cell formation, a third strategy was designed. The drawback of transfection by electroporation in NS cells is the low cell viability after electroporation, thus I felt necessary to test whether the absence of *Mbd3*$^{(-/-)}$ NS colonies after transfection was due to the inability for NS cells to survive in the absence of Mbd3 or to the combination of very low transfection efficiency and very low cell survival after electroporation. To test this hypothesis the nucleofection system was used instead. This system allows much higher cell viability after transfection and happens very rapidly because it delivers the DNA directly into the nucleus. The biggest benefit is, however the higher efficiency of transfection of 70% compared with 20% by electroporation.

Mbd3$^{(Flox/-)}$ NS cells were nucleofected with the CAG-Cre-IP and CAG-IP(as control) constructs. The transfected cells were separated in two groups, one was cultured in the presence of puromycin, and in another group Gfp positive cells were FACS sorted. These experiments were done in duplicate. In the first experiment drug selection was maintained for 5 days and in the second experiment drug selection was maintained for 3 days. Surprisingly, colonies were recovered in both groups, although in a much lower frequency than same cells electroporated with a Mock plasmid GFP-IP (table 5.1)

	CAG-CRE-IP	
	PURO	FACS sorted
Exp1 CRE	5.6%	22.29%
Exp2 CRE	17.53%	17.32%

Table 5.1 Percentage of recovered colonies after deletion of Mbd3 by Cre recombination of *Mbd3*$^{(Flox/-)}$ NS cells in two independent experiments. In experiment 1 puromycin selection was maintained for 5 days, while in experiment 2 puromycin selection was maintained for 3 days.

All the surviving colonies were picked, expanded and genotyped. The approximate frequency of *Mbd3*$^{(-/-)}$ colonies by genotyping PCR was 23%, and from them 5 were expanded and stocked (figure 5.18.A). Thus, Mbd3 is not required for NS cell viability.

5.7.4 *Mbd3*$^{(-/-)}$ NS astrocytic differentiation

Two *Mbd3*$^{(-/-)}$ NS cell lines were selected for astrocytic differentiation. *Mbd3*$^{(-/-)}$ NS cells, in the presence of serum and absence of EGF and bFGF, did differentiate into morphologically normal astrocytes. Immunocytochemistry confirmed that 100% of the cells were Gfap positive. Hence, astrocytes can be maintained in the absence of Mbd3 (figure 5.18.B)

In addition, I wanted to study whether deletion of Mbd3 would affect astrocytic viability. To determine this, *Mbd3*$^{(Flox/-)}$ were plated in astrocyte differentiation conditions for 7-10 days, and subsequently were harvested and co-transfected by nucleofection with Cre expression and Gfp constructs previously described. The survival after replating of differentiated astrocytes was proven to be very low independently of transfection, since non-transfected replated astrocytic cultures show similar recovery to transfected cultures. The efficiency of transfection, estimated by the number of Gfp positive cells, was as well very low, and there was no evidence for proliferation. Genotyping PCR showed no *Mbd3*$^{(-/-)}$ in the cultures. It is possible that in order the gene to be deleted in the nucleus the cell need to be in a proliferative state (figure 5.19.A).

In a independent experiment, *Mbd3*$^{(Flox/-)}$ NS were transfected with Cre expression construct and consequently exposed to serum to promote astrocytic differentiation at different time points. In addition, a set of clones were cultured with puromycin selection and other set without puromycin selection. Subsequently, these clones were genotyped by PCR. From these experiments I can conclude that *Mbd3*$^{(-/-)}$ Gfap positive astrocytes can be maintained *in vitro* (figure 5.19.B).

In summary my experiments demonstrate by two independent strategies that surprisingly, *Mbd3*$^{(-/-)}$ NS cells can differentiate into astrocytes.

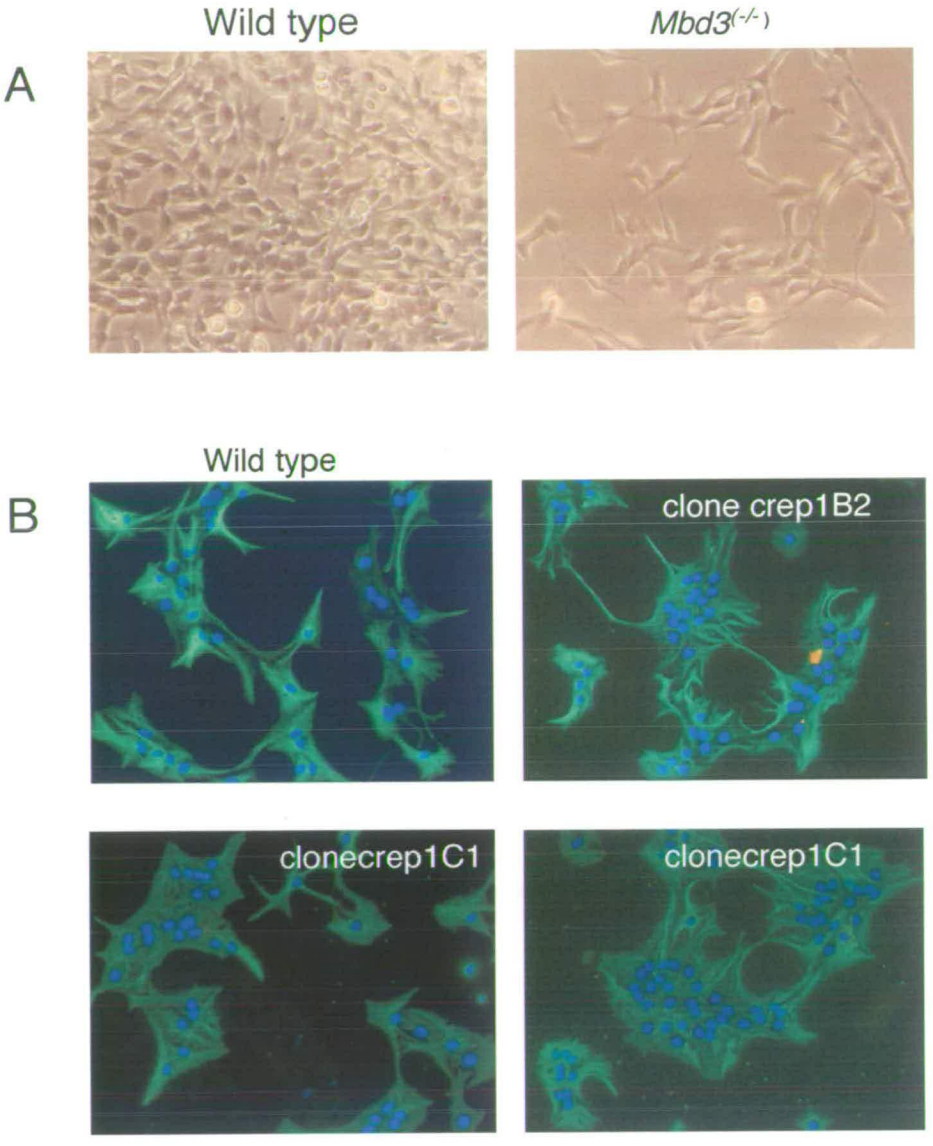
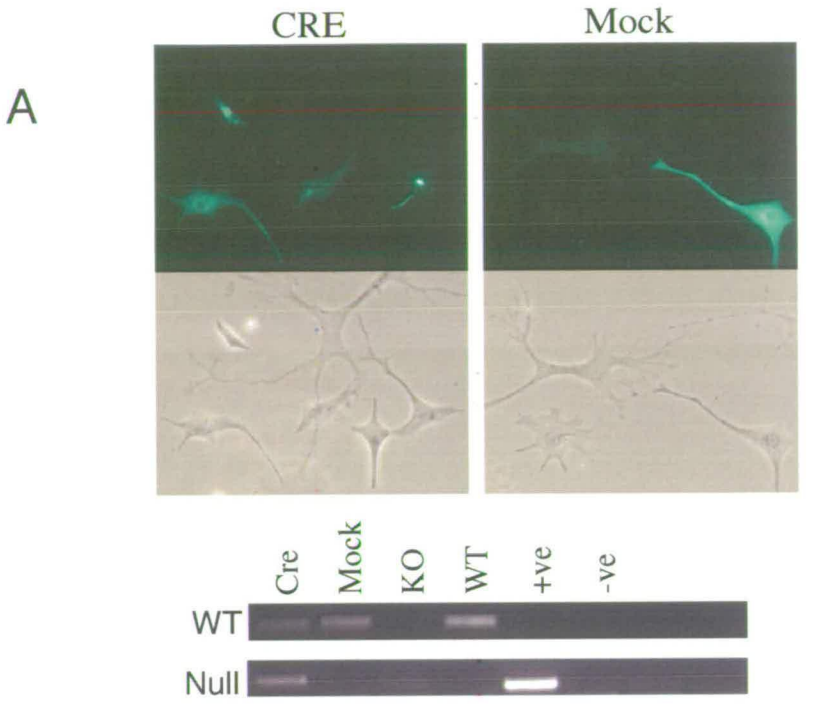
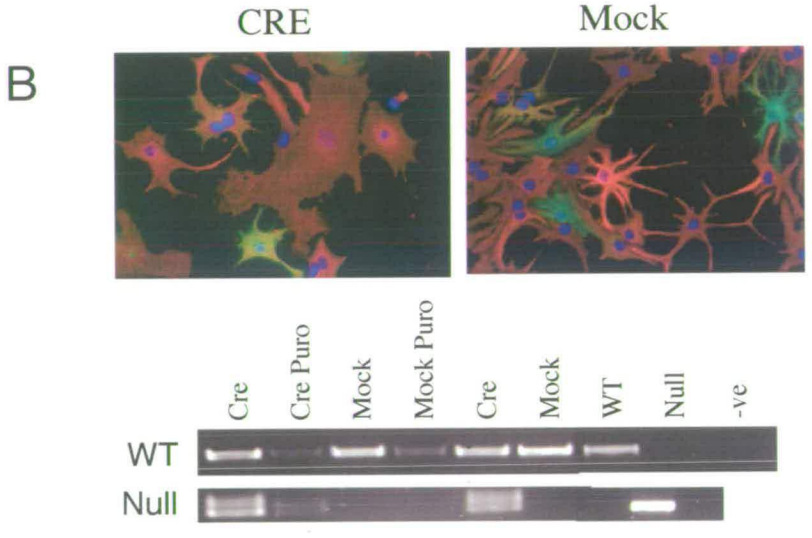


Figure 5.18. *Mbd3*$^{(-/-)}$ colonies can be expanded, and *Mbd3*$^{(-/-)}$ and undergo astrocytic differentiation, **A)** bright field Wild type versus *Mbd3*$^{(-/-)}$ colony. **B)** NS-derived astrocytes immunocytochemistry, Wild type astrocytes in top left, *Mbd3*$^{(-/-)}$ astrocytes derived from *Mbd3*$^{(-/-)}$ NS clone crep1B2 in top right and clone crep1C1 in the bottom. GFAP in green, Rc2 in red, Dapi in blue.


A
CRE
Mock
Cre
Mock
KO
WT
+ve
-ve
WT
Null


B
CRE
Mock
Cre
Cre Puro
Mock
Mock Puro
Cre
Mock
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Null

Figure 5.19

A) *Mbd3*$^{(3flox/-)}$ NS-derived astrocytes 10 days old are transfected with Cre or Mock plasmid. The efficiency of transfection is low. Below, genotyping PCR to amplify wild type allele (WT) and null allele (Null) for 40 cycles reveals no deletion of Mbd3. Samples from left to right: *Mbd3*$^{(3flox/-)}$ NS-derived astrocytes transfected with Cre plasmid (Cre), with Mock plasmid (Mock), *Mbd3*$^{(-/-)}$ ES as positive control, and negative control (-ve).

B) NS cells were transfected and subsequently serum was added after 24 hours. Immunoctychemistry reveals Gfap/Gfp coexpression (Gfap is in red and Gfp in green). Below, genotyping PCR reveals Cre deletion of Mbd3 in NS derived astrocytes. From left to right NS-derived astrocytes in the following conditions: Cre transfected (Cre), Cre transfected with puromycin selection (Cre Puro), Mock transfected (Mock), Mock transfected with puromycin selection (Mock Puro), Cre transfected (Cre), Mock transfected (Mock), wild type, *Mbd3*$^{(-/-)}$ ES as negative control (Null), and water sample as negative control (-ve).

5.8 Summary

Mbd3$^{(-/-)}$ ES cells show neuroectodermal differentiation impairment. *Mbd3*$^{(-/-)}$ ES cell neuronal differentiation efficiency is extremely low and they cannot differentiate into astrocytes in two different systems tested: serum free monolayer differentiation and PA-6 co-culture. *Mbd3*$^{(-/-)}$ cells do not die during differentiation since the number of caspase-3 positive cells is not higher, rather is lower, than wild type cells. *Mbd3*$^{(-/-)}$ cells can remain as undifferentiated ES cells positive for Oct-4 in the absence of LIF and serum for minimum of two weeks, and neural stem cell markers cannot be detected by RT-PCR. Overall, these experiments demonstrate that Mbd3 is necessary for ES cells to transit from Oct4 positive pluripotent cell to neural cell.

The efficiency of colony formation in conditionally deleted *Mbd3*$^{(-/-)}$ NS cells is much lower than the control. However, *Mbd3*$^{(-/-)}$ NS cells can be obtained, and can differentiate into Gfap positive astrocytes. Hence, Mbd3 is not essential for NS cell and astrocyte survival.

5.9 Discussion

5.9.1 Why only neurons?

Why are *Mbd3*$^{(-/-)}$ ES cells able to differentiate into post-mitotic neurons and not other cell types that could be marked by Gata4 or Gfap in the monolayer differentiation system? Is it because cells have an intrinsic neuronal differentiation default mechanism (Tropepe et al., 1999) and therefore, even in impaired differentiation conditions such as the lack of Mbd3 is still possible for the cell to become a neuron? Although Nestin is a marker for all neural precursors and there are TuJ1 positive cells, there is a negligible proportion of Nestin positive cells in the cultures. Is this transition specifically faster in the absence of Mbd3 so that Nestin positive cells are rarely detected?

5.8.2 Terminally differentiated astrocytes?

Interestingly, although *Mbd3*$^{(-/-)}$ ES cells did not differentiate into astrocytes, *Mbd3*$^{(-/-)}$ NS cells could, apparently healthily and efficiently. Is this because once the

null NS cell is developmentally beyond the Mbd3-dependent signal that prevents differentiation and maintains its pluripotency can function normally? This is unlikely to be the case given the extreme difficulties and low efficiency of *Mbd3*$^{(-/-)}$ NS cell derivation. On the other hand NS cells can differentiate into Gfap positive cells in less than 8 hours in the presence of Bmp4, and surprisingly, they can regain neural stem cell characteristics with Bmp4 withdrawal and bFGF/EGF signalling (S. Pollard, personal communication). Hence, it is possible that Gfap positive NS derived cells are quiescent progenitor cells and not fully differentiated post-mitotic astrocytes. Although Gfap is the key astrocytic marker in the field, it is increasingly clear that Gfap ''positiveness'' is not synonymous with terminal differentiated post-mitotic cell. Mouse post natal and adult radial glia, and human and primate radial glia are GFAP positive regardless of neurogenic capability (Choi and Lapham, 1978; Levitt and Rakic, 1980) (Malatesta et al., 2000). There are as well several reports where astrocytes have neurogenic potential (Casper and McCarthy, 2006; Garcia et al., 2004; Sanai et al., 2004). Therefore, I suggest that Gfap should be considered more as stem cell marker than as post-mitotic fully differentiated marker.

In conclusion, it may be possible that Gfap positive *Mbd3*$^{(-/-)}$ cells obtained after growth factor withdrawal and addition of serum are quiescent progenitor cells and not terminally differentiated *Mbd3*$^{(-/-)}$ astrocytes. If this would be the case, the question of whether astrocytes can survive without Mbd3 would remain to be answered.

5.8.3 Aberrant TuJ1 expression

In *Mbd3*$^{(-/-)}$ monolayer cultures, there are cells stained with the antibody TuJ1, and though the signal is not high, it is above the background. TuJ1 is a monoclonal antibody that reacts with c-terminal class III β tubulin, which is the earlier marker of neuronal differentiation after or during cell division (Lee et al., 1990b). There is evidence of TuJ1 positive cells in sertoli cells of the testis (Lewis and Cowan, 1988), transiently in non neuronal embryonic tissues (Lee et al., 1990a) and in some types of tumorigenic cells (squamous cell carcinomas, lymphomas and melanomas) (Scott et al., 1990). Perhaps, the immunoreactivity of *Mbd3*$^{(-/-)}$ ES cells to TuJ1 in differentiation conditions could be associated with its differentiation into another

type of embryonic tissue or its differentiation deficiency, which could lead to aberrant cell types TuJ1 positive.

5.8.4 Further directions

A key cell model to understand the function of Mbd3 in a non pluripotent cell is the use of NS cell technology. As explained in this chapter, to obtain *Mbd3*$^{(-/-)}$ NS cells is extremely difficult. However, I have demonstrated that *Mbd3*$^{(-/-)}$ NS cells can survive and can undergo apparent astrocytic differentiation. It will be very interesting to characterise these cells further and analyse their proliferation, self-renewal and differentiation into neuronal lineages.

CHAPTER 6

GENERAL DISCUSSION AND FUTURE DIRECTIONS

6.1 DNA methylation, MeCPs and history

Great discoveries in the field of DNA methylation have been achieved during the last few years. These frantic advances have lead to an evolution in the comprehension of the function of the methyl-CpG binding proteins. Biochemical analysis demonstrated the existence of four methyl-CpG binding proteins to interprete the DNA methylation signal (Cross et al., 1997; Hendrich et al., 1998). The MeCPs can induce transcriptional repression by the recruitment of chromatin remodelling complexes to the methylated DNA. The repression machinery partners for each MeCP have already been revealed (Jones et al., 1998; Nan et al., 1998; Ng et al., 1999; Sarraf and Stancheva, 2004; Wade et al., 1999; Yoon et al., 2003b).

Several years ago, researchers in the field were expecting global gene expression changes in the *MeCP2* null animal models (Nan et al., 1997;(Willard and Hendrich, 1999). Given that MeCP2 was shown to require only a single methylated CpG base pair to bind, it was predicted that this protein would act as a global transcriptional repressor, and its absence would cause transcriptional noise (Nan et al., 1997). This binding capability is also shared by Mbd1 and Mbd2 (Cross et al., 1997, Hendrich et al., 1998). Later, and progressively, it was understood how MeCP2 was most likely involved in silencing of individual targets (Chen et al., 2003; Martinowich et al., 2003). Recently, Klose and colleages discovered that MECP2 needs an A/T run in its binding site. This finding opens a new view for MeCP2 and its relation with the rest of the MeCPs. In the first place this specificity will allow to quickly find new targets for MeCP2, which before was believed to only need a single methylated mCpG (Klose et al 2006). Although MeCP2 can not occupy Mbd2 binding sites, Mbd2 can bind MeCP2 binding sites, thus Mbd2 could fit in the role of global transcriptional repressor. However, *Mbd2* null mice have proven this not to be the case (Hendrich et al, 2001). In contrast, specific target genes have been revealed to be regulated by Mbd2 (Hutchins et al., 2002; (Auriol et al., 2005). Mbd1 can bind specifically with its zinc finger domain to unmethylated CpGs as well as mCpGs

with its MBD (Jorgensen et al., 2001). Mbd1 was found to bind the human FGF2 CpG island promoter via its CxxC zinc finger domain (Ueba et al., 1999), although further studies have ruled out FGF2 as a target gene of Mbd1 (Jorgensen et al., 2001). The Kaiso binding site has two consecutive mCpG pairs and consequently, several candidate target genes have been proposed . However, *Kaiso* null mice have recently been shown to have no misregulation of these candidate genes (Prokhortchouk et al., 2006).

6.2 MeCP- redundancy or tailor-made MeCPs?

It has been widely hypothesised a possible functional redundancy between the MeCPs, as a clever mechanism to ensure silencing of methylated genes. The hypothesis was driven to answer two obvious questions: why does the vertebrate genome encode four methyl-CpG binding proteins, and why does the disruption of *Mbd1, Mbd2* or *Kaiso* cause mild phenotypes in mouse models (Zhao et al, 2003; Hendrich et al., 2001; Prokhortchouk et al., 2006)? In concordance with this hypothesis, studies carried out in our lab demonstrated how the mice survival to the RTT-like disease was directly regulated with the number of MeCPs that they still had left.

In disagreement with this hypothesis, and as discussed above, Mecp2 can not bind Mbd2 sites, and the knock down of *Mbd2* does not cause binding to Mbd2 sites by MeCP2 or Mbd1 (Ariol et al., 2005). It is possible that each MeCP targets repression to currently unkown individual target genes but as part of several other layers of transcriptional repression machinery to ensure correct silencing.

Surprisingly, this year was demonstrated the existence of another two methyl-CpG binding proteins, closely related with Kaiso and enlarging the family (Filion et al., 2006). What is more, this study opens the door to the possibility of many other unknown methyl-CpG binding proteins!

6.3 MeCPs and the central nervous system

Irrespective of whether there is functional redundancy between the 6 MeCPs or they play very specific silencing roles, what is still clear is that MeCPs have an important role in the central nervous system. This is where they are primarly

expressed, and the absence of MeCP2, Mbd1 or Mbd2 causes cognition-related dysfunctions. In addition to Rett Syndrome, there is a number of other neurological diseases caused by epigenetic dysfunction in which defects in synaptic circuits and neuronal maturation are involved (Zoghbi et al., 2003; Martin and Sun, 2004) . This demonstrates the consequences of the aberrant activation or silencing of epigenetically regulated genes that are crucial for neuronal function.

In this thesis I have investigated the hypothesis of whether MeCPs Mbd2, MeCP2 and Kaiso control neural stem cell decisions and whether this process occurs in a functionally redundant manner. This hypothesis was driven by the observations that MeCP2 control neural stem cell neurogenesis in *Xenopus laevis* (Stancheva et al., 2003), that Mbd1 is involved adult neural stem cell neurogenesis (Zhao et al., 2003) and that the Kaiso-containing NCoR complex is necessary to maintain undifferentiated neural stem cells (Jepsen et al., 2000, Hermanson et al., 2003).

My studies reveal that MeCPs are not necessary for neural stem cell function. This conclusion is driven by studies both from individual MeCPs as well as a triple knock out of the *MeCP2, Mbd2* and *Kaiso* genes. This conclusion agrees with parallel findings in the study of MeCP2 function in neurospheres where MeCP2 is found innocent of 'neural stem cell-ness matters' in mouse (Kishi and Macklis, 2004). In concordance with the lack of a role for Kaiso in neural stem cells, Kaiso has not been found to be a component of NCoR in mouse cells (Prokhortchouk, personal communication).

On the other hand, I found that MeCPs may be important in neuronal differentiation and maturation, following the train of recent discoveries in the field that appoint the most extensively studied MeCP, MeCP2 as an important player in neuronal maturation. Hence, although there may not be functional redundancy because the proteins have specific targets, it is possible that the maturation reflects several deficits accumulated within the cell to differentiate into neuronal fate. It will be very interesting to discern this question by studying the neuronal differentiation in MeCP2 and Mbd2 single null NS lines.

Once determined *in vitro* which protein or proteins are involved in neuronal maturation, it would be worth while to undertake detailed *in vivo* behavioural tests and learning and memory assays in mice to study whether the absence of MeCPs do

cause disruption of the complex neurocircuitry of the brain, in addition to what is already known for *MeCP2*-null mice.

6.4 Mbd3 rules pluripotency. What about multipotency?

In contrast with these findings, the only MBD protein that does not bind methylated DNA has been revealed in this study and in combination with the work of Dr. Hendrich and Dr. Kaji in the lab, indispensable for cell fate determination in embryonic stem cells. Mbd3 is the scaffold protein that brings together the NuRD corepressor complex. Although Mbd3-deficient ES cells present a severe defect in the differentiation into post-mitotic neurons, this differentiation pathway is not completely blocked and may correspond with a derepression of genes involved in this pathway by NuRD complex. Hence, the isolation of neural stem cell state in the absence of Mbd3 was proven to be possible, although inefficient. Since $Mbd3^{(-/-)}$ NS cells have been generated, it would be very interesting to use them as a base for microarray experiments comparing wild type and null NS cells to search for possible gene upregulations in $Mbd3^{(-/-)}$ NS cells that could aid the finding of candidate genes involved in neural stem cell function.

The next question to ask is whether Mbd3/NuRD regulates gene signalling involved in neural specification and/or neural self-renewal. Is Mbd3 only involved in early cell fate decisions so that the consequences are driven into blocking all types of tissue specification or is Mbd3 involved in most cell fate decisions? In agreement with this last hypothesis, Mbd3 is ubiquitously expressed throught the organism and MTA-3, a cell-specific subunit of NuRD is involved in B cell fate determination (Fujita et al., 2005). It would be very interesting to compare these results with the microarray studies performed previously in the lab between wild type and null ES cells and study whether there would be many or few overlapping upregulated candidate genes (figure 6.1)

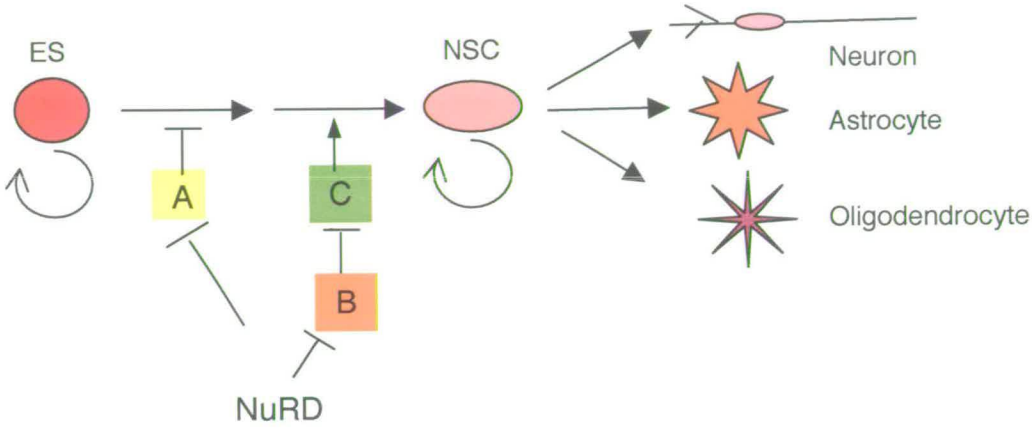

Figure 6.1 Diagram of hypothetical NuRD target genes. Mbd3-containing NuRD corepressor complex has been shown to be necessary for ES cell differentiation (Kaji et al., 2006). The next question to be resolved is whether NuRD plays a role in multipotency decisions. In the neural stem cell system, NuRD may mediate transcriptional silencing of genes involved in neural stem cell fate decisions. A and B represent hypothetical NuRD target genes and C represent a target gene of B gene.

6.5 Mbd3 and the central nervous system

Evidence accumulated over the past three years suggests a specific role for Mbd3 in neurogenesis ((Fan and Hutnick, 2005; Jung et al., 2003) and this thesis). Since the absence of Mbd3 results in such dramatic consequences from very early development, it it has not been possible to study the role of Mbd3 in the brain. It is demanding to develop conditional *Mbd3*$^{(-/-)}$ animal models to allow the study of the role of Mbd3 *in vivo* at different times of brain development and adult brain. It will be very interesting to study Nestin-Cre/*Mbd3*$^{(3flox/-)}$ animal models where Mbd3 will be deleted by Cre recombination once neural precursors start to be formed in the developing brain (E9-E10 dpc) (Bates et al., 1999; Trumpp et al., 1999), as well as αCamKII-Cre/*Mbd3*$^{(3flox/-)}$ mouse models where the deletion of Mbd3 occurs only in cells expressing αCamKII, which is a postmitotic specific kinase (Tsien et al., 1996). These animal models are in preparation in the lab. Further information would be retrieved by the generation of Cre-ER^{T2}/Mbd3$^{(3flox/-)}$ mice, to study selectively at a variety of time points through the development the consequences of Mbd3 deletion in the central nervous system.

Through the neuroectoderm differentiation model system used in my studies I found that in parallel with the *Mbd3*$^{(-/-)}$ ES differentiation deficiency, ES cells expressing Mbd3c isoform, *Mbd3*$^{(\Delta Ex1/-)}$ had impaired differentiation although less severe. Therefore, it would be very interesting to study the consequences of the exclusive presence of Mbd3c in mice, to determine the extent to which it can rescue the *Mbd3*-null phenotype.

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