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Epigenetic regulation of neurogenic differentiation of adipose tissue stem cells

60 study points

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LIST OF ABBREVIATIONS

5mC	5-methylcytosine
Ac	Acetylation
ADP	Adenosine diphosphate
ASC	Adipose tissue stem cell
BHA	Butylated hydroxyanisole
BSA	Bovine serum albumin
bFGF	Basic fibroblast growth factor
bHLH	Basic helix-loop-helix
bp	Base pair
BMMSC	Bone marrow mesenchymal stem cell
BMP	Bone morphogenic protein
cAMP	Cyclic adenosine monophosphate
ChIP	Chromatin immunoprecipitation
CNF-U	Colony forming units
CNS	Central nervous system
CpG	Cytosine-phosphate guanine dinucleotide
COUP-TF	Chicken ovalbumin upstream promoter transcription factor
DAPI	4', 6-diamidino-2-phenylindole
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DNMT	DNA methyl transferase
DPE	Downstream promoter element
EdU	5-ethynyl-2'-deoxyuridine
EGF	Epidermal growth factor
ESC	Embryonic stem cell
FCS	Fetal calf serum
FGF	Fibroblast growth factor
H3K	Histone 3 lysine
H4K	Histone 4 lysine
HAT	Histone acetyl transferase
HBSS	Hanks' balanced salt solution

HCP	High CpG promoter
HDAC	Histone deacetylase
HDMase	Histone demethylase
HMT	Histone methyl transferase
HP	Heterochromatin protein
IBMX	1-methyl-3-isobutylxanthine
ICP	Intermediate CpG promoter
IF	Intermediate filament
IgG	Immunoglobulin G
IL	Interleukin
KMT	Lysine methyl transferase
LCP	Low CpG promoter
LIF	Leukemia inhibitory factor
me1	Monomethylation
me2	Dimethylation
me3	Trimethylation
MBD	Methyl CpG binding domain
MBP	Methyl binding protein
mRNA	Messenger ribonucleic acid
MSC	Mesenchymal stem cell
NaPi	Sodium phosphate buffer
NF	Neurofilament
NGS	Normal goat serum
NPC	Neural progenitor cell
PBS	Phosphate buffer saline
PcG	Polycomb group
PCR	Polymerase chain reaction
POU	Pit-Oct-Unc
RA	Retinoic acid
RAR	Retinoic acid receptor
RNA	Ribonucleic acid
RT	Room temperature
RT-PCR	Reverse transcriptase polymerase chain reaction
RXR	Retinoid X receptor

SE	Secondary electron
SEM	Scanning electron microscopy
TR	Thyroid receptor
tRNA	Transfer ribonucleic acid
TSS	Transcription start site
TrxG	Trithorax group
VPA	Valproic acid

SUMMARY

Mesenchymal stem cells (MSCs) derived from adipose tissue are multipotent stem cells able to give rise into multiple cell types, not only of the mesodermal lineage, but also of the neuroectodermal lineage. In this study, we show that there is an epigenetic basis related to the capacity of adipose tissue stem cells (ASCs) to differentiate towards the neurogenic pathway. Neurogenic differentiation was induced by a step of mitogenic stimulation (step 1), followed by neurogenic induction (step 2), and shown at the gene and protein expression levels. Nestin has been extensively used as a marker for neurogenesis. The DNA methylation status of several regions of the nestin (*NES*) gene, including the promoter, a muscle-specific enhancer in the first intron, and a neural enhancer in the second intron, was determined by bisulphite genomic sequencing prior to and after induction of proliferation (step 1) and neurogenic differentiation (step 2). There was a global demethylation of the second intron upon proliferation induction and this was followed by a strong upregulation of nestin expression at the mRNA and protein level. We observed re-methylation of these regions after induction of neurogenic differentiation in vitro, and that was accompanied by a steep decline in nestin expression at the mRNA and protein level. These data are consistent with nestin being a marker of early neurogenesis. Analysis of post-translational histone modifications by chromatin immunoprecipitation reveals dynamic changes at the *NES* locus and on the promoter of a housekeeping gene (*GAPDH*) associated with step 1 and step 2. Our data suggest an epigenetic 'priming' of ASCs (at least at the *NES* locus) towards neurogenic differentiation, which is primarily elicited by mitogenic stimulation.

INTRODUCTION

1. STEM CELLS

Stem cells are unspecialized cells which have the ability to replenish themselves (self-renew) and can give rise to one or more specialized cell types (differentiate). Stem cells vary in their differentiation capacity and can be classified according to their grade of plasticity as totipotent, pluripotent, multipotent and unipotent. As they differentiate, their differentiation potential becomes more restricted **(Fig. I-1).** Totipotent stem cells have the biggest versatility from all the other stem cell types. In mammals, the fertilized egg and up to 4-8 cell stage blastomeres can be considered totipotent, meaning that they can give rise to an entire organism, including extra-embryonic tissues. Embryonic stem cells (ESCs) are derived from the inner cell mass which gives rise to the embryo itself (Evans and Kaufman, 1981;Thomson et al., 1998). ESCs are pluripotent in that they have the potential to give rise to all three germ layers: endoderm, mesoderm or ectoderm, but unlike totipotent stem cells they cannot give rise to extra-embryonic tissues. Multipotent stem cells have an even more limited differentiation potential and they can give rise to multiple cell types, preferably within a given lineage. Examples are hematopoietic stem cells which they can give rise to white blood cells, red blood cells and platelets. Unipotent stem cells, or progenitor cells, can differentiate into only one cell type. Erythroid progenitor cells are one of the many types of unipotent stem cells which exist in the body (Martinez-Agosto et al., 2007;Serafini and Verfaillie, 2006).

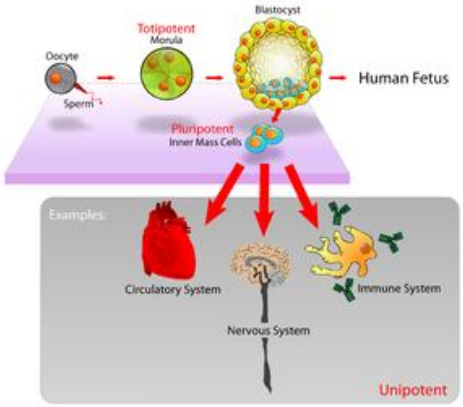

Fig. I-1. Developmental potency stages. As the differentiation proceeds the plasticity of stem cells becomes more restricted. Taken from www.wikipedia.org.

Multipotent stem cells are also known as somatic or adult stem cells. They are found in virtually all tissues and one possible function is to replenish cells that have died or lost their function, throughout an individual's life. They have been identified in many different tissue types such as muscle, liver, bone marrow, adipose tissue, retina, pancreas, central nervous system, dental pulp, blood, intestine and skin **(Fig. I-2).** Previously, it was thought that adult stem cells were restricted in their differentiation potential, giving rise only to cell types limited in their tissue of origin. Studies over the past years however suggest that adult stem cells from some tissues have the ability to differentiate into cell types from all three germ layers (Keating, 2006;Serafini and Verfaillie, 2006). This has caused great excitement, since they provide an easily accessible source of cells that could potentially treat degenerative diseases.

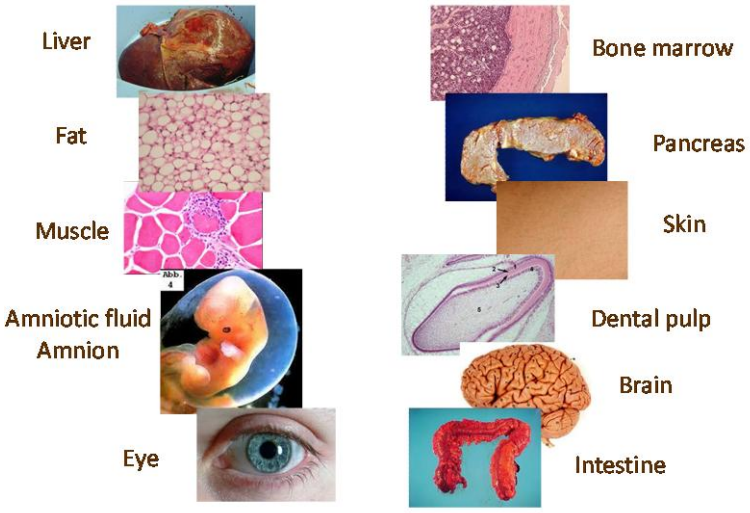

Fig. I-2. Sources of multipotent stem cells in the body. Source: Collas Lab.

1.1 Mesenchymal stem cells

Mesenchymal stem cells (MSCs) are multipotent stem cells which are found in the stroma of many adult tissues. They have the ability to differentiate *in vivo* and *in vitro* into mesenchymal lineages, such as adipogenic, osteogenic and chondrogenic lineages. They may also give rise to skeletal and cardiac muscle cells, endothelial cells (Pittenger et al., 1999;Serafini and Verfaillie, 2006), and to some extent, MSCs can be induced to exhibit neuronal and hepatic characteristics; this suggests a potential role in tissue repair and regeneration (Boquest et al., 2005;Sanchez-Ramos et al., 2000;Weng et al., 2003). These

observations also suggest that MSCs might be less committed in their differentiation potential than previously thought. An important fact about the usage of MSCs is that they can be isolated from individual patients, so the immune rejection issue of allogeneic tissue is eliminated (Ryan et al., 2005). Firstly MSCs were described by Friedenstein in 1974, as non-hematopoietic stem cells present in the bone marrow which were capable of osteogenesis (Friedenstein et al., 1974a;Friedenstein et al., 1974b). Since then, MSCs have been found and characterized in a wide number of tissues such as adipose tissue (Boquest et al., 2005;Zuk et al., 2001), umbilical cord (Wang et al., 2004), umbilical cord blood (Lee et al., 2004), amniotic fluid (In 't Anker et al., 2003), peripheral blood (Gronthos et al., 1994), dermal tissue and skeletal muscle (Williams et al., 1999).

Little is known about the function of MSCs in vivo because of their scarcity in different tissues and the lack of reagents required to isolate these cells with high purity and in large quantities (Boquest et al., 2005). Because of the lack of a common definition for this type of cell population, a series of minimum criteria has been developed for defining multipotent mesenchymal stromal cells. According to the International Society for Cellular Therapy, MSCs must be plastic-adherent under standard culture conditions and form fibroblast-like colonies (Lindroos et al., 2011). The cells must express a series of surface markers such as CD105, CD73 and CD90, and lack expression of CD45, CD34, CD14 or CD11b, CD79α or CD19 and HLA-DR surface molecules (Dominici et al., 2006). MSCs also lack expression of the endothelial cell marker CD31 (Chamberlain et al., 2007). Finally, MSCs must have the ability to differentiate into osteoblasts, adipocytes and chondroblasts *in vitro* (Dominici et al., 2006).

MSCs are thought to reside in a dynamic cellular microenvironment known as the stem cell niche (Fuchs et al., 2004;Jones and Wagers, 2008). These niches support the survival and growth of MSCs by providing them with the factors needed to maintain their viability. The niche provides a protective environment against toxins and irradiation and the presence of cell-adhesion proteins are important for retaining stem cells in the niche (Jones and Wagers, 2008).

1.2 Mesenchymal stem cells from adipose tissue

In order to use stem cells for clinical purposes, there must be a way to obtain them in high quantities. Even if adult stem cells have a lower plasticity than ESCs, the use of adult stem cells bypass the ethical concerns related to ES cell derivation (Denker, 2006) and potential issues of allogeneic immune rejection (Barry et al., 2005). Therefore, they may constitute a more attractive source to produce patient-specific cells for future clinical applications and drug development. Adipose tissue consists a rich and easily accessible source of MSCs (Mirsaidi et al., 2011). Adipose tissue stem cells (ASCs) have the ability to differentiate into cell types of mesodermal origin such as adipogenic, osteogenic, and chondrogenic lineages, as well as the myogenic lineage, leading to skeletal muscle, smooth muscle and cardiomyocytes (Gimble et al., 2007;Halvorsen et al., 2001;Zuk et al., 2001). Interestingly, ASCs display a gene expression profile which extends across all three germ layers, a feature considered to reflect a form of multilineage priming (Noer et al., 2006). It has previously been shown that ASCs have the capacity to differentiate into neuron-like cells and endothelial cells **(Fig. I-3)** (Boquest et al., 2005;Gimble et al., 2007;Jang et al., 2010;Safford et al., 2002;Zuk et al., 2002). However, whether ASCs can give rise to, or contribute to, functional tissues of these lineages remains the subject of many analyses (Boquest et al., 2007).

ASCs have similarities in their transcriptome and cell surface markers with bone marrow MSCs (BMMSCs), including CD44, CD105, CD73, CD90, and Stro-1, and they are both negative for CD45 and CD31 (Boquest et al., 2005;De Ugarte et al., 2003;Fraser et al., 2006;Strem et al., 2005). Even though BMMSCs have become a standard in stem cell research because of their high differentiation potential (Jaiswal et al., 2000;Johnstone et al., 1998;Pittenger et al., 1999), ASCs can be considered to be an attractive alternative source (Gimble et al., 2007;Zuk et al., 2001;Zuk et al., 2002). That is due to the fact that isolation of BMMSCs from patients is a painful procedure and the number of cells obtained upon harvest is limited (Lindroos et al., 2011). On the other hand, ASCs are abundant (>5 × 10^6 98% pure $CD45^-CD31^-$ cells can be purified from 100 ml of liposuction material in our laboratory; Boquest, unpublished data), they can be easily purified, and they do not require culture for

prospective isolation (Boquest et al., 2005;Noer et al., 2006;Zuk et al., 2001). Unlike BMMSCs, ASCs are more genetically stable in long term culture (Dahl et al., 2008;Meza-Zepeda et al., 2008) and they show higher CNF-U potential than BMMSCs (Kern et al., 2006;Strem et al., 2005). In our laboratory we use a combination of monoclonal antibodies to select against CD45+ and CD31+ cells from the stromal-vascular fraction, to purify uncultured ASCs before plating (Boquest et al., 2005).

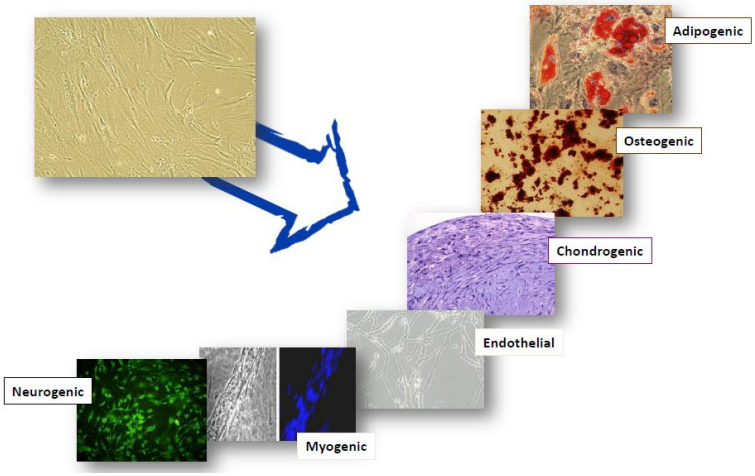

Fig. I-3. Adipose-derived MSCs display multilineage differentiation capacity. This panel exhibits examples of adipogenic (Oil Red-O staining), osteogenic (Alizarin red staining), chondrogenic (Toluidine blue staining), endothelial, myogenic and neurogenic differentiation (immunostaining for NEFH). Source: Collas lab.

2. NEUROGENIC DIFFERENTIATION

2.1. Neurogenic differentiation of embryonic stem cells

ESCs are pluripotent stem cells derived from the inner cell mass of the blastocyst (Evans and Kaufman, 1981;Thomson et al., 1995). ESCs have attracted enormous attention owing to their potential to differentiate into several cell types representing all three germ layers (ectoderm, endoderm and mesoderm); thus, they have been considered to be a powerful tool in regenerative medicine (Fathi et al., 2009;Guan et al., 2001;Wianny et al., 2011). Since 1995, when three independent groups published about the differentiation of mouse ESCs into neuronal cells in vitro (Bain et al., 1995;Fraichard et al., 1995;Strubing et al., 1995), many strategies have been devised to improve the differentiation of ESCs towards neurogenesis. These include the use of chemicals such as retinoic acid (RA) and neurogenic

medium (Fathi et al., 2009), lineage selection (Ying et al., 2003b), and stromal cell-derived inducing activity (Kawasaki et al., 2000). Neuronal cells which were RA-induced, expressed tissue specific genes in a developmentally controlled manner and acquired characteristics specific for postmitotic nerve cells, including complex electrophysiological and immunocytochemical properties (Guan et al., 2001). On the other hand, in vivo, RA is identified as a morphogenic and teratogenic compound (Rohwedel et al., 1999), which makes it unsuitable for therapeutics.

Thus, alternative strategies have been established involving multiple steps of differentiation and selection of neural progenitor cells. It has been demonstrated that monkey and human ESCs can easily be differentiated by prolonged culture without replacing feeder layers. The cells undergo a series of morphological changes, forming 'neural rosettes' reminiscent of the neuroectoderm and under the influence of appropriate developmental signals, they can differentiate into several neuronal and glial cell types (Wianny et al., 2011). Another strategy leading to neurogenic differentiation involves the formation of embryoid body intermediates, followed by induction of a neurogenic program using a combination of serum-free conditions, FGF2 stimulation and mechanical isolation (Wianny et al., 2011). The resulting neuronal precursors can be promoted to efficiently differentiate into multiple functional post-mitotic neuron types by the addition of neuronal differentiation factors (Bain et al., 1995;Okabe et al., 1996). All three main neural cell types have been identified, namely, neurons, astrocytes and oligodendrocytes, as seen from gene expression and electrophysiological studies (Wobus and Boheler, 2005).

In vivo, neuroectodermal differentiation of ESCs is regulated tightly by a network of transcription factors and co-regulators that coordinate the expression of many genes and proteins **(Fig. I-4)**. Oct3/4, Nanog, BMP-dependent SMAD and LIF-dependent JAK/STAT3 signal transduction pathways are key regulators determining differentiation in mouse ESCs (Wobus and Boheler, 2005). Leukemia inhibitory factor (LIF) is a soluble glycoprotein which belongs to the interleukin (IL)-6 family of cytokines. LIF acts as a regulator of a variety of cell functions through the STAT pathway (Burdon et al., 1999). Absence of LIF, together with the

absence of other members of IL-6 family or inactivation of STAT3, disrupts the maintenance of pluripotency in ESCs, inducing them to differentiate *in vitro* (Boeuf et al., 1997). Oct3/4 levels are important in determining differentiation or maintenance of pluripotency in ESCs (Matoba et al., 2006). Oct3/4 promotes self-renewal of ESCs while loss of Oct3/4 leads to trophectoderm formation (Niwa et al., 2000). Nanog works together with Oct3/4 to maintain the pluripotent state of ESCs and its forced expression acts to restrict the differentiation inducing potential of Oct3/4 (Chambers et al., 2003;Mitsui et al., 2003). Similarly, bone morphogenic protein (BMP) acts through the SMAD pathway to induce expression of Id genes, which are also involved in self-renewal of ESCs (Ying et al., 2003a).

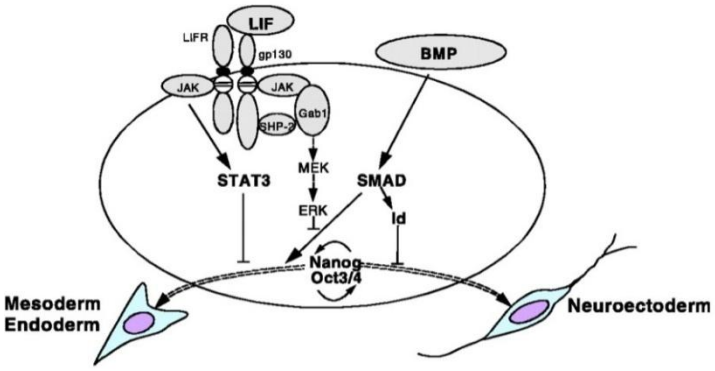

Fig. I-4. Regulation of the pluripotency state of ESCs by a number of transcription factors and different signaling pathways. Taken from (Wobus and Boheler, 2005)

On the other hand, the MEK/ERK pathway inhibits ESC self-renewal and promotes differentiation (Burdon et al., 1999;Burdon et al., 2002). Imbalance in the concentration of these signaling molecules can cause a loss of ESC identity and differentiation towards mesodermal, endodermal or neuroectodermal pathways (Wobus and Boheler, 2005). It is thought that there are three steps important for induction of neurogenesis: (i) repression of BMP signaling, (ii) activation of the fibroblast growth factor (FGF) and Wnt pathways, and (iii) accurate spatial and temporal expression of these factors distinguishes the newly induced neural plate from mesoderm, endoderm and epidermis (Diez del and Storey, 2001). In addition to these factors, there is a large number of transcription factors involved in embryonic neural development. These include Prominin1, Musashi, several POU-domain transcription factors such as Brn1, bHLH-domain-containing factors such as Numb and NeuroD, downstream effectors of Notch signaling, Hes1 and Hes5, Presenilins and Sox2 (Cai et al., 2002). It is thought that bHLH-domain transcription factors are key regulators of

neuronal determination and differentiation in ESCs (Kintner, 2002). Thus, the process of neurogenesis is the outcome of a complex interplay of temporally and spatially regulated factors.

2.2. Neurogenic differentiation potential of mesenchymal stem cells

MSCs display multilineage differentiation capacity, not only towards mesodermal but also towards neuroectodermal pathways (Jang et al., 2010;Kompisch et al., 2010;Wislet-Gendebien et al., 2005). Numerous 'neural induction' protocols have been developed using chemicals such as DMSO/BHA (Woodbury et al., 2000), treating cells with a combination of growth factors (Deng et al., 2001;Sanchez-Ramos et al., 2000) or co-culturing MSCs with neuronal tissues (Abouelfetouh et al., 2004). All these protocols have resulted in a change of MSCs towards a neuronal-like morphology accompanied by expression of neural markers varying for the different differentiation protocols. These include nestin, NSE, Tau, NeuN, glial fibrillary acidic protein (GFAP) and others (Krabbe et al., 2005;Lu et al., 2004;Neuhuber et al., 2004).

However, although the detection of neuronal markers suggests that MSCs display some neuronal characteristics, the cellular localization of some of these markers is often different from that of mature neurons (Krabbe et al., 2005). Nevertheless, *in vivo* studies have shown that intravascular administration of MSCs, and in particular BMMCs, significantly improves the functional outcome in rodents after induction of stroke (Chen et al., 2001), brain trauma (Lu et al., 2001), and Parkinson syndromes (Li et al., 2001). How the transplanted cells contribute to improving symptoms in these animal models and whether MSCs do harbor the ability to differentiate into mature, functional neurons remains however a matter of debate (Chen et al., 2006;Lu et al., 2004;Montzka et al., 2009).

3. NESTIN AND NEUROGENESIS

3.1. The intermediate filament protein nestin

Intermediate filaments (IFs) are one of the three components that, together with microfilaments and microtubules, constitute the cytoskeleton **(Fig. I-5)**. These three cytoskeletal elements work together to confer cell integrity, structure and shape, and organelle motility (Herrmann et al., 2007). One of the characteristics that make intermediate filaments unique comparing to the other filaments is the fact that this component is continuously remodeled during development of many tissues (Kim and Coulombe, 2007). An example is the developing central nervous system (CNS) where there is a specific spatial and temporal expression of IF genes (Dahlstrand et al., 1992a). There are six major types of IFs on the basis of similarities in their primary sequence (Liem, 1993;Yoon et al., 2001).

Nestin is an intermediate filament type VI protein (Lendahl et al., 1990;Steinert and Liem, 1990) which is expressed in neural progenitor cells of the developing CNS and in developing skeletal muscle (Michalczyk and Ziman, 2005). Comparison analysis of the rat and human introns in the nestin gene shows that nestin is more related to type IV IFs, which include neurofilament (NF) L, M or H and α-internexin (Dahlstrand et al., 1992b). In addition to these two IF classes which are

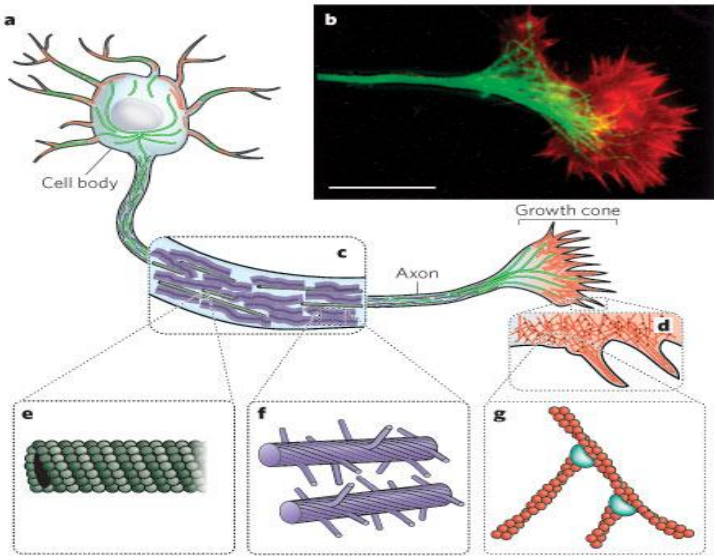

Fig. I-5. The three main components of the cytoskeleton. (A) Neurons exhibit extensive branching forming connections in the nervous system. They consist of three main polymers: microtubules (green), intermediate filaments (purple), and actin filaments (red). (B) A migrating growth cone as indicated by an immunofluorescence micrograph. (C) The neuronal axon where a structural matrix is formed by a class of neuronal intermediate filaments (neurofilaments) which embed microtubules. (D) Actin filament network in the growth cone. Taken from (Fletcher and Mullins, 2010).

expressed at various stages of CNS development, type III IFs are also expressed in the CNS and include vimentin, GFAP, peripherin and plasticin. It is possible to follow the stages of CNS development by observing the expression pattern of the various IFs. Vimentin and nestin are largely associated with mitotically active cells (Dahlstrand et al., 1995). Nestin expression is correlated with proliferating neural progenitor cells in many but not all proliferating regions of the CNS (Dahlstrand et al., 1995;Liem, 1993). Upon differentiation nestin is downregulated and replaced by tissue-specific IFs, including the three neurofilament proteins, NF-L, NF-M and NF-H in mature neurons and GFAP in astrocytes. Downregulation of nestin has been correlated with cells leaving the proliferative state and becoming post-mitotic (Dahlstrand et al., 1995;Michalczyk and Ziman, 2005).

Nestin has been extensively used as a marker of neuroepithelial stem cells, where it was first identified (Dubois et al., 2006;Lendahl et al., 1990). Multiple possible functions have been assigned to nestin. Previous studies on embryonic carcinoma cells suggest a role for nestin in growth cone guidance during axon elongation (Yan et al., 2001). Another function suggested for early expressed IFs, such as nestin, is the establishment of a de *novo* IF network. These early IFs, can be used as a scaffold by IFs expressed later in development to establish a more specialized system for long-term maintenance of a post-mitotic cell type (Dahlstrand et al., 1995). Nestin is important to promote the survival of neural progenitor cells facing oxidative stress and apoptosis (Kim and Coulombe, 2007;Sahlgren et al., 2006). This is plausible as nestin is able to regulate the activity of cyclin-dependent kinase 5 (cdk5) and its regulator p53 (Kim and Coulombe, 2007;Sahlgren et al., 2006).

The range of expression level of nestin is wider than previously thought; it is indeed not restricted to neural stem cells and neural progenitor cells (Wiese et al., 2004). Nestin is expressed, in a transient fashion, in many cell types during development, for example in migrating and proliferating cells that have the potential to develop in neuroectodermal, endodermal and mesodermal lineages (Wiese et al., 2004). This indicates that nestin is not only a marker for neural progenitor cells, but also a marker of multi-lineage differentiation potential in multipotent cells.

During adulthood, nestin expression does not persist and it is usually restricted to more defined regions. These areas contain a 'niche' of cells which have the ability to proliferate, differentiate and migrate after being activated (Wiese et al., 2004). In addition to progenitor cells, some mature cell types also express nestin. Some examples are pancreatic endothelial cells (Klein et al., 2003;Lardon et al., 2002), intestinal cells of Cajal (Tsujimura et al., 2001;Vanderwinden et al., 2002), retina (Mayer et al., 2003) and Schwann cells (Frisen et al., 1995;Hockfield and McKay, 1985). The role of nestin in these mature cell types, which are not in the proliferative state, remains unknown.

After injury, or in certain tumors, nestin is sharply upregulated or re-expressed; suggesting remodeling or reversion to a less mature state (Wiese et al., 2004). Indeed, nestin expression is thought to be involved in a wide range of cancers, including of the CNS (Dahlstrand et al., 1992a;Tohyama et al., 1992), colon (Teranishi et al., 2007), prostate (Kleeberger et al., 2007), breast (Liu et al., 2010) and pancreas (Matsuda et al., 2011). Nestin has been correlated with aggressive growth and metastasis in some tumors (Ishiwata et al., 2011). Recent studies have shown that nestin is able to regulate the migration rate of prostate cancer cells, the downregulation of which can cause up to a 5-fold retardation in metastasis (Kleeberger et al., 2007). Apart from cancer, nestin has also been implicated in many diseases, such as multiple sclerosis (Moreels et al., 2008), several neurodegenerative diseases such as Parkinson's (Buervenich et al., 2001), Alzheimer's (Pugliese et al., 2010), and Creutzfeldt-Jakob disease (CJD) (Mizuno et al., 2003), coronary heart disease (Suguta et al., 2007), and liver damage (Niki et al., 1999). In all these cases, there is an upregulation of nestin in the damaged tissues, supporting the idea that nestin may function in the regeneration of adult tissues.

3.2. Genomic organization and regulation of the nestin gene

The nestin gene (*NES*)

There is remarkable similarity in the organization of the human, mouse and rat nestin *(NES)* gene. The gene is conserved during evolution and contains four exons and three introns

(Zhong et al., 2008). Studies in transgenic mice have shown that tissue-specific expression of nestin in muscle and neural progenitor cells is regulated by two enhancer elements in the first and second intron, respectively (Jin et al., 2006;Zhong et al., 2008;Zimmerman et al., 1994). Interestingly, no cell-type specific regulatory elements have been identified in the upstream region of the *Nes* promoter up to position -3809 relative to the transcription start site (TSS). Nonetheless, the *Nes* promoter can drive nestin expression in various mammalian cell lines, thus showing no cell-type specificity. The minimal promoter region of the mouse *Nes* gene has been identified in region -11 to +183 relative to the TSS. Two transcription factors are essential for *Nes* promoter activity, namely Sp1 and Sp3 which bind to two adjacent Sp1 sites in the basal promoter region; these are notably involved in the transcriptional regulation of a number of 'housekeeping' genes (Cheng et al., 2004).

The intron 1 of *NES* contains a muscle-specific enhancer

From transgenic experiments in mice, the muscle-specific *Nes* enhancer has been localized in the +291 to +661 region of the *Nes* first intron. Two E-boxes reside in this area, which are considered to be essential for enhancer activity in differentiating myoblasts (Zhong et al., 2008). As is the case with neurogenesis, skeletal muscle development is also accompanied by changes in the composition of intermediate filaments (Zhong et al., 2008). Nestin is upregulated in proliferating myoblasts and it is sharply downregulated during differentiation, being replaced by desmin, another IF protein, in mature myofibers (Zhong et al., 2008). It has been proposed that nestin has the capacity to determine the progress of myoblast differentiation. The functionality of nestin in myogenesis has been evaluated in knock-down experiments in mice, in which nestin depletion accelerates myogenic differentiation (Pallari et al., 2011). MyoD, a transcription factor involved in determination and terminal differentiation of skeletal muscle (Berkes and Tapscott, 2005), binds to its consensus sequence CANNTG (E-box) in the regulatory region of muscle-specific genes, regulating their expression (Blackwell and Weintraub, 1990;Lassar et al., 1989). Recent studies suggest that MyoD is one of the proteins involved in the regulation of the first intron of the *Nes* gene: it binds to the

muscle-specific enhancer and directs nestin expression in muscle progenitor cells (Zhong et al., 2008).

The intron 2 of *NES* contains a neural-specific enhancer

The discovery of two neural enhancer elements in the second intron of the *Nes* gene has defined a hallmark in our understanding of the regulation of nestin expression in the CNS (Lothian et al., 1999). This enhancer region resides in the 3' portion of the second intron; it contains not only general but also region-specific enhancer elements, one for general CNS expression and another for expression in the midbrain (Kappen and Yaworsky, 2003;Lothian et al., 1999;Yaworsky and Kappen, 1999). These relevant CNS enhancer elements can act and be regulated independently of each other, and they show a high degree of conservation based on sequence comparisons between mouse, rat and human (Kappen and Yaworsky, 2003). This neural enhancer contains putative binding sites for a wide range of transcription factors including RXRs, SOX and POU domain transcription factors, which have been found in rodents to bind at different developmental time points to the *Nes* enhancer and regulate *Nes* expression in the CNS (Dubois et al., 2006;Josephson et al., 1998;Kappen and Yaworsky, 2003;Lothian et al., 1999;Tanaka et al., 2004).

A 714 base pair (bp) region in the 3' end of the second intron of *Nes*, which has the highest degree of similarity between species, is where important control elements reside (Lothian and Lendahl, 1997). Within that region, a 374 bp area is sufficient for enhancer activity and a 120 bp element is essential for nestin expression throughout the CNS. This region has binding sites for a number of nuclear hormone receptors such as TRs, RXR, RAR and COUP-TF, which are all important for mammalian development (Lothian et al., 1999). This suggests that nuclear hormone receptors play an essential role in nestin expression in the developing CNS (Lothian et al., 1999). These factors can positively or negatively regulate nestin expression (Lothian et al., 1999). This 120 bp region also contains a POU domain transcription factor binding site that can be bound by different members of the POU family at different developmental stages (Josephson et al., 1998;Lothian et al., 1999). According to

previous studies there is an interplay between POU and SOX family transcription factors, which elicit gene activation synergistically, and are essential for the regulation of the *Nes* gene **(Fig. I-6)** (Jin et al., 2009;Tanaka et al., 2004).

Nestin is thought to be regulated in a cell-cycle dependent manner: Downregulation of nestin during G2-M phase coincides with the cell-cycle dependent phosphorylation of an upstream regulator, Brn2, which leads to a reduced binding activity on the *Nes* CNS-specific enhancer. This suggests that nestin is absent in post-mitotic neurons due to the absence of Brn2 activity (Sunabori et al., 2008).

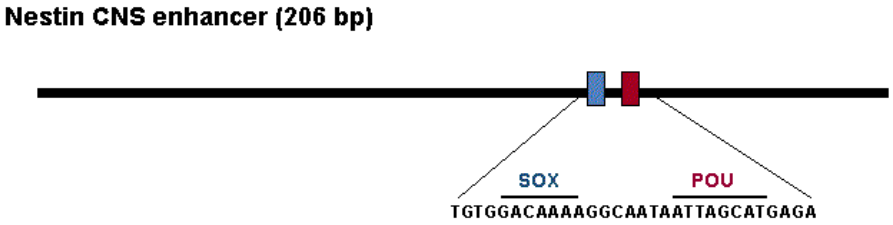

Fig. I-6. Potential SOX2 binding site found adjacent to the POU factor binding site and which are involved in regulation of the CNS enhancer (sequence of the mouse *Nes* gene). The human nestin enhancer sequence for SOX binding site is altered to GACAATG, while the sequence for the POU binding site is conserved between mouse, rat and human (Tanaka et al., 2004).

The ventral midbrain, where nestin is markedly expressed, is a source of dopaminergic neurons (Kappen and Yaworsky, 2003), the loss of which leads to neurodegenerative disorders such as Parkinson's disease. The second enhancer of *Nes*, specific for expression of nestin in the midbrain, is located upstream from the CNS-specific enhancer, within a 204 bp segment. This enhancer consists of two elements, a midbrain-specific and a tissue non-specific transcriptional potentiator **(Fig. I-7)** (Kappen and Yaworsky, 2003;Yaworsky and Kappen, 1999). The midbrain enhancer is activated early in development, however, unlike the CNS enhancer, its activity is not maintained at later developmental stages (Yaworsky and Kappen, 1999). There are putative binding sites for nuclear hormone receptors and with one mismatch, there is one site for binding of the orphan nuclear receptor Nurr1. Nurr1 is a transcription factor important for the generation of

dopaminergic neurons, so it may also function in the regulation of nestin (Kappen and Yaworsky, 2003;Yaworsky and Kappen, 1999). There have not been any putative regulatory sites found in the region of the potentiator element; however without this element, the midbrain-specific enhancer loses its transcriptional activity (Kappen and Yaworsky, 2003).

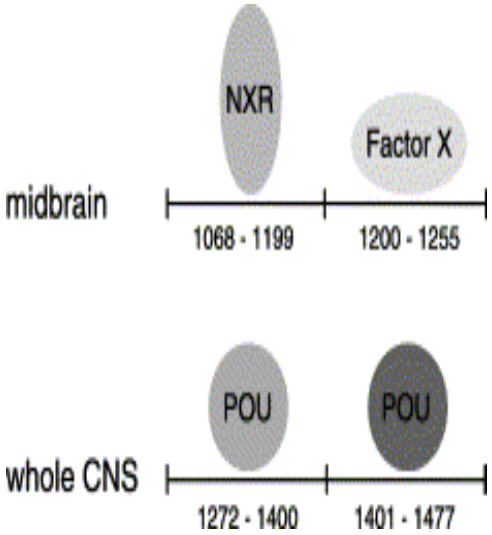

Fig. I-7. The second intron of nestin contains two enhancer elements: the midbrain enhancer and the CNS enhancer. The two enhancer elements require at least two sites for activity which are bound by distinct transcription factors. Two POU-domain transcription factors are essential for the CNS enhancer activity (Josephson et al., 1998) and two elements are required for the midbrain enhancer activity, one nuclear receptor protein and an unidentified factor. The numbers indicated are specific for the rat sequence. Taken from (Kappen and Yaworsky, 2003).

Cells of the midbrain can activate both midbrain and CNS enhancers, while other CNS cells can activate only the CNS-specific enhancer (Yaworsky and Kappen, 1999). This suggests that there must be some heterogeneity in the transcription factor repertoire in different cell types in the CNS, leading to differential regulation of the *Nes* neural enhancer. It has become clear that different populations of cells can be distinguished based on *Nes* enhancer activity (Yaworsky and Kappen, 1999). This suggests that nestin could potentially be used for the development of gene therapy strategies against neurodegenerative diseases.

4. EPIGENETIC REGULATION OF GENE EXPRESSION: ROLE OF DNA METHYLATION AND HISTONE MODIFICATIONS

4.1. Introduction to epigenetics

In multicellular organisms, nearly all the cells are genetically the same, meaning that they contain identical DNA. Nevertheless, there are dramatic differences, when it comes to function and structure, suggesting that these variations must be specified by information not encoded in the nucleotide sequence itself (Meehan, 2003). Superimposed upon the DNA sequence is a layer of 'heritable' epigenetic information, which results in differential gene expression for each cell type during development and is sustained throughout successive series of mitoses (Jaenisch and Bird, 2003). This field is known as 'epigenetics' and involves heritable modifications of DNA and chromatin which affect gene function but do not involve alterations of the DNA sequence itself (Collas et al., 2007).

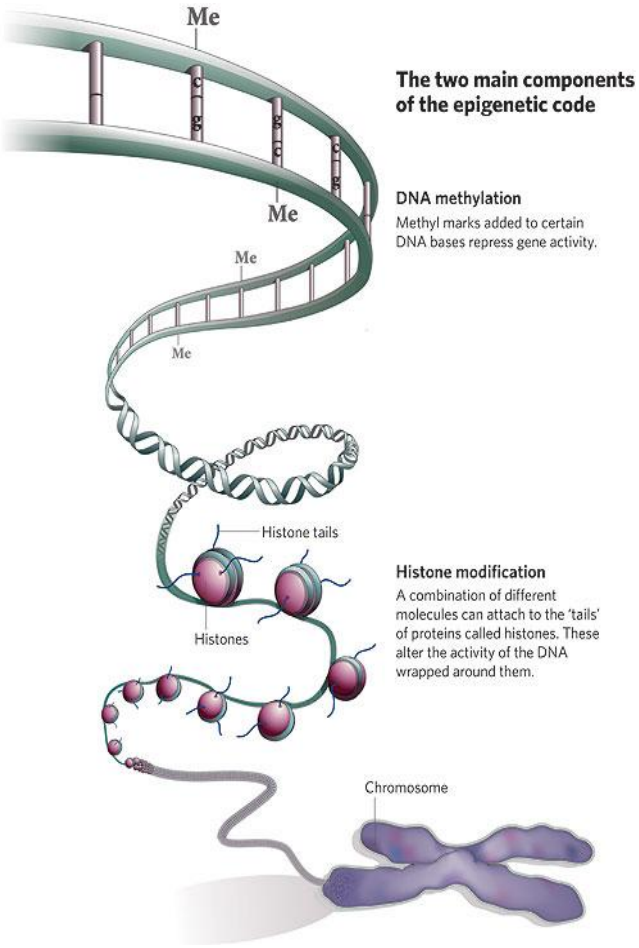

Fig. I-8. Overview of the two main components of the epigenetic code: DNA methylation and histone modifications. Taken from (Qiu, 2006).

Epigenetic modifications play a major role in development and cell differentiation, and are essential for the functional implication of extracellular stimuli (Jaenisch and Bird, 2003;Vaissiere et al., 2008). Epigenetic modifications fall into two main classes: DNA methylation and histone modifications **(Fig. I-8)**.

In vertebrates, DNA methylation occurs in the context of CpG dinucleotides and the majority of CpGs in the genome are methylated (Bird, 2002;Goll and Bestor, 2005). The methyl groups on CpG dinucleotides are recognized by proteins which may activate or repress the expression of a gene through regulation of chromatin structure (Jaenisch and Bird, 2003;Qiu, 2006). DNA methylation is often associated with gene silencing and heterochromatin formation, where there is a complex interplay between numerous systems, collectively forming a self-reinforcing network that promotes the spreading of the silenced chromatin (Beisel and Paro, 2011;Lande-Diner and Cedar, 2005).

Whereas DNA methylation constitutes a major component of the gene regulation machinery, additional regulatory effects are elicited by another major member of the epigenetic machinery, namely the post-translational modifications of histones. Histones and in particular their N-terminal tails have been shown to harbor a large number of modified residues. These modifications promote or are repressive against gene expression (Kouzarides, 2007). As DNA methylation, they also modulate the binding of transcriptional regulatory complexes to specific sequences (Li, 2002). Although histone modifications have received the most attention, chromatin may also be modified by the dynamic incorporation of histone variants such as the replication-independent deposition of H3.3 which marks active chromatin (Henikoff et al., 2004;Mito et al., 2005;Mito et al., 2007).

4.2. DNA methylation

DNA methylation is essential for important stages in mammalian development such as X-chromosome inactivation, genomic imprinting, long-term gene silencing and regulation of chromatin structure (Bird, 1997;Hore et al., 2007;Lande-Diner et al., 2007;Miranda and Jones, 2007;Reik, 2007;Suzuki and Bird, 2008;Yen et al., 2007). DNA methylation is a covalent modification which involves the addition of a methyl group in the 5 position of a cytosine at CpG dinucleotides (**Fig. I-9A**). These can be found randomly in the genome, in clusters, or in clusters within a CpG island (Collas, 2009). The methylation pattern is symmetrical in the two DNA strands (**Fig. I-9B**) (Klose and Bird, 2006). The human genome

is characterized by an underrepresentation of CpGs, because of the high mutational rate of CpG dinucleotides, since cytosine is vulnerable to deamination, and deamination of 5-methylcytosine (5mC) produces thymidine (Fryxell and Moon, 2005). However, this is not the case for CpG islands. Firstly proposed by Gardiner and Frommer in 1987, the definition of a CpG island is considered to be a 200-bp stretch of DNA with a G+C content greater than 50% and an observed/expected CG frequency greater than 0.6 (Gardiner-Garden and Frommer, 1987). A latter definition, which was proposed by Takai and Jones in 2002, provides a more concrete association of CpG islands with the 5' region of genes, excluding most Alu-repetitive elements. According to this definition, a CpG island is a region of DNA greater than 500 bp with a C+G content higher than 55% and an observed/expected CG frequency greater than 0.65 (Takai and Jones, 2002). CpG islands are commonly found in the 5' regulatory regions of housekeeping genes and they remain usually unmethylated (Weber et al., 2007). Aberrant DNA methylation of CpG islands has been associated with aging and carcinogenesis. CpG islands in the promoters of tumor suppressor genes which are normally unmethylated, are methylated in cancer cells, leading to the repression of these genes (Issa, 2000;Jones and Baylin, 2002;Laird, 2005). Generally, CpG islands in promoters are nucleosome destabilizing elements and facilitate establishment of a poised transcriptional state (Singh, 2009). They have certain characteristics which make them unique among other types of promoters, such as the lack of a functional TATA box or DPE elements, and the presence of multiple GC motifs which are usually bound by the ubiquitous transcription factor, Sp1 (Baumann et al., 2010).

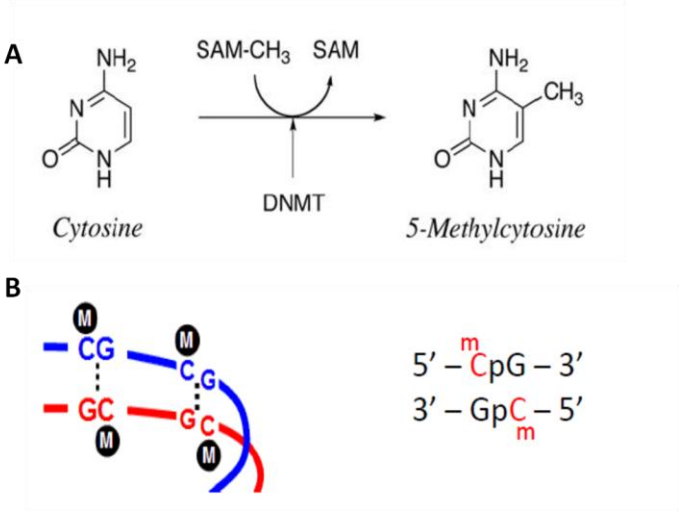

Fig. I-9. DNA methylation. (A) DNMTs catalyze the addition of a methyl group to the 5 position of a cytosine in a CpG dinucleotide. (B) DNA methylation is symmetrical and occurs on both DNA strands.

4.2.1. Targeted DNA methylation by DNMTs

DNA methylation is catalyzed by a conserved group of proteins namely, DNA methyltransferases (DNMTs). There are three different DNMTs currently known: DNMT1 which is responsible for maintenance methylation, and DNMT3a and DNMT3b which are involved in *de novo* methylation (Robertson, 2002). DNMT1 has a high affinity for hemimethylated substrates, which is important for the high-fidelity of the DNA methylation pattern onto the daughter strands after DNA replication. On the other hand, DNMT3a and DNMT3b have a strong preference for non-methylated DNA (Dhe-Paganon et al., 2011). DNMT3a and DNMT3b are important for the establishment of the de novo DNA methylation that takes place during development and differentiation, to repress genes that are no longer required after cell differentiation (Turek-Plewa and Jagodzinski, 2005). From current studies, it is also thought that DNMT3a and DNMT3b complete the methylation process and correct errors that might be left behind by DNMT1. This mechanism ensures that DNA methylation is maintained with high fidelity after replication (Dhe-Paganon et al., 2011).

There are two other DNMT-like proteins, but without any documented DNMT activity, DNMT2 and DNMT3L (Tang et al., 2003). DNMT2 has been shown to have cytoplasmic tRNA methyltransferase activity (Goll et al., 2006;Rai et al., 2007) and is important for the protection of tRNAs from stress-induced cleavage (Schaefer et al., 2010). DNMT3L, which is a member of the DNMT3 family, has been implicated to play an important role in maternal methylation imprinting by enhancing the catalytic activity of DNMT3A and DNMT3B through physical interactions (Suetake et al., 2004).

4.2.2. Transcriptional gene silencing by DNA methylation

DNA methylation is generally associated with long-term repression of gene expression (Boyes and Bird, 1991;Kass et al., 1997;Siegfried et al., 1999), and many studies have investigated the mechanisms that underpin this relationship. Two main models have emerged. In one model, methylation causes a physical hindrance of transcription factor

binding to their recognition motifs, inhibiting transcription **(Fig. I-10A)**. A second model involves the recruitment of transcriptional co-repressor complexes mediated by methyl CpG-binding domain (MBD) proteins which recognize methylated DNA and induce gene silencing **(Fig.I-10B)** (Klose and Bird, 2006). Firstly characterized in 1989, as mammalian proteins binding specifically to methylated CpGs (Meehan et al., 1989), MBPs constitute a family of five methyl-binding proteins characterized by the presence of a common methyl-CpG-binding domain (Klose and Bird, 2006). Four of this family members, MeCP2, MBD1, MBD2 and MBD4, specifically recognize methyl-CpG; MBD3, however, contains amino acid substitutions that prevent binding to methyl-CpG. Another MBP called Kaiso lacks the MBD but recognizes methylated DNA through zinc-finger domains (Prokhortchouk et al., 2001). DNMTs, in addition to their catalytic activity, can also interact with chromatin modifying enzymes such as histone deacetylates (HDACs) (Fuks et al., 2000;Fuks et al., 2001;Geiman et al., 2004), histone methyl transferases (HMTs) (Fuks et al., 2003;Geiman et al., 2004) and the ATP-dependent chromatin remodeling protein hSNF2H (Geiman et al., 2004) inducing chromatin alterations and transcriptional repression **(Fig I-10C)**. Lastly, DNA methylation may exert its repressive effects directly in the gene body, within intronic and exonic regions, resulting in reduced gene expression (Hsieh, 1997). This may be caused by reducing the occupancy of RNA polymerase II or by blocking the chromatin accessibility over the methylated gene body. The mechanism behind the repressive effect of DNA methylation in the gene body remains unclear but it is thought to involve the function of MBPs in inhibition of elongation **(Fig. I-10D)** (Klose and Bird, 2006).

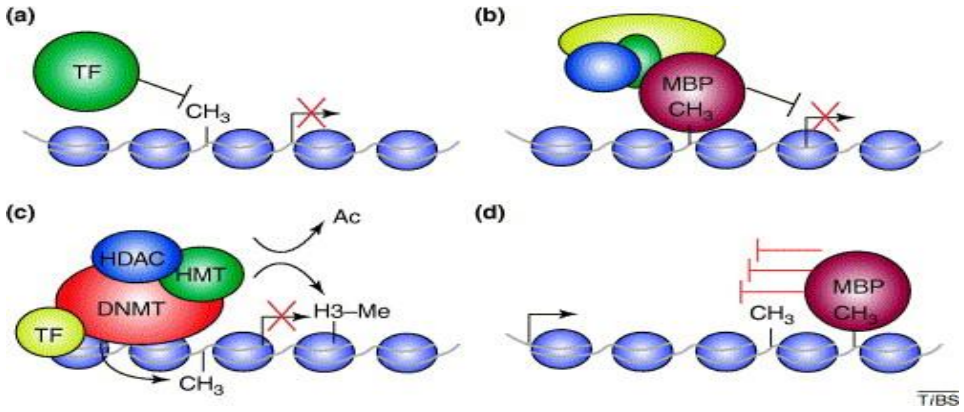

Fig. I-10. Mechanisms of DNA methylation-mediated repression. (A) Inhibition of transcription factor binding by DNA methylation. (B) Recruitment of a transcriptional co-repressor complex by MBPs. (C) Recruitment of histone modifying complexes (HDACs, HMTs) by DNMTs. (D) Inhibition of transcriptional elongation by of MBPs in the gene body. Taken from (Klose and Bird, 2006).

4.2.3. Transcriptional states in different CpG content promoters

Genome-wide DNA methylation profiling studies show that there is a correlation between promoter DNA methylation and promoter activity, depending on the CpG content of the promoter (Weber et al., 2007). High CpG content promoters (HCPs), which are often associated with housekeeping genes, and genes with more complex expression patterns such as those expressed during embryonic development (Mikkelsen et al., 2007), display no or weak methylation even when the promoter is inactive (Weber et al., 2007). Moreover, the expression pattern of genes with intermediate CpG content promoters (ICPs) is in general inversely correlated to the degree of methylation (Weber et al., 2007). Lastly, it seems that there is no correlation between promoter activity and the extent of DNA methylation in low CpG content promoters (LCPs). In fact, most LCPs are methylated regardless of transcriptional status (Weber et al., 2007), suggesting that other mechanisms regulate the activity of LCPs. Highly tissue-specific genes are considered to belong to this class of promoters (Mikkelsen et al., 2007). Overall, these results indicate that HCPs globally remain unmethylated regardless of transcriptional activity, ICPs are repressed when methylated and LCPs are usually methylated.

4.3 Post-translational modifications of histones

In the nucleus, DNA is packed into chromatin. The nucleosome is the fundamental building block of chromatin. It contains ~147 bp of DNA wrapped twice around a histone octamer that consists of two copies of each of the core histones H2A, H2B, H3, and H4 (Kouzarides, 2007). H1, the linker histone, is associated with the linker DNA between nucleosomes (Fan et al., 2003). Disordered N-terminal and C-terminal tails of histones protrude from the nucleosome through the minor groove channels and they are ideally located for covalent modifications (Khorasanizadeh, 2004). These freely protruding histone tails, are able to make interactions with other histone or non-histone proteins in the vicinity (Zheng and Hayes, 2003). Histones are among the most well conserved proteins in evolution and they are susceptible to a big variety of post-translational modifications which may have a positive or a negative effect on gene expression (Huang et al., 2009).

Different types of modifications have been identified on histone tails such as acetylation, methylation, phosphorylation, ubiquitylation, sumoylation, ADP ribosylation, deimination and proline isomerization (Tan et al., 2011). Various histone modifications are recognized by different proteins leading to the constitution of transcriptionally active or repressive regions in the nucleus (Cosgrove and Wolberger, 2005). The appearance of a given modification is dependent on signaling events prevailing in the cell at a specific time, for instance at a specific developmental time point (Vastenhouw et al., 2010; Lindeman et al., 2011).

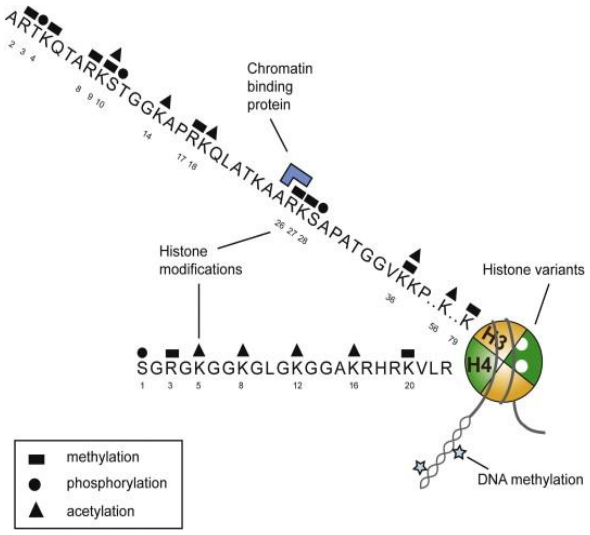

Fig. I-11. Post-translational histone modifications. Histone tails can be methylated, acetylated, phosphorylated, ubiquitylated or sumoylated to regulate gene expression. Together with histone variants and chromatin binding proteins are thought to constitute the 'histone code'. Taken from (Scharf and Imhof, 2011).

Post-translational modifications on

histones H3 and H4 and in particular, acetylation and methylation of lysine residues have been the best characterized so far **(Fig. I-11).** Lysine acetylation is a modification closely associated with transcriptional activation. It involves the reversible addition of an acetyl group to the lysine residues of the N-terminal tails of H3 and H4, resulting in the neutralization of the positively charged histone tails (Sterner and Berger, 2000). It is thought that acetylation makes nucleosomal DNA more accessible to the transcriptional machinery due to the disruption of the electrostatic interactions between DNA and histones (Bannister and Kouzarides, 2011). This can be illustrated by H4K16ac which has a significant negative effect on the formation of the 30 nm fiber, and disrupts the formation of higher order chromatin structures by impeding the ability of chromatin to form cross-fiber interactions (Shogren-Knaak et al., 2006). Acetylated lysines are recognized by bromodomains within nucleosome remodeling complexes, creating a chromatin conformation accessible by the transcriptional machinery (Bernstein et al., 2007). Acetylation is a dynamic process and is regulated by the opposing effects of two families of enzymes namely, histone acetyl transferases (HATs) and HDACs. HATs can be classified into three main families, GNAT, MYST and CBP/p300, depending on amino-acid sequence homology and conformational structure (Hodawadekar and Marmorstein, 2007). HDACs oppose the effects of HATs reversing acetylation of lysine residues and stabilizing local chromatin structure, potentially leading to transcriptional repression (Bannister and Kouzarides, 2011).

Histone methylation can induce either a positive or a negative effect in transcriptional regulation depending on the modified residue. Histone methylation mainly occurs on the side chains of lysines and arginines, where the former may be mono-, di-, tri- methylated, while the latter may be mono-, symmetrically or asymmetrically dimethylated. Unlike other modifications, such as acetylation and phosphorylation, histone methylation does not affect the charge of the histone proteins (Bannister and Kouzarides, 2011). Methylation of histone H3 lysine 4 (H3K4) and H3K36 are commonly associated with transcribed chromatin. H3K4 trimethylation (H3K4me3) and H3K9/K14 acetylation modifications in conjunction with H3K36me3 seem to constitute a signature of actively transcribed genes (Li et al., 2007).

Nonetheless, a large number of promoters harbor H3K4me3 at the TSS and are not transcriptionally active (Lenhard et al., 2012).

On the contrary, methylation of H3K9, H3K27, and H4K20 generally correlates with repression (Bernstein et al., 2007). In particular, methylated H3K9 and H3K27 are recognized by two different sets of proteins, heterochromatin protein 1 (HP1) and Polycomb group (PcG) proteins, respectively (Margueron et al., 2005), mediating the formation of repressive chromatin. HP1 shows preference for both di- and tri- methylation of H3K9 (H3K9me2, H3K9me3), while Polycomb preferentially interacts with H3K27me3 (Fischle et al., 2003). Histone methylation is catalyzed by histone lysine methyltransferases (HMTs), which are characterized by a conserved SET-domain essential for their activity (Bannister and Kouzarides, 2011). Recent studies have identified histone demethylases (HDMases) (Shi et al., 2004;Shi and Whetstine, 2007;Tsukada et al., 2006;Yamane et al., 2006), the existence of which has for long time been controversial (Kouzarides, 2007). The large number of histone modifications and the factors involved in their dynamic regulation create an enormous potential for functional responses.

Chromatin structure undergoes numerous alterations during development (Delaval and Feil, 2004;Margueron et al., 2005). Likewise, key developmental genes undergo dynamic changes in chromatin accessibility during ESC differentiation (Chambeyron and Bickmore, 2004;Perry et al., 2004), with lineage-specific genes being kept in a ‘poised’ but potentially-active state. Genome-wide and locus-specific ChIP analyses demonstrate that poised genes in mouse ESCs are associated with a ‘bivalent’ combination of modifications characterized by the presence of H3K4me3, a mark of active genes, and H3K27me3, a mark of repressed genes (Bernstein et al., 2006). This ‘bivalent’ state is established by an interplay between PcG and Trithorax group (TrxG) of proteins which mediate trimethylation of H3K27 and H3K4 respectively (Azuara et al., 2006;Bernstein et al., 2006;Delaval and Feil, 2004). Several transcription factors which are not expressed in mouse ESCs but at later stages of lineage specification are marked on their promoters by H3K4me3, H3K27me3 and H3K9ac

(Azuara et al., 2006) with H3K27me3 working as a transcriptional molecular brake in a context of transcriptionally permissive chromatin **(Fig. I-12)** (Bernstein et al., 2006).

Fig. I-12. The 'bivalency 'state. Lineage specific genes are repressed but poised for activation by a combination of H3K4m3 and H3K27m3. Adapted from (Noer et al., 2009).

4.4 Epigenetic mechanisms involved in neurogenic differentiation of ESCs

There is a great number of transcription factors identified which are involved in neurogenesis and neuronal differentiation and specification (Kintner, 2002). Epigenetic modifications are thought to play an major role in the selective expression or reversible silencing of genes at different stages of development **(Fig. I-13)** (Copray et al., 2009). Differentiation of ESCs into neural progenitor cells (NPCs) is accompanied by silencing of genes related to pluripotency and to non-neural lineage (Mohn et al., 2008). At the same time the 'poised' state at the promoters and enhancers of the neural genes is resolved by the loss of repressive marks as they become transcriptionally accessible (Mikkelsen et al., 2007).

It has been shown that stem cells contain a low level of DNA methylation at CpG-rich sequences. As differentiation proceeds, methylation of CpG island promoters and CpG-rich sequences outside promoter regions occurs with almost no detectable demethylation. Thus, DNA methylation increases during lineage specification (Mohn and Schubeler, 2009). One of the reasons for the selectivity of de novo DNA methylation of stem cell promoters could be that DNA methylation may stably repress the pluripotency program and prevent abnormal reactivation and de-differentiation during development (Mohn et al., 2008). This is supported by experimental data since reprogramming of somatic cells to a pluripotent state is overally

becoming more efficient by the use of DNMT inhibitors such as 5-aza-cytidine (Mikkelsen et al., 2008).

In addition to promoters, there is increasing evidence that distal regulatory regions such as enhancers and silencers might be dynamically regulated by DNA methylation and demethylation during neural differentiation of ESCs. In particular, distal regulatory elements that surround active developmental genes such as *Olig2*, undergo a CpG demethylation process and a gain of H3K4me2, a mark of active transcription, during neural differentiation (Meissner et al., 2008); this observation, together with recent studies of a genome-wide DNA methylation analysis at single-base resolution (Lister et al., 2009), raises the possibility that CpG methylation of distal regulatory elements also contributes to the poised state of developmental genes (Hirabayashi 2010).

Epigenetic regulation of neural differentiation of ESCs also involves dynamic changes on histone modifications. The transition of ESCs to NPCs is accompanied by the loss of H3K27me3 modification in the 'poised' promoters of many genes involved in the neural lineage (Mikkelsen et al., 2007). The loss of H3K27me3 and the maintenance of the H3K4me3 mark results in increased expression of these genes (Mikkelsen et al., 2007). Genes which are not neural-lineage related lose their bivalency mark, H3K4me3 and H3K27me3 (some may retain H3K27me3), reflecting the limited differentiation potential of NPCs (Mikkelsen et al., 2007). In addition, H3K4me3 loss is accompanied by a gain of the repressive H3K9me2 mark on the promoter of pluripotent genes (Golebiewska 2009); this may be followed by a gain of H3K9me3 leading to long-term repression in the terminally differentiated neurons (Hirabayashi 2010).

Although genes that function in the neural progenitor state are activated, genes involved in terminal neuronal differentiation become poised by the gain of H3K27me3 and H3K4me2 marks in NPCs. These remain silenced until they receive appropriate differentiation cues. At that time, these genes lose H3K27me3 to become expressed (Mohn 2008). This suggests that the differentiation potential of tissue-specific stem cells may also be determined by a set of genes resting in a bivalent 'poised' state. There has not been so

far any research regarding the epigenetic regulation of neurogenic differentiation of ASCs; this is the focus of this thesis.

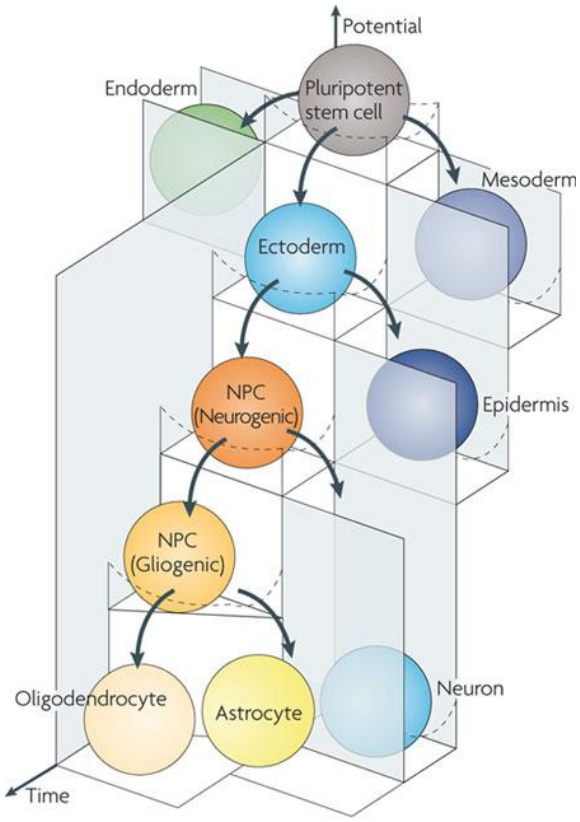

Fig. I-13. Differentiation capacity of stem cells in each developmental stage. Stem cells lose their differentiation potential sequentially as they acquire specific cell fates. Transition to NPCs is accompanied by silencing of genes related to pluripotency and to non-neural linage and activation of'poised' promoters and enhancers involved in the neural lineage. In turn, genes involved in terminal differentiation become 'poised' until they receive appropriate differentiation cues. Taken from (Hirabayashi and Gotoh, 2010).

AIMS OF THE STUDY

Little is known on the differentiation potential of adipose stem cells (ASCs) towards the neurogenic lineage and on associated epigenetic changes. The goal of this study was to determine whether ASCs have the ability to differentiate towards the ectodermal lineage, specifically towards the neurogenic lineage.

The aims of the study were to:

1. Determine the effect of proliferative stimulation and neurogenic differentiation of ASCs at the phenotypic level (light and scanning electron microscopy), gene expression level (RT-PCR), protein expression level (immunofluorescence labeling), and by monitoring cell cycle arrest (EdU staining)
2. Determine, by bisulphite genomic sequencing, DNA methylation changes upon proliferative stimulation and neurogenic induction of ASCs, in the promoter, muscle-specific enhancer and neural enhancer of the *NES* gene
3. Assess, by chromatin immunoprecipitation, histone modification changes on a gene associated with neurogenesis (*NES*) upon proliferative stimulation and neurogenic differentiation of ASCs.

In the course of this project, I was guided and assisted by Dr. Jean-Luc Boulland and Dr. Andrew Boquest. In particular, Dr. Boquest performed most of the cell culture and neurogenic differentiation. Dr. Boulland performed immunofluorescence and confocal imaging analysis of neurogenic-induced ASCs for the following proteins: NEFH, NEUROG1, TUJI, MAP2, SYNAPSIN, MUNC, SYNAPTOTAGMIN and GEPHYRIN (illustrated in Fig. R-4A, 4B, 4C and 4D)

MATERIALS AND METHODS

Cells

ASCs were isolated and purified from the stromal vascular fraction of human adipose tissue as reported earlier (Boquest et al., 2006b). Briefly, adipose tissue was obtained from liposuction material and stromal cells were isolated in a series of steps including collagenase and DNase digestion, sedimentation and straining. Stromal stem cells were separated from unwanted cells using magnetic cell sorting to eliminate CD45+ (hematopoietic) and CD31+ (endothelial) cells, resulting in the collection of CD45- CD31- cells; these cells have previously been shown to have MSC properties (Boquest et al., 2005) and are referred to as adipose stem cells (ASCs). The freshly isolated ASCs were firstly cultured in DMEM:F12 medium containing 50% fetal calf serum (FCS), and after ~16 h, the medium was replaced with DMEM:F12 containing 10% FCS. After 3 weeks, colonies were passaged by trypsinization at a split ratio if 1:3; cells were washed 2x in Hanks' Balanced Salt solution (HBSS) (Sigma-Aldrich), and treated with 0.25% trypsin-EDTA (Sigma-Aldrich) to induce cell detachment from the culture surface. Trypsinization was stopped by the addition of DMEM/F12 10% FCS and cells were replated into 162 cm^2 cell culture flasks (0.5 x 10^6 cells/flask) for an additional 7 day culture period. We used cells from one donor in this study, at passages 5-13. Cells were isolated and banked in liquid nitrogen prior to the start of the project.

Mitogenic stimulation of ASCs

The neurogenic induction protocol used in this study has been developed by Dr. Andrew Boquest, senior scientist in the laboratory, prior to the start of this project. This protocol consists of two distinct main steps, namely (1) induction of mitogenic proliferation of ASCs, followed by (2) induction of neurogenic differentiation per se.

Cells were cultured at ~70% confluency in DMEM/F12/10%FCS, passaged by trypsinization and cultured in stimulation medium to induce faster proliferation. The

stimulation medium consisted of Knockout DMEM (high glucose DMEM containing 4.5 g/l glucose and glutamax; Gibco) with 10% FCS, 1x B27 neural cell culture supplement (Gibco), 10 ng/ml EGF, 20 ng/ml bFGF, 1x L-glutamine, 1x penicillin/streptavidin. Cells were stimulated for 14 days during which they divided approximately 20-24 h, prior to induction of neurogenic differentiation. Media was left unchanged during a 7-day culture period.

Neurogenic induction

The second step of neurogenic differentiation consisted in neurogenic induction of the mitogenically stimulated cells *per se*. Stimulated cells were plated onto either 100-mm cell culture petri dishes ($0.5x10^6$ cells/dish) (BD Biosciences) for bisulphite sequencing, RT-PCR and ChIP analysis, or onto 24-well plates ($2x10^4$) (Nunc) for electron microscopy or immunohistochemical studies. Cultures were incubated for at least 6 h to allow cell attachment to the surface. After removal of stimulation culture media, cells were washed twice using serum-free Knockout DMEM, since differentiation should be carried out in a serum-free environment (Lowell et al., 2006). For neurogenic differentiation, cells were induced in neurobasal medium, designed both for long and short term maintenance of homogeneous population of neuronal cells without the need of an astrocyte feeder layer (Gibco) (Brewer, 1995). The neurobasal medium contained 0.5 mM 1-methyl-3 isobutylxanthine (IBMX), 1 µM dexamethasone, 10 µM forskolin, 0.2 mM 8CPT-cAMP (Biologic), 10 mM valproic acid (VPA), 1% FCS, 1x B27, 0.2% penicillin/streptavidin and 1x L-glutamine. Neuronal induction media was changed on a weekly basis.

Scanning electron microscopy (SEM)

Cells were cultured and/or differentiated into neuronal-like cells on sterile glass coverslips and then fixed with 2,5% glutaraldehyde in 0.1 M HEPES buffer, pH 7.4, and stored overnight at 0-4°C. The samples were rinsed 2 x 10 min in 0.1M HEPES buffer and this was followed by a stepwise dehydration procedure by exposure to increasing concentration of

ethanol: 70% for 10 min, 80% for 10 min, 90% for 10 min, 96% for 10 min and 4 x 100% each for 15 min. The samples were placed in suitable specimen holders and transferred to the Critical Point Dryer (Baltec CPD 030) to further dehydrate the biological tissue prior to examination to the scanning electron microscope. Samples were mounted and transferred to a Cressington 308UHR to be sputtered with platinum. This device is widely used for observing specimen surface morphology; metal coating aims to increase the signal to noise ratio and to make the surface electrically conductive. Samples were observed on a HITACHI S-4800 field-emission scanning electron microscope at the Electron Microscopy Unit of the Department of Molecular Biosciences, University of Oslo. Samples were analyzed using a built-in software. The secondary electron (SE) detector of the microscope collects SE from various sources and backscattered electrons. The HITACH S-4800 contains two SE detectors; the upper, placed above the objective lens and the lower, placed in the specimen chamber. Signals from these two detectors can be collected individually or mixed. The SE detector used in this project was mixed, the accelerating voltage varied between 5000 and 10000 Volt, the working distance was at 8500 μm and the emission current ranged from 7000 to 15600 nA.

Immunofluorescence

Cells were cultured and/or differentiated into neuronal-like cells on sterile glass coverslips and fixed with 4% paraformaldehyde in PBS for 1 h. The cells were rinsed 3 times in PBS and treated with 1 M ethanolamine-HCl in 0.1 M sodium phosphate buffer (NaPi), pH 7.4. The cells were blocked for one hour with 10% (v/v) normal goat serum (NGS), 3% (w/v) BSA in 0.1M Tris base and 0.3 M of NaCl, 0.5% (w/v) Triton X-100, pH 7.4 (TBST) and incubated overnight at room temperature (RT) with primary antibodies diluted in 3% NGS and 1% BSA in TBST. Neuronal and neurogenic proteins were detected using the following antibodies: anti-NEFH (Millipore, AB1989) at a 1/100 dilution, anti-NEUROGENIN-1 (Origene technologies, TA500305, clone 3F9) at 1/50, anti-SYNAPTOTAGMIN-1 (Synaptic Systems, 105002) at 1/300, anti-GEPHYRIN (Synaptic systems 147011) at 1/200, anti-SYNAPSIN 1-2

(Synaptic systems, 106004) at 1/1000, anti-MUNC-13-1 (Synaptic systems, 126103) at 1/300, anti-MAP2 (Millipore, AB5622) at 1/400, anti-TUJI (Sigma T8660) at 1/100, anti-NESTIN (Millipore, MAB5326) at 1/200 and anti-KI67 (BF550609) at 1/100. After rinsing with 3% NGS, 1% BSA in TBST, slides were incubated with secondary antibodies coupled to fluorescent dyes (Alexa-488, Alexa-555 and Alexa-633) for 1 h at RT. Cells were rinsed 1x 10 min in NaPi or PBS, 1x 5 min in NaPi (or PBS) – Hoescht (or DAPI) to counterstain DNA and finally 1x 10 min in NaPi or PBS. Samples were mounted on Fluoromount G water base with ProLong Gold Antifade (Invitrogen) and observed on a Zeiss Axiovert microscope equipped with LSM 510 Meta confocal unit (Zeiss).

Cell proliferation assessment with 5-ethynyl-2'deoxyuridine (EdU)

Detection of DNA synthesis in proliferating cells relies on the incorporation of labeled DNA precursors into cellular DNA during the S phase of the cell cycle (Bick and Davidson, 1974;Gratzner, 1982). A highly sensitive and fast method to detect DNA synthesis in proliferating cells, is based on the incorporation of EdU and its subsequent detection by a fluorescent azide through a Cu(I)-catalyzed [3 + 2] cycloaddition reaction ('click' chemistry) (Salic and Mitchison, 2008).

Briefly, cells were cultured and/or differentiated into neuronal-like cells on sterile glass coverslips and incubated with 10 µM EdU for 8 to 24 h at 37°C. The cells were fixed with 4% formaldehyde in NaPi for 15 to 60 min at RT. Cells were washed twice with 3% BSA in PBS and permeabilized with 0.5% Triton X-100 in PBS for 20 min at RT. For EdU detection, cells were washed twice with 3% BSA in PBS and incubated with Click-iT^{TM} reaction cocktail (Invitrogen), containing 1x Click-iT reaction buffer, $CuSO_4$, Alexa Fluor azide 488, and reaction buffer additive, for 30 min at RT, protected from light. The cells were rinsed 1x with 3x BSA-PBS for 10 min, 1x with PBS- Hoescht (or DAPI) for 5 min and 1x with PBS for 10 min and mounted with ProLong Gold Antifade (Invitrogen). Samples were visualized on a Zeiss Axioplan 2 microscope (Zeiss, Jena, Germany) with an AxioCam.

Reverse transcription polymerase chain reaction (RT-PCR)

RNA was purified from ~0.5x10^6 cultured cells using the RNeasy® Mini Kit (Qiagen RNeasy). cDNA was produced from a starting amount of 0.5 µg RNA using the Iscript cDNA synthesis kit (BioRad). In short, 20 µl of the cDNA synthesis reaction was subjected to the following conditions: 5 min at 25°C, 30 min at 42°C and 5 min at 85°C. cDNA is synthesized *in vitro*, from an mRNA template using the enzyme reverse transcriptase, resulting in single-stranded cDNA production. PCR was carried out on the produced cDNA to allow for detection of mRNA expression levels in the cells of interest. The PCR conditions are as follows: initialization step at 95˚C for 5 min, 30 or 35 cycles of denaturation step at 95˚C 30 sec, annealing step at 60˚C 30 sec and elongation step at 72˚C 30 sec, followed by the final elongation at 72˚C for 10 min to ensure that any remaining single-stranded DNA is fully extended. For most genes we used 30 PCR cycles, however, for those that were characterized by low expression levels, we used 35 cycles, as indicated.

RT-PCR products were visualized by 1% agarose gel electrophoresis using ethidium bromide staining. Products were electrophoresed for ~50-70 min at 70 V. Product size was determined using the 1 kb Plus DNA ladder (Invitrogen). Gels were visualized using a GEL DOC2000 (BioRad). The regions examined were downstream of the transcription start site (TSS) (Ensembl; www.ensembl.org/Homo_sapiens) and primers were designed to be exon spanning to ensure amplification was limited only to mRNA (http://frodo.wi.mit.edu/primer3/). Gene accession numbers, primer sequences as well as amplicon sizes are given in **Table M-1** below.

Bisulphite genomic sequencing

DNA was purified from ~0.5x10^6 cultured cells using the DNeasy Blood and Tissue Kit (Qiagen DNeasy), and bisulphite-converted as previously described (Noer et al., 2006) using the MethylEasy™ and MethylEasy™ Xceed kits (Human Genetic Signatures). Converted DNA was amplified by PCR, using primers designed with Methprimer

(www.urogene.org/methprimer/index1.html). Seven primer sets were designed for the neural progenitor marker, Nestin (*NES*). The primers are spanning regions of the proximal promoter, including the TSS or more upstream of the TSS, as well as the first and second intron (Ensembl; www.ensembl.org/Homo_sapiens) **(Fig. R-6)**. Primer sequences, amplicon sizes as well as CpG numbers are given in **Table M-2**. PCR was carried out with the following conditions: 95˚C for 5 min and 35 cycles of 95˚C 1min, 54/58˚C 2 min and 72˚C 2 min, followed by 15 min at 72˚C. PCR products were cloned into *E. coli* using TOPO TA cloning (Invitrogen). Briefly, the TOPOCloning reaction (PCR product and M13 vector) was added into a vial of One Shot® chemically competent *E. coli* and incubated on ice for 5 to 30 min. Cells were heat-shocked for 30 sec at 42°C and immediately transferred on ice. Cells were incubated for 1 h in S.O.C. medium (nourishing medium) and plated on selective X-Gal and LB plates for blue/white selection. White colonies were selected and individual clones were reverse sequenced (GATC Biotech and MWG Biotech), using reverse pUC/M13 sequencing primers, commonly used in our group. Reverse sequencing was used to unveil either an adenine (A) corresponding to an unmethylated cytosine (C) (converted to a uracil (U) by bisulphite then to a thymine (T) by PCR, reverse-sequenced as an A, complementary to T), or a guanine (G), corresponding to a methylated cytosine (unconverted by bisulphite and reverse-sequenced as a G, complementary to C).

Methylation profiles are shown as black dots (each dot representing a methylated C in a CpG dinucleotide) or white dots (each dot representing an unmethylated CpG) for each bacterial clone (rows). Each dot represents one CpG in the 5'-3' order with No. 1 being the 5'-most cytosine in the sequence examined. Average methylation of the regions examined and numbers of methylated cytosines for a given CpG were compared pairwise for different time points before and after induction of neurogenic differentiation using Fisher's exact test and two-tailed P values.

Chromatin immunoprecipitation (ChIP)

ChIP was performed on cultured ASCs as previously described (Dahl and Collas, 2007), prior to and after induction of neurogenic differentiation. Briefly, immediately before cell harvest, cells were resuspended with 20 mM Na-butyrate, a histone deacetylase inhibitor, in PBS. DNA and proteins were cross-linked by addition of 1% formaldehyde to stabilize these interactions in the cell population, and cells were incubated for 10 min at RT. Fixation was stopped by addition of 0.125 M glycine in PBS for 5 min at RT. Cross-linked cells were washed and lysed on ice in 50 mM Tris-HCl, pH 8, 10 mM EDTA, 1% SDS, protease inhibitors, 1 mM PMSF and 20 mM Na-butyrate for 5 min. Aliquots of 200 µl of chromatin were sonicated using a bath sonicator (Bioruptor UCD-200; Diagenode), 3 x 15 min, 30sec ON/OFF at high power to produce chromatin fragments of ~200 bp. The lysate was centrifuged, the supernatant collected and the amount of chromatin was measured by absorbance at A260 (DNA absorbance). Paramagnetic dynabeads (Protein A Dynabeads; Invitrogen) were washed twice in RIPA buffer (10 mM Tris-HCl, pH 8, 1 mM EDTA, 0.5 mM EGTA, 1% Triton X-100, 0.1% SDS, 0.1% Na-deoxycholate, 140 mM NaCl) and resuspended in 1 volume of RIPA buffer. Coupling of antibody to the magnetic beads was done by addition of the beads (10 µl) to 90 µl of RIPA buffer and 2.5 µg of primary antibody and followed by a 2-h incubation on a rotator at 40 rpm at 4°C. In these experiments, chromatin was diluted to a concentration of 0.8 A_{260} units in RIPA buffer containing 20 mM Na-Butyrate (0.8 units is equivalent to ~ 40,000 cells). Chromatin was mixed with antibody-bead complexes in RIPA buffer and the coupling was carried out overnight on a rotator at 40 rpm at 4°C. Immune complexes were washed 3x in RIPA buffer and 1x in TE buffer (10 mM Tris-HCl, pH 8.0, 10 mM EDTA). Each wash lasted for 4 min on a rotator at 40 rpm at 4°C. The contents were transferred to new tubes and the cross-linking was reversed and DNA eluted for 2 h at 68°C by addition of 150 µl elution buffer (20 mM Tris-HCl, pH 7.5, 5 mM EDTA, 20 mM sodium butyrate, 50mM NaCl) containing 1% SDS and 50µg/ml proteinase K. Elution buffer was recovered, ChIP material was re-extracted and both supernatants were added together. Another 200 µl elution buffer was added to the eluted ChIP material and

DNA was purified by phenol-chloroform-isoamylalcohol extraction and ethanol precipitation. Two independent ChIPs were done per experiment.

The antibodies used were against specific modifications of histone H3 and included, from Millipore, anti-H3K4me3 (No. 07-473), from Diagenode, anti-H3K27me3 (No. 069-050), and anti-H3K9me3 (No. 056-050). As a negative control we used IgG from Millipore (DAM 1437337), and it gave no enrichment on the level of the background as expected (data not shown).

Immunoprecipitated DNA was analyzed by duplicate real time PCR, by a MyiQ Real-time PCR Detection System using IQ SYBR® Green. PCR was carried out with the following conditions: 95°C for 3 min and 40 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. ChIP data are illustrated as mean±SD percent precipitated DNA relative to input. The primers are spanning regions of the proximal promoter of one neurogenic gene (*NES*) and one house-keeping gene (*GAPDH*) as well as the first and second intron of *NES*. Primer sequences as well as amplicon sizes are given in **Table M-3 .**

Table M-1. RT-PCR primers used in this study.

Gene	F: forward primer R: reverse primer	Amplicon size (bp)	Gene Accession Number
CACNA1A	F: CTGCACTGACGAGTCCAAAG R: GGTGAAGAGGGTCAGCAGAG	151	NM_000068
CDK5	F: TGAGGTGGTCACACTGTGGT R: CTGGTCATCGACATCATTGC	154	NM_004935
CHGA	F: AGGAGGAGGAGGAGGACAAC R: TTCTTCTCCTCGGGGTAGC	176	NM_001275
CNP	F: CTGAGCTGCAGTTTCCCTTC R: CACGGTACTTGTCCACGATG	138	NM_033133
ENG	F: GCCAGCATTGTCTCACTTCA R: ATGCGCAACAAGCTCTTTCT	180	NM_001114753
GAPDH	F: TCGGAGTCAACGGATTTGGT R: TTGCCATGGGTGGAATCATA	148	NM_002046
GFAP	F: GAAAGAGATCCGCACGCAGTAT R: ACTCCAGGTCGCAGGTCAAG	188	NM_002055
GNRHR	F: TTCTGCTCTCTGCGACCTTT R: ATCCAGTGGCATGACAATCA	158	NM_000406
KISS1	F: GCTACTGCTTTTCCTCTGTGC R: CAGTAGCAGCTGGCTTCCTC	161	NM_002256
KCNC1	F: GAGGACGAGCTGGAGATGAC R: TGAAGAAGAGGGAAGCGAAG	163	NM_001112741
XCL1	F: CTCCTTGGCATCTGCTCTCT R: AGCCTTCCGTGATGGTGTAG	139	NM_005283
MAP2	F: CGCTCAGACACCCTTCAGATAAC R: AAATCATCCTCGATGGTCACAAC	122	NM_002374
MBP	F: CCCCGTAGTCCACTTCTTCA R: TCCCTTGAATCCCTTGTGAG	145	NM_001025081
NEFH	F: GAGGAACACCAAGTGGGAGA R: TTCTGGAAGCGAGAAAGGAA	160	NM_021076
NES	F: CACCTGTGCCAGCCTTTCTTA R: TTTCCTCCCACCCTGTGTCT	170	NM_006617
NEUROD1	F: GCCCCAGGGTTATGAGACTA R: GCTCCTCGTCCTGAGAACTG	160	NM_002500
NEUROG1/2/3	F: ACCGCATGCACAACTTGA R: GCCAGAGCCCAGATGTAGTT	127	*
OLIG2	F: GACAAGCTAGGAGGCAGTGG R: CGGCTCTGTCATTTGCTTCT	111	NM_005806
RBFOX3	F: CAACGGCTGGAAGCTAAATC R: CGCAGCCCGAAATGTATTAT	160	NM_001082575
SCN1A	F: CTGGTGTTGGCTGGACTTCT R: CCTCATCCCTTCAAATCGAG	154	NM_001165963
TUBB3	F: GGCCTTTGGACATCTCTTCA R: ATACTCCTCACGCACCTTGC	241	NM_006086

**NEUROG1* NM_006161

NEUROG2 NM_024019

NEUROG3 NM_020999

Table M-2. Bisulphite sequencing primers used in this study.

Gene	F: forward primer R: reverse primer	Amplicon size (bp)	No. of CpGs
Bisulphite sequencing primers			
NES promoter	F1: GGGTATATTATAAAGGTTGGATAAATTTG	287	9
	R1: TTACCTAAATCTCCATCAAAAAAAA		
	F2: TTTTTGATTTTTAAGGGGTTAAGGT	345	24
	R2: AAACAACTCATTCTACTCCTCCAAC		
NES intron 1	F1: GGTTAGTTGGTTGTTGAATTAGGAG	481	12
	R1: ACCCAAACAAAAAACTATTTTCTCA		
	F2: TAGAATTTAGAATATGTGGGAAATGG	482	10
	R2: ATCCCTTCTATAAACCAACCTACCT		
NES intron 2	F1: GAAAGGAGGAGAGGTAAGGTTTAGA	425	7
	R1: ACCCTTCCCCTTAATAACTTCACTA		
	F2: AGATGAGGGTTTTGATTTTTAGTGA	366	6
	R2: CACACCCACACAAAACTTAAACTAA		
	F3: TGGAGATTTGAGTAATGTTTTGTGT	351	5
	R3: AATAAAAAACCACCCCTCCTTAAT		

Table M-3. ChIP primers used in this study.

Gene	F: forward primer R: reverse primer	Amplicon size (bp)
ChIP primers		
GAPDH	F: CTGAGCAGTCCGGTGTCACTAC	166
	R: GAGGACTTTGGGAACGACTGAG	
NES promoter	F: GCTGCCACTCTCTGACCTCT	158
	R: AGAGACCGACGGGGACAAT	
NES intron 1	F: AGTTCCTCACAGCCCTCCTT	165
	R: TCTCAGCTGCTGGTCACATC	
NES intron 2	F: TTCCCCAGAATCCTCTCCTT	167
	R: AGTCGGACATTGCCCTACAC	

RESULTS

1. Method of mitogenic stimulation and neurogenic differentiation of ASCs

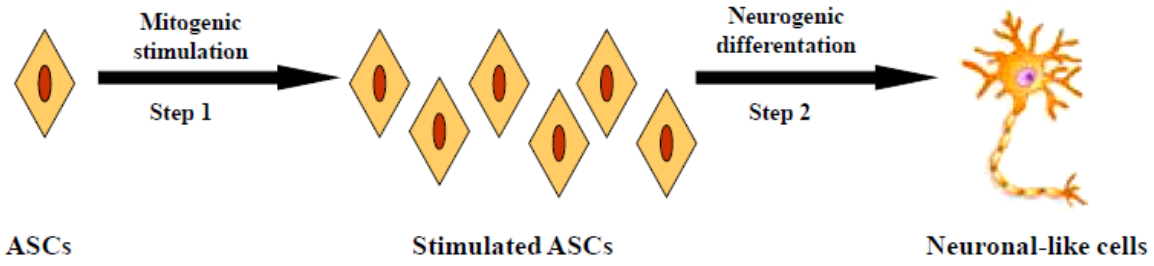

Fig. R-1. Steps leading to neurogenic differentiation of ASCs. Stimulation of proliferation leads to a dramatic increase in cell numbers. After 14 days in stimulation media 'stimulated' ASCs are induced to differentiate, giving rise to neuronal-like cells.

The ASC stimulation treatment (step1) substitutes standard growth medium with DMEM/F12 containing 10% FCS, with knockout DMEM (containing 4.5 g/L glucose and Glutamax), 20 ng/ml EGF, 20 ng/ml bFGF, 1 x B27 supplement. 'Stimulated ASCs', as referred in the current study, are ASCs which have been stimulated for 14 days. This has been a great advantage, in particular for stem cell culture since stem cells are isolated in limited amounts and do not grow as fast under standard conditions. Of note, culture of ASCs in stimulation medium does not alter ASC differentiation properties towards the adipogenic or osteogenic pathways, nor does it alter surface marker expression (Boulland et al., manuscript submitted). After 14 days of culture in stimulation medium, cells were subjected to neurogenic differentiation. Stimulated cells were plated either on 24 well plates or on 100 mm cell culture petri dishes and left for at least 6 h for attachment to occur. Neurogenic differentiation was induced by adding neurobasal medium containing 1% FCS, 1x B27, 0.5 mM 1-methyl-3-isobutylxanthine, 1 µM dexamethasone, 0.2 mM 8CPT-cAMP, 10 mM valproic acid and 10 µM forskolin **(Fig. R-1)**. Cells induced towards the neurogenic pathway were isolated on day 1, day 3, day 7, day 14 and day 21 for various experiments.

2. Morphological changes associated with neurogenic differentiation

We examined morphological changes associated with induction of neurogenic differentiation, by light microscopy and SEM **(Fig. R-2)**. For light microscopy, cells were induced to differentiate for 7 days **(Fig. R-2A, B, C)**; for SEM, cells were fixed and coated in platinum before and after neurogenic induction, on day 3 and after 3 weeks **(Fig. R-2D, E, F, G, H, I)**.

ASCs had a fibroblast-like appearance with a spindle-shape at confluency **(Fig. R-2A, B, D)**. Induction of neurogenic differentiation was followed by changes concerning cell size and shape over time. Early stages of differentiation (day 3) are accompanied by cells exhibiting a spherical cell-soma with mainly bipolar processes and in some cases tri- and multipolar extensions. Interestingly, we could observe terminal branching and intercellular contacts, forming a type of cellular network **(Fig. R-2E, G, H, I)**. At later stages of differentiation (day 21), cells acquired a more neuronal-like structure with thinner and bigger cell soma, extensive branching and longer and thinner protrusions which extend in different directions **(Fig. R-2F)**. These results collectively indicate that neurogenic induction elicits morphological changes in ASCs, suggestive of acquisition of a neuron-like phenotype.

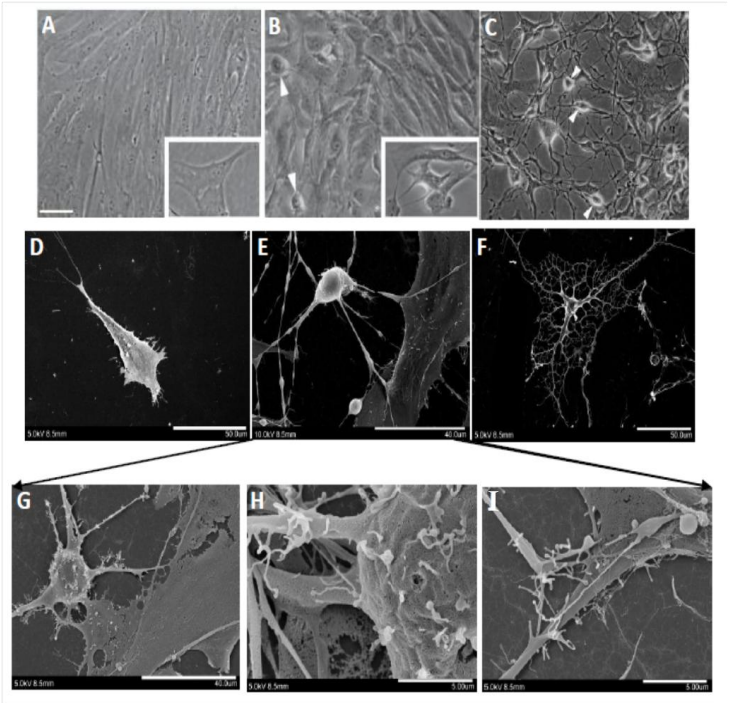

Figure R-2. Morphological changes associated with neurogenic differentiation. (A) Unstimulated ASCs. Bar, 50 µm. (B) Stimulated ASCs. Bar, 50 µm. (C) Neurogenic-induced ASCs, day 7. Bar, 37.5 µm. (D) Unstimulated ASCs, magnification 800. Bar, 50 µm. (E) Neurogenic-induced ASCs Day 3, magnification = 1300. Bar, 40 µm. (F) Neurogenic-induced ASCs Day 21, magnification 600. Bar, 50 µm. (G) Neurogenic-induced ASCs Day 3, magnification 1300. Bar, 40 µm. (H) Neurogenic-induced ASCs Day 3, magnification 8000. Bar, 5 µm. (I) Neurogenic-induced ASCs Day 3, magnification 7000. Bar, 5 µm.

3. Transcriptional changes associated with neurogenic differentiation

To further evaluate the extent of neurogenic differentiation of ASCs and assess whether the neurogenic-induced cells acquired a gene expression pattern similar to neurons, we investigated the gene expression level of some neural-specific genes. Neurogenic differentiation was evaluated by RT-PCR analysis of expression of several genes, markers of the neurogenic- immature early markers, regulators of neurogenesis- (*NESTIN*, *NEUROG1/2/3* [a designation reflecting the detection of a common transcript of the *NEUROG1*,*NEUROG2* and *NEUROG3 genes*], *NEUROD1*, *OLIG2)*, neuronal- mature markers of neurons- (*NEFH*, *RBFOX3*, *TUBB3*, *MAP2*, *CACNA1A*, *SCN1A*, *KCNC1*), glial-astrocytic, oligodendrocytic- (*GFAP*,*MBP*, *CNP*) and neuroendocrine (*CHGA*, *KISS1*, *XCL1*, *GNRHR*) pathways **(Fig. R-3A)**. These markers were evaluated prior to, and on day 1 and 7 of neurogenic induction. We also performed PCR without RT to control for DNA contamination using *NEUROG1/2/3* and *GAPDH* primers; as expected these gave no PCR products **(Fig.R-3B)**.

The stem cell marker *ENG* (CD105) (Pierelli et al., 2001) was clearly expressed in both unstimulated and stimulated ASCs as expected; upon induction of neurogenic differentiation, *ENG* became slightly downregulated, particularly at day 7. *CDK5*, a critical signaling determinant in development and regulator of nestin (Sahlgren et al., 2003), was upregulated upon mitogenic stimulation. Consequently, we detected strong nestin (*NES*) upregulation upon mitogenic stimulation. Expression of *NES*, a marker of neural progenitor cells (Lendahl et al., 1990), was maintained in the early stages of neurogenic induction (day 1) but steeply decreased thereafter. In addition, transcripts for *NEUROG1/2/3,* which encodes a transcription factor required for neurogenesis and is a regulator of NeuroD (Seo et al., 2007), were detected in unstimulated and stimulated ASCs. On day 1 of neurogenic induction, *NEUROG1/2/3* was slightly upregulated, yet its expression sharply declined by day 7. Of note, further transcript analysis using *NEUROG1*, *2* or *3*-specific RT-PCR primers indicate that whereas *NEUROG3* transcripts were not detected at any of the stages examined here, *NEUROG1* and *2* mRNAs were detected (data not shown), consistent with a

neurogenic induction process. The neurogenic *NEUROD1* and *OLIG 2* transcripts were not detected in ASCs. Upon differentiation however, there was a steep increase in the expression of *NEUROD1*, a transcription factor essential for survival and maturation of adult-born neurons (Gao et al., 2009), which coincided with the expression peak of *NEUROGENIN1/2/3*. *OLIG2*, a transcription factor important for motoneuron and oligodendrocyte fate specification and competitor of neurogenin (Lee et al., 2005b;Zhou et al., 2001), was also upregulated on day 1, with a dramatic increase on day 7. This suggests a sequential activation and repression of neurogenic genes leading to a signal cascade, and possibly determining the neural pathway of these cells.

We next determined changes in expression of a set of neuronal marker genes. We observed that with the exception of *CACNA1A* and to some extent *TUBB3* and *SCN1A* all other genes, namely *NEFH*, *RBFOX3*, *MAP2* and *KCNC1* were not detected in unstimulated ASCs. However, they were upregulated at later stages. In particular, *NEFH*, one of the major components of the neuronal cytoskeleton filaments (Lee and Cleveland, 1996), became detected upon mitogenic stimulation and remained expressed throughout the differentiation period. *RBFOX3* (encoding NeuN), a neuronal nuclear protein and a marker associated with the initiation of neuronal terminal differentiation (Mullen et al., 1992), was detectable only on day 1 after induction of neurogenic differentiation. *TUBB3*, a major constituent of microtubules important for the nervous system development and essential for proper axon guidance and maturation (Tischfield et al., 2010), was sharply induced upon mitogenic stimulation and remained expressed after neurogenic induction. *MAP2*, a microtubule assembly-promoting protein important for outgrowth of neuronal processes, synaptic plasticity and neuronal cell death (Sanchez et al., 2000), was also detected, although at low level, upon differentiation. Surprisingly, *CACNA1A*, a neuronal voltage gated calcium channel subunit which has been associated with a wide range of neurological disorders (Romaniello et al., 2010), was expressed in ASCs prior to and after induction of neurogenic differentiation. In addition, *SCN1A* and *KCNC1*, genes coding for voltage-sensitive ion channels (Lehmann-Horn and Jurkat-Rott, 1999), were upregulated upon induction of neurogenic differentiation,

followed by a decline on day 7. Collectively, this transcription analysis supports the view that ASCs may have the potential to differentiate towards the neuronal pathway *in vitro*.

Next, we investigated the expression pattern of glial cell marker genes. *GFAP*, an intermediate filament protein and astroglial-specific marker (Eliasson et al., 1999), was not expressed at detectable levels prior to or after induction of neurogenic differentiation. This was notably consistent with the induction of neurogenin, which is an inhibitor of *GFAP* (Sun et al., 2001). Interestingly, the oligodendrocytic markers *CNP* and *MBP* were expressed in ASCs. *CNP*, a microtubule-assembly myelin protein that directs process outgrowth in oligodendrocytes (Lee et al., 2005a), maintained nearly the same expression pattern at all stages. On the other hand, *MBP*, a gene involved in the formation of myelin sheaths and essential for oligodendrocyte morphogenesis at later stages of cell differentiation (Galiano et al., 2006), was downregulated upon induction of differentiation. This is consistent with neurogenin being an inhibitor of glial differentiation (Sun et al., 2001). This suggests that neurogenic-induced ASCs might repress a differentiation pathway towards the oligodendrocytic and astrocytic lineage.

The last set of genes examined included neuroendocrine cell marker genes. They were characterized by substantial variations in their expression patterns. *CHGA*, a secretory protein acting at the cross-road between the neuroendocrine and the cardiovascular systems (Di and Morganti, 2011), and *KISS1*, a gene encoding for kisspeptins which are potent stimulators of gonadotropin release in several mammalian species (Colledge, 2009), were upregulated upon neurogenic differentiation (*KISS1* to a larger extent). However, KISS1 expression did not persist at a later stage of differentiation. *XCL1* (Leptin), which has implications on brain development and glucose homeostasis (Jackson et al., 2011) and *GNRHR*, which in conjunction with *GnRH* is the primary regulator of reproduction in vertebrates (Hapgood et al., 2005), were also detectable in stimulated ASCs and until day 1 after induction of differentiation, suggesting their induction in a minor proportion of the cells. Overall, our data suggest that neurogenic-induced ASCs preferably differentiate towards the neuronal lineage, gradually repressing differentiation to other neural pathways.

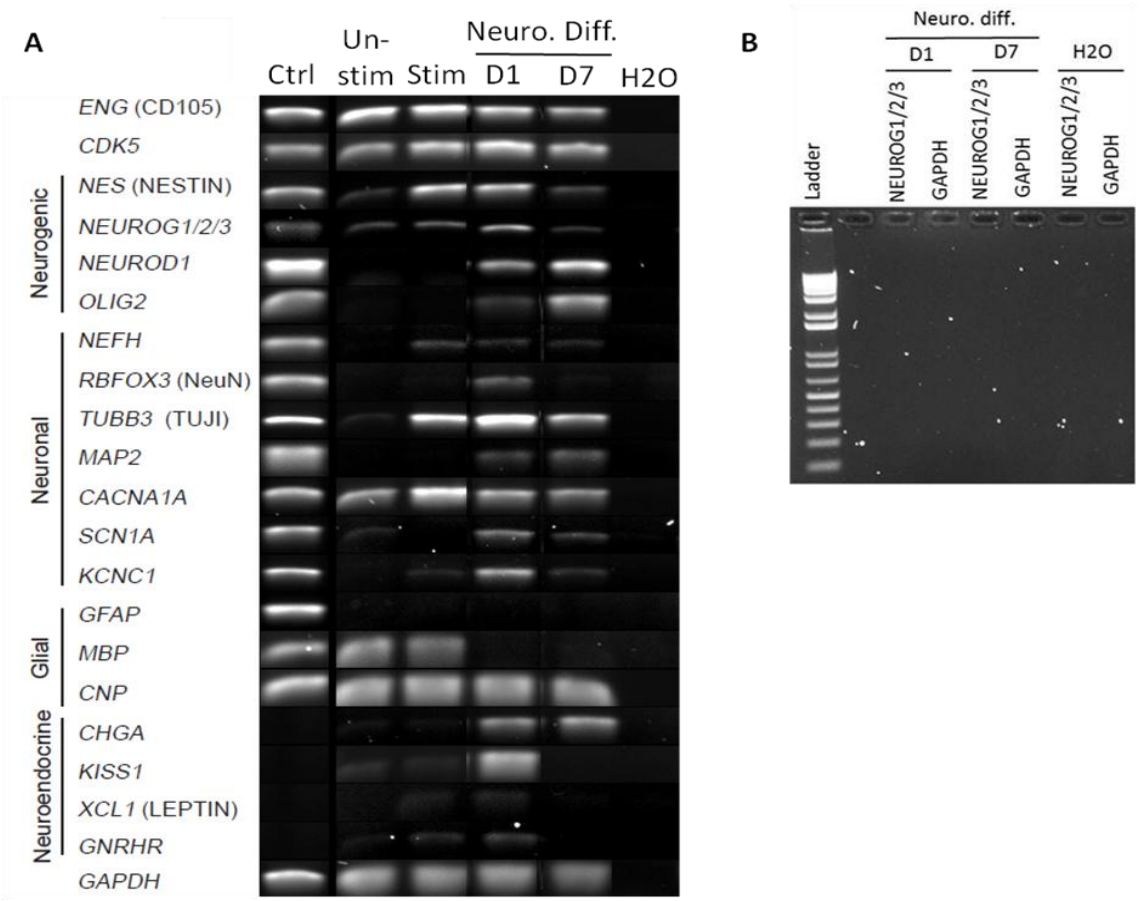

Fig. R-3. In vitro neurogenic differentiation of ASCs. (A) RT-PCR analysis of gene expression of neurogenic, neuronal, glial and neuroendocrine specific markers in unstimulated and stimulated ASCs and at day 1 and day 7 after induction of neurogenic differentiation. As a positive control for the primer-efficiency we used RNA isolated from retina, NPCs and white matter (left). Water was used as a negative control (-) (right). Genes were run at 30xcycles (*ENG*, *CDK5*, *NES*, *NEFH*, *TUBB3*, *MAP2*, *MBP*, *CNP*, *CHGA*, *KISS1*, *XCL1*, *GNRHR*, *GAPDH*) and at 35xcycles (*NEUROG1/2/3*, *NEUROD1*, *OLIG2*, *RBFOX3*, *CACNA1A*, *SCN1A*, *KCNC1*, *GFAP*). (B)–RT, no Reverse Transcription reaction control for day 1 and day 7 after induction of neurogenic differentiation for the genes *NEUROG1/2/3* and *GAPDH*.

4. Effect of neurogenic induction in the protein expression

Using immunofluorescence, we next analyzed the expression of different neurogenic (NEUROG1) and neuronal (NEFH, TUJI, MAP2, SYNAPSIN, MUNC, SYNAPTOTAGMIN and GEPHYRIN) proteins to further elucidate the neurogenic potential of ASCs after 14 days of culture in neurogenic medium **(Fig. R-4)**. These results are in agreement with the gene expression patterns, as we found immunoreactivity against NEUROG1, NEFH, TUJI (TUBB3) and MAP2. Double-immunolabeling of NEFH and NEUROG1 **(Fig. R-4A)**, gave

positive staining after induction of differentiation. NEFH, in correlation with being the most abundant cytoskeletal element in neuronal axons (Sasaki et al., 2009), was detected throughout the neurite-like processes in the neurogenic-induced ASCs. In contrast, NEUROG1, a nuclear transcription factor important for promotion of neurogenesis (Sun et al., 2001), was predominantly localized in the nucleus in neurogenic-induced ASCs. Next, we examined protein expression and localization of TUJI, a building block of neuronal microtubules with highest expression during periods of axon guidance and maturation (Tischfield et al., 2010), and MAP2, one of the most abundant proteins in the brain largely located in neuronal cell bodies and dendrites (Sanchez et al., 2000). Double-staining against those proteins **(Fig. R-4B)** showed strong expression upon induction of neurogenic differentiation, with localization of TUJI overall the neurite-like processes and MAP2 mostly concentrated in the perikaryon (cell-soma) and in the proximal part of the neurite-like processes. Of note, the majority of the neurogenic-induced ASCs stained for the above proteins. Our immunostaining experiments show that these neurogenic and neuronal proteins acquired relatively the expected distribution across the cell, suggesting that neurogenic-induced ASCs may acquire neuronal characteristics. To support this view, immunostaining analysis of human NPCs showed similar labeling patterns as in neurogenic-induced ASCs (Boulland et al., manuscript submitted).

Furthermore, we also examined the protein expression of three proteins, MUNC-13, SYNAPSIN 1-2, SYNAPTOTAGMIN, which are part of the machinery involved in the release of presynaptic vesicles; MUNC-13 is a family of proteins which are mostly expressed in brain and have a central role in synaptic vesicle priming (maturation to a fusion competent state) and is selectively targeted to presynaptic active zones (Koch et al., 2000). SYNAPSIN 1-2, are abundant synaptic-vesicle phosphoproteins that are known to regulate neurotransmitter release (Sun et al., 2006). SYNAPTOTAGMIN, is a Ca^{2+}-binding protein which participates in triggering neurotransmitter release at the synapse (Fernandez-Chacon et al., 2001). All three proteins stained strongly in neurogenic-induced ASCs. In particular, we detected MUNC-13 **(Fig. R-4C)** mostly in the perikaryon and proximal part of the neurite-like

processes. In contrast, SYNAPSIN **(Fig. R-4C)** was largely located at the distal-part of the neurite-like processes in comparison to the perikaryon. Another vesicle-associated protein, SYNAPTOTAGMIN **(Fig. R-4D)** was detected throughout the neurite-like processes as well as the perikaryon of the cells. Moreover, GEPHYRIN, a scaffolding protein often associated with clusters of postsynaptic GABAergic and glycine receptors (Kneussel and Betz, 2000), was detected in the perikaryon of some cells **(Fig. R-4D)**.

Notably, whereas localization of GEPHYRIN in the perikaryon was relatively as expected from its localization in neuronal cells (Kneussel and Betz, 2000), the localization of the presynaptic markers MUNC-13, SYNAPSIN 1-2, and SYNAPTOTAGMIN in the cytoplasm was unexpected. However, these presynaptic proteins were detected in clusters, a characteristic of synaptic vesicles in a resting presynaptic terminal in neurons (Pechstein and Shupliakov, 2010). Interestingly, immunolabeling of SYNAPTOTAGMIN in human NPCs showed similar labeling patterns (Boulland et al., manuscript submitted). These data suggest that there might be a lack of mature nerve-terminals or appropriate guiding signals in neurogenic-induced ASCs. To support this view, immunostaining analysis of SYNAPTOTAGMIN in individual cultured neurons also showed similar labeling patterns as in neurogenic-induced ASCs (Gardzinski et al., 2007). There was only a restricted number of cells which were stained for the above proteins (~10%), suggesting that cells do not respond in the same way when induced to differentiate.

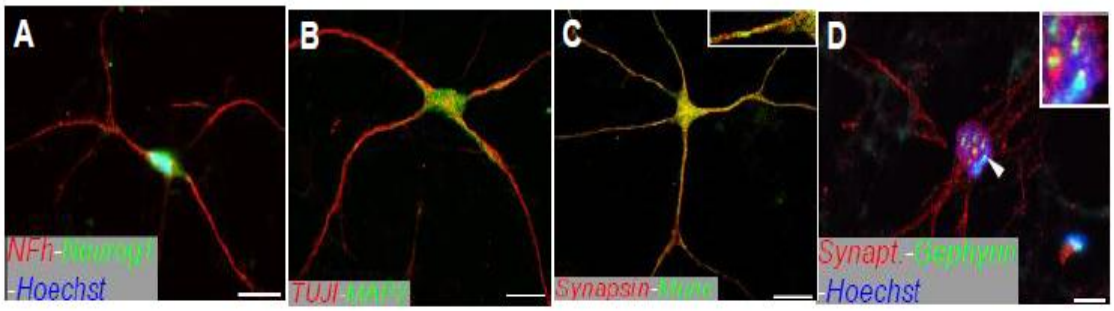

Fig. R-4. Localization of neurogenic and neuronal markers in neurogenic-induced ASCs as observed by immunofluorescence analyses using specific antibodies against NEFH, NEUROG1, TUJI, MAP2, SYNAPSIN, MUNC13, SYNAPTOTAGMIN, GEPHYRIN. Staining for these markers was performed after two weeks of differentiation. (A) Neurogenic-induced ASCs stain positive for the neuron-specific markers NEFH-NEUROG1. Localization of NEFH was detected in the neurite-like processes while NEUROG1 in the nucleus of the treated cells. Bar 10 μm. (B) Neurogenic-induced ASCs expressing TUJI and MAP2. TUJI was located in the neurite-like processes while MAP2 in the perikaryon and proximal part of the neurite-like processes. Bar 10 μm. (C) Staining of neurogenic-induced ASCs for SYNAPSIN and MUNC-13. SYNAPSIN was largely localized in the distal part of the neurite-like processes while detection of MUNC-13 was mostly found in the perikaryon and proximal part of the neurite-like processes. Bar 10 μm. (D) Double-labeling of SYNAPTOTAGMIN and GEPHYRIN gave positive staining, with SYNAPTOTAGMIN located both in the neurite-like processes and perikaryon of cells and GEPHYRIN located in the perikaryon. Bar 8 μm.

5. Effects of neurogenic induction on the cell cycle

Downregulation of *Nes* has been correlated with cells leaving the proliferative state and becoming post-mitotic (Dahlstrand et al., 1995). Using immunofluorescence, we investigated NESTIN expression prior and after induction of neurogenic differentiation **(Fig. R-5A,B,C,D)**. In particular, all cells examined were immunoreactive for NESTIN during mitogenic stimulation. This was followed by a gradual decline in immunoreactivity upon induction of differentiation. At later stages of differentiation (day 7) there was no detectable NESTIN expression. This strongly suggests that the cells are going towards a post-mitotic state upon induction of neurogenic differentiation.

To further evaluate the effects of neurogenic induction on the cell cycle, we used immunofluorescence for the detection of a cell-endogenous marker, tightly associated with cell proliferation, namely KI-67. KI-67 is a nuclear protein, expressed in all phases of the cell-cycle except the resting phase (G0), thus providing an effective marker of proliferation in the

initial phase of adult neurogenesis (Kee et al., 2002). Consistent with NESTIN expression pattern, the majority of the cells were stained for KI-67 **(Fig. R-5J, K, L)** at the mitogenic stimulation stage. Neurogenic induction was followed by a gradual decline in immunoreactivity with almost no detectable levels at day 7. These results suggest that the cells exit the cell-cycle and enter a resting phase or G0.

To strengthen this view, we also performed proliferation assessment by EdU incorporation, to allow microscopic analysis of the effect of the neurogenic differentiation treatment on the cell cycle, *in vitro* **(Fig. R-5E, F, G, H)**. In good agreement with NESTIN expression, there was an increase in the proliferation rate of ASCs upon mitogenic stimulation, followed by a dramatic decrease upon induction of neurogenic differentiation. Quantification studies confirm these data and show a steep decline in the proliferation rate of ASCs after neurogenic induction **(Fig. R-5-I).** Overall, our results indicate that neurogenic-induced ASCs become post-mitotic, a characteristic of terminally-differentiated cells.

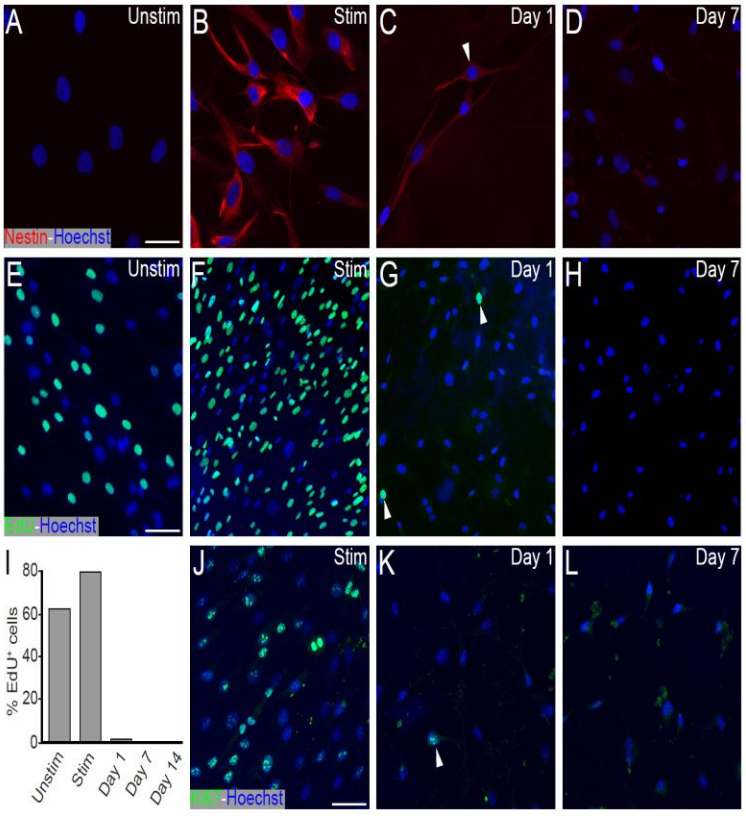

Fig. R-5. Evaluation of the effects of the neurogenic treatment on the cell cycle. (A-D) NESTIN immunostaining for unstimulated, stimulated and neurogenic-induced ASCs at day 1 and 7. The transition from a proliferating to a post-mitotic cell state is accompanied by a rapid decrease of NESTIN. Bar 25 µm. (E-H) Proliferation assessment by EdU incorporation for unstimulated, stimulated and neurogenic-induced ASCs at day 1 and 7. Neurogenic-induced ASCs are exiting the cell cycle, with no detectable EdU incorporation at Day 7. Bar 100 µm. (I) Percentage of EdU-positive cells for the different treatments and time points. (J-L) KI67 immunostaining for stimulated ASCs and day 1 and 7 after neurogenic induction. Bar 100 µm.

6. Epigenetic changes associated with ASC mitogenic stimulation and neurogenic induction

6.1. CpG methylation changes elicited by mitogenic stimulation and neurogenic induction

6.1.1. Nestin regions examined by bisulphite sequencing

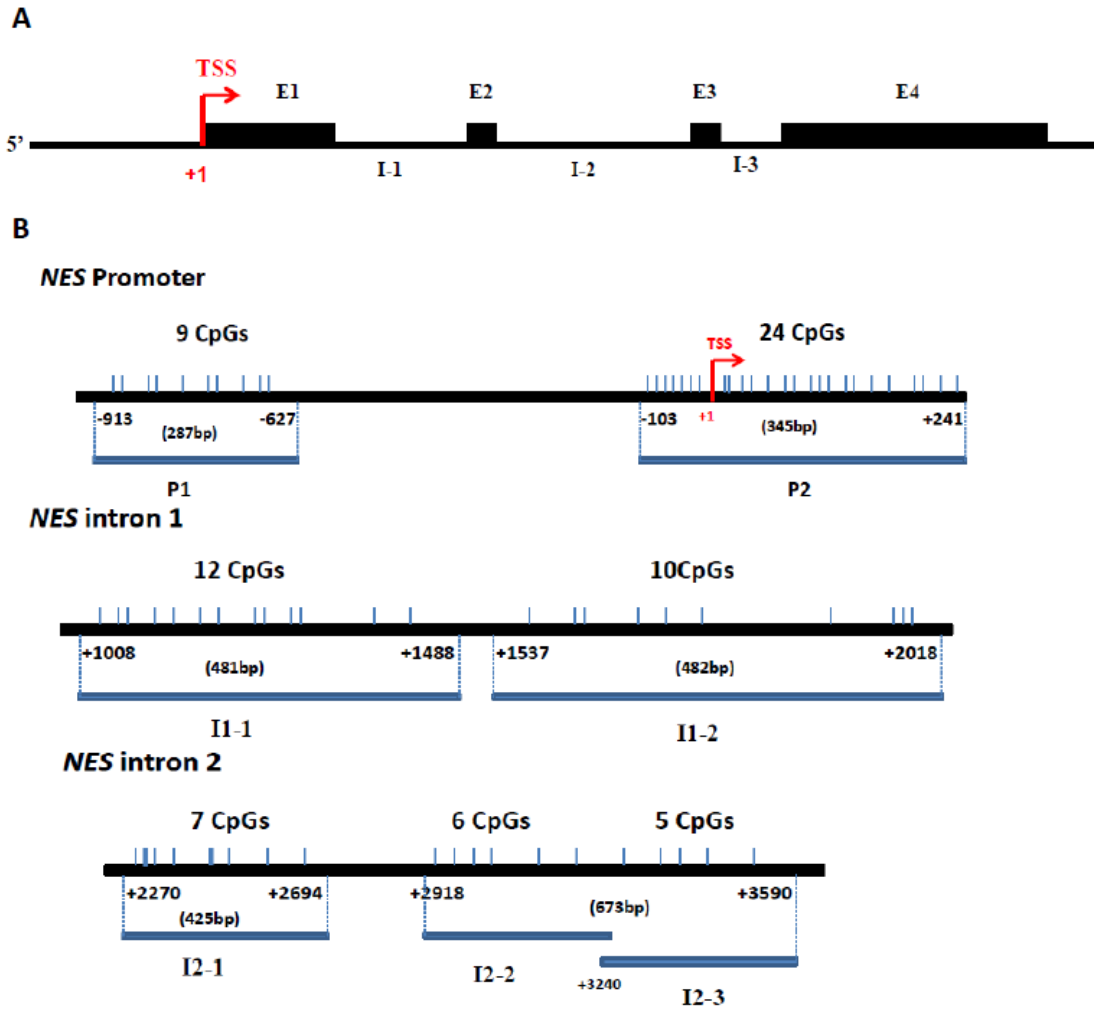

Fig. R-6. (A) An overview of the *NES* gene, consisting of four exons and three introns. (B) Genomic regions examined by bisulphite sequencing in the *NES* gene. TSS (+1), transcription start site. Numbers designate amplicon positioning relative to the TSS and the length of the regions examined is shown in bp.

PCR primers specific for bisulphite-converted DNA were designed to include several areas of the promoter region, first and second intron of *NES* (**Fig. R-6**). In the *NES* promoter, we have analyzed two regions, one more upstream from the TSS (+1) and one which spans the TSS together with a downstream region of exon 1. The first amplicon lies between nucleotides -913 to -627 relative to the TSS and covers a 287 bp region including 9 CpGs. The second amplicon is between nucleotides -103 to +241 relative to the TSS and covers a 345 bp region including 24 CpGs. This region is suggested to have the highest activity among other regions in the *Nes* promoter (Cheng et al., 2004). We also analyzed the DNA methylation pattern in the first intron of *NES* where there is a muscle-specific enhancer element important for regulation of myoblast differentiation (Zhong et al., 2008). The first primer set spans the region between nucleotides +1008 and +1488 relative to the TSS, with an amplicon size of 481 bp and 12 CpGs included in this region. The second primer set covers the region between nucleotides +1537 and +2018 relative to the TSS, with a product size of 482 bp and 10 CpGs in this region.

There are two neural enhancer elements residing in the second intron of *Nes,* one midbrain-specific and one CNS-specific (Yaworsky and Kappen, 1999). We analyzed three different regions in this second intron, one in the 5' region and two in the 3' region of the intron. The first primer set spans the region between nucleotides +2270 and +2694 relative to TSS, with an amplicon size of 425 bp containing 7 CpGs. A sequence comparison between the rat and human *NES* second intron revealed that this is the least conserved region of the intron (Yaworsky and Kappen, 1999). The second primer set spans the region between nucleotides +2918 and +3283 relative to the TSS, with an amplicon size of 366 bp containing 6 CpGs. This area is known to hold the enhancer that regulates *Nes* expression in the midbrain (Yaworsky and Kappen, 1999). The last primer set spans the region between nucleotides +3240 and +3590 relative to the TSS, with an amplicon size of 351 bp containing 5 CpGs. This is the region suggested to be specific for expression of *Nes* in the CNS (Kappen and Yaworsky, 2003).

6.1.2. CpG methylation profiles of *NES* promoter and *NES* intron 2

In this experiment, we were interested in analyzing the DNA methylation pattern for several regions of *NES* before and after induction of neurogenic differentiation. The promoter region examined in unstimulated ASCs and day 14 after induction of differentiation, remained globally unmethylated, consistent with its localization in a CpG-rich region **(Fig. R-7A)** (Appanah et al., 2007). This strongly suggests that the *NES* promoter region is not regulated by DNA methylation.

The second intron of *NES* (intron 2) was characterized by significant global changes ($P \leq 0.02$) in the methylation pattern, in particular in the region where the two enhancer elements are thought to reside **(Fig. R-7B, D)**. On the other hand, the changes in DNA methylation at the single CpG level were not significant **(Fig. R-7C)**. The first amplicon (I2-1) which represents the 5' region of the intron remained heavily methylated throughout the various treatments with no significant changes in the DNA methylation pattern ($P > 0.05$). The second (I2-2) and third (I2-3) amplicon, however, spanning the regions for the midbrain and CNS enhancers, respectively, were characterized by a global demethylation upon mitogenic stimulation. The two regions became fast remethylated upon induction of neurogenic differentiation and remained heavily methylated until day 14.

DNA methylation changes in I2-2 were extremely significant ($P < 0.0001$), upon mitogenic stimulation (step1) and induction of neurogenic differentiation (step2) in ASCs. There were no significant changes in the methylation pattern of neurogenic-induced ASCs during the differentiation treatment ($P > 0.05$). In I2-3, mitogenic stimulation was accompanied by a significant change in methylation ($P = 0.0207$), and a very significant change upon induction of differentiation ($P = 0.0023$). However, the changes in neurogenic-induced ASCs during the treatment were not significant ($P > 0.05$). Overall, these data show DNA methylation changes in the *NES* neural enhancer elicited by mitogenic stimulation and neurogenic induction. The data suggest that this enhancer might be regulated at the DNA methylation level, and that there might be putative transcription factor binding sites which become accessible upon global demethylation of this enhancer region.

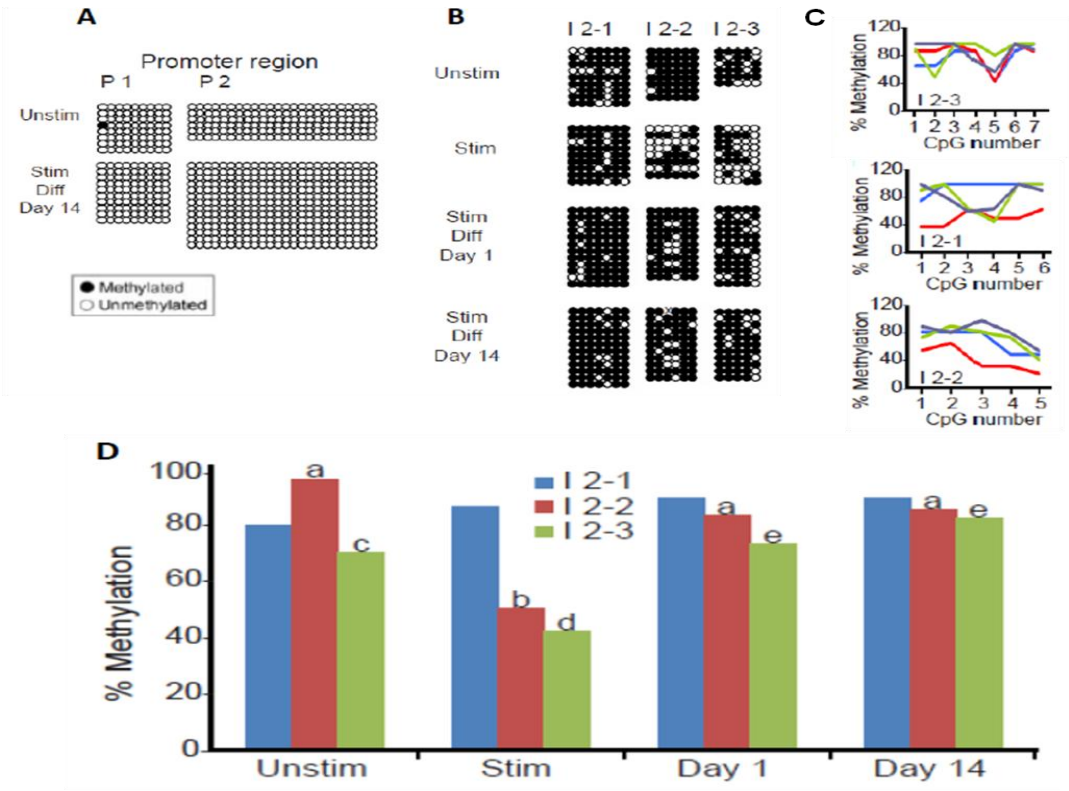

Fig. R-7. CpG methylation profiles of the *NES* locus. Diff stands for neurogenic differentiation (A) Bisulphite sequencing data for undifferentiated and neurogenic-induced ASCs for the promoter region. (B) Bisulphite sequencing data for unstimulated, stimulated and *in vitro* neurogenic-induced ASCs for the second intron. Each dot depicts one CpG in the 5'-3' order and each row of dots represents one bacterial clone (i.e., one genomic allele). Positioning of each CpG in each region is shown is Fig. R-6B. (C) DNA methylation profiles of the regions examined in the second intron, as indicated from (B). (D) Percentage of methylation in each region of the second intron and for each treatment, determined from (B). [a,b] $p < 0.001$, [c,d] $p = 0.02$ and [d,e] $p < 0.01$ (t-test).

6.1.3. CpG methylation profiles of *NES* intron 1

Previous studies in our group have established that ASCs do not posses the ability to differentiate towards the myogenic lineage (Sørensen et al., 2009). Therefore we were interested to investigate the DNA methylation state of the first intron of *NES*, important for myoblast differentiation (Zhong et al., 2008), and whether there are any differences elicited by the various treatments **(Fig. R-8A, B)**.

The first intron of *NES* (intron 1) was found to be heavily methylated in unstimulated ASCs. There were no significant global methylation changes upon mitogenic stimulation or induction of neurogenic differentiation (P>0.05). Interestingly, however, when we more closely examined the first amplicon (I1-1), we observed significant differences at the DNA methylation pattern at the local level. We detected a dynamic methylation and demethylation of some specific CpGs. In particular, CpG No. 3 was demethylated after mitogenic stimulation and this was followed by a gradual remethylation upon induction of differentiation, being almost completely methylated by day 14. The difference in the methylation status between stimulated ASCs and day 14 neuronal-like cells was significant (P=0,0237). There was also a significant difference in the methylation pattern between the various treatments for two CpGs (No. 5 and 6) with P values between unstimulated ASCs and day 14 neurogenic-induced ASCs equal to 0.03 and less than 0.0001, respectively. CpG No. 5 was almost completely unmethylated in unstimulated ASCs; however upon induction of differentiation it became gradually hypermethylated. On the contrary, CpG No. 6 was hypermethylated in unstimulated ASCs, however by day 14 was converted to a hypomethylated form. There were also some other CpGs which had a less significant change in there methylation pattern (No. 9, 11). CpG No. 9 was less methylated in unstimulated ASCs while upon mitogenic stimulation and after induction of neurogenic differentiation it remained completely methylated. CpG No. 11 was relatively unmethylated in the unstimulated, stimulated and day 1 neurogenic-induced ASCs; however it became heavily methylated by day 14. There were a few other CpGs which remained relatively unaltered at all time points (No. 2, 7, 8, 10, 12); in all cases these CpGs were hypermethylated. These dynamic alterations in the methylation of specific CpGs support the view for the presence of putative transcription factor binding sites important for regulation of the first intron of *NES.*

The second amplicon (I1-2) maintained relatively the same methylation pattern during the various conditions with no significant alterations at the global or local level, suggesting that it does not attain a dynamic role in nestin regulation upon mitogenic stimulation and differentiation. Interestingly, the presence of 3 CpGs being markedly hypomethylated suggests that there might be putative transcription factor binding sites essential for regulation of the first intron of *NES*, remaining consistently accessible for regulation.

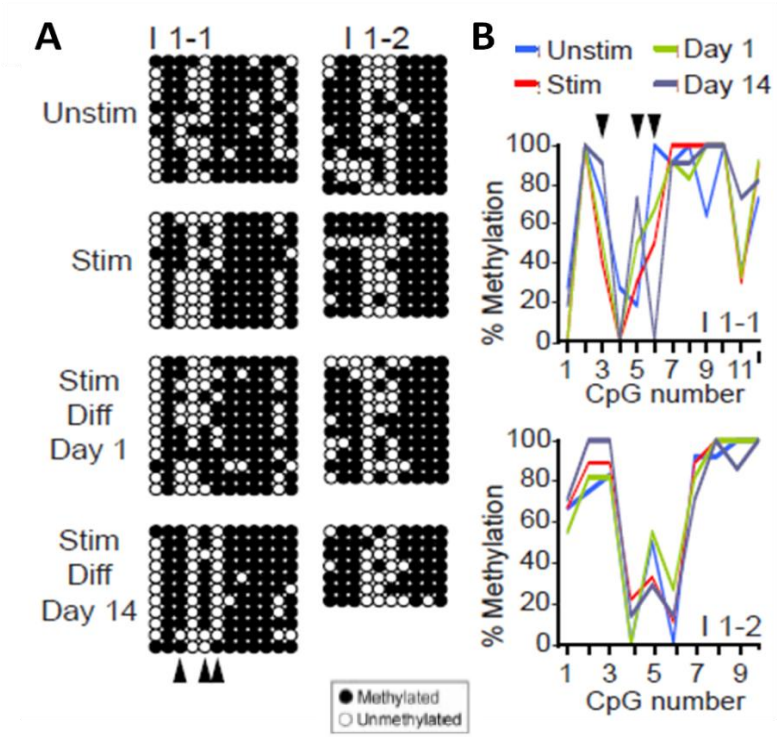

Fig. R-8. DNA methylation profiles in the *NES* first intron in unstimulated, stimulated and *in vitro* neurogenic-induced ASCs. Diff stands for neurogenic differentiation. (A) Bisulphite sequencing data for undifferentiated and neurogenic-induced ASCs. (B) DNA methylation profiles of the regions examined, as indicated from (A). The significance values of the three CpGs were: CpG No. 3, p=0.023 between stimulated and neurogenic-induced ASCs at day 14. CpG No. 5, p=0.03 between unstimulated and neurogenic-induced ASCs at day 14. CpG No. 6, p<0.0001 between unstimulated and neurogenic- induced ASCs at day 14.

6.2. Changes in histone modifications elicited by mitogenic stimulation and neurogenic induction

6.2.1. Regions examined by ChIP

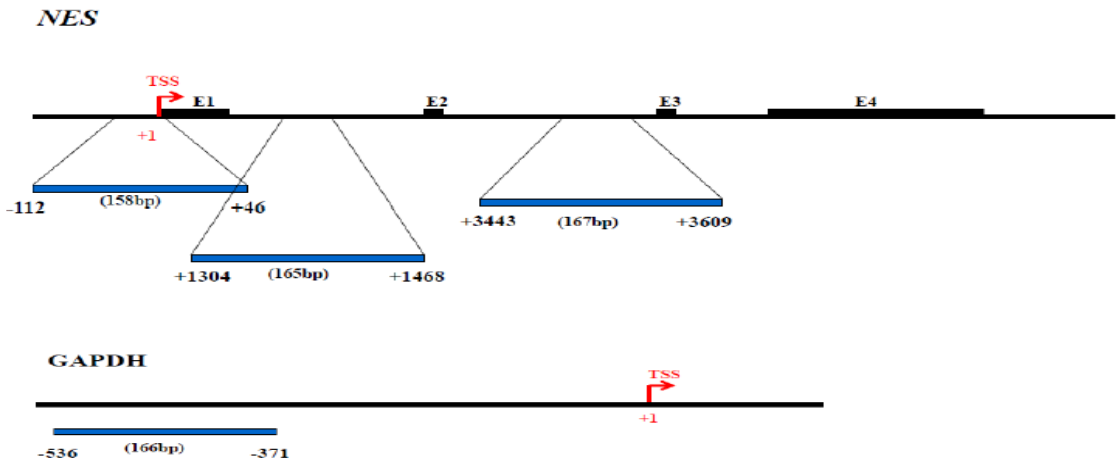

Fig. R-9. Genomic regions examined by Chromatin immunoprecipitation (ChIP) in *NES*, and *GAPDH*. TSS (+1), transcription start site. Numbers designate amplicon positioning relative to the TSS and the length of the regions examined is shown in bp.

We designed genomic DNA ChIP primers to include the proximal promoter regions of *NES* and of the house-keeping gene *GAPDH*. For the *NES* gene, we also examined a region in the first (muscle-specific enhancer) and second intron (neural enhancer) **(Fig. R-9)**.

In the *NES* promoter, we analyzed a region covering 158 bp ranging from nucleotides –112 to +46 relative to TSS. In the first intron, a 165 bp region was examined, spanning nucleotides +1304 to +1468 relative to the TSS. The second intron region examined spanned nucleotides +3443 to +3609 relative to the TSS, with an amplicon size of 167 bp. This area is important for the function of the CNS-specific enhancer and it contains a number of putative transcription factor binding sites (Lothian et al., 1999). Finally, the *GAPDH* promoter region examined spanned nucleotides –536 to –371 with an amplicon size of 166 bp.

6.2.2. Histone modification changes at the *NES* locus

DNA methylation has been shown to correlate with *Nes* repression; however DNA demethylation is not sufficient to mediate activation of *Nes* transcription (Han et al., 2009). To

expand our understanding of the epigenetic events associated with *NES* expression upon neurogenic induction of ASCs, we examined by ChIP whether transcriptionally repressive or permissive histone modifications on the *NES* locus were affected upon ASC mitogenic stimulation and neurogenic induction. We investigated the following histone modifications: H3K4me3, H3K27me3 and H3K9me3, three modifications commonly associated with, respectively, the TSS of most genes (active or inactive; H3K4me3), and with the promoter of transcriptionally inactive genes (H3K9me3 and H3K27me3). ChIP analysis was performed prior to and after mitogenic stimulation and following induction of neurogenic differentiation. Quantitative PCR was used to identify the enrichment of each modification on a specific genomic fragment, relative to input chromatin.

In undifferentiated ASCs prior to stimulation (unstimulated), all regions of *NES* examined were enriched in H3K4me3, H3K27me3 and H3K9m3, consistent with the very weak expression of *NES*. Upon mitogenic stimulation, H3K4me3 levels on the *NES* promoter remained unaltered while there was a steep decline in the repressive marks H3K27me3 and H3K9me3 **(Fig. R-10A)**, leading to an increase in the signal ratio of H3K4me3 to H3K27me3. As H3K4me3 marks most promoters regardless of their activity (Mikkelsen et al., 2007;Roh et al., 2006;Zhao et al., 2007), an increased H3K4me3/H3K27me3 ratio can be interpreted as an indicator of transcriptional activation (Noer et al., 2009). Induction of neurogenic differentiation was accompanied by an increase in H3K27me3 and H3K9me3, consistent with *NES* downregulation at the mRNA and protein levels **(Fig. R-10A)**. These data indicate that *NES* promoter activity correlates with dynamic changes in associated histone modifications.

The CNS enhancer in the second intron of *NES* (intron 2) displayed similar modification changes as the *NES* promoter. Upon mitogenic stimulation, H3K4me3 levels remained unaltered, while H3K27me3 and H3K9me3 exhibited a steep decline **(Fig. R-10B)**. Neurogenic induction was accompanied by an increase in H3K27me3 and H3K9me3, consistent again with *NES* repression on day 7 **(Fig. R-10B)**. This is in agreement with our DNA methylation data and supports the view that *NES* is under epigenetic control.

Overall, these results indicate a strong correlation between *NES* expression patterns and promoter enrichment in permissive and repressive histone marks. This also holds true for the CNS enhancer (intron 2) for these marks. This is consistent with previous data showing that the *NES* neural enhancer plays a major role in the regulation of *NES* expression (Zimmerman et al., 1994) and suggests a regulation of *NES* expression at the level of histone modifications (in addition to DNA methylation).

The *NES* muscle-specific enhancer in the first intron (intron 1), is also subjected to changes at the histone level. Whereas no changes were detected in H3K4me3 enrichment in this intron, mitogenic stimulation was associated with a decrease in H3K27me3 and H3K9me3, whereas re-enrichment occurred after neurogenic induction **(Fig. R-10C)**. These results suggest that the mitogenic stimulation treatment might initialize an active state on the *NES* muscle-specific enhancer, consistent with previous studies supporting that *Nes* intron 1 can induce reporter gene expression in both neural and muscle precursor cells (Zimmerman et al., 1994)

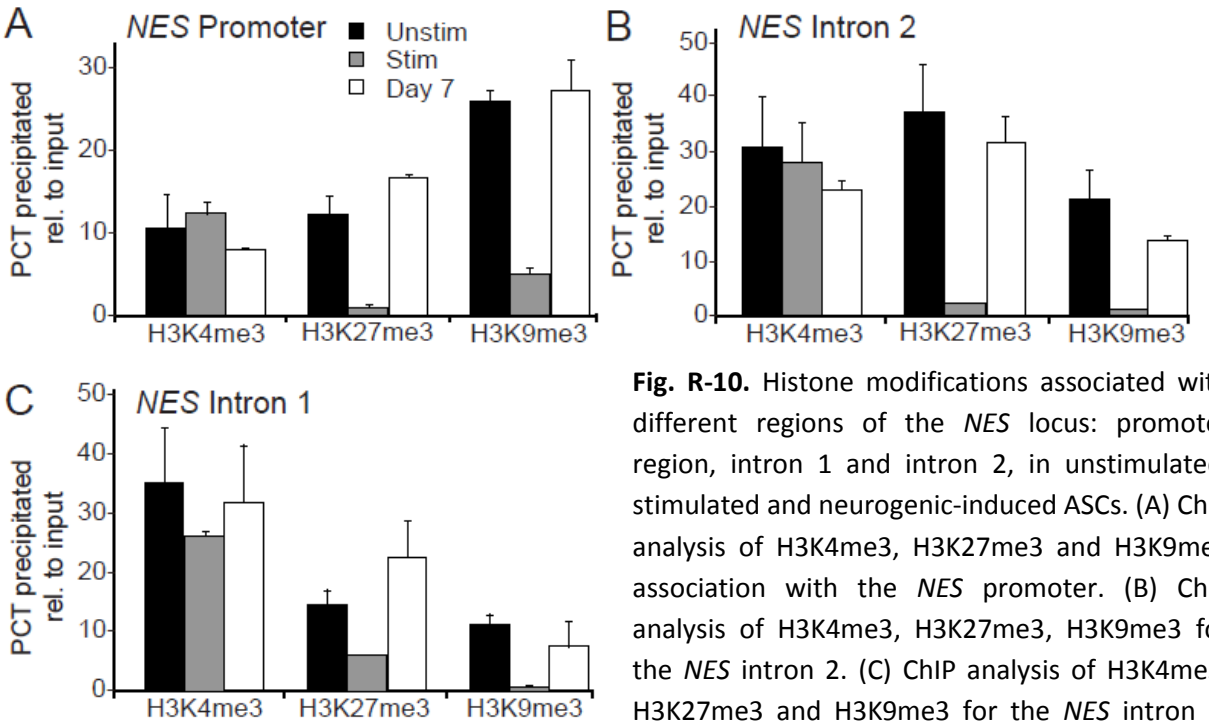

Fig. R-10. Histone modifications associated with different regions of the *NES* locus: promoter region, intron 1 and intron 2, in unstimulated, stimulated and neurogenic-induced ASCs. (A) ChIP analysis of H3K4me3, H3K27me3 and H3K9me3 association with the *NES* promoter. (B) ChIP analysis of H3K4me3, H3K27me3, H3K9me3 for the *NES* intron 2. (C) ChIP analysis of H3K4me3, H3K27me3 and H3K9me3 for the *NES* intron 1. Data are from two independent ChIPs each analyzed by duplicate quantitative PCRs.

6.2.3. Histone modification changes in the *GAPDH* promoter

In agreement with its constitutive activation, the *GAPDH* promoter was strongly enriched in H3K4me3 with no H3K27me3 or H3K9me3 in ASCs. H3K4me3 remained elevated after mitogenic stimulation and induction of differentiation. Interestingly, the *GAPDH* promoter became slightly enriched in the repressive histone marks H3K27me3 and H3K9me3 upon induction of neurogenic differentiation. The results were in good agreement with the mRNA expression profiles which indicated that there was a slight decrease in the expression of *GAPDH* at Day 14 (data not shown). Overall, these data suggest that these neuronal-like cells might become post-mitotic, low energy demanding cells, since *GAPDH* expression is higher in energy-demanding tissues (Barber et al., 2005).

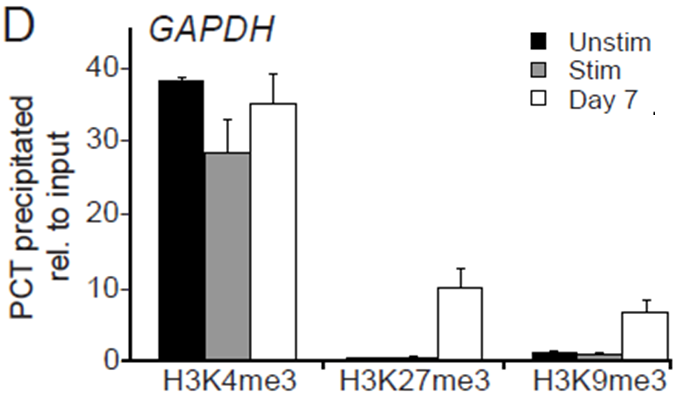

Fig. R-11. Histone modifications associated with the *GAPDH* promoter in undifferentiated and neurogenic-induced ASCs. ChIP analysis of H3K4me3, H3K27me3 and H3K9me3 association with the *GAPDH* promoter.

DISCUSSION

This project evaluates the capacity of ASCs to differentiate towards the neurogenic lineage and tests the hypothesis that there is an epigenetic basis related to the differentiation potential of ASCs towards the neurogenic pathway. Using the neurogenic marker, *NES*, as a model to study the epigenetic changes acquired upon induction of neurogenic differentiation, we show that there are dynamic changes in both DNA methylation and histone modifications along with dramatic changes in morphology and gene expression pattern before and after induction of neurogenesis in ASCs. Nevertheless, as MSCs from various sources have distinct differentiation capacities, different ASC clones similarly have distinct differentiation potential (Boquest et al., 2005). We show that there is a sequential activation and repression of neurogenic-specific genes taking place that might lead ASCs to differentiate towards the neurogenic pathway. Using bisulphite sequencing, we suggest that *NES* might be regulated at the DNA methylation level. In addition, using chromatin immunoprecipitation, we demonstrate that there are dynamic changes at the histone level following mitogenic stimulation and induction of neurogenic differentiation on the *NES* locus. Both our bisulphite sequencing and ChIP results are in agreement and are strengthened by the morphological and gene expression profiling data at the mRNA and protein level.

In vitro differentiation potential of ASCs

ASCs display a neuronal-like morphology after neurogenic induction

The possibility of generating neural cells from human ASCs (hASCs) *in vitro,* could be very promising for the treatment of neurodegenerative diseases since ASCs are easily isolated and they can be obtained in high amounts (Boquest et al., 2005). Neurogenic-induced ASCs, acquired dramatic changes in their structure and adopted a neuronal-like morphology. This was also accompanied by expression of neurogenic-specific genes and the significant reduction of cell proliferation upon differentiation, characteristics comparable to neural progenitors. These results, even though promising, must, however, be interpreted with

caution, in particular regarding morphological changes, as these might be caused by alterations in the cell cycle progression or even cytoskeletal collapse (Bertani et al., 2005;Kompisch et al., 2010).

Several protocols have been developed for induction of neurogenic differentiation of MSCs ranging from the use of chemicals (Woodbury et al., 2000), to more complex media containing growth factors (Deng et al., 2001;Sanchez-Ramos et al., 2000). Even when MSCs were induced by a simple treatment as chemicals, phenotypic alterations were taking place with the cells exhibiting a neuronal-like morphology followed by expression of only a few genes, such as *NSE*, *NEUN* and *TAU* (Bertani et al., 2005). In addition, immunoreactivity for certain neuronal proteins was observed, such as NEUN, but their localization was aberrant, supporting the idea that this change in cell morphology might be an artifact or rather a response of cells to environmental stress rather than a differentiation trait (Bertani et al., 2005). Therefore, whether MSCs can differentiate into functional neurons remains controversial.

ASCs display a unique gene expression pattern at the mRNA and protein level

Our work is in agreement with previous studies (Cardozo et al., 2010;Dhar et al., 2007;Jang et al., 2010;Safford et al., 2002), confirming that ASCs can undergo commitment towards a neurogenic fate. At the mRNA level, our results demonstrate that ASCs express MSC-specific markers such as *CD44*, *CD90* (data not shown) and *CD105* and upon neurogenic differentiation, they start acquiring the expression of neural markers followed by gradual dowregulation of the stem cell markers. In our experiment, we observed that ASCs tended to express neuronal and neurogenic genes and to a lesser extent oligodendrocytic markers. On the contrary, we did not detect expression of the astroglial marker *GFAP*, indicating that our ASCs were probably not differentiating towards the astrocytic pathway. This might be explained by the presence of epidermal growth factor (EGF), an agent used in the stimulation media, reported to restrict astrocytic lineages (Jang et al., 2010), or even by the presence of

the bHLH transcription factor Neurogenin 1 which inhibits the differentiation of neural stem cells into astrocytes (Sun et al., 2001).

It has been demonstrated that foskolin, a diterpene that activates adenylate cyclase in a variety of mammalian cells (Litosch et al., 1982), induces expression of neuronal markers such as NSE, NEFH and TUJI in human MSCs cultured in serum-free conditions (Kim et al., 2005;Rooney et al., 2009). Our work shows that neurogenic-induced ASCs exhibit increased immunoreactivity for the neuronal and neurogenic markers NEFH, NEUROG1, TUJI, MAP2, confirming results at the gene expression level (mRNA) as well as increased expression at the protein level of SYNAPTOTAGMIN, GEPHYRIN, SYNAPSIN and MUNC-13. We also observed expression at the mRNA level for a number of neurogenic genes such as *NES*, *NEUROG1/2*, *NEUROD1* and *OLIG2*, neuronal genes such as *RBFOX3* (NeuN), and oligodendrocytic markers such as *MBP* and *CNP*. Interestingly we found increased expression at the mRNA level for the three different ion channels *CACNA1A* (calcium channel, voltage-dependent, P/Q type, alpha 1A subunit), *SCN1A* (sodium channel, voltage-gated, type I, alpha subunit) and *KCNC1* (potassium voltage-gated channel, Shaw-related subfamily, member 1), which are important for the electrophysiological properties that characterizes neurons in the nervous system. Thus, ASCs have the ability to differentiate into neuronal cells in vitro and exhibit neuronal cell properties much like neuronal cells derived from other types of MSCs (Cho et al., 2005) or embryonic stem cells (Biella et al., 2007).

CpG methylation patterns in the *NES* gene

The *NES* promoter region houses a CpG island

The promoter region of the *NES* gene examined in this study was globally unmethylated both before and after induction of neurogenic differentiation, consistent with the observation that the TSS of this gene is located within a CpG island. The majority of mammalian gene promoters reside within regions of the genome called CpG islands. These regions are often protected from DNA methylation enabling the constitutive expression of these genes (Blackledge and Klose, 2011;Collas, 2009), aberrant methylation of which is tightly

associated with carcinogenesis. It was once thought that only housekeeping gene promoters were marked by CpG islands but it is now evident that many tissue-specific gene promoters are also encompassed within CpG islands (Blackledge and Klose, 2011), as in our case. These observations argue that the *NES* promoter is not regulated at the DNA methylation level, but it might use other mechanisms to regulate gene expression, for example, at the chromatin level.

Alternatively, one might also argue that the *NES* promoter might not be essential for the regulation of nestin expression. This possibility would be in agreement with the analysis of the *Nes* gene in transgenic mice which indicate that there are no regulatory elements for nestin specific expression identified in the region upstream of the first exon of *Nes* gene (Cheng et al., 2004).

Dynamic global changes in the neural enhancer of *NES*

Since the *Nes* promoter is unable to drive cell-type specific expression (Zimmerman et al., 1994), then what confers *Nes* its strict regulation and the CNS-specific expression pattern? Interestingly, regulation of *Nes*, together with several other intermediate filaments, is conferred by downstream enhancer elements (Zimmerman et al., 1994). Enhancers are distal elements which enhance the activity of the promoter. What characterizes enhancers is that they may act over long distances, independently of orientation and they can be upstream or downstream (e.g. in the *NES* gene) of the gene they regulate. They also contain multiple binding sites for diverse factors and determine the tissue-specificity of the gene they direct (Ong and Corces, 2011).

Two enhancer elements reside in the 3' region of the *Nes* second intron, one specific for the developing midbrain and one specific for expression of *Nes* in the CNS. The 204 bp midbrain enhancer resides between bases +1068 and +1271 and the 206 bp CNS enhancer between bases +1272 and +1477 relative to the start of the second intron (Yaworsky and Kappen, 1999). All three regions examined in the second intron of *NES* were strongly methylated in unstimulated ASCs, compatible with low level of transcription. However, in

stimulated ASCs, where cells expressed high levels of *NES* mRNA and protein, we found that the *NES* two enhancer elements (3' region) underwent a global demethylation, strongly suggesting that the *NES* neural enhancer may be regulated by DNA methylation. Our data also suggest that there might be putative transcription factor binding sites that regulate the expression of the gene during neural differentiation and central nervous system development (Jin et al., 2009;Josephson et al., 1998;Tanaka et al., 2004).

Midbrain cells expressing *Nes* can activate both enhancers, in comparison to other CNS cells which activate only the CNS-specific enhancer (Yaworsky and Kappen, 1999). This suggests that these cell types have different repertoires of transcription factors and apart from their physical proximity, these enhancer elements can be regulated in an independent manner (Kappen and Yaworsky, 2003;Yaworsky and Kappen, 1999). Prior to cell type specification or lineage restriction, the *Nes* enhancer elements are activated in progenitor cells. Although *Nes* was once thought to be a general marker of neural stem cells, it is feasible to identify and isolate distinct populations of progenitor cells by virtue of *Nes* enhancer activity (Yaworsky and Kappen, 1999). Our bisulphite sequencing data show that both enhancers are demethylated upon stimulation of proliferation suggesting that this treatment may ''prime'' *NES* for expression

The 5' most region examined in the second intron of *NES* remained heavily methylated throughout the different treatments, suggesting that it does not contain regulatory elements essential for *NES* expression. This region is the least conserved between rat and human compared to the 3' region which is highly conserved (Yaworsky and Kappen, 1999). A recent study in NSC-like cells and primary cultured astrocytes have shown that the 5' part of the intron also contains an enhancer element, however, it has quite distinct properties from the 3' portion (Zhang et al., 2005). It is thought that this enhancer element contributes to the transcriptional suppression of the neural enhancer in NSC-like cells but on the other hand it activates *Nes* expression in astrocytes. There are also multiple putative binding sites for transcription factors, which might play an important role in the regulation of this enhancer element (Zhang et al., 2005). In ASCs, the entire area examined was heavily methylated,

suggesting DNA-methylation-dependent repression of the enhancer. This is consistent with the inadequacy of ASC differentiation towards the astrocytic lineage.

Dynamic local changes in the muscle-specific enhancer of *NES*

Skeletal muscle development is also accompanied by changes in intermediate filament composition, with myogenic progenitor cells and myocytes expressing *Nes*, while upon maturation into myofibers nestin is exchanged for desmin (Pallari et al., 2011). *Nes* expression in the developing skeletal muscle is regulated by a muscle-specific enhancer in the first intron of *Nes* gene (Zhong et al., 2008). It has also been shown that the first intron can induce reporter gene expression in both neural and muscle progenitor cells in transgenic mice (Zimmerman et al., 1994).

Our bisulphite sequencing results show marked differences on specific CpGs for the different treatments, in comparison with the second intron of *NES* which shows changes at the global level. This indicates that there might be specific transcription factors which bind to the first intron and regulate nestin expression. Whether the first intron has an important role for expression of *NES* during induction of neurogenesis or whether the treated ASCs acquire a potential to differentiate towards the myogenic lineage remains uncertain. It would be interesting to determine whether these dynamic local changes in the methylation pattern could promote the myogenic potential of ASCs.

Transcription factor binding elements are unmethylated

CpG methylation is implicated to be essential for the maintenance of a silent chromatin state through recruitment of methyl binding proteins which can act as repressors or they can actively recruit repressor proteins (Jaenisch and Bird, 2003). In many cases, DNA methylation prevents binding of transcription factors to their recognition site, if these contain methylated CpGs (Kudo, 1998). The *Nes* core promoter region -161/+183 contains several putative cis-elements such as AP1 and AP2 binding sites (AP1 may not be essential for promoter activity), a TATA-like sequence (TATGAA) and two Sp1-binding sites which are

important for promoter activity (Cheng et al., 2004). The unmethylated state of the *NES* promoter in ASCs is consistent with an 'open' chromatin configuration compatible with potential binding of transcription factors. Moreover, Sp1 are transcription factors typically binding to GC-box motifs and they can trigger basal gene expression (Cheng et al., 2004). In agreement with this, *Nes* shows no cell-type specificity and it can function in different mammalian cell lines (Cheng et al., 2004).

A number of transcription factors have been identified to regulate the *Nes* neural enhancer. A 206-bp region, now termed the CNS enhancer, contains two POU-domain protein binding sites and a SOX-domain binding site. The SOX and POU binding elements are adjacent and essential for the regulation of the enhancer and synergic interactions between group B1/C SOX and class III POU within the nucleus determine *Nes* expression (Tanaka et al., 2004). The B1 *SOX* group of genes represented by *SOX2* and including *SOX1* and *SOX3* are transcription factors associated with early neurogenesis (Mizuseki et al., 1998;Pevny et al., 1998;Streit et al., 1997;Uchikawa et al., 2003). The class III POU transcription factors including *Brn1*, *Brn2*, *Brn4*, and *Oct-6* are also widely expressed in the developing CNS (Alvarez-Bolado et al., 1995;He et al., 1989). Together, various combinations of SOX and POU factors interplay to differentially regulate the *Nes* CNS enhancer (Tanaka et al., 2004). The region where these binding sites reside in the CNS enhancer undergo extensive demethylation upon mitogenic stimulation of ASCs making these sites potentially available for binding. Transcription factor binding in this enhancer region may protect it from re-methylation in the proliferative state.

Nuclear hormone receptors are important in development, differentiation and organ physiology (Eberl and Littman, 2003). Nuclear receptors such as TRs, RAR, RXR, and COUP-TF exert their effects by binding to motifs comparable to ER2 and IR1 in the *NES* CNS enhancer. Studies in transgenic mice have demonstrated that the *NES* CNS enhancer contains binding sites for all four members of the nuclear hormone receptor family implicating that nuclear hormone receptors are involved in regulation of the *NES* gene (Lothian et al., 1999). Depending on their combinatorial control can exert either positive or negative effects

on *NES* expression (Lothian et al., 1999). This might indicate that the *NES* neural enhancer is likely to be a hormone response element.

The 204 bp region upstream from the CNS enhancer has been demonstrated to harbor the midbrain-specific enhancer of *Nes* (Kappen and Yaworsky, 2003). A tissue-specific enhancer activity is plausible by the cooperation of two elements, a midbrain-specific element and a transcriptional potentiator element. Activation of the midbrain enhancer element is achieved by binding of a nuclear receptor-type protein in the midbrain-specific element, in conjunction with an unidentified potentiator element (Kappen and Yaworsky, 2003). It is speculated that Nurr1, an orphan nuclear receptor important for dopaminergic cell differentiation (Zetterstrom et al., 1997), is involved in *Nes* midbrain enhancer element regulation (Kappen and Yaworsky, 2003). Finally, Sonic hedgehog, a secreted factor which controls differentiation of specific cell types in the ventral midbrain (Chiang et al., 1996;Ericson et al., 1996), is also considered to control the midbrain enhancer activity (Lothian et al., 1999). Our bisulphite sequencing results show a global demethylation taking place in this area upon ASC mitogenic stimulation, further supporting the view that neurogenic-induced ASCs may differentiate towards midbrain-like neuronal cells. This could have great implications for the treatment of neurodegenerative diseases, since the ventral midbrain region is the main source of dopaminergic neurons, which are selectively lost in Parkinson's disease (Choi et al., 2005)

The muscle-specific enhancer of *Nes* resides in the first intron with the core enhancer being located to the +291- +661 region of the first intron (Zhong et al., 2008). Two E-boxes are essential for enhancer activity since their deletion abolishes activity. The second E-box is thought to contain a consensus sequence similar to that recognized by MyoD, a transcription factor important for differentiation of skeletal muscle (Zhong et al., 2008). Our results show that the CpGs around these E-boxes are heavily methylated probably inhibiting transcription factor binding. Nevertheless, we observe a dynamic change in the methylation pattern at a local level on other CpGs, suggesting transcription factor binding at other sites than the E-boxes. Unfortunately, no regulatory elements have been indentified in previous studies. It

would be interesting to determine whether these changes in CpG methylation in this region could induce the cells towards a myogenic fate or regulate their neurogenic differentiation.

These observations, in particular for the *NES* promoter and the *NES* muscle-specific enhancer, argue that in non-coding regulatory elements such as promoters and enhancers, unmethylated CpGs have the tendency to reside within or nearby binding sites for transcription factors. Through binding to their recognition motifs, these factors might mask these CpGs and keep them in their unmethylated form (Bird, 2002); by other mechanisms (e.g. H3K4me3), these CpGs may also be protected from DNA methylation, allowing transcription factor binding (Cedar and Bergman, 2009). This is in agreement with promoter studies which demonstrate that induced alterations on the methylation state of specific CpGs affect factor binding and transcription (Campanero et al., 2000;Comb and Goodman, 1990;Prendergast et al., 1991).

Although, the *NES* CpG promoter remains unmethylated invariably and the *NES* muscle-specific enhancer is characterized by CpG-specific changes in DNA methylation, this is not the case for the *NES* neural enhancer. In contrast to promoter CpG islands which are rarely methylated (Shen et al., 2007;Weber et al., 2007) irrespective of activity (Weber et al., 2007), the methylation pattern of enhancer elements is a common tissue-specific feature that correlates well with both chromatin accessibility and transcription factor binding (Wiench et al., 2011). Recent studies have shown a general decrease in methylation at active enhancers, suggesting that sustaining these elements in an unmethylated state might be important for their availability for protein-DNA interactions (Lister et al., 2009;Schmidl et al., 2009), consistent with the global demethylation of *NES* neural enhancer. In addition, methylation of CpGs are dynamic epigenetic marks that undergo extensive changes during cellular differentiation, particularly in regulatory regions outside core promoters (Meissner et al., 2008). The fast remethylation of the enhancer region upon neurogenic induction suggests that these dynamic epigenetic changes may be associated with enhancer inactivation. This is consistent with the dynamic downregulation of *NES* expression at the mRNA and protein level and the enrichment of repressive chromatin marks after neurogenic-induction.

Regulation of gene expression by histone modifications

NES as a predictor of differentiation capacity

In agreement with previous studies (Han et al., 2009), we observed that DNA methylation-dependent gene silencing regulates *NES* gene expression. Nevertheless, DNA methylation is not the primary determinant of gene expression (Boquest et al., 2006a;Collas, 2010;Noer et al., 2006), and DNA demethylation is not sufficient to induce activation of *Nes* transcription (Han et al., 2009). Then, what regulates activation of gene expression or activation potential, upon stimulation and induction of differentiation?

Another epigenetic mechanism, that is, post-translational histone modifications play a major role. Chromatin modifications are essential in the transfer of cell fate information during stem cell proliferation and differentiation (Kouzarides, 2007). The 'poised' state is of key importance for determining the differentiation potential of a cell (Hirabayashi and Gotoh, 2010). We show that the *NES* promoter and neural enhancer are bivalently marked suggesting that ASCs are epigenetically pre-patterned to differentiate toward a neural cell fate. The repressive marks H3K27me3 and H3K9me3 are decreased upon mitogenic stimulation, which is consistent with gene activation. On the other hand, *NES* downregulation is characterized by an increase in enrichment for H3K27me3 and H3K9me3 retrieving the repressive state of the gene. From previous studies in our laboratory, adipogenic promoters in undifferentiated ASCs were also enriched in H3K4me3 and H3K27me3 histone marks, while upon adipogenic differentiation, H3K27me3 on adipogenic promoters, was notably decreased (Noer et al., 2009). Together with the bivalent marks adipogenic promoters were also kept in an unmethylated state, as in our case. Taken together, these studies further support the view that ASCs might be epigenetically primed to differentiate towards the neurogenic lineage upon proliferation induction.

Genome-wide analysis of a range of histone modifications by CHIP-seq has revealed that although the chromatin signatures of promoters are remarkably similar for different cell types, epigenetic states associated with enhancers display more pronounced tissue-specificity. Enhancers are considered to be the most variable class of transcriptional

regulatory elements in a cell-type specific manner, suggesting that enhancers are essential in driving cell-type specific gene expression (Heintzman et al., 2009). Two histone modifications, which are thought to constitute an 'identity' for enhancers, are H3K27ac and H3K4me1. The neural and muscle-specific enhancers of *NES* contain both modifications which are highly enriched in ASCs throughout the different treatments (data not shown), consistent with previous studies which suggest that H3K27ac together with H3K4me1 is correlated with enhancers near active genes (Bulger and Groudine, 2011). Overall these data indicate that there is a complex interplay between histone modifications in the regulation of *NES* expression. They also argue that the *NES* gene in ASCs exists is an opened but 'poised' chromatin state, in which the proper cues might promote neurogenic differentiation.

Commitment of ASCs to the neurogenic lineage

The differentiation potential of a cell is based on two mechanisms of gene silencing at developmental gene loci; (i) transient silencing that confers a poised state, and (ii) long-term or permanent silencing (Hirabayashi and Gotoh, 2010). The 'bivalent' state of developmental genes acts as a brake preventing premature cell differentiation while maintaining the capacity of the genes to be activated in the future (Collas, 2009). Therefore, it may be used as an predictor to determine the differentiation potential of a cell. *NES*, a gene closely associated with neurogenesis, is characterized by a 'bivalency' in unstimulated ASCs, upon mitogenic stimulation though, is lost. Furthermore, DNA methylation may permanently repress genes associated with pluripotency and lineage-specification but it may also act transiently, as is the case of some distal enhancers of developmental genes in ESCs (Hirabayashi and Gotoh, 2010). *NES* becomes transiently demethylated upon mitogenic stimulation, suggesting that genes that are thought to be silenced through long-term or permanent silencing can be de-repressed under certain circumstances.

Further characterization of the chromatin states of master regulatory genes involved in neurogenesis at each stage of neural development and understanding the mechanisms underlying cell fate restriction will be of substantial value in the field of regenerative medicine.

This could lead to innovation of new strategies to expand fate restriction and enhance the plasticity of committed cells towards alternative cell fates.

REFERENCE LIST

Abouelfetouh,A., Kondoh,T., Ehara,K., and Kohmura,E. (2004). Morphological differentiation of bone marrow stromal cells into neuron-like cells after co-culture with hippocampal slice. Brain Res. *1029*, 114-119.

Alvarez-Bolado,G., Rosenfeld,M.G., and Swanson,L.W. (1995). Model of forebrain regionalization based on spatiotemporal patterns of POU-III homeobox gene expression, birthdates, and morphological features. J. Comp Neurol. *355*, 237-295.

Appanah,R., Dickerson,D.R., Goyal,P., Groudine,M., and Lorincz,M.C. (2007). An unmethylated 3' promoter-proximal region is required for efficient transcription initiation. PLoS. Genet. *3*, e27.

Azuara,V., Perry,P., Sauer,S., Spivakov,M., Jorgensen,H.F., John,R.M., Gouti,M., Casanova,M., Warnes,G., Merkenschlager,M., and Fisher,A.G. (2006). Chromatin signatures of pluripotent cell lines. Nat. Cell Biol. *8*, 532-538.

Bain,G., Kitchens,D., Yao,M., Huettner,J.E., and Gottlieb,D.I. (1995). Embryonic stem cells express neuronal properties in vitro. Dev. Biol. *168*, 342-357.

Bannister,A.J., and Kouzarides,T. (2011). Regulation of chromatin by histone modifications. Cell Res. *21*, 381-395.

Barber,R.D., Harmer,D.W., Coleman,R.A., and Clark,B.J. (2005). GAPDH as a housekeeping gene: analysis of GAPDH mRNA expression in a panel of 72 human tissues. Physiol Genomics. *21*, 389-395.

Barry,F.P., Murphy,J.M., English,K., and Mahon,B.P. (2005). Immunogenicity of adult mesenchymal stem cells: lessons from the fetal allograft. Stem Cells Dev. *14*, 252-265.

Baumann,M., Pontiller,J., and Ernst,W. (2010). Structure and basal transcription complex of RNA polymerase II core promoters in the mammalian genome: an overview. Mol. Biotechnol. *45*, 241-247.

Beisel,C., and Paro,R. (2011). Silencing chromatin: comparing modes and mechanisms. Nat. Rev. Genet. *12*, 123-135.

Berkes,C.A., and Tapscott,S.J. (2005). MyoD and the transcriptional control of myogenesis. Semin. Cell Dev. Biol. *16*, 585-595.

Bernstein,B.E., Meissner,A., and Lander,E.S. (2007). The mammalian epigenome. Cell *128*, 669-681.

Bernstein,B.E., Mikkelsen,T.S., Xie,X., Kamal,M., Huebert,D.J., Cuff,J., Fry,B., Meissner,A., Wernig,M., Plath,K., Jaenisch,R., Wagschal,A., Feil,R., Schreiber,S.L., and Lander,E.S. (2006). A bivalent chromatin structure marks key developmental genes in embryonic stem cells. Cell *125*, 315-326.

Bertani,N., Malatesta,P., Volpi,G., Sonego,P., and Perris,R. (2005). Neurogenic potential of human mesenchymal stem cells revisited: analysis by immunostaining, time-lapse video and microarray. J. Cell Sci. *118*, 3925-3936.

Bick,M.D., and Davidson,R.L. (1974). Total substitution of bromodeoxyuridine for thymidine in the DNA of a bromodeoxyuridine-dependent cell line. Proc. Natl. Acad. Sci. U. S. A. *71*, 2082-2086.

Biella,G., Di,F.F., Goffredo,D., Moiana,A., Taglietti,V., Conti,L., Cattaneo,E., and Toselli,M. (2007). Differentiating embryonic stem-derived neural stem cells show a maturation-dependent pattern of voltage-gated sodium current expression and graded action potentials. Neuroscience. *149*, 38-52.

Bird,A. (1997). Does DNA methylation control transposition of selfish elements in the germline? Trends Genet. *13*, 469-472.

Bird,A. (2002). DNA methylation patterns and epigenetic memory. Genes Dev. *16*, 6-21.

Blackledge,N.P., and Klose,R. (2011). CpG island chromatin: a platform for gene regulation. Epigenetics. *6*, 147-152.

Blackwell,T.K., and Weintraub,H. (1990). Differences and similarities in DNA-binding preferences of MyoD and E2A protein complexes revealed by binding site selection. Science. *250*, 1104-1110.

Boeuf,H., Hauss,C., Graeve,F.D., Baran,N., and Kedinger,C. (1997). Leukemia inhibitory factor-dependent transcriptional activation in embryonic stem cells. J. Cell Biol. *138*, 1207-1217.

Boquest,A.C., Noer,A., and Collas,P. (2006a). Epigenetic programming of mesenchymal stem cells from human adipose tissue. Stem Cell Rev. *2*, 319-329.

Boquest,A.C., Noer,A., Sorensen,A.L., Vekterud,K., and Collas,P. (2007). CpG methylation profiles of endothelial cell-specific gene promoter regions in adipose tissue stem cells suggest limited differentiation potential toward the endothelial cell lineage. Stem Cells *25*, 852-861.

Boquest,A.C., Shahdadfar,A., Brinchmann,J.E., and Collas,P. (2006b). Isolation of stromal stem cells from human adipose tissue. Methods Mol. Biol. *325*, 35-46.

Boquest,A.C., Shahdadfar,A., Fronsdal,K., Sigurjonsson,O., Tunheim,S.H., Collas,P., and Brinchmann,J.E. (2005). Isolation and transcription profiling of purified uncultured human stromal stem cells: alteration of gene expression after in vitro cell culture. Mol. Biol. Cell. *16*, 1131-1141.

Boyes,J., and Bird,A. (1991). DNA methylation inhibits transcription indirectly via a methyl-CpG binding protein. Cell. *64*, 1123-1134.

Brewer,G.J. (1995). Serum-free B27/neurobasal medium supports differentiated growth of neurons from the striatum, substantia nigra, septum, cerebral cortex, cerebellum, and dentate gyrus. J. Neurosci. Res. *42*, 674-683.

Buervenich,S., Olson,L., and Galter,D. (2001). Nestin-like immunoreactivity of corpora amylacea in aged human brain. Brain Res. Mol. Brain Res. *%19;94*, 204-208.

Bulger,M., and Groudine,M. (2011). Functional and mechanistic diversity of distal transcription enhancers. Cell. *144*, 327-339.

Burdon,T., Smith,A., and Savatier,P. (2002). Signalling, cell cycle and pluripotency in embryonic stem cells. Trends Cell Biol. *12*, 432-438.

Burdon,T., Stracey,C., Chambers,I., Nichols,J., and Smith,A. (1999). Suppression of SHP-2 and ERK signalling promotes self-renewal of mouse embryonic stem cells. Dev. Biol. *210*, 30-43.

Cai,J., Wu,Y., Mirua,T., Pierce,J.L., Lucero,M.T., Albertine,K.H., Spangrude,G.J., and Rao,M.S. (2002). Properties of a fetal multipotent neural stem cell (NEP cell). Dev. Biol. *251*, 221-240.

Campanero,M.R., Armstrong,M.I., and Flemington,E.K. (2000). CpG methylation as a mechanism for the regulation of E2F activity. Proc. Natl. Acad. Sci. U. S. A. *97*, 6481-6486.

Cardozo,A., Ielpi,M., Gomez,D., and Argibay,P. (2010). Differential expression of Shh and BMP signaling in the potential conversion of human adipose tissue stem cells into neuron-like cells in vitro. Gene Expr. *14*, 307-319.

Cedar,H., and Bergman,Y. (2009). Linking DNA methylation and histone modification: patterns and paradigms. Nat. Rev. Genet. *10*, 295-304.

Chamberlain,G., Fox,J., Ashton,B., and Middleton,J. (2007). Concise review: mesenchymal stem cells: their phenotype, differentiation capacity, immunological features, and potential for homing. Stem Cells. *25*, 2739-2749.

Chambers,I., Colby,D., Robertson,M., Nichols,J., Lee,S., Tweedie,S., and Smith,A. (2003). Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells. Cell. *113*, 643-655.

Chambeyron,S., and Bickmore,W.A. (2004). Chromatin decondensation and nuclear reorganization of the HoxB locus upon induction of transcription. Genes Dev. *18*, 1119-1130.

Chen,J., Li,Y., Wang,L., Zhang,Z., Lu,D., Lu,M., and Chopp,M. (2001). Therapeutic benefit of intravenous administration of bone marrow stromal cells after cerebral ischemia in rats. Stroke. *32*, 1005-1011.

Chen,Y., Teng,F.Y., and Tang,B.L. (2006). Coaxing bone marrow stromal mesenchymal stem cells towards neuronal differentiation: progress and uncertainties. Cell Mol. Life Sci. *63*, 1649-1657.

Cheng,L., Jin,Z., Liu,L., Yan,Y., Li,T., Zhu,X., and Jing,N. (2004). Characterization and promoter analysis of the mouse nestin gene. FEBS Lett. *565*, 195-202.

Chiang,C., Litingtung,Y., Lee,E., Young,K.E., Corden,J.L., Westphal,H., and Beachy,P.A. (1996). Cyclopia and defective axial patterning in mice lacking Sonic hedgehog gene function. Nature. *383*, 407-413.

Cho,K.J., Trzaska,K.A., Greco,S.J., McArdle,J., Wang,F.S., Ye,J.H., and Rameshwar,P. (2005). Neurons derived from human mesenchymal stem cells show synaptic transmission and can be induced to produce the neurotransmitter substance P by interleukin-1 alpha. Stem Cells. *23*, 383-391.

Choi,D.K., Pennathur,S., Perier,C., Tieu,K., Teismann,P., Wu,D.C., Jackson-Lewis,V., Vila,M., Vonsattel,J.P., Heinecke,J.W., and Przedborski,S. (2005). Ablation of the inflammatory enzyme myeloperoxidase mitigates features of Parkinson's disease in mice. J. Neurosci. *25*, 6594-6600.

Collas,P. (2009). Epigenetic states in stem cells. Biochim. Biophys. Acta *1790*, 900-905.

Collas,P. (2010). Programming differentiation potential in mesenchymal stem cells. Epigenetics *5*, 476-482.

Collas,P., Noer,A., and Timoskainen,S. (2007). Programming the genome in embryonic and somatic stem cells. J. Cell Mol. Med. *11*, 602-620.

Colledge,W.H. (2009). Kisspeptins and GnRH neuronal signalling. Trends Endocrinol. Metab. *20*, 115-121.

Comb,M., and Goodman,H.M. (1990). CpG methylation inhibits proenkephalin gene expression and binding of the transcription factor AP-2. Nucleic Acids Res. *18*, 3975-3982.

Copray,S., Huynh,J.L., Sher,F., Casaccia-Bonnefil,P., and Boddeke,E. (2009). Epigenetic mechanisms facilitating oligodendrocyte development, maturation, and aging. Glia. *57*, 1579-1587.

Cosgrove,M.S., and Wolberger,C. (2005). How does the histone code work? Biochem. Cell Biol. *83*, 468-476.

Dahl,J.A., and Collas,P. (2007). A quick and quantitative chromatin immunoprecipitation assay for small cell samples. Front. Biosci. *12*, 4925-4931.

Dahl,J.A., Duggal,S., Coulston,N., Millar,D.S., Melki,J., Shahdadfar,A., Brinchmann,J.E., and Collas,P. (2008). Genetic and epigenetic instability of human bone marrow mesenchymal stem cells expanded in autologous serum or fetal bovine serum. Int. J. Dev. Biol. *52*, 1033-1042.

Dahlstrand,J., Collins,V.P., and Lendahl,U. (1992a). Expression of the class VI intermediate filament nestin in human central nervous system tumors. Cancer Res. *52*, 5334-5341.

Dahlstrand,J., Lardelli,M., and Lendahl,U. (1995). Nestin mRNA expression correlates with the central nervous system progenitor cell state in many, but not all, regions of developing central nervous system. Brain Res. Dev. Brain Res. *84*, 109-129.

Dahlstrand,J., Zimmerman,L.B., McKay,R.D., and Lendahl,U. (1992b). Characterization of the human nestin gene reveals a close evolutionary relationship to neurofilaments. J. Cell Sci. *103*, 589-597.

De Ugarte,D.A., Alfonso,Z., Zuk,P.A., Elbarbary,A., Zhu,M., Ashjian,P., Benhaim,P., Hedrick,M.H., and Fraser,J.K. (2003). Differential expression of stem cell mobilization-associated molecules on multi-lineage cells from adipose tissue and bone marrow. Immunol. Lett. *89*, 267-270.

Delaval,K., and Feil,R. (2004). Epigenetic regulation of mammalian genomic imprinting. Curr. Opin. Genet. Dev. *14*, 188-195.

Deng,W., Obrocka,M., Fischer,I., and Prockop,D.J. (2001). In vitro differentiation of human marrow stromal cells into early progenitors of neural cells by conditions that increase intracellular cyclic AMP. Biochem. Biophys. Res. Commun. *282*, 148-152.

Denker,H.W. (2006). Potentiality of embryonic stem cells: an ethical problem even with alternative stem cell sources. J. Med. Ethics. *32*, 665-671.

Dhar,S., Yoon,E.S., Kachgal,S., and Evans,G.R. (2007). Long-term maintenance of neuronally differentiated human adipose tissue-derived stem cells. Tissue Eng. *13*, 2625-2632.

Dhe-Paganon,S., Syeda,F., and Park,L. (2011). DNA methyl transferase 1: regulatory mechanisms and implications in health and disease. Int. J. Biochem. Mol. Biol. *2*, 58-66.

Di,C.G., and Morganti,A. (2011). Chromogranin A: a novel factor acting at the cross road between the neuroendocrine and the cardiovascular systems. J. Hypertens. *29*, 409-414.

Diez del,C.R., and Storey,K.G. (2001). Markers in vertebrate neurogenesis. Nat. Rev. Neurosci. *2*, 835-839.

Dominici,M., Le,B.K., Mueller,I., Slaper-Cortenbach,I., Marini,F., Krause,D., Deans,R., Keating,A., Prockop,D., and Horwitz,E. (2006). Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy. *8*, 315-317.

Dubois,N.C., Hofmann,D., Kaloulis,K., Bishop,J.M., and Trumpp,A. (2006). Nestin-Cre transgenic mouse line Nes-Cre1 mediates highly efficient Cre/loxP mediated recombination in the nervous system, kidney, and somite-derived tissues. Genesis. *44*, 355-360.

Eberl,G., and Littman,D.R. (2003). The role of the nuclear hormone receptor RORgammat in the development of lymph nodes and Peyer's patches. Immunol. Rev. *195:81-90.*, 81-90.

Eliasson,C., Sahlgren,C., Berthold,C.H., Stakeberg,J., Celis,J.E., Betsholtz,C., Eriksson,J.E., and Pekny,M. (1999). Intermediate filament protein partnership in astrocytes. J. Biol. Chem. *%20;274*, 23996-24006.

Ericson,J., Morton,S., Kawakami,A., Roelink,H., and Jessell,T.M. (1996). Two critical periods of Sonic Hedgehog signaling required for the specification of motor neuron identity. Cell. *87*, 661-673.

Evans,M.J., and Kaufman,M.H. (1981). Establishment in culture of pluripotential cells from mouse embryos. Nature. *292*, 154-156.

Fan,Y., Nikitina,T., Morin-Kensicki,E.M., Zhao,J., Magnuson,T.R., Woodcock,C.L., and Skoultchi,A.I. (2003). H1 linker histones are essential for mouse development and affect nucleosome spacing in vivo. Mol. Cell Biol. *23*, 4559-4572.

Fathi,F., Altiraihi,T., Mowla,S.J., and Movahedin,M. (2009). Formation of embryoid bodies from mouse embryonic stem cells cultured on silicon-coated surfaces. Cytotechnology. *59*, 11-16.

Fernandez-Chacon,R., Konigstorfer,A., Gerber,S.H., Garcia,J., Matos,M.F., Stevens,C.F., Brose,N., Rizo,J., Rosenmund,C., and Sudhof,T.C. (2001). Synaptotagmin I functions as a calcium regulator of release probability. Nature. *410*, 41-49.

Fischle,W., Wang,Y., Jacobs,S.A., Kim,Y., Allis,C.D., and Khorasanizadeh,S. (2003). Molecular basis for the discrimination of repressive methyl-lysine marks in histone H3 by Polycomb and HP1 chromodomains. Genes Dev. *17*, 1870-1881.

Fletcher,D.A., and Mullins,R.D. (2010). Cell mechanics and the cytoskeleton. Nature. *463*, 485-492.

Fraichard,A., Chassande,O., Bilbaut,G., Dehay,C., Savatier,P., and Samarut,J. (1995). In vitro differentiation of embryonic stem cells into glial cells and functional neurons. J. Cell Sci. *108*, 3181-3188.

Fraser,J.K., Wulur,I., Alfonso,Z., and Hedrick,M.H. (2006). Fat tissue: an underappreciated source of stem cells for biotechnology. Trends Biotechnol. *24*, 150-154.

Friedenstein,A.J., Chailakhyan,R.K., Latsinik,N.V., Panasyuk,A.F., and Keiliss-Borok,I.V. (1974a). Stromal cells responsible for transferring the microenvironment of the hemopoietic tissues. Cloning in vitro and retransplantation in vivo. Transplantation. *17*, 331-340.

Friedenstein,A.J., Deriglasova,U.F., Kulagina,N.N., Panasuk,A.F., Rudakowa,S.F., Luria,E.A., and Ruadkow,I.A. (1974b). Precursors for fibroblasts in different populations of hematopoietic cells as detected by the in vitro colony assay method. Exp. Hematol. *2*, 83-92.

Frisen,J., Johansson,C.B., Torok,C., Risling,M., and Lendahl,U. (1995). Rapid, widespread, and longlasting induction of nestin contributes to the generation of glial scar tissue after CNS injury. J. Cell Biol. *131*, 453-464.

Fryxell,K.J., and Moon,W.J. (2005). CpG mutation rates in the human genome are highly dependent on local GC content. Mol. Biol. Evol. *22*, 650-658.

Fuchs,E., Tumbar,T., and Guasch,G. (2004). Socializing with the neighbors: stem cells and their niche. Cell *116*, 769-778.

Fuks,F., Burgers,W.A., Brehm,A., Hughes-Davies,L., and Kouzarides,T. (2000). DNA methyltransferase Dnmt1 associates with histone deacetylase activity. Nat. Genet. *24*, 88-91.

Fuks,F., Burgers,W.A., Godin,N., Kasai,M., and Kouzarides,T. (2001). Dnmt3a binds deacetylases and is recruited by a sequence-specific repressor to silence transcription. EMBO J. *20*, 2536-2544.

Fuks,F., Hurd,P.J., Deplus,R., and Kouzarides,T. (2003). The DNA methyltransferases associate with HP1 and the SUV39H1 histone methyltransferase. Nucleic Acids Res. *31*, 2305-2312.

Galiano,M.R., Andrieux,A., Deloulme,J.C., Bosc,C., Schweitzer,A., Job,D., and Hallak,M.E. (2006). Myelin basic protein functions as a microtubule stabilizing protein in differentiated oligodendrocytes. J. Neurosci. Res. *84*, 534-541.

Gao,Z., Ure,K., Ables,J.L., Lagace,D.C., Nave,K.A., Goebbels,S., Eisch,A.J., and Hsieh,J. (2009). Neurod1 is essential for the survival and maturation of adult-born neurons. Nat. Neurosci. *12*, 1090-1092.

Gardiner-Garden,M., and Frommer,M. (1987). CpG islands in vertebrate genomes. J. Mol. Biol. *196*, 261-282.

Gardzinski,P., Lee,D.W., Fei,G.H., Hui,K., Huang,G.J., Sun,H.S., and Feng,Z.P. (2007). The role of synaptotagmin I C2A calcium-binding domain in synaptic vesicle clustering during synapse formation. J. Physiol. *581*, 75-90.

Geiman,T.M., Sankpal,U.T., Robertson,A.K., Zhao,Y., Zhao,Y., and Robertson,K.D. (2004). DNMT3B interacts with hSNF2H chromatin remodeling enzyme, HDACs 1 and 2, and components of the histone methylation system. Biochem. Biophys. Res. Commun. *318*, 544-555.

Gimble,J.M., Katz,A.J., and Bunnell,B.A. (2007). Adipose-derived stem cells for regenerative medicine. Circ. Res. *100*, 1249-1260.

Goll,M.G., and Bestor,T.H. (2005). Eukaryotic cytosine methyltransferases. Annu. Rev. Biochem. *74:481-514.*, 481-514.

Goll,M.G., Kirpekar,F., Maggert,K.A., Yoder,J.A., Hsieh,C.L., Zhang,X., Golic,K.G., Jacobsen,S.E., and Bestor,T.H. (2006). Methylation of tRNAAsp by the DNA methyltransferase homolog Dnmt2. Science. *%20;311*, 395-398.

Gratzner,H.G. (1982). Monoclonal antibody to 5-bromo- and 5-iododeoxyuridine: A new reagent for detection of DNA replication. Science. *218*, 474-475.

Gronthos,S., Graves,S.E., Ohta,S., and Simmons,P.J. (1994). The STRO-1+ fraction of adult human bone marrow contains the osteogenic precursors. Blood. *84*, 4164-4173.

Guan,K., Chang,H., Rolletschek,A., and Wobus,A.M. (2001). Embryonic stem cell-derived neurogenesis. Retinoic acid induction and lineage selection of neuronal cells. Cell Tissue Res. *305*, 171-176.

Halvorsen,Y.D., Franklin,D., Bond,A.L., Hitt,D.C., Auchter,C., Boskey,A.L., Paschalis,E.P., Wilkison,W.O., and Gimble,J.M. (2001). Extracellular matrix mineralization and osteoblast gene expression by human adipose tissue-derived stromal cells. Tissue Eng *7*, 729-741.

Han,D.W., Do,J.T., Arauzo-Bravo,M.J., Lee,S.H., Meissner,A., Lee,H.T., Jaenisch,R., and Scholer,H.R. (2009). Epigenetic hierarchy governing Nestin expression. Stem Cells. *27*, 1088-1097.

Hapgood,J.P., Sadie,H., van,B.W., and Ronacher,K. (2005). Regulation of expression of mammalian gonadotrophin-releasing hormone receptor genes. J. Neuroendocrinol. *17*, 619-638.

He,X., Treacy,M.N., Simmons,D.M., Ingraham,H.A., Swanson,L.W., and Rosenfeld,M.G. (1989). Expression of a large family of POU-domain regulatory genes in mammalian brain development. Nature. *340*, 35-41.

Heintzman,N.D., Hon,G.C., Hawkins,R.D., Kheradpour,P., Stark,A., Harp,L.F., Ye,Z., Lee,L.K., Stuart,R.K., Ching,C.W., Ching,K.A., ntosiewicz-Bourget,J.E., Liu,H., Zhang,X., Green,R.D., Lobanenkov,V.V., Stewart,R., Thomson,J.A., Crawford,G.E., Kellis,M., and Ren,B. (2009). Histone modifications at human enhancers reflect global cell-type-specific gene expression. Nature. *459*, 108-112.

Henikoff,S., Furuyama,T., and Ahmad,K. (2004). Histone variants, nucleosome assembly and epigenetic inheritance. Trends Genet. *20*, 320-326.

Herrmann,H., Bar,H., Kreplak,L., Strelkov,S.V., and Aebi,U. (2007). Intermediate filaments: from cell architecture to nanomechanics. Nat. Rev. Mol. Cell Biol. *8*, 562-573.

Hirabayashi,Y., and Gotoh,Y. (2010). Epigenetic control of neural precursor cell fate during development. Nat. Rev. Neurosci. *11*, 377-388.

Hockfield,S., and McKay,R.D. (1985). Identification of major cell classes in the developing mammalian nervous system. J. Neurosci. *5*, 3310-3328.

Hodawadekar,S.C., and Marmorstein,R. (2007). Chemistry of acetyl transfer by histone modifying enzymes: structure, mechanism and implications for effector design. Oncogene. *26*, 5528-5540.

Hore,T.A., Rapkins,R.W., and Graves,J.A. (2007). Construction and evolution of imprinted loci in mammals. Trends Genet. *23*, 440-448.

Hsieh,C.L. (1997). Stability of patch methylation and its impact in regions of transcriptional initiation and elongation. Mol. Cell Biol. *17*, 5897-5904.

Huang,H., Maertens,A.M., Hyland,E.M., Dai,J., Norris,A., Boeke,J.D., and Bader,J.S. (2009). HistoneHits: a database for histone mutations and their phenotypes. Genome Res. *19*, 674-681.

In 't Anker,P.S., Scherjon,S.A., Kleijburg-van der Keur,C., Noort,W.A., Claas,F.H., Willemze,R., Fibbe,W.E., and Kanhai,H.H. (2003). Amniotic fluid as a novel source of mesenchymal stem cells for therapeutic transplantation. Blood. *102*, 1548-1549.

Ishiwata,T., Matsuda,Y., and Naito,Z. (2011). Nestin in gastrointestinal and other cancers: effects on cells and tumor angiogenesis. World J. Gastroenterol. *17*, 409-418.

Issa,J.P. (2000). CpG-island methylation in aging and cancer. Curr. Top. Microbiol. Immunol. *249:101-18.*, 101-118.

Jackson,P., Hougaard,K.S., Vogel,U., Wu,D., Casavant,L., Williams,A., Wade,M., Yauk,C.L., Wallin,H., and Halappanavar,S. (2011). Exposure of pregnant mice to carbon black by intratracheal instillation: Toxicogenomic effects in dams and offspring. Mutat. Res.

Jaenisch,R., and Bird,A. (2003). Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. Nat. Genet. *33 Suppl*, 245-254.

Jaiswal,R.K., Jaiswal,N., Bruder,S.P., Mbalaviele,G., Marshak,D.R., and Pittenger,M.F. (2000). Adult human mesenchymal stem cell differentiation to the osteogenic or adipogenic lineage is regulated by mitogen-activated protein kinase. J. Biol. Chem. *275*, 9645-9652.

Jang,S., Cho,H.H., Cho,Y.B., Park,J.S., and Jeong,H.S. (2010). Functional neural differentiation of human adipose tissue-derived stem cells using bFGF and forskolin. BMC. Cell Biol. *11:25.*, 25.

Jin,Z., Liu,L., Bian,W., Chen,Y., Xu,G., Cheng,L., and Jing,N. (2009). Different transcription factors regulate nestin gene expression during P19 cell neural differentiation and central nervous system development. J. Biol. Chem. *%20;284*, 8160-8173.

Jin,Z.G., Liu,L., Zhong,H., Zhang,K.J., Chen,Y.F., Bian,W., Cheng,L.P., and Jing,N.H. (2006). Second intron of mouse nestin gene directs its expression in pluripotent embryonic carcinoma cells through POU factor binding site. Acta Biochim. Biophys. Sin. (Shanghai). *38*, 207-212.

Johnstone,B., Hering,T.M., Caplan,A.I., Goldberg,V.M., and Yoo,J.U. (1998). In vitro chondrogenesis of bone marrow-derived mesenchymal progenitor cells. Exp. Cell Res. *238*, 265-272.

Jones,D.L., and Wagers,A.J. (2008). No place like home: anatomy and function of the stem cell niche. Nat. Rev. Mol. Cell Biol. *9*, 11-21.

Jones,P.A., and Baylin,S.B. (2002). The fundamental role of epigenetic events in cancer. Nat. Rev. Genet. *3*, 415-428.

Josephson,R., Muller,T., Pickel,J., Okabe,S., Reynolds,K., Turner,P.A., Zimmer,A., and McKay,R.D. (1998). POU transcription factors control expression of CNS stem cell-specific genes. Development. *125*, 3087-3100.

Kappen,C., and Yaworsky,P.J. (2003). Mutation of a putative nuclear receptor binding site abolishes activity of the nestin midbrain enhancer. Biochim. Biophys. Acta. *1625*, 109-115.

Kass,S., Landsberger,N., and Wolffe,A. (1997). DNA methylation directs a time-dependent repression of transcription initiation. Curr. Biol. *7*, 157-165.

Kawasaki,H., Mizuseki,K., Nishikawa,S., Kaneko,S., Kuwana,Y., Nakanishi,S., Nishikawa,S.I., and Sasai,Y. (2000). Induction of midbrain dopaminergic neurons from ES cells by stromal cell-derived inducing activity. Neuron. *28*, 31-40.

Keating,A. (2006). Mesenchymal stromal cells. Curr. Opin. Hematol. *13*, 419-425.

Kee,N., Sivalingam,S., Boonstra,R., and Wojtowicz,J.M. (2002). The utility of Ki-67 and BrdU as proliferative markers of adult neurogenesis. J. Neurosci. Methods. *115*, 97-105.

Kern,S., Eichler,H., Stoeve,J., Kluter,H., and Bieback,K. (2006). Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood or adipose tissue. Stem Cells *24*, 1294-1301.

Khorasanizadeh,S. (2004). The nucleosome: from genomic organization to genomic regulation. Cell. *116*, 259-272.

Kim,S., and Coulombe,P.A. (2007). Intermediate filament scaffolds fulfill mechanical, organizational, and signaling functions in the cytoplasm. Genes Dev. *21*, 1581-1597.

Kim,S.S., Choi,J.M., Kim,J.W., Ham,D.S., Ghil,S.H., Kim,M.K., Kim-Kwon,Y., Hong,S.Y., Ahn,S.C., Kim,S.U., Lee,Y.D., and Suh-Kim,H. (2005). cAMP induces neuronal differentiation of mesenchymal stem cells via activation of extracellular signal-regulated kinase/MAPK. Neuroreport. *16*, 1357-1361.

Kintner,C. (2002). Neurogenesis in embryos and in adult neural stem cells. J. Neurosci. *22*, 639-643.

Kleeberger,W., Bova,G.S., Nielsen,M.E., Herawi,M., Chuang,A.Y., Epstein,J.I., and Berman,D.M. (2007). Roles for the stem cell associated intermediate filament Nestin in prostate cancer migration and metastasis. Cancer Res. *67*, 9199-9206.

Klein,T., Ling,Z., Heimberg,H., Madsen,O.D., Heller,R.S., and Serup,P. (2003). Nestin is expressed in vascular endothelial cells in the adult human pancreas. J. Histochem. Cytochem. *51*, 697-706.

Klose,R.J., and Bird,A.P. (2006). Genomic DNA methylation: the mark and its mediators. Trends Biochem. Sci. *31*, 89-97.

Kneussel,M., and Betz,H. (2000). Receptors, gephyrin and gephyrin-associated proteins: novel insights into the assembly of inhibitory postsynaptic membrane specializations. J. Physiol. *525 Pt 1:1-9.*, 1-9.

Koch,H., Hofmann,K., and Brose,N. (2000). Definition of Munc13-homology-domains and characterization of a novel ubiquitously expressed Munc13 isoform. Biochem. J. *349*, 247-253.

Kompisch,K.M., Lange,C., Steinemann,D., Skawran,B., Schlegelberger,B., Muller,R., and Schumacher,U. (2010). Neurogenic transdifferentiation of human adipose-derived stem cells? A critical protocol reevaluation with special emphasis on cell proliferation and cell cycle alterations. Histochem. Cell Biol. *134*, 453-468.

Kouzarides,T. (2007). Chromatin modifications and their function. Cell *128*, 693-705.

Krabbe,C., Zimmer,J., and Meyer,M. (2005). Neural transdifferentiation of mesenchymal stem cells--a critical review. APMIS. *113*, 831-844.

Kudo,S. (1998). Methyl-CpG-binding protein MeCP2 represses Sp1-activated transcription of the human leukosialin gene when the promoter is methylated. Mol. Cell Biol. *18*, 5492-5499.

Laird,P.W. (2005). Cancer epigenetics. Hum. Mol. Genet. *14 Spec No 1:R65-76.*, R65-R76.

Lande-Diner,L., and Cedar,H. (2005). Silence of the genes--mechanisms of long-term repression. Nat. Rev. Genet. *6*, 648-654.

Lande-Diner,L., Zhang,J., Ben-Porath,I., Amariglio,N., Keshet,I., Hecht,M., Azuara,V., Fisher,A.G., Rechavi,G., and Cedar,H. (2007). Role of DNA methylation in stable gene repression. J. Biol. Chem. *%20;282*, 12194-12200.

Lardon,J., Rooman,I., and Bouwens,L. (2002). Nestin expression in pancreatic stellate cells and angiogenic endothelial cells. Histochem. Cell Biol. *117*, 535-540.

Lassar,A.B., Buskin,J.N., Lockshon,D., Davis,R.L., Apone,S., Hauschka,S.D., and Weintraub,H. (1989). MyoD is a sequence-specific DNA binding protein requiring a region of myc homology to bind to the muscle creatine kinase enhancer. Cell. *58*, 823-831.

Lee,J., Gravel,M., Zhang,R., Thibault,P., and Braun,P.E. (2005a). Process outgrowth in oligodendrocytes is mediated by CNP, a novel microtubule assembly myelin protein. J. Cell Biol. *170*, 661-673.

Lee,M.K., and Cleveland,D.W. (1996). Neuronal intermediate filaments. Annu. Rev. Neurosci. *19:187-217.*, 187-217.

Lee,O.K., Kuo,T.K., Chen,W.M., Lee,K.D., Hsieh,S.L., and Chen,T.H. (2004). Isolation of multipotent mesenchymal stem cells from umbilical cord blood. Blood. *103*, 1669-1675.

Lee,S.K., Lee,B., Ruiz,E.C., and Pfaff,S.L. (2005b). Olig2 and Ngn2 function in opposition to modulate gene expression in motor neuron progenitor cells. Genes Dev. *19*, 282-294.

Lehmann-Horn,F., and Jurkat-Rott,K. (1999). Voltage-gated ion channels and hereditary disease. Physiol Rev. *79*, 1317-1372.

Lendahl,U., Zimmerman,L.B., and McKay,R.D. (1990). CNS stem cells express a new class of intermediate filament protein. Cell. *60*, 585-595.

Lenhard,B., Sandelin,A., and Carninci,P. (2012). Metazoan promoters: emerging characteristics and insights into transcriptional regulation. Nat. Rev. Genet. *13*, 233-245.

Li,B., Carey,M., and Workman,J.L. (2007). The role of chromatin during transcription. Cell. *128*, 707-719.

Li,E. (2002). Chromatin modification and epigenetic reprogramming in mammalian development. Nat. Rev. Genet. *3*, 662-673.

Li,Y., Chen,J., Wang,L., Zhang,L., Lu,M., and Chopp,M. (2001). Intracerebral transplantation of bone marrow stromal cells in a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine mouse model of Parkinson's disease. Neurosci. Lett. *316*, 67-70.

Liem,R.K. (1993). Molecular biology of neuronal intermediate filaments. Curr. Opin. Cell Biol. *5*, 12-16.

Lindroos,B., Suuronen,R., and Miettinen,S. (2011). The potential of adipose stem cells in regenerative medicine. Stem Cell Rev. *7*, 269-291.

Lister,R., Pelizzola,M., Dowen,R.H., Hawkins,R.D., Hon,G., Tonti-Filippini,J., Nery,J.R., Lee,L., Ye,Z., Ngo,Q.M., Edsall,L., ntosiewicz-Bourget,J., Stewart,R., Ruotti,V., Millar,A.H., Thomson,J.A., Ren,B., and Ecker,J.R. (2009). Human DNA methylomes at base resolution show widespread epigenomic differences. Nature *462*, 315-322.

Litosch,I., Saito,Y., and Fain,J.N. (1982). Forskolin as an activator of cyclic AMP accumulation and secretion in blowfly salivary glands. Biochem. J. *204*, 147-151.

Liu,C., Chen,B., Zhu,J., Zhang,R., Yao,F., Jin,F., Xu,H., and Lu,P. (2010). Clinical implications for nestin protein expression in breast cancer. Cancer Sci. *101*, 815-819.

Lothian,C., and Lendahl,U. (1997). An evolutionarily conserved region in the second intron of the human nestin gene directs gene expression to CNS progenitor cells and to early neural crest cells. Eur. J. Neurosci. *9*, 452-462.

Lothian,C., Prakash,N., Lendahl,U., and Wahlstrom,G.M. (1999). Identification of both general and region-specific embryonic CNS enhancer elements in the nestin promoter. Exp. Cell Res. *248*, 509-519.

Lowell,S., Benchoua,A., Heavey,B., and Smith,A.G. (2006). Notch promotes neural lineage entry by pluripotent embryonic stem cells. PLoS. Biol. *4*, e121.

Lu,D., Li,Y., Wang,L., Chen,J., Mahmood,A., and Chopp,M. (2001). Intraarterial administration of marrow stromal cells in a rat model of traumatic brain injury. J. Neurotrauma. *18*, 813-819.

Lu,P., Blesch,A., and Tuszynski,M.H. (2004). Induction of bone marrow stromal cells to neurons: differentiation, transdifferentiation, or artifact? J. Neurosci. Res. *77*, 174-191.

Margueron,R., Trojer,P., and Reinberg,D. (2005). The key to development: interpreting the histone code? Curr. Opin. Genet. Dev. *15*, 163-176.

Martinez-Agosto,J.A., Mikkola,H.K., Hartenstein,V., and Banerjee,U. (2007). The hematopoietic stem cell and its niche: a comparative view. Genes Dev. *21*, 3044-3060.

Matoba,R., Niwa,H., Masui,S., Ohtsuka,S., Carter,M.G., Sharov,A.A., and Ko,M.S. (2006). Dissecting Oct3/4-regulated gene networks in embryonic stem cells by expression profiling. PLoS. ONE. *%20;1:e26.*, e26.

Matsuda,Y., Naito,Z., Kawahara,K., Nakazawa,N., Korc,M., and Ishiwata,T. (2011). Nestin is a novel target for suppressing pancreatic cancer cell migration, invasion and metastasis. Cancer Biol. Ther. *11*, 512-523.

Mayer,E.J., Hughes,E.H., Carter,D.A., and Dick,A.D. (2003). Nestin positive cells in adult human retina and in epiretinal membranes. Br. J. Ophthalmol. *87*, 1154-1158.

Meehan,R.R. (2003). DNA methylation in animal development. Semin. Cell Dev. Biol. *14*, 53-65.

Meehan,R.R., Lewis,J.D., McKay,S., Kleiner,E.L., and Bird,A.P. (1989). Identification of a mammalian protein that binds specifically to DNA containing methylated CpGs. Cell. *58*, 499-507.

Meissner,A., Mikkelsen,T.S., Gu,H., Wernig,M., Hanna,J., Sivachenko,A., Zhang,X., Bernstein,B.E., Nusbaum,C., Jaffe,D.B., Gnirke,A., Jaenisch,R., and Lander,E.S. (2008). Genome-scale DNA methylation maps of pluripotent and differentiated cells. Nature. *454*, 766-770.

Meza-Zepeda,L.A., Noer,A., Dahl,J.A., Micci,F., Myklebost,O., and Collas,P. (2008). High-resolution analysis of genetic stability of human adipose tissue stem cells cultured to senescence. J. Cell. Mol. Med. *12*, 553-563.

Michalczyk,K., and Ziman,M. (2005). Nestin structure and predicted function in cellular cytoskeletal organisation. Histol. Histopathol. *20*, 665-671.

Mikkelsen,T.S., Hanna,J., Zhang,X., Ku,M., Wernig,M., Schorderet,P., Bernstein,B.E., Jaenisch,R., Lander,E.S., and Meissner,A. (2008). Dissecting direct reprogramming through integrative genomic analysis. Nature. *454*, 49-55.

Mikkelsen,T.S., Ku,M., Jaffe,D.B., Issac,B., Lieberman,E., Giannoukos,G., Alvarez,P., Brockman,W., Kim,T.K., Koche,R.P., Lee,W., Mendenhall,E., O'Donovan,A., Presser,A., Russ,C., Xie,X., Meissner,A., Wernig,M., Jaenisch,R., Nusbaum,C., Lander,E.S., and Bernstein,B.E. (2007). Genome-wide maps of chromatin state in pluripotent and lineage-committed cells. Nature *448*, 553-560.

Miranda,T.B., and Jones,P.A. (2007). DNA methylation: the nuts and bolts of repression. J. Cell Physiol. *213*, 384-390.

Mirsaidi,A., Kleinhans,K.N., Rimann,M., Tiaden,A.N., Stauber,M., Rudolph,K.L., and Richards,P.J. (2011). Telomere length, telomerase activity and osteogenic differentiation are maintained in adipose-derived stromal cells from senile osteoporotic SAMP6 mice. J. Tissue Eng Regen. Med. 10.

Mito,Y., Henikoff,J.G., and Henikoff,S. (2005). Genome-scale profiling of histone H3.3 replacement patterns. Nat. Genet. *37*, 1090-1097.

Mito,Y., Henikoff,J.G., and Henikoff,S. (2007). Histone replacement marks the boundaries of cis-regulatory domains. Science *315*, 1408-1411.

Mitsui,K., Tokuzawa,Y., Itoh,H., Segawa,K., Murakami,M., Takahashi,K., Maruyama,M., Maeda,M., and Yamanaka,S. (2003). The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. Cell. *113*, 631-642.

Mizuno,Y., Takeuchi,T., Takatama,M., and Okamoto,K. (2003). Expression of nestin in Purkinje cells in patients with Creutzfeldt-Jakob disease. Neurosci. Lett. *352*, 109-112.

Mizuseki,K., Kishi,M., Matsui,M., Nakanishi,S., and Sasai,Y. (1998). Xenopus Zic-related-1 and Sox-2, two factors induced by chordin, have distinct activities in the initiation of neural induction. Development. *125*, 579-587.

Mohn,F., and Schubeler,D. (2009). Genetics and epigenetics: stability and plasticity during cellular differentiation. Trends Genet. *25*, 129-136.

Mohn,F., Weber,M., Rebhan,M., Roloff,T.C., Richter,J., Stadler,M.B., Bibel,M., and Schübeler,D. (2008). Lineage-specific polycomb targets and de novo DNA methylation define restriction and potential of neuronal progenitors. Mol. Cell *30*, 755-766.

Montzka,K., Lassonczyk,N., Tschoke,B., Neuss,S., Fuhrmann,T., Franzen,R., Smeets,R., Brook,G.A., and Woltje,M. (2009). Neural differentiation potential of human bone marrow-derived mesenchymal stromal cells: misleading marker gene expression. BMC. Neurosci. *10:16.*, 16.

Moreels,M., Vandenabeele,F., Dumont,D., Robben,J., and Lambrichts,I. (2008). Alpha-smooth muscle actin (alpha-SMA) and nestin expression in reactive astrocytes in multiple sclerosis lesions: potential regulatory role of transforming growth factor-beta 1 (TGF-beta1). Neuropathol. Appl. Neurobiol. *34*, 532-546.

Mullen,R.J., Buck,C.R., and Smith,A.M. (1992). NeuN, a neuronal specific nuclear protein in vertebrates. Development. *116*, 201-211.

Neuhuber,B., Gallo,G., Howard,L., Kostura,L., Mackay,A., and Fischer,I. (2004). Reevaluation of in vitro differentiation protocols for bone marrow stromal cells: disruption of actin cytoskeleton induces rapid morphological changes and mimics neuronal phenotype. J. Neurosci. Res. *77*, 192-204.

Niki,T., Pekny,M., Hellemans,K., Bleser,P.D., Berg,K.V., Vaeyens,F., Quartier,E., Schuit,F., and Geerts,A. (1999). Class VI intermediate filament protein nestin is induced during activation of rat hepatic stellate cells. Hepatology. *29*, 520-527.

Niwa,H., Miyazaki,J., and Smith,A.G. (2000). Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. Nat. Genet. *24*, 372-376.

Noer,A., Lindeman,L.C., and Collas,P. (2009). Histone H3 modifications associated with differentiation and long-term culture of mesenchymal adipose stem cells. Stem Cells Dev. *18*, 725-735.

Noer,A., Sørensen,A.L., Boquest,A.C., and Collas,P. (2006). Stable CpG hypomethylation of adipogenic promoters in freshly isolated, cultured and differentiated mesenchymal stem cells from adipose tissue. Mol. Biol. Cell *17*, 3543-3556.

Okabe,S., Forsberg-Nilsson,K., Spiro,A.C., Segal,M., and McKay,R.D. (1996). Development of neuronal precursor cells and functional postmitotic neurons from embryonic stem cells in vitro. Mech. Dev. *59*, 89-102.

Ong,C.T., and Corces,V.G. (2011). Enhancer function: new insights into the regulation of tissue-specific gene expression. Nat. Rev. Genet. *12*, 283-293.

Pallari,H.M., Lindqvist,J., Torvaldson,E., Ferraris,S.E., He,T., Sahlgren,C., and Eriksson,J.E. (2011). Nestin as a regulator of Cdk5 in differentiating myoblasts. Mol. Biol. Cell. *22*, 1539-1549.

Pechstein,A., and Shupliakov,O. (2010). Taking a back seat: synaptic vesicle clustering in presynaptic terminals. Front Synaptic. Neurosci. *2:143.*, 143.

Perry,P., Sauer,S., Billon,N., Richardson,W.D., Spivakov,M., Warnes,G., Livesey,F.J., Merkenschlager,M., Fisher,A.G., and Azuara,V. (2004). A dynamic switch in the replication timing of key regulator genes in embryonic stem cells upon neural induction. Cell Cycle. *3*, 1645-1650.

Pevny,L.H., Sockanathan,S., Placzek,M., and Lovell-Badge,R. (1998). A role for SOX1 in neural determination. Development. *125*, 1967-1978.

Pierelli,L., Bonanno,G., Rutella,S., Marone,M., Scambia,G., and Leone,G. (2001). CD105 (endoglin) expression on hematopoietic stem/progenitor cells. Leuk. Lymphoma. *42*, 1195-1206.

Pittenger,M.F., Mackay,A.M., Beck,S.C., Jaiswal,R.K., Douglas,R., Mosca,J.D., Moorman,M.A., Simonetti,D.W., Craig,S., and Marshak,D.R. (1999). Multilineage potential of adult human mesenchymal stem cells. Science. *284*, 143-147.

Prendergast,G.C., Lawe,D., and Ziff,E.B. (1991). Association of Myn, the murine homolog of max, with c-Myc stimulates methylation-sensitive DNA binding and ras cotransformation. Cell. *65*, 395-407.

Prokhortchouk,A., Hendrich,B., Jorgensen,H., Ruzov,A., Wilm,M., Georgiev,G., Bird,A., and Prokhortchouk,E. (2001). The p120 catenin partner Kaiso is a DNA methylation-dependent transcriptional repressor. Genes Dev. *15*, 1613-1618.

Pugliese,M., Rodriguez,M.J., Gimeno-Bayon,J., Pujadas,L., Billett,E.E., Wells,C., and Mahy,N. (2010). Alzheimer's disease modifies progenitor cell expression of monoamine oxidase B in the subventricular zone. J. Neurosci. Res. *88*, 2588-2597.

Qiu,J. (2006). Epigenetics: unfinished symphony. Nature. *441*, 143-145.

Rai,K., Chidester,S., Zavala,C.V., Manos,E.J., James,S.R., Karpf,A.R., Jones,D.A., and Cairns,B.R. (2007). Dnmt2 functions in the cytoplasm to promote liver, brain, and retina development in zebrafish. Genes Dev. *21*, 261-266.

Reik,W. (2007). Stability and flexibility of epigenetic gene regulation in mammalian development. Nature. *447*, 425-432.

Robertson,K.D. (2002). DNA methylation and chromatin - unraveling the tangled web. Oncogene. *21*, 5361-5379.

Roh,T.Y., Cuddapah,S., Cui,K., and Zhao,K. (2006). The genomic landscape of histone modifications in human T cells. Proc. Natl. Acad. Sci U. S. A *103*, 15782-15787.

Rohwedel,J., Guan,K., and Wobus,A.M. (1999). Induction of cellular differentiation by retinoic acid in vitro. Cells Tissues. Organs. *165*, 190-202.

Romaniello,R., Zucca,C., Tonelli,A., Bonato,S., Baschirotto,C., Zanotta,N., Epifanio,R., Righini,A., Bresolin,N., Bassi,M.T., and Borgatti,R. (2010). A wide spectrum of clinical, neurophysiological and neuroradiological abnormalities in a family with a novel CACNA1A mutation. J. Neurol. Neurosurg. Psychiatry. *81*, 840-843.

Rooney,G.E., Howard,L., O'Brien,T., Windebank,A.J., and Barry,F.P. (2009). Elevation of cAMP in mesenchymal stem cells transiently upregulates neural markers rather than inducing neural differentiation. Stem Cells Dev. *18*, 387-398.

Ryan,J.M., Barry,F.P., Murphy,J.M., and Mahon,B.P. (2005). Mesenchymal stem cells avoid allogeneic rejection. J. Inflamm. (Lond). *2:8.*, 8.

Safford,K.M., Hicok,K.C., Safford,S.D., Halvorsen,Y.D., Wilkison,W.O., Gimble,J.M., and Rice,H.E. (2002). Neurogenic differentiation of murine and human adipose-derived stromal cells. Biochem. Biophys. Res. Commun. *294*, 371-379.

Sahlgren,C.M., Mikhailov,A., Vaittinen,S., Pallari,H.M., Kalimo,H., Pant,H.C., and Eriksson,J.E. (2003). Cdk5 regulates the organization of Nestin and its association with p35. Mol. Cell Biol. *23*, 5090-5106.

Sahlgren,C.M., Pallari,H.M., He,T., Chou,Y.H., Goldman,R.D., and Eriksson,J.E. (2006). A nestin scaffold links Cdk5/p35 signaling to oxidant-induced cell death. EMBO J. *25*, 4808-4819.

Salic,A., and Mitchison,T.J. (2008). A chemical method for fast and sensitive detection of DNA synthesis in vivo. Proc. Natl. Acad. Sci. U. S. A. *%19;105*, 2415-2420.

Sanchez,C., Diaz-Nido,J., and Avila,J. (2000). Phosphorylation of microtubule-associated protein 2 (MAP2) and its relevance for the regulation of the neuronal cytoskeleton function. Prog. Neurobiol. *61*, 133-168.

Sanchez-Ramos,J., Song,S., Cardozo-Pelaez,F., Hazzi,C., Stedeford,T., Willing,A., Freeman,T.B., Saporta,S., Janssen,W., Patel,N., Cooper,D.R., and Sanberg,P.R. (2000). Adult bone marrow stromal cells differentiate into neural cells in vitro. Exp. Neurol. *164*, 247-256.

Sasaki,T., Ishiguro,K., and Hisanaga,S. (2009). Novel axonal distribution of neurofilament-H phosphorylated at the glycogen synthase kinase 3beta-phosphorylation site in its E-segment. J. Neurosci. Res. *87*, 3088-3097.

Schaefer,M., Pollex,T., Hanna,K., Tuorto,F., Meusburger,M., Helm,M., and Lyko,F. (2010). RNA methylation by Dnmt2 protects transfer RNAs against stress-induced cleavage. Genes Dev. *24*, 1590-1595.

Scharf,A.N., and Imhof,A. (2011). Every methyl counts--epigenetic calculus. FEBS Lett. *585*, 2001-2007.

Schmidl,C., Klug,M., Boeld,T.J., Andreesen,R., Hoffmann,P., Edinger,M., and Rehli,M. (2009). Lineage-specific DNA methylation in T cells correlates with histone methylation and enhancer activity. Genome Res. *19*, 1165-1174.

Seo,S., Lim,J.W., Yellajoshyula,D., Chang,L.W., and Kroll,K.L. (2007). Neurogenin and NeuroD direct transcriptional targets and their regulatory enhancers. EMBO J. *26*, 5093-5108.

Serafini,M., and Verfaillie,C.M. (2006). Pluripotency in adult stem cells: state of the art. Semin. Reprod. Med. *24*, 379-388.

Shen,L., Kondo,Y., Guo,Y., Zhang,J., Zhang,L., Ahmed,S., Shu,J., Chen,X., Waterland,R.A., and Issa,J.P. (2007). Genome-wide profiling of DNA methylation reveals a class of normally methylated CpG island promoters. PLoS. Genet. *3*, 2023-2036.

Shi,Y., Lan,F., Matson,C., Mulligan,P., Whetstine,J.R., Cole,P.A., Casero,R.A., and Shi,Y. (2004). Histone demethylation mediated by the nuclear amine oxidase homolog LSD1. Cell *119*, 941-953.

Shi,Y., and Whetstine,J.R. (2007). Dynamic regulation of histone lysine methylation by demethylases. Mol. Cell *25*, 1-14.

Shogren-Knaak,M., Ishii,H., Sun,J.M., Pazin,M.J., Davie,J.R., and Peterson,C.L. (2006). Histone H4-K16 acetylation controls chromatin structure and protein interactions. Science. *311*, 844-847.

Siegfried,Z., Eden,S., Mendelsohn,M., Feng,X., Tsuberi,B.Z., and Cedar,H. (1999). DNA methylation represses transcription in vivo. Nat. Genet. *22*, 203-206.

Singh,H. (2009). Teeing up transcription on CpG islands. Cell. *138*, 14-16.

Sørensen,A.L., Timoskainen,S., West,F.D., Vekterud,K., Boquest,A.C., Ährlund-Richter,L., Stice,S.L., and Collas,P. (2009). Lineage-specific promoter DNA methylation patterns segregate adult progenitor cell types. Stem Cells Dev. *19*, 1257-1266.

Steinert,P.M., and Liem,R.K. (1990). Intermediate filament dynamics. Cell. *60*, 521-523.

Sterner,D.E., and Berger,S.L. (2000). Acetylation of histones and transcription-related factors. Microbiol. Mol. Biol. Rev. *64*, 435-459.

Streit,A., Sockanathan,S., Perez,L., Rex,M., Scotting,P.J., Sharpe,P.T., Lovell-Badge,R., and Stern,C.D. (1997). Preventing the loss of competence for neural induction: HGF/SF, L5 and Sox-2. Development. *124*, 1191-1202.

Strem,B.M., Hicok,K.C., Zhu,M., Wulur,I., Alfonso,Z., Schreiber,R.E., Fraser,J.K., and Hedrick,M.H. (2005). Multipotential differentiation of adipose tissue-derived stem cells. Keio J. Med. *54*, 132-141.

Strubing,C., Ahnert-Hilger,G., Shan,J., Wiedenmann,B., Hescheler,J., and Wobus,A.M. (1995). Differentiation of pluripotent embryonic stem cells into the neuronal lineage in vitro gives rise to mature inhibitory and excitatory neurons. Mech. Dev. *53*, 275-287.

Suetake,I., Shinozaki,F., Miyagawa,J., Takeshima,H., and Tajima,S. (2004). DNMT3L stimulates the DNA methylation activity of Dnmt3a and Dnmt3b through a direct interaction. J. Biol. Chem. *279*, 27816-27823.

Suguta,M., Nakano,A., Hoshino,Y., Endoh,M., Hatori,T., Hasegawa,A., Aihara,M., Takeuchi,T., and Kurabayashi,M. (2007). Nestin, a maker for multilineage potential of cells from human primary and restenotic coronary artery plaques. Int. J. Cardiol. *121*, 53-56.

Sun,J., Bronk,P., Liu,X., Han,W., and Sudhof,T.C. (2006). Synapsins regulate use-dependent synaptic plasticity in the calyx of Held by a Ca2+/calmodulin-dependent pathway. Proc. Natl. Acad. Sci. U. S. A. *103*, 2880-2885.

Sun,Y., Nadal-Vicens,M., Misono,S., Lin,M.Z., Zubiaga,A., Hua,X., Fan,G., and Greenberg,M.E. (2001). Neurogenin promotes neurogenesis and inhibits glial differentiation by independent mechanisms. Cell. *104*, 365-376.

Sunabori,T., Tokunaga,A., Nagai,T., Sawamoto,K., Okabe,M., Miyawaki,A., Matsuzaki,Y., Miyata,T., and Okano,H. (2008). Cell-cycle-specific nestin expression coordinates with morphological changes in embryonic cortical neural progenitors. J. Cell Sci. *121*, 1204-1212.

Suzuki,M.M., and Bird,A. (2008). DNA methylation landscapes: provocative insights from epigenomics. Nat. Rev. Genet. *9*, 465-476.

Takai,D., and Jones,P.A. (2002). Comprehensive analysis of CpG islands in human chromosomes 21 and 22. Proc. Natl. Acad. Sci. U. S. A. *99*, 3740-3745.

Tan,M., Luo,H., Lee,S., Jin,F., Yang,J.S., Montellier,E., Buchou,T., Cheng,Z., Rousseaux,S., Rajagopal,N., Lu,Z., Ye,Z., Zhu,Q., Wysocka,J., Ye,Y., Khochbin,S., Ren,B., and Zhao,Y. (2011). Identification of 67 histone marks and histone lysine crotonylation as a new type of histone modification. Cell. *146*, 1016-1028.

Tanaka,S., Kamachi,Y., Tanouchi,A., Hamada,H., Jing,N., and Kondoh,H. (2004). Interplay of SOX and POU factors in regulation of the Nestin gene in neural primordial cells. Mol. Cell Biol. *24*, 8834-8846.

Tang,L.Y., Reddy,M.N., Rasheva,V., Lee,T.L., Lin,M.J., Hung,M.S., and Shen,C.K. (2003). The eukaryotic DNMT2 genes encode a new class of cytosine-5 DNA methyltransferases. J. Biol. Chem. *278*, 33613-33616.

Teranishi,N., Naito,Z., Ishiwata,T., Tanaka,N., Furukawa,K., Seya,T., Shinji,S., and Tajiri,T. (2007). Identification of neovasculature using nestin in colorectal cancer. Int. J. Oncol. *30*, 593-603.

Thomson,J.A., Itskovitz-Eldor,J., Shapiro,S.S., Waknitz,M.A., Swiergiel,J.J., Marshall,V.S., and Jones,J.M. (1998). Embryonic stem cell lines derived from human blastocysts. Science *282*, 1145-1147.

Thomson,J.A., Kalishman,J., Golos,T.G., Durning,M., Harris,C.P., Becker,R.A., and Hearn,J.P. (1995). Isolation of a primate embryonic stem cell line. Proc. Natl. Acad. Sci. U. S. A. *92*, 7844-7848.

Tischfield,M.A., Baris,H.N., Wu,C., Rudolph,G., Van,M.L., He,W., Chan,W.M., Andrews,C., Demer,J.L., Robertson,R.L., Mackey,D.A., Ruddle,J.B., Bird,T.D., Gottlob,I., Pieh,C., Traboulsi,E.I., Pomeroy,S.L., Hunter,D.G., Soul,J.S., Newlin,A., Sabol,L.J., Doherty,E.J., de Uzcategui,C.E., de,U.N., Collins,M.L., Sener,E.C., Wabbels,B., Hellebrand,H., Meitinger,T., de,B.T., Magli,A., Schiavi,C., Pastore-Trossello,M., Koc,F., Wong,A.M., Levin,A.V., Geraghty,M.T., Descartes,M., Flaherty,M., Jamieson,R.V., Moller,H.U., Meuthen,I., Callen,D.F., Kerwin,J., Lindsay,S., Meindl,A., Gupta,M.L., Jr., Pellman,D., and Engle,E.C. (2010). Human TUBB3 mutations perturb microtubule dynamics, kinesin interactions, and axon guidance. Cell. *140*, 74-87.

Tohyama,T., Lee,V.M., Rorke,L.B., Marvin,M., McKay,R.D., and Trojanowski,J.Q. (1992). Nestin expression in embryonic human neuroepithelium and in human neuroepithelial tumor cells. Lab Invest. *66*, 303-313.

Tsujimura,T., Makiishi-Shimobayashi,C., Lundkvist,J., Lendahl,U., Nakasho,K., Sugihara,A., Iwasaki,T., Mano,M., Yamada,N., Yamashita,K., Toyosaka,A., and Terada,N. (2001). Expression of the intermediate filament nestin in gastrointestinal stromal tumors and interstitial cells of Cajal. Am. J. Pathol. *158*, 817-823.

Tsukada,Y., Fang,J., Erdjument-Bromage,H., Warren,M.E., Borchers,C.H., Tempst,P., and Zhang,Y. (2006). Histone demethylation by a family of JmjC domain-containing proteins. Nature. *439*, 811-816.

Turek-Plewa,J., and Jagodzinski,P.P. (2005). The role of mammalian DNA methyltransferases in the regulation of gene expression. Cell Mol. Biol. Lett. *10*, 631-647.

Uchikawa,M., Ishida,Y., Takemoto,T., Kamachi,Y., and Kondoh,H. (2003). Functional analysis of chicken Sox2 enhancers highlights an array of diverse regulatory elements that are conserved in mammals. Dev. Cell. *4*, 509-519.

Vaissiere,T., Sawan,C., and Herceg,Z. (2008). Epigenetic interplay between histone modifications and DNA methylation in gene silencing. Mutat. Res. *659*, 40-48.

Vanderwinden,J.M., Gillard,K., De Laet,M.H., Messam,C.A., and Schiffmann,S.N. (2002). Distribution of the intermediate filament nestin in the muscularis propria of the human gastrointestinal tract. Cell Tissue Res. *309*, 261-268.

Wang,H.S., Hung,S.C., Peng,S.T., Huang,C.C., Wei,H.M., Guo,Y.J., Fu,Y.S., Lai,M.C., and Chen,C.C. (2004). Mesenchymal stem cells in the Wharton's jelly of the human umbilical cord. Stem Cells. *22*, 1330-1337.

Weber,M., Hellmann,I., Stadler,M.B., Ramos,L., Paabo,S., Rebhan,M., and Schubeler,D. (2007). Distribution, silencing potential and evolutionary impact of promoter DNA methylation in the human genome. Nat. Genet. *39*, 457-466.

Weng,Y.S., Lin,H.Y., Hsiang,Y.J., Hsieh,C.T., and Li,W.T. (2003). The effects of different growth factors on human bone marrow stromal cells differentiating into hepatocyte-like cells. Adv. Exp. Med. Biol. *534:119-28.*, 119-128.

Wianny,F., Bourillot,P.Y., and Dehay,C. (2011). Embryonic stem cells in non-human primates: An overview of neural differentiation potential. Differentiation. *81*, 142-152.

Wiench,M., John,S., Baek,S., Johnson,T.A., Sung,M.H., Escobar,T., Simmons,C.A., Pearce,K.H., Biddie,S.C., Sabo,P.J., Thurman,R.E., Stamatoyannopoulos,J.A., and Hager,G.L. (2011). DNA methylation status predicts cell type-specific enhancer activity. EMBO J. *30*, 3028-3039.

Wiese,C., Rolletschek,A., Kania,G., Blyszczuk,P., Tarasov,K.V., Tarasova,Y., Wersto,R.P., Boheler,K.R., and Wobus,A.M. (2004). Nestin expression--a property of multi-lineage progenitor cells? Cell Mol. Life Sci. *61*, 2510-2522.

Williams,J.T., Southerland,S.S., Souza,J., Calcutt,A.F., and Cartledge,R.G. (1999). Cells isolated from adult human skeletal muscle capable of differentiating into multiple mesodermal phenotypes. Am. Surg. *65*, 22-26.

Wislet-Gendebien,S., Hans,G., Leprince,P., Rigo,J.M., Moonen,G., and Rogister,B. (2005). Plasticity of cultured mesenchymal stem cells: switch from nestin-positive to excitable neuron-like phenotype. Stem Cells. *23*, 392-402.

Wobus,A.M., and Boheler,K.R. (2005). Embryonic stem cells: prospects for developmental biology and cell therapy. Physiol Rev. *85*, 635-678.

Woodbury,D., Schwarz,E.J., Prockop,D.J., and Black,I.B. (2000). Adult rat and human bone marrow stromal cells differentiate into neurons. J. Neurosci. Res. *61*, 364-370.

Yamane,K., Toumazou,C., Tsukada,Y., Erdjument-Bromage,H., Tempst,P., Wong,J., and Zhang,Y. (2006). JHDM2A, a JmjC-containing H3K9 demethylase, facilitates transcription activation by androgen receptor. Cell. *125*, 483-495.

Yan,Y., Yang,J., Bian,W., and Jing,N. (2001). Mouse nestin protein localizes in growth cones of P19 neurons and cerebellar granule cells. Neurosci. Lett. *%20;302*, 89-92.

Yaworsky,P.J., and Kappen,C. (1999). Heterogeneity of neural progenitor cells revealed by enhancers in the nestin gene. Dev. Biol. *205*, 309-321.

Yen,Z.C., Meyer,I.M., Karalic,S., and Brown,C.J. (2007). A cross-species comparison of X-chromosome inactivation in Eutheria. Genomics. *90*, 453-463.

Ying,Q.L., Nichols,J., Chambers,I., and Smith,A. (2003a). BMP induction of Id proteins suppresses differentiation and sustains embryonic stem cell self-renewal in collaboration with STAT3. Cell. *115*, 281-292.

Ying,Q.L., Stavridis,M., Griffiths,D., Li,M., and Smith,A. (2003b). Conversion of embryonic stem cells into neuroectodermal precursors in adherent monoculture. Nat. Biotechnol. *21*, 183-186.

Yoon,K.H., Yoon,M., Moir,R.D., Khuon,S., Flitney,F.W., and Goldman,R.D. (2001). Insights into the dynamic properties of keratin intermediate filaments in living epithelial cells. J. Cell Biol. *153*, 503-516.

Zetterstrom,R.H., Solomin,L., Jansson,L., Hoffer,B.J., Olson,L., and Perlmann,T. (1997). Dopamine neuron agenesis in Nurr1-deficient mice. Science. *276*, 248-250.

Zhang,Q., Qin,H., Lang,B., Liu,H., Han,H., and Ju,G. (2005). Different regions of the mouse nestin enhancer may function differentially in nestin expression in an NSC-like cell line and astrocytes. Neurosci. Lett. *379*, 90-95.

Zhao,X.D., Han,X., Chew,J.L., Liu,J., Chiu,K.P., Choo,A., Orlov,Y.L., Sung,W.K., Shahab,A., Kuznetsov,V.A., Bourque,G., Oh,S., Ruan,Y., Ng,H.H., and Wei,C.L. (2007). Whole-genome mapping of histone H3 Lys4 and 27 trimethylations reveals distinct genomic compartments in human embryonic stem cells. Cell Stem Cell *1*, 286-298.

Zheng,C., and Hayes,J.J. (2003). Intra- and inter-nucleosomal protein-DNA interactions of the core histone tail domains in a model system. J. Biol. Chem. *278*, 24217-24224.

Zhong,H., Jin,Z., Chen,Y., Zhang,T., Bian,W., Cui,X., and Jing,N. (2008). First intron of nestin gene regulates its expression during C2C12 myoblast differentiation. Acta Biochim. Biophys. Sin. (Shanghai). *40*, 526-532.

Zhou,Q., Choi,G., and Anderson,D.J. (2001). The bHLH transcription factor Olig2 promotes oligodendrocyte differentiation in collaboration with Nkx2.2. Neuron. *31*, 791-807.

Zimmerman,L., Parr,B., Lendahl,U., Cunningham,M., McKay,R., Gavin,B., Mann,J., Vassileva,G., and McMahon,A. (1994). Independent regulatory elements in the nestin gene direct transgene expression to neural stem cells or muscle precursors. Neuron. *12*, 11-24.

Zuk,P.A., Zhu,M., Ashjian,P., De Ugarte,D.A., Huang,J.I., Mizuno,H., Alfonso,Z.C., Fraser,J.K., Benhaim,P., and Hedrick,M.H. (2002). Human adipose tissue is a source of multipotent stem cells. Mol. Biol. Cell *13*, 4279-4295.

Zuk,P.A., Zhu,M., Mizuno,H., Huang,J., Futrell,J.W., Katz,A.J., Benhaim,P., Lorenz,H.P., and Hedrick,M.H. (2001). Multilineage cells from human adipose tissue: implications for cell-based therapies. Tissue Eng. *7*, 211-228.