Neuronal potential of umbilical cord blood non-hematopoietic multipotent stem cells

Hamad Ali

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Institute of Human Genetics

Newcastle University

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Declaration

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Acknowledgments

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Publications and abstracts

Publications:

ALI, H., FORRAZ, N., MCGUCKIN, C. P., JURGA, M., LINDSAY, S., IP, B. K., TREVELYAN, A., BASFORD, C., HABIBOLLAH, S., AHMAD, S., CLOWRY, G. J. & BAYATTI, N. 2011. In Vitro Modelling of Cortical Neurogenesis by Sequential Induction of Human Umbilical Cord Blood Stem Cells. *Stem Cell Rev*.

JURGA, M., FORRAZ, N., BASFORD, C., ATZENI, G., TREVELIAN, A. J., HABIBOLLAH, S., **ALI, H**., ZWOLINSKI, S. A. & MCGUCKIN, C. 2011. Neurogenic properties and a clinical relevance of multipotent stem cells derived from cord blood samples stored in the biobanks. *Stem Cells Dev*.

ALI, H. & BAHBAHANI, H. 2010. Umbilical cord blood stem cells - potential therapeutic tool for neural injuries and disorders. *Acta Neurobiol Exp (Wars),* 70**,** 316-24.

ALI, H., JURGA, M., KURGONAITE, K., FORRAZ, N. & MCGUCKIN, C. 2009. Defined serum-free culturing conditions for neural tissue engineering of human cord blood stem cells. *Acta Neurobiol Exp (Wars),* 69**,** 12-23.

MCGUCKIN, C., JURGA, M., **ALI, H**., STRBAD, M. & FORRAZ, N. 2008. Culture of embryonic-like stem cells from human umbilical cord blood and onward differentiation to neural cells in vitro. *Nat Protoc,* 3**,** 1046-55.

These papers are directly related to the research outlined in this thesis.

Abstracts:

- **Poster abstracts:**
    - **Ali H.**, Bayatti N., Trevelyan A., Ahmed S., Lindsay S., *In vitro* modelling of human corticogenesis using umbilical cord blood stem cells, March 13-14, 2010, 3rd Pan Arab Human genetic conference, Dubai, United Arab Emirates

- o **Ali H.,** Jurga M., Strbad M., Forraz N., McGuckin C., Engineering brains in laboratories: Are we there yet?, "Faculty of medical sciences poster competition", 2008, University of Newcastle, Newcastle, UK

- o **Ali H.,** Jurga M., Strbad M., Forraz N., McGuckin C., Neurogenic potential of pluripotent embryonic-like stem cells isolated from umbilical cord blood, "International society For stem cell research (ISSCR) 6th annual meeting", June 11-14, 2008, Philadelphia, PA, USA

- o Jurga M., Basford C., **Ali H.**, Forraz N., McGuckin C., 3D neuro-endothelial artificial tissue: Human umbilical cord blood tissue-engineering, "International society For stem cell research (ISSCR) 6th annual meeting", June 11-14, 2008, Philadelphia, PA, USA

- o **Ali H.**, Neuronal differentiation of two different pluripotent stem cell populations isolated from umbilical cord blood, "Stem cells and the brain", 2007, Research Beehive, Newcastle university, Newcastle, UK

- **Speaker abstracts:**
  - o **Ali H**., Bayatti N., Trevelyan A., Ahmed S., Lindsay S., Engineering human brain cells from umbilical cord blood: A potential tool for cell-therapy, "The third international conference of medical genetics" , 2010, Ministry of health, Kuwait

  - o **Ali H.,** Jurga M., Forraz N., McGuckin C., Production of pure functional mature neurons from umbilical cord blood stem cells, "North-east postgraduate research conference", 2008, Newcastle University, Newcastle, UK

  - o Jurga M., **Ali H.**, Forraz N., McGuckin CP., Umbilical cord blood stem cells for neural tissue-engineering, "4th ITERA-Life-Sciences forum workshop" 2008, Maastricht, Netherlands

o Jurga M., **Ali H.**, Martin P., McGuckin CP., Human umbilical cord blood: a source of stem cells for neural tissue engineering, "Stem cells and the brain" , 2007, Research Beehive, Newcastle university, Newcastle, UK

Abbreviations

µg	Microgram
µl	Microliter
µm	Micrometer
2D	Two dimensional
3D	Three dimensional
ABAT	4-Aminobutyrate aminotransferase
Ach	Acetylcholine
ACHE	Acetylcholinesterase
ACSF	Artificial cerebrospinal fluid
AD	Alzheimer's disease
ADI	Alzheimer's Disease International federation
AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
ANOVA	Analysis of variation
ANS	Autonomic nervous system
APP	Amyloid precursor protein
AT	Ataxia telangiectasia
ATP	Adenosine tri-phosphate
BDNF	Brain-derived neurotrophic growth factor
bFGF	Basic fibroblast growth factor
b-HLH	Basic helix-loop-helix
BrdU	5-bromo-2'-deoxyuridine
cAMP	Cyclic adenosine monophosphate
CBEs	Cord blood derived embryonic-like stem cells
CD	Cluster of differentiation
CHAT	Choline acetyltransferase
cm	Centimeter
CNS	Central nervous system
COMT	Catechol-O-methyltransferase
CP	Cortical plate
CPD-A	Citrate phosphate dextrose adenine

CSF	Cerebrospinal fluid
DAB	Diaminobenzidine
DAPI	4'-6-Diamidino-2-phenylindole
DDC	Dopa-decarboxylase
DIC	Differential interference contrast
DMEM	Dulbecco's modification of Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide phosphate
ECM	Extracellular matrix
EDTA	Ethylene diaminetetraacetic acid
EGF	Epidermal growth factor
ENS	Enteric nervous system
FACS	Fluorescence activated cell sorting
FCS	Foetal calf serum
FDA	Food and Drug Administration
FITC	Fluorescein isothiocyonate
GABA	Gamma aminobutyric acid
GAD1	Glutamate decarboxylase 1
GALC	Galactosylceramidase
GAP43	Growth associated protein 43
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GFAP	Glial fibrillary acidic protein
GvHD	Graft-versus-host diseases
HLA	Human leukocyte antigen
HSCs	Hematopoietic stem cells
ICC	Immuno-cytochemistry
ICM	Inner cell mass
IHC	Immuno-histochemistry
iPS cells	Induced pluripotent stem cells
ISVZ	Inner subventricular zone
IZ	Intermediate zone
Lin-Neg	Lineage negative
mAChR	Muscarinic-activated acetylcholine receptors
MAO	Monoamine oxidase
mGluRs	Metabotropic glutamate receptors
ml	Milliliter

mM	Millimolar
MNC	Mononuclear cell
MPC-I	Magnetic particle concentrator-I
MSCs	Mesenchymal stem cells
mV	Millivolt
MZ	Marginal zone
nAChR	Nicotinic-activated acetylcholine receptors
NeuN	Neural nuclei
NF200	Neurofilament 200
ng	Nanogram
NGF	Nerve growth factor
NIH	National Institute of Health
NM	Nanometer
NMDAR	N-methyl D-aspartate ionotropic reseptor
NSC	Neural stem cell
OB	Olfactory bulb
OCT4	Octamer-4
OSVZ	Outer subventricular zone
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PCW	Postconception week
PD	Parkinson's disease
PNS	Peripheral nervous system
POU5F1	POU class 5 homeobox 1
PSD95	Post synaptic density protein 95
Q-PCR	Quantative polymerase chain reaction (real time)
RA	Retinoic acid
RMS	Rostral migratory stream
RNA	Ribonucleic acid
RT	Reverse transcription
SC kit	Stem cell kit
SCID	Severe combined immune deficiency
SGZ	Subgranular zone
SLC	Salute carrier family
SNS	Somatic nerve system
SOX	SRY-related HMG-box

SP	Subplate
SSEA	Stage specific embryonic antigen
SVZ	Subventricular zone
TAE	Tris acetate/ethylene diaminetetraacetic acid
TBI	Traumatic brain injury
TH	Tyrosine hydroxylase
tPA	Tissue plasminogen activator
TPH	Tryptophan hydroxylase
TRA	Tumor rejection antigens
TrK	Tropomyosin receptor kinases
VSELs	Very small embryonic-like stem cells
VZ	Ventricular zone
WHO	World Health Organization

Abstract

The human central nervous system, one of the most complex organ systems anatomically and physiologically in the human body, is the body control center which manages and coordinates functions of all different organ systems. The lack of effective treatments and therapeutic intervention make injuries and disorders associated with the central nervous system one of the most dangerous and fatal health conditions worldwide.

The adult nervous system has a limited capability of self-repair and regeneration following either a neurological disorder or injury. Despite being limited and ineffective in initiating recovery from injuries and disorders, this property opened the doors for a new direction of research aimed at investigating the possibility of developing therapies and treatments for central nervous system injuries and disorders based on the concept of regeneration and cell transplantations.

Stem cells have gained significant public attention over the past decade due to their differentiation capabilities and potential utilization in clinical applications. The ability to differentiate stem cells into neural lineages including neurons and glial cells, highlighted their potential role as a therapeutic tool for central nervous system injuries and disorders.

The main aim of this thesis is to show that umbilical cord blood stem cells are a potential source of cells that could be used therapeutically in central nervous system injuries and disorders. A distinct population of cells has been purified from human umbilical cord blood. These cells have been characterized and differentiated *in-vitro* into neuron-like cells using fully defined sequential neuronal induction protocol. The differentiated cells were shown to have similar morphological and functional properties to developing central nervous system endogenous neurons using several different techniques, including immunocytochemistry, real-time PCR, cDNA arrays and calcium imaging. The results highlight the potential role of umbilical cord blood stem cells as a therapeutic tool for central nervous system injuries and disorders for which current mode of therapy is

inadequate. In addition, they might provide an *in-vitro* model of neural cells for toxicology and drugs testing research.

Chapter 1. Introduction

1.1 A historic perspective of stem cells

Although stem cell biology has gained significant public attention over the past decade, its history dates back to the 1950s. It was in 1954 when the differentiation characteristics of stem cells were reported for the first time through work on mice testicular teratoma. Upon analyzing the tumors, the presence of many different adult tissues and cell types were noted within the tumor (Stevens and Little, 1954). Continuing on from this, it was later shown that a single cell isolated from the tumor could generate many cell types once injected into a mouse (Kleinsmith and Pierce, 1964). It was clear by that time that some of the tumor cells could actually transform into many different cell types, however the identity and cell biology of those interesting cells was yet to be determined. Later *in-vitro* cell lines derived from mouse embryonic tumors were generated and their cell biology including morphology and differentiation properties were investigated (Rosenthal et al., 1970). Conclusions from these studies suggested the presence of stem cells that are able to differentiate into multiple cell types. However knowledge of the mechanisms governing these differentiation processes was still naïve. In 1981 two independent groups derived for the first time mouse embryonic stem cells from the blastocyst (Evans and Kaufman, 1981, Martin, 1981). Later those isolated cells were tested successfully for their ability to generate germline chimaeras and proved their pluripotent properties (Bradley et al., 1984). In 1998, James Thompson from the University of Wisconsin at Madison established the first human embryonic stem cell line isolated from human blastocyst. Embryos used for this study were created using *in-vitro* fertilization (IVF) (Thomson et al., 1998). The establishment of such cell lines allowed the scientists to study in greater detail the mechanisms and factors governing the differentiation of these stem cells into various cell types. In addition to embryonic stem cells, many other types of stem cells have been discovered including those from cord blood

tissues and adults stem cells. The field of stem cell biology has been advancing with a great pace in the past decade and attracted tremendous interest due to the potential it is offering that would revolutionize current medicinal practice by providing therapies for diseases and injuries for which current modes of therapy are inadequate.

1.2 What is a stem cell?

By definition, a "stem cell" is an unspecialized cell that is capable of:

- **Self-renewal**: This is the process by which a stem cell divides to produce one or two stem cells with similar characteristics to the original stem cell. This is achieved by either symmetrical or asymmetrical cell divisions. In symmetrical cell divisions, a cell divides to produce two identical cells, for example a stem cell can give rise to either two stem cells or two more differentiated cells; this kind of division is common in early stages of development (figure 1). On the other hand, in asymmetrical cell divisions, a cell divides into two cells, one of them being identical to the original cell and the other being a different type of cell, for example a stem cell can divide into two cells, one of them being a stem cell and the other being a more differentiated cell (figure 1). This kind of division maintains the pool of stem cells whilst producing more differentiated tissue specific cells, and is dominant in adult tissues (Verfaillie, 2009, Fortier, 2005, He et al., 2009, Stewart et al., 2006).
- **Differentiation**: This is the process by which a stem cell develops into a specialized cell with specialized functions.
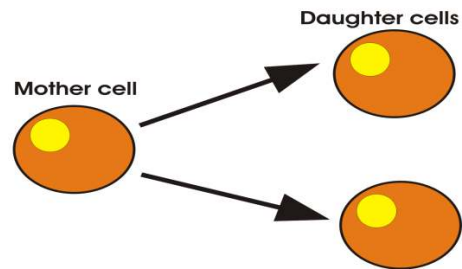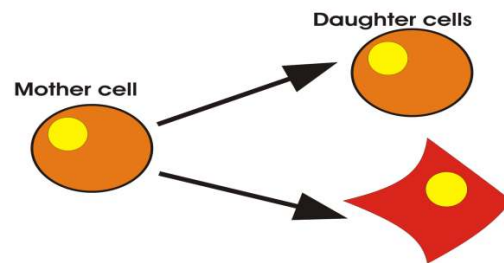
Figure 1. Symmetrical and asymmetrical cell divisions. In symmetrical cell division, one mother cell divides to give two daughter cells either both identical to the mother cell or both identical but more differentiated to the mother cell. In asymmetrical cell division, one mother cell divide to give one daughter cell identical to mother cell and one differentiated cell, and so the mother cell pool stays the same.

1.3 Classification of stem cells

1.3.1 *Stem cells potency*

There are many different types of stem cells that exist in human tissues and organs at different stages of development, starting from embryonic stages and into adulthood. Each type of stem cell has its own distinguishable differentiation properties. Stem cells can be classified based on their differentiation potential or what is known in cell biology as "cell potency" into:

- **Totipotent:** These are stem cells capable of forming all cell lineages of the three germ layers (endoderm, ectoderm, and mesoderm) that exist in a human body as well as extraembryonic tissue such as the trophoectoderm. Totipotency is a property maintained by the fertilized egg (zygote) to at least the 4-cell stage of the morula (figure 2) (Mitalipov and Wolf, 2009, Sills et al., 2005).

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- **Pluripotent:** These are stem cells that are capable of differentiating into all cell lineages of the three germ layers (endoderm, ectoderm, and mesoderm) that exist in the human body but cannot give rise to a complete human being because they lack the potential to form trophoectoderm that is needed for the development of extraembryonic tissues such as the placenta and umbilical cord. The best example of pluripotenct stem cells is embryonic stem cells (figure 2) (Mitalipov and Wolf, 2009, Smith et al., 2009).

- **Multipotent:** These are stem cells that can develop into a restricted subset of cell lineages. Hematopoietic stem cells are a good example of multipotent stem cells. They can develop into any cell type certainly within the limits of the hematopoietic system and possibly also a few other lineages (Muller-Sieburg et al., 2002, Fortier, 2005).

- **Unipotent:** These are stem cells that can only differentiate into a single type of specialized cells or cell lineage, for example adult muscle stem cells (Leeb et al., 2010).
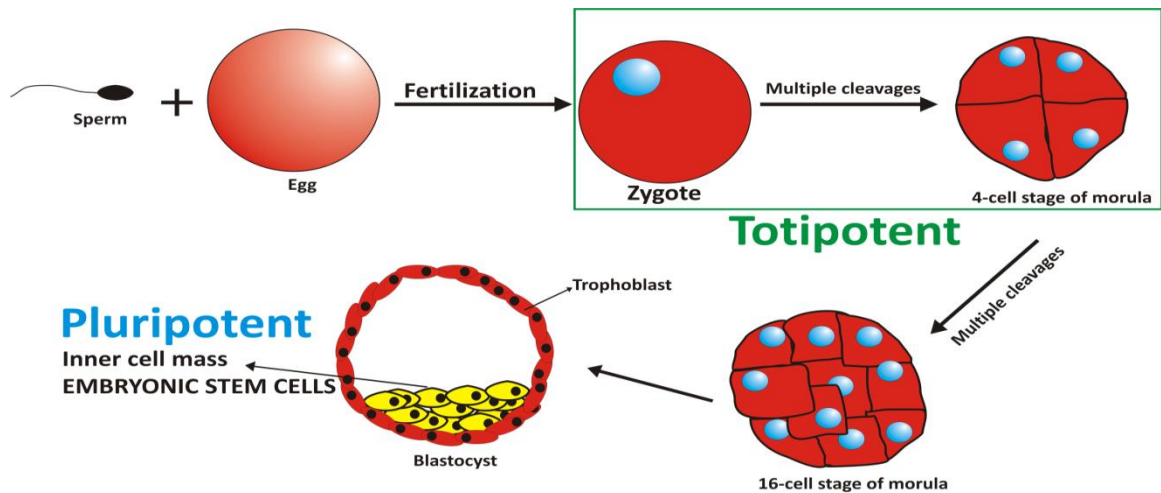
Figure 2. Human early embryonic development. After the sperm fertilize the egg, the zygote is formed which then divides rapidly (embryonic cleavage) with no significant growth in size producing the morula. The zygote and at least the 4-cell stage of morula demonstrate totipotent differentiation potential. When the morula reaches the 16-cells stage, the inner morula cells differentiate to become the inner cell mass from which embryonic stem cells with pluripotent differentiation potential are derived. The surface cells of morula differentiate to form the outer layer of the blastocyst known as trophoblast.

1.3.2 *Sources of stem cells at different stages of development*

Stem cells are also classified based on the developmental stage from which they are isolated, although this has been a source of debate amongst stem cell biologists: for example, whether umbilical cord blood is a source of adult stem cells (Tuch, 2006, Denner et al., 2007) or a source of stem cells different from both embryonic and adult stem cells (McGuckin et al., 2006, van de Ven et al., 2007). Umbilical cord blood stem cells demonstrated a number of differences to adult stem cells. They show longer telomeres and higher proliferation potential in comparison to adult stem cells (McGuckin and Forraz, 2008, Mayani, 2010). In addition, umbilical cord blood stem cells are isolated 9 months after the point of fertilization (postnatal, right after birth) in contrast to adult stem cells which are isolated from older individuals, highlighting different developmental stages at which adult and cord blood stem cells are obtained. Therefore, it can be reasonably argued that umbilical cord blood stem cells should be listed as a separate class of stem cells in addition to embryonic and adult stem cells:

- **Embryonic stem cells:** These are pluripotent stem cells derived from the inner cell mass (ICM) of 5-8 days old human blastocyst (figure 2) (Sills et al., 2005, Stewart et al., 2006). Their pluripotent properties make them capable of differentiating into any cell type in the human body.

- **Umbilical cord blood stem cells:** These stem cells are isolated from blood in the umbilical cord blood vessels and placenta right after birth (postnatally). Umbilical cord blood contains different types of stem cells including hematopoietic stem cells, non-hematopoietic stem cells (McGuckin et al., 2005) and mesenchymal stem cells (Lee et al., 2004). Each type of these stem cells has its own properties and differentiation potential (this will be discussed further in later sections).

- **Adult stem cells:** These stem cells are found in various tissues throughout the adult human body. Adult stem cells have different properties and differentiation potentials; in general, they vary from being multipotent to unipotent. Adult stem cells are crucial in maintaining tissue and organ mass during normal cellular turnover and tissue repair (Fortier, 2005). Examples of adult stem cells include neural stem cells which are multipotent stem cells that reside in neural tissue and

hepatic stem cells which are generally considered unipotent and reside in adult liver tissues.

1.4 Molecular control of pluripotency

Pluripotency and indefinite self-renewal potential are two characteristics associated with embryonic stem cells (He et al., 2009, Pardo et al., 2010, Stewart et al., 2006). These two distinct features of embryonic stem cells are established and regulated by a unique set of key transcription factors including OCT4, SOX2, and NANOG.

The POU domain transcription factor OCT4 (also known as POU class 5 homeobox 1, POU5F1) has been identified as an essential regulator highly involved in the establishment and maintenance of the pluripotent state of embryonic stem cells (Pardo et al., 2010). Studies on embryonic stem cells showed that over-expression of *OCT4* causes differentiation into primitive endoderm and mesoderm lineages while downregulation or loss of *OCT4* induces differentiation to trophectoderm lineage. This emphasizes the strict control required on levels of *OCT4* expression in maintaining the pluripotency of embryonic stem cells (Nichols et al., 1998, Niwa et al., 2000). OCT4 has been shown to occupy a central role in the core transcriptional regulatory circuitry that regulates the expression levels of other genes in order to establish and control pluripotency and self-renewal in embryonic stem cells (Boyer et al., 2006).

OCT4 interacts with other transcription factors in order to establish and maintain embryonic stem cell identity. Among these factors is SOX2, which is a transcription factor member of SOX (SRY-related HMG-box) family. It has been shown that OCT4 and SOX2 form a hetero-dimer transcription factor complex that regulates gene expression in embryonic stem cells (Rodda et al., 2005, Chew et al., 2005, Masui et al., 2007, Okumura-Nakanishi et al., 2005). Unlike OCT4 which functions predominantly in pluripotent stem cells, SOX2 is also involved in the maintenance of neural progenitor identity in neural tissues (Graham et al., 2003), in addition to its role in the maintenance of pluripotency and lineage specification (Boyer et al., 2006). SOX2 involvement in pluripotency regulation has been illustrated in studies where the loss of *SOX2* gene expression resulted in inappropriate differentiation and loss of the stem cell undifferentiated state (Fong et al., 2008).

In addition to OCT4 and SOX2, it has been shown that NANOG, a homeobox transcription factor, is also critically involved in maintenance of the pluripotent state and the self-renewal properties of embryonic stem cells (Hyslop et al., 2005). NANOG, which is normally expressed in pluripotent embryonic stem cells and in the developing germline, is also crucially required for early embryonic development. Deletion of *NANOG* in founder cells of the inner cell mass results in the failure of epiblast generation (Mitsui et al., 2003, Silva et al., 2009). Moreover, down-regulation of NANOG in embryonic stem cells results in loss of pluripotency and differentiation of these cells to extraembryonic endoderm lineages (Mitsui et al., 2003, Boyer et al., 2006).

OCT4, SOX2 and NANOG transcription factors are the key players involved in specifying the pluripotent status of embryonic stem cells as well as coordinating early fate decisions. Their collaborative interactions with each other and other transcription factors form the transcriptional regulatory network that controls the expression patterns and levels of many genes involved in pluripotency and fate specifications (figure 3) (Boyer et al., 2005, Liang et al., 2008). The cooperative interactions between OCT4, SOX2 and NANOG were revealed using genomic scale location analysis which showed that the three transcription factors co-occupied the promoters of a large numbers of genes involved in fate specification and development (Boyer et al., 2005, Loh et al., 2006). A large proportion of the co-targeted genes code developmentally important homeodomain transcription factors which in turn regulate other target genes. This forms a transcriptional regulatory hierarchy with OCT4, SOX2 and NANOG occupying the top position (Boyer et al., 2005, Boyer et al., 2006). Studies also reveal that OCT4, SOX2 and NANOG regulate the expression levels of their own genes forming auto-regulatory loops which stabilize genes expression and provide a fast response regulatory pathway (figure 3) (Chew et al., 2005, Okumura-Nakanishi et al., 2005, Rosenfeld et al., 2002). OCT4, SOX2 and NANOG expression and functions are highly linked to each other and their cooperative interaction is fundamental in the regulation of the pluripotent state of embryonic stem cells as well as fate specification during development.
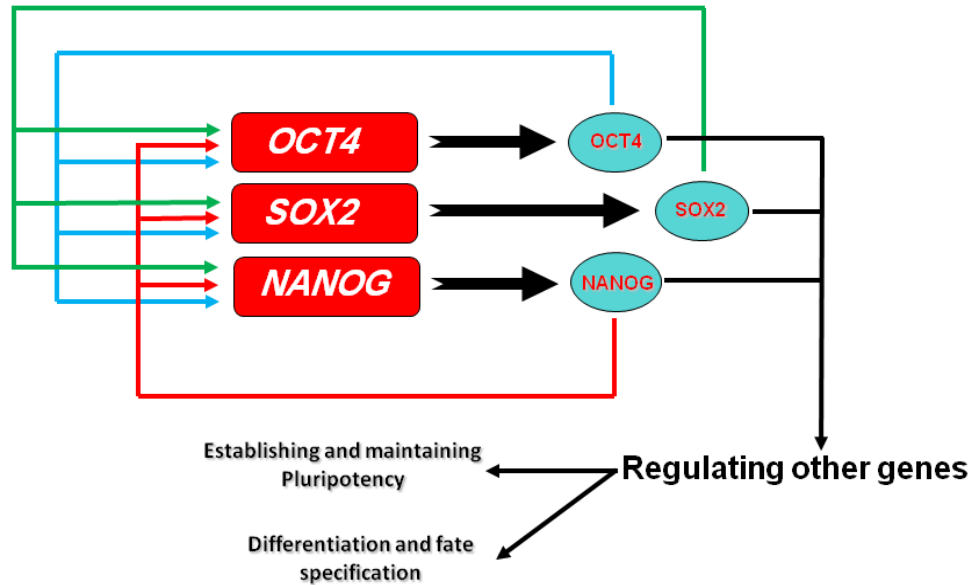
Figure 3. Core transcriptional regulatory networks in pluripotent stem cells. OCT4, SOX2 and NANOG transcription factors (blue spheres) form the core of the network. They regulate their own genes (red boxes) forming auto-regulatory circuits in addition to the expression of other genes to either establish and maintain the pluripotent status or regulating differentiation and fate specification. Adapted from (Boyer et al., 2005, Boyer et al., 2006).

1.4.1 *Induced pluripotent stem cells (iPS cells)*

Induced pluripotent stem cells (iPS cells) are pluripotent stem cells derived artificially from non-pluripotent cells, usually fully differentiated somatic cells, by introducing certain genes which reprogram the cell and de-differentiate it back to the pluripotent state (Yamanaka, 2008). iPS cells were first generated by Takashi and Yamanaka in 2006. They reprogrammed mouse fibroblasts to generate iPS cells by transfecting them with four genes (Oct4, Sox2, c-Myc and Klf4) using a retroviral delivery system. The iPS cells had similar morphology to embryonic stem cells and formed teratomas when transplanted into SCID mice (mice with severe combined immune deficiency) confirming their pluripotent properties (Takahashi and Yamanaka, 2006, Yamanaka and Takahashi, 2006). Although the generated cells showed pluripotent properties, they failed to produced chimeras when injected into developing embryos suggesting partial reprogramming (Yamanaka, 2008). This experiment was followed by three independent experiments that reported the successful production of iPS cells which this time also produced chimeras when injected

into developing mice embryos. The three groups used the same four genes for reprogramming (Oct4, Sox2, c-Myc and Klf4) but unlike Yamanaka who used Fbx15, a protein expressed by embryonic stem cells, as a selection marker for iPS cells, they used Nanog and produced germline-competent iPS cells with more similar ES-cell-like gene expression and DNA methylation patterns compared with iPS cells selected with Fbx15 (Maherali et al., 2007, Okita et al., 2007, Wernig et al., 2007).

In 2007, the first human iPS cells were generated by two independent groups. Yamanaka's group used the same set of markers they previously used on mice fibroblasts, to reprogram human fibroblast into iPS cells (Takahashi et al., 2007). The second group used OCT4, SOX2, NANOG and LIN28 to reprogram human fibroblasts into iPS cells (Yu et al., 2007). Both groups reported similarities between their generated iPS cells and human embryonic stem cells using a set of assays including morphological studies, epigenetic analysis, embryoid body formation, differentiation potential analysis and teratoma formation *in vivo* (Yu et al., 2007, Takahashi et al., 2007).

In following studies, different sets of factors were used to generate iPS cells. A group at Kyoto University in Japan used fibroblasts expressing endogenous c-Myc which were reprogrammed using Oct4, Sox2 and Klf4 only, without c-myc. Although they reported successful generation of iPS cells, the efficiency was low compared to induction with a full set of genes including c-Myc (Wernig et al., 2008, Nakagawa et al., 2008). Another experiment showed that mice neuronal progenitor cells expressing endogenous Sox2 and c-Myc were reprogrammed using only Oct4 and Klf4, Oct4 and c-Myc and finally Oct4 on its own. Successful generations of iPS cells were recorded for all three trials but again with lower efficiency rates compared to induction with all the four genes (Kim et al., 2008, Kim et al., 2009).

The iPS cell experiments provide further supporting evidence for the crucial role of OCT4 as a key central element in the pluripotency transcriptional network, based on the findings that OCT4 was the only factor that could not be substituted in the generation of iPS cells. The ability to induce and reprogram differentiated cells back to the undifferentiated pluripotent state by over-expression of certain markers including OCT4, SOX2 and

NANOG emphasizes the crucial role that all these factors have in the pluripotent properties of embryonic stem cells.

1.4.2 *Assessing pluripotency: the gold standards*

Scientific organizations around the world are trying to establish specific criteria for assessing and determining pluripotency of human cell lines in an attempt to standardize definitions within the field of stem cells. Nevertheless this issue continues to be a debatable focal point at both the scientific and political levels worldwide.

As mentioned earlier, the term "pluripotent" is defined as the potential of a stem cell to differentiate into any cell type from the three germ layers but not extraembryonic tissues. Therefore, the 'gold standard' way to test for pluripotency is by demonstrating that the tested cells can functionally produce all the cell types from all the germ layers.

In mice, this has been demonstrated by introducing the candidate cells into the blastocyst with the production of chimaeras that are germ line competent (Okita et al., 2007). In other studies, pluripotency has been demonstrated using "tetraploid complementation". In this protocol, a 4n blastocyst is generated by fusing the two cells of the two-cell stage blastocyst by applying an electrical charge. Then the candidate cells are introduced into the tetraploid blastocyst which will continue to divide and develop into mid to late stage embryos. Since the 4n cells cannot produce somatic cells, the generated embryo is composed entirely from the introduced candidate cells (Nagy et al., 1993, Hanna et al., 2008, Smith et al., 2009).

For human cells, generating chimaeras or using "tetraploid complementation" is undoubtedly unethical. Hence, scientists use the *in vivo* teratoma formation test in animal models as an alternative to assess the pluripotency of human candidate cells (Smith et al., 2009, Thomson et al., 1998, Odorico et al., 2001).

A teratoma is a tumor that is composed of cells derived from all three germ layers. In laboratories, they are produced by introducing the candidate cells into a (SCID) mouse by injection into specified sites, for example, subdermal, intratesticular, intramuscular or kidney-capsule. If the introduced cells are pluripotent, they will develop teratomas composed of cells from the three germ layers (Smith et al., 2009, Thomson et al., 1998).

Nevertheless, using teratoma formation as a test to assess pluripotency of human cells has its limitations:

- Significant histological expertise is required to determine the tumor compositions and cells lineages.
- Assessment of cells lineages is based on morphology and histology rather than function.
- Different injection sites and different mouse strains affect the frequency of teratoma formation and its composition (Prokhorova et al., 2009, Cooke et al., 2006).
- The formation of teratomas can take up to several weeks (Cooke et al., 2006).

Despite these limitations, teratoma formation is the current gold standard for determining the pluripotency of human cell lines. Many scientific organizations such as the National Institute of Health (NIH) in the USA have selected the teratoma formation assay as the main determinant of pluripotecy in combination with the expression profile of certain transcription factors and cell surface markers expressed by human embryonic pluripotent stem cells (Smith et al., 2009).

1.5 Umbilical cord blood
1.5.1 *Introduction*
Human umbilical cord blood is the blood found in the blood vessels of umbilical cord and placenta. The cord blood is regarded as the "life line" that supplies the developing fetus with the important nutrition elements and oxygen required for proper fetal development.

Beside its role in development, umbilical cord blood has been also involved in therapeutic applications which reported for the first time in 1972 by the pioneer doctors in the United States, Ende and Ende, to treat a lymphoblastic leukemia (Ende and Ende, 1972). It was later used regularly for transplantation in hematology setting for bone marrow replacement, following either hematological malignancy or bone marrow failure after any chemotherapy side effect. Umbilical cord blood was, for many years, considered to be restricted to blood

disease therapy (Gluckman et al., 1989, Slatter et al., 2006). However, advances in the production of tissue groups, from the three germ layers, has highlighted the additional potential of umbilical cord blood in treatment of other pathological disorders and medical applications including regenerative medicine and tissue engineering (McGuckin and Forraz, 2008, McGuckin et al., 2006, Watt and Contreras, 2005, Denner et al., 2007).

1.5.2 *Umbilical cord and placenta: structure, development and function*

- **Umbilical cord**

The umbilical cord is the cord that connects the developing fetus to the placenta. The umbilical cord originates from the same zygote as the fetus. It develops from the yolk sac and allantois by the 5th week of fetal development and replaces the yolk sac as the nutrient supplier for fetus (Exalto, 1995). The umbilical cord averages 50-60 cm in length and about 2 cm in diameter at the end of gestation (figure 4) (Di Naro et al., 2001). The umbilical cord contains three blood vessels, one vein and two arteries which coil around the vein in a helical configuration (figure 4) (Chaurasia and Agarwal, 1979). The vein supplies the fetus with nutrient-rich oxygenated blood from the placenta and the arteries takes the nutrient-depleted deoxygenated blood back to the placenta. The three blood vessels are insulated with a gelatinous substance called Wharton's jelly that protects these vessels and prevents their compression (figure 4A) (Ferguson and Dodson, 2009). The umbilical cord is connected to the fetus at the abdominal area at the point which after birth becomes the umbilicus. Once inside the fetus, the vein of the umbilical cord splits into two branches, one joins the hepatic portal vein which directs blood to the liver and the second allows the majority of blood to bypass the liver and directs it to the fetal heart via the left hepatic vein and inferior vena cava. The umbilical cord arteries branch from the fetal internal iliac artery which is the main artery in the pelvic area (Currarino et al., 1991).
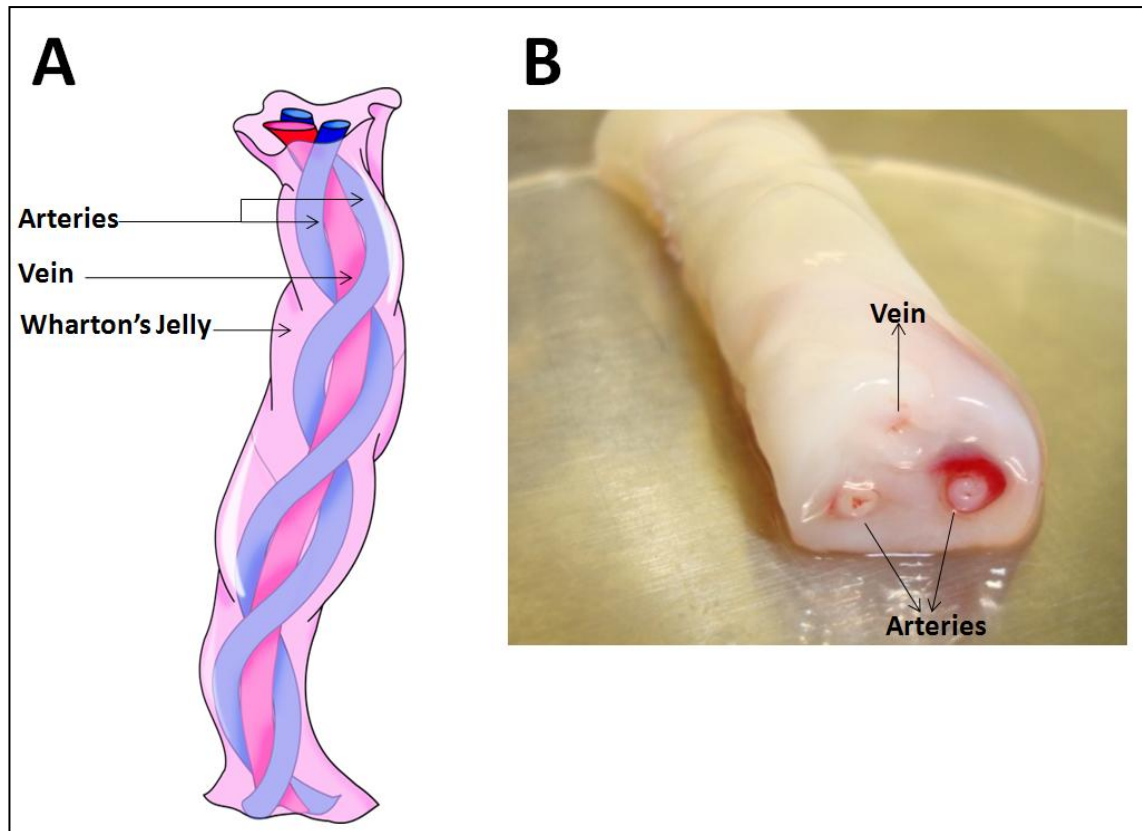
Figure 4. Anatomy of umbilical cord. (A) Umbilical cord has three blood vessels, two arteries and one vein that course through Wharton's jelly in a helical configuration. (B) Cross section of umbilical cord showing the vein and two arteries.

- **Placenta**

The placenta is the organ that connects the developing fetus via the umbilical cord to the maternal uterine wall carrying out nutritive, respiratory, and excretory functions (Desforges and Sibley, 2010). Similar to the umbilical cord, the placenta originate from the same zygote as the fetus. It begins to develop during implantation of the blastocyst into the maternal endometrium and grows throughout pregnancy (Cross et al., 2006). The placenta has a dark maroon color and round flat appearance. It averages around 20 cm in diameter and 2.5 cm in thickness at the end of gestation (figure 5).
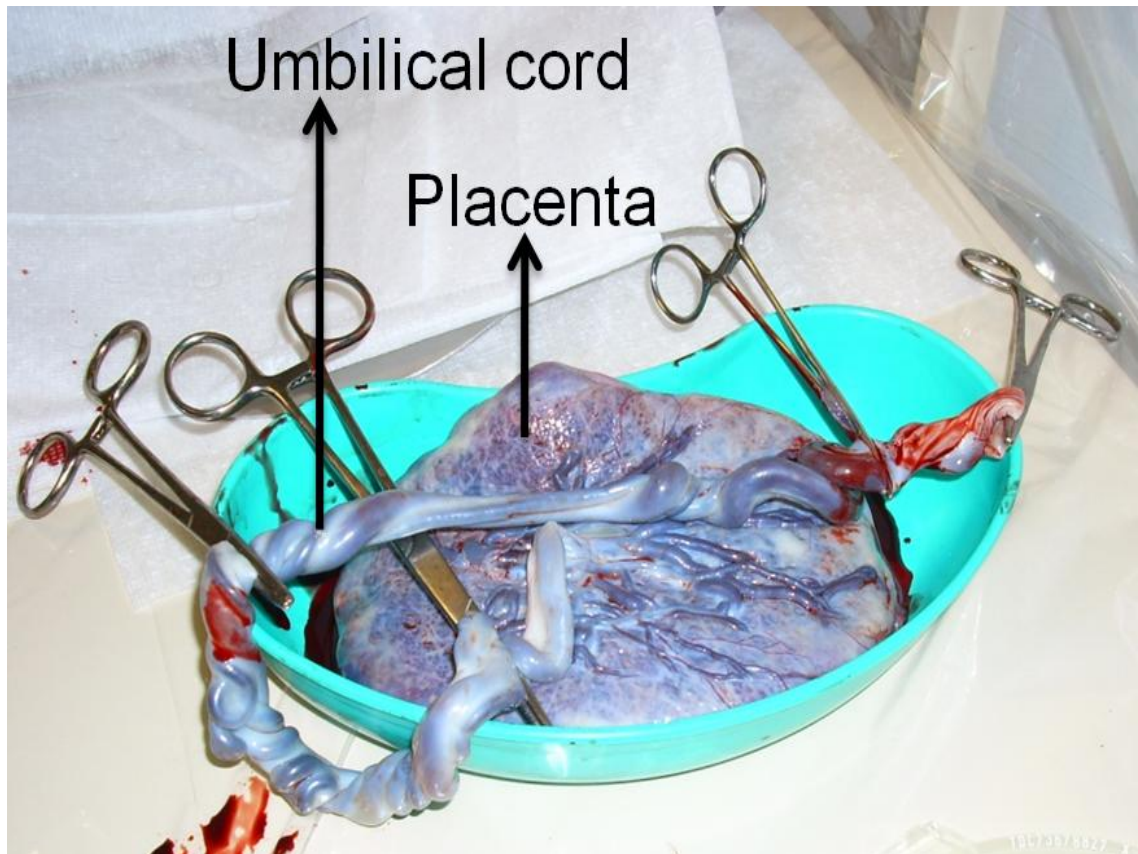
Figure 5. Human placenta and umbilical cord before cord blood collection. Photograph taken minutes after delivery.

The placenta is divided into two portions, the fetal portion and the maternal portion. The fetal portion consists of the chorionic villi which are villi that merge from the chorion to maximize the contact area with maternal blood. The maternal portion contains the intervillous space which is the space between the fetal chorionic villi and maternal blood vessels. The delicate walls of the chorionic villi allow the fetal blood to absorb nutrients and oxygen from the maternal blood and discard waste products into it without intermigration of the two blood currents (Cross et al., 2006, Desforges and Sibley, 2010).

1.5.3 *Umbilical cord blood contains different types of stem cells*

The increasing interest in umbilical cord blood after its involvements in hematological clinical applications in the past couple of decades focused efforts on analyzing and characterizing the constituents of umbilical cord blood. Beside the blood cells including erythrocytes, leukocytes and thrombocytes, the umbilical cord blood was found to contain different populations of stem cells, a unique feature not shared with peripheral blood. Scientists and researchers have characterized the following stem cell populations from umbilical cord blood; hematopoietic stem cells (HSCs), multipotent non-hematopoietic stem cells and mesenchymal stem cells (MSCs).

- **Hematopoietic stem cells (HSCs)**

Haematopoiesis is the process by which blood cells are formed. All blood cellular components are derived from a multipotent stem cell population called hematopoietic stem cells through a series of complex proliferation and differentiation events (figure 6) (Muller-Sieburg et al., 2002). Specialized hematopoietic cells can be categorized into erythrocytes (red blood cells), leukocytes (white blood cells) and thrombocytes (platelets) (table 1). The blood cellular components have been analyzed using special cell surface markers termed "the cluster of differentiation" or "CD" (table 2). These markers have been used to identify and define certain blood cell types and indicate the stage of differentiation (Zola et al., 2005, Weissman and Shizuru, 2008). For example CD133 has been associated with immature "naive" stem cells (Suzuki et al., 2010), CD34 has been used as a hematopoietic stem cell marker (Weissman and Shizuru, 2008, Kim et al., 1999) whilst other CD molecules have been associated with certain specialized hematopoietic cells (table 2).

Haematopoiesis takes place in different sites throughout development. At early embryonic stages, it occurs in the blood islands of the yolk sac, and later it moves to the fetal liver. Around the time of birth, the hematopoietic stem cells from the fetal liver colonize the bone marrow which will eventually becomes the main site of Haematopoiesis throughout adult life (Palis and Yoder, 2001, Zon, 1995).
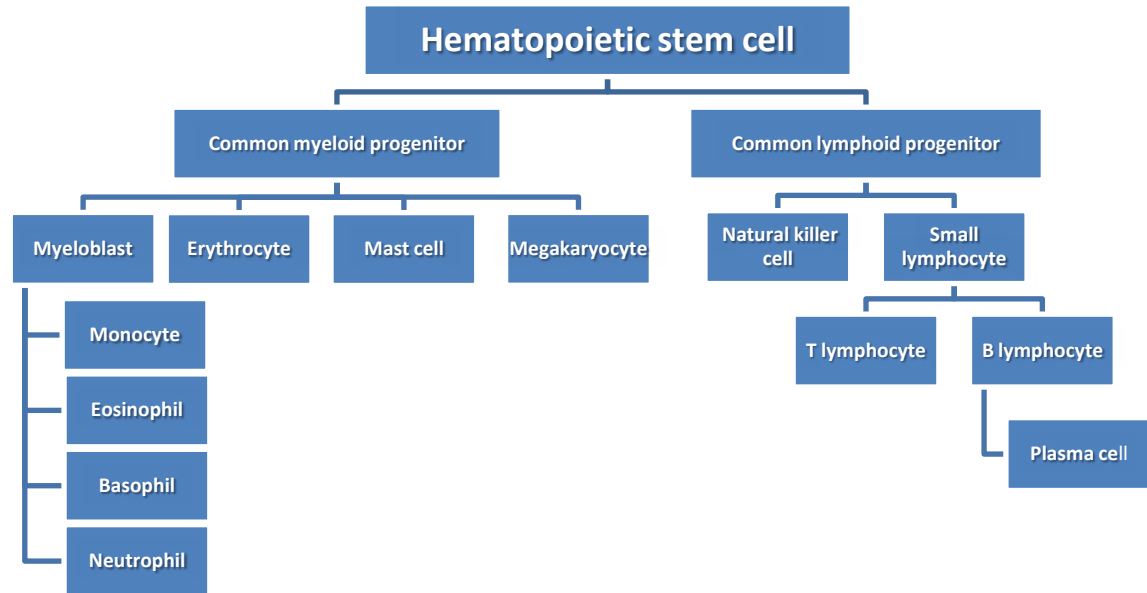
Figure 6. Human Haematopoiesis. Multipotent hematopoietic stem cells give rise to all lineages of blood cells. All hematopoietic specialized cells are derived from either myeloid or lymphoid progenitors which in turn are derived from the multipotent hematopoietic stem cells. Image adapted from http://commons.wikimedia.org/wiki/File:Hematopoiesis_simple.png.

Table 1. Hematopoietic cell types and their functions

Cell type		Function
Lymphocytes	Natural killer cells	Fighting tumor and viral infected cells
	T cells	Involved in adaptive immune response and cell-mediated immunity
	B cells	Involved in humoral immune response
Erythrocytes (red blood cells)		Carry oxygen to the body organs and tissues
Monocytes		Involved in initiation of immune reaction in response to inflammation signals. Production of macrophages and dendritic cells.
Macrophages		Involved in innate and adaptive immunity.
Dendritic cells		Antigen-presenting cells. Involved in both innate and adaptive immunity
Neutrophil		Fast immune response to microbial inflammations
Basophil		Immune response to inflammations related to allergies and asthma
Eosinophils		Combating multicellular parasites and also involved in fighting against viral infections
Thrombocytes (platelets)		Maintenance of hemostasis
Sources: (Schroeder, 2009, Hayashi et al., 2003, Hogan et al., 2008, Chaplin, 2010, Beaulieu and Freedman, 2010).		

Table 2. List of human CD antigens, cells on which they are expressed and brief description of their hematopoietic associated functions

Human CD antigen	Cell types expressed on	Function
CD2	T cells, thymocytes, Natural killer cells	Adhesion molecule and T cells activation
CD3	T cells, thymocyte subset	Associated with the T cell antigen receptor. Involved in T cell receptor (TCR) surface expression and signal transduction
CD4	Thymocyte subsets, monocytes, macrophages	MHC class II co-receptor, HIV receptor, T cell differentiation and activation
CD7	T cells, Natural killer cells	T cells interaction and activation
CD14	Monocytes, Macrophages	Act as a co-receptor for detection of bacterial lipopolysaccharide (LPS). Involved functionally in the innate immune system.
CD16	Neutrophils, Natural killer cells, Macrophages	Component of low affinity Fc receptor and mediate phagocytosis
CD19	B cells	B cells activation and differentiation
CD24	B cells, Granulocytes	Binds P-selectin
CD33	Myeloid progenitor cells, Monocytes	Regulation of human lymphoid and myeloid cells.
CD34	Hematopoietic stem/progenitor cells	Stem cell marker and adhesion
CD36	Platelets, Monocytes	Adhesion, phagocytosis
CD38	Variable levels on majority of leukocytes	Cell adhesion, signal transduction and calcium signaling.
CD45	Hematopoietic cells	Tyrosine phosphatase, enhance signals through T cells and B cells receptors
CD56	Natural killer cells and T subset	Adhesion
CD66b	Granulocytes	Activation and adhesion
CD133	Hematopoietic stem cell subset	Adhesion
CD235a (Glycophorin A)	Erythrocytes	Unknown

Sources : (Sempowski et al., 1999, McGuckin et al., 2007, Kitchens, 2000, Wilkins et al., 2003, Chen et al., 2004, Malavasi et al., 2008, Hernandez-Caselles et al., 2006, Yoon et al., 2007)

Umbilical cord blood has been shown to contain a population of hematopoietic stem cells (HSCs) at different stages of hematopoietic commitment characterized by their differential expression of hematopoietic antigens CD133, CD34 and CD45 according to a model previously described (figure 7) (McGuckin et al., 2003, McGuckin et al., 2007). It has been shown that cord blood hematopoietic stem cells can be selectively induced into specific hematopoietic lineages *in-vitro* including erythroid, megakaryocytic and monocytic lineages (Felli et al., 2010).
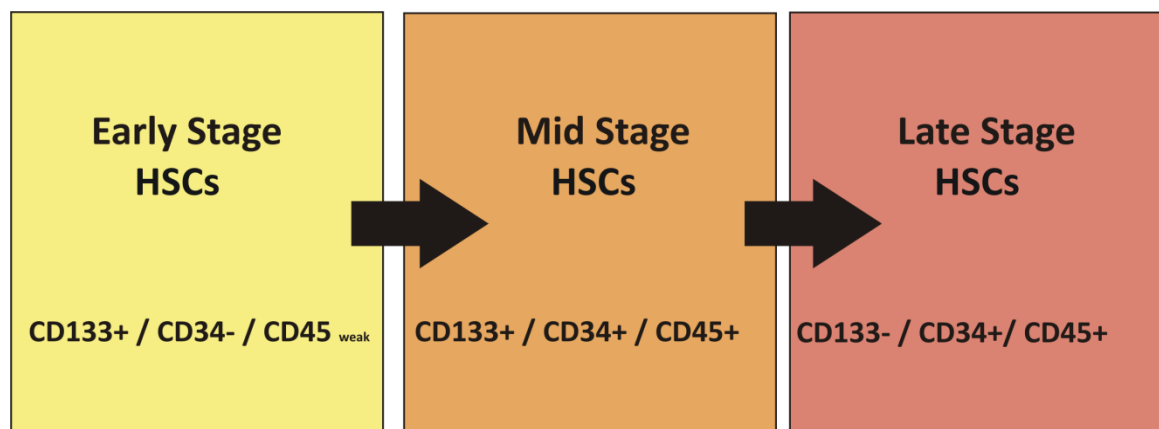
Figure 7. Subtypes of hematopoietic stem cells and their surface CD antigens. Differentiation of HSCs occurs in three sequential stages, each defined with specific surface antigen expression. At the early stage, the HSCs express CD133 while CD45 expression is weak and CD34 is absent. At the Mid stage, the HSCs express all the three antigens, CD133, CD34 and CD45. At the late stages of differentiation, HSCs express CD34, CD45 but not CD133. Image adapted from (McGuckin et al., 2007)**.**

- **Multipotent non-hematopoietic stem cells**

A unique population of multipotent non-hematopoietic stem cells has been identified in umbilical cord blood. These stem cells are small in size and exist at low density in cord blood and are negative for the major hematopoietic marker CD45 (Kucia et al., 2007, McGuckin et al., 2005, Zhao et al., 2006). This population of cells has been shown to express transcription factors normally expressed by embryonic stem cells including pluripotency key players OCT4, SOX2 and NANOG. In addition they expressed specific surface markers which have been used previously to characterize human embryonic stem cell lines. These markers include state-specific embryonic antigens, SSEA-3 and SSEA-4 in addition to tumor rejection antigens TRA1-60 and TRA1-80 (McGuckin et al., 2005, Kucia

et al., 2007, Zhao et al., 2006, Adewumi et al., 2007, Inamdar et al., 2009). These stem cells have been shown to differentiate into various cell types representing the three germ layers. Many groups have reported successful neural induction of cord blood stem cells representing ectodermal commitment (Ali et al., 2009, Zangiacomi et al., 2008, Habich et al., 2006), whilst other groups have successfully differentiated cord blood naïve stem cells into endodermic lineages including hepatic and pancreatic cells (McGuckin et al., 2005, Denner et al., 2007), and other groups have reported the successful generation of endothelial cells from cord blood naïve stem cells representing the mesodermal lineage (Senegaglia et al., 2010, Ma et al., 2006).

Due to their marker expression profile, McGuckin et al. (2005) named these cells cord blood derived embryonic-like stem cells (CBEs) while Kucia et al. (2007) named them very small embryonic-like stem cells (VSELs). Other groups have also named them cord blood pluripotent stem cells (Leeb et al., 2010, Kogler et al., 2004, Harris and Rogers, 2007). Besides the confusion caused by the introduction of different names and acronyms for these cells (due to the absence of standardized criteria for naming them,) the use of terms such as "embryonic-like" and "pluripotent" to describe these cells might be inaccurate and misleading. The term "embryonic-like" was given to the cells based on their expression of so-called embryonic stem cell markers such as OCT4, the main pluripotency key player in embryonic stem cells (Buitrago and Roop, 2007). It has been shown that OCT4 has two splice variants OCT4A and OCT4B that differ only in their N terminals whilst having identical C terminals. The splice variants have been shown to have different temporal and spatial expression patterns. Whilst OCT4A was expressed mainly in the nuclei of human embryonic stem cells, OCT4B was detected in many different types of differentiated cells (Cauffman et al., 2006, Lee et al., 2006). McGuckin et al. (2005), Kucia et al. (2007) and Zhao et al. (2006) have all used OCT4 antibodies that were not specific for the OCT4A variant in their studies, and such antibodies have been shown to give positive results on mature hematopoietic cells isolated from adult human peripheral blood (Zangrossi et al., 2007). Therefore, drawing conclusions based on such results could be inaccurate and misleading.

Using the term "pluripotent" to describe the differentiation potential of such cells based on the expression profiles of certain markers might not be appropriate even if the cells have been shown to differentiate into some but not all cell types representing the three germ

layers. The reason for this is that cord blood stem cells do not form teratomas after transplantation in SCID mice, which as mentioned before is a unique characteristic of pluripotent stem cells. These stem cells, therefore do not satisfy the current criteria for defining pluripotent stem cell (Sangeetha et al., 2010).

Hence to avoid confusion, in this thesis these cells are termed "non-hematopoietic multipotent stem cells" which defines their differentiation potential and distinguishes them from cord blood hematopoietic stem cells. Such terminology would avoid the inappropriate linking between these cells and embryonic stem cells and better describe their cell biology.

- **Mesenchymal stem cells (MSCs)**

Mesenchymal stem cells (MSCs) are a multipotent stem cell population found originally in the bone marrow (Short et al., 2003, Pittenger et al., 1999). These cells have demonstrated the ability of differentiation into osteogenic, adipogenic and chondrogenic lineages as well as non-mesodermal lineages including neural and hepatic lineages (Pittenger et al., 1999, da Silva Meirelles et al., 2006, Black and Woodbury, 2001, Krause et al., 2001). MSCs have been also isolated from umbilical cord blood (Erices et al., 2000, Kern et al., 2006, Bieback et al., 2004). Umbilical cord blood derived MSCs show high morphological and molecular similarities to bone marrow derived MSCs including the lacking of hematopoietic surface antigens CD133, CD34 and CD45 (Erices et al., 2000, Lee et al., 2004, Bieback et al., 2004, Lindner et al., 2010). Although the frequency of isolating MSCs from umbilical cord blood has been shown to be very low compared to the bone marrow, cord blood derived MSCs demonstrate higher proliferation capabilities than bone marrow MSCs (Bieback et al., 2004).

1.5.4 *Stem cells of choice: Why umbilical cord blood stem cells?*

There has been great debate on the stem cell source of choice for research and clinical applications. Embryonic stem cells, the least committed stem cells, have been shown to have high proliferation and extensive differentiation capacities which make them a powerful research platform for studying differentiation pathways and lineage commitment of stem cells (Thomson et al., 1998, Kogler et al., 2004). Nevertheless, when considering potential clinical applications, embryonic stem cells have shown some major limitations. Embryonic stem cells have high tumorigenic characteristics which might limit and delay any potential clinical use (Ben-David and Benvenisty, 2011). Further to this, embryonic

stem cells often lack the proper imprinting patterns and regulation of certain genes which might lead to spontaneous uncontrolled differentiation and developmental abnormalities (Sapienza, 2002). It has also been found that embryonic stem cells increase their immunogenicity by gaining human leukocyte antigens (HLA) during and after differentiation, which might increase the risk of rejection (Swijnenburg et al., 2008, Swijnenburg et al., 2005, Kofidis et al., 2005). Nevertheless, the ability to generate pluripotent stem cells from the patient's own cells (iPS cells) should offer an alternative to bypass this limitation. Moreover, the isolation of embryonic stem cells from the inner cell mass of the blastocyst involves the destruction of an embryo which itself creates ethical, religious and political problems.

Umbilical cord blood offers an alternative source of stem cells with both research and clinical advantages over other sources of stem cells. Moving toward effective clinical applications requires a readily abundant supply of stem cells to provide the needed amounts of stem cells. With the global birth rate reaching 200 millions/year, umbilical cord blood can be considered as one of the most abundant sources of stem cells (McGuckin et al., 2006, McGuckin et al., 2008). In addition, unlike embryonic stem cells, umbilical cord blood stem cells collection does not raise any ethical or religious concerns which makes it more appealing to both the research and clinical fields (Ballen et al., 2008, McGuckin et al., 2006, Watt and Contreras, 2005).

Umbilical cord blood stem cells also show a number of advantages over adult stem cells sources like bone marrow. The collection of umbilical cord blood stem cells is a safe and non-invasive procedure, unlike the collection of adult stem cells from bone marrow (McGuckin et al., 2006). Moreover, umbilical cord blood stem cells occupy an intermediate age stage between embryonic stem cells and adult stem cells, which leads to higher proliferating potential and longer telomeres than other adult stem cells (Pipes et al., 2006, Slatter and Gennery, 2006, Kim et al., 1999).

Allogenic transplantation with adult HSCs is regarded as a life-saving procedure in the treatment of severe hematological diseases such as hematopoietic malignancies, bone marrow failure syndromes and hereditary immunodeficiency disorders (Brunstein and Wagner, 2006). Yet, this procedure is limited by the availability of suitable HLA-matched

donors (Tse and Laughlin, 2005). Due to the immature HLA status of umbilical cord blood cells, transplantation with cord blood shows better tolerance for HLA-mismatching in comparison with adults HSCs (Liu et al., 2004). This unique feature of cord blood allows the safe use of unrelated and HLA-mismatched donor samples when HLA-identical donors are not available, thus providing the clinicians with alternative effective therapeutic options (Ringden et al., 2008, Slatter et al., 2006). Furthermore, transplantation with HLA-mismatched cord blood samples shows a lower risk of graft-versus-host diseases (GvHD) in comparison with bone marrow transplantation. This is also attributed to the fact that the cells transplanted from umbilical cord blood are more naïve and have lower (HLA) protein expression than adults stem cells including bone marrow stem cells (Fasouliotis and Schenker, 2000, Ringden et al., 2008, Slatter and Gennery, 2006, Mochizuki et al., 2008). In addition, umbilical cord blood transplantation was shown to be associated with a lower risk of infection transmission in comparison with bone marrow transplantation (Behzad-Behbahani et al., 2005).

On the other hand, the low number of stem cells per cord blood unit represents a limitation that is associated with delayed engraftment of these cells into host targeted tissues (Tse and Laughlin, 2005, Stanevsky et al., 2009). However this obstacle has been tackled with the possibility of combining multiple cord blood units in order to increase the final transplanted cell dose resulting in improved engraftment and survival of the transplanted cells (Brunstein and Wagner, 2006, Stanevsky et al., 2009, Ringden et al., 2008, Slatter et al., 2006).

Another advantage of umbilical cord blood is the ability to store and cryo-preserve cord blood units in cord blood banks for future use. This feature provides clinicians and patients with an immediate and abundant supply of cord blood units for transplantation. It also increases the chance of finding the right HLA-matched units for patients requiring allogeneic transplantation. Many cord blood banks have been established in the U.K, France and many other countries worldwide for such purposes (Solves et al., 2008, Lee et al., 2007, McGuckin et al., 2006, Watt and Contreras, 2005).

Umbilical cord blood has added advantages over other sources of stem cells (table 3), highlighting its potential as a promising therapeutic tool for many diseases and disorders for which the current form of treatment is inadequate.

Table 3. Summary of advantages and disadvantages of different stem cell sources.

	Advantages	Disadvantages
Embryonic stem cells	• Pluripotency: can differentiate into any cell type in the body. • Serve as a strong platform for pluripotency, developmental and lineage commitment studies.	• Ethical, religious and political concerns. • High chance of transformation into cancer cells (teratomas). • Cells instability *in-vitro* due to lack of proper imprinting patterns.
Umbilical cord blood stem cells	• No ethical, religious or political controversies. • Collection procedure is totally safe and non-invasive • Abundant supply. • Low viral contamination. • Ability to store cord blood units in cord blood banks. • No risk of teratoma formation. • Lower risk of graft-versus-host diseases (GvHD). • Tolerance of HLA-mismatching. • Higher proliferation capacity compared with adult stem cells.	• Limited number of stem cells per single cord blood unit.
Adult stem cells	• No ethical, religious or political controversies. • Effective in generation their tissue of origin. • No risk of teratoma formation. • Established clinical history.	• Often invasive collection procedure. • Limited cell numbers in human body tissues. • Limited differentiation capabilities. • Limited supply. • Limited availability of HLA-match donors.

1.5.5 *Neural potential of cord blood non-hematopoietic multipotent stem cells*

To evaluate the potential role of umbilical cord blood stem cells as a promising therapeutic tool for the treatment of neural diseases, extensive research has focused on the neural capability of these cells. Umbilical cord blood non-hematopoietic multipotent stem cells have shown high potential for neural differentiation *in-vitro*. The mononuclear fraction of umbilical cord blood which contains the non-hematopoietic multipotent stem cells has produced neuron-like cells expressing neuronal specific markers such as Neurofilament 200 (NF200), β-TUBULIN III and mature neuronal proteins including Neural Nuclei (NEUN) and post synaptic density protein 95 (PSD95) after controlled neuronal induction *in-vitro* (Ali et al., 2009, Neuhoff et al., 2007, Habich et al., 2006).

In order to further understand and analyze the neural differentiation potential and molecular pathways governing neural commitment in umbilical cord blood non-hematopoietic multipotent stem cells, scientists have utilized various techniques to purify this population of cells from cord blood hematopoietic cells and subject them to neural induction protocols *in-vitro,* followed by various neural characterization assays to evaluate the cellular neural potential. Several groups have purified cells that are negative for hematopoietic markers CD34 and CD45 and showed this purified cell population to be capable of producing neuron-like, astrocyte-like and oligodendrocyte-like cells characterized by their morphological features and specific proteins expression patterns including β-TUBULIN III as a neuronal marker, S100β as a marker of astrocytes and galactosylceramidase protein (GALC) as a marker of oligodendrocytes (Buzanska et al., 2006b, Jurga et al., 2006, Buzanska et al., 2002, Domanska-Janik et al., 2006). Other groups utilized negative depletion strategies (which will be further discussed in chapter 3) to enrich umbilical cord blood multipotent non-hematopoietic stem cells and show that these cells exhibit several neural morphologies including neuron-like, astrocyte-like and oligodendrocyte-like morphologies and express specific proteins for each neural subtype after *in-vitro* neural induction protocols (McGuckin et al., 2008, Chua et al., 2009).

Other research groups have investigated the functional properties of cord blood-derived neuron-like cells. They have shown that cord blood derived neuron-like cells expressed many functional neurotransmitter receptors and ion channels that are involved in neuronal signal transmission and the generation of an action potential (Zangiacomi et al., 2009, Sun et al., 2005, Buzanska et al., 2006a). Several other groups have demonstrated that cord

blood derived neurons can generate and transmit action potential in a similar pattern to that of primary cultures of neurons (Sun et al., 2005, Jurga et al., 2009). Other functional neuronal properties such as calcium-influx have also been confirmed in cord blood derived neuron-like cells (Zangiacomi et al., 2008).

Umbilical cord blood multipotent non-hematopoietic stem cells have demonstrated neural differentiation capabilities *in-vitro* including the generation of functional neurons capable of firing and transmitting action-potential signals in a similar manner to those of primary cultures of neurons. The neural differentiation capability of umbilical cord blood stem cells highlights its potential as a promising therapeutic tool for neural injuries and disorders for which current modes of therapies is inadequate.

1.6 The human nervous system

From a general perspective, the nervous system can be viewed as the control center of the human body which manages and coordinates functions of all different organ systems. The nervous system is also responsible for sensing and responding to different environmental stimuli, it receives information from sensory receptors, processes these information signals, determines a proper response and then sends commands to the appropriate destination to initiate the response. The nervous system is also responsible for sophisticated functions such as memory, learning, thinking and social communication.

The nervous system is the system that defines us as human beings by providing us with the ability to think, learn and communicate in a way that distinguishes us from all other species. It is one of the most complex organ systems anatomically and physiologically in the human body. The human central nervous system (CNS) forms the core of the entire nervous system. It is composed of two main structures, the brain and the spinal cord. The CNS is regarded as one of the most complex systems in the human body, from both anatomical and functional levels.

1.6.1 *The human adult brain*

The brain forms the core of the CNS. It resides in the head enclosed in the thick bone of the skull called the "cranium". The brain is suspended in cerebrospinal fluid (CSF) which is a clear bodily fluid that cushions the brain providing it with extra protection in addition to providing the brain with nutrients (Di Terlizzi and Platt, 2006, Johanson et al., 2008, Bear et al., 2007).

The human adult brain weighs an average of 1.5 kilograms and hosts about 100 billion neurons organized in distinct and complex functional and anatomical structures (Herculano-Houzel, 2009, Noctor et al., 2007, Doetsch, 2003a, Azevedo et al., 2009).

A medial cross section of the human adult brain reveales many of its deep parts and structures (figure 8). It shows the "brainstem" which consists of the "thalamus", "hypothalamus", "tectum","tegmentum", "pons" and "medulla" (Bear et al., 2007). These structures carry out several crucial functions. The "thalamus" is the largest structure of the "diencephalon" and is located on the top of the brainstem close to the center of the brain. It is involved in relaying sensory and motor inputs to the "cerebral cortex" (Guillery and Sherman, 2002). Beneath the "thalamus" lies the "hypothalamus" which is involved in the

control of the autonomic nervous system (ANS), the pituitary gland and other processes including body temprature, hunger and thirst (Chiamolera and Wondisford, 2009, Lopaschuk et al., 2010, Suzuki et al., 2010a, Nillni, 2010, Mebis and van den Berghe, 2009). Beneath the "thalamus" lies the midbrain which consists of the "tectum" and "tegmentum". The 'tectum" which is located in the dorsal region of the midbrain controls auditory and visual responses (Cappe et al., 2009, Bear et al., 2007). The "tegmentum" which forms the floor of the midbrain controls motor functions and is also involved in regulation of consciousness, mood, pleasure and pain (Taube, 2007, Iniguez et al., 2010, Sukhotinsky et al., 2006). The lower part of the brainstem consists of the "pons" and "medulla" which are parts of the hindbrain. The "pons" which is ventral to the "cerebellum" and above the "medulla" forms a bridge between the "cerebrum" and the "cerebellum" by relaying information signals between these two structures (Bear et al., 2007, Nieuwenhuys et al., 2008). Beneath the "pons" lies the "medulla" which forms the bottom part of the brainstem. The "medulla" hosts the cardiac, respiratory and vasomotor centers (Goodchild and Moon, 2009, Allen et al., 2009, Horiuchi et al., 2004). The posterior part of the bottom of the brain is formed by the "cerebellum" which is derived from the hindbrain. It is attached to the brainstem via the "pons". The "cerebellum" plays a key role in coordinating the body movements, balance and equilibrium (Bastian, 2006, Bear et al., 2007, Schmahmann, 2004).

The largest component of the brain is the "cerebrum" which is divided into two symmetrical left and right "cerebral hemispheres" residing in the lateral regions of the brain above the brainstem. The outside of each cerebral hemisphere is covered by a layer of folded "grey matter" called the "cerebral cortex". Deep inside the "cerebrum" lies a compact cluster of neurons forming the "basal ganglia" which is involved in learning and memory (Da Cunha and Packard, 2009, Packard and Knowlton, 2002). Medial to the lateral ventricle of the forebrain rests a specialized piece of cortex known as the "hippocampus" that is involved in long term memory functions (Bear et al., 2007, Axmacher et al., 2010). Collectively, the "cerebrum" is involved in controlling voluntary movement, sensory perception, learning, communication and memory (Bear et al., 2007, Zilles and Amunts, 2010, Nieuwenhuys et al., 2008).
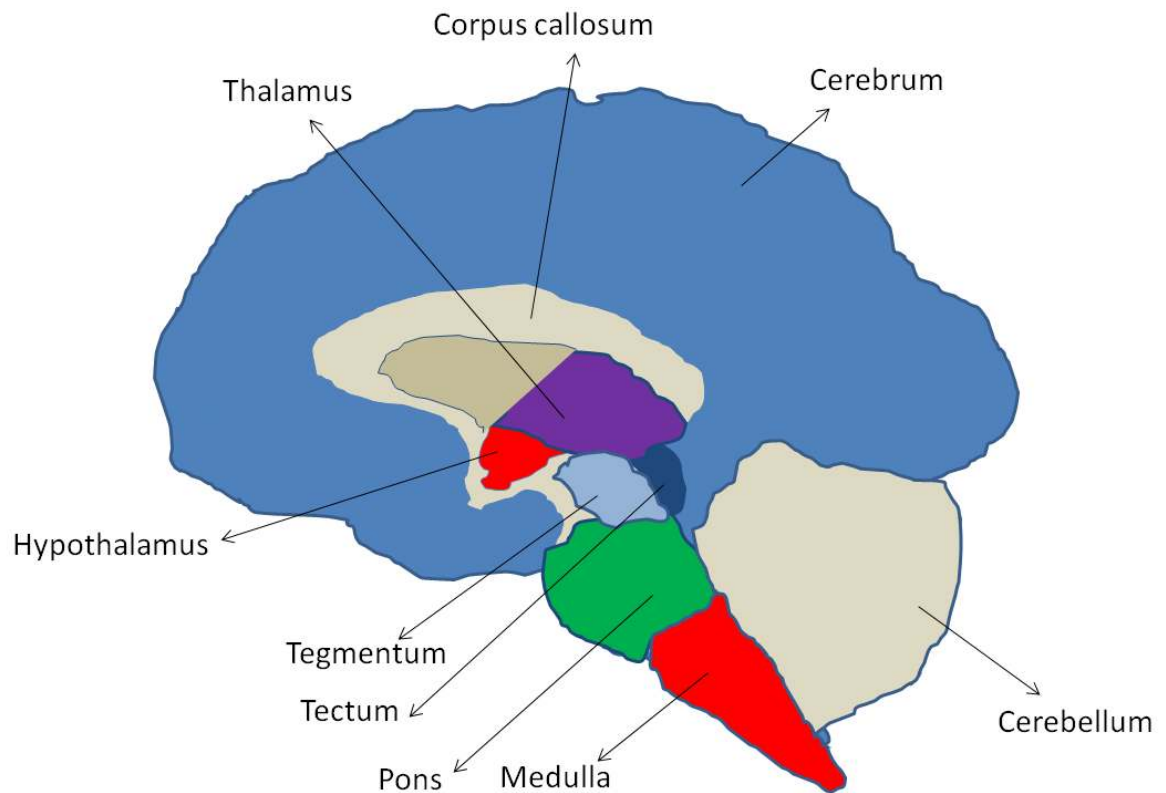
Figure 8. Cross section of human adult brain and associated structures. Image adapted from (Bear et al., 2007).

1.6.2 *The human nervous system: cellular units*

Each organ in the human body is made up of specific cells that share specified features and performing certain tasks and functions. The human nervous system is built up of unique types of cells. These cells are classified into two main categories: neurons, which are specialized neural cells defined by their capability to generate and transmit electric signals in the form of action potentials, and glial cells that do not directly transmit signals but have a supportive role for neural cells (figure 9). The human adult brain consists of around 100 billion neurons and 10 times more glial cells (Noctor et al., 2007, Nishiyama et al., 2005, Doetsch, 2003).
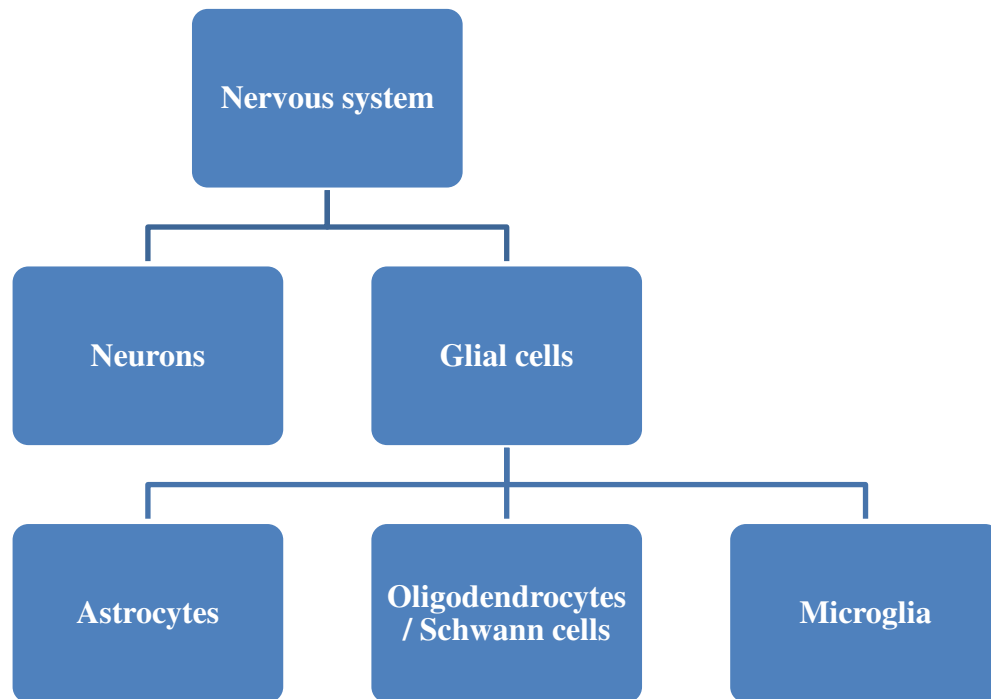
Figure 9. Cellular components of the nervous system. Neurons and glial cells are the building blocks of the nervous system.

Each cellular component of the nervous system has its unique roles, tasks and features. The collaborative regulated interaction between neurons and glial cells is essential for the nervous system to function properly (Fields and Stevens-Graham, 2002, Allen and Barres, 2009, Salmina, 2009). Below is a description of the main cellular component of the nervous system, the neurons. For information about glial cell, refer to table 18 in Appendix.I.

1.6.2.1 Neurons: the main cellular component of the nervous system

Neurons are the main functional and cellular component of the nervous system. They are defined as electrically excitable cells capable of processing and transmitting information signals in the form of action potentials. Neurons also transmit signals chemically through synaptic transmission from one neuron to the other (figure 10) (Bear et al., 2007, Allen and Barres, 2009, Barnett and Larkman, 2007).
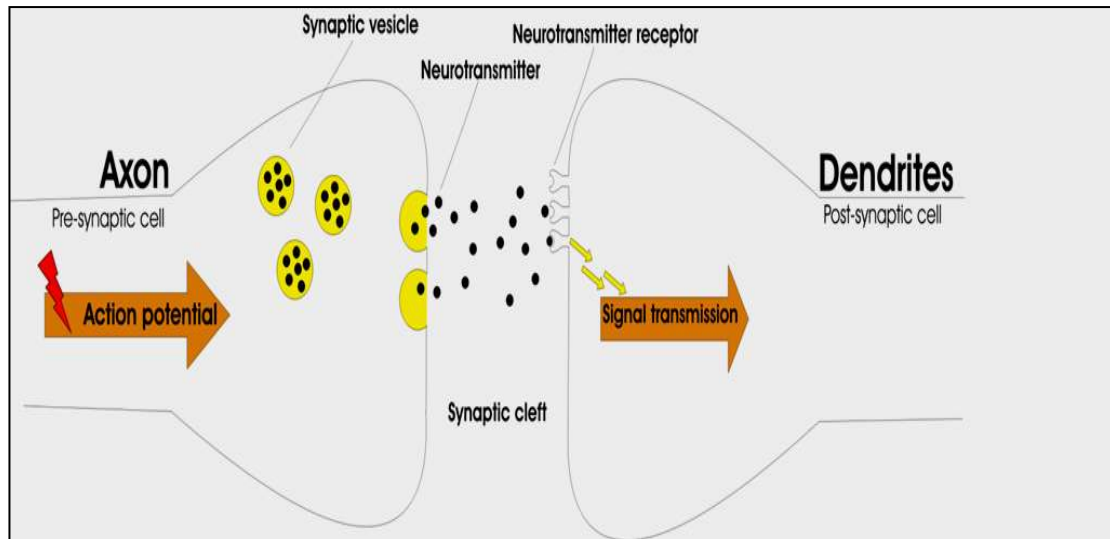
Figure 10. Neuronal synapse. Neurons transfer information between each other using neurotransmitters at the synapse. The action potential in the axon of the pre-synaptic cell triggers the release of neurotransmitters into the synaptic cleft from the synaptic vesicles. The neurotransmitters bind and activate their specific receptors on the dendrites of the post-synaptic cell. Depending on the type of the neurotransmitter (for example excitatory/inhibitory), specific response from the post-synaptic cell will be initiated (fire action potential or inhibit action potential firing).

There are many different types of neurons and understanding how each single neuron contributes functionally in the nervous system is quite difficult. However, dividing neurons into subgroups/categories based on either morphological features or functional properties can help ease the complexity of this issue by understanding the properties of each subgroup and its functional involvement in the nervous system (Bear et al., 2007, Bota and Swanson, 2007).

Neurons can be classified based on morphological features including the number of neurites, morphology of dendrites and axon length. From a functional perspective, neurons can be classified based on their functional connections into: "sensory neurons" that deliver signals from sensory parts of the body to the CNS (Duchamp-Viret et al., 2003, Bear et al., 2007). Neurons that deliver signals from the CNS to muscles and cause muscle contraction are classified as "motor neurons" (Lin et al., 1998, Bear et al., 2007). Neurons that are not sensory or motor neurons are termed "interneurons" (Ascoli et al., 2008, Markram et al., 2004, Bear et al., 2007).

Neurons can also be classified based on the neurotransmitter they produce and utilize (table 4) (note: only common neurotransmitters are listed here)

Table 4. Classification of neurons based on neurotransmitter production.

Neuron	Description
Glutamatergic neurons	These neurons utilize glutamate in signal transduction. Glutamate, a non-essential amino acid, is the principal and most common excitatory neurotransmitter in the CNS (Kaltschmidt et al., 1995, Meldrum, 2000, Greenamyre, 1986, Foster and Kemp, 2006). Neurons have 3 types of glutamate receptors AMPAR, NMDAR and mGluRs. The AMPAR and NMDAR complexes have ion channels which when activated allow Ca^{2+} and Na^+ into the cell mediating fast excitatory synaptic transmission (Nowak et al., 1984, Foster and Kemp, 2006). The mGluRs are coupled to G-protein complexes which when activated release Ca^{2+} from internal calcium storage involved in modulating synaptic transmission and mediating intracellular signal transduction (Elmslie, 2003, Mattson, 2008, Ryglewski et al., 2007, Meldrum, 2000, Palmada and Centelles, 1998, Foster and Kemp, 2006).
GABAergic neurons	These are the neurons that manufacture and utilize GABA as their neurotransmitter. GABA is synthesized in these neurons from glutamate through a chemical reaction mediated by the enzyme glutamate decarboxylase 1 (GAD1) (Foster and Kemp, 2006). GABA is the most abundant inhibitory neurotransmitter in the CNS (Zeilhofer et al., 2009, Foster and Kemp, 2006). GABA has two types of receptors, GABA A receptors and GABA B receptors. GABA A receptors are ligand-gated ion channels while GABA B receptors are G-protein coupled receptors (Enna and Möhler, 2007, Foster and Kemp, 2006).
Cholinergic neurons	These neurons manufacture and utilize acetylcholine (Ach) as neurotransmitter which is the major neurotransmitter at neuromuscular junctions (Zholos, 2006), while in the CNS it is more associated with anti-excitatory and modulary actions (Gulledge et al., 2009, Alkondon and Albuquerque, 2004, Gulledge et al., 2007). Ach has two types of receptors nAChR and mAChR (Gahring and Rogers, 2005). The nAChR are fast response ligand-gated ion channels that are permeable to ions such as Na^+, K^+ and Ca^{2+} and stimulated by nicotine and Ach. The mAChR are metabotropic receptors (G protein-coupled) stimulated by muscarine and Ach (Gahring and Rogers, 2005, Ishii and Kurachi, 2006).

Dopaminergic neurons	These neurons manufacture dopamine, a monoamine, and utilize it as their neurotransmitter. They biosynthesize dopamine from L-Tyrosine which is first converted to L-DOPA via the enzyme Tyrosine hydroxylase (TH), the rate limiting enzyme. Then the L-DOPA is converted to dopamine via the enzyme dopa-decarboxylase (DDC) (Borges et al., 2002, Lee et al., 2008). Dopamine receptors are metabotropic G protein-coupled receptors (Girault and Greengard, 2004).
Serotonergic neurons	Serotonergic neurons are the neurons that manufacture serotonin, a monoamine, and utilize it as their neurotransmitter. These neurons biosynthesize serotonin from L-tryptophan through a sequence of two successive reactions catalyzed by tryptophan hydroxylase (TPH), the rate limiting enzyme, and amino acid decarboxylase (DDC). Serotonin has two types of receptors: metabotropic G protein-coupled receptors and ligand-gated ion channels (Hannon and Hoyer, 2008, Bockaert et al., 2006). The receptors exert both excitatory and inhibitory neurotransmission effects on target neurons when activated (Saruhashi et al., 1994, Best and Regehr, 2008).

- **Neuronal action potential**

Action potentials are generated and utilized by neurons to transfer information signals from once place to the other in the nervous system. It is defined as a short fluctuation of membrane potential as a result of rapid opening and closing of voltage-gated ion channels. It propagates along the neuron's axon until reaching its terminals transferring information signals (Bear et al., 2007). In addition to neurons, action potentials are also generated by other types of cells such as muscle cells which utilize the action potential in muscle contractions (Williams and Ratel, 2009) and endocrine cells where it is involved in the release of hormones such as insulin (MacDonald and Rorsman, 2006).

In neurons, the action potential has a distinct pattern composed of certain parts as shown on the oscilloscope graph (figure 11). The first part is called the "rising phase" where the membrane potential increases rapidly above the resting potential (-70 mV) in response to a rapid depolarization of the membrane until it reaches a peak value around (40 mV). The part where the membrane potential has a positive value above (0 mV) is called the "overshoot phase". The "falling phase" of the action potential is a phase caused by a rapid repolarization of the membrane until the membrane potential reaches a value less than the resting potential in a phase called the "after-hyperpolarization". The membrane potential will gradually increase until the resting potential is once again restored. The action potential is a very rapid process; the entire process lasts for about 2 milliseconds (Barnett and Larkman, 2007, Bear et al., 2007, Nieuwenhuys et al., 2008).
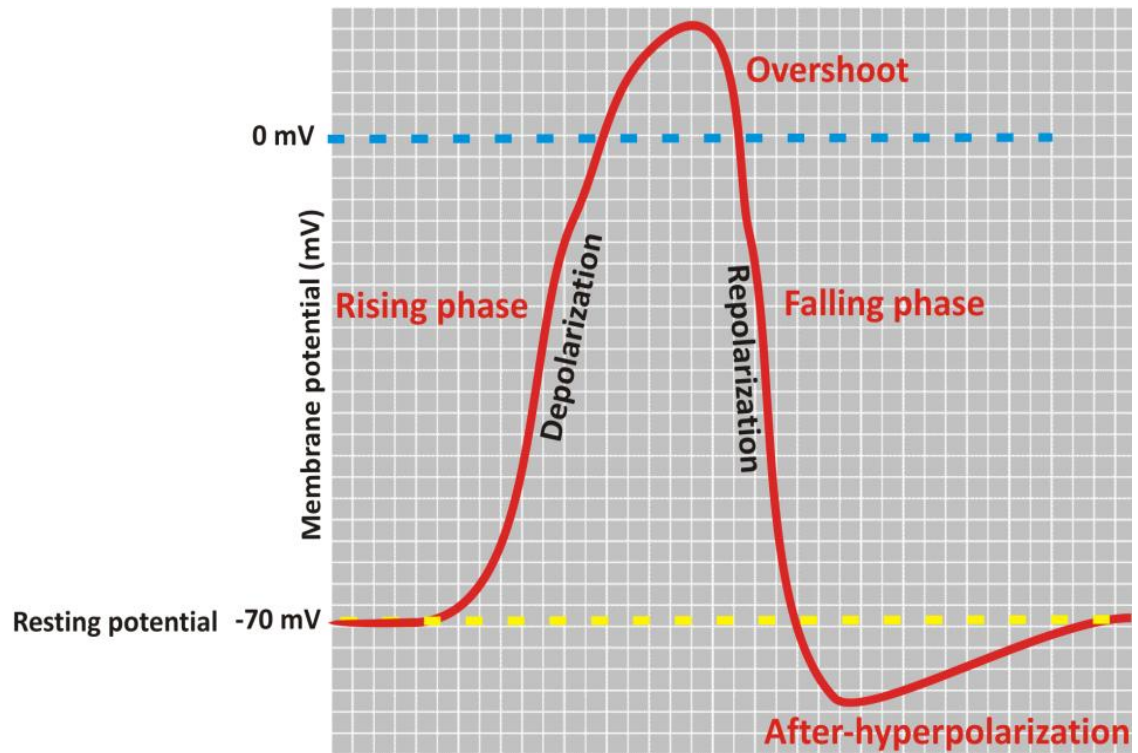
Figure 11. Neurons action potential. The depolarization phase is stimulated by the movement of Na$^+$ into the cell through the Na$^+$ voltage-sensitive ion channels until the K$^+$ voltage-sensitive gated channels open and initiate the repolarization phase mediated by the movement of K$^+$ outside the cell. Image adapted from (Bear et al., 2007).

The generation and propagation of an action potential is a highly systematic process. The membranes of neuronal axons host a high density of voltage-sensitive ion channels. The rapid movements of ions including Na$^+$ and K$^+$ across the membrane in response to changes in membrane potential forms the basis of the action potential mechanism.

The first phase of the action potential, which is characterized by rapid depolarization of the membrane, is mediated by rapid movement of Na$^+$ into the cell through the Na$^+$ voltage-sensitive ion channels. This event can be triggered in various ways, for example, in sensory neurons, a stretch of nerve fiber in skin by a mechanical force is sufficient to open special Na$^+$ channels allowing Na$^+$ into the cell (Bear et al., 2007). In the CNS, synaptic events which include the release of neurotransmitters are integrated into signals sufficient to initiate membrane depolarization (Barnett and Larkman, 2007). The initial depolarization which is mediated by an increase in membrane permeability to Na$^+$ results in an increase in the membrane potential which induces the opening of a small number of Na$^+$ voltage-

sensitive ion channels allowing more Na^+ to enter the cell since the voltage inside the cell is more negative than the voltage outside the cell. This influx of Na^+ results in what is called the "local depolarizing response" of the membrane. The influx of Na^+ continues to add to the depolarization of the membrane until it reaches a threshold (around -45 mV) which triggers rapid opening of more Na^+ voltage-sensitive ion channels adjacent to the previously opened channels and causing the rapid depolarization phase which overshoots the membrane potential above (0 mV) (Bear et al., 2007, Barnett and Larkman, 2007). The increased potential of the membrane triggers the opening of K^+ voltage-sensitive gated resulting in rapid movement of K^+ outside the neuron and causing the repolarization of membrane which would bring the membrane potential back to a negative value. The systematic termination of the depolarization and initiation of repolarization phases are explained by the properties of Na^+ and K^+ voltage-sensitive ion channels. Na^+ voltage-sensitive channels stay open for a short period of time after activation (about 1 millisecond) before they closed (inactivate). They will remain close (inactive) until the membrane potential returns back to a negative value near the threshold. As the K^+ voltage-sensitive channels continue to open and the Na^+ voltage-sensitive channels become inactive/closed, the membrane potential is driven back toward the resting potential. The activated K^+ channels start to close as the membrane potential hyperpolarizes, at this phase the membrane potential becomes slightly more negative than the resting potential before it gradually restores the resting potential (Barnett and Larkman, 2007, Bear et al., 2007).

Depolarization of a certain area on the axon's membrane by the action potential produces local currents that depolarize the adjacent membrane. If these currents are sufficient enough to depolarize these areas to the threshold, the action potential will propagate down the axon until it reaches the axon's terminal and initiates synaptic transmission.

1.7 Central nervous system: injuries and disorders

Beside its complex anatomy and physiology, the central nervous system is one of the most delicate systems in the entire human body. CNS injuries and disorders are one of the most dangerous and fatal health conditions worldwide. In a report published by the World Health Organization (WHO), CNS related injuries and disorders including Stroke and Alzheimer's disease (AD) were reported among the leading causes of death globally (http://www.who.int/mediacentre/factsheets/fs310_2008.pdf).

Despite the extensive efforts focused on developing treatments for CNS injuries and disorders, the current modes of therapy are inadequate and ineffective against such conditions. This highlights the need for an alternative approach to tackle this problem and offer effective treatments for CNS injuries and disorders.

1.7.1 *Neurodegenerative diseases: Alzheimer's and Parkinson's diseases*

Among the most devastating CNS disorders is a group of diseases collectively termed "neurodegenerative diseases". As the name implies, these diseases are caused and associated with progressive loss or death of neurons in the brain. Alzheimer's and Parkinson's diseases (AD and PD respectively) are among the most common examples of neurodegenerative diseases. Both diseases show late-onset (progress with age) and are caused by accumulation of toxic proteins leading to progressive loss of neuronal function and eventually neuronal death (Rubinsztein, 2006, Hashimoto et al., 2003).

- **Alzheimer's disease (AD)**

Alzheimer's disease (AD), the most common type of dementia, was described for the first time in 1906 by the German psychiatrist Alois Alzheimer and was named after him (Goedert and Spillantini, 2006). AD is a neurodegenerative disease that is associated with impaired cognitive skills including loss of memory (Goedert and Spillantini, 2006). According to Alzheimer's Disease International federation (ADI), in the United States alone, 5.3 million people have Alzheimer disease, 96% of these people are aged 65 and older (Maslow, 2010). In 2007, AD was reported as the sixth leading cause of death across all ages in the United States (Xu, 2009). These devastating figures demonstrate the seriousness of AD and reveal the current lack of effective therapies and treatments.

AD pathology is characterized by the presence of senile plaques, on the blood vessels and outside the neurons of the brain, composed mostly of amyloid-β, a 40-42 amino acid protein generated by the proteolytic cleavage of amyloid precursor protein (APP) (Neet

and Thinakaran, 2008, Goedert and Spillantini, 2006). These senile plaques have toxic effects on brain neurons which eventually lead to their death. The genetic basis of AD is very complex with many genes involved in the pathogenic pathway of the disease. Current understanding of the disease suggest that AD can be viewed as a consequence of the imbalance between amyloid-β production and clearance which leads to its accumulation outside the neurons and in the blood vessels of the brain triggering its neurodegenerative effect (Bettens et al., 2010, Goedert and Spillantini, 2006).

There are currently two classes of drugs for the treatment of AD approved by the US Food and Drug Administration (FDA), the cholinesterase inhibitors and the D-aspartic acid (NMDA) receptor antagonist memantine (Doody, 2003, Melnikova, 2007). Unfortunately, both classes of drugs have failed to either prevent or reverse the progression of the AD (Melnikova, 2007, Aderinwale et al., 2010).

While the number of deaths caused by other major causes of death including the number one cause of death, namely heart disease, has declined over the past few years in the United States, the number of deaths caused by AD continues to rise (Maslow, 2010, Xu, 2009). It has been estimated that by 2030 the number of people aged 65 and above with AD in the United States will reach 7.7 million individuals, that is more than a 50% rise (Maslow, 2010, Xu, 2009). Unless a major medical breakthrough provides successful and effective therapies to treat AD are found, the numbers of AD patients will continue to rise claiming more lives worldwide.

- **Parkinson's disease (PD)**

Parkinson's disease (PD) is named after the English apothecary James Parkinson who first described the disease in 1817. PD patients show various symptoms, most of them are associated with body motor functions, including resting tremor, bradykinesia, postural instability, gait difficulty and muscular rigidity (Thomas and Beal, 2007, Wood-Kaczmar et al., 2006). PD is the second most-common neurodegenerative disease in the Western world after AD, latest estimations revealed that in the United States alone, there are more than 1.5 million individuals affected with PD (Wood-Kaczmar et al., 2006, Thomas and Beal, 2007, Lesage and Brice, 2009). This devastating disease, which currently has no cure, is listed among the leading causes of death in the United States (Xu, 2009).

PD is a late-onset chronic neurodegenerative disorder characterized by selective degeneration and progressive death of brain's dopaminergic neurons and the presence of

Lewy bodies which are abnormal protein aggregates, in the surviving neurons in several brain regions (Thomas and Beal, 2007, Wood-Kaczmar et al., 2006).

The genetic basis of PD is very complex showing both dominant and recessive modes of inheritance (Lesage and Brice, 2009). A number of mutations in several genes have been shown to trigger and stimulate the pathogenic pathway of the disease which leads to the abnormal accumulation of α-synuclein-ubiquitin protein inside the neurons forming the Lewy bodies, a hallmark feature of PD (Lesage and Brice, 2009). This abnormal protein accumulation has a neurotoxic effect which leads to a progressive death of the neurons in the brain (Wood-Kaczmar et al., 2006, Lesage and Brice, 2009).

Current therapies for PD include administration of L-DOPA (which is transformed to dopamine in dopaminergic neurons), dopamine agonists and monoamine oxidase (MAO) B inhibitors. All these drugs are based on the concept of exogenous replacement of dopamine. Unfortunately, beside their significant side effects, none of these drugs are able to slow or treat the progression of the PD as their effect is limited to mild symptom relief (Schapira, 2005, Wood-Kaczmar et al., 2006).

1.7.2 *CNS injuries*

- **Neurovascular injuries: Stroke**

Stroke is defined as a sudden death of brain cells in a localized area due to disturbance in the blood supply to these brain areas. According to the World Health Organization (WHO), stroke is the second most common cause of death after heart disease and a major cause of disability worldwide (Donnan et al., 2008, Deb et al., 2010).

Stroke can be caused by either blockage or leakage of the blood supply to the brain tissues. Strokes caused by blockage are termed "ischemic strokes" which are the most common type of stroke. In ischemic stroke, the blood supply to a part of the brain is reduced, causing dysfunction and death of the neural tissues at this specific part. This reduction of blood supply is mostly caused by the underlying disease process of atherosclerosis, in which hardening and narrowing of the vessels promotes blood clotting and obstruction of blood flow. Blockage and reduction of blood supply to the brain tissues reduce the supply of oxygen and glucose to these tissues. Without sufficient amounts of oxygen and glucose supplied to the brain cells, these cells will stop functioning and die after a while leading to irreversible brain damage (Hall et al., 2009, Deb et al., 2010).

Stroke caused by leakage of blood from the blood vessels is termed "hemorrhagic stroke". This type of stroke occurs mostly in people with high blood pressures or in people with a congenital weakness in the blood vessels. This type of stroke occurs when the blood vessel is ruptured leading to bleeding within the brain tissue causing damage and death of the affected brain tissues. Hemorrhagic stroke is regarded as more dangerous than ischemic stroke because the pathogenic impact is not only caused by disrupted blood supply to the brain tissue but mostly because of irritant pressure symptoms and toxic effects of the released blood on surrounding brain tissue (Deb et al., 2010).

The symptoms of stroke vary depending on the affected area of the brain. The more brain regions affected, the more functions are affected and are likely to be lost, leading to various forms of disability and death. The irreversible damage and death of brain tissues caused by stroke underline its devastating outcomes (Deb et al., 2010, Donnan et al., 2008).

Currently, much of the efforts are focused on stroke prevention by altering life-style risk factors such as hypertension, smoking, physical inactivity and diet (Deb et al., 2010). Other serious stroke risk factors such as carotid stenosis and atrial fibrillation are managed and treated using anti-coagulants drugs such as aspirin and warfarin, or in some cases by surgical intervention, in order to minimize the likelihood of stroke occurrence (Deb et al., 2010, Mohr et al., 2001, Schwartz and Albers, 2010). However, when stroke occurs, therapeutic options become very limited. For acute ischemic stroke, the FDA has approved the use of thrombolysis with intravenous recombinant tissue plasminogen activator (tPA) but the effectiveness of this therapy is limited by stroke severity and the time of intervention after stroke occurrence (Deb et al., 2010, Katzan et al., 2004, Kimura et al., 2010). The damage and death of brain tissues caused by stroke is an irreversible process and responsible for the severe outcomes including death. Current treatments and therapies are ineffective when it comes to functional recovery of the damaged brain tissues.

- **Traumatic brain injuries (TBI)**

Traumatic brain injury (TBI) occurs as a result of external mechanical forces causing traumatic damage to the brain. TBI is regarded as one of leading cause of death and disability worldwide. In the United States alone, it is estimated that 1.6 million people sustain a form of TBI per year causing 52.000 deaths and around 80.000 patients with brain

damage and behavioral impairment (Maegele and Schaefer, 2008, Corrigan et al., 2010, Summers et al., 2009).

The pathological impact of TBI is subdivided into two categories, primary and secondary injuries. Primary injuries are caused by the direct mechanical insult to the brain tissue leading to neural tissue destruction and death and occur at the moment of trauma.

On the other hand, secondary injuries can develop over a period of hours or days following the TBI assault. The secondary cascade of events occurs as a result of altered gene expression and impairment of the regulation of certain cellular functions which contribute to the damaging impact of the injury. These events include excitotoxicity, dysregulation of calcium homeostasis and mitochondrial dysfunction (Maegele and Schaefer, 2008, Richardson et al., 2010, Povlishock and Katz, 2005).

Currently there is no effective treatment for TBI besides supportive care. Several clinical trials for using neuro-protective drugs that target the secondary cascade of events following TBI have failed to show any improvements in the outcomes (Maegele and Schaefer, 2008, Tolias and Bullock, 2004).

1.8 Stem cells: a potential therapeutic tool for CNS injuries and disorder

1.8.1 *Historic overview: the discovery of neural stem cells (NSC) and adult neurogenesis*

It has been long thought that the adult human CNS is incapable of self-renewal and generating new neurons. In 1928, Ramon Y. Cajal, a pioneer brain histologist, wrote "In adult centers the nerve paths are something fixed, ended, immutable. Everything may die, nothing may be regenerated" (Colucci-D'Amato et al., 2006). What also influenced the entire field to accept such conclusion was the relative lack of recovery noticed after a CNS injury or disorder. So it was widely accepted that neurogenesis was completely arrested after birth and doesn't occur in the adult brain.

In 1965, Joseph Altman utilized tritiated thymidine as a mitotic label and showed that cell divisions and proliferation occur constitutively in the adult brain, and specifically in the hippocampus (Altman and Das, 1965) and olfactory bulb (Altman, 1969). In 1981, Michel Kaplan reproduced Altman's results using the tritiated thymidine labeling in addition to electron microscopy (Kaplan, 1981). However, these results were not convincing to the scientific community because the criteria used to identify the new born neurons was mainly

based on morphological features due to the lack of neuronal specific immunocytochemical markers at that time. In the early 1990s, laboratories in Canada and Australia demonstrated for the first time that neural stem cells could be isolated from adult mammals' brains, expanded and differentiated *in-vitro* to neurons (Reynolds and Weiss, 1992, Richards et al., 1992). The advancing molecular technical tools during that time allowed both groups to utilize specific neuronal immunocytochemical markers to precisely and accurately identify and characterize neural stem cells and new born neurons. These isolated neural stem cells were later shown to have the potential to differentiate into neurons, astrocytes, and oligodendrocytes *in-vitro* (Reynolds and Weiss, 1996). These breakthrough results ended the previous dogma of neurobiology which restricted neurogenesis to embryonic brain tissues. It provided clear evidences that neurogenesis occurs in mammalian adult CNS. These findings established the basis of the new era of modern neuroscience from which more extensive studies and research were initiated to deeply investigate and understand the complex biology of the CNS.

1.8.2 *Adult neurogenesis and the neurogenic niches*

Adult neurogenesis has been shown to take place in two regions of the adult mammalian brain under normal conditions, the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the dentate gyrus in the hippocampus (Zhao et al., 2008, Abrous et al., 2005, Sohur et al., 2006). Whether or not neurogenesis occurs in other regions of the adult mammalian brain is still being debated. At the SVZ and SGZ, special stem cell niches have been identified, these structures are specialized microenvironments that host the neural stem cells which are capable of differentiating into new neurons and glia cells throughout adulthood (Riquelme et al., 2008, Taupin, 2006). The cell-cell interactions mediated by the niche's cytoarchitectural organization (Fuchs et al., 2004, Conover and Notti, 2008), the extracellular matrix proteins (ECM), including laminin and collagen , and the diffusible factors and signals in niche's microenvironment, including b-FGF and EGF, tightly regulate the balance between stem cell proliferation and differentiation (Kazanis, 2009, Riquelme et al., 2008, Doetsch, 2003b).

The SVZ niche is composed of four major cells types: ependymal cells, neural stem cells (Type B cells), transit amplifying progenitor cells (Type C cells) and neuroblasts (Type A

cells) (figure 12). The ependymal cells line the walls of the ventricles forming a physical barrier between the SVZ and the cerebrospinal fluid (CSF). They also serve as a filtration system for ions and factors involved in the process of neurogenesis from the CSF. The neural stem cells are located subjacent to the ependymal cells surrounding the neuroblasts that are organized into chains forming the roastral migratory stream (RMS) leading to the olfactory bulb (OB). Between the neuroblasts lie the highly proliferative transitory amplifying progenitor cells. The niche cells are surrounded with extracellular matrix (ECM) that provides them with structural organization and support. The differentiation sequence starts when the neural stem cells (Type B cells), expressing glial fibrillary acidic protein (GFAP), are activated to generate transit amplifying progenitor cells (Type C cells), which are negative for GFAP. These highly dividing progenitor cells then differentiate into migrating neuroblasts (Type A cells) destined to the olfactory blulb (OB) through the RMS (Conover and Notti, 2008, Doetsch, 2003b, Riquelme et al., 2008). Once reaching the OB, these newly born neurons start terminal differentiation and maturation to produce mainly mature inter-neurons which are then integrated into the existing complex neuronal circuitry (Alvarez-Buylla and Garcia-Verdugo, 2002, Zhao et al., 2008). In addition to neurons, oligodendrocytes can also be generated in the adult SVZ (Ahn and Joyner, 2005, Riquelme et al., 2008, Menn et al., 2006).
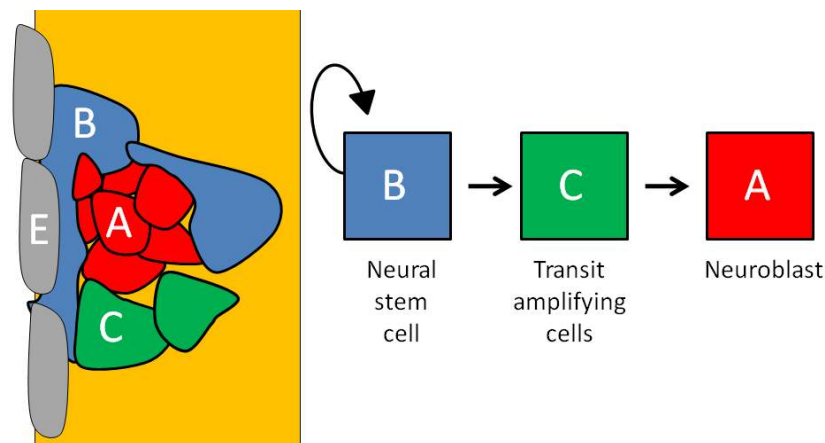
Figure 12. The SVZ neurogenic niche . It is composed of four main cell types: the ependymal cells (E) which separate the SVZ from the lateral ventricles, the neural stem cells (B) which surround the neuroblasts (A) and the transit amplifying cells (C). The neural stem cells (B) ,with self-renewal properties, divide to produce rapidly dividing transit amplifying cells (C) which differentiate into the neuroblasts (A) that migrate to the OB where they maturate and integrate into the brain's neuronal circuitry. Image adapted from (Riquelme et al., 2008).

At the SGZ of the dentate gyrus in the hippocampus, the stem cell niche is smaller compared with the SVZ. The niche is located in close proximate to blood vessels between the granule cell layer and the hilus (figure 13). It contains neural stem cells that extend processes into the granule cell layer, dividing precursor cells (Type D cells) and newly generated neurons. In the SGZ, newly born neurons migrate a shorter distance, compared with the neurons of the SVZ, and reside in the granule cell layer of the dentate gyrus where they are integrated into the brain's neuronal circuitry throughout adulthood. Similar to the SVZ, the newly generated neurons at the SGZ originate from neural stem cells expressing GFAP in addition to NESTIN, an intermediate filament associated with multi-lineage progenitor cells with high proliferation characteristics. The neural stem cells divide to produce precursor cells (Type D cells), expressing NESTIN but not GFAP, which in turn differentiate into neurons which migrate, mature and integrate in the granule cell layer of the dentate gyrus (Riquelme et al., 2008, Zhao et al., 2008, Doetsch, 2003b, Conover and Notti, 2008).
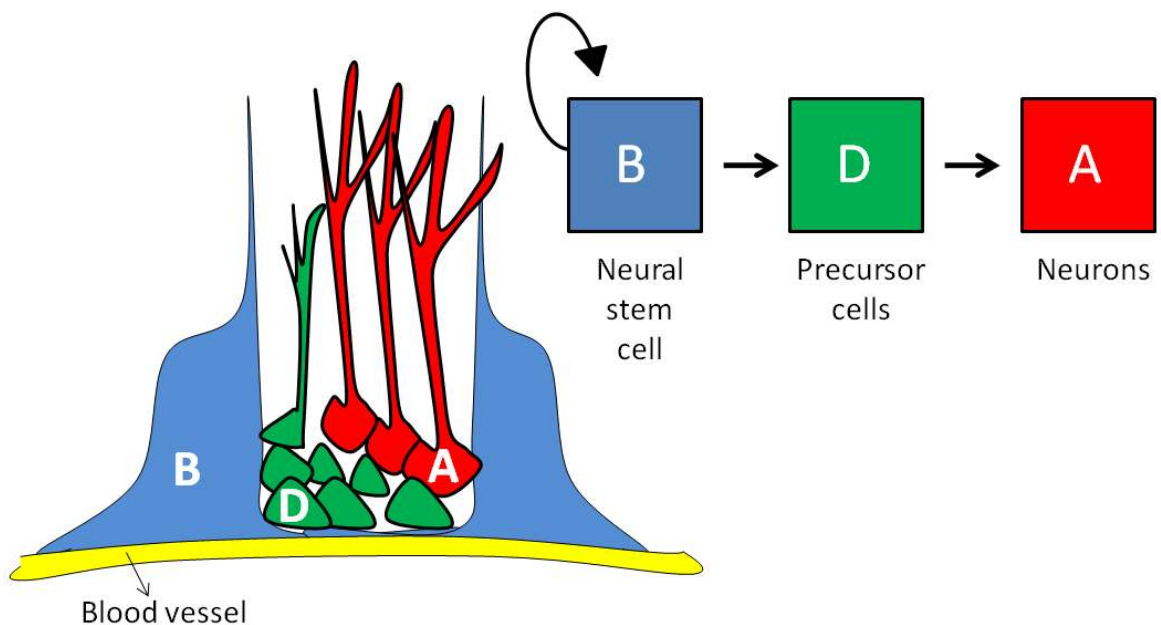
Figure 13. The SGZ neurogenic niche. The niche has the shape of a pocket adjacent to the blood vessels (shown in yellow). The neural stem cells (B) with, self-renewal properties, divide to give the precursor cells (D) which differentiate into neuronal cells (A) that migrate and integrate in the granule cell layer of the dentate gyrus. Image adapted from (Riquelme et al., 2008).

Given its restricted regional occurrence, adult neurogenesis is likely to have specific functional contributions. It is has been demonstrated that adult neurogenesis in SVZ and SGZ, under normal conditions, is involved in some forms of learning and memory functions throughout a person's life (Sohur et al., 2006, Zhao et al., 2008). Adult neurogenesis is stimulated and enhanced by physiological factors and pathological processes such as stroke (Jin et al., 2006) and Alzheimer's disease (Yu et al., 2009, Jin et al., 2004). Cell tracking studies showed that newly generated neurons in the SVZ migrate, and integrate at the lesions sites (Arvidsson et al., 2002, Jin et al., 2003). But despite these facts, adult neurogenesis is still insufficient to induce recovery from such diseases and conditions due to its limited levels (Hess and Borlongan, 2008, Kim and de Vellis, 2009). Understanding the dynamics of the neurogenic niche, its cytoarchitectural organization, cell-cell interactions and the factors involved in neurogenesis holds the key to our understanding of adult neurogenesis and neuronal development which is very important when wanting to develop potential cellular therapies for CNS injuries and disorders.

1.8.3 *Neuronal differentiation of stem cells: in-vitro strategies*

The discovery of neural stem cells and adult neurogenesis focused the efforts to investigate the neuronal potential of different types of stem cells to assess the effectiveness of potential stem cell based therapies aimed at treating CNS diseases and disorders. This lead to the development of different *in-vitro* methods and strategies to differentiate different types of stem cell into neural lineages (figure 14). Among the common strategies is the use of conditioned media to differentiate stem cells into neural lineages *in-vitro*. These media are designed to mimic the *in-vivo* neurogenic microenvironments by containing specific morphogens and mitogens known to be crucial for the development of CNS and neurogeneis in neurogenic niches. Commonly, b-FGF, EGF and Retinoic acid (RA) are used to differentiate different types of stem cells into neuronal cells. The stem cells include embryonic stem cells, (Abranches et al., 2009, Wichterle et al., 2002, Zhou et al., 2008, Li et al., 2008, Li et al., 2005), mesenchymal stem cells (Wang et al., 2007, Lee et al., 2007), neural stem cells (Takahashi et al., 1999, Zahir et al., 2009) and iPS cells (Hu et al., 2010).
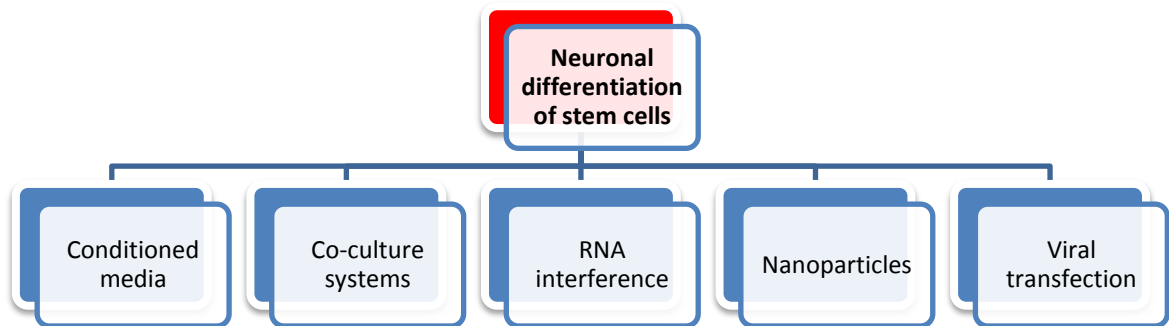
Figure 14. *In-vitro* **strategies for neuronal differentiation of stem cells.**

Another neural differentiation strategy is achieved by the co-culture of stem cells with neural tissues and cells. This differentiation strategy is based on the fact that differentiated glial cells such as astrocytes secrete a number of factors that mediate, direct and regulate neurogenesis of neural stem cells in neurogenic niches (Chang et al., 2003, Nern and Momma, 2006). Several groups have successfully differentiated different types of stem cells using the co-culturing system. Rat neural stem cells have been differentiated into neurons using a co-culture system with cortical astrocytes (Song et al., 2002, Oh et al., 2009). In another study, the co-culture of umbilical cord blood stem cells with astrocytes and oligodendrocytes promoted neuronal differentiation of the stem cells, while co-culturing with differentiated neurons induced oligodendrogliogenesis of the same population of stem cells (Markiewicz et al., 2011).

A relatively new strategy utilized RNA interference (RNAi) to differentiate stem cells into neuronal lineages *in-vitro*. This strategy is based on the concept of specific suppression of targeted gene translation by targeting its mRNA transcript using microRNA (miRNA). miRNA are short non-coding RNA molecules that are naturally found in many eukaryotic cells and are involved in post-transcriptional regulation of genes by binding to their

complementary sequences on corresponding mRNA molecules leading to translation suppression and gene expression silencing (Fire et al., 1998, Hannon, 2002, Bartel, 2004). It has been found that miRNA are involved in many biological processes including neuronal differentiation and adult neurogenesis (Papagiannakopoulos and Kosik, 2009, Shen and Temple, 2009, Cheng et al., 2009). Utilizing miRNA as an *in-vitro* tool to differentiate stem cells into neural lineages has attracted attention in recent years. Successful specific neuronal differentiation was reported when specific miRNA molecule (miRNA-125b) was ectopically expressed in human neural progenitor cells and neuroblastoma cell line (Le et al., 2009). In another study, the neuronal differentiation of neural stem cells, isolated from adult mouse forebrains, was enhanced when cells were transfected with the specific miRNA molecule (miR-9) (Zhao et al., 2009a). The fact that miRNA molecules are involved in regulating endogenous proliferation and differentiation of neural stem cells in the CNS offers a new *in-vitro* tool to direct the differentiation of stem cells into neuronal lineages (Perruisseau-Carrier et al., 2011). Nevertheless, more extensive research is required to fully understand the mechanisms of activity and pathways of miRNA molecules in stem cells and neuronal cells, which is crucial when considering potential cellular therapeutic applications.

Recently, nanotechnology has also been utilized to differentiate stem cells into neurons. This technology utilizes nanoparticles, which are small clusters of atoms that have at least one dimension measuring 100nm or less, as carriers to transport certain factors and morphogens into the cells (Jiang et al., 2008, Maia et al., 2011). Maia et al.(2011) showed that SVZ neural stem cells can be differentiated *in-vitro* into functional neurons using nanoparticles coated with RA. Such result demonstrates the effectiveness of nanoparticles in transporting and releasing the desired growth factors and morphogens into the cells which might aid the development of novel treatment strategies for certain neurological conditions.

The technology used to generate iPS cells was also utilized to produce neuronal cells from fibroblasts. Three genes (Mash1, Bm2 and Myt1) were introduced in rodent fibroblast cells using lentiviral vectors. The fibroblast cells converted into neurons *in-vitro*, after transfection with viral vectors, and were reported to generate action potentials and form functional synapses (Vierbuchen et al., 2010).

1.8.4 *Stem cells based therapies for CNS injuries and disorders*

The discovery of adult neurogenesis in addition to the development of *in-vitro* strategies to differentiate various types of stem cells into neural cells, including neurons and glial cells, established the basis of a new direction of potential therapies for CNS injuries and disorders, arguably the most difficult conditions to treat, based on stem cells. The aim of these potential therapies is to compensate the diseased or injured CNS with new neurons to replace the damaged or dead ones in order to stimulate and enhance the recovery and reduce the pathological impact of the targeted diseases and disorders. The development of such therapies for CNS injuries and disorders requires an extensive detailed knowledge of the pathological process of the disease and its molecular and physiological impact on the brain cells and tissues.

Stem cells have already been involved in studies aimed toward the development of potential therapies for CNS injuries and disorders and have shown promising results. In a rodent model of PD, embryonic stem cells have been transplanted into the striatum of the animals and results indicated that these transplanted cells differentiated into dopaminergic neurons. The study indicated gradual and sustained behavioral restoration of motor deficit in the animals after transplantation (Bjorklund et al., 2002). In another study on a mouse model of PD, embryonic stem cell derived dopaminergic neurons were transplanted into the animals and results reported indicated improvements for the behavioral and motor deficits (Barberi et al., 2003). Adult stem cells such as mesenchymal stem cells have also been used on mouse models of PD. The transplanted cells were reported to express tyrosine hydroxylase (TH) and the recipient animals demonstrated functional improvements (Li et al., 2001). In another study using a rat model of PD, human neural stem cells (NSC) were reported to stimulate significant behavioral improvements after transplantation into the striatum of the animals compared with the negative control (Yasuhara et al., 2006).

Development of therapies for Alzheimer's disease (AD) is relatively more challenging compared with other diseases, because the AD pathological impact affects multiple brain regions and cell types. In an attempt to tackle the disease, both gene and cell therapies have been integrated together to treat the neuronal degeneration and cell death in AD patients. In a phase 1 clinical trial, autologous fibroblast cells have been genetically modified to

express human nerve growth factor (NGF), which has previously been shown to have a neuronal protective role in animal models of ageing and amyloid toxicity (Tuszynski and Blesch, 2004), and these fibroblast were then implanted in the brains of eight patients with mild AD. After a mean follow-up period of 22 months in six of the eight patients, the cognitive decline rate was improved and brain autopsy from one patient demonstrated robust neuronal growth in response to NGF secreted by the transplanted fibroblasts (Tuszynski et al., 2005, Tuszynski, 2007). Stem cells, which can be genetically engineered to express the wanted genes (Kim, 2004), have been proposed as an alternative to fibroblasts due to their high migratory capacity after brain transplantation (Flexman et al., 2007, Kim and de Vellis, 2009). In a recent study on a mouse model of AD, neural stem cells were transplanted into the animals and improvements in cognitive function were reported and attributed to the enhancement of synaptic density in the hippocampus mediated by the brain-derived neurotrophic factor (BDNF) secreted by the transplanted cells (Blurton-Jones et al., 2009).

Stem cells have also been involved in research aimed towards development of effective treatments for stroke. Emerging evidences has shown that stroke induces and enhances endogenous neurogenesis in injured brains, indicated by the presence of cells expressing markers associated with newborn neurons near the injury sites (Jin et al., 2006). However, as mentioned earlier, adult neurogenesis is not sufficient to induce functional and tissue recovery after stroke. Hence, stem cells therapies have been proposed as a potential tool that would enhance neurogenesis at injury sites and hopefully stimulate functional and cellular recovery. Different types of stem cells have been transplanted into animal models of ischemic stroke and in general they show positive results on both cellular and functional levels. In one of the experiments, transplanted human neural stem cells in a rodent model of ischemic stroke have been shown to migrate to the damaged site and differentiate into neurons and glial cells replacing the lost neural cells which improved the condition of the animals (Chu et al., 2003, Chu et al., 2004). Bone marrow mesenchymal stem cells have also been transplanted into animal models of ischemic stroke. Results indicated migration and neuronal differentiation of the transplanted cells around the lesion sites inducing significant behavioral and functional recovery (Chen et al., 2008, Shen et al., 2007). Recently, the UK based biotechnology company "ReNeuron" (Sinden, 2006), announced

the start of phase I of the world's first clinical trial to treat ischemic stroke using *in-vitro* expanded human neural stem cells derived from 12 week aborted fetuses (Christie, 2009). The study aims to treat male patients 60 years old or over suffering from moderate to severe disabilities following ischemic stroke. The proposed treatment involves the injection of expanded neural stem cell line "ReN001" into the patient's brain surgically and under general anesthetic. The trial is currently active and recruiting participants (Christie, 2009).

1.8.5 *Umbilical cord blood stem cells for CNS injuries and disorders*

Umbilical cord blood has proven its worth as an effective therapeutic tool for hematological disorders and diseases. Its clinical involvement dates back to the early 1970s when it was utilized to treat a 16 year old male suffering from lymphoblastic leukemia (Ende and Ende, 1972). Since then, umbilical cord blood became a regular transplant in hematology at clinics and hospitals (Gluckman et al., 1989, Broxmeyer et al., 1990). However, the neural differentiation capability of umbilical cord blood stem cells has highlighted the further potential of cord blood as a promising therapeutic tool for CNS injuries and disorders.

Umbilical cord blood stem cells have shown positive results on many animal models of CNS injuries and disorders. A few research groups have transplanted umbilical cord blood stem cells into rats subjected to middle cerebral artery occlusion (MCAO) in order to induce focal ischemia like pathology. Results indicated improvement in the animal's functional recovery and behavioral deficits associated with the disease after transplantation (Chen et al., 2001, Newcomb et al., 2006). Umbilical cord blood stem cells have also yielded positive results when transplanted into a rat model of hemorrhagic stroke. The stroke was induced in the animals by intra-striatal injections of collagenase to cause bleeding in the striatum. Twenty four hours after inducing the injury, the animals received cord blood intravenously. Results revealed that transplantation of umbilical cord blood stem cells improved the neurologic deficits associated with the disease compared with the control groups (Nan et al., 2005).

Positive results have also been achieved on rat's model of traumatic brain injury (TBI) transplanted with umbilical cord blood. Intravenously transplanted cord blood stem cells were detected in injured brain, where some of the cells were integrated into the vascular walls in the boundary walls of the injured area. The transplanted cells tested positive for

neuronal specific markers NeuN and MAP2, demonstrating both migratory and neuronal capacities. More importantly, the transplanted cells significantly improved motor and neurological deficit when compared to the control group 4 weeks after the treatment (Lu et al., 2002). Similar positive outcomes were observed when umbilical cord blood was transplanted into the injury site of rat's model of spinal cord injury. Results from multiple studies show that transplanted cord blood cells induce significant functional/motor recovery and improved axonal regeneration at the injury site (Kuh et al., 2005, Saporta et al., 2003).

Umbilical cord blood stem cells have also been involved in studies on animal models of neurodegenerative diseases including Parkinson's and Alzheimer's diseases. In one study, the mononuclear (MNC) fraction of umbilical cord blood was transplanted into mouse models of PD. Results from this preliminary study revealed that animals from the control group which did not receive the treatment died significantly earlier than those treated with the cord blood suggesting an ameliorating effect of cord blood on disease progression (Ende and Chen, 2002). In another study conducted on a mouse model of AD, transplantation of umbilical cord blood into the animals significantly reduced the Amyloid-β plaques associated with AD in the brain tissues (Nikolic et al., 2008). The ability to reduce this pathological hallmark feature of AD represents a very important step toward the development of an effective treatment for the disease.

Umbilical cord blood has already been utilized in a few clinical trials aimed to treat neurological diseases. In a clinical trial sponsored by Duke University in the United States, autologous umbilical cord blood cells have been utilized to treat newborn infants with hypoxic-ischemic encephalopathy, a condition caused by inadequate oxygen supply to brain tissues leading to cells damage (Perlman, 2006). The study which was launched in January 2008 will carefully monitor and asses the treatment progress during the 12 month period following the infusion of cord blood into patients. The study is expected to be completed by January 2011 (www.clinicaltrials.gov, trial code: NCT00593242). In another clinical trial also sponsored by Duke University, autologous umbilical cord blood is being used to treat pediatric patients with spastic cerebral palsy, a condition caused by damage to the motor control centers in developing brain leading to movement abnormalities and physical disability (www.clinicaltrials.gov, trial code: NCT01072370 and NCT01147653) (Harris, 2009). Early studies on animal models of cerebral palsy have indicated that that

fusion of cord blood lessens disease progression and significantly alleviates the spastic paresis associated with the disease. This therapeutic effect was attributed to the fact that the transplanted cells were detected in the brain and were incorporated around the lesion site (Meier et al., 2006). The clinical trial which was launched in January 2008 entered phase II and is due for completion in July 2013. Official study results are yet to be published but several media reports have indicated positive beneficial outcomes following the treatment (Kurtzberg, 2009, Harris, 2009). In another recent clinical trial conducted in University of Texas Health Science Center, autologous cord blood is utilized to treat pediatric patients sustaining moderate/severe traumatic brain injury (TBI) (www.clinicaltrials.gov, trial code: NCT01251003). Clinical trials are still at its early stages but early indications suggest high potential and hope toward developing effective therapies for neural disorders and injuries using umbilical cord blood.

1.9 Stem cells: a potential tool for drug testing and toxicology studies

The process of drug development requires extensive research and testing to insure safety and effectiveness of any potential drug. Before any potential drug reaches clinical trials, extensive tests are performed mostly on animal models and *in-vitro* cultures, if available, to gather preliminary information about the drug's efficacy, toxicity and pharmacokinetics (figure 15). These pre-clinical studies are very important to determine whether or not the study shall proceed into clinical trials that involve testing on human beings. Pre-clinical studies are normally performed on animal models including rodents and mice. These studies offer a useful tool to evaluate the safety of the drugs tested. Nevertheless, the genomes, transcriptomes, proteomes and metabolomes between humans and animals differ. The molecular and physiological differences between humans and animal models might account for diverse reactions to a certain drug which might cause unpredicted dangerous reactions in humans (Zhao et al., 2009b, Rietjens et al., 2008). In addition, the physiological complexity of the animal models might not be helpful when it comes to simple toxicity tests and evaluation of drug toxic effects on the cellular level.

In-vitro cultures of human cells offer a potential tool to overcome the limitations and disadvantages of animal models in drug and toxicology testing. Because they are human cells, these *in-vitro* cultures offer a better way to evaluate and predict dangerous reactions

to certain drugs when compared to animal models. In addition, the simplicity of such cultures, compared with whole animals, offers a more reliable and direct tool to perform simple toxicity tests and evaluate the outcomes at the cellular level. Such cultures can be established using specific types of human cells such as fibroblast and hepatocytes, depending on the nature and target of the drug or toxin tested (Paulsen and Ljungman, 2005, Petit et al., 2008). Nevertheless, access to certain type of human cells including neural cells might be difficult due to limited supply of such cells.

Drugs

Animal models

Limitations:
•Do not always give accurate translation to human settings
•Very complex for simple toxicology assays

In-vitro human cell cultures

Limitation:
•Limited supply

Pre-clinical studies

Clinical studies

Routine clinical use
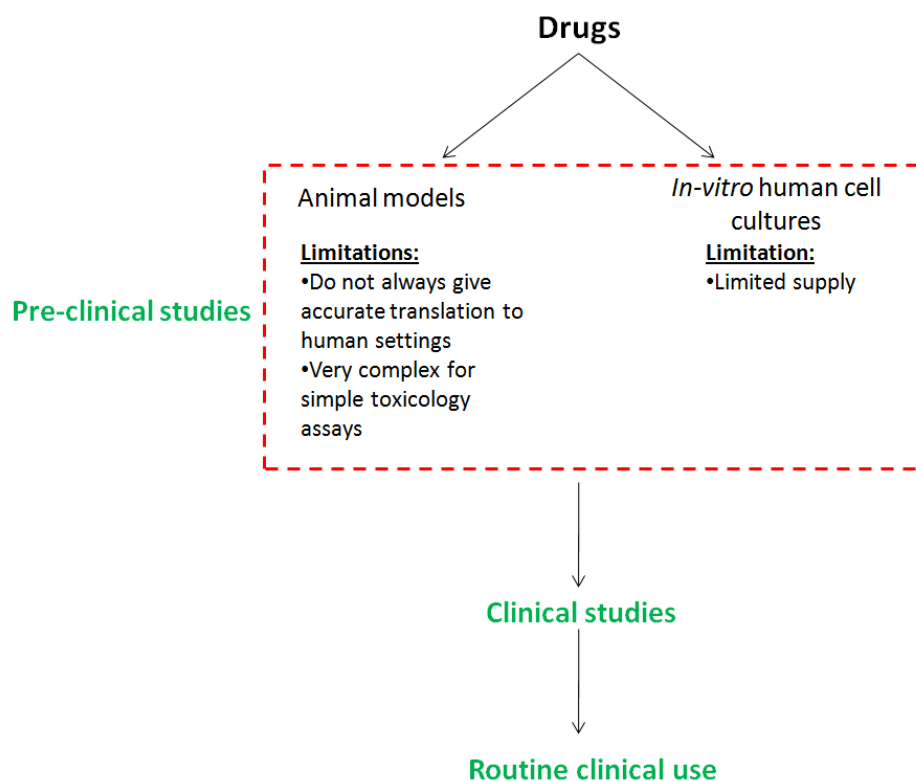
Figure 15. The current process of drug testing and its limitations. The first phase in drug testing is the pre-clinical phase (the red dotted box) in which the drug efficacy and safety are evaluated using animal models and *in-vitro* human cell cultures (if available). This phase is followed by the clinical phase where the drugs are tested on human subjects before reaching the shelves for routine clinical use.

Stem cells ability to differentiate into many cell types including neural lineages highlighted its potential in drug testing and toxicology studies. The ability to develop and establish effective protocols to produce neurons from human stem cells *in-vitro* offers a possible

solution to overcome the problems caused by the limitations of animal models and the limited supply of human neural cells needed for drug and toxicology testing targeting neural diseases and disorders. These cultures would allow deeper analysis of the drug effects on both cellular and molecular levels leading to better evaluation of its safety and efficacy before moving into clinical studies on human subjects.

1.10 Summary

Human umbilical cord blood is probably the largest, but under-utilized source of stem cells with the yearly global birth rate of 200 million per year (McGuckin et al., 2006, McGuckin et al., 2008). Cord blood non-hematopoietic multipotent stem cells, which demonstrated high potential for neural differentiation, are easily accessible, immunologically naive and are free from ethical controversies associated with other sources of stem cells. These added advantages made umbilical cord blood stem cells a potential candidate for CNS injuries and disorders clinical applications which current mode of therapy is inadequate. In addition, they might provide an *in-vitro* model of parenchymal neural cells for toxicology and drugs testing research. However, in order to assure potentially effective treatments and applications of umbilical cord blood stem cells, more research should be directed toward understanding the molecular properties of umbilical cord blood non-hematopoietic and their neuronal potential. This requires the development of effective purification and enrichment strategies of such cells to allow accurate analysis of the molecular mechanisms regulating the process of neuronal differentiation. It is also important to evaluate the neuronal functionality of generated cells to assess their therapeutic impact in potential clinical applications where the target is to recover brain functions lost a result of certain neural disease or condition.

1.11 Thesis layout

The thesis has four main aims: firstly to purify umbilical cord blood non-hematopoietic multipotent stem cells; secondly to characterize neuronal differentiation of the purified cells; thirdly, to compare between the process of umbilical cord blood purified cells undergoing neuronal differentiation *in-vitro* and the process of human cortical neurogenesis and fourthly, to evaluate the neuronal functionality of generated neuron-like cells. Each aim is introduced in a chapter in the following order:

- **Chapter 3**: Purification of umbilical cord blood non-hematopoietic multipotent stem cells.

- **Chapter 4:** Defined sequential neuronal differentiation protocol for umbilical cord blood non-hematopoietic multipotent stem cells and neural tissue engineering application.

- **Chapter 5:** *In vitro* modeling of human cortical neurogenesis using umbilical cord blood non-hematopoietic multipotent stem cells.

- **Chapter 6**: Functional analysis of umbilical cord blood derived neurons.

2. Materials and methods

2.1 Umbilical cord blood collection

2.1.1 *Inclusion criteria*

Ethical approval was sought and obtained from the Local Research Ethics Committee prior to any umbilical cord blood collection. Umbilical cord blood was stored in laboratories with a Human Tissue Authority license. A negative viral profile and infection status was required prior to any tissue collection. Informed consent from parents was obtained prior to delivery (refer to appendix.III for samples of collection and consent forms). Umbilical cord blood units were collected in the Caesarean sections Delivery Suite of the Maternity Unit in the Royal Victoria Infirmary at Newcastle University. All the ethical requirements required by hospital, university and National Health Service (NHS) have been fulfilled.

2.1.2 *Collection procedure*

For logistical and reasons of homogeneity of treatment, samples were only collected from caesarean section births. Umbilical cord blood collection was performed post partum, after the placenta was delivered. Clamping of the umbilical cord was performed by the surgeon according to standard protocols at the Royal Victoria Infirmary, Newcastle upon Tyne. The delivery procedure was not adjusted or modified for the purposes of this study (figure 16A). Three clamps were used for clamping the cord: one close to the cord-placenta joint, the second close to the baby and the third in the middle (figure 16B). Using a cone shaped collection vessel, the placenta was hung with the umbilical cord hanging downwards (figure 16B). Collection bags used for the procedure contained citrate phosphate dextrose adenine (CPD-A) as a anticoagulant and each bag had a needle attached [Baxter]. Using the needle and starting from the lowest point of the cord above the bottom clamp, the blood was drained into the bag using gravitational force (figure 16C,D). Once sample collection was complete, the unit was transported to the laboratory and processed the same day.
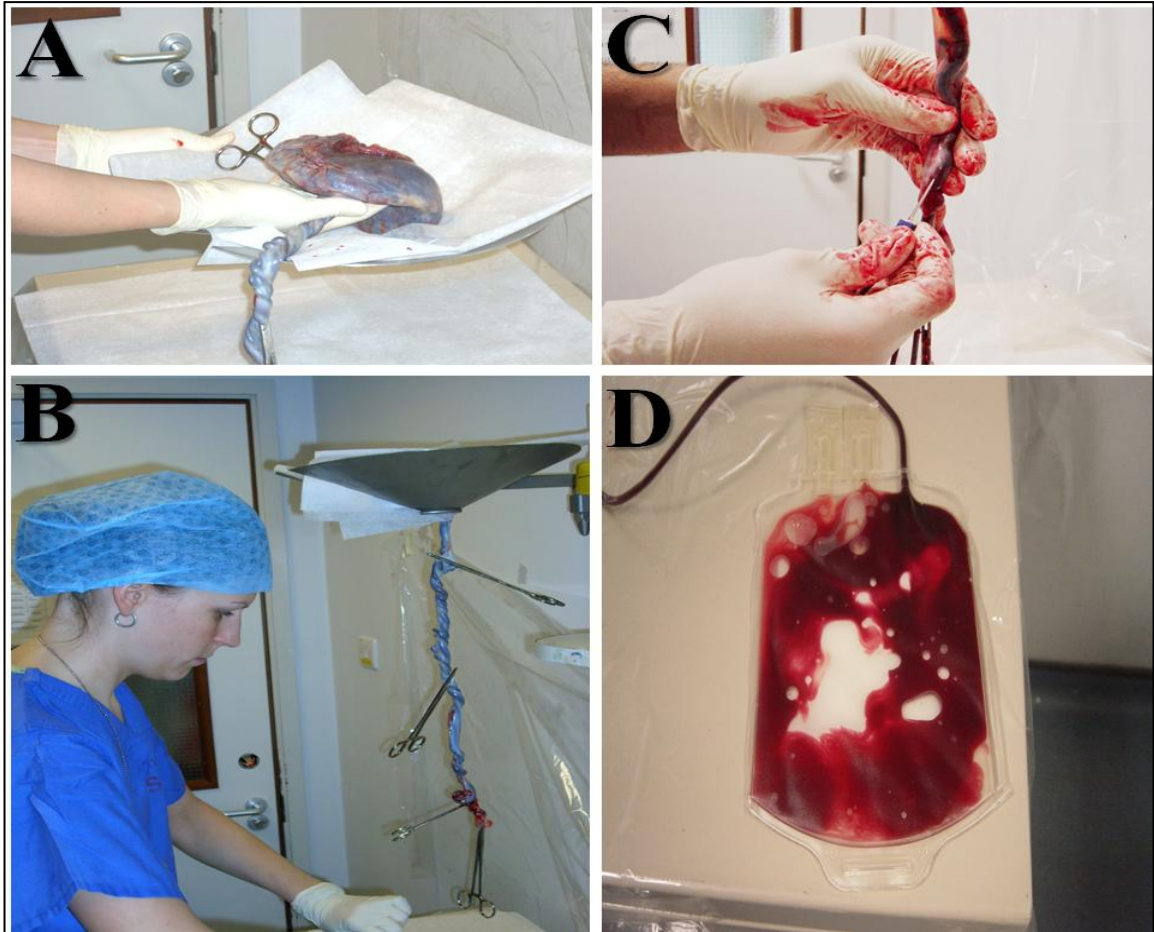
Figure 16 .Umbilical cord blood collection procedure. (A) The placenta is placed on the cone shaped collection vessel with the cord on the bottom. (B) The vessel is hung from a stand allowing the clamped umbilical cord to hang downwards. (C,D) The blood is drained into the collection bag using a needle to access the cord.

2.2 Umbilical cord blood mononuclear cells isolation

Mononuclear cells (MNC) were separated from whole umbilical cord blood using Ficoll-Paque™ PREMIUM [GE healthcare] on the same day as cord blood collection. Each 20 ml of blood was diluted with an equal volume of 1X phosphate buffered saline solution (PBS) [Sigma Aldrich]. The diluted sample was then layered very slowly on 10 ml of Ficoll-Paque™ PREMIUM (figure 17A). The layered samples were centrifuged at 400 X g for 30 minutes at room temperature with minimum acceleration and no deceleration. The resulting middle white layer containing the MNC was collected carefully using a sterile Pasteur pipette and placed in a 50 ml universal tube filled with PBS (Figure 17B). The resulting cell

suspension was centrifuged at 630 X g for 7 minutes at room temperature. The supernatant was discarded and cells were resuspended in 10 ml of PBS for a wash and the resulting suspension was centrifuged at 630 X g for 7 minutes at room temperate. The supernatant was again discarded and the cells were resuspended in either culture medium or PBS depending on the experimental needs. The cells were quantified using Coulter A^C T diff2 cell counter [Beckton Coulter].
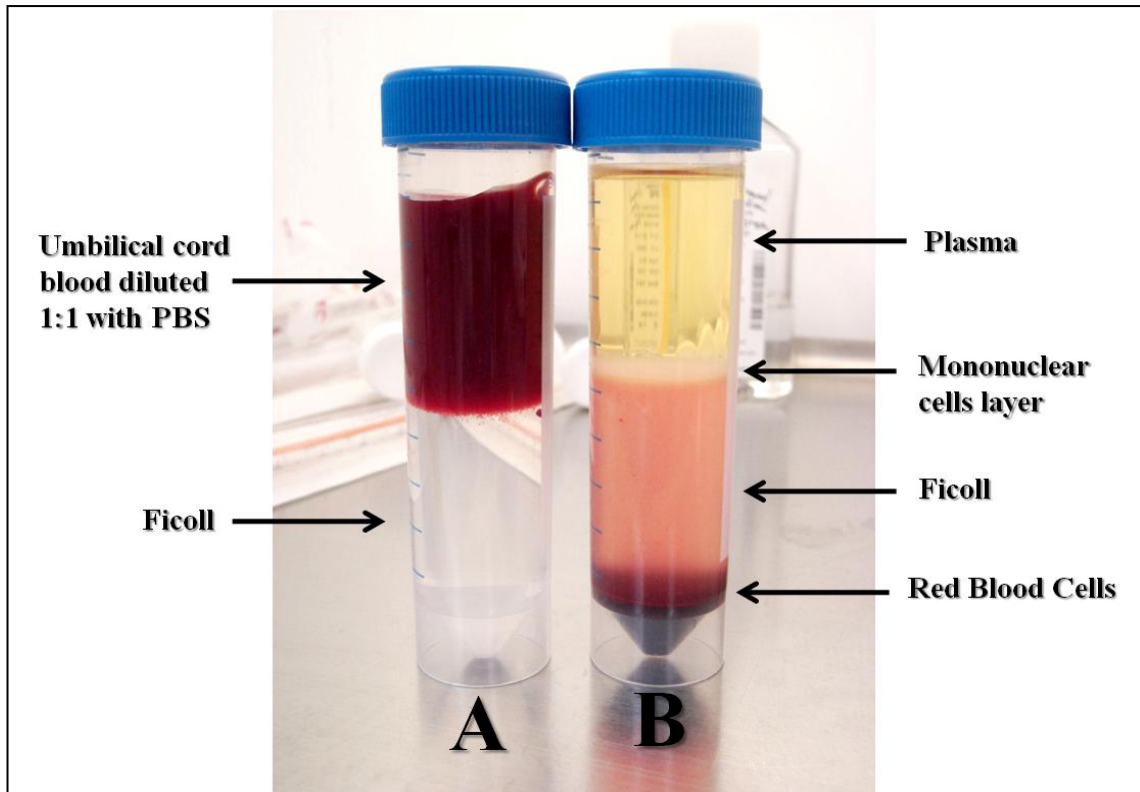
Figure 17. Density gradient separation of umbilical cord blood mononuclear cells using Ficoll. Tube (A) shows the diluted cord blood 1:1 with PBS layered over the Ficoll. Tube (B) shows the blood components after the centrifugation step. The red blood cells collect in the bottom of the tube, with a layer of Ficoll above. The top layer contains the plasma and the mononuclear cells rich white layer lies at the interface between the Ficoll and the plasma.

2.3 Purification of umbilical cord blood stem cells

2.3.1 *StemSep® Primitive Hematopoietic Progenitor Cell Enrichment Kit (SC kit)*

StemSep® Primitive Hematopoietic Progenitor Cell Enrichment Kit (SC kit) is a commercially available kit [Stemcell technologies, catalogue number 14067]. Separation medium was prepared using 2% human albumin [Bio Products Laboratory] in PBS. Previously isolated MNC were resuspended in this separation medium at a concentration of $5x10^7$ cells/ml. The StemSep® enrichment cocktail was added to this cell suspension (100µl of cocktail per 1 ml of cell suspension) and incubated at +4 ºC for 30 minutes. Magnetic colloids were added to the cell suspension (60 µl per 1ml of cells) and incubated at +4 ºC for 30 minutes. The resulting mixture was agitated gently every 10 minutes using a vortex. The mixture was transferred into a MACS® Cell Separation LS column [Miltenyi Biotec] and this placed within a specially designed magnet, MidiMACS ™ Separator [Miltenyi Biotec]. Cells were allowed to pass through the column by gravitational force. After all the mixture had passed through the column and into the collection tube, the column was washed with PBS. The cell suspension that passed through the column was centrifuged at 630 X g for 7 minutes at room temperature, the supernatant was discarded and the cell pellet was resuspended in culture medium. The cells were then counted and cultured.

2.3.2 *Lineage-Negative Separation Kit (Lin-Neg)*

MNCs were resuspended in 1ml of working buffer consisting of 0.2% human serum albumin [Bio Products Laboratory] in Hanks' balanced salt solution (HBSS) [GIBCO] in sterile 15 ml tube at a maximum cell density of $300x10^6$ cells. 500 µL of 2% human gamma-globulins [Fluka Chemie GmbH] diluted in PBS was added to the cell suspension and incubated for 20 minutes either at 4°C or on ice in order to block non-specific Fc receptors. Mouse monoclonal anti-human anti GlyA (CD235a) antibody was added at a concentration of 0.22 µg per $1X10^6$ cells and mouse monoclonal anti-human anti CD45 antibody was added at a concentration of 0.5 µg per $1X10^6$ cells (table 5). The cell suspension was incubated for 30 minutes either at 4°C or on ice with gentle agitation on a rocker. Excess antibodies were washed using a 0.2% human serum albumin and 10% dextran [Baxter Healthcare Ltd] in PBS (wash buffer). The cell suspension was centrifuged

at 630 X g for 7 minutes at 4°C with maximum acceleration and medium deceleration. The supernatant was discarded and the cell pellet was resuspended in 500 µl of working buffer at a final cell density of $1X10^8$ cells. DynaBeads® panmouse IgG [Dynal biotech, 110.42] were added to the cell suspension (50 µl of beads per $1X10^7$ cells) and incubated for 30 minutes either at 4°C or on ice with gentle agitation on a rocker. The resulting cell mixture was applied to a Dynal Magnetic Particle Concentrator (MPC-I) [Invitrogen, 120.01] for 2 minutes. The supernatant was collected into a new sterile tube labelled "GlyA/CD45 negative cells". The remaining positive cell fraction was rinsed twice with 4 ml of working buffer and the supernatant was added to the "GlyA/CD45 negative cells" tube. The final cell suspension in the "GlyA/CD45 negative cells" tube was re-applied to the MPC-I at least 3 more times to completely remove all the positive cells. The "GlyA/CD45 negative cells" cell suspension was centrifuged at 630 X g for 7 minutes at 4°C with maximum acceleration and medium deceleration. The supernatant was removed and the cells resuspended in 500 µl of working buffer. Monoclonal anti-human CD33 and monoclonal anti-human CD7 antibodies were added to the cell suspension each at a concentration of 1.0 µg per $1X10^6$ cells and incubated for 30 min either at 4°C or on ice (table 5). 10 ml of working buffer was added to the mixture to dilute the unbound antibodies. The resulting mixture was centrifuged at 630 X g for 7 minutes at 4°C with maximum acceleration and medium deceleration. The supernatant was removed and the cell pellet was resuspended in 500 µl of working buffer. Beads were applied to the sample as described previously at a concentration of 50 µl of beads per $1X10^7$ cells. This mixture was incubated for 30 min either at 4°C or on ice with gentle agitation using a rocker. The tube containing the mixture was applied to MPC-I for 2 minutes. The supernatant was collected into a new sterile tube labelled "Lineage-negative cells". The positive cell fraction was rinsed twice as previously described with 4 ml of working buffer and at each stage the supernatant was added to the "Lineage-negative cells" tube. The "Lineage negative cells" tube was re-applied to the MPC at least 3 more times to completely remove all the positive cells. The cells were counted and resuspended in either PBS or culture medium depending on the experimental needs. This lineage negative cell selection method has been previously described and published (McGuckin et al., 2008).

Table 5. Table of antibodies used for Lineage-Negative separation

Antibody	Host/isotype	Concentration per $1x10^6$ cells	Catalog number	Manufacturer
CD235a	Mouse IgG1	0.22 µg	M0819	Dako Cytomation
CD45	Mouse IgG1	0.5 µg	ABX252	Autogen Bioclear
CD33	Mouse IgG1	1.0 µg	AB163	Autogen Bioclear
CD7	Mouse IgG3	1.0 µg	AB164	Autogen Bioclear

2.4 Flow cytometry

Flow cytometry was performed using a Becton Dickinson LSR II machine. When processing umbilical cord blood, 100 µl of blood was used for each flow cytometric analysis. For purified samples, around 100,000 cells in 100 µl were used for analysis. After addition of fluorescent dye conjugated antibodies at the recommended concentrations (table 6), the tubes were incubated in dark at room temperature for 20 minutes. The whole umbilical cord blood samples were lysed and washed in BD FACS Lyse/Wash Assistant before being analyzed using BD LSRII in order . 7AAD was used to determine the cells viability to determine cells viability after ficoll and SC kit purification (n=4). Data was analyzed using BD FACSDiva™ 6.0 and cyflogic softwares. Negative control samples were used, where no antibodies were added, to determine the threshold of positive signals (refer to figure 66 in appendix III).

Table 6. Antibodies used for FACS analysis

Antibody	Host/isotype	Cat No.	Manufacturer	volume/100ul
CD34 (PE/Cy7)	Mouse IgG$_1$	348811	BD Pharmingen	5
CD45 (APC/Cy7)	Mouse IgG$_1$	557833	BD Pharmingen	5
CD133/1 (APC)	Mouse IgG$_1$	130-090-826	Miltenyi Biotech	5
SSEA-4 (PE)	Mouse IgG$_3$	FAB1435P	R&D Systems	5
7AAD	-	559925	BD Pharmingen	1:30 (dilution)

2.5 Culturing and differentiating protocol

Sterile plastic cover slips (15 mm diameter) [Nunc] were placed into the wells of a 24-wells plate [Greiner Bio-One]. Two different materials were tried for coating the cover slips, polylysine and collagen IV. Cells plated on polylysine died 48 hours after culturing (data not shown) while cells plated on collagen IV coated cover slips survived throughout the differentiation protocol. Collagen IV was used as primary coating material in this study. The cover slips in the wells were coated with 150 µm of collagen IV [Sigma Aldrich] and incubated for 3 hours at 37°C in 5% CO_2 to dry the collagen coating. This step has been performed to enhance the attachment of cells to the cover slips. Freshly isolated and purified cord blood stem cells were then cultured at a concentration of $1x10^6$ cells/ml in total of 500 µl of medium (figure 18). The cells were placed in neural early commitment medium for 7-10 days (table 7). Half of the medium was changed with fresh medium every 2-3 days. The cultured cells were then moved into neuronal differentiation medium (table 8). The medium was changed every other day for a one week period. After one week, the cells were moved into neuronal maturation medium (table 9). Half the medium was changed with fresh medium on a daily basis and the cells were kept in this medium for 7-10 days (figure 19). Cells were incubated at 37°C in 5% CO_2 and a humidified atmosphere throughout the differentiation protocol.
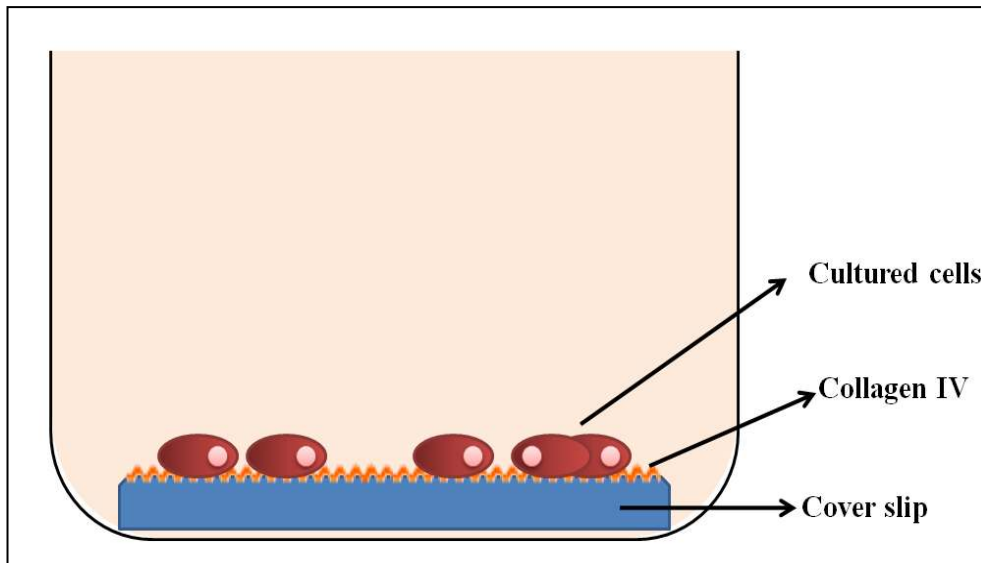
Figure 18. Culturing purified umbilical cord blood stem cells. A plastic cover slip is placed in each well of a 24-wells plate. The cover slip is coated with Collagen IV by drying before the cells are added to the well.
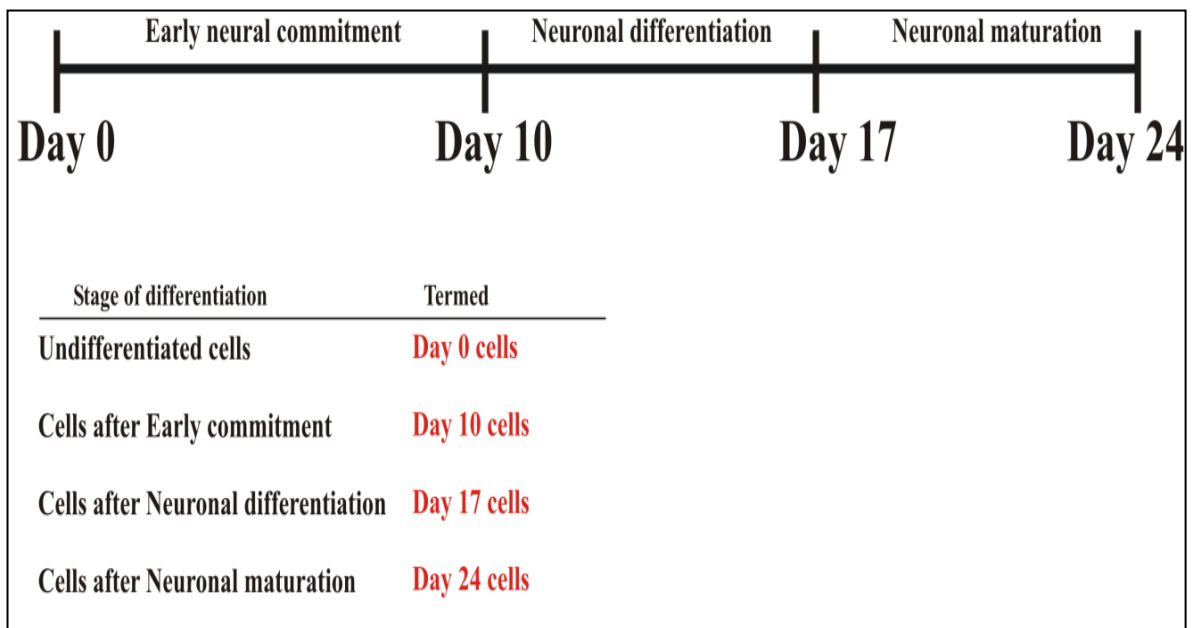
Figure 19. A diagram showing the neuronal differentiation protocol along a time line. For convenience, undifferentiated cells are termed "Day 0 cells", cells after early neural commitment are termed "Day 10 cells", cells after neuronal differentiation are termed "Day 17 cells" and cells after neuronal maturation are termed "Day 24 cells".

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Table 7. Early neural commitment medium components and their concentrations

Neural early commitment media			
Component	Working concentration	Manufacturer	Catalog number
DMEM/F-12 + GlutaMax™	-	Invitrogen	31331-093
B-27 Serum-Free Supplement (50X)	1:50	Invitrogen	17504-044
N-2 Supplement (100X)	1:100	Invitrogen	17502-048
Epidermal growth factor (EGF)	20 ng/ml	Immunotools	11343407
Basic fibroblast growth factor (bFGF)	20 ng/ml	Immunotools	11343627
Heparin	5µg/ml	Sigma Aldrich	H4784-1G
Fibronictin	5µg/ml	Sigma Aldrich	F0895-1MG
Penicillin-Streptomycin	1:100	Invitrogen	15070-063
Fungizone	1:100	Invitrogen	15290026

Table 8. Neuronal differentiation medium components and their concentrations

Neuronal differentiation media			
Component	Working concentration	Manufacturer	Catalog number
DMEM/F-12 + GlutaMax™	-	Invitrogen	31331-093
B-27 Serum-Free Supplement (50X)	1:50	Invitrogen	17504-044
N-2 Supplement (100X)	1:100	Invitrogen	17502-048
EGF	10 ng/ml	Immunotools	11343407
Brain Derived Neurotrophic Factor (BDNF)	10 ng/ml	Immunotools	11343375
Retinoic acid (RA)	1µM	Sigma Aldrich	R2625
Fibronictin	1µg/ml	Sigma Aldrich	F0895-1MG
Collagen IV	1µg/ml	Sigma Aldrich	C5533
Penicillin-Streptomycin	1:100	Invitrogen	15070-063
Fungizone	1:100	Invitrogen	15290026

Table 9. Neuronal maturation medium components and their concentrations

Neuronal Maturation media			
Component	Working concentration	Manufacturer	Catalog number
DMEM/F-12 + GlutaMax™	-	Invitrogen	31331-093
B-27 Serum-Free Supplement (50X)	1:50	Invitrogen	17504-044
N-2 Supplement (100X)	1:100	Invitrogen	17502-048
EGF	10 ng/ml	Immunotools	11343407
BDNF	5 ng/ml	Immunotools	11343375
Nerve growth factor (NGF)	10 ng/ml	Immunotools	11343354
Di-buthyryl cyclic AMP	1µM	Sigma Aldrich	D0627-25MG
Fibronictin	1µg/ml	Sigma Aldrich	F0895-1MG
Collagen IV	1µg/ml	Sigma Aldrich	C5533
Penicillin-Streptomycin	1:100	Invitrogen	15070-063
Fungizone	1:100	Invitrogen	15290026

2.6 Three-dimensional (3D) neuronal differentiation

We used scaffolds supplied by Protista but are not available commercially. The scaffolds were supplied as part of collaboration between the company and Newcastle center for cord blood represented by Professor Colin McGuckin.

The Protista scaffolds, which are composed of fibrinogen, dextran and LLA-HEMA, were cut into 300 µm slices using a microtome before use. Scaffolds were washed in 70% ethanol for 10 minutes for sterilization purposes and then PBS. Washed scaffolds were placed in a well of a 24 wells plate. 20µl of neural early commitment medium was added to wet the scaffold. Concentrated purified cord blood stem cells (100,000-500,000 cells) were placed on the scaffold gently and incubated at 37°C in 5% CO_2 for 2-4 hours to allow cell attachment to the scaffold's matrix. Then 1ml of the medium was added to the well containing the scaffold. The differentiation protocol described above was then followed in a similar way. After maturation (Day 24), the scaffolds were analyzed using immunocytochemistry and confocal imaging (Ali et al., 2009).

2.7 Cytogenetic analysis

In order to perform cytogenetic analysis, 0.2 ml of 1:30 KaryoMAX® Colcemid® Solution [Invitrogen] was added to the cells at Day10 which were already in a 25 cm^2 flask (Nunc) containing 6 ml of culture medium. This was incubated for 3 hours at room temperature. Trypsin-EDTA [Sigma] was warmed in a 37 ^{o}C incubator. 3ml of the warmed Trypsin-EDTA was added to the flask after the supernatant was transferred into a labeled centrifuge tube. The flask was incubated at 37 ^{o}C for approximately two minutes. Once the cells de-attached, the cell suspension was again collected with the previous supernatant and then centrifuged at 600 X g for 5 minutes. The resulting cell supernatant was removed from the cell pellet leaving 0.2-0.5ml of the supernatant to resuspend the pellet. 1ml of 75mM potassium chloride (KCl) solution was then added to the tube and mixed gently before placing it in a 37°C water bath for 5 minutes. The tube was centrifuged at 600 X g for 5 minutes. The KCl containing supernatant was then removed without disturbing the pellet. A few drops of fixative (Freshly made, 3:1 Methanol: Acetic Acid) were added and the cells mixed thoroughly. The tube was then filled up with fixative and then centrifuged at 600 X g

for 5 minutes. The supernatant was then removed and the cell pellet stored overnight at -20°C before sending it for analysis at the Northern Genetics Service at the Institute of Human Genetics at Newcastle University to prepare the slides and analyze and karyotype the samples (samples were analyzed by Dr Simon Zwolinski). A total of 3 different (SC kit) purified samples at day 10 were analyzed.

2.8 Cytospin slides preparation

In order to perform immunocytochemistry on freshly isolated and purified umbilical cord blood cells, cytospin slides were prepared using Shandon-thermo centrifuge at 90 x g for 3 minutes. The number of cells used for each cytospin was approximately 10,000-30,000 cells.

2.9 Immunocytochemistry

The protocol described below was applied to cells cultured on coated cover slips in 24-wells plate, cytospins slides and the scaffolds. The medium was removed from the wells and the cells then washed with PBS twice for 6 minutes each. PBS was added very slowly to the side of the well without any direct contact with the cells. The cells were then fixed with Accustain [Sigma Aldrich] and incubated at room temperature for 30 minutes. The fixative was removed and the cells were then washed with PBS 3 times for 10 minutes each to get rid of any ethanol in the fixative. The cells were permeabilized with 1% Triton®x100 [Sigma Aldrich] for 15 minutes at room temperature. Non-specific binding sites were blocked with 5% foetal calf serum (FCS), 5% goat serum [Zymed] and 0.1% Triton®x100 [Sigma Aldrich] all in PBS for 1 hour. Cells were washed with PBS twice for 6 minutes after this blocking step. The primary antibodies were added to the cells after being appropriately diluted in PBS and incubated overnight at +4°C (table 10). Cells were then washed with PBS twice for 6 minutes and the secondary antibody was added after being appropriately diluted in PBS and left for one hour at room temperature (table 11). Cells were washed again with PBS 3 times for 9 minutes at room temperature. Bis-benzimide [Sigma Aldrich] was added to the cells at a concentration of 1µg/ml in PBS and left for 10 minutes to enable visualization of nuclei. The cells were then washed with PBS 3 times for 9 minutes and the slides and cover-slips mounted using mounting media [Sigma aldrich, cat. no. G0918-20ML]. For negative control samples, the primary antibody step was

omitted and only secondary antibodies were used (refer to figure 70 in appendix III). Slides were stored in the dark at 4°C prior to viewing. Slides and plates were stored in the fridge for up to 4 weeks without any exposure to light. The slides were visualized using a DMRXA upright confocal microscope (Leica TCS SP2 UV CLSM) operating LCS 2.61 software [Leica Microsystems]. Slides and plates were stored in the fridge for up to 4 weeks without any exposure to light. A X40 na 0.85 lens was used to visualise the slides, 6 optical sections (1.3 µm steps) were taken from each image. Dapi (excitation 360 nm, emission 400-540 nm), FITC (excitation 488 nm, emission 500-550 nm) and Texas Red (excitation 543 nm, emission 580-650 nm) were imaged sequentially frame by frame to avoid crosstalk using two line averages. The resulting image stacks were viewed and saved using Max Projection with threshold and rescale turned off, and finally displayed as tiled or overlayed images.

To obtain the percentages of fluorescing cells, confocal images were obtained at 10x and 20x magnifications. Firstly, all the DAPI stained nuclei were counted. This refers to the total number of cells in each image. The fluorescence positive cells were then counted and percentages were generated for each marker at different stages using the formula: number of fluorescent cells / total number of cells X 100. The number of samples used to generate the statistics per each marker per each time point was 3 different samples (n=3), a biological triplicate from each sample.

Table 10. The primary antibodies used for immunocytochemistry (ICC) and immunohistochemistry (IHC) and their concentrations

Antibody	Host	Isotype	Dilution	Manufacturer	Catalog number
Pluripotency and proliferation markers					
OCT4	Goat	IgG	ICC 1:500	Abcam	Ab27985
SOX2	Mouse	IgG2a	ICC 1:50	R&D systems	MAB2018
NANOG	Goat	IgG	ICC 1:20	R&D systems	AF1997
KI-67	Mouse	IgG1	ICC 1:100	Novocastra	L111853
Neural development markers					
NESTIN	Mouse	IgG1	ICC 1:100	R&D systems	MAB1259
GFAP	Rabbit	IgG	ICC 1:1000	Abcam	Ab7779
NF200	Mouse	IgG1	ICC 1:800	Sigma	N1042
GAP43	Rabbit	IgG	ICC 1:500	Abcam	Ab75810
β-Tubulin III (TUJ1)	Mouse	IgG1	ICC 1:500	Abcam	Ab7751
β-Tubulin III (TUJ1)	Rabbit	IgG	ICC 1:2000	Abcam	Ab18207
PSD95	Rabbit	IgG	ICC 1:500	Abcam	Ab18258
NEUN	Mouse	IgG1	ICC 1:100	Chemicon	MAB377
Neurotransmitter transporters					
VGLUT1	Rabbit	IgG	ICC 1:800	Synaptic systems	135302
Neurotransmitter receptor					
NMDAR1	Rabbit	IgG	ICC 1:200	Abcam	Ab28669
Cortical development markers					
PAX6	Mouse	IgG1	ICC 1:50	Millipore	MAB5554
PAX6	Rabbit	IgG	IHC 1:300	Covance	PRB-278P
TBR2	Rabbit	IgG1	ICC/IHC 1:500	Abcam	Ab23345
TBR1	Rabbit	IgG1	ICC/IHC 1:200	Abcam	Ab31940

Table 11. Secondary antibodies used for immunocytochemistry (ICC)

Antibody	Type	Isotype	Dilution	Florochrome	Manufacturer	Catalog number
Alexa 488	Goat anti-mouse	IgG1	1:500	FITC	Invitrogen	A21121
Alexa 594	Goat anti-rabbit	IgG	1:500	TxRed	Invitrogen	A11012
Alexa 488	Rabbit anti-mouse	IgG	1:500	FITC	Invitrogen	A11059
Alexa 594	Rabbit anti-goat	IgG	1:500	TxRed	Invitrogen	A11080
Alexa 594	Goat anti-mouse	IgG2a	1:500	TxRed	Invitrogen	A21135
NorthernLights™ NL493	Donkey Anti-Goat	IgG	1:500	FITC	R&D systems	NL003

2.10 Statistical analysis

For two groups of data, the student's paired *t*-test was used to obtain probability (*p*) values. For three or more groups of univariate data, single-factor analysis of variation (ANOVA) was used to obtain *p* values. Results with *p* values of less than 0.05 were considered statistically significant.

2.11 Polymerase Chain Reaction (PCR)

2.11.1 *Primers*

OCT4, SOX2 and NANOG certified primers sets with confidential sequences were purchased from Sabiosciences (table 12). Primers presented in (table 13), except *ALB* and *GATA4*, were obtained from the PrimerBank website (http://pga.mgh.harvard.edu/primerbank) (Spandidos et al., 2008, Wang and Seed, 2003) and purchased from Sigma. For primer bank ID refer to (table 19 in appendix III). *ALB* and *GATA4* were provided by colleague Dr Saba Habibollah.

Table 12. List of certified primers used for real time PCR obtained from SABiosciences

Gene	Product size	GenBank Accession	Manufacturer	Catalog Number
POU5F1	138	NM_002701.4	SABiosciences	PPH2394E
SOX2	115	NM_003106.2	SABiosciences	PPH02471A
NANOG	148	NM_024865.2	SABiosciences	PPH17032E

Table 13. List of primers used for real time PCR and their product sizes

Gene	Primers	Product size	GenBank Accession	Location	
C-MYC	5'-TCGGAAGGACTATCCTGCTG-3' 5'-GTGTGTTCGCCTCTTGACATT-3'	133	NM_002467	1515	1534
				1647	1627
NEUROG1	5'-GCTCTCTGACCCCAGTAGC-3' 5'-GCGTTGTGTGGAGCAAGTC-3'	107	NM_006161	844	862
				950	932
NEUROG2	5'-TCCTCCGTGTCCTCCAATTC-3' 5'-AGGTGAGGTGCATAGCGGT-3'	125	NM_024019	997	1016
				1121	1103
NEUROD1	5'- GGATGACGATCAAAAGCCCAA-3' 5'-CGTCTTAGAATAGCAAGGCACC-3'	178	NM_002500	415	435
				592	571
NEUROD2	5'- TGCTACTCCAAGACGCAGAAG-3' 5'-CACGTAGGACACTAGGTCTGG-3'	120	NM_006160	648	668
				767	747
PAX6	5'-ATGTGTGAGTAAAATTCTGGGCA-3' 5'-GCTTACAACTTCTGGAGTCGCTA-3'	103	NM_000280	686	708
				788	766
TBR2	5'- CACCGCCACCAAACTGAGAT-3' 5'- CGAACACATTGTAGTGGGCAG-3'	109	NM_005442	841	860
				949	929
TBR1	5'- GCCTTTCTCCTTCTATCATGCTC-3' 5'-GTCAGTGGTCGAGATAATGGGA-3'	116	NM_006593	319	341
				434	413
DLG4 *(PSD95)*	5'- GGACCAGATCCTGTCGGTCA-3' 5'- CCTCGAATCGGCTGTACTCTT-3'	137	NM_001365	2398	2417
				2534	2514
GAPDH	5'-TGTTGCCATCAATGACCCCTT-3' 5'-CTCCACGACGTACTCAGCG-3'	202	NM_002046	192	212
				393	375
ALB	5'- AAGCTGCCTGCCTGTTGCCA-3' 5'- GCTCAGGCGAGCTACTGCCC-3'	140	NM_000477.5	666	685
				805	786
GATA4	5'- GCAGCAGCGAGGAGATGCGT -3' 5'- GGGGAGAGCTTCAGGGCCGA-3'	154	NM_002052.3	1621	1640
				1774	1775

2.11.2 *Total RNA isolation and preparation*

Total RNA was isolated from cells using Qiagen RNeasy® Plus Mini Kit [Qiagen, 74134] following the manufacturer's protocol. A DNase digestion step was incorporated for optimal purification of total RNA using RNase-Free DNase Set [Qiagen, 79254].

2.11.3 *cDNA Preparation*

cDNA was produced using a SuperScript ® VILO Kit [Invitrogen, 11754-050] in 20 µl total volumes using 1-2.5 µg of total RNA for each reaction and following the manufacturer's protocol and a MasterCycler® Gradient [Eppendorf]. The cDNA produced was quantified using NanoDrop 8000 [Thermo scientific]. Purity of the the cDNA was assessed using the 260/280 ratio provided by the NanoDrop. Samples with 260/280 ratio between 1.8 and 2.1 were considered optimally pure and used further for real time PCR analysis.

2.11.4 *Real time PCR*

All real-time PCR reactions were performed using RT^2 SYBR® Green/ROX™ qPCR Master Mix [Sabiosciences, PA-012-8] and a ABI7900HT machine [applied bio systems]. The final volume for each reaction was 10µl, composed of 5µl of the master mix, 1µl each of 10µM primers set (forward and reverse), 10-50 ng of cDNA (volume depending upon the concentration) and sterile distilled water (dH$_2$). Each reaction mix was transferred to a single well of a 384 well plate. The plates were centrifuged at 1000 X g for 1 minute to remove any air bubbles which might affect the efficiency of the PCR.

A two step cycling program was used for all reactions as follow: 95°C for 10 minutes and 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds. The melting curves were checked to confirm the presence of only one amplified product. The relative gene expression of each gene analysed was normalized to expression levels of housekeeping genes. All results were analyzed using qBase Software (Hellemans et al., 2007). PCR reactions of all genes have been optimised using cDNA samples generated from fetal cortical tissue (data not shown). In each analysis, the sample of the lowest expression was assigned the value of 1 and used as a caliborator.

As a control check, PCR reactions using *PAX6* and *TBR1* primers were performed on cDNA samples generated from fetal cortical tissue and adult liver tissue, in addition to day 0, day 10, day 17 and day 24 cells, in order to determine the tissue specificity of the primers (refer to figure 68 in appendix III).

2.11.5 *Agarose gel electrophoresis*

Agarose gels were run to confirm the product size of the amplified genes from the real time PCR. 1.5% agarose [Seachem, 1111] gel was prepared in Tris acetate/EDTA (TAE) buffer (40 mM Tris-acetate and 1 mM EDTA in distilled water at pH 8.0). The mixture was heated in microwave for about two minutes. The mixture was gently mixed until the agrose powder completely dissolved in the (TAE) buffer. SYPRO® Red protein gel stain (5000X concentrate in DMSO) was used to visualize the bands of DNA [Invitrogen, S-6653]. The gel was poured in the electrophoresis chamber and was allowed to set and then submerged in TAE buffer. 10µl of PCR product was mixed with 2µl loading buffer [GelPilot DNA Loading Dye, 5x, Qiagen, 239901] and then loaded into gel and electophoresed at 75 volts for 80 minutes. The gel was then visualized under a transilluminator [Gene Genius Bio Imaging System, Syngene, a division of Synoptics Ltd.]. GelPilot 100 bp plus DNA Ladder [Qiagen, 239045] was used to determine the product size for the genes except for *C-MYC* where 50 bp DNA Ladder [Qiagen, 239025] was used (refer to figure 67 in appendix III).

2.11.6 *Pathway PCR arrays*

Profiler™ PCR arrays were purchased from Sabiosciences [PAHS-060E and PAHS-036E] (table 14). Each array includes SYBR Green-optimized primer assays designed for neuronal specific functional aspct. Each array contains 84 genes involved in neural functioning in addition to 5 housekeeping genes. Quality controls were also integrated on each array including for genomic DNA contamination, reverse transcription and positive PCR controls. The reaction mixes for these controls were performed using an identical cycling program to the one described for real time PCR. cDNA samples generated from human fetal cortical tissue have been used as a positive control (data not shown).

Table 14. Real time PCR arrays

Array's name	Catalog number	Manufacturer
Human Neuroscience Ion Channels and Transporters RT² Profiler™	PAHS-036E	SABiosciences
Human Neurotransmitter Receptors and Regulators RT² Profiler™	PAHS-060E	

2.12 Functional analysis: calcium imaging

2.12.1 *Dye loading*

Cultures were bulk loaded with Oregon Green 488 Bapta 1 (OGB1)-AM ester, excitation at 494 nm and emission at 523 nm, using the following protocol. OGB1-AM [50µg vial, Molecular Probes] was mixed with 8µl DMSO [Sigma Aldrich] and 2µl pluronic acid F-127 solution [10% in DMSO, Molecular Probes], and this solution diluted by adding 90µl of culture medium. 12-20µl of this final mixture was then added to the culture well containing 3ml of medium. The final concentrations were OGB1-AM ester, ~12µM; 0.6% DMSO, 0.002% Pluronic F-127. The cultures were incubated for 30-40 minutes at 37°C and then transferred to medium without OGB1-AM for 20 minutes at 37°C prior to transferring the cultures to the recording chamber.

2.12.2 *Glutamate application*

In the recording chamber, the cultures were bathed in a continuously flowing stream (1-3ml / minute) of artificial cerebrospinal fluid (ACSF) in 125 mM NaCl, 26 mM NaHCO$_3$, 10 mM glucose, 3.5 mM KCl, 1.2 mM CaCl$_2$, 1.26 mM NaH$_2$PO$_4^-$, 1 mM MgSO$_4$,. Glutamate at a concentration of 1mM in ACSF was then applied directly using patch pipettes (5-7MΩ when filled with K-methylsulphate electrode filling solution) using a picospritzer pressure application system (10ms pulses; pressure = 20psi). The average bolus for a 10ms pressure application through these pipettes was approximately 1.6µl. The timing of the pressure applications was controlled using the Master 8 pulse stimulator. The pipette tip was positioned approximately 10-50µm from the cells in the open recording chamber. It is

unusual to detect any movement artefacts during the glutamate applications unless the pipette was broken, indicating that this means of stimulating cells was relatively atraumatic. Images for loaded cells with no glutamate induction were obtained to serve as a negative control (Trevelyan et al., 2010).

2.12.3 *Cell imaging*

The recording chamber was mounted on an upright Olympus BX51 DSU confocal microscope fitted with Scientifica Patchmaster micromanipulators. Images were collected at 10Hz, using a C9100/13 Hamamatsu camera connected to a Dell personal computer running Digital Pixel software.

2.12.4 *Data analysis*

Generated images were analyzed using ImageJ software which is a public domain, Java-based image processing program developed by the National Institutes of Health (NIH). Images were taken from multiple regions of each loaded cover slip. Single cells in each frame were analyzed individually to generate a final percentage of responsive cells using the formula: number of active cells / total number of cells X 100. Changes in intracellular calcium concentrations are correlated to the relative changes in fluorescence (F) detected by the fast camera before and after induction with glutamate. We used $\Delta F/F$ to normalize the data to that first data point. This is performed to control for small changes in non-biological variables such as the effective dye concentration. The student's t test was used to determine the significance of any differences between the data.

2.13 Human foetal brains samples

Human Foetal brains sections (7-8µm thick) and total RNA samples from human foetal brains were obtained from the MRC-Wellcome Trust Human Developmental Biology Resource at Newcastle University (HDBR, http://www.hdbr.org). Appropriate maternal written consent and approval from the Newcastle and North Tyneside NHS Health Authority Joint Research Ethics Committee had been obtained prior to collecting these samples. Samples obtained were 12 weeks postconception (PCW) (Bayatti et al., 2008b, Bayatti et al., 2008a).

2.14 Immunohistochemistry

Paraffin sections were dewaxed twice in xylene for 10 minutes and dehydrated twice in 100% ethanol for two minutes. Sections were treated with 3% hydrogen peroxide [Sigma Aldrich] in methanol for 10 minutes and boiled in 10mM citrate buffer. Sections were then incubated with primary antibodies (table 10) in 3% horse [Sigma Aldrich, H1138] and goat [Zymed] blocking serum in 0.1% Triton X/PBS (PBST) on the slides in a moist chamber at 4˚C overnight. After washing three times in 0.1% PBST for 5 minutes each, sections were incubated with biotinylated goat anti-rabbit IgG secondary antibody [Vector Laboratory, BA-1000] at 1:200 dilution in PBST at 4˚C for two hours. The slides were then washed 3 times in 0.1% PBST for 5 minutes each. Sections were incubated with streptavidin-horse radish peroxidase [Vector Laboratory] at 1:200 dilution in PBST at 4˚C for one hour. After these incubation steps, sections were washed in 0.1% PBST 3 times for 5 minutes each. Visualization of the immuno-reaction was achieved by adding a substrate of the enzyme complex 3, 3'-Diaminobenzidine tetrahydrochloride, also known as DAB chromogen, in diluent [Vector Laboratory] at a concentration of 30 µl/ml. This was left for colour development for up to 10 minutes before washing in PBS. All sections were then dehydrated in a series of ethanol (Two times in 95% ethanol, 10 seconds each and repeat in 100% ethanol), cleared in xylene and mounted in DPX mountant (Bayatti et al., 2008a, Bayatti et al., 2008b). For negative control, primary antibodies were omitted and only secondary antibody was used (refer to figure 71 in appendix III).

Chapter 3. Purification of umbilical cord blood non-hematopoietic multipotent stem cells

3.1 Introduction

Umbilical cord blood contains a highly heterogeneous mixture of cells. This mixture includes hematopoietic cells including erythrocytes and leukocytes, and in addition a population of hematopoietic stem cells (HSCs). It also contains a relatively low concentration of non-hematopoietic multipotent stem cells expressing SSEA-4, a surface marker expressed by embryonic stem cells (McGuckin et al., 2005, Kucia et al., 2007), and the transcription factors OCT4, SOX2 and NANOG normally expressed by pluripotent stem cells (McGuckin et al., 2008, Ali et al., 2009, Kucia et al., 2007) (see section 1.5.3). The potential use of this non-hematopoietic stem cell population in a range of applications underpins the efforts to further characterize and analyze the properties of this unique cell population. This required the development of purification strategies that allow the enrichment of this population in order to accurately analyze its molecular properties as well as the differentiation potential of these cells.

Many groups have developed different strategies in attempts to purify cord blood non-hematopoietic stem cells population (table 15). Buzanska et al. (2002) developed an immunomagnetic sorting strategy to purify the targeted population. They utilized magnetic beads that can specifically recognize CD34, a surface antigen expressed by hematopoietic stem cells (HSCs), and depleted the CD34-postive cells (Buzanska et al., 2002). The purified CD34-negative cell fraction was reported to express OCT4 and SOX2 (Buzanska et al., 2006a, Buzanska et al., 2006b, Habich et al., 2006). Forraz et al. (2004) utilized the same immunomagnetic depletion strategy used by Buzanska's group but instead of using a single antibody, they used a combination of hematopoietic antibodies including CD45, CD235a, CD38, CD7, CD33, CD56, CD16, CD3, and CD2 (table 2) in a sequential manner to purify the targeted population which were reported to make up around 0.1% of the total

mononuclear fraction of the cord blood (Forraz et al., 2004, McGuckin et al., 2004, McGuckin et al., 2005). Other groups utilized multi-parameter fluorescence-activated cell sorting (FACS) to purify the targeted population. They used a cocktail of antibodies including hematopoietic stem cell markers CD133, CD34 and the general hematopoietic marker CD45. The CD45-positive cells were eliminated and at the same time the CD133 and CD 34 positive cells were enriched using FACS sorting. Those enriched cells expressed OCT4 and NANOG in addition to surface embryonic marker SSEA-4. The cells were termed very small embryonic-like stem cells (VSEL) due to their small cell size and expression of markers (Kucia et al., 2007, Halasa et al., 2008).

Table 15. Reported purification and selection methods of umbilical cord blood non-hematopoietic stem cells

Name of isolated cells	Purification/selection method	Markers expressed	Neuronal differentiation	Reference
Human umbilical cord blood-neural stem cells (HUCB-NSC)	Continuous depletion of CD34 cells (cell line)	OCT4 SOX2	Reported	Buzanska et al. (2002) Buzanska et al. (2006a) Buzanska et al. (2006b) Habich et al. (2006)
Lineage-Negative Stem-Progenitor Cell Population	Immuno-magnetic depletion	TRA-1-60 TRA-1-81 SSEA-4 SSEA-3 OCT4	Reported	Forraz et al. (2004) McGuckin et al. (2004) McGuckin et al. (2005)
Very small embryonic-like stem cells (VSEL)	FACS sorting	SSEA-4 OCT4 NANOG	Not reported	Kucia et al. (2007) Halasa et al. (2008)
Progenitor cord blood cells	CD133 positive selection	NESTIN	Reported	Zangiacomi et al. (2008)
Cord blood derived embryonic-like stem cells (CBE)	Immuno-magnetic depletion	OCT4 SOX2 NANOG	Reported	McGuckin et al. (2008)
Lineage negative umbilical cord blood cells	Immuno-magnetic depletion	OCT4 SOX2	Reported	Chua et al. (2009)

In the studies outlined in this chapter, we compare two methods for purifying non-hematopoietic multipotent stem cells from umbilical cord blood. Both methods are based on immunomagnetic depletion strategy. The first method is an "in house" purification method which has been developed and used previously (McGuckin et al., 2008), and the second is a commercially available purification kit. For simplicity, we termed the "in house" kit (Lin-Neg) and the second kit (SC kit) (see section 2.3). We utilize FACS to analyze the purified fraction from each kit using four surface markers including, CD45, CD34, CD133 and SSEA-4. We further characterize the expression of pluripotency markers, OCT4, SOX2 and NANOG, in the enriched fractions using immunocytochemistry.

3.2 Study aims

- To purify and enrich umbilical cord blood non-hematopoietic multipotent stem cells.

- To characterize umbilical cord blood non-hematopoietic multipotent stem cells by analyzing their expression to proteins associated with pluripotency including OCT4, SOX2 and NANOG.

- To compare between the efficiency and practicality of two different protocols in purifying and enriching the cord blood non-hematopoietic multipotent stem cells and determine the one that will be used for the following experiments.

3.3 Results

3.3.1 *Lin-Neg and SC kit purification depleted CD45 positive cells and enriched SSEA-4 positive cells*

FACS analysis (see section 2.4) on freshly isolated umbilical cord blood revealed that 95.11±7.25% (n=6) of the cells were positive for CD45 while 24.29±8.79% of the cells expressed CD34 (n=6), 0.18±0.16% (n=6) expressed CD133 and 1.29±0.46% (n=6) expressed SSEA-4 (figure 20). After isolation of MNCs by depleting red blood cells using Ficoll density gradient centrifugation, the cells percentages did not show any significant changes compared to their percentages in unprocessed cord blood (figure 20). CD45 positive cells were 90.13±3.53% (n=6), CD34 positive cells were 24.74±7.06% (n=6), CD133 positive cells were 0.2±0.11% (n=6) and SSEA-4 positive cells were 1.39±0.37% (n=6).
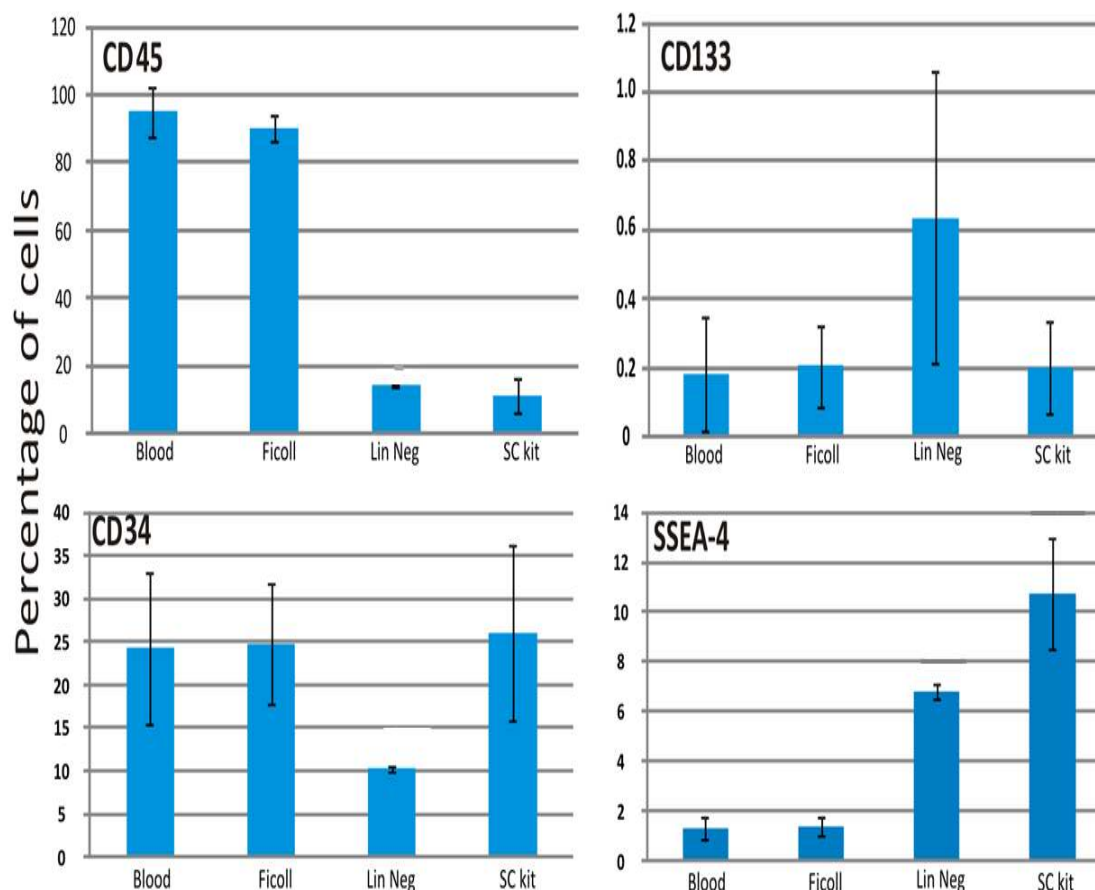
Figure 20. FACS analysis assessing the efficiency of negative depletion strategies through comparison with unprocessed umbilical cord blood. Changes in proportions of cells expressing CD45 in blood, Lin Neg and SC kit were statistically significant ($p<0.005$). Changes in proportions of cells expressing CD133 were also significant between blood, Lin Neg and SC kit ($p<0.05$). Changes in proportions of cells expressing CD34 were also statistically significant ($p<0.05$). Changes in proportion of cells expressing SSEA-4 in blood, Lin Neg and SC kit were statistically significant ($p<0.005$). p values were calculated using ANOVA (n=6).

The percentages of cells expressing the analyzed markers after Lin-Neg and SC kit purification steps have been compared to their calculated percentages in unprocessed umbilical cord blood using ANOVA in order to assess the efficiency of purification step by identifying significant differences (figure 20). After Lin-Neg isolation step, percentage of CD45 positive cells significantly decreased to $14.34\pm0.29\%$ (n=6). Percentage of cells expressing CD34 also decreased significantly to $10.27\pm0.35\%$ (n=6). Percentage of CD133 positive cells increased to $0.63\pm0.42\%$ (n=6). SSEA-4 positive cells were significantly enriched to $6.79\pm0.29\%$ (n=6).

After SC kit isolation step, percentage of CD45 positive cells decreased significantly compared to their percentage in freshly isolated cord blood reaching 11.36±4.97% (n=6). Percentage of CD34 positive cells increased to 25.97±10.16% (n=6). Percentage of CD133 positive cells stayed at 0.2±0.13% (n=6) and SSEA-4 positive cells were significantly enriched to 10.73±2.23% (n=6) (figure 20). The population of interest which expressed SSEA-4 was negative for CD45 (refer to figure 62 in appendix II).

3.3.2 *SC kit versus Lin-Neg : Statistical comparison*

Results obtained by SC kit and Lin-Neg isolations were statistically compared to each other in order to determine which kit is more efficient in purifying SSEA-4 positive cells by depleting cells expressing hematopoietic mature markers. Student's t-test was used to determine significant differences between the two kits (figure 21). FACS results showed that both methods significantly depleted CD45 positive cells compared to freshly isolated cord blood but the difference between the percentages obtained by the two methods was not statically significant. For CD34 positive cells, Lin-Neg showed significantly less CD34 positive cells percentage compared to SC kit (n=6, p<0.05) (figure 21). For CD133, differences between the two methods did not show statistical significant. For SSEA-4 marker, both methods showed statistically significant enrichment compared to freshly isolated cord blood but SC kit resulted in higher percentage of SSEA-4 positive cells compared to Lin-Neg with statistical significance (n=6 , p<0.05) (figure 21).
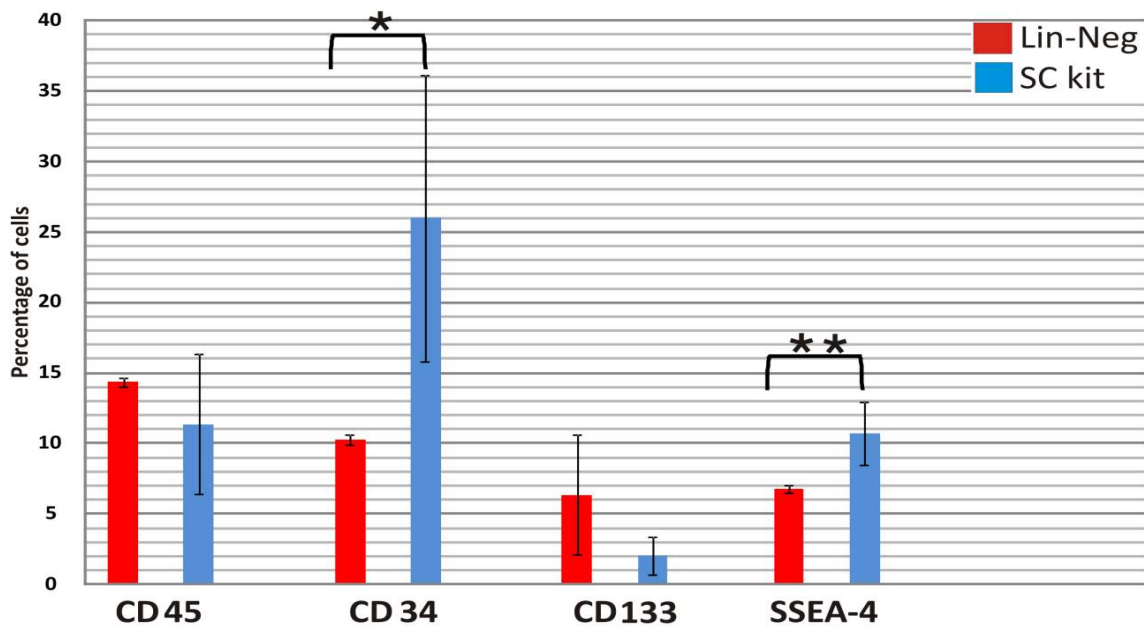
Figure 21. Statistical analysis between Lin-Neg and SC kit using FACS results to assess statistical significant differences between the two methods. Results showed that Lin-Neg had significantly fewer CD34 positive cells compared to SC kit (*) which also showed higher statistical significant enrichment rates of SSEA-4 positive cells and in comparison to Lin-Neg (**) ($p<0.05$). For the rest of markers, differences were not statistical significant. p values were calculated using Student's t-test (n=6). Values for CD133 were multiplied in 10 for presentation and scaling purposes.

3.3.3 *Purified cord blood stem cells expressed pluripotency markers*

Both isolation methods (SC kit and Lin-Neg) purified a relatively homogenous population of cells in comparison with MNCs after Ficoll centrifugation (figure 22, also refer to figure 62A in appendix II). The cells were round in shape with a diameter of 6-10µm and high nucleus to cytoplasm ratio.
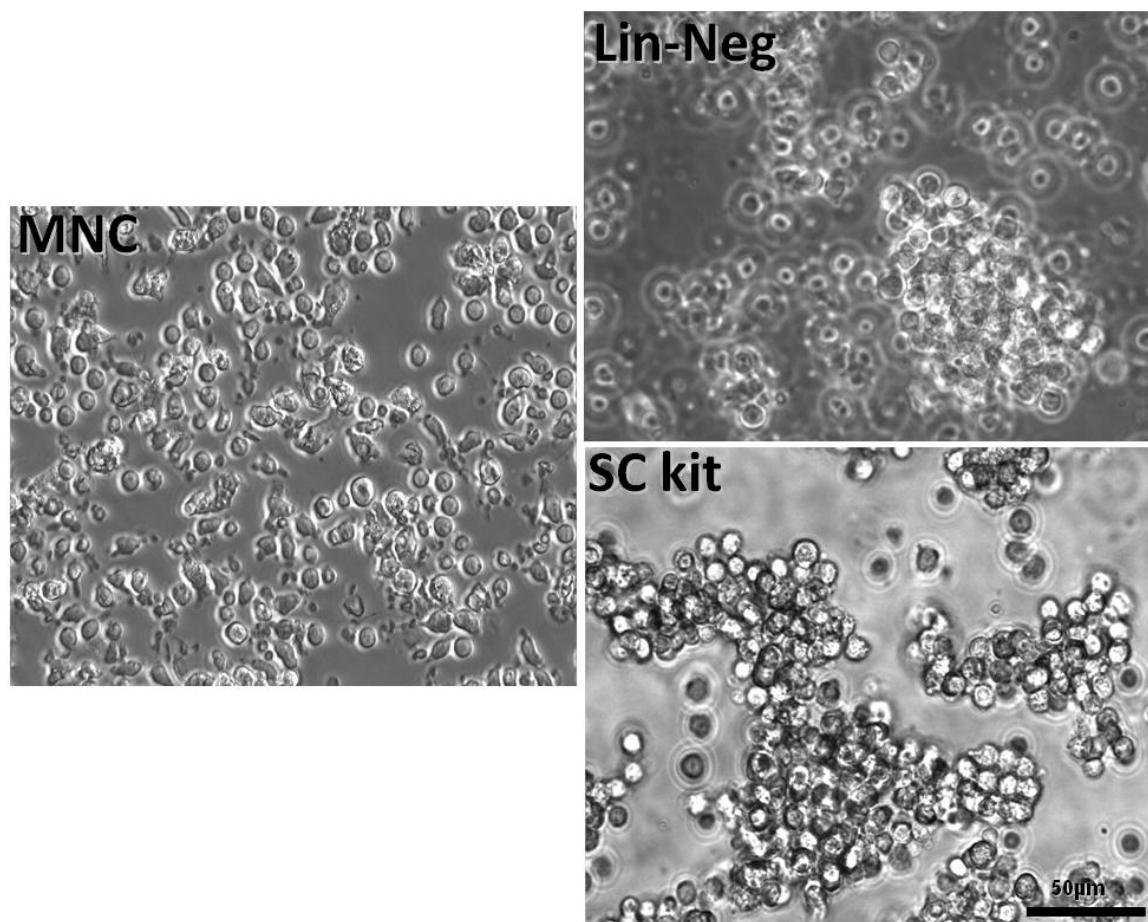
Figure 22. Purified umbilical cord blood stem cells using Lin-Neg and SC kit showed higher homogeneity compared with MNCs after Ficoll. MNC fraction contained a mixture of cells with different sizes and morphologies while both negative depletion methods (Lin-Neg and SC kit) yielded a relatively homogenous population of round-shaped cells with a diameter between 6-10 µm.

The purified cells co-expressed OCT4 and SOX-2 (figure 23). The two proteins were localized in the nuclei of the cells. They also expressed NANOG and KI67 in a similar manner (figure 24).
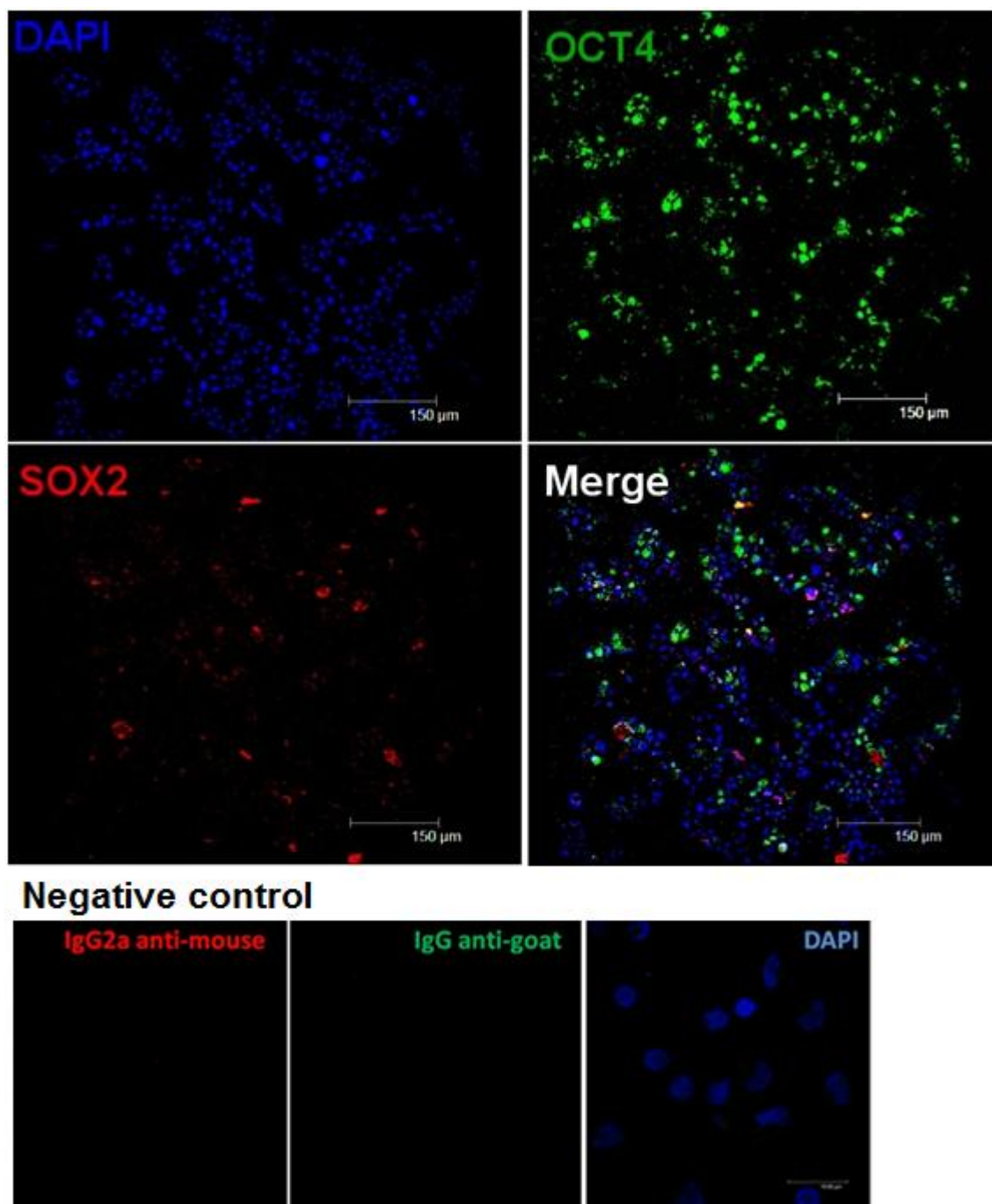
Figure 23. Freshly purified umbilical cord blood stem cells expressed OCT4 and SOX-2. Both proteins were localized in the nuclei of cells. Cells shown in this image were purified using SC kit. Negative controls are shown where only secondary antibodies were used. For additional images, refer to (McGuckin et al., 2008) attached at the end of this thesis.
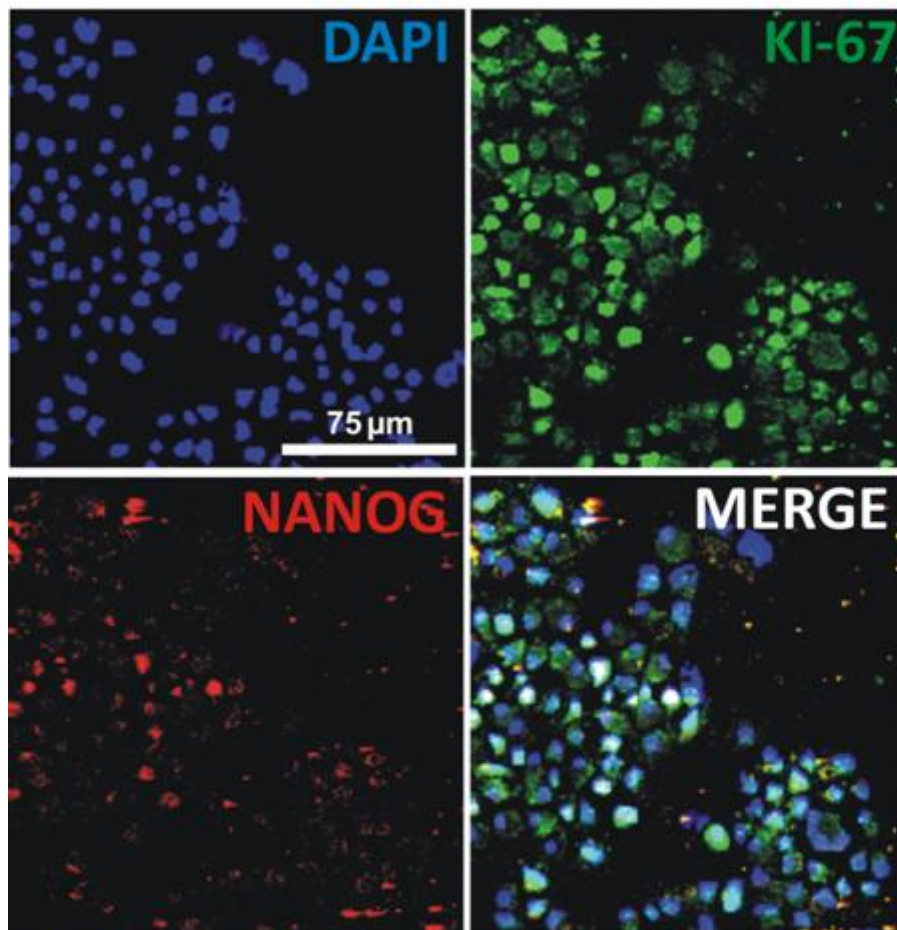
Figure 24. Freshly purified umbilical cord blood stem cells expressed KI-67 and NANOG. Both proteins were localized in the nuclei of cells. Cells shown in this image were purified using SC kit. Negative controls are shown where only secondary antibodies were used

3.4 Conclusions

- Umbilical cord blood contains a distinct population of non-hematopoietic multipotent stem cells expressing markers associated with embryonic stem cells.

- This distinct population of stem cells can be purified and enriched using an immuno-magnetic negative depletion strategy.

- SC kit demonstrated experimental and practical superiority to Lin-Neg kit. Therefore, we will utilize SC kit as the purification kit of choice for the rest of the research outlined in this thesis.

3.5 Discussion

The studies in this chapter compare two different methods to purify and enrich umbilical cord blood non-hematopoietic multipotent stem cells. Both methods utilized a negative depletion strategy by which specific antibodies with magnetic properties are used to label hematopoietic cells and then deplete them using a magnet to enrich the targeted non-hematopoietic multipotent stem cell population. The purified cell fraction was then characterized using FACS and immunocytochemistry. The purified cells by both methods showed significantly elevated expression levels of SSEA-4 when compared with cells prior to the purification step, indicating enrichment of the targeted non-hematopoietic stem cells. The purified cells also expressed markers normally associated with pluripotent stem cells including OCT4, SOX2 and NANOG. The SC kit showed number of advantages over the Lin-Neg kit. Beside its shorter purification time, simplicity and cost, it showed a statistically significant higher SSEA-4 positive cell enrichment when compared with Lin-Neg. Hence, further studies in this thesis utilized the SC kit as the purification kit of choice for research.

3.5.1 *Ficoll density gradient centrifugation eliminated umbilical cord blood erythrocytes*

The first step towards isolating and purifying umbilical cord blood non-hematopoietic multipotent stem cells involved the use of a Ficoll density gradient centrifugation to separate the mononuclear cell (MNCs) fraction, which contains the targeted cells, from the erythrocytes (red blood cells). Eliminating erythrocytes before negative depletion step is highly recommended to avoid any possible interference of these cells with the depletion step (Jaatinen and Laine, 2007). The diluted umbilical cord blood was layered above a Ficoll layer, which is composed of highly branched polysaccharides of high molecular weight, and then centrifuged (figure 17). This step separated blood cellular contents based on their density where denser cells like erythrocytes settle in the bottom of the tube whilst the less dense mononuclear cells (MNCs) fraction forms a buffy coat layer between the Ficoll layer and the plasma. This layer was then easily isolated and subjected to the negative depletion step. The FACS analysis results of unprocessed umbilical cord blood samples and samples after Ficoll density gradient centrifugation did not show any

significant changes which were expected because this step only eliminated the erythrocytes and did not involve any sub-fractioning of the mononuclear fraction (figure 20).

The mononuclear fraction of umbilical cord blood is a highly heterogeneous mixture of cells including various types of mature hematopoietic cells (table 1), hematopoietic stem cells and a low concentration of the targeted non-hematopoietic multipotent stem cells (see section 1.5.3). Many groups, including ours, have used the mononuclear cells (MNCs) fraction of cord blood in neural differentiation protocols without further purification of the non-hematopoietic stem cells and showed positive differentiation results (Ali et al., 2009, Lee et al., 2007, Neuhoff et al., 2007). However, one problem with such differentiation cultures is that the concentration of non-hematopoietic stem cells in the MNCs fraction is very low and consequently the concentration of derived neurons in cultures would also be low which might limit proper analysis of these cells. In addition the presence of different hematopoietic cells in cultures might affect differentiation and influence data analysis. Therefore, purification and enrichment of the non-hematopoietic multipotent stem cells prior to differentiation would allow accurate analysis of the cells molecular properties as well as their differentiation potential.

3.5.2 *Enrichment and purification of umbilical cord blood non-hematopoietic stem cells after negative depletion step*

CD45 is a transmembrane protein tyrosine phosphatases that is present on all differentiated hematopoietic cells except erythrocytes (Trowbridge and Thomas, 1994, Baldwin and Ostergaard, 2001, Arendt and Ostergaard, 1997). This explains the high proportions of cells expressing CD45 in both unprocessed cord blood samples and MNCs samples after Ficoll density gradient centrifugation (figure 20). The association of CD45 with different types of differentiated hematopoietic cell makes it one of the major markers targeted in the negative depletion step and both kits used contained an antibody specific for CD45.

In theory, the depletion of umbilical cord blood hematopoietic cells should leave behind the targeted non-hematopoietic stem cell population due to their negativity for the markers used in the depletion step resulting in their enrichment. To evaluate this, we used SSEA-4 as enrichment indicator in the FACS analysis. SSEA-4 is a surface marker widely used to characterize and select embryonic pluripotent stem cells (Thomson et al., 1998, Carpenter

et al., 2004, Mitalipova et al., 2003) and is also expressed by umbilical cord blood non-hematopoietic multipotent stem cells (McGuckin et al., 2005, Kucia et al., 2007). FACS analysis of purified cells from Lin-Neg and SC kit showed a significant decrease in the proportions of cells expressing the major hematopoietic marker CD45 and statistically significant increase in proportions of cells expressing SSEA-4 compared with unprocessed cord blood samples (figure 20). This indicates significant purification and enrichment of the targeted non-hematopoietic stem cell population.

3.5.3 *SC kit demonstrated number of experimental and practical advantages over Lin-Neg kit*

One of the major differences between the SC kit and Lin-Neg is that the SC kit which is manufactured by Stem cell technologies, is designed to enrich hematopoietic progenitor cells while Lin-Neg targets the purification of cord blood non-hematopoietic stem cells (McGuckin et al., 2008). This difference was clearly noticed after FACS analysis of CD34, a marker of hematopoietic stem cells. Lin-Neg depletion showed a decrease in CD34 positive cells unlike SC kit which did not show any significant changes in the proportion of CD34 positive cells when compared with the unprocessed umbilical cord blood samples (figure 20). The antibody content of each kit is different, whilst the SC kit contains a cocktail of 12 different antibodies covering only mature and fully differentiated hematopoietic stem cells; the Lin-Neg kit utilizes 4 antibodies including CD33 which is a marker of a subset of hematopoietic progenitor cells (table 2 and 4). The targeting of Lin-Neg kit to hematopoietic progenitor cells offers a possible explanation for the significant depletion of CD34, a marker of hematopoietic progenitor cells, positive cells unlike the SC kit where none of the progenitor cells are being targeted and CD34-postive cells proportions did not show any significant changes. On the other hand, when compared to each other, SC kit showed more significant enrichment of SSEA-4 positive cells compared to Lin-Neg (figure 21). Since SSEA-4 represent the direct marker associated with the targeted non-hematopoietic stem cells, the enrichment rate of these cells by SC kit represents a major experimental advantage over the Lin-Neg kit when considering the aim of this study.

From a practical point of view, the SC kit demonstrated a number of advantages over Lin-Neg kit. As mentioned earlier, the SC kit consists of a cocktail of 12 antibodies that are added all at one step to the MNCs fraction before performing the magnetic depletion of tagged cells using a specially designed column and magnet. The duration of the entire procedure is less than 90 minutes. On the other hand, the length of the Lin-Neg depletion procedure, which involves the use of only 4 antibodies, is around 4 hours because the antibodies are added at two separate steps resulting in a prolonged total procedure time (refer to section 2.3). Moreover, the cost of Lin-Neg depletion procedure is higher than SC kit depletion and this is because the antibodies and magnetic beads used in the procedure are purchased separately whereas the SC kit is commercially available and purchased as a complete kit.

The purification kits used in this study utilized negative selection strategies in which the antibodies used labeled the hematopoietic cells which were then depleted by the magnet leading to enrichment of the wanted non-hematopoietic multipotent stem cell population. Nevertheless, this strategy did not remove all the unwanted hematopoietic cells, indicated by the FACS analysis of CD45 in purified fractions (figure 21). Using higher concentration of CD45 antibodies in the purification kits might help improve the purity of the selected fraction. Moreover, utilizing positive selection strategies using FACS might also improve the purity of the wanted cells fraction. The mononuclear fraction after Ficoll isolation can be labeled with SSEA-4, as a positive marker of the wanted cells population, and CD45, as a negative marker. The cells then can be sorted using FACS where (SSEA-4-postive CD45-negative) fraction is selected. The purity of the selected fraction can be further assessed using multiplex qPCR, western blotting and immunocytochemistry.

3.5.4 *Expression of pluripotency markers and the misconception*

The enriched and purified fraction of cells from both kits expressed the main transcription factors shown previously to establish, maintain and regulate the pluripotent status of embryonic stem cells (Boyer et al., 2005, Boyer et al., 2006). OCT4 and SOX2 were co-localized in the nuclei of a subset the purified cells (figure 23), and such findings are consistent with current understanding of the interaction between these two transcription factors which form a heterodimer complex which regulates important gene expression (Rodda et al., 2005, Chew et al., 2005, Masui et al., 2007, Okumura-Nakanishi et al., 2005).

In addition, purified cells expressed NANOG, another pluripotency marker normally expressed by embryonic stem cells. NANOG was localized in the nuclei of a subset of the purified cells which also expressed Ki67, a transcription factor mainly expressed in proliferative cells (figure 24) (Brown and Gatter, 2002). Our results are consistent with the findings of other groups where they isolated similar cells from cord blood expressing OCT4, SOX2, NANOG, SSEA-4, TRA-1-60 and TRA1-81 (Kucia et al., 2007, McGuckin et al., 2005, McGuckin et al., 2008, Zhao et al., 2006, Zangiacomi et al., 2008, Chua et al., 2009).

The confocal images obtained for OCT4/SOX2 staining indicated that the expression of OCT4 did not correlate perfectly with DAPI in some cells. OCT4 is a transcription factor that is normally expressed in the nuclei of the stem cell. Nevertheless, OCT4 has different isoforms with different expression patterns (Cauffman et al., 2006). These isoforms include OCT4A and OCT4B which differ only in their N terminals whilst having identical C terminals. Whilst OCT4A was expressed mainly in the nuclei of human embryonic stem cells, OCT4B was mainly localized in the cytoplasm with no stemness properties (Cauffman et al., 2006, Lee et al., 2006). In this study we used an antibody that is not specific for certain OCT4 isoform (table 9), the antibody can bind to OCT4A and OCT4B as well which offer a possible explanation for the cytoplasmic localization of OCT4 in some cells. The quality of images obtained can be improved by using higher magnification lenses in order to accurately examine the localization of the transcription factors. The specific expression of *OCT4A* is further examined using qPCR in the next chapter. The activity of the SOX2 transcription factor is tightly regulated by post-translation modifications such as acetylation which affects its cellular distribution in embryonic stem cells (Baltus et al., 2009). This might offer a possible explanation for the weak cytoplasmic localization of SOX2 in some cells. It is also possible that the high concentrations of primary antibodies used contributed to the cytoplasmic weak staining observed in some confocal images for the markers including NANOG and KI67 where negative controls did not show any staining.

Many groups have described these non-hematopoietic stem cells of umbilical cord blood as being "embryonic-like" and pluripotent. Such conclusions have been drawn based on expression profiles of above-mentioned markers rather than meeting the widely accepted criteria used to define pluripotent stem cells (table 14) (McGuckin and Forraz, 2008, Harris

and Rogers, 2007, Lu and Ende, 1997, McGuckin et al., 2006, McGuckin et al., 2005, Kucia et al., 2007). As mentioned in the introduction, an important and widely accepted test of pluripotency is teratoma formation (see section 1.4.2). To date, there has not been a single reported case where cord blood stem cells formed teratoma when transplanted into SCID mice, therefore associating these cells with terms such as pluripotent and embryonic-like might not be appropriate. Expression profiles of markers by certain stem cells might not be the accurate and ideal way to describe and justify their "potency"; nevertheless, they can provide useful indications about the stemness and differentiation status of the cells.

 In our case, umbilical cord blood non-hematopoietic multipotent stem cells expressed OCT4, SOX2, NANOG, Ki67 and SSEA-4. The first three markers provided indications about a "stem cell" identity and suggested a possible wide range of differentiation capabilities rather than justifying pluripotent property. Expression of Ki67 suggested proliferative characteristics of the isolated cells, a property shared by different kinds of stem cells *in vitro*. Expression of SSEA-4 suggested the naïve undifferentiated status of the cells and distinguished them from the hematopoietic cell population of umbilical cord blood.

Chapter 4. Defined sequential neuronal differentiation protocol for umbilical cord blood non-hematopoietic multipotent stem cells and neural tissue engineering application

4.1 Introduction

Umbilical cord blood stem cells have been shown previously to have a high potential for neuronal differentiation (Buzanska et al., 2002, McGuckin et al., 2004, Jurga et al., 2006, Zangiacomi et al., 2008, Zangiacomi et al., 2009). This differentiation capability made umbilical cord blood stem cells a potential candidate for neuronal clinical applications including tissue engineering applications (see section 1.5.4). However, before regenerative medicine and neural tissue engineering applications can be considered in clinics, more information is needed about the biological potential of these cells and the possibility that they can be used safely in patients.

One of the most important issues that need to be taken into consideration is the safety of the *in-vitro* cell culturing protocols. It is necessary to design culturing and differentiation conditions free of animal products in order to reduce risk of contamination and possible infections by animal microbes (Unger et al., 2008). Animal serum is commonly used in laboratories as a supplement for the cell culture media. It contains a wide range of macromolecules including hormones, growth factors and different low molecular weight nutrients (Gstraunthaler, 2003). In stem cells applications, it is important to use well-defined culturing media in order to accurately study and precisely trigger targeted differentiation pathways, and undefined components of serum might interfere and exert unwanted effect on cells (Pick et al., 2007). It is important as well to keep the *in-vitro* culturing and differentiation durations as short as possible to reduce the risk of infections and chromosomal aberrations common in stem cell populations including embryonic stem cells (McGuckin et al., 2008, Corselli et al., 2008, Spits et al., 2008).

Moving into regenerative medicine and tissue engineering applications requires the development of an efficient 3-dimensional tissue engineering system including bio-compatible scaffolds that provide the cells with the mechanical support and organization to develop functional differentiated tissues. These systems must be accompanied with highly efficient and effective isolation and differentiation protocols in order to generate clinically functional tissues that could be can be transplanted into patients (McGuckin and Forraz, 2008). Hence, we incorporated our defined neuronal induction protocol with scaffold-based culturing conditions in order to evaluate the potential of using the purified cord blood stem cells in future regenerative medicine and neural tissue engineering applications.

In the studies outlined in this chapter, we present a defined serum-free sequential neuronal induction protocol to differentiate purified umbilical cord blood non-hematopoietic multipotent stem cells into neuron-like cells over a period of 24 days. The differentiation process is further characterized by analyzing the expression patterns and levels of certain markers thought to be involved in the process of neurogenesis (table 16). We then incorporate this differentiation protocol with a scaffold-based 3-dimensional culturing system and analyze the differentiation process.

Table 16. Markers used for characterization of neuronal development of umbilical cord blood purified stem cells.

	Marker	Expression pattern		References
Early neural commitment	NESTIN	An intermediate filament associated with multi-lineage progenitor cells with high proliferation characteristics.	Co-expression of NESTIN and GFAP is associated with proliferative neural stem cells in the SVZ of cerebral cortex and hippocampus.	Tavazoie et al., 2008, Wiese et al., 2004, Abrous et al., 2005
	GFAP	An intermediate filament expressed in astrocytes and a subset of neural stem cells in CNS.		
Neuronal markers	NF200	Structural proteins expressed in neurons and involved in regulation of neuronal cytoskeletal architecture.		Xiao et al., 2006
	B-TUBULIN III			Katsetos et al., 2003
	GAP43			Mishra et al., 2008
Later neuronal markers	NEUN	A DNA-binding neuron-specific protein associated with neuronal mature status in CNS.	Both markers are expressed in later stages of neuronal development.	Zhao et al., 2008, El-Husseini et al., 2000, Hata and Takai, 1999
	PSD95	Post synaptic density protein associated with receptors and cytoskeletal elements at the synapses and involved in maturation of functional synapses.		

4.2 Study aims

- To differentiate previously purified umbilical cord blood non-hematopoietic multipotent stem cells into neuron-like cells using defined serum-free sequential differentiation protocol *in vitro*.

- To characterize and analyze the neuronal commitment of umbilical cord blood non-hematopoietic multipotent stem cells using real time PCR and immunocytochemistry.

- To incorporate the neuronal differentiation protocol with a scaffold-based 3-dimensional culturing system to produce 3-dimensional neuronal-like tissue from umbilical cord blood non-hematopoietic multipotent stem cells.

4.3 Results

4.3.1 *Purified umbilical cord blood stem cells adopted neuronal-like morphology after neuronal induction*

Freshly purified umbilical cord blood non-hematopoietic stem cells using SC kit were found to make up around 1.2% (S.D=0.7, n=5) of total mononuclear cells after Ficoll density gradient step. Cell viability analysis using 7AAD showed that cells after SC kit had 81.87%±5.72 (n=4) viability while after Ficoll the percentage of viable cells was 87.10%±4.02 (n=4) (figure 25). These cells were round in shape with a diameter between 6-10µm (figure 26). The cells formed aggregates that varied in size. These aggregates began to attach firmly to the collagen IV coated cover slips after 24 hours of initial culturing (figure 26). By day 10, many cells lost their round morphology and became spindle in shape with clear thin projections merging from the cells bodies (figure 27). The majority of cells aggregates remained intact and the morphological transformation was noticed at the edges of the aggregates and in individual cells (figure 27).
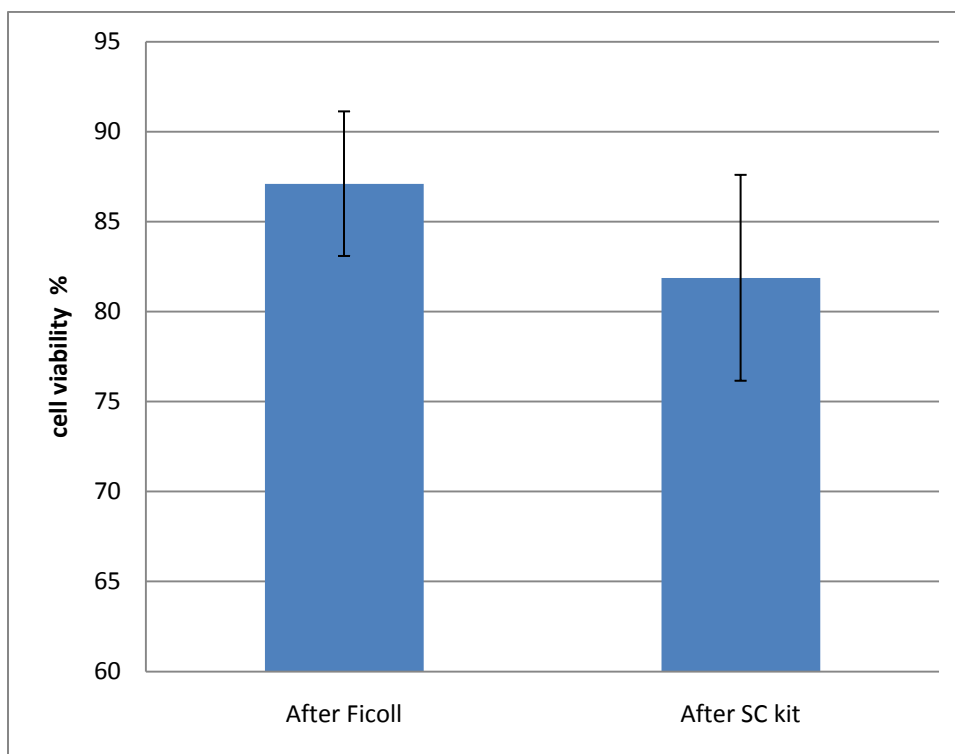
Figure 25. Cell viability after Ficoll isolation and SC kit purification. Cells viability was determined using FACS and 7AAD. Results indicated no statistically significant differences in percentages of viable cells between cells after Ficoll isolation and after SC kit purification. (Student t test, *p*>0.05, n=4).
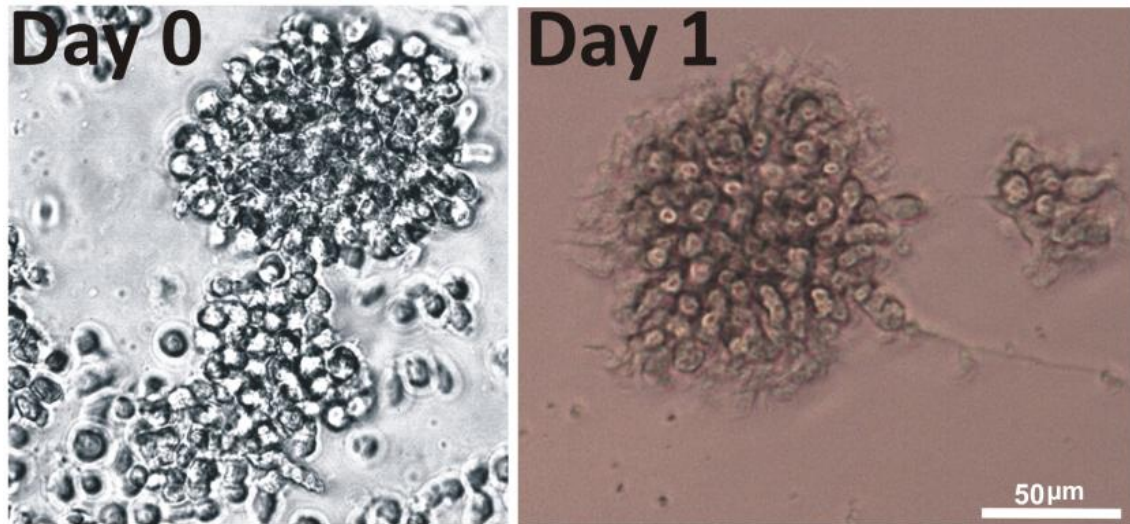
Figure 26. Attachment of the freshly purified umbilical cord blood stem cells. Freshly purified stem cells (Day 0) formed non-adherent aggregates. Within 24-48 hours of initial culturing (Day 1), these aggregates attached firmly to the collagen IV coated cover slips and fine and short processes appeared at the edges of these attached aggregates.
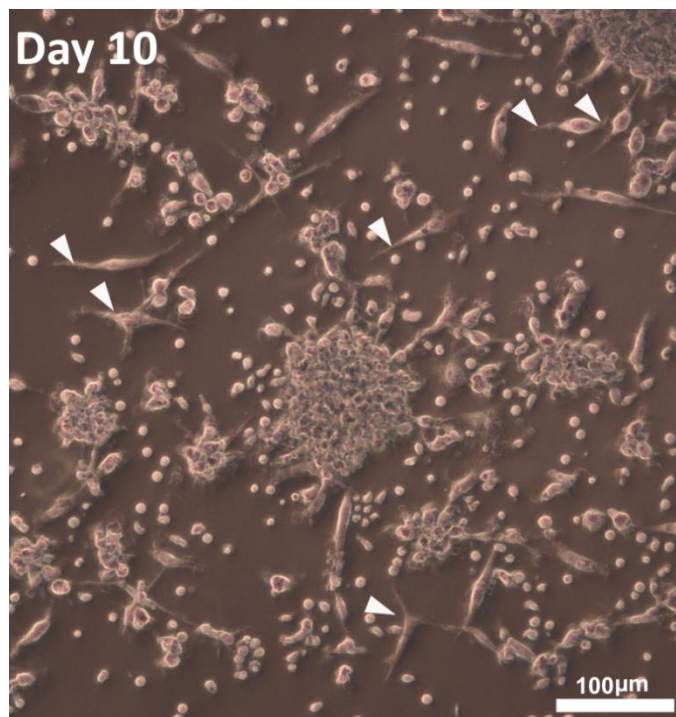
Figure 27. Morphological transformation of umbilical cord blood purified stem cells at day 10. After early neural commitment phase (Day 10), cells transformed their round spherical morphology into spindle-shape morphology with clear thin and long projections growing from the cell's body (white arrows). The morphological transformation was accompanied with noticeable increase in cell size. Note that the change in morphology can be seen in individual cells and not within the cells aggregates.

After neuronal differentiation (Day 17), cells adapted a clear neuronal-like morphology. They started to show polar structures with clear growing main axon-like and dendrite-like structures (figure 28).

Figure 28. Differentiated cells acquired a neuronal-like phenotype by day 17. Cells featured polarized neuronal-like morphology with thin long axon-like (lower arrow) and short dendrite-like structures (upper arrow) developed from cell bodies.

After neuronal maturation (Day 24), the cells acquired a more advanced neuronal-like morphology where the axons-like structures became thinner, longer (reaching over 100µm in length) and more visible (figure 29). Cells projections showed enhanced branching (figure 29C). Physical cell-cell connections formed complex networks and potential synaptic junctions between cells (figure 29C). The neuronal maturation process was also accompanied with cell death of differentiating cells (figure 29C1, C2). These cells showed clear multiple holes in their bodies which indicate dying cells.
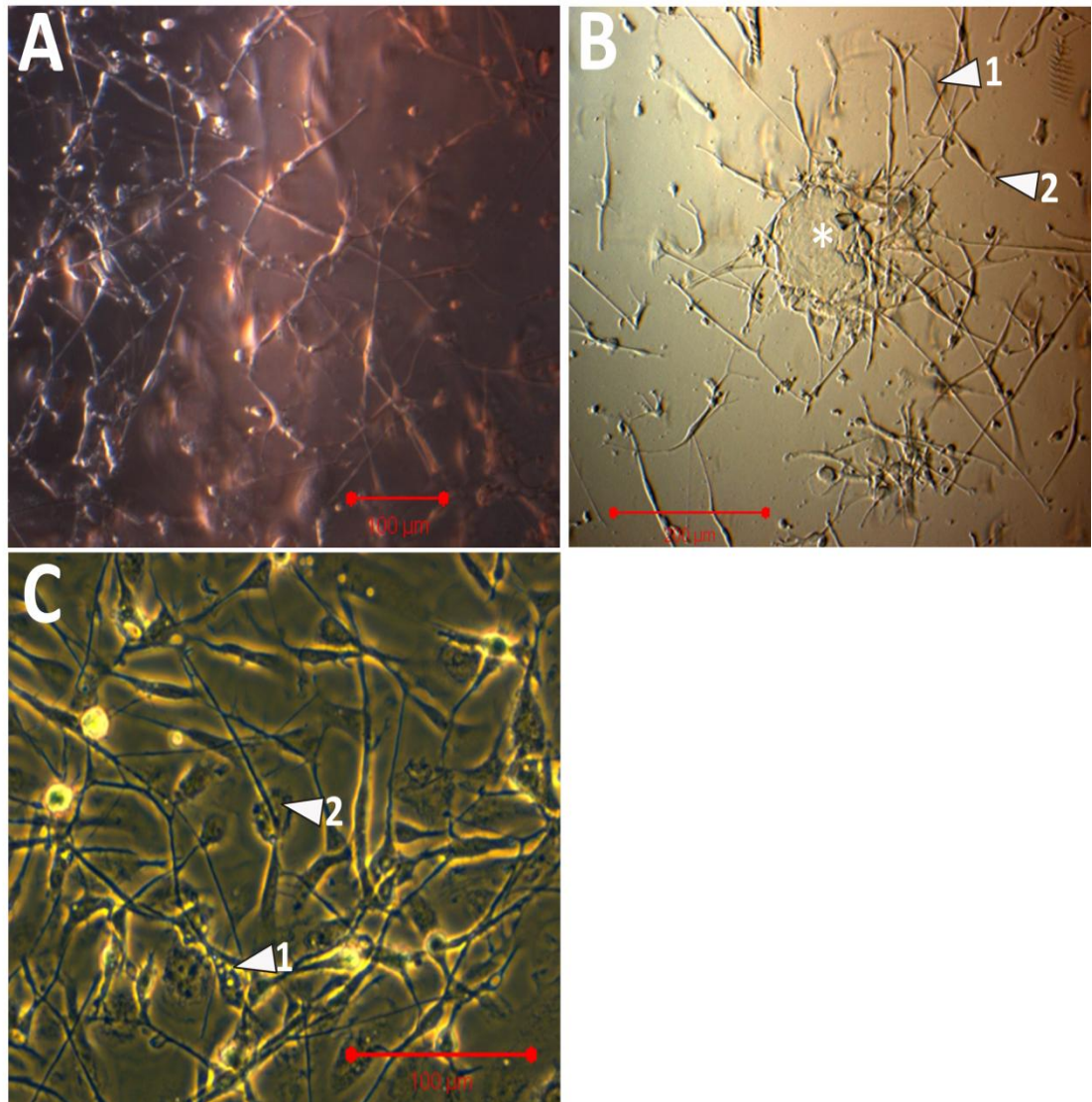
Figure 29. Cord blood stem cells acquired advanced neuronal-like morphology after the neuronal maturation phase (Day 24). Cells showed very thin and long neuronal-like processes (more than 100µm in length) (A). Cells featured polarized neuronal-like morphology with axon-like structures (B1) and dendrite-like structures (B2). Some cell aggregates were still tightly compacted at day 24 (B*). Physical cell-cell connections formed complex neuronal-like networks with possible synapses-like structures between cells (C). Dead cells were observed in the cultures after neuronal maturation (Day 24), some of these cells had a neuronal-like morphology (C1) while others were round in shape (C2).

4.3.2 *Down-regulation of pluripotency genes and up-regulation of neuronal genes during the 24 days differentiation period*

Quantitative real time PCR (Q-PCR) (see section 2.11.4) showed that *OCT4, SOX2, NANOG* and *C-MYC* gene expression levels, normalized to *GAPDH* expression, decreased significantly between undifferentiated cells (Day 0) and cells after neuronal maturation (Day 24) ($p < 0.05$, n=3, similar p value for all the genes) (figure 30).
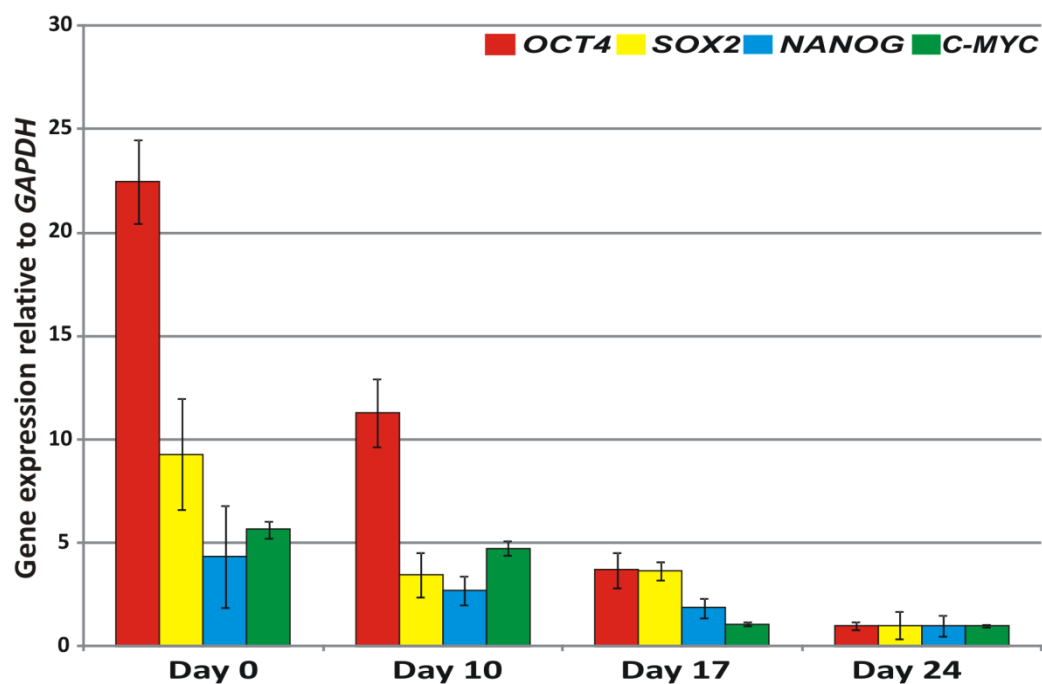
Figure 30. Q-PCR analysis of pluripotency genes (*OCT4, SOX2* and *NANOG*) and (*C-MYC*) expression patterns throughout the 24 days differentiation protocol. *OCT4, SOX2* and *NANOG* were highly expressed in undifferentiated cord blood stem cells (Day 0). Their expression levels were down-regulated gradually between day 0 and day 24. *C-MYC* was expressed by undifferentiated stem cells at day 0 and its expression level increased reaching its highest point by day 10 before being down-regulated at day 17 and further at day 24. Genes expression levels were normalized to the expression of GAPDH. The calibrator for the four genes is Day 24. Changes in genes expression levels for the four genes throughout the 24 days were statistical significant (ANOVA test, $p < 0.05$, n=3 apply for all genes).

On the other hand, Q-PCR results showed that *PSD95* gene expression level normalized to *GAPDH* was significantly up-regulated between (Day 0) and (Day 24) (p<0. 005, n=3) (figure 31).
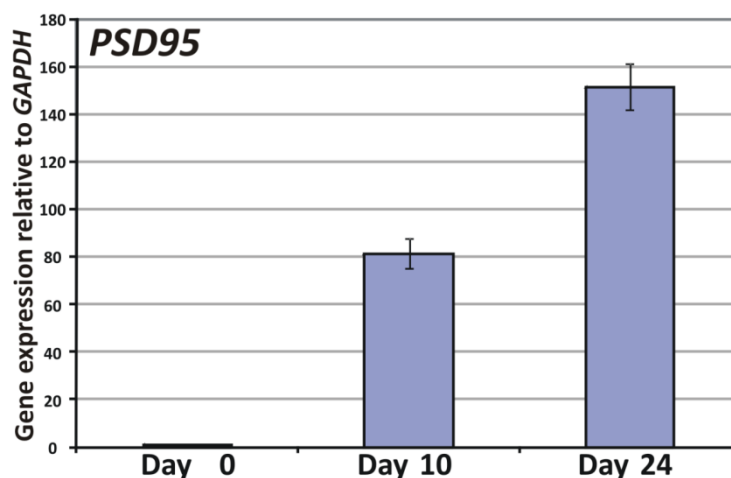
Figure 31. Q-PCR analysis of *PSD95* expression pattern between day 0 and day 24. *PSD95* expression level in undifferentiated cord blood stem cells (Day 0) was extremely low before being significantly up-regulated after early neural commitment (Day 10) and neuronal maturation (Day 24). Gene expression levels were normalized to the expression of GAPDH. The calibrator is Day 0. Changes in gene expression levels throughout the 24 days were statistical significant (ANOVA test, *p*<0.005, n=3 apply for all genes).

4.3.3 *Immunocytochemistry analysis of neuronal markers throughout the 24-day differentiation protocol*

The cord blood stem cells co-expressed NESTIN and GFAP in their cytoplasm (figure 32). The percentages of cells expressing NESTIN increased from 10.42±4.31% (n=3) at day 0 to reach 73.5±6.18% (n=3) at day 10 and 83.46±7.76% (n=3) at day 17 before decreasing to 32.12±8.16% (n=3) by day 24 (figure 33). The trend of percentages of cells expressing GFAP was similar to NESTIN where only 8.96±3.07% (n=3) expressed GFAP at day 0 before the percentage increased to 62.57±9.37% (n=3) at day 10 and 68.84±2.53% (n=3) at day 17 and then decreased to 30.06±8.79% (n=3) by day 24 (figure 33). Changes of

percentages over the 24 days period were statistically significant (ANOVA, p<0.005 for NESTIN and GFAP).
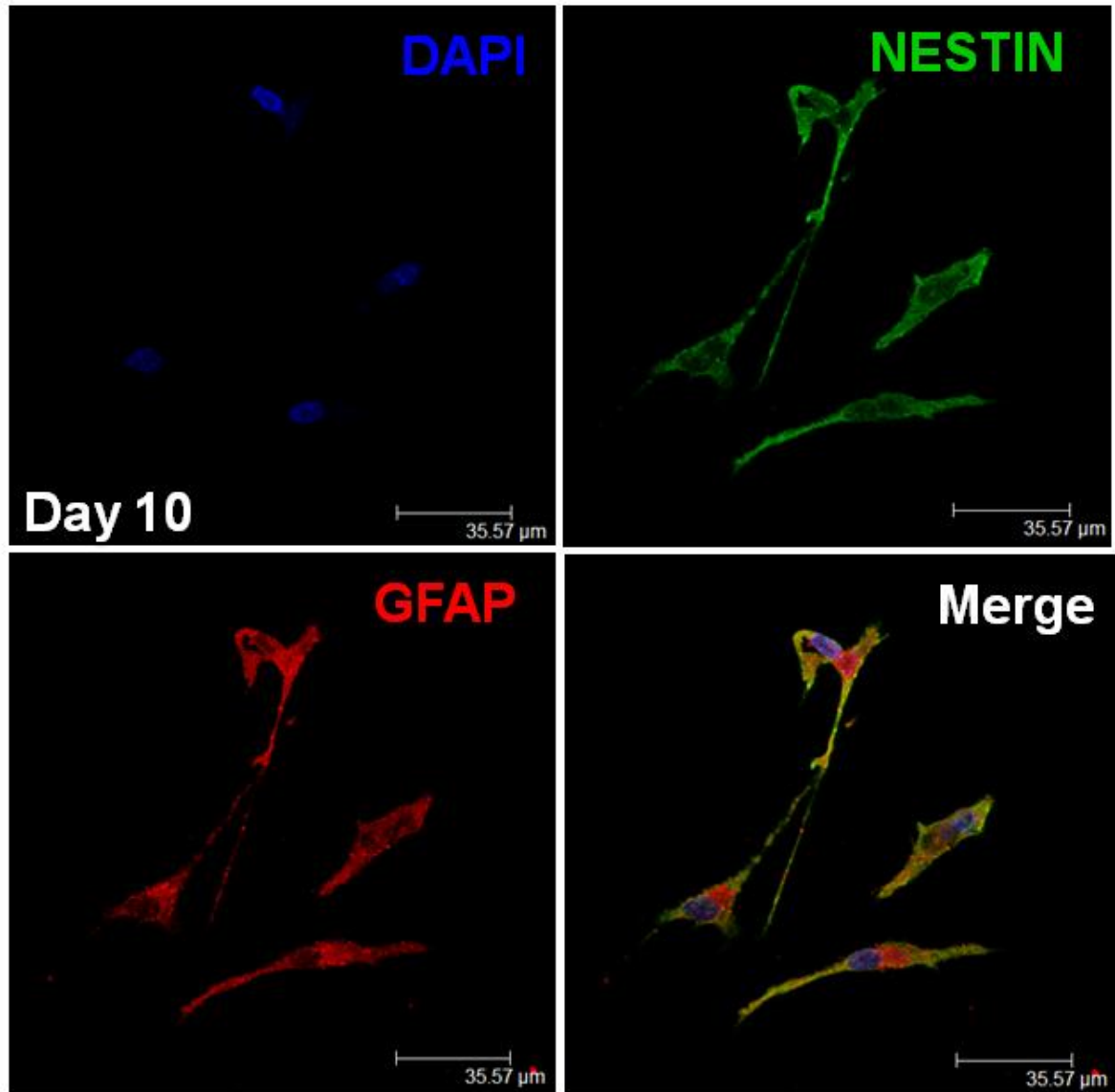
Figure 32. Umbilical cord blood purified stem cells co-expressed NESTIN and GFAP. Both markers were localized in the cells cytoplasm. Lower panel shows overlying of the markers with DAPI (Merge).Picture shown is taken from cells at (Day 10).
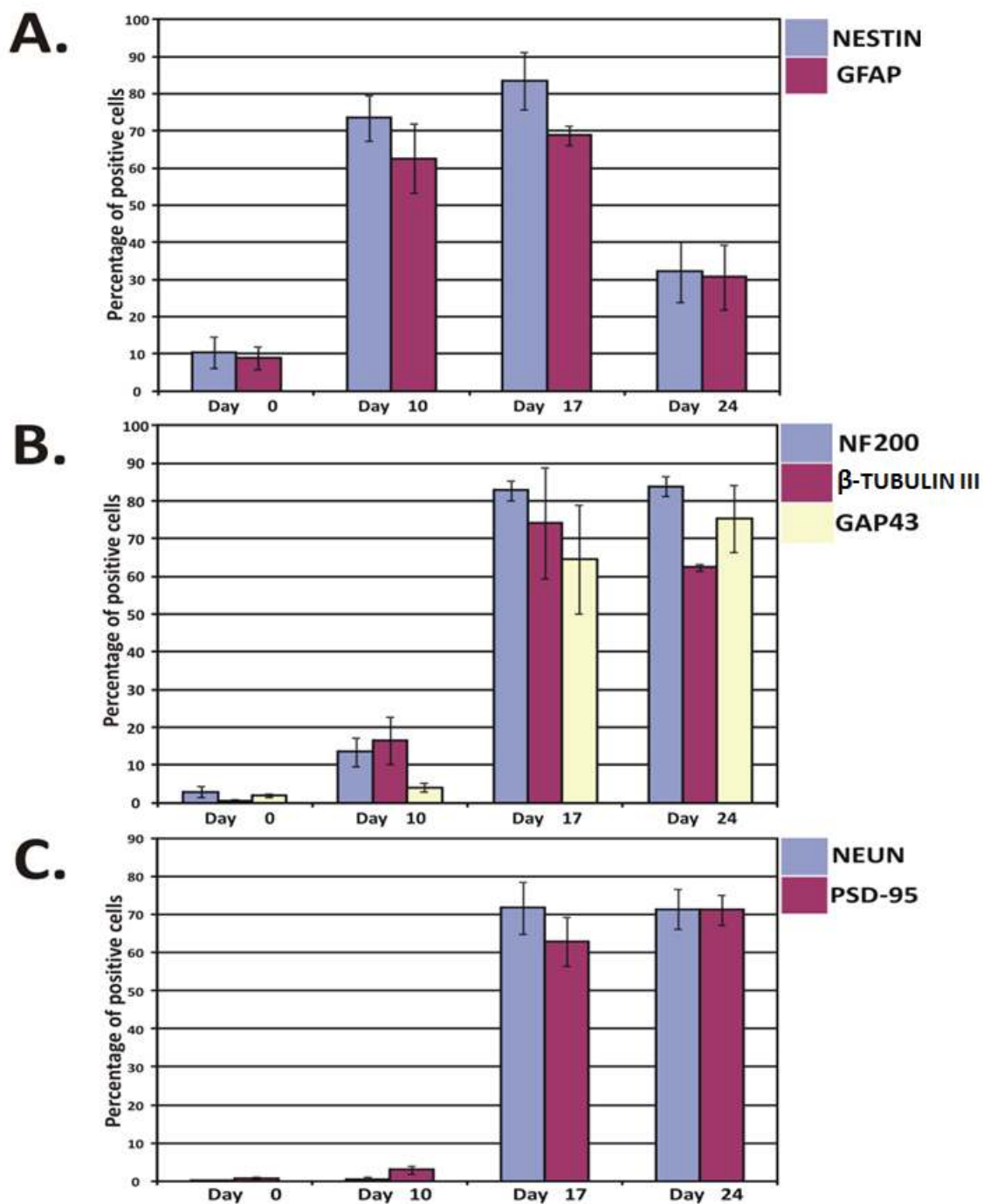
Figure 33. Expression analysis of early neural and neuronal specific markers during the 24-days neuronal induction protocol. Percentages of cells expressing NESTIN and GFAP increased between (Day 0) and (Day 10) before decreasing after maturation (Day 24) (A). Percentages of cells expressing neuronal-specific structural proteins NF200, β-TUBULIN III and GAP43 showed major increase after neuronal differentiation (Day 17) and stayed high after maturation (Day 24) (B). Percentages of cells expressing neuronal specific markers NEUN and PSD95 were extremely low between day 0 and day 10 before showing major increase after neuronal differentiation (Day 17). Changes in percentages over the 24 days period were statistically significant for all markers. (ANOVA, $p < 0.005$, $n = 3$, for all markers).

Percentages of cells expressing NF200, β-TUBULIN III and GAP43 were too low at day 0, 2.94±1.41% (n=3) for NF200, 0.57±0.4% (n=3) for β-TUBULIN III and 1.82±0.42% (n=3) for GAP43 (figure 33). Percentages remained relatively low at day 10, 13.48±3.82% (n=3) for NF200, 16.51±6.32% (n=3) for B-TUBULIN III and 4.11±1.14% (n=3) for GAP43. The percentages increased after differentiation at day 17 to 82.91±2.64% (n=3) for NF200, 74.32±14.72% (n=3) for β-TUBULIN III and 64.64±14.37% (n=3) for GAP43. At day 24, percentages stayed at roughly the same levels, 83.94±2.61% (n=3) for NF200, 62.49±0.88% (n=3) for β-TUBULIN III and 75.39±8.89% (n=3) for GAP43. Changes of percentages over the 24 days period were statistically significant (ANOVA, p<0.005 for NF200, β-TUBULIN III and GAP43). Differentiated cells expressed the three markers in their cytoplasm and noticeably in growing terminals like axons-like structures (figure 34, 35).
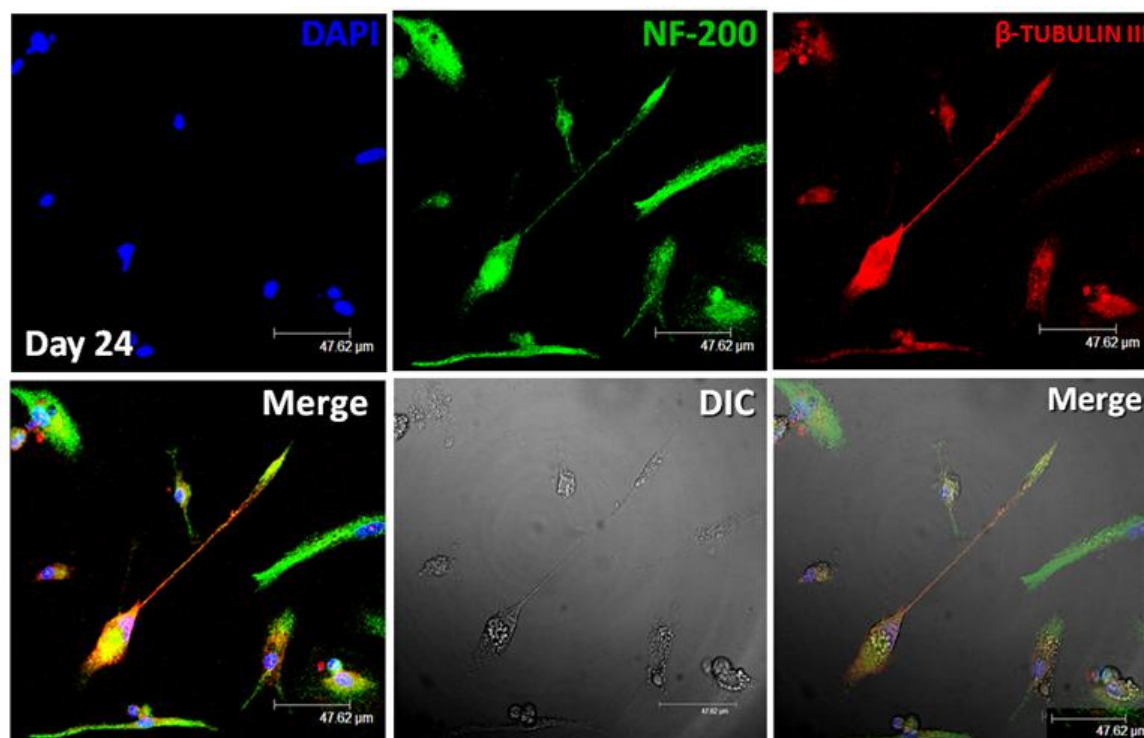
Figure 34. Immunocytochemistry analysis of NF200 and β-TUBULIN III. Differentiated (Day 17, data not shown) and maturated neuronal-like cells (Day 24) derived from umbilical cord blood stem cells highly expressed NF200 and β-TUBULIN III in growing axon-like structures and terminals. Lower panel shows overlying of the markers with DAPI (left Merge) and overlying of the markers with DAPI on DIC image of the cells (right Merge). Areas with strong co-expression of both markers appear in yellow. Images shown are for cells at day 24.
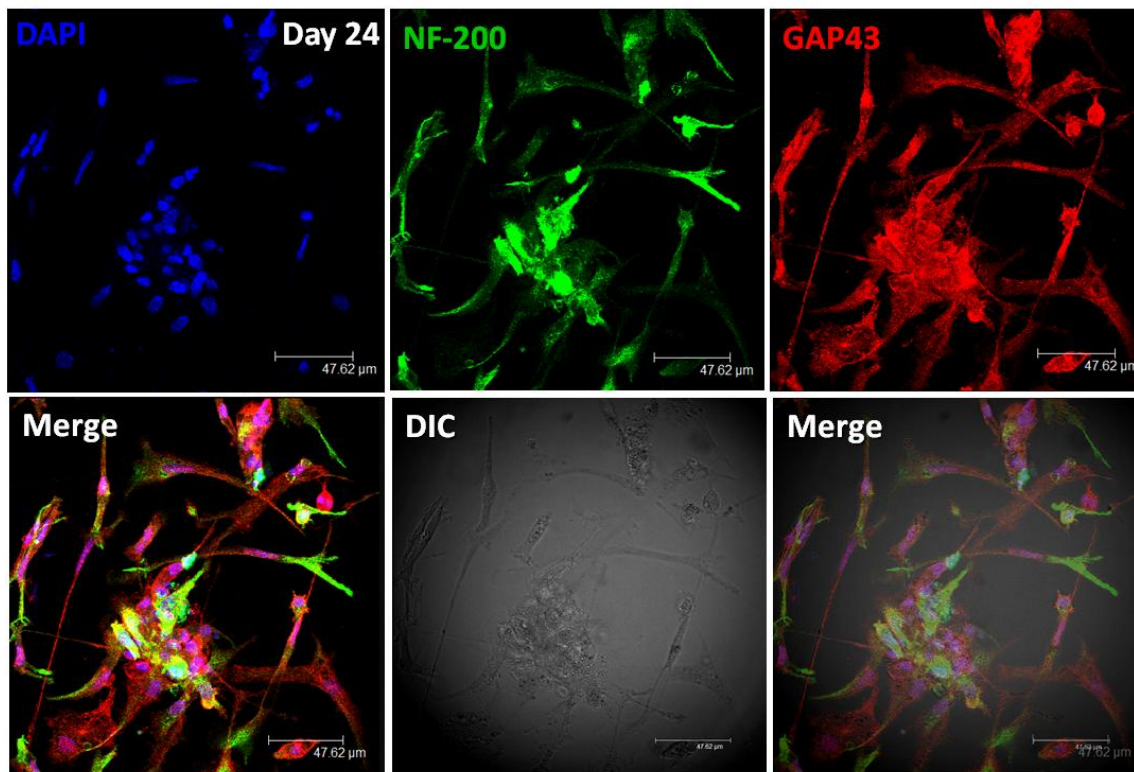
Figure 35. Immunocytochemistry analysis of NF200 and GAP43. Differentiated (Day 17, data not shown) and matured neuronal-like cells (Day 24) highly expressed NF-200 and GAP43 in growing axon-like structures and terminals. Lower panel shows overlying of the markers with DAPI (left Merge) and overlying of the markers with DAPI on DIC image of the cells (right Merge). Areas with strong co-expression of both markers appear in yellow. Images shown are for cells at day 24.

Differentiated cells expressed the more advanced neuronal markers NEUN and PSD95. NEUN was localized specifically in cell nuclei while PSD95 was expressed in the cytoplasm of cells (figure 36). Percentages of cells expressing NEUN increased significantly from 0.09±0.1% (n=3) at day 0 and 0.59±0.44% (n=3) at day 10 to 71.73±6.87% (n=3) at day 17 and 71.31±5.27% (n=3) at day 24. Proportions of cells expressing PSD95 followed the same trend. Percentages increased from only 0.82±0.22% (n=3) at day 0 and 3.01±1.11% (n=3) at day 10 to 62.92±6.42% (n=3) at day 17 and 71.18±3.97% (n=3) at day 24 (figure 33). Changes of percentages over the 24 days period were statistically significant for both markers (ANOVA, p<0.005).
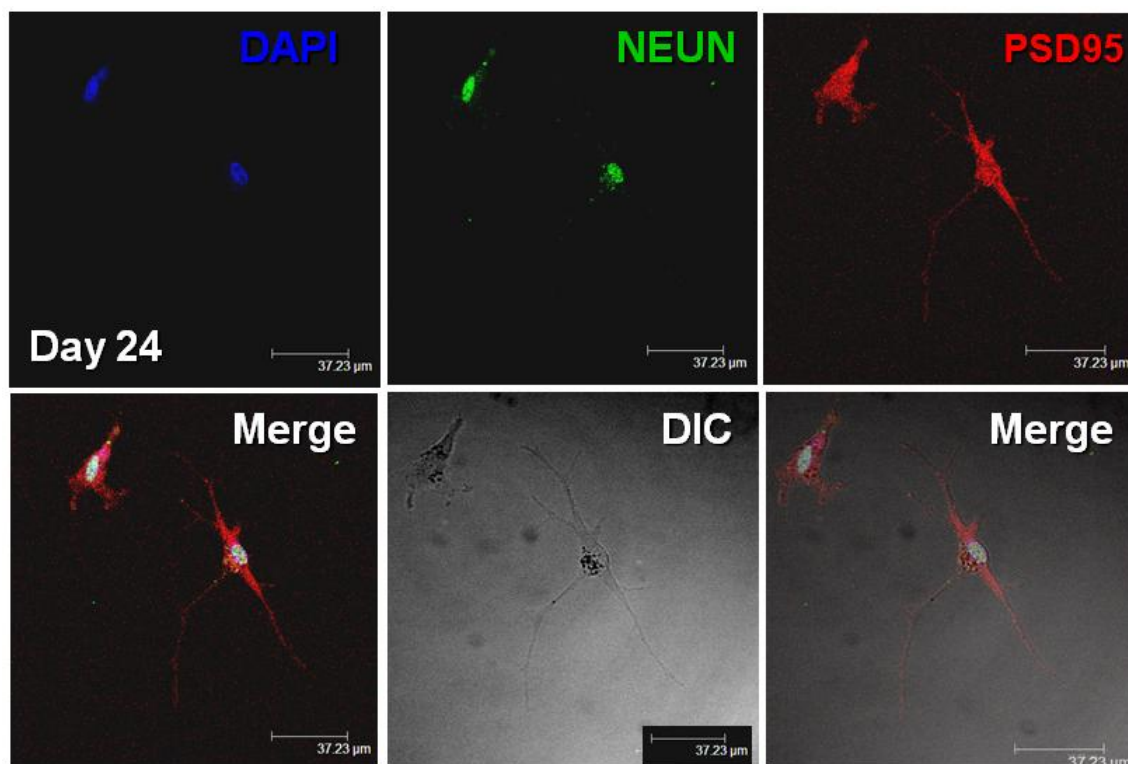
Figure 36. Differentiated and maturated cells expressed neuronal-specific markers NEUN and PSD95. NEUN is expressed specifically in cells nuclei and PSD95 is expressed in the cells bodies and terminals. Lower panel shows overlying of the markers with DAPI (left Merge) and overlying of the markers with DAPI on bright field image of the cells (right Merge). Images shown are for cells at day 24.

4.3.4 *Cells seeded into scaffolds expressed NF200 after the 24-days neuronal induction protocol*

Immunocytochemistry for cells differentiated and maturated in scaffolds showed that the scaffold provided the cells with mechanical and organizational support which allowed them to differentiate in a 3-D pattern inside the pores of the scaffold. Differentiated cells acquired neuronal-like phenotype and highly expressed NF200 (figure 37).
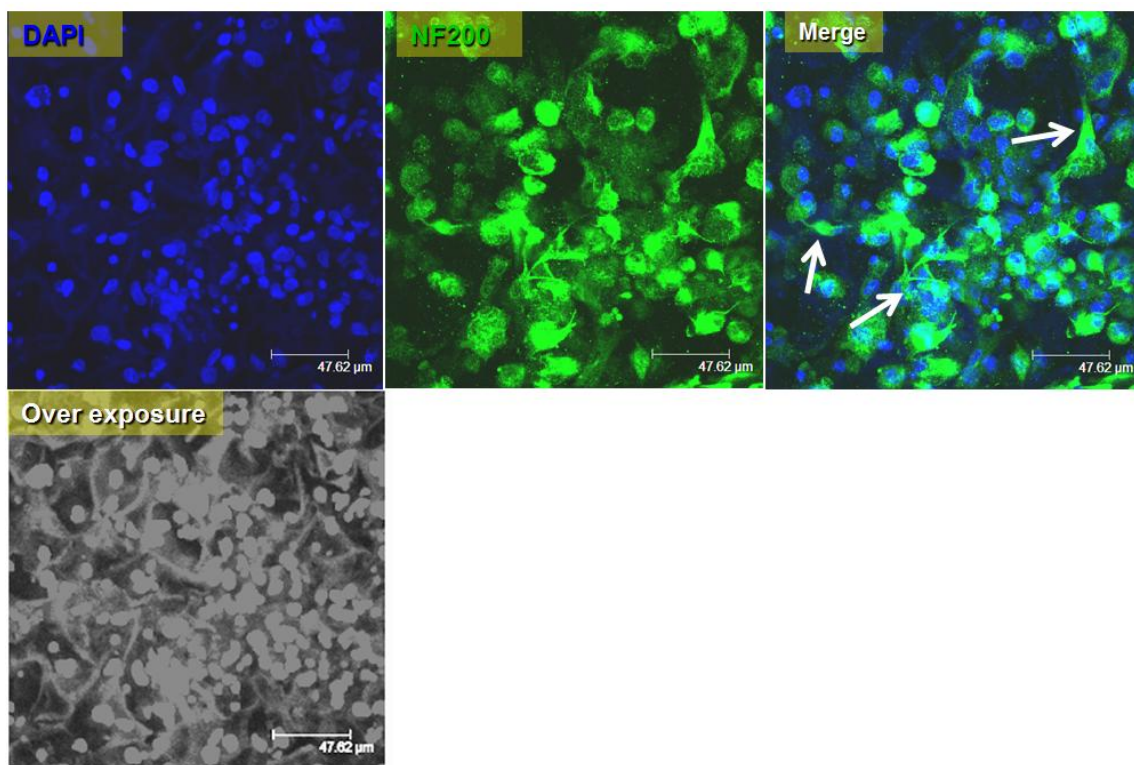
Figure 37. Cord blood stem cells seeded into scaffolds expressed NF200 after neuronal maturation (Day 24). The cells acquired neuronal-like morphology (white arrows in the merge image) highly expressed neuronal marker NF200. The (over exposure) image shows the scaffolds walls that provided mechanical and physical supports for the cells (represented with the bright DAPI spheres).

4.4 Conclusions

- Umbilical cord blood non-hematopoietic multipotent stem cells are capable of proper neuronal commitment and differentiation *in-vitro*.

- Our 24 days defined serum-free induction protocol efficiently differentiated umbilical cord blood non-hematopoietic multipotent stem cells into maturing neuron-like cells.

- Umbilical cord blood non-hematopoietic multipotent stem cells are a potential and promising candidate for clinical and future neural tissue engineering applications.

4.5 Discussion

In this chapter, the studies show that umbilical cord blood non-hematopoietic multipotent stem cells, purified by SC kit, can be differentiated into neuron-like cells *in-vitro*. A defined serum-free differentiation protocol is described, taking into account the requirements crucial for possible future clinical application. The neuronal commitment process of purified stem cells is characterized by analyzing the expression patterns of certain markers at both RNA and protein levels throughout the differentiation protocol. We then incorporated our differentiation protocol with 3-dimensional scaffold-based tissue engineering systems and showed that neuron-like cells could be produced in these scaffolds from seeded purified umbilical cord blood cells.

4.5.1 *Proper cell-cell interaction is essential for cells survival and proper neuronal differentiation*

Purified stem cells were cultured initially at a relatively high concentration (see section 2.5) because according to previous studies such high concentration of cells is necessary to initiate formation of cells aggregates that provide the cells with physical support and are important for cells proliferation and survival (Habich et al., 2006, Watt and Hogan, 2000). It has also been shown that close cell-to-cell contact is crucial for neuronal differentiation of stem cells isolated from umbilical cord blood. Zangiacome et al. (2008) showed that when stem cells isolated from cord blood where cultured at low concentration and were isolated from each other, they were unable to differentiation into neuron-like cells whereas at high concentration, the same cells were capable of neuronal differentiation upon induction.

In neurogenic niche, special molecules called extracellular matrix (ECM) provide the cells with structural and functional support that regulate their proliferation and differentiation (Raymond et al., 2009). We have coated the cover slips with collagen IV, one of the most common ECM molecules in human tissues including nervous tissues and commonly used for immobilization of neural stem cell and neuronal cultures *in-vitro*, to provide the plated cells with the physical support they need for proper differentiation (Lin et al., 2004, Raymond et al., 2009). It has been shown that collagen IV promotes and enhances neuronal differentiation of cortical progenitor cells (Ali et al., 1998). Collagen IV binds to special

receptors on cells membrane such as integrins causing changes in protein conformation which in-turn initiates signal transduction cascade involving in various cellular functions including proliferation and differentiation (Campos et al., 2004). The strategy we applied in our cultures aimed to mimic the microenvironment conditions in the neurogenic niches to direct the neuronal differentiation of the cultured cord blood purified stem cells.

4.5.2 *Fate commitment: purified stem cells adapting a neural fate*

To evaluate the neural commitment of cord blood purified stem cells, we investigated the expression levels patterns of NESTIN and GFAP. It was thought for a long time that Nestin, an intermediate filament, is only associated with neural progenitor cells and immaturity in the nervous system (Wiese et al., 2004). However it has been shown that Nestin expressing cells can be differentiated into many cell types such as hepatic cells (Kania et al., 2003), pancreatic cells (Denner et al., 2007) and neural cells (Jurga et al., 2006, Habich et al., 2006). Nestin expression can therefore be viewed as a property of multi-lineage progenitor cells with high proliferation characteristics (Wiese et al., 2004). Hence, using Nestin on its own as a marker of neural commitment might not be accurate. The co-expression of Nestin and GFAP by the same cell is stronger evidence of neural committed cells (figure 32) as co-expression of Nestin and GFAP is associated with proliferative neural stem cells in the SVZ of cerebral cortex and hippocampus (Abrous et al., 2005, Sohur et al., 2006, Zhao et al., 2008, Messam et al., 2002, Messam et al., 2000, Suh et al., 2007, Tavazoie et al., 2008). At day 10, a majority of cells co-expressed NESTIN and GFAP while only a small fraction expressed neuronal specific markers such as NF200 and β-TUBULIN III and none expressed more advanced neuronal markers such as NEUN (figure 33). These findings of limited expression of neuronal specific markers such as NF200 and β-TUBULIN III and the absence of advanced neuronal markers such as NEUN suggest that the cells at day 10 are still in early stages of neural development (figure 33). The changed from day 0 cultures is also supported by the results obtained from real time PCR analysis of *OCT4*, *SOX2* and *NANOG* which showed significant decrease in transcripts level of the three genes at day 10 compared with undifferentiated stem cells (Day 0) (figure 30). However, *C-MYC* expression level at day 10 was almost similar to its level at day 0 (figure 30). This suggests that neural committed cells (Day 10) retained their

proliferative characteristic despite being commitment to a possible neural fate, which resembles a stage of development highly similar to neural stem cells (figure 38).

The "early neural commitment" medium contained two important growth factors, basic fibroblast growth factor (b-FGF) and epidermal growth factor (EGF) (table 7). b-FGF is a member of the FGF family of growth factors that play essential roles in development. It has been shown that b-FGF stimulates the proliferation and survival of neural stem cells *in-vitro* and *in-vivo* as well (Zhao et al., 2008, Gremo and Presta, 2000). b-FGF acts as a mitogen that regulates the proliferation and commitment of neural stem cells and precursors without triggering neuronal differentiation (Bartlett et al., 1998, Murray and Dubois-Dalcq, 1997, Guan et al., 2001). EGF as well has important roles in regulation of neural stem cell proliferation and neuronal differentiation (Reynolds et al., 1992, Angenieux et al., 2006, Guan et al., 2001).

The role of b-FGF along with EGF as mitogens and neuro-regulators has been clearly observed in the experiments here where the proportion of cells expressing NESTIN and GFAP at day 10 increased significantly compared with day 0 and no similar increases were seen for neuronal specific markers such as NF200 and β-TUBULIN III indicating possible neural commitment but not yet neuronal differentiation (figure 33).

4.5.3 *Neuronal induction: molecular and morphological transformations*

After the neuronal differentiation phase (Day17), the cell morphology had changed to a clear neuronal-like morphology featuring growing thin long axon-like and short dendrite-like structures (figure 28). This change in morphology was also accompanied by a molecular transformation in which differentiating cells expressed neuronal specific markers in specific patterns. At day 17, cells expressed neuronal-specific structural proteins including NF-200, β-TUBULIN III and GAP43, proteins that are likely to be involved in the morphological transformation observed (figure 33, 34, 35). NF-200 is an intermediate filament found specifically in neurons and is involved in axonal growth and maintenance (Xiao et al., 2006). β-TUBULIN III is a microtubule protein specific for neurons and neuronal tissues (Katsetos et al., 2003b, Katsetos et al., 2003a). GAP-43 is a polypeptide expressed in neurons and is associated with axons growth and is involved in regulation of neuronal cytoskeletal architecture (Meiri et al., 1986, Mishra et al., 2008). The expression

patterns of these three markers and their concentrate localization in growing neurites suggest proper neuronal commitment of the differentiated cells (figure 34 & 35). Although the cells at this stage (Day 17) expressed mature neuronal markers such as NEUN and PSD95, the proportion of cells co-expressing NESTIN and GFAP was also high (figure 34). The expression of markers associated with early stages of neural development (NESTIN and GFAP) and markers associated with advanced neuronal stages of development (NEUN and PSD95) suggest a developmental transitional stage of the differentiating cells at day 17.

During this neuronal differentiation phase, two new important factors were introduced to the cells after day 10. The first factor was retinoic acid (RA) which is the oxidized form of Vitamin A (table 8). RA is known to have an essential role in controlling and patterning development in mammalian embryos and in particular the neural plate where it is involved in brain formation (Mey and McCaffery, 2004, Maden, 2006). It also has an essential role in maintenance and survival of adult neurons (Maden, 2007). RA is believed to be a general morphogen that induces and regulates differentiation to all neural lineages including astrocytes (Wuarin et al., 1990), oligodendrocytes (Noll and Miller, 1994) and neurons (Henion and Weston, 1994, Guan et al., 2001).

To increase the likelihood of neuronal induction in our cultures, we co-introduced RA along with brain-derived neurotrophic factor (BDNF), a member of a closely related family of proteins named "neurotrophins" that also includes nerve growth factor (NGF). It has been also shown that RA maximizes the cells' response to neurotrophins by up-regulating the expression of their receptors (Kobayashi et al., 1994, Takahashi et al., 1999). BDNF has been shown previously to selectively stimulate neuronal differentiation in neural stem cell, embryonic stem cell as well as umbilical cord blood stem cell cultures (Ahmed et al., 1995, Li et al., 2005, Habich et al., 2006, Murer et al., 2001). It is also critically involved in the regulation of synaptic development and plasticity in the CNS (Poo, 2001, Ji et al., 2005).

The co-introduction of RA and BDNF successfully directed the neural committed cells (Day 10) towards a neuronal fate (Day 17) indicated by the high proportions of cells expressing neuronal-specific markers and the morphological changes acquired by the cells after neuronal differentiation at day 17 of the differentiation protocol (figure 28 and 33).

4.5.4 *Neuronal maturation*

After one further week of culture in maturation medium (Day 24), the percentages of cells expressing neuronal mature markers NEUN and PSD95 stayed high (figure 33, 36). NEUN is a DNA-binding neuron-specific protein that has been routinely associated with neuronal mature status in human nervous system (Mullen et al., 1992, Sarnat et al., 1998, Zhao et al., 2008) while PSD-95 is a post synaptic density protein that is associated with receptors and cytoskeletal elements at the synapses and involved in the maturation of functional synapses in neurons (El-Husseini et al., 2000, Hata and Takai, 1999). The dominancy of cells expressing NEUN and PSD95 (figure 36) , the decreased proportion of cells expressing early neural markers NESTIN and GFAP (figure 33), the significant down-regulation of pluripotency markers *OCT4, SOX2* and *NANOG* and proliferative marker *C-MYC* (figure 30) and the significant up-regulation in *PSD95* (figure 31) in addition to the neuronal morphological features observed in cultures after day 24 (figure 29), all together offer a strong indication of advanced and maturing developmental status of generated neuron-like cells at day 24.

Two new factors have been introduced to differentiating cells at day 17, nerve growth factor (NGF) and cyclic AMP (cAMP) (table 8). NGF is a small protein that is involved in the maintenance, growth and survival of neurons (O'Keeffe et al., 2008) whilst cAMP is a second messenger that is involved in many cellular and biological functions and activities by relaying signals through specialized signal transduction cascades. In neurons, cAMP plays a key role in neuronal survival, plasticity and is involved in neuronal morphological maturation (Fujioka et al., 2004, Nakagawa et al., 2002). cAMP is also involved in the regulation of other important factors crucial for neuronal development and survival. It has been found that cAMP regulates the function of BDNF in hippocampal mature neurons through modulating the signaling and trafficking of its receptor (TrkB) in dendrites spines (Ji et al., 2005, Stewart et al., 2001). The co-introduction of NGF and cAMP at day 17 successfully enhanced and stimulated the maturation of differentiating cells which is suggested by the morphological features and protein expression patterns by day 24 (figure 29 & 33).

At day 24, dead cells were observed in cultures. The morphology of these cells varied, some of them showed clear neuronal-like morphology (figure 29C1) while other showed large round shape (figure 29C2). Defining the exact factors triggering the cell death observed needs further investigations.

4.5.5 *The sequential neuronal induction protocol reflects the dynamic process of neurogenesis*

Neuronal differentiation of the purified stem cells from human umbilical cord blood observed in our experiment reflected the dynamic process of neurogenesis *in vivo* which involves different stages including commitment, differentiation and maturation. Each stage is characterized with specific expression pattern of certain markers (Takahashi et al., 1999, Zhao et al., 2008, Duan et al., 2008, Ming and Song, 2005). Our differentiation protocol consisted of three phases (figure 38). During each phase the cells were provided with specific combination of factors and inducers.

During the first phase of our differentiation protocol, freshly purified cord blood non-hematopoietic stem cells were cultured in the presence of b-FGF and EGF (table 7) to stimulate neural commitment, suggested by the NESTIN and GFAP co-expression, the first step toward neuronal development (figure 37).

In the next phase, cells were placed in "neuronal differentiation" in which RA and BDNF were introduced and b-FGF was removed (table 8) to stimulate neuronal differentiation. Earlier studies have shown that the removal of b-FGF from neural stem cells cultures is sufficient to induce limited neuronal differentiation, suggesting that b-FGF is more involved in the regulation of neural stem cell proliferation and maintenance of their undifferentiated status rather than triggering differentiation (Ray et al., 1993, Zheng et al., 2010). After one week of differentiation (Day 17), significant down-regulation in pluripotency genes, in addition to *C-MYC*, were recorded, cells acquired neuronal-like morphology and expressed neuronal markers (including NF200, β-TUBULIN III, GAP43, NEUN, and PSD95) which suggest initiation of neuronal differentiation (figure 38).

 After maturation phase (Day 24) where cAMP and NGF were introduced, proportion of cells expressing early neural markers (NESTIN and GFAP) significantly decreased while

proportions of cells expressing neuronal specific markers stayed high which suggest a more advancing differentiation status of the cells (figure 38).
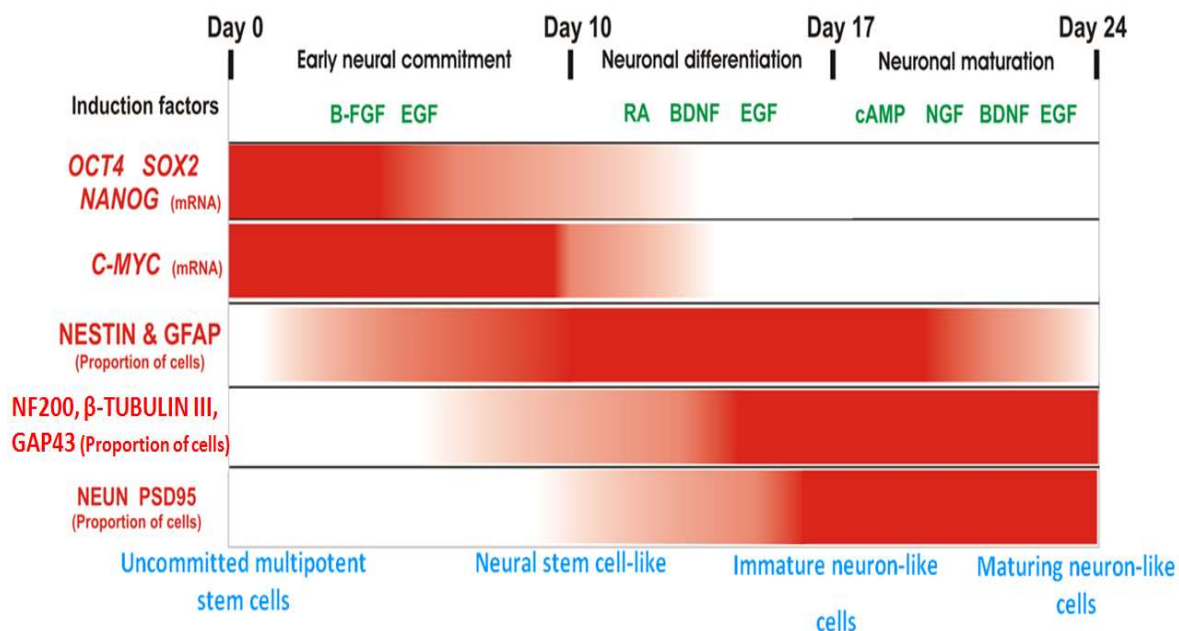
Figure 38. Summary of the sequential neuronal induction protocol. The top panel represents the experiment time line along with the key factors used in each phase (in green). The expression patterns of analyzed genes and proteins are shown in red thorough the experiment. The bottom panel (in blue) describes the developmental stage resembled at each time point suggested by the markers expression patterns.

4.5.6 *Fully defined short-term differentiation protocol, a step closer to clinics*

Animal serum has been commonly used as an essential growth supplement for human cell cultures. It has a complex "undefined" content of proteins, growth factors and nutrients that promote the growth of human cells in cultures (Gstraunthaler, 2003, Castle and Robertson, 1999, Martin et al., 2005). Despite being a useful cost-effective media supplement, the use of animal serum in human cultures involves some risks factors especially when considering potential clinical applications. The contamination with zoonotic agents including animal

microbes and viruses is among the most feared risk factors associated with use of animal products including serum (Castle and Robertson, 1999, Unger et al., 2008, Wessman and Levings, 1999). In addition, the use of animal serum in human stem cell cultures with potential use in transplantation applications could induce an unwanted immune response upon transplantation. Martin et al. (2005) showed that animal serum contain sialic acids, a family of acidic sugars expressed on surfaces of all cell types, that can be absorbed by human embryonic stem cells via a defined mechanism (Bardor et al., 2005), and humans have circulating antibodies specific for the absorbed sialic acid. So when the cultured human embryonic stem cells were exposed to human sera, antibodies in the sera bound to the sialic acid on the surface of the human embryonic stem cells which *in vivo* would lead to cell death due to an immune response (Martin et al., 2005). To avoid such risks, the media used here for culturing and differentiation were fully defined and free of animal serum. The animal serum was substituted with medium supplements B27 and N2 which are composed of defined amounts of certain components including amino acids, vitamins, hormones and antioxidants that provided our purified cord blood stem cells and derived neuron-like cells with nutrients necessarily for survival and growth (Fedoroff and Richardson, 2001, Wachs et al., 2003). The use of defined serum-free media allowed better control on the culturing environment and facilitated more precise analysis and characterization of the differentiation process of purified cord blood stem cells.

In addition to using defined serum-free culturing conditions, culturing protocol to be only for a relatively short time (24 days), without compromising its effectiveness, to reduce the risk of infections and possible chromosomal aberrations as reported by previous study where cord blood derived cells showed cytogenetic alterations after culturing and multiple passaging (Corselli et al., 2008). As a safety check, a cytogenetic analysis was performed on purified stem cells after the "early neural commitment" stage (Day 10) prior to differentiation, since chromosomal abnormalities are common in proliferating cells, and results showed normal karyotypes (figure 63 in appendix.II). The culturing system described in this thesis emphasizes a philosophy of increasing adherence toward clinical grade protocols for potential clinical applications by using short and fully defined culturing conditions free of animal serum.

4.5.7 3-dimensional (3D) differentiation: potential use of cord blood stem cells in neural tissue engineering applications

Standard 2-dimensional (2D) cultures are very important to understand and study the differentiation potentials of the stem cells. However when considering tissue engineering applications where the target is to produce tissues rather than cells, these standard (2D) cultures cannot represent the physical cell-cell interactions and mimic the 3-dimensional (3D) spatial organization of the cells within the targeted tissue or organ. Tissue engineering applications require proper mimicking of cell-cell interactions and organization in functional organs in order to develop potential functional tissues (Mahoney and Anseth, 2006, Mahoney and Anseth, 2007, Brannvall et al., 2007). In here, we used thin scaffolds (300 µm thick) and seeded the purified cord blood stem cells into the scaffolds right after purification. After the 24 days neuronal induction protocol, the cells differentiated successfully into neuronal-like cells, suggested by the phenotypic features and NF200 expression (figure 37), and were equally distributed inside the scaffolds indicating that the scaffolds provided mechanical support that allow proper cells organization and 3D cells growth and differentiation.

The long term goal of such a study is to be able to produce neuronal tissues suitable for transplantation into patients to compensate for brain tissues lost as a result of a neurological disease or injury. However, there are several requirements that need to be fulfilled in order to ensure potentially successful outcome of such applications. First, it is important to use safe and bio-degradable scaffolds to eliminate any possible toxic effect or unwanted immune response. Second, the generated tissues must be able to functionally integrate in the existing host brain networks. Third, the development of safe and efficient clinical procedures for transplanting the generated tissues into patients.

Here we have taken the first steps towards producing a scaffold-based 3-dimensional culture system in which neuronal tissues can develop and thrive. Refer to (Ali et al., 2009) attached at the end of this thesis.

Chapter 5. Umbilical cord blood non-hematopoietic multipotent stem cells model cortical neurogenesis *in-vitro*

5.1 Introduction

The human adult cerebral cortex is an intricately structured sheet of tissue organized into six distinct cortical layers. During development the cortex is formed in an inside-out manner in which the earliest born neurons are found in the deepest cortical layers while the later born neurons occupy locations within the superficial layers, reviewed in (Shipp, 2007, Meyer, 2001). It is composed mainly of two types of neurons: cortical pyramidal neurons and cortical interneurons. Pyramidal neurons are excitatory neurons that utilize glutamate as a neurotransmitter and they account for 80% of the cortex neuronal content. These neurons are derived from progenitor cells in the ventricular zone and migrate radially to the cortical plate (Guillemot et al., 2006, Campbell, 2005) . On the other hand, interneurons, which account for about 20% of total cortical neurons, are mainly inhibitory and utilize GABA as a neurotransmitter. Interneurons are derived from two locations: progenitor cells in the proliferative zones of the cortex which migrate radially to occupy different cortical layers and from progenitors in the ganglionic eminences that migrate tangentially into the cortex (Letinic et al., 2002, Meyer et al., 2000, Jones, 2009).

During development, the cortex increases its width from less than 1 mm by the 8 post conceptual week (PCW) stage to nearly 5 mm by 16 PCW stage (Bayatti et al., 2008a). At 12 PCW stage the human cortex is divided clearly into distinct layers. Just above the ventricles lies the ventricular zone (VZ) which hosts a population of neural progenitor cells that would give rise to the cortical neurons (Noctor et al., 2002). Above the (VZ) lies the subventricular zone (SVZ) which can be further divided into inner subventricular zone (ISVZ) and outer subventricular zone (OSVZ). The subplate/intermediate zone (SP/IZ) resides above the SVZ. It is the layer where the differentiating progenitor cells acquire their

neuronal identity (McConnell et al., 1989, Kanold and Luhmann, 2010). The SP/IZ is followed by the cortical plate CP and the marginal zone MZ.

Differentiating progenitor cells at the VZmigrate radially across cortical layers to the CP via the aid of radial glial cells (Rakic, 2003, Meyer, 2001). The corticogenesis of pyramidal neurons at embryonic stages is a complex but highly ordered process that is tightly regulated by specific genes, including *PAX6, TBR2* and *TBR1* which have attracted attention not only for their functional properties but also for their sequential expression patterns across the developing cortex (table 17) (Englund et al., 2005, Hevner et al., 2006). *PAX6* is expressed in the highly proliferative progenitor cells in the cortex where it regulates cell cycle and mitotic division and has an essential role in normal neuronal development (Gotz et al., 1998, Hevner et al., 2006, Quinn et al., 2010). *TBR2* defines intermediate progenitor cells (IPC) of the developing cortex where its expression pattern suggests its involvement in fate specification and differentiation of glutamatergic neurons (Sessa et al., 2008). *TBR1* is expressed primarily in postmitotic neurons of the cortex (Hevner et al., 2001, Hevner et al., 2006, Hong and Hsueh, 2007). The zonal and sequential expression patterns of these genes emphasize their important regulatory role in cortical neurogenesis (figure 39) (Hevner et al., 2006, Bayatti et al., 2008b, Englund et al., 2005, Bayatti et al., 2008a). Other transcription factors including basic helix-loop-helix (bHLH) transcription factors are also highly involved in the regulation of cortical neurogenesis and fate decisions at embryonic stages (Mattar et al., 2008, Bayatti et al., 2008b).

Table 17. Description of *PAX6, TBR2 and TBR1*

Gene	Functions	Knockout studies	Associated disorders (human)	Expression in CNS	References
PAX6	Transcription factor highly involved in the development of CNS, eye and pancreas.	*PAX* 6 knockout causes abnormal development in CNS including forebrain axonal connections.	Mutations in the gene have been found to cause aniridia and Peter's anomaly.	Expressed by the highly proliferative progenitor cells in cortex.	(Jones et al., 2002, Li et al., 2009, Quinn et al., 2010, Dansault et al., 2007)
TBR2	Transcription factor involved in trophoblast differentiation, gastrulation and brain development.	*TBR*2 knockout is lethal due to defects in trophoectoderm differentiation. Conditional knockout at early embryonic development cause microcephaly and severe behavioral deficits.	Mutations in the gene cause recessive microcephaly, polymicrogyria, corpus callosum agenesis, cognitive defects, and severe motor delay.	Expressed by intermediate progenitor cells (IPC) of the developing cortex.	(Sessa et al., 2010, Baala et al., 2007, Hodge et al., 2008)
TBR1	Transcription factor involved in brain development.	*TBR1* knockout causes abnormalities in cortical lamination, axon connections, and layer-specific differentiation.	Non reported.	Expressed by postmitotic neurons in cortex.	(Hevner et al., 2001, Englund et al., 2005)
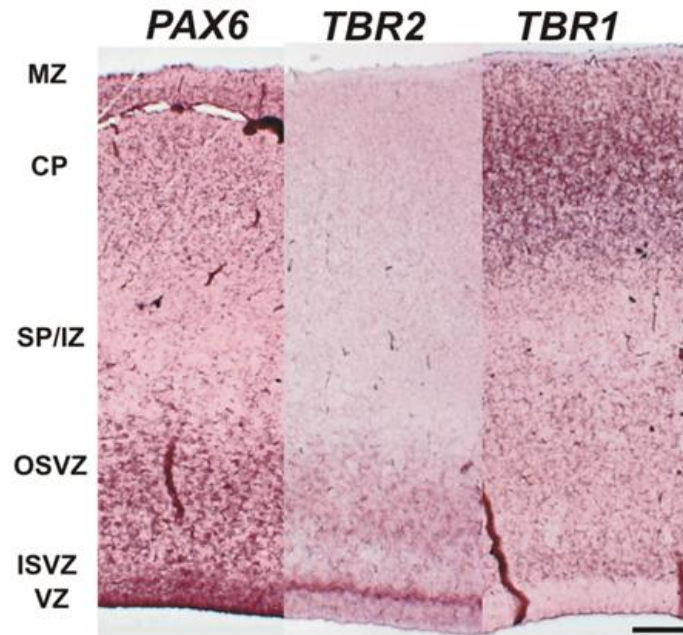
Figure 39. In situ hybridization localization of *PAX6, TBR2 and TBR1* across 12 PCW human neocortex. The three markers expressed in a sequence during cortical neurogenesis. PAX6 is mostly expressed by progenitor cells reside in the VZ, TBR2 expressed mostly in intermediate progenitor cells (IPC) residing mainly in ISVZ and TBR1 expressed mostly by post-mitotic neurons located at the CP (Bar = 200µm). Figure adapted from (Bayatti et al., 2008b).

The cortex, and in particular the motor cortex and/or corticospinal tract is a common site of damage and injury that might lead to severe neurological disorders, to which current modes of therapy are inadequate (Johnston and Hoon, 2006). Stem cells have been proposed as a potential therapeutic tool that offers a new level of therapeutic intervention which theoretically would open the doors for neural replacement strategies to compensate for the neurons lost as a consequence of the neurological condition. Such strategies rely on the ability of stem cells to produce neurons, with similar developmental and functional properties as the one targeted for replacement, which when integrated successfully in the lesion sites would stimulate functional and physiological recovery. Thus, it is important first to identify the molecules and mechanisms that determine and regulate the differentiation of endogenous cortical neurons and determine whether the same mechanisms can induce stem cells to produce neurons with similar characteristics to the ones targeted for therapy.

In this study, we show that the process of differentiating umbilical cord blood non-hematopoietic multipotent stem cells can provide an *in vitro* model of human cortical neurogenesis of glutamatergic neurons. We compare the expression patterns of markers previously found to be expressed sequentially across the 12 PCW human neocortex that are crucially involved in human cortical neurogenesis of neurons, and their expression patterns in cord blood purified stem cells throughout the differentiation protocol described previously (refer to section 2.5). We further investigate the expression of certain markers associated with glutamatergic neurotransmission, the dominating neurotransmission system in cortex, in cord blood derived neuron-like cells.

5.2 Study aims

- To assess the ability of umbilical cord blood non-hematopoietic multipotent stem cells to model cortical neurogenesis *in vitro* by analyzing the expression patterns of markers associated with cortical neurogenesis at mRNA and protein levels throughout the neuronal differentiation protocol.

- To investigate the expression of certain markers associated with glutamatergic neurotransmission in cord blood derived neuron-like cells.

5.3 Results

5.3.1 PAX6, TBR2 *and* TBR1 *are expressed in a sequence across the 12 PCW human neocortex*

In sections of 12 PCW fetal human neocortex, PAX6-immunoreactive cells were localized mainly in the VZ and to a lesser extent in the SVZ including ISVZ and OSVZ. No PAX6 immunoreactive cells were detected in either SP or CP (figure 40). TBR2-immunoreactive cells were largely confined to the ISVZ, with some positive cells in the OSVZ and also in the VZ but away from the ventricular surface. Similar to PAX6 positive cells, no TBR2 positive cells were detected in either the SP or the CP (figure 40). TBR1-immunoreactive cells were mainly localized in the CP and few reactive cells were detected in the SP. No TBR1 positive cells were detected in the VZ and the SVZ (figure 40). All three proteins showed nuclear localization.
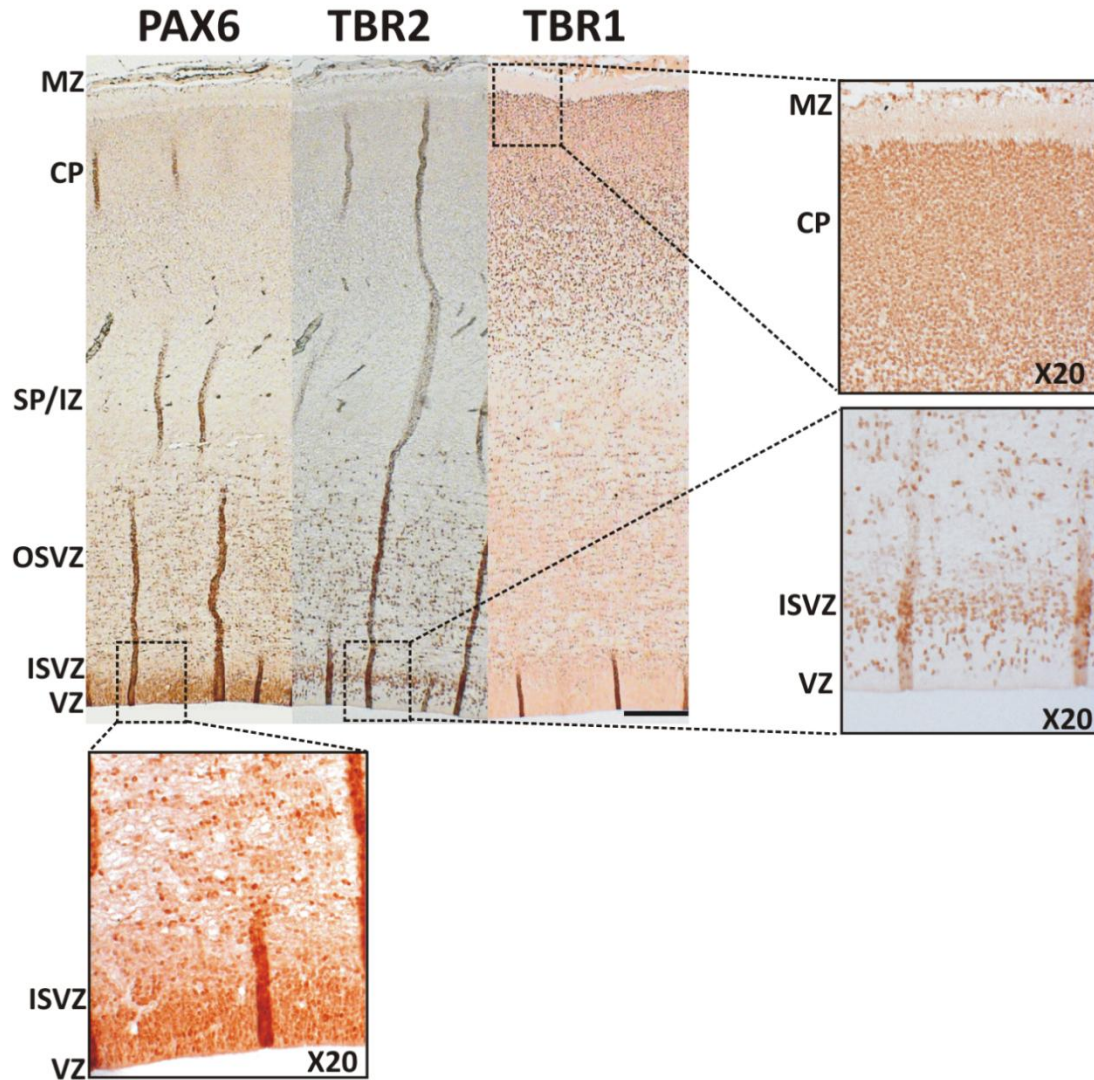
Figure 40. Immunohistochemistry analysis of PAX6, TBR2 and TBR1 at protein level in 12 PCW human neocortex. PAX6 positive cells were localized primarily in the ventricular zone (VZ) and in the sub-ventricular zone (SVZ) at lower extent. No PAX6 positive cells were detected in the sub-plate (SP) and the cortical plate (CP). TBR2 positive cells were mainly localized in the inner sub-ventricular zone (ISVZ) and some were localized in the outer sub-ventricular zone (OSVZ) and the ventricular zone (VZ) away from the ventricular surface. No TBR2 positive cells were detected in the sub-plate (SP) and the cortical plate (CP). TBR1 positive cells were confined in the cortical plate (CP) and some were localized in the sub-plate (SP). No TBR1 positive cells were detected in the ventricular zone (VZ) and the sub-ventricular zone (SVZ). All three markers showed nuclear localization. (Bar = 200μm).

5.3.2 *PAX6, TBR2 and TBR1 expression patterns define sequential neurogenesis in cord blood differentiating stem cells*

Q-PCR analysis of umbilical cord blood stem cells throughout the 24-days induction protocol showed that the normalized expression level of *PAX6* increased from day 0 to day 17 and decreased after maturation at day 24 (figure 41); these changes in expression pattern throughout the 24 days were statistically significant (ANOVA, $p<0.005$, n=3). *TBR2* expression normalized to *GAPDH* decreased between day 10 and day 17 and stayed low at day 24 (ANOVA, $p<0.005$, n=3) (figure 41). Normalized expression of *TBR1* increased gradually and significantly between day 0 and day 24 (ANOVA, $p<0.005$, n=3) (figure 41). Detection of PAX6, TBR2 and TBR1 immunoreactivity in cord blood stem cell cultures showed that the protein expression patterns strongly correlate with the corresponding mRNA expression patterns. $48 \pm 7.6\%$ of PAX6 positive cells were present at day 0 and $45 \pm 5.6\%$ on day 10 before decreasing to $15 \pm 4.4\%$ by day 24. These changes were statistically significant (ANOVA, $p<0.005$, n=4) (figure 42, 43). TBR2 positive cells increased from $5.2 \pm 4.4\%$ at day 0 to $17.7 \pm 3.7\%$ at day 10 and decreased by day 24d to $3.4 \pm 1.6\%$, again these changes were statistically significant (ANOVA, $p<0.05$, n=3) (figure 43, 44). TBR1 positive cells increased from $3.3 \pm 1\%$ at day 0 to$17.8 \pm 5.3\%$ at day 10 and $66 \pm 6.2\%$ at day 24 (figure 42, 43). These changes were statistically significant (ANOVA, $p<0.005$, n=3). Primers for *PAX6* and *TBR1* have been checked for neural specificity by testing on cDNA isolated from human adult liver, human fetal cortex and cord blood cells. PCR results on liver samples were negative for both markers while fetal cortex and cord blood samples showed positive results (refer to figure 68 in appendix III).
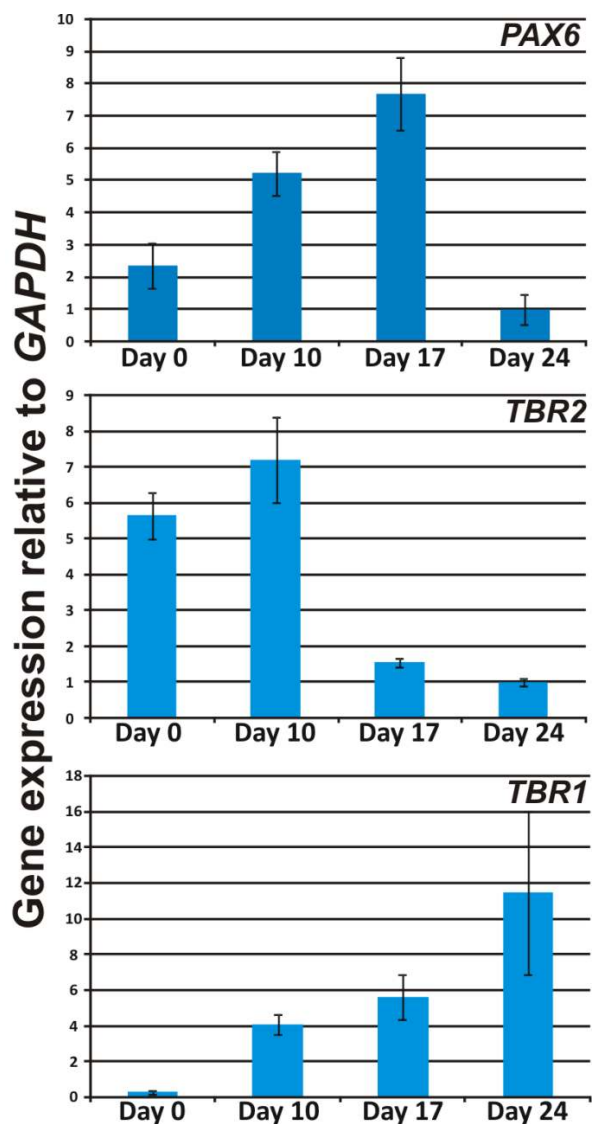
Figure 41. Q-PCR analysis of *PAX6, TBR2 and TBR1* expression pattern in umbilical cord purified stem cells through the 24-days neuronal induction protocol. *PAX6* expression level increased gradually between day 0 and day 17 before decreasing to its lowest level by day 24. *TBR2* expression level was high at day 0 and day 10 before decreasing after neuronal differentiation (Day 17) and maturation (Day 24). *TBR1* expression level increased gradually during the differentiation protocol reaching its highest level by day 24. Gene expressions levels were normalized to *GAPDH* expression level. For *PAX6* and *TBR2* the calibrator is Day 24, for TBR1 the calibrator is Day 0. Changes in expression levels of the three genes over the 24 days period are statistically significant (ANOVA, $p<0.005$, n=3, for the three genes).
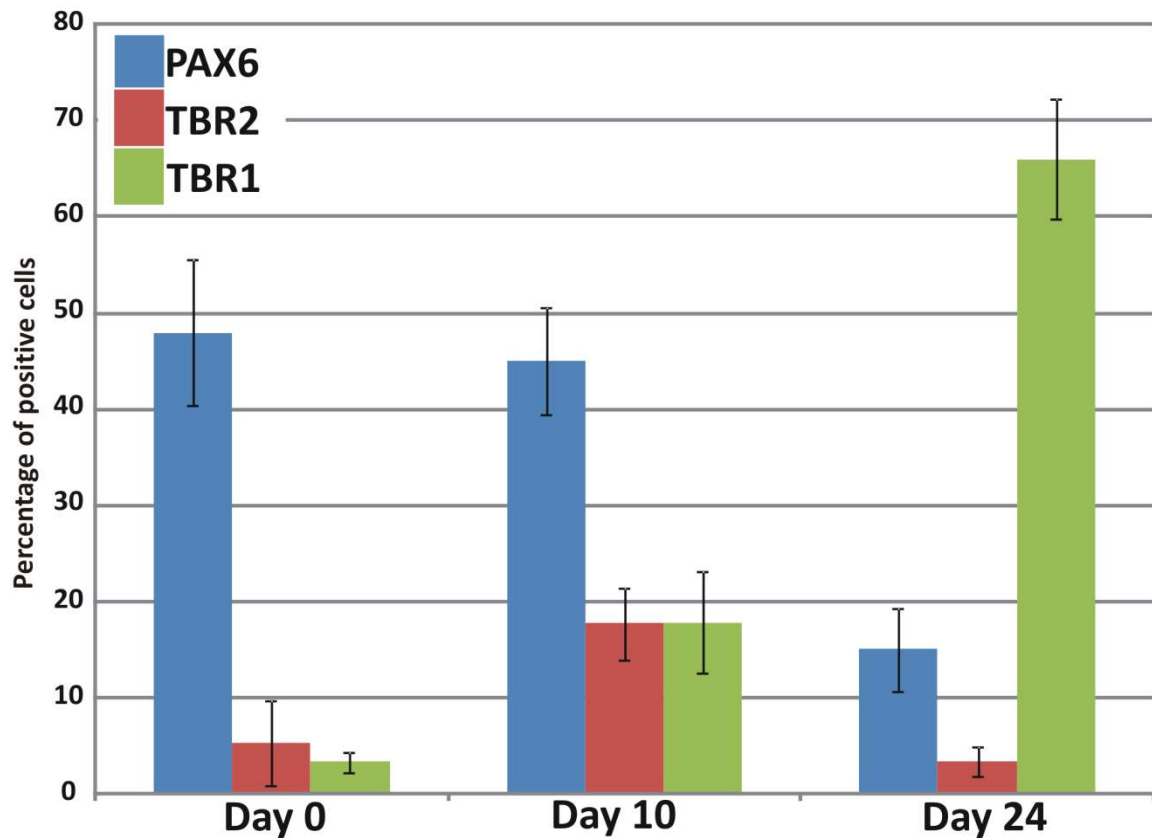
Figure 42. Expression analysis of PAX6, TBR2 and TBR1 during the 24-days neuronal induction protocol. Percentage of cells expressing PAX6 was at its highest at day 0 and day 10 before decreasing significantly by day 24. Proportion of cells expressing TBR2 increased between day 0 and day 10 before decreasing after neuronal maturation (Day 24). Percentage of cells expressing TBR1 increased gradually throughout the 24-days induction protocol reaching its highest point at day 24. Changes in percentages of positive cells over the 24 days period are statistically significant (ANOVA, $p<0.005$ for PAX6 and TBR1, $p<0.05$ for TBR2, n=3).
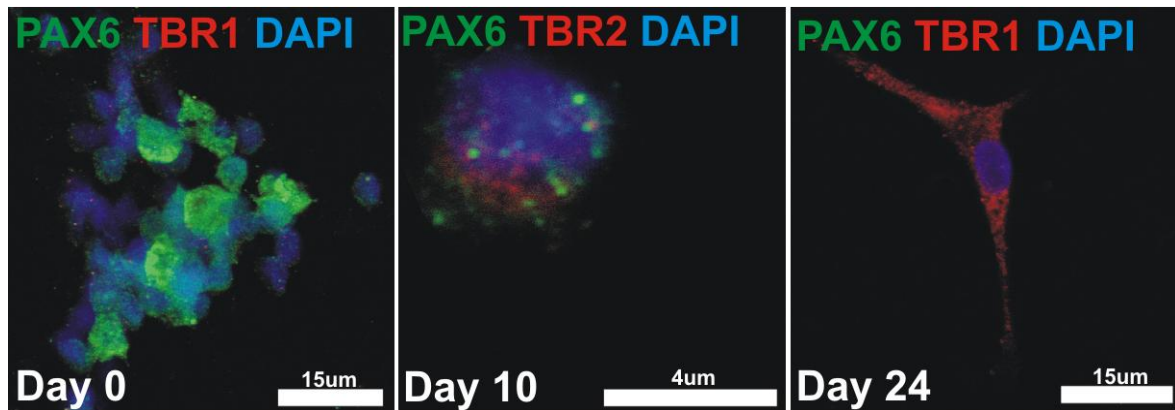
Figure 43. Localization of PAX6, TBR2 and TBR1 in cells throughout the differentiation protocol using immunocytochemistry. At day 0, cord blood stem cells strongly expressed PAX6 showing nuclear localization while TBR1 expression was weak compared to PAX6. At day 10, nuclear co-expression of PAX6 and TBR2 can be detected. At day 24, TBR1 was highly expressed in the cell's cytoplasm while PAX6 expression was greatly reduced compared with its expression at day 0.

5.3.3 *Cord blood differentiated stem cells expressed glutamate receptor and transporters*

After neuronal maturation (Day 24), cells expressed immunoreactivity for NMDAR1 in their cytoplasm (figure 44) and VGLUT1 (vesicular glutamate transporter 1) which appeared as punctuated staining on the surface of cells suggestive of nerve terminals (figure 46). No expressions of NMDAR1 and VGLUT1 in freshly purified cord blood stem cells (Day 0) were detected (refer to figure 64 and figure 65 in appendix II).

Quantitative PCR analysis showed a significant increase in glutamate transporter gene *SLC1A1* expression levels between day 0 and day 24 (figure 46) ($p<0.05$, n=3).
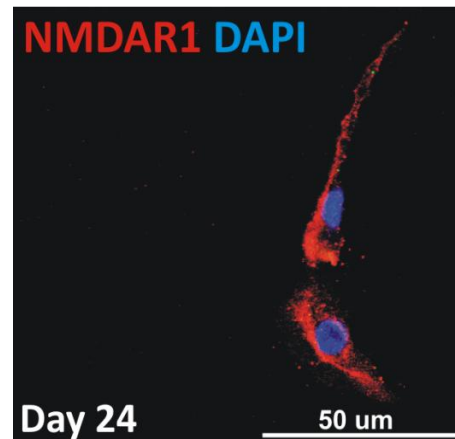
Figure 44. Generated neuron-like cells from umbilical cord blood stem cells expressed NMDAR1 in their cytoplasm at day24. No expression of NMDAR1 was detected in purified cord blood stem cells (Day 0), refer to (figure 64) in appendix II.
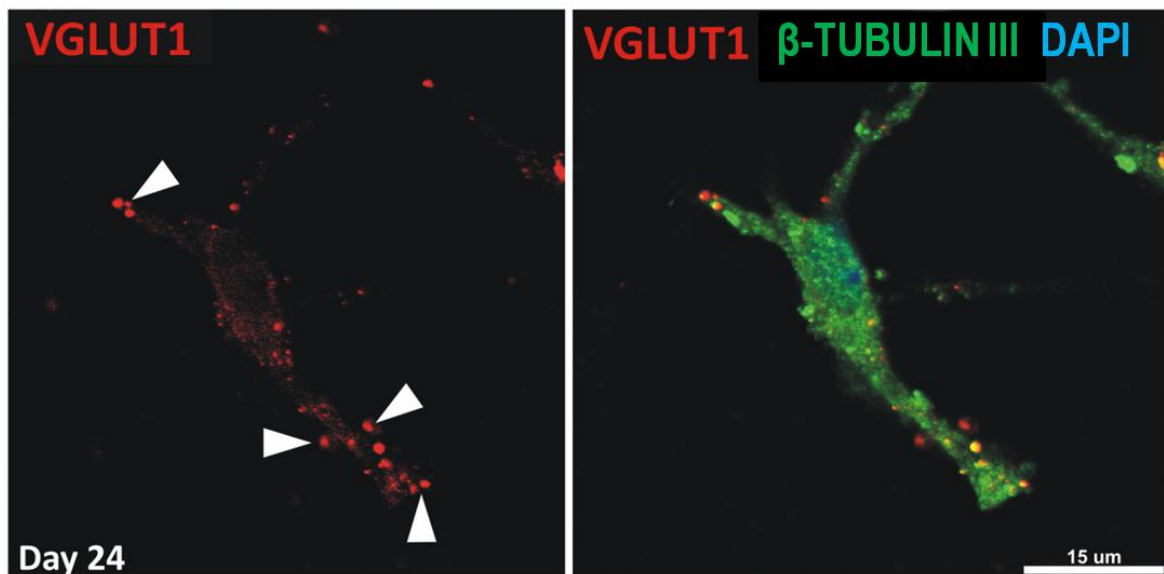
Figure 45. Cord blood generated neuron-like cells expressed vesicular glutamate transporter 1 (VGLUT1) at day24. VGLUT1 appeared as punctuated staining concentrated in the cell's terminals (white arrows)   suggestive of nerve terminals. Cells co-expressed VGLUT1 with neuron-specific tubulin β-TUBULIN III. No expression of VGLUT1 was detected in purified cord blood stem cells (Day 0), refer to (figure 65) in appendixII.
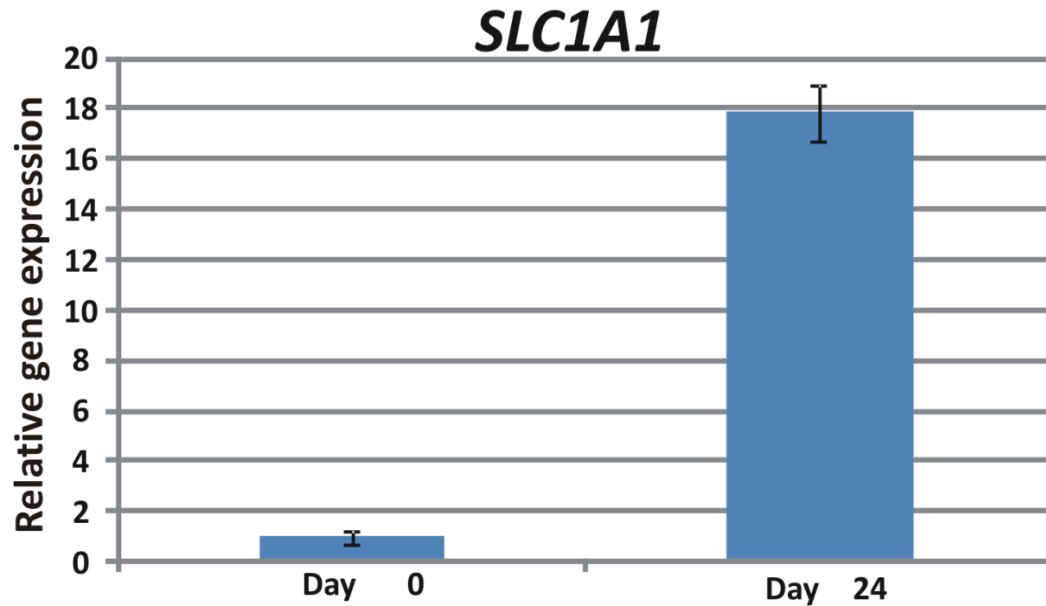
Figure 46. Q-PCR analysis of glutamate transporter *SLC1A1*expression pattern between (Day 0) and (Day 24). Expression of *SLC1A1* was extremely low in undifferentiated cord blood stem cells (Day 0) but increased significantly after neuronal maturation (Day 24). Calibrator is Day 0.Gene expression level was normalized to housekeeping genes *HPRT1, RPL13A* and *GAPDH*. Student's t-test (p<0.05, n=3).

5.3.4 *Expression patterns of basic helix-loop-helix (bHLH) genes throughout the 24-days induction protocol*

Q-PCR analysis showed that *NEUROD* normalized expression level was very weak at day 0 and slightly increased at day 10 and day 17 before reaching its highest expression level at day 24 (ANOVA, p<0.005, n=3) (figure 47). *NEUROD2* normalized expression level was very weak at day 0 and increased significantly at day 17 and day 24 (ANOVA, p<0.005, n=3) (figure 47). *NGN1* and *NGN2* expression patterns were relatively similar. Their normalized expression levels increased over the "neural early commitment" phase reaching their highest levels at day 10 before their expression levels decreased gradually after neuronal differentiation (Day 17) and neuronal maturation (Day 24) (ANOVA, p<0.005, n=3, for both genes) (figure 47).
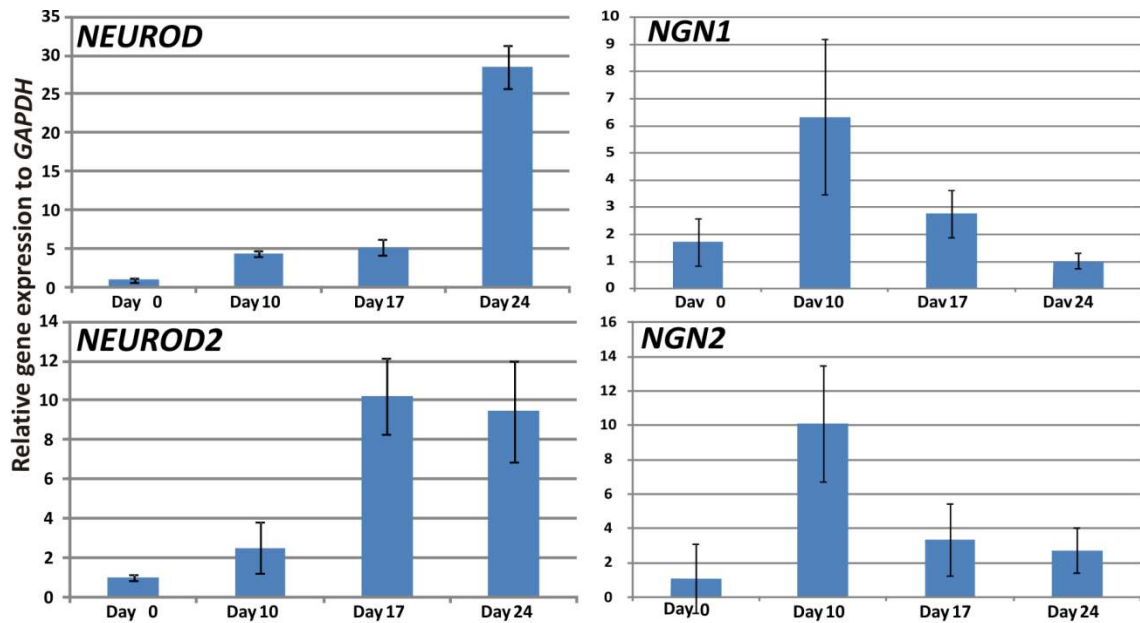
Figure 47. Q-PCR analysis of basic helix-loop-helix (bHLH) genes expression patterns in cord blood stem cells throughout the 24-day neuronal induction protocol. *NEUROD* expression level was weak at day 0, slightly increased at day 10 and day 17 and had increased substantially by Day 24. *NEUROD2* expression level was weak between day 0 and day 10 before significantly increasing by Day 17 and staying high at Day 24. *NGN1* and *NGN2* had relatively similar expression patterns, their highest expression levels were recorded at day10 before being down regulated at Day 17 Day 24. Expression levels of the five genes were normalized to *GAPDH* expression level. Calibrator for *NEUROD* and *NEUROD2* and *NGN2* is Day0, for *NGN1* is Day 24. Changes in expression levels of the five genes throughout the 24-days induction protocol were statistically significant (ANOVA, $p<0.005$, n=3, for the four genes).

5.4 Conclusions

- PAX6, TBR2 and TBR1 show sequential expression at early stages across the developing human neocortex.

- Umbilical cord blood non-hematopoietic multipotent stem cells undergoing our previously described neuronal induction protocol also show a similar sequential expression pattern of PAX6, TBR2 and TBR1 and produced neuron-like cells expressing markers associated with glutamatergic neurotransmission.

- Umbilical cord blood purified stem cells could provide an *in-vitro* model of human neurogenesis of glutamatergic neurons.

5.5 Discussion

In this chapter we have demonstrated that the induction of neuronal differentiation in human umbilical cord blood non-hematopoietic multipotent stem cells results in the recapitulation of gene expression patterns seen in the development of cortical neurons. The expression patterns of PAX6, TBR2 and TBR1 at both the transcriptional and translational levels in purified stem cells undergoing the previously described sequential neuronal induction protocol *in vitro,* resembled patterns of expression seen across the human developing neocortex from the proliferative zones to the cortical plate that characterize glutamatergic neurogenesis. Proper neuronal differentiation has also been suggested by expression patterns of basic helix-loop-helix transcription factors that are known for their regulatory role in neurogenesis. At the end of the neuronal induction protocol, the generated neuron-like cells properly expressed specific glutamatergic markers, including glutamate transporters and receptors, which provide evidence supporting their glutamatergic identity. Thus we have developed a possible *in vitro* model for human cortical neurogenesis.

5.5.1 PAX6, TBR2 *and* TBR1 *sequential expression across the human neocortex defines the corticogenesis of glutamatergic neurons*

Corticogenesis of glutamatergic neurons is a highly dynamic process in which proliferating progenitor cells at the (VZ) differentiate and migrate radially across cortical layers to reside in the (CP) with early born neurons occupying deeper layers (Rakic, 2003, Meyer, 2001, Angevine and Sidman, 1961). The fate commitment of progenitor cells and the differentiation process is regulated by genetic programs that involve certain transcription factors. It has been shown by several groups that PAX6, TBR2 and TBR1 are crucially involved in the regulation and fate commitment of glutamatergic cortical neurons (Bayatti et al., 2008a, Bayatti et al., 2008b, Hevner et al., 2006, Hevner, 2007). In the studies here we show, using immunohistochemical analysis on human neocortex, that PAX6, TBR2 and TBR1 are expressed in a sequence across the neocortical layers where PAX6 was mainly localized in VZ and SVZ that host a large subset of progenitor cells identified as radial glia (Hansen et al., 2010). TBR2 was localized mostly in the ISVZ and to a lesser degree in OSVZ, representing an additional pool of precursor cells termed intermediate progenitor

cells (IPC) which are derived from radial glia cells with neuronal-specified fate (Hevner et al., 2006). TBR1 was localized mainly in the CP where it specifies post-mitotic mature glutamatergic neurons (Hevner, 2007) (figure 39 and 40). The sequence of PAX6 → TBR2 → TBR1 observed across the neocortex reflects the process corticogenesis of glutamatergic neurons (figure 48). The exact function of each transcription factor in corticogenesis is not fully understood however their involvement can be predicted based on their expression patterns. The association of each transcription factor with certain cortical layers suggests their stage of involvement during corticogenesis. The expression pattern of PAX6 suggests that it is involved in early fate specification events where TBR2 is involved in neuronal subtype differentiation and migration and TBR1 is involved in neuronal maturation. The sequential expression of PAX6, TBR2 and TBR1 defines the corticogenesis of glutamatergic neurons in human fetal neocortex and plays a major role in specifying their fate and identity.

5.5.2 *Neuronal differentiation of umbilical cord blood purified stem cells recapitulated key events observed in the human fetal neocortex that regulate the development of glutamatergic neurons*

In order to evaluate the efficiency of our *in-vitro* system in modeling human cortico-neurogenesis, we investigated the expression patterns of PAX6, TBR2 and TBR1 in differentiating purified cord blood stem cells throughout the 24 days induction protocol and compared them to expression patterns observed in the developing neocortex at both the transcriptional and translational levels.

Quantative-PCR and immunocytochemistry analysis showed that PAX6 and TBR2 were more associated with early rather than late stages of differentiation unlike TBR1 which gradually and significantly increased during the 24 days neuronal induction protocol reaching its highest levels after neuronal maturation (Day 24) (figure 41 and 42). PAX6 was expressed by almost half of the freshly purified cells (Day 0). This transcription factor is expressed in the highly proliferative progenitor cells of cortex where it is involved in the regulation of cell cycle and mitotic division and plays a key role in neuronal development and differentiation (Gotz et al., 1998, Hevner et al., 2006, Quinn et al., 2010). So this population of cells at day 0 which highly expressed PAX6 but not TBR1 resembles the

population of progenitor cells (radial glia) located in the VZ of the neocrtex. After early neural induction (Day 10) the cells continued to express PAX6 in addition to TBR2 which was co-localized in the cells nuclei with PAX6 indicating advancing differentiation and commitment (figure 43). The protein co-expression pattern of PAX6 and TBR2 in cells at day 10 highly resembles the intermediate progenitor cells (IPC) observed in the OSVZ of neocortex where we observed clear overlapping of zonal expression patterns of the two markers at both transcriptional and transitional levels (figure 48).

As a transcription factor, TBR1 is expected to be expressed in nuclei of the cells and this was the case in the cells located in the CP of the neocortex. However, TBR1 was mainly localized in the cytoplasm of cells at day 24 (figure 44). Hong et al. (2007) have shown using DAB staining and confocal imaging that TBR1 at embryonic stages is mainly expressed in cellular nuclei before a significant amount of the protein is translocated to the cytoplasm in adult brain where it is thought to be involved in regulation of synaptic activity, indicating that the sub-cellular distribution of TBR1 in the brain is developmentally regulated. After neuronal maturation (Day 24) majority of cells expressed TBR1 and proportions of cells expressing PAX6 and TBR2 decreased compared with their proportions at day 10 (figure 48). Similar expression patterns were observed for the transcriptional levels of the three markers. These results, in addition to the morphological transformation, suggest that cells at day 24 resemble the post-mitotic neurons of the CP of the neocortex where the cells expressed TBR1 but were negative for PAX6 and TBR2.

The key events regulating glutamatergic neurogenesis within the neocortex were recapitulated in the developed *in-vitro* system where the sequential expression patterns of PAX6, TBR2 and TBR1 at both transcriptional and translational levels were resembled throughout the 24 days sequential induction protocol of cord blood purified stem cells.
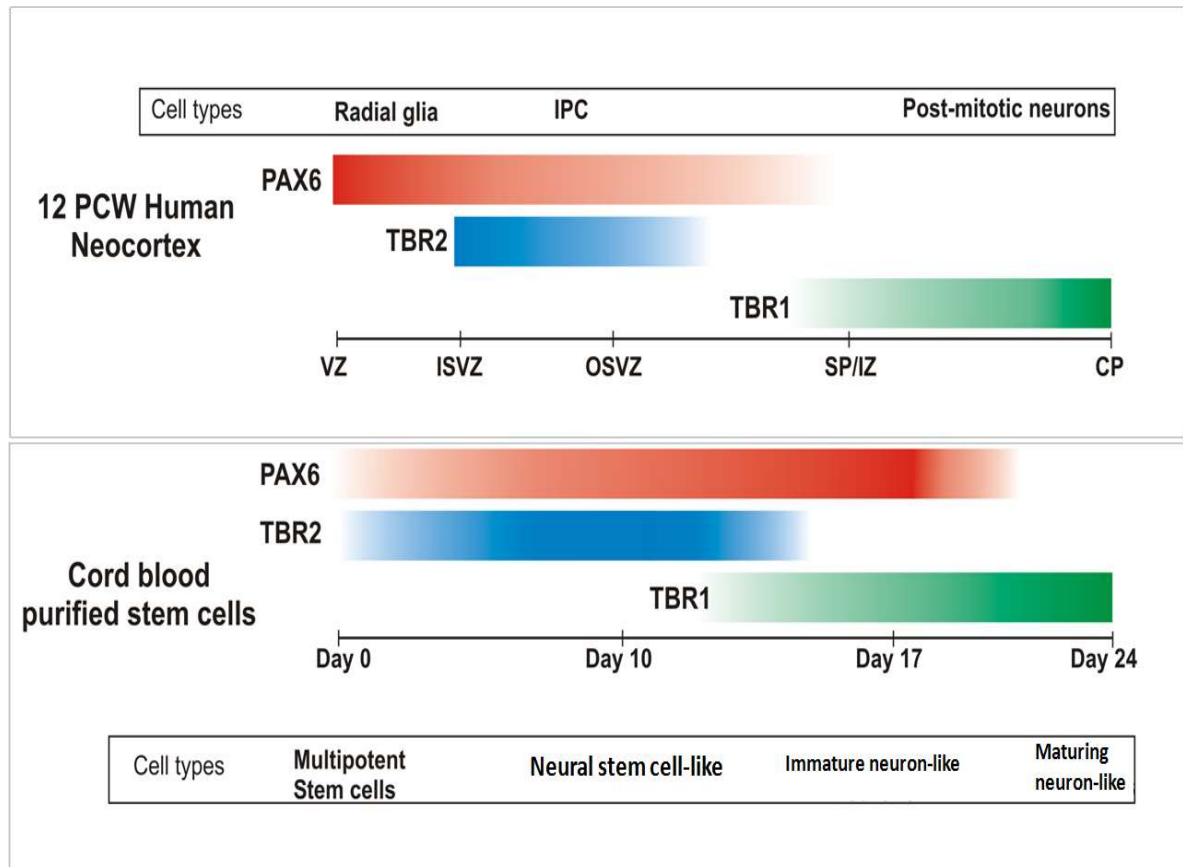
Figure 48. Summary of PAX6, TBR2, TBR1 expression patterns in 12 PCW human neocortex and *in-vitro* neuronal induction protocol of cord blood purified stem cells. The diagram represents markers expression patterns alongside a zonal axis in human neocortex and a time-line axis in cord blood differentiating cells establishing a "zonal/time-point" relationship between the neocortex based corticogenesis and cord blood stem cells based neuronal differentiation system.

5.5.3 *Umbilical cord blood derived neurons and the glutamatergic identity*

In order to assess whether our differentiated cord blood purified stem cells express glutamatergic specific markers normally expressed by cortical glutamatergic neurons, we analyzed the expression of common glutamate receptor NMDAR1 which is involved in synaptic transmission, synaptic plasticity and is essential for neuronal survival in the central nervous system (Martel et al., 2009, Platel et al., 2010, Mattson, 2007). Additionally, we examined expression of the vesicular glutamate transporter 1 (VGLUT1) which is predominantly expressed by glutamatergic neurons in human cerebral cortex and is partly responsible for uptake of glutamate from the synaptic space during synaptic transmission

and *SLC1A1* which encodes a high affinity glutamate transporter (neurotransmitter transporters will be further discussed in chapter 6) (Arnth-Jensen et al., 2002, Nie and Weng, 2009, Fremeau et al., 2004). Immunocytochemistry results indicated a robust induction of expression of both markers by day 24 (figure 45, 46), and Q-PCR results showed significant up-regulation in SLC1A1 transcript level at day 24 in comparison with day 0 (figure 46). Therefore at this time point (Day 24) the cells have undergone a differentiation process that has resulted in the sequential expression of genes known to be important in specifying glutamatergic identity, as well as expressing receptors and transporters for the neurotransmitter which confirm their neuronal glutamatergic identity.

5.5.4 *Basic helix-loop-helix (b-HLH) transcription factors are involved in cortical fate specification*

Basic helix-loop-helix (b-HLH) is a structural protein motif that consists of two α-helices connected by a loop which characterizes a large family of transcription factors named b-HLH class of transcription factors (Guillemot, 1999). Members of this structurally and functionally diverse family of transcription factors are involved in various developmental processes including neurogenesis, myogenesis and haematopoiesis (Morgenstern and Atchley, 1999, Porcher et al., 1999). A subgroup of b-HLH transcription factors termed pro-neural b-HLH transcription factors have been shown to play major roles in neocortex where they regulate neurogenesis and neuronal fate specification (Schuurmans et al., 2004). As mentioned earlier the human neocortex is composed of two types of cortical neurons: glutamatergic neurons and GABAergic neurons (Letinic et al., 2002, Au and Fishell, 2008, Guillemot et al., 2006, Campbell, 2005). The mechanisms regulating fate specification of cortical progenitor cells to either glutamatergic or GABAergic is tightly regulated by members of b-HLH family of transcription factors including MASH1, NGN1 and NGN2. It has been shown that MASH1 promotes progenitor cells in the VZ and SVZ to adapt a GABAergic fate where NGN1 and NGN2 promote glutamatergic fate (Campbell, 2005, Schuurmans et al., 2004, Mattar et al., 2008). Such effects have been demonstrated by a number of studies. In one study, it has been shown that repression of *mash1* in cortex is sufficient to prevent formation of GABAergic interneurons (Roybon et al., 2010). In another study, it was shown that knocking out *ngn1* and *ngn2* resulted in severe disruption of glutamatergic neurogenesis in the animal cortex (Schuurmans et al., 2004). So it is clear

that early specification of cortical identity is regulated by genetic programs involving MASH1, NGN1 and NGN2 which act as regulators for downstream genes.

In our study, we analyzed the transcript expression levels of four important b-HLH transcription factors throughout the 24 days induction protocol. Factor analyzed included *NGN1* and *NGN2* which are crucially involved in early stages of glutamatergic fate specification in the cortex, and NEUROD and NEUROD2 which are involved in late stages of cortical development of glutamatergic neurons(Schuurmans et al., 2004) (figure 47). Q-PCR results showed that *NGN1* and *NGN2* were up-regulated after early neural commitment stage (Day 10) before being down-regulated at following stages. The transitional expression of the two genes at early stage (day 10) recapitulated their early involvement in fate specification in human cortex where they regulate a cascade of downstream genes that are involved in the development and specification of cortical glutamatergic neurons (Letinic et al., 2002, Guillemot et al., 2006). We further analyzed the transcript expression patterns of *NEUROD* and *NEUROD2* in cord blood differentiating cells. Q-PCR results showed that *NEUROD* was significantly up-regulated after neuronal maturation (Day 24) where *NEUROD2* was up-regulated after neuronal differentiation (Day 17) and stayed high after maturation (Day 24). The expression patterns of both genes agree with their reported involvement in cortical neurogenesis where they have been shown to be involved in late stages of neuronal development regulating neuronal maturation and stimulating survival (Gao et al., 2009, Mattar et al., 2008). Bayatti et al. (2008b) have analyzed the expression pattern of *NEUROD* across a 12 PCW human neocortex, similar to the one used in our experiment, using ISH and showed that *NEUROD* expression was more restricted to the CP in a similar manner to *TBR1* which suggests its involvement at late stage of corticogenesis (figure 49). Our Q-PCR results on cord blood differentiating stem cells indicated that *NEUROD* and *TBR1* had similar expression patterns where their highest peaks recorded after neuronal maturation (day 24) which resemble their expression patterns observed across the neocortex. These results offer another strong indication that our *in-vitro* differentiation system properly recapitulated key corticogenesis events involved in regulation of fate specification and differentiation of glutamatergic cortical neurons.
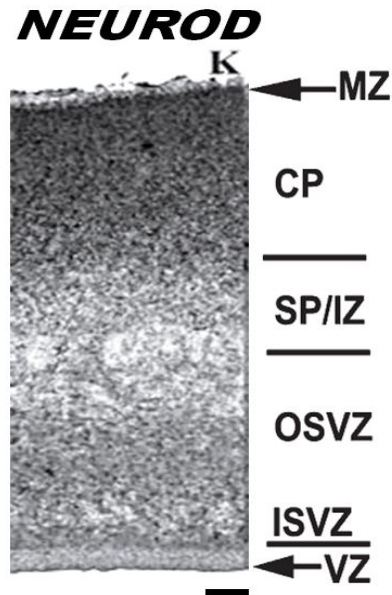
Figure 49. Laminar localisation of *NEUROD* in 12 PCW human neocortex. *NEUROD* expression is concentrated in the CP. Image adapted from Bayatti et al (2008b). Scale bar: 200µm

5.5.5 *Advantages of using human cord blood stem cells for cortical differentiation*

Two recent independent studies showed that mouse embryonic stem cells can be induced to recapitulate key cortical neurogenesis events *in vitro* producing specific cortical neuronal subtypes expressing certain cortical layers markers, such as Otx1 and Tbr1, following defined induction protocols. Both studies showed that the neurons produced were mostly excitatory and glutamatergic using electrophysiology and calcium imaging (Eiraku et al., 2008, Gaspard et al., 2008). Although both groups obtained similar results, the culturing systems utilized by each group were different. Gaspard et al. (2008) used a standard adherent monolayer approach similar to the one we utilized in our experiment. One of the advantages of this approach is the simplicity of the procedure; however it doesn't represent the layered physical organization of cells observed in the cortex. Eiraku et al. (2008) used embryoid body formation assay which is very complex when compared with the standard monolayer approach. However, the formation of three-dimensional structures *in-vitro* which highly resembled the spatial organization of cells observed in mouse cortex allowed a more precise analysis of stages of cortical development in a three-dimensional context.

This may provide insights on the effects of cellular spatial organization and interaction on fate specification and cortical development.

Both groups have used mouse embryonic stem cells to establish *in-vitro* models of corticogeneis. Such models are important in studying and investigating major mechanisms involved in regulation of mammalian corticogenesis in general due to the high degree of conservation across mammalian species. However there are some disadvantages to these approaches when wanting to target human development and disease. Although cortical neurogenesis in humans and mice share a lot of similarities, there are some major differences in structure and function that are underpinned by differences in cortical development (Bystron et al., 2008, Clowry et al., 2010). Examples in human include the presence of a larger and differentiated SVZ containing a larger pool of progenitors as compared to rodents, and differences in laminar expression patterns of genes involved in neurogenesis and laying down the early cortical map (Kornack, 2000, Hansen et al., 2010, Jones, 2009). Therefore using human stem cells to establish such models should more accurately mirror the developmental mechanisms regulating cortical neurogenesis in the human brain. Our *in vitro* system has recapitulated events occurring during cortical development which are important in the differentiation of a type of neuron commonly damaged in cerebral palsy as well as by stroke in adults. Such *in vitro* system should allow us to better understand and target potential brain repair strategies and provide *in vitro* models of neural diseases for toxicology and drug testing research.

In conclusion, umbilical cord blood non-hematopoietic multipotent stem cells undergoing our previously described neuronal induction protocol sequentially expressed genes crucially involved in human cortical neurogenesis of glutamatergic neurons, and expressed markers associated with glutamatergic neurotransmission. Such a stem cell-based system may aid the development of stem cell based therapeutic interventions for CNS injuries and disorders. Having an *in-vitro* model of human corticogenesis would also contribute towards a better understanding of the developmental mechanisms regulating neurogenesis in the cortex. In addition, they can provide cortical tissues with possible uses in toxicology and drugs testing research.

Chapter 6. Functional analysis of umbilical cord blood derived neurons

6.1 Introduction

One of the main targets of any stem cell differentiation protocol is the ability to generate cells with the same functional properties as the targeted cell type or tissue. This is very important when considering potential clinical applications where generated cells and tissues are used to replace diseased tissues. In the studies outlined in this chapter the neuronal functional properties of umbilical cord blood derived neuronal-like cells are evaluated. Using real time PCR arrays (refer to sections 2.11.6), we analyze the expression of ion channels genes involved in action potential generation and transmission (refer to section 1.7.3.1). We also analyze the gene expression of genes involved in modulating the biological processes of neurotransmitter biosynthesis, uptake, transport and signaling through neurotransmitter receptors (refer to section 1.7.3.1). In addition, we analyze the calcium influx activity in cord blood derived neuronal-like cells, a feature of neurons (refer to section 2.12).

6.2 Study aims

- To assess the functionality of cord blood derived neuron-like cells by analyzing the expression levels of genes coding ion channels involved in the generation and transmission of neuronal action potential.

- To analyze the subtypes of neuron-like cells produced from umbilical cord blood non-hematopoietic multipotent stem cells by analyzing the expression of genes involved in the biological processes of neurotransmitter biosynthesis, uptake, transport and signaling through neurotransmitter receptors.

- To evaluate the functionality of generated neuron-like cells by monitoring calcium influxes in cord blood derived neurons using calcium imaging system.

6.3 Results

6.3.1 *Q-PCR analysis of ion channels*

Q-PCR analysis of umbilical cord blood stem cells between day 0 and day 24 showed significant fold increase in ion channel genes expression levels (figure 50). Cells at day 24 also showed significant increase in expression levels of sodium channels genes including amiloride-sensitive channels *ACCN2* and *ACCN3* ($p<0.005$, $n=3$, for both genes), and voltage-gated channels *SCN1B* and *SCN8A* ($p<0.005$, $n=3$, for both genes).

Q-PCR also showed significant increase in expression levels of potassium channels genes at day 24 compared to day 0. Genes included inward rectifier channels *KCNJ1* and *KCNJ5* ($p<0.005$, $n=3$, for both genes), delayed rectifier channels *KCNA5* and *KCNS1* ($p<0.005$, $n=3$, for both genes), voltage-gated channels *KCNC4, KCND1, KCNH1* and *KCNQ3* ($p<0.005$, $n=3$, for all four genes) and calcium-activated potassium channel KCNMB1 ($p<0.005$, $n=3$).

Sodium Channels
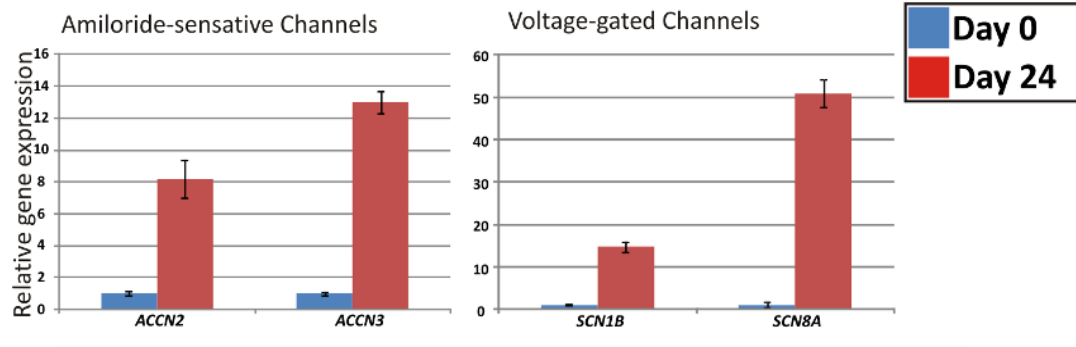
Potassium Channels
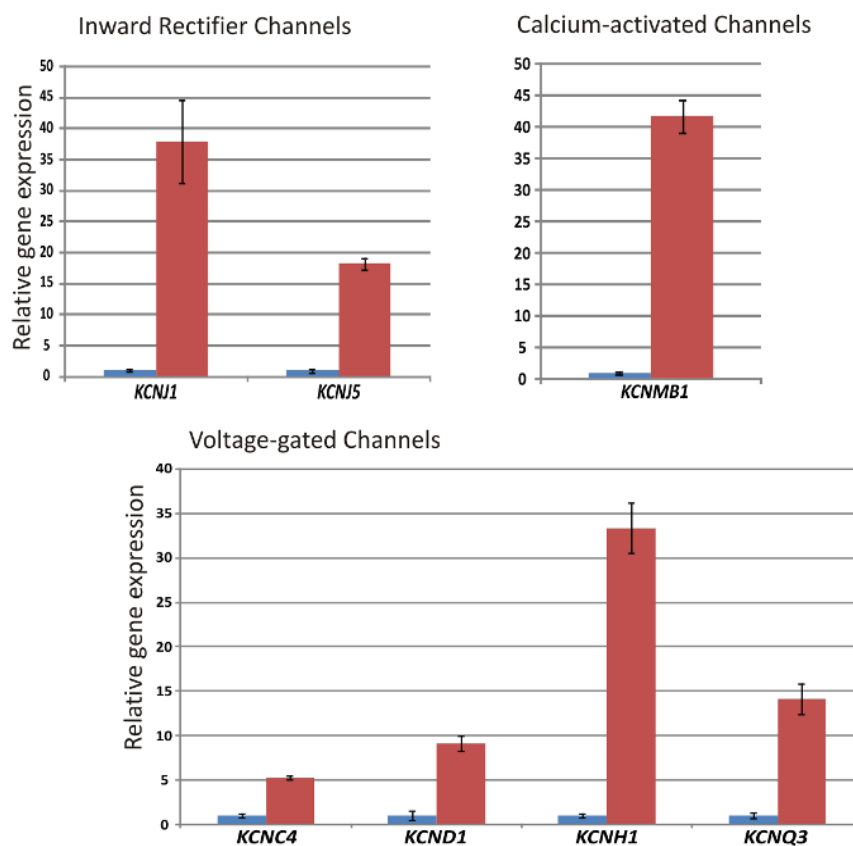
Figure 50. Q-PCR analysis of ion channels expression patterns between undifferentiated cord blood stem cells (Day 0) and cells after neuronal maturation (Day 24). Results showed significant increase in genes expression levels after neuronal maturation (Day 24) compared to undifferentiated stem cells (Day 0). Calibrator is Day 0 for all genes. All genes expression levels have been normalized to housekeeping genes *HPRT1, RPL13A* and *GAPDH*. Changes in expression levels for all shown genes between day 0 and day 24 are statistically significant, student's t-test ($p<0.005$, n=3).

6.3.2 *Q-PCR analysis of neurotransmitter regulatory enzymes*

Q-PCR results showed significant increase in expression level of neurotransmitter biosynthesis enzyme *GAD1* between days 0 and day24 ($p<0.005$, n=3) (figure 51). On the other hand, there were no detectable increases in expression levels of *CHAT* and *TPH1* between day 0 and day 24. For neurotransmitter catabolic enzymes, *ABAT*, *ACHE* and *COMT,* significant increase in expression levels were detected between undifferentiated stem cell stage (Day 0) and cells after neuronal maturation (Day 24) ($p<0.005$, n=3, for the three genes) (figure 51).
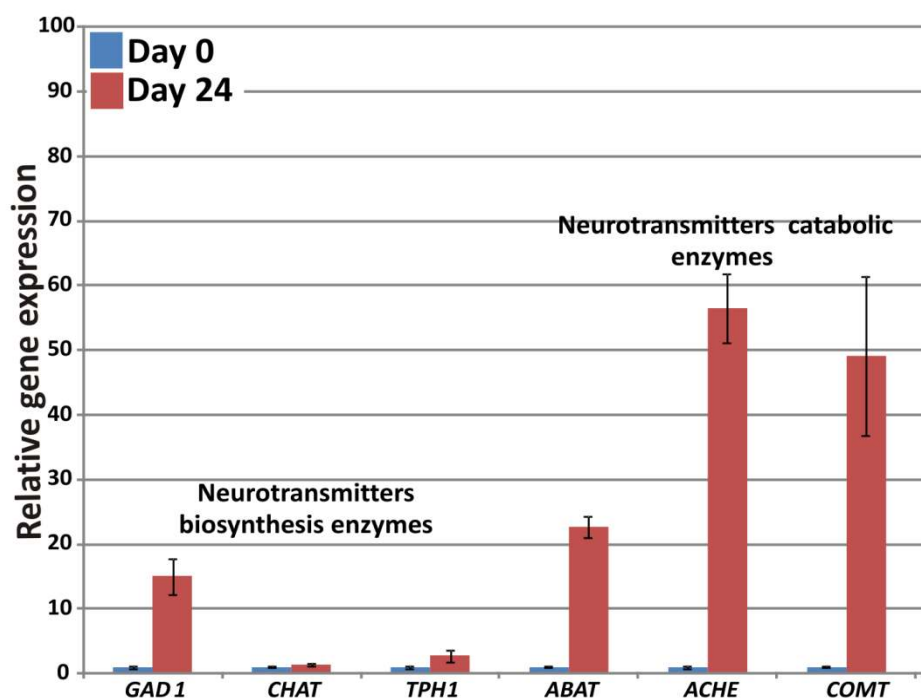
Figure 51. Q-PCR analysis of neurotransmitters regulatory enzymes genes between day 0 and day 24. For neurotransmitters biosynthesis enzymes, only *GAD1* showed significant increase in gene expression level on day 24 compared to day 0. *CHAT* and *TPH1* showed no detectable increases in gene expression levels after maturation. For neurotransmitter catabolic enzymes, *ABAT*, *ACHE* and *COMT*, all showed significant increase in gene expression levels after maturation (Day 24). Calibrator is Day 0 for all genes. All genes expression levels were normalized to housekeeping genes *HPRT1, RPL13A* and *GAPDH.* Changes in expression levels of shown genes, except *CHAT* and *TPH1,* between day 0 and day 24 were statistically significant, student's t-test ($p<0.005$, n=3).

6.3.3 *Q-PCR analysis of neurotransmitter transporters*

Q-PCR analysis on neurotransmitter transporters showed significant increase in expression levels of glutamate transporters *SLC1A1* (p<0.05, n=3) and *SLC1A2* (p<0.005, n=3) between day 0 and day 24. Similar significant increases were also found in GABA transporter *SLC6A12* (p<0.05, n=3) and serotonin transporter *SLC6A4* (p<0.005, n=3). On the other hand, there were no detectable increases in expression levels of dopamine transporter *SLC6A3* and glycine transporter *SLC6A* (figure 52).
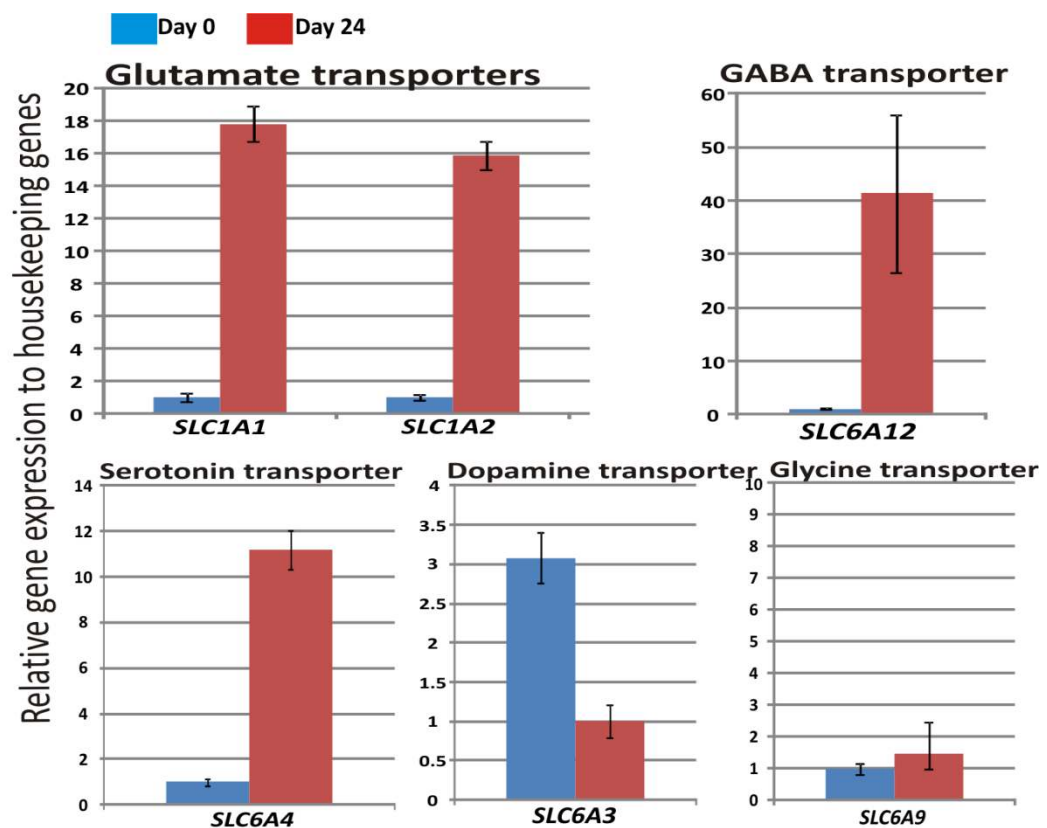
Figure 52. Q-PCR analysis of neurotransmitters transporters genes between day 0 and day 24. All transporters genes presented above showed significant increases in gene expression levels by day 24 compared to day 0 except for dopamine transporter *SLC6A3* and glycine transporter *SLC6A9*. Calibrator is Day 0 for all genes except SLC6A3 Day 24. All genes expression levels have been normalized to housekeeping genes *HPRT1, RPL13A* and *GAPDH*. Changes in expression levels of shown genes, except *SLC6A3* and *SLC6A9,* are statistically significant, student's t-test *SLCA1A* and *SLC6A12* (p<0.05, n=3) and rest of genes (p<0.005, n=3).

6.3.4 *Q-PCR analysis of neurotransmitter receptors*

Q-PCR analysis showed significant increase in gene expression levels between day 0 and day 24 for glutamate receptors *GRIA1* and *GRIN1* ($p<0.005$, n=3, for both genes), GABA receptors *GABRA3* and *GABRR2* ($p<0.005$, n=3, for both genes), acetylcholine receptors *CHRNA2*, *CHRNA5*, *CHRNB2*, *CHRNG* ($p<0.005$, n=3, for the four genes), dopamine receptors *DRD1*($p<0.05$, n=3) and *DRD2* ($p<0.005$, n=3) and glycine receptors *GLRA1* and *GLAR2* ($p<0.005$, n=3, for both genes) (figure 53).
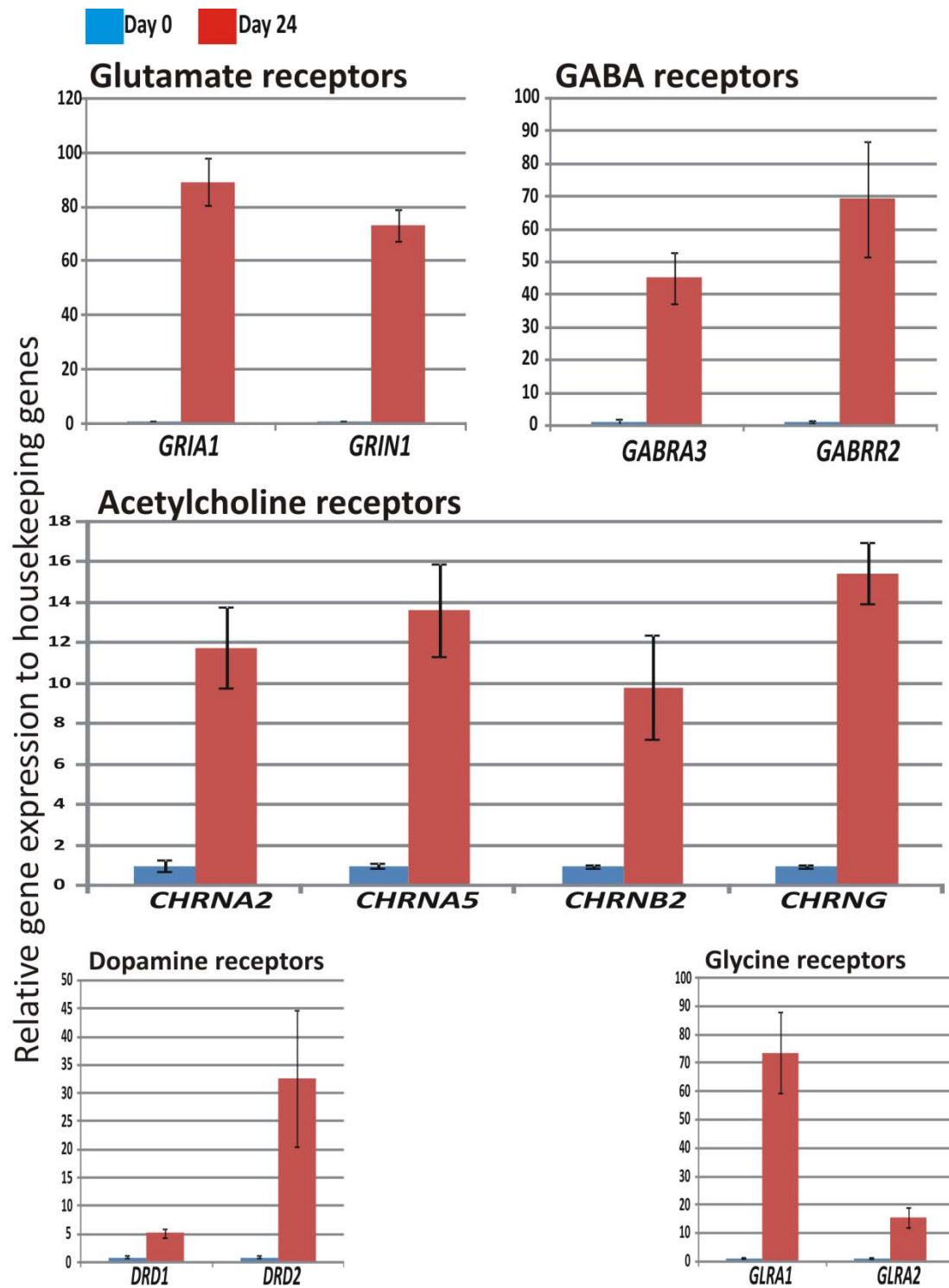
Figure 53. Q-PCR analysis of neurotransmitters receptors genes between day 0 and day 24. All the neurotransmitter receptor genes investigated showed significant increase in expression level after neuronal maturation (Day 24) compared to undifferentiated cord blood stem cells (Day 0). Calibrator is Day 0 for all genes. All gene expression levels have been normalized to housekeeping genes *HPRT1, RPL13A* and *GAPDH*. Changes in expression levels of shown genes are statistically significant, student's t-test, DRD1 ($p<0.05$, n=3) and rest of genes ($p<0.005$, n=3).

6.3.5 *Q-PCR analysis of neuropeptides receptors*

Q-PCR analysis showed significant increase in gene expression levels of neuropeptide receptors MCHR1, SORCS1 and SORCS2 between day 0 and day 24 ($p<0.005$, n=3, for the three genes), neuropeptide Y receptors *PPYR1* ($p<0.05$, n=3) and *NPY1R* ($p<0.005$, n=3) and galanin receptors *GALR2* ($p<0.05$, n=3) and *GALR3* ($p<0.005$, n=3) (figure 54).
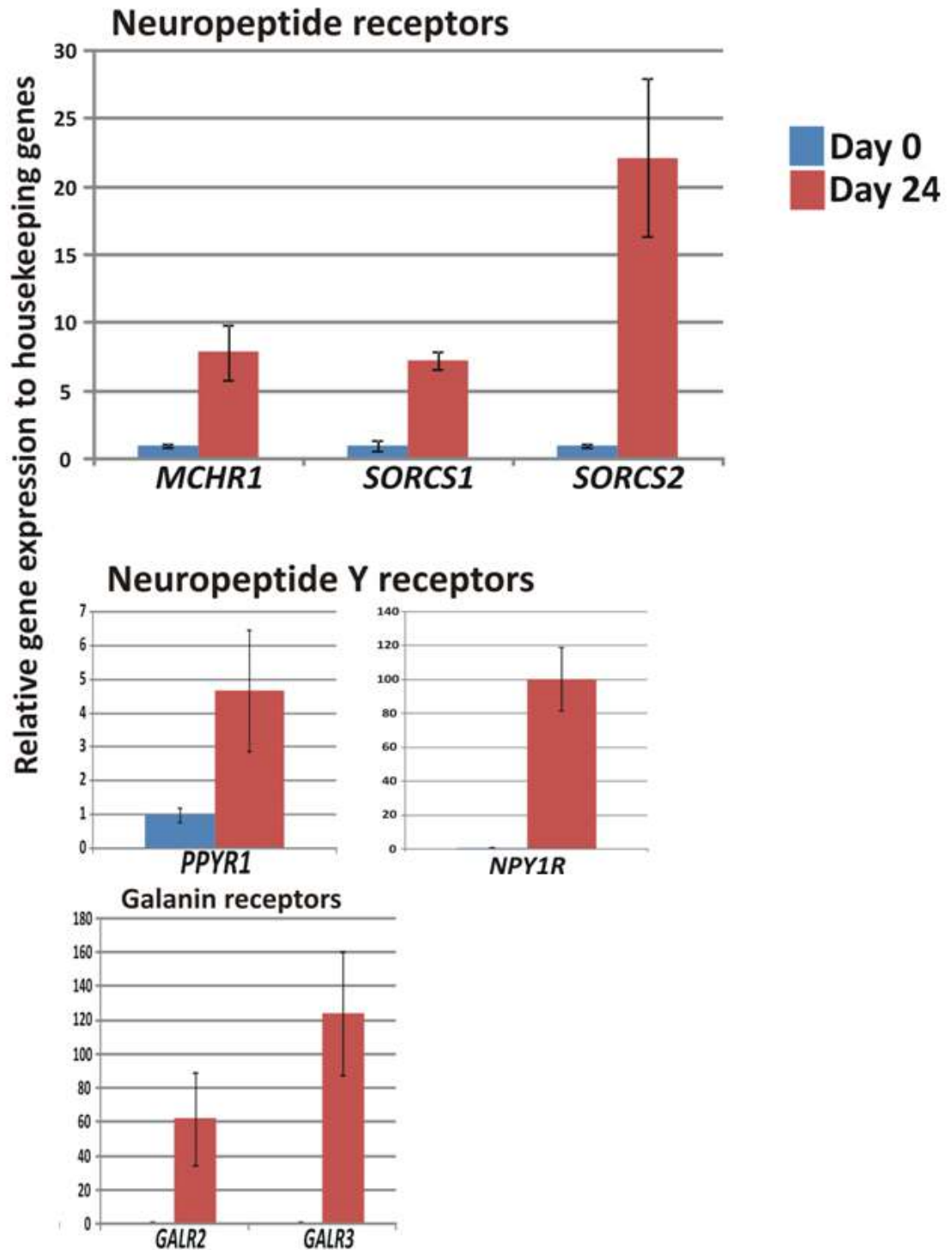
Figure 54. Q-PCR analysis of neuropeptide receptors genes between day 0 and day 24. All presented genes showed significant increase in gene expression levels after neuronal maturation (Day 24) compared to day 0. Calibrator is Day 0 for all genes. All genes expression levels were normalized to housekeeping genes *HPRT1, RPL13A* and *GAPDH*. Changes in expression levels of shown genes are statistically significant, student's t-test, *PPYR1* and *GALR2* ($p < 0.05$, n=3) and rest of genes ($p < 0.005$, n=3).

6.3.6 *Calcium Influx imaging*

Calcium imaging of neuronal induced cord blood stem cells at (Day 24) showed positive readings for fast calcium influxes inside the cells. Some cells demonstrated spontaneous fast calcium influxes without induction (figure 55).

Other cells generated fast calcium influxes only upon induction with glutamate. These influxes were recorded right after glutamate was applied to the cells via patch pipettes from a 10-50µm distance from the cells of interest. These influxes were not recorded when glutamate was not applied to these cells (figure 56, 57). In both spontaneous and glutamate-induced calcium influxes, the duration of a single influx (represented by the sharp peaks on graphs) was similar (about a second).
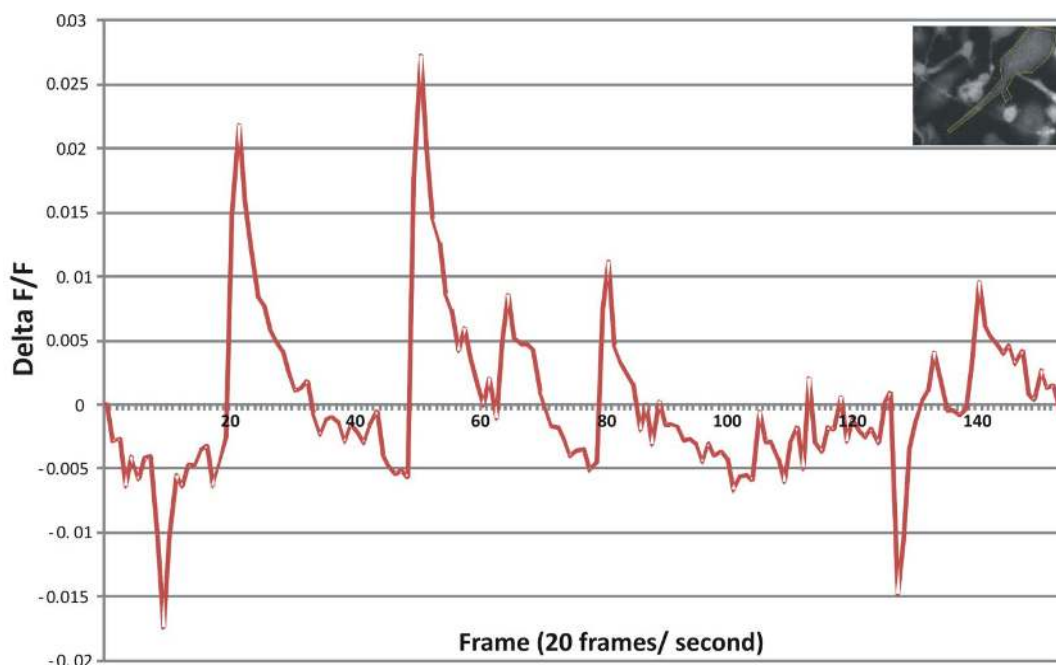
Figure 55. Spontaneous calcium influxes in cells after neuronal maturation (Day 24). Calcium influxes are represented by the peaks of ΔF/F florescence level graph. Top right picture represents the analyzed cell.
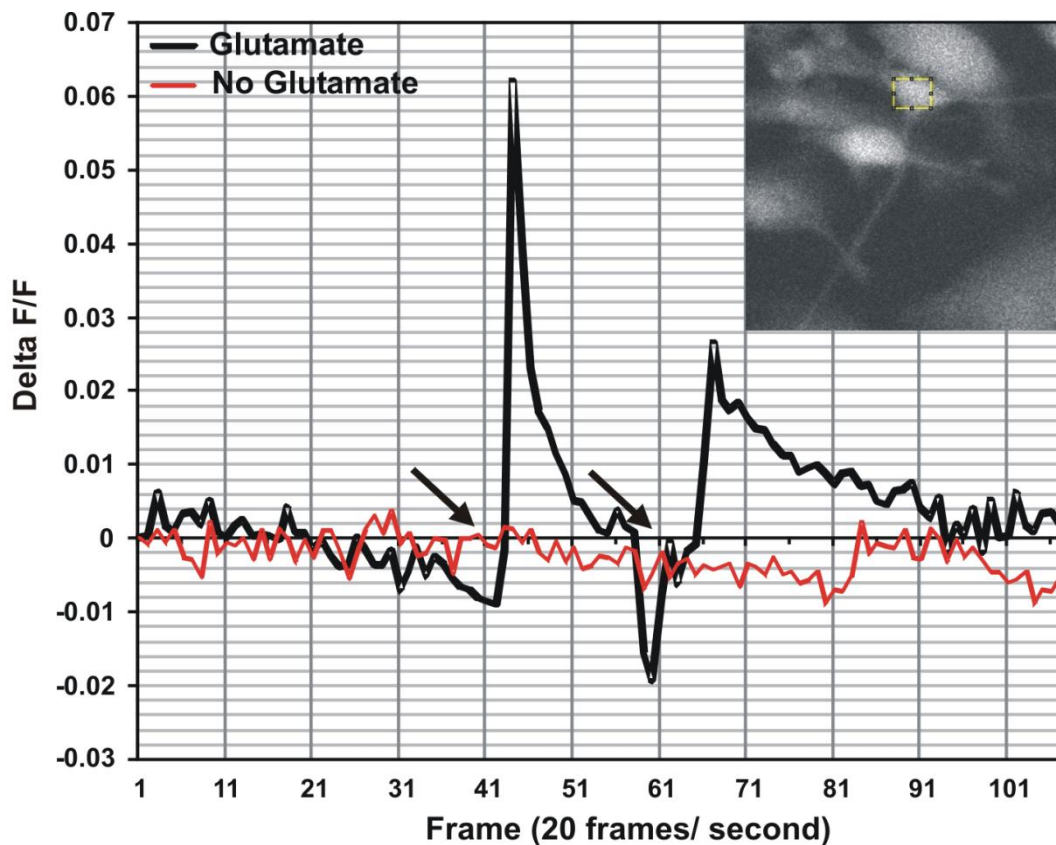
Figure 56. Glutamate-induced calcium influx in cord blood differentiated stem cells (Day 24). 1mM glutamate was applied twice, at frame 40 and frame 60 (black arrows). Cells responded to glutamate with calcium influxes represented by ΔF/F florescence level (black graph). Without glutamate, no calcium influxes were detected (red graph). The cell analyzed for this example shown on the top right corner.
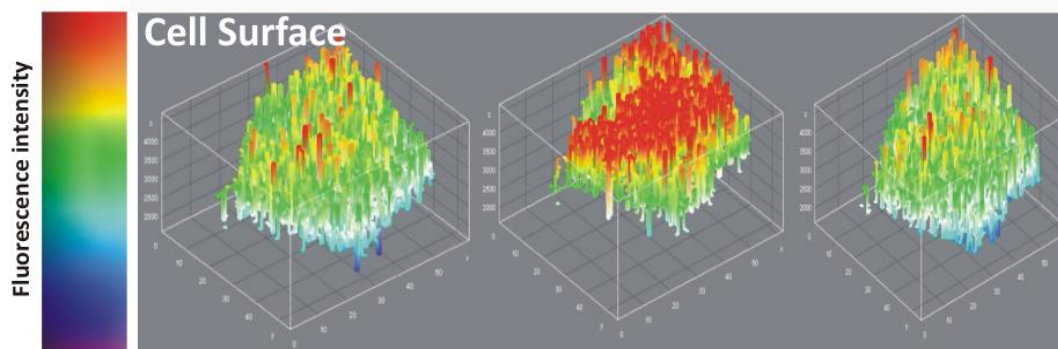
Figure 57. Three dimensional representations of the analyzed cell's surface upon induction with glutamate. The fluorescence intensity (purple to red) correlates with the intensity of the calcium influx.

The proportion of cells showing detectable calcium influx increased from only 4.88 ± 2.19% at day 0 to 40.42±7.92% (spontaneous) and 27.7±8.74% (induced) at day 24 (p<0.05, n=3, for both) (figure 58).
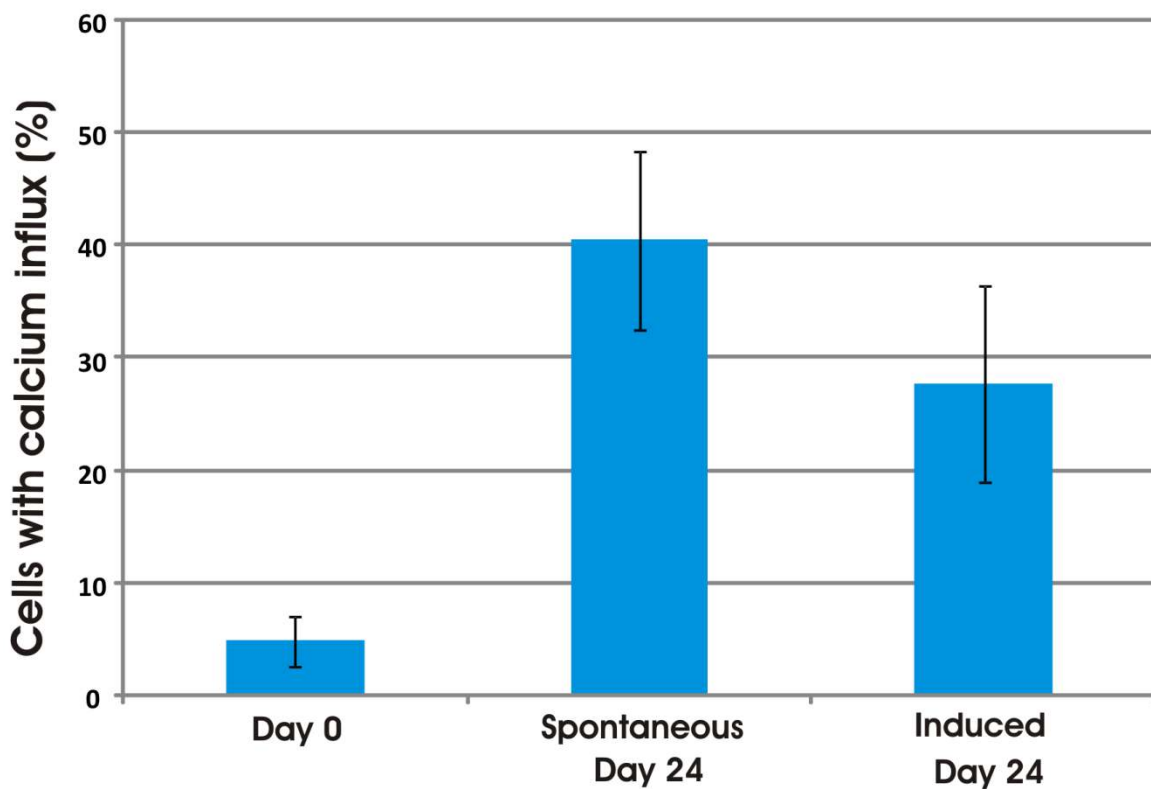
Figure 58. Proportion of cells generating spontaneous and induced Calcium influxes increased significantly after neuronal maturation (Day 24) compared to undifferentiated cord blood stem cells (Day 0). Results are statistical significant (student's *t*-test *p<0.05*, n=3).

6.4 Conclusion

- Umbilical cord blood non-hematopoietic multipotent stem cells yielded functional neuron-like cells after the 24 days *in-vitro* neuronal induction protocol as shown by the calcium influx studies.

- Expression of markers associated with the function of certain subtypes of neurons including glutamatergic, GABAergic and serotonergic neurons suggests neuronal subtype specification.

6.5 Discussion

In this chapter the neuronal functional properties of the umbilical cord blood derived neuron-like cells are evaluated. Expression patterns of genes involved in action potential generation and propagation are examined by comparing gene expression levels between undifferentiated stem cells (Day 0) and cells after neuronal maturation (Day 24) using Q-PCR. Similarly, the expression of genes involved in modulating the biological processes of neurotransmitter biosynthesis, uptake, transport and signaling through neurotransmitter receptors are also examined. We further evaluate the functionality of cord blood derived neuron-like cells using a direct approach that monitored and recorded spontaneous and induced calcium influxes in cells after neuronal maturation (Day 24). Gene expression patterns and calcium imaging suggest that the generated neuron-like cells have proper neuronal functional properties which further confirm their neuronal-like identity.

6.5.1 *Generated neuron-like cells expressed genes involved in action potential generation and propagation*

Ion channels are involved in a wide range of central nervous system functions including the generation and propagation of action potential in neurons. We compared the expression levels of gene transcripts using Q-PCR between cells after neuronal maturation (Day 24) and undifferentiated cells (Day 0) and found significant up-regulation in many genes coding ion channels after the neuronal induction protocol (figure 50). Up-regulated genes included Amiloride-sensitive cation channel 2 and 3 (*ACCN2* and *ACCN3*). These two channels are members of an H^+-gated subgroup of the degenerin/epithelial Na^+ channel (DEG/ENaC) family of cation channels highly expressed in neurons. ACCN2 is expressed dominantly in brain neurons where it regulates and mediates glutamate-independent Ca^{2+} entry into neurons upon acidosis (Askwith et al., 2004, Zha et al., 2009). ACCN3 is more associated with PNS neurons where it is involved in initiating responses to mechanical and painful stimuli (Wemmie et al., 2006, Hruska-Hageman et al., 2004). ACCNs are an important component of neuronal physiological regulatory system where their "functional sensitivity" to pH changes is thought to be involved in regulation of many neuronal functions.

Action potential is generated and propagated across neurons by rapid opening and closing of specialized sodium and potassium ion channels (refer to section 1.7.3.1). Q-PCR results showed significant up-regulation in gene transcripts coding various ion channels crucially involved in the generation and propagation of action potential. Among the up-regulated channels were sodium voltage-gated ion channels *SCNB1* and *SCN8A* which are subunits of trans-membrane channels specific for sodium and sensitive to voltage changes across the membrane. These channels are crucially involved in the depolarization phase of action potential where they open in response to increases in the membrane potential which allow Na^+ to enter the cell resulting in further increase in the membrane potential (Barnett and Larkman, 2007). Up-regulated genes also included potassium voltage-gated channels represented by *KCNC4, KCND1, KCNH1* and *KCNQ3* which are involved in the repolarization phase of the action potential where the increased membrane potential triggers their opening resulting in rapid movement of K^+ outside the neuron resulting in repolarization phase which would bring the membrane potential back to negative value (Yellen, 2002, Barnett and Larkman, 2007). Other more specialized potassium channels have also been significantly up-regulated after neuronal maturation (Day 24). These channels included inward rectifier channels represented by *KCNJ1* and *KCNJ5*. These channels differ from typical potassium channels in that they allow K^+ positive current to move inside the cells rather than outside the cell and play a key role in establishing the resting potential in neurons (Tao et al., 2009). Also significant up-regulation was recorded for potassium large conductance calcium-activated channel (*KCNMB1*). This channel can be activated by voltage changes as well as increases in concentration of intracellular calcium ion (Ca^{2+}) and plays a major role in regulation of neuronal excitability by modifying action potential duration, frequency and synaptic efficacy (Miller, 2000, Yuan et al., 2010, MacDonald et al., 2006).

The expression of ion channels involved in regulation of neuronal functions including the generation and propagation of action potential suggests that the cord blood derived neuron-like cells have reached a maturing neuronal-like functional status after neuronal maturation (Day 24). These results can be further analyzed and confirmed using patch clamping. This laboratory technique allows direct selective recording of currents that flow through ion channels during action potential transmission (Purves, 2004, Sun et al., 2005). Such

analysis offers a more direct approach to confirm the neuronal functional properties of the generated cells.

6.5.2 *Generated neuron-like cells expressed genes involved in neurotransmitter biosynthesis and regulation*

Neurotransmitters are chemicals released by neurons at the synapses to allow the transmission of action potential signal from one neuron to the other. Neurotransmitters are synthesized in neurons through chemical reactions which are catalyzed by certain enzymes. The expression of a particular enzyme by certain neuron can be used as marker to classify neurons based on the neurotransmitter they manufacture. The production of neurotransmitters is a fundamental component of the neuronal functional properties, hence we investigated the expression of certain genes coding enzymes involved in biosynthesis and regulation of neurotransmitters in our cord blood derived neuron-like cells (Day 24) (figure 51). We have selected the enzymes involved in biosynthesis of most common neurotransmitters in the central nervous system (refer to section 1.7.3.1) and found that *GAD1*, unlike *CHAT* and *TPH1*, was significantly up-regulated in cells after neuronal maturation (Day 24) compared with undifferentiated cells (Day 0). *GAD1* is the gene encoding glutamic acid decarboxylase which catalyzes the production of gamma-aminobutyric acid (GABA) from L-glutamic acid in GABAergic neurons (Dirkx et al., 1995). On the other hand, results did not show any significant up-regulation in levels of *CHAT*, which encodes choline acetyltransferase that catalyzes the production of acetylcholine from Acetyl-CoA and choline in cholinergic neurons (Blusztajn et al., 1987) and *TPH1*, which encodes tryptophan hydroxylase 1 which is the rate-limiting enzyme in the biosynthesis of serotonin from tryptophan in serotonergic neurons (Andreou et al., 2010). However, expression of *TPH1* is not restricted to neurons; it is also expressed by mononuclear leukocytes of peripheral and umbilical cord blood (Rahman, 1988). Our Q-PCR analysis compares the expression levels between undifferentiated purified stem cells (Day 0) and cells after neuronal maturation (Day 24). Any up-regulation at day 24 is measured relatively to the initial expression level of the gene at day 0. The negative depletion step does not eliminate all the leukocytes, suggested by the FACS analysis of CD45 expression after purification (figure 20), and it is possible that the purified fraction contained a portion of hematopoietic cells expressing *TPH1*. Such cells might have

interfered with the Q-PCR analysis of the neuronal induction between day 0 and day 24. In order to further investigate whether TPH1 is expressed by cord blood derived neuron-like cells or not, immunocytochemistry analysis of the enzyme and another neuronal general marker such NF200 or β-TUBULIN III would offer a very good opportunity to examine neuronal specific expression of TPH1. Such results suggest that our derived neuron-like cells contained a possible subgroup of GABAergic neuron-like cells but not cholinergic neurons. Whether or not cord blood derived neuron-like cells contained a subgroup of serotonergic neurons need to be further analyzed.

Up-regulated genes also included genes coding neurotransmitter catabolic enzymes including *ABAT*, *ACHE* and *COMT*. *ABAT* encode 4-aminobutyrate aminotransferase which is the enzyme responsible for catabolism of GABA, the most abundant inhibitory neurotransmitter in the CNS, into succinic semialdehyde at the synapses and therefore terminating the synaptic transmission of GABA (Larsson and Schousboe, 1990). *ACHE* encodes acetylcholinesterase which is the enzyme that degrades acetylcholine into choline and acetate group and thereby terminates its synaptic transmission (Descarries et al., 2004, Anglister et al., 2008). *COMT* is the gene that encodes Catechol-*O*-methyltransferase, the enzyme that degrades catecholamines including dopamine (Mannisto and Kaakkola, 1999). These catabolic enzymes perform a very important regulatory role in CNS by regulating synaptic transmission mediated by neurotransmitters.

The expression of genes involved in neurotransmitter biosynthesis and regulation not only reflects on the maturing neuronal-like status of our cells but also suggests proper neuronal-like functional regulatory properties acquired by the cells after neuronal maturation (Day 24).

6.5.3 *Generated neuron-like cells expressed various neurotransmitter transporters*

Another important component of the neuronal functional system is neurotransmitter transporters that are specialized proteins located on cellular membranes of the neurons and primarily involved in transporting neurotransmitters across membranes intra-cellularly. They carry out a crucial regulatory role that precisely controls the intensity and duration of synaptic transmission. When neurotransmitters are released at the synapses, they activate their receptors on the post-synaptic neurons and initiate synaptic transmission response.

This process is terminated when neurotransmitters are eliminated from the synaptic cleft by active transport of neurotransmitters by the neurotransmitter transporters (Masson et al., 1999). After neuronal maturation (Day 24), multiple genes encoding neurotransmitter transporters were significantly up-regulated (figure 52). These genes included neuron-specific glutamate transporter *SLC1A1* which encodes excitatory amino acid transporter 3 (EAAT3), the major neuronal glutamate transporter in the CNS (Foster and Kemp, 2006), and *SLC1A2* which encodes excitatory amino acid transporter 2 (EAAT2) that is expressed mostly by CNS glia cells (Chen and Swanson, 2003, Schmitt et al., 2002). Both transporters are Na^+ dependent, where the driving force of transport is provided by the trans-membrane gradient of Na^+ and also K^+ (Meldrum, 2000). Those high affinity glutamate transporters clear the excitatory neurotransmitter from the synaptic cleft in CNS. This process is very crucial to regulate glutamate-mediated synaptic transmission and to prevent neuronal damage and death as a result of excessive activation of glutamate receptors (Meldrum, 2000, Mattson, 2008).

Up-regulated genes also included multiple genes that encode transporters which belong to solute carrier family 6 (SLC6). These transporters function through a Na^+ and Cl^- dependent manner. Up-regulated genes included *SLC6A12* which encodes a high affinity GABA transporter. This transporter protein is located in the membrane of the pre-synaptic terminals and is involved in the regulation of GABA synaptic transmission through mediating fast GABA removal from synaptic cleft into the cell to avoid excessive activation of GABA receptors (Borden et al., 1995). Up-regulated genes also included *SLC6A4*, which encodes a serotonin transporter that regulates serotonin-mediated synaptic transmission and function in a similar manner to SLC6A12 where it reuptakes serotonin from the synaptic cleft and transports it into pre-synaptic terminals to be recycled and reutilized (Masson et al., 1999). It is logical that this transporter is expressed by neurons capable of producing serotonin (expressing TPH1). Based on such results, it is possible that our cord blood derived neuron-like cells contained a subgroup of serotonergic neurons. Nevertheless, the previously suggested immunocytochemistry analysis of TPH1 and another neuronal marker such as β-TUBULIN III or NF200 would provide a better way to confirm the identity of the such cells.

6.5.4 *Generated neuron-like cells expressed various neurotransmitter receptors*

Neurotransmitter receptors are an important component of the neuronal functional system. These membrane receptor proteins are involved in neuron-neuron chemical communication through synapses that facilitate neuronal signal transmission. They are expressed at the synapse of post- and pre-synaptic neurons. When neurotransmitters are released in the synaptic cleft from specialized vesicles on pre-synaptic neuron in response to appropriate stimuli, they interact and bind to their specific receptors initiating proper response in the post-synaptic neurons. After neuronal maturation (Day 24), cord blood derived neuron-like cells showed significant up-regulation in genes encoding different neurotransmitter receptors (figure 53). The up-regulated genes included *GRIA1* and *GRIN1* which encode glutamate receptors AMDAR1 and NMDAR1 respectively in addition to genes encoding GABA, acetylcholine, dopamine, galanin and glycine receptors. In CNS a single neuron can receive inputs from multiple sources utilizing different types of neurotransmitters and therefore it can express different neurotransmitter receptors (Clendening and Hume, 1990) which explains the diverse types of receptors expressed by our cells. In the same context, one neuron can express receptors for a particular neurotransmitter, which it does not produce; this explains the expression of the acetylcholine receptors by our cells while no significant *CHAT* expression was detected.

In addition to neurotransmitters, neurons also utilize small peptides for communication called "neuropeptides". Similarly to neurotransmitters, neuropeptides are expressed and released by neurons mediating synaptic transmission either in a direct or indirect manner (Li and Kim, 2008). In addition, neuropepetide plays a key role in the regulation of certain physiological functions including food intake, memory and learning (Wilding, 2002, Feany, 1996). Among the receptors expressed by cells at Day 24, Neuropeptide Y (NPY) receptors *PPYR1,* which is expressed predominantly in peripheral organs, and *NPY1R* which is expressed predominantly in CNS (Naveilhan et al., 1998). NPY is a peptide composed of 36 amino acids express widely in the brain. Beside its role in regulating food intake and energy balance (Gerald et al., 1996), NYP has also demonstrated neuroproliferative and neuroprotective properties (Xapelli et al., 2006, Thiriet et al., 2011). Neuroproliferative properties were demonstrated when genetic knockout of NYP receptors in mice decreased the number of proliferative neuroblasts in the SVZ (Stanic et al., 2008) while

intracerebroventricular injection of NPY enhanced the proliferation of neuroblasts in mice SVZ (Decressac et al., 2009). The neuroprotective properties were demonstrated in studies performed on organotypic slice cultures of rat hippocampus (Silva et al., 2003). In this study, selective activation of NYP receptors using agonists protected the neural cells from neuronal degeneration as a result of AMPA mediated excitotoxicity (Silva et al., 2003).

Our results showed significant up-regulation in transcripts of genes encoding neuropeptide receptors in cord blood derived neuron-like cells (Day 24) compared with undifferentiated cells (Day 0) (figure 54). The expression of neurotransmitter and neuropepetide receptors highlights the neuronal functional potential of generated neuron-like cells.

6.5.5 *Functional properties of generated neuron-like cells confirmed by spontaneous and induced calcium influxes*

In addition to the indirect approaches in analyzing neuronal functional properties of our cord blood derived neuron-like cells through mRNA expression and protein localization assays, we utilized a direct method to evaluate the functionality of generated neuron-like cells by monitoring the calcium ions (Ca^{2+}) influxes in the cells. Calcium ions play a major role in neuronal development and functionality. Neuronal calcium influx triggered by either neurotransmitters or electric signals is involved in regulation of many biological and functional processes in neurons including neuronal differentiation, neurites outgrowth, synaptic plasticity, action potential and neuronal survival (Elmslie, 2003, Mattson, 2008, Ryglewski et al., 2007).

We investigated the calcium activity in generated neuron-like cells after neuronal maturation (day 24) by bulk loading the cells with a dye that is sensitive to Ca^{2+} which allowed monitoring spontaneous and induced influxes of Ca^{2+}, a characteristic of functional neurons (refer to section 2.12). The calcium imaging procedure have been used previously to evaluate the functional properties of endogenous CNS neurons (Murayama et al., 2005, Johannssen and Helmchen, 2010) and neurons derived from stem cells (Zangiacomi et al., 2008, Eiraku et al., 2008).

We used glutamate ,the most abundance neurotransmitter in mammalian brain (Erecinska and Silver, 1990), to induce Ca^{2+} influxes and we recorded precise waves of Ca^{2+} influxes in response to glutamate. When glutamate was withdrawn, no Ca^{2+} influxes were recorded from the same cells which suggest that the recorded influxes were induced by glutamate (figure 56). Such process is thought to be involved in the stimulation of the neuronal depolarization phase of action potential. Our cells expressed two glutamate receptors AMPAR and NMDAR which are ligand-gated ion channels. When the receptors are activated by glutamate, the ion channels open and allow various ions into the cell such as Ca^{2+}. These receptors play a key role in mediating excitatory responses and controlling synaptic plasticity at the majority of CNS synapses (Mayer, 2005, Li and Tsien, 2009). Our results suggest that our derived neuron-like cells, which express glutamate receptors AMPAR and NMDAR, acquired an active functional status demonstrated by their precise responses to glutamate with Ca^{2+} influxes.

Calcium influxes can also be stimulated in neurons using KCI which activates calcium influxes through calcium voltage dependent channels (Zangiacomi et al., 2008). Additionally AMPA has also been used to stimulate calcium influxes in neurons (De Melo Reis et al., 2011). Using channel blockers such as tetrodotoxin (TTX), AMPA and NMDA antagonists would provide stronger evidence and indication of the channels mediating the observed Calcium influxes. In this study, we have not used such blockers due to insufficient time available on the equipment.

In addition to glutamate-induced Ca^{2+} influxes, we also recorded spontaneous influxes without using glutamate (figure 55). Such glutamate-independent influxes can occur through different channels, other than AMPAR and NMDAR, including the neuronal specific amiloride-sensitive cation channel 2 (ACCN2) which regulates and mediates glutamate-independent Ca^{2+} entry into neurons upon acidosis (Askwith et al., 2004, Zha et al., 2009). It is also possible that these influxes are mediated by calcium voltage-dependent ion channels (Jarvis and Zamponi, 2007).

Collectively, the proportion of cells showing detectable calcium influxes showed significant increase after neuronal maturation (Day 24) compared with undifferentiated purified stem cells (Day 0) (figure 58). This result suggests that the calcium-influx

characteristic was acquired in response to the neuronal induction protocol that stimulated neuronal-like molecular and morphological transformation, highlighting active neuronal-like functional status of the generated neuron-like cells.

6.5.6 *Putting the pieces together: the neuronal functional system*

The transmission of neuronal action potential in the human body is a very dynamic process that involves different components that contribute either directly to the process of signal transmission or by performing regulatory roles that regulate the over all process. These components collectively form the neuronal functional system which labels and defines the neuronal identity. Any therapeutic potential for stem cell-based therapies to treat neurological disorders relies on the ability of such cells to produce neurons capable of performing and carrying on neuronal functional duties similar to those of nervous system endogenous neurons.

Signal transmission in neurons occurs through two different mechanisms; chemically from one neuron to the other across synapses, and electrically along axons of neurons (figure 14). For chemical signaling, neurons produce and utilize neurotransmitters to transmit signals across synapses. In studies outlined in this chapter, it is shown that cord blood derived neuron-like cells expressed transcripts encoding enzymes involved in neurotransmitter production. When neurotransmitters are released across synapses, they bind to special neurotransmitter receptors which get activated and initiate proper response. This chemical signaling is regulated through catabolic enzymes which degrade the excess neurotransmitters and terminate the signaling and also by neurotransmitter transporters which reuptake neurotransmitters from the synaptic cleft and transport it into the neurons for recycling and reutilization. Depending on the neurotransmitter type (excitatory/inhibitory), activated receptors initiate proper response. In the case of excitatory effect, the activated receptor will stimulate an electric signal of action potential across the neuron as a result of rapid movement of ions across the neuron membrane through specialized ion channels. This entire process of chemical/electric signal transmission defines the functional identity of neurons. Cord blood derived neuron-like cells expressed genes encoding element involved in each stage of the signal transmission process. They

also directly demonstrated proper functionality by generating spontaneous and glutamate-induced calcium influxes, similar to nervous system endogenous neurons.

The concept of stem cell based therapies for CNS injuries and disorders is base on the ability to compensate and replace the dead or diseased neural tissues with new ones generated from stem cells. It is crucial that the stem cells used can produce functional neurons that are able of compensating for the dead/diseased tissues physically and functionally. Our results indicate that umbilical cord blood non-hematopoietic multipotent stem cells are capable of producing neuron-like cells with neuronal-like functional properties. Such results further highlight the potential of umbilical cord blood stem cells as a therapeutic tool for CNS injuries and disorders which current mode of therapy is inadequate.

Chapter 7. Concluding discussion

The work in this thesis has shown that the sequential neuronal induction protocol used here induces umbilical cord blood non-hematopoietic multipotent stem cells to adopt a neuronal-like fate. At the end of the differentiation protocol (Day 24), the cells adopted a neuronal-like morphology and expressed neuronal specific markers. We further showed that the process of neuronal differentiation of purified cells highly resembled the development of glutamatergic neurons in human neocortex. Indirect and direct functional analysis suggested that the generated cells have proper neuronal-like functional status. Such results highlight the potential of umbilical cord blood stem cells as a therapeutic tool for CNS injuries and disorders and also as a research tool for toxicology and drugs testing studies.

7.1 *In-vitro* model of cortical neurogenesis: GABAergic neurogenesis would complete the model

We have shown that the sequential induction of umbilical cord blood purified stem cells recapitulated key events taking place in human neocortex regulating cortical neurogenesis of glutamatergic neurons. We further showed that generated neurons expressed components involved in glutamatergic neurotransmission including glutamate receptor subunits and neuronal specific glutamate transporters (refer to chapter 5). In addition, the generated cells responded to glutamate induction with influxes of calcium demonstrating proper functional status (figure 56). As mentioned earlier the human cortex mainly hosts two kinds of neurons: glutamatergic neurons which accounting for 80% of the cortex neuronal content and GABAergic neurons which account for the remaining 20% (Letinic et al., 2002, Meyer et al., 2000, Guillemot et al., 2006, Jones, 2009). The excitatory and inhibitory systems, represented by both types of cortical neurons, function in a collaborative and coordinated manner regulating neuronal firing patterns, network activity and synaptic plasticity which contribute to the diverse and complex CNS functions (Foster and Kemp, 2006). We

showed that sequential induction of cord blood purified stem cells provided a good *in-vitro* model for the cortical excitatory system (chapter 5). However, including the GABAergic cortical neurogenesis as well would provide a more accurate and complete model of human cortical neurogenesis. Q-PCR results after neuronal maturation (Day 24) indicated that the cells expressed markers involved in GABA-mediated transmission and are routinely used to characterize GABAergic neurons. These markers included *GAD1* and high affinity GABA transporter (figure 51 and figure 52). These results suggest a possible existence of GABAergic neuron-like cells in cultures after neuronal maturation (Day 24). The possibility of studying and analyzing the commitment and differentiation processes of these cells *in-vitro* and comparing them with their differentiation pathways in human cortex would provide a more complete *in-vitro* representation of human corticogenesis. Such a stem cell-based system could lead to a better understanding of the developmental mechanisms regulating human corticogenesis. Such improved understanding may aid the development of therapeutic interventions for injuries and disorders affecting the human cortex in particular and CNS in general.

7.2 Umbilical cord blood non-hematopoietic stem cells: potential therapeutic tool for CNS injuries and disorders

As mentioned earlier, cell therapy has been proposed as a potential therapeutic tool for many CNS injuries and disorders (refer to section 1.9). The principle of the approach is based on the idea of restoring the function of the brain, lost and impaired due damage or disease, by replacing the diseased or dead neurons with new healthy generated neurons thorough transplantation. The effectiveness of this approach relies on several important factors (figure 60):

- The ability of stem cell used to generated functional neurons with similar characteristics and properties to the one targeted for replacement.
- The ability of the transplanted cells to survive and functionally integrate into the brain neuronal network.
- The development of efficient and safe transplantation procedures to deliver the cells to the targeted regions.
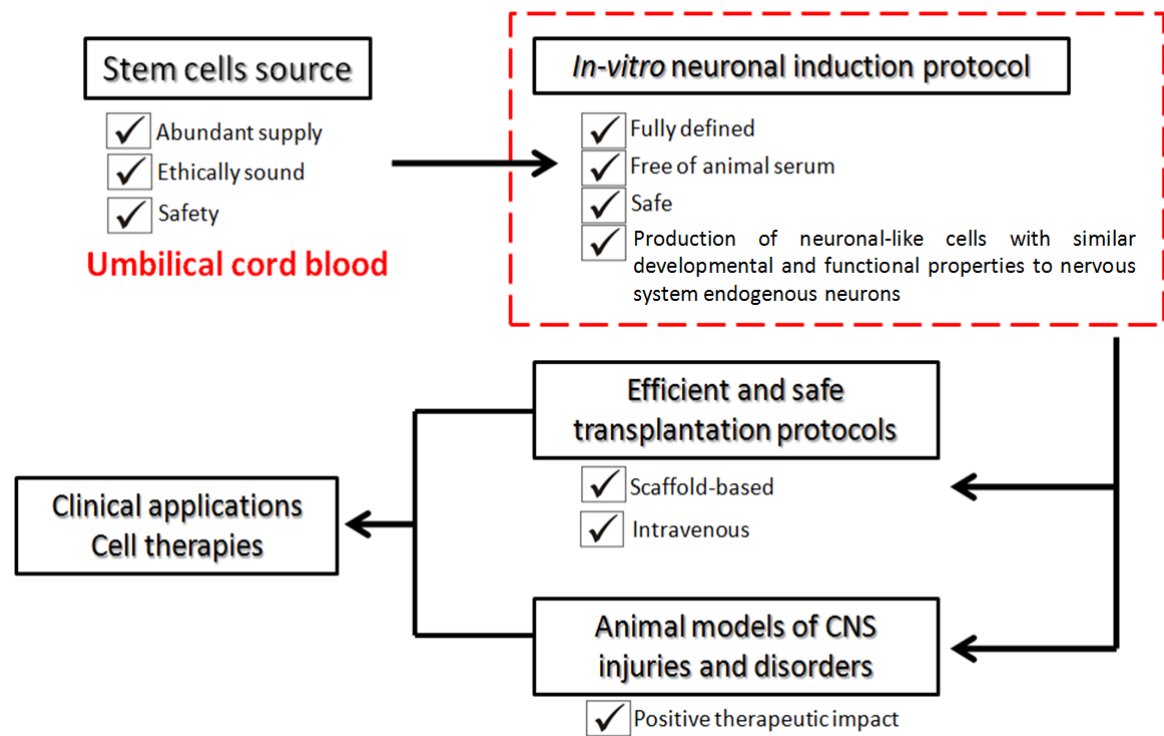
Figure 59. Umbilical cord blood and the pathway for CNS clinical applications. Effective clinical applications require an abundant and ethical supply of stem cells that are genetically stable (do not form teratoma). These stem cells must be accompanied with effective and safe *in-vitro* neuronal differentiation protocols (dotted red box highlights our protocol and results). Such protocols require efficient transplantation procedures to deliver the cells to the targeted regions. Extensive research must be conducted on animal models of CNS injuries and disorders to assess and evaluate the therapeutic impacts of such potential therapies before moving to clinical applications.

The studies outlined in this PhD thesis demonstrate that umbilical cord blood non-hematopoietic stem cells can produce neuron-like cells similar to CNS endogenous cortical neurons using a defined, short and safe differentiation *in-vitro* protocol (chapter 4, 5 and 6). Such results highlight the potential use of such cells as a therapeutic tool for many CNS injuries and disorders. Studies on rat's model of traumatic brain injury (TBI) have shown that intravenously transplanted cord blood stem cells were able to migrate to the brain and differentiated into neurons inducing motor and neurological improvements (Lu et al., 2002). Such results demonstrate that umbilical cord blood stem cells have both migratory and neuronal capacities which are important factors for any potential therapeutic application. Nevertheless, more extensive efforts should be aimed toward analyzing and

studying the cellular and functional integration of the transplanted cells into the brain networks which is crucial for long-term recovery and functional restoration. The possibility of producing functional glutamatergic neuron-like cells with similar characteristic to human endogenous cortical glutamatergic neurons, and possibly GABAergic neuron-like cells as well, from umbilical cord blood non-hematopoietic stem cells highlights it potential use for cell replacement therapies aimed to treat CNS injuries including stroke and TBI where the affected regions are likely to host glutamatergic and GABAergic neurons, the primary neurotransmission systems in the brain (Foster and Kemp, 2006).

One of the important issues that need to be taken into consideration when considering cell replacement therapies for CNS injuries and disorders is the cell dosage needed to replace the dead/diseased cells. We found that the umbilical cord blood non-hematopoietic stem cells make an average of 1.29±0.46% of total nucleated cells in umbilical cord blood sample. The average total nucleated cells per cord blood unit was estimated as 890×10^{6} cells (Reed et al., 2003). This means an average of 11.6 million non hematopoietic stem cells per cord blood unit. This low number of stem cells per cord blood unit represents a limitation when considering cell replacement therapies for neurodegenerative diseases such as AZ and PD which affect large portions of the brain. However this obstacle can be tackled with the possibility of combining multiple cord blood units in order to increase the final transplanted cell dose resulting in improved engraftment and survival of the transplanted cells (Brunstein and Wagner, 2006, Stanevsky et al., 2009, Ringden et al., 2008, Slatter et al., 2006). In addition, the development of safe and effective in-vitro strategies to expand umbilical cord blood non-hematopoietic stem cells before differentiation and transplantation may also contribute to the development of efficient therapies.

Another element that is crucial for any potential involvement in clinical applications is the safety of the proposed cell therapies. It is very important to ensure that the cells which will be used for transplantations are safe and do not pose any cancerous risk after transplantations into patients. Umbilical cord blood stem cells do not pose any risk of cancerous transformation unlike other types of stem cells such as fetal stem cells. In a recent clinical trial, a boy with ataxia telangiectasia (AT), a rare inherited

neurodegenerative disease, developed multifocal brain tumor after 4 years of treatment by human fetal neural stem cells injections. Cells from the tumor were non-host stem cells suggesting they were derived from the transplanted cells which was confirmed by microsatellite and HLA analysis (Amariglio et al., 2009). To date, there has not been a single reported case where cord blood stem cells formed teratoma when transplanted into SCID mice or humans. Many recent studies showed that human umbilical cord blood stem cells have positive impact on animal models of various neural injuries and diseases. In addition, umbilical cord blood stem cells have already reached the clinics in number of clinical trials aimed to treat neurological disorders including hypoxic-ischemic encephalopathy and cerebral palsy and showed promising results (refer to section 1.9.5). Such results further highlight the potential role of umbilical cord blood stem cells as a therapeutic tool aimed towards the development of safe treatments for CNS injuries and disorders for which current modes of therapy are ineffective.

7.3 Umbilical cord blood derived neuron-like cells: potential role in drug testing and toxicology studies

We have shown that umbilical cord blood non-hematopoietic multipotent stem cells can produce neuron-like cells similar to the CNS endogenous glutamatergic cortical neurons. Such *in-vitro* model offers a potential tool that can be used to test drugs and compounds targeting neural diseases and disorders, it can also be used for toxicology studies. Many drugs have been designed to target components of glutamate and GABA systems (such as neurotransmitter receptors and transporters) in attempts to develop treatments for a wide range of CNS disorders (Foster and Kemp, 2006). A number of drugs have already reached the markets including Namenda™ which is a drug that functions as NMDA antagonist helping in treating symptoms of Alzheimer's disease (AD) (Ferris, 2003). Others are still in clinical trials including SGS742, which is a drug that antagonizes GABA receptors as a potential treatment for AD symptoms (Froestl et al., 2004). More drugs are still in research and pre-clinical phases. The neuron-like cells we generated from umbilical cord blood purified cells expressed many molecular components targeted by potential drugs for a number of CNS diseases and disorders, such feature suggests possible involvement of such cells in studies aimed toward the development of drugs for various CNS disorders.

Our *in-vitro* model of cortical neurogenesis using umbilical cord blood non-hematopoietic multipotent stem cells would offer an additional tool allowing more extensive drug testing leading to a better understanding of the drug's molecular effects on the neurons before moving into clinical trials. This *in-vitro* model should not be regarded as a replacement for animal models in pre-clinical phases of drug development and testing, but it should be viewed as an extra tool which would allow more extensive molecular analysis and testing on human nervous tissues and cells resulting in more reliable information ahead of clinical trials.

7.4 Conclusion

Umbilical cord blood can be viewed as a promising source of stem cells for research and clinical applications. It is ethically sound, abundant supply, immunological immaturity and high plasticity made it superior to other sources of stem cells. Umbilical cord blood non-hematopoietic stem cells showed high potential for neuronal differentiation. We showed that these cells can produce neuron-like cells *in-vitro* with similar developmental and functional properties to endogenous cortical neurons. Pre-clinical trials using umbilical cord blood on animal models of neural injuries and disorders showed promising results. Clinical trials are still at its early stages but results obtained so far demonstrated high potential and hope toward developing effective therapies for various CNS injuries and disorders.

8. Future work

Investigating the molecular determinants of neuronal fate: glutamatergic versus GABAergic

Our Q-PCR results indicated significant up regulations in genes encoding markers normally associated with GABAergic neurotransmission. These genes included *GAD1* and GABA high affinity transporter (*SLC6A12*) (figure 51 and figure 52). These results suggest a possible existence of GABAergic neuron-like cells in addition to the glutamatergic neuron-like cells in cultures after neuronal maturation (Day 24). The co-existence of both types of neurons in the same culture which was exposed to the same culturing conditions over the 24 days duration suggests that the neuronal fate specification (glutamatergic versus GABAergic) was determined by molecular mechanisms independent from culturing conditions.

Previous studies have shown that the cortical fate specification (glutamatergic versus GABAergic) in the developing cortex is determined by certain molecules which decide the neuronal fate specification in the cortical progenitor's cells. Key molecules in this process are bHLH transcription factors neurogenin2 (NGN2) and MASH1 (Guillemot, 1999, Guillemot et al., 2006). According to the model of genetic programs controlling neuronal specification, NGN2 is thought to be involved in specifying the neuronal glutamatergic fate and MASH1 is involved in specifying the neuronal GABAergic fate (refer to section 5.5.4). These two transcription factors are involved in two cortical-specific differentiation programs involving a set of other specific factors that regulate the neuronal specification process (Schuurmans et al., 2004). We analyzed the glutamatergic fate commitment of our cells and showed that the *in-vitro* differentiation system offered a model for human glutamatergic neurogenesis. Similarly, GABAergic fate commitment can also be analyzed in cord blood cultures undergoing the same differentiation protocol. First, GABAergic neurons need to be further characterized using immunocytochemistry to localize GABAergic specific proteins (such as GAD1 and GABA transporters) to confirm their identity. Then we will analyze the expression patterns of markers including MASH1, DLX1 and DLX2 which define the GABAergic commitment process in the human cortex

(Guillemot et al., 2006, Cobos et al., 2007). It is also important to investigate the expression patterns of NGN2 and MASH1 using immunocytochemical co-staining in order to define the different progenitor populations and decide at which stage those mechanisms are involved.

Deeper analysis of those mechanisms and other molecular systems regulating developmental and functional processes in the cortex using neocortical tissues can be difficult due to the histological and cellular complexity of the neocortex (Gaspard et al., 2009). *In vitro* modeling of human corticogenesis would allow deeper analysis of molecular mechanisms regulating the specifications of neuronal subtypes in the cortex. Understanding these mechanisms will also aid the establishment of *in-vitro* culturing conditions specifying a certain neuronal fate which can be useful in the development of potential cell therapies targeting neural diseases or conditions that affect certain neuronal subtype.

Neuronal functional testing: patch clamping

We aim to confirm our Q-PCR results of ion channels involved in the generation and propagation of neuronal action potential using patch clamping, a laboratory technique that allows the direct selective recording of currents that flow through ion channels during action potential transmission (Purves, 2004, Sun et al., 2005). Such analysis offers a more direct approach to confirm the neuronal functional properties of the generated cells.

Investigating and characterizing the glial cellular content in cultures after neuronal maturation (Day 24)

Glial cells are an important component of the nervous system (refer to table 1 in appendix.I). These non-neuronal cells provide support for the neurons physically and functionally. For example, astrocytes are critically involved in formation of neuronal synapses and signal modulating in the nervous system. Our Q-PCR results indicated significant up-regulation in *SLC1A2* transcript which encodes which encodes excitatory amino acid transporter 2 (EAAT2) that is expressed mainly by CNS astrocytes (figure 52) (Chen and Swanson, 2003, Schmitt et al., 2002). This indicates possible existence of astocytic-like cells in the cultures. However, this result need to be further analyzed using immunocytochemistry using astrocytic specific markers such as S100β and EAAT2.

Additionally, we can also check for the existence of oligodendrocytes using specific markers such as myelin-oligodendrocyte glycoprotein (MOG) and Myelin Basic Protein (MBP). The ability of umbilical cord blood non-hematopoietic stem cells to generate glial cells in addition to neurons is important to insure proper neuronal functionality. Such feature can be very important when considering cell replacement therapies of CNS injuries and disorders such as stroke and TBI, where damaged cells include glial cells in addition to neurons.

Transplantation of neural tissues derived from umbilical cord blood non-hematopoietic cells into animal models of CNS injuries and disorders

Umbilical cord blood has already been utilized in a number of clinical applications aimed to treat hypoxic-ischemic encephalopathy and spastic cerebral palsy. In both trials, pediatric patients were transplanted with umbilical cord blood intravenously (refer to section 1.9.5). For other CNS injuries and disorders which affect certain regions of the brain, it might be more useful to transplant *in-vitro* pre-differentiated neural tissue directory into the lesion in order to stimulate faster recovery. It is really important to investigate the cellular physical and functional integration of the transplanted cells into the pre-existing brain networks. Successful integration and survival of the transplanted cells is necessary to stimulate functional recovery. As we showed, umbilical cord blood non-hematopoietic stem cells can be differentiated into neurons within scaffolds. Such differentiation system offers a useful and efficient way to transplant the differentiated neurons into the lesion sites with precision. Using animal models of CNS diseases and injuries for such studies is really important to evaluate the effectiveness of such procedures before moving into clinical trials.

9. Appendices

9.1 Appendix. I

Additional information

Table 18. cellular components of the nervous system

Cell type	Description
Astrocytes	Astrocytes are star-shaped glial cells residing in the brain and spinal cord. It has long been thought that glial cells including astrocytes have only a passive supporting role for neurons in the nervous system. However, following studies have shown that astrocytes are critically involved in the formation of synapses and neuronal signal modulation in the nervous system in addition to their physical and nutritive supportive role for neurons (Allen and Barres, 2009, Temburni and Jacob, 2001, Gross, 2006). Astrocyte processes form close associations with neurons at synaptic junctions. Like neurons, they express several neurotransmitter receptors including glutamate receptors. When neurons release glutamate at the synaptic junction in response to stimuli such as the action potential, astrocytic glutamate receptors get activated and Ca^{2+} influx triggers the release of glutamate from these astrocytes enhancing the synaptic transmission (Temburni and Jacob, 2001, Parpura and Haydon, 2000). In addition, astrocytes also remove and degrade excess neurotransmitters from the extracellular space which ensures precise and discrete synaptic transmission between neurons (Rothstein et al., 1996, Maciejewski and Rothman, 2008, Gross, 2006).
Oligodendrocytes and Schwann cells	These cells are mainly involved in insulation of neurons axons with a lipid-rich membrane called "myelin". This sheathing is crucial for rapid action potential conduction and propagation along neuron axons (Barkovich, 2000, Barnett and Larkman, 2007). In addition, the myelin sheath is important for proper formation of the axonal cytoskeleton (Brady et al., 1999) and it also provides the axons with a physical protective layer against potential injuries (Rodriguez, 2003). Myelination of neuron axons in the CNS is associated with oligodendrocytes whereas in the PNS it is associated with Schwann cells (Allen and Barres, 2009). The loss or damage of the myelin sheath around the neuron axons results in "demyelination" and this is associated with a number of neurological diseases such as multiple sclerosis, which emphasizes the essential role oligodendrocytes and Schwann cells have in nervous system (Mowry et al., 2009, Waxman, 1998).

Microglial cells	Microglial cells are the local immune cells of the nervous system. They are, perhaps, the only neural cells that are not derived from neural stem cells. Microglia are derived from monocytes which are in turn derived from hematopoietic stem cells in the bone marrow (Chan et al., 2007, Rock et al., 2004). They share morphological and functional similarities with the macrophages of hematopoietic system (Gehrmann et al., 1995, Rock et al., 2004). They form the first and main immune defensive line in the brain and spinal cord (Streit et al., 2004, Aloisi, 2001). They fight bacterial and viral infections in neural tissues via phagocytosis, and they also work as "housekeepers" removing dead cells and debris from neural tissues (Rock et al., 2004). Microglia become activated in several neurodegenerative diseases such as Alzheimer disease, but whether its activation is helpful or harmful in these cases is still a debatable issue (Rock et al., 2004, Allen and Barres, 2009).

9.2 Appendix. I I

Additional results
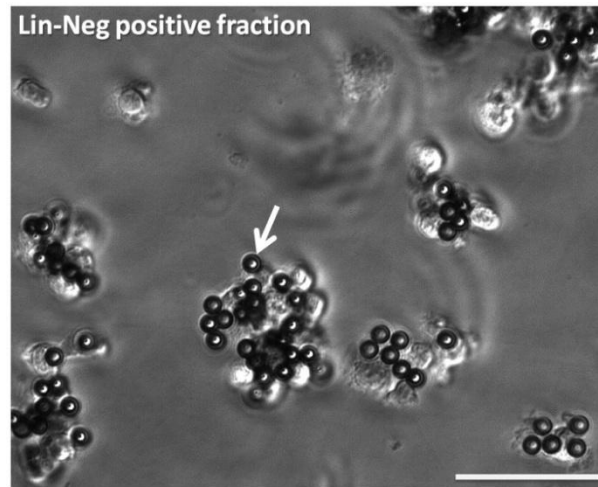
Figure 60. Positive cells fraction after Lin-Neg purification. The white arrow indicates the beads attached to cells after depletion. Scale bar 50 µm. (refer to section 2.3.2)
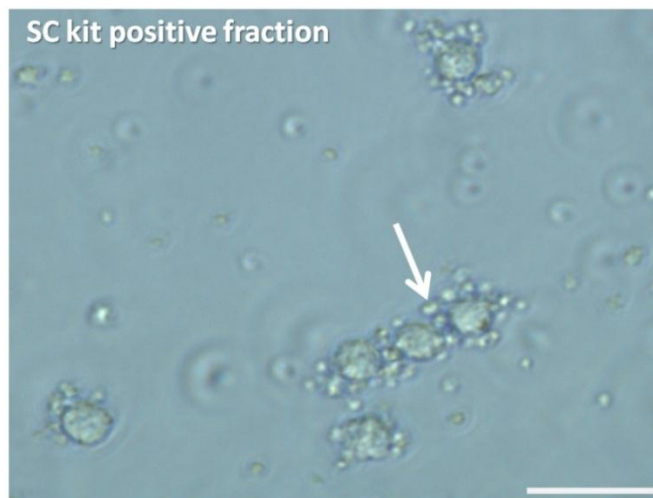
Figure 2. Positive cells fraction after SC kit purification. The white arrow indicates the beads attached to cells after depletion. Scale bar 50 µm. (refer to section 2.3.1)
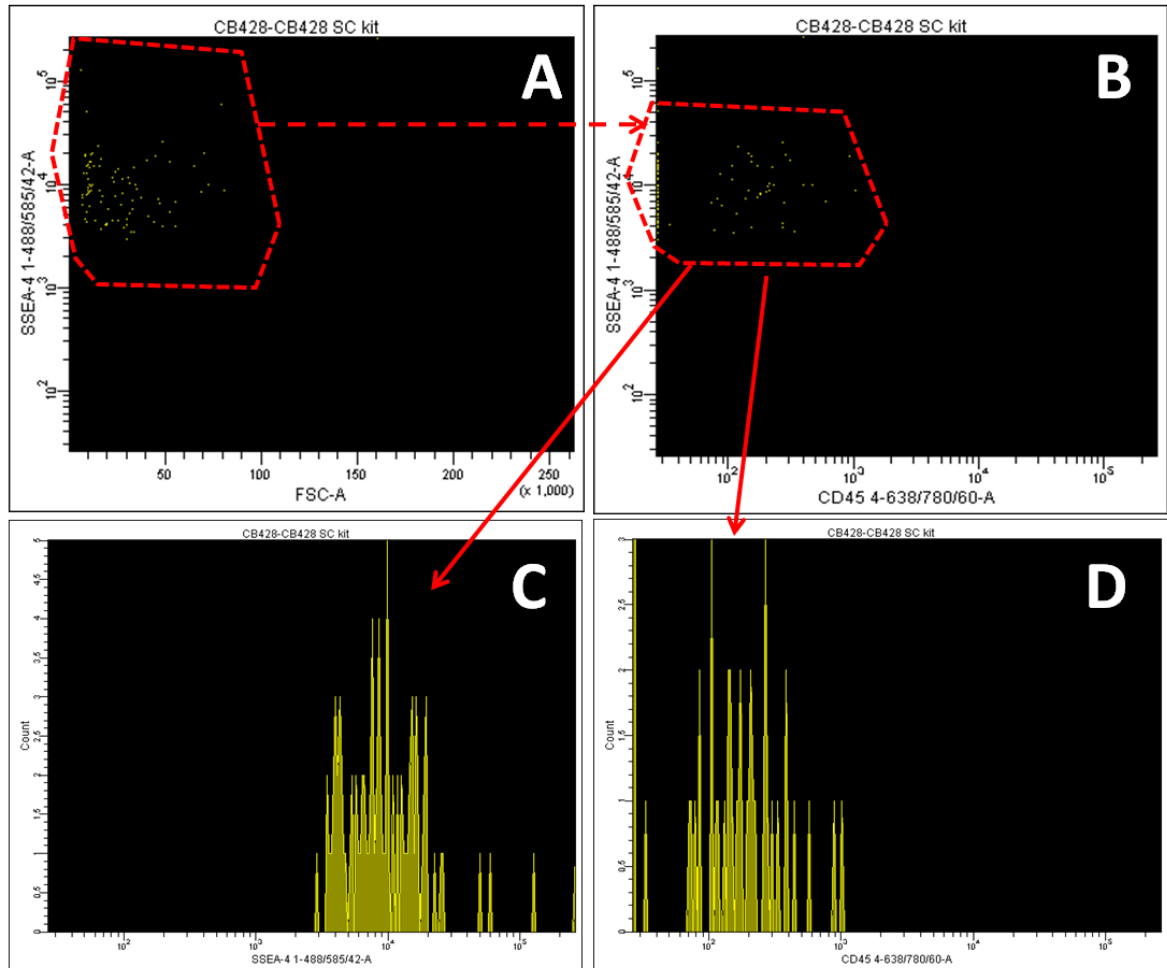
Figure 62. FACS analysis on SSEA-4 positive cells (yellow) showed that this subset of cells is negative for CD45. SSEA-4 positive cells were gated and then analyzed. Forward scattered (FSC-A) analysis showed that SSEA-4 positive cells are relatively small homogenous population of cells (A). The same population was negative for major hematopoietic marker CD45 (B) which is also demonstrated by histograms (C and D) where (C) indicates positivity for SSEA-4 and (D) negativity for CD45. Cells in this figure were purified using SC kit.
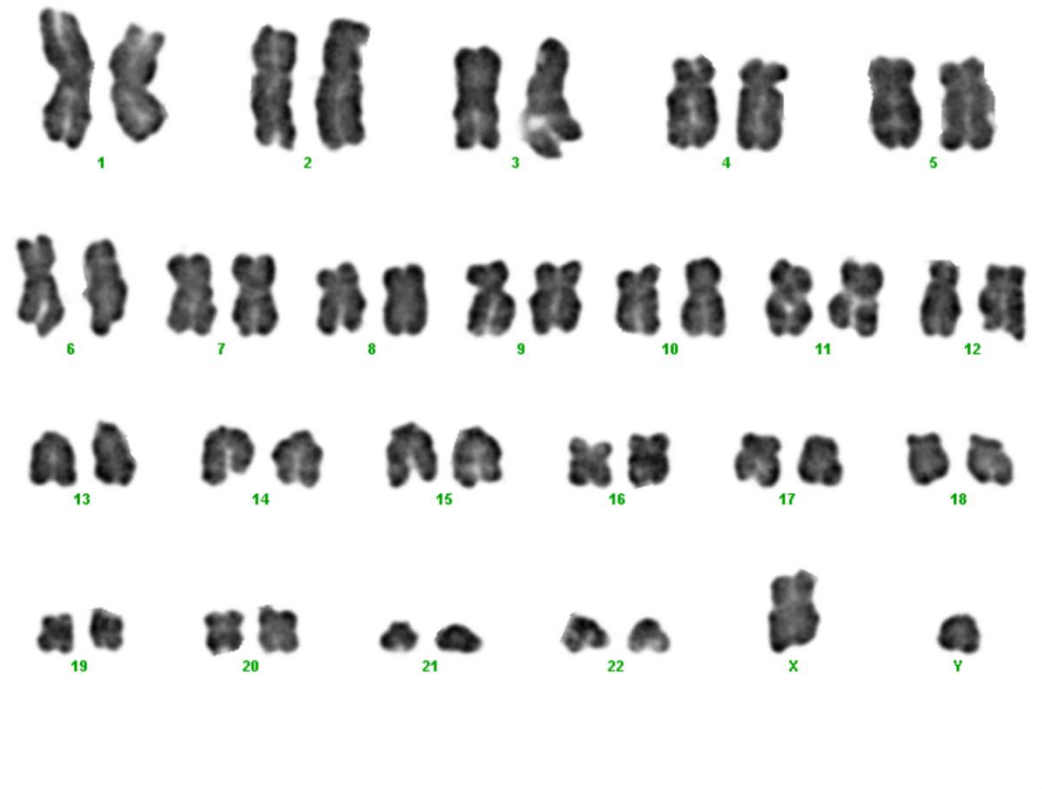
Figure 63. Cytogenetic analysis of cells after early neural commitment stage (Day 10). The results showed normal karyotype (n=3). (refer to section 2.7)
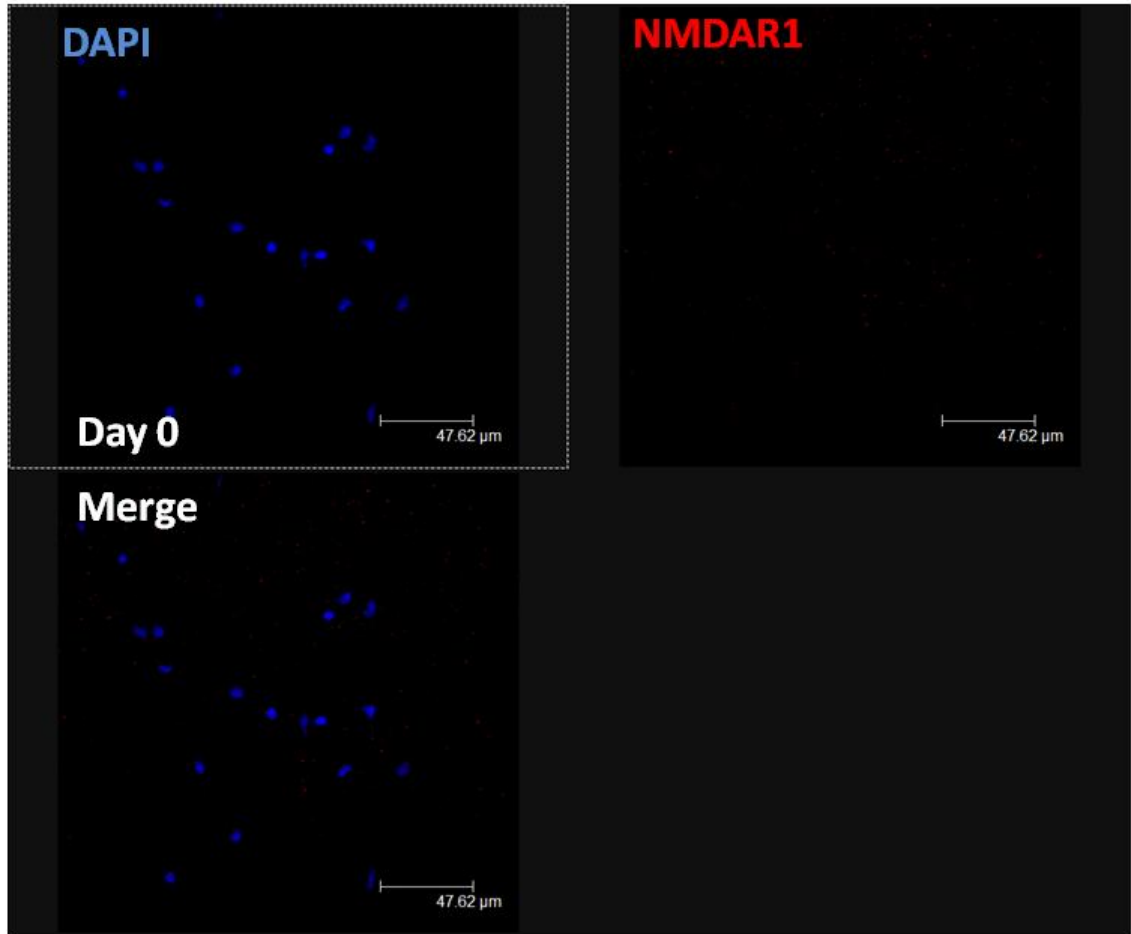
Figure 64. Freshly purified umbilical cord blood stem cells (Day 0) did not express NMDAR1.
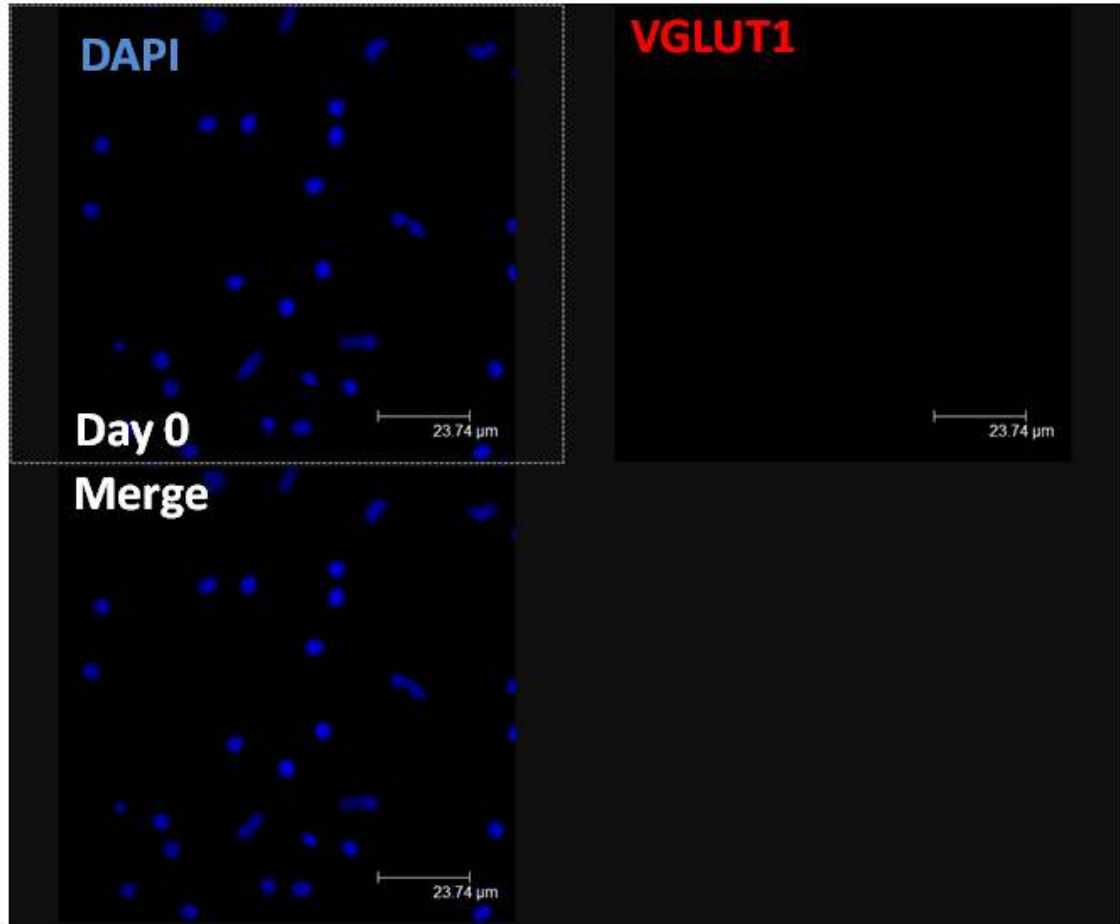
Figure 65. Freshly purified umbilical cord blood stem cells (Day 0) did not express VGLUT1.

9.3 Appendix. III

Addiotnal experimental data and controls
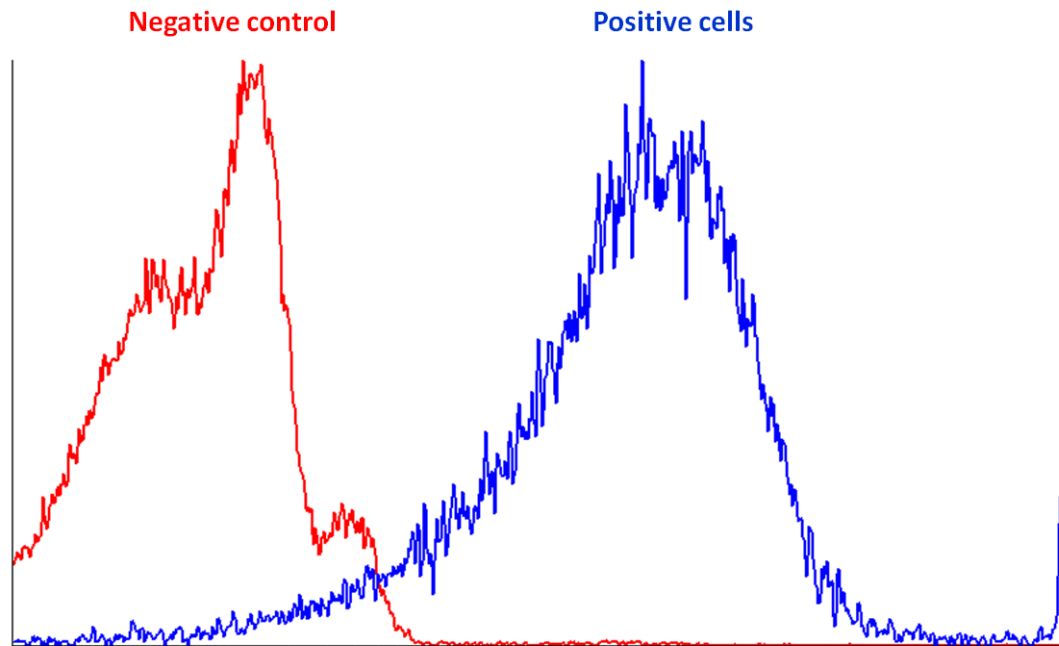
Figure 66. Negative control for FACS analysis. Unstained cells (without antibodies) have been used as negative control (red graph). In stained samples, signals located within the red graph are considered negative whilst signals detected outside the red histogram are considered positive (blue graph). This procedure has been performed for each marker used for FACS analysis. Example shown is for CD45.
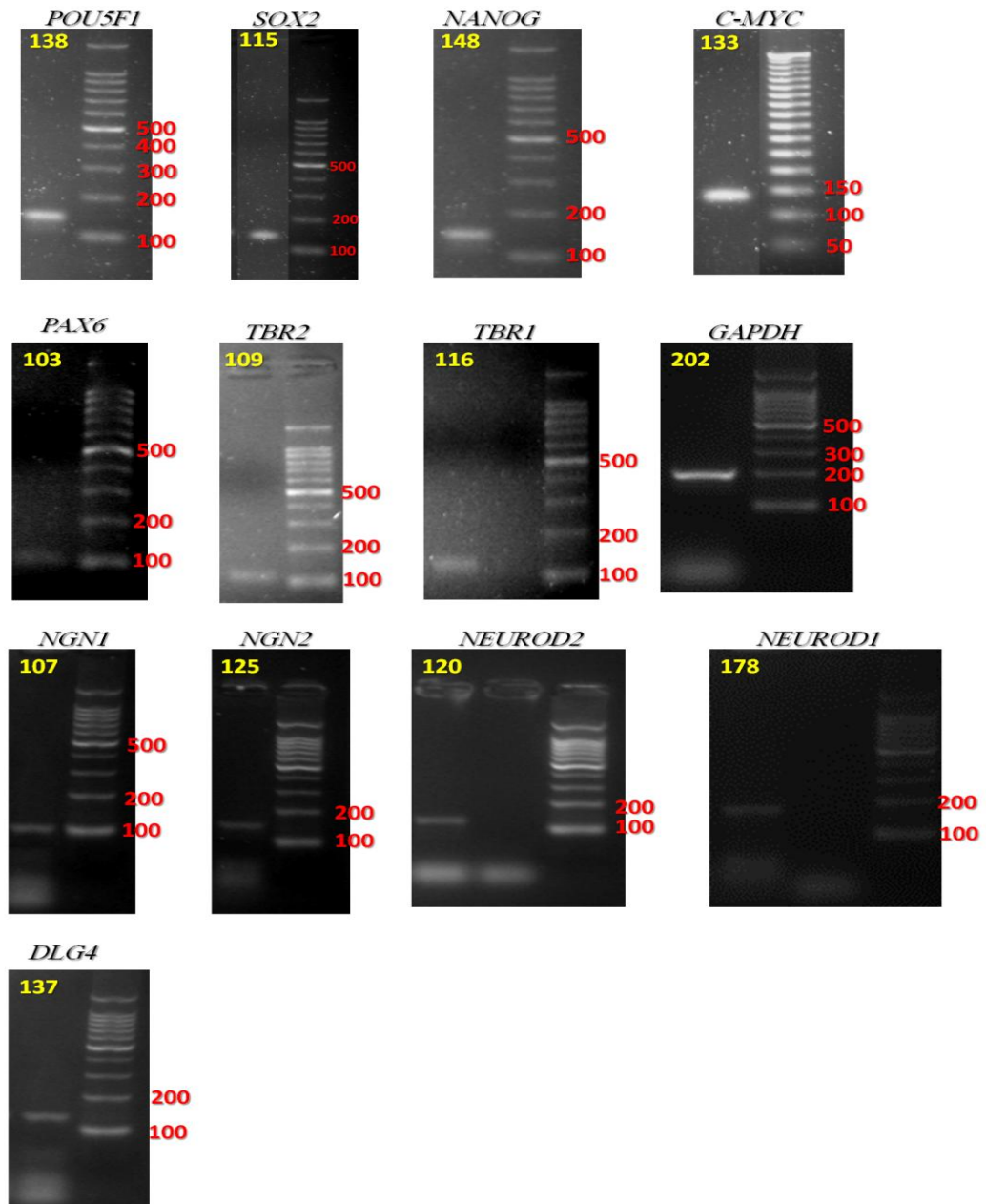
Figure 67. PCR product size confirmation. The product size is shown in yellow and the DNA ladder size in red. Refer to section (2.11.1)

Table 19. Primer Bank ID of primers obtained from primerbank website.
<http://pga.mgh.harvard.edu/primerbank/>

Gene	Primer Bank ID
C-MYC	12962935a2
NEUROG1	5453770a1
NEUROG2	31077092a3
NEUROD1	4505377a2
NEUROD2	21314638a1
PAX6	4505615a1
TBR2	22538470a2
TBR1	5730081a1
DLG4 (PSD95)	4557529a1
GAPDH	7669492a2
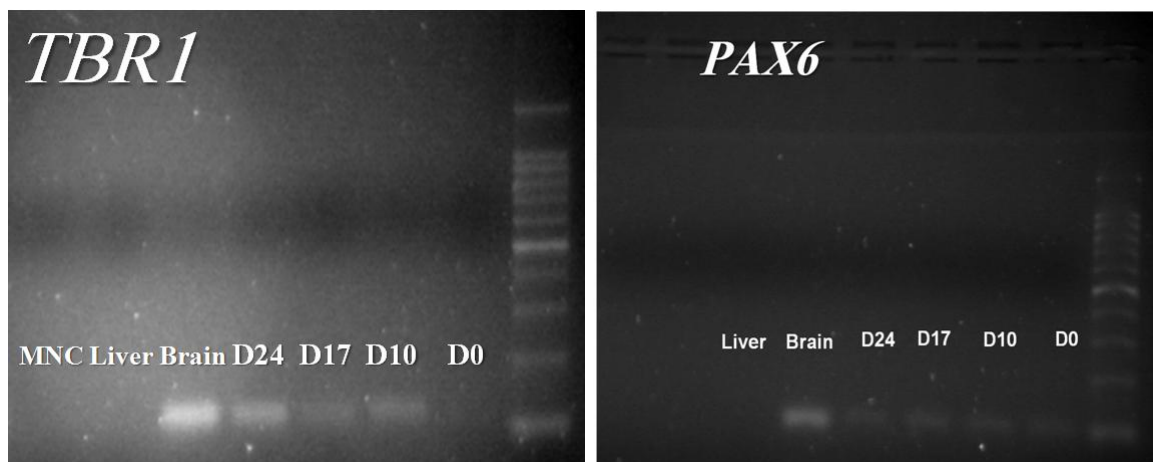
Figure 68. Primers tissue specificity check. *PAX6* and *TBR1* primers were tested on cDNA samples generated from umbilical cord blood purified cells samples from day 0 (D0), day 10 (D10), day 17 (D17) and day 24 (D24) "for *TBR1,* cDNA from mononuclear cell fraction was also used (MNC)". As a negative control, we used cDNA generated from adult liver tissue obtained from primer-design and results were negative for both genes. As a positive control, we used cDNA generated from fetal cortical tissue and results were positive for both genes. The obtained results from cord blood derived cells are consistent with *TBR1* and *PAX6* expressions in brain.
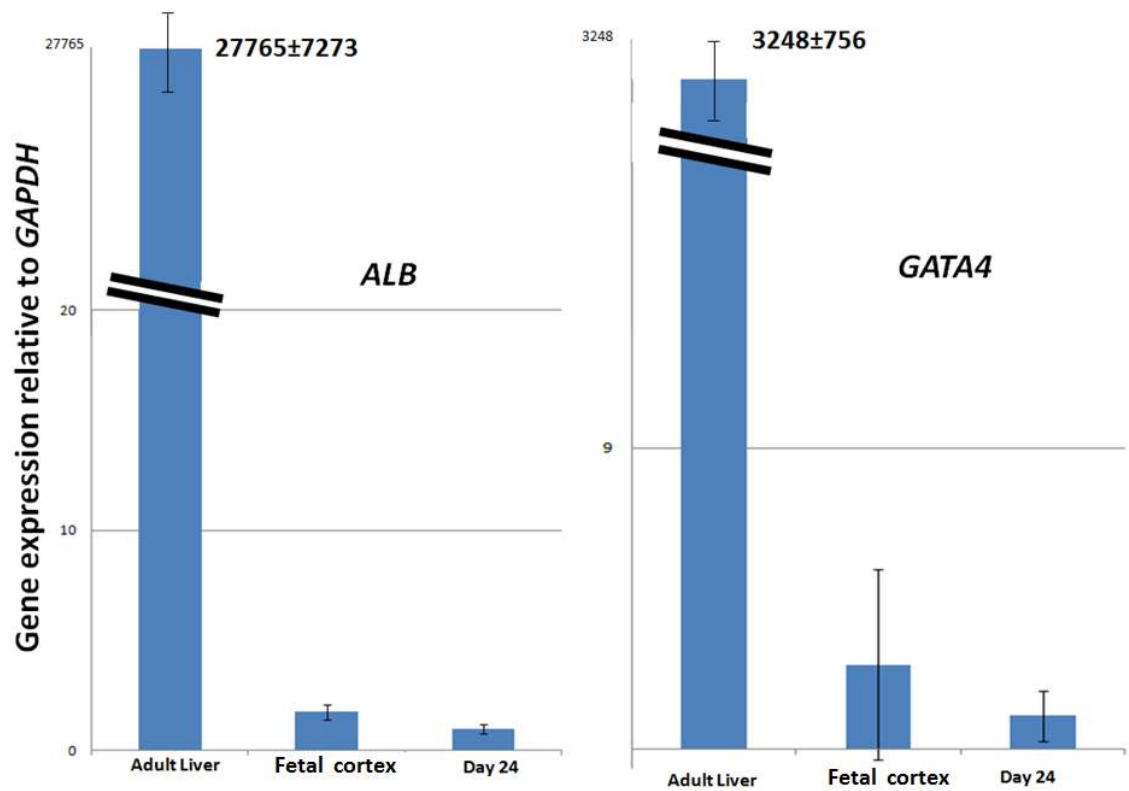
Figure 69. Q-PCR analysis of liver markers *ALB* and *GATA4*. Results show that *ALB* and *GATA4* are highly expressed in adult liver and extremely low in embryonic cortex and day 24 cells. This analysis has been performed as a negative control for the targeted neuronal fate.
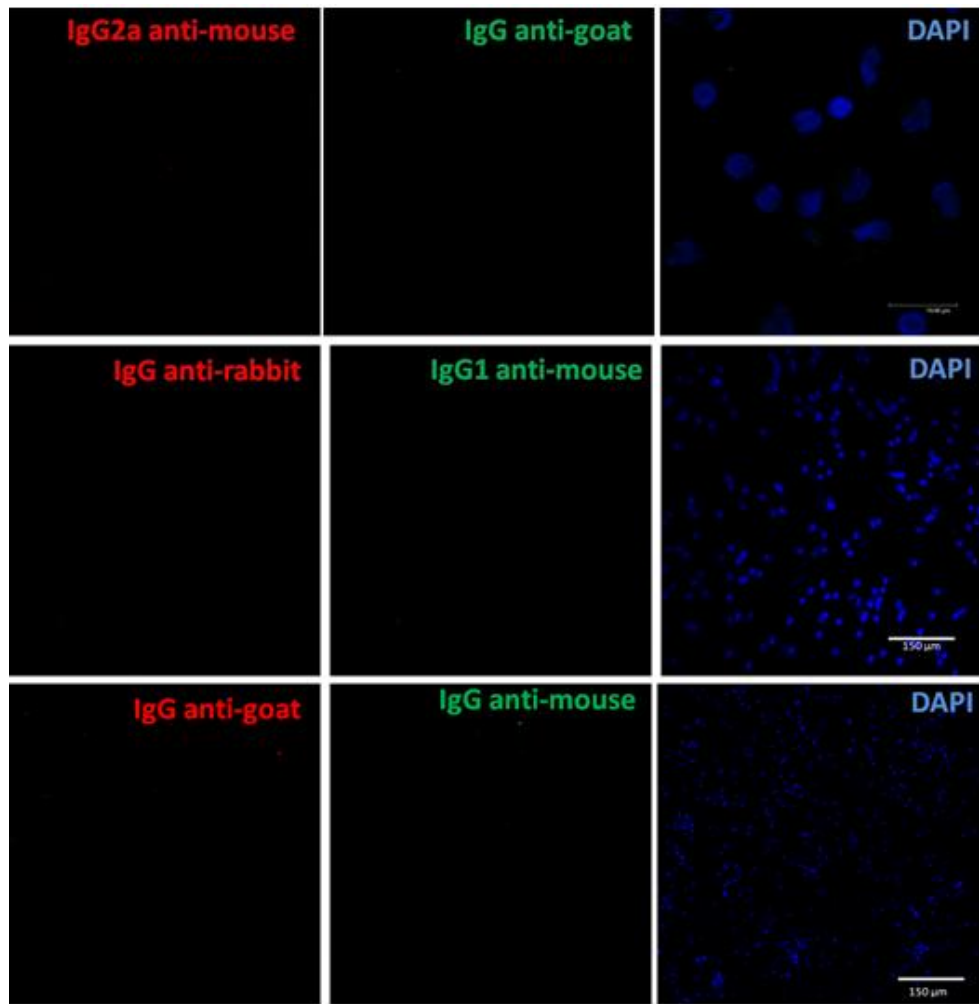
Figure 70. Negative control for immunocytochemistry. Only secondary antibodies were used along with DAPI. Refer to section 2.9.
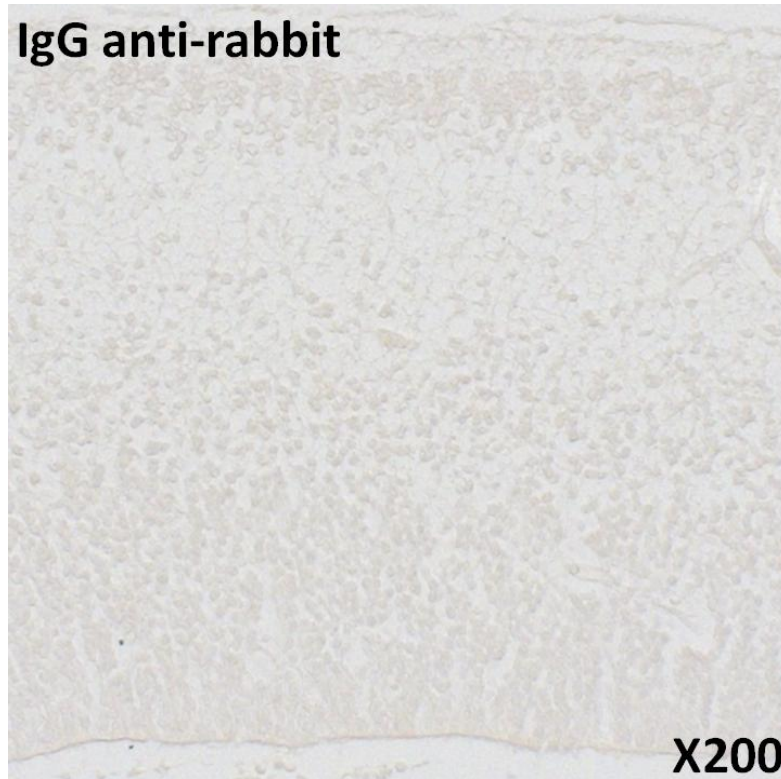
Figure 71. Negative control for immunohistochemistry. Only the secondary antibody was used. Refer to section 2.14.

9.4 Appendix.IV

Umbilical cord blood collection forms

- Participant Information Sheet

- Consent form

Participant Information Sheet

Evaluation of Cord Blood and Related Tissue Stem Cells
for processing, preservation and tissue engineering.

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully. Talk to others about the study if you wish.

- Part 1 tells you the purpose of this study and what will happen to you if you take part.

- Part 2 gives you more detailed information about the conduct of the study.

Ask us if there is anything that is not clear or if you would like more information. Please take time to decide whether or not you wish to take part.

_____

PART 1

What is the purpose of the study?

The purpose of the study is to learn more about the best ways to collect, process, and store cord blood and related tissue for transplantation and research.

This study will evaluate and develop products for the optimal processing of cord blood samples in order to improve the ways cord blood is separated into different blood cell groups and then stored in a freezer.

We will further investigate the potential of cord blood and related tissue stem cells for 'tissue engineering' whereby stem cells are grown in our laboratory and instructed by chemicals and materials to produce defined tissue structures such as blood or liver.

Basic information about Cord Blood and related tissues:

- Umbilical cord blood is the blood present in the umbilical cord which connects the baby to the placenta.

- Several research studies, including at the University of Newcastle, have shown that cord blood contains stem cells - the origin of all tissues and organs in the body – that can be turned into specific tissues in a laboratory offering high prospect for the advancement of medicine.

- Other related tissues including the cord, placenta and membranes also provide a source of stem cells

- Cord blood has been used clinically for over 20 years for the treatment of certain blood disorders (including leukaemia, lymphoma, and sickle cell disease) and immune deficiencies.

By donating your umbilical cord blood and related tissues to our research programme you will contribute the development of technologies and scientific discovery to better understand how umbilical cord blood and related tissue stem cells can help repair the human body.

Why have I been chosen?

You have been chosen because you are in the second or third trimester of your pregnancy. You may therefore wish to participate in this study by donating your cord blood and related tissues at the time of birth.

Do I have to take part?

No. It is up to you to decide whether or not to take part. **Cord blood and related tissue donation for this research is entirely voluntary**. If you decide to take part, you will be given this information sheet to keep and be asked to sign a consent form. You will be free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you will receive or the delivery of your baby.

What will happen to me if I take part?

 ˏ If you decide to take part in this study, you will be asked:

 -to read this patient information sheet carefully and ask us any question you might have with regard to this study.

 -to sign a consent form for cord blood and related tissue donation.

 ˏ Cord blood collection will occur **after birth with no harm to your baby or yourself**. The umbilical cord will be clamped when it is of no further use to your

 baby. Any samples will be taken after delivery of the placenta and cord and in a separate room by a member of staff. Blood will be drawn from the umbilical cord and placental vein. Samples of related tissues (such as cord, placenta and membranes) may sometimes be taken at this time.

 ˏ Donated cord blood and related tissue will be anonymised before it is used for the research study at the University of Newcastle. Laboratory researchers will not have access to your

 name, clinical records or any confidential information about your baby or yourself.

 ˏ As part of your routine clinical care you would have been or will be tested for sexually transmitted diseases. You may donate cord blood and related tissue only if you have been tested for sexually transmitted diseases. If any evidence of infection is found, you will receive advice and support from the NHS carers. Please ask a member of staff for more information and advice if you have any question on this matter.

 ˏ There is no cost to you for participating in this study. You will not receive payment for your participation in this study.

What do I have to do?

If you decide to take part in this research study by donating your cord blood and related tissue please:

- Read this Participant Information Form carefully.
- Complete and sign the consent form and return to:

Dr Majlinda Lako/Dr Lyle Armstrong, Newcastle Centre for Cord Blood, Institute of Human Genetics, International Centre for Life, Central Parkway, Newcastle Upon Tyne, NE1 3BZ

Phone: 0191 241 8817

Or to the Research Nurse during your next clinic visit..

What is the device / procedure that is being tested?

- In our laboratory at the University of Newcastle, we will investigate different products and methods to collect, to process and to store cord blood and related tissue for research and clinical applications.

- We will further develop techniques and methods to grow and transform cord blood and related tissue stem cells into different tissues including blood, brain and liver tissue.

What are the alternatives?

- The placenta, umbilical cord, and cord blood will be discarded as per hospital policy.

- You may also choose to arrange prior to your admission to the hospital, with a private company who will charge a fee to process and store your baby's cord blood. Please note that this is not currently facilitated by the NHS.

What are the side effects of any treatment received when taking part?

- No known side effects or discomforts are anticipated when taking part in this study.

- Cord blood and related tissues will be collected after birth with no harm to you or the baby. Your care and the delivery of your baby will not be altered if you decide to take part, or not, in this research study.

What are the other possible disadvantages and risks of taking part?

- You and your family will not be able to retrieve or access the cord blood, related tissue or derived-cells for personal or clinical use at a later stage.

What are the possible benefits of taking part?

> There are no direct benefits to you or your baby other than that the knowledge gained from this study may assist researchers in the discovery of improved methods for the collection, processing, and storage of cord blood and related tissue for transplant and research.

> The study may also lead to new ways of using umbilical cord blood and related tissue stem cells to detect or treat a wide variety of diseases in the future.

What happens when the research study stops?

> Donated cord blood and related tissue not used will be discarded at the end of the study.

> The information from this study will be collated with a view to understanding Cord Blood and related tissue better.

What if there is a problem?

> Any complaint about the way you have been dealt with during the study or any possible harm you might suffer will be addressed. The detailed information on this is given in Part 2.

Will my taking part in the study be kept confidential?

> Yes. All the information about your participation in this study will be kept confidential. The details are included in Part 2. However, if you wish to discuss your participation in the study with others you are free to do so.

Contact Details:

For further information please contact, Dr Majlinda Lako or Dr Lyle Armstrong

Newcastle Centre for Cord Blood, Institute for Human Genetics, International Centre for Life, Central Parkway, Newcastle Upon Tyne, Ne1 3BZ

Phone: 0191 241 8817 E-mail: cord.blood@newcastle.ac.uk

This completes Part 1 of the Information Sheet.

If the information in Part 1 has interested you and you are considering participation, please continue to read the additional information in Part 2 before making any decision.

What if relevant new information becomes available?

If new information about this study becomes available prior to your consent or donation, you will be approached by a member of staff with an up-to-date participant information sheet.

What will happen if I don't want to carry on with the study?

If you withdraw from the study we will not collect your cord blood or any other related tissues. You may then withdraw from the study until your sample has been anonymised. We will then dispose of your identifiable sample as per hospital policy. You will not have to give any reason for withdrawing from the study and it will in no manner affect your care or the delivery of your baby.

What if there is a problem?

If you have a concern about any aspect of this study, you should ask to speak with Dr Majlinda Lako or Dr Lyle Armstrong.

If you remain unhappy and wish to complain formally, you can do this through the NHS Complaints Procedure. Details can be obtained from the hospital.

NHS Indemnity does not offer no-fault compensation i.e. for non-negligent harm, and NHS bodies are unable to agree in advance to pay compensation for non-negligent harm. They are able to consider an ex-gratia payment in the case of a claim where the injury probably resulted from any test or procedure you received directly as part of this study. Any payment would be without legal commitment. (Please ask if you wish more information on this).

In the event that something does go wrong and you are harmed during the research study there are no special compensation arrangements. If you are harmed and this is due to someone's negligence then you may have grounds for a legal action for compensation against the Newcastle Hospitals NHS Trust. The normal National Health Service complaints mechanisms will still be available to you (if appropriate).

Will my taking part in this study be kept confidential?

Cord blood and related tissue will be anonymised before being used by laboratory research staff that will not have access to your name, your baby's name or your medical records.

Anonymised data regarding your delivery date, number of weeks in your pregnancy, the sex of your child, your age at delivery may be stored and used for statistical comparison only.

Any information about you which leaves the hospital/surgery will have your name and address removed so that you cannot be recognised from it.

Authorised University researchers, sponsors and regulatory authorities may have access to these data as granted by the Chief Investigator, Dr. Lako. Data will be stored for a maximum of 10 years on a university PC and secured server.

However, any information which is collected about you or your baby during the course of the research will be kept strictly confidential and be anonymised so that you and your baby cannot be recognised from it.

Handling, processing, storage and destruction of your data are compliant with the Data Protection Act 1998.

What will happen to any samples I give?

Cord blood will be drawn from the umbilical cord vein after your baby is born with no harm to you or your baby. Samples of placenta, cord or membranes may also be taken. Remaining tissues will be disposed of as per usual hospital policy. Your sample will be anonymised by a trained member of staff. Your cord blood and related tissue sample will then be dispatched to the Newcastle Centre for Cord Blood research staff.

Your donated cord blood and related tissue samples will be considered as a 'Gift' to the University of Newcastle in accordance with the Medical Research Council proposition.

Donated Cord Blood and related tissue will be used to evaluate techniques and commercial products to collect, process, and store cord blood for transplantation and research.

Cord blood and related tissue cells may also be tested for 'tissue engineering' (that is exposing cord blood cells to a range of chemicals and materials to direct the way they grow) applications and product development growing cells into specific tissue types for instance blood, liver or neural tissues.

Will any genetic tests be done?

Anonymised donated cord blood and related tissue cells may be tested for gene expression levels as required by the study, in order to monitor cells response to different growth conditions.

What will happen to the results of the research study?

The results and cells derived from the study may be used for academic scientific publications, commercial product development or validation, clinical application development and / or regulatory authorities' evaluation.

Results and progress of the study will be communicated through peer-reviewed scientific journals, international and regional conferences and also be disseminated to the general public with local seminars, media and the research group website (www.ncl.ac.uk/cordblood

or www.iscbrm.org). Because your cord blood and related tissue sample will be anonymised, you will not be identified in any report, publication or communication.

Who is organising and funding the research?

Our current research is organised by the University of Newcastle and funded by the University of Newcastle, OneNorth East, BioE Inc, StemCyte Inc and the Fondation Jerome Lejeune. This list may change during the study.

Who has reviewed the study?

This study was given a favourable ethical opinion for conduct in the NHS by the Newcastle and North Tyneside Local Research Ethics Committee.

You will be given a copy of the information sheet and a signed consent form for you to keep.

We thank you for considering taking part or taking time to read this sheet

	Newcastle Centre for Cord Blood	
Newcastle University	Institute of Human Genetics	**Life**
	International Centre for Life	
	Central Parkway	
North East England Stem Cell Institute	NE1 3BZ, United Kingdom Phone : 0191 241 8817 e-mail: cord.blood@newcastle.ac.uk	International Centre for Life

Consent Form

Patient Identification Number for this trial:

Title of Project: Evaluation of Cord Blood and Related Tissue Stem Cells for processing, preservation and tissue engineering.

Name of Researcher:

1. I confirm that I have read and understand the information sheet dated 16/01/2009 (version 9) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving reason, without my medical care or legal rights being affected. ☐
3. I understand that relevant sections of my medical notes and data collected during the study may be looked at by responsible individuals from the University of Newcastle, from regulatory authorities or from the NHS trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records.
4. I agree to take part in this study. ☐

_____   _____

Name of Participant   Date   Signature

_____   _____

Name of Person taking consent   Date   Signature

_____   _____

Researcher   Date   Signature

When completed and signed please retain one copy (1 for participant) and return the other copy (1 for researcher file) to: Dr. Majlinda Lako/Dr Lyle Armstrong Newcastle Cord Blood Centre, Institute of Human Genetics, International Centre for Life, Central Parkway, NE1 3BZ, Newcastle Upon Tyne

Phone: 0191 241 8817 Email: cord.blood@newcastle.ac.uk

Or return the signed consent form to the Research Nurse at your next clinic visit.

10. References

ABRANCHES, E., SILVA, M., PRADIER, L., SCHULZ, H., HUMMEL, O., HENRIQUE, D. & BEKMAN, E. 2009. Neural differentiation of embryonic stem cells in vitro: a road map to neurogenesis in the embryo. *PLoS One,* 4**,** e6286.

ABROUS, D. N., KOEHL, M. & LE MOAL, M. 2005. Adult neurogenesis: from precursors to network and physiology. *Physiol Rev,* 85**,** 523-69.

ADERINWALE, O. G., ERNST, H. W. & MOUSA, S. A. 2010. Current therapies and new strategies for the management of Alzheimer's disease. *Am J Alzheimers Dis Other Demen,* 25**,** 414-24.

ADEWUMI, O., AFLATOONIAN, B., AHRLUND-RICHTER, L., AMIT, M., ANDREWS, P. W., BEIGHTON, G., BELLO, P. A., BENVENISTY, N., BERRY, L. S., BEVAN, S., BLUM, B., BROOKING, J., CHEN, K. G., CHOO, A. B., CHURCHILL, G. A., CORBEL, M., DAMJANOV, I., DRAPER, J. S., DVORAK, P., EMANUELSSON, K., FLECK, R. A., FORD, A., GERTOW, K., GERTSENSTEIN, M., GOKHALE, P. J., HAMILTON, R. S., HAMPL, A., HEALY, L. E., HOVATTA, O., HYLLNER, J., IMREH, M. P., ITSKOVITZ-ELDOR, J., JACKSON, J., JOHNSON, J. L., JONES, M., KEE, K., KING, B. L., KNOWLES, B. B., LAKO, M., LEBRIN, F., MALLON, B. S., MANNING, D., MAYSHAR, Y., MCKAY, R. D., MICHALSKA, A. E., MIKKOLA, M., MILEIKOVSKY, M., MINGER, S. L., MOORE, H. D., MUMMERY, C. L., NAGY, A., NAKATSUJI, N., O'BRIEN, C. M., OH, S. K., OLSSON, C., OTONKOSKI, T., PARK, K. Y., PASSIER, R., PATEL, H., PATEL, M., PEDERSEN, R., PERA, M. F., PIEKARCZYK, M. S., PERA, R. A., REUBINOFF, B. E., ROBINS, A. J., ROSSANT, J., RUGG-GUNN, P., SCHULZ, T. C., SEMB, H., SHERRER, E. S., SIEMEN, H., STACEY, G. N., STOJKOVIC, M., SUEMORI, H., SZATKIEWICZ, J., TURETSKY, T., TUURI, T., VAN DEN BRINK, S., VINTERSTEN, K., VUORISTO, S., WARD, D., WEAVER, T. A., YOUNG, L. A. & ZHANG, W. 2007. Characterization of human embryonic stem cell lines by the International Stem Cell Initiative. *Nat Biotechnol,* 25**,** 803-16.

AHMED, S., REYNOLDS, B. A. & WEISS, S. 1995. BDNF enhances the differentiation but not the survival of CNS stem cell-derived neuronal precursors. *J Neurosci,* 15**,** 5765-78.

AHN, S. & JOYNER, A. L. 2005. In vivo analysis of quiescent adult neural stem cells responding to Sonic hedgehog. *Nature,* 437**,** 894-7.

ALI, H., JURGA, M., KURGONAITE, K., FORRAZ, N. & MCGUCKIN, C. 2009. Defined serum-free culturing conditions for neural tissue engineering of human cord blood stem cells. *Acta Neurobiol Exp (Wars),* 69**,** 12-23.

ALI, S. A., PAPPAS, I. S. & PARNAVELAS, J. G. 1998. Collagen type IV promotes the differentiation of neuronal progenitors and inhibits astroglial differentiation in cortical cell cultures. *Brain Res Dev Brain Res,* 110**,** 31-8.

ALKONDON, M. & ALBUQUERQUE, E. X. 2004. The nicotinic acetylcholine receptor subtypes and their function in the hippocampus and cerebral cortex. *Prog Brain Res,* 145**,** 109-20.

ALLEN, N. J. & BARRES, B. A. 2009. Neuroscience: Glia - more than just brain glue. *Nature,* 457**,** 675-7.

ALOISI, F. 2001. Immune function of microglia. *Glia,* 36**,** 165-79.

ALTMAN, J. 1969. Autoradiographic and histological studies of postnatal neurogenesis. 3. Dating the time of production and onset of differentiation of cerebellar microneurons in rats. *J Comp Neurol,* 136**,** 269-93.

ALTMAN, J. & DAS, G. D. 1965. Autoradiographic and histological evidence of postnatal hippocampal neurogenesis in rats. *J Comp Neurol,* 124**,** 319-35.

ALVAREZ-BUYLLA, A. & GARCIA-VERDUGO, J. M. 2002. Neurogenesis in adult subventricular zone. *J Neurosci,* 22**,** 629-34.

AMARIGLIO, N., HIRSHBERG, A., SCHEITHAUER, B. W., COHEN, Y., LOEWENTHAL, R., TRAKHTENBROT, L., PAZ, N., KOREN-MICHOWITZ, M., WALDMAN, D., LEIDER-TREJO, L., TOREN, A., CONSTANTINI, S. & RECHAVI, G. 2009. Donor-derived brain tumor following neural stem cell transplantation in an ataxia telangiectasia patient. *PLoS Med,* 6**,** e1000029.

ANDREOU, D., SAETRE, P., WERGE, T., ANDREASSEN, O. A., AGARTZ, I., SEDVALL, G. C., HALL, H., TERENIUS, L. & JONSSON, E. G. 2010. Tryptophan hydroxylase gene 1 (TPH1) variants associated with cerebrospinal fluid 5-hydroxyindole acetic acid and homovanillic acid concentrations in healthy volunteers. *Psychiatry Res,* 180**,** 63-7.

ANGENIEUX, B., SCHORDERET, D. F. & ARSENIJEVIC, Y. 2006. Epidermal growth factor is a neuronal differentiation factor for retinal stem cells in vitro. *Stem Cells,* 24**,** 696-706.

ANGEVINE, J. B., JR. & SIDMAN, R. L. 1961. Autoradiographic study of cell migration during histogenesis of cerebral cortex in the mouse. *Nature,* 192**,** 766-8.

ANGLISTER, L., ETLIN, A., FINKEL, E., DURRANT, A. R. & LEV-TOV, A. 2008. Cholinesterases in development and disease. *Chem Biol Interact,* 175**,** 92-100.

ARENDT, C. W. & OSTERGAARD, H. L. 1997. Identification of the CD45-associated 116-kDa and 80-kDa proteins as the alpha- and beta-subunits of alpha-glucosidase II. *J Biol Chem,* 272**,** 13117-25.

ARNTH-JENSEN, N., JABAUDON, D. & SCANZIANI, M. 2002. Cooperation between independent hippocampal synapses is controlled by glutamate uptake. *Nat Neurosci,* 5**,** 325-31.

ARVIDSSON, A., COLLIN, T., KIRIK, D., KOKAIA, Z. & LINDVALL, O. 2002. Neuronal replacement from endogenous precursors in the adult brain after stroke. *Nat Med,* 8**,** 963-70.

ASCOLI, G. A., ALONSO-NANCLARES, L., ANDERSON, S. A., BARRIONUEVO, G., BENAVIDES-PICCIONE, R., BURKHALTER, A., BUZSAKI, G., CAULI, B., DEFELIPE, J., FAIREN, A., FELDMEYER, D., FISHELL, G., FREGNAC, Y., FREUND, T. F., GARDNER, D., GARDNER, E. P., GOLDBERG, J. H., HELMSTAEDTER, M., HESTRIN, S., KARUBE, F., KISVARDAY, Z. F., LAMBOLEZ, B., LEWIS, D. A., MARIN, O., MARKRAM, H., MUNOZ, A., PACKER, A., PETERSEN, C. C., ROCKLAND, K. S., ROSSIER, J., RUDY, B., SOMOGYI, P., STAIGER, J. F., TAMAS, G., THOMSON, A. M., TOLEDO-RODRIGUEZ, M., WANG, Y., WEST, D. C. & YUSTE, R. 2008. Petilla terminology: nomenclature of features of GABAergic interneurons of the cerebral cortex. *Nat Rev Neurosci,* 9**,** 557-68.

ASKWITH, C. C., WEMMIE, J. A., PRICE, M. P., ROKHLINA, T. & WELSH, M. J. 2004. Acid-sensing ion channel 2 (ASIC2) modulates ASIC1 H+-activated currents in hippocampal neurons. *J Biol Chem,* 279**,** 18296-305.

AU, E. & FISHELL, G. 2008. Cortex shatters the glass ceiling. *Cell Stem Cell,* 3**,** 472-4.

AZEVEDO, F. A., CARVALHO, L. R., GRINBERG, L. T., FARFEL, J. M., FERRETTI, R. E., LEITE, R. E., JACOB FILHO, W., LENT, R. & HERCULANO-HOUZEL, S. 2009. Equal numbers of neuronal and nonneuronal cells make the human brain an isometrically scaled-up primate brain. *J Comp Neurol,* 513**,** 532-41.

BAALA, L., BRIAULT, S., ETCHEVERS, H. C., LAUMONNIER, F., NATIQ, A., AMIEL, J., BODDAERT, N., PICARD, C., SBITI, A., ASERMOUH, A., ATTIE-BITACH, T., ENCHA-RAZAVI, F., MUNNICH, A., SEFIANI, A. & LYONNET, S. 2007. Homozygous silencing of T-box transcription factor EOMES leads to microcephaly with polymicrogyria and corpus callosum agenesis. *Nat Genet,* 39**,** 454-6.

BALDWIN, T. A. & OSTERGAARD, H. L. 2001. Developmentally regulated changes in glucosidase II association with, and carbohydrate content of, the protein tyrosine phosphatase CD45. *J Immunol,* 167**,** 3829-35.

BALLEN, K. K., BARKER, J. N., STEWART, S. K., GREENE, M. F. & LANE, T. A. 2008. Collection and preservation of cord blood for personal use. *Biol Blood Marrow Transplant,* 14**,** 356-63.

BALTUS, G. A., KOWALSKI, M. P., ZHAI, H., TUTTER, A. V., QUINN, D., WALL, D. & KADAM, S. 2009. Acetylation of sox2 induces its nuclear export in embryonic stem cells. *Stem Cells,* 27**,** 2175-84.

BARBERI, T., KLIVENYI, P., CALINGASAN, N. Y., LEE, H., KAWAMATA, H., LOONAM, K., PERRIER, A. L., BRUSES, J., RUBIO, M. E., TOPF, N., TABAR, V., HARRISON, N. L., BEAL, M. F., MOORE, M. A. & STUDER, L. 2003. Neural subtype specification of fertilization and nuclear transfer embryonic stem cells and application in parkinsonian mice. *Nat Biotechnol,* 21**,** 1200-7.

BARDOR, M., NGUYEN, D. H., DIAZ, S. & VARKI, A. 2005. Mechanism of uptake and incorporation of the non-human sialic acid N-glycolylneuraminic acid into human cells. *J Biol Chem,* 280**,** 4228-37.

BARKOVICH, A. J. 2000. Concepts of myelin and myelination in neuroradiology. *AJNR Am J Neuroradiol,* 21**,** 1099-109.

BARNETT, M. W. & LARKMAN, P. M. 2007. The action potential. *Pract Neurol,* 7**,** 192-7.

BARTEL, D. P. 2004. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell,* 116**,** 281-97.

BARTLETT, P. F., BROOKER, G. J., FAUX, C. H., DUTTON, R., MURPHY, M., TURNLEY, A. & KILPATRICK, T. J. 1998. Regulation of neural stem cell differentiation in the forebrain. *Immunol Cell Biol,* 76**,** 414-8.

BAYATTI, N., MOSS, J. A., SUN, L., AMBROSE, P., WARD, J. F., LINDSAY, S. & CLOWRY, G. J. 2008a. A molecular neuroanatomical study of the developing human neocortex from 8 to 17 postconceptional weeks revealing the early differentiation of the subplate and subventricular zone. *Cereb Cortex,* 18**,** 1536-48.

BAYATTI, N., SARMA, S., SHAW, C., EYRE, J. A., VOUYIOUKLIS, D. A., LINDSAY, S. & CLOWRY, G. J. 2008b. Progressive loss of PAX6, TBR2, NEUROD and TBR1 mRNA gradients correlates with translocation of EMX2 to the cortical plate during human cortical development. *Eur J Neurosci,* 28**,** 1449-56.

BEAR, M. F., CONNORS, B. W. & PARADISO, M. A. 2007. *Neuroscience : exploring the brain,* Philadelphia, PA, Lippincott Williams & Wilkins.

BEAULIEU, L. M. & FREEDMAN, J. E. 2010. The role of inflammation in regulating platelet production and function: Toll-like receptors in platelets and megakaryocytes. *Thromb Res,* 125**,** 205-9.

BEHZAD-BEHBAHANI, A., POURANSARI, R., TABEI, S. Z., RAHIMINEJAD, M. S., ROBATI, M., YAGHOBI, R., NOURANI, H., RAMZI, M. M., FARHADI-ANDARABI, A., MOJIRI, A., RAHSAZ, M., BANIHASHEMI, M. & ZARE, N. 2005. Risk of viral transmission via bone marrow progenitor cells versus umbilical cord blood hematopoietic stem cells in bone marrow transplantation. *Transplant Proc,* 37**,** 3211-2.

BEN-DAVID, U. & BENVENISTY, N. 2011. The tumorigenicity of human embryonic and induced pluripotent stem cells. *Nat Rev Cancer,* 11**,** 268-77.

BEST, A. R. & REGEHR, W. G. 2008. Serotonin evokes endocannabinoid release and retrogradely suppresses excitatory synapses. *J Neurosci,* 28**,** 6508-15.

BETTENS, K., SLEEGERS, K. & VAN BROECKHOVEN, C. 2010. Current status on Alzheimer disease molecular genetics: from past, to present, to future. *Hum Mol Genet,* 19**,** R4-R11.

BIEBACK, K., KERN, S., KLUTER, H. & EICHLER, H. 2004. Critical parameters for the isolation of mesenchymal stem cells from umbilical cord blood. *Stem Cells,* 22**,** 625-34.

BJORKLUND, L. M., SANCHEZ-PERNAUTE, R., CHUNG, S., ANDERSSON, T., CHEN, I. Y., MCNAUGHT, K. S., BROWNELL, A. L., JENKINS, B. G., WAHLESTEDT, C., KIM, K. S. & ISACSON, O. 2002. Embryonic stem cells develop into functional dopaminergic neurons after transplantation in a Parkinson rat model. *Proc Natl Acad Sci U S A,* 99**,** 2344-9.

BLACK, I. B. & WOODBURY, D. 2001. Adult rat and human bone marrow stromal stem cells differentiate into neurons. *Blood Cells Mol Dis,* 27**,** 632-6.

BLURTON-JONES, M., KITAZAWA, M., MARTINEZ-CORIA, H., CASTELLO, N. A., MULLER, F. J., LORING, J. F., YAMASAKI, T. R., POON, W. W., GREEN, K. N. & LAFERLA, F. M. 2009. Neural stem cells improve cognition via BDNF in a transgenic model of Alzheimer disease. *Proc Natl Acad Sci U S A,* 106**,** 13594-9.

BLUSZTAJN, J. K., LISCOVITCH, M. & RICHARDSON, U. I. 1987. Synthesis of acetylcholine from choline derived from phosphatidylcholine in a human neuronal cell line. *Proc Natl Acad Sci U S A,* 84**,** 5474-7.

BOCKAERT, J., CLAEYSEN, S., BECAMEL, C., DUMUIS, A. & MARIN, P. 2006. Neuronal 5-HT metabotropic receptors: fine-tuning of their structure, signaling, and roles in synaptic modulation. *Cell Tissue Res,* 326**,** 553-72.

BORDEN, L. A., SMITH, K. E., GUSTAFSON, E. L., BRANCHEK, T. A. & WEINSHANK, R. L. 1995. Cloning and expression of a betaine/GABA transporter from human brain. *J Neurochem,* 64**,** 977-84.

BORGES, C. R., GEDDES, T., WATSON, J. T. & KUHN, D. M. 2002. Dopamine biosynthesis is regulated by S-glutathionylation. Potential mechanism of tyrosine hydroxylast inhibition during oxidative stress. *J Biol Chem,* 277**,** 48295-302.

BOTA, M. & SWANSON, L. W. 2007. The neuron classification problem. *Brain Res Rev,* 56**,** 79-88.

BOYER, L. A., LEE, T. I., COLE, M. F., JOHNSTONE, S. E., LEVINE, S. S., ZUCKER, J. P., GUENTHER, M. G., KUMAR, R. M., MURRAY, H. L., JENNER, R. G., GIFFORD, D. K., MELTON, D. A., JAENISCH, R. & YOUNG, R. A. 2005. Core transcriptional regulatory circuitry in human embryonic stem cells. *Cell,* 122**,** 947-56.

BOYER, L. A., MATHUR, D. & JAENISCH, R. 2006. Molecular control of pluripotency. *Curr Opin Genet Dev,* 16**,** 455-62.

BRADLEY, A., EVANS, M., KAUFMAN, M. H. & ROBERTSON, E. 1984. Formation of germ-line chimaeras from embryo-derived teratocarcinoma cell lines. *Nature,* 309**,** 255-6.

BRADY, S. T., WITT, A. S., KIRKPATRICK, L. L., DE WAEGH, S. M., READHEAD, C., TU, P. H. & LEE, V. M. 1999. Formation of compact myelin is required for maturation of the axonal cytoskeleton. *J Neurosci,* 19**,** 7278-88.

BRANNVALL, K., BERGMAN, K., WALLENQUIST, U., SVAHN, S., BOWDEN, T., HILBORN, J. & FORSBERG-NILSSON, K. 2007. Enhanced neuronal differentiation in a three-dimensional collagen-hyaluronan matrix. *J Neurosci Res,* 85**,** 2138-46.

BROWN, D. C. & GATTER, K. C. 2002. Ki67 protein: the immaculate deception? *Histopathology,* 40**,** 2-11.

BROXMEYER, H. E., GLUCKMAN, E., AUERBACH, A., DOUGLAS, G. W., FRIEDMAN, H., COOPER, S., HANGOC, G., KURTZBERG, J., BARD, J. & BOYSE, E. A. 1990. Human umbilical cord blood: a clinically useful source of transplantable hematopoietic stem/progenitor cells. *Int J Cell Cloning,* 8 Suppl 1**,** 76-89; discussion 89-91.

BRUNSTEIN, C. G. & WAGNER, J. E. 2006. Cord blood transplantation for adults. *Vox Sang,* 91**,** 195-205.

BUITRAGO, W. & ROOP, D. R. 2007. Oct-4: the almighty POUripotent regulator? *J Invest Dermatol,* 127**,** 260-2.

BUZANSKA, L., JURGA, M. & DOMANSKA-JANIK, K. 2006a. Neuronal differentiation of human umbilical cord blood neural stem-like cell line. *Neurodegener Dis,* 3**,** 19-26.

BUZANSKA, L., JURGA, M., STACHOWIAK, E. K., STACHOWIAK, M. K. & DOMANSKA-JANIK, K. 2006b. Neural stem-like cell line derived from a nonhematopoietic population of human umbilical cord blood. *Stem Cells Dev,* 15**,** 391-406.

BUZANSKA, L., MACHAJ, E. K., ZABLOCKA, B., POJDA, Z. & DOMANSKA-JANIK, K. 2002. Human cord blood-derived cells attain neuronal and glial features in vitro. *J Cell Sci,* 115**,** 2131-8.

BYSTRON, I., BLAKEMORE, C. & RAKIC, P. 2008. Development of the human cerebral cortex: Boulder Committee revisited. *Nat Rev Neurosci,* 9**,** 110-22.

CAMPBELL, K. 2005. Cortical neuron specification: it has its time and place. *Neuron,* 46**,** 373-6.

CAMPOS, L. S., LEONE, D. P., RELVAS, J. B., BRAKEBUSCH, C., FASSLER, R., SUTER, U. & FFRENCH-CONSTANT, C. 2004. Beta1 integrins activate a MAPK signalling pathway in neural stem cells that contributes to their maintenance. *Development,* 131**,** 3433-44.

CARPENTER, M. K., ROSLER, E. S., FISK, G. J., BRANDENBERGER, R., ARES, X., MIURA, T., LUCERO, M. & RAO, M. S. 2004. Properties of four human embryonic stem cell lines maintained in a feeder-free culture system. *Dev Dyn,* 229**,** 243-58.

CASTLE, P. & ROBERTSON, J. S. 1999. Summary and conclusion. Animal sera, animal sera derivatives and substitutes used in the manufacture of pharmaceuticals. *Dev Biol Stand,* 99**,** 191-6.

CAUFFMAN, G., LIEBAERS, I., VAN STEIRTEGHEM, A. & VAN DE VELDE, H. 2006. POU5F1 isoforms show different expression patterns in human embryonic stem cells and preimplantation embryos. *Stem Cells,* 24**,** 2685-91.

CHAN, W. Y., KOHSAKA, S. & REZAIE, P. 2007. The origin and cell lineage of microglia: new concepts. *Brain Res Rev,* 53**,** 344-54.

CHANG, M. Y., SON, H., LEE, Y. S. & LEE, S. H. 2003. Neurons and astrocytes secrete factors that cause stem cells to differentiate into neurons and astrocytes, respectively. *Mol Cell Neurosci,* 23**,** 414-26.

CHAPLIN, D. D. 2010. Overview of the immune response. *J Allergy Clin Immunol,* 125**,** S3-23.

CHAURASIA, B. D. & AGARWAL, B. M. 1979. Helical structure of the human umbilical cord. *Acta Anat (Basel),* 103**,** 226-30.

CHEN, C., HE, Z., SAI, P., FARIDI, A., AZIZ, A., KALAVAR, M., GRICIENE, P., GINTAUTAS, J. & STEIER, W. 2004. Inhibition of human CD24 binding to platelet-bound P-selectin by monoclonal antibody. *Proc West Pharmacol Soc,* 47**,** 28-9.

CHEN, J., SANBERG, P. R., LI, Y., WANG, L., LU, M., WILLING, A. E., SANCHEZ-RAMOS, J. & CHOPP, M. 2001. Intravenous administration of human umbilical cord blood reduces behavioral deficits after stroke in rats. *Stroke,* 32**,** 2682-8.

CHEN, J. R., CHENG, G. Y., SHEU, C. C., TSENG, G. F., WANG, T. J. & HUANG, Y. S. 2008. Transplanted bone marrow stromal cells migrate, differentiate and improve motor function in rats with experimentally induced cerebral stroke. *J Anat,* 213**,** 249-58.

CHEN, Y. & SWANSON, R. A. 2003. The glutamate transporters EAAT2 and EAAT3 mediate cysteine uptake in cortical neuron cultures. *J Neurochem,* 84**,** 1332-9.

CHENG, L. C., PASTRANA, E., TAVAZOIE, M. & DOETSCH, F. 2009. miR-124 regulates adult neurogenesis in the subventricular zone stem cell niche. *Nat Neurosci,* 12**,** 399-408.

CHEW, J. L., LOH, Y. H., ZHANG, W., CHEN, X., TAM, W. L., YEAP, L. S., LI, P., ANG, Y. S., LIM, B., ROBSON, P. & NG, H. H. 2005. Reciprocal transcriptional regulation of Pou5f1 and Sox2 via the Oct4/Sox2 complex in embryonic stem cells. *Mol Cell Biol,* 25**,** 6031-46.

CHRISTIE, B. 2009. Study on stem cells for stroke patients to start in Scotland later in 2009. *Bmj,* 338**,** b245.

CHU, K., KIM, M., JEONG, S. W., KIM, S. U. & YOON, B. W. 2003. Human neural stem cells can migrate, differentiate, and integrate after intravenous transplantation in adult rats with transient forebrain ischemia. *Neurosci Lett,* 343**,** 129-33.

CHU, K., KIM, M., PARK, K. I., JEONG, S. W., PARK, H. K., JUNG, K. H., LEE, S. T., KANG, L., LEE, K., PARK, D. K., KIM, S. U. & ROH, J. K. 2004. Human neural stem cells improve sensorimotor deficits in the adult rat brain with experimental focal ischemia. *Brain Res,* 1016**,** 145-53.

CHUA, S. J., BIELECKI, R., WONG, C. J., YAMANAKA, N., ROGERS, I. M. & CASPER, R. F. 2009. Neural progenitors, neurons and oligodendrocytes from human umbilical cord blood cells in a serum-free, feeder-free cell culture. *Biochem Biophys Res Commun,* 379**,** 217-21.

CLENDENING, B. & HUME, R. I. 1990. Expression of multiple neurotransmitter receptors by sympathetic preganglionic neurons in vitro. *J Neurosci,* 10**,** 3977-91.

CLOWRY, G., MOLNAR, Z. & RAKIC, P. 2010. Renewed focus on the developing human neocortex. *J Anat,* 217**,** 276-88.

COBOS, I., BORELLO, U. & RUBENSTEIN, J. L. 2007. Dlx transcription factors promote migration through repression of axon and dendrite growth. *Neuron,* 54**,** 873-88.

COLUCCI-D'AMATO, L., BONAVITA, V. & DI PORZIO, U. 2006. The end of the central dogma of neurobiology: stem cells and neurogenesis in adult CNS. *Neurol Sci,* 27**,** 266-70.

CONOVER, J. C. & NOTTI, R. Q. 2008. The neural stem cell niche. *Cell Tissue Res,* 331**,** 211-24.

COOKE, M. J., STOJKOVIC, M. & PRZYBORSKI, S. A. 2006. Growth of teratomas derived from human pluripotent stem cells is influenced by the graft site. *Stem Cells Dev,* 15**,** 254-9.

CORRIGAN, J. D., SELASSIE, A. W. & ORMAN, J. A. 2010. The epidemiology of traumatic brain injury. *J Head Trauma Rehabil,* 25**,** 72-80.

CORSELLI, M., PARODI, A., MOGNI, M., SESSAREGO, N., KUNKL, A., DAGNA-BRICARELLI, F., IBATICI, A., POZZI, S., BACIGALUPO, A., FRASSONI, F. & PIAGGIO, G. 2008. Clinical scale ex vivo expansion of cord blood-derived outgrowth endothelial progenitor cells is associated with high incidence of karyotype aberrations. *Exp Hematol,* 36**,** 340-9.

CROSS, J. C., NAKANO, H., NATALE, D. R., SIMMONS, D. G. & WATSON, E. D. 2006. Branching morphogenesis during development of placental villi. *Differentiation,* 74**,** 393-401.

CURRARINO, G., STANNARD, M. W. & KOLNI, H. 1991. Umbilical vein draining into the inferior vena cava via the internal iliac vein, bypassing the liver. *Pediatr Radiol,* 21**,** 265-6.

DA SILVA MEIRELLES, L., CHAGASTELLES, P. C. & NARDI, N. B. 2006. Mesenchymal stem cells reside in virtually all post-natal organs and tissues. *J Cell Sci,* 119**,** 2204-13.

DANSAULT, A., DAVID, G., SCHWARTZ, C., JALIFFA, C., VIEIRA, V., DE LA HOUSSAYE, G., BIGOT, K., CATIN, F., TATTU, L., CHOPIN, C., HALIMI, P., ROCHE, O., VAN REGEMORTER, N., MUNIER, F., SCHORDERET, D., DUFIER, J. L., MARSAC, C., RICQUIER, D., MENASCHE, M., PENFORNIS, A. & ABITBOL, M. 2007. Three new PAX6 mutations including one causing an unusual ophthalmic phenotype associated with neurodevelopmental abnormalities. *Mol Vis,* 13**,** 511-23.

DE MELO REIS, R. A., SCHITINE, C. S., KOFALVI, A., GRADE, S., CORTES, L., GARDINO, P. F., MALVA, J. O. & DE MELLO, F. G. 2011. Functional identification of cell phenotypes differentiating from mice retinal neurospheres using single cell calcium imaging. *Cell Mol Neurobiol,* 31**,** 835-46.

DEB, P., SHARMA, S. & HASSAN, K. M. 2010. Pathophysiologic mechanisms of acute ischemic stroke: An overview with emphasis on therapeutic significance beyond thrombolysis. *Pathophysiology,* 17**,** 197-218.

DECRESSAC, M., PRESTOZ, L., VERAN, J., CANTEREAU, A., JABER, M. & GAILLARD, A. 2009. Neuropeptide Y stimulates proliferation, migration and differentiation of neural precursors from the subventricular zone in adult mice. *Neurobiol Dis,* 34**,** 441-9.

DENNER, L., BODENBURG, Y., ZHAO, J. G., HOWE, M., CAPPO, J., TILTON, R. G., COPLAND, J. A., FORRAZ, N., MCGUCKIN, C. & URBAN, R. 2007. Directed engineering of umbilical cord blood stem cells to produce C-peptide and insulin. *Cell Prolif,* 40**,** 367-80.

DESCARRIES, L., KRNJEVI*C, K. & STERIADE, M. 2004. *Acetylcholine in the cerebral cortex,* Amsterdam ; Boston, Elsevier.

DESFORGES, M. & SIBLEY, C. P. 2010. Placental nutrient supply and fetal growth. *Int J Dev Biol,* 54**,** 377-90.

DI NARO, E., GHEZZI, F., RAIO, L., FRANCHI, M. & D'ADDARIO, V. 2001. Umbilical cord morphology and pregnancy outcome. *Eur J Obstet Gynecol Reprod Biol,* 96**,** 150-7.

DI TERLIZZI, R. & PLATT, S. 2006. The function, composition and analysis of cerebrospinal fluid in companion animals: part I - function and composition. *Vet J,* 172**,** 422-31.

DIRKX, R., JR., THOMAS, A., LI, L., LERNMARK, A., SHERWIN, R. S., DE CAMILLI, P. & SOLIMENA, M. 1995. Targeting of the 67-kDa isoform of glutamic acid decarboxylase to intracellular organelles is mediated by its interaction with the NH2-terminal region of the 65-kDa isoform of glutamic acid decarboxylase. *J Biol Chem,* 270**,** 2241-6.

DOETSCH, F. 2003a. The glial identity of neural stem cells. *Nat Neurosci,* 6**,** 1127-34.

DOETSCH, F. 2003b. A niche for adult neural stem cells. *Curr Opin Genet Dev,* 13**,** 543-50.

DOMANSKA-JANIK, K., HABICH, A., SARNOWSKA, A. & JANOWSKI, M. 2006. Neural commitment of cord blood stem cells (HUCB-NSC/NP): therapeutic perspectives. *Acta Neurobiol Exp (Wars),* 66**,** 279-91.

DONNAN, G. A., FISHER, M., MACLEOD, M. & DAVIS, S. M. 2008. Stroke. *Lancet,* 371**,** 1612-23.

DOODY, R. S. 2003. Current treatments for Alzheimer's disease: cholinesterase inhibitors. *J Clin Psychiatry,* 64 Suppl 9**,** 11-7.

DUAN, X., KANG, E., LIU, C. Y., MING, G. L. & SONG, H. 2008. Development of neural stem cell in the adult brain. *Curr Opin Neurobiol,* 18**,** 108-15.

DUCHAMP-VIRET, P., DUCHAMP, A. & CHAPUT, M. A. 2003. Single olfactory sensory neurons simultaneously integrate the components of an odour mixture. *Eur J Neurosci,* 18**,** 2690-6.

EIRAKU, M., WATANABE, K., MATSUO-TAKASAKI, M., KAWADA, M., YONEMURA, S., MATSUMURA, M., WATAYA, T., NISHIYAMA, A., MUGURUMA, K. & SASAI, Y. 2008. Self-organized formation of polarized cortical tissues from ESCs and its active manipulation by extrinsic signals. *Cell Stem Cell,* 3**,** 519-32.

EL-HUSSEINI, A. E., SCHNELL, E., CHETKOVICH, D. M., NICOLL, R. A. & BREDT, D. S. 2000. PSD-95 involvement in maturation of excitatory synapses. *Science,* 290**,** 1364-8.

ELMSLIE, K. S. 2003. Neurotransmitter modulation of neuronal calcium channels. *J Bioenerg Biomembr,* 35**,** 477-89.

ENDE, M. & ENDE, N. 1972. Hematopoietic transplantation by means of fetal (cord) blood. A new method. *Va Med Mon (1918),* 99**,** 276-80.

ENDE, N. & CHEN, R. 2002. Parkinson's disease mice and human umbilical cord blood. *J Med,* 33**,** 173-80.

ENGLUND, C., FINK, A., LAU, C., PHAM, D., DAZA, R. A., BULFONE, A., KOWALCZYK, T. & HEVNER, R. F. 2005. Pax6, Tbr2, and Tbr1 are expressed sequentially by radial glia,

intermediate progenitor cells, and postmitotic neurons in developing neocortex. *J Neurosci,* 25**,** 247-51.

ENNA, S. J. & MÖHLER, H. 2007. *The GABA receptors,* Totowa, N.J., Humana Press.

ERECINSKA, M. & SILVER, I. A. 1990. Metabolism and role of glutamate in mammalian brain. *Prog Neurobiol,* 35**,** 245-96.

ERICES, A., CONGET, P. & MINGUELL, J. J. 2000. Mesenchymal progenitor cells in human umbilical cord blood. *Br J Haematol,* 109**,** 235-42.

EVANS, M. J. & KAUFMAN, M. H. 1981. Establishment in culture of pluripotential cells from mouse embryos. *Nature,* 292**,** 154-6.

EXALTO, N. 1995. Early human nutrition. *Eur J Obstet Gynecol Reprod Biol,* 61**,** 3-6.

FASOULIOTIS, S. J. & SCHENKER, J. G. 2000. Human umbilical cord blood banking and transplantation: a state of the art. *Eur J Obstet Gynecol Reprod Biol,* 90**,** 13-25.

FEANY, M. B. 1996. Neuropeptide modulation of learning and memory processes. *Rev Neurosci,* 7**,** 151-64.

FEDOROFF, S. & RICHARDSON, A. 2001. *Protocols for neural cell culture,* Totowa, N.J., Humana Press.

FELLI, N., CIANETTI, L., PELOSI, E., CARE, A., LIU, C. G., CALIN, G. A., ROSSI, S., PESCHLE, C., MARZIALI, G. & GIULIANI, A. 2010. Hematopoietic differentiation: a coordinated dynamical process towards attractor stable states. *BMC Syst Biol,* 4**,** 85.

FERGUSON, V. L. & DODSON, R. B. 2009. Bioengineering aspects of the umbilical cord. *Eur J Obstet Gynecol Reprod Biol,* 144 Suppl 1**,** S108-13.

FERRIS, S. H. 2003. Evaluation of memantine for the treatment of Alzheimer's disease. *Expert Opin Pharmacother,* 4**,** 2305-13.

FIELDS, R. D. & STEVENS-GRAHAM, B. 2002. New insights into neuron-glia communication. *Science,* 298**,** 556-62.

FIRE, A., XU, S., MONTGOMERY, M. K., KOSTAS, S. A., DRIVER, S. E. & MELLO, C. C. 1998. Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. *Nature,* 391**,** 806-11.

FLEXMAN, J. A., CROSS, D. J., KIM, Y. & MINOSHIMA, S. 2007. Morphological and parametric estimation of fetal neural stem cell migratory capacity in the rat brain. *Conf Proc IEEE Eng Med Biol Soc,* 2007**,** 4464-7.

FONG, H., HOHENSTEIN, K. A. & DONOVAN, P. J. 2008. Regulation of self-renewal and pluripotency by Sox2 in human embryonic stem cells. *Stem Cells,* 26**,** 1931-8.

FORRAZ, N., PETTENGELL, R. & MCGUCKIN, C. P. 2004. Characterization of a lineage-negative stem-progenitor cell population optimized for ex vivo expansion and enriched for LTC-IC. *Stem Cells,* 22**,** 100-8.

FORTIER, L. A. 2005. Stem cells: classifications, controversies, and clinical applications. *Vet Surg,* 34**,** 415-23.

FOSTER, A. C. & KEMP, J. A. 2006. Glutamate- and GABA-based CNS therapeutics. *Curr Opin Pharmacol,* 6**,** 7-17.

FREMEAU, R. T., JR., VOGLMAIER, S., SEAL, R. P. & EDWARDS, R. H. 2004. VGLUTs define subsets of excitatory neurons and suggest novel roles for glutamate. *Trends Neurosci,* 27**,** 98-103.

FROESTL, W., GALLAGHER, M., JENKINS, H., MADRID, A., MELCHER, T., TEICHMAN, S., MONDADORI, C. G. & PEARLMAN, R. 2004. SGS742: the first GABA(B) receptor antagonist in clinical trials. *Biochem Pharmacol,* 68**,** 1479-87.

FUCHS, E., TUMBAR, T. & GUASCH, G. 2004. Socializing with the neighbors: stem cells and their niche. *Cell,* 116**,** 769-78.

FUJIOKA, T., FUJIOKA, A. & DUMAN, R. S. 2004. Activation of cAMP signaling facilitates the morphological maturation of newborn neurons in adult hippocampus. *J Neurosci,* 24**,** 319-28.

GAHRING, L. C. & ROGERS, S. W. 2005. Neuronal nicotinic acetylcholine receptor expression and function on nonneuronal cells. *AAPS J,* 7**,** E885-94.

GAO, Z., URE, K., ABLES, J. L., LAGACE, D. C., NAVE, K. A., GOEBBELS, S., EISCH, A. J. & HSIEH, J. 2009. Neurod1 is essential for the survival and maturation of adult-born neurons. *Nat Neurosci,* 12**,** 1090-2.

GASPARD, N., BOUSCHET, T., HOUREZ, R., DIMIDSCHSTEIN, J., NAEIJE, G., VAN DEN AMEELE, J., ESPUNY-CAMACHO, I., HERPOEL, A., PASSANTE, L., SCHIFFMANN, S. N., GAILLARD, A. & VANDERHAEGHEN, P. 2008. An intrinsic mechanism of corticogenesis from embryonic stem cells. *Nature,* 455**,** 351-7.

GASPARD, N., GAILLARD, A. & VANDERHAEGHEN, P. 2009. Making cortex in a dish: in vitro corticopoiesis from embryonic stem cells. *Cell Cycle,* 8**,** 2491-6.

GEHRMANN, J., MATSUMOTO, Y. & KREUTZBERG, G. W. 1995. Microglia: intrinsic immuneffector cell of the brain. *Brain Res Brain Res Rev,* 20**,** 269-87.

GERALD, C., WALKER, M. W., CRISCIONE, L., GUSTAFSON, E. L., BATZL-HARTMANN, C., SMITH, K. E., VAYSSE, P., DURKIN, M. M., LAZ, T. M., LINEMEYER, D. L., SCHAFFHAUSER, A. O., WHITEBREAD, S., HOFBAUER, K. G., TABER, R. I., BRANCHEK, T. A. & WEINSHANK, R. L. 1996. A receptor subtype involved in neuropeptide-Y-induced food intake. *Nature,* 382**,** 168-71.

GIRAULT, J. A. & GREENGARD, P. 2004. The neurobiology of dopamine signaling. *Arch Neurol,* 61**,** 641-4.

GLUCKMAN, E., BROXMEYER, H. A., AUERBACH, A. D., FRIEDMAN, H. S., DOUGLAS, G. W., DEVERGIE, A., ESPEROU, H., THIERRY, D., SOCIE, G., LEHN, P. & ET AL. 1989. Hematopoietic reconstitution in a patient with Fanconi's anemia by means of umbilical-cord blood from an HLA-identical sibling. *N Engl J Med,* 321**,** 1174-8.

GOEDERT, M. & SPILLANTINI, M. G. 2006. A century of Alzheimer's disease. *Science,* 314**,** 777-81.

GOTZ, M., STOYKOVA, A. & GRUSS, P. 1998. Pax6 controls radial glia differentiation in the cerebral cortex. *Neuron,* 21**,** 1031-44.

GRAHAM, V., KHUDYAKOV, J., ELLIS, P. & PEVNY, L. 2003. SOX2 functions to maintain neural progenitor identity. *Neuron,* 39**,** 749-65.

GREENAMYRE, J. T. 1986. The role of glutamate in neurotransmission and in neurologic disease. *Arch Neurol,* 43**,** 1058-63.

GREMO, F. & PRESTA, M. 2000. Role of fibroblast growth factor-2 in human brain: a focus on development. *Int J Dev Neurosci,* 18**,** 271-9.

GROSS, L. 2006. "Supporting" players take the lead in protecting the overstimulated brain. *PLoS Biol,* 4**,** e371.

GSTRAUNTHALER, G. 2003. Alternatives to the use of fetal bovine serum: serum-free cell culture. *ALTEX,* 20**,** 275-81.

GUAN, K., CHANG, H., ROLLETSCHEK, A. & WOBUS, A. M. 2001. Embryonic stem cell-derived neurogenesis. Retinoic acid induction and lineage selection of neuronal cells. *Cell Tissue Res,* 305**,** 171-6.

GUILLEMOT, F. 1999. Vertebrate bHLH genes and the determination of neuronal fates. *Exp Cell Res,* 253**,** 357-64.

GUILLEMOT, F., MOLNAR, Z., TARABYKIN, V. & STOYKOVA, A. 2006. Molecular mechanisms of cortical differentiation. *Eur J Neurosci,* 23**,** 857-68.

GUILLERY, R. W. & SHERMAN, S. M. 2002. Thalamic relay functions and their role in corticocortical communication: generalizations from the visual system. *Neuron,* 33**,** 163-75.

GULLEDGE, A. T., BUCCI, D. J., ZHANG, S. S., MATSUI, M. & YEH, H. H. 2009. M1 receptors mediate cholinergic modulation of excitability in neocortical pyramidal neurons. *J Neurosci,* 29**,** 9888-902.

GULLEDGE, A. T., PARK, S. B., KAWAGUCHI, Y. & STUART, G. J. 2007. Heterogeneity of phasic cholinergic signaling in neocortical neurons. *J Neurophysiol,* 97**,** 2215-29.

HABICH, A., JURGA, M., MARKIEWICZ, I., LUKOMSKA, B., BANY-LASZEWICZ, U. & DOMANSKA-JANIK, K. 2006. Early appearance of stem/progenitor cells with neural-like characteristics in human cord blood mononuclear fraction cultured in vitro. *Exp Hematol,* 34**,** 914-25.

HALASA, M., BASKIEWICZ-MASIUK, M., DABKOWSKA, E. & MACHALINSKI, B. 2008. An efficient two-step method to purify very small embryonic-like (VSEL) stem cells from umbilical cord blood (UCB). *Folia Histochem Cytobiol,* 46**,** 239-43.

HALL, A. A., LEONARDO, C. C., COLLIER, L. A., ROWE, D. D., WILLING, A. E. & PENNYPACKER, K. R. 2009. Delayed treatments for stroke influence neuronal death in rat organotypic slice cultures subjected to oxygen glucose deprivation. *Neuroscience,* 164**,** 470-7.

HANNA, J., MARKOULAKI, S., SCHORDERET, P., CAREY, B. W., BEARD, C., WERNIG, M., CREYGHTON, M. P., STEINE, E. J., CASSADY, J. P., FOREMAN, R., LENGNER, C. J., DAUSMAN, J. A. & JAENISCH, R. 2008. Direct reprogramming of terminally differentiated mature B lymphocytes to pluripotency. *Cell,* 133**,** 250-64.

HANNON, G. J. 2002. RNA interference. *Nature,* 418**,** 244-51.

HANNON, J. & HOYER, D. 2008. Molecular biology of 5-HT receptors. *Behav Brain Res,* 195**,** 198-213.

HANSEN, D. V., LUI, J. H., PARKER, P. R. & KRIEGSTEIN, A. R. 2010. Neurogenic radial glia in the outer subventricular zone of human neocortex. *Nature,* 464**,** 554-561.

HARRIS, D. T. 2009. Non-haematological uses of cord blood stem cells. *Br J Haematol,* 147**,** 177-84.

HARRIS, D. T. & ROGERS, I. 2007. Umbilical cord blood: a unique source of pluripotent stem cells for regenerative medicine. *Curr Stem Cell Res Ther,* 2**,** 301-9.

HASHIMOTO, M., ROCKENSTEIN, E., CREWS, L. & MASLIAH, E. 2003. Role of protein aggregation in mitochondrial dysfunction and neurodegeneration in Alzheimer's and Parkinson's diseases. *Neuromolecular Med,* 4**,** 21-36.

HATA, Y. & TAKAI, Y. 1999. Roles of postsynaptic density-95/synapse-associated protein 90 and its interacting proteins in the organization of synapses. *Cell Mol Life Sci,* 56**,** 461-72.

HAYASHI, F., MEANS, T. K. & LUSTER, A. D. 2003. Toll-like receptors stimulate human neutrophil function. *Blood,* 102**,** 2660-9.

HE, S., NAKADA, D. & MORRISON, S. J. 2009. Mechanisms of stem cell self-renewal. *Annu Rev Cell Dev Biol,* 25**,** 377-406.

HELLEMANS, J., MORTIER, G., DE PAEPE, A., SPELEMAN, F. & VANDESOMPELE, J. 2007. qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. *Genome Biol,* 8**,** R19.

HENION, P. D. & WESTON, J. A. 1994. Retinoic acid selectively promotes the survival and proliferation of neurogenic precursors in cultured neural crest cell populations. *Dev Biol,* 161**,** 243-50.

HERCULANO-HOUZEL, S. 2009. The human brain in numbers: a linearly scaled-up primate brain. *Front Hum Neurosci,* 3**,** 31.

HERNANDEZ-CASELLES, T., MARTINEZ-ESPARZA, M., PEREZ-OLIVA, A. B., QUINTANILLA-CECCONI, A. M., GARCIA-ALONSO, A., ALVAREZ-LOPEZ, D. M. & GARCIA-PENARRUBIA, P. 2006. A study of CD33 (SIGLEC-3) antigen expression and function on activated human T and NK cells: two isoforms of CD33 are generated by alternative splicing. *J Leukoc Biol,* 79**,** 46-58.

HESS, D. C. & BORLONGAN, C. V. 2008. Stem cells and neurological diseases. *Cell Prolif,* 41 Suppl 1**,** 94-114.

HEVNER, R. F. 2007. Layer-specific markers as probes for neuron type identity in human neocortex and malformations of cortical development. *J Neuropathol Exp Neurol,* 66**,** 101-9.

HEVNER, R. F., HODGE, R. D., DAZA, R. A. & ENGLUND, C. 2006. Transcription factors in glutamatergic neurogenesis: conserved programs in neocortex, cerebellum, and adult hippocampus. *Neurosci Res,* 55**,** 223-33.

HEVNER, R. F., SHI, L., JUSTICE, N., HSUEH, Y., SHENG, M., SMIGA, S., BULFONE, A., GOFFINET, A. M., CAMPAGNONI, A. T. & RUBENSTEIN, J. L. 2001. Tbr1 regulates differentiation of the preplate and layer 6. *Neuron,* 29**,** 353-66.

HODGE, R. D., KOWALCZYK, T. D., WOLF, S. A., ENCINAS, J. M., RIPPEY, C., ENIKOLOPOV, G., KEMPERMANN, G. & HEVNER, R. F. 2008. Intermediate progenitors in adult hippocampal neurogenesis: Tbr2 expression and coordinate regulation of neuronal output. *J Neurosci,* 28**,** 3707-17.

HOGAN, S. P., ROSENBERG, H. F., MOQBEL, R., PHIPPS, S., FOSTER, P. S., LACY, P., KAY, A. B. & ROTHENBERG, M. E. 2008. Eosinophils: biological properties and role in health and disease. *Clin Exp Allergy,* 38**,** 709-50.

HONG, C. J. & HSUEH, Y. P. 2007. Cytoplasmic distribution of T-box transcription factor Tbr-1 in adult rodent brain. *J Chem Neuroanat,* 33**,** 124-30.

HRUSKA-HAGEMAN, A. M., BENSON, C. J., LEONARD, A. S., PRICE, M. P. & WELSH, M. J. 2004. PSD-95 and Lin-7b interact with acid-sensing ion channel-3 and have opposite effects on H+- gated current. *J Biol Chem,* 279**,** 46962-8.

HU, B. Y., WEICK, J. P., YU, J., MA, L. X., ZHANG, X. Q., THOMSON, J. A. & ZHANG, S. C. 2010. Neural differentiation of human induced pluripotent stem cells follows developmental principles but with variable potency. *Proc Natl Acad Sci U S A,* 107**,** 4335-40.

HYSLOP, L., STOJKOVIC, M., ARMSTRONG, L., WALTER, T., STOJKOVIC, P., PRZYBORSKI, S., HERBERT, M., MURDOCH, A., STRACHAN, T. & LAKO, M. 2005. Downregulation of NANOG induces differentiation of human embryonic stem cells to extraembryonic lineages. *Stem Cells,* 23**,** 1035-43.

INAMDAR, M. S., VENU, P., SRINIVAS, M. S., RAO, K. & VIJAYRAGHAVAN, K. 2009. Derivation and characterization of two sibling human embryonic stem cell lines from discarded grade III embryos. *Stem Cells Dev,* 18**,** 423-33.

INIGUEZ, S. D., VIALOU, V., WARREN, B. L., CAO, J. L., ALCANTARA, L. F., DAVIS, L. C., MANOJLOVIC, Z., NEVE, R. L., RUSSO, S. J., HAN, M. H., NESTLER, E. J. & BOLANOS-GUZMAN, C. A. 2010. Extracellular signal-regulated kinase-2 within the ventral tegmental area regulates responses to stress. *J Neurosci,* 30**,** 7652-63.

ISHII, M. & KURACHI, Y. 2006. Muscarinic acetylcholine receptors. *Curr Pharm Des,* 12**,** 3573-81.

JAATINEN, T. & LAINE, J. 2007. Isolation of mononuclear cells from human cord blood by Ficoll-Paque density gradient. *Curr Protoc Stem Cell Biol,* Chapter 2**,** Unit 2A 1.

JARVIS, S. E. & ZAMPONI, G. W. 2007. Trafficking and regulation of neuronal voltage-gated calcium channels. *Curr Opin Cell Biol,* 19**,** 474-82.

JI, Y., PANG, P. T., FENG, L. & LU, B. 2005. Cyclic AMP controls BDNF-induced TrkB phosphorylation and dendritic spine formation in mature hippocampal neurons. *Nat Neurosci,* 8**,** 164-72.

JIANG, W., KIM, B. Y., RUTKA, J. T. & CHAN, W. C. 2008. Nanoparticle-mediated cellular response is size-dependent. *Nat Nanotechnol,* 3**,** 145-50.

JIN, K., PEEL, A. L., MAO, X. O., XIE, L., COTTRELL, B. A., HENSHALL, D. C. & GREENBERG, D. A. 2004. Increased hippocampal neurogenesis in Alzheimer's disease. *Proc Natl Acad Sci U S A,* 101**,** 343-7.

JIN, K., SUN, Y., XIE, L., PEEL, A., MAO, X. O., BATTEUR, S. & GREENBERG, D. A. 2003. Directed migration of neuronal precursors into the ischemic cerebral cortex and striatum. *Mol Cell Neurosci,* 24**,** 171-89.

JIN, K., WANG, X., XIE, L., MAO, X. O., ZHU, W., WANG, Y., SHEN, J., MAO, Y., BANWAIT, S. & GREENBERG, D. A. 2006. Evidence for stroke-induced neurogenesis in the human brain. *Proc Natl Acad Sci U S A,* 103**,** 13198-202.

JOHANNSSEN, H. C. & HELMCHEN, F. 2010. In vivo Ca2+ imaging of dorsal horn neuronal populations in mouse spinal cord. *J Physiol,* 588**,** 3397-402.

JOHANSON, C. E., DUNCAN, J. A., 3RD, KLINGE, P. M., BRINKER, T., STOPA, E. G. & SILVERBERG, G. D. 2008. Multiplicity of cerebrospinal fluid functions: New challenges in health and disease. *Cerebrospinal Fluid Res,* 5**,** 10.

JOHNSTON, M. V. & HOON, A. H., JR. 2006. Cerebral palsy. *Neuromolecular Med,* 8**,** 435-50.

JONES, E. G. 2009. The origins of cortical interneurons: mouse versus monkey and human. *Cereb Cortex,* 19**,** 1953-6.

JONES, L., LOPEZ-BENDITO, G., GRUSS, P., STOYKOVA, A. & MOLNAR, Z. 2002. Pax6 is required for the normal development of the forebrain axonal connections. *Development,* 129**,** 5041-52.

JURGA, M., LIPKOWSKI, A. W., LUKOMSKA, B., BUZANSKA, L., KURZEPA, K., SOBANSKI, T., HABICH, A., COECKE, S., GAJKOWSKA, B. & DOMANSKA-JANIK, K. 2009. Generation of functional neural artificial tissue from human umbilical cord blood stem cells. *Tissue Eng Part C Methods,* 15**,** 365-72.

JURGA, M., MARKIEWICZ, I., SARNOWSKA, A., HABICH, A., KOZLOWSKA, H., LUKOMSKA, B., BUZANSKA, L. & DOMANSKA-JANIK, K. 2006. Neurogenic potential of human umbilical cord blood: neural-like stem cells depend on previous long-term culture conditions. *J Neurosci Res,* 83**,** 627-37.

KALTSCHMIDT, C., KALTSCHMIDT, B. & BAEUERLE, P. A. 1995. Stimulation of ionotropic glutamate receptors activates transcription factor NF-kappa B in primary neurons. *Proc Natl Acad Sci U S A,* 92**,** 9618-22.

KANIA, G., BLYSZCZUK, P., CZYZ, J., NAVARRETE-SANTOS, A. & WOBUS, A. M. 2003. Differentiation of mouse embryonic stem cells into pancreatic and hepatic cells. *Methods Enzymol,* 365**,** 287-303.

KANOLD, P. O. & LUHMANN, H. J. 2010. The subplate and early cortical circuits. *Annu Rev Neurosci,* 33**,** 23-48.

KAPLAN, M. S. 1981. Neurogenesis in the 3-month-old rat visual cortex. *J Comp Neurol,* 195**,** 323-38.

KATSETOS, C. D., HERMAN, M. M. & MORK, S. J. 2003a. Class III beta-tubulin in human development and cancer. *Cell Motil Cytoskeleton,* 55**,** 77-96.

KATSETOS, C. D., LEGIDO, A., PERENTES, E. & MORK, S. J. 2003b. Class III beta-tubulin isotype: a key cytoskeletal protein at the crossroads of developmental neurobiology and tumor neuropathology. *J Child Neurol,* 18**,** 851-66; discussion 867.

KATZAN, I. L., HAMMER, M. D., HIXSON, E. D., FURLAN, A. J., ABOU-CHEBL, A. & NADZAM, D. M. 2004. Utilization of intravenous tissue plasminogen activator for acute ischemic stroke. *Arch Neurol,* 61**,** 346-50.

KAZANIS, I. 2009. The subependymal zone neurogenic niche: a beating heart in the centre of the brain: how plastic is adult neurogenesis? Opportunities for therapy and questions to be addressed. *Brain,* 132**,** 2909-21.

KERN, S., EICHLER, H., STOEVE, J., KLUTER, H. & BIEBACK, K. 2006. Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue. *Stem Cells,* 24**,** 1294-301.

KIM, D. K., FUJIKI, Y., FUKUSHIMA, T., EMA, H., SHIBUYA, A. & NAKAUCHI, H. 1999. Comparison of hematopoietic activities of human bone marrow and umbilical cord blood CD34 positive and negative cells. *Stem Cells,* 17**,** 286-94.

KIM, J. B., SEBASTIANO, V., WU, G., ARAUZO-BRAVO, M. J., SASSE, P., GENTILE, L., KO, K., RUAU, D., EHRICH, M., VAN DEN BOOM, D., MEYER, J., HUBNER, K., BERNEMANN, C., ORTMEIER, C., ZENKE, M., FLEISCHMANN, B. K., ZAEHRES, H. & SCHOLER, H. R. 2009. Oct4-induced pluripotency in adult neural stem cells. *Cell,* 136**,** 411-9.

KIM, J. B., ZAEHRES, H., WU, G., GENTILE, L., KO, K., SEBASTIANO, V., ARAUZO-BRAVO, M. J., RUAU, D., HAN, D. W., ZENKE, M. & SCHOLER, H. R. 2008. Pluripotent stem cells induced from adult neural stem cells by reprogramming with two factors. *Nature,* 454**,** 646-50.

KIM, S. U. 2004. Human neural stem cells genetically modified for brain repair in neurological disorders. *Neuropathology,* 24**,** 159-71.

KIM, S. U. & DE VELLIS, J. 2009. Stem cell-based cell therapy in neurological diseases: a review. *J Neurosci Res,* 87**,** 2183-200.

KIMURA, K., IGUCHI, Y., SHIBAZAKI, K., SAKAMOTO, Y. & WATANABE, M. 2010. Intravenous Tissue Plasminogen Activator Thrombolysis in Patients without Major Arterial Occlusion Seems to Be Safe and Effective. *Eur Neurol,* 64**,** 258-264.

KITCHENS, R. L. 2000. Role of CD14 in cellular recognition of bacterial lipopolysaccharides. *Chem Immunol,* 74**,** 61-82.

KLEINSMITH, L. J. & PIERCE, G. B., JR. 1964. Multipotentiality of Single Embryonal Carcinoma Cells. *Cancer Res,* 24**,** 1544-51.

KOBAYASHI, M., KURIHARA, K. & MATSUOKA, I. 1994. Retinoic acid induces BDNF responsiveness of sympathetic neurons by alteration of Trk neurotrophin receptor expression. *FEBS Lett,* 356**,** 60-5.

KOFIDIS, T., DEBRUIN, J. L., TANAKA, M., ZWIERZCHONIEWSKA, M., WEISSMAN, I., FEDOSEYEVA, E., HAVERICH, A. & ROBBINS, R. C. 2005. They are not stealthy in the heart: embryonic stem cells trigger cell infiltration, humoral and T-lymphocyte-based host immune response. *Eur J Cardiothorac Surg,* 28**,** 461-6.

KOGLER, G., SENSKEN, S., AIREY, J. A., TRAPP, T., MUSCHEN, M., FELDHAHN, N., LIEDTKE, S., SORG, R. V., FISCHER, J., ROSENBAUM, C., GRESCHAT, S., KNIPPER, A., BENDER, J., DEGISTIRICI, O., GAO, J., CAPLAN, A. I., COLLETTI, E. J., ALMEIDA-PORADA, G., MULLER, H. W., ZANJANI, E. & WERNET, P. 2004. A new human somatic stem cell from placental cord blood with intrinsic pluripotent differentiation potential. *J Exp Med,* 200**,** 123-35.

KORNACK, D. R. 2000. Neurogenesis and the evolution of cortical diversity: mode, tempo, and partitioning during development and persistence in adulthood. *Brain Behav Evol,* 55**,** 336-44.

KRAUSE, D. S., THEISE, N. D., COLLECTOR, M. I., HENEGARIU, O., HWANG, S., GARDNER, R., NEUTZEL, S. & SHARKIS, S. J. 2001. Multi-organ, multi-lineage engraftment by a single bone marrow-derived stem cell. *Cell,* 105**,** 369-77.

KUCIA, M., HALASA, M., WYSOCZYNSKI, M., BASKIEWICZ-MASIUK, M., MOLDENHAWER, S., ZUBA-SURMA, E., CZAJKA, R., WOJAKOWSKI, W., MACHALINSKI, B. & RATAJCZAK, M. Z. 2007. Morphological and molecular characterization of novel population of CXCR4+ SSEA-4+ Oct-4+ very small embryonic-like cells purified from human cord blood: preliminary report. *Leukemia,* 21**,** 297-303.

KUH, S. U., CHO, Y. E., YOON, D. H., KIM, K. N. & HA, Y. 2005. Functional recovery after human umbilical cord blood cells transplantation with brain-derived neutrophic factor into the spinal cord injured rat. *Acta Neurochir (Wien),* 147**,** 985-92; discussion 992.

KURTZBERG, J. 2009. Update on umbilical cord blood transplantation. *Curr Opin Pediatr,* 21**,** 22-9.

LARSSON, O. M. & SCHOUSBOE, A. 1990. Kinetic characterization of GABA-transaminase from cultured neurons and astrocytes. *Neurochem Res,* 15**,** 1073-7.

LE, M. T., XIE, H., ZHOU, B., CHIA, P. H., RIZK, P., UM, M., UDOLPH, G., YANG, H., LIM, B. & LODISH, H. F. 2009. MicroRNA-125b promotes neuronal differentiation in human cells by repressing multiple targets. *Mol Cell Biol,* 29**,** 5290-305.

LEE, J., KIM, H. K., RHO, J. Y., HAN, Y. M. & KIM, J. 2006. The human OCT-4 isoforms differ in their ability to confer self-renewal. *J Biol Chem,* 281**,** 33554-65.

LEE, J. J., JIN, C. M., KIM, Y. K., RYU, S. Y., LIM, S. C. & LEE, M. K. 2008. Effects of anonaine on dopamine biosynthesis and L-DOPA-induced cytotoxicity in PC12 cells. *Molecules,* 13**,** 475-87.

LEE, M. W., MOON, Y. J., YANG, M. S., KIM, S. K., JANG, I. K., EOM, Y. W., PARK, J. S., KIM, H. C., SONG, K. Y., PARK, S. C., LIM, H. S. & KIM, Y. J. 2007. Neural differentiation of novel multipotent progenitor cells from cryopreserved human umbilical cord blood. *Biochem Biophys Res Commun,* 358**,** 637-43.

LEE, O. K., KUO, T. K., CHEN, W. M., LEE, K. D., HSIEH, S. L. & CHEN, T. H. 2004. Isolation of multipotent mesenchymal stem cells from umbilical cord blood. *Blood,* 103**,** 1669-75.

LEEB, C., JURGA, M., MCGUCKIN, C., MORIGGL, R. & KENNER, L. 2010. Promising new sources for pluripotent stem cells. *Stem Cell Rev,* 6**,** 15-26.

LESAGE, S. & BRICE, A. 2009. Parkinson's disease: from monogenic forms to genetic susceptibility factors. *Hum Mol Genet,* 18**,** R48-59.

LETINIC, K., ZONCU, R. & RAKIC, P. 2002. Origin of GABAergic neurons in the human neocortex. *Nature,* 417**,** 645-9.

LI, C. & KIM, K. 2008. Neuropeptides. *WormBook***,** 1-36.

LI, F. & TSIEN, J. Z. 2009. Memory and the NMDA receptors. *N Engl J Med,* 361**,** 302-3.

LI, P. C., YAO, Q., REN, X., ZHANG, M. C., LI, H., LIU, J. Y., SHENG, S. Y., WANG, Q. & LIU, M. G. 2009. [Analysis of PAX6 gene in a Chinese family with congenital aniridia]. *Zhonghua Yan Ke Za Zhi,* 45**,** 931-4.

LI, X. J., DU, Z. W., ZARNOWSKA, E. D., PANKRATZ, M., HANSEN, L. O., PEARCE, R. A. & ZHANG, S. C. 2005. Specification of motoneurons from human embryonic stem cells. *Nat Biotechnol,* 23**,** 215-21.

LI, X. J., HU, B. Y., JONES, S. A., ZHANG, Y. S., LAVAUTE, T., DU, Z. W. & ZHANG, S. C. 2008. Directed differentiation of ventral spinal progenitors and motor neurons from human embryonic stem cells by small molecules. *Stem Cells,* 26**,** 886-93.

LI, Y., CHEN, J., WANG, L., ZHANG, L., LU, M. & 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.

LIANG, J., WAN, M., ZHANG, Y., GU, P., XIN, H., JUNG, S. Y., QIN, J., WONG, J., COONEY, A. J., LIU, D. & SONGYANG, Z. 2008. Nanog and Oct4 associate with unique transcriptional repression complexes in embryonic stem cells. *Nat Cell Biol,* 10**,** 731-9.

LIN, H. J., O'SHAUGHNESSY, T. J., KELLY, J. & MA, W. 2004. Neural stem cell differentiation in a cell-collagen-bioreactor culture system. *Brain Res Dev Brain Res,* 153**,** 163-73.

LIN, J. H., SAITO, T., ANDERSON, D. J., LANCE-JONES, C., JESSELL, T. M. & ARBER, S. 1998. Functionally related motor neuron pool and muscle sensory afferent subtypes defined by coordinate ETS gene expression. *Cell,* 95**,** 393-407.

LINDNER, U., KRAMER, J., ROHWEDEL, J. & SCHLENKE, P. 2010. Mesenchymal Stem or Stromal Cells: Toward a Better Understanding of Their Biology? *Transfus Med Hemother,* 37**,** 75-83.

LIU, E., LAW, H. K. & LAU, Y. L. 2004. Tolerance associated with cord blood transplantation may depend on the state of host dendritic cells. *Br J Haematol,* 126**,** 517-26.

LOH, Y. H., WU, Q., CHEW, J. L., VEGA, V. B., ZHANG, W., CHEN, X., BOURQUE, G., GEORGE, J., LEONG, B., LIU, J., WONG, K. Y., SUNG, K. W., LEE, C. W., ZHAO, X. D., CHIU, K. P., LIPOVICH, L., KUZNETSOV, V. A., ROBSON, P., STANTON, L. W., WEI, C. L., RUAN, Y., LIM, B. & NG, H. H. 2006. The Oct4 and Nanog transcription network regulates pluripotency in mouse embryonic stem cells. *Nat Genet,* 38**,** 431-40.

LU, D., SANBERG, P. R., MAHMOOD, A., LI, Y., WANG, L., SANCHEZ-RAMOS, J. & CHOPP, M. 2002. Intravenous administration of human umbilical cord blood reduces neurological deficit in the rat after traumatic brain injury. *Cell Transplant,* 11**,** 275-81.

LU, S. & ENDE, N. 1997. Potential for clinical use of viable pluripotent progenitor cells in blood bank stored human umbilical cord blood. *Life Sci,* 61**,** 1113-23.

MA, N., LADILOV, Y., KAMINSKI, A., PIECHACZEK, C., CHOI, Y. H., LI, W., STEINHOFF, G. & STAMM, C. 2006. Umbilical cord blood cell transplantation for myocardial regeneration. *Transplant Proc,* 38**,** 771-3.

MACDONALD, P. E. & RORSMAN, P. 2006. Oscillations, intercellular coupling, and insulin secretion in pancreatic beta cells. *PLoS Biol,* 4**,** e49.

MACDONALD, S. H., RUTH, P., KNAUS, H. G. & SHIPSTON, M. J. 2006. Increased large conductance calcium-activated potassium (BK) channel expression accompanied by STREX variant downregulation in the developing mouse CNS. *BMC Dev Biol,* 6**,** 37.

MACIEJEWSKI, P. K. & ROTHMAN, D. L. 2008. Proposed cycles for functional glutamate trafficking in synaptic neurotransmission. *Neurochem Int,* 52**,** 809-25.

MADEN, M. 2006. Retinoids and spinal cord development. *J Neurobiol,* 66**,** 726-38.

MADEN, M. 2007. Retinoic acid in the development, regeneration and maintenance of the nervous system. *Nat Rev Neurosci,* 8**,** 755-65.

MAEGELE, M. & SCHAEFER, U. 2008. Stem cell-based cellular replacement strategies following traumatic brain injury (TBI). *Minim Invasive Ther Allied Technol,* 17**,** 119-31.

MAHERALI, N., SRIDHARAN, R., XIE, W., UTIKAL, J., EMINLI, S., ARNOLD, K., STADTFELD, M., YACHECHKO, R., TCHIEU, J., JAENISCH, R., PLATH, K. & HOCHEDLINGER, K. 2007. Directly reprogrammed fibroblasts show global epigenetic remodeling and widespread tissue contribution. *Cell Stem Cell,* 1**,** 55-70.

MAHONEY, M. J. & ANSETH, K. S. 2006. Three-dimensional growth and function of neural tissue in degradable polyethylene glycol hydrogels. *Biomaterials,* 27**,** 2265-74.

MAHONEY, M. J. & ANSETH, K. S. 2007. Contrasting effects of collagen and bFGF-2 on neural cell function in degradable synthetic PEG hydrogels. *J Biomed Mater Res A,* 81**,** 269-78.

MAIA, J., SANTOS, T., ADAY, S., AGASSE, F., CORTES, L., MALVA, J. O., BERNARDINO, L. & FERREIRA, L. 2011. Controlling the neuronal differentiation of stem cells by the intracellular delivery of retinoic acid-loaded nanoparticles. *ACS Nano,* 5**,** 97-106.

MALAVASI, F., DEAGLIO, S., FUNARO, A., FERRERO, E., HORENSTEIN, A. L., ORTOLAN, E., VAISITTI, T. & AYDIN, S. 2008. Evolution and function of the ADP ribosyl cyclase/CD38 gene family in physiology and pathology. *Physiol Rev,* 88**,** 841-86.

MANNISTO, P. T. & KAAKKOLA, S. 1999. Catechol-O-methyltransferase (COMT): biochemistry, molecular biology, pharmacology, and clinical efficacy of the new selective COMT inhibitors. *Pharmacol Rev,* 51**,** 593-628.

MARKIEWICZ, I., SYPECKA, J., DOMANSKA-JANIK, K., WYSZOMIRSKI, T. & LUKOMSKA, B. 2011. Cellular environment directs differentiation of human umbilical cord blood-derived neural stem cells in vitro. *J Histochem Cytochem,* 59**,** 289-301.

MARKRAM, H., TOLEDO-RODRIGUEZ, M., WANG, Y., GUPTA, A., SILBERBERG, G. & WU, C. 2004. Interneurons of the neocortical inhibitory system. *Nat Rev Neurosci,* 5**,** 793-807.

MARTEL, M. A., WYLLIE, D. J. & HARDINGHAM, G. E. 2009. In developing hippocampal neurons, NR2B-containing N-methyl-D-aspartate receptors (NMDARs) can mediate signaling to neuronal survival and synaptic potentiation, as well as neuronal death. *Neuroscience,* 158**,** 334-43.

MARTIN, G. R. 1981. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc Natl Acad Sci U S A,* 78**,** 7634-8.

MARTIN, M. J., MUOTRI, A., GAGE, F. & VARKI, A. 2005. Human embryonic stem cells express an immunogenic nonhuman sialic acid. *Nat Med,* 11**,** 228-32.

MASLOW, K. 2010. 2010 Alzheimer's disease facts and figures. *Alzheimers Dement,* 6**,** 158-94.

MASSON, J., SAGNE, C., HAMON, M. & EL MESTIKAWY, S. 1999. Neurotransmitter transporters in the central nervous system. *Pharmacol Rev,* 51**,** 439-64.

MASUI, S., NAKATAKE, Y., TOYOOKA, Y., SHIMOSATO, D., YAGI, R., TAKAHASHI, K., OKOCHI, H., OKUDA, A., MATOBA, R., SHAROV, A. A., KO, M. S. & NIWA, H. 2007. Pluripotency governed by Sox2 via regulation of Oct3/4 expression in mouse embryonic stem cells. *Nat Cell Biol,* 9**,** 625-35.

MATTAR, P., LANGEVIN, L. M., MARKHAM, K., KLENIN, N., SHIVJI, S., ZINYK, D. & SCHUURMANS, C. 2008. Basic helix-loop-helix transcription factors cooperate to specify a cortical projection neuron identity. *Mol Cell Biol,* 28**,** 1456-69.

MATTSON, M. P. 2007. Calcium and neurodegeneration. *Aging Cell,* 6**,** 337-50.

MATTSON, M. P. 2008. Glutamate and neurotrophic factors in neuronal plasticity and disease. *Ann N Y Acad Sci,* 1144**,** 97-112.

MAYANI, H. 2010. Biological differences between neonatal and adult human hematopoietic stem/progenitor cells. *Stem Cells Dev,* 19**,** 285-98.

MAYER, M. L. 2005. Glutamate receptor ion channels. *Curr Opin Neurobiol,* 15**,** 282-8.

MCCONNELL, S. K., GHOSH, A. & SHATZ, C. J. 1989. Subplate neurons pioneer the first axon pathway from the cerebral cortex. *Science,* 245**,** 978-82.

MCGUCKIN, C., FORRAZ, N., BARADEZ, M. O., BASFORD, C., DICKINSON, A. M., NAVRAN, S. & HARTGERINK, J. D. 2006. Embryonic-like stem cells from umbilical cord blood and potential for neural modeling. *Acta Neurobiol Exp (Wars),* 66**,** 321-9.

MCGUCKIN, C., JURGA, M., ALI, H., STRBAD, M. & FORRAZ, N. 2008. Culture of embryonic-like stem cells from human umbilical cord blood and onward differentiation to neural cells in vitro. *Nat Protoc,* 3**,** 1046-55.

MCGUCKIN, C. P., BASFORD, C., HANGER, K., HABIBOLLAH, S. & FORRAZ, N. 2007. Cord blood revelations: the importance of being a first born girl, big, on time and to a young mother! *Early Hum Dev,* 83**,** 733-41.

MCGUCKIN, C. P. & FORRAZ, N. 2008. Potential for access to embryonic-like cells from human umbilical cord blood. *Cell Prolif,* 41 Suppl 1**,** 31-40.

MCGUCKIN, C. P., FORRAZ, N., ALLOUARD, Q. & PETTENGELL, R. 2004. Umbilical cord blood stem cells can expand hematopoietic and neuroglial progenitors in vitro. *Exp Cell Res,* 295**,** 350-9.

MCGUCKIN, C. P., FORRAZ, N., BARADEZ, M. O., NAVRAN, S., ZHAO, J., URBAN, R., TILTON, R. & DENNER, L. 2005. Production of stem cells with embryonic characteristics from human umbilical cord blood. *Cell Prolif,* 38**,** 245-55.

MCGUCKIN, C. P., PEARCE, D., FORRAZ, N., TOOZE, J. A., WATT, S. M. & PETTENGELL, R. 2003. Multiparametric analysis of immature cell populations in umbilical cord blood and bone marrow. *Eur J Haematol,* 71**,** 341-50.

MEIER, C., MIDDELANIS, J., WASIELEWSKI, B., NEUHOFF, S., ROTH-HAERER, A., GANTERT, M., DINSE, H. R., DERMIETZEL, R. & JENSEN, A. 2006. Spastic paresis after perinatal brain damage in rats is reduced by human cord blood mononuclear cells. *Pediatr Res,* 59**,** 244-9.

MEIRI, K. F., PFENNINGER, K. H. & WILLARD, M. B. 1986. Growth-associated protein, GAP-43, a polypeptide that is induced when neurons extend axons, is a component of growth cones and corresponds to pp46, a major polypeptide of a subcellular fraction enriched in growth cones. *Proc Natl Acad Sci U S A,* 83**,** 3537-41.

MELDRUM, B. S. 2000. Glutamate as a neurotransmitter in the brain: review of physiology and pathology. *J Nutr,* 130**,** 1007S-15S.

MELNIKOVA, I. 2007. Therapies for Alzheimer's disease. *Nat Rev Drug Discov,* 6**,** 341-2.

MENN, B., GARCIA-VERDUGO, J. M., YASCHINE, C., GONZALEZ-PEREZ, O., ROWITCH, D. & ALVAREZ-BUYLLA, A. 2006. Origin of oligodendrocytes in the subventricular zone of the adult brain. *J Neurosci,* 26**,** 7907-18.

MESSAM, C. A., HOU, J., BERMAN, J. W. & MAJOR, E. O. 2002. Analysis of the temporal expression of nestin in human fetal brain derived neuronal and glial progenitor cells. *Brain Res Dev Brain Res,* 134**,** 87-92.

MESSAM, C. A., HOU, J. & MAJOR, E. O. 2000. Coexpression of nestin in neural and glial cells in the developing human CNS defined by a human-specific anti-nestin antibody. *Exp Neurol,* 161**,** 585-96.

MEY, J. & MCCAFFERY, P. 2004. Retinoic acid signaling in the nervous system of adult vertebrates. *Neuroscientist,* 10**,** 409-21.

MEYER, G. 2001. Human neocortical development: the importance of embryonic and early fetal events. *Neuroscientist,* 7**,** 303-14.

MEYER, G., SCHAAPS, J. P., MOREAU, L. & GOFFINET, A. M. 2000. Embryonic and early fetal development of the human neocortex. *J Neurosci,* 20**,** 1858-68.

MILLER, C. 2000. An overview of the potassium channel family. *Genome Biol,* 1**,** REVIEWS0004.

MING, G. L. & SONG, H. 2005. Adult neurogenesis in the mammalian central nervous system. *Annu Rev Neurosci,* 28**,** 223-50.

MISHRA, R., GUPTA, S. K., MEIRI, K. F., FONG, M., THOSTRUP, P., JUNCKER, D. & MANI, S. 2008. GAP-43 is key to mitotic spindle control and centrosome-based polarization in neurons. *Cell Cycle,* 7**,** 348-57.

MITALIPOV, S. & WOLF, D. 2009. Totipotency, pluripotency and nuclear reprogramming. *Adv Biochem Eng Biotechnol,* 114**,** 185-99.

MITALIPOVA, M., CALHOUN, J., SHIN, S., WININGER, D., SCHULZ, T., NOGGLE, S., VENABLE, A., LYONS, I., ROBINS, A. & STICE, S. 2003. Human embryonic stem cell lines derived from discarded embryos. *Stem Cells,* 21**,** 521-6.

MITSUI, K., TOKUZAWA, Y., ITOH, H., SEGAWA, K., MURAKAMI, M., TAKAHASHI, K., MARUYAMA, M., MAEDA, M. & YAMANAKA, S. 2003. The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. *Cell,* 113**,** 631-42.

MOCHIZUKI, K., KIKUTA, A., ITO, M., AKAIHATA, M., SANO, H., OHTO, H. & HOSOYA, M. 2008. Successful unrelated cord blood transplantation for chronic granulomatous disease: A case report and review of the literature. *Pediatr Transplant*.

MOHR, J. P., THOMPSON, J. L., LAZAR, R. M., LEVIN, B., SACCO, R. L., FURIE, K. L., KISTLER, J. P., ALBERS, G. W., PETTIGREW, L. C., ADAMS, H. P., JR., JACKSON, C. M. & PULLICINO, P. 2001. A comparison of warfarin and aspirin for the prevention of recurrent ischemic stroke. *N Engl J Med,* 345**,** 1444-51.

MORGENSTERN, B. & ATCHLEY, W. R. 1999. Evolution of bHLH transcription factors: modular evolution by domain shuffling? *Mol Biol Evol,* 16**,** 1654-63.

MULLEN, R. J., BUCK, C. R. & SMITH, A. M. 1992. NeuN, a neuronal specific nuclear protein in vertebrates. *Development,* 116**,** 201-11.

MULLER-SIEBURG, C. E., CHO, R. H., THOMAN, M., ADKINS, B. & SIEBURG, H. B. 2002. Deterministic regulation of hematopoietic stem cell self-renewal and differentiation. *Blood,* 100**,** 1302-9.

MURAYAMA, M., MIYAZAKI, K., KUDO, Y., MIYAKAWA, H. & INOUE, M. 2005. Optical monitoring of progressive synchronization in dentate granule cells during population burst activities. *Eur J Neurosci,* 21**,** 3349-60.

MURER, M. G., YAN, Q. & RAISMAN-VOZARI, R. 2001. Brain-derived neurotrophic factor in the control human brain, and in Alzheimer's disease and Parkinson's disease. *Prog Neurobiol,* 63**,** 71-124.

MURRAY, K. & DUBOIS-DALCQ, M. 1997. Emergence of oligodendrocytes from human neural spheres. *J Neurosci Res,* 50**,** 146-56.

NAGY, A., ROSSANT, J., NAGY, R., ABRAMOW-NEWERLY, W. & RODER, J. C. 1993. Derivation of completely cell culture-derived mice from early-passage embryonic stem cells. *Proc Natl Acad Sci U S A,* 90**,** 8424-8.

NAKAGAWA, M., KOYANAGI, M., TANABE, K., TAKAHASHI, K., ICHISAKA, T., AOI, T., OKITA, K., MOCHIDUKI, Y., TAKIZAWA, N. & YAMANAKA, S. 2008. Generation of induced pluripotent stem cells without Myc from mouse and human fibroblasts. *Nat Biotechnol,* 26**,** 101-6.

NAKAGAWA, S., KIM, J. E., LEE, R., MALBERG, J. E., CHEN, J., STEFFEN, C., ZHANG, Y. J., NESTLER, E. J. & DUMAN, R. S. 2002. Regulation of neurogenesis in adult mouse hippocampus by cAMP and the cAMP response element-binding protein. *J Neurosci,* 22**,** 3673-82.

NAN, Z., GRANDE, A., SANBERG, C. D., SANBERG, P. R. & LOW, W. C. 2005. Infusion of human umbilical cord blood ameliorates neurologic deficits in rats with hemorrhagic brain injury. *Ann N Y Acad Sci,* 1049**,** 84-96.

NAVEILHAN, P., NEVEU, I., ARENAS, E. & ERNFORS, P. 1998. Complementary and overlapping expression of Y1, Y2 and Y5 receptors in the developing and adult mouse nervous system. *Neuroscience,* 87**,** 289-302.

NEET, K. E. & THINAKARAN, G. 2008. Thematic minireview series on the molecular basis of Alzheimer disease. *J Biol Chem,* 283**,** 29613-4.

NERN, C. & MOMMA, S. 2006. The realized niche of adult neural stem cells. *Stem Cell Rev,* 2**,** 233-40.

NEUHOFF, S., MOERS, J., RIEKS, M., GRUNWALD, T., JENSEN, A., DERMIETZEL, R. & MEIER, C. 2007. Proliferation, differentiation, and cytokine secretion of human umbilical cord blood-derived mononuclear cells in vitro. *Exp Hematol,* 35**,** 1119-31.

NEWCOMB, J. D., AJMO, C. T., JR., SANBERG, C. D., SANBERG, P. R., PENNYPACKER, K. R. & WILLING, A. E. 2006. Timing of cord blood treatment after experimental stroke determines therapeutic efficacy. *Cell Transplant,* 15**,** 213-23.

NICHOLS, J., ZEVNIK, B., ANASTASSIADIS, K., NIWA, H., KLEWE-NEBENIUS, D., CHAMBERS, I., SCHOLER, H. & SMITH, A. 1998. Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. *Cell,* 95**,** 379-91.

NIE, H. & WENG, H. R. 2009. Glutamate transporters prevent excessive activation of NMDA receptors and extrasynaptic glutamate spillover in the spinal dorsal horn. *J Neurophysiol,* 101**,** 2041-51.

NIEUWENHUYS, R., VOOGD, J. & HUIJZEN, C. V. 2008. *The human central nervous system,* New York, Springer.

NIKOLIC, W. V., HOU, H., TOWN, T., ZHU, Y., GIUNTA, B., SANBERG, C. D., ZENG, J., LUO, D., EHRHART, J., MORI, T., SANBERG, P. R. & TAN, J. 2008. Peripherally administered

human umbilical cord blood cells reduce parenchymal and vascular beta-amyloid deposits in Alzheimer mice. *Stem Cells Dev,* 17**,** 423-39.

NIWA, H., MIYAZAKI, J. & SMITH, A. G. 2000. Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. *Nat Genet,* 24**,** 372-6.

NOCTOR, S. C., FLINT, A. C., WEISSMAN, T. A., WONG, W. S., CLINTON, B. K. & KRIEGSTEIN, A. R. 2002. Dividing precursor cells of the embryonic cortical ventricular zone have morphological and molecular characteristics of radial glia. *J Neurosci,* 22**,** 3161-73.

NOCTOR, S. C., MARTINEZ-CERDENO, V. & KRIEGSTEIN, A. R. 2007. Contribution of intermediate progenitor cells to cortical histogenesis. *Arch Neurol,* 64**,** 639-42.

NOLL, E. & MILLER, R. H. 1994. Regulation of oligodendrocyte differentiation: a role for retinoic acid in the spinal cord. *Development,* 120**,** 649-60.

NOWAK, L., BREGESTOVSKI, P., ASCHER, P., HERBET, A. & PROCHIANTZ, A. 1984. Magnesium gates glutamate-activated channels in mouse central neurones. *Nature,* 307**,** 462-5.

O'KEEFFE, G. W., GUTIERREZ, H., PANDOLFI, P. P., RICCARDI, C. & DAVIES, A. M. 2008. NGF-promoted axon growth and target innervation requires GITRL-GITR signaling. *Nat Neurosci,* 11**,** 135-42.

ODORICO, J. S., KAUFMAN, D. S. & THOMSON, J. A. 2001. Multilineage differentiation from human embryonic stem cell lines. *Stem Cells,* 19**,** 193-204.

OH, J., RECKNOR, J. B., RECKNOR, J. C., MALLAPRAGADA, S. K. & SAKAGUCHI, D. S. 2009. Soluble factors from neocortical astrocytes enhance neuronal differentiation of neural progenitor cells from adult rat hippocampus on micropatterned polymer substrates. *J Biomed Mater Res A,* 91**,** 575-85.

OKITA, K., ICHISAKA, T. & YAMANAKA, S. 2007. Generation of germline-competent induced pluripotent stem cells. *Nature,* 448**,** 313-7.

OKUMURA-NAKANISHI, S., SAITO, M., NIWA, H. & ISHIKAWA, F. 2005. Oct-3/4 and Sox2 regulate Oct-3/4 gene in embryonic stem cells. *J Biol Chem,* 280**,** 5307-17.

PALMADA, M. & CENTELLES, J. J. 1998. Excitatory amino acid neurotransmission. Pathways for metabolism, storage and reuptake of glutamate in brain. *Front Biosci,* 3**,** d701-18.

PAPAGIANNAKOPOULOS, T. & KOSIK, K. S. 2009. MicroRNA-124: micromanager of neurogenesis. *Cell Stem Cell,* 4**,** 375-6.

PARDO, M., LANG, B., YU, L., PROSSER, H., BRADLEY, A., BABU, M. M. & CHOUDHARY, J. 2010. An expanded Oct4 interaction network: implications for stem cell biology, development, and disease. *Cell Stem Cell,* 6**,** 382-95.

PARPURA, V. & HAYDON, P. G. 2000. Physiological astrocytic calcium levels stimulate glutamate release to modulate adjacent neurons. *Proc Natl Acad Sci U S A,* 97**,** 8629-34.

PAULSEN, M. T. & LJUNGMAN, M. 2005. The natural toxin juglone causes degradation of p53 and induces rapid H2AX phosphorylation and cell death in human fibroblasts. *Toxicol Appl Pharmacol,* 209**,** 1-9.

PERLMAN, J. M. 2006. Summary proceedings from the neurology group on hypoxic-ischemic encephalopathy. *Pediatrics,* 117**,** S28-33.

PERRUISSEAU-CARRIER, C., JURGA, M., FORRAZ, N. & MCGUCKIN, C. P. 2011. miRNAs stem cell reprogramming for neuronal induction and differentiation. *Mol Neurobiol,* 43**,** 215-27.

PETIT, E., LANGOUET, S., AKHDAR, H., NICOLAS-NICOLAZ, C., GUILLOUZO, A. & MOREL, F. 2008. Differential toxic effects of azathioprine, 6-mercaptopurine and 6-thioguanine on human hepatocytes. *Toxicol In Vitro,* 22**,** 632-42.

PICK, M., AZZOLA, L., MOSSMAN, A., STANLEY, E. G. & ELEFANTY, A. G. 2007. Differentiation of human embryonic stem cells in serum-free medium reveals distinct roles for

bone morphogenetic protein 4, vascular endothelial growth factor, stem cell factor, and fibroblast growth factor 2 in hematopoiesis. *Stem Cells,* 25**,** 2206-14.

PIPES, B. L., TSANG, T., PENG, S. X., FIEDERLEIN, R., GRAHAM, M. & HARRIS, D. T. 2006. Telomere length changes after umbilical cord blood transplant. *Transfusion,* 46**,** 1038-43.

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. & MARSHAK, D. R. 1999. Multilineage potential of adult human mesenchymal stem cells. *Science,* 284**,** 143-7.

PLATEL, J. C., DAVE, K. A., GORDON, V., LACAR, B., RUBIO, M. E. & BORDEY, A. 2010. NMDA receptors activated by subventricular zone astrocytic glutamate are critical for neuroblast survival prior to entering a synaptic network. *Neuron,* 65**,** 859-72.

POO, M. M. 2001. Neurotrophins as synaptic modulators. *Nat Rev Neurosci,* 2**,** 24-32.

PORCHER, C., LIAO, E. C., FUJIWARA, Y., ZON, L. I. & ORKIN, S. H. 1999. Specification of hematopoietic and vascular development by the bHLH transcription factor SCL without direct DNA binding. *Development,* 126**,** 4603-15.

POVLISHOCK, J. T. & KATZ, D. I. 2005. Update of neuropathology and neurological recovery after traumatic brain injury. *J Head Trauma Rehabil,* 20**,** 76-94.

PROKHOROVA, T. A., HARKNESS, L. M., FRANDSEN, U., DITZEL, N., SCHRODER, H. D., BURNS, J. S. & KASSEM, M. 2009. Teratoma formation by human embryonic stem cells is site dependent and enhanced by the presence of matrigel. *Stem Cells Dev,* 18**,** 47-54.

PURVES, D. 2004. *Neuroscience,* Sunderland, Mass., Sinauer Associates, Publishers.

QUINN, J. C., MOLINEK, M., NOWAKOWSKI, T. J., MASON, J. O. & PRICE, D. J. 2010. Novel lines of Pax6-/- embryonic stem cells exhibit reduced neurogenic capacity without loss of viability. *BMC Neurosci,* 11**,** 26.

RAHMAN, A.-U. 1988. *Studies in natural products chemistry,* Amsterdam ; New York, Elsevier.

RAKIC, P. 2003. Developmental and evolutionary adaptations of cortical radial glia. *Cereb Cortex,* 13**,** 541-9.

RAY, J., PETERSON, D. A., SCHINSTINE, M. & GAGE, F. H. 1993. Proliferation, differentiation, and long-term culture of primary hippocampal neurons. *Proc Natl Acad Sci U S A,* 90**,** 3602-6.

RAYMOND, K., DEUGNIER, M. A., FARALDO, M. M. & GLUKHOVA, M. A. 2009. Adhesion within the stem cell niches. *Curr Opin Cell Biol,* 21**,** 623-9.

REED, W., SMITH, R., DEKOVIC, F., LEE, J. Y., SABA, J. D., TRACHTENBERG, E., EPSTEIN, J., HAAZ, S., WALTERS, M. C. & LUBIN, B. H. 2003. Comprehensive banking of sibling donor cord blood for children with malignant and nonmalignant disease. *Blood,* 101**,** 351-7.

REYNOLDS, B. A., TETZLAFF, W. & WEISS, S. 1992. A multipotent EGF-responsive striatal embryonic progenitor cell produces neurons and astrocytes. *J Neurosci,* 12**,** 4565-74.

REYNOLDS, B. A. & WEISS, S. 1992. Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. *Science,* 255**,** 1707-10.

REYNOLDS, B. A. & WEISS, S. 1996. Clonal and population analyses demonstrate that an EGF-responsive mammalian embryonic CNS precursor is a stem cell. *Dev Biol,* 175**,** 1-13.

RICHARDS, L. J., KILPATRICK, T. J. & BARTLETT, P. F. 1992. De novo generation of neuronal cells from the adult mouse brain. *Proc Natl Acad Sci U S A,* 89**,** 8591-5.

RICHARDSON, R. M., SINGH, A., SUN, D., FILLMORE, H. L., DIETRICH, D. W., 3RD & BULLOCK, M. R. 2010. Stem cell biology in traumatic brain injury: effects of injury and strategies for repair. *J Neurosurg,* 112**,** 1125-38.

RIETJENS, I. M., BOERSMA, M. G., ZALESKA, M. & PUNT, A. 2008. Differences in simulated liver concentrations of toxic coumarin metabolites in rats and different human populations evaluated through physiologically based biokinetic (PBBK) modeling. *Toxicol In Vitro,* 22**,** 1890-901.

RINGDEN, O., OKAS, M., UHLIN, M., UZUNEL, M., REMBERGER, M. & MATTSSON, J. 2008. Unrelated cord blood and mismatched unrelated volunteer donor transplants, two alternatives in patients who lack an HLA-identical donor. *Bone Marrow Transplant,* 42**,** 643-8.

RIQUELME, P. A., DRAPEAU, E. & DOETSCH, F. 2008. Brain micro-ecologies: neural stem cell niches in the adult mammalian brain. *Philos Trans R Soc Lond B Biol Sci,* 363**,** 123-37.

ROCK, R. B., GEKKER, G., HU, S., SHENG, W. S., CHEERAN, M., LOKENSGARD, J. R. & PETERSON, P. K. 2004. Role of microglia in central nervous system infections. *Clin Microbiol Rev,* 17**,** 942-64, table of contents.

RODDA, D. J., CHEW, J. L., LIM, L. H., LOH, Y. H., WANG, B., NG, H. H. & ROBSON, P. 2005. Transcriptional regulation of nanog by OCT4 and SOX2. *J Biol Chem,* 280**,** 24731-7.

RODRIGUEZ, M. 2003. A function of myelin is to protect axons from subsequent injury: implications for deficits in multiple sclerosis. *Brain,* 126**,** 751-2.

ROSENFELD, N., ELOWITZ, M. B. & ALON, U. 2002. Negative autoregulation speeds the response times of transcription networks. *J Mol Biol,* 323**,** 785-93.

ROSENTHAL, M. D., WISHNOW, R. M. & SATO, G. H. 1970. In vitro growth and differetiation of clonal populations of multipotential mouse clls derived from a transplantable testicular teratocarcinoma. *J Natl Cancer Inst,* 44**,** 1001-14.

ROTHSTEIN, J. D., DYKES-HOBERG, M., PARDO, C. A., BRISTOL, L. A., JIN, L., KUNCL, R. W., KANAI, Y., HEDIGER, M. A., WANG, Y., SCHIELKE, J. P. & WELTY, D. F. 1996. Knockout of glutamate transporters reveals a major role for astroglial transport in excitotoxicity and clearance of glutamate. *Neuron,* 16**,** 675-86.

ROYBON, L., MASTRACCI, T. L., RIBEIRO, D., SUSSEL, L., BRUNDIN, P. & LI, J. Y. 2010. GABAergic differentiation induced by Mash1 is compromised by the bHLH proteins Neurogenin2, NeuroD1, and NeuroD2. *Cereb Cortex,* 20**,** 1234-44.

RUBINSZTEIN, D. C. 2006. The roles of intracellular protein-degradation pathways in neurodegeneration. *Nature,* 443**,** 780-6.

RYGLEWSKI, S., PFLUEGER, H. J. & DUCH, C. 2007. Expanding the neuron's calcium signaling repertoire: intracellular calcium release via voltage-induced PLC and IP3R activation. *PLoS Biol,* 5**,** e66.

SALMINA, A. B. 2009. Neuron-glia interactions as therapeutic targets in neurodegeneration. *J Alzheimers Dis,* 16**,** 485-502.

SANGEETHA, V. M., KALE, V. P. & LIMAYE, L. S. 2010. Expansion of cord blood CD34 cells in presence of zVADfmk and zLLYfmk improved their in vitro functionality and in vivo engraftment in NOD/SCID mouse. *PLoS One,* 5**,** e12221.

SAPIENZA, C. 2002. Imprinted gene expression, transplantation medicine, and the "other" human embryonic stem cell. *Proc Natl Acad Sci U S A,* 99**,** 10243-5.

SAPORTA, S., KIM, J. J., WILLING, A. E., FU, E. S., DAVIS, C. D. & SANBERG, P. R. 2003. Human umbilical cord blood stem cells infusion in spinal cord injury: engraftment and beneficial influence on behavior. *J Hematother Stem Cell Res,* 12**,** 271-8.

SARNAT, H. B., NOCHLIN, D. & BORN, D. E. 1998. Neuronal nuclear antigen (NeuN): a marker of neuronal maturation in early human fetal nervous system. *Brain Dev,* 20**,** 88-94.

SARUHASHI, Y., YOUNG, W., HASSAN, A. Z. & PARK, R. 1994. Excitatory and inhibitory effects of serotonin on spinal axons. *Neuroscience,* 61**,** 645-53.

SCHAPIRA, A. H. 2005. Present and future drug treatment for Parkinson's disease. *J Neurol Neurosurg Psychiatry,* 76**,** 1472-8.

SCHMITT, A., ASAN, E., LESCH, K. P. & KUGLER, P. 2002. A splice variant of glutamate transporter GLT1/EAAT2 expressed in neurons: cloning and localization in rat nervous system. *Neuroscience,* 109**,** 45-61.

SCHROEDER, J. T. 2009. Basophils beyond effector cells of allergic inflammation. *Adv Immunol,* 101**,** 123-61.

SCHUURMANS, C., ARMANT, O., NIETO, M., STENMAN, J. M., BRITZ, O., KLENIN, N., BROWN, C., LANGEVIN, L. M., SEIBT, J., TANG, H., CUNNINGHAM, J. M., DYCK, R., WALSH, C., CAMPBELL, K., POLLEUX, F. & GUILLEMOT, F. 2004. Sequential phases of cortical specification involve Neurogenin-dependent and -independent pathways. *EMBO J,* 23**,** 2892-902.

SCHWARTZ, N. E. & ALBERS, G. W. 2010. Dabigatran challenges warfarin's superiority for stroke prevention in atrial fibrillation. *Stroke,* 41**,** 1307-9.

SEMPOWSKI, G. D., LEE, D. M., KAUFMAN, R. E. & HAYNES, B. F. 1999. Structure and function of the CD7 molecule. *Crit Rev Immunol,* 19**,** 331-48.

SENEGAGLIA, A. C., BARBOZA, L. A., DALLAGIOVANNA, B., AITA, C. A., HANSEN, P., REBELATTO, C. L., AGUIAR, A. M., MIYAGUE, N. I., SHIGUNOV, P., BARCHIKI, F., CORREA, A., OLANDOSKI, M., KRIEGER, M. A. & BROFMAN, P. R. 2010. Are purified or expanded cord blood-derived CD133+ cells better at improving cardiac function? *Exp Biol Med (Maywood),* 235**,** 119-29.

SESSA, A., MAO, C. A., COLASANTE, G., NINI, A., KLEIN, W. H. & BROCCOLI, V. 2010. Tbr2-positive intermediate (basal) neuronal progenitors safeguard cerebral cortex expansion by controlling amplification of pallial glutamatergic neurons and attraction of subpallial GABAergic interneurons. *Genes Dev,* 24**,** 1816-26.

SESSA, A., MAO, C. A., HADJANTONAKIS, A. K., KLEIN, W. H. & BROCCOLI, V. 2008. Tbr2 directs conversion of radial glia into basal precursors and guides neuronal amplification by indirect neurogenesis in the developing neocortex. *Neuron,* 60**,** 56-69.

SHEN, L. H., LI, Y., CHEN, J., CUI, Y., ZHANG, C., KAPKE, A., LU, M., SAVANT-BHONSALE, S. & CHOPP, M. 2007. One-year follow-up after bone marrow stromal cell treatment in middle-aged female rats with stroke. *Stroke,* 38**,** 2150-6.

SHEN, Q. & TEMPLE, S. 2009. Fine control: microRNA regulation of adult neurogenesis. *Nat Neurosci,* 12**,** 369-70.

SHIPP, S. 2007. Structure and function of the cerebral cortex. *Curr Biol,* 17**,** R443-9.

SHORT, B., BROUARD, N., OCCHIODORO-SCOTT, T., RAMAKRISHNAN, A. & SIMMONS, P. J. 2003. Mesenchymal stem cells. *Arch Med Res,* 34**,** 565-71.

SILLS, E. S., TAKEUCHI, T., TANAKA, N., NERI, Q. V. & PALERMO, G. D. 2005. Identification and isolation of embryonic stem cells in reproductive endocrinology: theoretical protocols for conservation of human embryos derived from in vitro fertilization. *Theor Biol Med Model,* 2**,** 25.

SILVA, A. P., PINHEIRO, P. S., CARVALHO, A. P., CARVALHO, C. M., JAKOBSEN, B., ZIMMER, J. & MALVA, J. O. 2003. Activation of neuropeptide Y receptors is neuroprotective against excitotoxicity in organotypic hippocampal slice cultures. *Faseb J,* 17**,** 1118-20.

SILVA, J., NICHOLS, J., THEUNISSEN, T. W., GUO, G., VAN OOSTEN, A. L., BARRANDON, O., WRAY, J., YAMANAKA, S., CHAMBERS, I. & SMITH, A. 2009. Nanog is the gateway to the pluripotent ground state. *Cell,* 138**,** 722-37.

SINDEN, J. D. 2006. ReNeuron Group plc. *Regen Med,* 1**,** 143-7.

SLATTER, M. A., BHATTACHARYA, A., FLOOD, T. J., ABINUN, M., CANT, A. J. & GENNERY, A. R. 2006. Use of two unrelated umbilical cord stem cell units in stem cell transplantation for Wiskott-Aldrich syndrome. *Pediatr Blood Cancer,* 47**,** 332-4.

SLATTER, M. A. & GENNERY, A. R. 2006. Umbilical cord stem cell transplantation for primary immunodeficiencies. *Expert Opin Biol Ther,* 6**,** 555-65.

SMITH, K. P., LUONG, M. X. & STEIN, G. S. 2009. Pluripotency: toward a gold standard for human ES and iPS cells. *J Cell Physiol,* 220**,** 21-9.

SOHUR, U. S., EMSLEY, J. G., MITCHELL, B. D. & MACKLIS, J. D. 2006. Adult neurogenesis and cellular brain repair with neural progenitors, precursors and stem cells. *Philos Trans R Soc Lond B Biol Sci,* 361**,** 1477-97.

SONG, H., STEVENS, C. F. & GAGE, F. H. 2002. Astroglia induce neurogenesis from adult neural stem cells. *Nature,* 417**,** 39-44.

SPANDIDOS, A., WANG, X., WANG, H., DRAGNEV, S., THURBER, T. & SEED, B. 2008. A comprehensive collection of experimentally validated primers for Polymerase Chain Reaction quantitation of murine transcript abundance. *BMC Genomics,* 9**,** 633.

SPITS, C., MATEIZEL, I., GEENS, M., MERTZANIDOU, A., STAESSEN, C., VANDESKELDE, Y., VAN DER ELST, J., LIEBAERS, I. & SERMON, K. 2008. Recurrent chromosomal abnormalities in human embryonic stem cells. *Nat Biotechnol,* 26**,** 1361-3.

STANEVSKY, A., GOLDSTEIN, G. & NAGLER, A. 2009. Umbilical cord blood transplantation: pros, cons and beyond. *Blood Rev,* 23**,** 199-204.

STANIC, D., PARATCHA, G., LEDDA, F., HERZOG, H., KOPIN, A. S. & HOKFELT, T. 2008. Peptidergic influences on proliferation, migration, and placement of neural progenitors in the adult mouse forebrain. *Proc Natl Acad Sci U S A,* 105**,** 3610-5.

STEVENS, L. C. & LITTLE, C. C. 1954. Spontaneous Testicular Teratomas in an Inbred Strain of Mice. *Proc Natl Acad Sci U S A,* 40**,** 1080-7.

STEWART, R., STOJKOVIC, M. & LAKO, M. 2006. Mechanisms of self-renewal in human embryonic stem cells. *Eur J Cancer,* 42**,** 1257-72.

STEWART, R. J., CHEN, B., DOWLATSHAHI, D., MACQUEEN, G. M. & YOUNG, L. T. 2001. Abnormalities in the cAMP signaling pathway in post-mortem brain tissue from the Stanley Neuropathology Consortium. *Brain Res Bull,* 55**,** 625-9.

STREIT, W. J., MRAK, R. E. & GRIFFIN, W. S. 2004. Microglia and neuroinflammation: a pathological perspective. *J Neuroinflammation,* 1**,** 14.

SUH, H., CONSIGLIO, A., RAY, J., SAWAI, T., D'AMOUR, K. A. & GAGE, F. H. 2007. In Vivo Fate Analysis Reveals the Multipotent and Self-Renewal Capacities of Sox2(+) Neural Stem Cells in the Adult Hippocampus. *Cell Stem Cell,* 1**,** 515-528.

SUKHOTINSKY, I., REINER, K., GOVRIN-LIPPMANN, R., BELENKY, M., LU, J., HOPKINS, D. A., SAPER, C. B. & DEVOR, M. 2006. Projections from the mesopontine tegmental anesthesia area to regions involved in pain modulation. *J Chem Neuroanat,* 32**,** 159-78.

SUMMERS, C. R., IVINS, B. & SCHWAB, K. A. 2009. Traumatic brain injury in the United States: an epidemiologic overview. *Mt Sinai J Med,* 76**,** 105-10.

SUN, W., BUZANSKA, L., DOMANSKA-JANIK, K., SALVI, R. J. & STACHOWIAK, M. K. 2005. Voltage-sensitive and ligand-gated channels in differentiating neural stem-like cells derived from the nonhematopoietic fraction of human umbilical cord blood. *Stem Cells,* 23**,** 931-45.

SUZUKI, S., TERAUCHI, M., UMEZU, T., KAJIYAMA, H., SHIBATA, K., NAWA, A. & KIKKAWA, F. 2010. Identification and characterization of cancer stem cells in ovarian yolk sac tumors. *Cancer Sci*.

SWIJNENBURG, R. J., SCHREPFER, S., GOVAERT, J. A., CAO, F., RANSOHOFF, K., SHEIKH, A. Y., HADDAD, M., CONNOLLY, A. J., DAVIS, M. M., ROBBINS, R. C. & WU, J. C. 2008.

Immunosuppressive therapy mitigates immunological rejection of human embryonic stem cell xenografts. *Proc Natl Acad Sci U S A,* 105**,** 12991-6.

SWIJNENBURG, R. J., TANAKA, M., VOGEL, H., BAKER, J., KOFIDIS, T., GUNAWAN, F., LEBL, D. R., CAFFARELLI, A. D., DE BRUIN, J. L., FEDOSEYEVA, E. V. & ROBBINS, R. C. 2005. Embryonic stem cell immunogenicity increases upon differentiation after transplantation into ischemic myocardium. *Circulation,* 112**,** I166-72.

TAKAHASHI, J., PALMER, T. D. & GAGE, F. H. 1999. Retinoic acid and neurotrophins collaborate to regulate neurogenesis in adult-derived neural stem cell cultures. *J Neurobiol,* 38**,** 65-81.

TAKAHASHI, K., TANABE, K., OHNUKI, M., NARITA, M., ICHISAKA, T., TOMODA, K. & YAMANAKA, S. 2007. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell,* 131**,** 861-72.

TAKAHASHI, K. & YAMANAKA, S. 2006. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell,* 126**,** 663-76.

TAO, X., AVALOS, J. L., CHEN, J. & MACKINNON, R. 2009. Crystal structure of the eukaryotic strong inward-rectifier K+ channel Kir2.2 at 3.1 A resolution. *Science,* 326**,** 1668-74.

TAUBE, J. S. 2007. The head direction signal: origins and sensory-motor integration. *Annu Rev Neurosci,* 30**,** 181-207.

TAUPIN, P. 2006. Adult neural stem cells, neurogenic niches, and cellular therapy. *Stem Cell Rev,* 2**,** 213-9.

TAVAZOIE, M., VAN DER VEKEN, L., SILVA-VARGAS, V., LOUISSAINT, M., COLONNA, L., ZAIDI, B., GARCIA-VERDUGO, J. M. & DOETSCH, F. 2008. A specialized vascular niche for adult neural stem cells. *Cell Stem Cell,* 3**,** 279-88.

TEMBURNI, M. K. & JACOB, M. H. 2001. New functions for glia in the brain. *Proc Natl Acad Sci U S A,* 98**,** 3631-2.

THIRIET, N., AGASSE, F., NICOLEAU, C., GUEGAN, C., VALLETTE, F., CADET, J. L., JABER, M., MALVA, J. O. & CORONAS, V. 2011. NPY promotes chemokinesis and neurogenesis in the rat subventricular zone. *J Neurochem,* 116**,** 1018-27.

THOMAS, B. & BEAL, M. F. 2007. Parkinson's disease. *Hum Mol Genet,* 16 Spec No. 2**,** R183-94.

THOMSON, J. A., ITSKOVITZ-ELDOR, J., SHAPIRO, S. S., WAKNITZ, M. A., SWIERGIEL, J. J., MARSHALL, V. S. & JONES, J. M. 1998. Embryonic stem cell lines derived from human blastocysts. *Science,* 282**,** 1145-7.

TOLIAS, C. M. & BULLOCK, M. R. 2004. Critical appraisal of neuroprotection trials in head injury: what have we learned? *NeuroRx,* 1**,** 71-9.

TREVELYAN, A. J., KIRBY, D. M., SMULDERS-SRINIVASAN, T. K., NOOTEBOOM, M., ACIN-PEREZ, R., ENRIQUEZ, J. A., WHITTINGTON, M. A., LIGHTOWLERS, R. N. & TURNBULL, D. M. 2010. Mitochondrial DNA mutations affect calcium handling in differentiated neurons. *Brain,* 133**,** 787-96.

TROWBRIDGE, I. S. & THOMAS, M. L. 1994. CD45: an emerging role as a protein tyrosine phosphatase required for lymphocyte activation and development. *Annu Rev Immunol,* 12**,** 85-116.

TSE, W. & LAUGHLIN, M. J. 2005. Umbilical cord blood transplantation: a new alternative option. *Hematology Am Soc Hematol Educ Program***,** 377-83.

TUCH, B. E. 2006. Stem cells--a clinical update. *Aust Fam Physician,* 35**,** 719-21.

TUSZYNSKI, M. H. 2007. Nerve growth factor gene therapy in Alzheimer disease. *Alzheimer Dis Assoc Disord,* 21**,** 179-89.

TUSZYNSKI, M. H. & BLESCH, A. 2004. Nerve growth factor: from animal models of cholinergic neuronal degeneration to gene therapy in Alzheimer's disease. *Prog Brain Res,* 146**,** 441-9.

TUSZYNSKI, M. H., THAL, L., PAY, M., SALMON, D. P., U, H. S., BAKAY, R., PATEL, P., BLESCH, A., VAHLSING, H. L., HO, G., TONG, G., POTKIN, S. G., FALLON, J., HANSEN, L., MUFSON, E. J., KORDOWER, J. H., GALL, C. & CONNER, J. 2005. A phase 1 clinical trial of nerve growth factor gene therapy for Alzheimer disease. *Nat Med,* 11**,** 551-5.

UNGER, C., SKOTTMAN, H., BLOMBERG, P., DILBER, M. S. & HOVATTA, O. 2008. Good manufacturing practice and clinical-grade human embryonic stem cell lines. *Hum Mol Genet,* 17**,** R48-53.

VAN DE VEN, C., COLLINS, D., BRADLEY, M. B., MORRIS, E. & CAIRO, M. S. 2007. The potential of umbilical cord blood multipotent stem cells for nonhematopoietic tissue and cell regeneration. *Exp Hematol,* 35**,** 1753-65.

VERFAILLIE, C. 2009. Pluripotent stem cells. *Transfus Clin Biol,* 16**,** 65-9.

VIERBUCHEN, T., OSTERMEIER, A., PANG, Z. P., KOKUBU, Y., SUDHOF, T. C. & WERNIG, M. 2010. Direct conversion of fibroblasts to functional neurons by defined factors. *Nature,* 463**,** 1035-41.

WACHS, F. P., COUILLARD-DESPRES, S., ENGELHARDT, M., WILHELM, D., PLOETZ, S., VROEMEN, M., KAESBAUER, J., UYANIK, G., KLUCKEN, J., KARL, C., TEBBING, J., SVENDSEN, C., WEIDNER, N., KUHN, H. G., WINKLER, J. & AIGNER, L. 2003. High efficacy of clonal growth and expansion of adult neural stem cells. *Lab Invest,* 83**,** 949-62.

WANG, T. T., TIO, M., LEE, W., BEERHEIDE, W. & UDOLPH, G. 2007. Neural differentiation of mesenchymal-like stem cells from cord blood is mediated by PKA. *Biochem Biophys Res Commun,* 357**,** 1021-7.

WANG, X. & SEED, B. 2003. A PCR primer bank for quantitative gene expression analysis. *Nucleic Acids Res,* 31**,** e154.

WATT, F. M. & HOGAN, B. L. 2000. Out of Eden: stem cells and their niches. *Science,* 287**,** 1427-30.

WATT, S. M. & CONTRERAS, M. 2005. Stem cell medicine: umbilical cord blood and its stem cell potential. *Semin Fetal Neonatal Med,* 10**,** 209-20.

WEMMIE, J. A., PRICE, M. P. & WELSH, M. J. 2006. Acid-sensing ion channels: advances, questions and therapeutic opportunities. *Trends Neurosci,* 29**,** 578-86.

WERNIG, M., MEISSNER, A., CASSADY, J. P. & JAENISCH, R. 2008. c-Myc is dispensable for direct reprogramming of mouse fibroblasts. *Cell Stem Cell,* 2**,** 10-2.

WERNIG, M., MEISSNER, A., FOREMAN, R., BRAMBRINK, T., KU, M., HOCHEDLINGER, K., BERNSTEIN, B. E. & JAENISCH, R. 2007. In vitro reprogramming of fibroblasts into a pluripotent ES-cell-like state. *Nature,* 448**,** 318-24.

WESSMAN, S. J. & LEVINGS, R. L. 1999. Benefits and risks due to animal serum used in cell culture production. *Dev Biol Stand,* 99**,** 3-8.

WICHTERLE, H., LIEBERAM, I., PORTER, J. A. & JESSELL, T. M. 2002. Directed differentiation of embryonic stem cells into motor neurons. *Cell,* 110**,** 385-97.

WIESE, C., ROLLETSCHEK, A., KANIA, G., BLYSZCZUK, P., TARASOV, K. V., TARASOVA, Y., WERSTO, R. P., BOHELER, K. R. & WOBUS, A. M. 2004. Nestin expression--a property of multi-lineage progenitor cells? *Cell Mol Life Sci,* 61**,** 2510-22.

WILDING, J. P. 2002. Neuropeptides and appetite control. *Diabet Med,* 19**,** 619-27.

WILKINS, A. L., YANG, W. & YANG, J. J. 2003. Structural biology of the cell adhesion protein CD2: from molecular recognition to protein folding and design. *Curr Protein Pept Sci,* 4**,** 367-73.

WILLIAMS, C. A. & RATEL, S. 2009. *Human muscle fatigue,* London ; New York, Routledge.

WOOD-KACZMAR, A., GANDHI, S. & WOOD, N. W. 2006. Understanding the molecular causes of Parkinson's disease. *Trends Mol Med,* 12**,** 521-8.

WUARIN, L., SIDELL, N. & DE VELLIS, J. 1990. Retinoids increase perinatal spinal cord neuronal survival and astroglial differentiation. *Int J Dev Neurosci,* 8**,** 317-26.

XAPELLI, S., AGASSE, F., FERREIRA, R., SILVA, A. P. & MALVA, J. O. 2006. Neuropeptide Y as an endogenous antiepileptic, neuroprotective and pro-neurogenic peptide. *Recent Pat CNS Drug Discov,* 1**,** 315-24.

XIAO, S., MCLEAN, J. & ROBERTSON, J. 2006. Neuronal intermediate filaments and ALS: a new look at an old question. *Biochim Biophys Acta,* 1762**,** 1001-12.

XU, J., KOCHANEK, K., SCOTT, C., TEJADA-VERA, B 2009. Deaths: Preliminary Data for 2007. *National Vital Statistics Reports,* 58**,** 58.

YAMANAKA, S. 2008. Pluripotency and nuclear reprogramming. *Philos Trans R Soc Lond B Biol Sci,* 363**,** 2079-87.

YAMANAKA, S. & TAKAHASHI, K. 2006. [Induction of pluripotent stem cells from mouse fibroblast cultures]. *Tanpakushitsu Kakusan Koso,* 51**,** 2346-51.

YASUHARA, T., MATSUKAWA, N., HARA, K., YU, G., XU, L., MAKI, M., KIM, S. U. & BORLONGAN, C. V. 2006. Transplantation of human neural stem cells exerts neuroprotection in a rat model of Parkinson's disease. *J Neurosci,* 26**,** 12497-511.

YELLEN, G. 2002. The voltage-gated potassium channels and their relatives. *Nature,* 419**,** 35-42.

YOON, J., TERADA, A. & KITA, H. 2007. CD66b regulates adhesion and activation of human eosinophils. *J Immunol,* 179**,** 8454-62.

YU, J., VODYANIK, M. A., SMUGA-OTTO, K., ANTOSIEWICZ-BOURGET, J., FRANE, J. L., TIAN, S., NIE, J., JONSDOTTIR, G. A., RUOTTI, V., STEWART, R., SLUKVIN, II & THOMSON, J. A. 2007. Induced pluripotent stem cell lines derived from human somatic cells. *Science,* 318**,** 1917-20.

YU, Y., HE, J., ZHANG, Y., LUO, H., ZHU, S., YANG, Y., ZHAO, T., WU, J., HUANG, Y., KONG, J., TAN, Q. & LI, X. M. 2009. Increased hippocampal neurogenesis in the progressive stage of Alzheimer's disease phenotype in an APP/PS1 double transgenic mouse model. *Hippocampus,* 19**,** 1247-53.

YUAN, P., LEONETTI, M. D., PICO, A. R., HSIUNG, Y. & MACKINNON, R. 2010. Structure of the human BK channel Ca2+-activation apparatus at 3.0 A resolution. *Science,* 329**,** 182-6.

ZAHIR, T., CHEN, Y. F., MACDONALD, J. F., LEIPZIG, N., TATOR, C. H. & SHOICHET, M. S. 2009. Neural stem/progenitor cells differentiate in vitro to neurons by the combined action of dibutyryl cAMP and interferon-gamma. *Stem Cells Dev,* 18**,** 1423-32.

ZANGIACOMI, V., BALON, N., MADDENS, S., LAPIERRE, V., TIBERGHIEN, P., SCHLICHTER, R., VERSAUX-BOTTERI, C. & DESCHASEAUX, F. 2008. Cord blood-derived neurons are originated from CD133+/CD34 stem/progenitor cells in a cell-to-cell contact dependent manner. *Stem Cells Dev,* 17**,** 1005-16.

ZANGIACOMI, V., BALON, N., MADDENS, S., TIBERGHIEN, P., VERSAUX-BOTTERI, C. & DESCHASEAUX, F. 2009. Human cord blood-derived hematopoietic and neural-like stem/progenitor cells are attracted by the neurotransmitter GABA. *Stem Cells Dev,* 18**,** 1369-78.

ZANGROSSI, S., MARABESE, M., BROGGINI, M., GIORDANO, R., D'ERASMO, M., MONTELATICI, E., INTINI, D., NERI, A., PESCE, M., REBULLA, P. & LAZZARI, L. 2007. Oct-4 expression in adult human differentiated cells challenges its role as a pure stem cell marker. *Stem Cells,* 25**,** 1675-80.

ZEILHOFER, H. U., MOHLER, H. & DI LIO, A. 2009. GABAergic analgesia: new insights from mutant mice and subtype-selective agonists. *Trends Pharmacol Sci,* 30**,** 397-402.

ZHA, X. M., COSTA, V., HARDING, A. M., REZNIKOV, L., BENSON, C. J. & WELSH, M. J. 2009. ASIC2 subunits target acid-sensing ion channels to the synapse via an association with PSD-95. *J Neurosci,* 29**,** 8438-46.

ZHAO, C., DENG, W. & GAGE, F. H. 2008. Mechanisms and functional implications of adult neurogenesis. *Cell,* 132**,** 645-60.

ZHAO, C., SUN, G., LI, S. & SHI, Y. 2009a. A feedback regulatory loop involving microRNA-9 and nuclear receptor TLX in neural stem cell fate determination. *Nat Struct Mol Biol,* 16**,** 365-71.

ZHAO, L., KRISHNAN, S., ZHANG, Y., SCHENKMAN, J. B. & RUSLING, J. F. 2009b. Differences in metabolite-mediated toxicity of tamoxifen in rodents versus humans elucidated with DNA/microsome electro-optical arrays and nanoreactors. *Chem Res Toxicol,* 22**,** 341-7.

ZHAO, Y., WANG, H. & MAZZONE, T. 2006. Identification of stem cells from human umbilical cord blood with embryonic and hematopoietic characteristics. *Exp Cell Res,* 312**,** 2454-64.

ZHENG, Y. L., LI, B. S., RUDRABHATLA, P., SHUKLA, V., AMIN, N. D., MARIC, D., KESAVAPANY, S., KANUNGO, J., PAREEK, T. K., TAKAHASHI, S., GRANT, P., KULKARNI, A. B. & PANT, H. C. 2010. Phosphorylation of p27Kip1 at Thr187 by cyclin-dependent kinase 5 modulates neural stem cell differentiation. *Mol Biol Cell,* 21**,** 3601-14.

ZHOLOS, A. V. 2006. Regulation of TRP-like muscarinic cation current in gastrointestinal smooth muscle with special reference to PLC/InsP3/Ca2+ system. *Acta Pharmacol Sin,* 27**,** 833-42.

ZHOU, J. M., CHU, J. X. & CHEN, X. J. 2008. An improved protocol that induces human embryonic stem cells to differentiate into neural cells in vitro. *Cell Biol Int,* 32**,** 80-5.

ZILLES, K. & AMUNTS, K. 2010. Centenary of Brodmann's map--conception and fate. *Nat Rev Neurosci,* 11**,** 139-45.