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Intrinsic factors controlling stem cell proliferation and differentiation in the central nervous system

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A thesis submitted to the University of London in fulfilment of the requirements for the degree of Doctor of Philosophy

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DECLARATION

I, Cynthia Lilian Andoniadou, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

DEDICATION

Στους γονείς μου Τζούλη και Λάμπη, που με στήριξαν και μου συμπαραστάθηκαν όλα αυτά τα χρόνια- σας ευχαριστώ.

To my parents, Julie and Lambi, who were always there for me these past years- thank you.

ABSTRACT

Populations of neural stem cells (NSCs) residing in different locations of the central nervous system (CNS) are predicted to have similar gene expression profiles, which identify them as stem cells. The best *in vitro* model to study NSCs is the neurosphere – a spherical colony generated from a single NSC. Currently, there is a lack of markers to characterise NSCs and distinguish them from lineage-committed progenitor cells. One promising candidate is *Sox2*, which is a member of the *Sox* family of transcription factors and is expressed within the CNS. $Sox2^{\beta geo/+}$ mice carry a β-galactosidase/ neomycin fusion gene in the *Sox2* locus. Homozygous $Sox2^{\beta geo}$ mutants exhibit peri-implantation lethality since *Sox2* is essential for the maintenance of the pluripotent epiblast cells in the embryo. I have established that the neurosphere-generating cellular component, throughout the developing CNS, resides within the *Sox2* expressing population. Through neomycin selection, neurosphere cultures derived from $Sox2^{\beta geo/+}$ mice have been enriched for NSCs and have been used to characterise NSCs.

Differences in the gene expression profiles of cells expressing and not expressing *Sox2* have been carried out through microarray experiments. To investigate the possible function of *Sox2* in NSCs I have depleted SOX2 by RNA interference, followed by microarray analysis, to identify potential targets of *Sox2* that may further affect the proliferation or differentiation of stem cells. The influence of the niche surrounding stem cells within the neurosphere has been addressed through a series of culture experiments comparing NSC-enriched to non-enriched neurospheres.

In order to elucidate the genes responsible for the identity of stem cells, enriched NSC cultures have been compared to their tissue of origin through microarray analysis. These studies have revealed genes expressed at significantly elevated levels within the stem cell cultures compared to the tissue samples - a predominantly differentiating population. Pair-wise comparisons between neurospheres generated from the spinal cord (11.5 dpc, 14.5 dpc) and dorsal telencephalon (14.5 dpc) were used to refine candidate genes and provide an insight into the spatial and temporal properties of NSCs. Several candidate NSC markers, including transcription factors and extracellular matrix molecules common to all populations, have been identified.

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LIST OF ABBREVIATIONS

ATP	adenosine 5'-triphosphate
βgal	β-galactosidase
βgeo	β-galactosidase/neomycin fusion gene
bp	base pairs
BSA	bovine serum albumin
CNS	central nervous system
cm	centimetre
CMFDG	5-chroromethylfluorescein di-β-D-galactopyranoside
Cy3	cyanine 3
Cy5	cyanine 5
°C	degrees Celsius
DAPI	diaminido-2-phenylindole
dATP	2'-deoxyadenosine 5'-triphosphate
db-cAMP	dibutyryl cyclic adenosine monophosphate
dCTP	2'-deoxycytidine 5'-triphosphate
ddH_2O	double distilled water
dGTP	2'-deoxyguanosine 5'-triphosphate
DMSO	dimethylsulfoxide
DMEM	Dulbecco's Modified Eagle Medium
DMEM F-12	Dulbecco's Modified Eagle Medium Nutrient Mixture F-12
DMF	*N,N*-Dimethylformamide
DNA	deoxyribonucleic acid
dpc	days post coitum
DT	dorsal telencephalon
dTTP	2'-deoxythymidine 5'-triphosphate
DTT	dithiothreitol
E	embryonic day
EC cells	embryonic carcinoma cells
EDTA	ethylene diamine tetraacetic acid
EGTA	ethylene glycol-bis(β-amino-ethylether)-N,N,N',N'-tetraacetic acid
EPC	endothelial progenitor cell
ES cells	embryonic stem cells
F	farads
FACS	fluorescent-activated cell sorting
FCS	fetal calf serum
g	average acceleration
g	grams
HSC	haematopoietic stem cell
kb	kilobase
LB	Lurea broth
M	molar
MEM	MOPS, EGTA, magnesium sulfate
μg	micrograms
μl	microlitres
μm	micrometres
mm	millimetres

MOPS	3-(N-morpholino) propanesulphonic acid
MSC	mesenchymal stem cell
NDM	neurosphere differentiation medium
neo	neomycin gene
NPC	neural precursor cell
NPM	neurosphere proliferation medium
NSC	neural stem cell
OPC	oligodendrocyte precursor cell
PB	phosphate buffer
PBS	phosphate-buffered saline
PBS-A	phosphate-buffered saline (Dulbecco's solution A)
PBS-CMF	phosphate-buffered saline- calcium and magnesium free
PCR	polymerase chain reaction
PETG	phenylethyl β-D-thiogalactopyranoside
PIPES	piperazine-N-N'-bis (2-ethanesulphonic acid)
PKA	protein kinase A
PNS	peripheral nervous system
PreI	pre-immune
RMS	rostral migratory stream
RNA	ribonucleic acid
RNAi	ribonucleic acid interference
RPC	retinal progenitor cell
rpm	revolutions per minute
SC	spinal cord
SDS	sodium dodecyl sulphate
SSC	standard saline citrate
SVZ	subventricular zone
tRA	all-trans-retinoic acid
Tris	tris (hydroxylmethyl) aminomethane
U	enzymatic units
UV	ultraviolet
V	volts
VZ	ventricular zone
w/v	weight to volume ratio
X-gal	5-Bromo-4-chloro-3-indoyl-β-D-galactopyranoside

Prefixes

ds	double-stranded
si	short-interfering
ss	single-stranded

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CHAPTER 1

Introduction

1.1 Principles of Stem Cells

A stem cell is a cell that can divide to generate one daughter cell with stem cell properties and another daughter cell that produces differentiated descendants. Some stem cell types are unipotent, giving rise to a single type of differentiated cell, such as spermatogonial stem cells, where ultimate descendants are sperm, while others can give rise to many, such as bone marrow stem cells. Transit amplifying progenitor cells are more committed than true stem cells and while they may divide, they do not strictly self-renew as they progress down one or more pathways of differentiation. However, it is not always easy to distinguish between these two cell types. To make a definition of stem cells more practical, the range of cell types produced by a progenitor has to be considered alongside self-renewal, where the original potential also needs to be maintained. Also, in theory, in conditions that promote self-renewal, single stem cells should be able to give rise to populations of equivalent cells that can be serially passaged for an extended number of divisions. This would reflect the continued presence of stem cells *in vivo*, often throughout the life of the adult animal, but conditions to allow this to happen *in vitro* are only known for a few stem cell types. True stem cells may be totipotent i.e. have the ability to generate all the cell types within the organism. Such cells may exist in plants and in some animal species, but almost certainly not in mammals where the only truly totipotent cells are the fertilized egg and blastomeres of early cleavage stage embryos, which do not self-renew on division. A more common usage of the stem cell definition includes cells of more restricted potential, such as the vast array of cell types of multipotent character

that proliferate after gastrulation in the embryo and can give rise to all differentiated cells in their lineage. Although it is arguable whether most of these are truly stem cells *in vivo*, since their properties evolve as the embryo becomes more complex. Once the potential of a cell becomes restricted, and typically begins to express markers associated with more differentiated progeny, it can be referred to as a precursor or a committed progenitor. These cell types may retain the capacity to divide giving rise to cells of more than one type (although this capacity is more restricted in this lineage) or they may terminally and irreversibly differentiate.

If stem cells divide symmetrically to give rise to two identical daughter stem cells, this will result in an exponential increase of cell numbers. Such divisions are more common in the embryo as they are associated with growth. In the adult, stem cells/progenitors will normally undergo asymmetric divisions, giving rise to a multipotent daughter cell and one cell that will commit or is already committed to differentiate. This type of division maintains cell numbers in the adult fairly constant since the numbers of new cells that are generated are similar to the numbers of differentiating or dying cells. It is likely that there are feedback mechanisms whereby the differentiating cells regulate division of the stem cells. See section 1.1.4 for further discussion.

1.1.1 Stem cells in the early mouse embryo

Embryonic Stem (ES) cells can be isolated from the inner cell mass of blastocyst stage embryos within the narrow time frame of about 3.5 to 4.5 dpc in the mouse. In terms of differentiation capacity, they behave as the *in vitro* equivalent of the early epiblast. They are therefore able to give rise to all cell types of the embryo and the

adult mouse and to some extraembryonic cell types, but not to trophectoderm derivatives. While they can give extraembryonic visceral endoderm *in vitro*, they do so very inefficiently *in vivo* after re-introduction into preimplantation embryos. They are therefore pluripotent rather than totipotent- although the latter term is often used erroneously. ES cells were first isolated from the inner cell mass of murine blastocysts in 1981 (Evans and Kaufman 1981; Martin 1981), following pioneering observations that pluripotent cells can be derived from teratocarcinomas generated from embryos transferred to extrauterine sites. Stem cell-like cells derived from teratocarcinomas were termed embryonal carcinoma (EC) cells and have properties similar to ES cells but they are often aneuploid. Some EC cells can contribute to normal embryonic development in chimeras after their introduction into normal host embryos at the blastocyst stage, however their contribution is often sporadic and less extensive than that of ES cells (Gardner 1968). With very rare exceptions, they have been unable to contribute to the germ cell lineage (Papaioannou and Rossant 1983). On the other hand, ES cells have the ability to contribute readily to all cell types of the developing embryo including the germ line. Their pluripotency has been demonstrated further through the generation of completely ES-cell derived normal mice through the production of ES cell-tetraploid embryo aggregation chimeras, where the tetraploid component is excluded from the embryo proper but will form functional extraembryonic cell types (Nagy et al. 1993). More recently, ES cells have been derived from human blastocysts (Thompson et al. 1998) and from some primates, but there has been no success in their isolation from cows, sheep or rats. Mouse, but not human, ES cells in culture require leukaemia inhibitory factor (LIF), which promotes self-renewal, either as an additive to the medium or supplied by fibroblasts acting as feeder cells. They are both usually sustained in supplemented

foetal calf serum but in mouse ES cell culture, bone morphogenic protein 4 (BMP4) has been identified as being the factor within the serum that is sufficient to maintain ES cells together with LIF (Ying and Smith 2003). ES cells can differentiate into a wide range of cell types in culture. This will happen spontaneously, especially when they are not passaged and have formed large clumps of cells. Although their differentiation is disorganised, it tends to follow routes taken by normal early embryo differentiation. Thus, the first differentiated cell type to be identified in culture is extraembryonic endoderm. ES cells as well as EC cells have the ability to form aggregates in non-adherent culture conditions, and these are known as embryoid bodies. Cells cultured in this way readily differentiate and, to some extent, can be directed by external factors to differentiate into specific cell lineages e.g. retinoic acid favours differentiation into neural cell types. It is also possible to use very defined conditions, whereby the withdrawal of LIF and BMP can lead to the majority of the cells following a neural pathway. This is not strictly a default pathway as it involves autocrine signalling via FGFs made by ES cells (Ying et al. 2003).

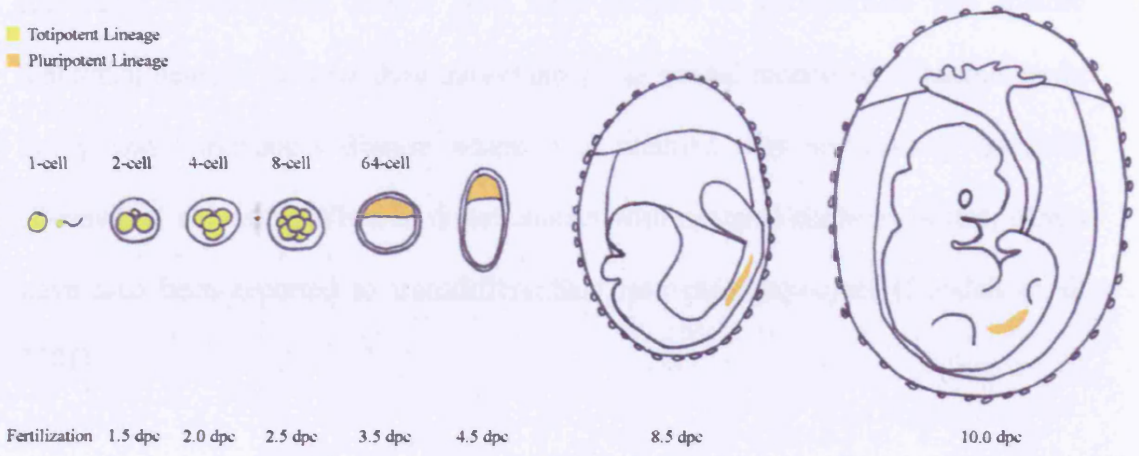
From fertilisation until the 8-cell stage, all the blastomeres divide symmetrically and all progeny are totipotent. This remains a property of the ICM in the blastocyst (4.0 dpc). The ICM gives rise to extraembryonic endoderm, epiblast, which remains pluripotent until gastrulation (the process that leads to the establishment of the primitive germ layers), ectoderm, mesoderm and endoderm, as well as to primordial germ cells. Although cells of the ICM lose their ability to form trophoblast cells *in vivo* and are pluripotent, in a few abnormal situations, cultured ICM cells have been shown to generate trophoderm cells, for example after the loss of *Oct4* (Nichols et al. 1998) or *Sox2* (Avilion et al. 2003). The outer cells of the blastocyst are the first cells

to undergo lineage commitment, where their fate becomes restricted to that of the trophectoderm lineage. The trophoblast cells in contact with the ICM are known as polar trophectoderm and they are induced to divide in a relatively undifferentiated state, remaining diploid, and giving rise to extraembryonic ectoderm, whereas the mural trophectoderm cells undergo endoreduplication and differentiate as giant cells. The extraembryonic ectoderm gives rise to the chorionic ectoderm, which is essentially a population of stem cells able to give rise to all the differentiated trophectoderm derivatives of the placenta. In culture, trophoblast cells can be maintained as undifferentiated trophoblast stem (TS) cells in the presence of fibroblast feeders and FGF4. TS cells can also be isolated from blastocysts, extraembryonic ectoderm at 6.0-7.0 dpc and from chorionic ectoderm at 7.5 dpc. TS cells can differentiate into all cell types of the trophectoderm lineage including trophoblast giant cells, when either FGF4 or the feeder cells are removed (Tanaka et al. 1998). More recently, it has been possible to culture a third type of stem cell from the late blastocyst. These are called XEN cells and are able to give rise to extraembryonic endoderm cell types *in vitro*, or in chimeras (Kunath et al. 2005). After gastrulation, the generation of ectoderm, endoderm and mesoderm results in a restriction of potential in each layer, but many of their component cells retain multipotent properties as they can generate all the cell types required for specific structures. Ectoderm gives rise to skin, dermal appendages, brain and neural tissues; endoderm gives rise to the gastrointestinal tract and internal glandular organs (such as liver, lung) and mesoderm gives rise to connective tissue, muscle, bone and blood vessels (Arey 1974).

As gastrulation proceeds, the majority of the cells in the embryo commit to a restricted fate. By 7.25 dpc approximately 30-50 cells that reside outside the embryo proper, and which are derived from the epiblast, become committed as primordial germ cells (PGCs) (Ohinata et al. 2005). These cells are excluded from somatic specification and restriction. Although not pluripotent as such, they are fated to make either sperm or oocytes, which are both highly specialised cell types. However, they retain some properties reminiscent of early epiblast. Notably, they express certain marker genes (*Sox2*, *Oct4*) and their potential can be realised by fertilisation. Furthermore, *in vitro*, they can give rise to embryonic germ (EG) cells, which are very similar to ES cells (Matsui et al. 1992). PCGs proliferate and re-enter the embryo from the extraembryonic mesoderm, migrating through the hindgut and dorsal mesentery to arrive at the gonadal primordium, the genital ridge, by 11.5 dpc. PGCs proliferate until shortly after gonadal sex determination, where in the female they enter meiosis between 13 and 15 dpc (McLaren 1984), and in the male they undergo mitotic arrest between 13 and 14 dpc and become primitive spermatogonia. These do not resume mitosis until 3 days postnatally (Nebel et al. 1961), giving rise to spermatogonial stem cells. These are true, unipotential stem cells, which on division give rise to transit amplifying spermatogonial cell types which then progress into spermatocytes, spermatids and sperm. PGCs isolated throughout gestation until after their arrival in the genital ridge, can be cultured as EG cells from both male and female embryos (Matsui et al. 1992; Resnick et al. 1992). As with ES cells, their long-term culture requires the presence of LIF, as well as the mitogen basic fibroblast growth factor (bFGF) and Steel factor (SF), the *c-kit* ligand. Most EG cell lines contribute to chimeras in a similar way to ES cells and can also give germ line transmission (Labosky et al. 1994; Stewart et al. 1994). PGCs from 10.5 dpc

embryos have been used to obtain full-term foetuses by nuclear transfer (Miki et al. 2005). Although they share many similar characteristics with ES cells in culture, they display considerable differences in imprinting of specific genes. This reflects the normal erasure of genomic imprinting.

Figure 1.1 Cartoon outlining the in *in vivo* totipotent (yellow) and pluripotent (orange) lineages in the early mouse embryo. Adapted from Papaioannou and Hadjantonakis, Stem Cells Handbook (2004). The PGCs are labelled pluripotent here, only because of their ability to generate EG cells *in vitro*.



1.1.2 Stem cells in the late embryo and in the adult

As development progresses, the plasticity of stem cells tends to become more and more restricted. This results in populations of specialised stem cells and progenitors that *in vivo* give rise to a restricted range of differentiated cell types. One example is mesenchymal stem cells (MSCs), which are the adult progenitors of the mesenchymal cells found in bone marrow, skin, muscle and adipose tissue. They are also referred to as bone marrow stromal cells and can indirectly give rise to two multipotent progenitors: multipotent haematopoietic stem cells (HSCs) and stromal progenitors, which can give rise to fibroblasts, adipocytes, smooth muscle,

chondrocytes and osteoblasts. MSCs have also been isolated from adult human dental pulp, primary teeth and periodontal ligament. All dental MSC populations can be cultured *in vitro* and have the ability to regenerate human dental tissues when transplanted into mice (Shi et al. 2005). The therapeutic potential of MSCs also extends to the possibility for manipulation to treat cartilage disorders. They have the potential to become cartilage both *in vivo* and *in vitro* and it has been recently demonstrated that insulin-like growth factor I has chondrogenic effects on MSCs (Spagnoli et al. 2005). However, more surprisingly, and against the rules of normal embryonic development, MSCs have been induced to differentiate into mature functional neurons *in vitro*, then transplanted into animal models of ischaemic brain injury and Parkinson's disease where it is claimed they successfully integrate (Dezawa et al. 2005). When in direct contact with neonatal cardiomyocytes, MSCs have also been reported to transdifferentiate into cardiomyocytes (Goodell et al. 2001).

HSCs are thought to give rise to two multipotent progeny, the hemocytoblast that enters the blood cell pathway, and the lymphoid stem cell that enters the lymphocytic pathway, giving rise to T-lymphocytes and B-lymphocytes. HSCs arise after gastrulation 7.5 dpc), but when isolated from such an early stage embryo they do not have the same properties as those isolated from the adult. Cells resembling adult HSCs can first be isolated from the aorta-gonad-mesonephros (AGM) region of the embryo at 10.5 dpc. It has been reported that single adult HSCs can reconstitute the haematopoietic system in mice (as well as to contribute to endoderm-derived tissues) (Krause et al. 2001). HSCs have also been reported to give rise to muscle (Ferrari et al. 1989; Jackson et al. 1999; Goodell et al. 2001) and brain (Bjornson et

al. 1999; Brazelton et al. 2000). The generation of liver from HSCs may have therapeutic potential (Peterson et al. 1999), as bone marrow-derived hepatocyte transplantation in mouse models of hereditary tyrosinemia type 1 results in complete metabolic correction.

In humans, bone marrow transplants from healthy, matching donors are used to replenish the adult stem cell population after chemotherapy for the treatment of leukaemia and various blood disorders. Cancer chemotherapy may utilise combinations of several chemotherapeutic agents as well as radiation therapy. Some treatments require very high doses that also ablate HSCs (myeloablation) to levels from which they cannot successfully regenerate in the remaining lifespan. Another approach is to remove bone marrow from patients prior to chemotherapy, and to use a fluorescence activated cell sorter (FACS) to purify HSCs using specific stem cell marker antibodies. This, has been reported to deplete contaminating cancer cells by 100,000 fold (Manz et al. 2004) and the HSCs can be returned to the patient after the completion of the treatment. This manipulation of HSCs in therapy has been reported in three clinical trials for breast cancer, non-Hodgkin's lymphoma and multiple myeloma treatments (Michallet et al. 2000; Negrin et al. 2000; Vose JM 2001).

The epithelium of the skin epidermis is composed of the interfollicular epidermis, which surrounds hair follicles, sebaceous glands and sweat glands. A layer of proliferating stem cells attached to the underlying basal lamina maintains skin homeostasis. These detach from the basal lamina and terminally differentiate until they are eventually lost from the surface of the skin (Watt 2002). Hair follicles host at least two stem and progenitor cell populations, which reside in the external outer

root sheath known as the bulge. These stem cell populations are in contact with the basal lamina and are therefore known as the basal stem cells of the bulge. Cells with progenitor properties have also been isolated from the suprabasal bulge and sebaceous glands, although these are thought to be progenitor cells that have migrated from the basal bulge to populate other areas. The stem cells of the interfollicular epidermis can contribute to all keratinocyte derivatives: hair follicles, interfollicular epidermis and sebaceous glands when grafted *in vivo* and demonstrate self-renewal *in vitro* (Blanpain et al. 2004). The success incurred through grafts of bulge stem cells in mice may provide sources of treatment for human patients such as post-operational skin cancer patients and burns victims.

The retina is derived from the optic sulci, which form from the anterior neural plate when this evaginates in the region of the presumptive diencephalon. Retinal progenitor cells (RPCs) are responsible for the generation of all retinal cell types and during retinogenesis, their potential is influenced by environmental cues such as EGF, SHH, LIF, and CNF (Fischer and Reh 2003, Wang et al. 2005). In the late embryonic retina, RPCs can be cultured as CNS stem cells to reveal that the progenitor pool consists of mainly differentiating or commited progenitors and a small amount of proliferating stem cells, similar to other regions of the developing CNS (Tropepe, 2000). In the retina of amphibians and fish, a specialized proliferative zone called the ciliary marginal zone (CMZ) harbours the stem cells of the retina (CMZ stem cells), which remain active throughout life. In higher vertebrates, proliferative activity has been observed in late embryonic and early postnatal stages. However the adult retina of mammals does not exhibit a capacity for *in vivo* regeneration. Retinal progenitor cells (RPCs) have recently been isolated *in vitro*

from the pigmented ciliary margin (PCM) of adult mice (Tropepe 2000). These form neurosphere-like colonies in culture and are both neurogenic and gliogenic and are able to give rise to the majority of retinal cell types (Taranova et al. 2006). The successful re-introduction of these cells in host retinas has not yet been demonstrated. The role of RPCs in the adult mammalian retina has not been determined so far. Progenitors in the post-natal rat retina have been shown to respond through proliferation *in vivo* to growth factors such as FGF2 (Zhao et al. 2005). Their identification supports the idea that tissue-specific stem cells in non-proliferative regions of the adult may be inhibited from proliferating by environmental cues.

1.1.3 Brief overview of stem cell markers

To date, no factor has been identified that can exclusively mark all stem cell populations in the mouse and it is very unlikely that this will ever be the case. Several stem cell populations may have markers in common, but these may also be expressed in lineage-restricted progenitors of other populations, or other non-proliferative cell types.

Table 1.1 Summary of commonly used markers for the identification of various murine stem cell lineages.

Stem Cell Type	**Markers Expressed**
ES	Sox2, Oct3/4, Nanog
Hemangioblast (HSC & EPC progenitor)	Flk-1, Slc, Tie-1, Tie-2, CD34, Tal-1, Hex, c-kit
EPC	Flk-1, Slc, Tie-1, Tie-2, CD34, CD133
HSC	CD34, Sca1, c-kit
Basal Bulge	CD34, alpha6-integrin, beta1-integrin, Sca-1, K14, K19, K15, K5
Hepatocyte	OV-6, c-met

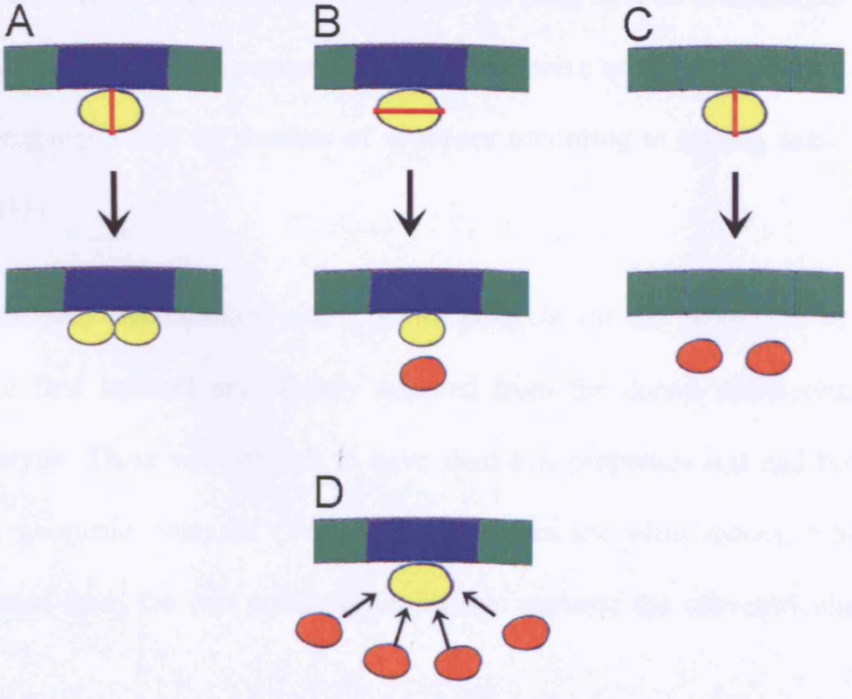
1.1.4 A requirement for a niche

Extrinsic factors can affect cells in multiple ways. They can induce differentiation, proliferation, migration or death. Cell-cell contact and diffusible factors are responsible for conducting correct patterning throughout development alongside intrinsic cues. The microenvironment around cells or, as it also known, the niche, has been shown to play a crucial role in the homeostasis of several stem cell populations and their descendants. If not controlled, stem cells could overpopulate tissues leading to a high number of proliferative progeny, risking the introduction of mutations that can lead to tumor formation. If the rate of differentiation becomes greater than the rate of self-renewal, the stem cell population may eventually become depleted. The concept of the existence of a stem cell niche was first proposed for hematopoietic stem cells by Schofield (Schofield 1978). Niches have been proposed for numerous tissues since then, and some of the best defined are within the testis, hematopoietic system, gut epithelium and epidermis.

If a stem cell divides symmetrically such that both daughter cells retain contact with the niche then each will remain a stem cell. This also happens in culture if stem cells are grown in conditions that favour self-renewal. If they divide symmetrically but move away from their niche, then both will differentiate. Alternatively, a stem cell can divide asymmetrically to give rise to progeny with different fates. This can occur through two mechanisms: (i) the cell can contain determinants already localised in an asymmetric fashion, such that the two daughter cells will have different fates; or (ii) the plane of division can locate one of the progeny in a position remote from the niche, where different extrinsic cues result in an altered fate to that of its sister cell that has remained in contact. In the hypothetical stem cell niche, signals from the

surrounding microenvironment promote the stem cell state. Once removed from their niche, stem cells may lose their potential, as is the case with HSCs. Hence, stem cell numbers are limited by the size, availability and location of one or multiple niches. In proposed stem cell niche systems, the stem cells are in contact with other cells, such as supporting cells, and basement membranes. These seem important to contain stem cells within the niche, specify polarity and provide cues that promote proliferation and inhibit differentiation. However, the differentiated progeny of the stem cells also contribute to the niche, as they will feed back signals to the stem cells (directly or indirectly via supporting cells) to control their rate of division and perhaps also the plane of division.

Figure 1.2 Cartoon outlining the theoretical effects of the niche in the fate of progeny based on the plane of division. If a stem cell (yellow) in contact with the niche (blue) divides symmetrically, then both progeny will remain stem cells (A). If a stem cell in contact with the niche divides asymmetrically, then the daughter cell still in contact with the niche will remain a stem cell and the daughter cell that loses contact with the niche will differentiate (orange, (B)). In the absence of a niche, both progeny of a stem cell will differentiate irrespective of the plane of division (C). The effect of differentiated progeny surrounding stem cells must be taken into account as they may influence aspects such as the plane and timing of division (D).



1.2 Neural stem cells

The mammalian CNS is composed mainly of astrocytes, oligodendrocytes and neurons. The latter are the main functional unit of the CNS, but astrocytes and oligodendrocytes provide critical support and modulate their activity. During embryonic development, the ability to generate new neurons (neurogenesis) declines, which led to the idea that the adult CNS is mitotically quiescent, and unable to repair tissues that have been damaged due to disease or injury. The multipotent stem cell of the CNS, the neural stem cell, was once thought to reside only in neurogenic regions of the embryo and in the early postnatal period. Although *in vivo* studies generally supported this theory, there were exceptions that identified a proliferative zone of the adult brain, the forebrain subependyma (Altman 1962). This was believed to be species-specific. Work on rodents revealed that during adolescence, when neurogenesis has largely subsided, limited regions of the brain, such as the hippocampus, remain neurogenic (Bayer 1980). Further significant evidence that neurogenesis persists in adult vertebrates came from studies in songbirds, where the two telencephalic song control nuclei of adult male canaries were found to significantly enlarge during spring when the birds have to demonstrate stable singing and acquire a new repertoire. These findings were attributed to a cycling increase in neurogenesis and the number of synapses according to mating season (Nottebohm 1981).

Apparently uncommitted cell types responsible for the generation of new neurons were first isolated and serially cultured from the dorsal telencephalon of mouse embryos. These were shown to have stem cell properties and had both neurogenic and gliogenic potential (Temple 1989). From the adult mouse, NSCs were also isolated from the two principal neurogenic regions: the subventricular zone (SVZ)

and the dentate gyrus of the hippocampus (Reynolds and Weiss 1992; Lois and Alvarez-Buylla 1993). However other regions of the CNS not known to be neurogenic, have also been identified as sites in which NSCs reside and from where they can be isolated, such as the embryonic spinal cord (McKay 1997; Rao 1999; Gage 2000) and the adult striatum and septum (Palmer et al. 1995). NSCs isolated from these regions have neurogenic potential *in vitro*. Even more surprisingly, cells exhibiting similar properties in culture to NSC have been isolated from neural crest, a peripheral nervous system derivative (Stemple and Anderson 1992).

Figure 1.3 Cartoon outlining areas from which NSCs can be isolated in the brain (yellow). In the adult mouse brain (A) Proliferation is highest in the SVZ and the SGZ (blue). In the embryo, represented here by a transverse section through one hemisphere of the 14.5 dpc forebrain (B), NSC-rich regions line the lateral ventricles.

A **B**

Knowledge of expression of markers common to NSCs and their *in vivo* morphology are limited, hence their isolation cannot depend on these. Instead, the culture conditions used encourage the survival of proliferating cells from a pool of progenitors and differentiated cells, which are normally found in the CNS tissue. The surviving cells include NSCs, which can be identified through functional features such as their proliferation capacity and self-renewal, their multipotentiality, which

can be sustained over long time periods, and by the generation of a large number of progeny, most probably through one or more transiently amplifying populations of progenitors.

Being able to distinguish between stem cell and transit amplifying progenitors would be a step toward understanding what attributes make neural stem cells unique. This knowledge could be exploited to manipulate the proliferation and differentiation of NSCs *in vitro* or *in vivo*, to establish strategies for therapies for damage of CNS tissue from disease or injury.

Stem cells are often quiescent, i.e. they rarely divide, but when they do they give rise to a transit amplifying progenitor cell type. In most culture systems, a distinction is rarely made between the transit amplifying progenitors and the true stem cells. NSCs require the presence of mitogens to stimulate their proliferation both *in vivo* and *in vitro* (Richards et al. 1992; Kilpatrick and Bartlett 1993; Gritti et al. 1996; Gritti et al. 1999), and differentiate in their absence. Various studies have used culture medium containing epidermal growth factor (EGF), which seems to be sufficient for the long-term sustainability of NSC-derived colonies (Reynolds et al. 1992; Reynolds and Weiss 1992; Reynolds and Weiss 1996). Others have used basic fibroblast growth factor (bFGF) without EGF in the same way, generating cultures with a higher fraction of proliferating cells (Palmer et al. 1995; Gritti et al. 1996; Qian et al. 1997). However when the two growth factors are provided in combination, they appear to have a synergistic effect on neural stem cells resulting in higher numbers of rapidly proliferating cells (Vescovi et al. 1993; Weiss et al. 1996; Ciccolini and Svendsen 1998). Studies in 14.0 dpc murine striatal cultures indicate

that the initial responsiveness of NSCs is only to bFGF, but that upon culture with bFGF they then become responsive to EGF. Consistent with this, NSCs of the mammalian embryonic CNS acquire bFGF receptors earlier in development, and only subsequently express EGF receptors. In high concentrations, bFGF can promote *in vitro* neurogenesis of typically non-neurogenic regions (Palmer et al. 1995; Shihabuddin et al. 1997; Palmer et al. 1999). The bFGF concentration has also been reported to regulate the switch between neuronal, oligodendroglial and glial fates, mimicking the *in vivo* order of the generation of neurons, then glia (Qian et al. 1997).

Several markers of NSCs have been proposed mainly from *in vitro* studies, as outlined in Table 1.2. As is the case for other stem cell populations, it is unlikely that a single marker will be sufficient for the identification of the true stem cell type or types of the CNS. Moreover, many of these markers are not exclusive to NSCs as they are expressed at other sites, within or outside the CNS. Several groups have therefore proposed combinations of expressed markers, or even the lack of expression of some genes, aiming to distinguish the stem cells from more committed progenitors.

Table 1.2 Table summarising markers for the identification of NSCs that have been proposed to date ('+' denotes expressed, '-' denotes note expressed and 'lo' denotes expressed at low levels).

Marker	**Organism**	**Reference**
LeX/Ssea-1^{+}	Mouse	(Capela and Temple 2000)
Nestin^{+}	Mouse	(Kawaguchi 2001)
Sox2^{+}	Mouse	(Zappone et al. 2000; Cai et al. 2002)
CD133^{+}5E12^{+}CD34^{-}CD45^{-}CD24$^{-/lo}$	Human fetal	(Uchida 2000)
Prominin1/CD133^{+}	Mouse	(Lee et al. 2005)
Mcm2^{+}	Mouse (NSC subpopulation)	(Maslov et al. 2004)
Nestin^{+}PNA$^{-/lo}$CD24^{-}	Mouse (adult periventricular region)	(Rietze 2001)

1.2.1 Neural Stem Cell niches in the mouse

In the developing CNS, the predominant cell type identified as a neural stem cell is the radial glial cell (Malatesta et al. 2000; Alvarez-Buylla et al. 2001; Campbell and Gotz 2002; Doetsch 2003; Kriegstein and Gotz 2003; Merkle et al. 2004). Radial glia within the ventricular zone line the lateral ventricles and proliferate to generate glial and neural derivatives. Their processes, along which differentiating progenitors migrate, extend up to the pial surface of the cortex, enabling the formation of organised layers where the last cells to be generated occupy positions furthest from the ventricular layer. The proliferating NSCs of the adult brain have been identified as radial glial-derived astrocytes, residing in the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the hippocampal dentate gyrus. By lineage tracing they have been shown to give rise to neurons, glia,

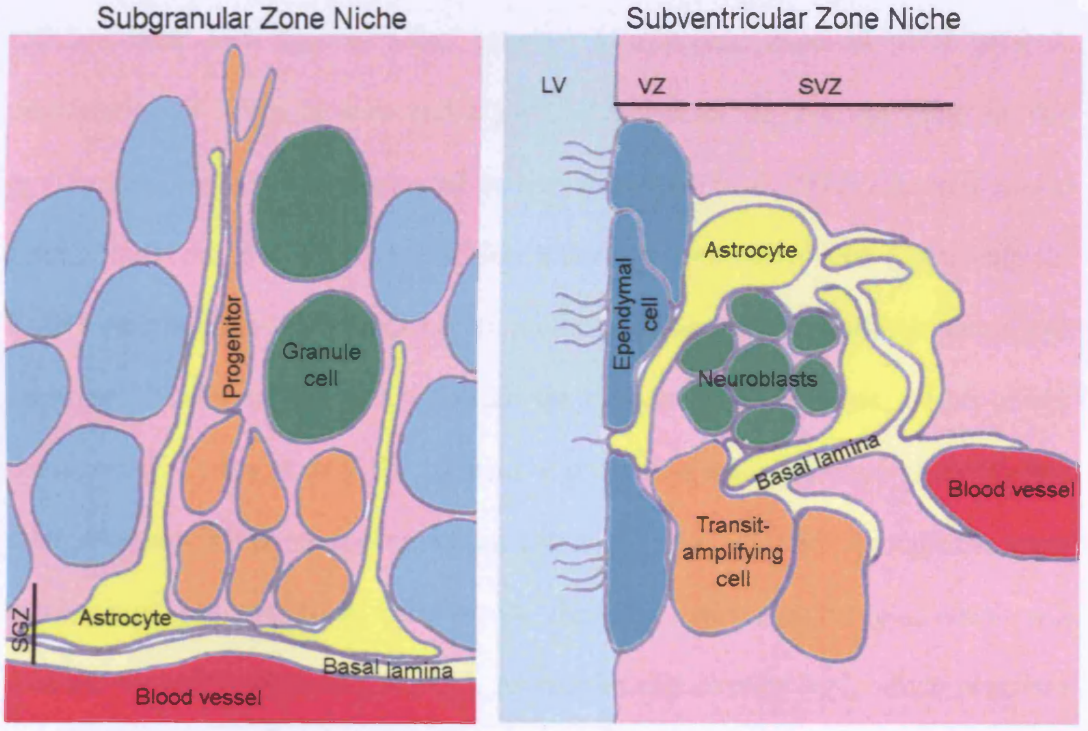
oligodendrocytes as well as to ependymal cells (Merkle et al. 2004). Astrocytes in the brain express *Gfap* and *s-100β*. Cells proposed to be NSCs in the SVZ uniquely express *Gfap* suggesting that they comprise a distinguishable stem cell subpopulation (Ma et al. 2005). Astrocytes from the dentate gyrus, apart from providing structural support, have also been shown to contribute to the niche through producing factors that promote proliferation and neurogenesis *in vitro* (Song et al. 2002). It is likely that at different developmental stages, cells within the niche will respond in distinct ways to particular extrinsic cues or have unique requirements.

Also contributing to the niche are the basal lamina and endothelial cells of adjacent blood vessels. It has been proposed that, the NSC population within the SVZ, which has a glial phenotype, is in contact with the basement membrane (Doetsch et al. 2002; Mercier et al. 2003). In the SGZ it has been shown that proliferation is highest around associated blood vessels (Palmer et al. 2000). The extracellular matrix has also been shown to provide signals that can affect NSC proliferation, differentiation and migration. In the adult, the basal lamina is in close association with the neural stem cells in the SVZ in the space between the stem cells and blood vessels and this may also be the case in the dentate gyrus (Alvarez-Buylla and Lim 2004). As surrounding blood vessels enter the parenchyma, the associated basal lamina invaginates from the pial surface towards the ventricles. The basal lamina is rich in laminin 1 and collagen and maintains extensive contact with radial glial cells throughout development.

Endothelial cells of the blood vessels produce vascular endothelial growth factor (VEGF), which when infused into the adult brain, induces proliferation of neurogenic

precursors. *In vitro*, endothelial cells produce soluble factors that promote proliferation and neurogenesis in embryonic NSCs, as demonstrated through co-culture experiments (Shen et al. 2004).

Figure 1.4 Cartoon outlining NSC niches for the adult brain that have been proposed in the literature by others. Possible architecture of the proposed adult NSC niches in the mouse SVZ and SGZ (Adapted from (Alvarez-Buylla and Lim 2004)). In the SGZ, astrocytic NSCs with projections extending toward the pial surface generate progenitor cells, which mature into granule cells that integrate into the granule cell layer. Progenitor cells may have the ability to migrate along the stem cell scaffold. In the SVZ, the astrocytic NSCs are in close contact with their transit-amplifying progeny and neuroblasts, generated from the transit-amplifying cells, which migrate along the RMS differentiating into olfactory bulb interneurons. Some astrocytes extend projections to contact the ventricle and display a single cilium. Ependymal cells in the ventricular zone produce factors critical for the niche. In both niches, a strong association with the basal lamina and associated vasculature is proposed.



LeX (also referred to as *Ssea-1* or CD15) is a carbohydrate moiety that is expressed in adult astrocytes that behave as stem cells *in vitro* (Capela and Temple 2002). It is also expressed in blood vessel endothelial cells in the brain. In the early embryo it is thought to be associated with blastocyst adhesion. It is likely that it has a role in the niche to promote adhesion of NSCs and transit-amplifying progenitors.

Mitogens such as EGF and FGF have proliferative effects on NSCs and transit-amplifying progenitors *in vitro* and *in vivo*. EGF is not endogenously expressed in the adult SVZ, but its function may be replaced by TGFα, which can also activate the EGF receptor. In addition, activation of Notch signalling in the SVZ inhibits neuronal differentiation and may lead to greater numbers of stem cells (Chambers et al. 2001). SVZ astrocytes express both FGFs and Notch ligands suggesting that they regulate their own fate to some extent. Morphogens such as SHH promote proliferation of NSCs *in vitro* and a partial dependence on *Shh* signalling for this proliferation has been demonstrated *in vivo* (Machold et al. 2003; Ahn and Joyner 2005). Early on, BMPs promote differentiation to non-neural cell types, whereas BMP signaling in the embryonic cortex promotes gliogenesis and differentiation into astrocytes. BMPs are thought to induce the expression of *Id* genes, which inhibit neurogenesis. In the adult SVZ, the stem or progenitor cells express both BMPs and their receptors which may promote an astrocytic stem cell fate through the same mechanisms, but ependymal cells secrete the BMP antagonist Noggin, which will then permit neurogenesis (Lim 2000). Astrocytes also express *Ngl*, which promotes neurogenesis in the adult hippocampus (Ueki et al. 2003).

It remains unclear if the role of the NSC niche is simply to inhibit differentiation or whether it has a more complex role to actively promote both proliferation and induction into neuronal lineages. However, some evidence suggests that the latter may be the case. Studies reveal that astrocytes not residing within niche regions of the adult CNS have neurogenic potential when transplanted into appropriate domains, but this is not evident within their original location (Lie et al. 2004; Emsley et al. 2005). When SGZ progenitors are transplanted into the RMS, where normally only SVZ neuroblast progenitors migrate towards the olfactory bulb and differentiate, they behave in a similar way to the endogenous cell population and generate tyrosine hydroxylase expressing interneurons.

1.3 Sox2 as a marker of stem cell populations

One gene that is widely expressed in stem cell generating regions and specific stem cell populations is *Sox2*. Its expression is necessary for the ES cell state and the maintenance of pluripotency, alongside *Nanog* and *Oct3/4* (Avilion et al. 2003; Chambers et al. 2003; Mitsui et al. 2003) Through co-operation with *Oct3/4*, *Sox2* has been shown to regulate *Nanog* (Kuroda et al. 2005; Rodda et al. 2005; Wu da and Yao 2005). It has been demonstrated that *Sox2* is expressed in several stem cell-generating regions of the CNS. A subset of *Sox2* expressing cells from such regions has been shown to be capable of forming neurospheres in culture (Zappone et al. 2000; Cai et al. 2002). Neural precursors derived from embryonic stem (ES) cells have been isolated through selecting for *Sox2* expression (Li et al. 1998). This evidence suggests *Sox2* may play a role in neural stem cell initiation and maintenance as well as in the properties of the stem cell. In the early post-gastrulation embryo, *Sox2* is expressed in the neuroepithelium throughout the CNS

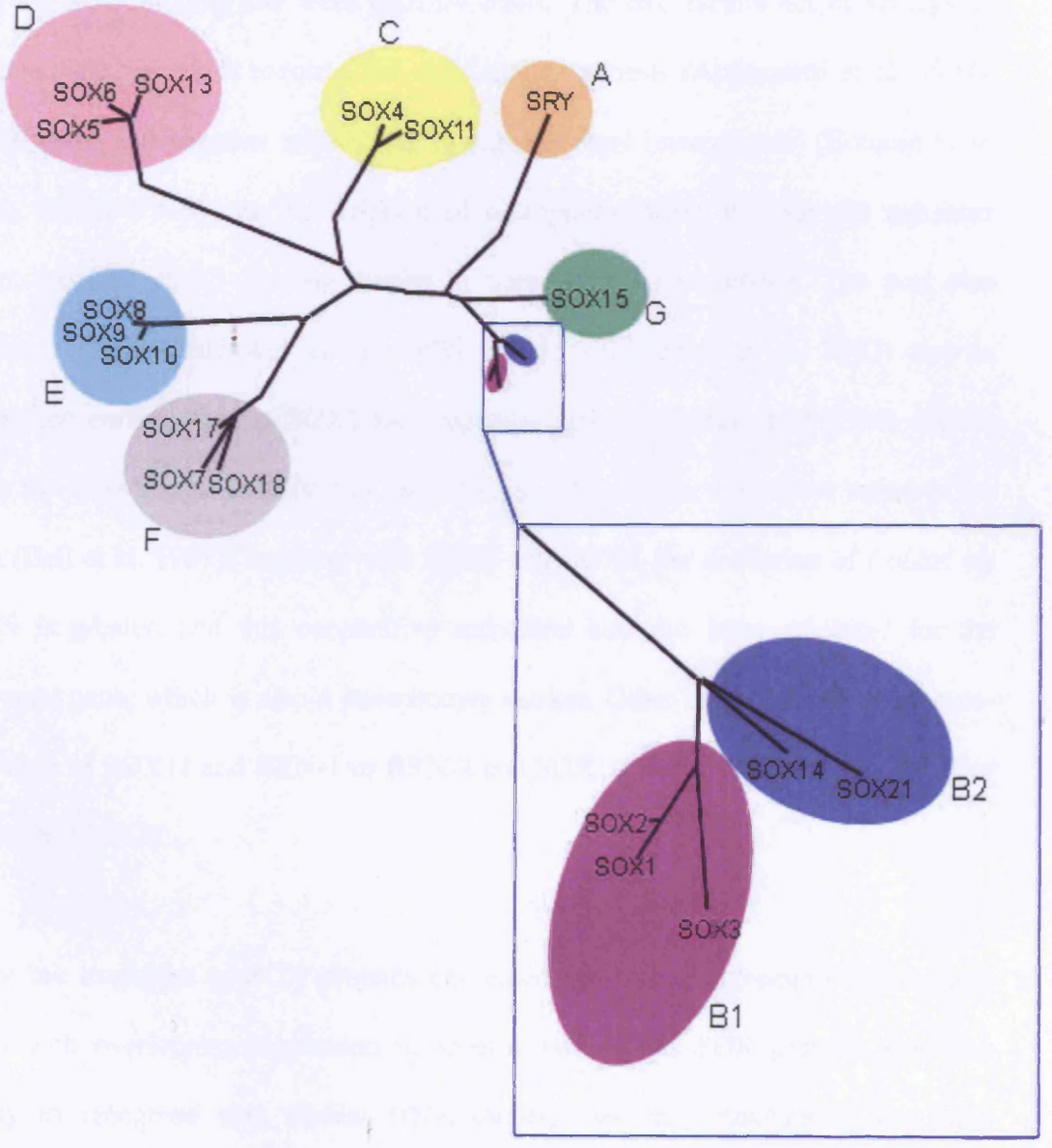
(Li et al. 1998). At later stages of the developing embryo, *Sox2* is expressed in the forebrain (dorsal telencephalon), midbrain, hindbrain, spinal cord, in the olfactory epithelium, in the lens and otic placodes. It is also expressed in some cell types outside the CNS known to contain stem cells, such as the bulge region of the hair follicles and gut endoderm. The expression of *Sox2*, in many regions where stem cells are found, renders it a good candidate as a stem cell marker.

1.4 Sox genes

The SOX (SRY-related HMG box) family encodes transcription factors related primarily by homology within their high-mobility group (HMG) box DNA binding motif including specific signature amino acids (Gubbay et al. 1990). The high mobility group (HMG) domain sequence ranges in different proteins between 70-80 amino acids (79 amino acids long in SOX proteins). Proteins containing an HMG box belong to the HMG superfamily. This superfamily includes two families- the TCF/SOX/MATA family where the members have single HMG domains, and the HMG/UBF family that contains members with multiple domains (Soullier et al. 1999) including non-histone chromosomal proteins HMG1 and HMG2, UBF; a nucleolar RNA polymerase I transcription factor and mtTF; a mitochondrial transcription factor. Members of the second family are found throughout eukaryotic species and are widely expressed. They have no particular sequence specificity but have an affinity for bent or structured DNA. In contrast, members of the TCF/SOX/MATA family are mainly tissue-specific transcription factors recognising specific DNA sequences. MATA proteins are found in yeast and TCF/SOX in animals. SOX proteins bind a heptameric consensus motif that has been characterised as: 5'-(A/T)(A/T)CAA(A/T)G-3'. They bind to the minor groove of DNA (Laudet et

al. 1993), unlike most other transcription factors, which bind the major groove. When SOX proteins bind, they induce a 70-80° bend in the DNA, a property that is essential for their role in regulating gene expression (Scaffidi and Bianchi 2001). Vertebrate SOX genes can be classified into distinct groups, based on sequence comparisons of their HMG domains, as well as non-box sequences (Laudet et al. 1993; Wright et al. 1993; Soullier et al. 1999; Wegner 1999; Bowles et al. 2000; Schepers et al. 2002). In the mouse, there are 20 *Sox* genes classified into 8 groups (A to G, including B1 and B2) (Bowles et al. 2000). The majority of SOX proteins have HMG domains with at least 50% identity to the HMG domain of SRY (Bowles et al. 2000). Mouse SOX2 has 50% HMG sequence identity with that of SRY (Collignon et al. 1996), but some of the most recently classified members such as hu-SOX30 only have 48% homology with SRY (Osaki et al. 1999). Many of the *Sox* genes found in vertebrates are thought to have arisen by genome duplication (Soullier et al. 1999; Wegner 1999; Bowles et al. 2000). This is probably true of the closely related genes *Sox1*, *Sox2* and *Sox3*, which are classified in the SOX B1 subgroup (Kamachi et al. 2000), but the duplication must be ancient, because each gene maps to a different chromosome (8, 3 and X, respectively).

Figure 1.5 An unrooted phylogeny for the mouse SOX proteins. Adapted from Bowles et al (2000), where it was computed based on SOX HMG amino acid sequences by distanced based phylogenetic analysis. Different colours are used to identify individual groups. Most recent members SOX16, SOX12 and SOX30 have not been included in this diagram as there is insufficient information available on them.



The ability of SOX proteins to act as transcription factors depends on their interaction and co-operative activity with other transcription factors. SOX2 is expressed in the ICM and epiblast of murine blastocysts and then later in the forming neuroepithelium. Its expression pattern in the early embryo overlaps with that of OCT3/4- a POU transcription factor (encoded by the *Pou5f1* locus and is also referred to as *Oct3* and *Oct4*). SOX2 binds the *Fgf4* enhancer in exon 3 of the gene, separated by only 3bp from the POU binding site were OCT3/4 binds. The two factors act in synergy to regulate *Fgf4*, which is required for early embryogenesis (Ambrosetti et al. 1997). OCT3/4 also acts together with SOX2 to regulate *Spp1* (*osteopontin*) (Botquin et al. 1998). OCT3/4 activates transcription of *osteopontin* when it binds the enhancer alone, whereas SOX2 binding results in transcriptional repression. The pair also regulate UTF1 (Nishimoto et al. 1999), *Fbx15* (Tokuzawa et al. 2003) and as mentioned earlier, *Nanog*. SOX2 also regulates *Nestin* (Tanaka et al. 1998). SOX9 binds the chondrocyte-specific *Col2a1* (collagen II) enhancer in the first intron of the gene (Bell et al. 1997). Together with SOX6 and SOX5, the activation of *Col2a1* by SOX9 is greater, and this cooperative activation has also been exhibited for the *aggrecan* gene, which is also a chondrocyte marker. Other examples include the co-operation of SOX11 and BRN-1 or BRN-2 and SOX10 with PAX3, CREB, MITF or TST1/OCT6/SCIP.

There are examples of SOX proteins expressed throughout development, in many cases with overlapping expression in specific tissues. As SOX proteins have the ability to recognise very similar DNA motifs, they may function in the same processes. Moreover, for members of a subfamily, with a high degree of sequence similarity, they may have very similar functions. Indeed, functional redundancy has

been demonstrated for several SOX proteins. One well-characterised subgroup whose members exhibit functional redundancy is B1 (*Sox1*, *2* and *3*). The three genes are expressed throughout the nervous system from its earliest stage. Inhibition of SOX2 function leads to delamination of neural progenitors from the ventricular zone and exit from the cell cycle, a phenotype that can be rescued by co-expression of *Sox1* (Graham et al. 2003). *Sox1*, *2* and *3* have all been shown to suppress neurogenesis in order to maintain CNS cells in an undifferentiated state (Bylund et al. 2003; Graham et al. 2003). Outside the CNS, other SOX proteins have also been shown to act in similar ways: SOX10 (group E) inhibits differentiation of neural crest progenitors (Kim et al. 2003) and SOX15 (group G) inhibits muscle differentiation (Beranger et al. 2000). Group B also contains the subgroup B2 members *Sox14* and *Sox21*, whose encoded proteins are similar to each other but resemble B1 proteins only in the HMG box and immediately proximal to the HMG box in their C-terminal domains. As shown in the chick, SOX21 promotes neuronal differentiation, thus counteracting the activity of the SOXB1 subgroup factors (Sandberg et al. 2005). This study also demonstrated that, although *Sox21* is upregulated by proneural bHLH factors, its proneural action is independent of their expression. Members of other groups also have overlapping expression patterns, such as the genes *Sox4*, and *Sox11*, both members of group C. When co-expressed, members of the same group may show redundancy. However they can have unique roles in other tissues, and it remains possible that they also have subtly different roles even within the same tissue.

In the chicken, *Sox11* (group C) is co-expressed with *Sox1*, *Sox2* and *Sox3* in specific regions of the neuroepithelium and is transiently up-regulated in maturing neurons after they leave the neuroepithelium and as they down-regulate *Sox2* and *Sox3*

(Uwanogho et al. 1995). This switch in *Sox* genes indicates that *Sox11* may be involved in the differentiation or maturation of particular neuronal populations. Its expression in the murine CNS is strong during development and it persists in differentiating neuronal populations in the cortical plate and the inferior colliculus (Kuhlbrodt et al. 1998). *Sox11* shows high sequence homology with another group C gene, *Sox4*. Like *Sox11*, *Sox4* expression seems associated with maturing cells of the CNS and the two genes have an overlapping expression pattern. The expression of *Sox4* is sustained for longer periods than *Sox11* in the brain, but the opposite occurs in the spinal cord. (Cheung et al. 2000).

1.4.1 Expression of SOXB1 subgroup genes

The expression patterns of the *Sox* Group B members have been extensively described elsewhere (Collignon 1992; Ishii et al. 1998; Uchikawa et al. 1999; Wood and Episkopou 1999; Cheung et al. 2000; Avilion et al. 2003; Pevny and Rao 2003; Bell 2004). *Sox2* is the first of the genes to be expressed in the embryo where it is found in the inner cell mass of blastocysts at 3.5 dpc (Avilion et al. 2003). By 6.0 dpc, *Sox3* is expressed together with *Sox2* in the extraembryonic ectoderm and throughout the epiblast. Their expression becomes restricted during gastrulation to the chorion and anterior ectoderm. By early headfold stages (7.25 dpc), *Sox3* is downregulated in the extraembryonic component whilst *Sox2* maintains expression (Wood and Episkopou 1999). At this stage *Sox1* expression can also be detected in the neural plate. The three genes show overlapping expression in the neuroectoderm at the onset of somitogenesis (Collignon 1992; Wood and Episkopou 1999). The expression of *Sox2* does not overlap with *Sox1* or *Sox3* in the floor plate and roof plate regions of the neural tube. *Sox1* and *Sox2* are largely restricted to the neuroectoderm until

midgestation and *Sox2* in expressed in the surface ectoderm region from where the sensory placodes will form, from the six somite stage onwards, and then becomes resticted to the placodes within this area (Collignon 1992). *Sox2* and *Sox3* are both expressed in the olfacory placodes but *Sox2* alone is expressed in the otic placodes (Wood and Episkopou 1999). During lens formation, *Sox2* is expressed at the earliest stages in the optic cup and prospective lens tissue, being replaced in the latter by *Sox1* from about 11.5 dpc (Kamachi et al. 1998). *Sox2* and *Sox3* expression patterns partially overlap in the endoderm of the posterior foregut (Collignon 1992; Kamachi et al. 1998; Wood and Episkopou 1999) whilst *Sox2* is also expressed throughout the gut and lung endoderm and by 11.5 dpc also in the oesophagus. In developing hair follicles *Sox2* is expressed in the dermal papilla from 12.5 dpc and later in the dermal sheath and in epithelial cells within the bulge region (Bell 2004). *Sox2* in also expressed in the nasal epithelium at 12.0 dpc, in the prospective sensory placodes in the ear and in the prospective fungiform papillae in the tongue by 13.5 dpc (Bell 2004). In the CNS *Sox2* expression persists and from 13.5 dpc onwards it becomes more restricted as cells differentiate. It remains in the ventricular layer and by 16.0 dpc also in the subventricular layer of the forebrain, the ependymal layer of the spinal cord and the olfactory epithelium. This expression is maintained in the adult. Cells migrating along the rostral migratory stream (RMS) express *Sox2*, as do cells migrating from the ventricular zones of the lateral ganglionic eminence to populate the nucleus accumbens and olfactory tubercle (Bell 2004). In the latter population, *Sox1* and *Sox3* are also expressed. *Sox1* expression is more extensive in the adult olfactory tubercle than that of the other B1 subgroup genes.

1.5 Microarrays

Determining the set of genes that are active in neural stem cells in comparison with those that are active in their differentiated derivatives, is a prerequisite in the attempt to understand the factors necessary to define the stem cell state. Expression analysis using northern blotting or RT-PCR employs hybridisation of oligonucleotide probes to mRNA. Single probes are used to hybridise with the sample and signals revealed in such a way as to yield quantitative information about each gene. Spatial information on gene expression can be obtained through *in situ* hybridisation analysis. Microarray technology revolutionises the speed and effectiveness of these assays, as several thousand probes are immobilised on a substrate and assayed at the same time. This reverses the situation in northern blot analysis, where the target is immobilised and the probes are added individually. Fluorescent labeled target cRNA or cDNA samples, prepared from the tissues under investigation are then allowed to hybridise with the probes, and a confocal laser scanner is used to detect the fluorescence emitted by the successfully hybridised probes. An expression profile is obtained for each sample, providing information in a qualitative and quantitative manner on thousands of mRNAs according to their extent of hybridisation with the immobilised probes. A variety of sources have been used to compile this introductory section to microarrays, including books (Knudsen 2002; Bowtell and Sambrook 2003), publications (The Chipping Forecast I, II and III, 1999, 2002 and 2005, respectively) and manuals (GeneSpring User Manual 2002, Ambion TechNotes articles on Array Analysis), as well as personal experience.

Two types of probes are commonly used for microarrays: cDNA and long oligonucleotides (short oligonucleotides are also used but have progressively become

less common due to their low specificity). Both of these can be immobilised in spots on a glass substrate (usually coated with poly-L-lysine) using a robot spotter. Their major advantage is that they can be custom made and tailored according to need, allowing specific pools of genes to be analysed in different areas of research. For routinely used sets of spotted arrays, the cost is low, but a custom-made chip with thousands of probes can be very expensive. Problems regarding spotted arrays usually arise from their printing. Spotting of the same probe over several slides may not be uniform, and within one slide, smearing and irregularity of spots can occur due to various printing problems, resulting in the loss of information from large portions of the array. The ideal spotted array has round, equidistant, evenly sized spots of uniform concentration and volume, generating images of low background fluorescence and uniform spot intensity across the entire slide. Successful hybridisations on spotted arrays of high quality can provide a relative measure of the expression levels of genes assayed by the entire probe set. Binding of labeled target to a probe spot is an indirect measure of the expression of that gene, as the fluorescence intensity emitted is proportional to how many copies of labeled target bind, the latter having been generated from the expressed mRNA.

cDNA probes are usually amplified from clone sets by high-throughput PCR. Unreliability of the probe sets can be a result of inefficient PCR and/or contamination by primers or enzymes used in their production. Oligonucleotide probes are more cost-effective and reliable. Long oligonucleotide probes are now commonly used, and these can be designed accurately and specifically against regions with low homology to related genes. Their synthesis per base is lower than that of cDNA probes and they typically represent 50 to 75 nucleotide fragments, making them highly specific.

Appropriate positive and negative controls for the specific probe set are normally incorporated on the slide. Spotted arrays are normally used for the simultaneous assessment of two targets (representing all the transcripts of two cell or tissue samples). This is useful in order to compare the relative expression of genes between them. Different fluorescent labeling of the targets (Cy3 and Cy5) allows the samples to be distinguished after scanning. When such a protocol is followed, dye-incorporation can be biased giving a stronger intensity signal for e.g. the Cy3 labeled sample compared to the Cy5 labeled sample. This can lead to errors in the interpretation of their expression profiles. To minimise this error it is usual to perform dye-swaps in two-colour experiments. Where one target sample (A) is labeled with Cy3 and the other (B) with Cy5 in one hybridisation, in a replicate hybridisation their dyes are reversed (A labeled with Cy5 and B with Cy3). Any intensity bias can be normalised after scanning, by using the dye-swap sample as a reference. This type of quality control in an experiment is essential to generate reliable results. Another important aspect of quality control is the replication of the experiment. Replicates can be of technical or biological significance. Technical replicates would involve repetition of a protocol at any stage of the sample preparation, such as a hybridisation using independent cDNA transcription or an independent round of labeling of the target sample. However, error is less likely to be introduced in the handling of the sample than it is in the generation of the sample itself. When comparing a treated sample versus a normal sample in an experiment, the treated sample may not be affected in an identical manner when the treatment is reproduced. Therefore at least one biological replicate is required in a two-colour experiment. Minimising biological variability by pooling samples prior to or after RNA extraction is not advisable, because any significant outlier values cannot be traced to a specific sample and

eliminated. This could bias the experiment. However, if performed carefully, pooling can increase the reliability of the experiment. For example, in mouse studies involving outbred strains, pooling can average differences due to genetic heterogeneity. It is appropriate in cases where starting material is limited and the classification of samples is clear-cut; e.g. treated versus non-treated. For two-colour experiments, 3 replicates are normally performed, including a dye-swap and an independent biological repeat. In this way, if there is differential expression for one gene between two repeats, the third one may be used to clarify the situation. In all cases, subsequent validation of the microarray results using independent samples and a different assay (e.g. quantitative real-time PCR (qRT-PCR)) is required.

A more reliable microarray platform than spotted arrays is provided by Affymetrix GeneChip technology. GeneChips consist of oligonucleotide probes, synthesised *in situ* on a glass surface. Each probe that is perfectly complementary to the mRNA of that gene (perfect match probe) is represented more than once on the array, and it is synthesised adjacent to a series of probes with a single nucleotide change in a central position of the probe, to a complementary nucleotide (mismatch probe). The mismatch oligonucleotides are used to detect background and non-specific hybridisation. For perfect match probe design, a region of the gene with low homology to other genes is selected. For eukaryotic genomes they are chosen with a bias to the 3' end to minimise effects of partial mRNA degradation and incomplete cDNA or cRNA synthesis. 11 to 20 perfect match probes, typically 25-mer oligonucleotides, are designed against this region along with their mismatch probes. All together, these form one probe-set specific for a target gene. Target genes may be represented in independent probe sets more than once, typically against alternate

published sequences for the gene. Controls such as bacterial spikes, housekeeping and consistently expressed genes are represented in such a way so the mRNA input quality can be assessed, based on a ratio of hybridisation to 3' end and 5' end specific probes. The reliability of this technology extends to the high reproducibility in array manufacturing. The probes are synthesised at high densities (400,000 different probes in a 20 x 20 mm area) using photolithography in conjunction with combinatorial chemistry (McGall et al. 1996). Photolithography uses light to remove photolabile protecting groups from linkers bound to a glass substrate, which enables nucleoside phosphoramidite addition in specific locations specified by a mask. For the synthesis of an array, around 80 cycles of this are required.

A disadvantage of Affymetrix GeneChip arrays is their relative high cost. Hybridisation protocols involving GeneChips, which are encased in protective plastic throughout the hybridisation and washing, require GeneChip- specific hardware such as a fluidics station and scanner, which may not be accessible to all users. They can however, cut costs through the high specificity and the nature of the hybridisations. There is less of a requirement to perform technical replicates for a hybridisation, as the GeneChip methodology is highly reproducible. However, it is still recommended that an independent biological repeat is performed, or alternatively pooling of sample material. In a single hybridisation, one biotin-labeled target sample is used to hybridise one GeneChip. Other target samples are compared to it through subsequent software analysis. When a larger number of samples are to be compared, this system results in fewer hybridisations in relation to spotted arrays, where samples have to be compared pair-wise, with no flexibility to create new comparisons through software analysis. However, the design of spotted array experiments can be modified to

overcome this problem; a reference sample can be used to hybridise alongside the target samples. By keeping the reference constant, more targets can be incorporated in the study and compared to each other in any combination using appropriate software.

From both spotted and GeneChip platforms, results have to be interpreted and normalised in order to be analysed further. The initial collection of the results is in the form of fluorescence intensity emitted from the hybridisations. The scanning process generates an image of the array chip with assigned colours to represent the fluorescent hybridised probes. For spotted arrays, this image has to be processed in order to let the software (such as ImaGene) know where the spots are located. Pre-designed grids are available for commercially available spotted arrays. A manual quality control has to take place in order to define the spot location and size as plenty of variation occurs during robotic spotting. Spot locations with no probe, merged spots or smears have to be 'flagged' (tagged with a label in the software) so that they are not processed as true data. Intensities are given arbitrary values, usually ranging from 0 to around 50,000. Two-colour experiments receive independent values for the Cy3 and Cy5 channels. Each probe is then assayed for signal intensity after background intensity has been subtracted. In spotted arrays, raw intensities under 200 are considered potential background (depending on the specific slide), and in Affymetrix arrays this number is generally lower. (In this thesis, this number ranges between 50 and 100, always taking the value providing highest stringency, depending on expression levels of known negative controls.) Each probe or probe set in the case of GeneChip is assigned a label as detected (Present, P), not detected (Absent, A) or marginally detected (Marginal, M). For probe sets, P is assigned only when the majority of probes against a specific

target are in agreement. Changes of intensity 1.8 times higher than background noise are generally detected as P.

Comparison analysis of two-colour experiments takes place by dividing the intensity of one channel by the other and generating a relative value. For one-colour GeneChip experiments, probe pairs in a set are checked for changes between two experiments and are labeled as increasing (I), decreasing (D) or showing no change (NC), the latter incorporating probes that were previously deemed marginal. In order to calculate fold change of expression levels between target samples, a minimum possible intensity denominator above zero is set, which is based on the levels of noise.

In order for different target samples to be compared, their intensity values need to be adjusted to a baseline. This primary analysis can be done in the GCOS software of the Affymetrix platforms. For spotted arrays, the normalisation is performed in downstream analysis software such as GeneSpring. A measurement of variance has to be calculated between samples to identify whether differences between them are likely to be significant or not. For two samples, a Student's t-test is commonly applied and for multiple samples Analysis of Variance (ANOVA) tests are appropriate. Their performance can be specified in the software and are automatically performed by the software upon data entry between probe replicates on a slide or between the same probes across slides with replicate hybridisations. They are used to identify outliers, which may be an entire chip that deviates from its replicates, or one or more genes deviating from the same genes on other samples. Outliers are given a low significance and can be removed from the analysis.

Normalisations of samples needs to be specified and typically involves a per chip normalisation, and a per gene normalisation. A per chip normalisation can be carried out with reference to a median, percentile or positive control e.g. normalising each measurement to the 50^{th} percentile of the total measurements in that sample. A per gene normalisation can be carried out with reference to the median of the measurements in all samples. If, after normalisation, sample intensities do not follow a comparable normal distribution when plotting normalised log intensity against all samples (i.e. non-continuous mode), e.g. if there is an abnormal shift in the distribution of log intensities, various further statistical correction methods may be applied before the samples are compared.

Data can be represented in various forms such as a graph of normalised intensities across samples or a scatter plot of logarithmic intensities of one sample compared to another. In scatter plots, expression differences can be assessed as fold change. Fold change is calculated by dividing the expression level of a gene in one sample (A) by the expression level of the same gene in another sample (B). Genes whose expression does not change between samples have a fold change of 1 and follow a linear distribution on the plot. Genes expressed higher in sample A will have a value over 1, e.g. 2-fold higher in A = 2 (2/1), and genes expressed higher in B will have a value under 1, e.g. 2-fold higher in B = 0.5 (1/2). For this type of data analysis to be useful it has to be calculated on a logarithmic scale, otherwise the scale becomes asymmetric as up-regulated genes can range from 1 to infinity and down-regulated genes can only range from 1 to 0. Problems with fold change values arise if an expression value is zero or even negative. To overcome this problem, a data transformation in the normalisation step can be applied to assign a set value to such data, e.g. setting

measurements of less than 0.01 to 0.01. Data points, which are expressed at different levels between samples, are seen to deviate from the linear distribution. A significant fold change value has to be set for each experiment. Typically, in the experiments described in this thesis, and in line with other reports (Geschwind et al. 2001; Ahn et al. 2004) a 2-fold change in expression when comparing two target samples is considered significant. In order to determine what fold change is significant, the change in expression has to be compared to the standard deviation for each gene and the more it exceeds the standard deviation, the more significant it is (Tusher et al. 2001). For higher expressed genes, which have a low variance between replicates, a fold change of even less than two can be determined as significant. For very weakly expressed genes, a much higher fold change is required to reach significance.

The first point of data analysis, as discussed, is looking at genes expressed significantly higher and lower between two samples. Further analysis, which becomes essential when more than two experimental samples are included, involves grouping genes into clusters that behave in a similar fashion under different conditions. There are several types of clustering including hierarchical clustering, *K*-means clustering and the creation of self-organising maps. Hierarchical clustering calculates the distance between all the genes, taking account of the number of samples as the number of different dimensions, to produce a distance matrix. Genes that are closest together in this hypothetical space are grouped in the same cluster. A point falling in the middle of each cluster, or centroid, is used to calculate the distance of each gene to it and to other centroids to verify the cluster that each gene should be in (if it is closer to another centroid, it will be reassigned to this cluster). For large numbers of genes, *K*-means clustering may be more appropriate. It will classify genes into clusters but

not compute the relationship between clusters. In this algorithm, a user-defined number of clusters are set and all genes are randomly assigned in clusters. Genes are then reassigned to the cluster whose centroid they are closest to and the centroids are re-calculated after every reassignment. Once the value of the centroids does not change, the algorithm stops. Self-organising maps are similar to *K*-means clustering, but constrain centroids in a two-dimensional grid. For the experiments described in this thesis, only *K*-means clustering has been applied.

At the time when the experiments for this thesis were initiated, cost limitations and lack of support facilities ruled out the possibility of using Affymetrix GeneChips. Oligonucleotide spotted arrays were selected as a platform due to increasing evidence of their reliability at the time and because they were available free of charge through the HGMP. The 'Mouse Known Gene Oligo' array was selected incorporating 7,445 known gene sequences and 79 controls (bacterial spike probes and negative spots, ubiquitously expressed genes, tissue-specific genes and pure Cy3 spots termed 'landing lights' to aid in orientation and Cy3-labeled target hybridisation quality control). These sequences were represented by 65-mer oligonucleotides that showed average cross-homology of 30.5%. Each probe was represented in duplicate on the array. The experiments were performed at the Medical Research Council Mammalian Genetics Unit, which runs a 'hoteling' facility, allowing microarray users to perform their experiments using the provided reagents, hardware and software. In 2004, the HGMP stopped making these arrays available. However, by this time an accessible Affymetrix GeneChip facility had been established at NIMR. From this point, all microarray experiments were performed on the Mouse 430A GeneChip, containing 22,690 known genes. The experiments performed are described through Chapters 4, 5

and 6. Following guidelines set out by Brazma and co-workers (Brazma et al. 2001) a 'Minimum Information About a Microarray Experiment' (MIAME) protocol was compiled for each experiment and is accompanied by an experimental outline. MIAME information is an integral feature of microarray experiments in order to aid in the reproducibility and understanding of the experimental design. This information will be deposited on publication of any work arising from this thesis.

1.6 Aims of Thesis

General Aim

The aim of this thesis is to explore and understand the characteristics of neural stem cells and lineage-restricted progenitor cells and to determine if there are distinct neural stem cell populations within the CNS or a single stem cell type that displays diverse potential in response to environmental signals.

Specific Aims

- To standardise optimal culture conditions for different NSC populations so as to characterise them and perform comparative studies.
- To investigate the role of SOX2 in NSCs by:
  - (i) Characterisation of the expression pattern of SOX2 in neural stem cell generating regions as well as it's sub-cellular localisation in proliferating and differentiating NSCs
  - (ii) Determining the effects of reducing the levels of SOX2 in NSCs; this can also be used to indentify potential downstream target genes.
- To compare the properties and gene expression profiles of stem cell-enriched neurosphere cultures to non-enriched cultures in order to identify properties of NSCs.
- To identify novel candidates that can be used as markers for neural stem cells.

CHAPTER 2

Establishment of neural stem cell culture conditions

2.1 Background

2.1.1 Neurospheres as a culture system to study stem cells

Monolayer culture was the first method used to allow the survival of neuroepithelial cells, including NSCs. When these neuroepithelial cultures where plated under non-adherent conditions, some cells had the ability to survive in suspension and form clonal, spherical colonies which are known as neurospheres (Reynolds and Weiss 1992). These methods were then adapted to select for floating neurosphere colonies, eliminating the differentiating adherent cells. The neurosphere system is the best-characterised *in vitro* assay for neural stem cells and a good model to study their differentiation and to search for new cell type-specific markers, but they do not recapitulate the *in vivo* situation. The cultures may encourage NSCs with a particular phenotype to survive, and suppress others. Also, such spherical structures do not exist in the mouse CNS and stem cells reside in distinct zones alongside differentiated cells. Also, any interactions observed between cells in the neurosphere will not necessarily take place *in vivo*. Moreover, neurospheres contain a mixed population of NSCs and their differentiated progeny (see Results). All this has to be borne in mind when interpreting experiments.

The presence of neural stem cells can be identified through the formation of neurosphere colonies from single cells in culture that subsequently differentiate into cells of all major neural types i.e. neurons, glia and oligodendrocytes. However, there is a lack of markers to distinguish neural stem cells from committed progenitor cells

that are incapable of self-renewal. There is a lot of circumstantial evidence that neural stem cells express the neuroepithelial marker Nestin as well as SOX2- indeed the former is a direct transcriptional target of the latter (Tanaka et al. 2004). However, some differented cells can also express stem cell markers. For example, cells of the ependymal zone of the adult CNS express both SOX2 and Nestin (Ferri et al. 2004; Dahlstrand et al. 1995). There is evidence that radial glial cells are the stem cells in the embryonic CNS from midgestation stages and that these subsequently give rise to specific astrocytes that act as stem cells in the adult (Malatesta et al. 2000; Merkle et al. 2004). However, it is not clear wether all radial glia are NSCs and radial glial cell morphology is not seen in cells of the neurosphere unless the cells are allowed to attach and differentiate. Moreover, not all astrocytes will have neural stem cell properties in the adult, so further markers are required to characterise this subpopulation. For example, GFAP marks astrocytic stem cells but also committed astrocytes. It is also not clear if cells other than astrocytes and radial glia can act as stem cells. This poses a major problem in isolating murine NSCs based on marker expression and pure populations of NSC cannot presently be achieved through neurosphere culture. A recent study has described the culture of radial glia-like NSCs as a pure stem cell population grown as a monolayer (Conti et al. 2005), but this method was published only late during the progress of this thesis. Apart from expressing Nestin and *Sox2*, we know that NSCs of the adult forebrain expressing *Ssea-1* (also referred to as *LeX* or CD15) have the ability to generate neurospheres (Capela and Temple 2002).

2.1.2 NSC in vitro culture methods

Protocols for NSC or neuroepithelial precursor culture have been extensively described in the literature. Culture conditions differ for progenitors or NSCs isolated from different regions of the CNS and at different developmental stages. When evaluating NSCs isolated from different regions or developmental stages, it is important to minimise the differences between protocols so that the resulting populations are comparable. For the experiments in this thesis, standard protocols were applied and were modified in order to improve survival efficiency and to increase the proportion of the undifferentiated cells being cultured, as will be described in this chapter. Serum free media has commonly been used in the expansion of neural stem and progenitor cell lines. Some groups have introduced the addition of serum only for the first few days of culture to increase survival. Serum however, encourages growth of the cells as a monolayer attached to a substrate, such as poly-D-lysine coated surfaces, but is not suitable for long-term suspension cultures. DMEM-F12 is used as the basic medium as it has a high glucose to lactate ratio (3:1) that encourages CNS cell growth. The HAMS F12 component of the DMEM-F12 contains a range of supplements and amino acids. Alongside the antibiotics penicillin and streptomycin to prevent infection, N2 supplement is often added, which specifically encourages neural progenitor growth and is a commercially available hormone mix (containing insulin, human transferrin, putrascine and progesterone). Another supplement that can be added is B27, which increases survival of proliferating cells as it contains antioxidants (Svendsen et al. 1995), an attribute especially advantageous in the initial establishment of primary cultures. The mitogens EGF or bFGF can also be used alone or in combination, while the addition of heparin to the medium can enhance the mitogenic effects of bFGF on neural progenitors (Caldwell and Svendsen

1998). NSCs isolated from early murine CNS (~12.0 dpc) initially respond only to bFGF as they do to not have EGF receptors. NSCs isolated from later stages respond to both bFGF and EGF (Kilpatrick et al. 1995). Studies suggest that bFGF-dependent neural stem cells give rise to progenitors that are also able to respond to EGF (Ciccolini and Svendsen 1998). Progenitors only responsive to EGF have also been characterised as stem cells (Reynolds and Weiss 1996).

Growing NSCs as neurospheres under clonal conditions provides several advantages. The greatest one is that an assessment of proliferating cells from the initial cell population can be achieved through counting the numbers of neurospheres generated. Passaging neurospheres weekly results in an expansion of the stem cell population. Although serial passaging without a decrease in stem cell activity should be a characteristic of a healthy and established stem cell line, certain factors in the methodology can affect this outcome. The action of enzymes such as trypsin to dissociate neurospheres into single cells induces spontaneous differentiation when applied long-term, however it is useful for short-term studies involving a couple of passages. When performed to completion, mechanical dissociation of neurospheres bypasses the problem of excess spontaneous differentiation but results in high levels of cell death, probably due to damage. Passaging of neurospheres too frequently also results in differentiation and a decline in proliferation rates. This may be attributed to regular passing of cells to a post-mitotic type or to the frequent removal of cell-cell contact that seems to play an important role in NSC maintenance and/or proliferation.

Dissociation of neurospheres and embryonic CNS tissue can be achieved by mechanical dissociation or short enzymatic treatment. Adult CNS tissue requires

extensive enzymatic as well as mechanical treatment using a fine-polished Pasteur pipette, or similar. Papain treatment is widely used, frequently in conjunction with DNase. More gentle enzymes are also used, such as the neutral metalo-protease dispase from *Bacillus polymyxal*, which cleaves fibronectin, collagen IV and to a lesser extent collagen I. Since it does not cleave collagen V and laminin, it is often combined with collagenase from *Vibrio algionolyticus*. The latter two enzymes are not inhibited by serum that may be used in the culture. When cells are plated at a very low density, proliferation and cell survival rates drop. As an optimal guideline, neural stem cells should be cultured as neurospheres at 2 x 10^5 cells per ml (Lanza et al. 2004). To encourage single-cell proliferation when cultured as a monolayer during clonal analysis, the addition of 10% chick embryo extract (CEE) has been suggested (Stemple and Anderson 1992; Kalyani et al. 1997).

Table 2.1. Outline of pioneering culture conditions described in the literature, for the generation of neurospheres from CNS regions at various stages of development.

Age	Region	Medium	Supplement	Mitogens	References
10.0 dpc	Mesencephalon	DMEM+ 1% or 10% FBS	N2/ none	bFGF + heparin	Murphy et al, 1990
10.0 dpc	Telencephalon	DMEM+ 1% or 10% FBS	N2/ none	bFGF + heparin	Kilpatrick et al, 1993
14.5 dpc	Striatum	DMEM-F12	N2	EGF	Reynolds et al, 1992 Vescovi et al, 1993
3-18 months	Striatum	DMEM-F12	N2	EGF/ bFGF + heparin	Reynolds & Weiss 1992, Gritti et al, 1996

Culture conditions need to be standardised as much as possible to avoid both culture artefacts and selective expansion or diminution of particular cell types, both of which could significantly distort or complicate the interpretation of any experimental result. The aim of this chapter is to determine the optimal treatment for healthy isolation and culture on neural stem cells so that spatial and temporal comparisons can be performed with minimal bias.

2.2 Results

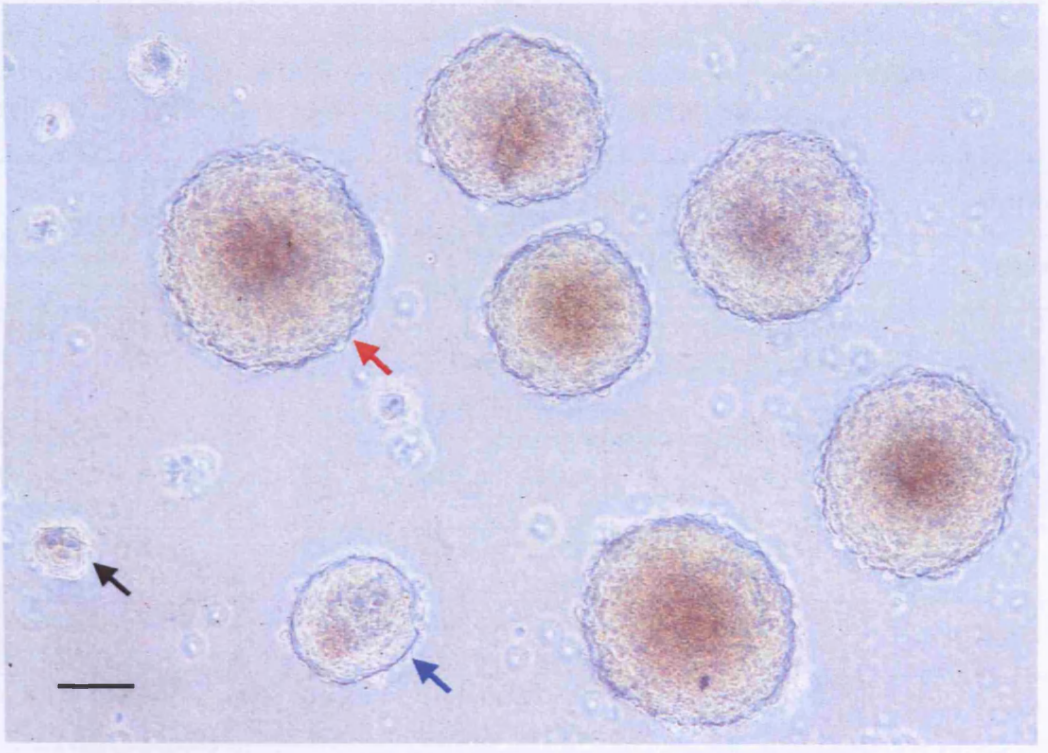
2.2.1 Establishment of optimal culture conditions

Neurospheres from the 14.5 dpc dorsal telencephalon are first identifiable after 60 hours of culture. After 4 days in culture, new neurospheres are seen, smaller in size than the primary neurospheres that initially formed. This secondary population appears to grow at the same rate as the primary population. Approximately three days later (i.e. day 7 in culture), a new set of neurospheres is observed even if primary and secondary neurospheres are selectively picked and re-plated to eliminate remaining single cells and debri from the culture. These waves are not frequent after 10 days of culture and become irregular and are demonstrated in Figure 2.1. Possibilities for their generation may be the proliferation of latent cells (either NSCs or more committed progenitors with proliferative capacities) in culture at delayed points, slower proliferation rates of some NSCs or the detachment of NSCs from primary neurospheres that subsequently generate secondary structures.

A range of different methods was used in order to establish the optimal conditions for neurosphere generation by CNS stem cells. Preliminary results from wild type dorsal telencephalon-derived neurospheres from 14.5 dpc suggest that the combined addition

of the mitogens EGF and bFGF during the entire length of culture leads to increased numbers of initial neurospheres formed and of secondary and tertiary sets, in comparison to using bFGF or EGF alone. I aimed to define a set of culture conditions as similar as possible to each other, in order to culture different NSC populations with spatial and temporal differences and use these in comparative investigations.

Figure 2.1 Sequential waves of wild type 14.5 dpc dorsal telencephalon neurospheres. After 8 days of culture in proliferation medium, three generations of neurospheres can be seen (distinguished by the red, blue and black arrows) Scalebar represents 250 μm.



Variations in the methods used to dissociate primary tissue and to culture NSCs and the efficiency of each method is outlined in Tables 2.2 and 2.3 below. In summary, mechanical dissociation of tissue and culturing in DMEM-F12 with the addition of N2, B27, EGF and bFGF (termed 'proliferation medium') lead to an increased

number of primary neurospheres from various CNS regions at embryonic stages. For the generation of neurospheres from the adult subventricular zone, enzymatic dissociation of the tissue using papain yields the highest neurosphere numbers when cultured in proliferation medium as described above. For all populations, the dissociation of neurospheres in culture during passaging to generate secondary neurospheres is most successful after digestion with trypsin for 2 minutes followed by mechanical dissociation.

Table 2.2 Summary of the methods of tissue dissociation for the generation of primary neurospheres, rated overall (- to ****) for enhanced neurosphere formation and low levels of differentiation based on morphological observations.

Treatment (optimal culture medium)	**Observations**	**Rating**
Mechanical	High neurosphere formation, little differentiation	****
Trypsin (1.33 mg/ml)	High neurosphere formation, medium differentiation- best for 2ry neurosphere dissociation	***
Trypsin (1.33 mg/ml) /Hyaluronic acid(0.7mg/ml)/ Kynureic acid (0.2 mg/ml)	Low neurosphere formation, high differentiation	-
Dispase (1U/ml)	Neurosphere formation, medium differentiation	*
Collagenase (0.1U/ml)/Dispase (0.8 U/ml)	Neurosphere formation, low differentiation	**
Papain (adult SVZ only ****) (75U/5ml + 12µg/ml DNase)	High neurosphere formation, low differentiation	***

Table 2.3 Summary of results using different compositions of culture medium. Rated overall (* to ****) for efficiency of neurosphere formation and low levels of differentiation based on observations of cellular morphology in culture.

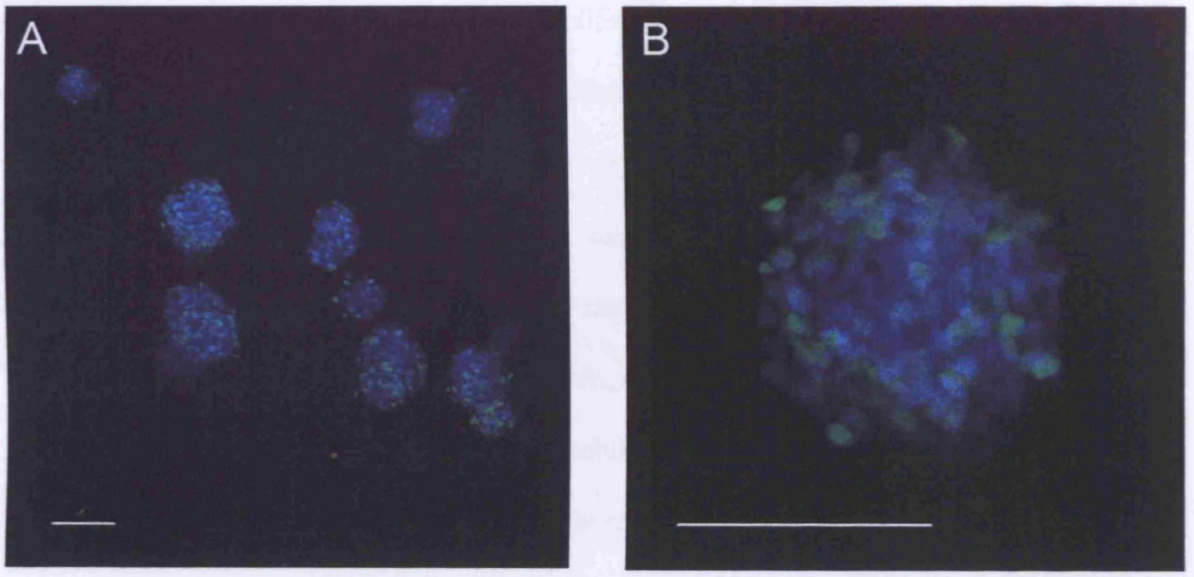
	Culture Medium	Observations	Rating
Dorsal telencephalon (14.5 dpc)	NSC hormone mix in DMEM-F12	Neurosphere formation	***
	B27 in DMEM-F12	Neurosphere formation, sustainable for > 2 weeks	**
	N2 + B27 in DMEM-F12	High neurosphere formation	****
Spinal cord (14.5 dpc)	N2 + CEE + bFGF in DMEM-F12. E9	Adherent neuroepithelial culture only	*
	N2 + B27 + CEE +bFGF in DMEM-F12	Adherent culture, high differentiation. Some neurosphere formation	**
	N2 + B27 + bFGF/EGF in DMEM-F12	High neurosphere formation	****
Subventricular zone (adult)	NSC hormone mix in DMEM-F12	Very low neurosphere formation	*
	B27 in DMEM-F12	Low neurosphere formation	**
	N2 + B27 in DMEM-F12	Neurosphere formation. High numbers only with papain dissociation (see below)	****

2.2.2 Establishment of clonal density

Preliminary observations on neurosphere cultures suggest that the density at which primary neural cells are plated affects the total number of primary neurospheres that form: when plated at clonal density (less than 1 x 10^3 cells/ml) or as single cells per individual well, 0.3% of cells give rise to neurosphere colonies (standard error of mean ± 0.09%). These neurospheres appear unhealthy, i.e. individual cells within the structure appear loosely adhered and the spheres do not appear completely round due to cells detaching from the periphery. By one week of culture they either attach to the well surface and differentiate or die. However, when wild type single cells are plated at a density of 1 x 10^4 cells/ml, 1% (embryonic 14.5 dpc spinal cord) to 4% (embryonic dorsal telencephalon) of the cells generate neurospheres that are healthy in appearance and have the ability to give rise to secondary neurospheres upon dissociation. I aimed to determine if these primary neurospheres are generated from single cells or from several cells that have adhered to form clusters prior to proliferation. For this, $R26^{Eypf/Eyfp}$ 14.5 dpc dorsal telencephalon cells, which ubiquitously express EYFP, were plated together with wild type 14.5 dpc dorsal telencephalon cells at equal numbers, at a range of cell densities. At the densities of 1 x 10^4, 1 x 10^5, and 1 x 10^6 but not 1 x 10^3 cells/ml, chimeric neurospheres formed (Table 2.4), incorporating both fluorescent and wild type cells (Figure 2.2 A and B). More detailed analysis involving intermediate densities, determined that the initial plating density of dissociated primary CNS cells has to be less than 5 x 10^3 cells/ml in order to ensure the clonal generation of neurospheres. No neurospheres incorporating both fluorescent and non-fluorescent cells were seen at this density (n = 54).

Figure 2.2 Establishment of clonal density in neurosphere cultures. (A,B) Example of chimeric neurospheres formed at a 1 x 10^4 plating density. Confocal imaging of these neurospheres, showing Rosa26-EYFP fluorescent in green and the nuclei of all cells in blue, stained with DAPI (A). Higher magnification of one such neurosphere (B) reveals the extent of aggregation and random distribution of the fluorescent cells. Scalebars represent 100μm.

Table 2.4 summarises the numbers of chimeric neurospheres formed at different densities, expressed as a percentage of the total number of neurospheres in the culture. Neurospheres that are formed through aggregation of fluorescent and non-fluorescent cells were used as an indicator of the extent of aggregation at various densities.



	Cell density/ml			
	1 x 10^3	1 x 10^4	1 x 10^5	1 x 10^6
% Chimeric neurospheres	0%	4.2%	35.9%	97.0%

2.3 Discussion

2.3.1 Establishment of neurosphere culture conditions

General observations of wild type neurosphere formation suggest that not all NSCs proliferate to generate neurospheres at the same time. In primary cultures from embryonic or adult CNS, secondary waves of neurospheres form after the initial primary neurospheres have become established (Figure 2.1). The possibility that there are quiescent NSCs in culture that do not proliferate initially is likely, based on high numbers of viable single cells in the culture that do not become incorporated in the primary neurospheres. These cells may become activated in culture in response to secreted factors, or they may have a distinct stem/progenitor profile from the initial neurosphere-forming NSCs as well as slower rates of proliferation. However, at even later stages, a tertiary neurosphere wave is seen, even when only whole neurospheres are re-plated. This suggests that cells from established neurospheres become detached and proliferate forming independent neurospheres. Indeed, from BrdU analysis, it is known that proliferating cells do reside on the surface of the neurosphere (data not shown). It is likely that delayed growth and detachment from established neurospheres are both taking place in long-term cultures.

As determined from the results summarised in Tables 2.2 and 2.3, embryonic neurospheres from the dorsal telencephalon and spinal cord can be cultured under identical conditions and the tissue dissociated using the same methods. For adult SVZ neurospheres, the same culture conditions as for embryonic neurospheres can be used but mechanical dissociation alone is not sufficient to effectively obtain a single-cell suspension. It frequently results in more cell damage than with the generation of embryonic neurospheres, due to repeated mechanical treatment, which in turn can

lead to higher levels of differentiation in culture. For this reason papain was determined to be the most appropriate enzymatic treatment for the dissociation of adult primary SVZ tissue.

2.3.2 Establishment of clonal density

The clonal plating density at which NSCs are able to give rise to neurospheres at a regular frequency was established so that subsequent experiments, assaying neurosphere formation as a proportional measure of the numbers of proliferating NSCs in culture, were not influenced by the potential aggregation of several NSCs giving rise to a single neurosphere. At very low densities, such as 1 x 10^3 cells per ml, long-term neurosphere cultures are not successful. The same applies to true clonal density assays when single cells are picked and plated in individual wells. This suggests that there may be a requirement for soluble factors, secreted by the cultured cells into the medium, which only reach threshold levels above a particular cell density, or that efficient survival and growth only occur when colonies are formed by more than one cell. By mixing equal numbers of fluorescent and non-fluorescent cells, any aggregation from the two populations is evident. However, at low cell plating densities, there may still be low levels of aggregation, which can pass undetected in the experiments outlined in section 2.2.2 if these events occur between same-phenotype cells. To overcome this, a larger sample size needs to be examined. Here, the highest density at which no aggregation was seen was determined by a 20-fold smaller sample size compared to the regular experimental scale used in other experiments this thesis. Therefore, the value of clonal density as less than 5 x10^3 cells per ml has been used as a guideline and not an assurance for the lack of aggregation.

CHAPTER 3

SOX2 in neural stem cells

3.1 Background

3.1.1 Tools to study Sox2 expression

The *Sox2*$^{\beta geo/+}$ mutant allele, generated by homologous recombination in ES cells, contains a *βgeo* sequence, encoding a β-galactosidase-neomycin fusion (Friedrich and Soriano 1991), which has replaced the open reading frame of the *Sox2* locus and is under the transcriptional control of *Sox2* regulatory elements (Avilion et al. 2003). Mice, which are homozygous null for *Sox2* die around implantation as the epiblast fails to form in the absence of *Sox2*. Neurosphere cultures derived from heterozygote targeted mutants show no apparent differences with wild type cultures. Hence, in the neurosphere experiments in this study *Sox2*$^{\beta geo/+}$ animals were used. The expression of β-galactosidase in *Sox2*$^{\beta geo/+}$ heterozygous mice can be used to monitor the expression of *Sox2* as these mice are viable and do not demonstrate any phenotype that may interfere with these studies. This allows for the visualisation of cells in which the *Sox2* promoter is active, through performing X-gal staining to assay for β-galactosidase activity. This assay cannot reveal the sub-cellular localisation of SOX2, so immunofluorescence staining using an antibody raised against SOX2 can be used. It does however, have another advantage: as the *βgeo* cassette confers neomycin resistance, the cells that do not express *Sox2* can be eliminated from the culture through the addition of G418. This tool will be used to investigate the role of SOX2 in NSCs, through assaying the differentiation and proliferation capacities of cells selected for *Sox2*$^{\beta geo}$ expression, as well as to aid in determining the expression

pattern of *Sox2*$^{\beta geo}$ in the regions of neural stem cell generation used in this study and in cultured neurosphere populations.

3.1.2 The association between the sub-cellular localisation of SOX2 and the state of differentiation

Several of the regions of the adult CNS that retain *Sox2* expression harbour neural stem cells, as defined by their culture properties and neurosphere generation capability. Some regions of the CNS however, still express *Sox2* in terminally differentiated populations such as purkinje neurons in the cerebellum where SOX2 is cytoplasmic, and in ependymal cells of the lateral ventricles and some neurons of the cortex, in both of which SOX2 localises in the nucleus (Lovell-Badge, unpublished data). Therefore the persistence of *Sox2* expression in such cell types is not an indicator of the stem cell state. However, the sub-cellular localisation of SOX2 may provide an insight into the differentiation state of the cells, depending on the region or tissue. In undifferentiated, pluripotent cells of the early embryo up until blastocyst stages, SOX2 localises in the nucleus. The first differentiation event in the early embryo leads to the formation of trophectoderm. In this tissue, *Sox2* is downregulated and the protein translocates to the cytoplasm. *In vivo*, nuclear SOX2 is generally observed in multipotent lineages while it tends to be seen in the cytoplasm of terminally differentiated cells.

The HMG box sequence of *Sox2* has two nuclear localisation signals (NLS), as do other members of the *Sox* family. One of these signals (NLS2) overlaps a consensus phosphorylation site for the cyclic AMP (cAMP) dependent protein kinase A (PKA). Through kinase assays on mutated forms of SOX2, it has been demonstrated that SOX2 is an *in vitro* substrate of PKA (Sockanathan et al, unpublished data). Initial

experiments suggested that treatment with db-cAMP induces the nuclear export of SOX2 to the cytoplasm, presumably through the activation of the PKA pathway. Other members of the SOX family (SRY and SOX9) exhibit nuclear-cytoplasmic shuttling and there is evidence to suggest that this is mediated by calmodulin (Harley et al. 1996). Calmodulin is an intracellular calcium receptor and is responsible for the inhibition and activation of numerous factors. It interacts directly with bHLH factors and inhibits their DNA binding capacity. Both SRY and HMG1 have been demonstrated to bind calmodulin (Harley et al. 1996). Therefore, it may be possible for SOX2 to also interact with calmodulin.

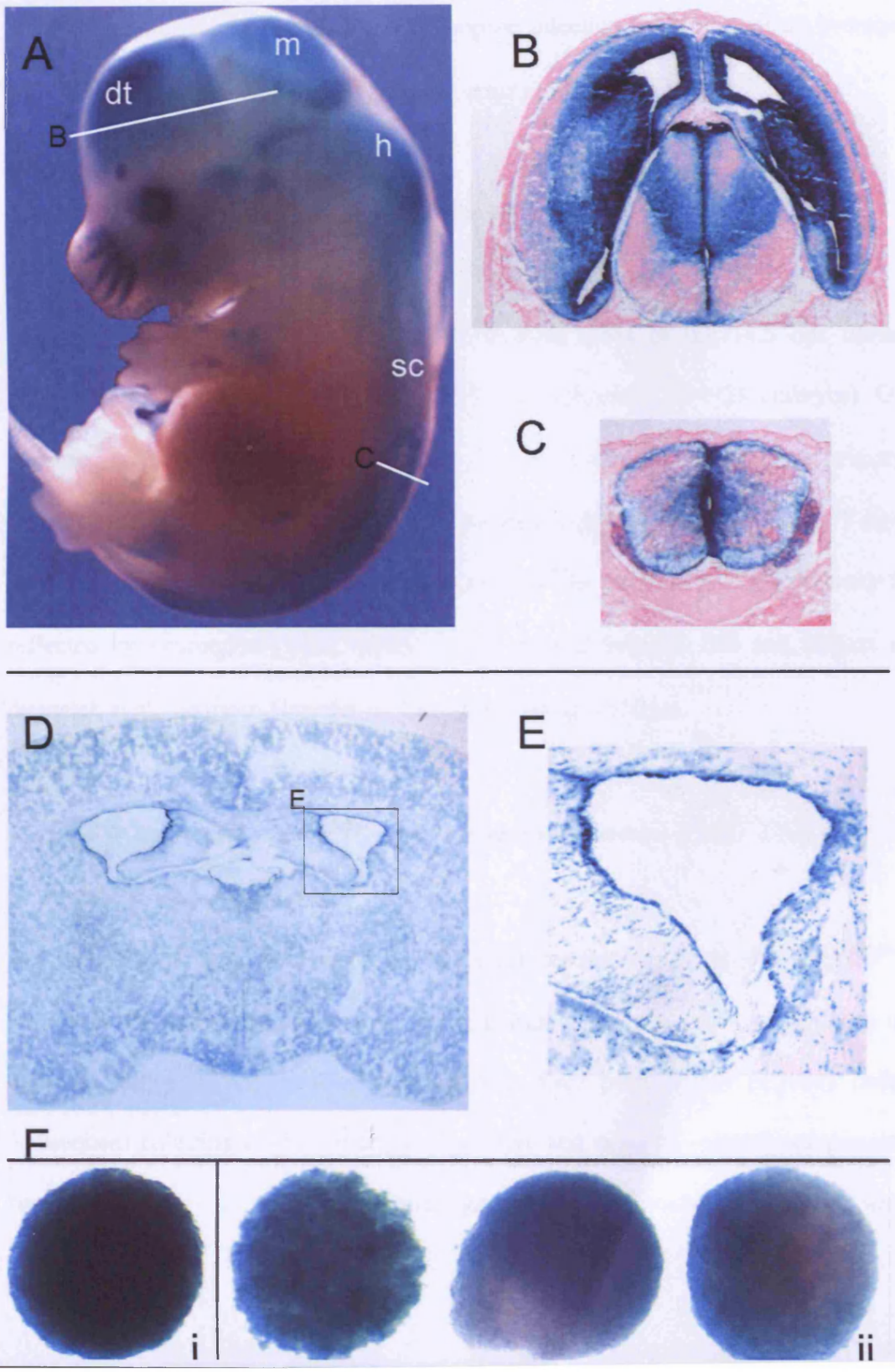
In this chapter, I aim to investigate the role of SOX2 in NSCs selected for *Sox2*$^{\beta geo}$ expression and to determine the expression pattern of *Sox2*$^{\beta geo}$ in regions of neural stem cell generation. Furthermore, I aim to characterise the sub-cellular localisation of SOX2 in proliferating and differentiating NSCs and to gain insight on the mechanism of SOX2 translocation from the nucleus to the cytoplasm.

3.2 Results

3.2.1 Sox2 in NSC-generating regions and in populations of neurospheres from the dorsal telencephalon at 14.5dpc

Wholemount embryos at 14.5 dpc where fixed and assayed for β-galactosidase activity through X-gal staining. This recapitulated the endogenous expression pattern of *Sox2* (Figure 3.1 A). X-gal staining on paraffin sections of adult brain reveals prominent staining in the SVZ (Figure 3.1 D and E). To obtain a stronger and more detailed expression pattern in the neural stem cell generating regions than by staining on paraffin sections, X-gal staining was performed following cryosectioning of fixed 14.5 dpc embryos (Figure 3.1 B and C). The expression of $Sox2^{\beta geo}$ can also be seen in neurospheres generated from NSC-generating regions that are defined by *Sox2* expression. When neurospheres from the 14.5 dpc dorsal telencephalon are selected for $Sox2^{\beta geo}$ expression in culture using G418, all the cells appear blue after X-gal staining (Figure 3.1 Fi). In neurospheres that have been cultured in the absence of selection, just as wild type neurospheres would be, a variable staining pattern is obtained (Figure 3.1 Fii). This ranges from 10% of the cells in the neurosphere being positive for LacZ in 5% of the cases to 98% of the cells being LacZ positive in 60% of the neurospheres studied. Commonly, large patches of cells expressing *βgeo* are seen together, and less often, a salt and pepper distribution of expressing and non-expressing cells.

Figure 3.1 The expression pattern of *Sox2βgeo* in $Sox2^{\beta geo/+}$ mice and neurosphere cultures. *Sox2βgeo* expression can be visualised through X-gal staining in the 14.5 dpc $Sox2^{\beta geo/+}$ embryo (A) and specifically in NSC generating regions such as the dorsal telencephalon (B) and the spinal cord (C). *Sox2βgeo* is expressed in the adult brain (D) and most prominently in the subventricular zone (E). In neurospheres derived from the 14.5 dpc dorsal telencephalon all cells stain when they are selected for the expression of *Sox2βgeo* (i) and in the non-selected situation *Sox2βgeo* cells show variable distribution patterns (ii).



Sox2$^{\beta geo/+}$ cells can be analysed by flow cytometry, through treating the cells with a substrate for β-galactosidase (CMFDG) that generates a fluorescent product. When G418-selected neurospheres (homogenously expressing *Sox2*) undergo FACS analysis by this method, two overlapping populations are seen, one with high levels of *Sox2* expression (60%) and one with low levels (40%). FACS analysis of *Sox2*$^{\beta geo/+}$ neurospheres that have not undergone selection indicates that on average, 79% of cells are *Sox2*-expressing (standard error of mean ± 1).

3.2.2 NSCs and the generation of neurospheres

3.2.2.1 Neurosphere formation under proliferation conditions

The average number of cells cultured from both lobes of the 14.5 dpc dorsal telencephalon is $1.4x10^6$ (result from FACS quantifications, $N = 24$ embryos). On average, after 3 days in culture 0.95% of cells (± 0.12%) give rise to primary neurospheres ($N > 40$). The number of cells in each neurosphere varies and by 7 days in culture neurospheres can be composed of $1x10^3$ to $1x10^7$ cells. This diversity is reflected by neurosphere size, which after 7 days is between 200 and 800μm in diameter, with the mean diameter of the spheres being ~550μm.

3.2.2.2 FACS analysis of Sox2$^{\beta geo/+}$ *neurospheres to determine if Sox2 is required for neurosphere generation*

To determine if *Sox2* is required by NSCs for neurosphere generation, *Sox2*$^{\beta geo/+}$ neurospheres were flow sorted after being cultured in the absence of selection in order to allow for the establishment of both *Sox2* positive and negative cells. Subsequent culturing of the sorted *Sox2* positive and negative cellular components reveals that the *Sox2* positive component generated neurospheres whereas the *Sox2*

negative component did not (Table 3.1). The generation of neurospheres from the *Sox2*-expressing cells was found to be 10-fold reduced compared to neurosphere generation from primary tissue. The neurospheres that formed survived in culture for approximately 7 days without selection pressure. After this time they either adhered to the surface of the culture dish and differentiated, or the neurosphere dissociated and the individual cells died. Cells that survived in culture from the *Sox2* negative component adhered to the dish surface and showed signs of differentiation (projections, elongated morphology). These results indicate that the multipotent NSCs that have the ability to proliferate, reside within the *Sox2* positive component of the neurosphere. Similar values were obtained when neurosphere populations derived from spinal cords of $Sox2^{\beta geo/+}$ 14.5 dpc embryos and adult SVZ were used (no neurospheres could be cultured from the *Sox2* negative component in all cases).

Table 3.1 Generation of neurospheres after FACS sorting. *Sox2βgeo* expressing and non-expressing cellular components of non-selected primary neurospheres, where cultured separately in the absence of selection. Three repeats where carried out.

	Total cells FACS sorted	**Cells sorted Sox2 +ve**	**Neurospheres from +ve**	**Cells sorted Sox2 -ve**	**Neurospheres from –ve**
Trial 1	3.5×10^6	2.7×10^6	3521 (0.13%)	1.3×10^5	0
Trial 2	1.7×10^6	1.3×10^6	2715 (0.21%)	4.3×10^5	0
Trial 3	4.8×10^6	3.9×10^6	4326 (0.11%)	7.7×10^5	0

3.2.2.3 Secondary neurosphere formation as an indicator of NSC numbers

When the single cells dissociated from primary neurospheres are re-plated under proliferation conditions, they give rise to secondary neurospheres. The numbers of secondary neurospheres that form are a good retrospective indicator of the number of NSCs present in the primary culture. On average, 1.3% of cells (± 0.07%) from

neurospheres that have been maintained under selection for the expression of *Sox2βgeo* give rise to secondary neurospheres.

3.2.2.4 BrdU incorporation as an indicator of proliferating NSCs

G418-selected neurospheres where treated in culture with a 40-minute pulse of BrdU before fixation and immunofluorescence staining using an anti-BrdU antibody (results summarised in Table 3.2). The average number of BrdU labelled cells (15.98% of the total cell in a neurosphere) is much higher than the percentage of cells that gives rise to secondary neurospheres in culture (1.3%, determined in section 3.2.2.3) indicating that not all proliferating cells are capable of neurosphere generation in the presently used culture conditions.

Table 3.2 Numbers of BrdU positive cells in $Sox2^{\beta geo/+}$ selected neurospheres. The numbers of BrdU positive cells are expressed as a percentage of the total cells, in four independent trials. The average of all four trials is highlighted in red.

Neurosphere Population	% BrdU +ve (repeat 1)	% BrdU +ve (repeat 2)	% BrdU +ve (repeat 3)	% BrdU +ve (repeat 4)	Average% BrdU +ve
$Sox2^{\beta geo/+}$ Selected	15.7%	16%	18%	14.2%	**15.98%**

3.2.3 Immunofluorescence analysis on proliferating and differentiating 14.5 dpc dorsal telencephalon neurospheres

3.2.3.1 Proliferation

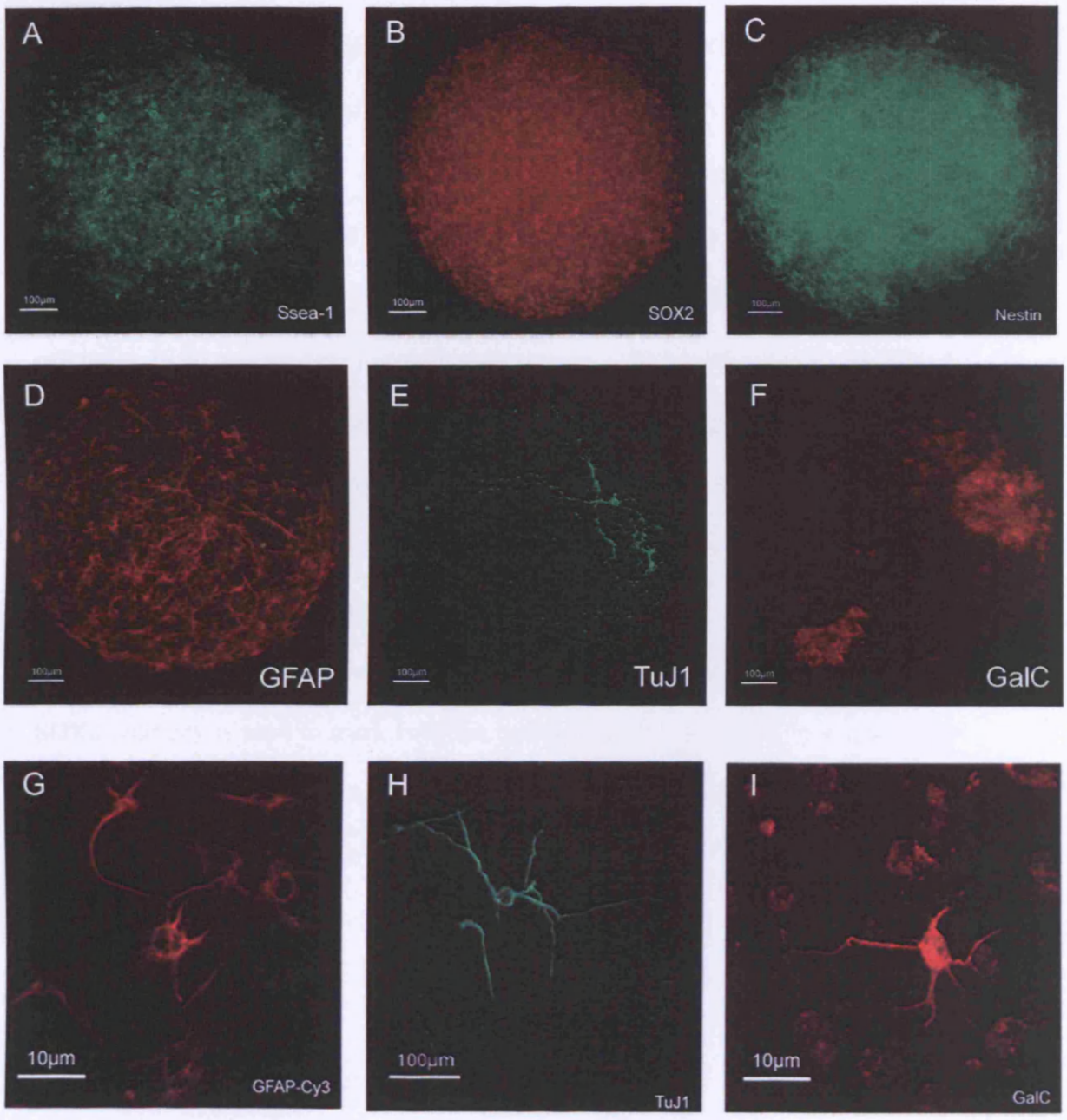
Immunofluorescence using antibodies against SOX2 in G418-selected neurospheres confirms the homogenous *Sox2* expression, previously seen through X-gal staining in Figure 3.1 (Figure 3.2 B). Other markers reported to identify neural stem cells such as the uncommitted neuroepithelial marker Nestin and the surface antigen Ssea-1,

which has been described as an adult subventricular zone stem cell marker are also expressed in neurospheres derived from the dorsal telencephalon of *Sox2*$^{\beta geo/+}$ mice (Figure 3.2 A-C). These mostly co-localise in cells expressing *Sox2*. In neurospheres cultured under proliferation conditions, some differentiated progeny are also seen (Figure 3.2 D to F). High numbers of GFAP positive glial cells are seen, located all over the sphere. TuJ1 immunoreactive neurons as well as some oligodendrocytes are typically seen in proliferating neurospheres. The latter are commonly seen localised in patches rather than spread equidistantly around the sphere (as seen in Figure 3.2 F). To establish if differentiated cells still express *Sox2*, neurospheres were induced to differentiate as described in section 3.2.3.2, but with the continuous addition of G418 in the medium in order to eliminate *Sox2*-negative cells from the culture. Glia and oligodendrocytes persisted in the culture, but at lower numbers compared to neurospheres differentiating in the absence of selection. A small number of TuJ1 positive neurons were observed (12 across 25 differentiating neurospheres) with no identifiable axons.

3.2.3.2 Differentiation

To determine if NSCs in *Sox2*$^{\beta geo/+}$ dorsal telencephalon neurospheres are multipotent, differentiation is encouraged by the attachment of spheres on a support matrix. For these studies, a Matrigel support has been used, as it does not promote preferential differentiation into a particular lineage as found for other supports such as laminin. In the absence of the mitogens EGF and bFGF, NSCs rapidly differentiate. After 5-days under differentiation conditions, neurospheres have given rise to neurons, glia and oligodendrocytes (Figure 3.2 G to I).

Figure 3.2 Immunofluorescence analysis of selected *Sox2*$^{\beta geo/+}$ neurospheres. Cells in the neurosphere are immunoreactive for the candidate stem cell markers Ssea-1 (A), SOX2 (B) and Nestin (C). They also contain low numbers of differentiated progeny (D to F). Once in differentiation conditions, neurospheres have the ability to generate glia (G), neurons (H) and oligodendrocytes (I). Scalebars represent 100μm except in G and I where they represent 10μm.

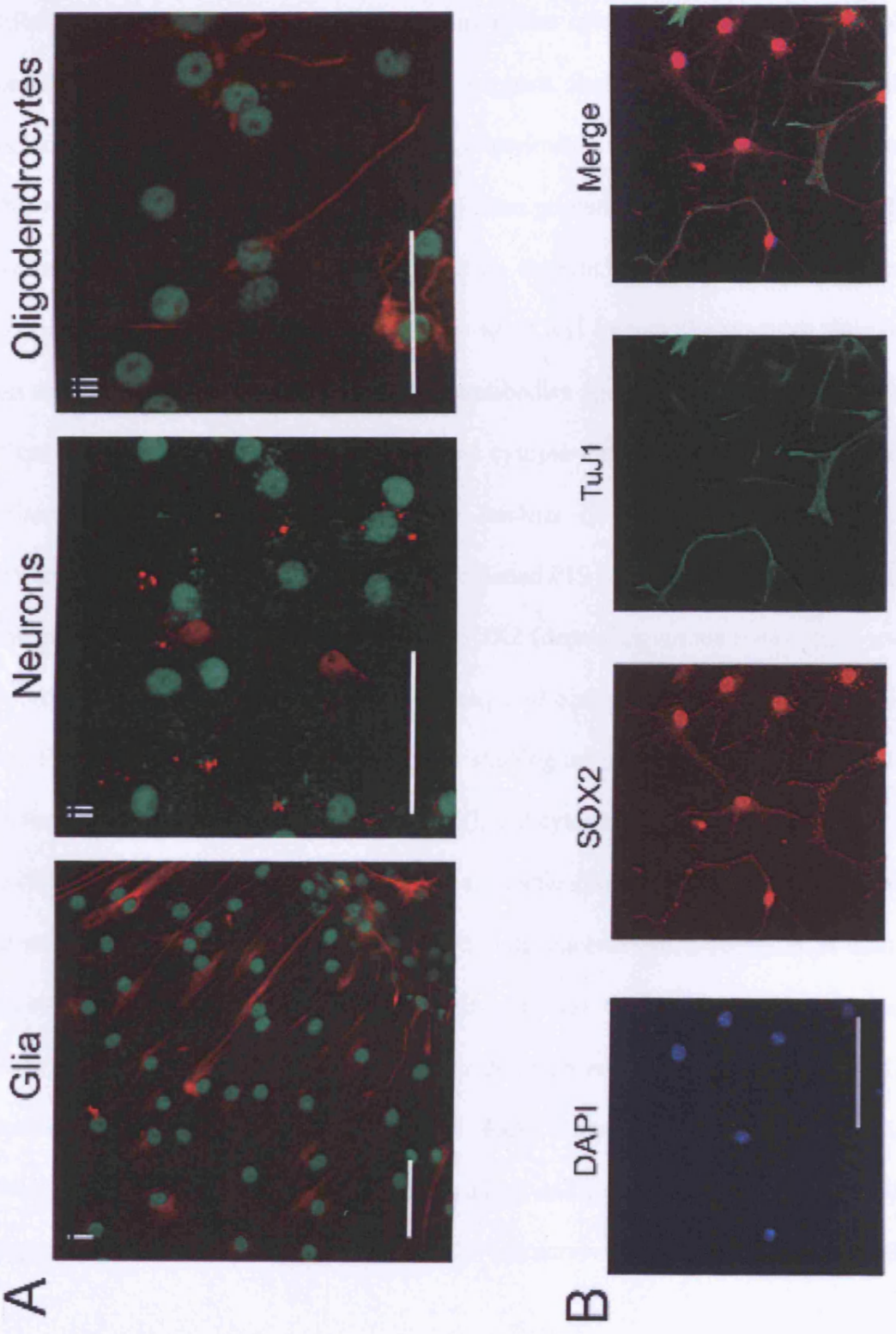


3.2.4 The sub-cellular localisation of SOX2

3.2.4.1 Immunofluorescence analysis to determine the sub-cellular localisation of SOX2 in neurosphere cultures

SOX2 is expected to localise in the cytoplasm of most terminally differentiated cell types. Double immunofluorescence antibody staining was performed against SOX2 and against known markers of differentiated neural cells (GFAP, RC2, S100β, TuJ1, CNPase, O4, GalC). These cell types were analysed in neurosphere populations derived from embryonic and adult CNS tissue. After 5 days under differentiation conditions, SOX2 localises predominantly in the cytoplasm of these differentiated cell types. In all neurosphere populations examined, SOX2 localises in the nucleus of a subpopulation of GFAP and/or RC2 positive glial cells (Figure 3.3 A, i). It also localises in the nucleus of some GalC positive oligodendrocytes (Figure 3.3 A, iii). SOX2 is not normally seen in neurons (Figure 3.3 A, ii) but when it is expressed in neurons derived from embryonic neurospheres, it localises in the cytoplasm. Interestingly, when this experiment was performed using adult SVZ cultures, the SOX2 antibody is seen to mark both the nucleus and the cytoplasm in mature TuJ1 positive neurons differentiating for 5 days (Figure 3.3 B). After 10 days however, its localisation becomes mostly cytoplasmic.

Figure 3.3 Localisation of SOX2 in differentiated cell types. SOX2 (green in A, red in B) is localised in the nucleus of some GFAP positive glia (red, A, i) and GalC positive oligodendrocytes (A, iii) but it is not expressed in neurons (A, ii) differentiating from 14.5 dpc dorsal telencephalic neurospheres. In neurospheres isolated from the adult SVZ, *Sox2* is expressed in neurons differentiating for 5 days and the protein localises transiently in the nucleus as well as in the cytoplasm (B). Scalebars represent 100 μm.

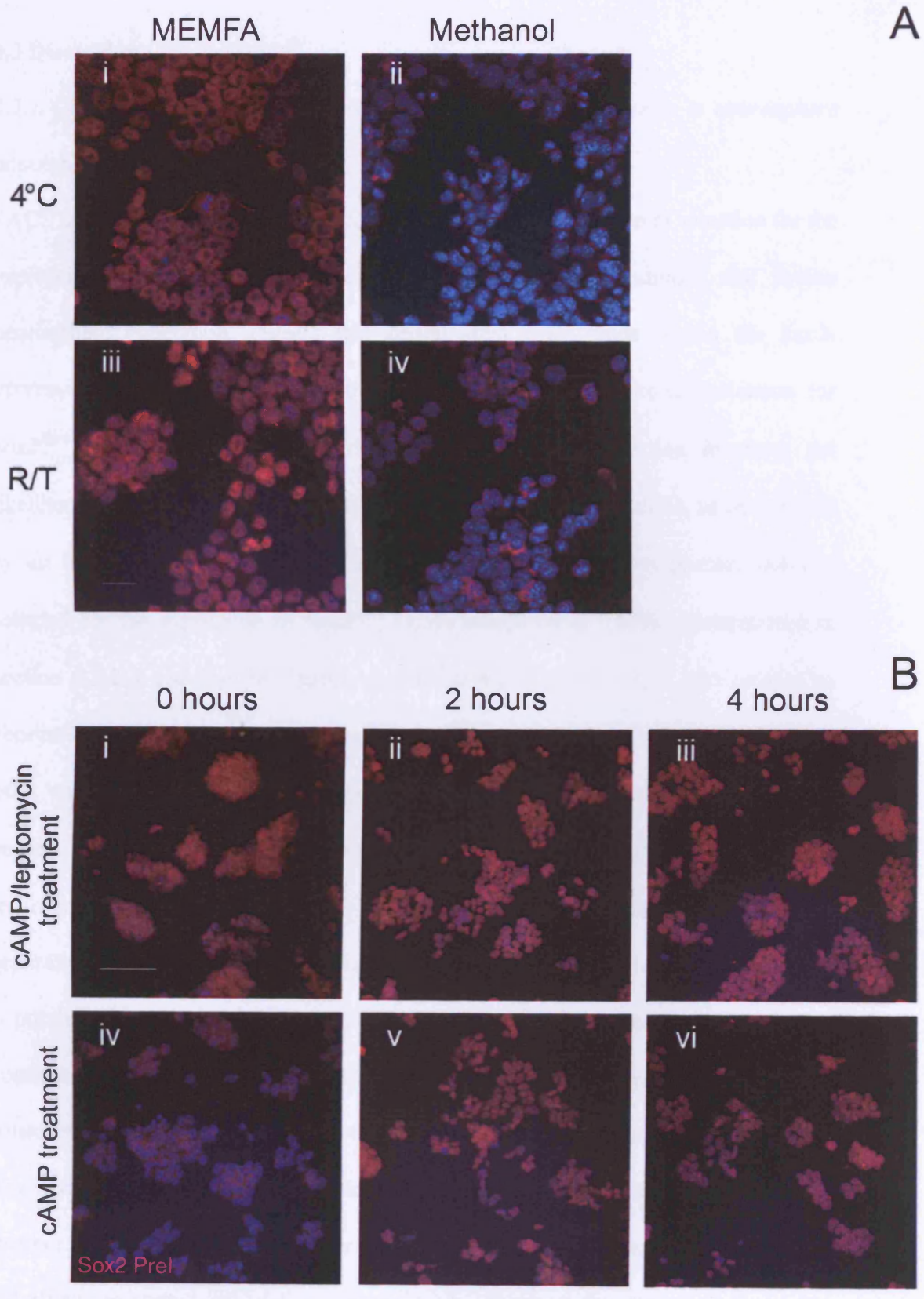


3.2.4.2 Investigating the mechanism of SOX2 nuclear-cytoplasmic shuttling

To determine the pathway used by SOX2 to translocate from the nucleus to the cytoplasm, the P19 embryonal carcinoma (EC) cell line was used. When neural differentiation of P19 cells is induced through the addition of *trans*-retinoic acid (tRA), SOX2 translocates from the nucleus to the cytoplasm (Remboutsika et al, unpublished data). Since previous work suggests that treatment with db-cAMP induces the nuclear export of SOX2, these experiments were reproduced to confirm this result. Initially, SOX2 was found to localise primarily in the cytoplasm of both treated and untreated cells, which was not an expected finding. The experimental conditions employ methanol as the fixation agent and immunofluorescence staining on sections of cryopreserved tissue, using antibodies against SOX2, has shown that fixation with methanol results in artefactual cytoplasmic SOX2 staining in regions where SOX2 is known to be in the nucleus (Dr Ariel Avilion, personal communication). Since fixation of undifferentiated P19 cells with methanol results in cytoplasmic or perinuclear localisation of SOX2 (depending on the temperature and length of fixation), the fixation conditions required optimisation (Figure 3.4 A ii and iv). Fixation with MEMFA leads to nuclear staining using an antibody against SOX2 in untreated P19 cells (Figure 3.4 A i and iii), and cytoplasmic SOX2 staining in P19 cells treated with tRA, so was chosen as a reliable fixative. When P19 cells were treated with cAMP, SOX2 remained located in the nucleus over a period of 24 hours (shown here for up to 4 hours, Figure 3.4 B v and vi). Cells that have been treated with tRA and subsequently with cAMP for 24 hours retain the cytoplasmic SOX2 localisation induced by tRA. Leptomycin B blocks the nuclear export of proteins via the cAMP-mediated PKA pathway and was added as a control alongside cAMP in all experiments for a maximum of 4 hours (P19 cell survival is limited to 4 hours with

leptomycin treatment). As expected, there was no effect on the localisation of SOX2 in cells treated with leptomycin B (Figure 3.4 B i-iii). These results demonstrate that the addition of cAMP to P19 cells does not induce the translocation of SOX2 between the nucleus and the cytoplasm, indicating that the nuclear-cytoplasmic shuttling of SOX2 is independent of the cAMP-dependent PKA pathway.

Figure 3.4 Localisation of SOX2 in P19 EC cells after cAMP treatment. Fixation is important to determine the correct localisation of SOX2. MEMFA fixation leads to nuclear fixation in undifferentiated P19 cells where SOX2 is known to be nuclear (A, i, iii) whereas methanol fixation leads to cytoplasmic localisation (A, ii, iv), irrespective of temperature conditions. Treatment with db-cAMP does not result in nuclear-cytoplasmic shuttling of SOX2 (B, v, vi) and is also not inhibited by leptomycin, which blocks cAMP-dependent shuttling (B, i to iii). As a negative control for the immunofluorescence, SOX2 pre-immune antibody was used (iv). Scalebars represent 100μm


A
MEMFA
Methanol
i
ii
4°C
iii
iv
R/T
B
0 hours
2 hours
4 hours
i
ii
iii
cAMP/leptomycin treatment
iv
v
vi
cAMP treatment
Sox2 Prel

3.3 Discussion

3.3.1 Characterisation of Sox2 expressing and non-expressing cells in neurosphere populations

FACS sorting primary neurosphere cells maintained in the absence of selection for the expression of *Sox2*, and subsequently culturing them in conditions that favour neurosphere generation, reveals that neural stem cells reside within the *Sox2*-expressing component, as demonstrated in section 3.2.2.2. Through selection for $Sox2^{\beta geo}$, neurospheres can be enriched for NSCs. This selection increases the likelihood that higher numbers of stem cells are present in the culture, as determined by an increase in the numbers of secondary neurospheres from primary cultures selected for the expression of $Sox2^{\beta geo}$ (1.3% compared to 0.95%, demonstrated in section 3.2.2.3 and also in Chapter 4, section 4.2.2). However, it also creates an abnormal situation where no *Sox2* negative cells are present. Assays involving pulsing cells with BrdU reveal that the percentage of BrdU incorporation is higher than percent neurosphere formation in $Sox2^{\beta geo}$ -selected neurospheres (Table 3.2). This result indicated that not all of the proliferating cells are capable of neurosphere generation. There are at least three, non-mutually exclusive explanations fro this: (i) it is possible that proliferating progenitor cells cannot form neurospheres in culture, in contrast to neural stem cells; (ii) all proliferating cells may be capable of initiating neurosphere formation, but the survival of neurospheres may be impaired for some originating cell types; (iii) aggregation of cells in culture leads to a falsely reduced number of neurospheres. If the latter is true, it should be a constant error and should not affect the assessment of fluctuations in NSC numbers through assays involving secondary neurosphere formation. BrdU incorporation assays have to be performed with an extension of the BrdU treatment time in order to obtain more accurate Figures

for the number of proliferating cells in the neurosphere. X-gal staining on *Sox2*$^{\beta geo/+}$ neurospheres reveals that the number of *Sox2* negative cells varies significantly between neurospheres in a single culture, indicating that there is no requirement for a specific proportion of *Sox2* negative cells (Figure 3.1 Fii). This variation in the non-selected neurospheres poses a problem, as it is difficult to accurately reproduce comparisons between individual *Sox2*$^{\beta geo/+}$ selected and non-selected neurospheres. In both neurosphere populations, the neuroepithelial stem cell marker Nestin is expressed by the *Sox2* positive cells, consistent with their stem cell character (this is not surprising as it has been shown that Nestin is regulated by *Sox2* (Tanaka et al. 1998)), and both cultures are capable of differentiating into neurons, glia and oligodendrocytes (Figure 3.2, shown for *Sox2*$^{\beta geo/+}$ selected cultures). They also express *Ssea-1*, which has been identified as a stem cell marker in the adult SVZ (Capela and Temple 2002). Aside from the inability to from neurospheres, after FACS sorting, *Sox2* negative cells also show signs of differentiation. However, the absence of *Sox2* is not an essential criterion for differentiation. When selected neurospheres were induced to differentiate with continuing selection for five days, cells expressing differentiation markers for glia and oligodendrocytes and on some occasions, neurons were present (3.2.3.1). It may be the case that the persistence of neomycin phosphotransferase permitted the survival of these cells, irrespective of their SOX2 status. The observation that there were only very few TuJ1-immunoreactive neurons and that these had no axons, suggests that the maintenance of *Sox2* expression inhibits neurogenesis, consistent with findings that ectopic expression of *Sox2* in chick neural tubes inhibits neurogenesis (Bylund et al. 2003; Graham et al. 2003). The overall lower numbers of differentiated cells, in the situation where selection for *Sox2* continues, can be attributed to two possible reasons: (i) *Sox2* non-expressing cells are

being eliminated from the cultures as predicted; (ii) the continued expression of *Sox2* exerts pressure to maintain an uncommitted character.

3.3.2 Sub-cellular localisation of SOX2

In undifferentiated neurospheres, nuclear SOX2 is seen in the majority of the cells. SOX2 localises predominantly in the cytoplasm of differentiated cells, as revealed through double immunofluorescence analysis on neurospheres cultured under differentiation conditions. At least in some cases, SOX2 shuttles from the nucleus of undifferentiated cells to the cytoplasm, upon differentiation. This observation is supported by the fact that after 5 days in differentiating culture conditions, neurons derived from adult SVZ neurosphere cultures exhibit both nuclear and cytoplasmic SOX2, which becomes fully cytoplasmic after a further 5 days (Figure 3.3 B). One exception with respect to cytoplasmic SOX2 localisation, where differentiated cells have nuclear SOX2, is in a subpopulation of glial cells that are also immunoreactive for RC2 and/or GFAP (Figure 3.3 A). Cells within this population exhibit both astrocytic and radial glial morphologies. A possible explanation for these results is the NSC identity of some embryonic radial glial cells, which are capable of generating astrocytic progeny that retain stem cell properties. The nuclear-cytoplasmic shuttling of SOX2 is a common feature of several SOX proteins as discussed earlier. Experiments to investigate if SOX2 nuclear export occurs through induction of the cAMP-mediated PKA pathway suggest that this pathway is not responsible (section 3.2.4.2). It is possible the PKA pathway was not correctly induced in these experiments so to refine the experimental design a positive control is required. Ideally, the localisation of a known protein whose export depends on the PKA pathway and which is expressed in P19 cells should be examined alongside the

localisation of SOX2. Other signaling pathways such as *Wnt* signaling may be responsible for SOX2 shuttling. The possibility of calmodulin-mediated shuttling of SOX2 should be next investigated, as calmodulin is involved in the localisation of other SOX family members (Harley et al. 1996). It would be possible to demonstrate the involvement of calmodulin in the localisation of SOX2 through inhibiting calmodulin signaling, but a suitable method could not be established in order to proceed with these experiments.

CHAPTER 4

Comparisons between NSC cultures homogeneously expressing *Sox2* and cultures that have not been selected for *Sox2* expression

4.1 Background

If NSCs require cues through cell-cell interactions to maintain their state or function, an altered environment within the neurosphere may provide cues for a particular fate. We can manipulate this environment in neural stem cell cultures, to select for cells expressing *Sox2βgeo*, generating a population that homogenously expresses *Sox2*. Comparing these cultures that homogenously express *Sox2* to a mixed population of cells similar to wild type cultures hence more comparable to published literature, can help characterise the rôles of the *Sox2* expressing and non-expressing cells. Besides proliferation and differentiation assays for the two populations, their global gene expression profiles can be investigated through microarray analysis, in order to obtain further insight into their cellular character. Two different array platforms were used: the Mouse 430A GeneChip (Affymetrix)- an oligonucleotide array encompassing 44,000 known mouse genes and their mismatch controls and the Mm_SGC_Av2 spotted oligonucleotide array (named Mouse 'Known' Gene Oligo Array, produced by Codelink-Amersham and the MRC Rosalind Franklin Centre for Genomic Research- former HGMP), which represents 7,500 genes including controls. The initial availability of the spotted array allowed for several pilot studies to be performed, and these preliminary results were then compared to results from the more reliable GeneChip platform.

In this chapter, the differences between the differentiation and proliferation properties as well as in the gene expression profiles of neurospheres homogenously expressing *Sox2*, and neurospheres composed of a mixed population with respect to *Sox2* status will be investigated. This chapter also aims to examine the effects of varying the proportion of *Sox2*-expressing cells, in the neurosphere microenvironment, on proliferating NSCs.

4.2 Results

4.2.1 Comparisons between Sox2$^{\beta geo/+}$ neurospheres that have or have not undergone selection for the expression of Sox2

4.2.1.1 Differences in differentiation into glia, neurons and oligodendrocytes

Cells derived from embryonic and adult CNS tissues (14.5 dpc dorsal telencephalon, 14.5 dpc spinal cord and adult subventricular zone) from wild type mice (MF1) do not survive in culture past ten days with the addition of G418 in the proliferation growth medium. This confirms that selection for 10 days with G418 is sufficient to ablate any *Sox2* negative cells resulting in neurospheres homogenously expressing *Sox2*. Indirect immunofluorescence analysis of differentiation markers reveals differences in the unrestricted differentiation of neural stem cell progeny in homogeneous and heterogeneous populations between selected and non-selected population with respect to *Sox2* expression. Neurospheres cultured under proliferation conditions for 3 weeks, in the absence of selection, were allowed to differentiate for 5 days in the absence of growth factors on an

adherent matrix. Under these conditions, cells from these neurospheres give rise to *Gfap* expressing glia, at higher numbers than in neurospheres cultured under selection during proliferation. Semi-quantitative PCR for *Gfap* expression (Figure 4.1 B) as well as FACS sorting analysis using an anti-GFAP antibody on proliferating neurospheres also confirm this observation (Figure 4.1 A). Higher numbers of TuJ1 positive neurons are also observed in the majority of differentiating neurospheres not enriched for *Sox2* expression (Figure 4.2 C and D). Differentiation into these cell types can be attributed to the *Sox2* negative cellular component. There appears to be no significant difference in the numbers of oligodendrocytes (marked by antibodies against O4, CNPase and GalC), but in enriched neural stem cell cultures selected for *Sox2* expression, mature oligodendrocytes are seen alongside cells with perinuclear O4 localisation without oligodendroglial projections that resemble more immature oligodendrocytes or oligodendrocyte precursor cells (OPCs). In cultures not selected for the expression of *Sox2*, only the latter cells have been observed (Figure 4.2 A and B). In 14.5 dpc spinal cord-derived neurospheres, higher numbers of oligodendrocytes are seen in differentiating neurospheres that have not undergone selection, which highlights a regional difference.

Figure 4.1 Differences in the number of GFAP positive cells between 14.5 dpc neurospheres from the dorsal telencephalon selected or not selected for the expression of *Sox2βgeo*. FACS sorting reveals that the GFAP positive component is higher in neurospheres that have not been selected (iii) than neurospheres that homogenously express *Sox2βgeo* (ii). Cells in the non-selected population also show higher levels of fluorescence intensity (iv green), than in their *Sox2βgeo* selected counterpart population (iv red) indicating higher levels of GFAP. Differences in the levels of *Gfap* expression where confirmed by semi-quantitative RT-PCR (B). Neurospheres from the 14.5 dpc dorsal telencephalon not selected (B i, 'DT-') express *Gfap* at higher levels than selected neurospheres (B i, 'DT+'). Levels of expression of *Actb* (β-Actin) where used as a control to ensure no differential loading of template (B ii).

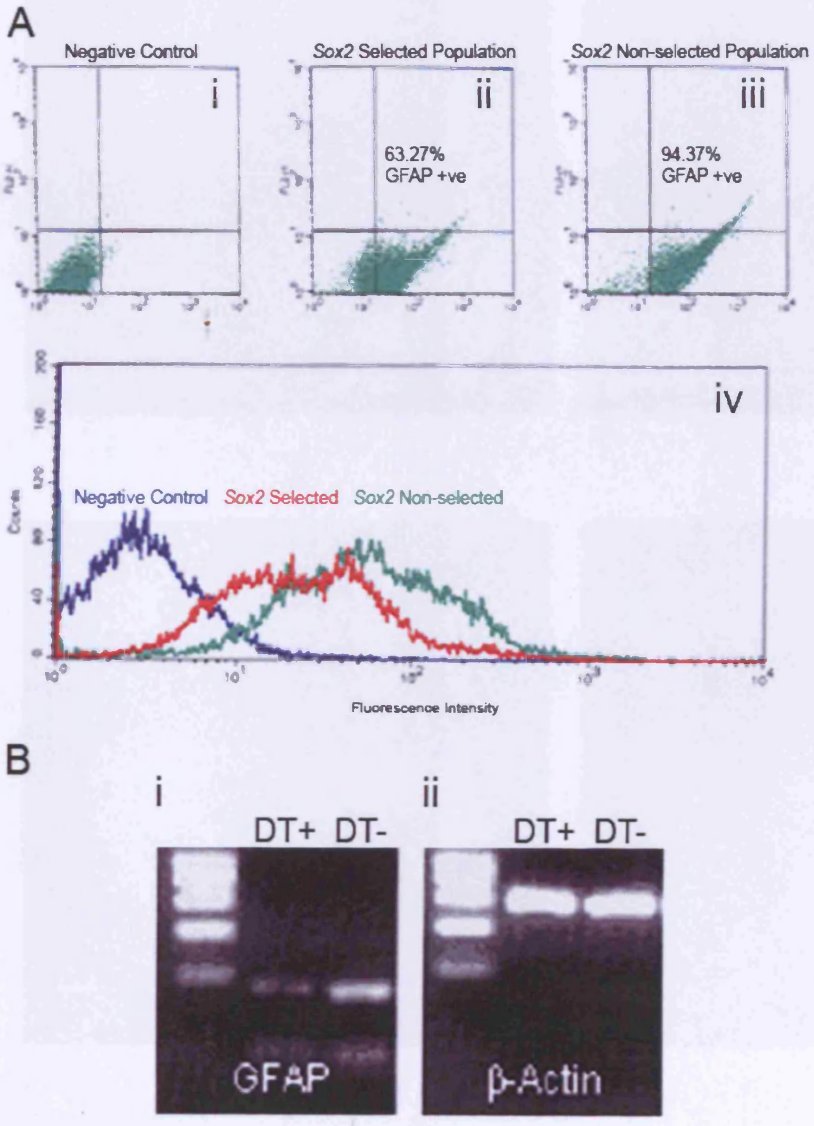
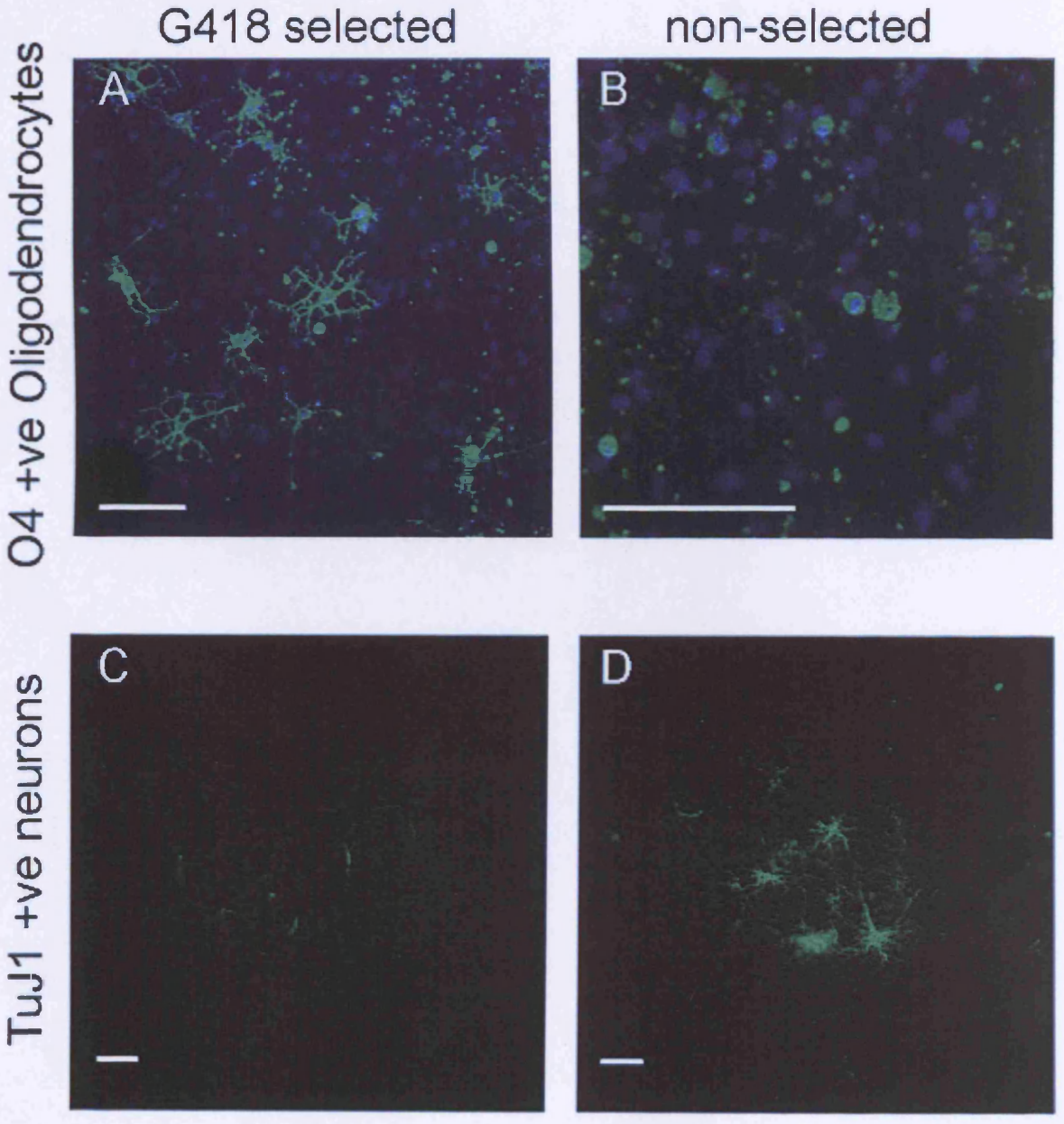


Figure 4.2 Differences in oligodendrocytes and neurons between G418 selected and non-selected neurospheres from the 14.5 dpc dorsal telencephalon of *Sox2*$^{\beta geo/+}$ mice. Although the numbers of oligodendrocytes do not change significantly between the two cultures, neurospheres that have undergone selection generate more mature oligodendrocytes (A) than non-selected neurospheres (B) as revealed by immunofluorescence staining using the O4 antibody. More TuJ1 positive neurons are seen in non-selected neurospheres (D) than in selected neurospheres (C). Scalebars represent 100μm.



4.2.1.2 Differences in proliferation

BrdU incorporation assays on neurosphere-derived cells indicate higher levels of proliferation in neurosphere populations that have been selected for the expression of *Sox2βgeo* compared to neurospheres that have not undergone selection (Table 4.1).

Table 4.1 Proliferation in selected and non-selected neurospheres. BrdU incorporation was used as an indicator of proliferation and was expressed as a percentage of BrdU labelled cells of the total cells in the population. More cells in neurospheres selected for *Sox2βgeo* expression incorporate BrdU than cells in neurospheres not selected for the expression of *Sox2βgeo*. The average percentage of the four repeats is highlighted in red. After angular transformation of the percentages (angle = arcsin√percentage), a one-way analysis of variance test (ANOVA) was performed. In the ANOVA table: SS is sum of squares, df is degrees of freedom, MS is mean squares (ratio SS/df), F is F-statistics (ratios of the mean squares). The *p*-value generated is lower than 0.05, confirming that the two sets of experiments yield significantly different results (*p*-value in blue).

Neurosphere Population	% BrdU +ve (repeat 1)	% BrdU +ve (repeat 2)	% BrdU +ve (repeat 3)	% BrdU +ve (repeat 4)	Average% BrdU +ve
$Sox2^{\beta geo/+}$ Selected	15.7%	16%	18%	14.2%	**15.98%**
$Sox2^{\beta geo/+}$ Non-selected	12%	11.8%	13.6%	9.1%	**11.63%**

SUMMARY

Groups	Count	Sum	Average	Variance
Selected	4	94.16	23.54	1.4784
Non-selected	4	79.56	19.89	2.891933

ANOVA

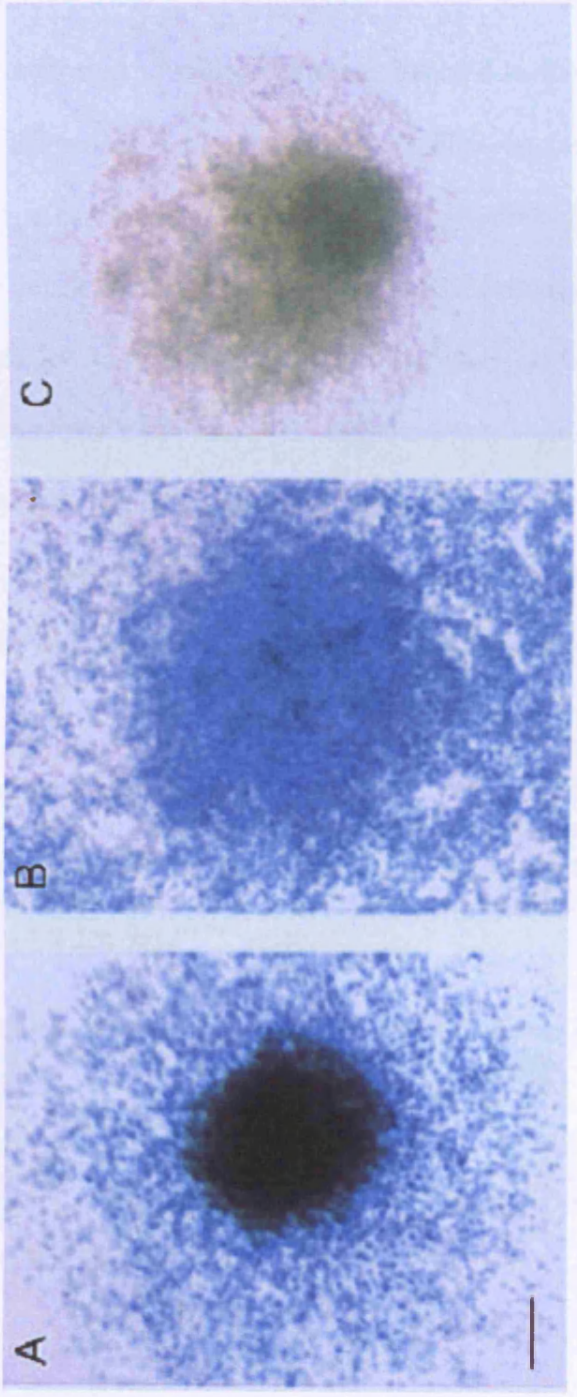
Source of Variation	SS	df	MS	F	*p*-value	F crit
Between Groups	26.645	1	26.645	12.19358	**0.012953**	5.987374
Within Groups	13.111	6	2.185167			
Total	39.756	7				

Cells from neurospheres that have been maintained under selection for the expression of *Sox2βgeo* give rise to higher numbers of secondary neurospheres compared to non-selected primary neurosphere cells. On average 1.3% of cells (± 0.07%) give rise to secondary neurospheres after G418 selection whereas only 0.4% of cells (± 0.11%) give rise to secondary neurospheres without selection.

4.2.1.3 Differences in the migration patterns of differentiating cells

The attachment of neurospheres on an adherent matrix and the removal of growth factors, results in the differentiation of the cells and their migration outward from the centre of the sphere. When neurospheres that have been maintained under selection for the expression of *Sox2* undergo this induced differentiation (Figure 4.3 A), cells that migrate away from the neurosphere do so in a homogenous manner, forming an organised periphery. Cell-to-cell contact in this radius gives the appearance of a lattice structure. The migration patterns of $Sox2^{\beta geo/+}$ neurospheres not selected for the expression of *Sox2* and of wild type neurospheres are more disorganised (Figure 4.3 B and C). In these, cells may predominantly migrate in single directions around the sphere and rarely appear to form organised lattice structures.

Figure 4.3 Differentiation and subsequent X-gal staining in neurospheres derived from the 14.5 dpc dorsal telencephalon of *Sox2*$^{\beta geo/+}$ mice and selected (A) or not selected (B) for the expression of *Sox2βgeo*, and in neurospheres derived from wild type mice (C). More organised migration patterns are seen in the periphery of selected neurospheres (A) than in the other two conditions. Scalebar represents 250μm.



4.2.1.4 Differences in the gene expression profiles determined through microarray analysis

Differences in the gene expression profiles between cells positive and negative for the expression of *Sox2* have been investigated through microarray analysis according to the experimental outline in Figure 4.4 and the microarray minimum information documents in Tables 4.2 and 4.3. Neurosphere cultures selected for the expression of *Sox2βgeo* were compared to neurospheres maintained in the absence of selection. Any significant differences in gene expression profiles can be attributed to the *Sox2* negative cells of non-selected neurospheres. It is possible that these cells have an influence on the gene expression profiles of the *Sox2* expressing cells. General gene expression trends in these experiments conform with predictions generated from immunofluorescence and real-time PCR experiments previously performed (Figure 4.1) e.g. *Gfap* is expressed at significantly higher levels in non-selected populations (average 6-fold increase in experiments performed using the Affymetrix Mouse 430A chip and the Mm_SGC_Av2 2-colour spotted oligonucleotide array chip).

Figure 4.4 Microarray experimental outline of comparing $Sox2^{\beta geo/+}$ cultures +/- G418. Experiment to determine the differences between NSC-enriched neurospheres, which have been selected for the expression of *Sox2βgeo* and neurospheres from that have not been treated. Both the populations where derived from the dorsal telencephalon of 14.5 dpc $Sox2^{\beta geo/+}$ mice.

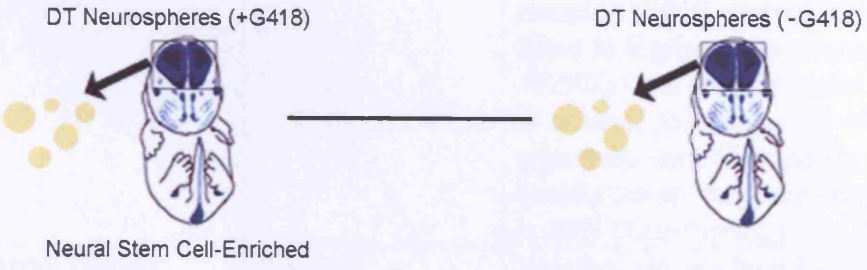


Table 4.2 MIAME: Comparing untreated neurospheres with NSC-enriched neurospheres using the spotted oligonucleotide platform

Experiment Design	Type	Normal neurospheres v Treated neurospheres (NSC enriched)
	Experimental Factors	Selection (for NSC enriched) or no selection of primary and subsequent secondary neurospheres for *Sox2βgeo* expression with neomycin (G418)
	Number of Hybridisations	1 two-colour
	Reference Sample	None
	Hybridisation Design	Neural stem cell (NSC) enriched v non enriched neurosphere cultures, see diagram
	Quality Control	3 replicates, one biological repeat, one dye-swap
Samples	Origin	14.5 dpc murine dorsal telencephalon of $Sox2^{\beta geo/+}$ mice (MF1 outbred background)
	Manipulation	Mechanical tissue dissociation, culture in NPM as neurospheres. Continuous addition of neomycin (G418) after 72 hours for NSC enriched sample, 2^{ry} neurosphere generation on day 10, culture of 2^{ry} spheres for a further 7 days
	Sample Preparation	Extraction of total RNA using RNeasy Midi kit (Qiagen). DNase treatment using DNA-free (Ambion). One-cycle synthesis of cDNA using Transcriptor First Strand cDNA Synthesis kit (Roche)
	Labeling Protocol	Klenow labeling with Cy3 or Cy5
Hybridisation Procedures	Parameters	See Methods for overnight hybridisation and post-hybridisation washes protocols
Measurement Specifications	Hardware Information	Two-colour 428 scanner (Affymetrix), BioAnalyzer 2100 (Agilent Technologies) for RNA quality control and quantitation
	Software Information	ImaGene 4.0 (BioDiscovery), GeneSpring 7.2 (Silicon Genetics)
	Measurements used	Data transformation: Dye swap. Per spot and per chip: intensity dependent normalisaion (Lowess). Lowess curve fitted to log-intensity versus log-ratio plot. 40.0% of the data was used to calculate the fit at each point. This curve was used to adjust the control value for each measurement. Set measurements < 0.01 set to 0.01 (GeneSpring)
Array Design	Platform	Spotted oligonucleotide array
	Type	Mm_SGC_Av2: Mouse Known Oligo Array on CodeLink Activated Slides (Amersham Biosciences)

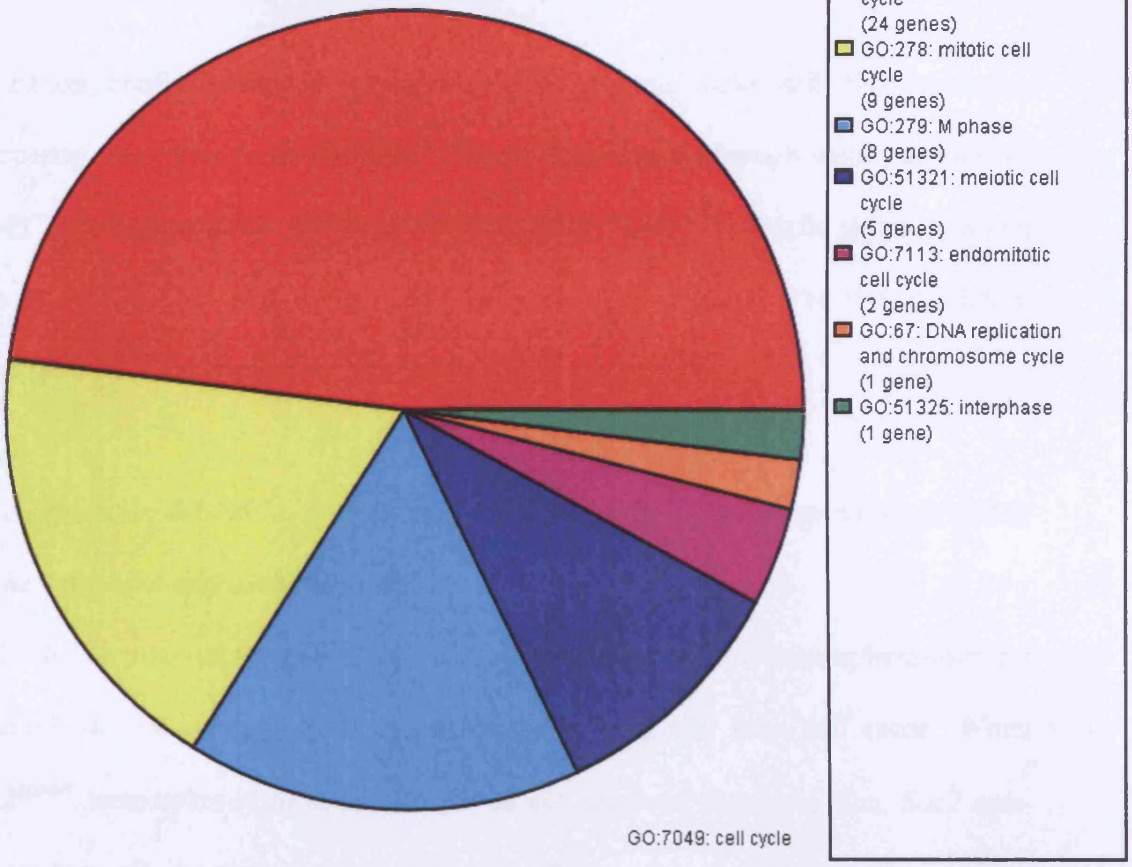
Table 4.3 MIAME: Comparing untreated neurospheres with NSC-enriched neurospheres using the GeneChip platform

Experiment Design	Type	Normal neurospheres v Treated neurospheres (NSC enriched)
	Experimental Factors	Selection (for NSC enriched) or no selection of primary and subsequent secondary neurospheres for *Sox2βgeo* expression with neomycin (G418)
	Number of Hybridisations	2 one-colour
	Reference Sample	None
	Hybridisation Design	Neural stem cell (NSC) enriched v non enriched neurosphere cultures, see diagram
	Origin	14.5 dpc murine dorsal telencephalon of $Sox2^{\beta geo/+}$ mice (MF1 outbred background)
	Quality Control	2 replicates, repeated on another array platform (see later)
Samples	Manipulation	Mechanical tissue dissociation, culture in NPM as neurospheres. Continuous addition of neomycin (G418) after 72 hours for NSC enriched sample, 2^{ry} neurosphere generation on day 10, culture of 2^{ry} spheres for a further
	Sample Preparation	Extraction of total RNA using RNeasy Midi kit (Qiagen). DNase treatment using DNA-free (Ambion)
	Labeling Protocol	Biotin labeling
	Parameters	Affymetrix 'Eukaryotic Sample and Array Processing' guide followed for all protocols (see Methods)
Hybridisation Procedures	Hardware Information	GenAmp PCR System 9700 (Applied Biosystems), GeneChip Hybridization Oven 640, GeneChip Fluidics Station 450 and GeneChip Scanner (all Affymetrix). NanoDrop ND-1000 spectrophotometer (Labtek) and BioAnalyzer 2100 (Agilent Technologies) for RNA quality control and quantitation
Measurement Specifications	Software Information	GCOS (Affymetrix), GeneSpring 7.2 (Silicon Genetics)
	Measurements used	Raw values after GC RMA Normalisation, per chip: normalise to 50^{th} parcentile, per gene: normalise to median and data transformation: set measurements < 0.01 set to 0.01 (GeneSpring)
Array Design	Platform	Affymetrix GeneChip
	Type	Mouse 430A

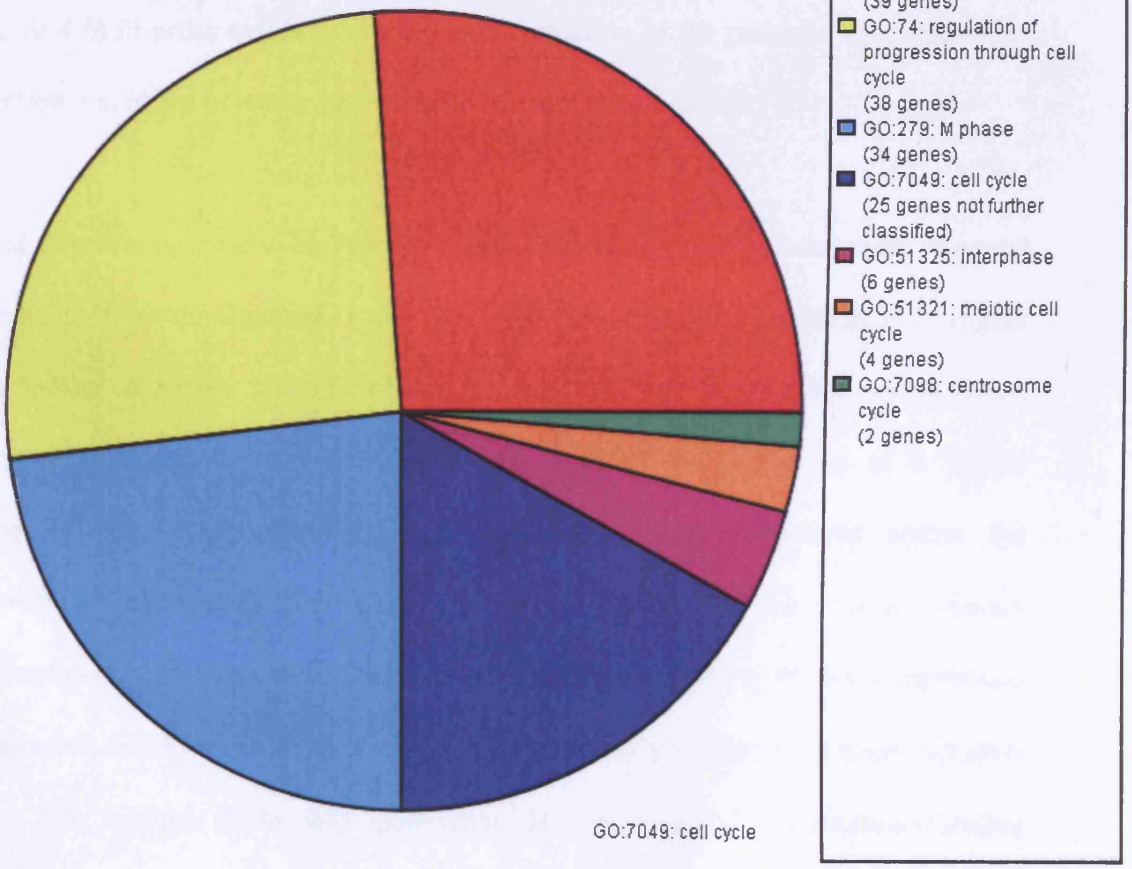
Sox2 expression remains at the same levels between the two populations, on both microarray platforms used. In the Affymetrix GeneChip platform, after GCRMA normalisation, the levels of *Sox2* transcripts are higher in non-selected neurospheres but not over the 2-fold significance limit imposed (1.2-fold increase from 9,383 arbitrary units in selected neurospheres to 11,181 in non-selected neurospheres), as are levels of expression of genes involved in myelination and nerve ensheathment. Compared to non-selected populations, selected $Sox2^{\beta geo/+}$ neurospheres show an increase in expression levels of genes classified by the Gene Ontology Consortium as being implicated in cell cycle (108 genes compared to 50 genes in non-selected populations, Figure 4.5). This observation suggests that higher levels of proliferation are taking place in pure *Sox2* expressing populations, which was previously suggested by secondary neurosphere formation numbers and BrdU incorporation assays. Further inspection of the ontologies mentioned, reveals that a large proportion of the genes expressed at significantly higher levels in neurosphere populations enriched for neural stem cells, are involved in double-strand break repair of DNA and are mainly expressed in the second growth phase of the cell cycle (G2)

Figure 4.5 Gene Ontology classifications of microarray results. Pie charts depicting a break down of the categories of the Gene Ontologies classification of genes involved in the regulation of the cell cycle, for genes expressed 2-fold significantly higher in either selected (B) or non-selected $Sox2^{\beta geo/+}$ neurospheres (A). More genes are involved in these processed in selected neurospheres (108) compared to non-selected (50). The key can be found on the side of each pie chart.

A Cell cycle associated genes expressed over 2-fold higher in *Sox2*$^{\beta geo/+}$ neurospheres than *Sox2*$^{\beta geo/+}$ selected neurospheres



B Cell cycle associated genes expressed over 2-fold higher in *Sox2*$^{\beta geo/+}$ selected neurospheres than *Sox2*$^{\beta geo/+}$ neurospheres



Validation confirms that the results generated from these and the remaining microarray experiments in this thesis can be reproduced through semi-quantitative RT-PCR and quantitative real-time RT-PCR (qRT-PCR) for specific genes, and can also be reproduced using independent RNA samples. Examples of this validation using qRT-PCR can be found in Figure 9.2.1 of the Appendix.

4.2.2 Effects on the NSCs, of varying the numbers of cells that express Sox2 within the neurosphere microenvironment

Cells that are not expressing *Sox2* and which do not show neurosphere-forming potential in culture may have a role in maintaining the stem cell niche. When $Sox2^{\beta geo/+}$ neurospheres cultured with G418 are removed from selection, *Sox2* non-expressing cells emerge. To investigate the effects of the neurosphere composition with respect to *Sox2* expression on NSCs, an experimental outline was developed (Figure 4.6) in order to assess neurosphere formation in the presence and absence of selection, i.e. in the presence and absence of *Sox2* positive cells.

Initial selection appears to be important in the maintenance of high numbers of neural stem cells. When the selection is removed in the subsequent generation as in D, higher neurosphere numbers may form due to the withdrawal of G418 cytotoxicity. However, tertiary neurospheres from these populations that form at a higher percentage (H) imply that the *Sox2* negative cellular component within the neurosphere encourages stem cell enrichment. This is surprising as in primary neurospheres, where the neurosphere-forming stem cells reside in the *Sox2* expressing component, G418 selection of $Sox2^{\beta geo/+}$ NSC cultures provides enrichment for stem cells. The reasons as to why population H has a higher neurosphere-forming

component have not been investigated. The stem cells giving rise to tertiary neurospheres are unlikely to reside in the *Sox2* non-expressing component, so it seems that higher numbers of *Sox2* expressing cells retain their stem cell properties in the presence of a more differentiated population. These results indicate that the initial stem cell microenvironment can have a significant influence on the stem cells and their future progeny even after passaging. In this situation, the differences in the niche have been dictated by whether cells express *Sox2* or not. This is also known to influence differentiation into the three neural lineages. Thus, changes in the neurosphere microenvironment with respect to differentiated cell types, affects stem cell properties.

In order to assess whether the differentiation of secondary neurospheres that have been cultured in the absence of selection from primary neurospheres maintained under selection is similar to that of neurospheres that have never been selected, the neurospheres from conditions C, D, E and F were differentiated for 5 days. Secondary neurospheres generated as in D differentiate in a similar way to primary and secondary neurospheres A and C, which have been cultured under selection. They generate a low number of TuJ1 positive neurons and show a regular lattice formation in the periphery of the neurosphere, which arises from cells that migrate away from the centre and differentiate. Secondary neurospheres cultured in the absence of selection generated from similar primary neurospheres as in F, exhibit typical differentiation patterns for non-selected $Sox2^{\beta geo/+}$ neurospheres, such as those differentiating from condition B. However, secondary neurospheres from condition E, cultured with G418 selection show lower levels of differentiation into neurons than in F, but higher than in A, C or D. These observations suggest that pressure to maintain

the expression of *Sox2βgeo* as in E, restricts but does not completely inhibit differentiation into neurons. This may be the case, as cells that have stopped expressing *Sox2* prior to selection and have initiated neurogenesis, would not survive the G418 treatment.

Figure 4.6 Varying the selection for *Sox2βgeo* in subsequent neurosphere generations. This was performed on dorsal telencephalon neurospheres from 14.5 dpc $Sox2^{\beta geo/+}$ mice. Flow chart (A'): blue panels (B, D, F, G, H, I and J) indicate the absence of selection and grey panels (A, C and E) indicate selection with G418. The results of how many neurospheres were generated in each condition are shown as percentages on the panels, calculated in Table 4.4. The outline for the experiment is shown in B'.

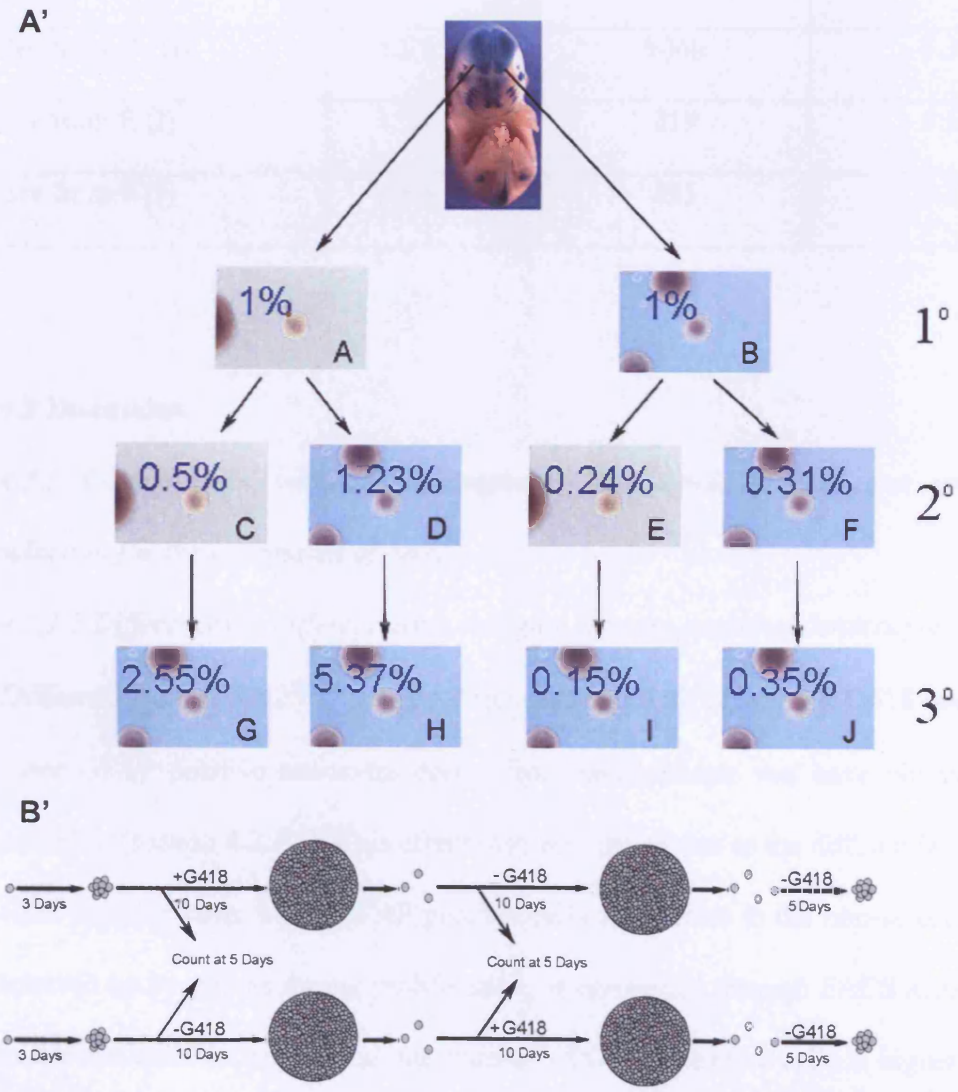


Table 4.4 Neurosphere counts for the experiment outlined in Figure 4.6. Neurosphere forming units are expressed as a percentage of the total number of cells plated in each condition.

Condition	Cells plated	Neurospheres counted	Neurosphere forming units %
2ry +G418→ +G418 (C)	1.2×10^6	6146	0.5
2ry +G418→ -G418 (D)	1.2×10^6	3675	1.23
2ry -G418→ +G418 (E)	3.5×10^6	2128	0.24
2ry -G418→ -G418 (F)	3.5×10^6	2744	0.31
3ry from C (G)	1.5×10^5	3525	2.35
3ry from D (H)	1.5×10^5	8066	5.37
3ry from E (I)	1.5×10^5	219	0.15
3ry from F (J)	1.5×10^5	525	0.35

4.3 Discussion

4.3.1 Comparisons between neurospheres that have or have not undergone selection for the expression of Sox2

4.3.1.1 Differences in differentiation into glia, neurons and oligodendrocytes

Differentiation of *Sox2*$^{\beta geo/+}$ neurospheres cultured with or without G418 reveals that more GFAP positive astrocytes derive from neurospheres that have not undergone selection (section 4.2.1.1). This effect may not just be due to the differentiation of the *Sox2* negative cells. More GFAP positive cells are present in the non-selected versus selected neurospheres during proliferation, as confirmed through FACS analysis, and in the non-selected population, the number of GFAP positive cells is higher than the average total number of SOX2 negative cells (up to 70% GFAP positive versus 20-30% SOX2 negative). It is possible that *Sox2* negative cells influence the *Sox2*

expressing component, an effect which would be absent in the stem cell-enriched, selected neurospheres (also demonstrated in section 4.2.2). RT-PCR shows that the levels of *Gfap* transcripts are higher in the non-selected neurospheres. However, FACS analysis reveals that there are two distinct GFAP positive cell types, with either high or low expression, in both selected and non-selected neurospheres, so any quantitative analysis needs to take into account their relative potential. If as proposed in the literature, *Gfap* expressing cells can have distinct NSC or astrocytic characters, the two distinct populations differentiated by FACS sorting could correspond to these. There is evidence to suggest that LIF-mediated signaling promotes *Gfap* positive NSCs and that BMP mediated signaling through BMP4 leads to *Gfap* positive astrocytes (Bonaguidi et al. 2005). As will be demonstrated in Chapter 5, *Stat3* has been identified as a candidate NSC marker, which is in agreement with the notion that LIF promotes a NSC fate. Furthermore, from the microarray analysis performed in this chapter between *Sox2βgeo* selected and non-selected populations (section 4.2.1.4), non-selected cultures where found to express higher levels of *Bmp4* than selected neurospheres (3.75-fold difference), consistent with the high proportion of astrocytes seen by immunofluorescence in *Sox2βgeo* non-selected cultures and by the increased levels of *Gfap* determined through RT-PCR.

The observation that non-selected neurospheres give rise to higher numbers of neurons compared to very few and sometimes no neurons in differentiating cultures from selected neurospheres (Figure 4.2) suggests that the *Sox2* negative cells are mainly responsible for neurogenic differentiation. Indeed it has been previously suggested that the absence of *Sox2* promotes neurogenesis (Kondo and Raff 2000).

No differences in the numbers of oligodendrocyte generation were observed between selected and non-selected neurospheres derived from the dorsal telencephalon. However cells with a more mature oligodendroglial morphology were observed with those originally in selection than without (Figure 4.2). Repeating the same experiments using spinal cord-derived cultures, a clear increase in the numbers of oligodendrocytes was seen from non-selected cultures, which could again be attributed to the presence of the *Sox2* negative component.

These results highlight both intrinsic regional differences and the possibility that more differentiated cells can influence fate choices made by the stem cells (or vice versa). It is likely that in non-selected neurospheres the influence on *Sox2* expressing cells by the *Sox2* negative component prevents maturation of oligodendrocytes or terminal differentiation, whereas this influence is absent in selected neurospheres. This speculation does not correlate with data yielded from the spinal cord cultures (demonstrated in Chapter 5), highlighting potential regional differences in the intrinsic differentiation mechanism. When dissociated into single cells, both selected and non-selected cultures give rise to secondary neurospheres. Therefore, both of these cultures meet the requirements for containing self-renewing, multipotent NSCs. This leads to the conclusion that the composition of the *Sox2* negative component may be responsible for creating an influential microenvironment for the NSCs. This affects differentiation capacity and potential, as well as proliferation, as demonstrated by the experiments in this chapter that have studied the effects of varying the composition of the neurosphere with respect to *Sox2* expression status.

4.3.1.2 Differences in the migration patterns of differentiating cells

The migration of cells away from the neurosphere under differentiation conditions highlights the differences between selected and non-selected $Sox2^{\beta geo/+}$ neurospheres (Figure 4.3). The disorganised migration seen in non-selected neurospheres is similar to that of wild type cultures, suggesting that the $Sox2^{\beta geo/+}$ selected neurosphere has a more homogenous array of cell types migrating away from the centre. A similar organised lattice arrangement has been described by Conti and co-workers, generated by ES-derived homogenous populations of radial glial-like cells (Conti et al. 2005). $Sox2^{\beta geo/+}$ selected neurospheres are enriched for cells expressing radial glial markers (Remboutsika et al, unpublished data), which may explain this phenotype. Given that radial glia have been described as stem cells in the embryonic brain (Alvarez-Buylla et al. 2001; Campbell and Gotz 2002; Doetsch 2003; Kriegstein and Gotz 2003), these findings combined with higher numbers of NSCs in the selected neurospheres indicate that a higher proportion of radial glia may be found in neurospheres derived from the dorsal telencephalon and maintained under selection for the expression of *Sox2βgeo*.

4.3.1.3 Differences in the gene expression profiles determined through microarray analysis

Microarray analysis to investigate differences between the selected and non-selected $Sox2^{\beta geo/+}$ neurosphere populations generated similar results from both the spotted oligonucleotide and the GeneChip platforms (section 4.2.1.4). For a more detailed bioinformatic analysis of the results, the GeneChip array data were used as they incorporate a much larger number of genes, are more reliable due to the unique specific and mismatch probe system and have updates available for the annotations of the sequences (the Mm_SGC_Av2 chips have been discontinued). Considering the

stem cell-enriched population has more cells that express *Sox2* than the non-selected population, we would expect it to show a bias towards higher relative levels of *Sox2*. Surprisingly however, this has not been the case in either platform and in all replicates. It is a fact that more cells are expressing *Sox2* in the selected cultures; hence the levels of expression of *Sox2* in the *Sox2*-expressing cells of the non-selected cultures must be proportionately higher. This may be due to underlying differences in the cell types composing the neurosphere, for example an increased number of highly *Sox2*-expressing astrocytes in the non-selected neurospheres compared to an increased number of radial glia in the selected neurospheres. The large group of genes involved in cell cycle control and promoting mitosis, seen at significantly elevated levels in *Sox2* selected neurospheres suggests that a higher proportion of cells are undergoing mitosis, hence proliferation in this population is increased, as other functional assays in this chapter have suggested (Figure 4.5, also section 4.2.1.2). Differences were also seen in the expression of components that may be involved in maintaining the NSC niche. Several genes responsible for the production of laminin (*Lamb1-1*, *Lamc1*, *Lama4*, *Lama5*) are expressed at significantly higher levels (over 2-fold expression) in NSC-enriched neurosphere populations. Similarly, genes controlling the expression of collagen (*Col11a1*, *Col4a6*, *Col4a3bp*, *Col4a5*, *Col9a1*) are preferentially expressed at higher levels in non-enriched populations suggesting that non-NSC's, in the neurosphere produce more collagen than laminin. In NSC-enriched populations, *Itgb1* is expressed over 2-fold higher that in the non-selected population. This gene encodes β1-integrin, a receptor of laminin and fibronectin. The expression of β1-integrin has been proposed to mark NSCs although its expression is not required for the growth of neurospheres (Leone et al. 2005). Similarly, the levels of expression of *Tnc* are significantly increased in NSC-enriched neurospheres. *Tnc* has been identified as a

NSC candidate gene in Chapter 5, being expressed at significantly higher levels in neurosphere populations of the dorsal telencephalon and spinal cord compared to their respective tissues of origin. These results suggest that NSCs are responsible for the production of components associated with positive regulation of NSCs in their niche.

4.3.2 The effects, of varying the numbers of cells that express Sox2, within the neurosphere microenvironment, on the NSCs

Selection for the expression of *Sox2βgeo* impacts on the subsequent fate of cells within neurospheres. Experiments involving consecutive periods of selection and no selection with successive passaging of neurospheres were used to establish if the composition of the neurosphere had an effect on stem cell potential. From these experiments, it is clear that an initial selection for *Sox2βgeo* in primary neurospheres results in an overall enrichment for NSCs in subsequent cultures, irrespective of downstream selection. However in subsequent generations, selection for *Sox2βgeo* does not necessarily result in enrichment. It appears that when pure $Sox2^{\beta geo/+}$ populations are cultured as secondary and tertiary neurospheres with the removal of selection, they are able to give rise to higher proportions of neurosphere-forming cells. One hypothesis is that the cytotoxicity of G418 used to select for *Sox2βgeo* expressing cells, is compromising the full potential of the neural stem cells in these cultures. This hypothesis has not been tested, but can be done so using cells from mice ubiquitously expressing a neomycin resistance gene in the ROSA26 locus. Primary neurospheres from these mice can be grown with or without G418, which will not have an impact on the cellular composition of the neurospheres as all cells are resistant. The generation of a secondary neurosphere population from these cells and an assessment of their neurosphere-forming capacity would be sufficient to determine

if G418 is responsible for lower neurosphere formation when comparing populations with or without G418 that are derived from primary neurospheres treated in the same way. Reservations for this experiment are that strain-specific differences may impair the results and an MF1 out-bred background (as is the background of the *Sox2*$^{\beta geo/+}$ mice used) ubiquitously resistant to neomycin should be used if possible. If G418 cytotoxicity is responsible for the compromised ability to generate neurospheres, further experiments will be necessary to determine if the extended culture of a pure population of *Sox2* expressing cells results in NSC enrichment. The current results suggest that NSCs have a long-term requirement for *Sox2* non-expressing cells to maintain their stem cell status.

CHAPTER 5

Identification of candidate genes that mark neural stem cells

5.1 Background

5.1.1 Comparisons between CNS neurospheres

In Chapter 3, characterisation of neurospheres from the dorsal telencephalon of 14.5 dpc mice was performed. In this chapter, these neurospheres will be compared to 14.5 dpc spinal cord neurospheres in order to obtain information that will lead to the identification of candidate genes that commonly define several populations of neural stem cells. As established in Chapter 4, selection for the expression of $Sox2^{\beta geo}$ leads to enrichment in the numbers of NSCs in culture. Hence, the culture of $Sox2^{\beta geo/+}$ neurospheres under G418 selection will be employed in all further studies.

5.1.2 Formation of brain and spinal cord- changes in properties of NSCs

During gastrulation, the neural plate is patterned from a portion of the ectoderm. The ventral part of the ectoderm is induced to become epidermis, in response to BMP signalling, which blocks neural differentiation. BMP inhibitors, such as noggin, chordin and follistatin, produced by the organizer, lead to the specification of neural tissue, as this portion inhibits BMP signals. In vertebrates, the neural plate is initially composed of a population of neural progenitors with uniform capabilities in their properties i.e. their fate. However, they may have already acquired anterior-posterior patterning. During primary neurulation, the ectoderm becomes divided into the externally positioned epidermis of the skin, neural crest cells and the neural tube. For the formation of the latter, proliferation in the cells of the neural plate (in the dorsal part of the ectoderm) ceases to be uniform, resulting in folding to form the neural

groove and subsequent closure of the neural folds to form the neural tube. The rostral neural tube forms three brain vesicles: the prosencephalon (forebrain), from which the telencephalon and diencephalon later form, the mesencephalon (midbrain) and the rhombencephalon (hindbrain), from which the metencephalon and the myelencephalon later form. At this three-vesicle stage, the brain flexes at the junction between the hindbrain and the caudal neural tube, which is the prospective spinal cord (cervical flexure) and at the midbrain-hindbrain junction (cephalic flexure). Later, another flexure, called the pontine flexure, develops between the cephalic and cervical flexures. The cephalic flexure is the only one that remains prominent throughout development and causes the longitudinal axis of the forebrain to differ from that of the brain stem and spinal cord.

Figure 5.1 Cartoon depicting the major regional specifications of the murine CNS at 11.5 dpc. Each region has been coloured separately: purple- telencephalon, blue-diencephalon, green-metencephalon, orange-myelencephalon, yellow-spinal cord.

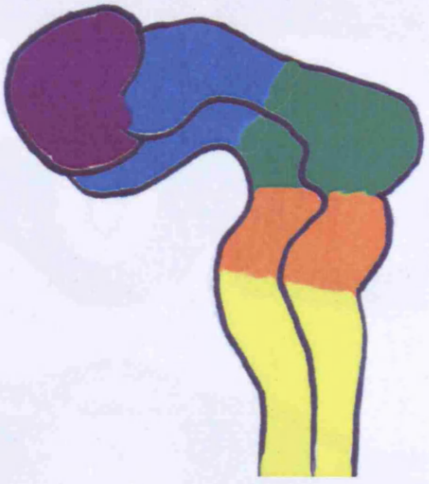


Figure 5.2. Cartoon outlining the major stages in the formation of the neural tube, based on chick development.

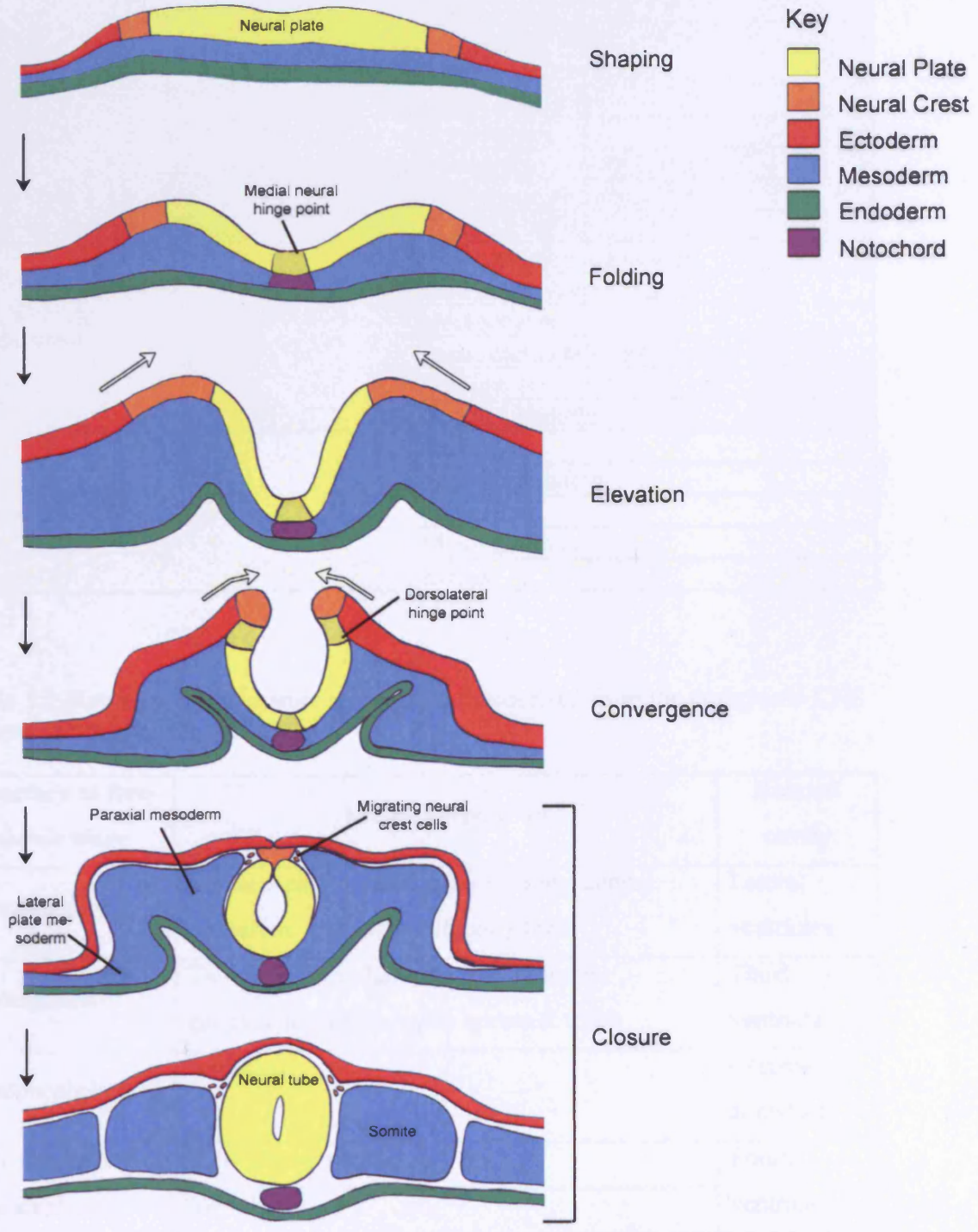


Table 5.1 Outline of the derivatives of the three major domains of the ectoderm germ layer (adapted from (Gilbert 2000)).

Epidermis (outer ectoderm)	Epidermis
	Hair
	Nails
	Sebaceous glands
	Olfactory epithelium
	Mouth epithelium
	Lens, cornea
	Otic vesicle
	Neurogenic placodes
	Cranial ganglia
Neural crest	Peripheral nervous system
	Enteric nervous system
	Melanocytes
	Facial cartilage/bone/muscle
	Dentine
	Adrenal medulla
Neural tube	Brain
	Posterior pituitary
	Spinal cord
	Motor neurons
	Retina

Table 5.2 Summary of the mature adult structures derived from the embryonic CNS (adapted from (Kandel et al. 2000)).

Structure at five-vesicle stage	**Major derivatives**	**Related cavity**
Telencephalon	Cerebral cortex, basal ganglia, hippocampal formation, amygdala, olfactory bulb	Lateral ventricles
Diencephalon	Thalamus, hypothalamus, subthalamus, epithalamus, retina, optic nerves & tracts	Third ventricle
Mesencephalon	Midbrain	Cerebral aqueduct
Metencephalon	Pons and cerebellum	Fourth ventricle
Myencephalon	Medulla	
Caudal neural tube	Spinal cord	Central canal

During the development of the nervous system in the mouse, a period of extensive cellular proliferation is followed by a period of enhanced neurogenesis (~ 13.5 dpc) and later gliogenesis (~17.5 dpc). Patterning of the neural tube results in spatially well-defined areas of specialised differentiation of neuroepithelial precursors into neurons. In the adult mouse, several hundreds of distinct neuronal cell types are thought to exist. Proneural factors such as the bHLH neurogenin proteins play an important role in NSC fate decisions. They promote expression of neuronal differentiation genes, activate neuronal subtype genes, promote cell-cycle exit and inhibit glial differentiation by suppressing glial-specific genes such as *Gfap*. This commitment to precursor or terminally differentiated cells leads to the progressive restriction of zones where undifferentiated neural stem cells can be found. In the late mouse embryo, regions lining the ventricles and cavities throughout the CNS are rich in proliferating cells, the most abundant being the dorsal telencephalon. In the adult, the proliferative regions in the brain are restricted to the subventricular zone of the lateral ventricles and to the subgranular layer of the dentate gyrus in the hippocampus.

In this study, similarities between spatially different progenitor populations have been investigated, in order to identify genes that are important in the stem cell state. Alongside the intrinsic programming of NSCs, the influence of environmental cues on these populations has been taken into account. If neural stem cells are able to retain properties arising from the impact of environmental cues, their unique differentiation patterns will be reflected in culture.

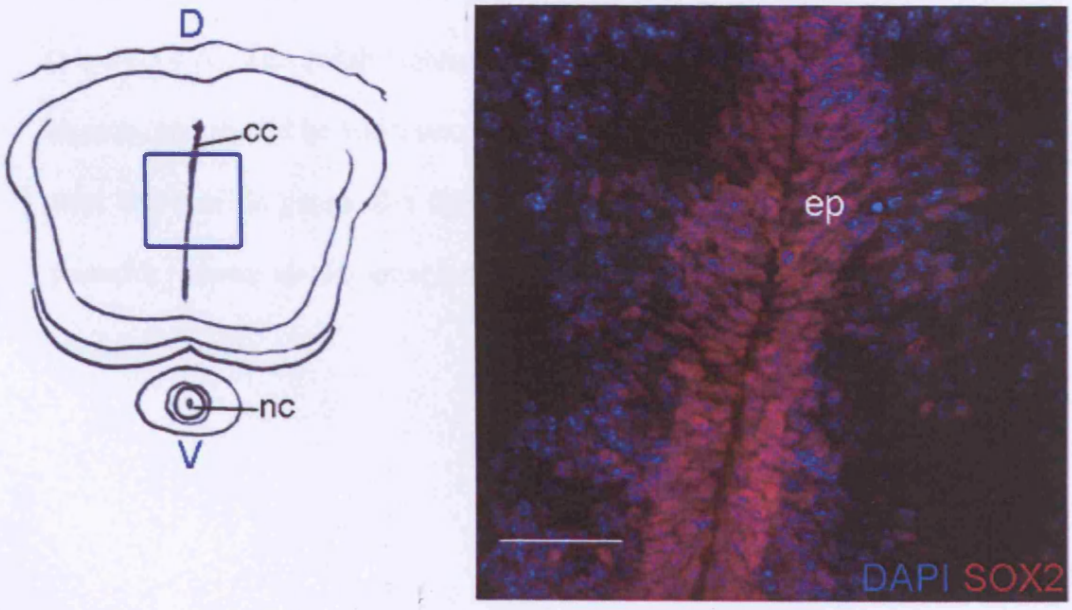
5.2 Results

5.2.1 14.5 dpc spinal cord neural stem cells

5.2.1.1 Localisation of *Sox2* expressing cells in the spinal cord

The proliferating NSCs are thought to reside in the ependymal layer surrounding the central canal of the spinal cord. Immunofluorescence staining using antibodies against SOX2 reveals that cells expressing *Sox2* are found mainly in the ependymal layer and SOX2 localises in the nucleus of these cells (Figure 5.3). Isolated SOX2 immunoreactive cells are also sometimes seen in the tissue surrounding the ependymal layer but their incidence is low.

Figure 5.3 Immunofluorescence staining for SOX2 on a section through the 14.5 dpc spinal cord. SOX2 localises in the nucleus of cells in the ependymal layer (*ep*). The part of the spinal cord shown is indicated by the blue box in the cartoon, where D is dorsal, V is ventral, *cc* is central canal and *nc* is notochord. Scalebar represents 100μm.



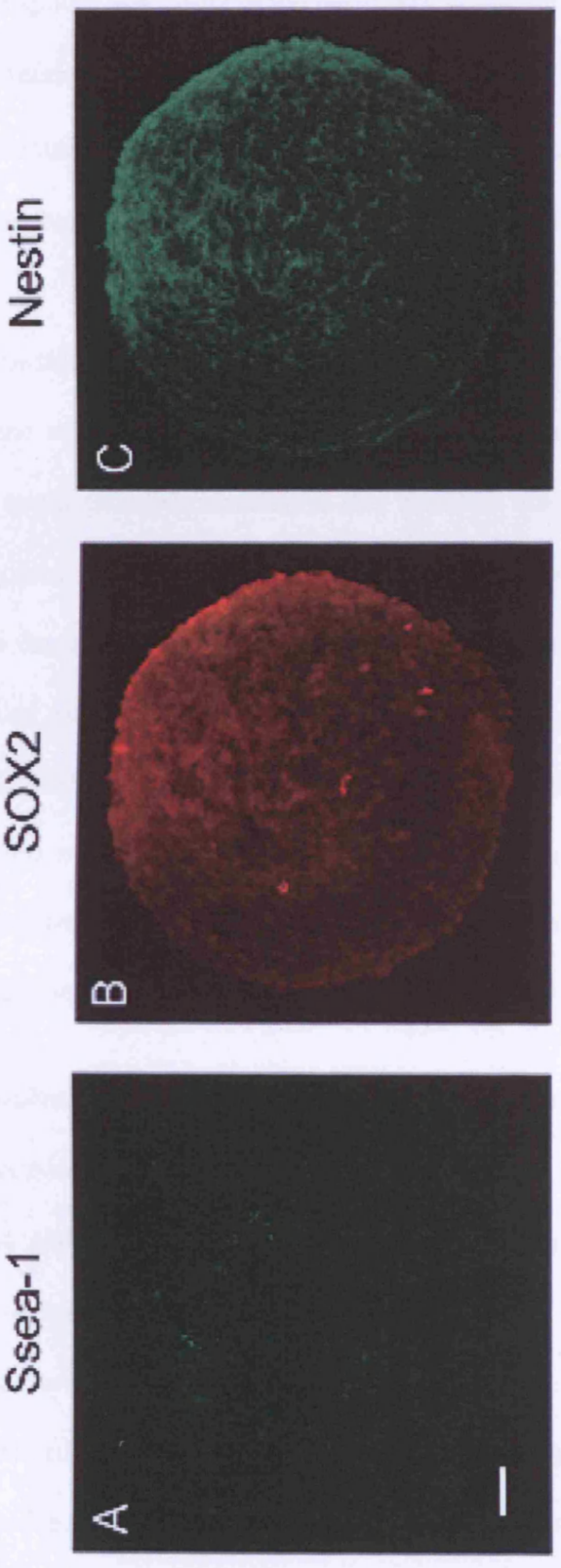
5.2.1.2 Generation of neurospheres from the 14.5 dpc spinal cord

For the generation of neurospheres derived from the 14.5 dpc spinal cord, whole spinal cords were dissociated and cultured under optimal conditions as determined in Chapter 2. Only 0.2% of cultured spinal cord cells at 14.5 dpc generate neurospheres in culture. The inclusion of many cells in the culture that lie outside of the restricted proliferative ependymal zone is likely to be responsible for this reduced proportion of neurosphere generating cells compared to 14.5 dpc dorsal telencephalon. Preliminary results of spinal cord culture at different embryonic stages reveal that similar neurosphere formation levels to the 14.5 dpc dorsal telencephalon cells can be obtained using 11.5 dpc spinal cords where the proportion of undifferentiated cells is likely to be much higher.

5.2.1.3 Proliferation marker analysis of spinal cord neurospheres

The majority of cells in stem cell-enriched *Sox2*$^{\beta geo/+}$ neurospheres express SOX2 and Nestin as shown by immunofluorescence staining (Figure 5.4 B and C). Ssea-1, which is used as a brain stem cell-specific marker, is not expressed in these cultures (Figure 5.4 A). This result highlights differences in stem cells isolated from different regions, and should be taken into account when aiming to identify ubiquitous neural stem cell-specific genes. But this also highlights regional differences and anterior-posterior patterns already established, and that cells have a memory that reflects their region of origin.

Figure 5.4 Immunofluorescence staining in neurospheres derived from the 14.5 dpc spinal cord of *Sox2*$^{\beta geo/+}$ mice that have been selected for *Sox2βgeo* expression. Cells are immunoreactive for SOX2 (B) and Nestin (C), which co-localise. Ssea-1 levels are not detectable (A). Scalebar represents 100μm.



5.2.1.4 Differentiation marker analysis of spinal cord neurospheres

Differentiation analysis of 14.5 dpc stem cell-enriched spinal cord cultures confirms their potential to form neurons, glia and oligodendrocytes. Neurons are often seen in the main body of the sphere, but rarely in the periphery. Neurospheres that have been selected for the expression of $Sox2^{\beta geo}$, differentiate to give TuJ1 positive neurons with long axons, in contrast with TuJ1 positive neurons differentiating from $Sox2^{\beta geo}$ selected neurospheres from the dorsal telencephalon.

5.2.2 Selection of candidate neural stem cell markers

By analysing the gene expression patterns of several neural stem cell populations, it was hoped that we could establish similarities that could be attributed to their stem cell character. Comparisons between different NSC populations will highlight many similarities between them, other than the expression of genes unique to neural stem cells, such as similar expression of housekeeping genes and genes required for metabolic pathways. Such genes can be partly eliminated through the use of a non-stem cell reference. As well as identifying similarities, these comparisons will also highlight differences between the populations, including some that define the regions and stages from which the NSCs where originally isolated.

5.2.2.1 Direct and indirect comparisons of NSCs using a reference sample: spotted oligonucleotide array platform

As a pilot study to test this experimental approach, 14.5 dpc $Sox2^{\beta geo/+}$ dorsal telencephalon and spinal cord-derived neurospheres selected with G418 were indirectly compared through pair-wise analysis using a reference sample. The reference sample selected was 14.5 dpc whole CNS tissue from MF1 out-bred mice. In order to recruit additional cell populations in the study for future comparisons, a

highly reproducible reference sample was chosen. The similarities between the dorsal telencephalon (DT) and spinal cord (SC) cultures were compared indirectly (DT v reference, SC v reference) and directly (DT v SC) as outlined in Figure 5.5 and based on the parameters outline in the microarray minimum information document outlined in Table 5.3.

Figure 5.5 Microarray Experimental Outline of a pilot experiment to determine candidate factors of NSCs. Neurospheres from the dorsal telencephalon and from the spinal cord of 14.5 dpc *Sox2*$^{\beta geo/+}$ mice where selected for the expression of *Sox2βgeo* for NSC enrichment. These two populations where compared directly and indirectly through a reference sample (14.5 dpc CNS).

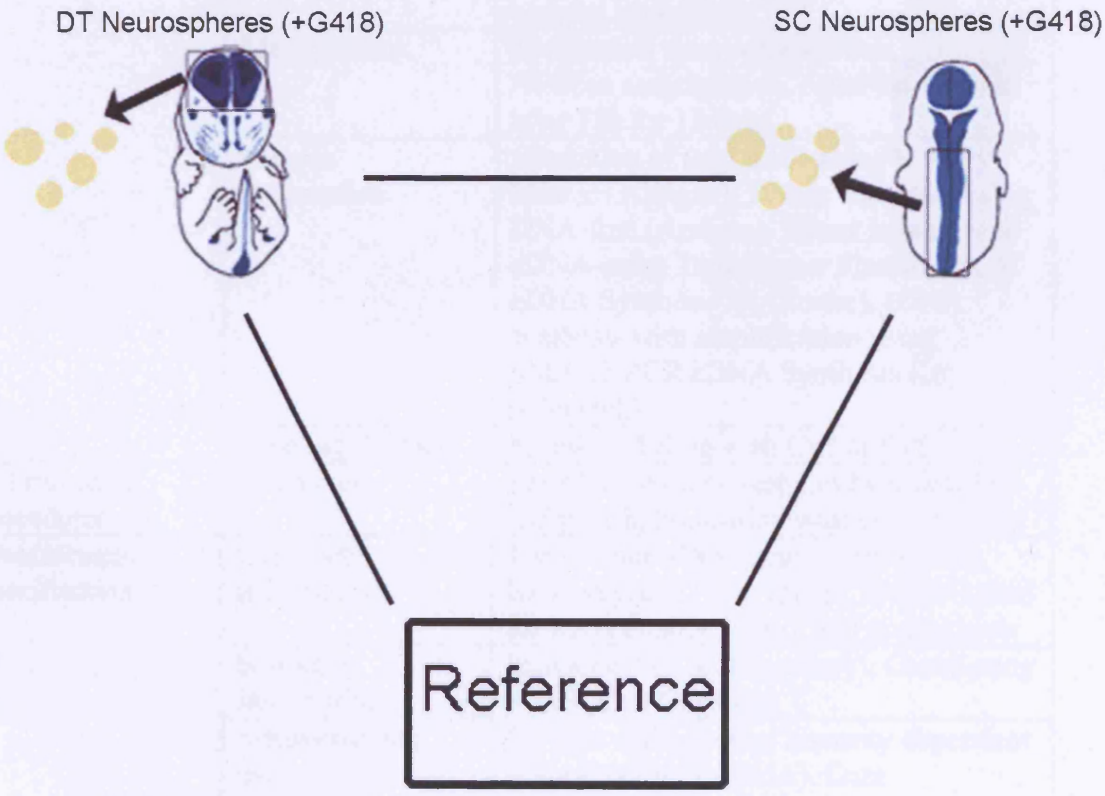


Table 5.3 MIAME: Determining NSC candidates using the spotted platform (A)

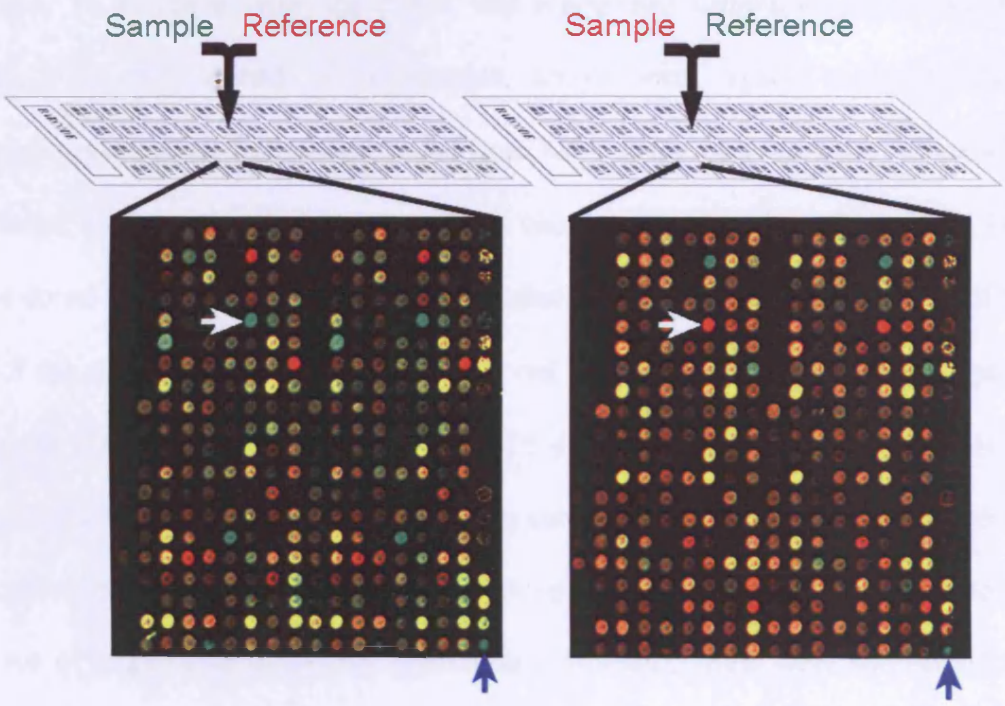
Experiment Design	Type	Cultured NSCs from spinal cord v Cultured NSCs from dorsal telencephalon
	Experimental Factors	Neurosphere culture of neural stem cells and enrichment for NSCs through selection for *Sox2βgeo* expression
	Number of Hybridisations	3 two-colour
	Reference Sample	14.5 dpc whole CNS (wild type MF1 outbred)
	Hybridisation Design	Indirect: Dorsal telencephalon neurospheres v reference, spinal cord neurospheres v reference, Direct: dorsal telencephalon neurospheres v spinal cord (see diagram)
	Quality Control	3 replicates, one independent cDNA synthesis with amplification, one dye-swap of for unamplified synthesis.
Samples	Origin	14.5 dpc dorsal telencephalon, 14.5 dpc spinal cord of $Sox2^{\beta geo/+}$ mice (MF1 outbred background)
	Manipulation	Mechanical tissue dissociation, culture in NPM as neurospheres. Addition of G418 after 72h for 15 days
	Sample Preparation	Extraction of total RNA using RNeasy Midi kit (Qiagen). DNase treatment using DNA-free (Ambion). Direct synthesis of cDNA using Transcriptor First Strand cDNA Synthesis kit (Roche), cDNA synthesis with amplification using SMART PCR cDNA Synthesis Kit (Clontech)
	Labeling Protocol	Klenow labeling with Cy3 or Cy5
Hybridisation Procedures	Parameters	See Methods for overnight hybridisation and post-hybridisation washes protocols
Measurement Specifications	Hardware Information	Two-colour 428 scanner (Affymetrix), BioAnalyzer 2100 (Agilent Technologies) for RNA quality control and quantitation
	Software Information	ImaGene 4.0 (BioDiscovery), GeneSpring 7.2 (Silicon Genetics)
	Measurements used	Per spot and per chip: intensity dependent normalisaion (YASMA). Data transformation: Dye swap. Set measurements less than 0.01 in the control channel were set to 0.01 (GeneSpring)
Array Design	Platform	Spotted oligonucleotide array
	Type	Mm_SGC_Av2: Mouse Known Oligo Array on CodeLink Activated Slides (Amersham Biosciences)

For efficient microarray interpretation using spotted oligonucleotide arrays, 3 replicates are normally performed for each sample to ensure the validity of the results. cDNA is synthesized from total RNA samples and at least one independent synthesis reaction is performed, that can serve as one replicate, preferably using an independent RNA source. The most likely source of variation in microarray experiments is between biological replicates, as variation between technical replicates is unlikely if experiments are performed in parallel. Each cDNA sample is labelled with either Cy3 or Cy5 and hybridised onto the chip alongside the sample it will be compared to, which has been labelled with the alternate dye. To ensure no dye bias in the hybridisations, a dye-swap was performed, an example of which, taken from the hybridisations performed for this experiment, is shown in Figure 5.6. Limitations in the RNA yield for this set of experiments made it necessary to use a method of cDNA synthesis that employs amplification (SMART cDNA synthesis), which served as the independent replicate. This also provided a means to assess the reliability of amplified cDNA compared to direct cDNA synthesis.

Using different methods to generate cDNA should not affect the end result, given that the same RNA template was used. However, studying replicates with and without amplification demonstrated that these two methods are not comparable within this study. Comparisons between amplified and unamplified cDNA hybridisations for all targets resulted in little overlap in the genes that were significantly expressed at higher or lower levels compared to a reference target. The highest number of genes shared between candidate lists generated by the two different methods was 25 in a total sample size of 7,500. This led to the conclusion that the amplified results cannot be taken into consideration, as error muse have been

introduced during the amplification (PCR amplification cycles range from 25 to 32). This left an insufficient number of replicates to conclude the study. However, as the similarities between the two populations were of direct interest, and the differences less so, this pilot study highlighted an experimental flaw: the expression of over 5,000 genes remained significantly similar between the two populations, which are too many to process in order to identify NSC candidates.

Figure 5.6 Outline of a dye-swap experiment. The two samples ('sample' and 'reference') are labelled with Cy3 or Cy5 in one hybridisation and the reciprocal in the other. The individual grids from two chips illustrate the dye swap. Spots to which 'sample' probe preferentially hybridises to is green in A and red in B (white arrows). When both probes show the same affinity for a spot it is yellow. Blue arrows indicate control spots of only Cy3 (landing lights), which serve as hybridisation controls.



5.2.2.2 Comparing enriched-NSC neurospheres to their tissue of origin: spotted oligonucleotide array platform

A very large number of genes were commonly expressed at significantly high levels between neurosphere populations in the previous study. To refine this list of genes that potentially define NSCs, one approach is to introduce an increasing number of different neurosphere populations as regional and temporal differences are bound to reduce the similarities. However an alternative approach was taken to identify genes commonly expressed between neural stem cell populations. When compared to their tissue of origin, stem cell-enriched neurospheres should exhibit higher levels of expression for genes important in neural stem cells. Therefore, the experimental design was changed to compare neurospheres populations to their respective tissue of origin. To minimise error, the cDNA was synthesised without amplification. The Mm_SGC_Av2 spotted oligonucleotide arrays were again used for these experiments. Labeled cDNA generated from RNA of the 14.5 dpc *Sox2*$^{\beta geo/+}$, G418-selected dorsal telencephalon neurospheres was hybridised against cDNA from 14.5 dpc dorsal telencephalon tissue. Similarly, labeled cDNA generated from RNA of the 14.5 dpc *Sox2*$^{\beta geo/+}$, G418-selected spinal cord neurospheres was hybridised against labeled cDNA generated from RNA of 14.5 dpc spinal cord tissue, as outlined in Figure 5.7 and according to the parameters summarised in Table 5.4. Genes whose expression level was two-fold higher in each neurosphere population compared to the tissue of origin were considered significantly different. These were short-listed for each of the neurosphere and spinal cord samples and the overlapping genes from both of these shortlists from the neurosphere populations were identified as candidate intrinsic factors, important in neural stem cells (Figure 5.8). These 48 candidate markers are listed in Figure 5.10 and are classified based on their molecular function.

Figure 5.7 Microarray Outline to determine candidate markers of NSCs. *Sox2βgeo*-selected neurospheres (NSC enriched) from the dorsal telencephalon (DT) and from the spinal cord (SC). They where compared directly to their tissue of origin and genes significantly higher in both populations compared to their respective tissue where short-listed as NSC candidates.

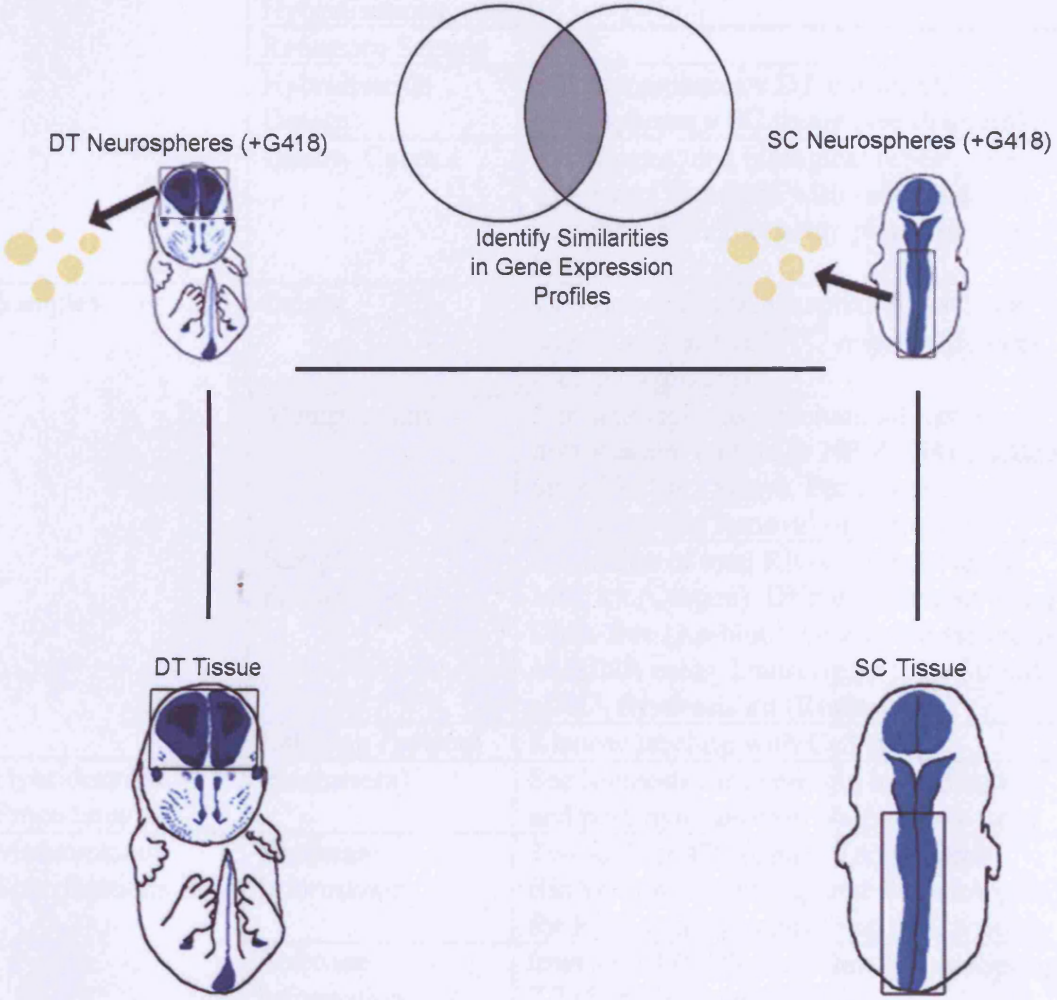


Table 5.4 MIAME: Determining NSC candidates using the spotted platform (B)

Experiment Design	Type	Cultured cells v Tissue of origin
	Experimental Factors	Neurosphere culture of neural stem cells and enrichment for NSCs through selection for *Sox2βgeo* expression. Tissue of origin of neurosphere cultures remained untreated
	Number of Hybridisations	2 two-colour
	Reference Sample	None
	Hybridisation Design	DT neurospheres v DT tissue, SC neurospheres v SC tissue (see diagram)
	Quality Control	3 replicates, one biological repeat, one dye-swap. Repeated with independent samples on another array platform (see later)
Samples	Origin	14.5 dpc dorsal telencephalon, 14.5 dpc spinal cord of $Sox2^{\beta geo/+}$ mice (MF1 out-bred background)
	Manipulation	For neurospheres: mechanical tissue dissociation, culture in NPM. G418 added after 72h for 15 days. For tissue: dissection and removal of membranes
	Sample Preparation	Extraction of total RNA using RNeasy Midi kit (Qiagen). DNase treatment using DNA-free (Ambion). One-cycle synthesis of cDNA using Transcriptor First Strand cDNA Synthesis kit (Roche)
	Labeling Protocol	Klenow labeling with Cy3 or Cy5
Hybridisation Procedures	Parameters	See Methods for overnight hybridisation and post-hybridisation washes protocols
Measurement Specifications	Hardware Information	Two-colour 428 scanner (Affymetrix), BioAnalyzer 2100 (Agilent Technologies) for RNA quality control and quantitation
	Software Information	ImaGene 4.0 (BioDiscovery), GeneSpring 7.2 (Silicon Genetics)
	Measurements used	Data transformation: Dye swap. Per spot and per chip: intensity dependent normalisaion (Lowess). Lowess curve fitted to log-intensity versus log-ratio plot. 40.0% of the data used to calculate the fit at each point. The curve was used to adjust the control value for each measurement. Set measurements < 0.01 set to 0.01 (GeneSpring)
Array Design	Platform	Spotted oligonucleotide array
	Type	Mm_SGC_Av2: Mouse Known Oligo Array on CodeLink Activated Slides (Amersham Biosciences)

Figure 5.8 Overlap of candidate markers shared between 14.5 dpc dorsal telencephalon and spinal cord NSCs using data generated from experiments performed on the spotted microarray platform. Venn diagram outlining the overlap between the two gene lists generated on the Mm_SGC_Av2 spotted oligonucleotide platform. The 48 overlapping genes are short-listed as candidates for their significance in neural stem cells and are listed in Figure 5.9.

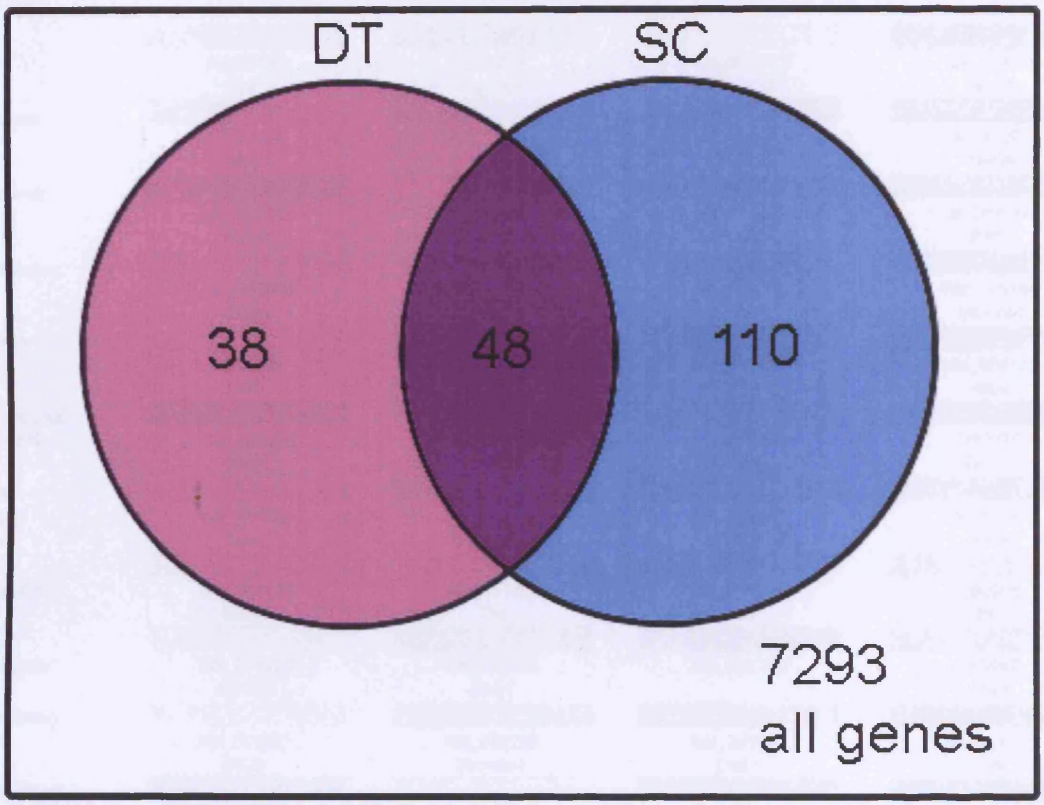


Figure 5.9 Candidate markers shared between 14.5 dpc dorsal telencephalon and spinal cord NSCs. These genes were selected for future further investigation into their significance in neural stem cells. This list was generated by comparing candidates expressed higher in enriched stem cell populations of the spinal cord and of the dorsal telencephalon as described in Figures 5.7 and 5.8. Their Gene Ontology classification according to their known molecular function is indicated by colour. Known markers of committed progenitors/differentiating cells are included under one category (Differentiation markers). Genes with multiple function where only included in one category.

5.2.2.3 Comparing enriched-NSC neurospheres to their tissue of origin: Affymetrix GeneChip platform

The experiments described in section 5.2.2.2 were repeated using the Affymetrix Mouse 430A GeneChip arrays (22,690 genes), which are high-density oligonucleotide probe arrays. As stated in section 1.5, this type of platform is more reliable than spotted arrays and does not require the same level of technical repetition. Each sample was hybridised individually on one chip and different targets were compared digitally. One hybridisation was performed for each sample and independent samples from the previous experiment were used (acting as biological replicates for the genes investigated). Some genes on this platform are represented more than once by probe sets against independent sequences on the array. A larger list of candidates than for the spotted arrays was expected, reflecting the greater total number of genes (22,690 compared to 7,500 genes). GCRMA normalisation was performed using GeneSpring 7.2 and 7.3 (Silicon Genetics) software and the 'Raw' value data were used for all comparisons. Following the experimental outline in Figure 5.7 and using the parameters in Table 5.5, a list of 339 candidates of significance to neural stem cells was identified (535 prior to removal of duplicates, Figure 5.10) that had 2-fold significantly higher expression levels in the neurosphere populations compared to the tissue of origin, and that had expression levels of over 100 arbitrary units to eliminate false-positives (maximum expression level reached in this experiment is 47,500 arbitrary units). To refine this list further, genes were filtered on expression levels of over 1,000 arbitrary units in order to obtain the highest expressing candidates. There are some genes, which are represented by more than one probe set on the arrays. These where manually removed, and after their exclusion, 96 candidates were short-listed, listed in Table 5.6.

Table 5.5 MIAME: Determining NSC candidates using the GeneChip platform

Experiment Design	Type	Cultured cells v Tissue of origin
	Experimental Factors	Neurosphere culture of neural stem cells and enrichment for NSCs through selection for *Sox2βgeo* expression. Tissue of origin of neurosphere cultures remained untreated
	Number of Hybridisations	4 one-colour
	Reference Sample	None
	Hybridisation Design	Dorsal telencephalon neurospheres v dorsal telencephalon tissue, spinal cord neurospheres v spinal cord tissue
	Quality Control	Repeated on another array platform (see later)
Samples	Origin	14.5 dpc dorsal telencephalon, 14.5 dpc spinal cord of $Sox2^{\beta geo/+}$ mice (MF1 out-bred background)
	Manipulation	For neurospheres: mechanical tissue dissociation, culture in NPM as neurospheres. Addition of G418 after 72h for 15 days. For tissue: dissection and removal of membranes
	Sample Preparation	Extraction of total RNA using RNeasy Midi kit (Qiagen). DNase treatment using DNA-free (Ambion)
	Labeling Protocol	Biotin labeling
Hybridisation Procedures	Parameters	Affymetrix 'Eukaryotic Sample and Array Processing' guide followed for all protocols (see Methods)
Measurement Specifications	Hardware Information	GenAmp PCR System 9700 (Applied Biosystems), GeneChip Hybridization Oven 640, GeneChip Fluidics Station 450 and GeneChip Scanner (all Affymetrix). NanoDrop ND-1000 spectrophotometer (Labtek) and BioAnalyzer 2100 (Agilent Technologies) for RNA quality control and quantitation
	Software Information	GCOS (Affymetrix), GeneSpring 7.2 & 7.3 (Silicon Genetics)
	Measurements used	Raw values after GC RMA Normalisation, per chip: normalise to 50th parcentile, per gene: normalise to median and data transformation: set measurements < 0.01 set to 0.01 (GeneSpring)
Array Design	Platform	Affymetrix GeneChip
	Type	Mouse 430A

Figure 5.10 Overlap of candidate markers (prior to removal of duplicates) between 14.5 dpc dorsal telencephalon and spinal cord NSCs using data generated from experiments performed on the Mouse 430A GeneChip platform.

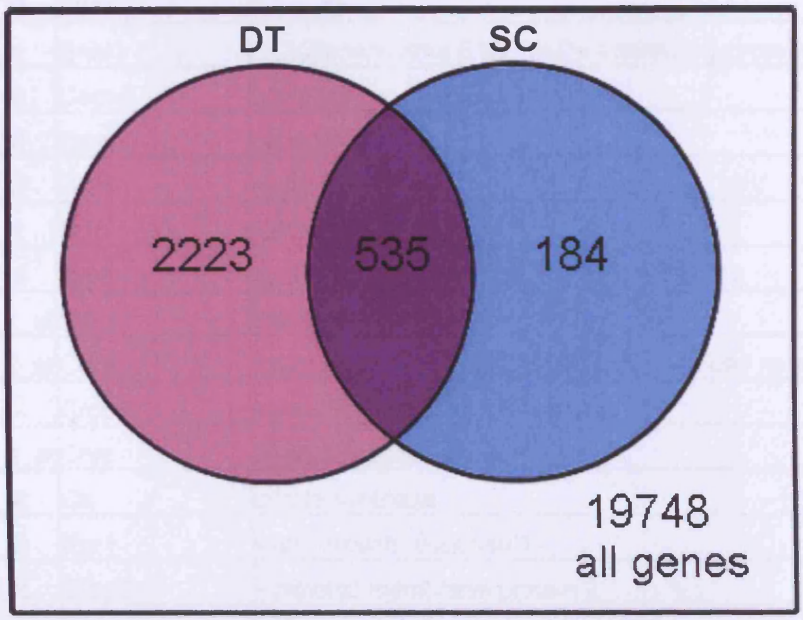


Table 5.6 Short-list of candidate markers. This list was determined from comparisons of NSC-enriched neurosphere populations to their tissue of origin, on the Mouse 430A GeneChip platform. All genes are expressed significantly higher in the NSC-enriched populations with expression levels > 1,000 arbitrary units. They have been arranged in alphabetical order of their common gene name.

Gene Name	Common Name	Description
1448412_a_at	0610009M14Rik	RIKEN cDNA 0610009M14 gene
1423909_at	0610011I04Rik	RIKEN cDNA 0610011I04 gene
1418004_a_at	1810009M01Rik	RIKEN cDNA 1810009M01 gene
1460344_at	2310033F14Rik	pre-B-cell leukemia transcription factor interacting protein 1
1437671_x_at	2310046G15Rik	BB378796 RIKEN full-length enriched, 16 days embryo head
1428288_at	2310051E17Rik	basic transcription element binding protein 1
1451385_at	2310056P07Rik	RIKEN cDNA 2310056P07 gene
1426856_at	2610207I16Rik	RIKEN cDNA 2610207I16 gene
1452354_at	2810459M11Rik	RIKEN cDNA 2810459M11 gene
1434542_at	4631422C05Rik	RIKEN cDNA 4631422C05 gene
1416315_at	Abhd4	abhydrolase domain containing 4
1448987_at	Acadl	acetyl-Coenzyme A dehydrogenase, long-chain
1416094_at	Adam9	a disintegrin and metalloproteinase domain 9 (meltrin gamma)
1432466_a_at	Apoe	apolipoprotein E
1434449_at	Aqp4	clone:A330077C20 product:aquaporin 4. Adult male spinal cord cDNA

1452308_a_at	Atp1a2	ATPase, Na+/K+ transporting, alpha 2 polypeptide
1435148_at	Atp1b2	ATPase, Na+/K+ transporting, beta 2 polypeptide
1435559_at	BC029719	myosin VI
1416718_at	Bcan	brevican
1422470_at	Bnip3	BCL2/adenovirus E1B 19kDa-interacting protein 1, NIP3
1422845_at	Canx	calnexin
1417327_at	Cav2	caveolin 2
1450017_at	Ccng1	cyclin G1
1416066_at	Cd9	CD9 antigen
1424638_at	Cdkn1a	cyclin-dependent kinase inhibitor 1A (P21)
1437458_x_at	Clu	clusterin
1437341_x_at	Cnp1	BB251922 RIKEN full-length enriched 7day neonate cerebellum
1450966_at	Crot	carnitine O-octanoyltransferase
1438610_a_at	Cryz	crystallin, zeta
1422577_at	Cs	citrate synthase
1417065_at	Egr1	early growth response 1
1433670_at	Emp2	epithelial membrane protein 2
1422438_at	Ephx1	epoxide hydrolase 1, microsomal
1449324_at	Ero1l	Transcribed sequences
1416209_at	Glud	glutamate dehydrogenase
1433485_x_at	Gpr56	G protein-coupled receptor 56
1416368_at	Gsta4	glutathione S-transferase, alpha 4
1448330_at	Gstm1	glutathione S-transferase, mu 1
1448429_at	Gyg1	glycogenin 1
1426522_at	Hadhb	hydroxyacyl-Coenzyme A dehydrogenase beta subunit
1455972_x_at	Hadhsc	L-3-hydroxyacyl-Coenzyme A dehydrogenase, short chain
1423780_at	Hibadh	3-hydroxyisobutyrate dehydrogenase
1418072_at	Hist1h2bc	histone 1, H2bc
1416344_at	Lamp2	lysosomal membrane glycoprotein 2
1449893_a_at	Lrig1	leucine-rich repeats and immunoglobulin-like domains 1
1433532_a_at	Mbp	myelin basic protein
1420911_a_at	Mfge8	milk fat globule-EGF factor 8 protein
1415897_a_at	Mgst1	microsomal glutathione S-transferase 1
1448139_at	Mlc1	megalencephalic leukoencephalopathy w subcortical cysts 1 (Hum Homl)
1424534_at	Mmd2	monocyte to macrophage differentiation-associated 2
1416632_at	Mod1	malic enzyme, supernatant
1422557_s_at	Mt1	metallothionein 1
1428942_at	Mt2	metallothionein 2
1433942_at	Myo6	myosin VI
1417568_at	Ncald	neurocalcin delta
1448154_at	Ndrg2	N-myc downstream regulated 2

1416816_at	Nek7	NIMA (never in mitosis gene a)-related expressed kinase 7
1460303_at	Nr3c1	nuclear receptor subfamily 3, group C, member 1
1427475_a_at	Nrap	Mus musculus cDNA clone IMAGE:3495171, partial cds.
1416149_at	Olig1	oligodendrocyte transcription factor 1
1416232_at	Olig2	oligodendrocyte transcription factor 2
1454714_x_at	Phgdh	Sequence with similarity to protein sp:Q61753 (M.musculus)
1430700_a_at	Pla2g7	phospholipase A2, group VII
1417288_at	Plekha2	pleckstrin homology domain-containing, family A member 2
1416178_a_at	Plekhb1	pleckstrin homology domain containing, family B member 1
1434180_at	Plekhc1	pleckstrin homology domain containing, family C member 1
1425468_at	Plp	proteolipid protein (myelin)
1448908_at	Ppap2b	phosphatidic acid phosphatase type 2B
1433691_at	Ppp1r3c	protein phosphatase 1, regulatory (inhibitor) subunit 3C
1423223_a_at	Prdx6	peroxiredoxin 6
1426246_at	Pros1	protein S (alpha)
1416211_a_at	Ptn	pleiotrophin
1427019_at	Ptprz1	protein tyrosine phosphatase, receptor type Z, polypeptide 1
1417481_at	Ramp1	receptor (calcitonin) activity modifying protein 1
1415850_at	Rasa3	RAS p21 protein activator 3
1423254_x_at	Rps27l	ribosomal protein S27-like
1460235_at	Scarb2	scavenger receptor class B, member 2
1415964_at	Scd1	stearoyl-Coenzyme A desaturase 1
1415823_at	Scd2	stearoyl-Coenzyme A desaturase 2
1420764_at	Scrg1	scrapie responsive gene 1
1449084_s_at	Sh3d19	SH3 domain protein D19
1417600_at	Slc15a2	solute carrier family 15 (H+/peptide transporter), member 2
1426340_at	Slc1a3	solute carrier family 1 (glial high affinity glutamate transporter), member 3
1434210_s_at	Slc25a26	solute carrier family 25 (mitochondrial & phosphate carrier), member 26
1430542_a_at	Slc25a5	solute carrier family 25, member 5
1452071_at	Slc4a4	solute carrier family 4 (anion exchanger), member 4
1448321_at	Smoc1	SPARC related modular calcium binding 1
1435192_at	Sox3	AV306664 RIKEN full-length enriched, 8 days embryo
1449264_at	Syt11	synaptotagmin 11
1449929_at	Tcte1l	t-complex-associated-testis-expressed 1-like
1423405_at	Timp4	tissue inhibitor of metalloproteinase 4
1416342_at	Tnc	tenascin C
1426641_at	Trib2	expressed sequence AW319517
1422694_at	Ttyh1	tweety homolog 1 (Drosophila)
1433916_at	Vamp3	vesicle-associated membrane protein 3

The changes in the expression profiles of these candidate markers across the different conditions are shown in Figure 5.11. The candidates have been classified based on their biological process and their molecular function according to the Gene Ontology Consortium classifications (Figure 5.12). A degree of overlap between categories in each classification is seen. The largest proportion of the candidates involved in similar biological processes fall under 'cellular physiological process', which includes cell proliferation and cell cycle regulation, 'cell communication', and 'cell differentiation' (only 6 out of the 63 genes fall into this category). From the classification according to molecular function, the largest proportion of gene products is categorized as having a role in binding, which includes protein and nucleic acid binding.

Figure 5.11 Graph of changes in the expression of candidate NSC markers. The expression of 535 NSC candidate genes is measured as arbitrary normalised intensity across the different target conditions (14.5 dpc dorsal telencephalon NSC-enriched neurospheres (DT+), 14.5 dpc dorsal telencephalon tissue (DTt), 14.5 dpc spinal cord NSC-enriched neurospheres (SC+) and 14.5 dpc spinal cord tissue (SCt). Values are shown after GC RMA normalisation of data generated on the GeneChip platform.

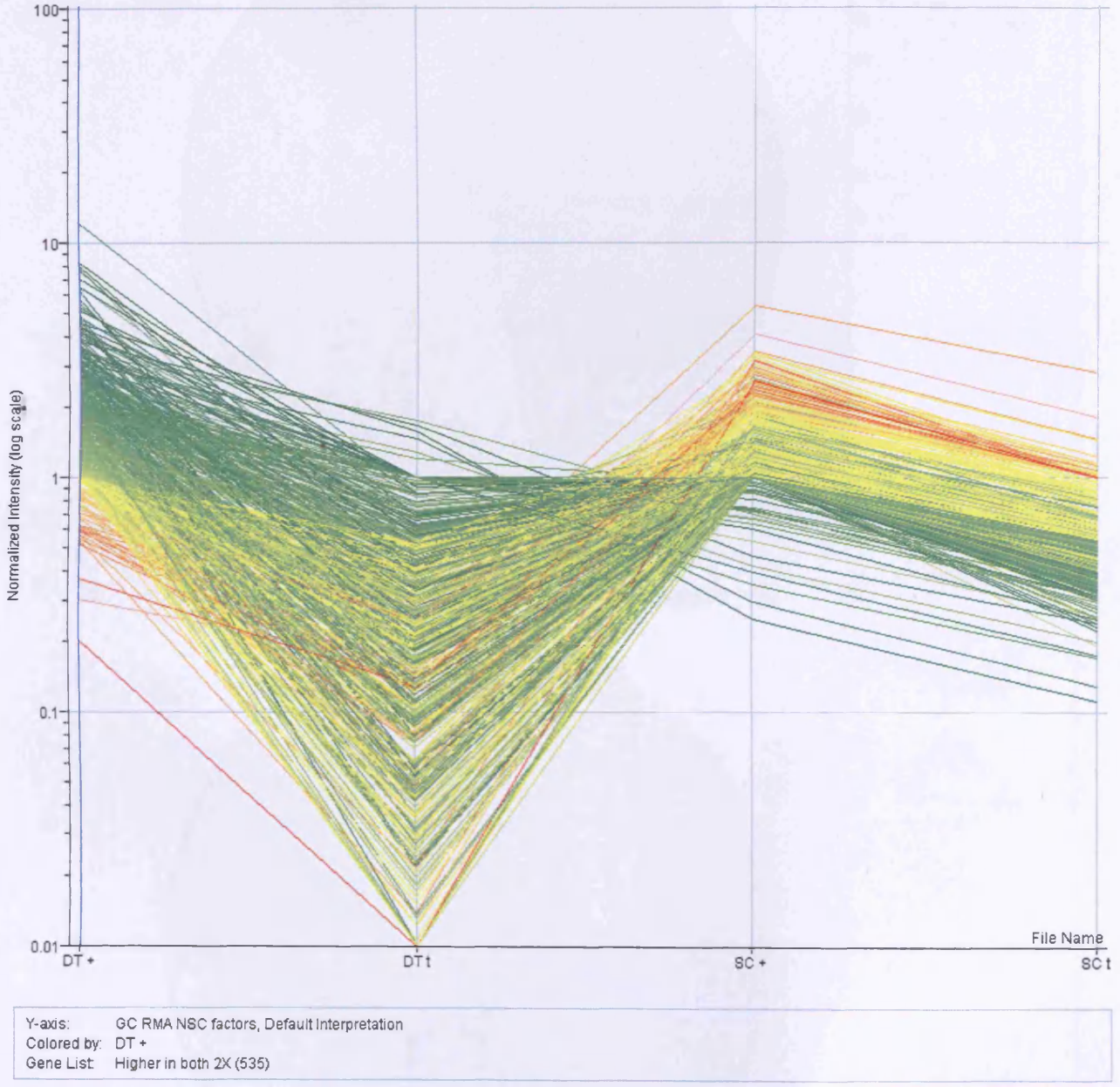
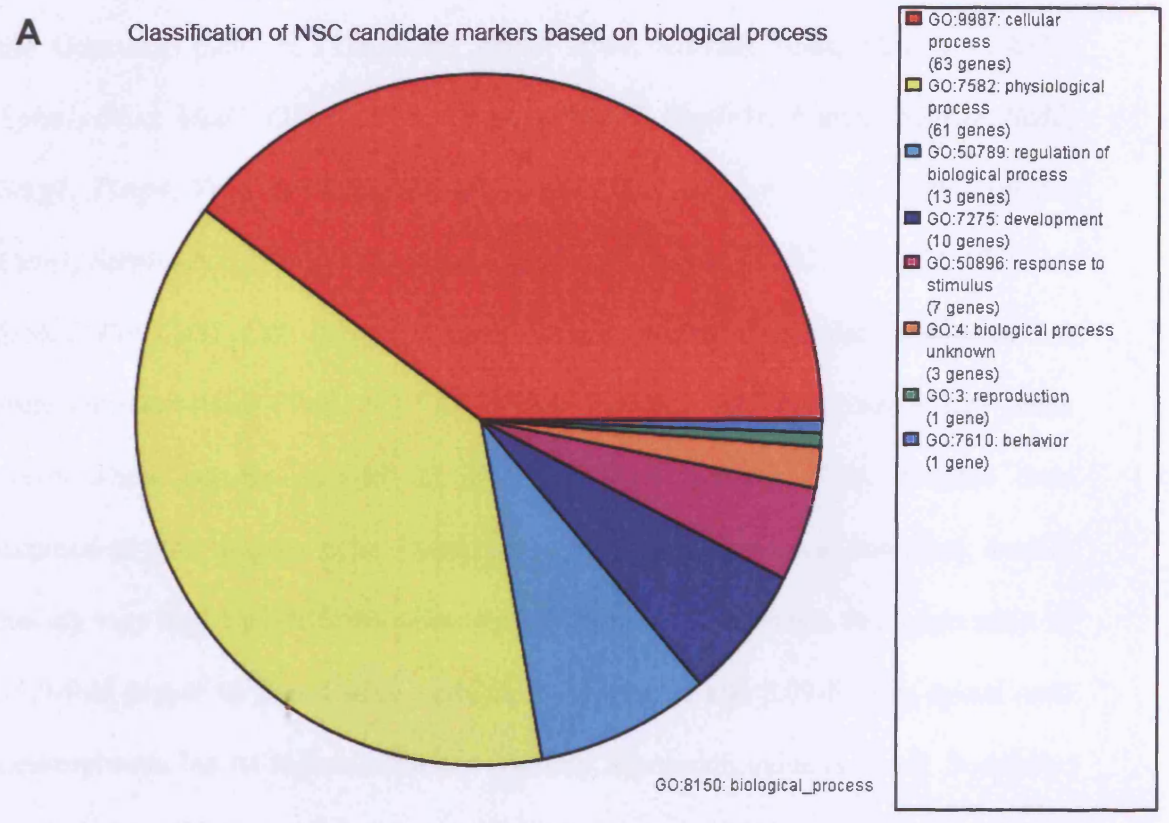
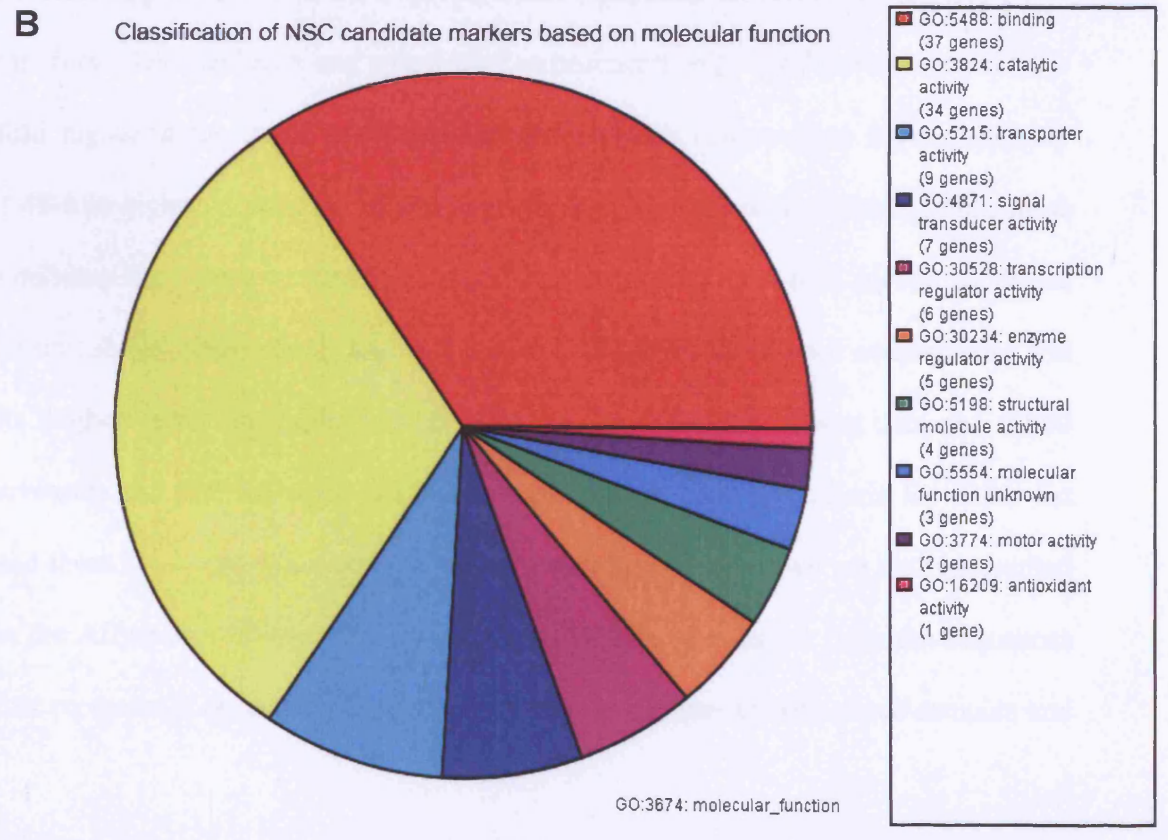


Figure 5.12 Pie charts of classification of candidate NSC markers by Gene Ontology
NSC candidate marker genes are classified into groups based on their biological process (A) and molecular function (B), as classified by the Gene Ontology Consortium. The colour key is on the side of each pie chart.





There is a significant level of overlap of the candidate genes between the two different microarray platforms. 20 out of the 46 short-listed genes (that have annotations) from the list generated on the spotted platform are present in the second list generated on the GeneChip platform (These are *Acadl*, *Apoe*, *Atp1b2*, *Bcan*, *Ccng1*, *Cdkn1a*, *Ephx1*, *Glud*, *Mod1*, *Olig1*, *Olig2*, *Pgam1*, *Pla2g7*, *Ppp1r3c*, *Ramp1*, *Scarb2*, *Scd2*, *Scrg1*, *Timp4*, *Tnc*). A further 21 genes from this list (*Lxn*, *Stat3*, *Gfap*, *Gpm6b*, *Cntn1*, *Serpine2*, *Gpi1*, *Slc38a3*, *Glul*, *Igfbp4*, *Odf2*, *Pgk1*, *Nab2*, *S100a13*, *Slc12a4*, *Slc6a1*, *Ptn*, *Cd81*, *Ckb*, *Ldh1*, *Qk*) are included in the list of potential candidates that were not short-listed (Table 9.2.1 of Appendix). There are three reasons why these genes where not be included in the main candidates list. First, because their expression level may be below 1,000 arbitrary units on the GeneChip array despite having very high fold-differences in expression e.g. *Slc12a4* has a fold expression of 15.9-fold higher in dorsal telencephalon neurospheres and 2.09-fold in spinal cord neurospheres, but its highest arbitrary intensity expression value is 714.8. Secondly, because they did not meet the imposed 2-fold expression difference criteria in both of the dorsal telencephalon and spinal cord experiments, e.g. *Stat3* is expressed 12.83-fold higher in the dorsal telencephalon neurospheres compared to tissue, but only 1.49-fold higher in spinal cord neurospheres compared to tissue. Finally, both these attributes may have occurred, e.g. *Nab2* is expressed 14.9-fold higher in dorsal telencephalon neurospheres and only 1.85-fold higher in spinal cord neurospheres and its highest arbitrary expression value is 463.15, which is lower than the 1,000 arbitrary unit filtering level. Only five genes did not meet the criteria for either list and these are *Mobp*, *Pfkm*, *Scn8a Cd63* and *Pde3b*. The latter two are not represented in the Affymetrix Mouse 430A chip. *Mobp* shows no expression from the sequences that represent it on the array, *Scn8a* is surprisingly higher in both tissue samples and

Pfkm is expressed higher in both neurosphere populations but under the 2-fold significance level. These differences may be products of the variation in sensitivity and accuracy between the two platforms. Overall, results from both platforms were comparable and this fact increases the reliability of the results, providing confidence in the list of candidate NSC markers.

5.2.3 Bioinformatic analysis of expression traits of NSCs

5.2.3.1 Genes revealing potential interaction of NSCs with endothelial cells in the niche

Microarray data sets generated from stem cell-enriched NSC cultures derived from the 14.5 dpc dorsal telencephalon and spinal cord compared to their corresponding tissues of origin were inspected for the expression of genes of likely importance in interactions with cells that may surround their *in vivo* environment such as endothelial cells, and for the expression of genes that may reveal responsiveness to factors controlling the NSC niche. After computational filtering to eliminate factors with low expression levels (less than 100 arbitrary units in all conditions), the expression of 12 genes of known potential importance to niche interactions was found to be higher in one or both of the NSC-enriched neurosphere populations compared to the more differentiated tissue of origin in both telencephalon and spinal cord populations (Table 5.7).

Table 5.7 List of genes with elevated expression in NSC-enriched neurospheres, important to niche interactions. The following genes, which are potentially involved in niche interactions, show an increase in expression levels in NSC-enriched neurospheres compared to their tissue of origin in microarray experiments on the GeneChip platform.

Gene	Fold-expression higher in D.T. NSC than D.T. tissue	Fold-expression higher in S.C. NSC than S.C. tissue
Vcam1	1.29	1.58
Gja1	3.76	1.44
Tjp1	1.72	1.41
Vegfa	7.96	1.88
Bmp4	6.5	1.84
Pdgfra	17.23	1.88
Bmpr1a	1.56	1.94
Smo	2.51	1.43
Itgav	7.3	3.3
Lamb2	1.79	1.54
Abca1	2.57	2.95
Abcg2	1.87	1.45

For the genes *Vegfb*, *Pdgfa*, *Fgfr1* and *Rarb*, an increase was seen in the neurosphere population compared to tissue only for the dorsal telencephalon and not the spinal cord. The endothelial-specific genes *Flt1*, *Flt4* and *Tek* (*Tie-2*) had low levels of expression in neurosphere populations (lower than 50 arbitrary units potentially indicating no expression). As described, previous reports indicate that NSCs can be stimulated to express the endothelial specific markers PECAM-1, VE-cadherin and

Flk-1 (Oishi et al. 2004). Endothelial cells present membrane-bound bFGF, which can induce proliferation of NSCs, also supporting the notion that NSCs external to the vessels are in direct contact with endothelial cells. Alvarez-Buylla and co-workers have provided evidence that NSCs of the adult SVZ are radial glial-derived astrocytes (Merkle et al. 2004). Consistent with this, these results suggest that NSCs residing outside of blood vessels may be in direct contact with endothelial cells. These cells could have an astrocytic character as astrocytes form tight junctions with endothelial cells to establish and maintain the blood-brain barrier. An increase in expression levels of *Tjp1* (tight junction protein 1) is seen from the microarray data to define the neural stem cell populations. NSC-enriched neurospheres were also shown to express *Stat3* higher than their tissue of origin (the role of STAT3 is examined in more detail in section 5.2.4). STAT3 has been shown to regulate proangiogenic *Vegf* expression in embryonic and adult endothelial cells (Hilfiker-Kleiner et al. 2005), which may be a requirement in the immediate vascular niche to the NSCs. ES cells and haematopoietic stem cells can be enriched through FACS sorting based on their Hoechst efflux properties. Dual wavelength analysis, by flow cytometry, of bone marrow cells labeled with Hoechst 33342 reveals a distinct side-population of cells that readily efflux Hoechst dye mediated by high expression of MDR (multi-drug resistance protein)-like transporters, such as ABC-binding cassette (ABC) transporters. Side-population (SP) cells make up 0.03% to 0.07% of total cells and 85% of these express long-term HSC lineage markers. The side-population cells have bona fide stem cell activity and cells with the highest dye efflux are the most potent in vivo (Goodell et al. 1997). Higher levels of ABC-transporter expression in the NSC-rich populations (*Abca1*, *Abcg2*) reinforces the concept that some NSCs may have dye-efflux properties and can appear as the side population in scatter profiles in blue

and red spectra after Hoechst labeling. A side population of NSCs through Hoechst 33342 staining has previously been reported (Hulspas and Quesenberry 2000). In this study it was reported that the side population is capable of neurosphere generation but that the dye-efflux phenotype cannot be used for neurosphere-generating NSC enrichment, as is the case with HSCs. A small side population is observed when this assay was performed on *Sox2*$^{\beta geo/+}$ neurospheres (data not shown) although these failed to form neurospheres in culture. This property may define cells that have no neurosphere-generating potential or perhaps more interestingly, quiescent neural stem cells that would not necessarily generate neurospheres in short-term culture and under the conditions used.

5.2.3.2 Genes revealing potential interaction of NSCs *with the basal lamina in the niche*

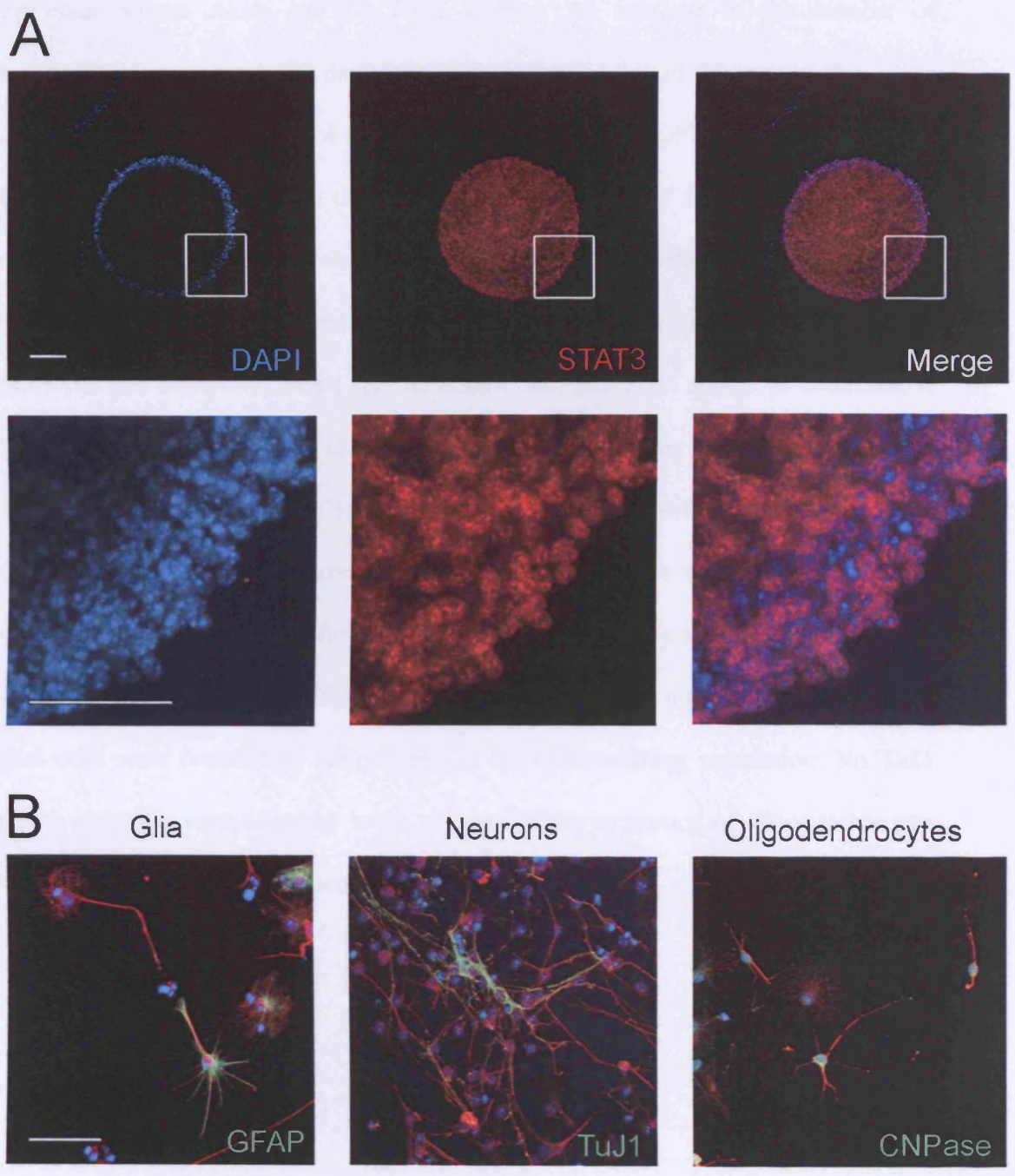
Direct contact between the basal lamina and NSCs has previously been proposed (Mercier et al. 2002) and our data supports this hypothesis. NSC-enriched neurosphere populations, compared to their tissue of origin, preferentially express some extracellular matrix components that have been shown to encourage the stem cell state (laminin genes, *Itgb1* and *Tnc*). The expression of these transcripts is found at higher levels in NSC-enriched neurospheres compared to non-enriched neurospheres, supporting the idea that NSCs contribute to the extracellular matrix that in turn also has a role in the stem cell niche. It has been reported that Tenascin-C is expressed by astrocytes in the adult CNS in regions of stem cell generation (Jankovski and Sotelo 1996) and by embryonic radial glia (Malatesta 2003). Since it has been demonstrated that both of these cell types have stem cell properties our findings through microarray analysis that *Tnc* is a potential candidate marker, highly expressed

in *in vitro* NSC-enriched cultures, is in support of the reported *in vivo* phenotype of NSCs.

5.2.4 NSC candidate marker analysis-the role of STAT3

From the short-listed candidates, I decided to investigate the role of *Stat3* in neural stem cells. The necessity for STAT3 to drive ES cell proliferation through LIF-mediated signalling is well defined. This led to the association that *Stat3* expression may be playing an important role in NSC proliferation. The localisation of STAT3 was investigated in proliferating stem cell-enriched $Sox2^{\beta geo/+}$ neurospheres as well as in differentiated cell types from these cultures. As expected from a predominantly undifferentiated population, STAT3 localises in the nucleus of the majority of cells in the neurosphere (Figure 5.13 A). In differentiated glia, neurons and mature oligodendrocytes, STAT3 localises mainly in the cytoplasm (Figure 5.13 B).

Figure 5.13 STAT3 in neural stem cell cultures derived from the 14.5 dpc dorsal telencephalon. STAT3 mainly localises in the nucleus of cells in neurospheres under proliferating conditions (A) and in the cytoplasm of differentiated cell types generated under differentiation conditions (B). STAT3 is in red in all panels, differentiation markers in green and nuclei in blue, stained with DAPI. Scalebars represent 100μm.



To test the effects of over-expressing and under-expressing *Stat3* in cultured neurospheres, dissociated single cells from primary neurospheres were electroporated with constitutive active and dominant negative forms of *Stat3* (kind gifts from Professor Shizuo Akira and Dr Toru Kondo). An increase in the number of neurospheres was observed 3 days following electroporation of the constitutive active construct, compared to control electroporations using the EGFP-N1 vector. Cultures that were electroporated with the dominant negative form of *Stat3* showed a severe decrease in the number of neurospheres and a large proportion of cell death. The results are summarized in Table 5.8. Chi-Square tests were performed between the Stat3-DN and EGFP-N1 results and the Stat3-C and EGFP-N1 results, to determine if neurospheres were generated at significantly lower and higher numbers respectively. In both cases, p has a value of less than 0.001, hence both distributions are significant (Table 5.9). Following electroporation with the constitutive active construct, cells were induced to differentiate for 2 days through attachment on an adherent matrix and withdrawal of the mitogens EGF and bFGF from the culture medium. GFAP positive glial cells were detected as the majority of the differentiating population. No TuJ1 positive neurons were observed in the culture and the proportion of oligodendrocytes was not assayed (data not shown).

Table 5.8 Effects of dominant negative inhibition of STAT3 on neurosphere formation. The EGFP-N1 vector was used as a transfection control.

Plasmid	Cells plated after electroporation	Neurospheres after 72h	% Formation
Stat3-DN	1.7×10^5	58	0.03%
Stat3-C	1.7×10^5	2231	1.3%
EGFP-N1 (control)	1.7×10^5	1603	0.9%

Table 5.9 Chi-Square test results for Stat3-DN compared to EGFP-N1 (A) and Stat3-C compared to EGFP-N1 (B).

A	Column 1	Column 2	Total	B	Column 1	Column 2	Total
Row 1	58	1603	1661		2231	1603	3834
Row 2	169942	168397	338339		167769	168397	336166
Total	170000	170000	340000		170000	170000	340000

A: Stat3-DN

Chi-square value = 1444.15627

B: Stat3-C

Chi-square value = 104.038075

In both cases, there is one degree of freedom and since Chi-square values are greater than 10.827 (see Chi-square Table 5.10 below) p is less than 0.001

Table 5.10 Chi-Square values for one degree of freedom (df).

For df = 1	P = 0.05	P = 0.01	P = 0.001
Chi-square value:	3.841	6.635	10.827

5.3 Discussion

5.3.1 SOX2 in dorsal telencephalon and spinal cord cultures

X-gal staining of $Sox2^{\beta geo/+}$ embryos (Figure 3.1) confirms that *Sox2* is expressed in stem cell-generating regions of the embryonic CNS such as the dorsal telencephalon and spinal cord. In the spinal cord, cells in the ependymal layer express *Sox2*, and as expected, the SOX2 protein localises in the nucleus (Figure 5.3). The low proportion of *Sox2* expressing cells in the spinal cord as opposed to the dorsal telencephalon can account for the reduced numbers of neurospheres generated from the dissociated spinal cord. This presents problems in comparing spinal cord primary neurosphere formation to primary dorsal telencephalon cultures but not when comparing spinal cord $Sox2^{\beta geo/+}$ stem cell-enriched population to other spinal cord NSC cultures (e.g. wild type). However, dorsal telencephalon neurospheres can be directly compared to spinal cord neurospheres with respect to secondary neurosphere formation. When culturing 11.5 dpc spinal cord NSCs, a higher percentage of cells are capable of neurosphere formation in culture (data not shown and Charlotte Scott, personal communication). At this earlier stage of spinal cord development a larger proportion of cells retain undifferentiated neuroepithelial properties and the *Sox2* expression domain is not as restricted as in 14.5 dpc embryos. Investigation of gene expression profiles of 14.5 dpc spinal cord and dorsal telencephalon reveals differences that can be attributed to differentiation of the more mature progenitors of the spinal cord. Genes with expression differences highlighted in this way have to be excluded from lists of potential neural stem cell marker genes, which should be similarly expressed in both populations. This does not negate the fact that expression of some genes uniquely in one population is a characteristic of NSCs from that region, although it does not help with the identification of candidates for pan-NSC markers.

5.3.2 NSC markers in dorsal telencephalon and spinal cord cultures

Marker analysis through immunofluorescence showed that spinal cord neurospheres express Nestin and SOX2 and that their expression patterns largely co-localise as with dorsal telencephalon neurosphere cultures (section 5.2.1.3). Spinal cord cultures do not express SSEA-1, however, which has been characterised as a forebrain stem cell marker (Capela and Temple 2000), eliminating this gene as a candidate pan-NSC marker. It has been reported that spinal cord cultures derived from 14.5 dpc embryos express *Ssea-1* (Terry Kelly, personal communication). In this case the researchers were using different culture conditions (mechanical as well as enzymatic dissociation for passaging every 3 days as well as addition of FCS in the medium) from those reported here, which may result in the acquisition of this marker. SSEA-1 is upregulated in CNS cultures in response to injury, which may be induced by mechanical dissociation (Professor Masato Nakafuku, personal communication). Other genes such as *Musashi1*, *Dlx2* and *Prom1* (Prominin-1, CD133) are also implicated in the literature as candidate NSCs markers (Kaneko et al. 2000; Lee et al. 2005). Microarray analysis could not provide information on *Musashi-1* and *Dlx2*, however *Prom1* is downregulated in dorsal telencephalon cultures compared to dorsal telencephalon tissue (3.8-fold difference) suggesting that the *Prom1 in vivo* expression domain may be including a larger proportion of cells that the total *in vitro* progenitors. In spinal cord cultures the levels of *Prom1* expression do not change between neurosphere cultures and tissue indicating that if *Prom1* expression is not unique to NSCs, this may be forebrain-specific. The prominin surface antigen is also expressed in other tissues and stem cell populations and its expression has been used

to isolate endothelial progenitors (Bussolati et al. 2005) and human HSCs (Miraglia et al. 1997; Yin et al. 1997).

5.3.3 Selection of candidate NSC markers

There are many factors to consider when identifying genes that play an important role in all undifferentiated NSC populations. Ideal outcomes from the experimental plan are if these genes are expressed with similar patterns in NSCs both *in vivo* and *in vitro*, if they are only expressed in proliferating progenitors or also in differentiating cells (perhaps at lower levels or with varying sub-cellular protein localisation) and if they are involved in the promotion of self-renewal or the suppression of differentiation. Microarray analysis has previously been used to characterise neural stem cell/ CNS progenitor populations (Geschwind et al. 2001; Ivanova et al. 2002; Ramalho-Santos et al. 2002; D'Amour and Gage 2003; Hu et al. 2004; Abramova et al. 2005). It is difficult to compare the data presented in this thesis with these studies, as the approaches taken are different. One study comparing proliferating and differentiating neurospheres from the postnatal SVZ (Karsten et al. 2003) yields results with similar trends to the NSC-enriched neurosphere populations derived from the 14.5 dpc dorsal telencephalon compared to their tissue of origin. Apart from differences in gene expression arising from different developmental stages, a custom-made cDNA array platform was used, with large probe set differences compared to the Affymetrix Mouse 430A GeneChip. The use of markers to enrich stem cell populations for microarray analysis has also been previously performed: the expression of *Sox2-Gfp* has been used to purify NSCs and to compare them to *Sox2* expressing ES cells in order to identify common 'stemness' genes (D'Amour and Gage 2003). Similarly, Abramova and co-workers used the expression of the surface

marker *LeX* (*Ssea-1*) to purify populations of neural stem and progenitor cells from the embryonic cortex (10 dpc, 13 dpc and 17 dpc) and the adult SVZ and compared these through microarray analysis to ES cells (Abramova et al. 2005). The use of LeX as a marker resulted in NSC enrichment but not purification and allowed for the identification of developmental stage-dependent gene expression differences within the LeX positive population. Further studies have compared NSCs to ES cells and to HSCs (Ivanova et al. 2002; Ramalho-Santos et al. 2002) in order to identify genes enriched in all three populations. These studies aim to characterise the similarities across stem cell populations, however NSCs may have properties that are essential for the stem cell state that are not shared by other populations of cultured stem cells. There is a low degree of overlap between the stem cell candidate gene lists of these studies (Abramova et al. 2005). Differences in statistical approaches can generate highly dissimilar results. This was demonstrated by Ivanova and co-workers through reanalysing their data using the statistical methods of Ramaldho-Santos and co-workers, resulting in a 65.7% overlap from an initial 10% overlap ((Ivanova et al. 2002) and reviewed in (Li and Akashi 2003)). The low overlap correlation may also highlight a potential pitfall in the methodologies: using stem cell populations from different ages or locations may lead to short-listing of genes specific to the temporal and spatial identity of the NSC populations as 'stemness' genes. The aim of the microarray experiments between NSC-enriched populations and their tissue of origin described in this chapter (section 5.2.2) is to identify a shortlist of candidate genes shared amongst all NSC populations irrespective of their developmental status and origin. The modular properties of the Affymetrix GeneChip system, allowing for more NSC populations to be incorporated in the study in the near future, can help

establish this list, which can then be used to compare 'stemness' properties across stem cell populations of different tissues.

The mixed composition of the neurospheres has to be also taken into account when interpreting expression-profiling results. Microarray analysis for neural stem cell cultures can only be performed using a mixed, impure stem cell population as starting material, except if the recently reported culture of a pure ES cell-derived radial-glial like stem cell population was used (Conti et al. 2005). Therefore, it may be impossible to attribute the expression of some genes to a particular cell type without further analysis, especially in cases where gene function is unknown and the expression has not been described in the literature. Pilot experiments on spotted oligonucleotide arrays, comparing neurosphere populations from the 14.5 dpc dorsal telencephalon and spinal cord to a reference sample, helped identify various pitfalls in the experimental design (section 5.2.2.1). Using a reference sample provides a distinct advantage for future experiments in that more neurosphere populations can be incorporated into the study through performing hybridisations against the reference sample. The reference sample can then be statistically eliminated from the results and aid in expression level normalisations, allowing the neurosphere samples to be compared to one-another. Through comparing neurosphere samples to a suitable reference of non-stem cell origin, genes involved in metabolism and other housekeeping genes, which will be common between all populations can be identified and excluded to allow lists of more interesting stem cell candidates common to neurosphere populations to emerge. However, comparing just two neurosphere populations via a reference sample was not sufficient to detect similarities unique to NSCs, as the list of genes expressed in common between spinal cord and dorsal

telencephalon neurospheres was very large. This experiment also helped to determine that the amplification of cDNA was not suitable for this study. After reverse transcription, each cDNA sample underwent a different number of amplification cycles, deemed optimal for the particular sample. However, the number of cycles may have been high enough to introduce error in the hybridisation probes. Gene expression profiles obtained via hybridisation with non-amplified cDNA probes were very dissimilar to those generated from amplified cDNA probes even if they were generated from the same starting total RNA. This suggests that errors have been introduced through the cDNA preparation, as all other steps were kept identical. Taking these issues into account, the experimental plan was altered. Only non-amplified cDNA was used for the remaining experiments of spotted arrays. Since G418-selected *Sox2*$^{\beta geo/+}$ neurospheres are enriched for proliferating stem cells (section 4.2.1.2) and more secondary neurospheres form from primary enriched neurospheres rather than from dissociated tissue (section 4.2.2), comparing enriched neurospheres to their tissue of origin should demonstrate stem cell-related genes being expressed at higher levels in the neurosphere populations. The tissue of origin of each neurosphere population had to specifically be used, as using whole CNS (as was used as a reference sample in the pilot experiments) would dilute region-specific differences in gene expression profiles that may be retained in the neurospheres. Thus, by overlapping lists of genes enriched for their expression in the neurospheres, which eliminates regional differences, a set of candidates expressed specifically in neurosphere populations, hence in NSCs, was established (Figure 5.8). This list can be refined further by incorporating data from neurosphere populations with further spatial and temporal differences compared to their tissue of origin. One potential disadvantage of this experimental plan is that 14.5 dpc neurospheres are not only

enriched for NSC, but also contain populations of committed progenitors as well as terminally differentiated cells (Figure 3.2 D-F). Although the proportion of differentiated cells to NSCs and progenitors may be low, the types and extent of terminal differentiation found in the neurospheres is less likely to be found in the tissue, as at 14.5 dpc the majority of differentiating cells are more likely to be committed to progenitor stages rather than be terminally differentiated. The array data confirms this concern, as within the list of candidate genes are factors that are known to mark differentiating lineages. This does not exclude the possibility that these factors are implicated in aspects of the stem cell state. For example, differentiated astrocytes are GFAP positive but radial glia of the embryonic and astrocytes of the adult brain, both characterised as NSCs, express GFAP (a subset of radial glia are an exception to this). The subsequent availability of the Affymetrix GeneChip arrays allowed for these experiments to be repeated on this platform, with the advantages of both higher specificity and a larger gene library (section 5.2.2.3). Similar results were obtained with this platform, although more stringent criteria than normal had to be applied to generate a manageably sized candidate list. If these criteria had not been applied, and the same criteria were used across both platforms, then 41 out of 43 candidates comprising the first list would have been shared between the two platforms, revealing the high level of reliability and reproducibility of the results.

5.3.4 Stat3 as a promising NSC candidate

Out of both lists of candidates, only one identifiable factor known to be associated with multipotent lineages was found. *Stat3* is one of the short-listed candidates from the spotted array data. This gene was not included in the candidate list from the GeneChip array as its expression in the spinal cord neurosphere populations was

higher compared to spinal cord tissue but not above the significance threshold. However, in dorsal telencephalon neurospheres on the GeneChip platform, it shows an increase of over 12-fold compared to the dorsal telencephalon tissue.

Recently, through chromatin immunoprecipitation and subsequent microarray analysis, it was demonstrated that SOX2 together with OCT4 and NANOG all bind active STAT3 in undifferentiated human ES cells (Boyer et al. 2005). The potential regulation of STAT3 by these transcription factors is also likely to take place in murine ES cells, where the expression of *Stat3* is essential for the regulation of self-renewal (Chambers and Smith 2004). The overlap in expression patterns of *Stat3* and *Sox2* in the developing CNS and NSC cultures derived from the CNS, make it increasingly likely that an association between these two genes may play an important role in the control of the stem cell state.

Binding of the gp130 receptor by IL-6 family cytokines results in the activation of Janus kinase type tyrosine kinases (JAKs), which phosphorylate STAT3. In retinal explants, it has been shown that STAT3 can be activated in response to extrinsic factors, including CNTF, LIF, FGF1, FGF2, EGF, IFNα and IFNγ (Zhang et al. 2005). Phosphorylated STAT3 dimerizes, translocates to the nucleus and binds to target genes acting as a transcriptional activator. In response to activated STAT3, retina precursor cells demonstrate an increase in proliferation (Zhang et al. 2005). STAT3 has been strongly implicated in the promotion of gliogenesis. Transfection of rat NSCs with a dominant negative form of STAT3 results in the promotion of neurogenesis and a significant decrease of GFAP positive cells (Gu et al. 2005). This is consistent with results presented in this chapter, where overexpression of *Stat3*

leads to an increase in GFAP positive glial cells. CNTF can activate the Jak-Stat signaling pathway and promote glial differentiation of rat cerebral cortical precursors through the activation of the *Gfap* promoter (Bonni et al. 1997; Rajan and McKay 1998). Also, oncostatin M (OSM) has been shown to contribute to the differentiation of astrocytes in mouse neuroepithelial cultures through downstream activation of *Stat3* (Yanagisawa et al. 1999).

There is evidence to suggest that *Hes1* and *Hes5* are associated with the proliferative NSC state (Ohtsuka et al. 2001). HES proteins, which are Notch effectors, promote STAT3 phosphorylation by associating with both JAK2 and STAT3 and they suppress the expression of proneural genes (Kageyama and Nakanishi 1997). In the presence of active Notch, STAT3 is activated while suppression of *Hes1* reduces the phosphorylation of STAT3 (Kamakura et al. 2004). The dominant negative inhibition of STAT3 has been shown to result in a decrease of *Hes5* mRNA levels (Gu et al. 2005). From the microarray experiments in section 5.2.2.3, designed to establish a list of potential NSC candidate markers, *Hes1* and *Hes5* mRNA levels where found to be higher in both neurosphere populations (*Hes1* by 3.3-fold in dorsal telencephalon cultures and 1.79-fold in spinal cord cultures; *Hes5* by 3.87-fold in dorsal telencephalon cultures and 2.32-fold in spinal cord cultures). *Hes5* was not identified as a potential candidate despite having a significant expression increase in the neurosphere populations, as the arbitrary intensity levels were lower than 1,000 units in 3 out of the 4 array conditions. These findings substantiate a potential correlation between HES proteins and an association with the proliferative NSC state, as enriched NSC populations express *Hes1* and *Hes5* at higher levels, as they do *Stat3*.

Our group, through the use of conditional Stat3 mutant mice, is investigating the role of STAT3 in neural stem cells further. Mice deficient for *Stat3* in the CNS can be used to derive neurospheres from the telencephalon, demonstrating that *Stat3* is not the sole factor required for neurosphere generation. However, we hypothesise that activated STAT3 does have a role in NSC proliferation and neurosphere generation *in vitro*, because dominant negative inhibition of *Stat3* in NSC cultures leads to a reduction in the number of secondary neurospheres, while transfection with a constitutive active form of *Stat3* leads to an increase. The effect of STAT3 may be masked by the expression of genes complementing its function. One likely candidate is *Sox2*. A reduction in neurosphere generation is seen following the introduction of a dominant negative form of *Sox2* (section 6.2.1.1). If SOX2 can activate targets of STAT3 such as *Id2*, independent of Stat3 expression (which has not been demonstrated), then a reduction in the levels of SOX2 in the absence of STAT3 may lead to a more severe phenotype than the sole absence of STAT3. This can be achieved through the generation of mice null for *Stat3* in the CNS (targeted deletion of *Stat3* leads to early embryonic lethality (Takeda et al. 1997)) and heterozygous for *Sox2βgeo*. This can be done through breeding $Stat3^{flox/flox}$ animals (Alonzi et al. 2001) with Nestin::Cre mice (Nestin driving *Cre* expression. Nestin is expressed in the CNS from 11.5 dpc), and then breeding conditionally null *Stat3* mutants ($Stat3^{ConΔ/ConΔ}$) with $Sox2^{βgeo/+}$ animals. The prediction would be that the numbers of neurospheres that could be derived from these mice would be reduced compared to mice mutant for *Stat3* but wild type for *Sox2*. However, we have to consider functional redundancy between the SOXB1 subgroup genes, as this may mask the severity of any phenotype. We have the means to generate embryos conditionally null for *Stat3*, heterozygous null for *Sox2*, homozygous null for *Sox1* and conditionally null for *Sox3*. The

generation of these mice, however, has not been successful as mice heterozygous for *Sox2* and conditionally null for *Sox3* die during embryonic stages and exhibit a truncation of the anterior CNS (Rizzoti et al, unpublished data). Therefore, other strategies need to be employed to overcome this problem, such as the conditional deletion of *Sox3* at later stages of development to overcome the lethality, in order to determine the roles of *Stat3* and *Sox2* in NSCs.

CHAPTER 6

Effects of lowering SOX2 levels in NSCs

6.1 Background

The ideal way to investigate the role of SOX2 in neural stem cells would be to characterise the stem cell populations in its absence. Since *Sox2* null mutants exhibit peri-implantation lethality, two further approaches are possible: 1) the generation of a conditional knockout, 2) lowering the levels of SOX2 through the *in vitro* post-transcriptional silencing of SOX2 by RNA interference (RNAi) or by dominant-negative strategies. In this study, the second, *in vitro* approach was chosen to investigate the role of SOX2 in cultures of embryonic neural stem cells and to identify candidate transcriptional targets of SOX2.

6.1.1 RNA interference

RNAi can be an effective means of achieving the down-regulation of protein products. It is the process by which epigenetic gene silencing effects occur, triggered by double-stranded RNA (dsRNA). 'RNAi' is the term used in animals, but a similar mechanism identified in plants is termed 'posttranscriptional gene silencing' (PTGS), and in the fungus *Neurospora crassa*, 'quelling'. The stability of eukaryotic genomes is often under threat from random integration of transposable elements and transgenes, as well as by the infection of viruses. In such situations, the invaders frequently produce dsRNA, during viral replication or by aberrant transcription from promoters located near transgene insertion sites. RNAi is thought to have developed as a cellular defence mechanism, responding to dsRNA, to rapidly process it into small fragments that direct the sequence-specific degradation of the single-stranded

mRNAs of the invading genes (Elbashir et al. 2001). The injection of antisense RNA in *Caenorhabditis elegans* embryos results in the extinction of homologous endogenous gene expression (Guo and Kemphues 1995). However, dsRNA molecules act as more potent inducers of interference than antisense single-stranded RNA (ssRNA) (Fire et al. 1998). This mechanism has been described amongst others in *Paramecium* (Ruiz et al. 1998), trypanosomes (Ngo et al. 1998), *Drosophila* (Kennerdell and Carthew 1998; Misquitta and Paterson 1999), and mammals (Wianny and Zernicka-Goetz 2000; Elbashir et al. 2001). The process of RNAi takes place predominantly in the cytoplasm, so pre-mRNA in the nucleus remains unaffected. Evidence suggests that either amplification of the signal or catalytic activity is involved, as very low levels of dsRNA are capable of triggering interference. In some species e.g. *C. elegans* and *Artemia*, it can act systemically-reaching every cell in the organism, and in some cases may even be transmitted to the offspring (Tabara et al. 1998). Critical characteristics of siRNA duplexes for the silencing function are 5' phosphate and 3' hydroxyl termini on each strand (Zamore et al. 2000) and 2-nucleotide 3' overhangs (Elbashir et al. 2001). We know that it is RNA and not DNA that is targeted, as sequences designed against introns are not effective (Montgomery and Fire 1998). dsRNA structures are cleaved to ~21bp fragments directly through the action of an RNase type III nuclease termed Dicer (Bernstein et al. 2001). It is thought that the antisense siRNAs are incorporated in a larger RNase complex (the RNA-induced silencing complex (RISC)), which targets homologous mRNA leading to their degradation (Hammond et al. 2000). Such ribonucleotide fragments can be synthesised *in vitro* specific to a target gene sequence and introduced in mammalian cells. Their cleavage by Dicer or RNase III into small interfering RNAs (siRNAs) results in the post-transcriptional silencing of

the target gene. siRNAs bind with target mRNA and interact with proteins to form the dsRNA-induced silencing complex (RISC).

6.1.2 Construct design and sequence selection

Several methods of delivery have been described to establish RNA-mediated silencing in mammalian cell culture. The most efficient system at the time that this study commenced was through vector delivery. The pSUPER vector was used, which contains a sequence that forms a hairpin, allowing Dicer to excise the dsRNA that forms the hairpin arm (Brummelkamp et al. 2002). Sequence selection had to be vigorous however, with the efficiency of finding a successful sequence being 1 in 4. Promoters typically used to drive the expression cassette are either U6 or H1, both members of the type III polymerase promoters (Medina and Joshi 1999). They are located upstream of the transcribed region, eliminating the need for promoter sequence to be included in the siRNA design. Inserts shorter than 400 nucleotides can be transcribed, which is ideal for the expression of siRNA hairpin cassettes. The delivery of siRNA sequences into a cell instead of long dsRNA is advantageous, as siRNA does not elicit a non-target dependent interferon response that is potentially cytotoxic. Long dsRNA activates the cellular enzymes Protein Kinase R and 2'-5'oligoadenylate synthetase related to the interferon system.

This section aims to demonstrate the effects of lowering the levels of SOX2 in NSCs as well as to identify likely downstream targets of SOX2 by identifying changes in the expression profiles in lowered SOX2 conditions.

6.2 Results

6.2.1 Reducing the levels of SOX2 in NSCs

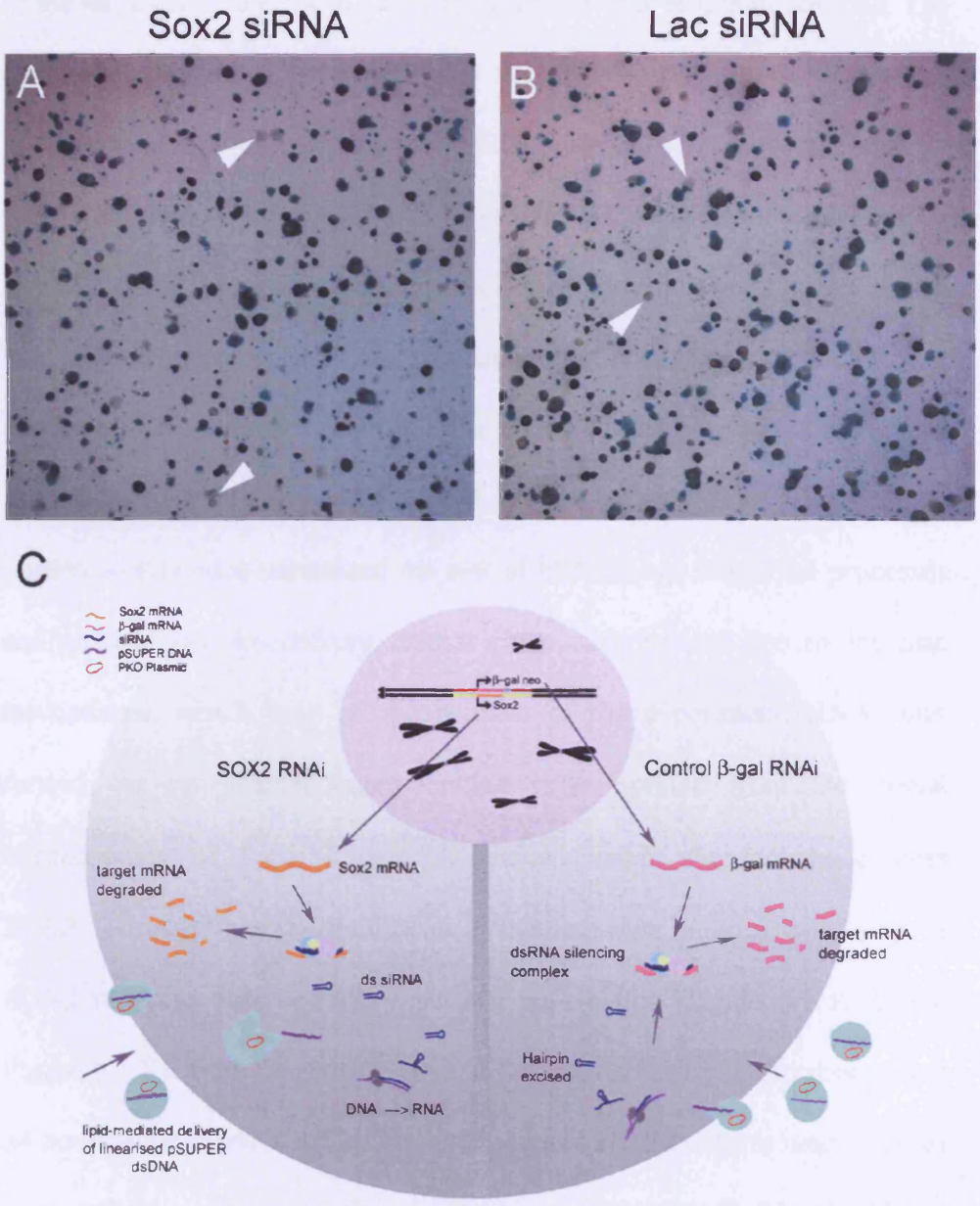
6.2.1.1 Electroporation of dominant negative constructs

To determine the effects of lowering SOX2 levels on neurosphere formation, single cells from dissociated primary neurospheres that had previously been selected for *Sox2* expression, were electroporated using dominant negative constructs of *Sox2*. As a control, the EGFP-N1 vector was used in parallel electroporations on an aliquot of the same cells. Two different dominant negative constructs were used: A truncated form of *Sox2* lacking the HMG box sequence, cloned into the pCMV/*myc*/nuc vector (Dr Karine Rizotti) and a fusion of *Sox2* with the engrailed repressor domain, cloned into the pCI-neo vector (Dr Ariel Avilion). Both of these constructs have been previously used to demonstrate a dominant negative effect on SOX2 in ES cells (Dr Robin Lovell-Badge, unpublished data). After electroporation of the *Sox2*-engrailed repressor domain fusion construct, no effect in the reduction of neurosphere numbers was observed. However 36 hours after electroporation with the *Sox2* HMG-truncation construct, a 6-fold reduction in neurosphere numbers was seen compared to the control electroporation (control EGFP-N1 vector 188 neurospheres, where standard error from the mean is ± 4.25, *Sox2* HMG-truncation construct 32 neurospheres, where standard error from the mean is ± 4.84. Experiments were performed in triplicate). The surviving neurospheres looked like typical proliferating cultures, without exhibiting elevated adherence to the culture dish surface or signs of grossly differentiated cellular morphology.

6.2.1.2 RNAi by delivery of a hairpin vector

Sequences from the *Sox2* and control *LacZ* genes were selected based on established methods as being good predicted targets for RNAi (Brummelkamp et al. 2002). Four sequences for each gene (two from the N-terminal region of each gene and two from the HMG box for *Sox2* and C-terminal region for *LacZ*) were selected and each was cloned into the EcoRI/XhoI sites of the pSUPER vector. The post-transcriptional knockdown activity of each siRNA sequence was assessed in $Sox2^{\beta geo/+}$ ES cells. Downregulation of *Sox2* in the embryo by targeted deletion causes a failure to isolate ES cells from the ICM due to their rapid differentiation (Avilion et al. 2003). Hence, differentiation was chosen as an indicator that the *Sox2* siRNA constructs were having an effect. After ampicilin selection, X-gal staining of $Sox2^{\beta geo/+}$ ES cells transfected with control LacZ siRNA constructs was used as an indicator of RNA interference. As expected, white colonies were observed in cells transfected with LacZ constructs (Figure 6.1 B), but in cells transfected with *Sox2* constructs, which should remain blue, white colonies were also observed (Figure 6.1 A). High levels of differentiation into typical ES cell derivatives were seen under both conditions. To investigate the presence of white colonies in the ES cells transfected with the *Sox2* construct, as a control, non-transfected $Sox2^{\beta geo/+}$ ES cells were subjected to X-gal staining. This assay revealed that a proportion of white colonies were present in the stock ES $Sox2^{\beta geo/+}$ line. The latter results probably reflect a deletion of an enhancer sequence 3' to the *Sox2* open reading frame (ORF), which compromises early expression (Avilion et al. 2003). This result, together with high levels of differentiation using construct against both genes, prevented the validation of the selected sequences. Transfection of the same constructs in neural stem cells showed no significant change in neurosphere numbers.

Figure 6.1 RNAi in *Sox2*$^{\beta geo/+}$ ES cells against SOX2 and β-galactosidase. siRNA sequences used for silencing of SOX2 and β-galactosidase at the post-transcriptional level were tested in ES cells prior to delivery in NSCs. No effects were seen using Sox2 siRNA sequences (A). White colonies were seen after transfection with Lac siRNA sequences against β-galactosidase and subsequent X-gal staining (white arrowheads B), but these were also present in ES cells transfected with Sox2 siRNA (white arrowheads A). The mechanisms underlying this experiment are illustrated in cartoon C.



6.2.1.3 RNAi by delivery of siRNA sequences

To increase the likelihood of selecting a good target sequence leading to protein knockdown, siRNA sequences were generated *in vitro*, prior to delivery. A region of the *Sox2* ORF exhibiting low homology with other B1 group genes was transcribed and recombinant Dicer was used for cleavage of the sequence into random 19-21 bp fragments, the length of siRNAs. The entire *Egfp* sequence from the Egfp-N1 vector (800bp) was used as a control to assess transfection efficiency by EGFP protein knockdown in *Egfp*-expressing cells. Determining EGFP intensity via fluorescence microscopy enabled quick progress assessment during the experiment, offering an advantage over using LacZ control sequences. Through regular monitoring of EGFP, the maximum effect of protein knockdown was determined at 48 hours (Figure 6.2 E, F and Figure 6.3 C). The use of a non-vertebrate protein as a control minimized the risk of interference with vital processes and ensured that knockdown effects observed were not due to intrinsic mechanisms, which may be independent of the experiment. RNAi was carried out on neurosphere-generating cells isolated from the dorsal telencephalon of $Sox2^{\beta geo/+};B5^{EGFP/+}$ mice, which ubiquitously express EGFP. Through immunofluorescence staining, maximum knockdown of SOX2 was also observed 48 hours post transfection (Figure 6.2 K, L and Figure 6.3 E). Cells were initially cultured as neurospheres and dissociated 24 hours prior to transfection. Their subsequent ability to form neurospheres after reduction in the levels of SOX2 was investigated. No significant decrease in neurosphere number or size was observed in cells that had undergone RNAi for SOX2 compared to the EGFP control.

Figure 6.2 RNAi against SOX2 and EGFP in NSCs (1). After transfection with siRNA sequences against EGFP, NSC cultures derived from R26-EYFP fluorescent mice show very little EGFP fluorescence (E, F) compared to untransfected controls (B, C). RNAi against SOX2 reveals lower levels of the protein as seen by immunofluorescence staining 48h post-transfection (K, L) compared to untransfected controls (H, I). Scalebar is 100μm (applies to all panels).

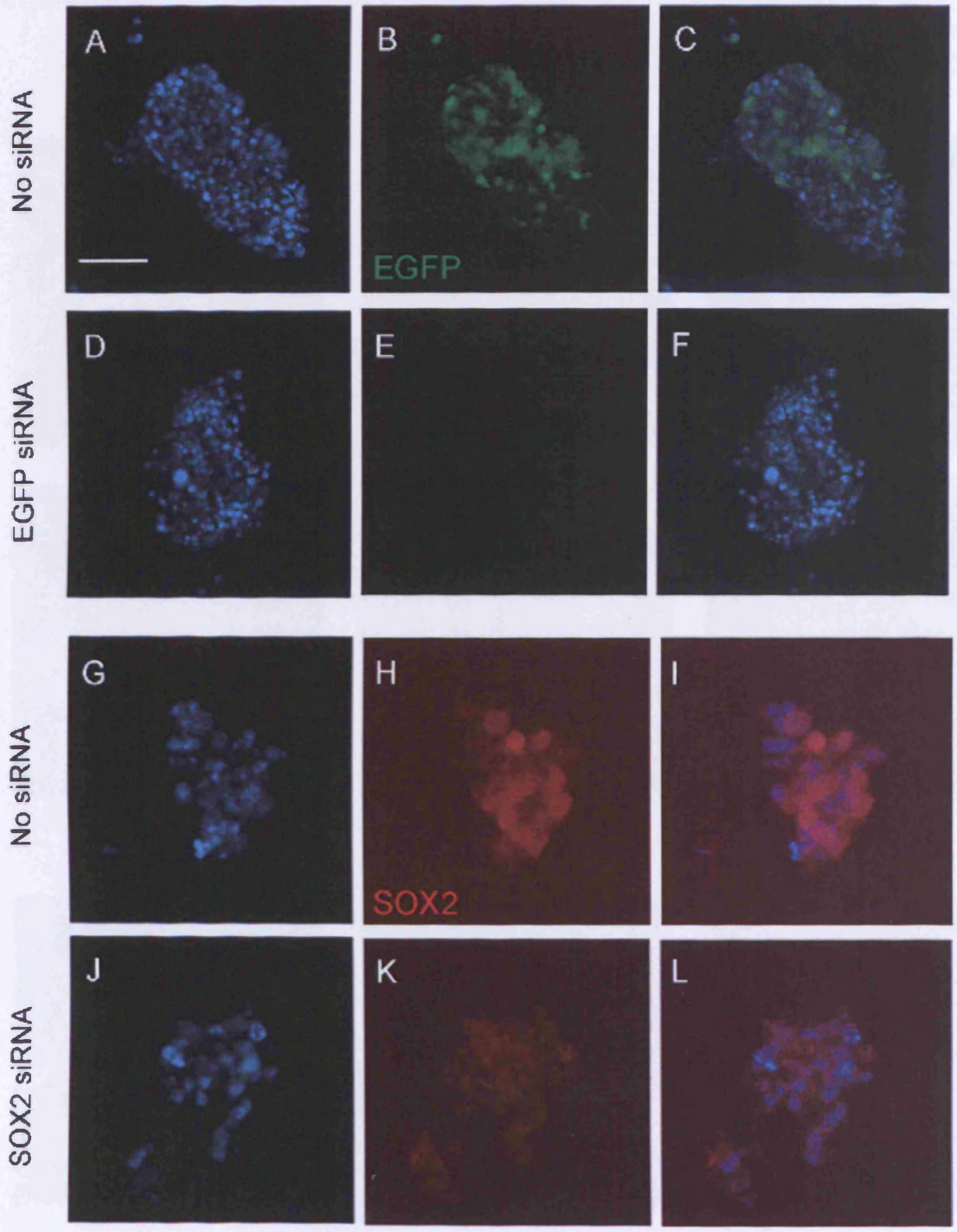
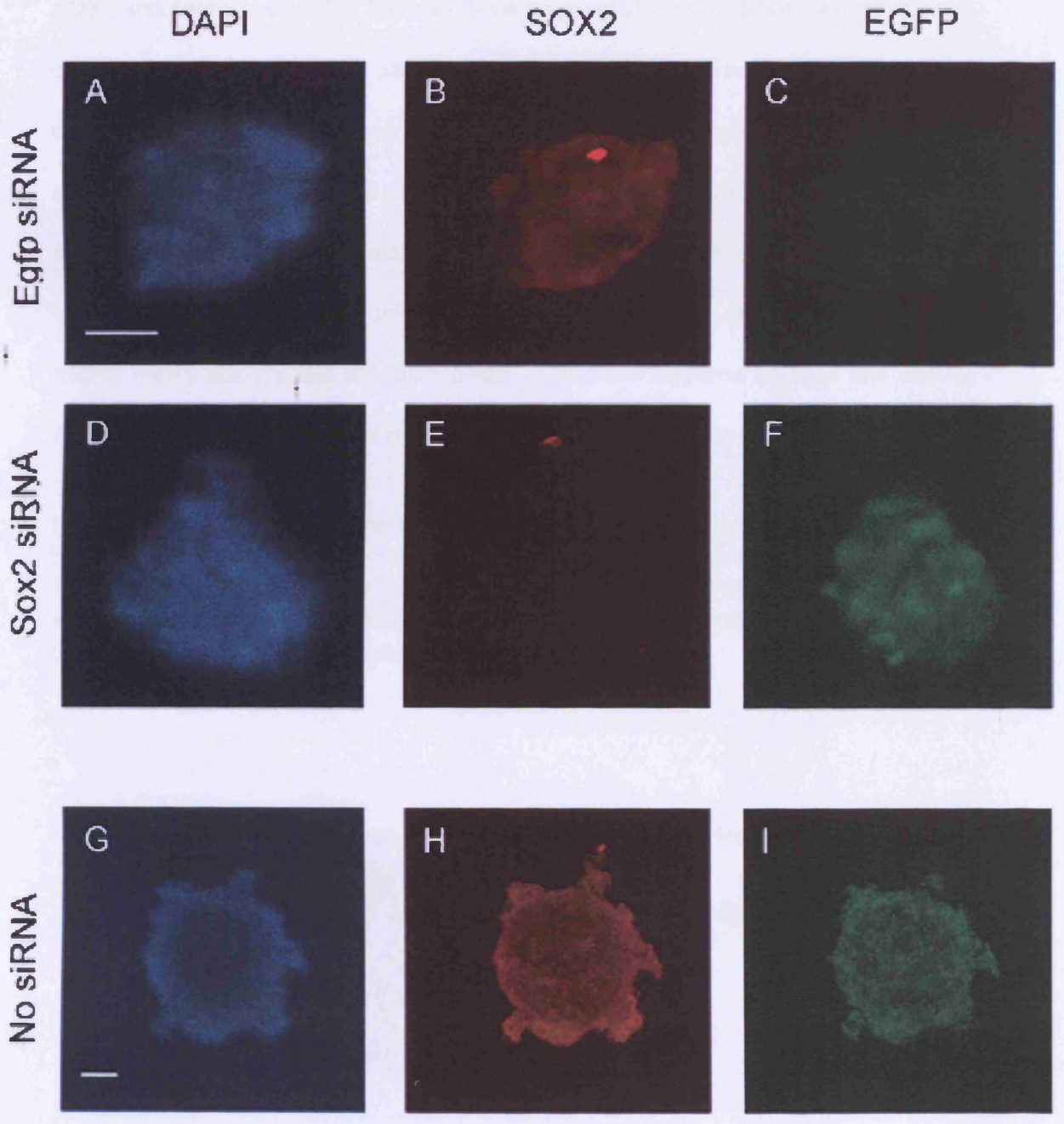


Figure 6.3 RNAi against SOX2 and EGFP in NSCs (2). Independent example of an RNAi experiment identical to the one in Figure 2.9. The quality of this image is poor (due to a failing confocal laser) but it is important to show that the levels of SOX2 remain unaffected in cells treated for RNAi against EGFP (A to C) and the levels of EGFP remain unaffected in cells treated for RNAi against SOX2 (D to F). Control transfections without siRNA sequences show that the protein levels for both SOX2 and EGFP do not change (G to I). Scalebars represent 100μm. Scalebar in A applies in panels A to F and scalebar in G applies in panels G to I.



6.2.2 Microarray analysis on neurospheres that have been subjected to RNA interference against SOX2

6.2.2.1 Determining SOX2 target candidates through fold-change in expression levels

The gene expression profiles of neurospheres that have undergone RNAi against SOX2 and neurospheres that have not been transfected with an RNAi sequence were compared through microarray analysis on the Mouse 430A GeneChip platform. The experimental outline is shown in Figure 6.4 and the parameters for the experiment are summarised in Table 6.1. The control used was a population treated in an identical fashion as the RNAi-treated sample except that no siRNA sequence was used. This control offers the following advantage over a control that employs a population treated with a non-specific sequence: a likely interferon response or other side effects in the RNAi treated population can be detected in the microarray comparisons.

Figure 6.4 Microarray Experiment Outline: microarray analysis after RNAi. Microarray analysis was performed on NSCs derived from the dorsal telencephalon of 14.5 dpc *Sox2*$^{\beta geo/+}$ mice after they had undergone RNA interference against SOX2, and compared to untreated NSCs. Both populations had previously been selected for the expression of *Sox2βgeo* to provide NSC enrichment.

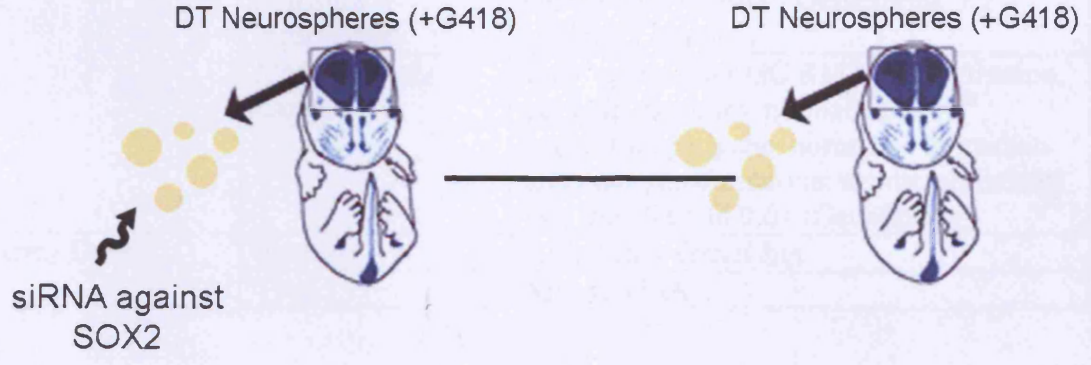
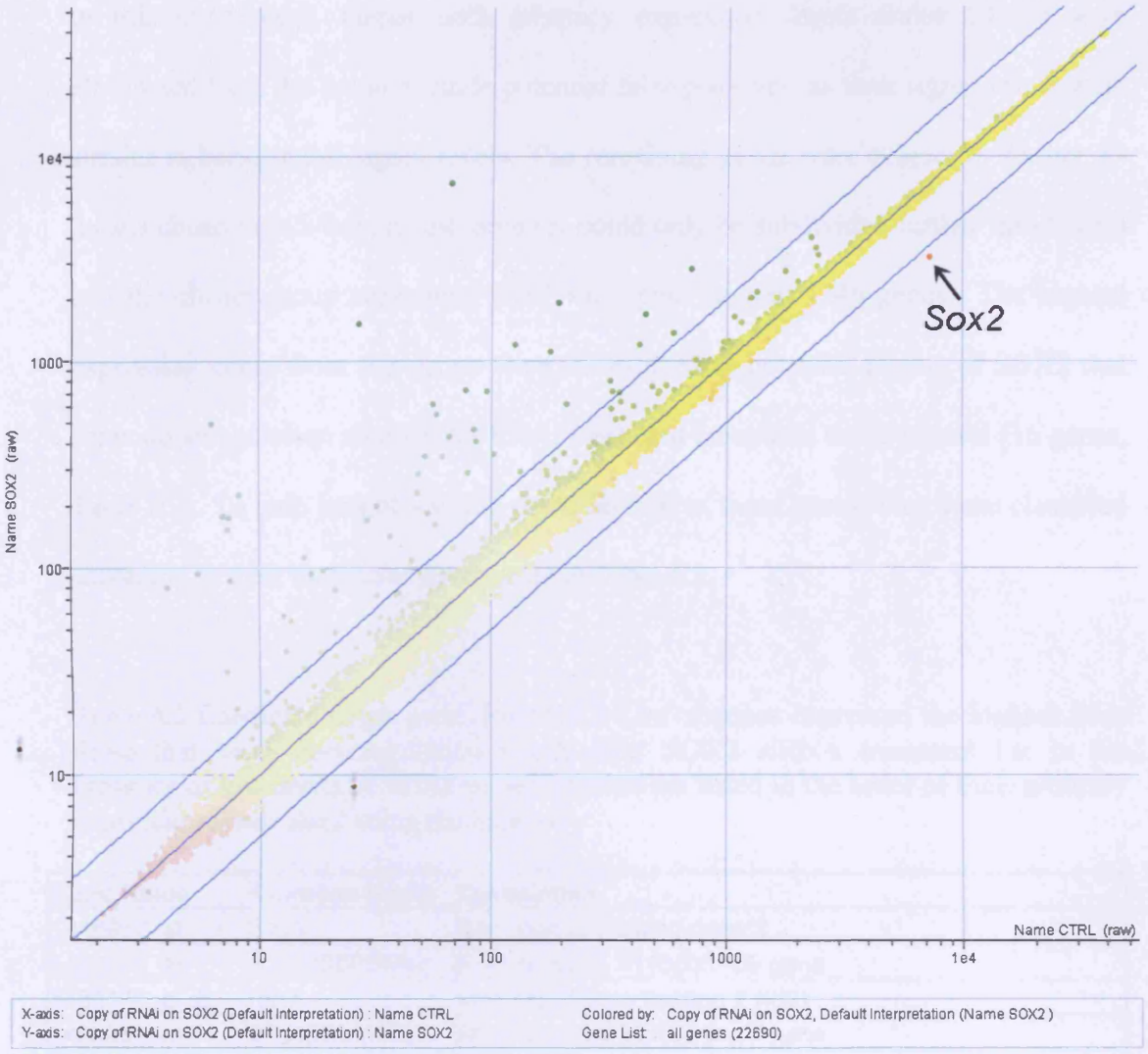


Table 6.1 Determining potential targets of SOX2 through RNAi interference

Experiment Design	Type	Normal neurospheres v Treated neurospheres (RNAi)
	Experimental Factors	48h treatment with siRNA (against SOX2)
	Number of Hybridisations	2
	Reference Sample	None
	Hybridisation Design	RNAi treated (SOX2)- Control (No siRNA), see diagram
	Quality Control	2 replicates
Samples	Origin	14.5 dpc murine dorsal telencephalon of *Sox2*$^{\beta geo/+}$ mice (MF1 outbred background)
	Manipulation	Mechanical tissue dissociation, culture in NPM as neurospheres. Selected with neomycin (G418) for 10 days, dissociated into single cells on day 10 and transfected on day 11. Lipid based transfection (sIMPORTER (Ambion)) with siRNA sequences (or no siRNA) for 48 hours
	Sample Preparation	Extraction of total RNA using RNeasy Midi kit (Qiagen). DNase treatment using DNA-free (Ambion)
	Labeling Protocol	Biotin labeling
Hybridisation Procedures	Parameters	Affymetrix 'Eukaryotic Sample and Array Processing' guide followed for all protocols (see Methods)
Measurement Specifications	Hardware Information	GenAmp PCR System 9700 (Applied Biosystems), GeneChip Hybridization Oven 640, GeneChip Fluidics Station 450 and GeneChip Scanner (all Affymetrix). NanoDrop ND-1000 spectrophotometer (Labtek) and BioAnalyzer 2100 (Agilent Technologies) for RNA quality control and quantitation
	Software Information	GCOS (Affymetrix), GeneSpring 7.2 (Silicon Genetics)
	Measurements used	Raw values after GC RMA Normalisation, per chip, per chip: normalise to 50th parcentile, per gene: normalise to median and data transformation: set measurements less than 0.01 to 0.01 (GeneSpring)
Array Design	Platform	Affymetrix GeneChip
	Type	Mouse 430A

A 1.5-fold or higher level of expression in the control slides compared to SOX2 RNAi treated cells (i.e. 1.5-fold lower levels in SOX2 RNAi treated cells) was imposed as a cut-off to determine potential targets of SOX2 whose expression would be downregulated when levels of the SOX2 protein are low (14 genes short-listed). Genes that were short-listed and did not exhibit the same pattern in all replicates were eliminated (6 genes eliminated, 8 genes remaining). The expression levels of the short-listed genes were compared to those of genes known to be absent from neurosphere populations as determined from previous array studies. Genes that had similar arbitrary levels of expression to these negative controls were eliminated as these where showing expression differences of over 1.5-fold due to their differences with the expression of background signal that can have high fold changes when the numbers are very low. After elimination of these unreliable genes as, only one gene remained as having significantly downregulated expression in SOX2 RNAi treated cells and that gene is *Sox2*. This was also the only gene to display higher than a two-fold difference in levels of expression between the two conditions (2.26-fold).

Figure 6.5 Scatter plot of microarray results comparing cells that have and have not undergone RNAi against SOX2. Raw values of data from the sample of cells undergone RNAi against SOX2 (y-axis) were plotted against raw data from the sample of cells that were transfected without any siRNA sequences (x-axis). The line of best fit intercepting zero indicates a relative expression value of 1 i.e. genes that remain unchanged between the two conditions are plotted along this line. Blue lines on either side indicate 2-fold significant changes in relative gene expression favouring either condition. Dots represent individual genes and are coloured by expression where yellow marks genes with similar expression levels between the two samples, green marks genes that are expressed higher in the SOX2 RNAi-treated sample (as is the case for interferon response-related genes) and orange/red marks genes that are expressed higher in the control sample. As indicated, only SOX2 expression is over the 2-fold significance cut-off in the control sample indicating lower numbers of mRNA transcripts with siRNA treatment. The strength of the colours is relative to the arbitrary expression levels of individual genes.



6.2.2.2 Determining SOX2 target candidates through clustering analysis

An alternative approach was applied to identify potential targets of SOX2 that have been affected in SOX2 RNAi treated cells. All genes with an expression difference higher that one-fold in the control slides (11,545 genes), i.e. have higher levels in the control than in the SOX2 RNAi-treated, were subject to 10-way *K*-means clustering analysis. The cluster set containing *Sox2* was isolated (1217 genes) as it included genes that had the most similar expression pattern to *Sox2* across all array chips used

in this experiment. Genes with arbitrary expression levels under 100.00 were eliminated from the list to exclude potential false positives, as their signal levels were similar to background signal levels. The remaining genes were subject to further *K*-means clustering (5-way, as the gene set could only be subdivided further into 5 sets) and the cluster group containing *Sox2* was again isolated (246 genes). The highest expressing genes from this group were short-listed as potential targets of SOX2 that show downregulation after SOX2 RNAi treatment compared to the control (16 genes, Table 6.2). To gain insight into the potential role of these genes, they were classified according to their molecular function as in Table 6.3.

Table 6.2 Candidate target genes for SOX2. List of genes expressed the highest from those that show downregulation trends after SOX2 siRNA treatment i.e. in the presence of low levels of SOX2 protein. Genes are listed in the order of their arbitrary expression levels *Sox2* being the highest.

Gene Name	Common Name	Description
1416967_at	Sox2	SRY-box containing gene 2
1426679_at	3110006P09Rik	RIKEN cDNA 3110006P09 gene
1435176_a_at	Idb2	inhibitor of DNA binding 2 (Id2)
1428080_at	2610528A17Rik	RIKEN cDNA 2610528A17 gene
1418816_at	2810405I11Rik	RIKEN cDNA 2810405I11 gene
1424586_at	AF424697	DNA sequence AF424697
1424571_at	Ddx46	DEAD (Asp-Glu-Ala-Asp) box polypeptide 46
1423921_at	C77668	expressed sequence C77668
1428612_at	Apg7l	autophagy 7-like (S. cerevisiae)
1448985_at	Dusp22	dual specificity phosphatase 22
1418088_a_at	Stx8	syntaxin 8
1421177_at	9030625G08Rik	RIKEN cDNA 9030625G08 gene
1425090_s_at	Kcnc4	potassium voltage gated channel, Shaw-related subfamily, member 4
1426607_at	3110070M22Rik	Transcribed sequences
1450295_s_at	D7Ertd458e	DNA segment, Chr 7, ERATO Doi 458, expressed
1422647_at	Ring1	ring finger protein 1

Table 6.3 Gene Ontology classifications of candidate target genes of SOX2. Genes, as listed in Table 2.3, are classified according to molecular function.

Common Name	Gene Ontology Classification
Sox2	Cell fate specification: DNA binding, transcription factor activity
3110006P09Rik	--
Idb2	Development
2610528A17Rik	Metabolism: catalytic activity
2810405I11Rik	--
AF424697	--
Ddx46	Helicase activity
C77668	--
Apg7l	Ubiquitin activating enzyme activity
Dusp22	Phosphatase activity, positive regulation of JNK cascade
Stx8	Transporter activity
9030625G08Rik	DNA binding, nucleic acid binding
Kcnc4	Potassium ion transport: cation channel activity
3110070M22Rik	--
D7Ertd458e	Receptor activity: cell-cell adhesion, cell migration; signal transducer
Ring1	Chromatin modification, transcriptional repressor activity

6.2.2.3 Further analysis of candidate target genes- Id2

From the list of candidate target genes of SOX2, *Idb2* (*Id2*) was the most interesting gene for further study, as it is highly expressed in neurospheres and it has been previously implicated in the maintenance of the undifferentiated state and the suppression of neurogenesis (discussed in section 6.3.3). Analysis of the promoter sequence for *Id2* did not reveal any putative binding sites of the SOX consensus binding motif (A/T)(A/T)CAA(A/T)G. Computational analysis 10Kb further upstream of this promoter exposed five potential binding sites for SOX2 (Table 6.4). These sites may be located within potential *cis*-regulatory element sequences. These sites however, are not clustered in one location, which would suggest that the region might be a potential enhancer with multiple binding sites. In the *Id2* promoter sequence, there are two sites of sequence ACCAATG –'C' in bold font differs from the SOX consensus binding sequence, but this sequence may also have the potential to act as SOX2 binding sites (Table 6.4). One of these two sites in the promoter region

overlaps with a functional binding site for C/EBPβ (Karaya et al. 2005). The location of these sites in the *Id2* promoter encourages the hypothesis that *Id2* may be a direct target of SOX2 involved in the maintenance of an undifferentiated state in neural stem cells.

Table 6.4 Potential binding sites for SOX2 upstream of the murine *Id2* gene. Sequences in black are putative binding sites. Sequences in red indicate potential sites in the *Id2* promoter sequence.

Sequence	Site location from transcriptional start
AACAATG	-9941 to –9934
AACAATG	-8858 to –8851
TACAAAG	-8104 to –8097
TTCAAAG	-3325 to –3315
AACAATG	-5386 to –5379
ACCAATG	-126 to –120
ACCAATG	-82 to –76

6.3 Discussion

6.3.1 Dominant negative approaches in neurosphere cultures

RNAi interference against *Sox2* in murine dissociated neurospheres via transfection siRNA sequences in pSUPER did not reveal an apparent phenotype in the cells during the 48-hour period post-transfection (section 6.2.1.2). When a dominant negative form of SOX2 was electroporated into dissociated primary neurosphere cells, a reduction in the number of secondary neurospheres generate was observed (section 6.2.1.1). In this dominant negative approach, the construct employed a truncation in the HMG box, which makes it likely not to be specific for SOX2 and to also work against SOX1 and SOX3. Electroporation using an engrailed repressor domain fusion to SOX2 acting as dominant negative did not reveal a reduction in neurosphere numbers. This construct did function to reduce neuropoiesis in a *Xenopus* embryo assay (Karine Rizzoti, personal communication) but perhaps it was too short-lived in the transfected

neurospheres or too specific against *Sox2* (if functional redundancy is an issue) to give a noticeable reduction in secondary neurosphere generation. To assess this, subsequent detailed differentiation assays of the electroporated cells are required. It is likely that functional redundancy between the B1 subgroup genes reduces the severity of the phenotype seen through the engrailed repressor domain construct. There are data to suggest that SOX1 and SOX3 are not required for the neural stem cell state (unpublished data). This does not mean that they would not be able to function in the place of SOX2 when this is absent. To determine if functional redundancy is taking place, the engrailed repressor domain-SOX2 fusion construct can be introduced in *Sox1* null, *Sox3* conditional null neurospheres. As discussed earlier, the transient nature of the siRNA transfections limits the effects that can be seen in the cells therefore alternate approaches where required.

6.3.2 RNA interference against SOX2

There is increasing evidence that SOX2 is important in the maintenance of the neural stem cell state (Zappone et al. 2000; Bylund et al. 2003; Graham et al. 2003; Kondo and Raff 2004). From experiments using rat neural stem cell and oligodendrocyte precursor-derived neural stem cells it was shown that it's activation depends on the recruitment of *Brca1* and *Brm* (Brahma) to an enhancer in the *Sox2* promoter region (Kondo and Raff 2004). However, other more specific transcription factors are also likely to be involved in *Sox2* expression, perhaps including SOX2 itself in an autoregulatory loop. RNAi on *Sox2* in rat neural stem cells induces neurogenesis (Kondo and Raff 2004). The microarray analysis presented here on RNAi treated murine neural stem cell cultures of the 14.5 dpc dorsal telencephalon does not indicate significant upregulation of genes associated with neuronal differentiation such as neurogenins, neurofilaments and specific markers characteristic of differentiating

neurons (Figure 6.5). The analysis of the gene expression profiles in these cells took place 48 hours post transfection, where maximum levels of SOX2 protein knock-down are seen through immunofluorescence (western blots were also performed but a low specificity of the antibodies used in this method made it difficult to quantify the reduction in protein levels)(Figures 6.2 and 6.3). However, this effect was only transient, with levels of SOX2 returning to normal after 4 days. Perhaps if a stable transfection system had been used, the indirect impact on neurogenesis and gliogenesis would have been observed. Post-RNAi differentiation assays were performed by Kondo and co-workers after 5 days under differentiation promoting conditions. A requirement for SOX2 to inhibit neurogenesis is supported by results described earlier, where reduced numbers of neurons differentiate from *Sox2*$^{\beta geo/+}$ selected neurospheres (Figure 4.2) and in the presence of G418 during differentiation culture (section 3.2.3.1). However, this is not absolute as some differentiated neurons in the adult brain maintain *Sox2* expression.

6.3.3 Candidate transcriptional targets of SOX2

As indicated from the genes significantly upregulated in the siRNA-transfected cells, there was an interferon response, most likely due to remaining dsRNA that was not cleaved by Dicer into siRNA fragments. This is likely, as no method to eliminate uncleaved dsRNA was employed prior to transfection of the sequences. This response, makes it difficult to determine which, if any, genes may be normally repressed by SOX2. To determine the genes normally positively regulated directly or indirectly by SOX2, higher transcript levels would be expected in the control non-transfected population compared to the RNAi-treated sample. Apart from immunofluorescence analysis to determine the efficiency of transfections, microarray

hybridisations of RNA from SOX2 siRNA-transfected cells against RNA from non-transfected control cells revealed that the levels of *Sox2* mRNA are significantly lower in the siRNA transfected cells (Figure 6.5). The levels of expression of other SOX B1 subgroup genes (*Sox1* and *Sox3*), as well as of other *Sox* genes, were unaffected, highlighting the specific nature of the siRNA, which was generated against a non-homologous region of the *Sox2* ORF excluding the HMG box. It has been previously proposed that SOX2 acts as a transcriptional activator for its own regulation (Tomioka et al. 2002; Boyer et al. 2005). This remains a possibility although it cannot be determined if the lower expression levels are solely due to mRNA degradation or not. Although the *in silico* method of designing siRNA sequences was efficient, in that it elicited specific protein knock-down effects, the particular siRNA sequences responsible for this effect cannot be determined unless all of the potential short RNA sequences are individually designed and assayed in numerous combinations. Since only *Sox2* showed significantly lower levels of expression after RNAi treatment, other bioinformatic methods were used to determine candidate target genes that may be positively regulated by SOX2. The highest expressing genes that showed similar expression to *Sox2* across the arrays were short-listed as described in the results section of this chapter. The highest expressing known gene in this list, apart from *Sox2*, is *Idb2* (which from here on will be referred to as *Id2*). *Id2* encodes a well-characterised inhibitory bHLH protein that has an inhibitory impact on neurogenesis. Unlike other bHLH proteins, Id family members are unable to bind DNA. They contain a dimerization domain and can therefore form non-functional dimers with other bHLH proteins (Benezra et al. 1990). Proneural bHLH proteins (as are neurogenins), which normally bind DNA as heterodimers with the ubiquitously expressed E proteins, form non-functional dimers with Id proteins

(Coppe et al. 2003). This results in the inhibition of proneural protein activity; the Id proteins negatively regulating the differentiation of neural precursors. The overexpression of *Id2* is also known to inhibit the expression of neuron-specific genes and to promote the apoptosis of cortical progenitors (Toma et al. 2000). Subsequent inhibition or targeted deletion of *Id2* protects neurons from apoptosis (Gleichmann et al. 2002). An involvement of Id proteins in the Notch signalling pathway (which is important in regulating neurogenesis) has been shown by Reynaud-Deonauth and co-workers (Reynaud-Deonauth et al. 2002). Also, BMPs can lead to the induction of *Id* genes in neuroepithelial cells (Nakashima et al. 2001).

6.3.4 The possible relationship between Id2 and Sox2 in NSCs

In the microarray pair-wise comparison experiments of non-selected *Sox2*$^{\beta geo/+}$ and neural stem cell-enriched populations selected for the expression of *Sox2*, *Id2* levels are significantly higher in the former (2.7-fold difference) (section 4.2.1.4). *Id2* is expressed in oligodendrocyte precursor cells (OPCs) prior to lineage commitment. At this early OPC stage, the expression of the bHLH factor *Olig2* is inhibited by ID2 (as well as ID4), which localises in the nucleus (Gokhan et al. 2005). Its expression continues in oligodendrocytes undergoing lineage specification, where nuclear ID2 and its inhibitory action is overcome through the synergistic expression of the bHLH factors *Olig1*, *Mash1*, the homeodomain protein-coding gene *Nkx2.2* and *Sox10*. These factors are all able to activate the myelin basic protein (MBP) promoter and those of other myelination genes (Gokhan et al. 2005). Through these microarray comparisons it became apparent that a high proportion of genes involved in myelination are also expressed at higher levels in the non-selected neurospheres. The increase in the expression of *Id2* may be a result of a larger proportion of

oligodendrocyte precursor cells undergoing commitment. To understand the relationship between *Sox2* and *Id2*, the region 10kb upstream of the *Id2* gene was analysed. In the promoter region, two sites were found that could act as SOX2 binding sites, although they differ in one nucleotide from the consensus binding motif (section 6.2.2.3). Functional analysis of these sites needs to be performed to determine if SOX2 does indeed bind to regulate the transcription of *Id2*.

6.3.5 The relationship between Stat3 and Id2

There are several reports that suggest a direct activation of *Id2* by STAT3 (Gu et al. 2005; Sekkai et al. 2005). From the microarray analysis performed on SOX2 RNAi-treated cells, *Id2* was identified as a potential target of SOX2. SOX2 and STAT3 may therefore both be involved in the activation of *Id2*. From the recent report mentioned which identifies STAT3 as a potential SOX2 target in human ES cells, the expression of *Stat3* is expected to be lower in SOX2 RNAi-treated cells than in the control. Surprisingly, the expression of *Stat3* is higher in these cells relative to the control. However, as discussed earlier, an interferon response is elicited in the RNAi-treated population. Extracellular interferons are known to activate *Stat* genes and this is likely to be happening in the RNAi treated cells. IFNα and IFNγ have been shown to specifically induce *Stat3* activation in mouse retinal explants (Zhang et al. 2005). Supporting this hypothesis, *Stat1* shows highly increased expression levels in the RNAi treated cells (10-fold increase), as do several known interferon-induced genes (*Ifit3*, *Ifit1*, *Ifitm*, *Ifi35*, *Ifi1*, *Isgf3g* and *Igtp*). It is possible that the small increase in the levels of *Stat3* (1.2-fold increase) is buffered by a potential reduction in *Stat3* expression by lower levels of SOX2, if this regulatory mechanism is also in place in NSCs. One way of establishing if endogenous *Sox2* activates *Stat3* in these cells is to

perform an EGFP RNAi control transfection alongside SOX2 RNAi. If an interferon response is generated from the EGFP RNAi, then *Stat3* should be activated. If the levels of *Stat3* mRNA in the EGFP RNAi treated cells are significantly higher than in the SOX2 RNAi treated cells, this would suggest that SOX2 contributes to the activation of *Stat3* in NSCs.

To determine if there is a STAT3-independent action of SOX2 on *Id2* expression, the following can be performed: *Stat3* conditional null embryos can be used to derive NSCs, which can undergo RNAi for SOX2 and subsequent microarray analysis 48 hours post-transfection. We expect the levels of *Id2* mRNA to be lower in *Stat3* null cells than in wild type cells. If in SOX2 RNAi treated, *Stat3* null cells, the levels of *Id2* mRNA are even lower, then this would confirm that SOX2 can regulate *Id2* in a STAT3-independent manner.

CHAPTER 7

Conclusions and Future Directions

The initial aims of this thesis were to examine differences between neural stem cells and lineage-restricted progenitors, and to determine if NSCs isolated from separate regions or stages during CNS development are essentially identical, but behave differently according to extrinsic cues, or if they are innately distinct. Through this work, novel candidate markers for NSCs have been identified, which should contribute to our understanding of their biology. During the time of this PhD research, the glial character of at least some NSCs has been confirmed in the literature by other researchers, and their plasticity in giving rise to each of the major CNS lineages, as well as to other cell types has been demonstrated. NSCs in the embryo have been characterised as radial glia, while those in the adult are a subpopulation of astrocytes derived from radial glia. Despite a lineage relationship and both having stem cell properties, these clearly correspond to distinct NSC populations. However, one aspect of neural stem cell identity that had not been examined was whether distinct NSC populations co-exist at the same developmental stage in different regions of the CNS. This thesis has highlighted some of the differences in the character of NSCs residing in the embryonic spinal cord and dorsal telencephalon. As highlighted from microarray analysis, neurosphere cultures from the embryonic spinal cord and dorsal telencephalon have very similar expression profiles and they share a large common subset of genes, which are preferentially expressed at high levels in NSC-enriched cultures. At the same time, they retain some of their spatial and temporal properties. It remains unclear, if these two NSC types are interchangeable *in vivo*, having the competence to respond correctly to

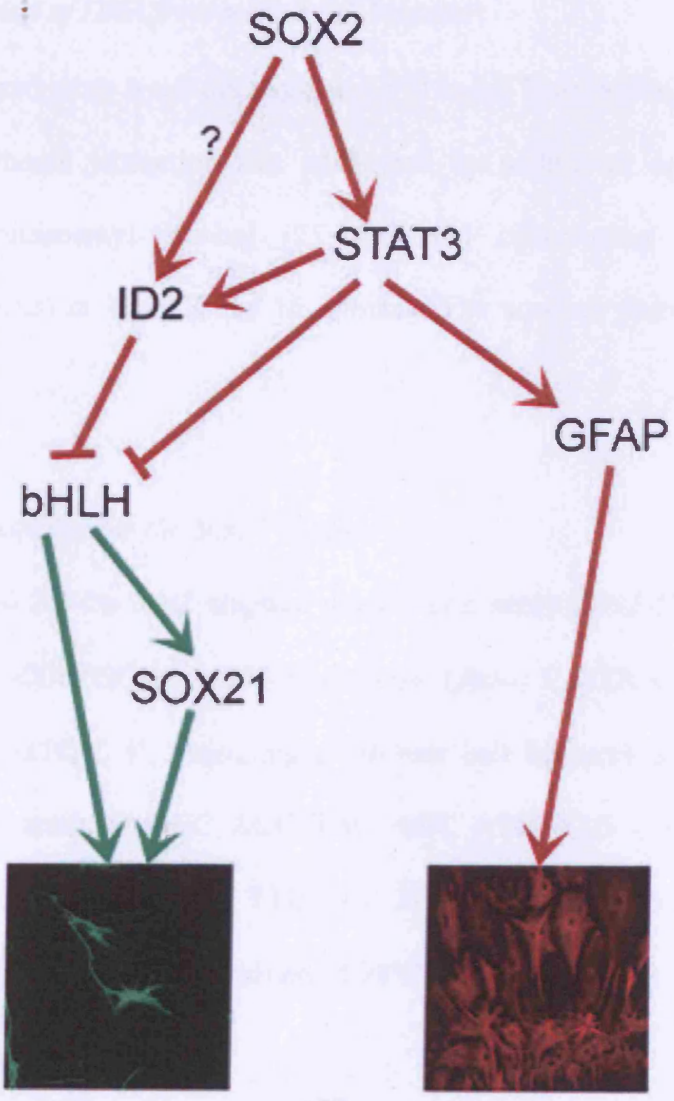
local environmental cues. Assessment of this would be the next step towards determining the plasticity and intrinsic character of NSCs.

Selection for the expression of *Sox2βgeo* provided an initial enrichment for NSCs *in vitro*, but it is likely that the removal of differentiated subsets of cells has had an impact on the proportion of the NSCs themselves. This seems to include a change of long-term potential. These experiments support the notion that extrinsic cues provided by differentiated cells *in vivo* might play a role in the maintenance of the stem cell state. The altered environment within selected *Sox2βgeo* neurospheres may result in the loss of the ability of NSCs to respond to particular extrinsic cues, highlighting the importance of the microenvironment.

Amongst the short-list of candidate markers generated to aid in the process of deconstructing stemness, there are candidates already implicated in the maintenance of the stem cell state, such as *Stat3*. The interactions between STAT3 and SOX2 are being investigated further and recently, SOX2 has been shown to regulate *Stat3*. The expression of *Sox2* was shown to be of importance for the generation of neurospheres. Its role appears to be essential for the maintenance of the stem cell state and for inhibition of neurogenesis. Candidate target genes for SOX2 were identified through RNAi against SOX2, followed by microarray analysis. One interesting candidate target gene is *Id2*, which inhibits neurogenesis through the formation of non-functional dimers with bHLH proteins. STAT3 has been shown to regulate *Id2*, but in these RNAi experiments, the levels of *Stat3* mRNA were raised, probably due to an interferon response. Nevertheless, this leads to the hypothesis that SOX2 may regulate *Id2* in a STAT3-independent manner. Experiments following on

from this thesis will aim to determine if SOX2 and STAT3 have unique and independent roles in NSCs or if their action to inhibit differentiation and promote their stem cell state is synergistic. A working model incorporating some of the factors involved in neurogenesis and gliogenesis is outlined below. This is almost certainly an over-simplification as it does not take into account Notch signalling or the role of regional fate determinants such as *Pax6*. However, it provides a few additional connections that can now be tested by experimentation.

Figure 7.1 Potential pathways leading to neurogenesis or gliogenesis. A glial character has been associated with neural stem cells. The interactions between the factors shown here have previously been proposed in the literature by others apart from the direct regulation of ID2 by SOX2. Data in this thesis has led to the proposal of this interaction.



CHAPTER 8

Materials and Methods

8.1 ANIMALS

8.1.1 Animal care

Animals were kept on a 12 hour light-dark cycle and food and water where provided *ad libidum*. The day of detection of a vaginal plug was taken as 0.5 days *post coitum*, assuming the time of conception was half way through the dark phase. All methods where carried out under Home Office approval.

8.1.2 Genotyping

8.1.2.1 Preparation of DNA from murine tail biopsies

Tail pieces (25mm) were lysed overnight at 55°C in tail lysis buffer, with 100μg/ml proteinase K. Phenol extraction was performed by adding an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) and centrifuging on a desktop centrifuge (Heraeus) at 16,000*g* for 15 minutes. The aqueous phase was used for PCR.

8.1.2.2 PCR genotyping for the Sox2$^{\beta\text{-}geo}$ allele

The primers used for the *Sox2* targeted allele were: sense (*Sox2* 5'UTR) 5' CAC AGT CCT GGC CGG GCC GAG G 3',antisense (*βgeo*) 5' GTA GAT GGG CGC ATC GTA ACC GTG C 3', generating a 336 base pair fragment, and for the *Sox2* wild type allele: sense 5' GGC AGC TAC AGC ATG ATG CAG GAG C 3', antisense 5' CTG GTC ATG GAG TTG TAC TGC AGG 3', generating a 130 base pair fragment. The PCR profile consisted of 94°C for 1 minute (1 cycle), 92°C for 1

minute, 60°C for 1 minute, 72°C for 2 minutes 30 seconds (36 cycles) and 72°C for 5 minutes (1 cycle). A Peltier Thermal Cycler (MJ Research) was used for all reactions.

8.1.2.3 PCR genotyping for the Stat3$^{flox/+}$ allele

The primers used for the *Stat3* floxed allele were: sense (APRF_11_UP) 5' CAC CAA CAC ATG CTA TTT GTA GG 3', antisense (APRF_11_DOWN) 5' CCT GTC TCT GAC AGG CCA TC 3', generating a 400 base pair fragment, and for the Stat3 wild type allele: sense (APRF_11_UP) as before, antisense (APRF_14_DOWN) 5' GCA GCA GAA TAC TCT ACA GCT C 3', generating a 250 base pair fragment. The PCR profile consisted of 94°C for 4 minutes (1 cycle), 94 °C for 1 minute, 60°C for 2 minutes, 71°C for 3 minutes (35 cycles) and 72°C for 5 minutes (1 cycle). A Peltier Thermal Cycler (MJ Research) was used for all reactions.

8.2 CELL CULTURE & TREATMENT

8.2.1 Neurosphere generation from the dorsal telencephalon of 14.5 dpc *Sox2*$^{\beta-geo/+}$ mice

Sox2$^{\beta-geo/+}$ males were crossed with wild type MF1 females and checked for the presence of vaginal plugs. At day 14.5 of gestation, pregnant dames were killed using schedule 1 methods and the embryos were dissected free from their decidua in PBS-A. Complying with schedule 1 methods, the head of each embryo was severed from the body. Both eyes of each embryo was dissected out and stained for β-galactosidase activity to identify *Sox2*$^{\beta-geo/+}$ positive animals. The heads were

transferred into DMEM F-12 media containing 100U/ml penicillin G and 100μg/ml streptomycin sulfate (both Gibco). Both lobes of the dorsal telencephalon were dissected out, and surrounding membranes were removed. Telencephalons from *Sox2*$^{\beta\text{-}geo/+}$ positive mice were placed on ice in neurosphere proliferation medium (NPM). Up to four lobes were combined and dissociated into one 6cm tissue culture dish. Tissue was mechanically triturated into a single-cell suspension using fire-polished Pasteur pipettes. The dissociated cells were passed through a 40μm cell strainer (Falcon). The cellular flow-through, a suspension culture, was incubated at 37°C, 5% CO_2. Cells were re-dissociated after 24 hours, and passed through a 70μm strainer (Falcon) without a medium change. 72 hours after initial culturing, the medium was replaced with fresh NPM. From the following day and for a minimum of 10 days, daily media changes were performed, using NPM with 250 μg/ml G418, in order to select for *Sox2*$^{\beta\text{-}geo/+}$ expressing cells for the duration of the culture. For the generation of non-selected *Sox2*$^{\beta\text{-}geo/+}$ neurospheres, G418 was not added in the medium. Wild type neurospheres were generated from wild type littermates of *Sox2*$^{\beta\text{-}geo/+}$ mice.

8.2.2 Generation of neurospheres from the spinal cord of 14.5 dpc mice

Whole spinal cords from *Sox2*$^{\beta\text{-}geo/+}$ mice were dissected out and attached somites were removed. Neurospheres were generated as described above, in identical conditions to those used for generation of neurospheres from the dorsal telencephalon.

8.2.3 Generation of neurospheres from the SVZ of adult mice

Eight *Sox2*$^{\beta geo/+}$ adult males were killed using schedule 1 methods and cells from the subventricular zone (SVZ) were isolated in PIPES solution. The dissected tissue was pooled and the PIPES solution was replaced with activated Papain solution, filtered through a 22 μm syringe filter. After the addition of DNase I (20U), the tissue was incubated at 37°C for 1 hour with gentle agitation. The tissue was centrifuged at 1200 rpm for 10 minutes at 4°C and the enzyme solution was replaced with 1ml DMEM-F12. The tissue was triturated into a single cell suspension using a fire-polished Pasteur pipette. A further 7 ml DMEM F-12 were added and the cells were centrifuged at 1200 rpm for 10 minutes at 4°C. The DMEM F-12 was replaced with NPM plated on a 6 cm culture dish. To select for *Sox2*$^{\beta geo/+}$ expressing cells, G418 was added in the medium at a concentration of 250 μg/ml, 72 hours after initial culturing.

8.2.4 Differentiation of neurospheres

18 day old neurospheres were placed onto Matrigel-coated Sonic Seals and allowed to settle and adhere overnight in neurosphere differentiation media (NDM) at 37°C in 5% CO_2. Differentiating neurospheres were cultured for five days, the medium being replaced daily with fresh NDM. On the fifth day they were fixed under appropriate conditions for subsequent immunofluorescence/β-galactosidase activity staining.

8.2.5 Embryonic Carcinoma (EC) cell culture

P19.6 EC cells (passage 12) were stored in liquid nitrogen in 0.5ml aliquots in 10% DMSO in foetal calf serum (FCS). Cells were thawed following standard protocols and resuspended at a density of $3x10^6$ cells per 10ml P19 cell medium. Cells were

cultured on gelatin-coated 10cm culture dishes at 37°C, 5% CO_2 and passaged following trypsinisation at a 1:9 ratio every 48 hours.

8.2.6 Embryonic Stem (ES) cell culture

Feeder-independent, LIF-dependent *Sox2*$^{\beta\text{-}geo}$ ES cells (passage 24) were stored in liquid nitrogen in 0.5ml aliquots in 10% DMSO in FCS. Cells were thawed following standard protocols and resuspended at a density of $3x10^6$ cells per 10ml ES cell medium. Cells were cultured on gelatin-coated 10 cm culture dishes at 37°C, 5% CO_2 and passaged following trypsinisation at a 1:3 ratio every 48 hours.

8.2.7 Fluorescent Activated Cell Sorter Analysis

For Fluorescent Activated Cell Sorter (FACS) analysis on *Sox2*$^{\beta geo/+}$ cells, 1-2 x 10^7 cells were dissociated into a single cell suspension, washed in PBS-CMF and resuspended in 200µl NPM with 10% FCS. The CMFDG LacZ staining kit (Invitrogen) was used as follows: 1% of the total volume CMFDG reagent and 0.1% verapamil (to reduce the efflux of the product) were added and cells were incubated at 37°C for 45 minutes. To stop the reaction, cells were diluted to 1 ml in ice-cold serum-containing medium and 2% of total volume PETG (competitive inhibitor of β-galactosidase) was added. Cells were strained through a 40µm cell strainer (Falcon) and sorted into positives and negatives with a FlowJo in the FL1 channel.

For FACS analysis using specific antibodies, single cells were washed in PFN and fixed in 2% PFA for 20 minutes on ice, washed briefly in PBS-0.5% saponin (Sigma), then incubated in PBS-0.5% saponin for 20 minutes on ice. The wash was replaced with the appropriate dilution of primary antibody in PBS-0.1% saponin, and

the cells were incubated for 30 minutes on ice. Cells were washed briefly three times in PBS-0.1% saponin and incubated in an appropriate dilution of secondary antibody (anti-mouse or anti-rabbit Alexa Fluor 488 (Molecular Probes)) in PBS-0.1% saponin for 30 minutes at room temperature. The secondary antibody solution was rinsed three times in PBS-0.1% saponin and cells were resuspended in PFN. Cells were analysed using a BD FACSCalibur in the FL1 channel.

For both methods, compensation was performed using the Beckton-Dickinson CELLQuest software using either a wild type cell sample (i.e. non-*βgeo*) stained in the same way or a negative control incubated only in secondary and not in primary antibody solution. Data was further analysed using the FlowJo 5.5 software.

8.2.8 Bromodeoxyuridine (BrdU) assay on neurospheres

Neurospheres were incubated for 4 hours in NPM containing 10μM BrdU in PBS (10mg/ml stock). After washing briefly in PBS Neurospheres were dissociated and single cells were plated on poly-D-lysine coated slides before treatment and subsequent BrdU antibody staining.

8.2.9 BrdU assay on cortical cells- *in vivo* labeling

100μg of BrdU in PBS-A per gram of mouse weight were administered by intra-peritoneal injection to pregnant dames. After 30 minutes, pregnant dames were killed using schedule 1 methods and embryos were harvested and fixed for subsequent sectioning.

8.2.10 BrdU assay on cortical cells- *in vitro* labeling

Cells were pulsed with DMEM-F12 medium containing 10μM BrdU in PBS-A for 45 minutes at 37°C, 5% CO_2.

8.2.11 Electroporation of neural stem cells to introduce plasmid vectors

Neural stem cells were mechanically dissociated into a single-cell suspension using fire-polished Pasteur pipettes. Cells were centrifuged for 5 minutes at 1000rpm and resuspended in 800μl ice-cold PBS-CMF at a density of 1.5 x 10^6 cells per ml. The cell suspension was mixed with 20μg of appropriate circular plasmid in 0.4 cm electroporation cuvettes (Bio-Rad) and electroporated at 300V, 500μF using a Bio-Rad Gene Pulser, then allowed to recover on ice for 30 minutes before plating in pre-warmed NPM.

8.3 HISTOLOGY

8.3.1 Preparation of embryos for paraffin sections

Sox2$^{\beta\text{-}geo/+}$ embryos stained for β-galactosidase activity and stored in 50% glycerol were placed in 80% ethanol in for 20 minutes and then in 70% ethanol overnight. They were then progressively dehydrated for 20 minutes in washes of 80%, 90% and three washes in 100% ethanol at room temperature. To clear, embryos were passed once through 50%/50% ethanol/xylene and twice in 100% xylene for 20 minutes. The embryos were equilibrated in a mixture of 1:1 xylene:fibrowax (BDH) for 20 minutes at 60°C. The solution was replaced with pure molten fibrowax three times for 20 minutes at 60°C, using a Blockmaster Embedding Centre (Reynard-Lamb). The embryos were transferred to fresh wax in dispomoulds (CellPath), which were then allowed to cool for the wax to set.

8.3.2 Sectioning and counterstaining of paraffin-embedded embryos

14.5 dpc *Sox2*$^{\beta\text{-}geo/+}$ embryos were sectioned at 6μm thickness, using a Leica RM 2165 microtome. Wax sections were floated on a 50°C section-mounting water bath (Electrothermal) and then mounted on Superfrost+ slides (BDH) and allowed to dry. Slides were de-waxed by twice immersing in Histoclear for 10 minutes at room temperature. The slides were rehydrated through a decreasing ethanol series (3 x 100%, 1 x 90%, 1 x 80%, 1 x 70%, 1 x 50% 1 x 20%), then washed in distilled water. Eosin counterstaining was performed followed by an ethanol dehydration series (briefly through 1 x 20%, 1 x 50%, 1 x 70%, 1 x 80%, 1 x 90%, 3 x 100%). Slides were rinsed in Histoclear two times for 5 minutes and mounted with DPX mounting medium (Raymond Lamb) under glass coverslips.

8.3.3 Preparation and sectioning of paraffin-embedded neurospheres

Fixed neurospheres were set in 4% low melting point agarose in PBS and cut in 0.3cm diameter blocks. The neurosphere-containing blocks were then treated as described above (Preparation of embryos for paraffin sectioning). Blocks were sectioned at 3μm thickness, using a Leica RM2165 microtome as described above (Sectioning and counterstaining of paraffin-embedded embryos).

8.3.4 Preparation and Cryosectioning of 14.5 dpc embryos

Embryos were fixed in 4% PFA (30 minutes for neurospheres, 2 hours for 14.5 dpc embryos) and washed in PBS-A. They were placed in 20% sucrose in PBS for 4 hours (until fully sunk). Embryos were transferred into OCT Compound which was replaced after 10 minutes. Samples were then transferred into fresh OCT compound

in Dispomoulds and frozen on dry ice. They were stored at -80°C until required for cryosectioning. 10-15μm (12μm) sections were cut using a Cryostat (Leica).

8.4 PROTEIN METHODS

8.4.1 Fixation of cells/neurospheres

Samples were washed with PBS-CMF. They were fixed in either: 4% PFA for 20 minutes on ice, or in MEMFA for 15 minutes on ice, or in ice cold 100% methanol for 15 or 30 minutes on ice or in ethanol/acetic acid for 15 minutes at room temperature, then briefly washed with PBS containing 0.1% Triton-X.

8.4.2 Staining for β-galactosidase activity

14.5 dpc embryos were fixed with Glutaraldehyde/Formaldehyde fixative for 15 minutes on ice. Neurospheres and embryonic stem (ES) cells were fixed under the same conditions for 10 minutes. After fixation, all samples where washed in buffer L_0. LacZ Staining Solution was added, through a 0.22μm syringe filter to cover the samples and the enzymatic reaction allowed to proceed in the dark at 37°C overnight. Embryos were stored in 50% glycerol. Stained neurospheres and ES cells were post-fixed in 4% paraformaldehyde (PFA) and stored in PBS-A at 4°C. Images of neurospheres were captured using a Leica MZ75 dissection microscope at 5x magnification and of embryos at 0.8x magnification, using the Leica IM50 Software.

8.4.3 Immunofluorescence antibody staining on cells/neurospheres

Fixed samples were washed briefly in Blocking Buffer. The primary antibody, diluted in blocking buffer containing 1% sheep serum was added to the sample and incubated overnight with gentle rocking at 4°C. The excess primary antibody

solution was removed and the sample washed three times with Blocking Buffer. The secondary antibody diluted in Blocking Buffer containing 1% sheep serum, was added and incubated for 30 minutes at room temperature, on a shaker, in the dark (for fluorescent secondary antibodies). The excess secondary antibody solution was removed and the sample rinsed three times with Blocking Buffer. One drop of Vectashield with 4',6-Diamidino-2-phenylindole (DAPI) (Vector Laboratories) was added on Superfrost slides and the samples were mounted. All immunofluorescence images were obtained with a Leica TCS SP confocal using a UV 10x/0.4 or 40x/0.5 NA dry HC-PLAPO lens (Leica) and were composed of 16 sections of 4 accumulates per section unless otherwise specified. Images were processed using the ImageJ 1.30v (National Institutes of Health) and Adobe Photoshop CS v8.0 (Adobe Systems) software packages.

8.4.4 Immunofluorescence antibody staining on cryosections

Slides were air-dried and washed for 2 minutes in PBS-CMF. They were blocked for 1 hour in PBS-CMF containing 0.1% Triton and 10% heat-inactivated sheep serum. Primary antibody was added and incubated overnight at 4°C, in Blocking Buffer containing 1% sheep serum. Slides were washed 3 times for 5minutes in PBS-T (PBS + 0.1% Triton X-100). The appropriate secondary antibody was added and incubated for 30 minutes in the dark at room temperature in Blocking Buffer containing 1% sheep serum. Slides were washed twice for 5 minutes. Excess solution was removed and 22x64 mm coverslips (BDH) were mounted using Vectashield with DAPI (Vector laboratories).

8.4.5 TUNEL assay on neurosphere sections

Wax sections were air-dried for 24 hours, heated to 60°C for 15 minutes then cooled to ambient temperature. They were passed twice through Histoclear for 10 minutes, once through 50% Histoclear/50% Ethanol for 5 minutes, then through a progressive ethanol series (twice in 100%, 90%, 70%, 50% for 5 minutes in each) before rinsing in 0.1% Triton-X in 0.1% sodium citrate. They were permeabilized on ice for 8 minutes in 0.1% Triton-X in 0.1% sodium citrate ad finally rinsed in PBS-A. Slides were incubated in TUNEL Labeling solution (terminal deoxynucleotidyl transferase in reaction buffer containing labeled nucleotide mix (both Roche)) for 1 hour at 30°C in the dark then rinsed in PBS-A, air-dried and mounted. Fluorescence detection was performed using standard settings for fluorescein.

8.4.6 Pre-treatment of sections/slides for BrdU antibody staining

After immunofluorescence labeling using appropriate primary and secondary antibodies, slides were washed 3 times for 5 minutes each in Blocking Buffer and post-fixed in 4% PFA for 5 minutes. Sections or cells were rinsed in PBS and treated with 0.05% pepsin (Sigma) in 0.01M HCl for 1 minute at 37°C. They were incubated in 20M HCl for 30 minutes at 37°C and rinsed in borate buffer (0.1M boric acid pH8.5), then PBS. Anti-BrdU antibody was added at an appropriate dilution in Blocking Buffer and staining was performed following protocols described (Immunofluorescence Antibody Staining on Cells/Neurospheres or Cryosections).

8.4.7 Western blotting

Samples were lysed in RIPA buffer, vortexed for 20 seconds and placed on ice for 10 minutes. They were loaded onto QIAshredders (Qiagen) and centrifuged for 5

minutes at 16,000*g* to homogenise. Dilutions of 20μg, 10μg and 5μg of each sample were loaded onto a NuPage 10% or 4%-12% Bis-Tris gel (Invitrogen) alongside SeeBlue Plus2 protein standard (Invitrogen) in NuPage MOPS SDS running buffer (Invitrogen). Gels were run at 200V for 1 hour and transferred at 30V for 2 hours using an Xcell SureLock Mini-Cell tank with a PowerEase 500 power pack (both Invitrogen). Membranes were washed in PBS-T (PBS-0.1% tween) and blocked for 1 hour in PBS-T with 10% skimmed milk powder (Nestlé). Membranes were incubated overnight at 4°C in primary antibody solution at the appropriate dilution in PBS-T containing 5% milk powder. Membranes were washed four times for 10 minutes each, in PBS-T and incubated for 1 hour at room temperature with either anti-rabbit IgG or anti-mouse IgG peroxidase conjugate secondary antibodies (both Sigma). After four 5 minute washes in PBS-T membranes were incubated for 1 minute with Detection Reagent (Pierce) according to manufacturers protocols and visualized after 1, 3 and 15 minute exposures with Kodak X-OMAT AR film in a dark room and developing using standard protocols.

8.5 DNA METHODS

8.5.1 Transformation of DH5α cells

Luria-Bertani (LB) agar plates containing ampicillin (100μg/ml) were warmed in a 37°C oven. DH5α *E.coli* competent cells (Stratagene), stored at -80°C were thawed on ice and divided into two cooled eppendorf tubes- each to be transformed by a different plasmid. Plasmids pSUPER and PKO PURO (both 1μg/μl) were diluted to 1ng/μl in water. Cells and plasmids were mixed for 30 minutes on ice, and then heat shocked at 42°C on a dry heat block, for 90 seconds and then cooled on ice for 2 minutes. LB broth was added to the cells, which were then allowed to recover for 30

minutes in a shaker at 37°C. 200µl of cells were plated onto ampicillin plates using glass beads. Plates were incubated in a 37°C oven overnight.

8.5.2 Picking of single colonies and inoculation of starter culture

Individual bacterial colonies were picked from the ampicillin resistant plates, using sterile P200 pipette tips, and inoculated in 2 ml of LB broth containing 50µg/ml ampicillin. They were incubated at 37°C in a Brunswick Scientific Incubator Shaker for 8 hours at 200 rpm.

8.5.3 Expansion of starter culture

The starter culture was diluted in 90ml LB containing 50 µg/ml ampicillin and incubated at 37°C in a Brunswick Scientific Incubator Shaker for 16 hours at 250 rpm.

8.6 RNA METHODS

8.6.1 RNA extraction

Fresh samples were lysed in lysis buffer RLT (Qiagen) and homogenized either by loading on QIAshredders (Qiagen) and centrifuging for 5 minutes at 16,000*g* (for neurospheres) or through the Ultra Turrax T25 IKA-Labortechnik tissuemiser (Janke & Kunkel GMBH & Co) at 24,000 vibrations per minute for 5 minutes (for tissue). Total RNA was extracted from samples according to the Qiagen RNease Midi protocol for animal cells/animal tissues. RNA was eluted in nuclease-free H_20 and treated with DNase I at 37°C for 30 minutes using the DNA-free kit (Ambion) according to the manufacturer's instructions. The RNA was quantified using a

NanoDrop ND-1000 spectrophotometer (Labtek) and/or a BioAnalyzer 2100 (Agilent Technologies). RNA was precipitated by ethanol precipitation and resuspended in nuclease-free H_20 at a concentration of 2μg/μl.

8.6.2 Microarray methods for spotted arrays

8.6.2.1 SMART cDNA Synthesis

The SMART PCR cDNA Synthesis Kit (Clontech) was used according to manufacturer's instructions. 1μg of starting RNA was used for each reaction. The first strand was synthesized using a modified oligo(dT) primer to selectively prime mRNA. A specialized SMART oligonucleotide (provided) was used to extend the template creating a synthetic adaptor. Second strand synthesis took place, using primers specific to the synthetic adaptor sequences generated by the SMART oligonucleotide, ensuring that full-length cDNA species were produced. Each 1st strand reaction was amplified through PCR and the appropriate optimal number of cycles for each sample was determined (ranging from 25 to 32).

8.6.2.2 Roche cDNA Synthesis

The Transcriptor First Strand cDNA Synthesis kit (Roche) was used according to manufacturer's instructions. 20 μg of starting RNA was used for each reaction. The first strand was synthesized using a 5' modified oligo(dT) primer to selectively prime mRNA and second strand synthesis using DNA polymerase I with E.coli ligases and RNase H.

8.6.2.3 Klenow labeling

An appropriate amount of target cDNA (one single 50µl cDNA reaction per hybridisation) was incubated at 100°C with Random Octamers (Invitrogen) in Reaction Buffer (Invitrogen), according to manufacturer's protocols, for 5 minutes, then rapidly cooled on ice. Low dCTP dNTP mix (100µM dCTP final, 200µM dATP, dTTP, dGTP final) and 60U Klenow fragment [Large Fragment of DNA Polymerase I] (Invitrogen) were added to the mix together with either Cy3-dCTP or Cy5-dCTP. Samples were incubated in the dark at 37°C for 4 hours. The reaction was stopped by adding EDTA pH8.0 to a final concentration of 50µM. Each labeling mix was loaded onto a sephadex G50 Probequant column (Codelink) and centrifuged for 2 minutes at 4000*g*. Each labeling mix pair to be hybridised on the same slide was combined and Mouse C_0t_1 DNA (Invitrogen) was added at a final concentration of 0.3µg/µl to block non-specific hybridisation. Samples were desiccated using a SpeedVac Concentrator SPD131DDA (Thermo) and resuspended in 12.5 µl ddH_20. Microarray Hybridisation Buffer was added to 50µl and each hybridisation mix was incubated at 85°C for 5 minutes.

8.6.2.4 Hybridisation

The Mouse Known Oligo Array (provided by the MRC-HGMP, now RFCGR) on CodeLink Activated Slides (Amersham Biosciences) (Serial numbers 0807038078-85 and 0803048028-48) were placed in hybridisation chambers (Qiagen) with a small amount of water and pre-warmed at 42°C for 1 hour. Each hybridisation mix was pre-warmed by incubating at 42°C for 1 hour with dry heat and centrifuged for 2 minutes at 16,000*g*. Hybridisation mix was loaded onto array slides and cover slips were mounted avoiding air bubbles. Arrays were hybridised overnight in a 48°C

water bath. Slides were washed in Wash solution A until the cover slips came off. They were transferred rapidly into Wash solution B with vigorous shaking for 5 minutes, then Wash solution C for 2 minutes. During washing, slides were not allowed to dry or exposed to air and the arrays were not touched on any surface or with forceps. Slides were centrifuged for 5 minutes or until dry at 75 x g and scanned using a two-colour 428 scanner (Affymetrix).

8.6.3 Microarray methods for Affymetrix arrays

Total RNA was extracted and quantified from tissue or cell culture samples, as described previsouly. All the reactions for the preparation of samples and array processing were performed according to the Affymetrix 'Eukaryotic Sample and Array Processing' guide. One-cycle cDNA synthesis was performed on 8μg total RNA starting material and a GenAmp PCR System 9700 (Applied Biosystems) was used for all reactions. Synthesis of biotin-labeled cRNA was carried out for 18 hours at 37°C and labeled cRNA was quantified using a NanoDrop ND-1000 spectrophotometer (Labtek). 20μg of labeled cRNA were used for fragmentation and quantified on a BioAnalyzer 2100 (Agilent Technologies). Overnight hybridisation to the Mouse 430A GeneChips (Affymetrix) took place in a GeneChip Hybridisation Oven 640 (Affymetrix). Post-hybridisation washes of GeneChips took place in a GeneChip Fluidics Station 450 (Affymetrix) and GeneChips were scanned using a GeneChip Scanner (Affymetrix) and the GCOS Software. GeneSpring 7.2 was used for downstream analysis.

8.6.4 Reverse-Transcription PCR

Total RNA samples were treated with DNase I (Promega) for 30 minutes at 37°C and reverse transcribed for 1 hour at 37°C using Omniscript RT (Qiagen) with Random Decamers (Ambion). 2μg of starting cDNA were used for each PCR reaction, employing gene-specific primers. The PCR profile consisted of 94°C for 15 minutes (1 cycle), 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 1 minute (30 cycles) and 72°C for 8 minutes (1 cycle). A Peltier Thermal Cycler (MJ Research) was used for all reactions.

8.6.5 Real-time Reverse-Transcription PCR

For all samples, cDNA templates were prepared as described (see Reverse Transcription PCR) and used at a concentration of up to 30ng per 25μl reaction. All reactions were repeated in triplicate using gene-specific primers. A no primer control and non-reverse transcribed sample control were performed for each reaction set. Primers were designed using the ABI Prism Primer Express version 2.0.0 software (Applied Biosystems) and their efficiency was determined through absolute quantification reactions of a dilution series of template. A standard curve of C_T (threshold cycle) value against logarithm of template concentration and the slope of each standard curve was determined. C_T values of primers that were efficient (slope between –3.3 to –3.8) were used to determine if they had comparable amplification efficiencies to the endogenous control. The difference in C_T values (C_TTarget – C_TControl) was plotted against the logarithm of the template amount and primers that generated slope values of less that 0.1 were deemed comparable to primers against the endogenous control. Efficient primers for target genes were used to perform relative quantification across samples against glyceraldehyde-3-phosphate

dehydrogenase (*Gapdh*) amplification acting as the endogenous control. The fluorescent dye ROX was used as a passive reference calibrator to correct well-to-well variation. The PCR profile consisted of 50°C for 2 minutes (1 cycle), 95°C for 15 minutes (1 cycle), 95°C for 15 seconds and 60°C for 1 minute (40 cycles). The ABI 7000 and ABI 7500 Real Time PCR Machines (Applied Biosystems) were used for all reactions. The normalized target gene expression levels between samples were relatively quantified, as described in the Appendix.

8.7 RNA INTERFERENCE

8.7.1 Selection of siRNA sequences for inclusion in the pSUPER vector

Sequences were selected following guidelines provided by Brummelkamp *et al* (2002) against the target *LacZ* gene (Lac1 and Lac2), the N-terminal of the target *Sox2* sequence (Sox2nt) and the *Sox2* HMG box (Sox2HMG) as follows:

Lac1	5'-GATCCCCTTATCGATGAGCGTGGTGGTTCAAGAGACCACCACG CTCATCGATAATTTTTGGAAA-3'
Lac2	5'-GATCCCCCGGGCGCTGGGTCGGTTACTTCAAGAGAGTAACCGA CCCAGCGCCCGTTTTTGGAAA-3'
Sox2nt	5'-GATCCCCGAGGCCCATGAACGCCTTCTTCAAGAGACTCCGGGT ACTTGGGGAAGTTTTTGGAAA-3'
Sox2HMG	5'-GATCCCCGTACACGCTTCCCGGAGGCTTCAAGAGACATGTGCG AAGGGCCTCCGTTTTTGGAAA-3'

Where: 5'[spacer]-[19bp target sense]-[spacer]-[19bp target αsense]-[spacer]3'

Only sense construct sequences are shown. Each construct is designed to form a hairpin structure upon excision from the vector for cleavage by Dicer *in vivo* to generate small interfering RNA (siRNA).

8.7.2 Construction of vectors for siRNA delivery

The pSUPER vector was digested with restriction enzymes BglII and HindIII (Promega). The digested DNA was dephosphorylated using shrimp alkaline phosphatase (SAP) (Roche) in a 37°C water bath for 1 hour. The forward and reverse pairs of oligonucleotides Sox2HMG, Sox2nt, Lac1 and Lac2 (custom synthesized by Oswell) were annealed in annealing buffer for 4 minutes at 95°C, 10 minutes at 70°C, using a Biometra Personal Cycler, then cooled gradually to room temperature overnight. Annealed oligonucleotides were phosphorylated using 1X T4 PNK, 1mM ATP in 1X T4 PNK buffer (New England Biolabs), for 30 minutes at 37°C, 10 minutes at 70°C, then cooled to 4°C using a Biometra Personal Cycler. Each set of annealed, phosphorylated oligonucleotides (Sox2HMG, Sox2nt, Lac1, Lac2) was ligated into linearised, dephosphorylated pSUPER vector using T4 DNA ligase (New England Biolabs) for 1 hour at 16°C using a Biometra Personal Cycler. DH5α bacteria were transformed with each resulting plasmid and starter cultures were inoculated. Maxi-preps were performed for each plasmid (following protocols from the Qiagen Plasmid Purification Handbook). A 1:100 dilution of each Maxi-prep DNA plasmid product in TE pH 8.0 was used to quantify the amount of DNA in each sample using a NanoDrop ND-1000 spectrophotometer (Labtek).

8.7.3 Transfection of ES cells

ES *Sox2*$^{\beta\text{-}geo}$ cells were cultured to 90% confluency in 6-well plates. Each well was transfected with the appropriate pSUPER plasmid which was previously linearised with XhoI. The PKO PURO plasmid was co-transfected at 1/10th of the concentration of each pSUPER plasmid to allow further puromycin selection. The plasmid combinations transfected were: Lac1, Lac2, Lac1+Lac2 (mixed 1:1), Sox2HMG, Sox2nt, Sox2HMG + Sox2nt (mixed 1:1), no plasmid (control). Transfections were carried out using the cationic lipid transfection reagent Lipofectamine 2000 (Invitrogen), according to the manufacturers protocols.

8.7.4 Culture of transfected cells

The day following transfection, each well of a 6-well dish was transferred onto a 10 cm culture dish. The ES medium was replaced with ES medium containing 5μg/ml puromycin (Sigma). Cells underwent selection for 48 hours (at which point the control cells had not survived), after which 50 colonies were picked from each plate into 96-well culture dishes and cultured in the absence of selection. Each colony was expanded to 4 x 96-well wells to be further used for DNA isolation and subsequent PCR, immunofluorescence and β-galactosidase activity staining and for freezing.

8.7.5 PCR analysis of transfected ES cells

Primers for the ampicillin resistance sequence of the pSUPER vector were designed using the DNA STAR PrimerSelect programme and synthesised by Sigma-Genosys: forward 5'GGG CCG AGC GCA GAA GTG G3', reverse 5' TGA CGC CGG GCA AGA GCA 3', generating a 422 base pair fragment. The PCR profile consisted of 3 minutes at 94°C (1 cycle), 1 minute at 94°C, 1 minute at 58 °C, 1 minute at 72°C (30

cycles), and 3 minutes at 72°C (1 cycle). A Peltier Thermal Cycler (MJ Research) was used for the reactions.

8.7.6 Selection of sequences for generation of short double-stranded RNA

Following guidelines accompanying the siRNA generation kit (GTS), sequences between 600 and 800bp in length were selected for transcription from each template. Gene specific primers to amplify 770bp of the *Sox2* sequence (nucleotides 1287-2057 of the Sox2 1015 vector (RLB lab), 524 bp downstream of the HMG box) and 700bp of the *Egfp* sequence (nucleotides 681-1381 of the pEGFP-N1 vector (BD Biosciences Clontech), which includes the entire *Egfp* sequence) were designed to add T7 promoter flanking sequences to both ends of each DNA template as follows:

SOX2_fw

5'-GCGTAATACGACTCACTATA**G**GGAGAGTTTGCCTTAAACAAGACCA-3'

SOX2_rv

5'-GCGTAATACGACTCACTATA**G**GGAGACACATGGCCCAGCACTACCA-3'

EGFP-N1_fw

5'-GCGTAATACGACTCACTATA**G**GGAGAGGTGAGCAAGGGCGAGGAGC-3'

EGFP-N1_RV

5'-GCGTAATACGACTCACTATA**G**GGAGACTTGTACAGCTCGTCCATGCCGAG-3'

Where: 5'-[Leader]-[T7 promoter sequence] [AGA]-[gene specific sequence]-3' and **G** = +1 of transcription

8.7.7 Generation of short double-stranded RNA

50ng of DNA template were used in each PCR reaction designed to add T7 promoter flanking sequences to both ends of each template using the gene-specific primers outlined above. The PCR profile consisted of 94°C for 3 minutes (1 cycle), 94°C for 30 seconds, 58°C for 30 seconds, 68°C for 1 minute (35 cycles) and 68°C for 5 minutes (1 cycle). An MJ Research Peltier Thermal Cycler was used. PCR products were purified by ethanol precipitation using standard protocols and the DNA was resuspended in 20µl nuclease-free H_20. 1µg of template DNA was used for transcription using Turboscript T7 Enzyme Mix (GTS) and transcribed for 4 hours at 37°C. The dsRNA generated was treated with DNase I (Promega) for 15 minutes at 37°C. dsRNA was digested into 22bp fragments using 1 unit of Recombinant Dicer Enzyme (GTS) per 1µg dsRNA for 15 hours at 37°C.

8.7.8 Delivery of siRNA into neural stem cell cultures

Neurospheres were dissociated into a single cell suspension the night prior to siRNA delivery and 5 x 10^5 cells were plated per well in 24-well plates (Corning). On the day of transfection, proliferation medium was replaced with 500µl OptiMEM (Gibco). For each transfection, 3.5µl of sImporter (Ambion) (or RiboJuice transfection reagent (Novagen) or GeneSilencer reagent (GTS) both of which have a lower transfection efficiency) were mixed with 25 µl OptiMEM to form the Transfection solution. 500ng of appropriate diced siRNA was diluted in water to 10µl and mixed with 15µl OptiMEM for 5 minutes to form the d-siRNA solution. The Transfection and d-siRNA solutions were mixed together and incubated at room temperature for 5 minutes to allow RNA/lipid complexes to form, then applied drop-

wise onto the cells. After 4 hours, 1 ml of NPM was added and the following day replaced completely with fresh NPM.

8.7.9 Assessment of protein knock-down through RNAi

48 hours post transfection, transfected and control cells were fixed for 15 minutes in 4% PFA and used for immunofluorescent antibody staining. Anti-SOX2 primary antibody using Alexa-594 secondary antibody, was used to assess the knock-down of *Sox2*. EGFP control knock-down was assessed based on EGFP intensity, viewed with a Leica TCS SP confocal.

8.7.10 Assessment of RNAi through microarray analysis

Cells were harvested 48 hours post transfection and washed in PBS-CMF. RNA was extracted and treated with DNase I as described previously.

CHAPTER 9

Appendices and Bibliography

9.1 APPENDIX I

9.1.1 GENOTYPING

9.1.1.1 Tail Lysis Buffer

Tris-HCl pH 8.5	100mM
EDTA	5mM
SDS	0.2%
Sodium chloride	200mM

In ddH_20

9.1.1.2 PCR reaction mix

Taq DNA polymerase	1.25U
Buffer IV (10X)	1X
Magnesium chloride	1.5mM
dNTP mix (4N)	0.2mM
Primer (each)	125ng/µl
DMSO	20%
DNA (upper phase of phenol extraction)	2µl

Total volume of 25µl in ddH2O

9.1.2 CELL CULTURE

9.1.2.1 NSC hormone mix

Putrascine	9.6µg/ml
Progesterone	6.3ng/ml
Sodium selenite	5.2mg/ml
Insulin	25µg/ml
Transferrin	100µg/ml

9.1.2.2 Neurosphere proliferation medium (NPM)

N2 (5X)*	1X
B27 (10X)	1X

Glucose	0.6% (w/v)
Penicillin G	100U/ml
Streptomycin sulfate	100μg/ml
L-glutamine	2mM
Human recombinant bFGF	20ng/ml
Human recombinant EGF	20 ng/ml

In Dulbecco's Modified Eagle's Medium (DMEM) F-12 NUT (nutrient) MIX (All from Gibco, glucose from Sigma)

*N2 components include insulin, human transferrin, putrascine, progesterone

9.1.2.3. Neurosphere differentiation medium (NDM)

N2 (5X)	1X
B27 (10X)	1X
Glucose	0.6%
Penicillin G	100U/ml
Streptomycin sulfate	100μg/ml
L-glutamine	2mM

In DMEM F-12 NUT (nutrient) MIX (All from Gibco, glucose from Sigma)

9.1.2.4. Preparation of Matrigel-coated Sonic Seals

Matrigel (BD Biosciences) was diluted 10-fold in DMEM-F12 (Gibco), using pipettes cooled at -20°C and stored in aliquots at –20°C. Prior to use, aliquots were thawed at 4°C and diluted 5-fold in DMEM-F12. 4-chamber Sonic Seals were coated with 200 μl per chamber of diluted Matrigel, and incubated for 1 hour at 37°C until set. The excess liquid was aspirated off.

9.1.2.5. Preparation of gelatin-coated cell culture dishes

PBS Medium with gelatin (GIBCO) [0.1 (w/v) gelatin solution] was used to cover the surface of tissue culture dishes. The medium was left on for 30 minutes at room temperature, after which, the excess was aspirated.

9.1.2.6 PIPES Solution

PIPES (Sigma)	0.1M
In PBS	
pH to 6.8 using 1M Na-pyruvate	

9.1.2.7 Papain Solution

75U papain in 1 ml DMEM F-12 containing 4.3 mg L-cysteine

Activated for 30 minutes at 37°C

4ml PIPES Solution added after activation

9.1.2.8 PFN

PBS-A	1X
Fetal calf serum (Gibco)	2%
Sodium azide (Sigma)	0.1%

9.1.2.9 P19 cell medium

Penicillin G (Gibco)	100U/ml
Streptomycin sulfate (Gibco)	100 μg/ml
L-glutamine (Gibco)	2mM
β-mercaptoethanol (Sigma)	90 μM
Fetal calf serum (Gibco)	15%
In DMEM (Sigma)	

9.1.2.10 ES cell medium

Penicillin G (Gibco)	100U/ml
Streptomycin sulfate (Gibco)	100 μg/ml
L-glutamine (Gibco)	2mM
β-mercaptoethanol (Sigma)	90 μM
Leukaemia Inhibitory Factor (Chemicon)	1000U/ml
ES qualified fetal calf serum (ESQ) (Gibco)	15%
In DMEM (Sigma)	

9.1.2.11 Picking ES colonies

Individual colonies were picked under a microscope using sterile P200 pipette tips. They were transferred in gelatin-coated 96-well plates containing 25µl trypsin and incubated at 37°C for 5 minutes. 200 µl ES cell medium was added to each well and the colonies were mechanically dissociated using a multipipettor. They were cultured at 37°C, 5% CO_2, the medium being replaced daily.

9.1.3 FIXATIVES

9.1.3.1 Paraformaldehyde (PFA) fixative

4%

Freshly prepared 4% (w/v) paraformaldehyde was dissolved in PBS-A in a 67°C water bath. Aliquots were stored at –20°C for up to 2 months.

2%

4% paraformaldehyde	50% (v/v)
PBS-A	50% (v/v)

9.1.3.2 Ethanol/Acetic Acid fixative

Ethanol 100%	95%
Acetic acid	5%

9.1.3.3 MEMFA fixative

10X MEM stock was prepared as follows:

MOPS pH7.4 (filtered)	1M
EGTA (autoclaved)	20mM
Magnesium sulfate (autoclaved)	10mM

In ddH_20

Aliquots were stored at 4°C. Prior to use, one part 10X MEM stock and one part formaldehyde 37% were added to 8 parts ddH_20.

9.1.3.5 Glutaraldehyde/Formaldehyde fixative

Glutaraldehyde	0.2%	
Formaldehyde	2%	In Buffer L_0

9.1.4 X-GAL STAINING

9.1.4.1 PB

Sodium phosphate pH 7.2 (filtered)	0.1M

9.1.4.2 PBS-T

Tween-20	0.1%
in PBS-A	

9.1.4.3 Buffer L

PB pH7.2	100mM
Magnesium chloride	2mM
EGTA	5mM
NP40	0.2% (0.02% for cells)
Na-Deoxycholate	0.1% (0.01% for cells)

9.1.4.4 Buffer L_0

PB pH7.2	100mM
Magnesium chloride	2mM
EGTA	5mM

9.1.4.5 Staining Solution

$K_3Fe(CN)_6$	10mM
$K_4Fe(CN)_6.3H_20$	10mM
In Buffer L	

9.1.4.6 X-gal Stock

X-gal	50mg/ml
in DMF	

9.1.4.7 LacZ Staining Solution

X-gal	1mg/ml
In Staining Solution	

9.1.5 PROTEIN METHODS

9.1.5.1 Blocking buffer

Triton-X	0.1%
Glycine	0.15%
BSA	2 mg/ml
In PBS-A	

9.1.5.2 TUNEL Labeling solution

4µl enzyme, 36µl buffer for each sample

9.1.5.3 RIPA Buffer

Sodium chloride	150mM
SDS	0.1%
NP40	1%
Sodium deoxycholate	0.5%
Tris pH7.5-8.0	50mM
Sodium fluoride	2mM
Sodium orthovanadate	1mM

Table 9.1.A Primary antibodies used for immunofluorescence

Primary Antibody	*Host spp*	*Poly-/ Mono-clonal*	*Dilution*	*Antigen Recognized*	*Source*
Sox2 (Purified)	Ra	P	1:2500	SOX2	Lovell-Badge lab
Nestin Rat 401	Mo	M	1:75	Intermediate Filament (I.F.)	D.S.H.B.
GFAP	Mo	M	1:100	Glial Fibrillary Acid (I.F.)	Chemicon
GFAP-Cy3	Ra	P	1:100	Glial Fibrillary Acid (I.F.)	Sigma
GFAP	Mo	M	1:100	Glial Fibrillary Acid (I.F.)	Sigma
TuJ1	Mo	M	1:1000	β-III Tubulin (I.F.)	Covance
GalC ascites	Ra	P.	1:100	Galactocerebroside	Sigma
O4	Mo	M		Oligodendrocyte marker O4	Chemicon
CNPase ascites	Mo	M	1:100	2',3'-cyclic nucleotide-3'-phosphodiesterase	Santa Cruz
Ssea-1	Mo	M	1:75	Surface carbohydrate 3-fucosyl-N-acetyllactosamine	Santa Cruz
Oct4	Mo	M	1:250	OCT3/4 protein	Santa Cruz
Stat3 (K-15)	Ra	P	1:500	STAT3 p92 and STAT3b	Santa Cruz
Sox9	Ra	P	1:500	SOX9	Gift from Silvana Guioli
Sox10	Ra	P	1:200	SOX10	Gift from Matin Cheung
Sox2	Ra	P	1:200	SOX2	Chemicon
RC2	Mo	M	1:100	RC2	D.S.H.B.

Table 9.1.B Secondary antibodies used for immunofluorescence

(All from Moecular Probes)

Secondary Antibody	*Dilution*	*Primary Antibody Recognized*
Goat Anti-Mouse Alexa 594 IgG	1:350	Mouse Monoclonal
Goat Anti-Mouse Alexa 488 IgG	1:350	Mouse Monoclonal
Goat Anti-Mouse Alexa 488 IgM	1:350	Mouse Monoclonal
Goat Anti-Rabbit Alexa 594 IgG	1:350	Rabbit Polyclonal
Goat Anti-Rabbit Alexa 488 IgG	1:350	Rabbit Polyclonal

9.1.6 DNA METHODS

9.1.6.1 Annealing buffer

Potassium acetate	100 mM
HEPES-KOH pH 7.4	30 mM
Magnesium acetate	2 mM

9.1.6.2 P1

Tris-Cl (pH 8.0)	50mM
EDTA	10mM
in ddH_2O	

9.1.6.3 Solution I (TEG)

Glucose	50mM
Tris-Cl (pH 8.0)	25mM
EDTA	10mM
in ddH_2O	

9.1.6.4 P2/Solution II

Sodium hydroxide	0.2N
SDS	1%
in ddH_2O	

9.1.6.5 P3/Solution III

Porassium acetate (pH5.5)	3M
Glacial acetic acid	2M
in ddH_2O	

9.1.6.7 QBT

MOPS (pH 7.0)	50mM
Sodium chloride	750mM
Isopropanol	15%
Triton X-100	0.15%
in ddH_2O	

9.1.6.8 QC

MOPS (pH 7.0)	50 mM
Sodium chloride	1M
Isopropanol	15%
in ddH_2O	

9.1.6.9 QF

Tris-Cl (pH 8.5)	50mM
Sodium chloride	1.25M
Isopropanol	15%
in ddH_2O	

9.1.6.10 Isolation of DNA from ES colonies

Cells were washed twice in PBS-CMF and 50 µl lysis buffer (10mM Tris pH7.5, 10mM EDTA pH8.0, 10mM NaCl, 0.5% Sarcosyl , 1mg/ml proteinase K) was added per well. The plates were wrapped in parafilm and incubated overnight at 60°C in a humid box. 100µl of fresh 75mM NaCl in 100% ethanol was added to each well for 30 minutes at room temperature. The solution was discarded and the DNA (stuck onto the plate) washed 5 times in 70% ethanol. Wells were air-dried and DNA resuspended in 20µl TE pH8.0

9.1.6.11 Freezing 96-well plates

Wells were washed twice in PBS-CMF and 25µl of trypsin added to each well. Cells were incubated for 5 minutes at 37°C after which the plates were gently tapped to dislodge the cells and then returned to 37°C for another 5 minutes. 75µl ES cell medium was added to each well and a single cell suspension generated by pipetting up and down. 100µl of 20% DMSO in FCS was mixed into each well. 50µl filter-sterilized mineral oil was added to cover each well and the lid sealed with parafilm. Each plate was placed in a polystyrene box and frozen at -80°C.

9.1.7 RNA METHODS

9.1.7.1 DEPC treatment of ddH_20

0.1% DEPC added to ddH_20 and allowed to evaporate overnight in a loose-capped bottle.

The treated solution was autoclaved for 15 minutes per liter.

(This treatment is not appropriate for solutions containing Tris, MOPS, HEPES or PBS).

9.1.7.2 Spotted Array Solutions

9.1.7.2.1 Microarray Hybridisation Buffer

Formamide	42%
SSC	5X
SDS	0.1%

Filtered through a 0.22µm sterile syringe filter

9.1.7.2.2 Wash solution A

SSC	2X

Filtered through a 0.2µm sterile filter

9.1.7.2.3 Wash solution B

SSC	
SDS	0.1%

Filtered through a 0.2µm sterile filter

9.1.7.2.4 Wash solution C

SSC	0.1X

Filtered through a 0.2μm sterile filter

9.1.7.3 Reverse Transcription PCR mix

Buffer RT 10X (Qiagen)	1X
dNTP mix (Qiagen)	4μl
Random decamers (Ambion)	8μl
Rnase inhibitor (Ambion)	0.5 μl
DTT (Promega)	0.44μl
Omniscript RT (Qiagen)	2.0 μl
RNA template	2μg

In ddH_20 to 40 μl

9.1.7.4 Real time PCR mix

SYBR Green ROX mix(2X) (ABgene)	1X
DNA	7-30ng
Primer forward (10μM stock)	150nM
Primer reverse (10 μM stock)	150 nM

In ddH_2O to 25μl

9.1.7.5 Relative quantification of amplification of target genes

Out of each group of samples that were compared, one sample acted as the calibrator (in this example B). The following equations were followed in order:

ΔC_T(sample A) = C_T target gene – C_T endogenous reference gene

ΔC_T(sample B)= C_T target gene – C_T endogenous reference gene

$\Delta\Delta C_T$(sample A)= ΔC_T(A) – ΔC_T(B)

$\Delta\Delta C_T$(calibrator B)= ΔC_T(B) – ΔC_T(B) = 0

Normalised target gene expression level = $2^{-\Delta\Delta C_T}$, where for calibrator = 1

Table 9.1.C List of Primers used for RT-PCR

Target	Direction	Sequence	Product length
Casr-rs5	Forward	5'-ATTCAAGGCCACTGTTCCAG-3'	209bp
	Reverse	5'-TGTGGAAGGCAACTGTTGAA-3'	
Dcx	Forward	5'-TCCCCAACACCTCAAAAGAC-3'	236bp
	Reverse	5'-ATGGAATCGCCAAGTGAATC-3'	
Foxg1	Forward	5'-GAACGGCAAGTACGAGAAGC-3'	197bp
	Reverse	5'-TCACGAAGCACTTGTTGAGG-3'	
Nsg2	Forward	5'-TTGGCTTGACTCTGCTCTGA-3'	178bp
	Reverse	5'-CAGCCAGCTGCAAATGATTA-3'	
Gfap	Forward	5'-AGAAAACCGCATCACCATTC-3'	184bp
	Reverse	5'-TCACATCACCACGTCCTTGT-3'	
Mela	Forward	5'-ACTGTGTCCTGGTCGAGCTT-3'	184bp
	Reverse*	5'-ACTAGGGCGGTAGTCCCTGT-3'	
Sacs	Forward	5'-AGGGACTGCCCCTACTCATT-3'	195bp
	Reverse	5'-CAGCAAAGCTGGAAATGTCA-3'	
Lag	Forward	5'-CGGGAGAATGTCTGGACCTA-3'	217bp
	Reverse	5'-AGCAGACAGCTGTGGTGATG-3'	
Ttr	Forward	5'-TTTCACAGCCAACGACTCTG-3'	153bp
	Reverse	5'-ATGGGATGCTACTGCTTTGG-3'	
Olfr65	Forward	5'-GAGCAAAGGCTCTCAACACC-3'	195bp
	Reverse	5'-TTTGCTTGGTCTTGATGCTG-3'	
Plp	Forward	5'-GGCGACTACAAGACCACCAT-3'	154bp
	Reverse	5'-CAAACTTGTCGGGATGTCCT-3'	
Pten	Forward	5'-ACACCGCCAAATTTAACTGC-3'	170bp
	Reverse	5'-TACACCAGTCCGTCCCTTTC-3'	
mFat1	Forward	5'-TCAGGAGACAGCGAAAACCT-3'	152bp
	Reverse	5'-GCTTTCTCCACTGCCTTGAC-3'	
Aprf (Stat3)	Forward	5'-GACCCGCCAACAAATTAAGA-3'	215bp
	Reverse	5'-TCGTGGTAAACTGGACACCA-3'	
Oda8	Forward	5'-ATGTACAGCGGTGTGCTGAG-3'	183bp

	Reverse	5'-TGACTCTGGTGTGCCTCTTG-3'	
Pdgfa	Forward	5'-GAGATACCCCGGGAGTTGAT-3'	238bp
	Reverse	5'-AAATGACCGTCCTGGTCTTG-3'	
Actb	Forward	5'-TCATGCCATCCTGCGTCTGGACCT-3'	600bp
	Reverse	5'-CCGGACTCATCGTACTCCTGCTTG-3'	

Table 9.1.D List of Primers for qRT-PCR

Target	Direction	Sequence
Sox2	Forward	5'-CTGTTTTTTCATCCCAATTGCA-3'
	Reverse	5'-CGGAGATCTGGCGGAGAATA-3'
Stat3	Forward	5'-GGGCCAGGCCAACCA-3'
	Reverse	5'-CCGGACATCCTGAAGATGCT-3'
Gfap	Forward	5'-CGTTTCTCCTTGTCTCGAATGA-3'
	Reverse	5'-CCCGGCCAGGGAGAAGT-3'
Tnc	Forward	5'-CCTTGCCCCTTCTGGAAAA-3'
	Reverse	5'-GCCGTCCAGGAAACTGTGA-3'
Ptn	Forward	5'-CCTGTGGCAAGCTCAGGAA-3'
	Reverse	5'-TTCTTGCCTTCCTTTTTCTTCTTC-3'
Actb	Forward	5'-TGACCGAGCGTGGCTACA-3'
	Reverse	5'-TCTCTTTGATGTCACGCACGAT-3'
Gapdh	Forward	5'-GGGAAGCCCATCACCATCTT-3'
	Reverse	5'-GCCTTCTCCATGGTGGTGAA-3'

9.1.8 RNA INTERFERENCE

9.1.8.1 Master mix for amplification of DNA template (RNAi)

Buffer IV (10X)	1X
Magnesium chloride	2.0μM
dNTPs	100 μM
DNA (circular)	1μg
Forward primer	10ng/μl
Reverse primer	10ng/μl
Taq DNA polymerase	5U

In nuclease-free ddH_20, to 100μl

9.1.8.2 Master mix for transcription of template for dsRNA generation

ddH_20	1μl
NTP mix	8μl
T7 Reaction buffer	2μl
PCR template	7μl
T7 Enzyme mix	2μl

Reaction was assembled in the above order at room temperature.

9.1.8.3 Recombinant Dicer Enzyme Digestion mix

dsRNA	1μg
ATP	1mM
$MgCl_2$	2.5mM
Dicer Reaction Buffer (2.5X)	1X
Recombinant Dicer Enzyme	1U

in nuclease-free ddH_20

Reaction buffer kept at room temperature prior to assembly to avoid precipitation

9.1.9 STATISTICS

9.1.9.1 Standard Deviation

$$S.D. = \sqrt{\frac{\sum_{s=1}^{m}\sum_{i=1}^{n}(y_{is} - M)^2}{(n_y - 1)}}$$

$$M = \frac{\sum_{s=1}^{m}\sum_{i=1}^{n} y_{is}}{n_y}$$

9.9.1.2 Standard Error

$$S.E. = \sqrt{\frac{\sum_{s=1}^{m}\sum_{i=1}^{n} y_{is}^2}{(n_y - 1)(n_y)}}$$

9.9.1.3 Chi Square Test

$$\chi^2 = \sum \frac{(O-E)^2}{E}$$

9.9.1.4 ANOVA

$$SS_T = \sum x^2 - \frac{(\sum x_T)^2}{N}$$

$$SS_b = \sum \frac{(\sum x.)^2}{n} - \frac{(\sum x_T)^2}{N}$$

$$SS_w = SS_T - SS_b$$

$$df_b = \text{(number of groups - 1)}$$

$$df_T = \text{(number of subjects - 1)}$$

$$df_w = df_T - df_b$$

$$MS_b = \frac{SS_b}{df_b}$$

$$MS_w = \frac{SS_w}{df_w}$$

$$F = \frac{MS_b}{MS_w}$$

Where:

s	= series number
i	= point number in series *s*
m	= number of series for point *y* in chart
n	= number of points in each series
y_{is}	= data value of series *s* and the i^{th} point
N_y	= total number of data values in all series
M	= arithmetic mean
Σ	= sum of
χ	= Chi
O	= observed
E	= expected
SS	= sum of squares
df	= degrees of freedom
T	= total
b	= between groups
w	= within groups
MS	= mean squares
F	= F-statistics

9.9.10 PLASMID VECTORS

Figure 9.1.10.A pSUPER vector (Brummelkamp *et al*, 2002)

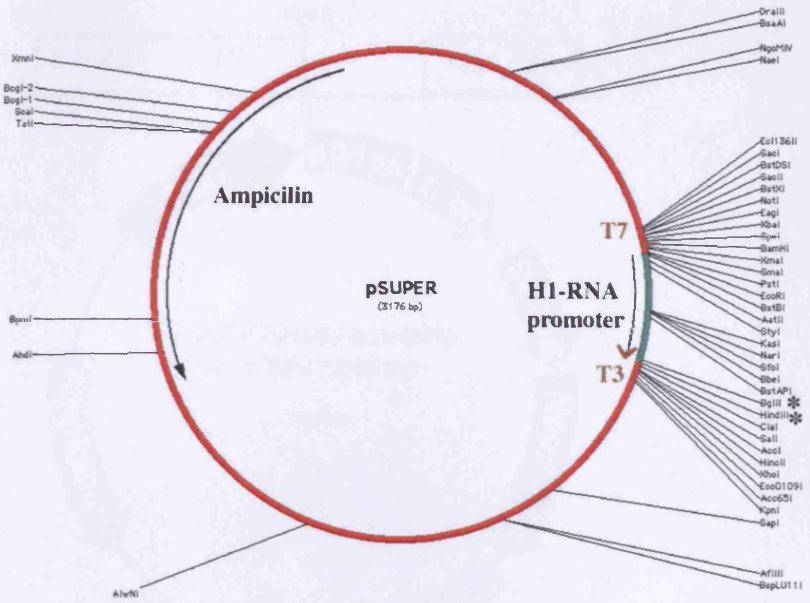


Figure 9.1.10.B *Sox2* dominant negative (engrailed repressor domain fusion) in pCI-neo vector [kind gift from Dr. Ariel Avilion]:

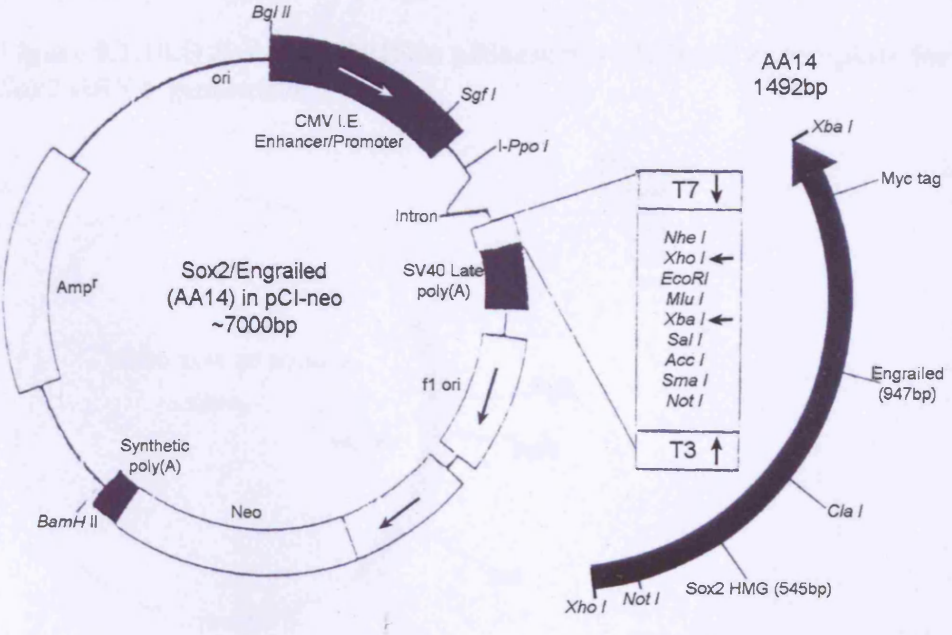


Figure 9.1.10.C *Sox2* dominant negative (truncated HMG box) in pCMV/*myc*/nuc vector [kind gift from Dr. Karine Rizzotti]:

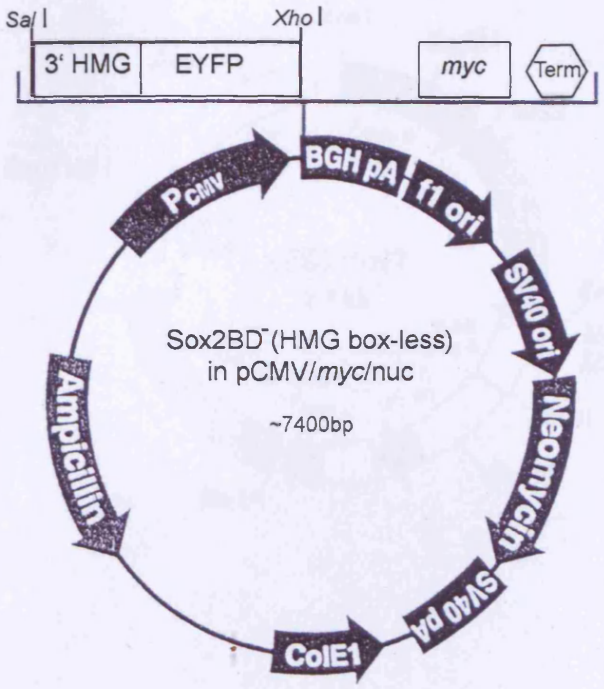


Figure 9.1.10.D *Sox2* 4C #1015 in pBluescript SK- used as template for *Sox2* siRNA generation

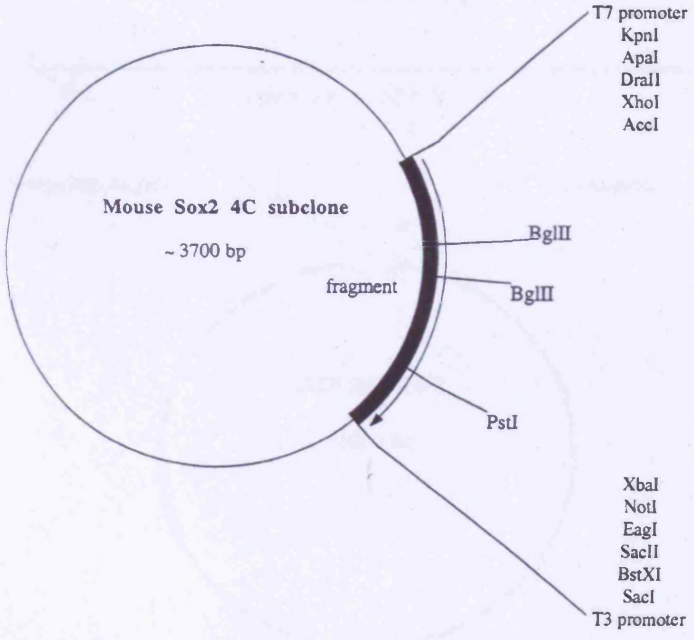


Figure 9.1.10.E pEGFP-N1 vector (Clontech)- used as template for *Egfp* siRNA generation:

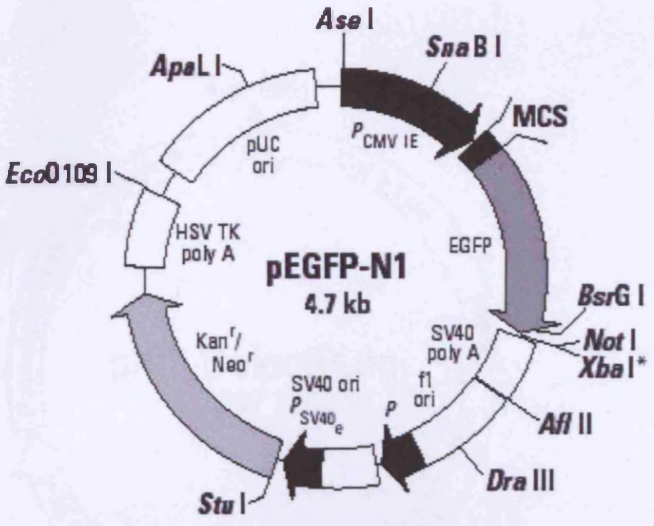


Figure 9.1.10.F *Stat3*-DN plasmid. *Stat3* dominant negative (Y705F) in pMY-IRES-GFP [Kind gift from Professor Shizuo Akira] :

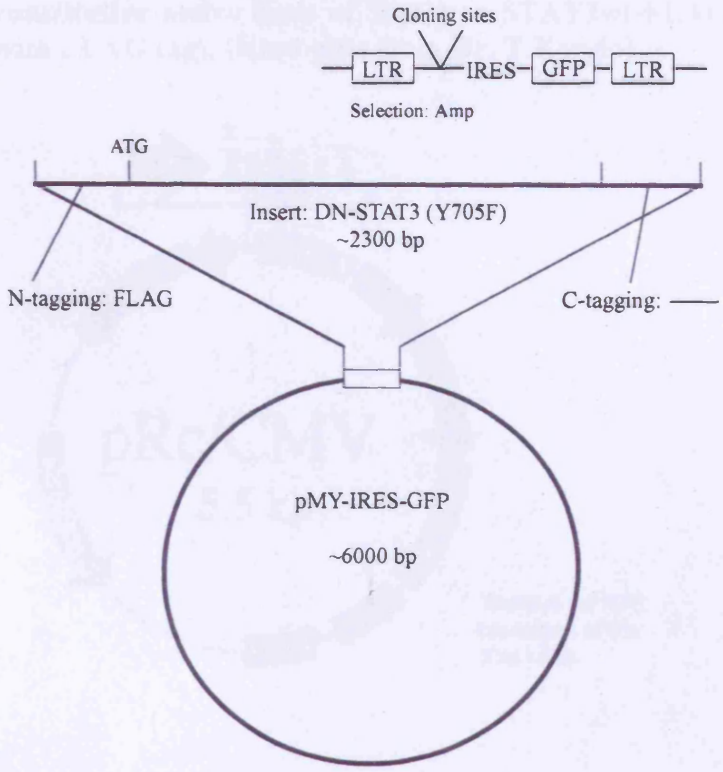


Figure 9.1.10.G pKO SelectPuro vector- used as transfection control to introduce puromycin selection:

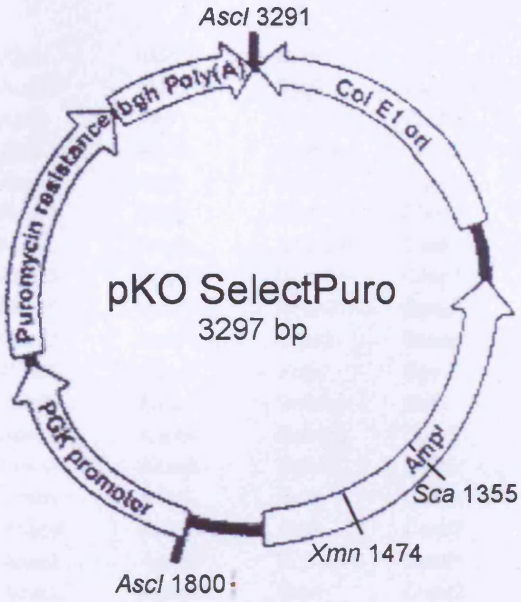
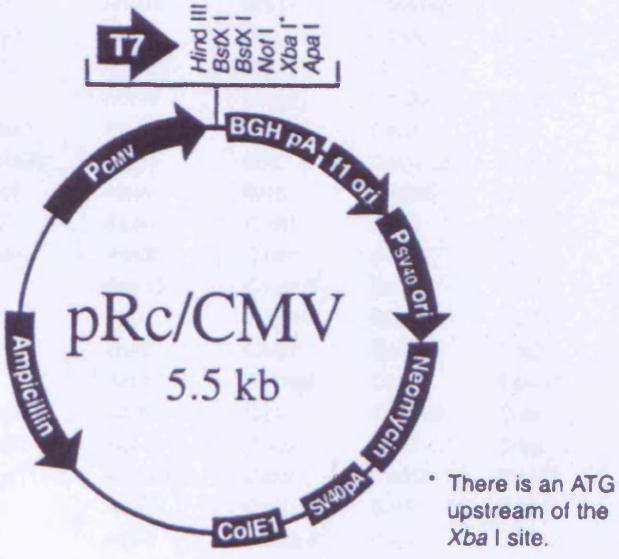


Figure 9.1.10.H pRC/CMV vector containing either STAT3-C (a constitutive active form of Stat3) or STAT3wt-FLAG (wild type Stat3 with FLAG tag). [Kind gifts from Dr. T.Kondo] :



9.2 APPENDIX II

Table 9.2.A Potential candidate NSC markers, not shortlisted. List of genes not included in the candidate NSC marker list generated from the Affymtrix GeneChip platform approach. It is likely that several genes in this list should be considered NSC candidates.

A2m	Aldo1	B2m	Capn5	Cklfsf6	Cspg2	Dhrs8	Elovl5
Aaas	Aldo3	Bag2	Capns1	Clcn3	Cspg3	Dia1	Elovl6
Aard	Alg1	Bag3	Car12	Cldn10	Cspg5	Diap3	Emb
Aars	Alg12	Baiap2	Car13	Cldn11	Csrp1	Dio2	Emid2
Abca2	Alg3	Bak1	Car3	Clic1	Cst3	Dip3b	Emp1
Abca3	Alg5	Bbc3	Car8	Cln2	Ctbs	Dlat	Endog
Abcc1	Amph	Bcap29	Cask	Cln3	Ctdsp1	Dleu2	Eno1
Abcd3	Angptl2	Bcap31	Casp1	Cln5	Ctdsp2	Dlg7	Eno2
Abhd1	ank	Bcap37	Casp7	Cln6	Cte1	Dlst	Enpp2
Abhd3	Antxr1	Bcar3	Catns	Cml1	Ctsb	Dmd	Enpp5
Abhd4	Anxa1	Bche	Cav	Cnnm2	Ctsc	Dmrtb1	Entpd5
Abhd5	Anxa2	Bckdha	Cbfb	Cntn1	Ctsd	Dnahc8	Epb4.1l2
Acaa2	Anxa4	Bckdhb	Cbl	Cntn3	Ctsh	Dnaja3	Epb4.1l4a
Acadm	Anxa6	Bcl10	Cbln3	Coasy	Ctsl	Dnajb11	Epb7.2
Acads	Aox1	Bcl3	Ccnb1	Col11a1	Ctsz	Dnajb12	Epn2
Acadvl	Ap3m2	Bcl6	Ccnb2	Col16a1	Cx3cl1	Dnajc3	Epn2
Acas2	Apba3	Bcl7c	Ccnd1	Col18a1	Cxxc5	Dnase2a	Eral1
Acas2l	Apeg1	Bdnf	Ccnd2	Col19a1	Cycs	Dnm1l	Ercc4
Aco2	Apln	Bet1	Ccnd3	Col4a5	Cyfip1	Dnm2	Erf
Acox1	Aplp2	Bet1l	Ccnf	Col9a1	Cyp4f15	Dock1	Erp29
Acsl5	Apoa1bp	Bicc1	Ccs	Col9a3	Cyp4f16	Dorz1	Ethe1
Actc1	Apobec1	Bicd2	Cd151	Commd1	Cyp4v3	Dph2l1	Ets2
Adam12	Apobec3	Blvra	Cd164	Commd6	Cyp51	Dr1	Etv4
Adam15	Araf	Blvrb	Cd38	Commd9	Cyp7b1	Drpla	Evi5
Adam17	Arc	Bmp1	Cd44	Comt	Cyr61	Dsc2	Ext2
Adam19	Areg	Bmp4	Cd81	Copeb	Dab2	Dscam	Extl3
Adamts1	Arhgap18	Bmp7	Cdc20	Cops7a	Dad1	Dtna	Eya1
Adamts4	Arhgdia	Bre	Cdc42	Coq3	Dag1	Dtr	Eya2
Adamts5	Arl10c	Brp17	Cdc42ep1	Cotl1	Dap	Dullard	F8a
Adcy3	Arl4	Bsg	Cdgap	Cox15	Dbi	Dusp14	Fads1
Add3	Arl6ip2	Btbd14a	Cdh13	Cox6a1	Dbp	Dusp16	Fads2
Adk	Armet	Bteb1	Cdh22	Cox8a	Dbt	Dusp6	Fancc
Adora1	Arpc1b	Btg2	Cdh4	Cp	Dcamkl1	Dusp7	Fasn
Adora2a	Arpc4	Btrc	Cdk5rap2	Cpd	Dck	DXImx39e	Fastk
Adrb1	Arts1	Bzrp	Cdkal1	Cplx2	Dcxr	Ece1	Fbn1
Adsl	Asah1	C1ql1	Cdv3	Cpne2	Ddit3	Ecgf1	Fbn2
Aebp1	Asah2	C1qr1	Cebpb	Cpne3	Ddit4	Echdc1	Fbp1
Aes	Asb13	C1qtnf5	Cecr5	Cpox	Ddit4l	Edem1	Fbxl3a
Afg3l1	Ascl1	C1qtnf6	Centg3	Cpt1a	Ddost	Edil3	Fbxo32
Afg3l2	Asns	Cabc1	Cerk	Cpt2	Ddr1	Eef1d	Fbxo33
Agl	Asph	Cacng5	Cflar	Cpxm1	Ddt	Eef2k	Fbxo36
Agpat3	Asrgl1	Cad	Chchd3	Crat	Ddx41	Efcbp2	Fbxw4
Agpt2	Ass1	Calca	Chd1	Creg	Ddx54	Efemp1	Fgd6
Ahcyl1	Atad3a	Calcrl	Chd1l	Creld1	Deb1	Efemp2	Fgf11
Ak1	Atf5	Cald1	Chl1	Crim1	Decr1	Efs	Fgfr1
Ak2	Atf6	Calmbp1	Chm	Crkl	Dedd2	Egfr	Fgfr1
Ak3l	Atp1a1	Calml4	Chn2	Crtap	Defcr3	Egln1	Fgfr3
Ak5	Atp1a2	Calr	Chrdl1	Cryz	Degs	Egln3	Fgfr3
Akap12	Atp1b1	Calu	Chrna4	Cs	Dep1	Egr2	Fhit
Akap2	Atp2a2	Camk1	Chst2	Csad	Depdc1	Ehd2	Fip1l1

Akr1b3	Atp4a	Camk2b	Cib1	Csda	Dgka	Ehd4	Fjx1
Akr7a5	Atp5g2	Camk2d	Cited1	Csf1	Dgkz	Eif2s1	Fkbp10
Aldh6a1	Atp6v0a2	Camkk2	Ckap4	Csnk1g2	Dhcr7	Elf1	Fkbp11
Aldh7a1	Avpi1	Camta2	Ckb	Csnk2a1	Dhh	Elk3	Fkbp2
Aldh9a1	Axl	Canx	Cklfsf3	Csnk2a2	Dhrs7	Elovl1	Fkbp7
Fkbp9	Gla	Gtpbp3	Idb1	Kif20a	Ltbr	Mrpl39	Ndufs8
Fliih	Glce	Gtse1	Idb3	Kif2c	Luzp1	Mrpl4	Ndufv1
Flot2	Glg1	Gus	Ide	Kifc3	Lxn	Mrpl40	Nek7
Fnbp1	Glo1	Gypc	Idh3a	Kifc5a	Lyl1	Mrpl48	Nek8
Fnbp2	Glrx1	Gys3	Idh3g	Kitl	Lyn	Mrpl51	Nek9
Fndc4	Gltp	H2-Ab1	Idi1	Klf13	Lynx1	Mrpl9	Nes
Fntb	Glud	H2-DMa	Ier2	Klf3	Lysal1	Mrps16	Neto1
Fosl2	Glul	H2-K1	Ifi1	Klf5	Lztr1	Mrps18a	Neu1
Foxc1	Gm2a	H2-Ke6	Ifitm2	Kns2	Mad1l1	Mrps18b	Nfatc1
Foxm1	Gmfb	H2-T10	Ifnar2	Kntc2	Mad2l2	Mrps25	Nfia
Fth	Gmpr2	Hadha	Igfbp2	Kpnb1	Mad4	Mrps28	Nfic
Fthfd	Gna12	Hadhb	Igfbp3	Krtcap2	Mag	Mrps36	Nfkb2
Fuca	Gna13	Hadhsc	Igfbp4	Kua	Man2b1	Mrps6	Nfkbia
Fut8	Gnai2	Has2	Igfbp5	L259	Manba	Mrps7	Nfs1
Fxc1	Gnb1	Hbp1	Il10rb	Lama5	Maoa	Mrsb	Ninj1
Fxr1h	Gne	Hccs	Il11ra1	Lamc1	Map2k1	Mrvldc1	Nmi
Fyco1	Gng12	Hdgf	Il18	Lamp2	Map3k1	Msn	Nmnat
Fyn	Gng5	Hes1	Il1rl1l	Laptm4b	Map4k4	Mt3	Nol3
Fzd1	Gnpda1	Hexa	Il6st	Lass2	Mapk12	Mtap	Nola2
Fzd4	Gns	Hexb	Impdh2	Lbh	Mastl	Mtap4	Nolc1
G2an	Golph2	Hey1	Incenp	Lcat	Matn2	Mthfd1	Notch1
G6pc3	Golph3	Hfe	Inhbb	Ldh1	Mbd2	Mthfd2	Notch3
Gaa	Gorasp2	Hibadh	Inpp5a	Ldlr	Mbd3l1	Mthfs	Npc1
Gab1	Got1	Hibch	Ipo4	Lfng	Mbtps1	Mtm1	Npc2
Gadd45a	Got2	Hif1a	Iqgap1	Lgals1	Mcee	Mtmr2	Npl
Gadd45b	Gpc6	Hig1	Irak1	Lgals3bp	Mcm5	Mtmr6	Npn3
Gal3st1	Gpd1	Hip1	Irf2	Lgals8	Mecp2	Mut	Npnt
Galc	Gpd2	Hipk2	Irx3	Lgmn	Meig1	Mvd	Nptx1
Galk1	Gphn	Hipk3	Irx5	Lifr	Mela	Mybph	Nqo1
Galns	Gpi1	Hist1h2bc	Itga6	Limd1	Mertk	Myc	Nqo2
Galnt1	Gpm6b	Hist1h3d	Itgav	Lims1	Mettl1	Myd116	Nr1i3
Galnt10	Gpr23	Hk1	Itgb1	Lin7c	Mfhas1	Myd88	Nr2f6
Galnt11	Gpr56	Hlf	Itgb1bp1	Lip1	Mfn1	Myh9	Nr4a1
Galnt2	Gprc5b	Hlx	Itgb5	Litaf	Mgll	Mynn	Nrbf1
Galnt7	Gpx3	Hmg20b	Itm2b	Lman1	Mina	Myo10	Nrm
Gamt	Gpx7	Hmgcs1	Itm2c	Lmna	Mint	Nab1	Nt5c
Gart	Grb14	Hmmr	Itpr5	Lmo1	Mkks	Nab2	Ntn1
Gas1	Grcc3f	Hnrpl	Jarid1c	Lnx1	Mlf2	Naglu	Ntn4
Gas7	Grhpr	Hnt	Jun	Lor	Mlycd	Nap1l5	Ntrk2
Gba	Gria3	Hod	Junb	Loxl3	Mmab	Nbl1	Ntrk3
Gca	Gria4	Hoxa1	Jundm2	Lpin1	Mmp15	Nbn	Nubp1
Gcat	Grid2	Hoxa7	Jup	Lpo	Moxd1	Ncald	Nucb
Gclm	Grim19	Hoxc6	Kai1	Lpp	Mpdu1	Ncam1	Nudt9
Gcn5l2	Grina	Hrasls3	Kcna2	Lpxn	Mpp1	Ncam2	Nup210
Gcs1	Grn	Hrsp12	Kcnc1	Lrp1	Mpp5	Ncl	Nupr1
Gdf1	Grp58	Hs2st1	Kcnd2	Lrp10	Mpp6	Ncor2	Nxph1
Gdf15	Gsn	Hsd17b12	Kcne1l	Lrp4	Mpra	Ncstn	Odf2
Gdpd2	Gsr	Hsd17b4	Kcnmb4	Lrrc4	Mpv17	Ndel1	Ogdh
Gem	Gstk1	Hsd3b7	Kctd4	Lrrc8	Mras	Ndst2	Ogg1
Gfer	Gstm1	Hspa1a	Kdelr2	Lrrfip1	Mrpl10	Ndufab1	Omg
Gfpt2	Gstm5	Hspa1b	Kdelr3	Lrrn1	Mrpl12	Ndufaf1	Oprs1
Ggh	Gstp1	Hspa9a	Keap1	Lss	Mrpl17	Ndufb2	Orc2l
Ggta1	Gstz1	Hspb1	Kif13a	Ltb4dh	Mrpl27	Ndufs2	Osbpl10

Ghitm	Gtf2h4	Hspb8	Kif18a	Ltbp1	Mrpl35	Ndufs5	Osbpl1a
Gja1	Gtl3	Htatip2	Kif1c	Ltbp3	Mrpl36	Ndufs7	Osbpl6
Ostf1	Pgd	Polr3c	Purb	Rgs7	Sdc2	Slc1a3	Socs5
Otub1	Pgk1	Polr3g	Pus1	Rhbdf1	Sdc3	Slc1a4	Sod2
Oxr1	Pgls	Pop5	Pvrl2	Rhoa	Sdc4	Slc20a2	Sorcs1
P2rx4	Pgm2	Ppap2b	Pvrl3	Rhoc	Sdf2l1	Slc22a17	Sorcs2
P4ha2	Pgrmc2	Ppargc1a	Pxmp2	Rhog	Sdf4	Slc24a3	Sort1
P4hb	Phgdh	Ppargc1b	Pxmp4	Rhoj	Sdfr2	Slc25a10	Sox10
Pa2g4	Phka1	Ppat	Pxn	Rhoq	Sdh1	Slc25a25	Sox3
Pacrg	Phkb	Ppfibp2	Pygb	Rnaset2	Sdha	Slc25a5	Sox6
Pacsin2	Phlda1	Ppgb	Pygl	Rnf128	Sdhc	Slc27a3	Sox9
Pald	Phlda3	Ppib	Qdpr	Rnf13	Sdhd	Slc29a1	Sp1
Papss1	Phospho1	Ppic	Qk	Rnf141	Sec14l1	Slc2a1	Spag5
Pard3	Phxr4	Ppp1r14b	Qscn6	Rnf26	Sec14l2	Slc2a3	Sparc
Parva	Phyhipl	Ppp2r4	Rab10	Rnpepl1	Sec61a1	Slc2a8	Sparcl1
Pax6	Pigk	Ppp2r5a	Rab13	Rod1	Sec8l1	Slc2a9	Spata6
Pcbp2	Pigm	Ppp2r5c	Rab14	Rorb	Sel1h	Slc31a1	Spec1
Pcca	Pigq	Ppp3cc	Rab34	Rp2h	Sema4b	Slc35a2	Spg20
Pcdh10	Pigs	Ppp5c	Rab3b	Rpl36al	Sema6a	Slc35a4	Spg7
Pcdh7	Pigt	Ppt1	Rab5c	Rpl37	Sep-15	Slc35b1	Spon1
Pcdhb10	Pik3r1	Pqlc2	Rab6	Rpn1	Sepm	Slc35f5	Spp1
Pcdhb11	Pim2	Prdx1	Rab7l1	Rpn2	Sepn1	Slc37a4	Spr
Pcdhb21	Pip5k2a	Prdx3	Rab9	Rpo1-3	Sepp1	Slc38a3	Spred1
Pcdhb9	Pkd1	Prdx4	Rabl3	Rps20	Sep-02	Slc39a1	Spred2
Pcdhga12	Pkm2	Prelp	Rad18	Rps27l	Sep-08	Slc39a7	Spry1
Pcnx	Pkn1	Prep	Rad23a	Rps6ka4	Sep-09	Slc3a2	Spry2
Pcolce	Pla2g12a	Prkaca	Rad51ap1	Rras	Serhl	Slc41a1	Spry4
Pcolce2	Pla2g6	Prkca	Rad51c	Rrbp1	Serpine2	Slc4a4	Sqrdl
Pcp4l1	Plat	Prkcb	Rad51l3	Rrm2	Serpinh1	Slc6a1	Srcasm
Pctk1	Plcb3	Prkcb	Raet1a	Rsu1	Sertad1	Slc6a8	Srd5a2l
Pcx	Plcd	Prkcdbp	Ralb	Rtkn	Sesn2	Slc6a9	Srebf1
Pcyt1a	Pld2	Prkcm	Ramp2	Rtn4ip1	Sesn3	Slc7a5	Srebf2
Pdcd6ip	Plec1	Prkcn	Ranbp9	Runx2	Sf3a2	Slc9a1	Sri
Pde4b	Plekha3	Prkwnk1	Rangnrf	Ryk	Sfrs9	Slc9a3r1	Srm
Pdgfa	Plekha4	Prnp	Rap1a	S100a1	Sfxn1	Slc9a3r2	Srprb
Pdgfra	Plekhe1	Prps2	Rap1b	S100a10	Sgce	Slitl2	Srr
Pdha1	Plekhk1	Prss15	Rap1ga1	S100a11	Sgpl1	Slmap	Ssb4
Pdir	Plk1	Psa	Rap1gds1	S100a13	Sgpp1	Smfn	Ssbp4
Pdk2	Plod1	Psap	Rap2b	S100a6	Sh3bp4	Smo	Sspn
Pdk3	Plod2	Psmb10	Rap2c	Sall2	Sh3d19	Smox	Ssr3
Pdk4	Plod3	Pstpip2	Rapgef3	Sall3	Sh3glb1	Smpd1	Ssr4
Pdlim3	Plp	Ptdsr	Rarb	Sardh	Shc3	Smpd2	Stard10
Pdlim4	Plscr1	Ptdsr	Rasgrf1	Sas	Shmt1	Smpdl3a	Stard5
Pdxk	Plvap	Ptdss2	Rassf3	Sat1	Shmt2	Smpdl3b	Stat1
Pdxk	Plxdc2	Pten	Rbms2	Sc4mol	Siah2	Smtn	Stat3
Pea15	Pmaip1	Pter	Rbpms	Scamp2	Siat10	Smyd2	Stat5b
Pecr	Pmf1	Ptgds	Rdh11	Scamp4	Siat6	Snag1	Stc1
Pelo	Pmm1	Ptk7	Rdh5	Scamp5	Siat7d	Snap23	Steap
Per1	Pmp22	Ptn	Rec8L1	Scap	Sirt2	Snapc2	Stk11ip
Per2	Pnkp	Ptp4a3	Recc1	Scarb1	Skp2	Sncaip	Stk17b
Perp	Pnn	Ptpla	Relb	Scd1	Slc12a4	Sntg2	Stk3
Pes1	Pnp	Ptpn13	Renbp	Schip1	Slc12a9	Snx3	Stoml2
Pfdn1	Pold2	Ptpns1	Repin1	Scn1b	Slc13a3	Snx5	Stx4a
Pfkfb3	Pold4	Ptpra	Rerg	Scotin	Slc16a2	Snx5	Stxbp3
Pfkl	Pole2	Ptprz1	Rest	Scpep1	Slc16a3	Snx6	Sulf2
Pfkp	Polg	Pttg1ip	Rga	Scube2	Slc19a2	Snx9	Suox
Pgam1	Polr2a	Pura	Rgl1	Sdbcag84	Slc1a1	Soat1	Surf4
Svil	Tm4sf7	Tspyl	Ysg2		B930041F14Rik		D15Ertd405e

Swap70
Syne1
Syngr1
Syngr2
Synj2
Sypl
Syt11
Sytl2
T2bp
Taf10
Taf7
Tagln2
Tap2
Tapbp
Tbc1d1
Tbc1d15
Tbc1d8
Tbl2
Tbrg4
Tceb3
Tcf4
Tcf7l2
Tcfcp2l1
Tcfl5
Tcirg1
Tcn2
Tdrd7
Tegt
Tenc1
Tesc
Tex15
Tex264
Tgfb2
Tgoln1
Thap7
Thrsp
Tieg1
Timm44
Timm8a
Timp1
Timp3
Tiparp
Tjp2
Tkt
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Tnk2
Tnrc15
Tom1
Tomm40
Tor2a
Tpcn1
Tpd52
Tpi
Tpk1
Tpm1
Tpm3
Tpmt
Tpr
Tra1
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Traf3
Traf4
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Trdn
Trib1
Trib3
Trim21
Trim47
Trim8
Trp53
Trps1
Tst
Ttyh2
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Txndc4
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Txnip
Tyro3
Ube2r2
Ubl3
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Ugt8
Upp1
Uqcr
Uqcrc2
Usp2
Usp31
Vamp3
Vangl1
Vapb
Vav3
Vcam1
Vcl
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Vdac2
Vegfa
Vegfb
Vim
Vkorc1
Vldlr
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Vrk2
Wasf2
Wasl
Waspip
Wbp2
Wdr1
Wdr10
Wfs1
Wig1
Wnt4
Wnt5a
Wwox
Xist
Xrcc5
Yaf2
Yes
Yif1
Ywhab
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Zdhhc2
Zdhhc6
Zfhx1a
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Zfp191
Zfp277
Zfp294
Zfp54
Zfp64
Zfp67
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AI181996
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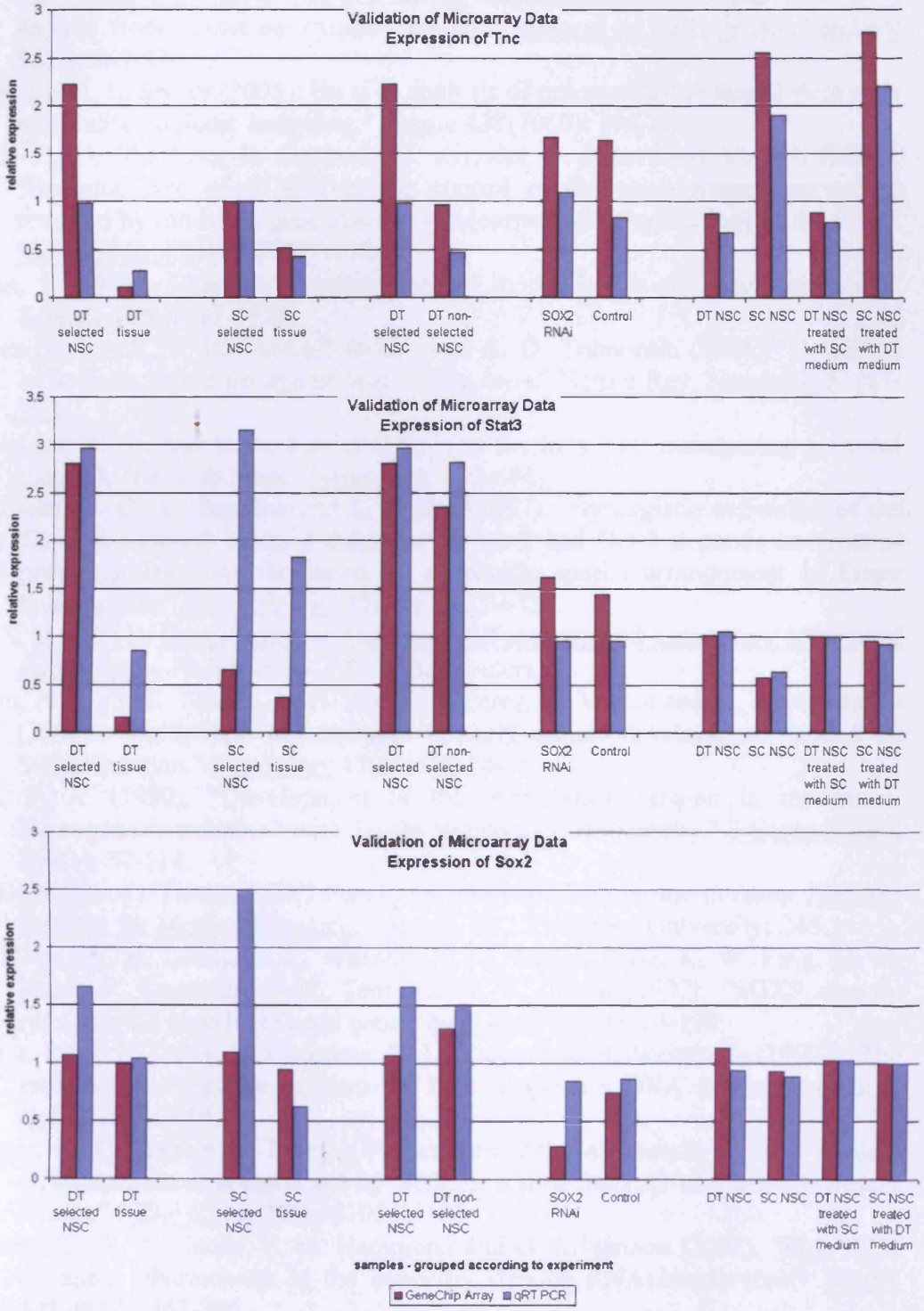
C76683
C87777
C920006C10Rik
D0HXS9928E
D10Bur2e
D10Bwg0940e
D10Ertd214e
D10Ucla1
D11Ertd498e
D11Ertd730e
D11Ertd99e
D11Wsu68e
D130038B21Rik
D15Ertd747e
D15Wsu75e
D16Bwg1494e
D16Wsu65e
D16Wsu65e
D17Wsu104e
D17Wsu92e
D17Wsu94e
D19Ertd678e
D19Ertd721e
D19Wsu12e
D1Bwg1363e
D1Ucla4
D2Ertd217e
D2Ertd750e
D430028G21Rik
D530020C15Rik
D530030D03Rik
D630048P19Rik
D6Wsu176e
D730049H07Rik
D7Ertd156e
D7Ertd458e
D830019K17Rik
D8Ertd325e
D8Ertd354e
D930010J01Rik
E030024M05Rik
E130016I23Rik
E130107N23Rik
E130112L23Rik
E130209G04Rik
E130307C13
E430007K15Rik
E430019H13Rik
E430021N18Rik
E430036I04Rik
LOC14433
LOC215866
MGC18837

Figure 9.2.1 qRT-PCR validation of all microarray experiments. Relative expression changes are shown for the selected representative genes *Tnc*, *Stat3* and *Sox2*, which largely reflect changes from the microarray analysis. Target hybridisations (x-axis) have been grouped together based on the experiment and the relative expression from the microarray analysis is shown next to the relative expression from the qRT-PCR analysis. The values are relative between all conditions for each gene investigated.



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