MONASH University

Multipotency and Stability of Mouse Embryonic Stem Cell Derived Neural Stem Cells

Thesis submitted for the degree of

DOCTOR OF PHILOSOPHY

By

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2014

Amendments

Page 27, line 5
delete "neurospheres consists of similar cell types to found from stem cell niches" and read "neurospheres consist of similar cell types found from stem cell niches"

Page 46, line 4
delete "hypothesised" and read "hypothesis"

Page 133 line 2
insert "we show that" and read "In this study we show that NS/NP cells can be derived from both monolayer and PA6 derived cultures"

Page 133, line 5
delete "ultimately differentiate these cells towards a GABAergic or dopaminergic fate" and read "ultimately these cells can be differentiated towards a GABAergic or dopaminergic fate".

Page 133, line 9
delete "gives rise" and read "give rise"

Page 137, line 18
delete "This study aims to generate neurosphere cultures from both monolayer and PA6 NI derived NS/NP cells were propagated as individuals up to three rounds" and read "This study aims to generate neurosphere cultures from both monolayer and PA6 NI derived NS/NP cells. These cells were propagated up to three rounds"

Page 165, line 11
delete "differentiates" and read "differentiate"

Page 166, line 4
delete "the presence of EGF and FGF2 is a poor method in the maintenance of Lmx1a-positive NS/NP cells" and read "the presence of EGF and FGF2 is a poor method for the maintenance of Lmx1a-positive NS/NP cells".

Page 169, line 10
delete "potentially lead to downstream effects" and read "it could potentially lead to downstream effects"

Page 172, line 11
delete "possesses" and read "possess"

Page 172, line 12
delete "individual neurosphere expanded cultures possesses different degrees of neurogenic and gliogenic potential was defined by immunoreactivity to βIII-tubulin and GFAP" and read "individual neurosphere expanded cultures possesses different degrees of neurogenic and gliogenic potential as defined by immunoreactivity to βIII-tubulin and GFAP"

Page 173, line 1
delete "This indicates that in the proportion of "stem" cells also rapidly decreased with each successive round of neurosphere formation" and read "that the proportion of "stem" cells also rapidly decreased with each successive round of neurosphere formation"

Page 174, line 12
delete "Lmx1a and at least one more regional specific marker would allow the isolation of enriched NS/NP populations possibly through gene expression analysis, and identification of selectively expressed surface markers" and read "Lmx1a and at least one more regional specific marker would allow the isolation of enriched NS/NP populations and identification of selectively expressed surface markers possibly through gene expression analysis"

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General Declaration

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes two original papers in peer reviewed journals The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.

Chapter 3: “Extended periods of neural induction and propagation of embryonic stem cell-derived neural progenitors with EGF and FGF2 enhances Lmx1a expression and neurogenic potential” are parts of the paper published in Neurochemistry International (2011)

Chapter 4: “Lmx1a Allows Context-Specific Isolation of Progenitors of GABAergic or Dopaminergic Neurons During Neural Differentiation of Embryonic Stem Cells” are parts of the paper published in Stem Cells (2012)

Wendy R. Zeng

Date

In the case of Chapter 3, my contribution to the work involved the following:

Name	**Nature of Contribution**
Wendy Zeng	Execution of experimental work, composition of manuscript (Est. 90% of total work)
Stewart Fabb	Generation of reporter cell lines (Est. 10% of total work)
John Haynes	Consultative role in planning and execution of experimental work, reviewing of manuscript drafts
Colin Pouton	Consultative role in planning and execution of experimental work, reviewing of manuscript drafts

In the case of chapter 4, my contribution to the work involved the following:

Name	**Nature of Contribution**
Wendy Zeng	Execution of experimental work (Est. 5% of total work)
Colin Su	Planning and execution of experimental work, composition of manuscript (Est. 35% of total work)
Christian Nefzger	Planning and execution of experimental work, composition of manuscript (Est. 35% of total work)
Stewart Fabb	Generation of reporter cell lines (Est. 10% of total work)
Brigham Hartley	Execution of experimental work (Est. 7.5% of total work)
Siew Beh	Execution of experimental work (Est. 7.5% of total work)
John Haynes	Consultative role in planning and execution of experimental work, reviewing of manuscript drafts
Colin Pouton	Consultative role in planning and execution of experimental work, reviewing of manuscript drafts

Declaration by co-authors

The undersigned hereby certify that:

1. They meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;

2. They take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;

3. There are no other authors of the publication according to these criteria;

4. Potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and

5. The original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

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Wendy Zeng	[redacted]	15/5/12
J	[redacted]	
C	[redacted]	

List of Publications

The following papers, resulting from the studies conducted in the thesis, were published in peer reviewed journals:

Wendy R. Zeng, Stewart R. Fabb, John M. Haynes, Colin W. Pouton.

Extended periods of neural induction and propagation of embryonic stem cell-derived neural progenitors with EGF and FGF2 enhances Lmx1a expression and neurogenic potential.

Neurochemistry International. 2011 Sep;59(3):394-403

Christian M. Nefzger, Colin T. Su, Stewart A. Fabb, Brigham J. Hartley, Siew J. Beh, **Wendy R. Zeng**, John M. Haynes, Colin W. Pouton.

Lmx1a Allows Context-Specific Isolation of Progenitors of GABAergic or Dopaminergic Neurons During Neural Differentiation of Embryonic Stem Cells.

Stem Cells. 2012 Apr 11. 2012 Jul; 30(7): 1349-1361

The following communications, resulting from the studies conducted in this thesis, were presented as conference abstracts:

***Wendy R. Zeng**, Stewart R. Fabb, Colin W. Pouton, John M. Haynes.*

Long term maintenance directs ES cell-derived neural stem cells towards a forebrain GABAergic phenotype.

Australian Neuroscience Society (ANS) Conference 2012

January 29-February 1 (Gold Coast, Australia)

***Wendy R. Zeng**, Stewart R. Fabb, Colin W. Pouton, John M. Haynes.*

Long term maintenance directs ES cell-derived neural stem cells towards a forebrain GABAergic phenotype.

Australasian Society for Stem Cell Research (ASSCR) Conference 2011

October 23-29 (Leura, Australia)

***Wendy R. Zeng**, Warren S. Raye, Colin W. Pouton, John M. Haynes.*

Effect of Duration of Neural Conversion on Multipotency of Mouse Neural Stem Cells.

Australasian Society for Stem Cell Research (ASSCR) conference 2009

November 22-24 (Canberra, Australia)

***Wendy R. Zeng**, Warren S. Raye, Colin W. Pouton, John M. Haynes.*

The Multipotency of Mouse Neural Stem Cells.

Monash Annual Postgraduate Research Symposium 2009

September 30 (Parkville, Australia)

***Wendy R. Zeng**, Warren S. Raye, Colin W. Pouton, John M. Haynes.*

Effect of Duration of Neural Conversion on Multipotency of Mouse Neural Stem Cells.

International Society for Stem Cell Research (ISSCR) conference 2009

July 8-11. (Barcelona, Spain)

Acknowledgements

First and foremost I wish to express my sincere gratitude to my primary supervisor, Dr. John M. Haynes for his continuous support of my PhD study and research. I appreciate all his stimulating suggestions, patience and guidance at various stages of my research. He has provided growth for my professional career over the years in more ways than I can express. I am also grateful to my co-supervisor Prof. Colin W. Pouton for his invaluable insights and helpful suggestions.

Very special thanks to the stem cell group; the group have contributed immensely to my personal and professional time at Monash. You have been a source of friendships as

well as good advice and collaborations. I would like to particularly acknowledge the contribution of Christian Nefzger, Simer Khaira and Ben Finnin for introducing me to different experimental techniques and also for your helpful ideas and support.

Amongst my fellow postgraduate students at Monash, I owe my deepest gratitude to my close friends who provided a stimulating and fun environment in which to learn and grow, Jen La, Colin Su, Thiam Chua and Ann Lim, thank you for the countless memorable moments we shared during this tough journey, the 2009 Parkville Postgraduate Association will always be the best committee!

Lastly, my loving thanks go to my family for all their support and encouragement; emotionally, morally and financially. For my parents who raised me with a love of science and supported me in all my pursuits, for the personal sacrifices they made to allow me to reach new levels of excellence and most of all, for my loving, supportive and encouraging fiancé Quang (my go to and cry person), your faithful support during my PhD study is deeply appreciated. No matter what happens today, “Tomorrow’s another day...”

Summary

Neuronal cell loss is a common feature of many neurological disorders, including stroke, Parkinson's disease, Alzheimer's disease and traumatic brain injury. Embryonic stem (ES) cells and ES cell-derived neural stem cells or progenitor (NS/NP) cells may provide a number of new ways for studying and treating diseases and injuries in the brain. NS/NP cells derived from embryonic stem cells, isolated from the fetal or adult central nervous system (CNS) are defined by their abilities to self-renew and differentiate into neurons, astrocytes and oligodendrocytes. Their growth and specialisation is dependent on environmental cues, such as media formulation and exposure to patterning growth factors. It is important to study the propagation and differentiation characteristics of ES

cell derived NS/NP cells because contamination of ES cell derived cultures with pluripotent cells or unwanted phenotypes is a practical problem that may result in false positive hits in high through-put screening and tumour formations in replacement therapies. If NS or NP cells are used as a starting point, the likelihood of contamination is reduced. These cells potentially represent an unlimited source for neuron replacement therapies; they are also a suitable source of differentiated cells for studying functional genomics, proteomics or for drug screening; and they allow us to study early brain development.

The first experimental chapter (Chapter 3) investigated the effect of altering the period of neural induction (NI) by exposing ES cells to the neural inducing media N2B27 for up to 10 days prior to forming neurospheres and growing cells in EGF and FGF2 conditions which are widely used to propagate NS cells. The ability of the ES cell-derived NS/NP cells to generate dopaminergic, serotonergic, cholinergic and gamma aminobutyric acid (GABA)ergic neurons was assessed using immunocytochemistry. Extending the NI period to 10 days, prior to the generation of neurospheres, and subsequent expansion as monolayer cultures, gave rise to more multipotent NS/NP cells that were able to generate phenotypically diverse neurons with very small numbers of residual NS/NP cells and astrocytes.

The second experimental chapter (Chapter 4) explored the effect of long term maintenance of ES cell derived NS/NP cells in the presence of EGF and FGF2, and investigated their ability to give rise to catecholaminergic and GABAergic neurons after extended propagation. Lmx1a reporter cells were used to identify cells at the NP stage using flow cytometry. It was found that the presence of EGF and FGF2 was not sufficient

to stabilise Lmx1a-positive NP cells in either monolayer or PA6 co-culture during long term maintenance. Although the neurogenic potential of these cells remained stable over 10 passages; the percentage of catecholaminergic neurons reduced dramatically.

The last experimental chapter (Chapter 5) investigated the isolation and propagation of clonal NS/NP populations via neurospheres using low numbers of cells. Neurosphere formation in the presence of EGF and FGF2 was shown to be a poor method for the maintenance of Lmx1a-positive NP cells since the proliferation and neurosphere formation capability of these cells declined dramatically over the three passages. When neurospheres were formed with 10 cells, progressive loss of neurogenic potential with passaging and poor yield of neurons after exposure to patterning cues indicated that this culture method was unable to adequately support the propagation of NS/NP cells.

List of Abbreviations

5HT	Serotonin
AA	Ascorbic acid
ALDH	Aldehyde dehydrogenase
APC	Adenomatous polyposis coli
Ascl1	Achaete-scute homolog 1
BDNF	Brain neurotrophic factor
bHLH	Basic helix-loop-helix
BLBP	Brain lipid binding protein
BMP	Bone morphogenic protein
BSA	Bovine serum albumin

CCF	Cephalosporin linked 7-hydroxycoumarin fluorescein
ChAT	Choline acetyltransferase
CNS	Central nervous system
cRET	RET receptor tyrosine kinase
DAT	Dopamine transporter
DG	Dentate gyrus
DKK1	Dickkopf
Dlx2	Distal-less homeobox 2
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DVL	Dishevelled
EB	Embryoid body
EC cells	Embryonal carcinoma cells
EFNB1	Ephrin B1
EGF	Epidermal growth factor
eGFP	Enhanced green fluorescent protein
EGFR	Epidermal growth factor receptor
En1	Engrailed 1
En2	Engrailed 2
ES cell	Embryonic stem cell
FACS	Fluorescent activated cell sorting
FGFR	Fibroblast growth factor receptor
FGF	Fibroblast growth factor
Forse1	Forebrain surface embryonic 1
FoxA1	Forkhead box protein A1
FoxA2	Forkhead box protein A2

FoxG1	Forkhead box protein G1
Fz	Fizzled
GABA	Gamma aminobutyric acid
GAD1	Glutamic acid decarboxylase 1
Gbx2	Gastrulation brain homeobox 2
GDF6	Growth and differentiation factor 6
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
GLAST	Glutamate astrocyte-specific transporter
Gli2	GLI family zinc finger 2
gp130	Signal transducer glycoprotein 130
GSK3β	Glycogen synthase kinase 3β
hA	Horizontal astrocytes
hES cell	Human embryonic stem cell
HMBA	Hexamethylene bisacetamide
Hoxb4	Homeobox protein Hox-2.6
Hoxb9	Homeobox protein Hox-2.5
ICM	Inner cell mass
Id	Inhibitor of differentiation
IGF2	Insulin like growth factor 2
iPS cell	Induced pluripotent stem cell
Irx3	Iroquois homeobox 3
Islet1	Insulin gene enhancer protein 1
JAK	Janus kinase
JNK	Jun N-terminal kinase
LEF1	Lymphoid enhancer-binding factor 1

LIF	Leukaemia inhibitory factor
LIFR	Leukaemia inhibitory factor receptor
Lmx1a	LIM homeobox transcription factor 1α
Lmx1b	LIM homeobox transcription factor 1β
MAPK	Mitogen activated protein kinase
MEK	Mitogen activated protein kinase/extracellular signal regulated kinas
MEF	Mouse embryonic fibroblast
mES cell	Mouse embryonic stem cell
MHO	Mid-hindbrain organizer
Msx1	Msh homeobox 1
NCAM	Neural cell adhesion molecule
NEP cell	Neuroepithelial progenitor cell
NeuN	Neuronal nuclei
NG2	Neuron-glial antigen 2
NGF	Nerve growth factor
Ngn2	Neurogenin 2
NI	Neural induction
NS cell	Neural stem cell
Nurr1	Nuclear receptor related factor 1
OB	Olfactory bulb
Oct3	Octamer binding transcription factor 3
Oct4	Octamer binding transcription factor 4
Otx2	Orthodenticle homeobox 2
Pax2	Paired box protein 2
Pax6	Paired box protein 6

Pitx3	Paired homeodomain transcription factor 3
PSA-NCAM	Polysialylated neuronal cell adhesion molecule
PTN	Pleiotrophin
rA	Radial astrocytes
RA	Retinoic acid
rG	Radial glia
RNA	Ribonucleic acid
rNS cell	Rosette neural stem cell
RMS	Rostral migratory stream
RRF	Retrorubal field
SatB2	Special AT-rich sequence-binding protein 2
SDF1	Stromal cell-derived factor 1
SDIA	Stromal cell derived inducing activity
SGZ	Subgranular zone
Shh	Sonic hedgehog
SMAD	Sma and Mad related family
SNpc	Substantia nigra pars compacta
Sox1	Sex determining region Y box 1
Sox2	Sex determining region Y box 2
STAT 3	signal transducer and activation of transcription 3
SVZ	Subventricular zone
T3	Triiodthyrone hormone
TGFβ	Transforming growth factor β
TH	Tyrosine hydroxylase
TNFα	Tumor necrosis factor α
VMAT2	Vesicular monoamine transporter 2

VTA	Ventral tegmental area
Wnt	Wingless
ZO1	Apocal zonula occludent 1

Units

nm	Nanometre
µm	Micrometre
cm	Centimetre
µL	Microliter
mL	Millimetre
L	Litre
ng	Nanogram
µg	Microgram
g	Gram
µM	Micromolar
mM	Millimolar
M	Molar
U	Unit
xg	g-force
v/v	Volume per unit volume

Chapter One

1. General Introduction

1.1 Stem cells

The term “stem cell” originated from the German word “Stammzellen” used by Max Askanazy in 1907 in one of the earliest publications to describe stem cells (Maehle, 2011). In the beginning of the last century, the concept of stem cells was proposed by Russian histologist Alexander Maximow, confirming the theory of hematopoiesis (Konstantinov, 2000). Thomas et al. (1957) and McCullouch and Till (1960) then established in both rodents and humans that self-renewable cells in the bone marrow gave rise to different types of blood cells. It was not until the mid-1960s that Altman and Das (Altman & Das, 1965b, 1966, 1967) reported neurogenesis in the brain and

the first pluripotent cells were isolated from highly malignant tumours that arose when mouse embryos are transplanted to an extrauterine site in a histocompatible host. These cells are known as embryonal carcinoma (EC) cells. These cells are able to be propagated in the presence of feeder cells and able give rise to all of the primary germ layers: endoderm, mesoderm and ectoderm (G. M. Keller, 1995; Martin & Evans, 1974). EC cells accumulated abnormal karyotypes during development, restricting the stability of these cells to differentiate into mature cells (Smith, 2001), A great breakthrough in stem cell research was made when two groups, Evans and Kaufman (1981) and Martin (1981) reported the existence of a small population of cells found within the blastocyst of a pre-implantation embryo, that retained the essential properties of teratocarcinoma stem cells when cultured in EC cell conditioned medium. These extraordinary cells were diploid, capable of tumour formation when injected in athymic mice and had the ability to generate the entire embryo, which provided the foundation for stem cell research (Martin, 1981).

1.1.1 Definition and classification

Stem cells are characterised by broad differentiation potential and indefinite capacity for self-renewal (Baizabal, Furlan-Magaril, Santa-Olalla, & Covarrubias, 2003; G. M. Keller, 1995). They are the origin of every tissue and organ of the body and are sub-grouped according to their differentiation potential and are defined by their ability to self-renew for long periods while remaining unspecialised. Stem cells are able to divide symmetrically, giving rise to two daughter cells that are identical to the parent, retaining their stem cell identity; or also asymmetrically, generating one identical daughter stem cell and a more specialised cell (Figure 1.1) (Gotz & Huttner, 2005; Morrison, Shah, & Anderson, 1997). The adoption of speciality or differentiation for a stem cell is a complex process during which, certain genes become activated and

others become inactivated via numerous signalling pathways, as a result, differentiated cells develop defined structures and perform specialised functions.

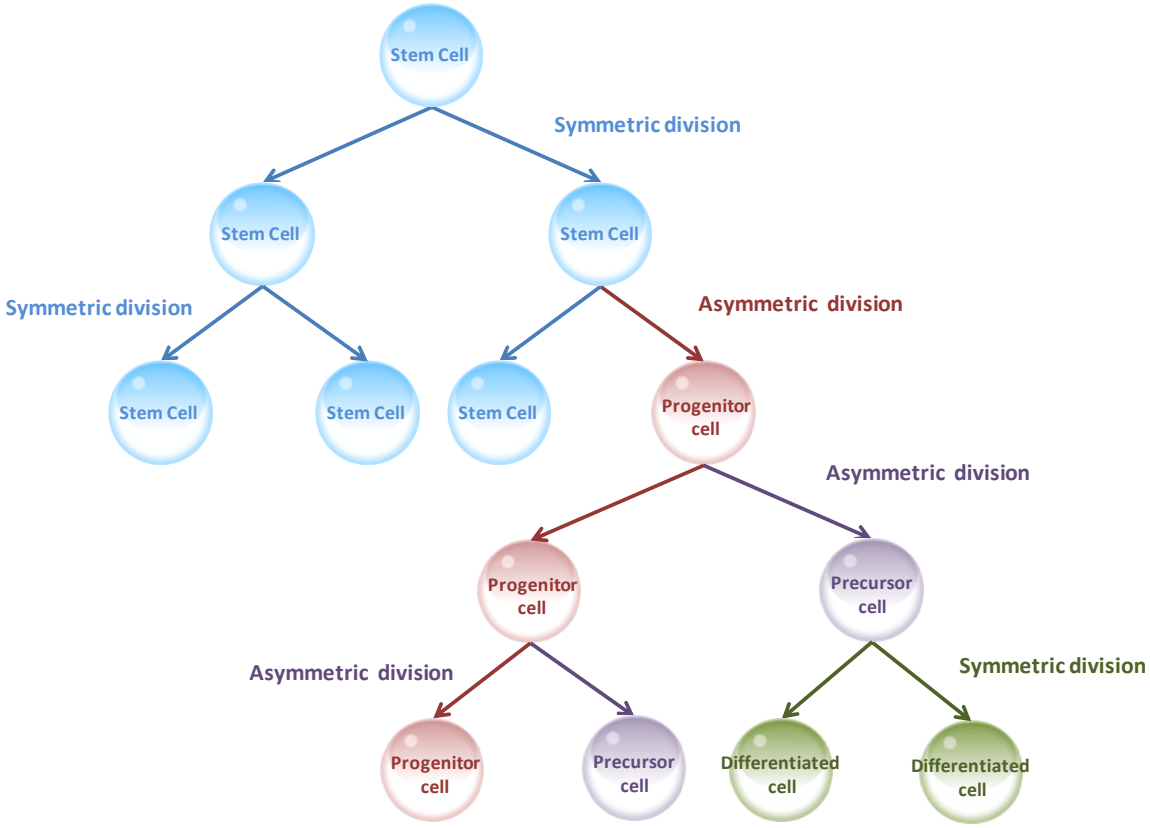


Figure 1.1 Symmetric cell division gives rise to two identical daughter stem cells. Asymmetric cell division gives rise to one daughter stem cell and one differentiated precursor cell.

The different subgroups of stem cells are: totipotent, pluripotent, multipotent and unipotent and their differentiation potential become more restricted as they differentiate. The fertilised oocyte (the zygote) and the daughter cells of the first few divisions are totipotent stem cells. They are able to give rise to all cell types of the body as well as extra-embryonic tissues, such as the trophoblasts of the placenta.

Four days later, these totipotent cells begin to differentiate and form a hollow ball of cells, known as the blastocyst. Within this cluster of cells lies the inner cell mass (ICM), which can be isolated and maintained indefinitely as an embryonic stem (ES) cell culture (Alison, Poulsom, Forbes, & Wright, 2002; Bishop, Buttery, & Polak, 2002; Gottlieb, 2002). However, unlike totipotent cells, ES cells are unable to generate the extra-embryonic tissues.

Within the tissues of the adult, certain cells types have the capacity to generate cells of limited range of cell lineages specific the tissue where they reside. This type of stem cell is referred to as an adult stem cell, examples of these cells include haematopoietic, mesenchymal and neural stem (NS) cells. Unlike pluripotent cells, multipotent adult stem cells are found in almost all organs and tissues. They are rare and are often located in special niches that provide a suitable environment for regulating the stem cell. Adult stem cells respond to the needs of their surroundings, enabling tissue repair and maintaining homeostasis within the organ. Today, we are aware that adult stem cells exist within almost all organs in the human body. Organs such as skin and blood that have a high turnover of cells have stem cells with much higher proliferative capacity than those that are better protected such as the heart or central nervous system (CNS). Multipotent cells can differentiate further into unipotent stem cells, cells that are capable of generating one specific cell type whilst maintaining their own population. They are classified as stem cells since they are able to self-renew indefinitely and are commonly found in skin and liver. Ultimately, unipotent cells give rise to terminally differentiated cells of adult tissues or organs that lack the ability to divide (Figure 1.2).

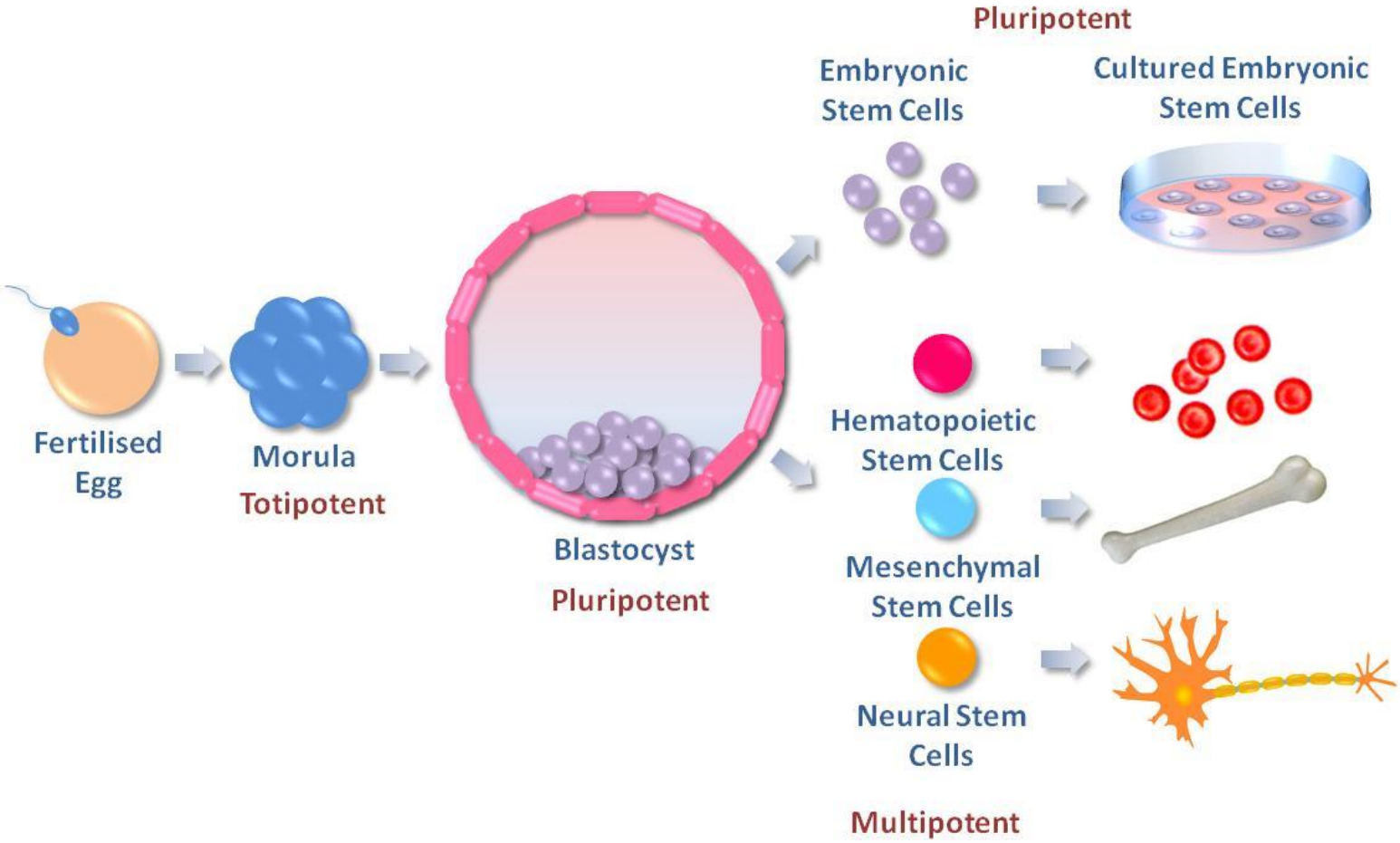


Figure 1.2 Pluripotent ES cells isolated from the ICM of a blastocyst can be maintained in an undifferentiated state *in vitro*. They are able to give rise to multipotent stem cells and ultimately generate all cell types found in the body. Only totipotent stem cells isolated from the morula are able to give rise to all cell types of the body as well as extra embryonic tissues. As development proceeds, cells lose their capacity to proliferate and differentiate into specialised cell types.

1.1.2 Stem cell application

Stem cells hold great promise for therapeutics, drug discovery and for the treatment of diseases, including, cardiovascular diseases, Parkinson's disease, blood cell diseases, and certain types of liver diseases, that currently lack effective treatments (Barberi et al., 2003; Brustle et al., 1999; G. M. Keller, 1995; J. H. Kim et al., 2002; Lee, Lumelsky, Studer, Auerbach, & McKay, 2000; Morizane et al., 2006; O'Shea, 1999; Okano, Yoshizaki, Shimazaki, & Sawamoto, 2002; Perrier & Studer, 2003).

ES cells can be used for the establishment of *in vitro* models to study the cascade of gene expression during lineage development and differentiation (O'Shea, 1999). One of the benefits of ES cells is that they are very amenable to genetic mutations, amongst these manipulations, lacZ, green fluorescent protein (GFP) or luciferase can be targeted to loci that are activated during development (Baizabal et al., 2003; Friedrich & Soriano, 1991; G. M. Keller, 1995; Thomas & Capecchi, 1987; Ying, Stavridis, Griffiths, Li, & Smith, 2003). ES cell models also have widespread applications in drug discovery. Cell types such as cardiomyocytes and hepatocytes generated from human ES (hES) cells can now be used for high throughput screening and predictive toxicology studies that used to rely on animal models (G. Keller, 2005). However the lack of homogeneous populations of differentiated cells, limits their use (Bibel et al., 2004). Furthermore, there are ethical concerns around the production of ES cell lines which requires the destruction of embryos. The generation of adult stem cell lines for therapeutic purposes is less controversial, since it does not involve the destruction of embryos. Arguably, adult stem cells were the preferred type of stem cells used in therapeutics. In recent times, the emergence of induced pluripotent (iPS) cell reprogramming technology may have addressed many of the problems associated with the use of ES cells. In addition, customised patient specific cells can be made for patients with Parkinson's disease,

Huntington's disease, amyotrophic lateral sclerosis and spinal muscular atrophy (Dimos et al., 2008; Ebert et al., 2009; Osakada & Takahashi, 2011; Soldner et al., 2009).

1.2 Embryonic Stem Cells

1.2.1 Mouse embryonic stem cells

Following the discovery of mouse ES (mES) cells, they have been routinely cultured on a layer of mitotically inactive mouse embryonic fibroblasts (MEFs) to maintain self-renewal and pluripotency (Evans & Kaufman, 1981; Martin, 1981). Amongst the presence of different factors that maintains mES cell pluripotency, leukaemia inhibitory factor (LIF) plays a pivotal role (Smith et al., 1988; Williams et al., 1988). LIF binds to the low affinity LIF receptor (LIFR), which together with the signal transducer glycoprotein 130 (gp130) forms a heterodimer (Matsuda et al., 1999), that activates the Janus kinase-signal transducer and activator of transcription 3 (JAK/STAT3) pathway, to maintain mES cell self-renewal (Boulton, Stahl, & Yancopoulos, 1994). However, the activation of LIFR alone is insufficient for mES cell maintenance (Niwa, Burdon, Chambers, & Smith, 1998; Starr et al., 1997), bone morphogenetic proteins (BMPs) signalling is also required through the Sma and Mad Related Family (SMAD) proteins (Ying, Nichols, Chambers, & Smith, 2003). SmAD signalling promotes the expression of inhibitor of differentiation (Id), helix-loop-helix domain proteins that dimerise, and inhibit the function of, helix-loop-helix transcription factors that regulate fate determination (Ying, Nichols, et al., 2003). LIF/JAK/STAT3 and BMP/SmAD/Id signalling pathways work together to prevent the differentiation of mES cells in culture by inhibiting the effect of mitogen-activated protein kinase (MAPK) signalling, which is known to promote differentiation (Ying, Nichols, et al., 2003). In addition, bone morphogenic protein 2 (BMP2) and growth and differentiation factor 6 (GDF6) has also shown to be an alternative chemically defined serum free culture condition for mES cell maintenance (Ying, Nichols, et al., 2003).

1.2.2 Human embryonic stem cells

Few mammalian species have yielded long term cultures of ES cells. The first primate ES cells were derived from a rhesus monkey by Thomson (1995) and three years later, the same group derived the first hES cells (Thomson et al., 1998). The ultimate potency test for stem cells is whether they have the capacity to give rise to germline transmission upon injection into a blastocyst or morula stage embryo. While this test can be performed for rodent ES cells, it cannot be carried out on hES cells due to ethical reasons, leaving room for speculation concerning the capacity of hES cells (Smith, 2001).

Unlike mES cells, hES cell self-renewal cannot be maintained in the presence of LIF and serum (Thomson et al., 1998). Factors such as fibroblast growth factor (FGF) and transforming growth factor β (TGFβ)/Activin/Nodal signalling pathways have been identified as key signalling regulators involved in hES cell self-renew and pluripotency (Beattie et al., 2005; James, Levine, Besser, & Hemmati-Brivanlou, 2005; Vallier, Alexander, & Pedersen, 2005; C. Xu et al., 2005). These factors allow for feeder free, serum free propagation of hES cells. The addition of fibroblastic growth factor 2 (FGF2), is crucial in the maintenance of hES cells (Amit et al., 2000; Diecke, Quiroga-Negreira, Redmer, & Besser, 2008; Levenstein et al., 2006). FGF2 maintains hES cells by working in conjunction with Wingless (Wnt), TGFβ, Hedgehog and Notch pathways to promote self-renewal, and antagonises BMP pathway which promotes trophoblasts formation (Delaune, Lemaire, & Kodjabachian, 2005; Dhara et al., 2008; Greber, Lehrach, & Adjaye, 2007; R. H. Xu et al., 2002). Controversy in literature still exists over the dependence of the mitogen activated protein kinase/extracellular signal regulated kinase (MEK) pathway (Diecke et al., 2008; Dvorak & Hampl, 2005; Li et al., 2007). Other studies suggest that by cooperating with Activin A, FGF2 modulates transforming growth factor β1 (TGFβ1) signalling to support self-renewal (Beattie et al., 2005; Greber et al.,

2007). The activation of TGFβ1 signalling such as Activin and Nodal stimulates the SMAD pathway (James et al., 2005). Ultimately the combination of the Activin/Nodal cooperates with the FGF pathway to maintain the self-renewal of hES cells (Vallier et al., 2005).

1.3 Neural Stem Cells

Long before the emergence of stem cell research, vertebrates such as fish and newts were found to have continuous neural regenerative capabilities through their entire adulthood. Mammals were believed to have lost this ability through evolution since the human brain is considered too sophisticated to maintain this primitive function. In the beginning of the last century, it was a long held belief within the scientific community that "nothing may be generated" in the CNS following initial development. This idea was widely accepted until 1965, when the presence of neurogenesis in the adult mammalian brain was reported (Altman & Das, 1965a) and was later confirmed by Kaplan (1981) and Gratzner (1982). Stem-like cells were subsequently isolated from the embryonic mammalian CNS (Cattaneo & McKay, 1990; Kilpatrick & Bartlett, 1993; Reynolds & Weiss, 1992; Temple, 1989). Initially, NS cells from the forebrain were cultured *in vitro* as free floating clusters known as neurospheres, which gave rise to secondary spheres, displaying self-renewing behaviour. They also showed multipotent characteristics once subject to differentiation (Reynolds & Weiss, 1992, 1996; Richards, Kilpatrick, Bartlett, & Murphy, 1992). Following this discovery, NS cells were isolated from the basal forebrain, cerebral cortex, hippocampus, cerebellum and spinal cord (Gage, 2000; Laywell, Rakic, Kukekov, Holland, & Steindler, 2000; McKay, 1997; Rao, 1999). They have also been extracted from the neural crest (Stemple & Anderson, 1992) and were able to generate sympathetic and sensory neurons, and Schwann cells. It was then realised that NS cells have great potential in replacement therapies, drug discovery and developmental studies. NS cells meet the significant requirements for clinical and drug discovery applications, they are: readily available, expandable and karyotypically stable *in vitro*; differentiate in an appropriate manner and are able to generate functional neural cells that integrate appropriately and consequently mediate functional repair. Not only can NS cells be derived from brain tissues, they can also be derived from pluripotent ES and iPS cells

and reprogrammed fibroblasts (Brustle et al., 1999; Thomson et al., 1998; Wernig et al., 2008).

1.3.1 Neural stem/progenitor cells *in vivo*

NS cells and neural progenitors (NPs) from different stages of neural development can be found in the fetal and adult brain. Mammalian neurogenesis begins with neural induction (NI), followed by neural plate formation and subsequent neural tube formation. This is then followed by neural patterning, specification and maturation. The identification of early stage-specific neural markers allowed the identification of NS cells, NPs and neural precursors during neural development.

1.3.1.1 Neural stem/progenitors in the developing brain

One of the earliest known neural progenitor markers is sex determining region Y box 1 (Sox1) (Pevny, Sockanathan, Placzek, & Lovell-Badge, 1998). Sox1-positive neuroepithelial progenitor (NEP) cells are the most primitive stage after NI (Tropepe et al., 2001). At E5.5-7.5 in mouse embryos, LIF dependent “primitive” NPs with similar antigenic and functional properties with forkhead box protein G1 (FoxG1)-positive NEP cells can be isolated. However, these cells are transient and cannot be maintained *in vitro,* and readily differentiate into a later stage NEP cells known as rosette NS (rNS) cells. rNS cells form a unique rosette like structure that express the tight junction marker apical zonula occluden 1 (ZO1) and anterior NEP marker FoxG1. They can be maintained *in vitro* in the presence of sonic hedgehog (Shh) and Notch receptor antagonists. By utilising cell sorting methods, it has been shown that rNS cells can be isolated using the forebrain surface embryonic 1 (Forse1) epitope and N-cadherin markers from E8.25 anterior neural plate tissue or neuralised hES cells. Paired box

protein 6 (Pax6) positive-radial glia (rG) is another type of neural progenitor cell type found in the neural tube (E9.5 in mice), like NEPs, they are transient heterogeneous populations. Efforts have been made in the isolation of these cells *in vivo* using a transgenic mouse line, expressing eGFP under the control of rG makers such as glutamate astrocyte-specific transporter (GLAST), brain lipid binding protein (BLBP) and human glial fibrillary acidic protein (GFAP) by fluorescence activated cell sorting (FACS) methods. rG are heterogeneous *in vivo*; isolated from E14.5-16.5 in mice they give rise to predominantly neurons (60-70%), however by E18 rGs gives are largely gliogenic in nature (Figure 1.3).

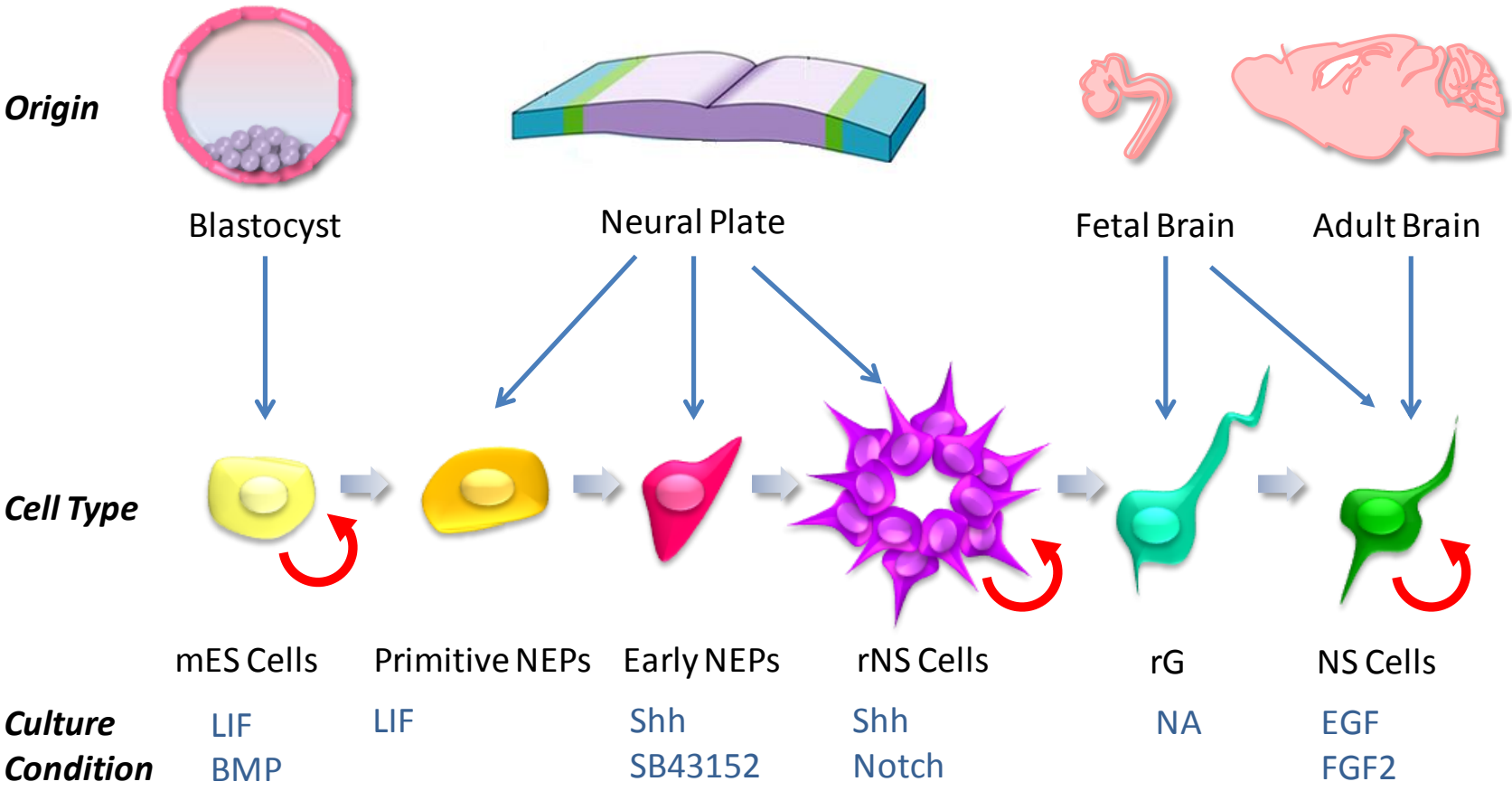


Figure 1.3 Specific NS/NP cells can be derived from different stages of neural development. These cells have different characteristics and require different culture conditions.

1.3.1.2 Neural stem/progenitors in the adult brain

In the adult CNS, NS cells are found only in two major tissue specific niches in the subventricular zone (SVZ) of telencephalic lateral ventricles (Figure1.4A and B) and the subgranular zone (SGZ) of the hippocampal dentate gyrus (DG) (Figure1.4 A and C) (Cassidy & Frisen, 2001; Doetsch, Garcia-Verdugo, & Alvarez-Buylla, 1999). NS cells from the Subventricular zone/olfactory bulb (SVZ/OB) give rise to oligodendrocytes (Jackson & Alvarez-Buylla, 2008) while the NS cells from the DG give rise to SGZ astrocytes (Suh et al., 2007). Other than these regions, NS like cells can also be found in other areas but show lower differentiation potential (Gage, 2000; Jensen & Parmar, 2006; Kornblum, 2007; McKay, 1997; Temple, 2001).

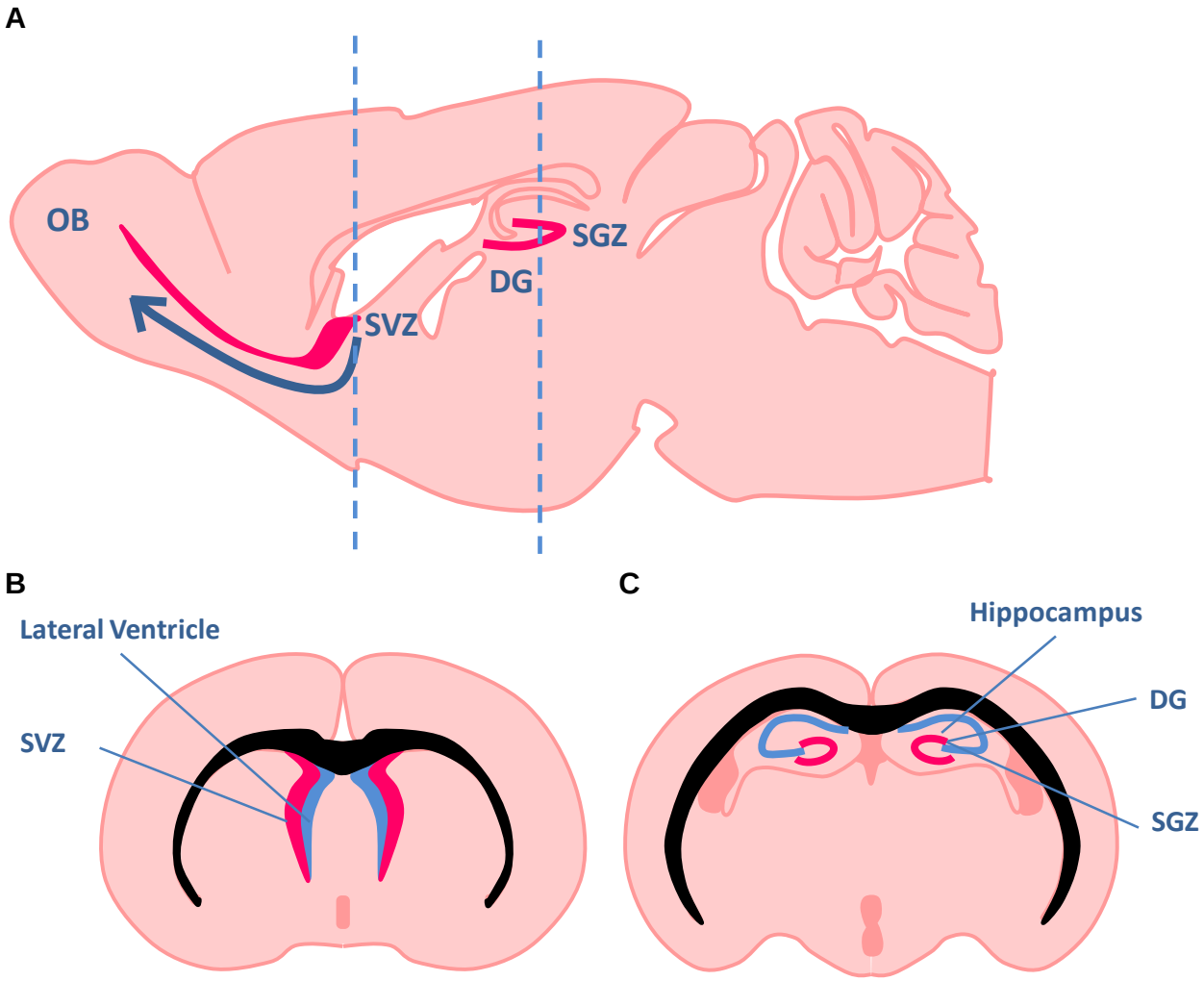


Figure 1.4 (A) Sagittal and (B and C) coronal sections of the mouse brain. NS cells (type B cells) can be found in along the lateral ventricles in the SVZ (B) or the hippocampus at the SGZ (C) in the adult brain. The SVZ NS cells follow the RMS to the OB and the SGZ NS cells migrates to the DG where they differentiate into mature OB or DG neurons.

The lack of unambiguous stem cell marker has made it difficult to study the stem cell population. NS cells are now identified by a set of markers and functional characteristics, such as localisation, morphology, cell cycle properties together with stem cell characteristics: multipotency and self-renewal. NS cells are identified by their immunoreactivity to the intermediate filament protein, Nestin, and the ribonucleic acid (RNA) binding protein Musashi1 (Lendahl, Zimmerman, & McKay, 1990), BLBP

(Rousselot, Heintz, & Nottebohm, 1997), GFAP (Doetsch, Caille, Lim, Garcia-Verdugo, & Alvarez-Buylla, 1999; Doetsch, Garcia-Verdugo, et al., 1999), sex determining region Y box 2 (Sox2) (D'Amour & Gage, 2003), and Prominin1 (Coskun et al., 2008; Uchida et al., 2000). However, NS cells from different regions display different properties. For example, NS cells found in the SVZ express the mature astrocyte marker GFAP and the ependymal and stem cell marker Prominin1 (Marzesco et al., 2005; Weigmann, Corbeil, Hellwig, & Huttner, 1997) and are found intermingled between the ependymal cells in the lateral wall, extending one single cilia into the ventricle (Doetsch, Caille, et al., 1999; Garcia-Verdugo, Doetsch, Wichterle, Lim, & Alvarez-Buylla, 1998; Mirzadeh, Merkle, Soriano-Navarro, Garcia-Verdugo, & Alvarez-Buylla, 2008; Shen et al., 2008). Subpopulations of these GFAP positive cells are proliferative and display bipolar properties and are able to give rise to mature neurons populating both the OB and hippocampus (Doetsch, Caille, et al., 1999; Garcia, Doan, Imura, Bush, & Sofroniew, 2004; Imura, Kornblum, & Sofroniew, 2003; Laywell et al., 2000; Seri, Garcia-Verdugo, McEwen, & Alvarez-Buylla, 2001).

1.3.1.2.1 Subventricular zone

The SVZ niche consists of at least four different types of cells: ependymal cells, NS cells (type B cells), transient amplifying cells (type C cells) and neuroblasts (type A cells). The multiciliated ependymal cells are post mitotic cells that line the lateral ventricle that directs neuroblast migration along the lateral wall with their beating cilia (Mirzadeh, Han, Soriano-Navarro, Garcia-Verdugo, & Alvarez-Buylla, 2011). The NS cells, also known as type B cells are adjacent to the ependymal cell layer and are GFAP expressing, astroglial-like cells that give rise to the highly proliferative transient amplifying cells, also known as type C cells (Doetsch, Caille, et al., 1999). Type C cells express the protein markers Dlx2 (Distal-less homeobox 2), Ascl1 (Achaete-scute homolog 1) and fibroblastic growth factor receptor (FGFR) and gives rise to the polysialylated neuronal cell adhesion molecule (PSA-NCAM) positive neuroblasts, also known as type A cells. Type A cells then travels through the rostral migratory stream (RMS) into the granular and periglomerular layers and ultimately differentiate into olfactory granule neurons at the OB (Figure 1.4A and 1.5) (Doetsch, Garcia-Verdugo, & Alvarez-Buylla, 1997; Garcia-Verdugo et al., 1998).

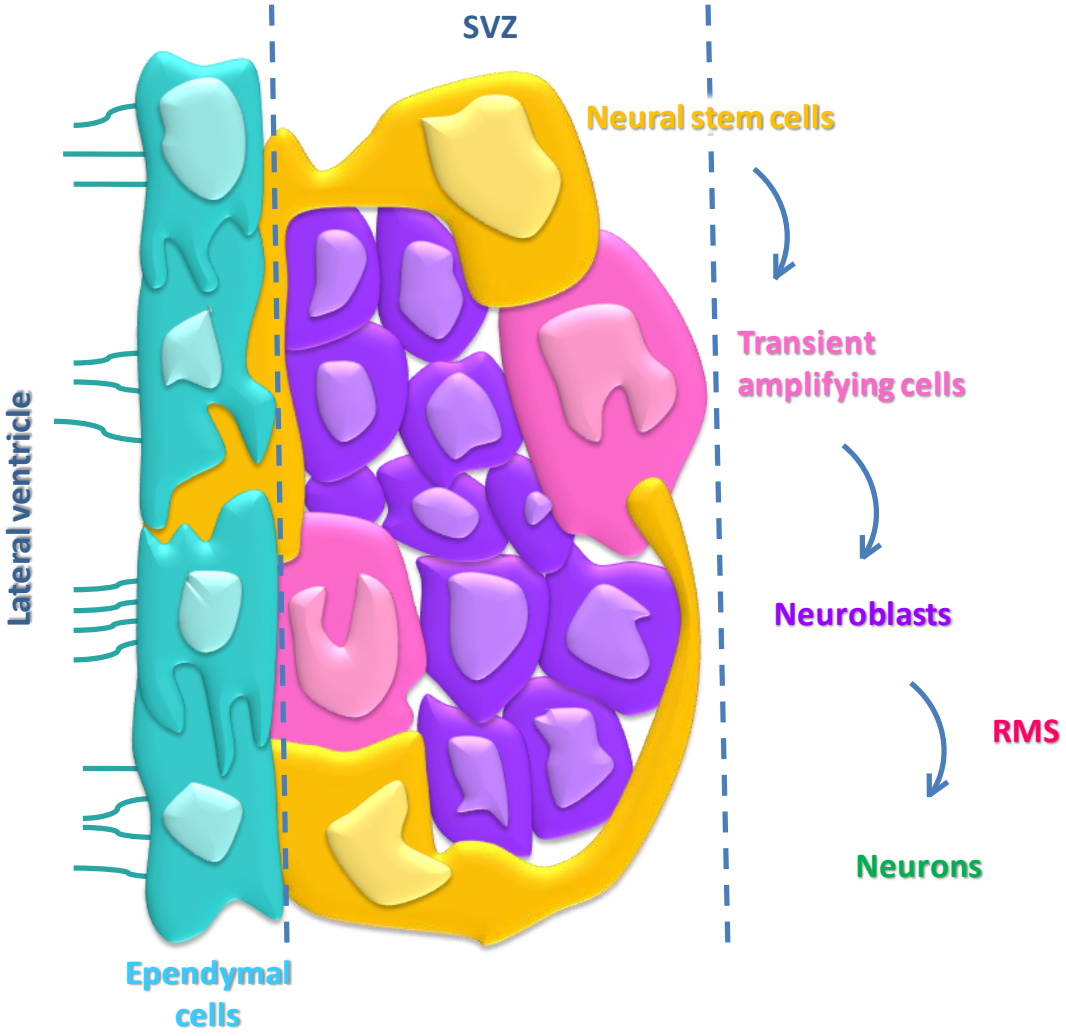


Figure 1.5 There are 3 types of stem-like cells in the SVZ: The slowly dividing SVZ NS cells (type B cells) gives rise to fast dividing transient amplifying cells (type C cells). Type C cells then gives rise to neuroblasts (type A cells), which migrates along the RMS to the olfactory bulb becoming olfactory bulb neurons.

The phenotype of the interneurons is decided in the SVZ, prior to migration to the OB. Different regions of the SVZ has shown to generate different types of interneurons. A recent study have also shown that NS cells isolated from the dorsal regions of the SVZ are able to differentiate into glutamatergic neurons (Brill et al., 2009).

1.3.1.2.2 Subgranular zone

Throughout the lifetime of an organism, although with gradual decline with age, new neurons can be found within the hippocampus (Kuhn, Dickinson-Anson, & Gage, 1996). These NS cells reside in the SGZ in a narrow 2-3 cell layer between the granular cell layer and the hilus region in the DG of the hippocampus (Burns, Verfaillie, & Low, 2009). Like NS cells of the SVZ, these NS cells also display astrocytic features; they express GFAP and have long radial process making contact with blood vessels (Palmer, Willhoite, & Gage, 2000). This NS cell population can also be divided into 2 types: the radial astrocytes (rA) and the horizontal astrocytes (hA) (Seri, Garcia-Verdugo, Collado-Morente, McEwen, & Alvarez-Buylla, 2004). The rAs have radial process that penetrate the granule cell layer and extend towards the surface of the DG, hAs on the other hand exhibit no radial process, but a thin basal lamellae that orientates tangentially along the SGZ. They both express astrocytic and rG markers such as Sox2, Vimentin, BLBP and Musashi1. Further studies demonstrated that the Sox2 expressing populations are capable of self-renewal and generate identical Sox2 positive daughter cells. They are able to give rise to SGZ astrocytes and also form glutamatergic excitatory DG neurons (Figure 1.6) (Suh et al., 2007).

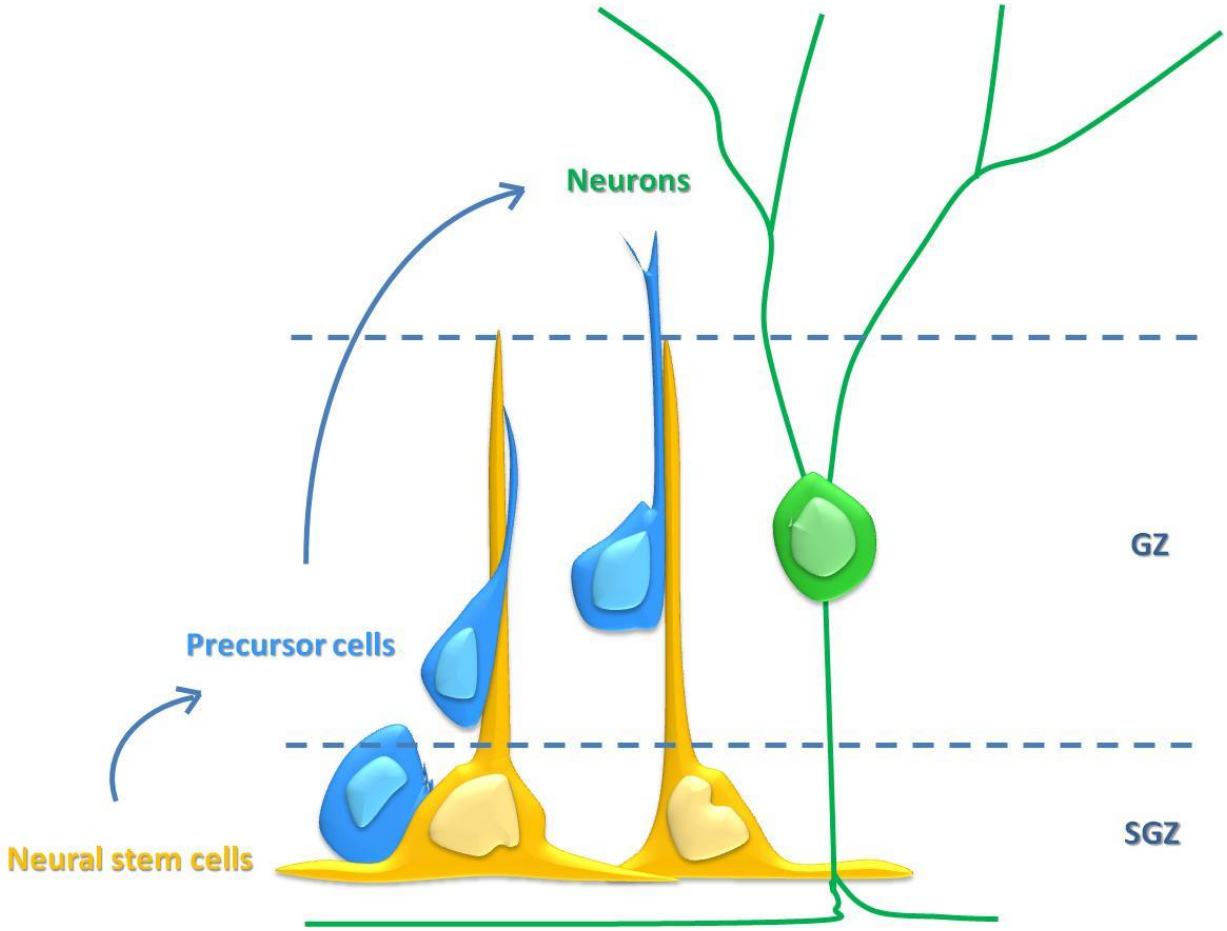


Figure 1.6 SGZ structure. In the SGZ, the SGZ NS cells gives rise to precursor cells, which migrate up the radial projection of type B cells to become the dentate granule neurons.

The maturing neurons migrate to the granule cell layer and generate mainly local glutamatergic excitatory granule neurons with axons that extend into the hippocampus (Hastings & Gould, 1999) and dendrites into the DG (Henze et al., 2000).

1.3.2. Neural stem/progenitor cells *in vitro*

In vitro intrinsic factors and extrinsic cytokines direct ES cells to undergo progressive lineage restrictions (Pankratz et al., 2007; Ying & Smith, 2003) that generate neurons,

astrocytes and oligodendrocytes. Neurogenesis was found to be the “default” pathway of ES cell differentiation and various groups have had different levels of success in directing ES cells towards specific neuronal or glia phenotypes.

1.3.2.1 Neural epithelial cells

NEP cells can be induced, within 24 hours, from mES cells by the withdrawal of extrinsic factors such as LIF and serum. Continuous cultivation in this condition induces the formation of colonies that are classified as the LIF dependent ‘primitive’ NEP cells. These primitive NEP cells are the intermediate stage between ES cells and committed NS cells. They retain the pluripotent marker octamer binding transcription factor 4 (Oct4) and are able to give rise to a broad range of differentiated cell types in chimeras (Tropepe et al., 2001). NEP cells are transient *in vitro* and cannot be maintained in culture. The exposure of the Sox1 positive transient NEP cells to retinoic acid (RA) prevents the development of rostral neuron phenotypes (Glaser & Brustle, 2005) that results in the posterior regional specificity of NEP cells. Furthermore, prolonged passaging switches them into a Notch dependent rNS cell type that are immunoreactive to ZO1 followed by a more committed precursor cell type that is dependent on FGF2.

1.3.2.2 Rosette neural stem cells

Like NEPs, rNS cells also respond to patterning factors and have the ability to give rise to a range of neuronal phenotypes (Elkabetz et al., 2008a) and can be maintained in the presence of Shh and Notch receptor agonists (Elkabetz et al., 2008a). Once these rNS cells are exposed to the commonly used mitogens for NS cell maintenance, FGF2 and epidermal growth factor (EGF), they convert to a Sox1-negative rG-like cells that are more phenotype restricted (Elkabetz et al., 2008a).

1.3.2.3 Radial glia

rGs are readily available from ES cells and rGs are an intermediate stage between ES cells and neurons (Bibel, Richter, Lacroix, & Barde, 2007; Bouhon, Joannides, Kato, Chandran, & Allen, 2006; Conti et al., 2005; Liour et al., 2006; Lowell, Benchoua, Heavey, & Smith, 2006; Nat et al., 2007). They are fate restricted as near homogeneous populations of Pax6 positive cells which differentiate only into glutamatergic neurons (Bibel et al., 2007; Bibel et al., 2004). A different population of rG can also be generated by exposing Sox1 positive cells to EGF and FGF2, like NS cells are homogeneous Sox1 negative, Nestin, BLBP and Pax6 positive cells and can be passaged as a monolayer culture for over 100 passages (Conti et al., 2005). These NS cells retain the markers expressed by rG and also the capacity to generate neurons and glia; both cell types can be generated *in vitro* from ES cells. The only difference between the two is that the generation of rG does not involve an amplification step that causes fast proliferation and differentiation of ES cells into glutamatergic neurons, while NS cells requires the addition of mitogens for continued proliferation (Haubensak, Attardo, Denk, & Huttner, 2004; Miyata et al., 2004; Noctor, Martinez-Cerdeno, Ivic, & Kriegstein, 2004).

1.4 Long-Term Propagation of Neural Stem/Progenitor Cells

Over the years, a lack of understanding of the physiological states of NS cells has meant that their isolation, purification and expansion has been problematic. Much effort has been made in propagation methods and growth factor combinations to maintain the NS cell phenotype. Amongst these EGF and FGF2 have been commonly used in cell culture conditions to sustain prolonged division of cells with NS cell characteristics in both neurosphere and monolayer systems (Laywell, Kukekov, & Steindler, 1999; Reynolds & Weiss, 1992).

1.4.1 Neurosphere system.

Neurospheres are free-floating aggregates of neural progenitors each, ideally, derived from a single NS cell. Their generation relies on tissue dissection or dissociation of neuralised pluripotent cells such as ES or iPS cells (Chojnacki & Weiss, 2008). Mouse or rat neurospheres are commonly derived from E10.5–E18.5 neural tissues. Neurosphere formation is induced in low-attachment tissue culture dishes in serum-free media, supplemented with 10–20 ng/mL of FGF2 and/or EGF (Singec et al., 2006). Under these conditions, NS and NP cells are expected to proliferate driven to dissociation and re-plating to generate secondary neurospheres. This method can be repeated to isolate and expand NS cell populations (Reynolds & Weiss, 1992). Neurospheres consist of heterogeneous populations of stem cells, progenitor cells, cells at different stages of maturation and also necrotic cells. The inner core can be made up of more differentiated and necrotic cells depending on neurosphere size. Differentiating or differentiated cells are not expected to survive and in most cases die due to lack of support. This may be due to the low access to mitogens or oxygen in the environment (Bez et al., 2003; Ostenfeld et al., 2002). Generally, as sphere size increases, heterogeneity also increases (Parmar, Skogh, & Englund, 2003; Reynolds & Weiss, 1996; Suslov, Kukekov,

Ignatova, & Steindler, 2002). *In vivo*, the stem cell niche represents a cell-specific microdomain with precise organisation orientation that assists in the regulation of NS cell maintenance, repair, migration, differentiation, and ultimately tissue generation, (Campos, 2004; Campos, Decker, Taylor, & Skarnes, 2006). Neurosphere forming cells isolated from the adult rodent SVZ consist of both GFAP positive type B cells and neuron-glial antigen 2 (NG2) and Dlx2 positive type C cells (Doetsch, Petreanu, Caille, Garcia-Verdugo, & Alvarez-Buylla, 2002), a variable proportion of cells also express other NS cell markers, such as Sox2 and Prominin1, and rG markers, such as RC2 (also known as Ifaprc2; intermediate filament-associated protein), GLAST and BLBP (Hartfuss, Galli, Heins, & Gotz, 2001). Long-term expansion has only been thoroughly studied for neurospheres derived from GFAP positive cells isolated from adult rodents (Doetsch et al., 2002). In these neurospheres, EGF signalling was sufficient to regulate both the generation and the differentiation of morphologically, antigenically and functionally defined rG from mouse neurospheres when cultured as a monolayer (Gregg, Chojnacki, & Weiss, 2002). This was also observed in human neurospheres exposed to a high EGF concentration (Nelson, Suzuki, & Svendsen, 2008) and indicates that the presence of EGF, neurospheres generated from cells isolated from the adult SVZ have rG identity. Many studies have also looked at the *in vitro* properties of extracted NS cells by the neurosphere formation method (Golmohammadi et al., 2008; Marshall, Reynolds, & Laywell, 2007). However, the consistency of this process may be questionable, since this method involves intrinsic signalling processes that are very difficult to control (Singec et al., 2006). It has been suggested that initial cell aggregation determines multipotency within heterogeneous populations of NS cells (Jessberger, Clemenson, & Gage, 2007; Mori, Fujitani, Kanemura, Kino-Oka, & Taya, 2007; Singec et al., 2006), however, although the number of spheres generated at the early stage cannot be regarded as a

formal index of the number of NS cells in the tissue unless single-cell analysis is carried out (Wachs et al., 2003).

Electron microscopy has revealed that neurospheres consist of similar cell types to found in stem cell niches, *in vivo,* but with no specificity in organisation between cells. Thus culture conditions allow the propagation of dividing cells without instructive cues from their niches (Seaberg & van der Kooy, 2003). As a result, the primary spheres are likely to be composed of a mixture of cells with only a small percentage capable of self-renewal, and only a fraction of these meet the definition of NS cells (Reynolds & Weiss, 1992). Each round of neurosphere formation also leads to cell loss and may diminish the population of NS cells (Kornblum, 2007). Although these cells display multipotent potential, over the successive passages, they have shown to be biased towards gliogenesis (Chojnacki & Weiss, 2008; Grandbarbe et al., 2003; Tropepe et al., 1999a; Weiss & Orkin, 1996).

1.4.2 Monolayer system.

Attempts have been made to culture NS/NPs derived from different brain regions have been cultured in adherent monolayer conditions in order to understand the molecular mechanism that allows the maintenance of NS cells *in vitro* (Johe, Hazel, Muller, Dugich-Djordjevic, & McKay, 1996). However, one of the only NS/NP lines that could be successfully cultured was from the rat hippocampus (Palmer, Takahashi, & Gage, 1997). These Nestin and Sox2 positive cells showed some heterogeneity, with undifferentiated progenitors continuously undergoing asymmetrical cell division to generate early neurons; and also symmetrical cell division to replenish the undifferentiated population (Palmer et al., 1997; Takahashi, Palmer, & Gage, 1999). Other strategies have also been employed

for the derivation of NS cell lines that are stable and are able to withstand long term propagation from both rodent (Conti et al., 2005; Pollard, Conti, Sun, Goffredo, & Smith, 2006) and human (Sun et al., 2008). It was shown that by exposing NPs to EGF and FGF2, they can be expanded as adherent, clonal, almost homogeneous populations of NS cell lines. In this condition, the cells are able to retain their multipotent differentiation capacity following long term maintenance (Conti et al., 2005; Elkabetz et al., 2008a; Koch, Opitz, Steinbeck, Ladewig, & Brustle, 2009). The presence of EGF and FGF2 is crucial in the derivation and propagation of adherent cultures from either ES cells or fetal/adult brain (Palmer et al., 1997) and this NS cell population shows antigenic properties that is very similar to forebrain rG (Conti et al., 2005; Pollard, Benchoua, & Lowell, 2006; Pollard, Conti, et al., 2006). The monolayer system is efficient in the propagation an enrichment of near homogeneous NS cell populations that prevent lineage restriction and minimize spontaneous differentiation (Conti et al., 2005). Extended culturing of NS cell population can be problematic, where increased proliferative capacity at the expense of differentiation has been reported. Generally these cultures display a bias toward an astrocytic fate and a reduced capacity to generate neurons and oligodendrocytes after long term maintenance (Chang, Hung, Chuang, & Jong, 2004). This may be due to selection from the culture conditions, by the fast dividing cells or a general loss of stem cell capacity over extended passages (Ferron et al., 2004). Some studies showed that intrinsic regional specification is maintained to a certain degree (Hitoshi, Tropepe, Ekker, & van der Kooy, 2002; Ostenfeld et al., 2002; Parmar, Skogh, Bjorklund, & Campbell, 2002), however, others reported the lack of regional specification (Hack, Sugimori, Lundberg, Nakafuku, & Gotz, 2004; Santa-Olalla, Baizabal, Fregoso, del Carmen Cardenas, & Covarrubias, 2003). Differences in NS cell cultural conditions may be the reason behind the discrepancies.

1.5 Neural Differentiation *In vivo*

The brain and spinal cord, is one of the first organ systems formed during vertebrate embryo development (Gilbert & Lasley, 2002). Following fertilization, multiple cell divisions generate a large number of cells from the fertilised oocyte. This is followed by complex cellular movements during gastrulation which gives rise to the three germ layers of the embryo: endoderm, mesoderm and ectoderm. The innermost layer, the endoderm gives rise to the gut, lungs and liver. The middle layer, the mesoderm, gives rise to connective tissue, muscle and vascular system. The outermost layer, the ectoderm, gives rise to the central and peripheral nervous system.

The fate of ectodermal cells is dependent on axial position; the most dorsal ectoderm thickens to form the neural plate, which then folds to give rise to the neural tube and ultimately the CNS. During neurulation, the ectodermal cells at the ventral edges of the neural plate fold towards the dorsal surface of the neural tube. These cells become the neural crest cell that migrate away from the neural tube and give rise to the peripheral nervous system (PNS). The ectodermal cells more ventral to the cranial neural plate become placodes, which in turn becomes the sensory ganglia, the most ventral ectodermal cells give rise to the epidermis. Therefore, the establishment of the different regions of ectoderm along the different regions of the embryo is crucial to the generation phenotypically distinct neurons (Kandel & Squire, 2000).

During the formation of the CNS, multiple signalling factors dictate regionalization, from neural induction to development of the brain and spinal cord. Independent patterning of two axes: anterior-posterior and dorsal-ventral, establish the structure of the CNS (Simon et al., 1995). Signals from the notochord and the mesoderm lateral to the neural tube are major influences in shaping the dorsal-ventral structure of the neural tube (Wilson &

Maden, 2005). Secreted factors are largely responsible for directing differentiation and there are four major factors: BMP, FGF, Wnt and RA.

1.5.1 Anterior-posterior patterning

The inhibition of BMP signalling by Chordin, Noggin, or Follistatin is a crucial step in neural induction where epidermal ectoderm differentiation is prevented when SMAD4 expression is downregulated. Also a member of the TGFβ superfamily; Nodal must also be inhibited by overexpression of Lefty or Cerberus for neural induction (Sonntag et al., 2005; Vallier, Reynolds, & Pedersen, 2004). This is supported by the finding that mouse Nodal mutants show early adoption of neural fates (Camus, Perea-Gomez, Moreau, & Collignon, 2006). However, inhibition of BMP and Nodal is not sufficient to induce neural fate in the chick and mouse (Figure 1.7) (Delaune et al., 2005). The activation of additional signals such as FGF is also required. It has been demonstrated that the inhibition of FGF signalling with the FGF receptor tyrosine kinase antagonist SU5402 inhibited neural induction in the chick embryo (Delaune et al., 2005). In the Xenopus model, only explants that have been exposed to FGFs adopt neural fates (Delaune et al., 2005). FGF signalling has been demonstrated to induce neurogenesis by activation of the MAPK dependent inhibition of SMAD1 (Kuroda, Fuentealba, Ikeda, Reversade, & De Robertis, 2005). FGFs are involved in the induction of posterior neural tissue by enabling ectodermal cells to respond to Wnt signalling (Domingos et al., 2001).

Wnt has also been proposed to have a crucial role in NI. Wnt signalling is involved in a vast array of cellular processes, including development of the CNS (Ciani & Salinas, 2005; Moon, Kohn, De Ferrari, & Kaykas, 2004). Three different Wnt-signalling pathways have been identified: the canonical or β-cantenin dependent pathway; the non-canonical Wnt Jun N-terminal kinase (JNK) and Wnt-Ca^{2+} pathways. The canonical Wnt-signalling

pathway is activated when a Wnt molecule binds to Frizzled (Fz) receptors, leading to the formation of the LRP5/6, Dishevelled (DVL) and adenomatous polyposis coli (APC) complex. This complex then blocks the activity of glycogen synthase kinase 3β (GSK3β), allowing β-catenin to enter the nucleus to interact with lymphoid enhancer-binding factor 1 (LEF1) and activate Wnt target genes. It has been suggested that Wnt regulates BMP expression via the β-cantenin dependent Wnt pathways (Klaus et al., 2012; Soshnikova et al., 2003). The non-canonical Wnt pathways Wnt-JNK and Wnt-Ca^{2+} pathways, are independent of β-catenin (Inestrosa & Arenas, 2010). It has also been demonstrated that Wnt inhibits neurogenesis in chick embryos. Therefore Wnt expression must be present before its subsequent inhibition to induce neurogenesis (Figure 1.7) (G. M. Keller, 1995; Wessely, Agius, Oelgeschlager, Pera, & De Robertis, 2001).

In the developing Xenopus embryo, Wnt and RA signalling are expressed in a posterior-to-anterior gradient. The Xenopus Wnt1 related gene, Xwnt8 is expressed from the posterior end where it posteriorises the neural plate while Frzb or Dickkopf1 (DKK1) are expressed from the anterior end, inhibiting the Wnt signalling and anteriorsing the neural plate (Domingos et al., 2001; Kiecker & Niehrs, 2001). FGFs are involved in the induction of posterior neural tissue by enabling ectodermal cells to respond to Wnt signalling (Domingos et al., 2001). Like Wnt signalling, RA is also expressed from the posterior end of the neural plate and spreads in a concentration dependent manner (Chen, Huang, & Solursh, 1994; Cho & De Robertis, 1990; Sive & Cheng, 1991).

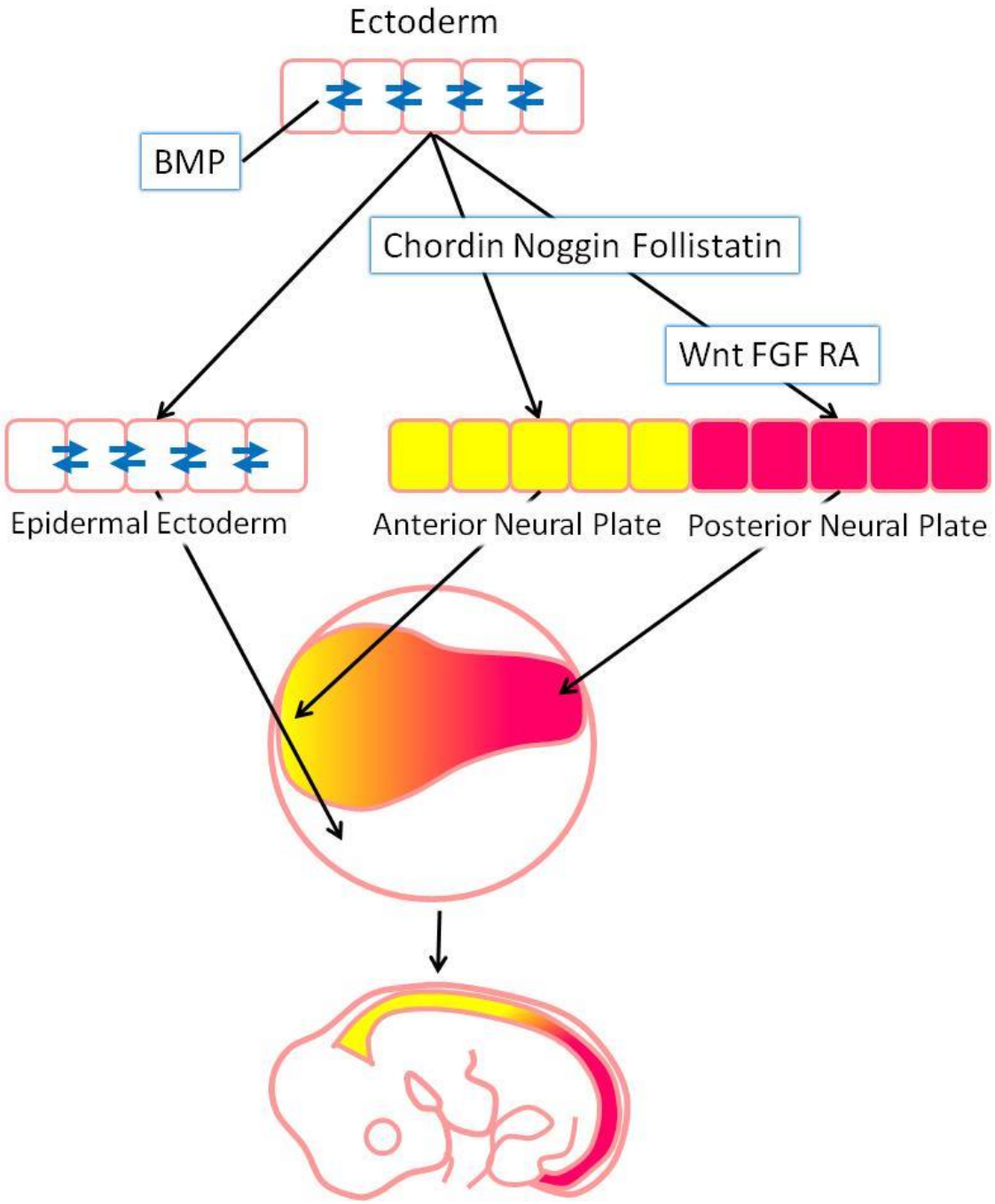


Figure 1.7 Patterning of early neural tube in anterior-posterior-axis. Ectodermal cells exposed to BMP4 signalling differentiate into epidermal ectodermal cells. Inhibition of BMP4 signalling by Chordin, Noggin, or Follistatin induces the formation of anterior neural plate tissue. Exposure of this tissue to Wnt, RA or FGF leads to the generation of posterior neural plate tissue. (Kandel & Squire, 2000).

1.5.2 Dorsal-ventral patterning

At the same time, dorsal-ventral axis patterning also takes place. Similar to the anterior-posterior patterning of the neural plate, dorsal-ventral patterning is also dependent on the expression of factors in a concentration dependent manner. Shh is expressed from the ventral pole from the notochord and later from the floor plate. TGFβs such as BMP4, bone morphogenic protein 7 (BMP7) and Activin are expressed from the dorsal ectoderm and later from the roof plate, creating an opposing gradient of morphogens along the dorsal-ventral axis (Garcia-Campmany & Marti, 2007; Liem, Tremml, & Jessell, 1997; Novitch, Wichterle, Jessell, & Sockanathan, 2003; Pituello, Yamada, & Gruss, 1995; Wilson, Gale, & Maden, 2003). As cells are exposed to different concentrations of factors they adopt different phenotypes. The most dorsal cells are exposed to higher concentrations of BMP signalling while the most ventral cells are exposed to higher concentrations of Shh signalling. Nkx2.2 expressing neural progenitors found adjacent to the floor plate eventually become ventral V3 neurons (Briscoe et al., 1999). Nkx6.1 expressing progenitors on the other hand show a broader expression pattern, they give rise to motor neurons and V2 and V3 interneurons (Sander et al., 2000). Cells adjacent to the ventral neuron domain express transcription factors encoding Nkx6.1 and Pax6 and in turn become motor neurons. Cells exposed to from the dorsal side low Shh and high BMP become V2 and V1 interneurons (Ericson et al., 1995).

Since the telencephalon is devoid of motor neurons, has no floor plate, and does not sit above the notochord, Shh spreads rostrally in a planar fashion from the dicephalic floor region towards the ventral telencephalon. The telencephalon is divided into the dorsal and ventral pallia regions. Shh signalling from the ventral midline influences the ventromedial patterning of the medial and lateral ganglionic eminence. The BMP and Wnt signalling expressed from the roof plate and the medial pallium and later from the

cortical hem is important in the specification of the pallium. Although Wnt and RA signalling are implicated in dorsal-ventral patterning, their roles in pallium specification is not well understood (Figure 1.8).

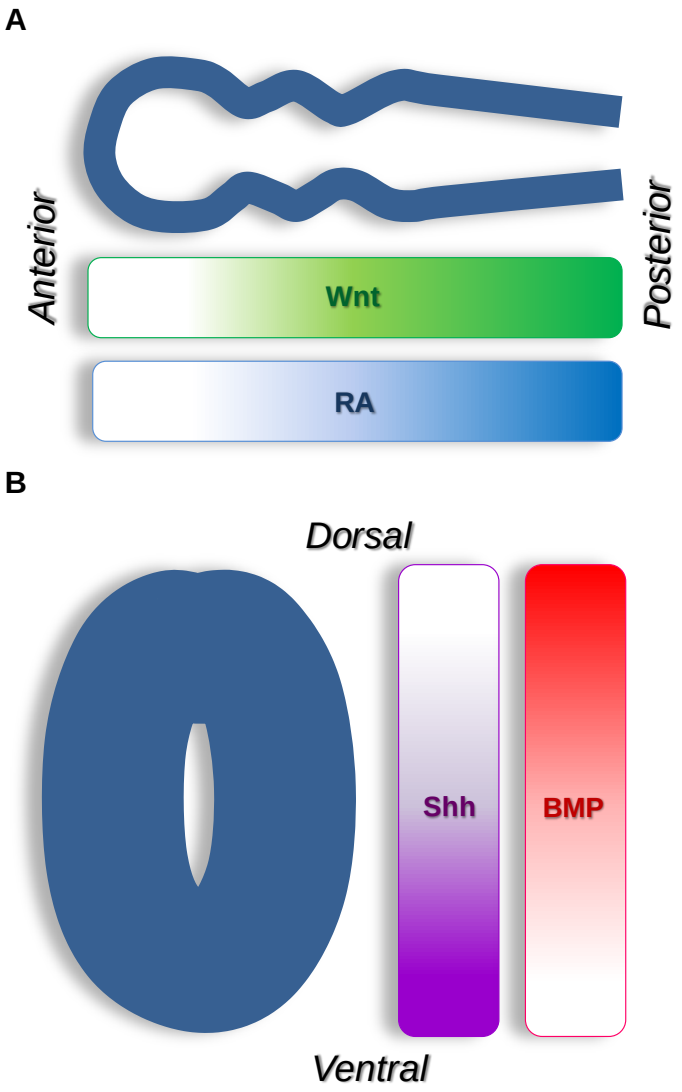


Figure 1.8 Patterning of the nervous system. A gradient of Wnt and RA expression from the posterior to the anterior specifies the anterior-posterior axis of the neural tube. Wnt and RA posteriorise the neural tube and Wnt and RA antagonists anteriorises of the neural tube. A gradient of BMP expression from the dorsal to ventral and Shh expression from ventral to dorsal specifies the dorsal-ventral axis of the neural tube.

1.5.3 Midbrain dopaminergic patterning

Dopaminergic neurons are found in multiple locations in the central nervous system; the forebrain, OB, retina and midbrain. The midbrain dopaminergic neurons make up 75% of all dopaminergic neurons in the adult brain. They are located in the ventral regions of the mescephalon and are organised laterally in the retrorubal field (RRF; A8 neurons) and the substantia nigra pars compacta (SNpc, A9 neurons), and medially in the ventral tegmental area (VTA; A10 neurons). SNpc neurons project to the dorsolateral striatum and form the nigrostriatal pathway involved in the control of voluntary movements. VTA neurons project to the ventromedial striatum, cortical and subcortical areas forming the mesocorticolimbic system, which is involved in emotional behaviour and motivation and reward mechanisms. RRF neurons project to the dorsal striatum and connect with the SNpc, VTA neurons (Gale & Li, 2008).

The development of midbrain dopaminergic neurons is dependent on the integration of anterior, posterior and dorsal signals. These signals specify the generation of the midbrain region. The two key morphogens essential and sufficient for the induction of ventral midbrain dopaminergic neurons during neurogenesis are Shh, released by the floor plate, and FGF8, secreted by the mid-hindbrain organizer (MHO or isthmus) (Hynes, Porter, et al., 1995; Hynes, Poulsen, Tessier-Lavigne, & Rosenthal, 1995; Ye, Shimamura, Rubenstein, Hynes, & Rosenthal, 1998). Wnt1 and TGFβs are crucial factors involved in signalling cascades that lead to the generation midbrain dopaminergic neurons (Prakash et al., 2006). During midbrain formation and midbrain dopaminergic specification, genes encoding Otx2, Engrailed 1 and 2 (En1 and 2), FoxA2, LIM homeobox transcription factor 1α and β (Lmx1a and b), Msh homeobox 1 (Msx1) and neurogenin 2 (Ngn2) are expressed (Andersson, Jensen, Parmar, Guillemot, & Bjorklund, 2006; Andersson, Tryggvason, et al., 2006; Bonilla et al., 2008; Ferri et al., 2007; Kele et

al., 2006; Kittappa, Chang, Awatramani, & McKay, 2007; Puelles et al., 2003; Puelles et al., 2004; Simon, Saueressig, Wurst, Goulding, & O'Leary, 2001; Thompson et al., 2006; Vernay et al., 2005). This is followed by nuclear receptor related factor 1 (Nurr1) and the paired homeodomain transcription factor 3 (Pitx3) as midbrain dopaminergic progenitors undergo maturation and become post-mitotic neurons (Figure 1.9) (Hwang, Ardayfio, Kang, Semina, & Kim, 2003; Maxwell, Ho, Kuehner, Zhao, & Li, 2005; Nunes, Tovmasian, Silva, Burke, & Goff, 2003; Saucedo-Cardenas et al., 1998; Smidt, Smits, & Burbach, 2004; Wallen et al., 1999). Recently, novel reporter ES cell lines targeting the expression of Lmx1a, Lmx1b, Msx1 and Pitx3 have been developed to track the appearance of dopaminergic progenitors and dopaminergic neurons (Hedlund et al., 2008; Jaeger et al., 2011; Nefzger et al., 2012; Roybon, Hjalt, Christophersen, Li, & Brundin, 2008; Watmuff, Pouton, & Haynes, 2012). In particular, Lmx1a have been identified as prominent during dopaminergic differentiation and it's expressed at day 4 of NI (Nefzger et al., 2012). However, Lmx1a expression has also been associated with cortical hem of the forebrain, roof plate of the developing cerebellum and other non-neural progenitors during neural tube formation (Millonig, Millen, & Hatten, 2000; Mishima, Lindgren, Chizhikov, Johnson, & Millen, 2009b). Pitx3 is a post-mitotic dopaminergic neuron marker that is found in 2-5% of live cells after enzymatic dissociation following dopaminergic differentiation protocol (Hedlund et al., 2008; Watmuff et al., 2012).

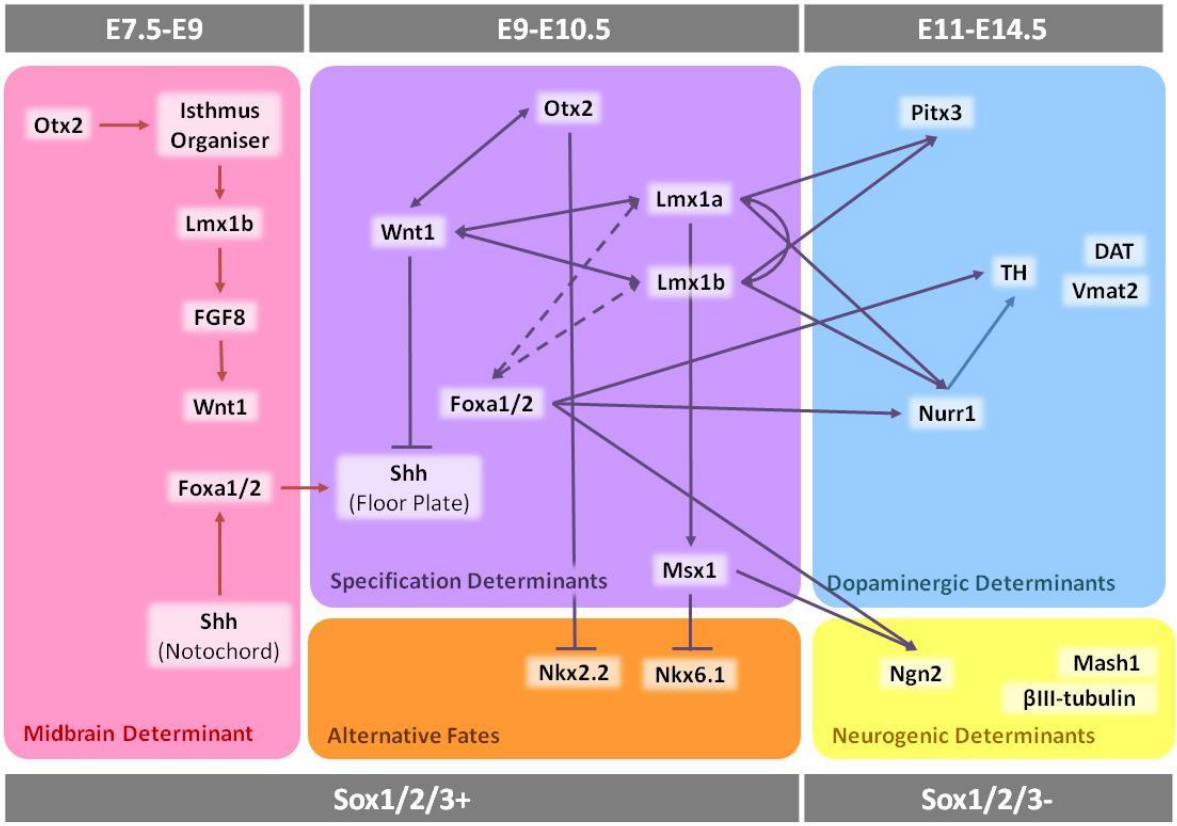


Figure 1.9 Molecular determinants in dopaminergic differentiation.

The homeodomain transcription factor Otx2 is critical in the specification of the midbrain region and the subsequent differentiation of midbrain dopaminergic neurons and is first expressed in the epiblast and anterior visceral endoderm. During gastrulation, Otx2 expression becomes progressively restricted to the anterior epiblast that defines the forebrain and midbrain (Broccolini, Engel, & Askanas, 1999; Simeone, Puelles, & Acampora, 2002). At the end of gastrulation (E7.5), Otx2 and the Gbx2 from the posterior epiblast combine and give rise to the isthmus at the midbrain and hindbrain border (Wurst & Bally-Cuif, 2001). This is followed by the expression of the paired box gene2 (Pax2) at the interface of Otx2/Gbx2 domain and Wnt1 at the Otx2 domain. FGF8 is then expressed (E8) at the Gbx2 domain which later is restricted to the isthmus (E9). This expression is controlled by the LIM homeodomain transcription factor Lmx1b, which also

plays an important role in the regulation of the other isthmus genes such as Wnt1 and En1/2 (Guo et al., 2007); and maintained by the transcription factors En1 and 2 throughout the Otx2/Gbx2 domain. En1/2 expression is regulated by Wnt1 and is crucial in early development for the generation and survival of midbrain dopaminergic neurons (Simon et al., 2001).

The mirrored interaction between FGF8 and Wnt1 signalling leads to isthmus activation and the establishment of the midbrain. Ultimately, this leads to the generation of committed midbrain dopaminergic precursors. Wnt1 expression has also been shown to be controlled by FGF activity in the ventral midbrain (Saarimaki-Vire et al., 2007), in the ectopic induction of dopaminergic neurons in the forebrain and hindbrain (Prakash et al., 2006). The coordinated conversion of floor plate cells into neuronal progenitors is dictated by canonical Wnt signalling, activated by Otx2 in the midbrain (Omodei et al., 2008; Ono et al., 2007). At E10.5, Shh expression is suppressed by Wnt1 via an Msx1 mediated mechanism (Joksimovic, Patel, Taketo, Johnson, & Awatramani, 2009) that induces the expression of Ngn2 (Ono et al., 2007). This is also supported by FoxA1 and A2 expression (Ferri et al., 2007), which reinforces the midbrain dopaminergic regulatory pathway. It has also been suggested that Otx2 represses Nkx2.2, a Shh responsive gene necessary for specification of ventral cell types in the both the hindbrain and spinal cord (Prakash & Wurst, 2006).

During isthmus formation, Shh from the notochord induces the formation of the floor plate along the neural tube (Yamada, Placzek, Tanaka, Dodd, & Jessell, 1991). This is mediated by a Shh activity dependent GLI family zinc finger 2 (Gli2) transcription factor binding site in the FoxA2 promoter in the floor plate cells (Sasaki, Hui, Nakafuku, &

Kondoh, 1997). It has been demonstrated that Gli2 regulates Gli1 expression and both Gli1 and 2 induce FoxA2 expression, which in turn regulates Shh expression in the midbrain (Matise, Epstein, Park, Platt, & Joyner, 1998). FoxA2 induces Shh expression in the ventral domain of mesencephalon to specify ventral neural precursors (Jeong & Epstein, 2003). It has been shown that floor plate cells that express Shh become midbrain dopaminergic neurons (Ono et al., 2007). Downstream of Shh signalling, FoxA1 and A2 inhibit the expression of the gene encoding Nkx2.2 and Nkx6.1 that leads to the generation of GABAergic neurons (P. Y. Lin, Hinterneder, Rollor, & Birren, 2007). The gene encoding Nkx6.1 is present in the midbrain dopaminergic domain but is suppressed by Msx1 in the ventral midbrain (Anderson, Bergner, & Murphy, 2006).

Lmx1a and Lmx1b are amongst the first markers that identify midbrain dopaminergic neurons and have important roles in their specification (Andersson, Tryggvason, et al., 2006; Smidt, Smits, & Burbach, 2003). Lmx1a cooperates with Lmx1b to regulate specification, proliferation and differentiation of midbrain dopaminergic progenitors (Yan, Levesque, Claxton, Johnson, & Ang, 2011). Lmx1a is essential for induction and neurogenesis, however Lmx1b is not crucial (Guo et al., 2007; Yan et al., 2011). Lmx1a is expressed at the ventral midline of mesencephalon (E9) and is essential in dopaminergic differentiation in the chick and mouse (Andersson, Tryggvason, et al., 2006; Ono et al., 2007). Lmx1a activates the Msx1 which acts synergistically with Lmx1a to activate expression of Ngn2, a proneural gene, while repressing Nkx6.1, a homeodomain protein broadly expressed in ventral progenitor cells (Andersson, Jensen, et al., 2006). Ngn2 is part of a family of bHLH transcriptional regulators involved in neurogenesis but not specification of midbrain dopaminergic fates while repressing Nkx6.1, a homeodomain protein broadly expressed in ventral progenitor cells (Andersson, Jensen, et al., 2006). Otx2 together with FoxA2 have been found to induce and maintain

Lmx1a expression and possibly also Lmx1b expression (W. Lin et al., 2009; Omodei et al., 2008; Ono et al., 2007). Wnt1 have also been suggested to be responsible for the expression of Lmx1a through the canonical Wnt pathway forming an autoregulatory loop (Chung et al., 2009). Lmx1a specification of midbrain dopaminergic fate is restricted to FoxA2 expressing progenitors (Nakatani, Kumai, Mizuhara, Minaki, & Ono, 2010). FoxA2 and Lmx1a cooperate to promote Ngn2 and Nurr1 expression, targets important in midbrain dopaminergic differentiation. However, FoxA2 alone directly inhibits Nkx2.2, and is sufficient to regulate TH expression (Ferri et al., 2007; W. Lin et al., 2009). Lmx1b on the other hand has two roles: the formation and maintenance of isthmus by directly and indirectly controlling fibroblast growth factor 8 (FGF8), Wnt1 and both En1 and 2 as mentioned earlier; and dopaminergic neuron generation. It has been reported that Lmx1b expression has been detected before the emergence of dopaminergic neurons and is maintained in the SNc and VTA until adulthood (Smidt et al., 2000).

One of the earliest markers identified for proper midbrain dopaminergic neuron development is the orphan nuclear receptor Nr4a2/Nurr1. It is also required for the expression of several proteins involved in dopamine synthesis and regulation such as TH, vesicular monoamine transporter 2 (VMAT2), dopamine transporter (DAT) and RET receptor tyrosine kinase (cRET) (Saucedo-Cardenas et al., 1998; Wallen et al., 1999; Zetterstrom et al., 1997).

Expression of Ngn2 is required for the generation of Nurr1 positive, post-mitotic immature neurons which further differentiate into TH positive mature neurons (Andersson, Jensen, et al., 2006; Kele et al., 2006). This is immediately followed by the expression of the orphan nuclear receptor Nr4a2/Nurr1 and Pitx3. These genes encode transcription

factors involved in defining the dopaminergic phenotype, such as TH (Ferri et al., 2007; Kim et al., 2003; Saucedo-Cardenas et al., 1998; Smidt et al., 2003; Zetterstrom et al., 1997). Nurr1 and Pitx3 have also been shown to maintain survival of midbrain dopaminergic neurons, especially in the SNpc and the VTA (Hwang et al., 2003; Nunes et al., 2003; Smidt et al., 2004; van den Munckhof et al., 2003). The expression of Nurr1 in immature neurons is regulated by the combined action of FoxA1, FoxA2 and Lmx1a, which also function to directly activate TH expression in mature neurons. As well as establishing the midbrain border at early stages, En1 and En2 also play an important role in the survival of midbrain dopaminergic neurons (Alberi, Sgado, & Simon, 2004; Simon et al., 2001; Simon, Thuret, & Alberi, 2004).

1.6 Neural Differentiation *In vitro*

1.6.1 Neural Differentiation of ES Cells

Inhibitors of BMP, Nodal, and Wnt signalling that act as neural inducers *in vivo* also promote *in vitro* differentiation of ES cells into neural cells. FGF signalling is involved in both *in vivo* neural induction and *in vitro* neural differentiation of ES cells (Figure 1.10). The inhibition of BMP signalling is required but not sufficient to induce neural differentiation in ES cells (Kawasaki et al., 2000; Tropepe et al., 2001; Watanabe et al., 2005; Ying & Smith, 2003). Therefore, most *in vitro* neural induction requires low or no serum environments to remove BMPs found in serum.

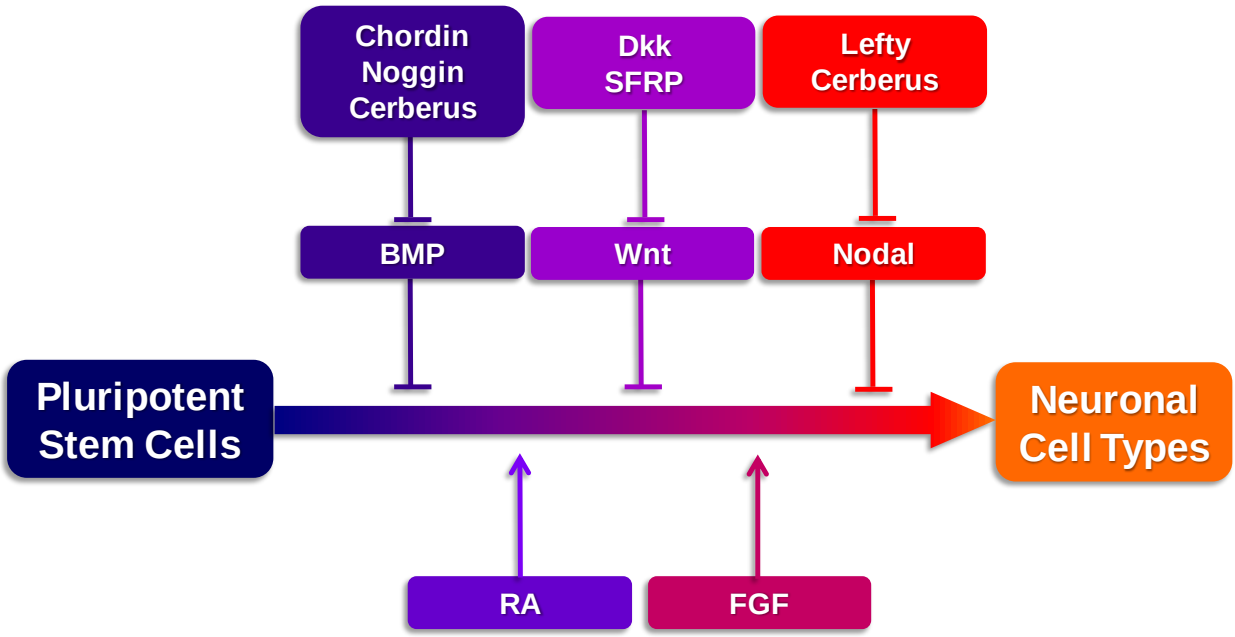


Figure 1.10 Neural induction in pluripotent stem cells. Inhibitors of BMP, Nodal, and Wnt signalling that act as neural inducers *in vivo* also promote *in vitro* differentiation of ES cells into neural cells. FGF signalling is involved in both *in vivo* neural induction and *in vitro* neural differentiation of ES cells. In contrast, RA, which promotes neural induction in ES cells, is not known to be important for neural induction *in vivo*.

1.6.1.1 Retinoic acid treatment

The potent neural inducing factor, RA can be added to suppress BMP signalling (Kawasaki et al., 2000; Tropepe et al., 2001; Watanabe et al., 2005; Ying & Smith, 2003). This method involves the formation of embryoid bodies (EBs) with the addition of RA. However, the developmental pattern capacity of RA-induced neurons were limited to GABAergic neurons (Strubing et al., 1995).

1.6.1.2 The 5 step protocol

The 5 step protocol is a widely used method that first involves expansion of undifferentiated ES cells (stage 1), generation of EBs (stage 2), plating on adhesive substrate in serum free medium for neuronal precursor selection (stage 3), expansion of precursors in the presence of mitogen such as FGF2 (stage 4), and further differentiation by removal of the mitogen (stage5) (Lee et al., 2000). After withdrawal of FGF2, glutamatergic and GABAergic neurons can be generated. Expanded neuronal progenitors from stage 3 can also be cryopreserved and retain 50% viability and differentiation potential (Hancock, Wetherington, Lambert, & Condie, 2000). The EB formation stage in this protocol offers the advantage of providing three dimensional structures, enhancing cell to cell interaction that may be important during neuronal development. However these structures can also cause an increase in endodermal and mesodermal contamination that can be carried throughout the differentiation process (Kawasaki et al., 2000; O'Shea, 1999).

1.6.1.3 Stromal cell derived inducing activity method

A serum free, RA free neural induction method has been established by Kawasaki et al. (2000) that involves the co culture of ES cells with PA6 stromal cells, known as the

stromal cell derived inducing activity (SDIA) method. It is very efficient in promoting neural differentiation and especially efficient in the generation of tyrosine hydroxylase (TH) positive dopaminergic neurons. Later, four factors were found to be responsible for the effect of PA6 stromal cells, these are stromal cell-derived factor 1 (SDF1), pleiotrophin (PTN), insulin like growth factor 2 (IGF2), and ephrin B1 (EFNB1) (Vazin et al., 2009). Stromal or mesenchymal cells have neuronal inducing activity when co-cultured with ES cells as feeders. (Kawasaki et al., 2000; Morizane, Takahashi, Takagi, Sasai, & Hashimoto, 2002). SDIA-induced cultures are able to differentiate into a wide range of CNS cell types that correlate with phenotypes found along the dorsal-ventral and anterior-posterior axes. Cultures derived from the SDIA method predominately express the forebrain marker Otx2, midbrain-hindbrain border marker engrailed 2 (En2), and the rostral hindbrain marker gastrulation brain homeobox 2 (Gbx2). This method is very efficient in generating TH positive dopaminergic neurons with very little expression of the spinal cord markers Homeobox protein Hox-2.6 (Hoxb4), Homeobox protein Hox-2.5 (Hoxb9), and HB9. It also allows the growth of ES cells as colonies from single cells in two dimensional serum free conditions that mimic the development of midbrain during early neurogenesis. Kawasaki et al. (2000) obtained dopaminergic, GABAergic, cholinergic and serotonergic neurons on day 12 of neural induction from this method and Morizane et al. (2002) generated 20-30% dopaminergic neurons. Barberi et al. (2003) is able to modify this protocol with patterning cues for the selective generation of dopaminergic, serotonergic, cholinergic, GABAergic and motor neurons, as well as NS cells, astrocytes and oligodendrocytes. Although the SDIA method has been shown to be an efficient method for neuronal differentiation, the isolation of ES cell derived cells from the stromal cells presents a problem.

1.6.1.4 Adherent monolayer culture

Tropepe et al. (2001) found that ES cell survival in serum free, feeder free conditions is density dependent. When ES cells were cultured at low density, they did not adopt an epidermal fate, approximately 70% of cells died within 24 hours, leaving Sox1 and Nestin expressing survivors. When cultured at high density, however, fewer neural markers were expressed and more ES cell markers are present. A chemically defined, serum free, feeder free culture condition, where FGF signalling played a pivotal role was then established by Ying et al. (2003) which showed more efficient neural commitment and differentiation. However, monolayer derived cultures were limited to anterior neuronal cell types (Gaspard et al., 2008; Konstantoulas, Parmar, & Li, 2010).

1.6.2 Neural differentiation of neural stem/progenitor cells

It has been suggested that *in vivo*, NS cells can differentiate into neurons in areas that support neurogenesis, but only into glia in regions that are non-neurogenic (Shihabuddin, Horner, Ray, & Gage, 2000), suggesting that these cells have the ability to respond to extracellular signals present in local brain environments (Ming & Song, 2005). Differentiation of monolayer ES cell derived NS cells by mitogen withdrawal shows that these cells readily adopt glutamatergic or GABAergic fates with limited dopaminergic and motor neuron generation capability (Bibel et al., 2007; Bibel et al., 2004; Brill et al., 2009; Spiliotopoulos et al., 2009). Direct differentiation of neurospheres cultures has shown that although these cells have multipotent differentiation potential in the generation of neurons and glia, the neurogenic potential is limited to less than 20% (Chojnacki & Weiss, 2008; Grandbarbe et al., 2003; Tropepe et al., 1999a; Weiss & Orkin, 1996).

1.7 General Hypothesis

Differentiation of ES cells into neurons is a highly complex and orchestrated process, which involves the activation and deactivation of genes at various developmental stages. This thesis investigates the hypothesised that NS/NP cells isolated at different time points during the neural induction stage are able to give rise to NS/NP cells capable of generating different neuronal phenotypes.

1.8 General aims

NS cells obtained from fetal or adult brain (*in vivo*) and pluripotent cells (*in vitro*) are able to generate neurons, astrocytes and oligodendrocytes. The generation and maintenance of NS cells have been extensively studied. However, the neurogenic, phenotypic and gliogenic potential have not been thoroughly investigated. In this study, reporter cell lines were utilised using adherent monolayer and SDIA differentiation protocols to examine the phenotypic potential, stability and clonality of ES cell derived NS cells.

1.8.1 Specific Aims

1. Examine the effect of altering the period of neural induction (a crucial step in NS cell derivation) on the phenotypic potential of these cells.

2. Investigate the effect of long term maintenance of ES cell derived NS/NP cells in the presence of EGF and FGF2 on dopaminergic and GABAergic neuron generation

3. Explore the clonal growth and expansion of ES cell derived NS/NP cells

Chapter Two

2. General Methods

2.1 Cell Culture

The pluripotent mouse ES cell lines E14TG2a, Lmx1a-AMP-IRES-eGFP, Lmx1a-eGFP and Pitx3-eGFP were used for this study. E14TG2a is a subclone of the E14 line derived from the inbred strain 129 (Downing et al. 2004). The Lmx1a-AMP-IRES-eGFP was targeted using Lmx1a-AMP-IRES-eGFP-FneoF and targeting vector and was designed to replace exon 1 of the respective genes with genes for AMP (β-lactamase), and enhanced green fluorescent protein (eGFP), separated by an internal ribosomal entry site (IRES), and a neomycin (neo) gene cassette flanked by flippase recognition target (Frt) sites. The Pitx3-eGFP cell line was kind a gift from Dr. C. O'Brien (Zhao et al., 2004).

Generation of targeted reported ES cells Lmx1a-AMP-IRES-eGFP and Lmx1a-eGFP were carried out by Dr Stewart Fabb, detailed protocol is outlined in Appendix A1.

2.1.1 Maintenance of Mouse ES cells

Pluripotent mES cells were maintained at 37°C in a 5% CO_2 incubator in ES growth medium, which consists of supplemented Dubeco's Modified Essential Medium (DMEM; Invitrogen, Australia) with 15% (v/v) ES cell qualified fetal bovine serum (FBS; Invitrogen, Australia), 100 U/mL penicillin G-streptomycin sulpfate (Pen-Strep; Invitrogen, Australia), 0.1 mM β-mercaptoethanol (Sigma, Australia), 4 mM GlutaMAX-1 (Invitrogen, Australia) and 1000 U/mL recombinant mouse LIF (Millipore, Australia). Routine passages were performed every 48-72 hours as cells approached 70% confluency. To passage, cells were washed twice in phosphate buffed saline (PBS, containing 136.9 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4 adjusted to pH 7.4 with 1 M NaOH) before StemPro® Accutase® (Sigma, Australia) was added to the culture vessel (0.1 mL/cm^2) and incubated at 37°C for 2 to 5 minutes to dissociate cells from the vessel. Cells were then resuspended in supplemented DMEM before transferred to a Falcon conical tube and centrifuged for 5 minutes at 200 *xg*. The supernatant was aspirated using a vacuum and discarded and the cell pellet was resuspended with ES growth media. The cells were seeded onto gelatin coated (0.1% (v/v) gelatin in PBS) tissue culture flasks at 2.5x10^4 cells/cm^2. All tissue culture flasks and plates were coated with 0.1% (v/v) gelatin solution (Sigma, Australia) for 15 minutes for 37°C, which was aspirated immediately prior to use.

2.1.2 Differentiation of ES cells into NS/NP cells

Two methods of NI were used in this study, monolayer and PA6 co-culture. For monolayer NI, NS/NP cells were cultured using a modification of the method described

by Conti et al. (2005). Once ES cells reach approximately 70% to 80% confluency ES cells were seeded at 1.5×10^4 cells/cm^2 on gelatin coated culture plastic dishes in ES growth media and LIF. After 24 hours the ES growth media was removed, washed twice with PBS and replaced with N2B27 media; a 1:1 mixture of DMEM/F12 medium (Invitrogen, Australia) and Neurobasal medium (Invitrogen, Australia) supplemented with 5 mL/L N2 supplement (Invitrogen, Australia), 10 mL/L B-27 supplement (Invitrogen, Australia), 10 mg/mL insulin (Sigma, Australia) and 25 mg/L bovine serum albumin (BSA) fraction V (Invitrogen, Australia). The cells were cultured from 4 to 10 days with half media changes every day.

For PA6 co-culture, the PA6 cells (Ricken, Japan) were plated at $5x10^4$ cells/cm^2 in α-MEM medium (Invitrogen, Australia) supplemented with 10% FCS and 50 units/mL-50 µg/mL Pen-Strep, the next day ES cells were seeded at 100 cells/cm^2 onto PA6 cells and the media was replaced with GMEM medium supplemented with 15% Knockout Serum Replacement (KSR), 2 mM Glutamax, 1 mM sodium pyruvate, 0.1 mM MEM non-essential amino acids and 0.1 mM β-mercaptoethanol. Media was replaced every day. On day 8, the media was replaced with N2B27 up to day 10. Once the culture is dissociated, PA6 cells were separated using a 40µm filter and discarded.

On the day of use, the dissociated cells were seeded at 4×10^4 cells/cm^2 on ultra-low attachment plates for 4 to 6 days to allow neurosphere formation. For media changes, half of the existing media was replaced with fresh supplemented NS culture media every second day. At least 140 neurospheres (a single well of a 6 well plate; Figure A3) were used for the next step. The neurospheres were then collected by pipetting the neurosphere suspension gently onto a 40 µm filter. The separated neurospheres were

then placed onto gelatin coated and dried plate in supplemented NS culture media. Expansion of the now adherent neurospheres was allowed for another 4 to 6 days, with media changes every second day.

Once extensive outgrowth of NS/NP cells was observed, the expanded neurospheres can be collected for NS/NP cell expansion and maintenance. The cells were seeded as single cells onto 0.1% (v/v) gelatin coated and dried culture plates, these cells can then be routinely passaged. All culture vessels or plates are coated with 0.1% gelatin for 15 minutes at 37°C and dried completely with an aspirator.

2.1.3 Maintenance of NS/NP cells

2.1.3.1 Adherent monolayer culture

During monolayer cultivation, NS/NP cells were passaged every 48 to 72 hours. Cells were washed with PBS and dissociated with StemPro® Accutase®. After centrifugation, the supernatant was discarded and the cell pellet was resuspended in NS supplemented culture media at a density of 6×10^4 -8×10^4 cells/cm^2 on gelatin coated and dried plates.

2.1.3.2 Non adherent suspension culture

During the suspension process, the neurospheres were collected from the ultra-low attachment plates and centrifuged to remove the existing culture medium. StemPro® Accutase® was then added to the neurospheres and incubated at 37°C for 5 minutes. The neurospheres were then dispersed by gentle pipetting using a 1 mL micropipette. Single cells were then seeded at 4×10^4 cells/cm^2 in ultra-low attachment plates. Neurospheres were passaged every 4 to 6 days in the presence of EGF and FGF2.

2.1.3.3 10 cell suspension cultures

For 10 cell neurosphere maintenance, neurospheres were collected, dispersed and single cells were seeded at 10 cells per well in 96 well ultra-low attachment plates in supplemented NS medium. In some cases single neurospheres were picked and dissociated with StemPro® Accutase® and allowed to form neurospheres in 24 well ultra-low attachment plates. 10 cell neurospheres were passaged approximately every 10 to 20 days.

2.1.4 Cryopreservation-thawing Cells

For long-term storage in liquid nitrogen, cells were dissociated with StemPro® Accutase® and centrifuged as described above. ES cells were resuspended in a mixture of 10% DMSO, 25% FCS and 65% ES growth medium and NS/NP cells were resuspended in 10% DMSO and 90% NS culture medium. The cells were frozen to -80°C at approximately 1°C per minute using “Mr Frosty” (Nalgene, Australia) for 24 hours before transferred to storage in liquid nitrogen dewar.

When required, both ES and NS/NP cells were removed from liquid nitrogen and thawed quickly to 37°C. Once thawed completely, the cell suspension was diluted in ES growth media and centrifuged. The supernatant was aspirated and removed and the cells were seeded on gelatin coated plates. Cells were allowed one passage to recover before used in differentiation experiments.

2.1.5 Differentiation of ES cells into neurons

In all cases neurons were cultured using modifications of the methods described by Barberi et al. 2003. On the day of use, ES cells were dissociated into a single cell suspension and plated onto freshly gelatin coated plates at a density of 1.5×10^4 cells/cm^2 in ES culture medium supplemented with LIF. The cells were allowed to attach to the plate for 24 hours before they were washed twice with PBS and the media is changed to N2B27. The cells were exposed to N2B27 for 7 days with half medium changes every day. After 7 days of N2B27 exposure, the cells were dissociated with StemPro® Accutase® as described above and cell pellet was resuspended in N2B27 supplemented with patterning factors and seeded onto culture plates coated with 1 µg/cm^2 laminin in 0.1% (v/v) gelatin solution, which was allowed to dry for 2 to 3 hours before use. The cells were exposed to patterning factors as described in protocol 1, protocol 2 and protocol 3 for 6 days with media changes every 2 to 3 days before the media was replaced with maturation inducing and neural protective factors: 20 ng/mL brain neurotrophic factor (BDNF) and 200 µM l(+)-ascorbic acid (AA) for a further 10 days. Medium was replaced every 2 to 3 days

2.1.6 Differentiation of NS/NP cells into neurons

On the day of differentiation NS/NP cells were dissociated into single cells using StemPro® Accutase®, and resuspended in NS culture medium supplemented with EGF and FGF2 and plated at 8×10^4-$1x10^5$ cells/cm^2 on gelatin and laminin-coated plates (Figure 2.1).

2.1.6.1 Protocol 1

Dopaminergic neurons: The cells were initially exposed to N2B27 media supplemented with 200 ng/mL Shh, 20 ng/mL FGF2 and 100 ng/mL fibroblastic growth factor 8 (FGF8) during the patterning step (Barberi et al., 2003).

2.1.6.2 Protocol 2

Serotonergic neurons: Cells were exposed to 200 ng/mL Shh, 20 ng/mL FGF2 and 100 ng/mL fibroblastic growth facto 4 (FGF4). After 3 days 100 ng/mL FGF8b replaced FGF4. The incubation was continued for another 3 days during the patterning step (Barberi et al., 2003).

2.1.6.3 Protocol 3

Motor neurons: Cells were exposed to 500 ng/mL Shh, 10 ng/mL FGF2 and 1 μM RA for 4 days, followed by Shh and FGF2 only for 3 days during the patterning step (Barberi et al., 2003).

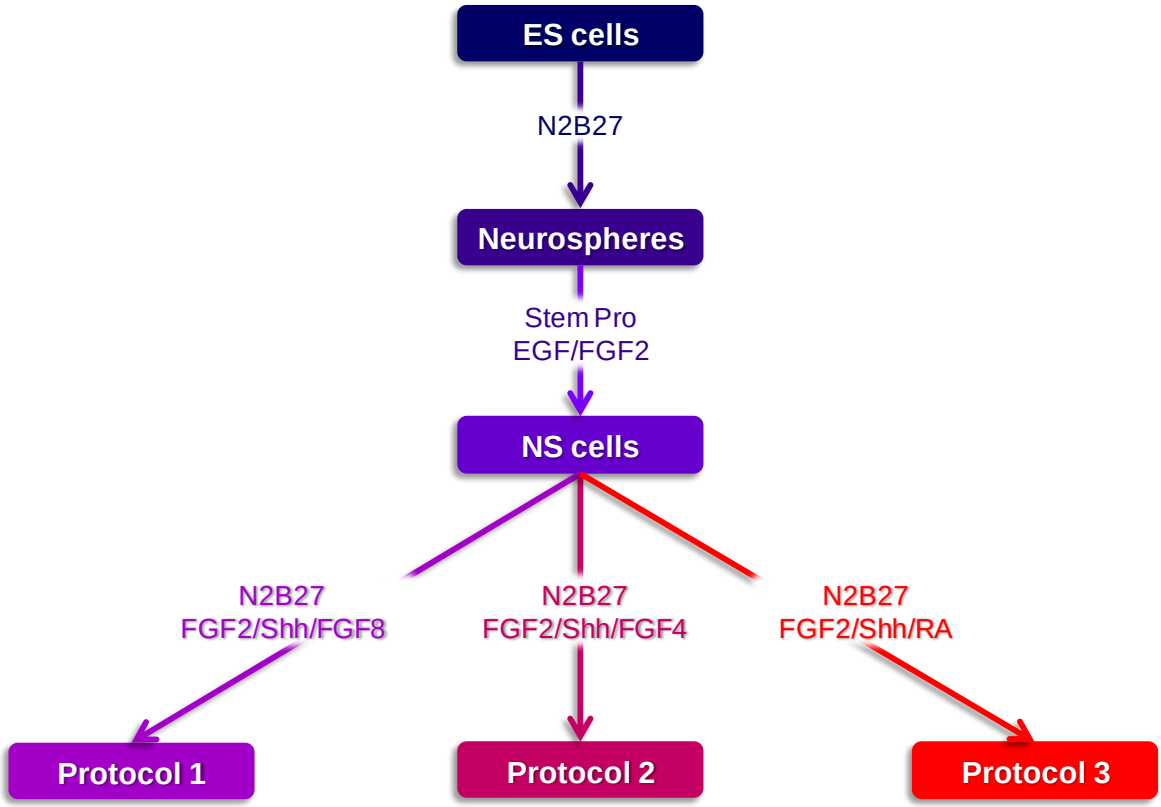


Figure 2.1 Schematic diagrams of differentiation protocols that describe the differentiation of ES cells into NS cells and their subsequent differentiation into neurons following the three different protocols.

2.2 Immunofluorescence Labelling

2.2.1 Immunocytochemistry

Live cells were washed once with PBS and fixed in 4% paraformaldehyde (Sigma, Australia) at room temperature for 30 min, then washed twice with PBS (10 min per wash) and incubated in PBS with 0.5% (v/v) Triton-X (Sigma, Australia; PBS-T) for 30 min at room temperature. The cells were washed twice with PBS and incubated in PBS with 2% (v/v) donkey serum and 2% (v/v) goat serum (Sigma, Australia) for 30 min at room temperature followed by another three washes in PBS. The cells were then incubated with primary antibodies diluted in PBS-T (Table 2.1) overnight at 4 °C before they were washed three times with PBS. They were subsequently incubated with secondary antibodies diluted in PBS-T (Table 2.2) for 2.5 h at room temperature before washing three more times with PBS. The cells were incubated with DAPI (50 ng/mL) for 5 min at room temperature and washed twice with PBS immediately prior to visualisation. Testing of each the primary and secondary antibodies were carried out on ES cell derived samples and mouse brain slices. Positive controls of the primary antibodies listed in table 2.1 were carried out on mouse brain slices (Figure A10A-D). Negative controls of the secondary antibodies listed in table 2.2 were carried out on samples used for analysis without primary incubation (Figure A10E).

2.2.2 Immunohistochemistry

For the preparation of mouse brain slices, 4% PFA perfused and fixed mouse brain was cut into 8 µm slices using the Leica CM1850 cryostat then mounted onto glass slides and allows to dry for 1 hour prior to primary and secondary antibody incubation as described above.

In some cases the neurospheres were extracted from media. The neurosphere suspension was allowed to settle for 30 minutes and the supernatant removed carefully. The neurospheres were then transferred to a tissue embedding mould and Tissue-Tek O.C.T. was added prior to storage at −80 °C. The frozen neurospheres were cut into 8 µm slices then mounted onto glass slides and allowed to dry for 1 h. The sections were fixed with 4% paraformaldehyde at room temperature for 30 min prior to primary and secondary antibody incubation as described above.

Table 2.1 Conditions and suppliers of primary antibodies used in immunocytochemistry, immunohistochemistry and quantitative immunocytochemistry experiments.

Antibodies	Dilutions	Manufacturer	References
Rabbit anti-tyrosine hydroxylase (TH)	1:200	Chemicon	Collo et al. 2013; Trisch et al. 2012; McCutcheon et al. 2012; Katori et al. 2012; Ladewig et al. 2012; Traschenberger et al. 2012
Rabbit anti-serotonin (5HT)	1:3000	Sigma	Fouad et. al 2010; Hatami et. al 2009; Podrygajlo et. Al 2009; Koch et. Al 2009
Rabbit anti-βIII-tubulin (TUJ-1)	1:800	Covance	Locher et al. 2013; Schoenmann et al. 2010
Rabbit anti-musashi1	1:300	Abcam	Nizzardo et al. 2014; Kanwar et al. 2010; Takehara et al. 2009; Le et al. 2009
Rabbit anti-tight junction protein 1 (ZO-1)	1:300	Zymed	Assawachananont et al. 2014; Esmailpour et al. 2012; Xu et al. 2012; Lamba et al. 2010; Koch et al. 2009
Rabbit anti-brachyury (Bry)	1:200	Abcam	Goh et al. 2013; Tarafdar et al. 2013; Wheadon et al. 2011; Pekkanen-Mattila et al. 2010; Arpornmaeklong et al. 2009
Rabbit anti-α1-fetoprotein	1:200	Abcam	Xin et al. 2014; Zhu et al. 2014; Gong et al. 2013; Ritner et al. 2011
Rabbit anti-oct3/4 (Pou5f1)	1:200	Sigma	Gopalakrishna-Pillai et al. 2011; Okamura et al. 2008; Hoof et al. 2006
Rabbit anti-gamma-aminobutyric acid (GABA)	1:1000	Sigma	Serre et. al 2012; Li et. al 2011; Liu et. 2011; Tanaka et al. 2011; Desfeux et. al 2010
Mouse anti-TH	1:800	Immunostar	Fitzgerald et al. 2012; Bernácer et al. 2012; Mast et al. 2012
Mouse anti-βIII-tubulin	1:800	Covance	Dugan et al. 2008; Jouhilahti et al. 2008; Chen et al. 2007; Jepsen et al. 2000
Mouse anti-NeuN	1:100	Chemicon	Weiner et al. 2008; Erzurumlu et al. 2006; Bloechlinger et al. 2004; Fricker-Gates et al. 2004
Mouse anti-nestin	1:200	Sigma	Schira et al. 2012; Beck et al. 2011; Conti et al. 2005
Mouse anti-choline acetyltransferase (ChAT)	1:200	Abcam	Sims et al. 2013; Akten et al. 2011; Sibaev et al. 2009
Mouse anti-glial fibrillary acidic protein (GFAP)	1:300	Chemicon	Bao et al. 2012; Gray et al. 2011; Ibrahim et al. 2011; Wall et al, 2010
Goat anti-vimentin	1:50	Sigma	Yu et al. 2011; Shime et al. 2002; Risco et al. 2002

Table 2.2 Conditions and suppliers of secondary antibodies used in immunocytochemistry, immunohistochemistry and quantitative immunocytochemistry experiments.

Antibodies	Dilutions	Manufacturer
Donkey-Alexa Fluor 594 anti-rabbit	1:1000	Molecular Probes
Donkey-Alexa Fluor 567 anti rabbit	1:1000	Molecular Probes
Donkey-Alexa Fluor 594 anti rabbit	1:1000	Molecular Probes
Donkey-Alexa Fluor 488 anti-mouse	1:1000	Molecular Probes
Donkey-Alexa Fluor 594 anti-mouse	1:1000	Molecular Probes
Donkey-ALexa Fluor 637 anti-mouse	1:1000	Molecular Probes
Goat-Alexa Fluor 488 anti-rabbit	1:1000	Molecular Probes

2.2.3 Imaging

Cells were viewed using a Nikon TE2000U microscope coupled to a Coolsnap-fx low light camera and illuminated using a Sutter Instruments DG-4 light box. Cells were illuminated at 482, 594 and 647nm. Emission wavelengths were detected at 520-540, 590-610 and 650-670 nm, respectively. Cultures were also viewed using a Nikon A1R laser scanning confocal microscope.

The percentage of neurons and or specific neuronal phenotypes was obtained by counting the number of DAPI stained cells and the number of cells that were immunoreactive to the antibody of interest in the same field of view with the Metamorph® imaging software (Universal Imaging Co., USA). At least three fields of view were captured within each well from three separate experiments (Figure 2.2). Comparisons of

manual cell count and Metamorph® imaging software were carried out. The data obtained indicate no significant difference between the methods (Figure A1).

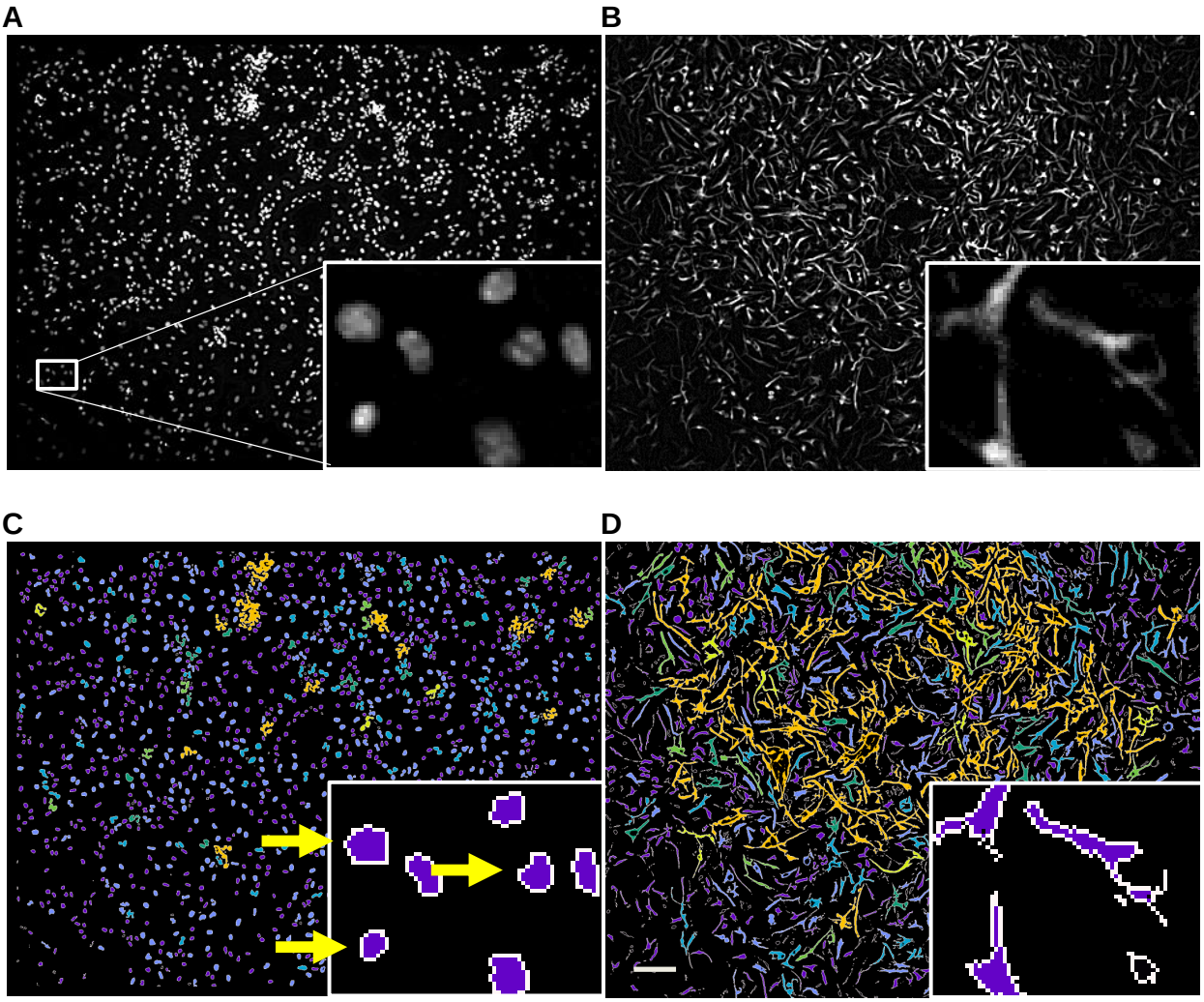


Figure 2.2 Cells immunoreactive to antibodies were quantitated using Metamorph®. 50 individual DAPI positive cells were first selected at random to obtain the average surface area of individual cells. The overall area of DAPI positive cells was then measured and the total number of cells in the field of view was then calculated. The procedure was then carried out on Nestin, Musashi1, βIII-tubulin, GFAP, TH, 5HT or NeuN positive cells in the same field of view. (A and B) Example showing view flattened background of (A) DAPI and (B) GFAP positive cells, and quantitated image of (C) DAP cells and (D) GFAP positive cells in the same field of view. All cells in the field of view were counted. (Purple coloured cells indicate the presence of single cells and colours other than purple, such as blue, green and yellow indicate the presence of more than one cell. Yellow

arrows indicate DAPI positive cells that are also GFAP positive. All images are of the same scale; Scale bar 100 µm)

2.3 Flow Cytometry

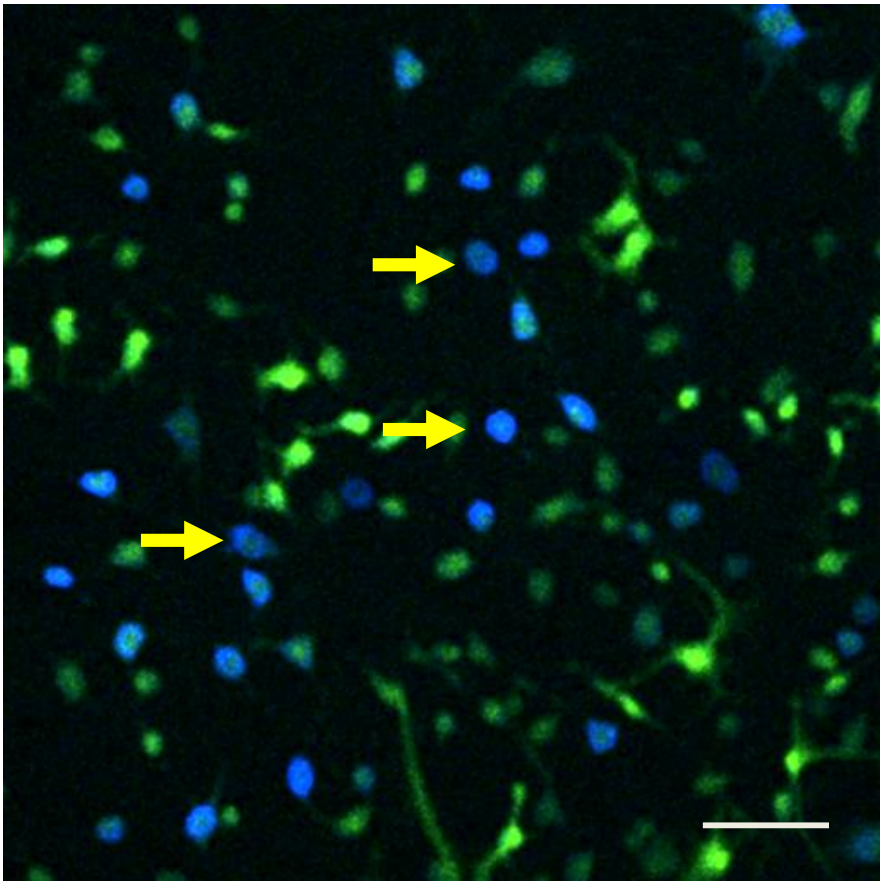
2.3.1 β-lactamase and eGFP Detection

For β-lactamase and eGFP detection using the Lmx1a-β-lactamase-IRES-eGFP and Lmx1a-eGFP neurospheres and monolayer cultures were first washed twice with PBS and dissociated with StemPro® Accutase® and were collected via centrifugation. The dissociated Lmx1a-β-lactamase-IRES-eGFP cells pellet was resuspended in the LiveBLAzer™ FRET – B/G with CCF4AM loading solution and incubated in the dark for 2.5 hours. PBS was then added to cells to dilute the loading solution and the suspension was centrifuged and the supernatant was discarded. This step was skipped for Lmx1a-eGFP cells. The cell pellet was resuspended and incubated in 1000 U/mL Sytox red with PBS in the dark at 4°C for 15 minutes for dead cell detection. The samples were then analysed using a BD FACS Canto II™ flow cytometer. Cells were illuminated at 405 nm with emissions recorded at 450 nm and 520 nm (Figure 2.3). Wild-type E14Tg2a ES cells treated under the same condition were used as control for gating positive cells. For GFP expression analysis, cells were dissociated and resuspended in PBS. Wild-type E14Tg2a ES cells treated under the same condition were used as control for gating positive cells. For all FACS experiments, gates were set where 99.9% of wild-type cells were determined to be negative (Figure 2.4).

2.3.2 β-lactamase and eGFP separation

For FACS separation experiments, day 10 neural induction cultures were dissociated with Accutase® and resuspended in PBS with 5% FCS (v/v). Prior to FACS extraction, the resuspended sample was filtered through a 40μm strainer (BD Biosciences, Australia) to remove aggregates. Flow cytometry was performed with a FACS Aria I (BD Biosciences, Australia) where the highest 25% of cells expressing β-lactamase were

collected. Post FACS sorting, Lmx1a positive fractions were used immediately or neurosphere formation at 4×10^4 cells/cm^2 on ultra-low attachment plates for neurosphere formation. After neurosphere formation neurospheres were collected and passaged as described in section 2.1.3.



Pacific blue/GFP

Figure 2.3 Cells incubated with the CCF2/4 β-lactamase substrate. GFP positive cells indicate cells that have taken up the substrate and Pacific Blue positive cells indicate Lmx1a positive cells (Yellow arrows). Example showing passage 2 day 10 monolayer cultures. (Scale bar 100 µm).

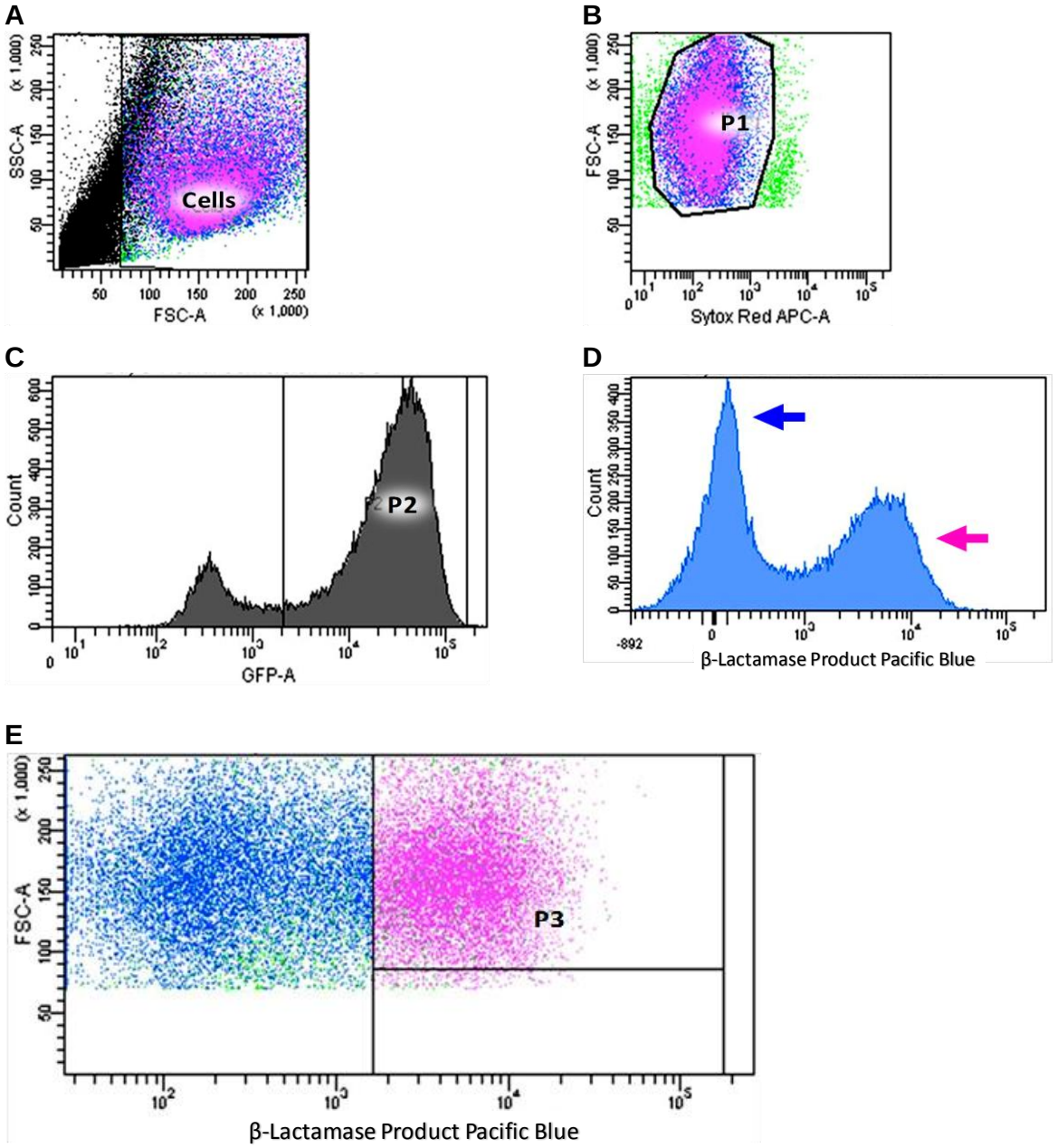


Figure 2.4 FACS analysis of Lmx1a positive populations. (A)The "Cells" population was gated by selecting the dominant population excluding the debris. "Cells". (B) Cells that have taken up the CCF4 substrate are GFP positive hence GFP positive cells "P1" were then selected as a subpopulation of "Cells". (C) Live cells that were Sytox red negative "P2" were selected as a subpopulation of "P1". (D and E). The Pacific blue positive cells "P3" were then selected as a subpopulation of "P2" as the final percentage. Histogram (D) and dot plot (E) of Pacific blue positive cells. The blue arrow indicates Pacific blue negative population and the pink arrow indicates the Pacific blue positive population. (Example showing cultures sorted on day 10 of neural induction)

2.3.3 Aldehyde dehydrogenase detection

For ALDFLUOR® staining, the cells were washed and dissociated as single cells as described above, the ALDFLUOR® assay buffer containing the aldehyde dehydrogenase (ALDH) substrate (BAAA) was added. The samples were incubated for 1 hour at 37°C and the cells were centrifuged at 4°C for 5 minutes and resuspended in cold assay buffer. Sample incubated with both the ALDH substrate and ALDH enzyme inhibitor were used as control for gating positive cells.

2.4 Quantitative Polymerase Chain Reaction

2.4.1 RNA extraction and cDNC synthesis

Total RNA was extracted from >10^6 cells per sample using the RNeasy micro extraction kit (Qiagen) following the manufactures specification. RNA samples were quantified using the Nanodrop® ND-1000 (Thermo Scientific, USA) spectrophotometer. Complementary DNA was transcribed using SuperScript III transcriptase kit (Invitrogen, Australia) using oligo-dT as a primer with 200 ng of RNA as specified by the manufacturer in a total volume of 20 µL. The samples were used immediately or stored at -80°C.

2.4.2 Primer sequences and qPCR conditions

PCR was performed using the HotStar Taq PLymerase PCR kit (Qiagen) on a Corbett Research thermal cycler using primer concentrations of 0.2 µM. General PCR conditions were 94°C for 10 minutes followed by 45 cycles consisting of 94°C for 1 minute, 53°C to 60°C for 1 minute and 72°C for 1 minute. Primer sequences and PCR conditions have been described by Nefzger et al. (2012).

All quantitative Polymerase Chain Reactions (qPCR) were performed on 3 technical replicates and 3 biological replicates. SYBR Green I Master Mix (Roche, Australia) and the Light Cycler 480 System (Roche, Australia) were used for the analysis of primers outlined in Table 2.3. 1 µl of cDNA was added to a PCR Mix containing 7 µl PCR-grade water, 2 µl PCR primer pairs and 10 µl 2x Master Mix. General PCR conditions were 95°C for 10 minutes followed by 50 cycles with 95°C for 10 s, 60°C for 30 seconds. For the En1 primers, conditions for the 50 cycles were 95°C for 10 seconds, 60°C for 20 seconds and 72°C for 30 seconds. After PCR amplification, samples were maintained at

4°C for 10 seconds. Specificity of the used primer pairs was verified by DNA sequencing of products (Micromon, Australia). Lmx1a and FoxA2 primer sequences were previously described by Čajánek *et al.* (2009) and the En1 primer sequences by Kim *et al.* (2002). All other primer pairs were designed using the program “Primer Premier 3.0.

qPCR analyses of βIII-tubulin (Mm00727586_s1), TH (Mm00447557_m1), GAD1 (Mm00725661_s1), FGFR1 (Mm00438930_m1) and EGFR (Mm00433023_m1) (TaqMan® Probe) was carried out. Amplification was performed in a single step from isolated RNA with the Superscript™ III Platinum® One-Step quantitative RT-PCR System (Invitrogen, Australia) according to the manufacturer's instructions. 0.75 µL of RNA was used in 15 µL reactions.

Data analysis was performed using the $2^{-\Delta\Delta CT}$ method (Livak *et al.*, 2001). Relative quantification values were obtained by standardising C_T values of each target gene to averaged C_T values of two housekeeping genes (β-actin and TATA box binding protein). Figure 2.5 shows typical amplification and melting curves of qPCR experiments.

Table 2.3 Primer sequences and conditions for quantitative PCR.

Primer	Sequence	Size (bp)	Annealing Temperature (°C)
β-actin	5'-CTAAGGCCAACCGTGAAAAG-3' 3'-ACCAGAGGCATACAGGGACA-5'	104	60
TATA box binding protein	5'-CTGCTGTTGGTGATTGTTGG-3' 3'-AACTGGCTTGTGTGGGAAAG-5'	100	60
Lmx1a	5'-GAGACCACCTGCTTCTACCG-3' 3'-CCTCCTTCAGGACAAACTCG-5'	211	60
Lmx1b	5'-GCCAAGAGGTTCTGTCAAGC-3' 3'-GCTACTTCCGTAGGGGCTCT-5'	110	60
Foxa2	5'–CATCCGACTGGAGCAGCTA-3' 3'–CATAGGATGACATGTTCATGGAG-5'	171	60
Foxg1	5'-CTGACGCTCAATGGCATCTA-3' 3'-TCACGAAGCACTTGTTGAGG-5'	118	60
En1	5'-TCAAGACTGACTCACAGCAACCCC-3' 3'-CTTTGTCCTGAACCGTGGTGGTAG-5'	381	60
Nestin	5'-CTCGAGCAGGAAGTGGTAGG-3' 3'-GCCTCTTTTGGTTCCTTTCC-5'	140	60
BMP2	5'-GAACCCAGGTGTCTCCAAGA-3' 3'-TGACGCTTTTCTCGTTTGTG-5'	143	60
Notch1	5'-TGTTGTGCTCCTGAAGAACG-3' 3'-GCAACACTTTGGCAGTCTCA-5'	110	60
Sox1	5'-AGACAGCGTGCCTTTGATTT-3' 3'-TGGGATAAGACCTGGGTGAG-5'	124	60
Sox2	5'-GAACGCCTTCATGGTATGGT-3' 3'-TCTCGGTCTCGGACAAAAGT-5'	125	60

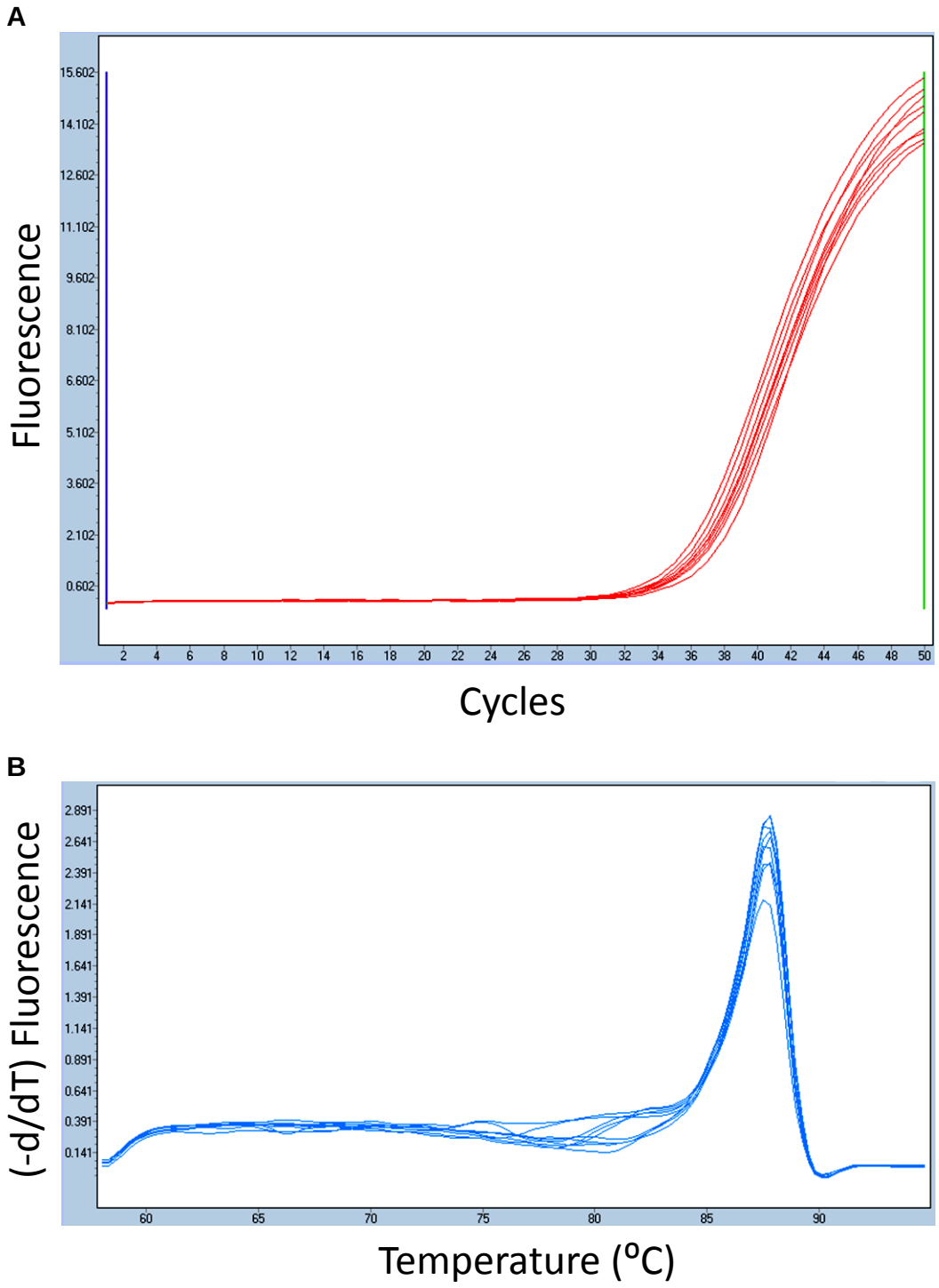


Figure 2.5 Typical (A) amplification and (B) melting curves of qPCR experiments. (Example showing day 10 monolayer derived cultures reacting to Lmx1a)

2.5 Numerical and statistical analysis

Statistical analyses were carried out using PRISM v5.00 and statistical significances were determined using the One-way or Two-way ANOVA followed by post hoc Dunnett's or Bonferroni's tests. Numerical results are presented as mean ± SEM of three separate fields of view (from 4 separate experiments for differentiation of ES cells into NS/NP cells; and 3 separate experiments for differentiation of NS/NP cells into neurons). $p<0.05$ was taken to be statistically significant.

Chapter Three

3. The Effect of Neural Induction on Phenotypic Potential of Neural Stem/Progenitor Cells

3.1. Introduction

Pluripotent ES cells have been differentiated into specific neural cell types such as dopaminergic, serotonergic, GABAergic and motor neurons, as well as astrocytes and oligodendrocytes (Barberi et al., 2003; McKay, 1997; Wichterle, Lieberam, Porter, & Jessell, 2002). Generally these neuronal differentiation protocols, irrespective of whether they utilize EB formation, monolayer or co-culture methods, include a specific period of time for NI, when the cells are exposed to neurobasal medium in the absence of mitogens. The NI period gives rise to NS cells, which are multipotent cells that can be differentiated further under the influence of growth factors and neuroprotective agents

during a fourteen to twenty day period (Kawasaki, Mizuseki, & Sasai, 2002; Lee et al., 2000; Ying & Smith, 2003). Such protocols require long periods of cultivation and are complex, requiring specific conditions at precise time points to direct them towards the desired neural lineage. In addition the use of pluripotent ES cells as the starting point may result in contamination of neural cultures with cells of mesodermal or endodermal origin (Pollard et al., 2006). It would be more desirable to commence differentiation from NS or neural NP cell source that is able to self-renew as well as differentiate into the desired cell types. NS cells can be generated from ES cells, and they can also be isolated from fetal and adult brain tissue. Although the phenotype of NS cells grown in culture may be an artefact of the cell culture conditions (Conti & Cattaneo, 2010), there is no doubt NS cells will be a valuable source of cells for biological research and possibly therapeutic uses. Isolated NS cells can be maintained in monolayer culture or aggregates, in the presence of exogenous factors such as EGF (Reynolds & Weiss, 1992) or both EGF and FGF (Conti et al., 2005). NS cells have often been cultured in aggregated spheroid structures, referred to as neurospheres. These are complex three-dimensional structures consisting of heterogeneous mixtures of stem cells, progenitor cells and mature cell types. This complex structure is difficult to define and control the early phase of neuronal differentiation. Although Conti et al. (2005) showed that NS cells generated from neurospheres were multipotent when grown in the presence of FGF2 and EGF (Glaser & Brustle, 2005; Sun et al., 2008), the focus of these studies was on whether the cells could produce neurons and glia, rather than on whether various or all subtypes of neurons could be produced. A recent follow up study using ES cell-derived NS cells indicated that their phenotypic potential, at least under the culture conditions used by these authors, appeared to be largely restricted to GABAergic neurons and glia (Spiliotopoulos et al., 2009). From a practical perspective it would be desirable to establish methods for culture of multipotent NS cells and reliable methods for directing

their differentiation, or to at least establish methods for isolation and expansion of panels of committed NP cells, each of which can generate specific phenotypes.

This chapter investigates the hypothesis that the length of NI prior to neurosphere formation in EGF and FGF2 may influence the subsequent multipotency of cultures.

3.2. Aims

This study aims to generate NS/NP cell cultures following different periods of NI (4, 7 and 10 days) by exposing ES cells to the neural inducing media N2B27; and subsequently differentiate these ES cell-derived NS/NP cells using protocols that have been shown to generate different neuronal phenotypes such as dopaminergic, serotonergic and cholinergic neurons. The extent of differentiation will be assessed both qualitatively and quantitatively with a combination of neuronal phenotype specific markers *via* FACS analysis and immunocytochemistry.

3.3 Methods

The pluripotent stem cell reporter line Lmx1a-AMP-IRES-eGFP was used to track expression of Lmx1a; an early gene associated with neural development, which is essential for the development of dopaminergic neurons, during NI and terminal differentiation. The experimental methods used in this chapter, including the differentiation of ES cells into NS/NP cells and terminal differentiation have been described in Chapter 2.

3.4. Results

3.4.1. The effect of neural induction on Lmx1a expression

To investigate the time course of neural induction (NI), LIF and serum was withdrawn from ES cells and used immunocytochemistry to track, over a 13-day period, the expression of Oct3/4 (ES cells); Nestin and Mushashi-1 (NS cells); βIII-tubulin (immature neurons) and GFAP (astrocytes). Figure 3.1 shows the time course of expression and Figure 3.2 shows typical examples of immunolabelling. Oct3/4 positive cells were present in undifferentiated cultures and were clearly visible until day two of NI (Figure 3.1A and B). Nestin expression was apparent one day after removal of LIF, and was sustained in the majority of cells in cultures beyond day 10; most of the cells may have differentiated beyond the NS stage (Figure 3.1A and C). After day 3 of NI the majority of cells expressed Musashi1 until at least day 13 (Figure 3.1A and D). On day 4 a few cells expressed βIII-tubulin and there was a steady increase in expression up to day 13 of NI (Figure 3.1A and D). Low levels of GFAP were detectable during neural NI (Figure 3.1A and E).

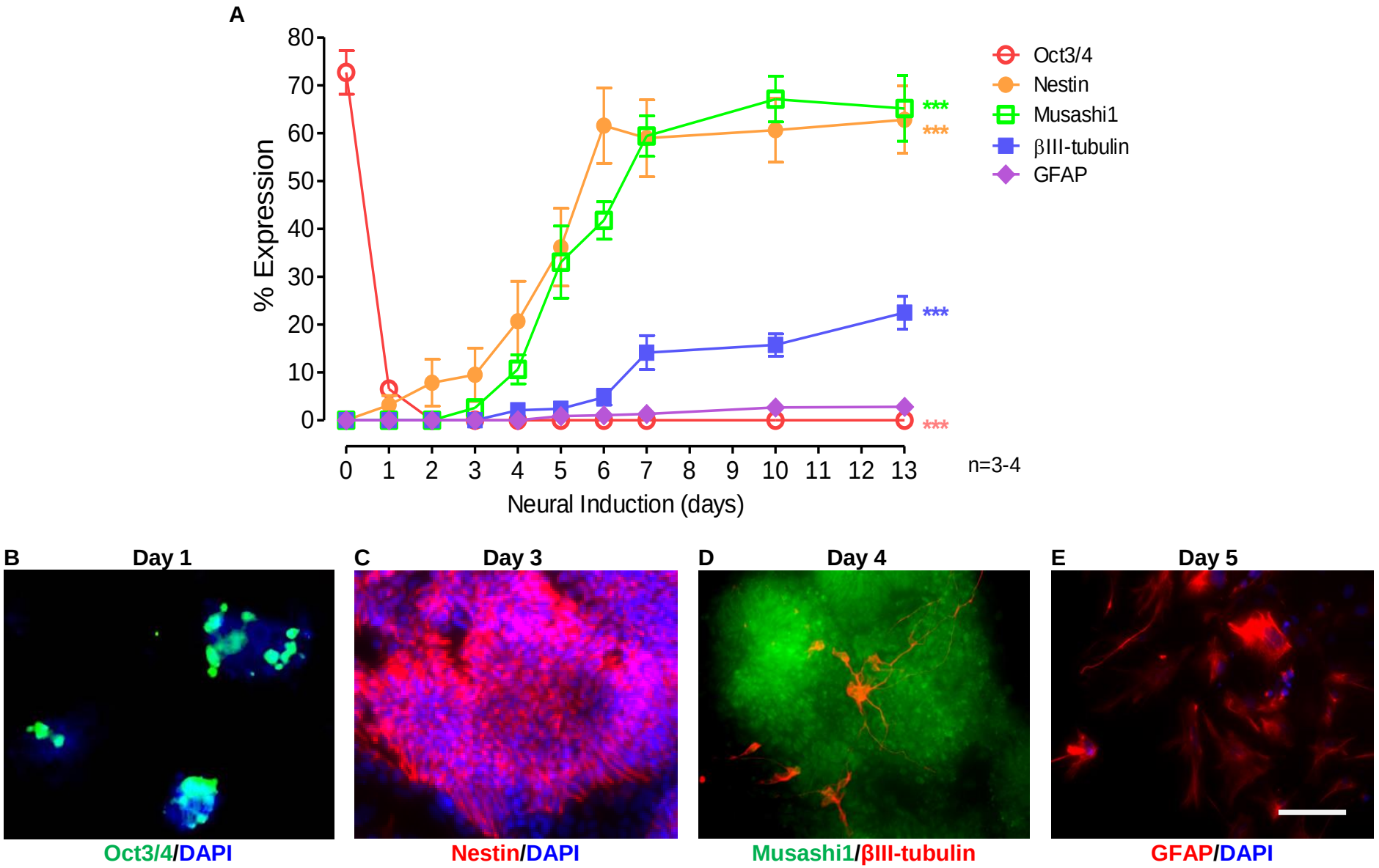

A
% Expression
Neural Induction (days)
Oct3/4
Nestin
Musashi1
βIII-tubulin
GFAP
***
***
***
***
n=3-4
B
Day 1
Oct3/4/DAPI
C
Day 3
Nestin/DAPI
D
Day 4
Musashi1/βIII-tubulin
E
Day 5
GFAP/DAPI

Figure.3.1 (A) Time course of protein expression during neural induction as indicated by immunocytochemistry. Oct3/4 immunoreactivity was essentially absent by day 3. Proneural genes, Nestin, Musashi1, βIII-tubulin and GFAP were detected from days 1, 3, 4, and 5, respectively during neural induction. By day 13, significant decrease in Oct3/4 positive cells and significant increase in Nestin, Musashi1 and βIII-tubulin positive cells were evident in the cultures (n=3-4; ***p<0.001; two-way ANOVA followed by Bonferroni's test compared to day 0 cultures). Examples of typical immunolabelled of NI cultures. (B) Day one cultures included Oct3/4 positive cells. (C) Typical Nestin staining in day three cultures. (D) At day 4 the majority of the cells were Musashi1 positive, and a few βIII-tubulin positive cells co-localised with Musashi1 positive cells. (E) Typical GFAP staining at day five (All images are of the same scale; Scale bar 100 µm).

Using the targeted Lmx1a-β-lactamase reporter ES cell line, the relative expression of β-lactamase over a 13-day NI period was compared. The population of β-lactamase positive cells increased up to day 13 of NI (Figure. 3.2). Between days 4 and 7, the period within which NS cells have been derived previously, a 20% of cells expressed β-lactamase but at a lower level than typical expression levels on days 10–13 (Figure 3.3 shows typical FACS plots of 2 and 10 day NI, respectively). Taken together these data suggest that ES cells exposed to 10 days of NI included 40% of NPs, which had differentiated beyond the NS cell stage. As a result, 3 different time points were chosen as starting points for NS/NP cell isolation and propagation. Day 4 and day 7 of NI represents time points were the initial neural and neuronal parkers were first expressed and plateaued (Figure 3.1A); and day 10 represent a peak in Lmx1a expression during the 13 day NI analysed (Figure 3.2).

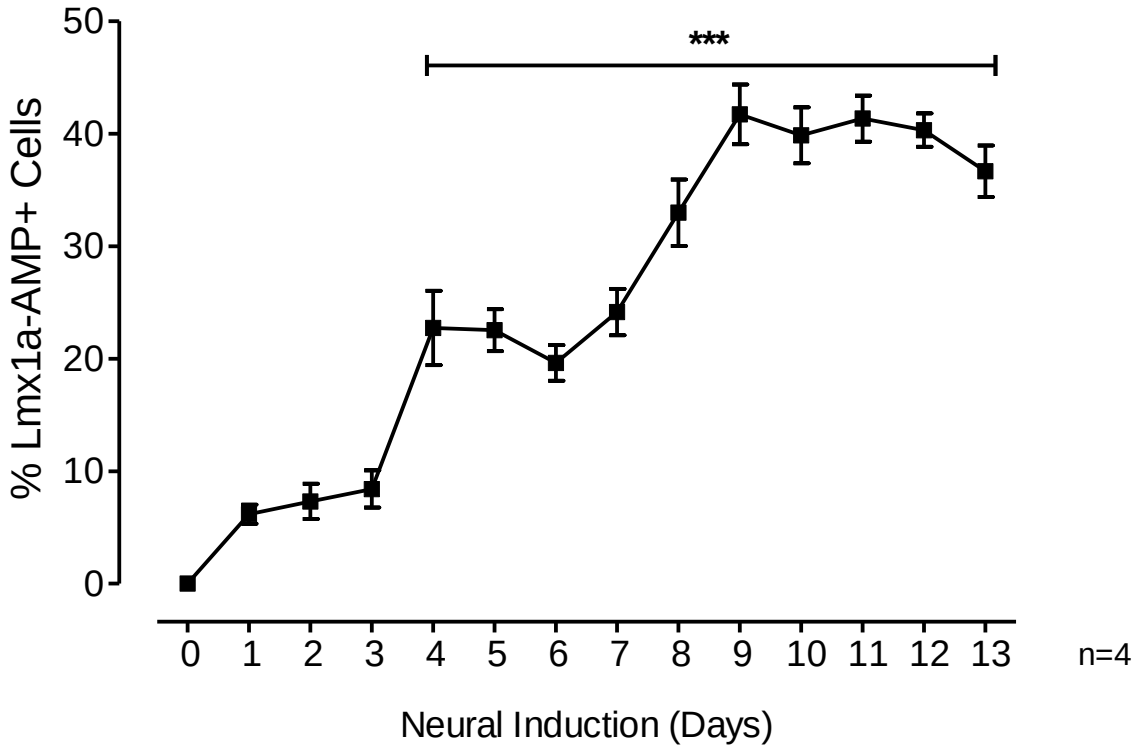


Figure 3.2 Lmx1a expression was examined during neural induction. FACS analysis was used to determine the percentage of Lmx1a expressing cells on successive days during NI (determined by expression of β-lactamase, the cDNA of which was targeted to exon 1 of one allele of Lmx1a). (n=4; ***p<0.001, two-way ANOVA followed by Bonferroni's test compared to day 0 cultures)

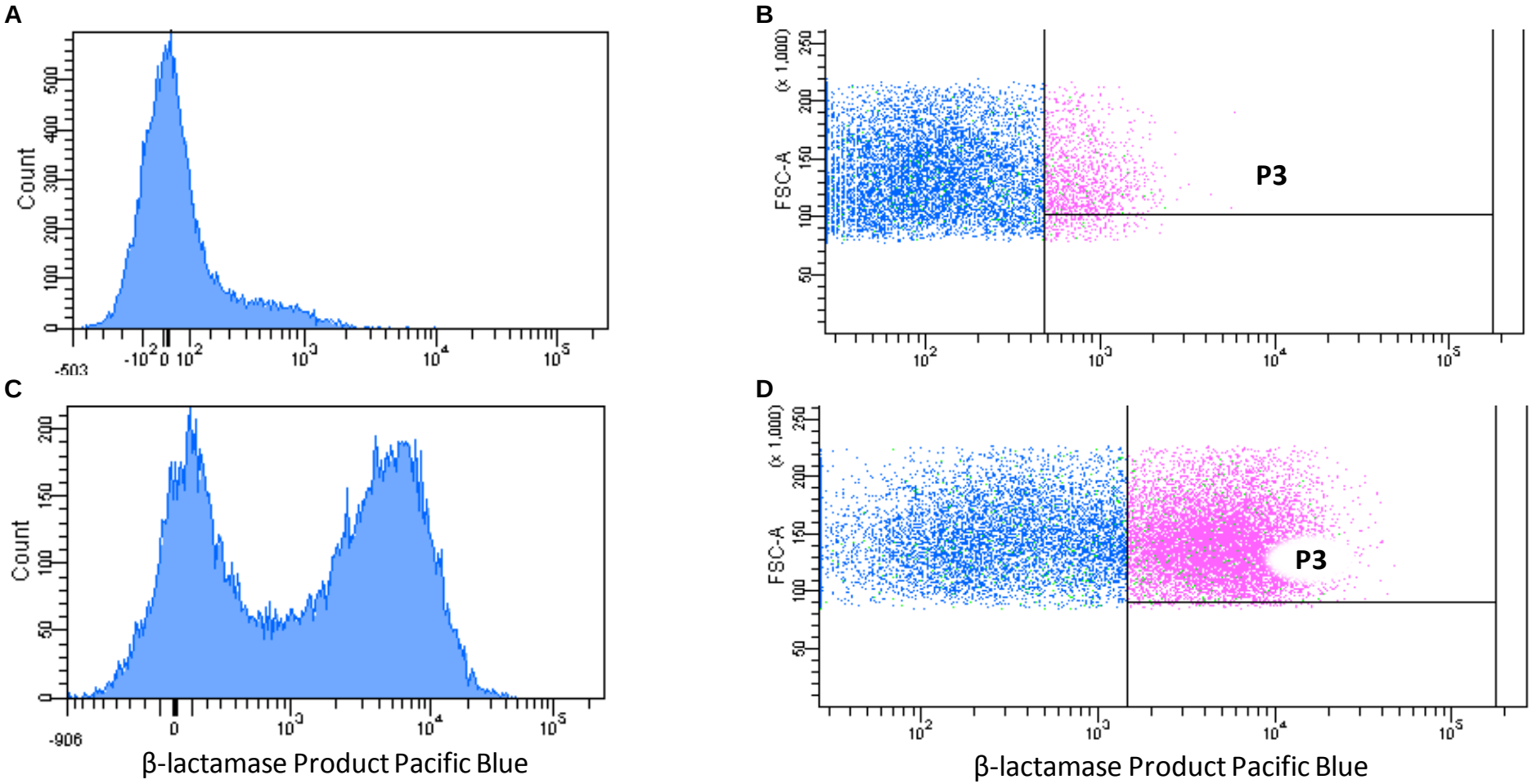


Figure 3.3 Typical histogram and dot plot of Lmx1a expression during neural induction. (A and B) Typical histogram (A) and dot plot (B) of day two cultures. (C and D) Typical histogram (C) and dot plot (D) of day 10 cultures (Lmx1a positive population was gated according to 0.1% Lmx1a expression in wild type NI cultures under the same culture conditions)

3.4.2 The effect of EGF and FGF2 propagation on 4, 7 and 10 day NS/NP cells

Using a method adapted from Conti et al. (2005), neurospheres in non-adherent conditions from ES cells cultures were derived following 4, 7 or 10 days of NI. Neurospheres formed after 4, 7 and 10 days revealed foci, which were immunoreactive for the neural rosette marker, ZO1, typically surrounded by Nestin positive cells (Figure 3.4A; Data not shown from day 4 and 7 EBs). This indicated that the neurospheres contained NS/NP cells present in rosette form. The neurospheres were positive for Musashi1 throughout the spheres (Figure. 3.4B) and typically had βIII-tubulin positive cells at the periphery around a core of Nestin-positive cells (Figure. 3.4C).

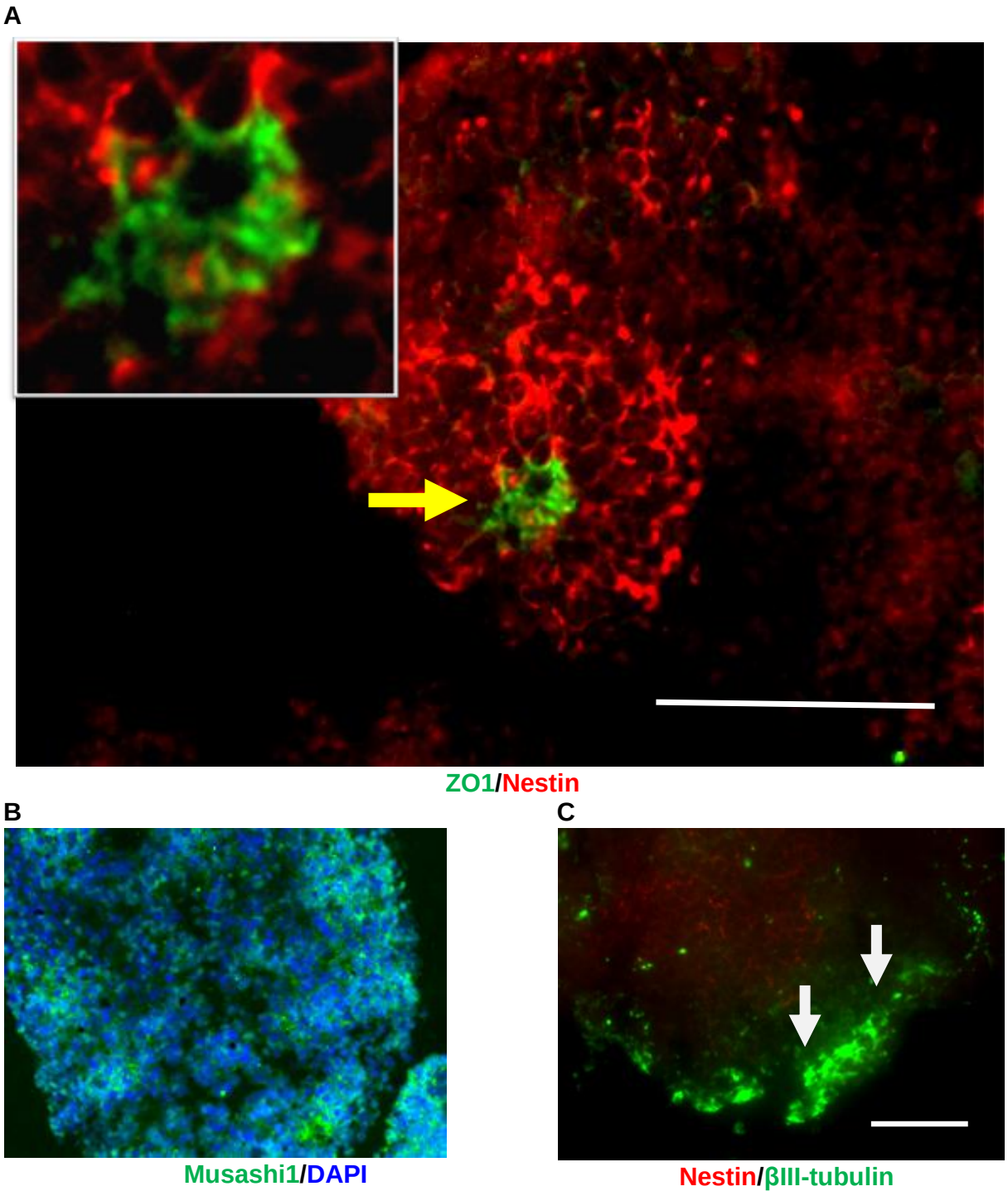
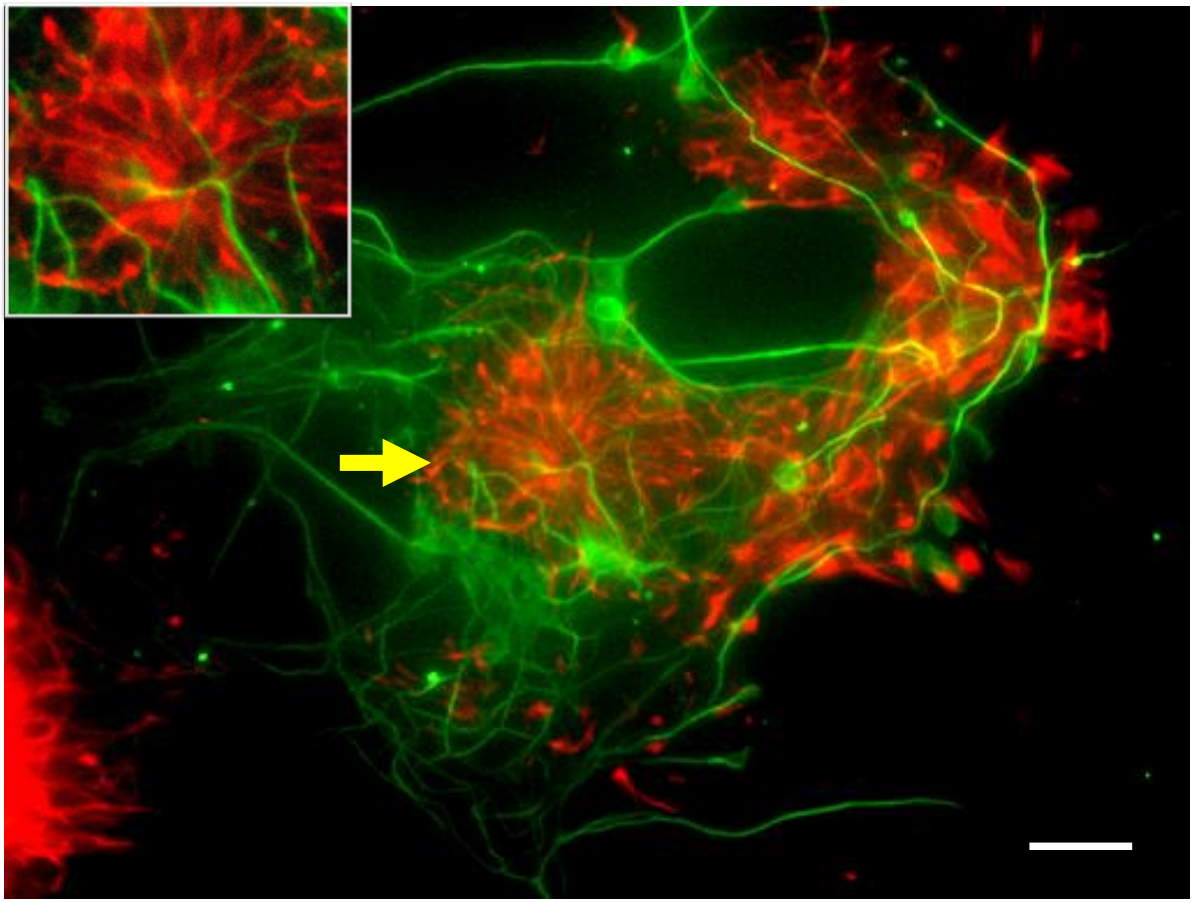


Figure 3.4 Immunocytochemistry of sectioned neurospheres derived after 10 days of neural induction. (A) Immunocytochemistry indicated the presence of ZO1 positive central regions of rosettes surrounded by Nestin positive cells. (B) Typical pattern of Musashi1 expression throughout the neurospheres. (C) βIII-tubulin positive cells were typically present on the outer surface of neurospheres (Scale bar 100 µm).

Following plating of neurospheres on laminin, and expansion with EGF and FGF2, neurosphere-derived cells migrated and spread onto the laminin surface. Only the neurospheres derived after 10 days NI gave rise to the structures shown in Figure 3.5, regions rich in Nestin-positive neural rosette structures, surrounded by immature neurons. Some cultures, up to 7 days of NI, contained mesodermal cells, as indicated by the presence of the mesodermal marker, Brachyury (Figure 3.6); and the presence of spontaneously contracting aggregates, which may be cardiac myocytes. Cells derived from endoderm were not evident following immunolabelling with anti-a1-fetoprotein. This indicates that 4 days was an insufficient period to guarantee neural induction.



Nestin/βIII-tubulin

Figure 3.5 Immunocytochemistry of expanded neurospheres derived from 10 days of neural induction. The neurospheres were expanded in EGF and FGF2 and subsequently plated onto laminin coated plates, still in the presence of EGF and FGF2, whereupon they spread onto the substrate and the cell numbers continued to expand. Immunocytochemistry for βIII-tubulin and Nestin in neurospheres formed after 10 days NI, following plating under adherent conditions in the presence of EGF and FGF2, indicated the presence of rosette structures, and the development of neurons at the periphery (Scale bar 100μm).

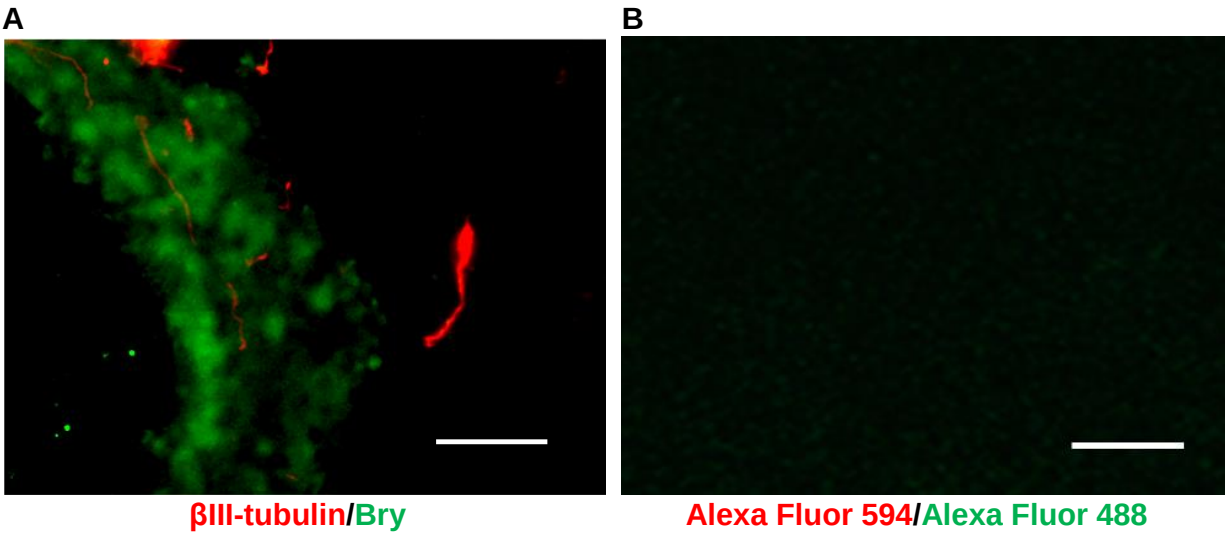


Figure 3.6 (A) Immunocytochemistry of cultures on day four of neural induction showed immunoreactivity to the mesodermal marker Brachyury with small numbers of βIII-tubulin positive cells. This was not observed in day seven or ten cultures. (B) Negative control: day four cultures incubated with secondary antibodies only with the same acquisition and illumination parameters (Scale bar 100 µm)

When these cultures were detached, resuspended and grown as monolayers on laminin in the presence of EGF and FGF2, after two passages, cultures derived from 4, 7 and 10-day neurospheres adopted different morphologies. The 4-day NS cells produced a flatter, spindle-shaped morphology, more commonly associated with glial cells (Figure. 3.7A), while the 7 and 10-dayNS/NP cells typically adopted a more bipolar appearance with rounder soma (Figure 3.7B and C). The properties of the NS/NP cells derived after the three induction periods were compared using immunocytochemistry (Figure. 3.8; 3.9). The 4-day NS cells included limited populations of βIII-tubulin positive (4.6±0.7%; Figure. 3.8A). GFAP positive (2.0±0.4%; Figure. 3.8C) and Musashi1 positive cells (10±1%; Figure 3.8D) and a large population of Nestin positive cells (84±6%, Figure 3.8E), suggesting that most of these cells had differentiated towards an early stage neural phenotype. The 7-day NI cultures also contained few βIII-tubulin cells (0.53±0.2%; Figure.

3.8A), but included many more GFAP positive cells (16±4%; Figure. 3.8C; examples of immunolabelling is shown in Figure 3.9), most of these cells showed co-localization with Vimentin, indicating that they are rG (Figure 3.10). The majority of the cells were both Nestin positive (76±3%; Figure 3.8E) and Musashi1 positive (95±1%, Figure 3.8D). The 10-day NI cells included a larger population of βIII-tubulin positive neurons (20±5%; Figure. 3.8A). Most of the cells were Musashi1 positive (96±1%; Figure 3.8D) and many were still Nestin positive (65±3%; Figure 3.8E). Very few GFAP positive cells were present following a 10-day NI (1.3±0.3%; Figure. 3.8C).

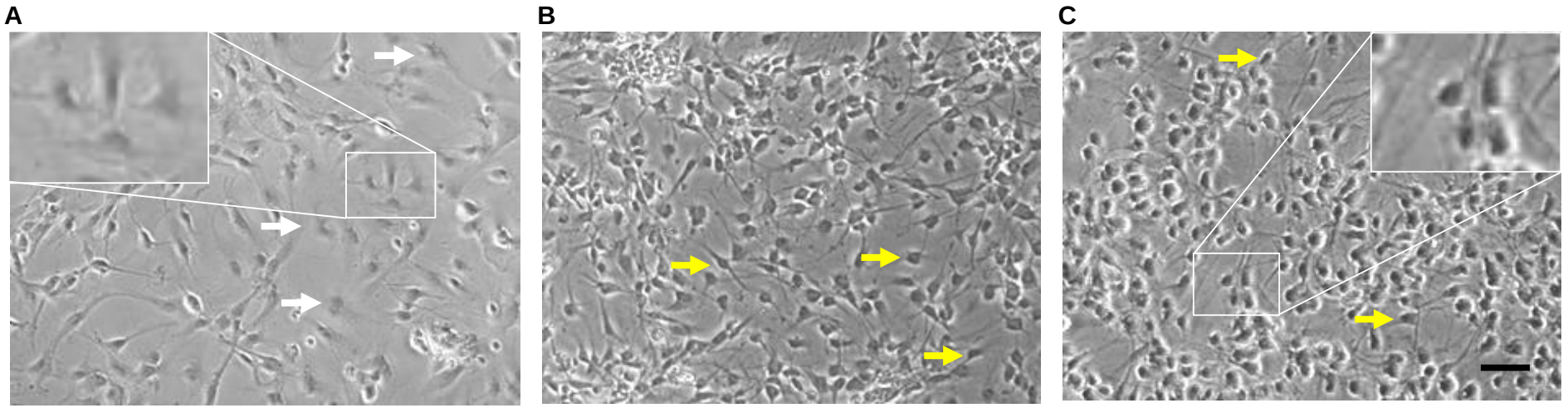


Figure 3.7 Morphological differences between NS/NP cells replated as monolayers from neurospheres derived after 4, 7 or 10 days of NI. (A) 4 day NS/NPs adopted a flatter and more glial appearance indicated by white arrows; (B) 7 day NS/NPs and (C) 10 day NS/NPs adopted bipolar or tripolar neural structure indicated by yellow arrows with fewer flat cells. (All images are of the same scale; Scale bars 100 µm)

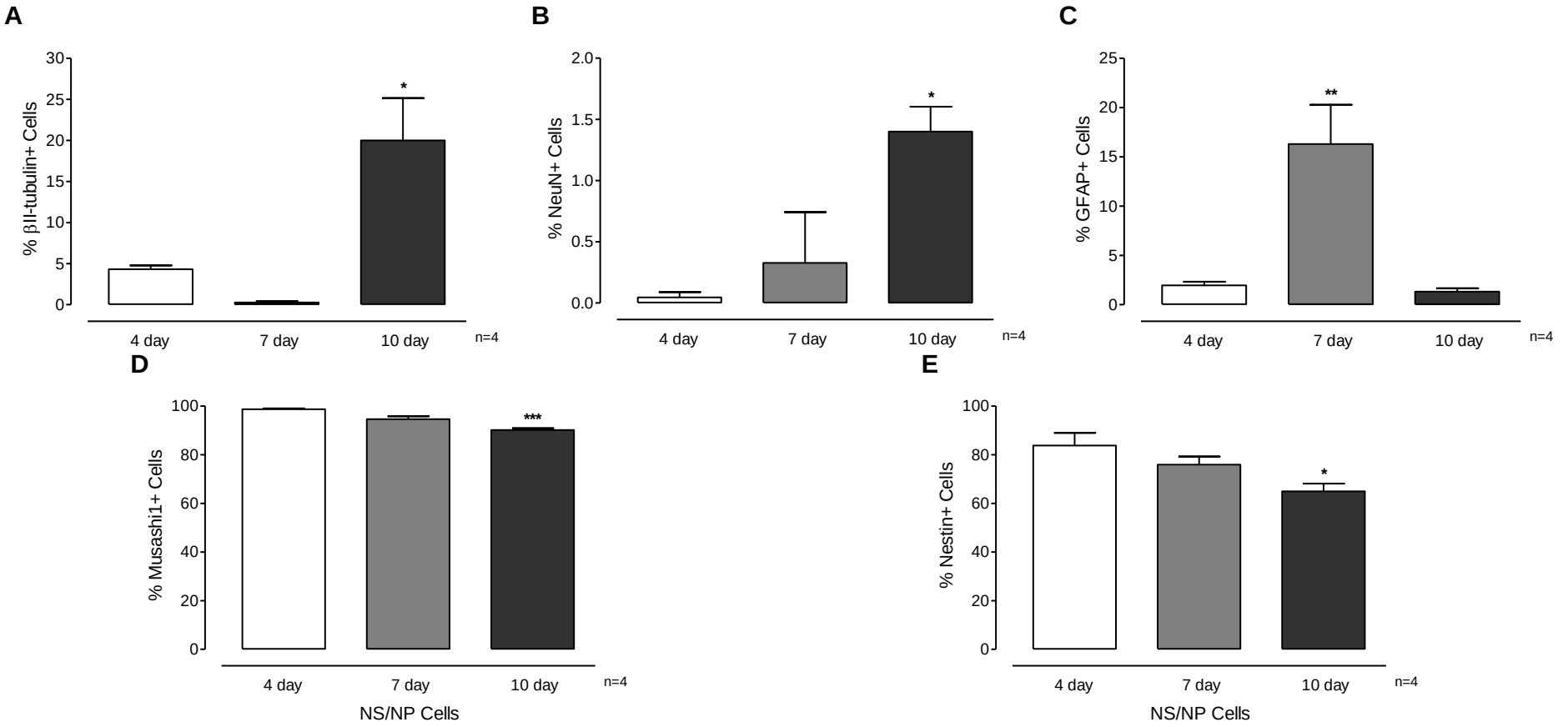


Figure 3.8 Percentage of NS/NP cells immunoreactive for neuronal and astrocyte cell markers. The NS/NP cells are derived from neurospheres originally isolated after 4, 7 or 10 days of NI and grown as monolayers in EGF and FGF2. By day 10, 20% of cells were βIII-tubulin positive. There was a peak in incidence of GFAP immunoreactivity at day 7. The percentage of more mature NeuN positive neurons was limited, increasing to 1.3% by day 10, indicating that the bulk of the cells were neural progenitors or neuroblasts. (n=4, *p<0.05, **p<0.005, ***p<0.001, one-way ANOVA followed by post hoc Dunnett's test comparing days 7 and 10 to respective day 4 cultures).

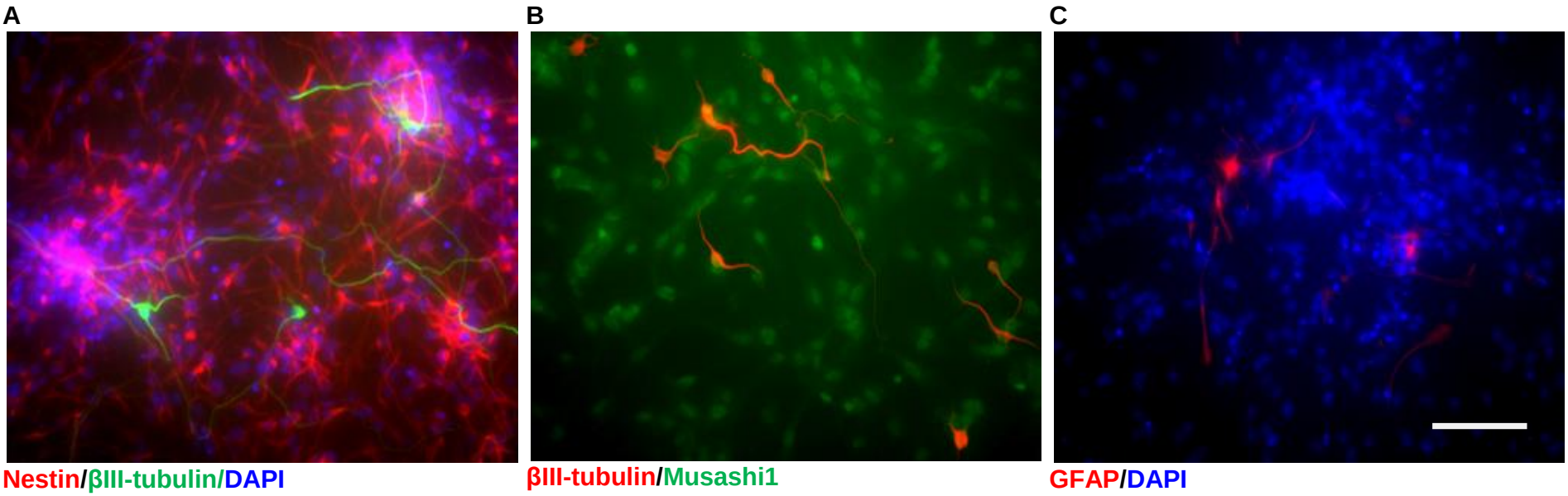


Figure 3.9 Typical immunocytochemical staining of NS/NP cells isolated from day 4, 7 or 10 days of neural induction. (A and B) Immunocytochemistry indicated the majority of the cells present were immunoreactive to Nestin (A) and Musashi1 (B), with a small population βIII-tubulin (A and B) and GFAP positive cells (C). (All images are of the same scale; Scale bar 100 µm)

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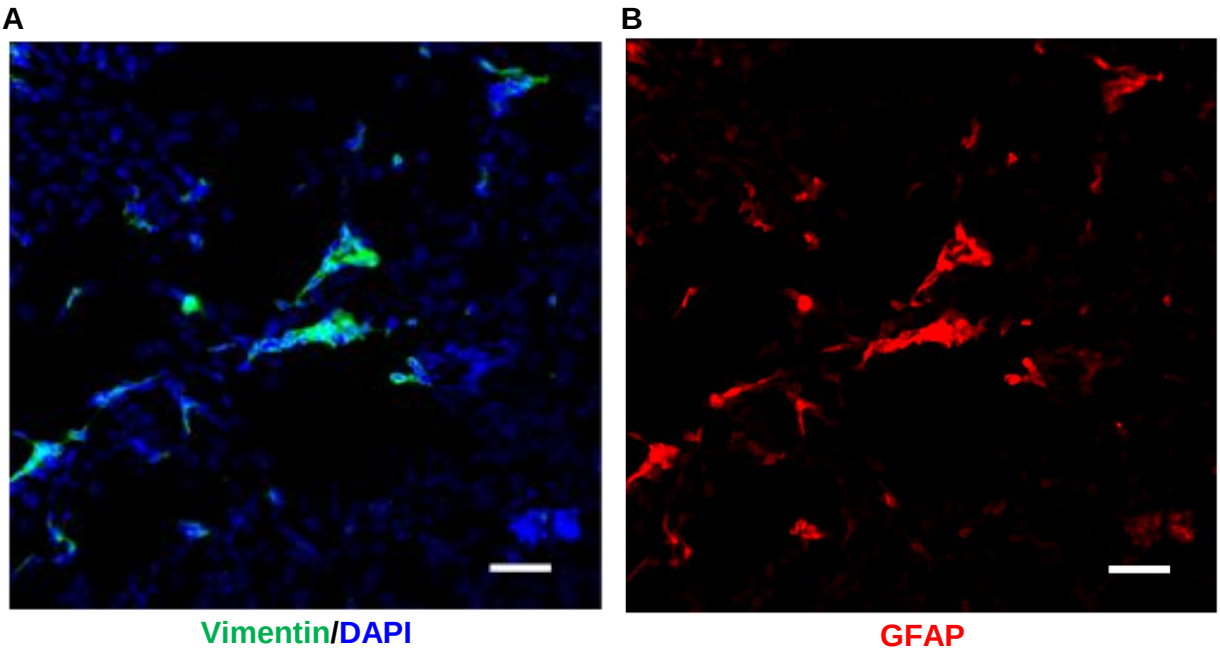


Figure 3.10 NS/NP cells isolated from day 7 of neural induction showed more GFAP positive cells compared to day 4 and 10, most of these cells showed co-localization with Vimentin, indicating that they were radial glia. (All images are of the same scale; Scale bar 100 µm)

3.4.3 The phenotypic potential of 4, 7 and 10 day NS/NP cells

Since cultures derived after 4-day, 7-day and 10-day NI all included a large proportion of Nestin positive cells, the neuronal differentiation potential of these cultures using three differentiation protocols (1, 2 and 3) were investigated, each of which has been reported to induce development of particular neural lineages; dopaminergic, serotonergic and cholinergic, respectively (Barberi et al., 2003).

The dopaminergic neuron differentiation protocol (Protocol 1) after 16 or 17 days of differentiation remained predominantly Nestin (Figure. 3.11A), Musashi1 (Figure. 3.11B) and GFAP positive (Figure 3.11D), with a small population of βIII-tubulin positive cells (Figure 3.11C) many of which were GABA positive (Figure. 3.12A) for day 4 and 7 NI

cultures. In contrast, cells derived after 10-day NI differentiated into populations containing comparatively few Musashi1, Nestin or GFAP positive cells and many βIII-tubulin positive cells (Figure 3.11C). Around 20% of these βIII-tubulin positive cells were also GABA positive (Figure. 3.12A). Of more significance is the finding that after 10 days of NI, Protocol 1 gave rise to a rich population (24%) of tyrosine hydroxylase (TH) positive neurons (Figure 3.12A), as well as 13% 5-HT positive neurons (Figure 3.12B). Similarly, the serotonergic neuron protocol (Protocol 2) also gave rise to predominantly Nestin, Musashi1 and GFAP positive cells and very few βIII-tubulin positive cells in day 4 and 7 NI cultures (Figure 3.11A-D). This protocol showed very few TH positive neurons and only 5% 5-HT positive cells in day 10 NI cultures (Figure. 3.12A and B). The majority of βIII-tubulin positive cells derived from protocols 1, 2 and 3 were also GABA positive (Figure. 3.12C). The motor neuron differentiation protocol (Protocol 3) gave rise to a reduced proportion of β3-tubulin positive cells in all of day 4, 7 and 10 NI cultures. No choline acetyltransferase (ChAT) positive neurons were evident following differentiation with any of the protocols (Data not shown). Figure 3.14 shows typical images from immunocytochemistry studies after differentiation of 4, 7 and 10-day derived NS/NP cells. When each of the patterning protocols were applied to NS/NP cells derived after 4 days or 7 days of NI, the result was limited expression of βIII-tubulin (Figure 3.13A-C; F and G) and a wealth of GFAP (Figure 3.13B and D), indicating that the majority of the cells had a glial phenotype. In contrast differentiation of NS/NP cells derived after 10 days produced cultures rich in βIII-tubulin and GABA with regions that were clearly immunopositive for TH or 5-HT (Figure. 3.13E-G).

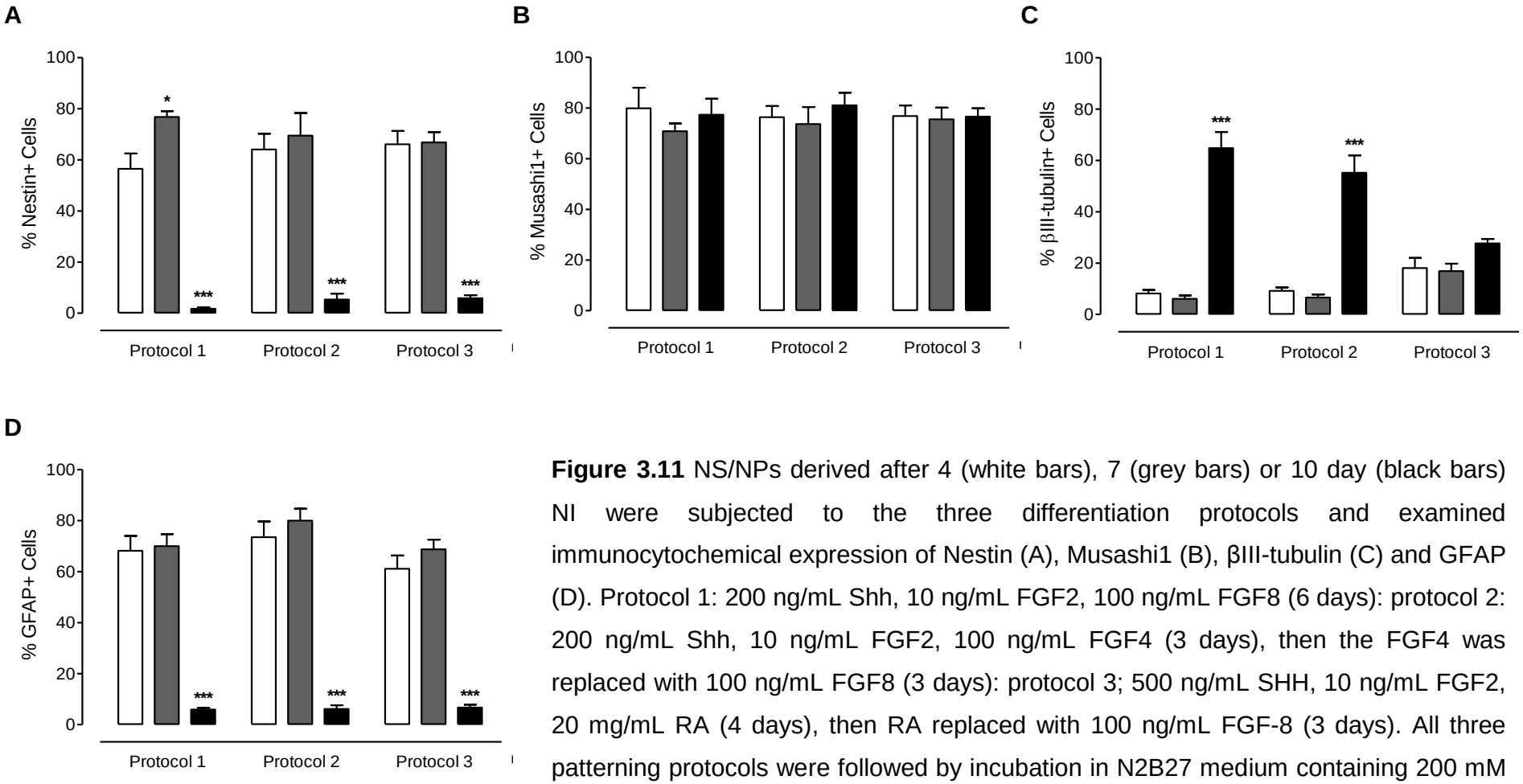


Figure 3.11 NS/NPs derived after 4 (white bars), 7 (grey bars) or 10 day (black bars) NI were subjected to the three differentiation protocols and examined immunocytochemical expression of Nestin (A), Musashi1 (B), βIII-tubulin (C) and GFAP (D). Protocol 1: 200 ng/mL Shh, 10 ng/mL FGF2, 100 ng/mL FGF8 (6 days): protocol 2: 200 ng/mL Shh, 10 ng/mL FGF2, 100 ng/mL FGF4 (3 days), then the FGF4 was replaced with 100 ng/mL FGF8 (3 days): protocol 3; 500 ng/mL SHH, 10 ng/mL FGF2, 20 mg/mL RA (4 days), then RA replaced with 100 ng/mL FGF-8 (3 days). All three patterning protocols were followed by incubation in N2B27 medium containing 200 mM L(+)-ascorbic acid and 20 ng/mL BDNF for a further 10 days. (n=3; *p<0.05; ***p<0.001, two-way ANOVA followed by Bonferroni's test comparing days 7 and 10 to respective day 4 cultures).

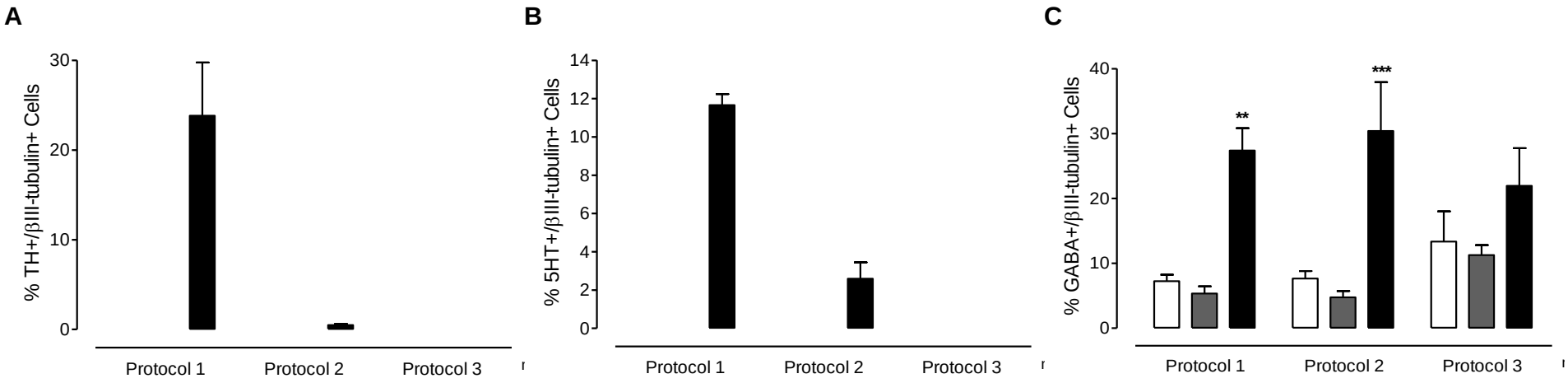


Figure 3.12 NS/NPs derived after 4 (white bars), 7 (grey bars) or 10 day (black bars) NI were subjected to the three differentiation protocols and then examined by immunocytochemistry for expression of TH (A), 5-HT (B) and GABA (C). Differentiated cells were obtained by using protocols outlined previously (see caption for Figure 3.13). 4 day and 7 day-derived NS cultures resulted in similar outcomes producing GFAP positive cells and no TH or 5-HT positive cells. 10-day NS/NPs produced βIII-tubulin positive neurons using protocols 1 and 2, and protocol 1 led to over TH and 5HT positive neurons with less GFAP positive glial cells (n=3; **p<0.01; ***p<0.001, two-way ANOVA followed by Bonferroni's test comparing day 7 and 10 to respective day 4 cultures).

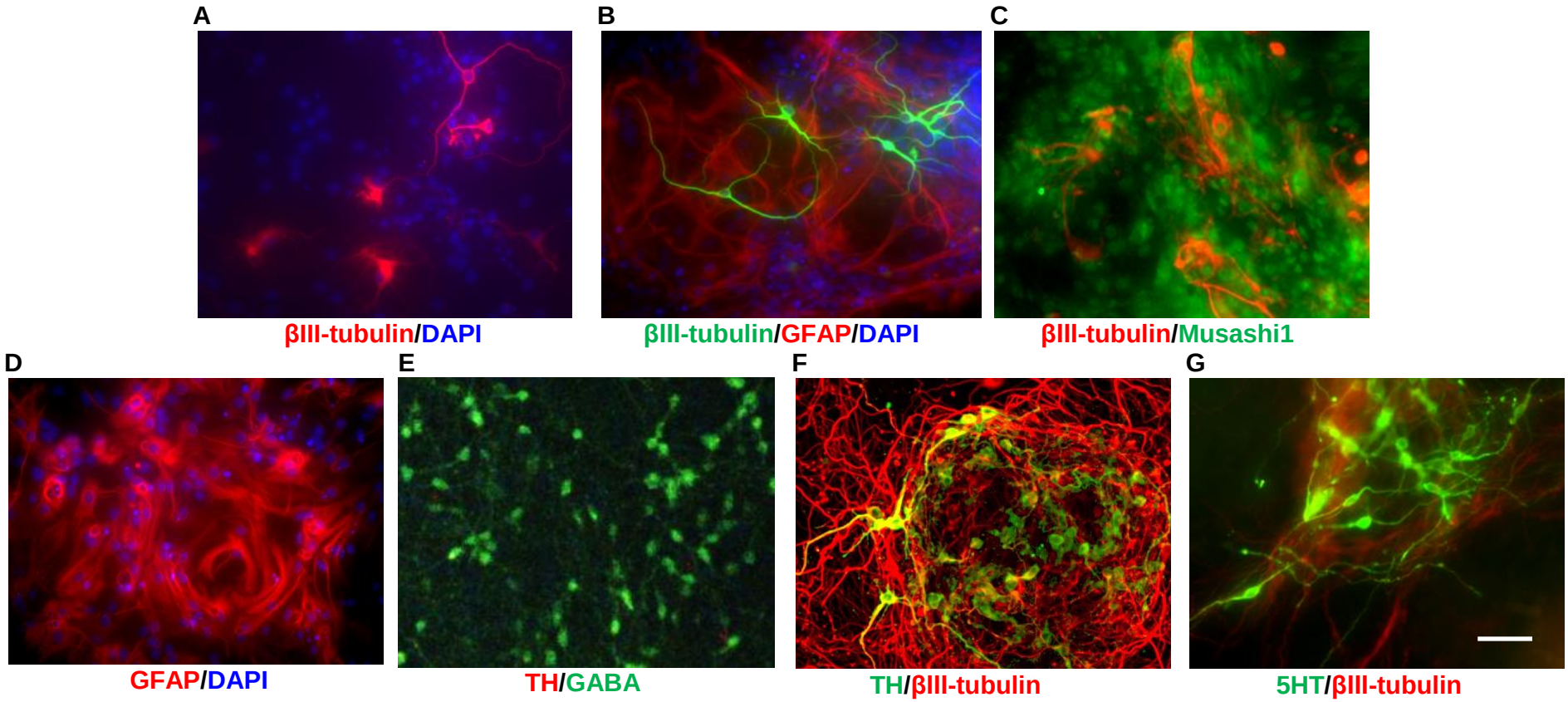


Figure 3.13 Immunocytochemistry of differentiated cells at day 16 of differentiation Typical differentiated cultures produced using protocol 1 with NS/NP cells derived after 4-days NI (A and B – showing limited βIII-tubulin and many GFAP positive cells), 7 days NI (C and D) or 10 days NI (E-G – showing a wealth of βIII-tubulin and GABA; and specific TH and 5-HT immunoreactivity). (All images are of the same scale; Scale bar 100 μm).

3.5. Discussion

The use of NS/NP cells as a starting point for generating mature neurons offers distinct advantages over ES cells, since the terminal differentiation process is shorter, and the starting population is likely to be considerably more homogenous (Pouton & Haynes, 2007). If populations of NP cells could be stabilised, expanded and stored as frozen stocks, they would be an ideal source of neurons for drug discovery and cell therapy. Indeed, defined and committed NPs may be the cells of choice for cell therapy. Propagation of NS cells has been described both from adult brain and also from ES cells (Pollard, Conti, et al., 2006). The publication of a method for derivation of NS cells from mES cells after 7 days of NI in N2/B27 medium was a significant development (Conti et al., 2005), allowing stabilisation of NS cells in EGF and FGF2 for over 100 passages. A recent study investigated the differentiation of NS cell lines after withdrawal of EGF (Spiliotopoulos et al., 2009). The results of differentiation of early and very late passage NS cells were the same, which is encouraging, but the NS cells appeared to be lineage restricted, rather than multipotent, and the neurons produced were predominantly GABAergic. During neurogenesis *in vivo*, ES cells differentiate into NEP cells, then rG, which in turn become neural intermediate progenitor cells, and then neurons (Kriegstein and Alvarez-Buylla, 2009). Similarly, *in vitro*, ES cells also appear to differentiate into NEP cells, rG, and NS cells, which are capable of both symmetrical and asymmetrical cell division. The NS cells then become NP cells, which are only capable of asymmetric division, producing neural precursors, which then expand symmetrically and subsequently differentiate into post mitotic neurons (Juliandi, Abematsu, & Nakashima, 2010). One published protocol for derivation of NS cells (Conti et al., 2005) calls for 7-days of NI, so neurospheres were derived after 4, 7 or 10 days using a method, that differed only by the use of StemPro NS cell culture medium (Invitrogen), rather than NS-A medium (Euroclone) as the base of the medium for neurosphere formation and NS/NP

expansion. Neurospheres were derived after 10 days NI based on the time course of expression of the Lmx1a reporter. Consistent with this finding it was also shown that after 10 days of NI there is no expression of either Brachyury, an early mesoderm marker (Vidricaire, Jardine, & Mcburney, 1994) or α1-fetoprotein which marks primitive and definitive endoderm during mouse development (Kwon et al., 2006), indicating the absence of early mesoderm and endodermal lineages. Lmx1a is expressed in the developing nervous system, in the roof plate of the neural tube, the notochord and the otic vesicles (Failli, Bachy, & Retaux, 2002). Lmx1a is known to be essential for development of dopaminergic neurons in the mesodiencephalon (Smidt & Burbach, 2007), and is expressed in mitotic dopaminergic progenitors, precursors and mature dopaminergic neurons (Andersson, Tryggvason, et al., 2006). Lmx1a works in cooperation with Lmx1b (W. Lin et al., 2009) to specify dopaminergic development in the floorplate (Ono et al., 2007), and is involved in an autoregulatory loop with Wnt1 (Chung et al., 2009). Forced expression of Lmx1a has been used to enhance the yield of dopaminergic neurons from differentiating cultures of ES cells (Andersson, Tryggvason, et al., 2006; Friling et al., 2009). In the differentiation experiments, expression of Lmx1a reached a peak at 9–10 days, which indicated that NP cells were present after 1 day of NI. There were clear differences between the cultures transferred to medium containing EGF and FGF2 after different periods of NI, although in all the methods, the bulk of the cells were immunopositive for Nestin. On withdrawal of EGF the 4 and 7-day derived NS cells gave rise to 60–80% GFAP positive cells, indicating glial phenotypes, and less than 20% neurons. At day7 many of these GFAP positive cells were also immunoreactive to the Vimentin, indicating the presence of rG. 4 and 7 day NI cultures were unable to give rise to dopaminergic or serotonergic neurons. In contrast the cells derived after 10 days of NI already contained some βIII-tubulin positive cells, and were capable of producing up to 60% neurons including 23% dopaminergic neurons (Figure 3.12C and 3.13A). The

10-day derived cells were most likely to be a mixture of NP cells and NS cells, given that the cells were not necessarily at the same stage of development throughout each neurosphere. The neurospheres derived after 10 days of NI in the study clearly included expanded rosettes with ZO1 positive central regions (Figure. 3.5A, 3.6) (Elkabetz et al., 2008a). The current hypothesis is that the 10-day cultures contain rosettes which are rich for NPs of specific phenotypes, and that the cultures could potentially be used to derive cultures of specific NPs by picking out individual rosettes or by forming neurospheres with fewer cells. At this stage it is not clear whether individual NS/NP cells can be cloned by forming colonies from single cells. Two groups have reported that expansion of neural rosettes, followed by treatment with Shh and FGF8, results in a rich source of dopaminergic neurons (Cho & De Robertis, 1990; Perrier et al., 2004). Most research groups have had difficulty producing such high yields of dopaminergic neurons. One explanation may be that these two studies involved fortuitous expansion of cultures from rosettes, which were already committed to the dopaminergic phenotype. Indeed it may be that selection of appropriate specified rosettes is the key to enriching cultures for specific neuronal phenotypes. Published neural patterning protocols were used to investigate whether the NS/NP cells were responsive to different growth factors (Barberi et al., 2003). Shh, and FGF8 have often been used to mimic the neural patterning *in vivo*. During floor plate neurogenesis, Wnt1-mediated inhibition of β-catenin degradation modulates Shh expression, and inhibits neurogenesis. Thus, when Shh is present during the early stages, neurogenesis is repressed (Fasano & Studer, 2009). This study hypothesises that following a short period of NI, the resultant early NS cells respond to Shh with an inhibition of neurogenesis, rather than patterning. This finding is largely consistent with the findings of Conti et al. (2005) who reported that 7-day NS cells gave rise to 30–40% neurons after EGF withdrawal. This number is greater than those generated from the 7-day NI protocol. However, following withdrawal of EGF, Conti et al.

(2005) used FGF to enhance self-renewal and survival, rather than begin patterning with Shh. In this study immediate exposure to Shh following withdrawal of EGF was used, this absence of FGF2 expansion may explain the low number of neurons, which developed. More importantly the 10 day NS/NP cells did indeed respond to different patterning protocols although not the same extent as described by Barberi et al. (2003), who generated 50% TH and 5HT positive cells, 23% dopaminergic neurons were generated with the appropriate patterning protocol (Barberi et al., 2003). Barberi et al. (2003) employed a co-culture NI method that involves seeding mES cells on a monolayer of PA6 stromal cells. This method is widely known to induce the differentiation mES cells towards the midbrain dopaminergic phenotype (Perrier et al., 2004). The 2D monolayer NI method was used in this study to generate NS/NP cells because this method enables better definition of the differentiation environment.

3.6 Conclusions

Published protocols for differentiation of ES cells to neural phenotypes vary in length of NI, format (monolayer versus aggregate or co-culture) and the timing of additions of differentiation and/or growth factors (Barberi et al., 2003; Kawasaki et al., 2000; Khaira, Nefzger, Beh, Pouton, & Haynes, 2011; Khaira, Pouton, & Haynes, 2009; Lee et al., 2000; Raye, Tochon-Danguy, Pouton, & Haynes, 2007; Wichterle et al., 2002). Often, the length of NI varies considerably and, although many cells derived using such protocols express NS cell markers, such as Nestin and Musashi1, the cultures are difficult to characterise in more detail. The data suggest that although the cultured NS cells from the shorter periods of NI give rise to Nestin positive monolayer cultures, such cultures are lineage restricted, possibly as a result of early exposure to EGF and FGF2. 4-day NS cells are not responsive to patterning protocols, and treatment with Shh and FGF8 results in limited differentiation into neurons. Extending the NI period to 10 days prior to

the generation of neurospheres in EGF and FGF2, and subsequent expansion as monolayer cultures, enables expansion of NS/NP cells which respond to patterning cues, and are able to generate phenotypically diverse neurons with very small numbers of residual NS/NP cells and astrocytes.

Chapter Four

4. Long Term Maintenance of Neural Stem/Progenitors

4.1. Introduction

NS cells are self-renewing populations found in the developing and adult brain; they can also be derived from pluripotent ES cells. These cells are multipotent, thus they are able to generate neurons and glia, including astrocytes and oligodendrocytes both *in vivo* and *in vitro*. They are also one of the ideal cell types used in the treatment to neurological diseases such as Parkinson's disease and schizophrenia in replacement therapies or as models for high throughput screening. The use of NS cells is advantages compared to ES cells since they are less likely to contain mesodermal and endodermal contamination; however differentiation of NS cells into neurons does not completely eliminate the

presence of undifferentiated cells. Undifferentiated cell contamination in grafts may results in tumour formation (L. M. Bjorklund & Isacson, 2002; Nishimura et al., 2003). It is also possible that non-neural contaminations in cultures used may results false positive hits in high throughput screening. It is hence important that the NS cell sources used for these purposes are multipotent, self-renewing and most importantly stable and have predictable differentiation patterns. Over the years, *in vitro* NS/NP cells with different neurogenic potential such as NEP, rNS and rG cells have been generated from mES or hES cells. For example, NEP and rG cells are transient and cannot be maintained, rNS cells can be maintained in the presence of Shh and Notch antagonists (Dll4 and Jagged1) for a limited number of passages (Elkabetz et al., 2008a); while NS cells can be maintained from more than 100 passages in the presence of EGF and FGF2 (Conti et al., 2005; Koch et al., 2009). However the phenotypic potential and stability of these cell types have yet to be investigated. Under the culture conditions described by the authors these cell types predominantly give rise to GABAergic and glutamatergic neurons with limited proportions of dopaminergic and motor neurons *in vivo* (Bibel et al., 2007; Bibel et al., 2004; Brill et al., 2009). The determination of the best sources for *in vitro* derivation of NS/NP cells and optimizing protocols for stable, clonal proliferation are still one of the main objectives in the stem cell field. The pluripotent reporter cell line Lmx1a-AMP-IRES-eGFP derived from mouse E14Tg2a ES cells was used in this chapter to track expression of Lmx1a, which is a key transcription factor in dopaminergic neuron differentiation. However, Lmx1a is also expressed in other neural and non-neural tissues such as the non-neurogenic roof plate of the neural tube (Chizhikov & Millen, 2004; Millonig et al., 2000), the roof plate of the developing cerebellum (Chizhikov et al., 2010; Chizhikov & Millen, 2004; Mishima, Lindgren, Chizhikov, Johnson, & Millen, 2009a) and the cortical hem of the forebrain (Chizhikov et al., 2010). During midbrain dopaminergic differentiation, Lmx1a cooperates with other transcription factors that are essential for the

specification of midbrain dopaminergic neurons such as Msx1, Wnt1, Otx2, Lmx1b and FoxA1/2 (Figure 1.12) (Andersson, Tryggvason, et al., 2006; Chung et al., 2009; W. Lin et al., 2009; Nakatani et al., 2010; Yan et al., 2011), in particular the Wnt1-Lmx1a autoregulatory loop with the Shh-FoxA2 pathway (Chung et al., 2009). Previously in chapter 3, it was demonstrated that by extending the NI period to 10 days prior to the generation of NS/NP cells, they are able to respond to patterning cues and give rise to phenotypically diverse neurons (Zeng, Fabb, Haynes, & Pouton, 2011).

This chapter investigates the hypothesis that NS/NP cells can be propagated in the presence of EGF and FGF2 over a number of passages without loss of multipotency.

4.2 Aim

Generate NS/NP cells from 2D monolayer and PA6 co-culture maintain these cells in the presence of EGF and FGF2 up to 25 passages and address their ability to differentiate into dopaminergic and GABAergic neurons using protocols that have been shown to generate dopaminergic and GABAergic neurons.

4.3 Methods

The pluripotent Lmx1a-AMP-IRES-eGFP and Lmx1a-IRES-eGFP reporter cell lines were used in this study to track expression of Lmx1a during long term maintenance in the presence of EGF and FGF2. The experimental methods used in this chapter, including the differentiation of ES cells into NS/NP cells and terminal differentiation are described in Chapter 2.

4.4. Results

4.4.1. The effect of long term propagation on monolayer derived NS/NP cells

NS/NP cells derived from Lmx-AMP-IRES-eGFP or Lmx1a-eGFP reporter cell lines were propagated in the presence of EGF and FGF2 over a 25 passage period and the expression of Lmx1a was determined using the targeted Lmx1a-β-lactamase reporter ES cell line. This pluripotent cell line was used in previously the generation of NS/NP cells following 10 days of NI (Zeng et al., 2011). After plating of neurospheres the expression of β-lactamase positive cells increased to 66±10% by passage seven and remained constant up to passage 10 before it rapidly declined by passage 14 to 5±2% (Figure 4.1). Figure 4.2 show typical FACS plots of passage 2 and 10 respectively. This data suggests that growth in EGF and FGF2 was unable to maintain the cells at the same stage of development. Alternatively it is possible that the Lmx1a expressing cells were more resilient during the passaging process and thus were enriched up to passage 10. The decrease in % β-lactamase positive cells after passage 11 suggests that enrichment due to resilience was unlikely to be the explanation. Next ALDH expression was also investigated to determine the populations of NS/NP cells retained multipotential up to passage 16.

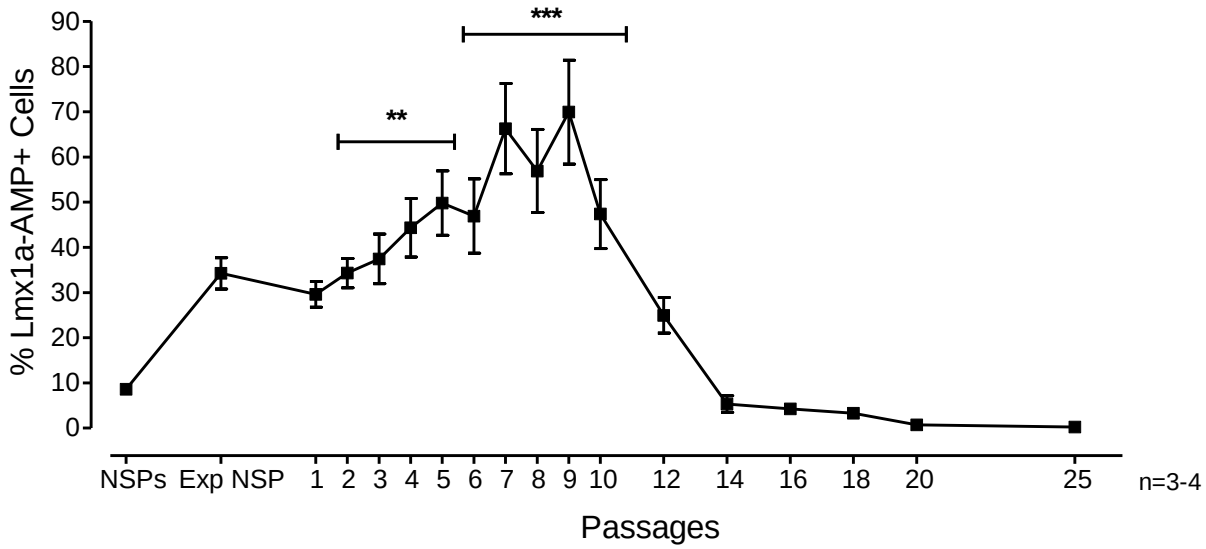


Figure 4.1 Lmx1a expressions during 10 day NS/NP cell maintenance. FACS analysis was used to determine the percentage of Lmx1a expressing cells on successive passages during NS/NP cell maintenance (determined by expression of β-lactamase, the cDNA of which was targeted to exon 1 of one allele of Lmx1a). (n=3-4; **p< 0.005; ***p<0.001, two-way ANOVA followed by Bonferroni's test compared to neurosphere (NSP) cultures)

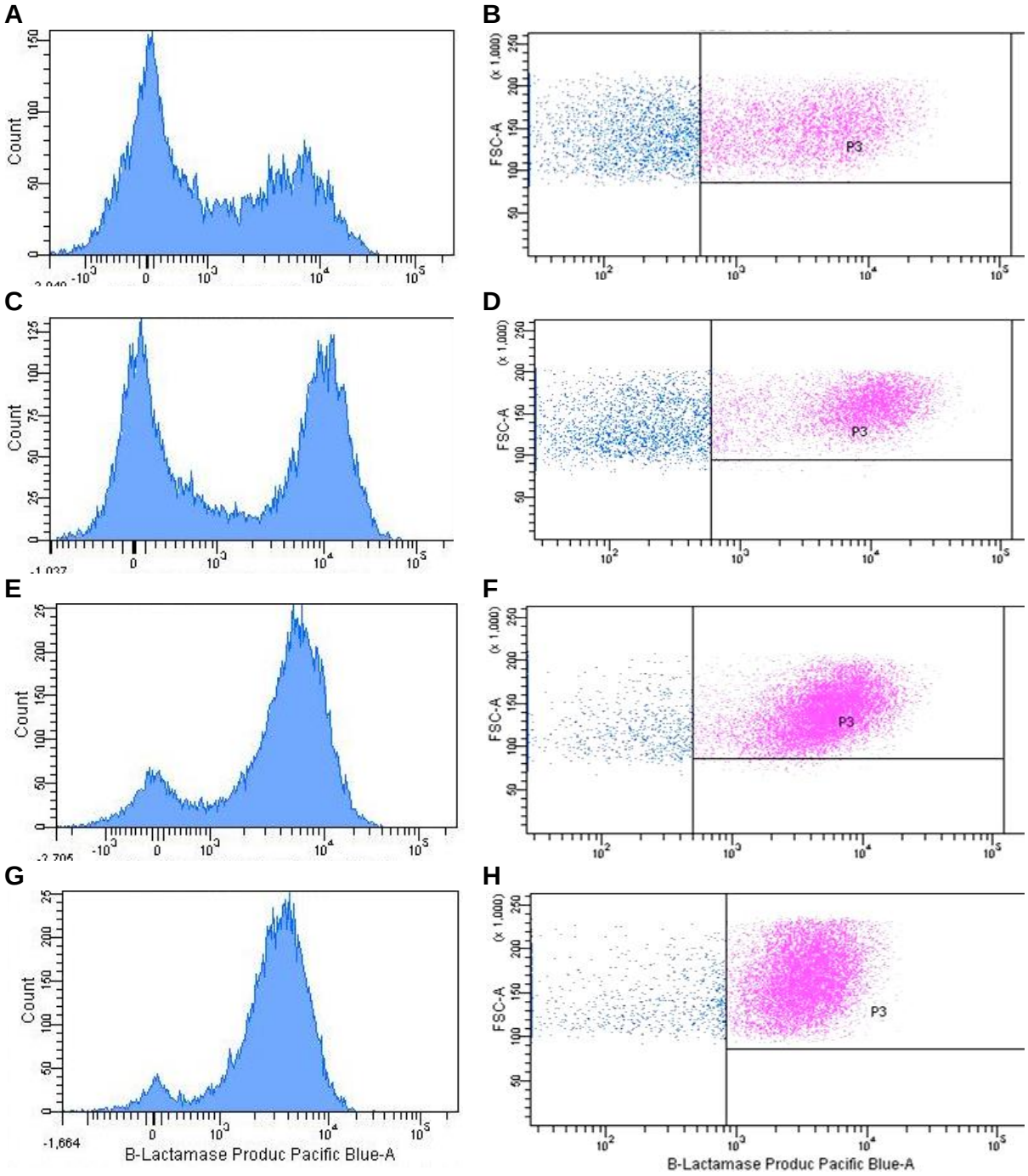


Figure 4.2 Typical FACS plots of passage 2, 5, 7 and 10 of Lmx1a expression during NS/NP cell maintenance. (A and B) Typical histogram (A) and dot plot (B) of passage two cultures. (C and D) Typical histogram (C) and dot plot (D) of passage 5 cultures. (E and F) Typical histogram (E) and dot plot (F) of passage 7 cultures. (G and H) Typical histogram (G) and dot plot (H) of passage7 cultures. “P3” represents β-lactamase pacific blue positive, Lmx1a positive population. (Lmx1a positive population was gated according to 0.1% Lmx1a expression in wild type NI cultures under the same culture conditions)

ALDH is an enzyme crucial in aldehyde oxidation in the generation of RA in dopaminergic neurons (McCaffery & Drager, 1994) and also associated with other stem and progenitor cells including mesenchymal, endothelial, hematopoietic and cancer stem cells. ALDH expression during the long term propagation of NS/NP cells in the presence of EGF and FGF2 was determined using the ALDH substrate BAAA (BODIPY®-aminoacetaldehyde) allowing analysis of ALDH positive cells by FACS analysis. The population of cells that accumulated the product BODIPY™-aminoacate increased up to 18±3% at passage 9 and decreased to basal levels (equivalent to the original neurospheres) by passage 12 (3±1%; Figure 4.3). Approximately one third of the cells that were Lmx1a positive were also ALDH positive between passages 7 to 10. Passages 2, 5 and 7 cultures, which were used in differentiation experiments, included 6-12% ALDH positive cells. Figure 4.4 shows a typical FACS plot of passage 9 NS/NP cells.

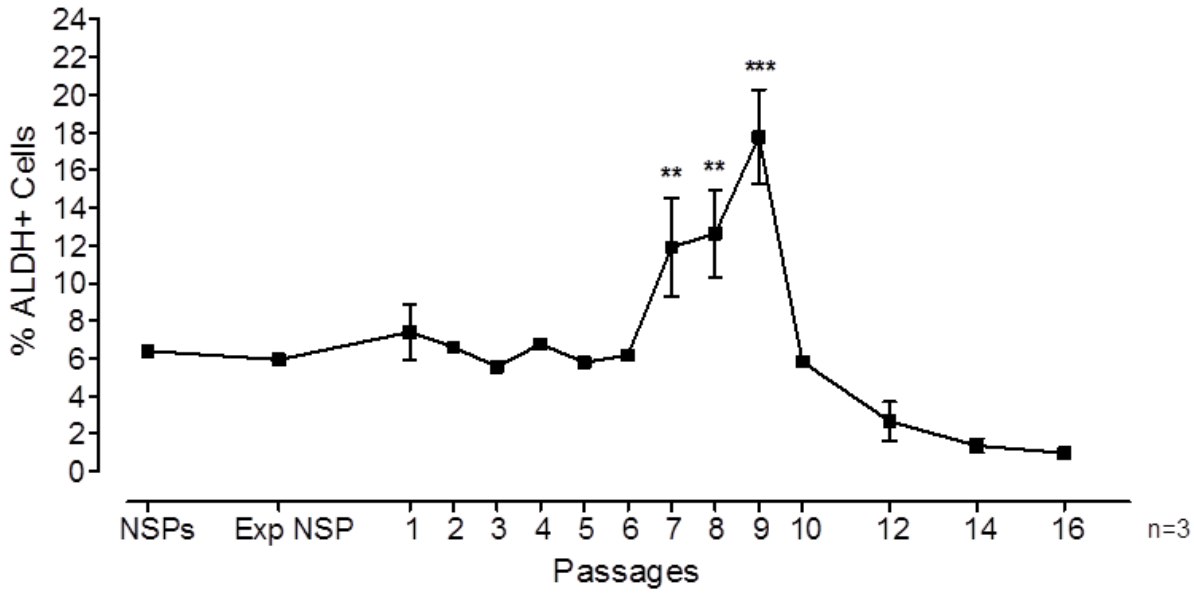


Figure 4.3 The percentage of ALDH expression was evaluated during 16 passages of NS/NP cell propagation in the presence of EGF and FGF2 using FACS analysis (ALDH is detected using the ALDH substrate (BAAA)). The population of ALDH positive cells increased up to 18% at passage 9 and decreased back to basal levels by passage 12. (n=3; ***p<0.001; two-way ANOVA followed by Bonferroni's test compared to NSP cultures)

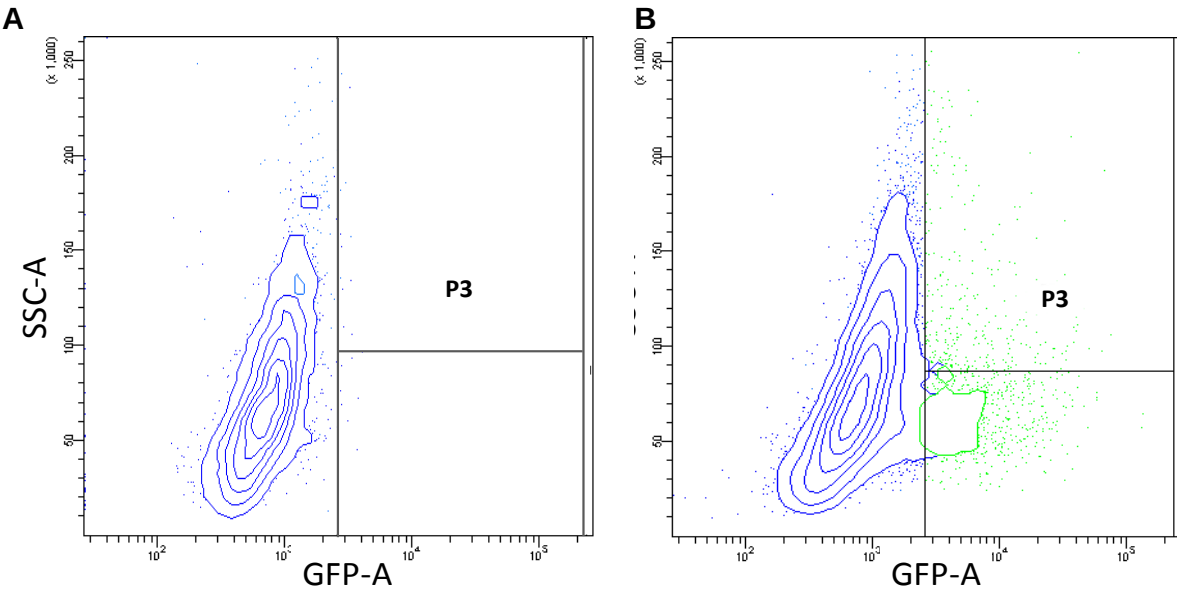


Figure 4.4 Typical dot plot of ALDH expression of passage 2 (A) and passage 9 (B) during NS/NP cell maintenance. "P3" represents the "GFP positive" which is equivalent to cells that accumulated the BAAA cleavage product, i.e. the ALDH positive population. (The ALDH positive population was gated to mark 0.1% ALDH expression in cells incubated with both the ALDH substrate and enzyme inhibitor under the same culture conditions)

4.4.2. Molecular characteristics of monolayer derived NS/NP cells

Given that the propagation of ES cell derived NS/NP cells in the presence of EGF and FGF2 resulted in enrichment in Lmx1a positive cells, four time points were chosen. Passage 2 is the earliest time point possible for analysis, passage 5 showed intermediate Lmx1a expression and low ALDH expression, passage 7 showed intermediate Lmx1a and ALDH expression levels and passage 10 showed the highest Lmx1a and ALDH expression. NS/NP cells isolated at passages 2, 5, 7 and 10 were investigated further to determine whether the enrichment in Lmx1a expression correlated with an enrichment in progenitors of the midbrain dopaminergic neurons.

qPCR analysis of NS/NP cells derived by neurosphere formation after 10 days of NI then grown in EGF and FGF2 was carried out with cells from passages 2, 5, 7 and 10. Expression profiles of a series of genes associated with regional neural specification or NS/NP phenotype were determined. Genes included Nestin and Sox2 (NS cells), Sox1 (neural ectoderm), Notch (rNS cells) FoxA2 (ventral roof plate progenitors), BMP2 (dorsal roof plate progenitors), FoxG1 (forebrain progenitors), En1 (midbrain progenitors), βIII-tubulin (immature neurons), GFAP (rG and astrocytes), TH (catecholaminergic neurons), GAD1 (GABAergic neurons) as well as Lmx1a and Lmx1b. Figure 4.5 shows the time course of gene expression. A dramatic increase in Lmx1a expression was expected and indeed the Lmx1a transcription level rose 301±48 fold at passage 7 compared to cells tested on day 1 of NI. This was followed by a decline to 30±11 fold at passage 10. The downstream target Lmx1b was also over-expressed showing a 68-167 fold increase in expression over the series of passages analysed (Figure 4.5A). As the cells were passaged in EGF and FGF2 in monolayer culture, a rapid decrease was observed in genes associated with the different stages of NS/NP development: Sox1 (60 to 2 fold), Sox2 (4 to 0.3 fold) and Notch1 (19 to 0.7 fold; Figure 4.5B). Nestin expression peaked at passage 5 (128±16 fold; Figure 4.5B). Analysis of regional specific markers showed a bias towards the forebrain phenotype. The early forebrain marker FoxG1 peaked at passage 5 (83±16 fold) while the early midbrain marker En1 decreased (36 to12 fold) from passage 2 to 10 (Figures 4.5C). However the ventral marker FoxA2 peaked at passage 7 (104±13 fold) though this gene is not limited to ventral midbrain and the dorsal marker BMP2 increased up to 29±4 fold by passage 10 (Figure 4.5C).

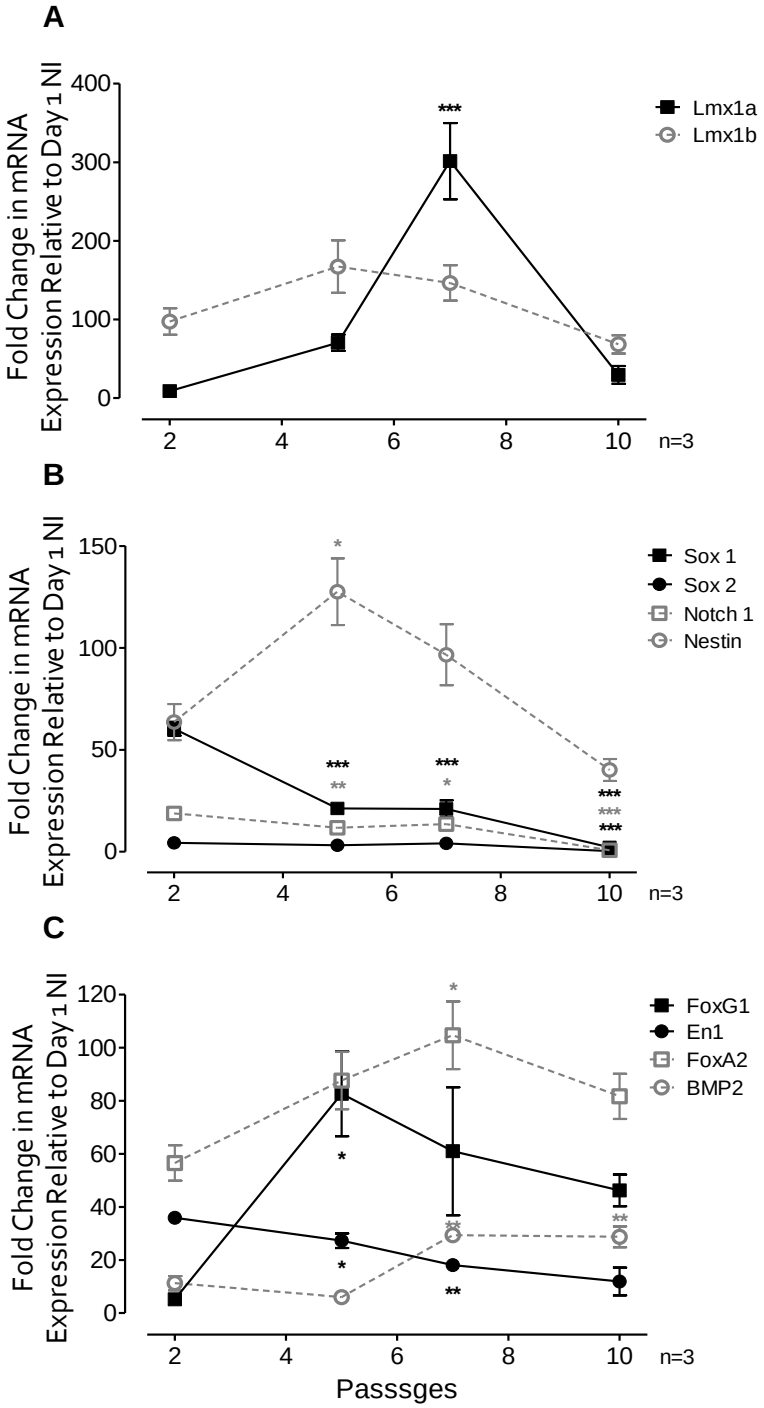


Figure 4.5 Expression profiling (qPCR) of passage 2, 5, 7 and 10 NS/NP cells propagated in the presence of EGF and FGF2. (A) Lmx1b expression remained constant while Lmx1a expression peaked at passage 7. (B) The NS/NP markers Sox1, Sox2 and Notch decrease over the 10 passages. (C) The ventral markers FoxA2 peaked at passage 5 and BMP2 increased up to passage 10. (n=3; *p<0.01; **p<0.05; ***p<0.001; two-way ANOVA followed by Bonferroni's test compared to day 1 cells during NI)

As an indicator of neuronal multipotency, the neuronal marker βIII-tubulin increased up to 46±19 fold, was observed at passage 10 (Figure 4.6A). The expression of EGFR increased up to 191±40 fold while FGFR1 levels remained constant over the cultural period (3 to 9 fold; Figure 4.6A). Although there were relatively low expression levels of the catecholaminergic transcript of TH (2 to 6 fold), there was an enormous increase in the expression of the GABAergic marker GAD1 (2 to 561 fold; Figure 4.6B). Collectively, these qPCR data indicate a shift towards the ventral roof plate and the GAD1 expression strongly suggests the presence of GABAergic progenitors. In order to further investigate whether these NS/NP cells are phenotypically restricted by passage 10, passages 2, 5, 7 and 10 NS/NP cells were terminally differentiated into neurons.

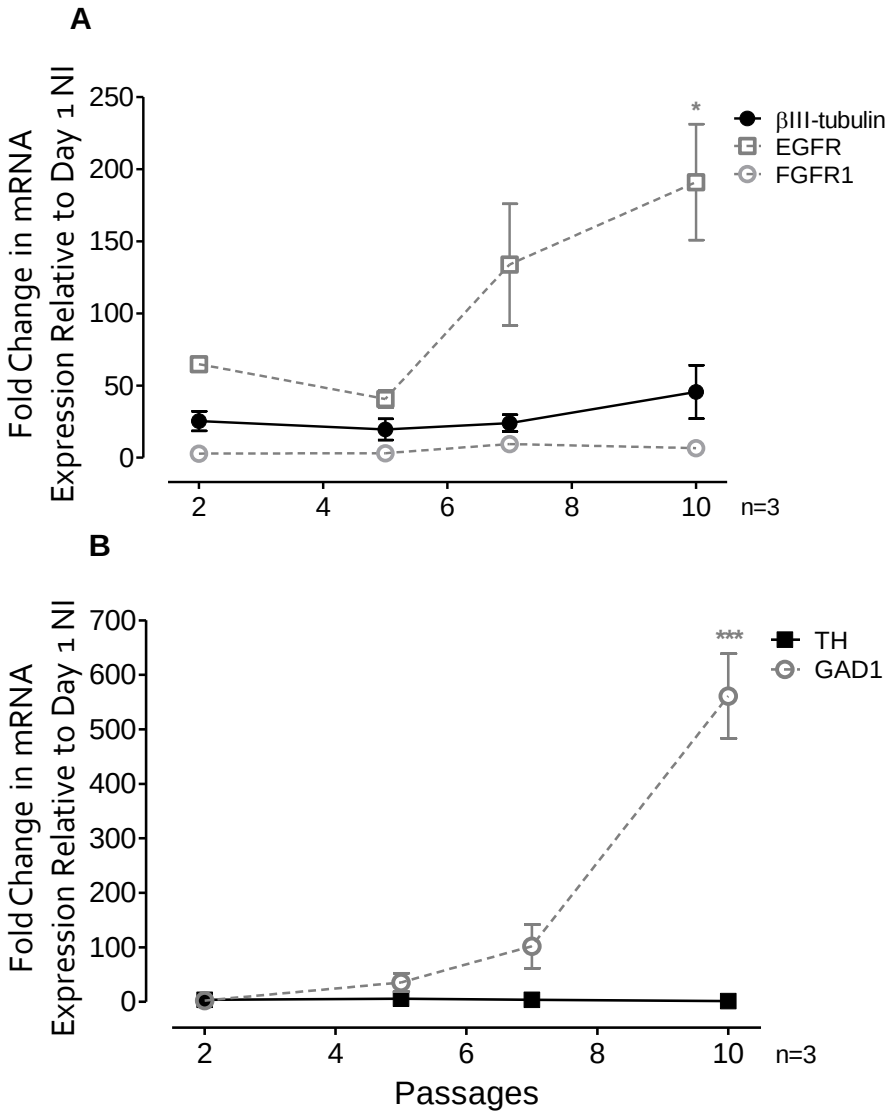
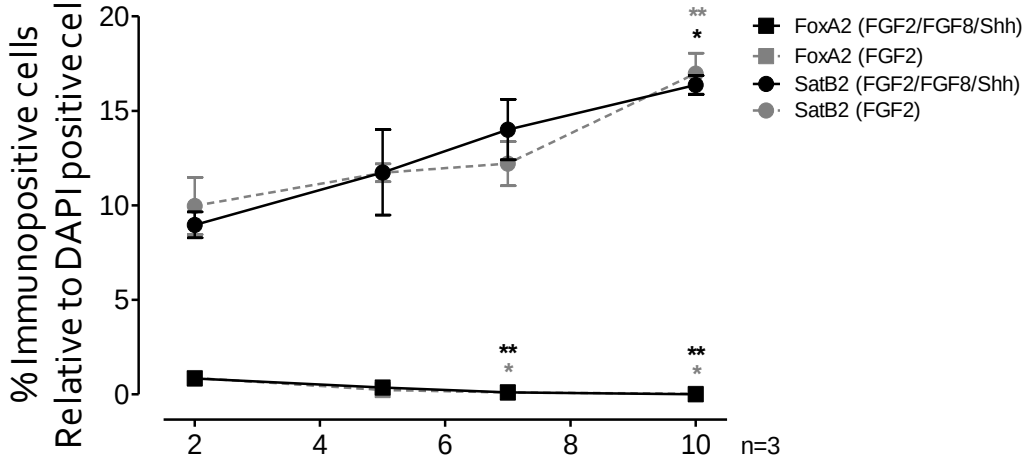


Figure 4.6 Expression profiling (qPCR) of passage 2, 5, 7 and 10 monolayer derived NS/NP cells propagated in the presence of EGF and FGF2. (A) The expression of βIII-tubulin and EGFR increased while expression of EGFR1 remained constant over the passages. (B) Although low expression levels TH was observed, an enormous increase in GAD1 expression at passage 10. (n=3; *p<0.05; **p<0.01; ***p<0.001; two-way ANOVA followed by Bonferroni's test compared to day 1 NI cultures)

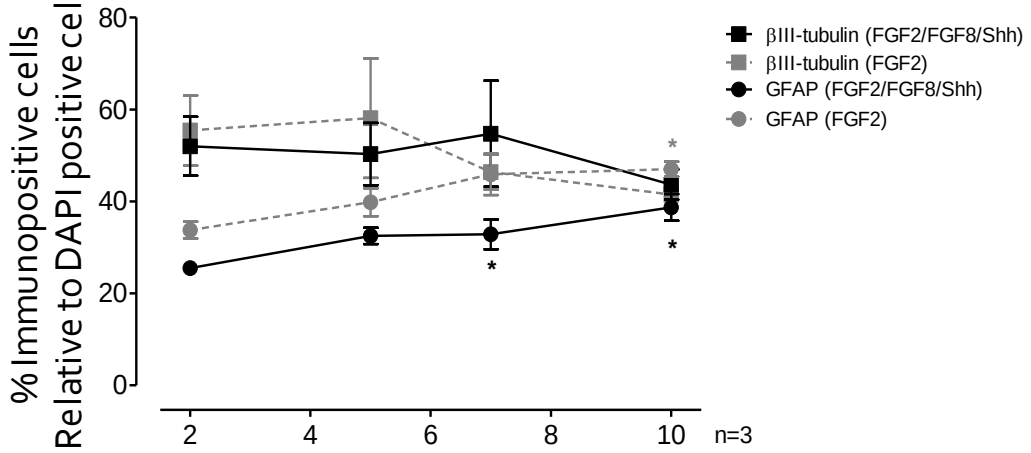
4.4.3. Differentiation potential of monolayer derived NS/NP cells

The NS/NP cells were exposed to two differentiation conditions: (i) the addition of patterning factors (Protocol 1: FGF2, FGF8 and Shh) that have been reported to direct ES and NS/NP cells towards a dopaminergic phenotype (Barberi et al., 2003) or, (ii) the withdrawal of EGF (FGF2 only), a method commonly for default differentiation into GABAergic neurons (Barberi et al., 2003; Spiliotopoulos et al., 2009). Immunocytochemistry of differentiated cultures at day 16 revealed that regardless of the patterning factor combination, the terminally differentiated cultures showed stable immunoreactivity to βIII-tubulin (41 to 58%; Figure 4.7B, Figure 4.8A and B) which co labelled with the GABAergic marker GABA. The astrocyte marker GFAP increased in later passages (26 to 47%; Figure 4.7B). Of the βIII-tubulin positive cells, only 1 to 13% were TH positive dopaminergic neurons (Figure 4.7C) while 25-42% were GABA positive (Figure 4.7C). Later passages were immunoreactive for the forebrain marker SatB2, which increased to 16.4±0.5% in differentiated passage 10 cultures. Very few FoxA2 (0.005-0.86%) ventral midbrain specific cells were detected (Figure 4.7A). Furthermore, the majority of cells that labelled for GABA also co labelled with SatB2 (Figure 4.8D) while TH positive cells did not co-label with the very limited number of FoxA2 positive cells (Figure 4.8C). Consistent with the qPCR results, with progressive passages, majority of neurons were GABAergic phenotype by passage 10. Surprisingly this did not correspond with the Lmx1a expression data showing an enrichment of Lmx1a by passage 10 (Figure 4.1). The presence of SatB2 and the lack of FoxA2 positive cells suggest the presence of forebrain phenotypes. As a result, the PA6 co-culture (an SDIA) NI method previously shown to generate large populations of midbrain dopaminergic neurons was then employed to assess the relationship between Lmx1a expression and the presence of midbrain dopaminergic progenitor cells.

A


% Immunopositive cells
Relative to DAPI positive cells
20
15
10
5
0
2
4
6
8
10
n=3
FoxA2 (FGF2/FGF8/Shh)
FoxA2 (FGF2)
SatB2 (FGF2/FGF8/Shh)
SatB2 (FGF2)

B


% Immunopositive cells
Relative to DAPI positive cells
80
60
40
20
0
2
4
6
8
10
n=3
βIII-tubulin (FGF2/FGF8/Shh)
βIII-tubulin (FGF2)
GFAP (FGF2/FGF8/Shh)
GFAP (FGF2)

C

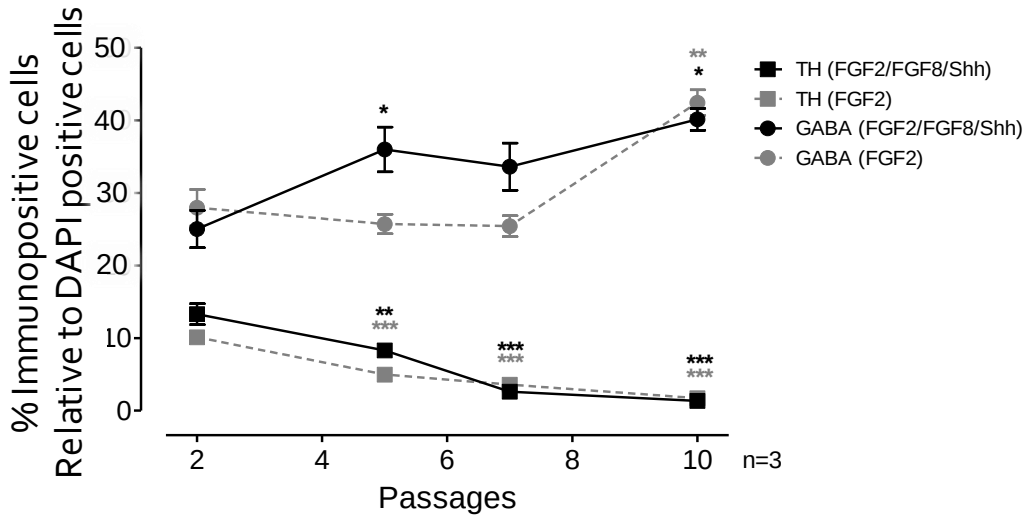

% Immunopositive cells
Relative to DAPI positive cells
50
40
30
20
10
0
2
4
6
8
10
n=3
Passages
TH (FGF2/FGF8/Shh)
TH (FGF2)
GABA (FGF2/FGF8/Shh)
GABA (FGF2)

Figure 4.7 Immunocytochemical analyses of monolayer derived cultures to determine the percentage of cells immunoreactive to (A) FoxA2 and SatB2; (B) βIII-tubulinand GFAP; and (C) TH and GABA. Passage 2, 5, 7 NS/NP cells derived from monolayer NI were subjected to differentiation in the presence of N2B27 supplemented with patterning factors: (i) 200 ng/mL Shh, 10 ng/mL FGF2, 100 ng/mL FGF8 (6 days), or (ii) 10 ng/mL FGF2 only, used to pattern NS/NP cells for 6 days. Following patterning, cells were cultured in N2B27 medium containing 200 mM L(+)-ascorbic acid and 20 ng/mL BDNF for the remaining 10 days. (n=3; *p<0.05; **p<0.01; ***p<0.001; two-way ANOVA followed by Bonferroni's test. Passage 5, 7 and 10 NS/NP cells were compared to passage 2 NS/NP cultures).

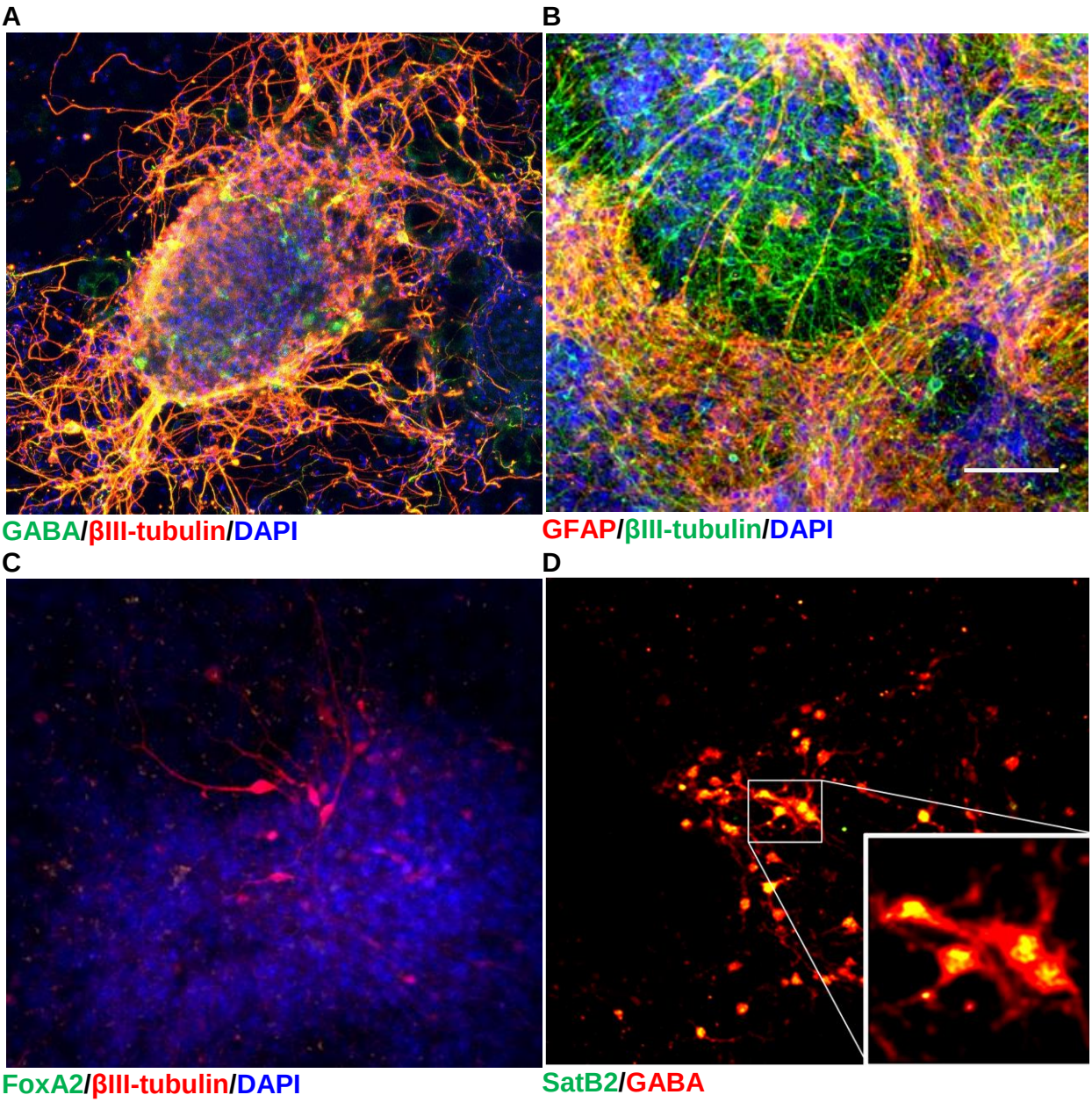


Figure 4.8 Immunocytochemistry studies of differentiated cells produced on day 16 using protocols outlined previously (see caption for Figure 4.7). Typical differentiated cultures showed a large population of βIII-tubulin positive cells that also co-localised with (A) GABA, (B) GFAP positive cells, (C) TH positive cells that did not co-localised with the very limited population of FoxA2 positive cells and (D) GABA positive cells that largely co-labelled with SatB2 positive cells. (All images are of the same scale excluding the enlarged panel insert; Scale bar 100 μm).

4.4.4. The effect of long term propagation on PA6 derived NS/NP cells

The SDIA method is known to generate dopaminergic neurons at high frequencies with ES cell differentiation cultures was used (Barberi et al., 2003; Kawasaki et al., 2000), by introducing PA6 stromal cells as a co-culture into the NS/NP cell differentiation protocol. Analysis of Lmx1a expression during long term EGF and FGF2 propagation did not show a transient peak similar to that observed when the monolayer protocol was used instead there was a gradual decrease from a high at passage 7 (10±4%) down to basal levels by passage 14 (4±2%; Figure 4.9). The ALDH expression in these cells was transient. The percentage of cells contained the BAAA cleavage product peaked at passage 6 (17±3%) before gradually declined to 2±0.3% by passage 14 (Figure 4.10). Like the monolayer NI derived NS/NP cells, qPCR, terminal differentiation followed by immunochemical analyses were carried out on passages 2, 5, 7 and 10 PA6-coc-culture NI derived NS/NP cells.

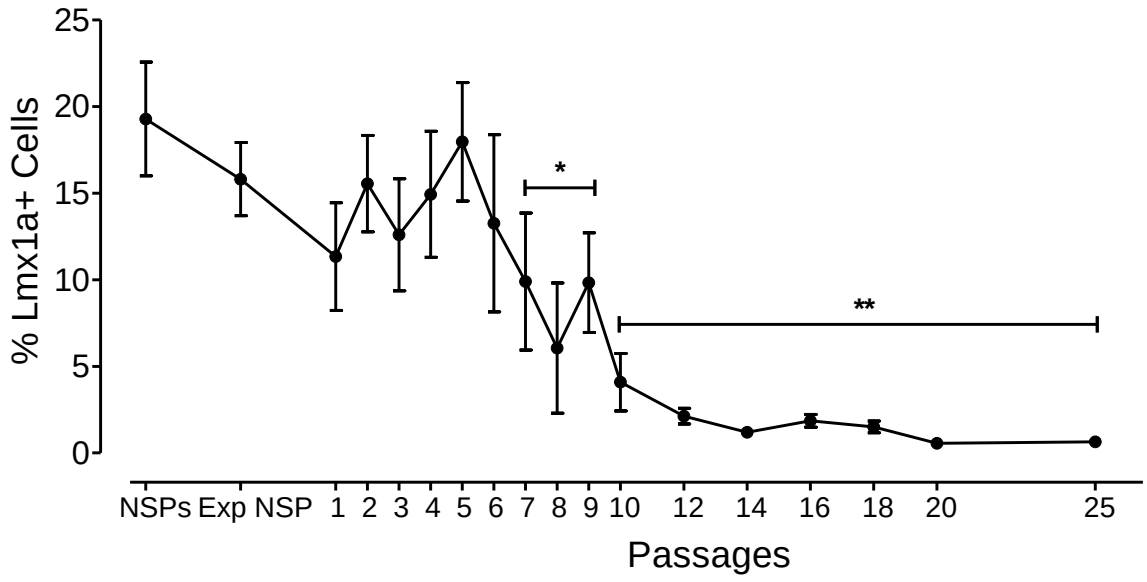


Figure 4.9 Lmx1a expression during passaging of 10 day NS/NP cells first produced using PA6 cell mediated NI FACS analysis was used to determine the percentage of

Lmx1a expressing cells on successive passages during NS/NP cell maintenance (n=3-4; *p<0.01; **p<0.05; two-way ANOVA followed by Bonferroni's test compared to NSP cultures)

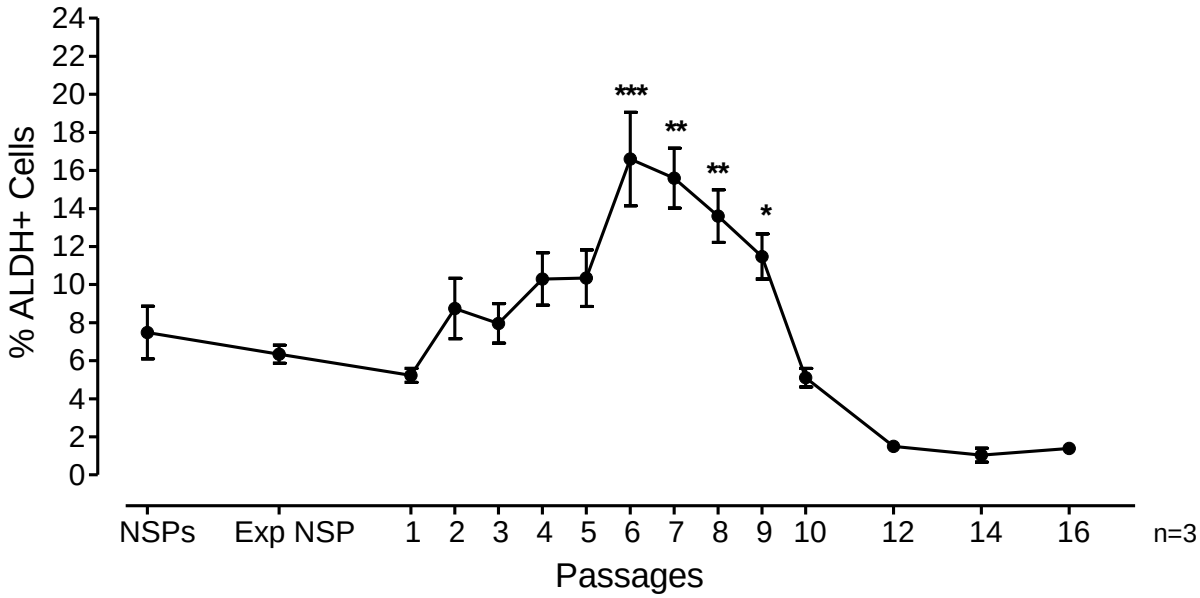


Figure 4.10 The percentage of the stem cell marker ALDH was evaluated during 16 passages of NS/NP cells first produced using PA6 cell-mediated NI. Cells were propagated in the presence of EGF and FGF2 was analysed using FACS analysis. The population of the ALDH substrate BAAA bound cells peaked at passage 6 before gradually declined to basal levels. (n=3; *p<0.05; **p<0.01; ***p<0.001; two-way ANOVA followed by Bonferroni's test compared to NSP cultures)

4.4.5 Molecular characteristics of PA6 derived NS/NP cells

qPCR analysis was carried out to investigate the expression of Nestin, Sox1, Sox2, Notch, FoxA2, FoxG1, BMP2, En1, Lmx1a, Lmx1b, βIII-tubulin, GFAP, TH and GAD1 in PA6-co-culture NI derived NS/NP cells over passages 2, 5, 7 and 10. Lmx1a expression from PA6 cell-derived cultures peaked at passage 7 (22±9 fold increase compared to day 1 NI cultures) while the Lmx1b expression in passage 5, 7 and 10 cultures was not significantly different from passage 2 (Figure 4.11A). In common with cultures derived from monolayer NI, the expressions of the stage specific NS/NP markers (Sox1, Sox2,

Notch1 and Nestin) decreased dramatically from passage 2 to 10 for PA6 derived cultures (Figure 4.11B). FoxG1 expression in passage 2, 5, 7 and 10 cultures was not significantly different from each other (6 to 11 fold), while En1 expression peaked at passage 5 (27±4 fold; Figure 4.11C). However, FoxA2 expression increased dramatically up to 185±24 fold from passages 5 to 10, while no significant difference was observed in the expression of BMP2 (Figure 4.11C). At the same time, a decrease was observed in the NS/NP marker Nestin from 133-34 fold while the expressions of the neuronal marker βIII-tubulin (5 to 11 fold) and the receptors for EGF, EGFR (30 to 72 fold) was not significantly different in passage 2, 5, 7 and 10 cultures (Figure 4.12A). Unlike monolayer derived NS/NP cells the expression of there were no significant changes in the dopaminergic and the GABAergic markers TH (2 to 13 fold) and GAD1 (4 to 13 fold) respectively over the different passages (Figure 4.12B). These results indicate that a strong shift towards the ventral roof plate phenotype was observed in these cultures from passage 5 onwards (double the expression level found in monolayer cultures; Figure 4.5C and Figure 4.11C). Terminal differentiation was then carried out on the NS/NP cells to assess whether these cells were able to differentiate into dopaminergic and GABAergic neurons.

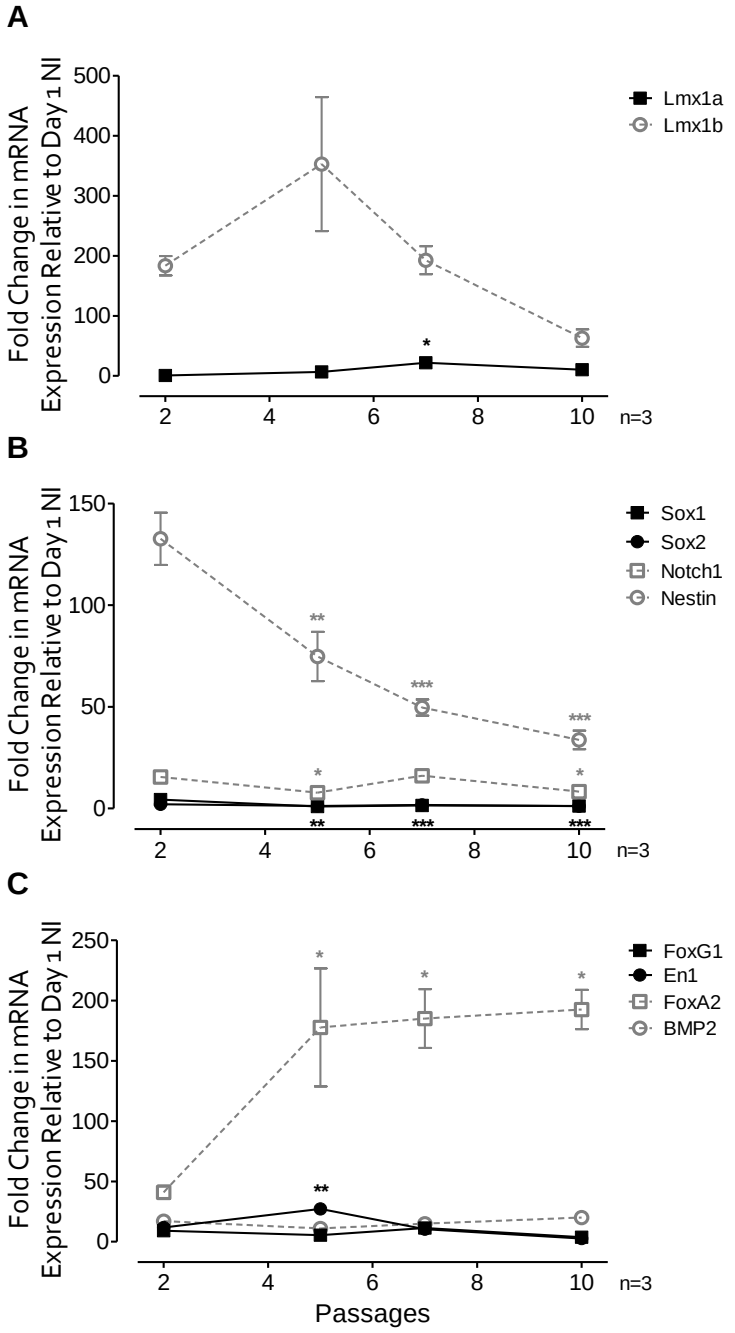


Figure 4.11 Expression profiling (qPCR) of passage 2, 5, 7 and 10 PA6 derived NS/NP cells propagated in the presence of EGF and FGF2. (A) Lmx1b expression peaked at passage 5 while Lmx1a expression remained constant. The NS/NP markers Sox1, Sox2 and Notch1 decreased over the passages. (C) FoxG1 expression was not significantly different across the passages while En1 expression peaked at passage 5. However, FoxA2 expression increased from passages 5 to 10, while no significant difference was observed in the expression of BMP2. (n=3; *p<0.05; **p<0.01; ***<0.001; two-way ANOVA followed by Bonferroni's test. Passage 5, 7 and 10 NS/NP cells were compared to passage 2 NS/NP cells)

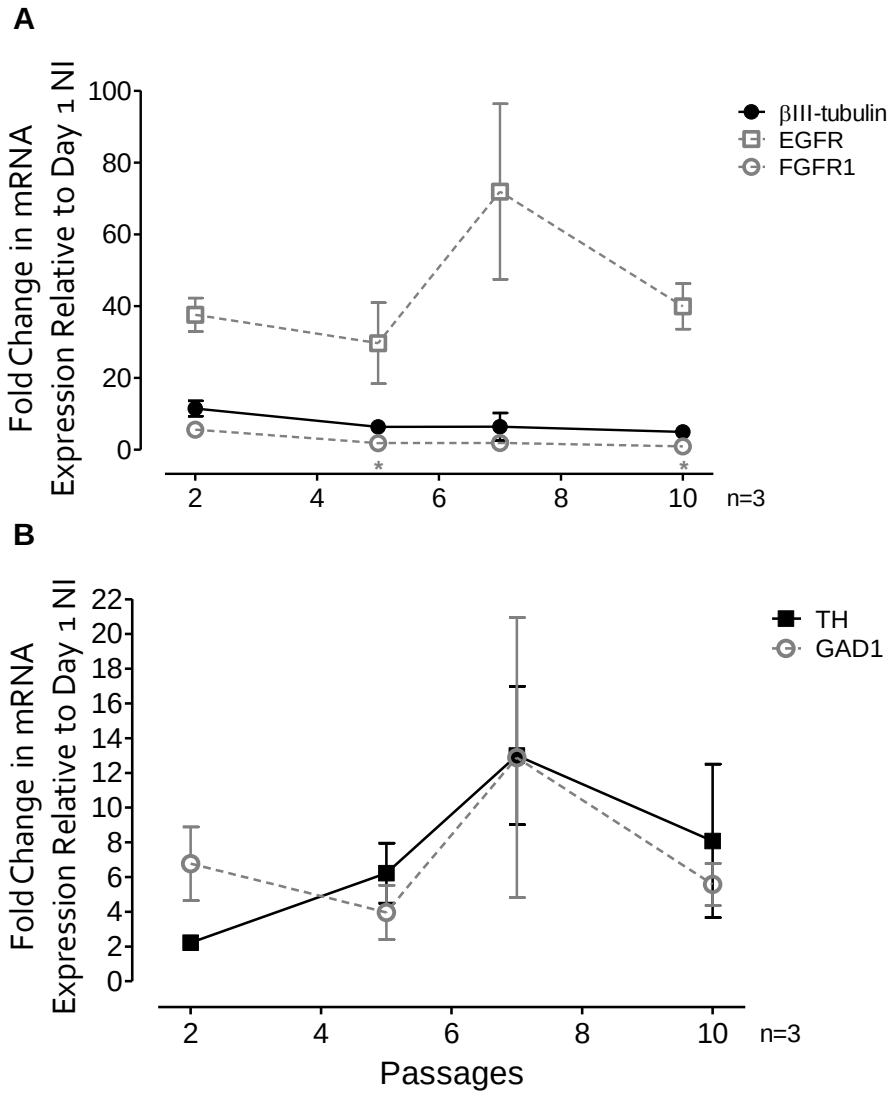


Figure 4.12 Expression profiling (qPCR) of passage 2, 5, 7 and 10 PA6 derived NS/NP cells propagated in the presence of EGF and FGF2. (A) The expressions of βIII-tubulin, EGFR was not significantly different throughout the passages, FGFR1 decreased at passages 5 and 10. (B) The expression of TH and GAD1 sis not change over the different passages (n=3; *p<0.05; **p<0.01; ***<0.001; two-way ANOVA followed by Bonferroni's test. Passage 5, 7 and 10 NS/NP cells were compared to passage 2 NS/NP cells)

4.4.6 Phenotypic potential of PA6 derived NS/NP cells

The differentiation potential of PA6 derived NS/NP cells was investigated over different passages using immunocytochemistry to identify the regional and phenotypic specificity of the differentiated cultures. The percentage of cells immunoreactive to neuronal specific markers during the different passages was very similar to that of differentiated monolayer cultures (Figure 4.13). Over the passages, the neurogenic potential of these NS/NP cells was not significantly different (as defined by Figure 4.13A), markers associated with midbrain dopaminergic development FoxA2 and TH decreased over the different passages (Figure 4.13B) while markers associated with forebrain GABAergic neurons increased (Figure 4.13C). GFAP-positive cells also increased with passaging (Figure 4.13A; Figure 4.14B). Although a large population of GABA-positive cells showed co-localisation with βIII-tubulin (Figure 4.14A), they did not necessarily co-localise with SatB2-like differentiated monolayer cultures (Figure 4.14D). Conversely, a population of TH-positive cells that co-localised with FoxA2 was also observed (Figure 4.14C). This suggests the presence of both FoxA2 positive midbrain and SatB2 positive forebrain phenotypes within the cultures instead of dominating populations of forebrain phenotypes found in monolayer NI derived cultures.

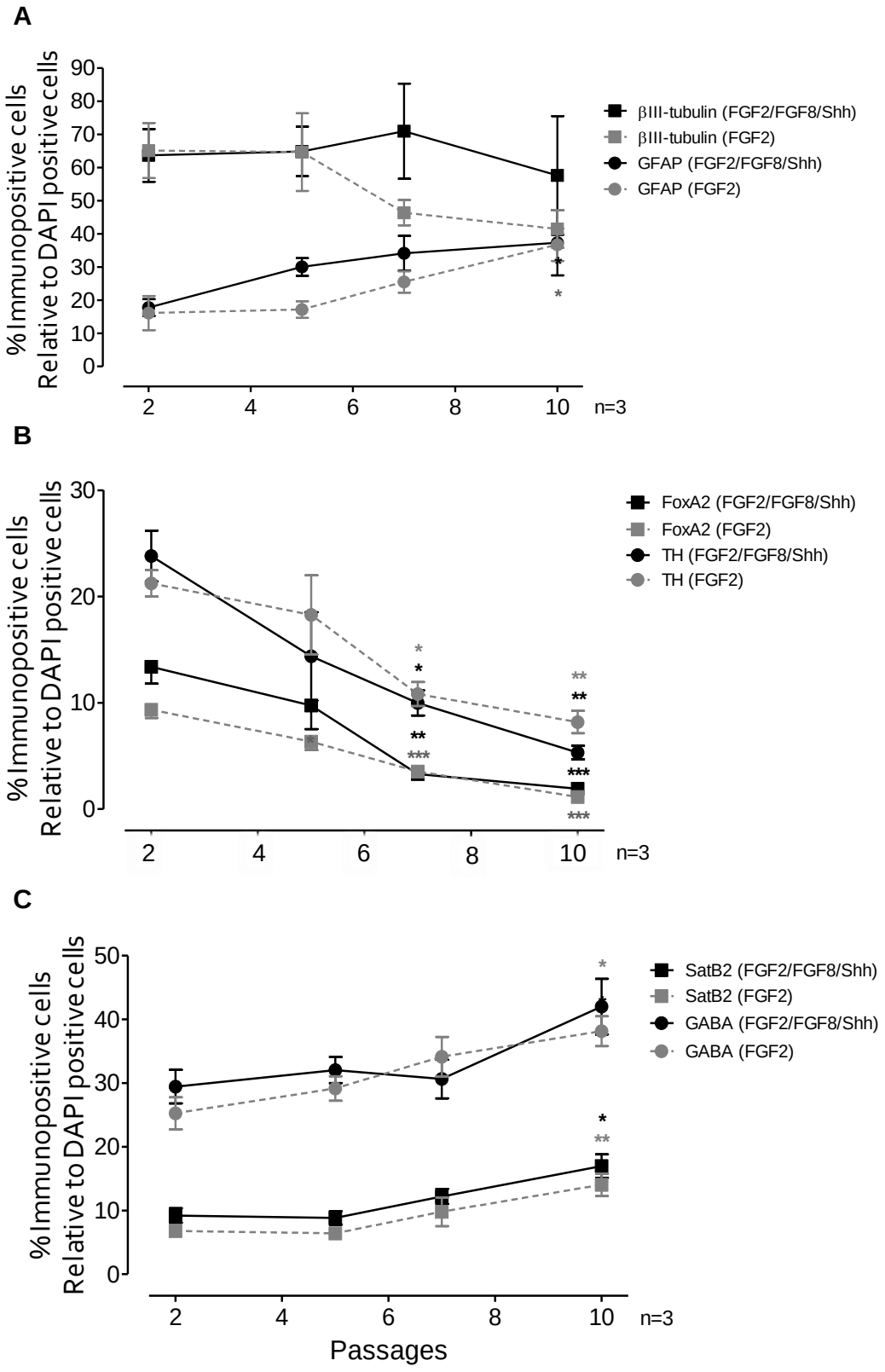


Figure 4.13 Immunocytochemical analyses to determine the percentage of PA6 derived cultures immunoreactive to (A) FoxA2 and SatB2; (B) βIII-tubulin and GFAP; and (C) TH and GABA. Passage 2, 5, 7 NS/NP cells derived from monolayer NI were subjected to

differentiation in the presence of N2B27 supplemented with patterning factors: (i) 200 ng/mL Shh, 10 ng/mL FGF2, 100 ng/mL FGF8 (6 days), or (ii) 10 ng/mL FGF2 only, used to pattern NS/NP cells for 6 days. Following patterning, cells were cultured in N2B27 medium containing 200 mM L(+)-ascorbic acid and 20 ng/mL BDNF for the remaining 10 days. (n=3; *p<0.05; ***p<0.001; two way ANOVA followed by Bonferroni's test).

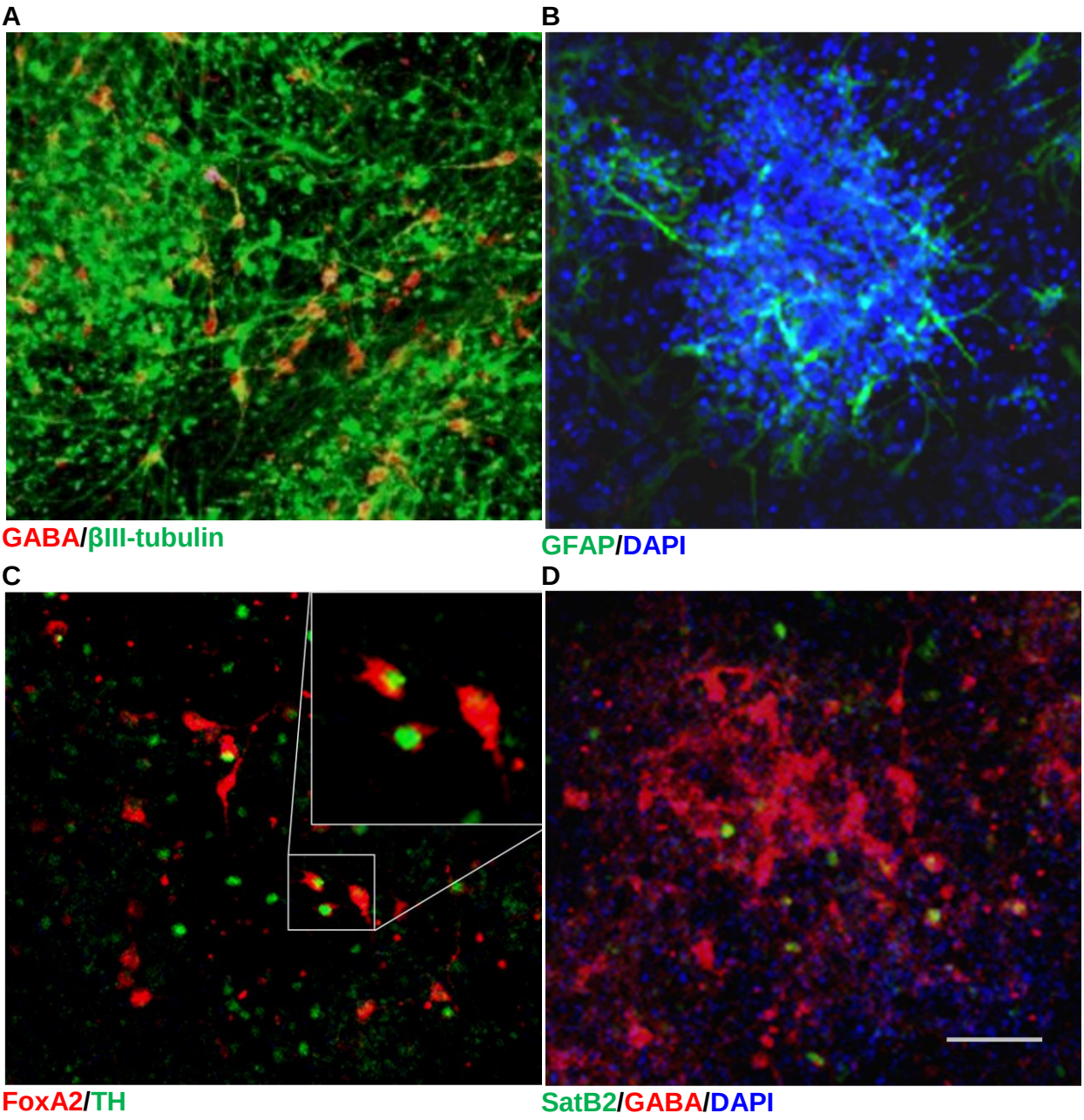


Figure 4.14 Immunocytochemistry studies of differentiated cells produced on day 16 using protocols outlined previously (see caption for Figure 4.15). (A) Typical differentiated cultures showed a population of βIII-tubulin positive cells that co-localised with GABA, (B) GFAP positive cells, (C) TH and FoxA2 positive cells and (D) GABA positive cells that did not necessarily co-localise with SatB2 positive cells. (All images are of the same scale excluding the enlarged panel insert; Scale bar 100 μm)

The late stage NS/NP cells (passage 20), where Lmx1a expression was found only at basal levels was exposed to patterning factors to investigate the neurogenic potential of these late stage cells. Only very small percentages of βIII-tubulin and GFAP positive cells were present. This suggests that the decrease in Lmx1a expression may indicate lack of neurogenic potential.

4.4.7 The effect of EGF and FGF2 on Lmx1a positive cultures

In order to investigate the effect of EGF and FGF2 on Lmx1a-positive cultures, the Lmx1a-AMP-IRES-eGFP reporter ES cells were used to sort eGFP expressing cells in day 10 NI monolayer cultures. These cells were sorted using FACS and the enriched production of Lmx1a-positive cells allowed to form neurospheres and passaged in the presence of (i) FGF2 and EGF or (ii) FGF2, Hedgehog signalling agonist (SAG); and Notch antagonists (Jagg1 and Dll4) for four passages. Irrespective of whether the NS/NP cells were exposed to the different factors, the number of Lmx1a-positive cells decreased dramatically over the initial four passages including neurosphere formation and expansion (Figure 4.15). This suggests that Lmx1a-positive NS/NP cells cannot be maintained in the presence of FGF2 and EGF, or growth factors known to maintain rNS cultures: FGF2, SAG, Jagg1 and Dll4.

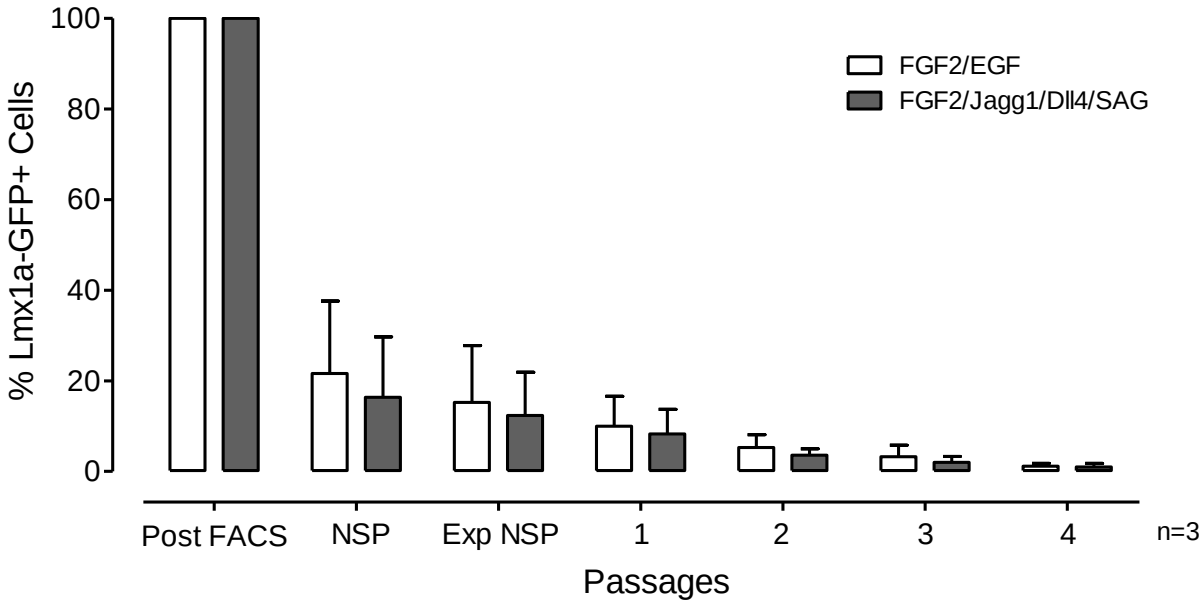


Figure 4.15 Isolation of enriched populations of Lmx1a positive cells on 10 day cultures. These Lmx1a positive cells were allowed to form neurospheres, expanded and propagated on laminin coated wells in the presence of (i) EGF and FGF2 or (ii) FGF2, SAG, Jagged1and Dll4. A dramatic decrease was observed during the initial neurosphere formation and expansion until a basal level was reached at approximately passage 3 and 4. (n=3)

4.5 Discussion

Considerable efforts have been made to identify the most suitable cells for use as cell transplants for the treatment of Parkinson's disease. Amongst the candidates, NS cells are considered to be a suitable cell source for transplantation since they are less likely to be contaminated with pluripotent cells or cells derived from other germ layers (Sanberg, 2007). However, upon implantation, NS cells have yet to demonstrate the ability to generate dopaminergic neurons (Ourednik, Ourednik, Lynch, Schachner, & Snyder, 2002). Thus, it appears that multipotent cells which are not phenotypically committed and may require exposure to specific patterning factors to direct them towards a dopaminergic fate. The Lmx1a reporter cell line was established to enable us to track midbrain differentiation since Lmx1a has been associated with the identification of midbrain dopaminergic progenitors (Chizhikov et al., 2010; W. Lin et al., 2009; Nakatani et al., 2010). It has also been demonstrated that forced expression of Lmx1a in monolayer cultures results in the generation of large populations of dopaminergic neurons (Friling et al., 2009). It would be advantageous to enrich and maintain committed dopaminergic progenitors as a reliable and unlimited source of midbrain dopaminergic neurons. In the previous chapter it was established that NS/NP cells derived from 10 days of monolayer NI were able to give rise to dopaminergic, serotonergic and GABAergic neurons. In this chapter, the stability of Lmx1a-positive cells during long term propagation was explored. Continuous propagation was carried out and surprisingly, it was found that during EGF and FGF2 propagation of monolayer derived NS/NP cells, enrichment of Lmx1a-positive cells was observed at passage 9. This was also supported by a peak expression in ALDH at passage 9. However, following terminal differentiation, high Lmx1a and ALDH expression did not correlate to increased midbrain dopaminergic neurons, instead GABA-positive SatB2-positive cortical neurons were generated, most likely to be forebrain neurons (Gaspard et al., 2008; Hevner et al., 2003).

Recent studies in our laboratory have confirmed that Lmx1a is a poor indicator of dopaminergic progenitors when monolayer NI is used (Nefzger et al., 2012). The TH-positive neurons present did not show co-localisation with FoxA2, which is regarded as a necessary indicator of precursors of midbrain dopaminergic neurons (A. Bjorklund & Dunnett, 2007) and may indicate other dopaminergic or catecholaminergic neurons. This is consistent with evidence that monolayer NI generates anterior neuronal cell types (Gaspard et al., 2008; Konstantoulas et al., 2010). Two independent transcription factor pathways have been identified as essential for midbrain dopaminergic neuronal development: namely Lmx1a and (Shh induced) FoxA2 (Chung et al., 2009). Although it was found that extended propagation in the presence of EGF and FGF2 increased Lmx1a expression of these cells, it did not correspond to midbrain dopaminergic neurons, even in the presence of Shh during terminal differentiation. Instead, early expression of Shh and FGF8 contributed to patterning events and the establishment of ALDH expressing dopaminergic progenitors. As the cells stop proliferating, they express Nurr1, followed by downstream markers such as Pitx3 and TH. However, ALDH was also found in other highly proliferative cells such as hematopoietic and cancer stem cells of different origins (Hynes, Porter, et al., 1995; Wallen et al., 1999; Ye et al., 1998).

Both Lmx1a and BMP2 are primarily expressed in the cortical hem during forebrain development (Chizhikov et al., 2010; Grove & Tole, 1999). In this study, an increase in both Lmx1a and BMP2 expression increased from passage 7 in monolayer derived NS/NP cells and overall these markers were upregulated in the monolayer derived NS/NP cells compared to PA6 derived cultures. Although a significant increase in FoxA2 expression was observed in monolayer NS/NP cells, a decrease in TH-positive cells was observed. Furthermore, GAD1 expression increased dramatically in monolayer derived NS/NP cells and over the extended passages, indicating upregulation of the GABAergic

phenotype. An increase in EGFR was observed in these cells at passage 10. It has been suggested that EGF responsive progenitors were previously FGF responsive and that exposure of the more primitive NS/NP cells such as NEP and rNS cells to EGF results in the generation of the EGF responsive rGs, which are found to generate largely glutamatergic and GABAergic neurons (Goldman, 1995; Lois & Alvarez-Buylla, 1993; Luskin, 1993; Spiliotopoulos et al., 2009). Increased EGFR signalling in the SVZ results in the expansion of the NP cell pool, and reduces NS cell number and self-renewal. This occurs through a non-cell autonomous mechanism involving EGFR-mediated regulation of Notch signalling (Aguirre, Rubio, & Gallo, 2010). In my experiments, it appears that after 10 days of monolayer NI followed by up to 10 passages of EGF and FGF2 propagation, these cells may be restricted towards the forebrain GABAergic phenotype. Terminal differentiation of the NS/NP cells resulted in the generation of SatB2/GABA-positive cells that largely co-localised with GABA-positive neurons. Although GABAergic interneurons do not express SatB2 (Britanova et al. 2011, Alcamo et al. 2008), it has been reported that a subpopulation of upper layer callosal projection neurons are also GABA-positive (Lodato et al., 2011). In addition, GABAergic interneurons labelling for SatB2 can also be found in the mammalian retina (Kay, Voinescu, Chu, & Sanes, 2011).

In contrast NS/NP cells derived from NI in the presence of PA6 cells showed an overall upregulation in the midbrain dopaminergic specific markers, FoxA2 and TH when compared to monolayer derived cultures. This is supported by an increase in the number of FoxA2/TH-positive neurons. Thus the PA6 co-culture method generated cultures that gave rise to the midbrain dopaminergic phenotype (Perrier et al., 2004). Although GABA-positive neurons were also generated at high frequencies compared to TH-positive cells, the incidence of SatB2 and GABA co-localisation occurs at a much lower rate compared to monolayer-derived cultures. These NS/NP cells are therefore likely to have a different

regional specificity compared to monolayer derived NS/NP cells. Over progressive passages in the presence of EGF and FGF2, the cultures produced an increase in SatB2-positive and GABA-positive cells that did not necessarily co-localise. Sorting experiments revealed that neither EGF and FGF2 in combination; or the presence of Notch antagonists were able to support the proliferation of Lmx1a-positive NS/NP cells, although EGF and FGF2 are known to regulate NS/NP cell self-renewal through Notch mediated pathways (Faux, Turnley, Epa, Cappai, & Bartlett, 2001). Like EGF and FGF2 dependent rG or type B cells (*in vivo* NS cells), the Notch dependent rNS cells are also found to differentiate towards a forebrain, anterior fate (Alves, Barone, Engelender, Froes, & Menezes, 2002; Elkabetz et al., 2008a). This indicates that regardless of the origin of the NS/NP cells, monolayer or PA6, prolonged propagation of NS/NP cells in the presence of EGF and FGF2 phenotypically restricts these cells from becoming dopaminergic neurons.

4.6 Conclusion

In this study the NS/NP cells can be derived from both monolayer and PA6 derived cultures, and can be propagated in the presence of EGF and FGF2 for more than 25 passages. By employing a reporter line, the changes in Lmx1a expression can be monitored during long-term maintenance and ultimately differentiate these cells towards a GABAergic or dopaminergic fate. It was found that Lmx1a cannot be used solely as a marker for dopaminergic neurons during propagation; it also indicates the presence of progenitors of the forebrain in both monolayer and PA6 derived cultures, which when exposed to patterning factors gives rise to GABAergic neurons. In addition, the presence of EGF and FGF2 was not sufficient to stabilise Lmx1a positive cells in both monolayer and PA6 derived NS/NP cells during long term maintenance. Although over the time course of 10 passages, the NS/NP cells were able to give rise to a stable percentage of βIII-tubulin positive neurons, the ability of these cells to produce catecholaminergic neurons over this time frame is greatly compromised. Under the influence of EGF and FGF2 both NS/NP cells derived from monolayer and PA6 co culture NI displayed bias towards the forebrain GABAergic phenotype.

Chapter Five

5. Clonal Propagation of Neural Stem/Progenitor Cultures

5.1 Introduction

Physical injuries and neurodegenerative diseases of the CNS are extremely and permanently debilitating since the CNS has only limited ability to replace lost or diseased neurons. NS/NP cells are the most versatile and promising cell source for the regeneration of injured neurons since they can be isolated and expanded as a large-scale, using both monolayer and neurosphere assays, and differentiated into the three major cell types of the CNS: astrocytes, oligodendrocytes and neurons. These characteristics could make NS/NP cells an invaluable renewable source of cells for investigations of cell physiology; drug screening; toxicology and also for cell replacement

therapies for many neurological diseases. There are some unresolved concerns that need to be addressed before the power of stem cells can be fully utilised. These unresolved issues revolves around the consistency of methods to ensure maintenance of multipotency have not been established, and evidence of propagation induced lineage restriction (Temple. 1989; Qian et al. 2000). Also, in practice differentiated progenies of NS cells are highly heterogeneous producing predominance of astrocytes following differentiation and low yields of neurons and oligodendrocytes (Temple. 1989; Qian et al. 2000). Cell therapies also face many problems related to cell survival, control of cell fate and proper cell engraftment after transplantation limiting the use of NS/NP cells in clinical applications.

One of the methods used in the propagation of NS/NP cells is neurosphere formation. The neurosphere culture was the first *in vitro* system to enable the propagation of NS cells (Reynolds & Weiss, 1992, 1996), and it has become the method of choice not only for the expansion of NS/NP cells, but also to determine the presence of stem cells from cell populations *in vitro* (Reynolds & Weiss, 1992, 1996). Testing for neurosphere forming capacity over serial clonal passaging followed by *in vitro* differentiation is widely used to show multipotency of individual spheres and provides the best functional assay for stem cell-like properties in NS/NP cells (Gritti et al., 1996; Rietze et al., 2001; Tropepe et al., 1999a; Uchida et al., 2000). However, other cell types also have the capacity to form neurospheres; including NP cells, O2A cells, oligodendrocyte precursors, and possibly astrocytes (Engstrom et al., 2002; Gritti et al., 1996). While the neurosphere assay is ideal for the expansion of NS/NP cells, it is not capable of discriminating stem cells from other sphere-forming cell types. The presence of NS and NP cells are often assessed based on their proliferative potential and only the large colonies (diameter >2 mm) are believed to be derived from cells exhibiting stem cell characteristics (Louis et al.,

2008). Therefore, the frequency of large colony can be used as an indicator of NS cell frequency. It was established in chapter 3 that 10 days of NI was efficient in the generation of multipotent NS/NP cells. In the previous chapter it was also found that 10 day NS/NP cells can be generated using both monolayer and PA6 co-culture methods. They can be maintained in the presence of EGF and FGF2 up to 10 passages with a stable neurogenic potential. However, it is no surprise that these NS/NP cultures consist of heterogeneous populations of NS and NP cells at different stages of development. In this chapter the neurosphere forming potential of NS/NP cells was explored using two methods of NI and using the Lmx1a and Pitx3 reporter cell lines. Both the neurogenic and phenotypic potential of individual neurospheres was investigated, with a focus on the generation of dopaminergic and GABAergic neurons.

This chapter investigates the hypothesis that individual neurosphere cultures. The propagation of these individual neurospheres may allow the isolation of clonal propagation and expansion of committed dopaminergic or GABAergic neurons.

5.2 Aim

This study aims to generate neurosphere cultures from both monolayer and PA6 NI derived NS/NP cells were propagated as individual up to three rounds via spontaneous neurosphere formation or minimal cell numbers (1, 2, 5, 10, 50 and 100). The neurogenic and phenotypic potential of these individual neurospheres were assessed using FACS analysis and immunocytochemistry, specifically the dopaminergic and GABAergic phenotype, by applying protocols that have been shown to generate dopaminergic and GABAergic neurons.

5.3 Methods

The pluripotent Lmx1a-AMP-IRES-eGFP, Lmx1a-eGFP and Pitx3-eGFP reporter cell lines were used in this study to track the expression of Lmx1a and Pitx3 during neurosphere propagation in the presence of EGF and FGF2. The experimental methods used in this chapter, including the differentiation of ES cells into NS/NP cells and terminal differentiation have been described in Chapter 2.

5.4 Results

5.4.1 Phenotypic diversity in individual neurospheres

To investigate the neurogenic diversity in individual neurospheres formed after NI, a detailed study of 12 neurospheres was carried out. ES cells were initially subjected to 10 days of NI followed by neurosphere formation. Individual neurospheres (1-12) were then selected at random and expanded on gelatin. The expanded neurospheres were then dissociated and passaged once as monolayer cultures in the presence of EGF and FGF2. The expression of proteins associated with neural differentiation: NS/NP cells (Nestin), immature neurons (βIII-tubulin), mature neurons (NeuN) and astrocytes (GFAP) by these monolayer cultures was investigated (Figure 5.1). Figure 5.2 shows typical immunoreactivity to these markers. Neurospheres 4 and 5 contained the greatest proportion of βIII-tubulin-positive cells (30±2% and 28±3% respectively; Figure 5.1B; 5.2B and C), neurosphere 6 contained the greatest proportion of GFAP-positive cells (66±2%; Figure 5.1D; 5.2H), while expression of the mature neuron marker NeuN was minimal (0-0.4%; Figure 5.1C; 5.2 E-H). Expression of the NS/NP cell marker Nestin remained high across the 12 neurospheres (47-87%; Figure 5.1A; 5.2A-D). This suggests that individual neurospheres are made up of heterogeneous populations of differentiated and undifferentiated NS/NP cells. In order to assess whether this

heterogeneity leads to phenotypic diversity, theses individual neurospheres were differentiated in the presence of specific growth factors.

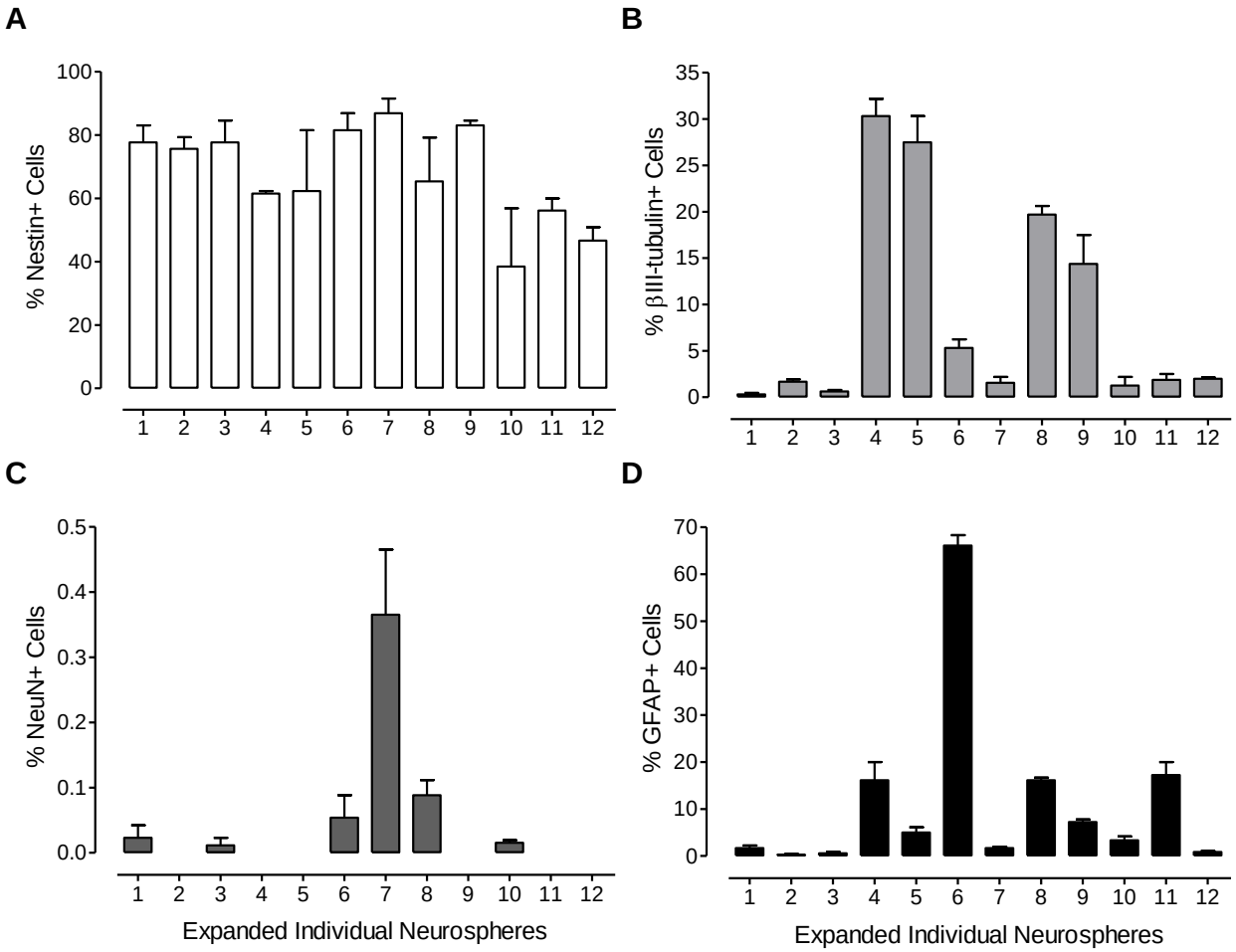


Figure 5.1 Percentage of NS/NP cell immunoreactive for neuronal and astrocyte markers. 12 neurospheres were chosen at random and expanded in the presence of EGF and FGF2. Cells immunoreactive to Nestin (A), βIII-tubulin (B), NeuN (C) and GFAP (D) was analysed. Neurospheres 4 and 5 contained the greatest proportion of βIII-tubulin-positive cells, neurosphere 6 contained the greatest proportion of GFAP-positive cells, while expression in the mature neuron marker NeuN is at a minimal level and the NS/NP cell marker Nestin remained high across all neurospheres.

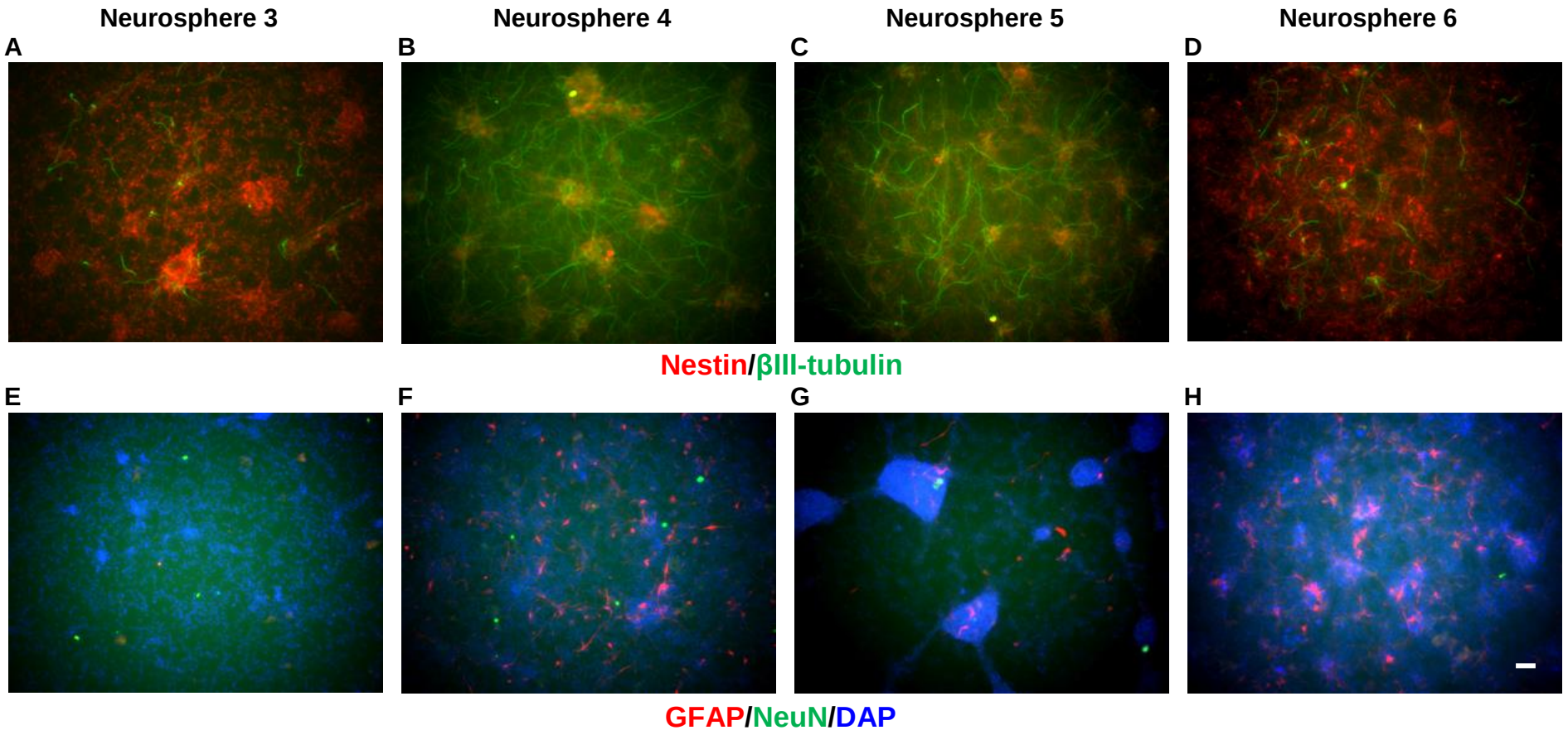


Figure 5.2 Typical immunocytochemical staining of replated individually NS/NP cells following 10 days of monolayer NI, neurosphere formation and expansion: neurosphere 3 (A and E), neurosphere 4 (B and F), neurosphere 5 (C and G); and neurosphere 6 (D and H). Immunocytochemistry indicated presence of Nestin and βIII-tubulin (A-D); and GFAP and NeuN (E-H). The proportion of cells expanded from individual neurospheres that immunoreactive to these markers vary (All images are of the same scale; Scale bar 100μm).

The differentiation potential of individual neurospheres exposed to patterning factors was then investigated using a midbrain differentiation protocol (Protocol 1, Patterning factors: FGF2, FGF8 Shh; See Chapter 2). Neurospheres quickly gave rise to different proportions of βIII-tubulin-positive (Figure 5.3A and B) and GFAP-positive cells (Figure 5.3F). Only small numbers of TH-positive cells were generated from differentiated neurospheres using this protocol (Figure5.3C and D), these cells also retained Musashi1 and Nestin expression during differentiation (Figure 5.3E and F) indicating that different neurospheres have different neurogenic and phenotypic potentials. This may also indicate the presence of fate restricted NS/NP cells that are either more likely to generate neurons rather than glia or vice versa. These studies indicate the advantage of a reporter cell line, which enables NS/NP tracking without destroying neurons.

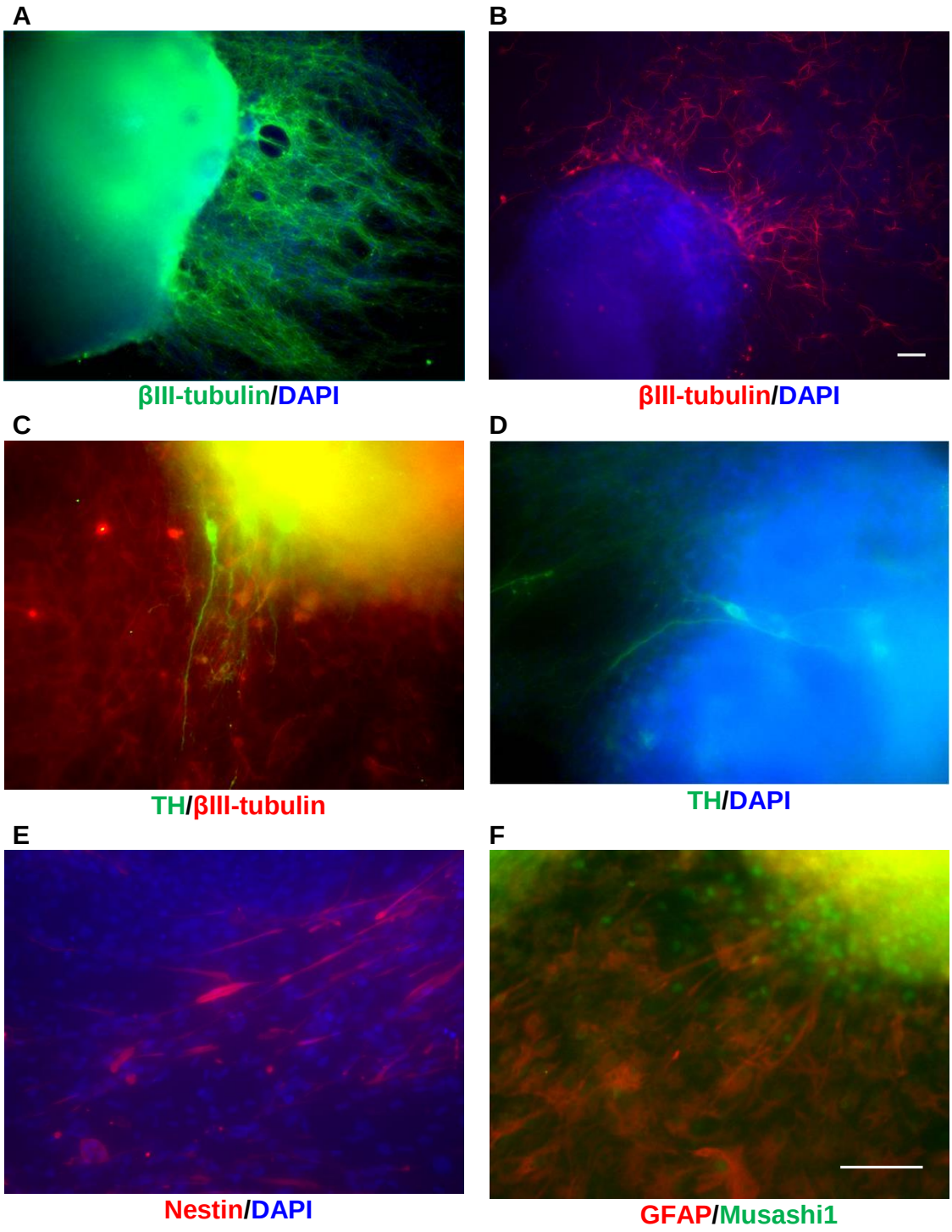


Figure 5.3 Typical immunocytochemistry of differentiated neurospheres. Individual neurospheres were chosen at random, plated on laminin in the presence of patterning factors: 10 ng/mL FGF2, 100 ng/mL FGF8 and 200 ng/mL Shh (6 days), followed by 200 mM L(+)-ascorbic acid and 20 ng/mL BDNF (10 days). These neurospheres quickly give rise to different proportions of βIII-tubulin (A and B), TH (C and D) and GFAP-positive cells (F). They also retained Musashi1 and Nestin expression (E and F). (Scale bar 100 µm)

5.4.2 Lmx1a expression in individual neurospheres

The Lmx1a-β-lactamase reporter cell line was used to investigate the expression of Lmx1a by individual neurospheres and their progeny. FACS analysis of monolayer expanded neurospheres was carried out. 42 neurospheres were chosen at random and expanded in the presence of EGF and FGF2. The proportion of cells that were positive for β-lactamase was variable. 27 neurospheres had a <15% β-lactamase-positive cells while only 6 had >35% β-lactamase-positive cells (Figure 5.4A). Some neurospheres had no β-lactamase-positive cells, which could reflect an early NS phenotype with few NP cells in the neurosphere. These neurospheres could not be used further.

From the individual neurospheres, two lower β-lactamase expressing neurospheres (<10% Lmx1a expression; B and E) and two higher β-lactamase expressing neurospheres (>35% Lmx1a expression; C and D) were expanded as monolayers and used to form secondary neurospheres. Four neurospheres were chosen at random amongst the secondary neurospheres and analysed as before (Figure 5.4B, C, D and E). The low β-lactamase expressing neurospheres generated daughter neurospheres that were also low β-lactamase expressing neurospheres (0.1-14%; Figure 5.4B and E) while high β-lactamase expressing neurospheres generated a variety of daughter neurospheres that were both low (0.6-6%) and high expressing neurospheres (53-91%; Figure B and D). Figure 5.5 Shows example of typical FACS histogram (Figure 5.5A and C) and scatter (Figure 5.5B and D) plots of daughter neurospheres. This demonstrates that neurospheres are made up of cells that have different levels of Lmx1a expression, which would potentially lead to different proportions of neurons once differentiated.

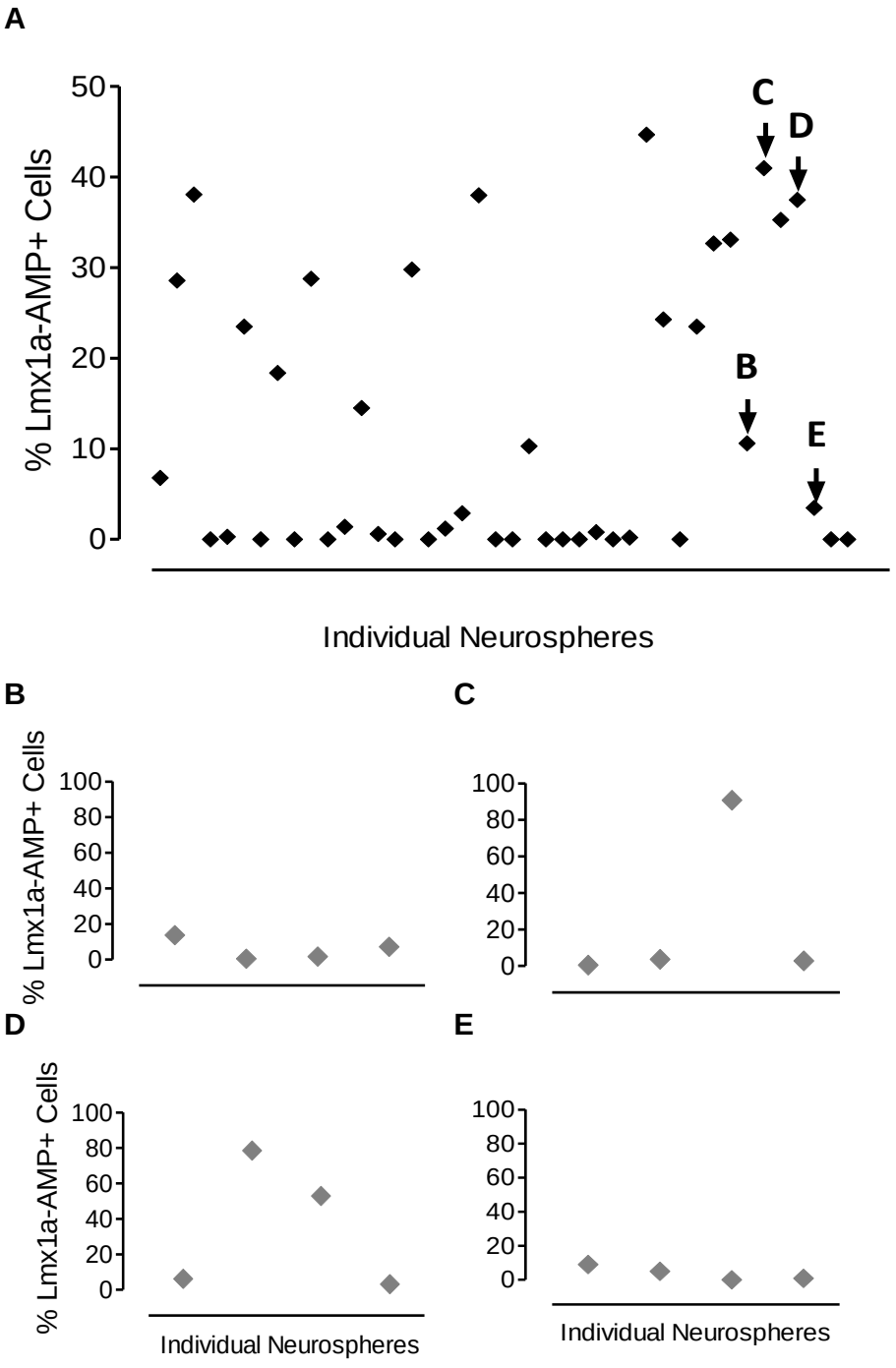


Figure 5.4 Lmx1a expression was examined in individual neurospheres and their corresponding daughter neurospheres. Amongst a mixture of 42 neurospheres (A), two low expressing (B and E) and two high expressing (C and D) neurospheres were chosen and allowed to form secondary neurospheres, in which four neurospheres were then chosen and analysed. It was found that the low expressing neurospheres generated daughter neurospheres that also express Lmx1a at a low level while high expressing

neurospheres generated daughter neurospheres that express Lmx1a at both low and high levels.

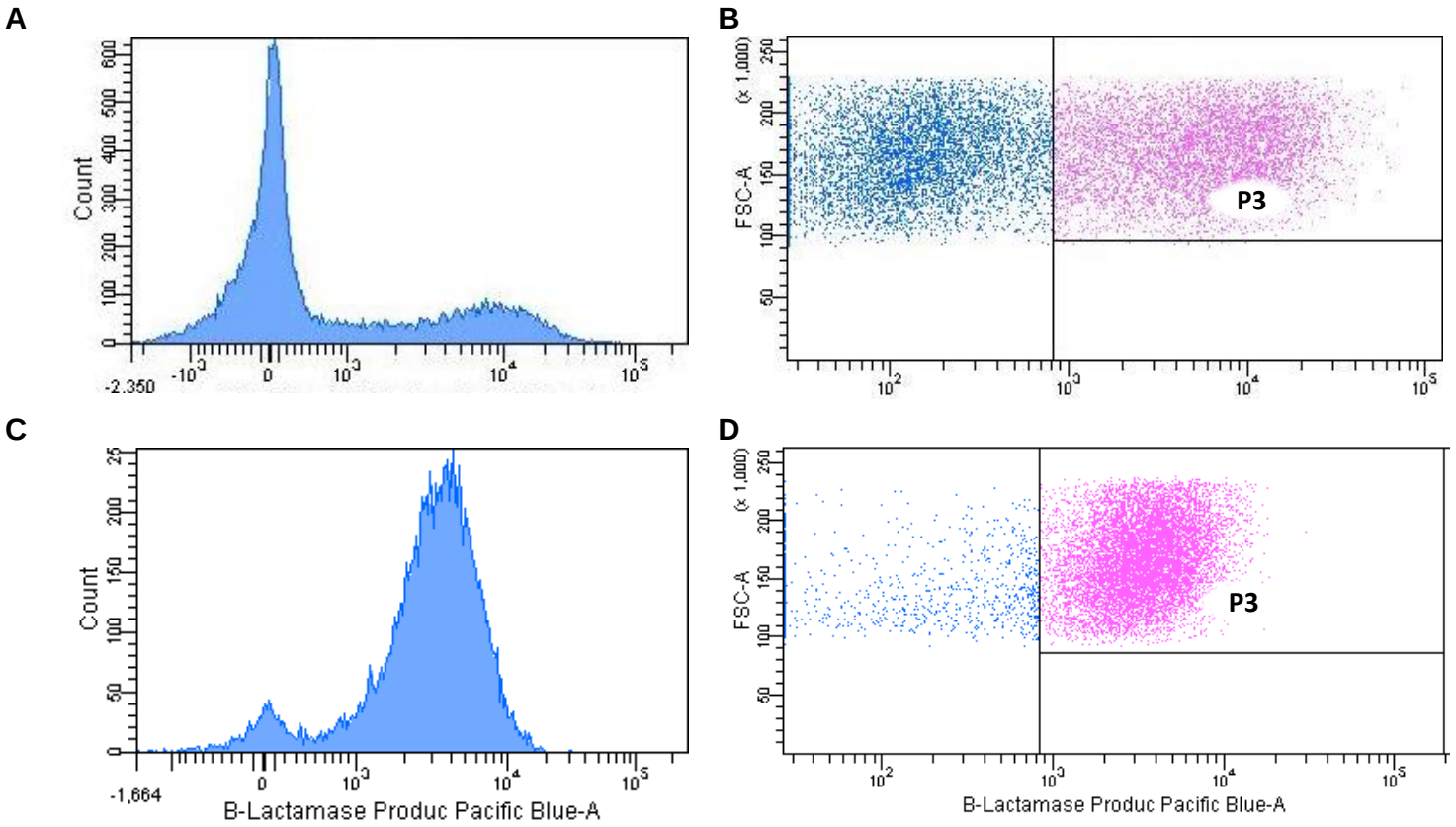


Figure 5.5 Examples of typical FACS plots of individual neurospheres. Neurospheres were selected at random after monolayer NI and subsequent four day neurosphere formation in the presence of EGF and FGF2. The selected neurospheres were then expanded as monolayer cultures before half the culture was analysed and the other half is allowed to form daughter neurospheres. Examples show typical histogram (A) and dot plot (B) of a low expressing neurosphere; and typical histogram (C) and dot plot (D) of a high expressing neurosphere. “P3” represents β-lactamase pacific blue-positive; Lmx1a-positive population is gated according to 0.1% Lmx1a-positive cells in wild type neurosphere cultures under the same culture conditions.

In order to visualise Lmx1a-positive neurospheres during EGF and FGF2 propagation, the Lmx1a-eGFP reporter cell line was used. This line had the AMP-IRES sequences removed so that expression of eGFP was stronger in the Lmx1a-AMP-IRES-eGFP reporter line. Again neurospheres were selected at random after the initial monolayer NI and subsequent four days neurosphere formation. FACS analysis was carried out on neurospheres expanded in the presence of the growth factors. During the first round of neurosphere formation, the neurospheres showed overall low levels of Lmx1a expression (5.6±0.6%; Figure 5.6). The secondary neurospheres also showed minimal level of Lmx1a expression (4.3±0.7%; Figure 5.6); however, tertiary neurospheres showed almost no Lmx1a expression levels (1.3±0.2%; Figure 5.6). Furthermore, during the third round of neurosphere formation, the size of these neurospheres as well as the rate of proliferation as monolayer cultures was greatly reduced. Figure 5.7 shows typical FACS plots of an example of a high Lmx1a expressing and a low Lmx1a expressing neurospheres. It is likely that prolonged propagation in the presence of EGF and FGF2 may result in a decrease in neurosphere forming NS/NP cells.

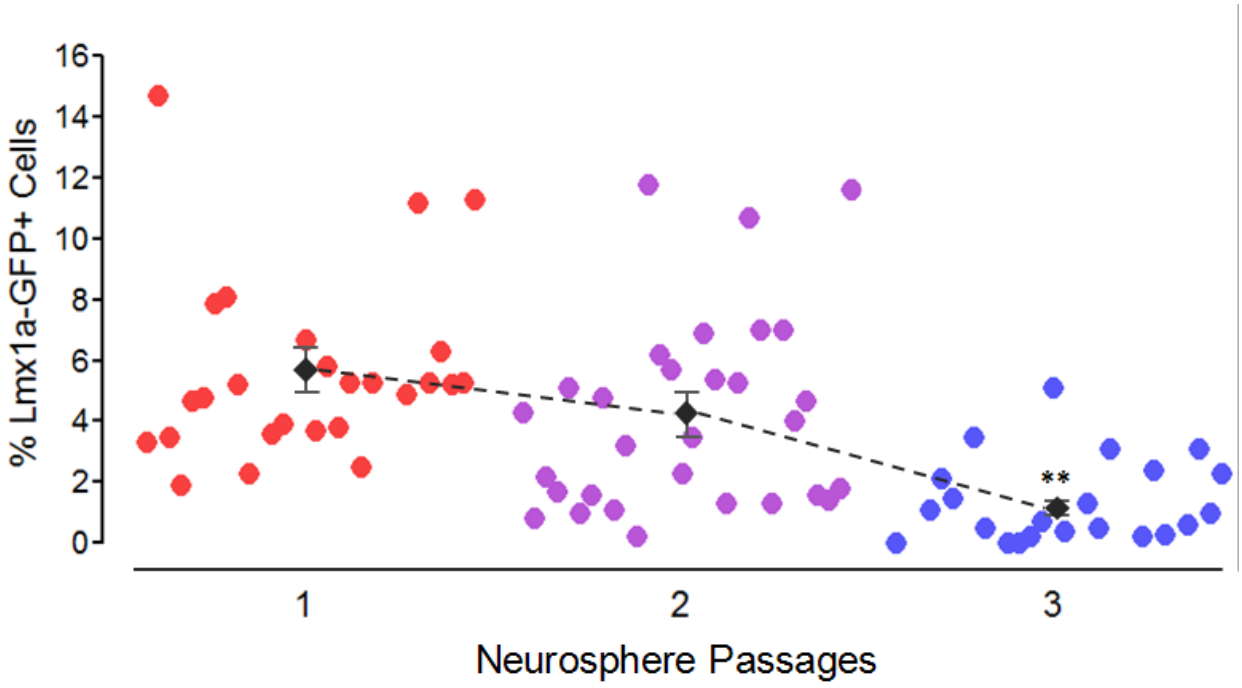


Figure 5.6 The Lmx1a-eGFF reporter line was used to assess the stability of neurosphere cultures in the presence of EGF and FGF2.Lmx1a expression for passages 1, 2 and 3 of individual expanded neurospheres are assessed. With each progressive passage, Lmx1a expression level decrease. (n=30; **p<0.001; two way ANOVA followed by Bonferroni's test. Passage 2 and 3 neurospheres compared to passage 1 neurospheres).

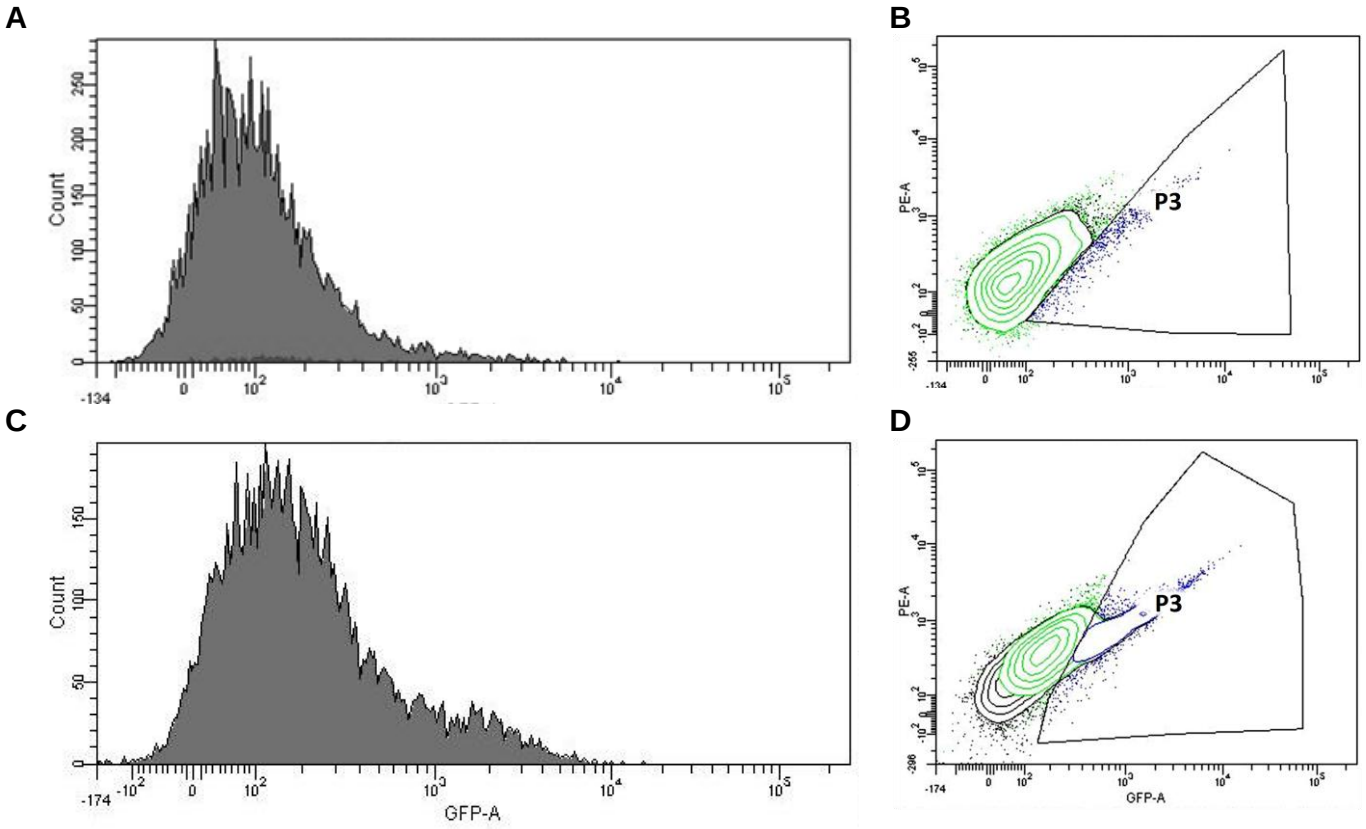


Figure 5.7 Examples of typical FACS plots of individual neurospheres. Neurospheres were selected at random after monolayer NI and subsequent four day neurosphere formation in the presence of EGF and FGF2. The selected neurospheres were then expanded as monolayer cultures before half the culture was analysed and the other half allowed to form daughter neurospheres. Examples show typical histogram (A) and dot plot (B) of a low expressing neurosphere; and typical histogram (C) and dot plot (D) of a high expressing neurosphere. "P3" represents the eGFP-positive, Lmx1a-positive population. (Lmx1a-positive population is gated according to 0.1% Lmx1a-positive cells in wild type neurosphere cultures under the same culture conditions)

5.4.3 Phenotypic diversity in 10 cell neurospheres

Because of the variability in past NI phenotypes, it was thought that by reducing the number of cells during neurosphere formation, NS/NP cultures with a restricted phenotypic fate might be generated. Neurospheres were generated from 1, 2, 5, 10, 50 and 100 cells by plating a fixed number of cells into individual non-adherent wells (from monolayer cultures or PA6 cultures). The neurospheres formed were expanded in the presence of EGF and FGF2. Attempts to generate neurospheres from 1 and 2 cells failed. Neurospheres were generated at low frequency from 5 cell cultures (2 neurospheres in 24 wells). Increasing the number of cells plated into each well resulted in an increase in neurosphere formation from both monolayer and PA6 derived cultures (Figure 5.8), indicating that not all cells resulting from NI were capable of producing neurospheres. Figure 5.9 shows GFP fluorescent images of neurospheres generated from 5, 10 and 50 cells generated either using the regular monolayer protocol or alternatively from co-culture with PA6 stromal cells. Given that 10 cell per well neurospheres formed frequently, this protocol was used for a closer examination of the clonality of monolayer and PA6 derived NS/NP cells.

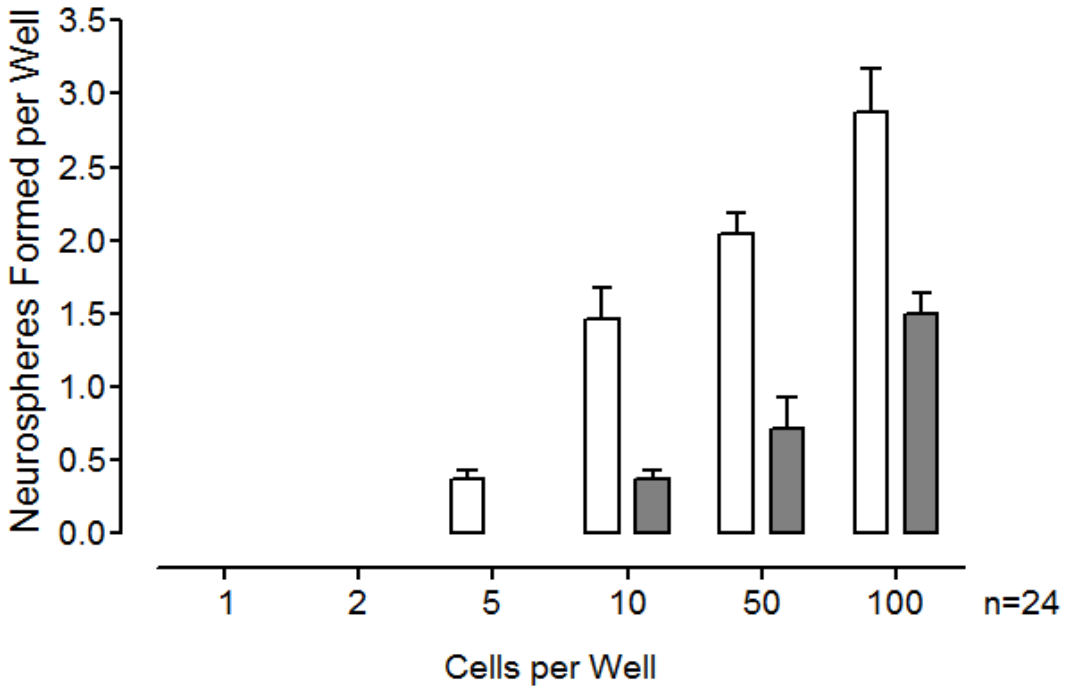


Figure 5.8 Generation of neurospheres. 1, 2, 5, 10 or 100 Monolayer or PA6 derived cells were placed in individual wells to allow neurosphere formation. The average number of neurospheres formed per well was assessed. Neurospheres could only be generated by the aggregation of >5 cells in monolayer cultures and >10 cells in PA6 derived cultures. (n=24)

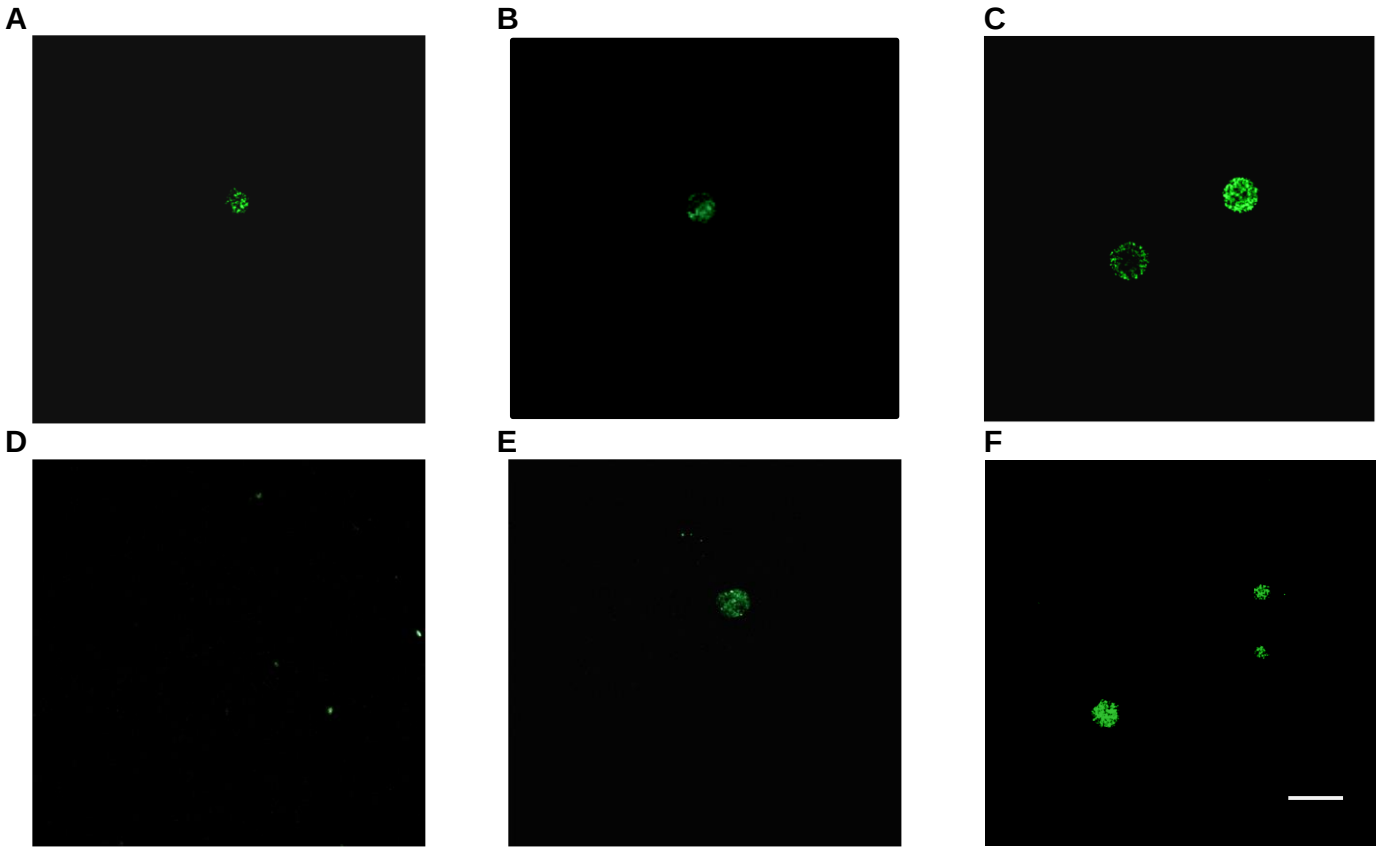


Figure 5.9 Lmx1a-positive neurospheres generated from monolayer (A-C) and PA6 (D-F) NI. Neurospheres formed from 5 (A and D), 10 (B and E) and 50 cells (C and F). Although neurospheres can be generated from 5 cells from monolayer cultures (A), neurospheres cannot be generated from PA6 cultures (D). Generally more than one neurosphere was formed from 50 cells taken from either monolayer or PA6 cultures. (All images are of the same scale; Scale bar 100µm)

To assess the neurosphere forming ability of these NS/NP cells, the initial primary 10 cell neurosphere populations were dissociated without expansion and allowed to reform 10, 50, 100, 1000 or 10,000 cell secondary, tertiary and quaternary neurospheres. The number of neurospheres formed per well was quantitated and the diameter of neurospheres formed was measured. During the initial neurosphere formation step, both monolayer and PA6 derived NS/NP cells readily formed secondary neurospheres, but did not form tertiary and quaternary neurosphere. The incidence of tertiary and quaternary neurospheres formation, even at 10,000 cells per well was low (Figure 5.10 A and C). This suggests that the number of cells with the ability to form neurospheres reduced during passaging in EGF and FGF2.

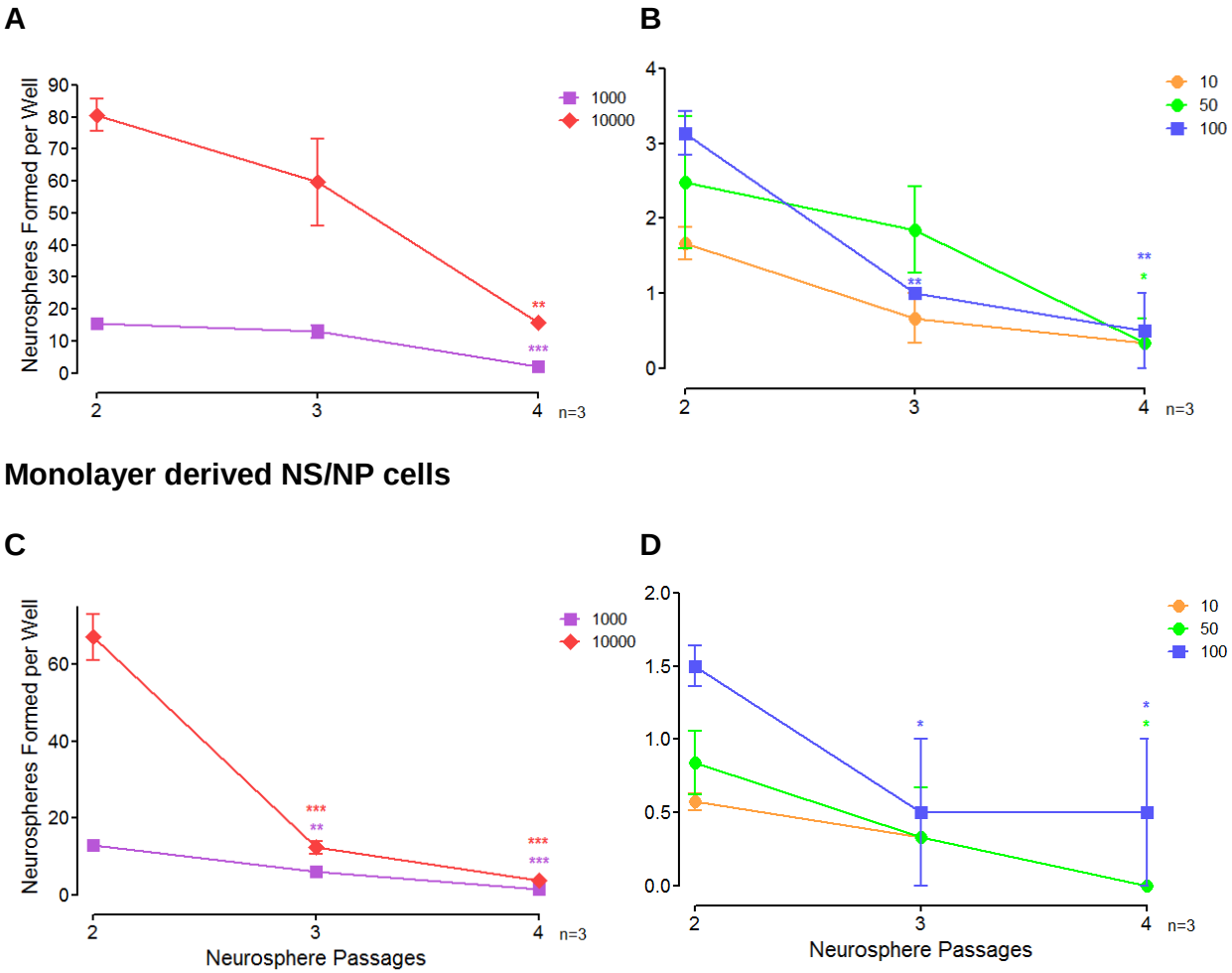


Figure 5.10 The number of neurospheres formed during three neurosphere passages in monolayer (A and B) and PA6 (C and D) derived NS/NP cells. NS/NP cells were suspended at different densities: (A and C) 10000 and 1000 cells; (B and D) 100, 50 and 10 cells to allow primary neurosphere formation. The primary neurospheres were then dissociated and resuspended at the different densities to allow secondary neurosphere formation, followed by tertiary neurosphere formation. Neurosphere numbers decreased dramatically over the passages. (Statistical analysis was by two-way ANOVA by Bonferroni's test compared to passage one neurospheres; n=3; *p<0.05; **p<0.005; ***p<0.001)

Neurosphere sizes over successive passages were also assessed. Monolayer derived neurospheres (1044±36 µm) were considerably larger than PA6 derived neurospheres (381±20 µm; Figure 5.11). In both monolayer and PA6 cultures, neurosphere size

decreased rapidly over the passages (from 1044 to 155 µm in monolayer and 381 to 63 µm in PA6 derived neurospheres). This suggests that during the three rounds of neurosphere formation, the NS/NP cells may be differentiating towards a mature phenotype with a limited neurosphere formation and/or proliferative potential, despite the presence of the EGF and FGF2.

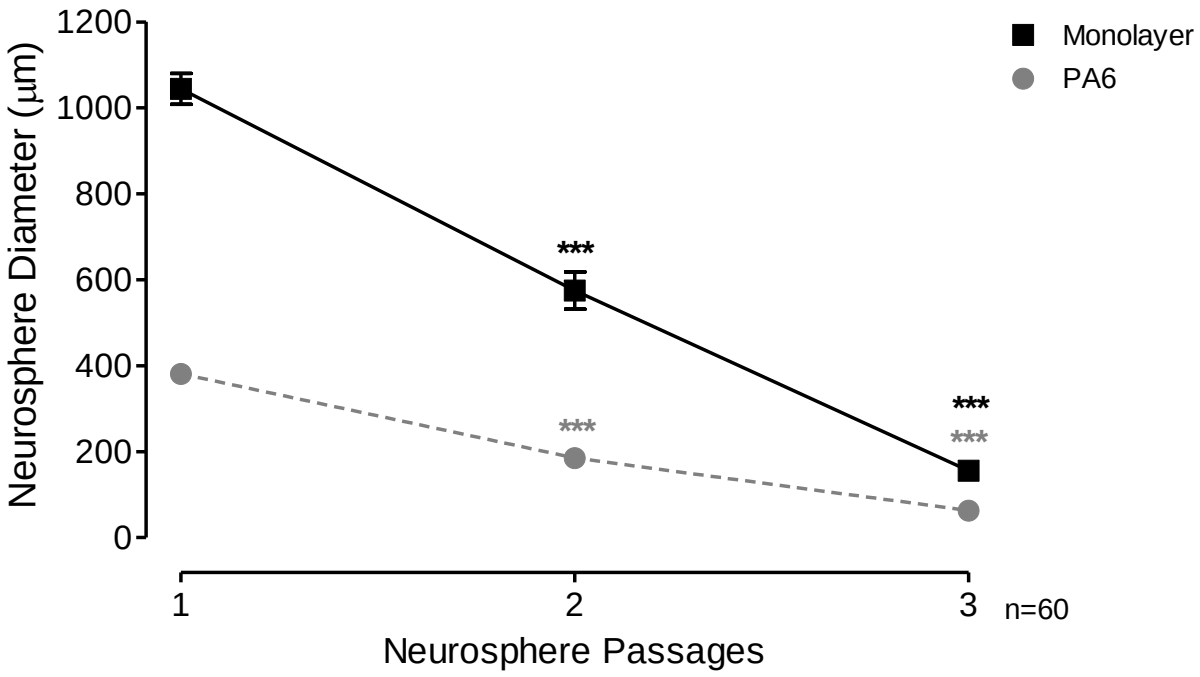


Figure 5.11 10 cell neurospheres derived from both monolayer and PA6 NI were passaged for three passages. A decrease in the diameter of the neurospheres was observed. (Two-way ANOVA by Bonferroni's test compared to passage one neurospheres; n=60; ***p<0.001)

The expression of markers of neural differentiation was examined using immunocytochemistry. Typically, individual neurospheres contained Nestin-positive cells within the centre of the neurospheres (Figure 5.12C) and βIII-tubulin-positive cells predominantly around the edges (Figure 5.12A and B). It was observed that βIII-tubulin-positive cells around the edges of neurospheres co-localised with GABA-positive cells, although small numbers of βIII-tubulin-positive, TH-positive cells can also found within the centre of neurospheres (Figure 5.12A).

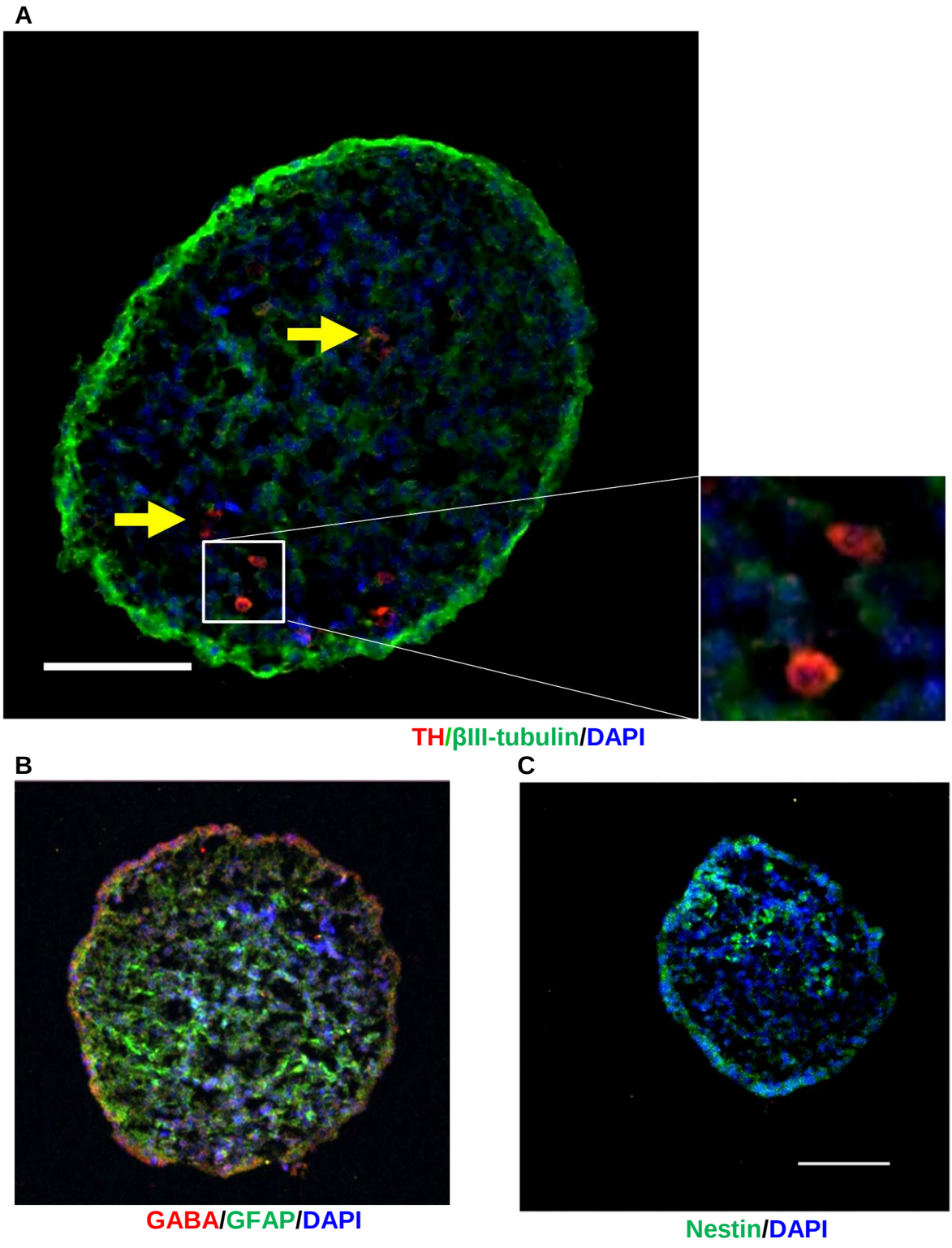


Figure 5.12 Immunocytochemistry of sectioned PA6 (A) and monolayer (B and C) derived neurospheres. Immunocytochemistry indicated the presence of TH-positive cells that co-labelled with βIII-tubulin (A; indicated by yellow arrows), GABA-positive cells at the peripheral of the neurospheres (B) and Nestin-positive cells within the centre of neurospheres (C). (Scale bar 100 µm)

5.4.4 Phenotypic diversity in 10 cell neurospheres

To explore the phenotypic potential of the 10 cell neurospheres after primary, secondary and tertiary neurosphere generation, individual 10 cell neurospheres were allowed to differentiate in the presence of patterning factors (Protocol 1). 20 neurospheres were selected at random from both monolayer and PA6 derived neurospheres. The ability of neurospheres to generate TH-positive neurons reduced significantly over the three rounds of neurosphere formation: Neurospheres that were able to give rise to TH-positive cells decreased from 58% to 5% in monolayer derived cultures; and from 79% to 10% in PA6 derived cultures (Figure 5.13). Figure 5.14 shows the expression levels of TH and GABA in 10 cell neurospheres over the three neurosphere passages and Figure 5.15 shows examples of typical immunocytochemistry images of the differentiated neurospheres. Although both monolayer and PA6 derived neurospheres displayed immunoreactivity to TH and GABA over the passages, they were expressed at different ratios. Cells immunoreactive to GABA decreased in PA6 derived secondary neurospheres, but the proportion of TH-positive cells remained at a similar level to that of primary neurospheres (Figure 5.14B and D). By the third passage, most of the differentiated neurospheres showed a decrease in GABA-positive cells and no TH-positive cells whether these cultures were derived from monolayer or PA6 co-culture (Figure 5.14E and F; Figure 5.15C and F).

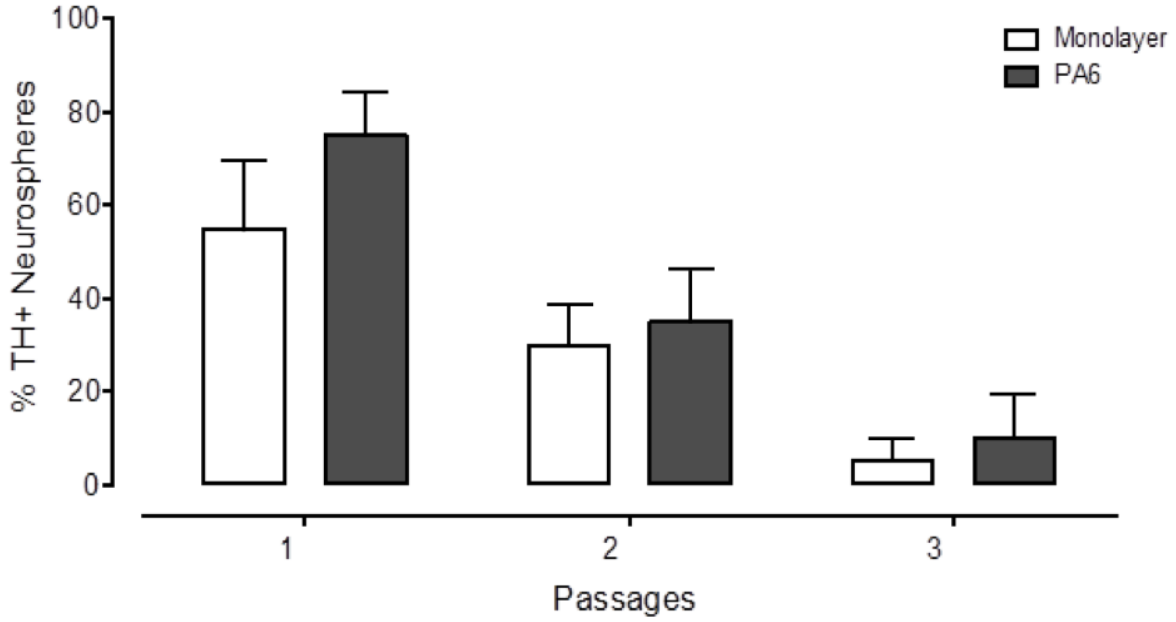


Figure 5.13 Primary, secondary and tertiary neurospheres derived from monolayer and PA6 co-cultures were exposed to patterning factors: 10 ng/mL FGF2, 100 ng/mL FGF8 and 200 ng/mL Shh (6 days), followed by 200 mM L(+)-ascorbic acid and 20 ng/mL BDNF (10 days). The number of neurospheres able to generate TH-positive cells decreased rapidly after each passage.

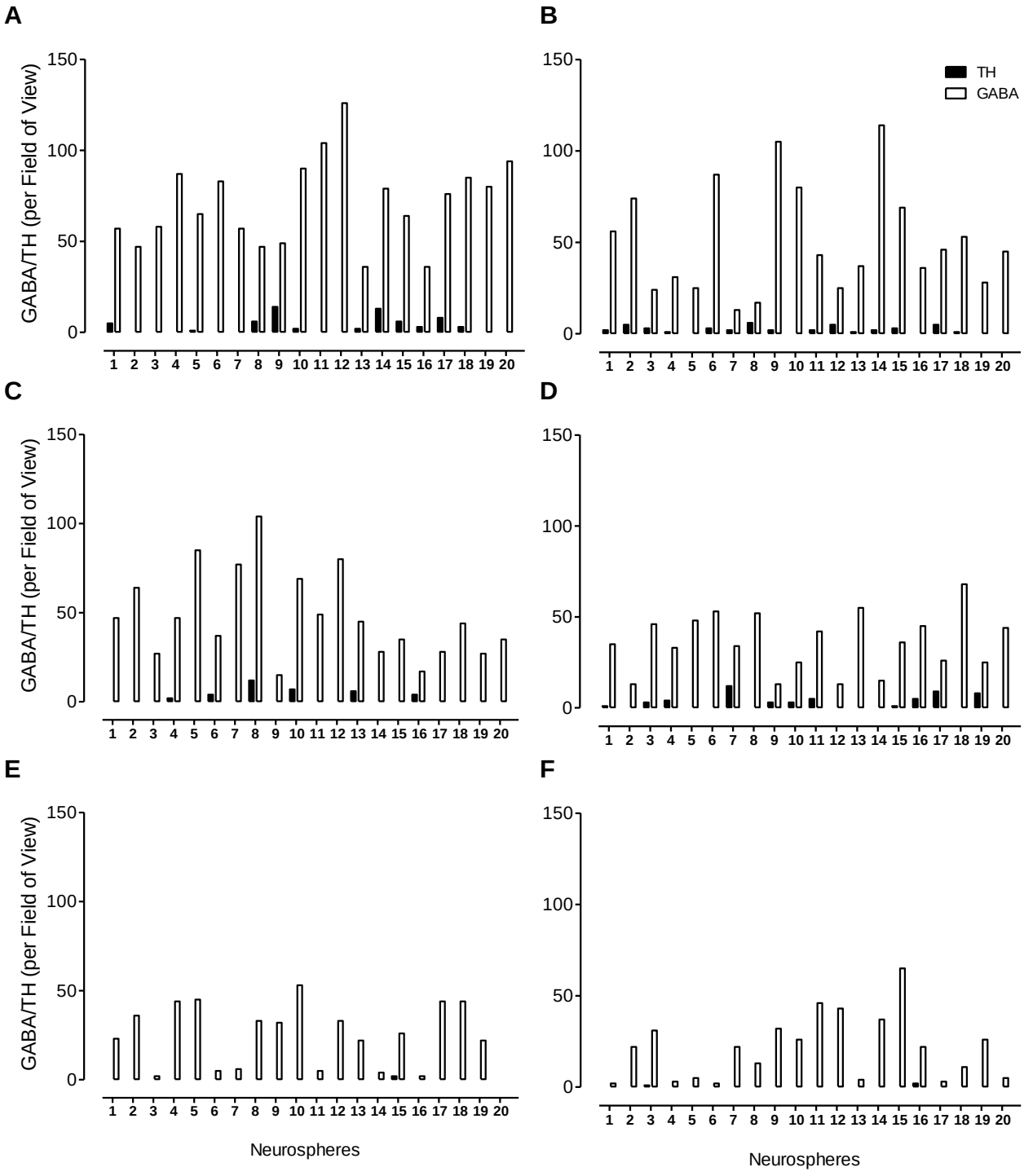


Figure 5.14 Differentiated 10 cell neurospheres derived from monolayer (A, C and E) and PA6 co-culture (B, D and F). 20 Primary (A and B), secondary (C and D) and tertiary (E and F) neurospheres were differentiated in the presence of 10 ng/mL FGF2, 100 ng/mL FGF8 and 200 ng/mL Shh (6 days), followed by 200 mM L(+)-ascorbic acid and 20 ng/mL BDNF (10 days). The number of cells immunoreactive to TH and GABA were counted per neurosphere per field of view.

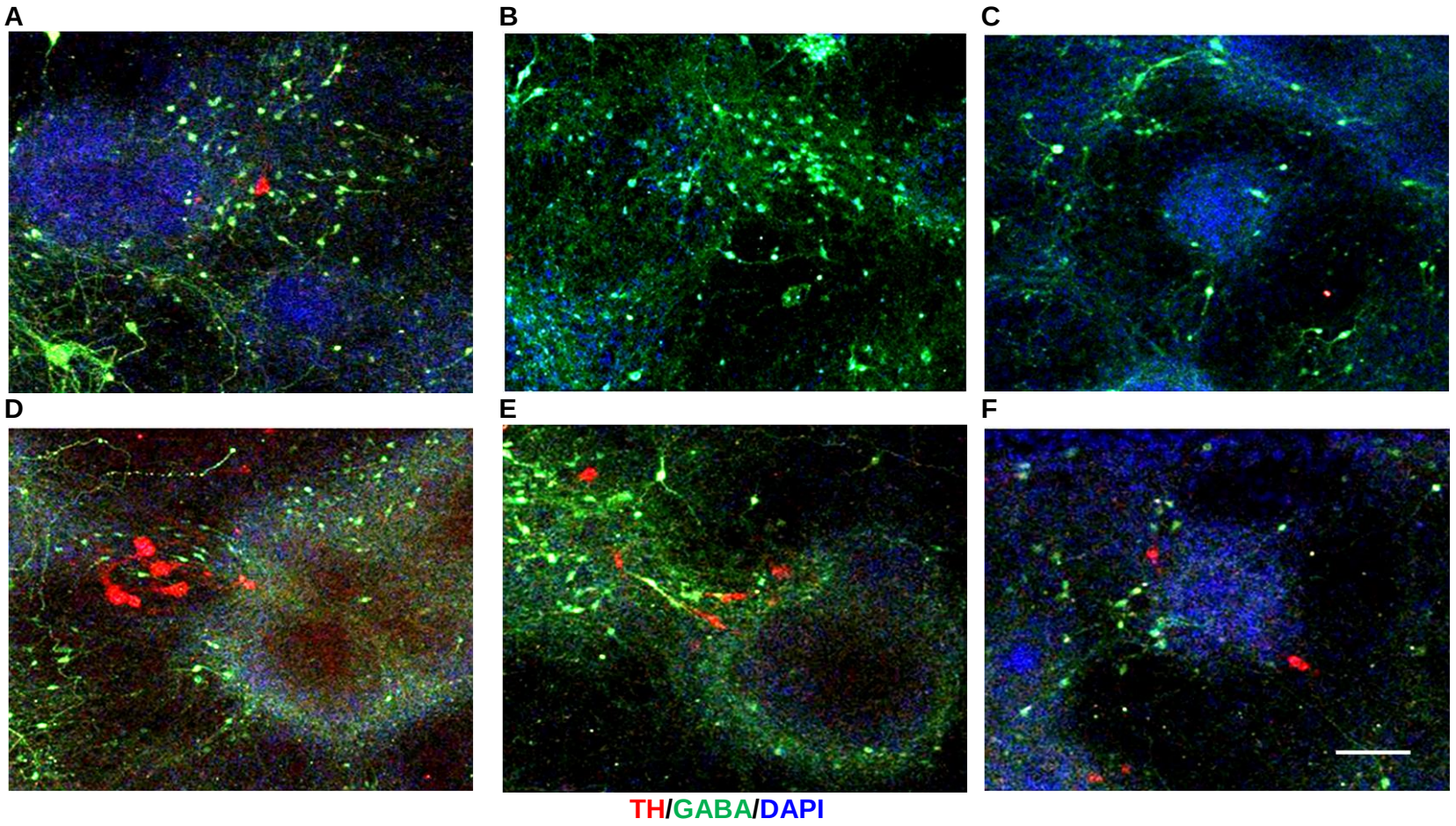


Figure 5.15 Typical immunocytochemistry of differentiated primary (A and D), secondary (B and E), tertiary (C and F) monolayer (A-C) and PA6 (D-F) derived neurospheres. Differentiated 10 cell neurospheres showed different proportions of GABA and TH-positive cells. (All images are of the same scale; Scale bar 100 µm)

5.5 Discussion

Recent studies have shown that neurosphere cultures of fetal mesencephalic tissue can be used as a source of dopaminergic neurons for transplantation into patients with Parkinson's disease (Dunnett, Bjorklund, & Lindvall, 2001; Lindvall & Bjorklund, 2004). This work supported the use of NS cells as a source of dopaminergic neurons in models of Parkinson's disease (Nikkhah et al., 1994; Winkler, Kirik, & Bjorklund, 2005). Pluripotent hES cells in particular have the potential to be used in cell replacement therapies but suffer from ethical issues relating to the destruction of embryos. Therefore it would be desirable to obtain a standardised and readily expandable source of dopaminergic progenitors for transplantation. Methods for stabilisation of NS/NP cells have not yet been established, and their multipotency remains an issue. It is possible that even at the NS/NP cell stage cells are already phenotypically restricted. It was hypothesised that neurosphere cultures might allow isolation of the clonal propagation and expansion of committed dopaminergic or GABAergic progenitors. These results show that during primary neurosphere formation, individual neurospheres already exhibit different degrees of neurogenic and gliogenic potential when maintained as monolayer cultures in the presence of EGF and FGF2. Once EGF is withdrawn and patterning factors are added, these neurospheres showed a greater diversity in phenotypic potential. Consistent with this finding is evidence that primary neurosphere cultures generate early projection neurons (Okano & Temple, 2009). The differences observed across different expanded neurospheres cultures are perhaps due to the complex structures of neurospheres, which even at a very early stage, appear made up of NS, NP and precursor neural and glial cells types, indicative of different stages of neurodevelopment (Barraud, Thompson, Kirik, Bjorklund, & Parmar, 2005). The neurospheres, therefore represent a mixed population of NS/NP cells at different stages of development that maybe dominated by the NS/NP cell type that possess the most rapid rate of proliferation.

In this study, my aim was to generate neurospheres and establish whether the NS cells could be isolated and propagated over successive neurosphere generation. The transcription factor Lmx1a has been shown to be a key regulator of midbrain dopaminergic as well as forebrain development (Chizhikov et al., 2010; W. Lin et al., 2009; Nakatani et al., 2010). The sensitive AMP reporter was used to analyse individual neurospheres as well as their progeny during secondary neurosphere formation. During the first round of neurosphere formation, variability in Lmx1a expression was observed. Secondary neurosphere formation also showed variability in Lmx1a expression indicating that high Lmx1a expressing neurospheres were made up of a mixture of cells capable of forming both high and low expressing neurospheres. Primary, secondary and tertiary neurosphere formation was also carried out using the GFP reporter for visualisation purposes. The apparent difference between expression levels using the two reporters is reflective of eGFP being less sensitive than AMP reporter. Variability in Lmx1a expression was also observed during primary and secondary neurosphere formation. However, during tertiary neurosphere formation, Lmx1a expression was almost non-existent. This may be due to a decrease in dopaminergic and GABAergic progenitors, since it was found that neurogenic capacity of the neurosphere expanded cells decline after extended numbers of passages (Fricker et al., 1999).

In an effort to minimise the heterogeneity of neurosphere cultures, the ability of both monolayer and PA6 derived NS/NP cells to form single cell cultures was assessed by allowing neurosphere formation using 10 cells or less. However, it was not possible for both monolayer or PA6 derived NS/NP cells. 10 or more cells were required for neurosphere formation; indicating that it is possible that only 1 in 10 cells within these cultures is able to survive low-density stress and have neurosphere forming capabilities through clonal self-renewal. During neurosphere propagation, the number of cells that

were able to form neurospheres decreased over passage in the presence of EGF and FGF2 (Figure 5.10). In addition, there was as a rapid decrease in neurosphere diameter. This indicates that in the proportion of “stem” cells was also rapidly decreasing with each successive round of neurosphere formation as the diameter of neurospheres is often associated with the presence of “stem” cells (Louis et al., 2008). Terminal differentiation of 10 cell neurospheres, over the three rounds of neurosphere formation, showed a decrease in the number of GABA-positive as well as TH-positive neurons although in very small numbers. It is not clear whether this is because progenitors with capacity to form neurons are outnumbered by glial progenitors and thus progressively lost, or whether the intrinsic properties of the cells within the neurospheres change over time. It has been demonstrated that neurosphere cultures differentiates mainly into glial cells, rather than neurons when grafted into the striatum (Hitoshi, Alexson, et al., 2002; Jensen, Bjorklund, & Parmar, 2004; Klein, Butt, Machold, Johnson, & Fishell, 2005). However, PA6 derived 10 cell neurospheres in contrast to monolayer derived neurospheres, continued to give rise to TH-positive cells even after the third round of neurosphere formation. This is consistent with the findings that monolayer cultures are a poor source of midbrain dopaminergic neurons (Gaspard et al., 2008; Konstantoulas et al., 2010). Reports have indicated that neurosphere culture systems appear sensitive to variations in cell density (Tropepe et al., 1999a), intrinsic and extrinsic cues (Hack et al., 2004), different media formulations (Arsenijevic et al., 2001; Caldwell et al., 2001; Irvin, Dhaka, Hicks, Weinmaster, & Kornblum, 2003) and both the method and frequency of passaging (Caldwell et al., 2001).

5.6 Conclusion

In this chapter I have shown that both monolayer and PA6 derived NS/NP cells can be maintained as neurosphere cultures. However, neurosphere formation in the presence of EGF and FGF2 is a poor method in the maintenance of Lmx1a-positive NS/NP cells since the proliferative ability and neurosphere formation capability of these cells decreases dramatically over three passages. Although neurosphere culture system has been shown to be useful for biological studies of developmental processes (Alexson, Hitoshi, Coles, Bernstein, & van der Kooy, 2006a; Klein et al., 2005), the heterogeneous characteristics of neurospheres even at the 10 cell level, the progressive loss of neurogenic potential with passaging and the poor yield of neurons after exposure to patterning cues shows this culture method is inadequate for the large scale generation of midbrain dopaminergic neurons.

Chapter Six

6. General Discussion

This work investigated the generation of ES cell derived NS/NP cells, NS/NP cell maintenance and the isolation of phenotypic specific progenitors. The neurogenic, phenotypic potential of NS/NP cells; their stability during long term monolayer culture; and their ability to generate clonal, phenotypically distinct progenitors was investigated using reporter cell lines, qPCR and immunocytochemistry. In this study, Lmx1a reporter cell lines were used to track the presence of dopaminergic and GABAergic progenitors during different lengths of NI, neurosphere formation and long-term monolayer cultures; qPCR was used to investigate the expression of regional specific genes during NS/NP cell maintenance; and immunocytochemistry was used to track the expression various

proteins during neural differentiation. This work contributes to the characterisation of *in vitro* derived NS/NP cells, their phenotypic potential, long term propagation and clongenic potential over time that need further investigation.

6.1 Neural Induction

In both *in vivo* and *in vitro* systems, NI is the first decisive step in neurogenesis. Generally, published protocols for the differentiation of ES cells into NS/NP cells use periods of NI anywhere between 5 to 7 days in length (Barberi et al., 2003; Kawasaki et al., 2000; Khaira et al., 2011; Lee et al., 2000; Raye et al., 2007; Wichterle et al., 2002). Since NI is the first step during differentiation of ES cells into neurons, it could potentially to lead to downstream effects such as differences in the responsiveness of cells to the addition of patterning factors. Factors such as Shh and FGF8 have often been applied after the 5-7 day period of NI, though recent protocols favour early addition of patterning factors during NI (Fasano, Chambers, Lee, Tomishima, & Studer, 2010; Kriks et al., 2011).

In the first experimental chapter, the effect of different days of NI (days 4, 7 and 10) on the multipotency of NS/NP cells was explored. The Conti et al. (2005) method for derivation of NS cells from mES cells utilised a 7 day monolayer NI followed by neurosphere formation, expansion and propagation in the presence of EGF and FGF2. Much emphasis has been placed on the generation of neuroepithelial progeny from pluripotent cell types (Elkabetz et al., 2008b; Roy et al., 2006). In this study it was hoped that by selecting cells at earlier and later stages, it would be possible to optimise the timespan of NI. Day 10 NI cultures expressed the NS and early neural markers Nestin, Musashi1 and βIII-tubulin; and did not express the mesodermal marker Brachyury or the

endodermal marker α1-fetoprotein. Day 10 cultures also expressed the dopaminergic and GABAergic progenitor marker Lmx1a at a higher level compared to day 4 and 7 cultures. At the neurosphere stage, 10 day neurospheres expressed the rosette NS marker ZO1 and Nestin expression adopted a rosette NS cell-like morphology, surrounding ZO1-positive centres. It is possible that day 10 NI cultures are heterogeneous mixtures of NS and NP cells at different stages of differentiation. When neurospheres from 10 day NI were expanded in the presence of EGF and FGF2, the culture represented a mixture of NS/NP cells at different stages of development. This was demonstrated by the presence of βIII-tubulin-positive early neurons within the NS/NP cultures. Once EGF was withdrawn and the NS/NP cells were exposed to patterning factors, some gave rise to dopaminergic, serotonergic and GABAergic neurons. It is possible that NS/NP cells derived from day 10 NI cultures may contain more committed NP and precursor cells that are able to respond more readily to specific growth factors and differentiate efficiently into different neuronal phenotypes established in other laboratories (Drury-Stewart et al., 2013; Guan et al., 2013).

6.2 Monolayer Maintenance

NS/NP cell stability during culture *in vitro* is another important factor in the utilisation of these cells in practice since it would be more desirable to commence differentiation from an NS/NP cell source that is able to self-renew as well as differentiate into the desired cell types. There are many culture methods for NS/NP cells, the most common being monolayer culture in the presence of both EGF and FGF2 (Conti et al., 2005), or FGF2 alone (Reynolds & Weiss, 1992). In the second experimental chapter, the stability of 10 day NS/NP cells during 25 passages of monolayer maintenance (in the presence of EGF and FGF2) was explored. Enrichment in Lmx1a expression was observed in monolayer derived cultures before a rapid decrease; but a gradual decrease in Lmx1a expression

was observed in PA6 derived cultures. This indicated that EGF and FGF2 were not able to maintain Lmx1a-positive NS/NP cells in monolayer cultures. This loss of Lmx1a expression was also demonstrated following replating of FACS sorted homogenous Lmx1a-positive cultures. A rapid decrease in Lmx1a expression was observed over only 4 monolayer passages in the presence of EGF and FGF2. These NS/NP cells also expressed the stem cell marker ALDH up to passage 10-12 indicating the presence of highly proliferative “stem” cells (Hynes, Poulsen, et al., 1995; Wallen et al., 1999; Ye et al., 1998). Although monolayer and PA6 derived NS/NP cells could be maintained under the same conditions, monolayer derived NS/NP cells generate predominantly SatB2 and GABA-positive, forebrain GABAergic phenotype with small proportions of FoxA2-negative, TH-positive dopaminergic neurons, most likely forebrain specific (A. Bjorklund & Dunnett, 2007). PA6 derived NS/NP cells on the other hand generated more TH-positive neurons that are also FoxA2-positive indicating a midbrain phenotype. However, prolonged monolayer propagation in the presence of EGF and FGF2 should a bias towards GABA-positive neurons. With increase in passages numbers, the qPCR data NS/NP cultures also supported this finding, a more pronounced upregulation in the GABAergic neuron marker GAD1 as well as and BMP2 was observed in the monolayer derived NS/NP cells. Although the number of βIII-tubulin-positive neurons generated by both monolayer and PA6 derived NS/NP cells remained stable over 10 passages, the ability of these NS/NP cells to produce catecholaminergic neurons over was greatly compromised with both NS/NP cultures showed a bias towards the GABAergic phenotype. This supports the recent finding that NS cell self-renewal is regulated by Notch and EGF activated signalling pathways (Aguirre et al., 2010; Alexson, Hitoshi, Coles, Bernstein, & van der Kooy, 2006b). Independently, EGF has been shown to modulate proliferation and migration of transit-amplifying cells that generate largely generate astrocytes and GABAergic neurons (Mira et al., 2010; Scheffler et al., 2005).

6.3 Neurosphere Formation

With the desire to generate enriched populations of phenotypically distinct neurons for replacement therapies and models for drug discovery, much effort have been made in refining differentiation protocols to generate homogenous neuronal phenotypes such as the generation of GABAergic, glutamatergic, cholinergic phenotypes (Bain, Kitchens, Yao, Huettner, & Gottlieb, 1995; Bibel et al., 2007; Bibel et al., 2004; Brill et al., 2009; Finley, Kulkarni, & Huettner, 1996; Spiliotopoulos et al., 2009; Strubing et al., 1995), and in particular dopaminergic neurons for the treatment of Parkinson's disease (Barberi et al., 2003; Kawasaki et al., 2000; Morizane et al., 2002). In the third experimental chapter, the generation of clonal NS/NP cultures from PA6 and monolayer derived NS/NP cells was explored. It was found that individual neurosphere expanded cultures possesses different degrees of neurogenic and gliogenic potential was defined by immunoreactivity to βIII-tubulin and GFAP. Terminal differentiation of individual neurospheres showed further diversity in neurogenic potential with the evidence of neurospheres that generated different ratios of TH and GABA-positive cells and some only generate GABA-positive cells. However, direct differentiation from neurospheres gave rise to very small numbers of TH-positive neurons. Lmx1a-AMP and GFP reporter cells were used to track Lmx1a expression during primary, secondary and tertiary neurosphere formation. It was found that individual neurospheres cultures were heterogeneous in nature and expressed Lmx1a at different levels. It was found that neurospheres that expresses Lmx1a at high levels are made up of cells that are able to form both high and low expressing neurospheres. Both primary and secondary neurosphere formation showed diversity in Lmx1a expression, tertiary neurosphere formation showed a reduced Lmx1a expression. The ability of these NS/NP cells to generate clonal cultures was investigated. It was found that 10 cells are required for the generation of monolater and PA6 derived neurospheres and the incidence of neurosphere formation rapidly decreased with each

neurosphere passage. The neurosphere size also decreased. This indicates that in the proportion of “stem” cells also rapidly decreased with each successive round of neurosphere formation (Louis et al., 2008; Tropepe et al., 1999a). The ability of these neurospheres to generate of TH and GABA-positive neurons was then assessed. Although individual neurospheres showed different proportions of TH and GABA-positive cells, an overall decrease in both TH and GABA-positive cells was observed over the passages, this may be due to a decrease in neurogenic potential or an increase in glial cell types. EGF and FGF2 have shown to promote NS cell proliferation in both early and late stage NS cells and together they also promote β1-integrin expression, important in cell attachment and neurosphere formation (Leone et al., 2005; Suzuki, Yanagisawa, Yagi, Nakatani, & Yu, 2010; Tropepe et al., 1999b). A decrease in neurosphere size over the three successive passages may be due to differentiation into mature cell types that express lower levels of β1-integrin (Campos et al., 2004; Hall, Lathia, Miller, Caldwell, & Ffrench-Constant, 2006)

6.4 Future Directions

It would be ideal to characterise and isolate the different types of NS/NP cells present in the cultures such as NEP, rNS or rG cells to generate homogenous cultures to further investigate the differentiation potential of these cells. The NEP, rNS and rG specific markers Pax6, FoxG1, Forse1, N-Cadherin, ZO1, BLBP, Vimentin, GLAST, RC2 and Prominin1 could be used to identify these individual NS/NP phenotypes. By characterising the NS/NP cells in the cultures it may be possible to isolate NS/NP cells of appropriate lineage commitment and direct them towards a desired phenotype. Early NS/NP cells are likely to generate neurons but not glial cells, whereas later and adult NS/NP cells are able to generate both neurons and glia. However, these late NS/NP cells may not be able to generate a diversity of neurons: forebrain cholinergic neurons,

midbrain dopaminergic neurons, and spinal motor neurons. In addition, NS cells maintain, to some extent, the regional identity of their origin such as the difference between monolayer and PA6 cultures. Therefore it would be advantageous to isolate the different NS/NP cell types through FACS sorting. Furthermore, use of growth factors and small molecules such as LIF, Notch ligands, EGF, FGF2 will need to be optimised for the maintenance of these NS/NP cells to stabilise these cultures, it would then be possible to store stocks of cells for clinical use. At present the standard conditions for propagation in EGF and FGF2 do not stabilise the cells for unlimited expansion.

More specific markers for GABAergic and dopaminergic progenitor are required for the isolation of these progenitors: Lmx1a and at least one more regional specific marker would allow the isolation of enriched NS/NP populations possibly through gene expression analysis, and identification of selectively expressed surface markers.

It would be necessary to demonstrate that the GABA-positive and TH-positive cells are express all the markers present in CNS such as dopamine-β-hydroxylase, dopamine transporter, GABA transporters, glutamate decarboxylase and vesicular GABA transporter to ensure they are deemed equivalent to those found in the adult brain. Functional studies will be required to show if these TH and GABA-positive neurons are capable of dopamine or GABA uptake and release so they can be used in cell therapy to replace damaged or lost neurons. Furthermore, functional experiment experiments are required to ensure these differentiated neurons are able to respond to extracellular signalling in a similar fashion to those found *in vivo*. Calcium imaging could be used to examine whether NS/NP cell derived TH and GABA-positive cells are able to respond to

neurotransmitters and KCl and electrophysiological experiments could provide information on the electrical activity of these differentiated neurons.

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Appendix

A1 Generation of Reporter ES cell lines

A1.1 Targeting Vectors

Lmx1a-AMP-IRES-eGFP-FneoF (Lmx1a-AMP-IRES-eGFP) targeting vectors were designed to replace exon 1 of their respective genes with the gene for AMP (β-lactamase), enhanced green fluorescent protein (eGFP), separated by an internal ribosomal entry site (IRES), and a neomycin (neo) gene cassette flanked by flippase recognition target (Frt) sites. A Rosa26-floxed-stop-mCherry (Rosa26-mCherry) targeting vector, which contained a neo gene cassette flanked by Cre recombinase sites followed by the fluorescent gene mCherry, was targeted to the Rosa26 locus 1.

A1.2 Electroporation

All reagents were purchased from Invitrogen unless stated otherwise. For genetic modification E14Tg2a ES cells (ATCC) were grown in high glucose Dulbecco's Modified Eagle's Medium containing Glutamax™, supplemented with 15% ES cell qualified foetal bovine serum, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, 100 mM β-mercaptoethanol, 100 U/mL of penicillin, 100 mg/ml of streptomycin and 2i 2. Approximately $8x10^6$ cells were electroporated in Opti-MEM® medium with 25 µg of linearised vector at 240 V, 500 µF in a GenePulser XCell electroporator (Biorad). Cells were plated onto mitomycin C (Sigma) treated neomycin-resistant MEF feeder cells (Cell Biolabs, Inc.) in 10 cm diameter dishes. The cells were placed under G418 selection (200 µg/mL; Merk) after 2 days and the medium changed every 1-2 days. After 8-10 days colonies were picked into duplicate 96-well plates. The Rosa26-mCherry/Lmx1a-AMP-IRES-eGFP double knock-in cell line was made by first targeting the Rosa26-mCherry vector, removal of the Cre flanked neo cassette (see below), and subsequent targeting using the Lmx1a-AMP-IRES-eGFP targeting vector. The Cre flanked neo cassette was removed by electroporating (as above) 10-40 µg of uncut pEFBOS ‑ creIRESPuro vector 3 into the Rosa26 ‑ mCherry cell line. Plating and culture conditions were as above. After 24 hr 1 µg/mL puromycin (InvivoGen) was added to the media and the cells incubated for 48 hr. After 8-10 days colonies were picked into duplicate plates, one containing G418 the other without. Cells that died in the plate containing G418 were deemed to be negative for the selection cassette.

A1.3 Validation by Southern blotting

Genomic DNA was isolated essentially as described by 4. Pooled colonies were initially screened by PCR for correct integration, using the Expand Long Template PCR System (Roche). Clones that were positive were further screened by Southern blotting. Digested genomic DNA was electrophoresed, transferred to Hybond-N+ membranes (GE Healthcare) and hybridised with external probes complimentary to sequences upstream of the 5' homology arm of the vector and downstream of the 3' homology arm, as well as an internal (neo) probe. Probes were labelled with α-32P-dATP (Perkin Elmer) using a DECAprime™ II kit (Ambion) and hybridised in ULTRAhyb™ hybridisation buffer (Ambion). The signal was detected using Kodak BioMax MS film in a BioMax Cassette with BioMax TranScreen HE (Sigma).

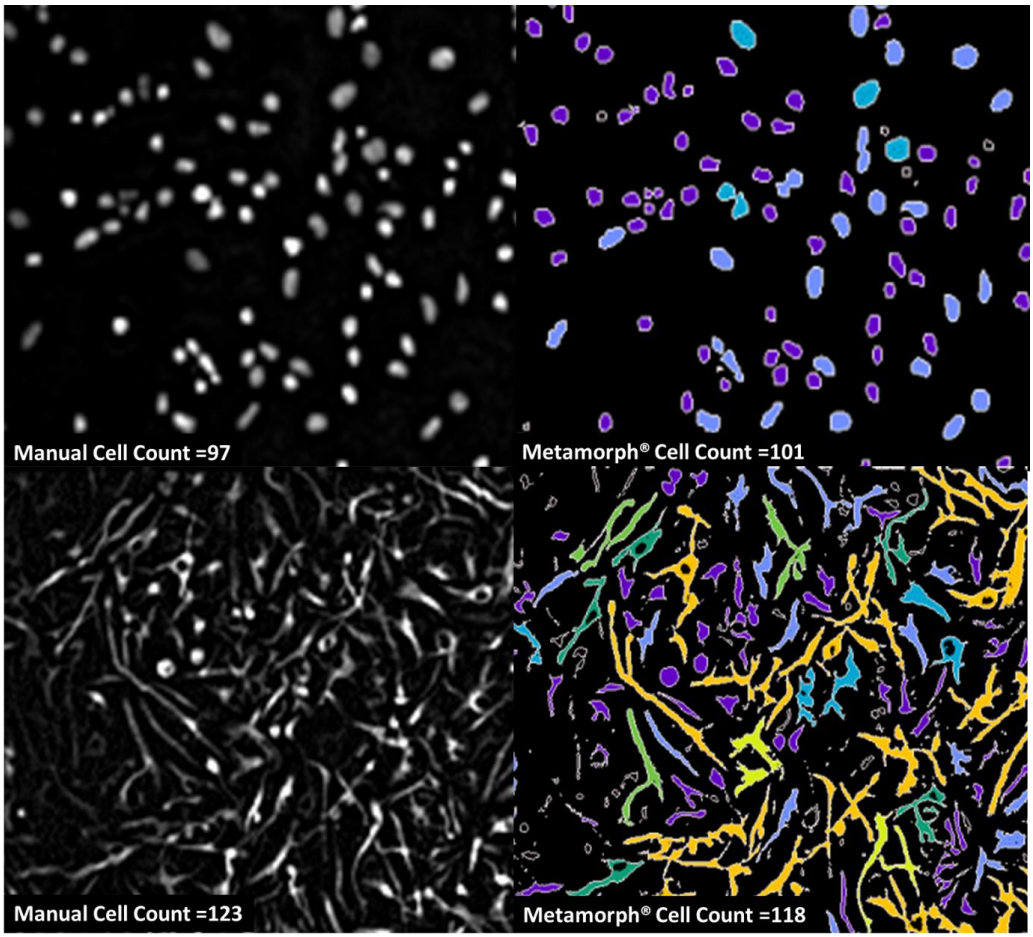
A1.4 Immunocytochemistry

Cells were fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton-X, blocked with 5% donkey serum and stained with the following primary antibodies: rabbit anti‐tyrosine hydroxylase (TH) (1:200, Millipore), mouse anti-FoxA2 (1:4000, DHSB), mouse anti-βIII-tubulin (1:200, Covance), mouse anti-Nestin (1:200, Millipore), mouse anti-glial fibrillary acid protein (GFAP) (1:200, Millipore), rabbit anti-GABA (1:1000, Sigma), mouse anti-SatB2 (1:40, Abcam), mouse anti-GAD67 (1:1000, Millipore), rabbit anti-ZO1 (1:100, Invitrogen), rabbit anti-TBR1 (1:400, Abcam), rabbit anti-Wnt1 (1:200, Thermo-Fisher,), rabbit anti-p73 (1:500; Calbiochem/Merk). After labelling with primary antibodies cells were incubated with secondary antibodies (all purchased from Invitrogen) Alexa Fluor 405 goat anti-mouse (1:200), Alexa Fluor 405 goat anti-rabbit (1:200), Alexa Fluor 488 donkey anti-mouse (1:500), Alexa Fluor 567 donkey anti-mouse (1:500), Alexa Fluor 567 donkey anti-rabbit (1:500), Alexa Fluor 594 donkey anti-rabbit (1:500), Alexa Fluor 637 donkey anti-mouse (1:200) or Alexa Fluor 637 donkey anti-rabbit (1:200) and afterwards exposed to nuclear counter stains TOPRO3 (1 µM, Invitrogen) or DAPI (0.5 µg/mL, Sigma).

A1.5 RNA extraction and quantitative‐PCR

Total RNA was extracted from >1 million cells per sample using the RNeasy Mini Kit (Qiagen). RNA was quantified using a Nanodrop® ND-1000 (Thermo Scientific). For generation of cDNA, 100 ng of RNA was used in 10 µl reaction mix together with oligodTs (SuperScript III First-Strand Synthesis kit, Invitrogen, Melbourne, Australia) according to the manufacturer's specifications. All quantitative PCR reactions were performed on 3 biological

replicates in triplicate using SYBR Green I Master Mix (Roche) and the Light Cycler 480 System (Roche). General PCR conditions were 95°C for 10 minutes followed by 50 cycles (95°C for 10s, 60°C for 30s). Specificity of the used primer pairs was verified by DNA sequencing of products (Micromon). Data analysis was performed using the $2^{-\Delta\Delta CT}$ method. Relative quantification values were obtained by standardising C_T values of each target gene to averaged C_T values of two housekeeping genes (β-actin and TATA box binding protein).



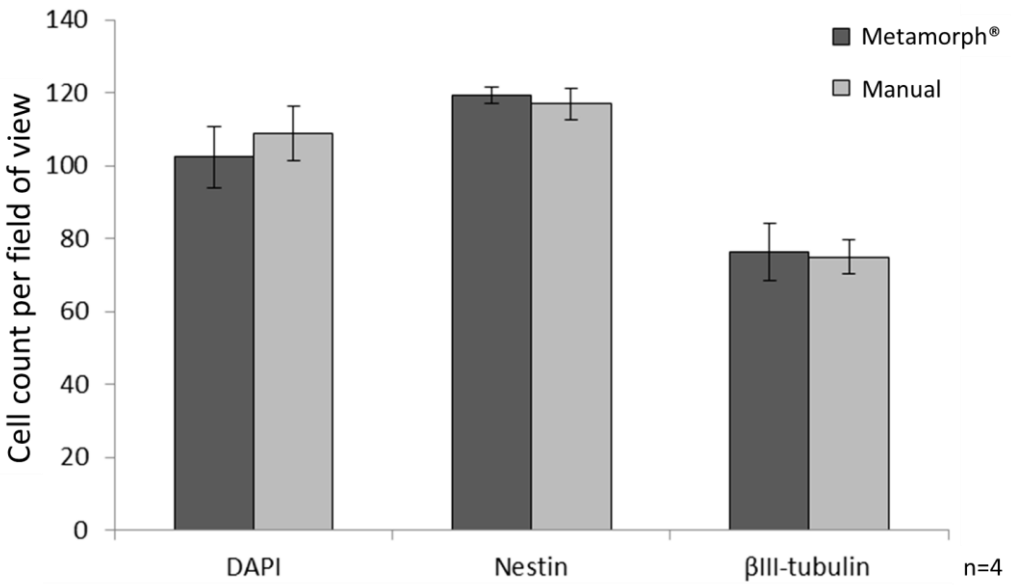


Figure A1 Comparison between manual cell count and Metamorph® cell count. No significant difference was found between the two methods (n=4).

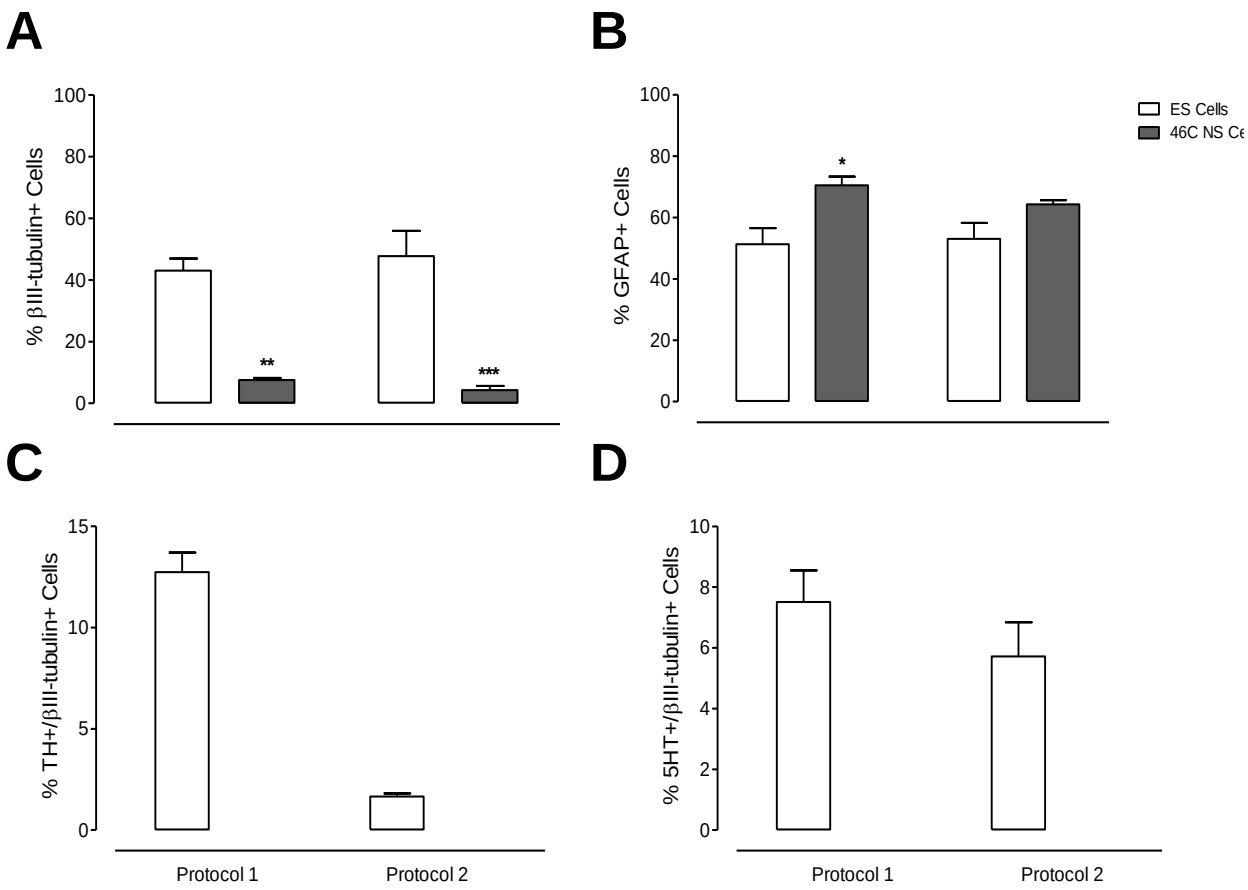
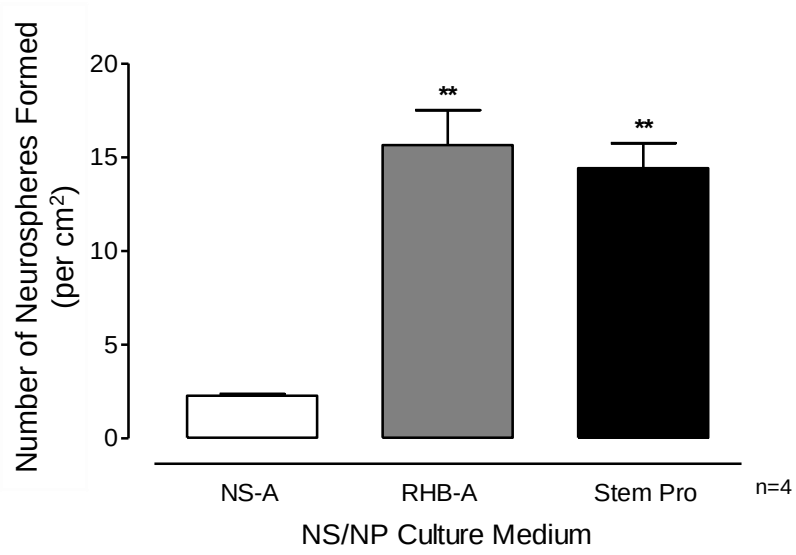


Figure A2 ES and the previously Sox1 positive 46 C NS were subjected to two protocols and examined immunocytochemical expression of the neuronal marker (A) βIII-tubulin, astrocyte marker (B) GFAP; catecholaminergic markers (C and D) TH and 5HT expressed as a percentage of βIII-tubulin. Protocol 1; 200 ng/mL Shh, 10 ng/mL FGF2, 100 ng/mL FGF8 (6 days): protocol 2; 200 ng/mL Shh, 10 ng/mL FGF2, 100 ng/mL FGF4 (3 days), then the FGF4 was replaced with 100 ng/mL FGF8 (3 days). Both patterning protocols were followed by incubation in N2B27 medium containing 200 mM L(+)-ascorbic acid and 20 ng/mL BDNF for a further 10 days. The differentiation of ES cells included an additional 7 days of NI prior to growth factor addition. (n=3; *p<0.05; ***p<0.001, two-way ANOVA followed by Bonferroni's test comparing days 7 and 10 to respective day 4 cultures)



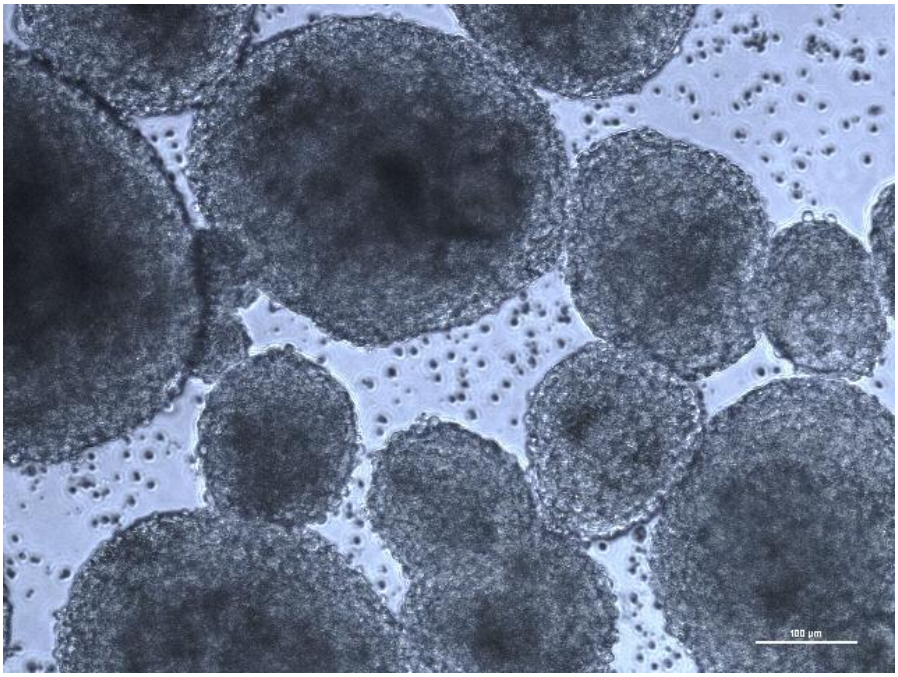


Figure A3 NS/NP cells derived from 10 days of monolayer NI was allowed to form neurospheres in the presence of three different types of culture media: NS-A (Euroclone; Conti et al. 2005), RHB-A (StemCells, Inc) and Stem Pro (Invitrogen), supplemented with EGF and FGF2. (n=4, **p<0.005, one-way ANOVA followed by post hoc Dunnett's test comparing RHB-A and Stem Pro to NS-A medium) and typical brightfield image of neurospheres after 4 days of neurosphere formation in the presence of Stem Pro, supplemented with FGF2 and EGF (Scale bar 100µm).

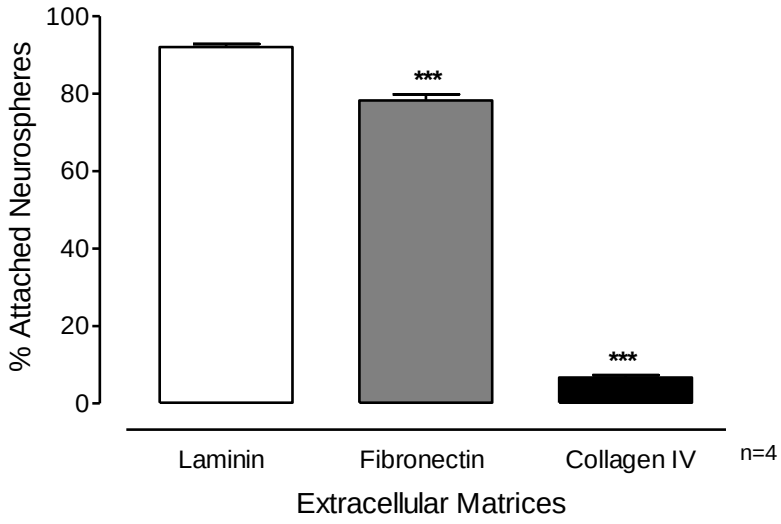


Figure A4 Three different types of ECM: Laminin, Fibronectin and Collagen IV, was used for the monolayer expansion of neurospheres. 20 neurospheres were plated on these ECMs and the number of neurospheres that attached and expanded was counted and expressed as a percentage of the plated neurospheres.(n=4, ***p<0.001, one-way ANOVA followed by post hoc Dunnett's test comparing Fibronectin and Collagen IV to Laminin).

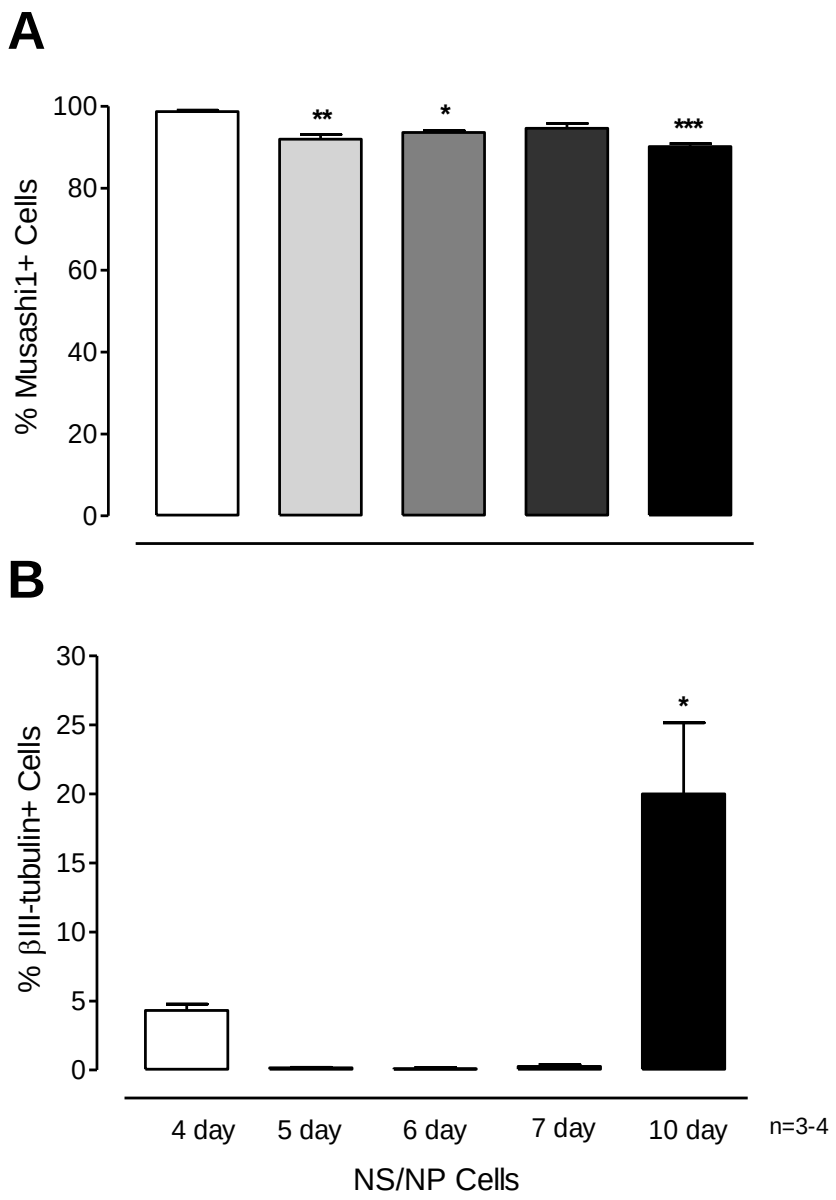


Figure A5 Percentage of NS/NP cells immunoreactive for the NS cell marker (A) Musashi1 and the neuronal marker (B) βIII-tubulin (B). NS/NP cells were derived from 4, 5, 6, 7 and 10 days of monolayer NI in the presence of EGF and FGF2. (n=3-4, *p<0.05, **p<0.005, ***p<0.001, one-way ANOVA followed by post hoc Dunnett's test comparing days 5, 6, 7 and 10 to respective day 4 cultures)

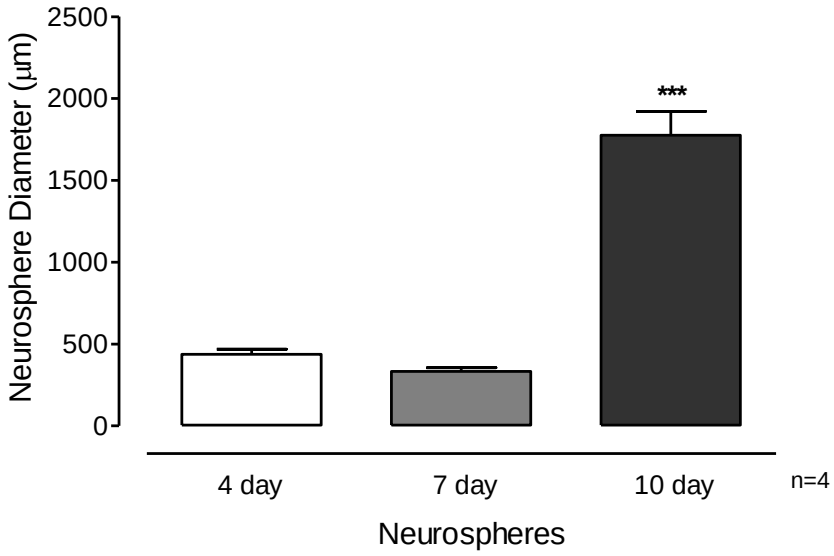


Figure A6 The average diameter of neurospheres derived from day 4, 7 and 10 monolayer NI cultures. (n=4, ***p<0.001, one-way ANOVA followed by post hoc Dunnett's test comparing days 7 and 10 to respective day 4 cultures).

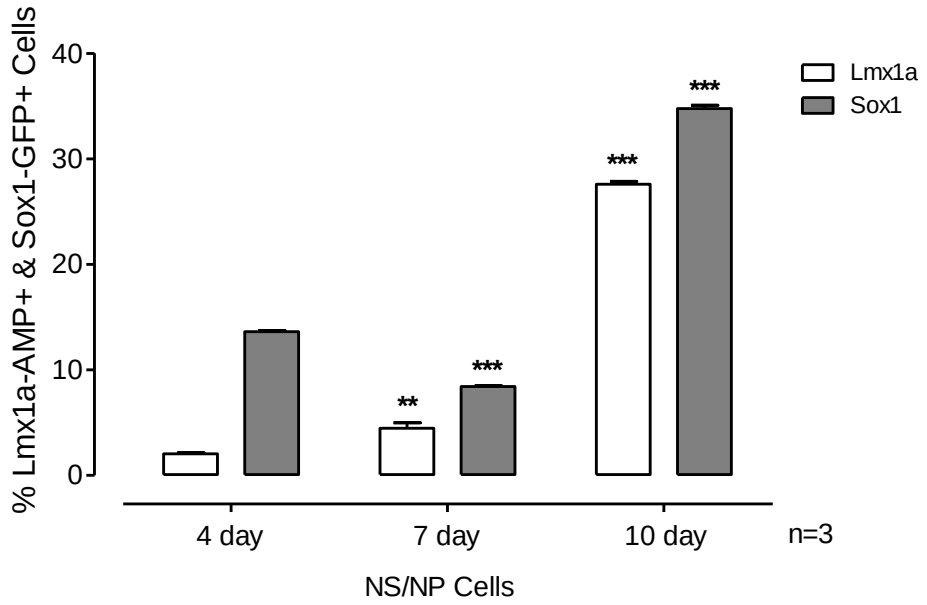


Figure A7 Percentage of Lmx1a and Sox1 expression in 4, 7 and 10 day NS/NP cells. FACS analysis was used to determine the expression of β-lactamase for Lmx1a expression and eGFP for Sox1 expression. (n=3, **p<0.005; ***p<0.001, one-way ANOVA followed by post hoc Dunnett's test comparing days 7 and 10 to respective day 4 cultures).

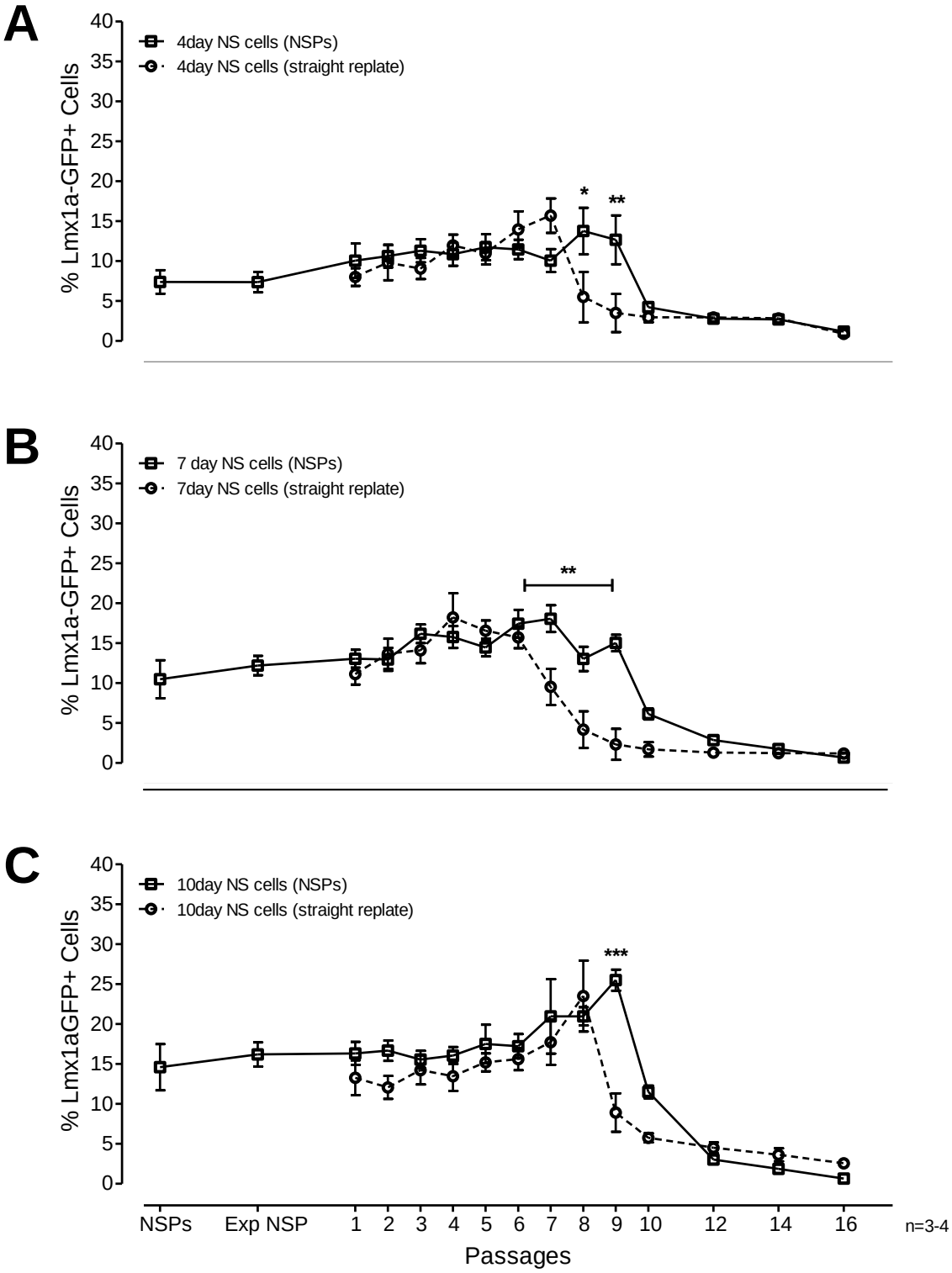


Figure A8 Lmx1a expressions during (A) 4, (B) 7 and (C) 10 day NS/NP cell maintenance. FACS analysis was used to determine the percentage of Lmx1a expressing cells on successive passages during NS/NP cell maintenance (determined by eGFP expression). (n=3-4; *p<0.01; **p<0.005; ***p<0.001, two-way ANOVA followed by Bonferroni's test compared to NSP cultures)

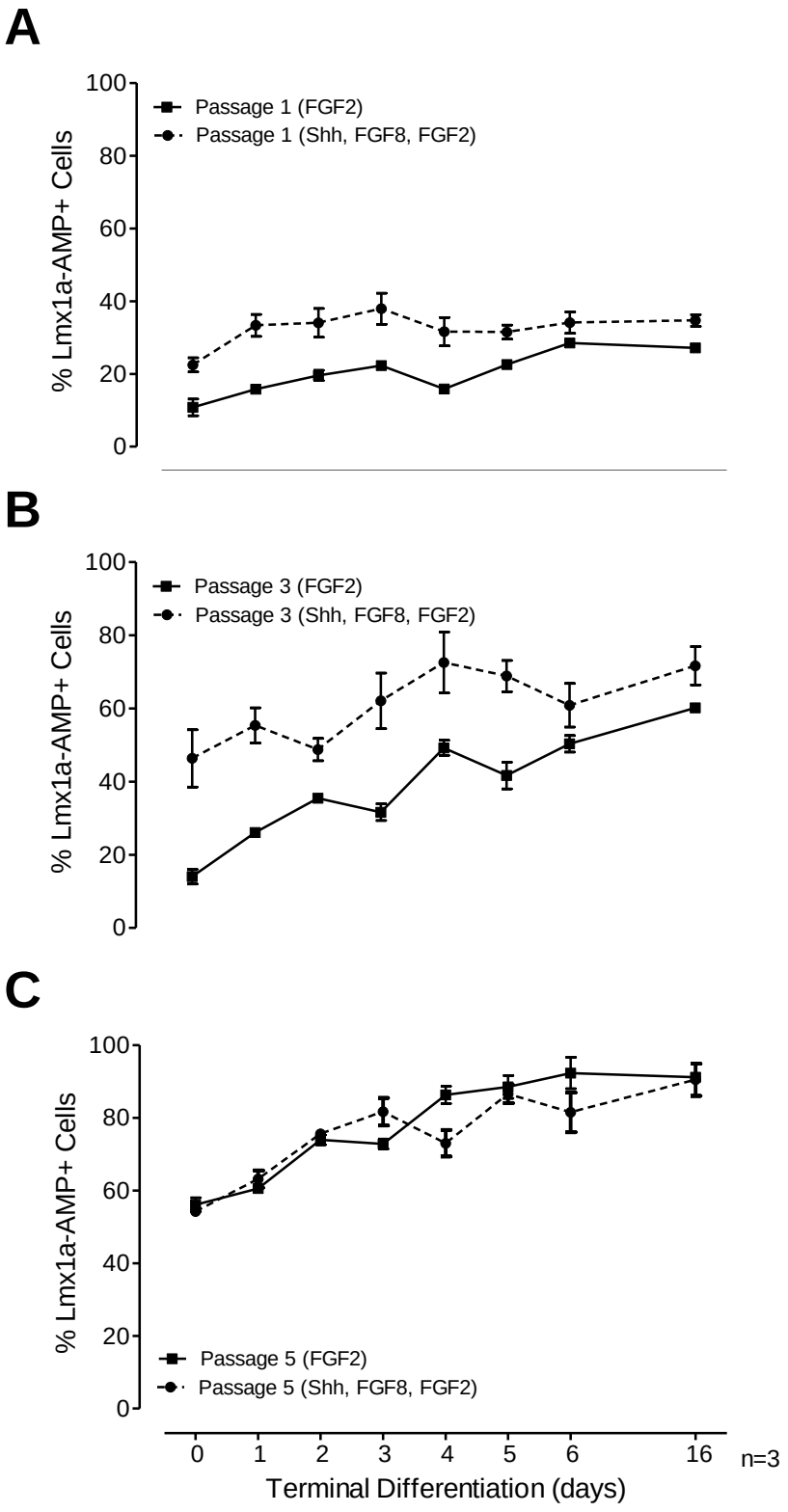


Figure A9 Lmx1a expression during differentiation of 10 day NS/NP cell at (A) passage 1, (B) passage 3 and (C) passage 5 during maintenance in the presence of EGF and FGF2. FACS analysis was used to determine the percentage of Lmx1a expressing cells on successive passages during NS/NP cell maintenance (determined by β-lactamase expression). (n=3)

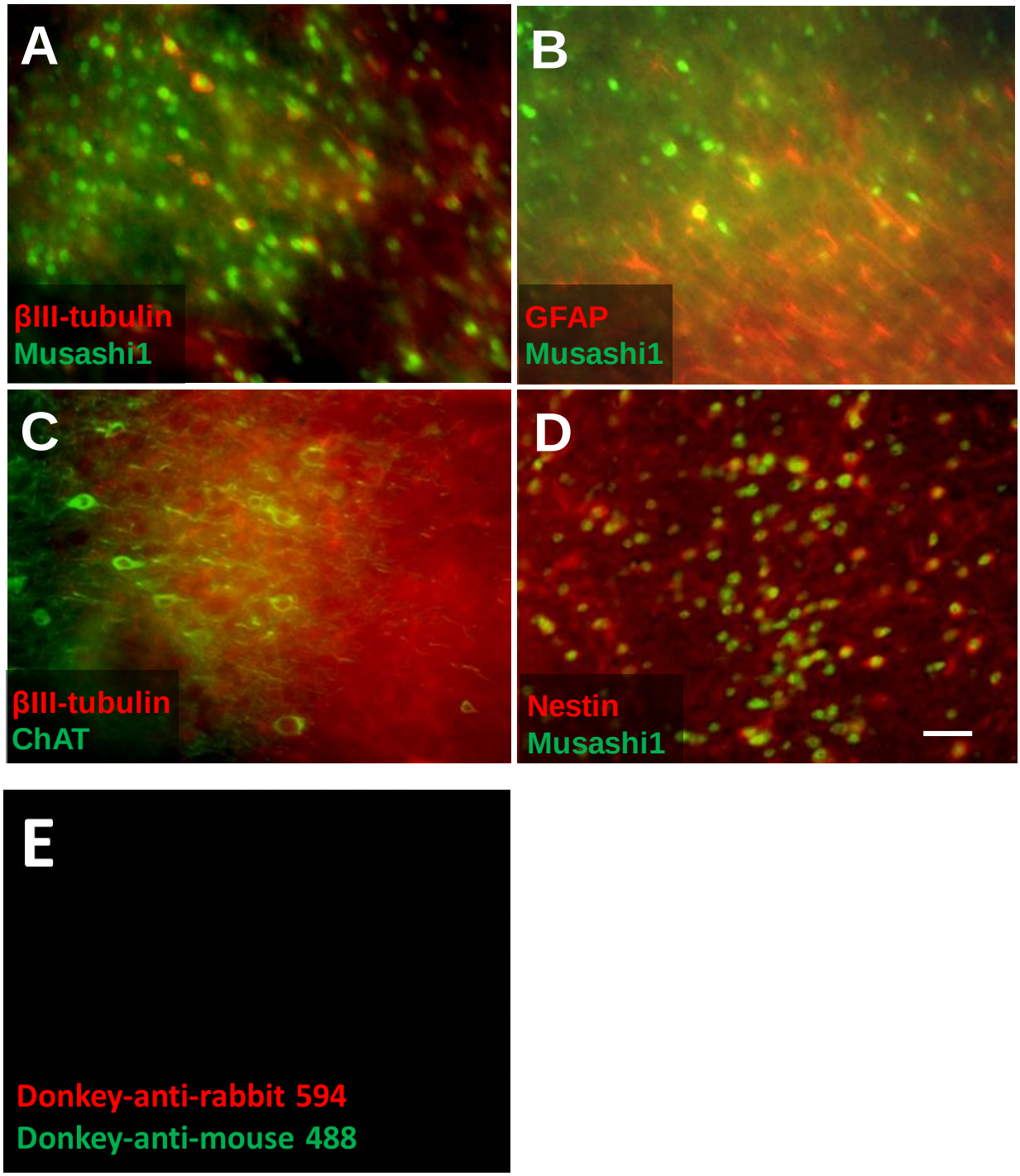


Figure A10 Typical immunocytochemical staining of adult mice brain slices. Immunocytochemistry indicated the presence of minimal βIII-tubulin-Musashi1 (A), GFAP-Musashi1 co-localisation (B) and the presence of ChAT (C) and Nestin (D) positive cells. (E) Negative control (Secondary antibody only) (Scale bar 100 µm)

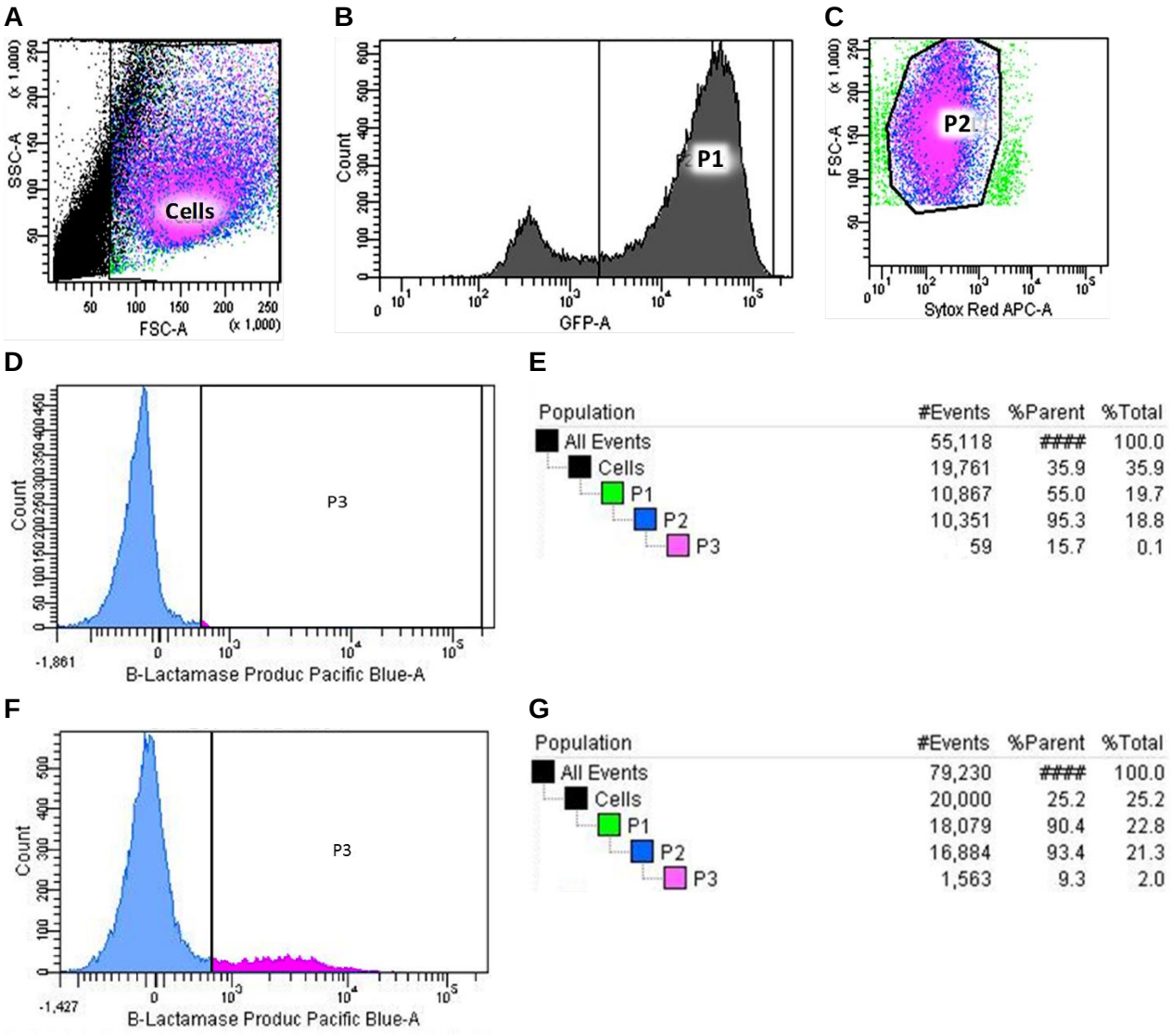


Figure A11 Typical FACS data of NS/NP cells. (A) The "Cells" population was gated by selecting the dominant population excluding the debris. "Cells". (B) Cells that have taken up the CCF4 substrate are GFP positive hence GFP positive cells "P1" were then selected as a subpopulation of "Cells". (C) Live cells that were Sytox red negative "P2" were selected as a subpopulation of "P1". (D and E). Control sample where "P3" were selected as subpopulation of "P2", gated at 0.1% positive. Histogram (D) and raw data (E) of Pacific blue positive cells. (F and G). Test sample where "P3" were selected as subpopulation of "P2" at 2% positive. Histogram (F) and raw data (G) of Pacific blue positive cells.

Neurochemistry International 59 (2011) 394–403

Contents lists available at ScienceDirect

Neurochemistry International

journal homepage: www.elsevier.com/locate/neuint

Extended periods of neural induction and propagation of embryonic stem cell-derived neural progenitors with EGF and FGF2 enhances Lmx1a expression and neurogenic potential

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ARTICLE INFO

Article history:
Received 8 October 2010
Received in revised form 28 March 2011
Accepted 21 April 2011
Available online 23 May 2011

Keywords:
Neural stem cells
Neural progenitors
Embryonic stem cells
In vitro differentiation
Dopaminergic
Multipotency

ABSTRACT

Neural stem (NS) cells are multipotent cells defined by their capacity to proliferate and differentiate into all neuronal and glial phenotypes. NS cells can be obtained from specific regions of the adult brain, or generated from embryonic stem cells (ESCs). NS cells differentiate into neural progenitor (NP) cells and subsequently neural precursors, as transient steps towards terminal differentiation into specific mature neuronal or glial phenotypes. When cultured in EGF and FGF2, ESC-derived NS cells have been reported to be stable and multipotent. Conditions that enable differentiation of NS cells through the committed progenitor and precursor stages to specific neuronal subtypes have not been fully established. In this study we investigated, using Lmx1a reporter ESCs, whether the length of neural induction (NI) dictated the phenotypic potential of cultures of ESC-derived NS cells or NP cells. Following 4, 7 or 10 day periods of NI, ESCs in monolayer culture were harvested and cultured as neurospheres, prior to replating as monolayer cultures for several passages in EGF and FGF2. The NS/NP cultures were then directed towards mature neuronal fates over 16–17 days. 4 and 7-day NS cell cultures could not be differentiated towards dopaminergic, serotonergic or cholinergic fates as determined by the absence of tyrosine hydroxylase, 5-HT or choline acetyltransferase (ChAT) immunolabelling. In contrast NS/NP cultures derived after 10 days of NI were able to generate tyrosine hydroxylase and 5-HT positive neurons (24 ± 6 and 13 ± 1% of the βIII-tubulin positive population, respectively, $n = 3$). Our data suggest that extended periods of neural induction enhanced the potential of mouse ESC-derived NS/NP cells to generate specific subtypes of neurons. NS/NP cells derived after shorter periods of NI appeared to be lineage-restricted in relation to the neuronal subtypes observed after removal of EGF.

1. Introduction

Pluripotent embryonic stem cells (ESCs) are defined by their ability to be maintained indefinitely and differentiate into any cell types found in the three primary germ layers (Martin and Rickets, 1981). ESCs have been differentiated into specific neural cell types such as dopaminergic, serotonergic, GABAergic and motor neurons, as well as astrocytes and oligodendrocytes (Barberi et al., 2003; Chatzi et al., 2009; McKay et al., 1997; Wichterle et al., 2002). Generally these neuronal differentiation protocols, irrespective of whether they utilize embryoid body formation, monolayer or co-culture methods, include a specific period of time for neural induction (NI), when the cells are exposed to neurobasal medium in the absence of mitogens. The NI period gives rise to neural stem (NS) cells, which are multipotent cells that can be differentiated further under the influence of growth factors and neuroprotective agents during a fourteen to twenty day period (Kawasaki et al., 2002; Lee et al., 2000; Ying and Smith, 2003). Such protocols require long periods of cultivation and are complex, requiring specific conditions at specific time points to direct them towards the desired neural lineage. In addition the use of pluripotent ESCs as the starting point may result in contamination of neural cultures with cells of mesodermal or endodermal origin (Pollard et al., 2006). It would be more desirable to commence differentiation from a NS or neural progenitor (NP) cell source that is able to self-renew as well as differentiate into the desired cell types.

NS cells can be generated from ESCs, and they can also be isolated from fetal and adult brain tissue. The phenotype of NS cells grown in culture may be an artefact of the cell culture conditions, an issue that has been addressed in a recent review (Conti and Cattaneo, 2010), but no doubt NS cells will be a valuable source of

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doi:10.1016/j.neuint.2011.04.002

cells for biological research and possibly therapeutic uses. Isolated NS cells can be maintained in monolayer culture or aggregates, in the presence of exogenous factors: EGF (Reynolds et al., 1992) or both EGF and FGF (Conti et al., 2005). NS cells have often been cultured in aggregated spheroid structures, referred to as neurospheres. These are complex three-dimensional structures consisting of heterogeneous mixtures of stem cells, progenitor cells and mature cell types. This complex structure makes it difficult to define and control the early phase of neuronal differentiation. Although Conti et al. (2005) showed that NS cells generated from neurospheres were multipotent when grown in the presence of FGF2 and EGF (Glaser et al., 2007; Sun et al., 2008), the focus of these studies was on whether the cells could produce neurons and glia, rather than on whether various or all subtypes of neurons could be produced. A recent follow up study using ESC-derived NS cells indicated that their phenotypic potential, at least under the culture conditions used by these authors, appear to be largely restricted to GABAergic neurons and glia (Spiliotopoulos et al., 2009).

From a practical perspective it would be desirable: (a) to establish methods for culture of multipotent NS cells and reliable methods for directing their differentiation, or (b) to establish methods for isolation and expansion of panels of committed NP cells, each of which can generate specific phenotypes. In this study we investigated the effect of altering the period of neural induction on the ability of ESC-derived NS/NP cells to generate dopaminergic, serotonergic and cholinergic neurons. We used an ESC reporter line to track expression of Lmx1a; an early gene associated with neural development, which is essential for the development of dopaminergic neurons. Our data indicates that an extended period of neural induction in N2/B27 medium, results in an increasing proportion of cells expressing Lmx1a. Cells selected after an extended induction period are likely to include a higher proportion of NP cells. Our studies indicate that these NP cells are able to give rise to neuronal lineages which have not yet been obtained from early NS cells. Thus, we hypothesise that the transfer of NS cells produced during the early stages of NI into medium containing EGF and FGF2 may be effective in halting the further differentiation of the NS cells, but may restrict their subsequent lineage potential following withdrawal of EGF.

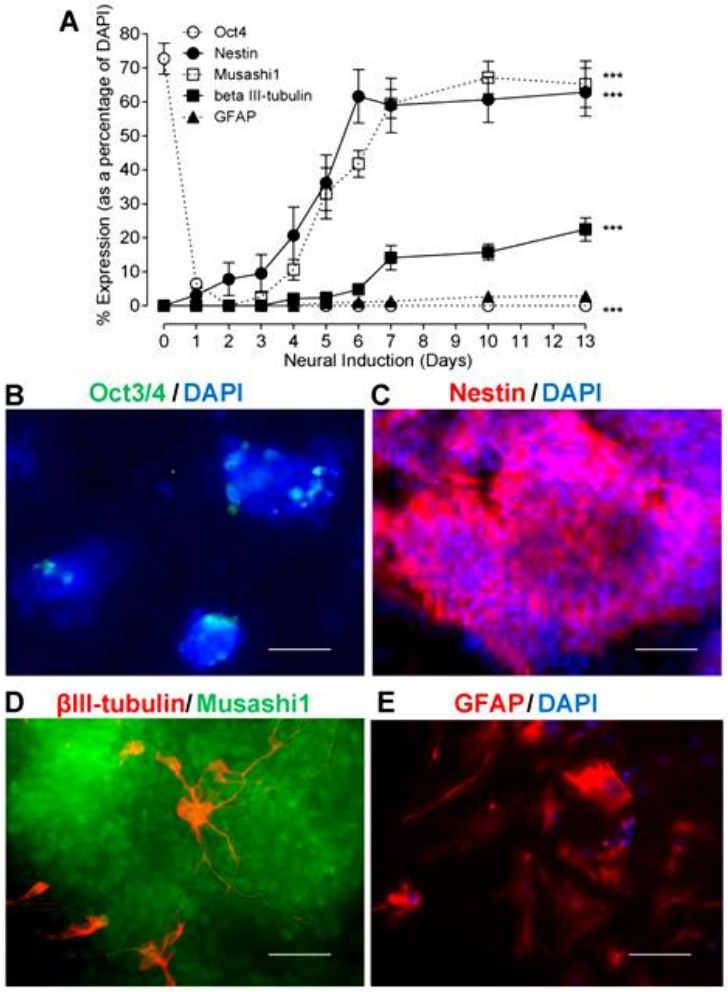

Fig. 1. (A) Time course of protein expression during neural induction as indicated by immunocytochemistry. Oct3/4 immunoreactivity was essentially absent by day 3. Proneural genes, nestin, musashi1, βIII-tubulin and GFAP were detected from days 1, 3, 4, and 5, respectively during neural induction. By day 13, 60% of nestin, 65% of musashi1 and 20% of βIII-tubulin was detected (statistical analysis was by two-way ANOVA followed by Bonferroni's test; $n = 3$; $^{*}p < 0.05$; $^{***}p < 0.001$). (B) Day one cultures included Oct3/4 positive cells. (C) Typical nestin staining in day three cultures. (D) At day 4 the majority of the cells were musashi1 positive, and a few βIII-tubulin positive cells co-localised with musashi1 positive cells. (E) Typical GFAP staining at day five (scale bar 100 μm).

2. Materials and methods

2.1. Lmx1a-β-lactamase reporter mESCs

An Lmx1a-AMP-IRES-eGFP-FneoF vector was constructed for homologous recombination. This vector was introduced into mouse E14Tg2a ESCs by electroporation with a GenePulser XCell™ instrument (BioRad, USA). The AMP is the beta-lactamase gene which is driven directly by the Lmx1a promoter, this is then followed by an internal ribosomal entry site (IRES) and then the eGFP gene. The IRES allows both genes (AMP and eGFP) to be driven from the same promoter, however, because the eGFP is after the IRES the expression levels will be lower than the AMP gene. Finally, the FneoF stands for frt-neo-frt (frt, flippase recognition target) which allows the neomycin gene to be excised using the flippase recombination enzyme. G418-positive colonies were first screened for correct targeting by PCR, and subsequently confirmed by Southern blot analysis. Ninety-three G418 positive Lmx1a-β-lactamase targeted colonies were screened by PCR. Of those colonies, 15 (~16%) were correctly targeted to the Lmx1a start codon. Positive clones were validated by Southern blot analysis, expanded and frozen down for further study.

2.2. Differentiation of ESCs into NS/NP cells

Pluripotent mESCs (E14TG2a, ATCC, USA) and Lmx1a-β-lactamase-IRES-eGFP reporter ESCs were routinely passaged every 48 h in mESC growth medium: DMEM supplemented with 10% (v/v) fetal bovine serum, 0.1 mM β-mercaptoethanol, 4 mM GlutaMAX-1 and 1000 U/ml recombinant mouse leukaemia inhibitory factor (LIF). For neural induction: ESCs were seeded at 1.5×10^4 cells/cm^2 on 0.1% (v/v) gelatin coated culture plastic dishes in mESC growth medium and LIF. After 24 h the medium was replaced with N2B27; a 1:1 mixture of DMEM/F-12 supplemented with N2 additives, 50 μg/ml bovine albumin fraction V, 25 ng/ml insulin and Neurobasal media supplemented with B-27 serum-free additive. The cells were cultured from 4 to 10 days and the medium was changed every day. The cells were dissociated with StemPro® Accutase® and resuspended at 4×10^4 cells/cm^2 on ultra low attachment plates in StemPro® neural stem cell culture medium (Invitrogen) supplemented with 20 ng/ml FGF2 and 20 ng/ml EGF for 4 days to allow neurosphere formation. The neurospheres were then harvested using a 40 μm filter and subsequently plated onto 0.1% (v/v) gelatin-coated culture plastic. Expansion of the neurospheres was allowed for another 4 days, with media changes every second day. Neurospheres were then dissociated and replated as single cells onto 0.1% (v/v) gelatin-coated plastic. mNS cells were then routinely passaged every 48 h in neural stem cell culture medium supplemented with 20 ng/ml of both FGF2 and EGF (Conti et al., 2005).

2.3. Differentiation of NS/NP cells into neurons

In all cases the differentiation of mNS/NP cells into neurons was commenced by plating cells at 8.0×10^4 cells/cm^2 on 0.1% (v/v) gelatin and 2 μg/cm^2 laminin-coated culture plastic. The cultures were incubated in a 5% CO_2 humidified environment at 37 °C for 24 h in NS cell culture medium and 20 ng/ml of EGF and FGF-2. After the appropriate time the NS cell culture medium was replaced with N2B27 medium supplemented with patterning factors, as described in protocols 1, 2 and 3. Following patterning, the growth factors were withdrawn and replaced with N2B27 medium containing 200 μM L(+)-ascorbic acid and 20 ng/ml BDNF for a further 10 days, with media changes every second day. These methods and the following specific patterning protocols are adapted from (Barberi et al., 2003).

Protocol 1: Dopaminergic neurons. N2B27 media was supplemented with 200 ng/ml sonic hedgehog (Shh), 20 ng/ml recombinant human fibroblast growth factor 2 (FGF2) and 100 ng/ml FGF8b for 6 days. The growth medium was replenished on day 3.

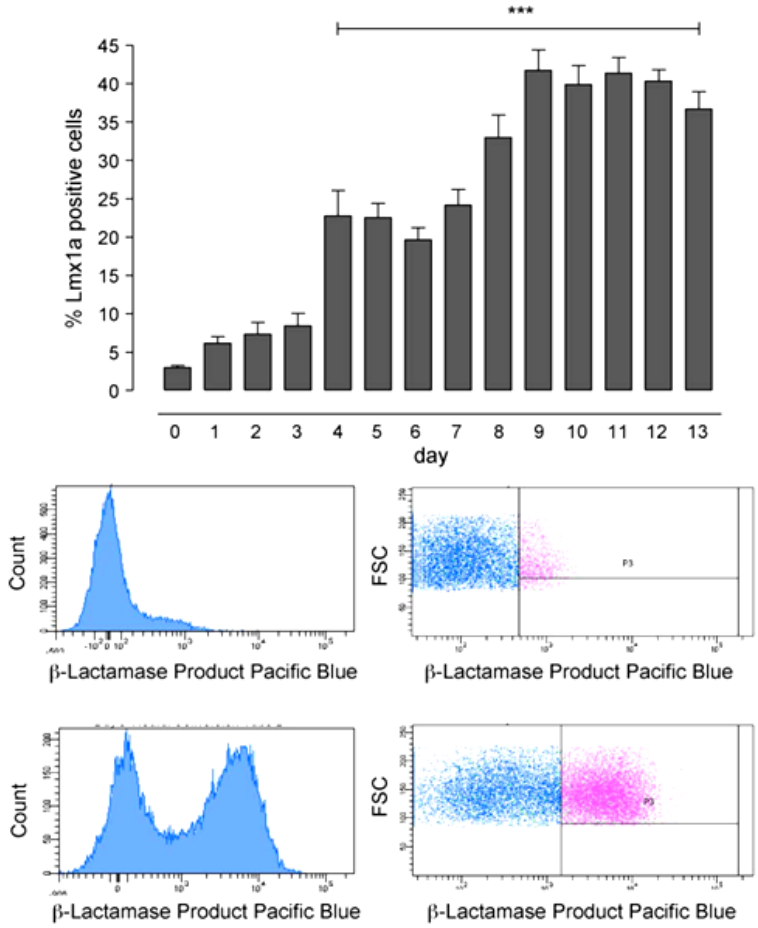

Fig. 2. (A) FACS analysis was used to determine the percentage of Lmx1a expressing cells on successive days during neural induction (determined by expression of β-lactamase, the cDNA of which was targeted to exon 1 of one allele of Lmx1a). (B and C) Typical histogram (B) and dot plot (C) of day two cultures. (D and E) Typical histogram (D) and dot plot (E) of day 10 cultures ($n = 3$; $^{***}p < 0.05$).

Protocol 2: Serotonergic neurons. The cells were initially exposed to Shh, FGF2 and 100 ng/ml FGF4. After 3 days FGF8b replaced FGF4. The incubation was continued for another 3 days.

Protocol 3: Cholinergic neurons. Cells were exposed to 500 ng/ml Shh, 10 ng/ml FGF2 and 1 μM retinoic acid for 4 days, followed by Shh and FGF2 only for 3 days.

2.4. Immunocytochemistry

Immunocytochemistry was performed with the following primary antibodies; rabbit anti-tyrosine hydroxylase (TH; 1:200, Chemicon), mouse anti-tyrosine hydroxylase (TH; 1:800, Immunostar), rabbit anti-serotonin (5HT; 1:3000; Sigma), mouse and rabbit anti-βIII-tubulin (TUJ-1; 1:800, Covance), mouse anti-NeuN (1:100, Chemicon), mouse anti-nestin (1:200, Sigma), mouse anti-choline acetyl transferase (ChAT; 1:200, Abcam), rabbit anti-musashi1 (1:300, Abcam), mouse anti-glial fibrillary acidic protein (GFAP; 1:300, Chemicon), Rabbit anti-tight junction protein-1 (ZO-1; 1:300; Zymed), Rabbit anti-Brachyury (Bry; 1:200; Abcam), Rabbit anti-α1-fetoprotein (1:200; Abcam), Rabbit anti-Oct3/4 (Pou5f1; 1:200; Sigma), Goat anti Vimentin (1:50; Sigma). The secondary antibodies were goat-Alexa Fluor 488 anti-rabbit (1:1000, Molecular Probes) or donkey-Alexa Fluor 488 anti-mouse (1:1000, Molecular Probes) and donkey-Alexa Fluor 594 anti-mouse or anti-rabbit (1:1000, Molecular Probes). Primary and secondary antibodies were diluted in phosphate buffered saline (PBS) with Triton X-100 (0.5% (v/v), PBS-T). Live cells were washed once with PBS and fixed in 4% paraformaldehyde at room temperature for 30 min, then washed twice with PBS (10 min per wash) and incubated in PBS-T for 30 min at room temperature. The cells were washed twice with PBS and incubated in PBS with 2% (v/v) donkey serum and 2% (v/v) goat serum for 30 min at room temperature followed by another three washes in PBS. The cells were then incubated with primary antibodies overnight at 4 °C before they were washed three times with PBS. They were subsequently incubated with secondary antibodies for 2.5 h at room temperature before washing three more times with PBS. The cells were incubated with DAPI (50 ng/ml) for 5 min at room temperature and washed twice with PBS immediately prior to visualisation. Cells were viewed using a Nikon TE2000U microscope coupled to a Coolsnap-fx low light camera and illuminated using a Sutter Instruments DG-4 light box. Cells were illuminated at 390, 482 and 592 nm. Emission wavelengths were detected at 446, 532 and 646 nm, respectively. Cultures were also viewed using a Nikon A1R laser scanning confocal microscope. In some cases the neurospheres were extracted from media and cooled in Tissue-Tek O.C.T. prior to storage at −20 °C. The frozen neurospheres were cut into 8 μm slices then mounted onto glass slides and allowed to dry for 1 h. The sections were fixed with 4% paraformaldehyde at room temperature for 30 min prior to primary and secondary antibody incubation as described above.

2.5. Fluorescent staining and FACS analysis

Neurospheres and monolayer cultures were dissociated with StemPro® Accutase® and cell suspensions incubated in the LiveBLAzer™ FRET – B/G (with CCF-4AM) loading solution (in the dark for 2.5 h). Cells were then analysed using a BD FACSCanto II™ flow cytometer. Cells were illuminated at 405 nm with emissions recorded at 450 nm and 520 nm.

2.6. Numerical and statistical analysis

The percentage of neurons and or specific neuronal phenotypes was obtained by counting the number of DAPI stained cells and the number of cells that were immunoreactive to the antibody of interest in the same field of view. At least four fields of view were captured within each well. Statistical analyses were carried out using PRISM v5.00 and statistical significances were determined using the One-way ANOVA or Two-way ANOVA followed by post hoc Dunnett's or Bonferroni's tests. Numerical results are presented as mean ± SEM of three separate fields of view (from 4 separate experiments for differentiation of ESCs into NS/NP cells; and 3 separate experiments for differentiation of NS/NP cells into neurons). $p < 0.05$ was taken to be statistically significant.

3. Results

3.1. 10 days of neural induction is required to reach peak expression of Lmx1a

To investigate the time course of neural induction (NI), we withdrew LIF and serum from mESCs and used immunocytochemistry to track, over a 13-day period, the expression of the following proteins that are associated with differentiation: Oct3/4 (ES cells); nestin and mushashi-1 (neural stem cells); βIII-tubulin (immature neurons) and GFAP (astrocytes). Fig. 1 shows the time course of expression (A) and typical examples of immunolabelling (B–E). Oct3/4 positive cells were present in undifferentiated cultures and were clearly visible until day two of NI. Nestin expression was apparent as early as day 1 (i.e., one day after removal of LIF), and was sustained in the majority of cells in our cultures beyond day 10 (60%),

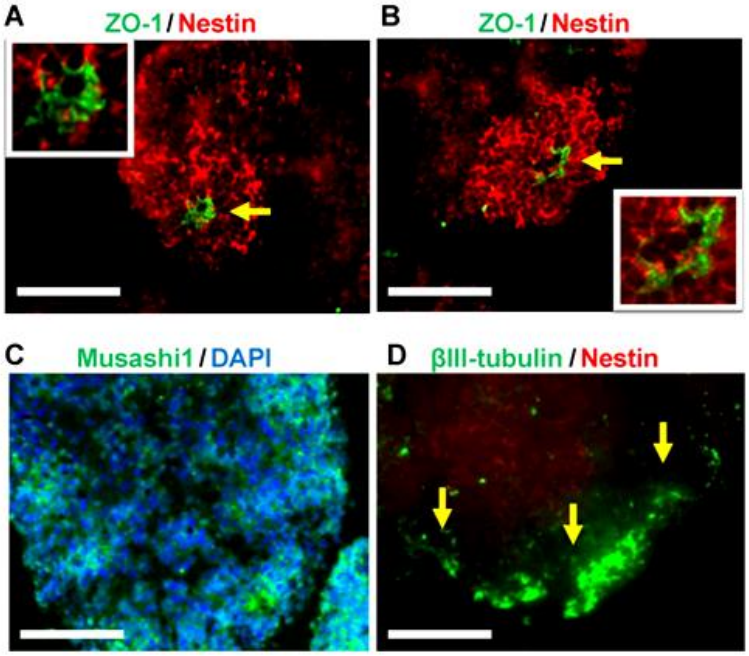

Fig. 3. Immunocytochemistry of neurospheres derived after 10 days of neural induction. The neurospheres were expanded in EGF and FGF2 and subsequently plated onto laminin-coated plates, still in the presence of EGF and FGF2, whereupon they spread onto the substrate and the cell numbers continued to expand. (A and B) Immunocytochemistry indicated the presence of ZO-1 positive central regions of rosettes surrounded by nestin positive cells. (C) Typical pattern of musashi1 expression throughout the neurospheres. (D) βIII-Tubulin positive cells were typically present on the outer surface of neurospheres which retained a nestin-positive core (scale bar 100 μm).

when we believe that most of the cells had differentiated beyond the NS stage. After day 3 of NI the majority of cells expressed musashi1 until at least day 13 (65%). On day 4 a few cells (2%) expressed βIII-tubulin and there was a steady increase in expression up to day 10 (15%), and 22% of the cells were positive for this marker of immature neurons by 13 days of NI (Fig. 1A). Expression of GFAP was detectable during neural induction but they were not significant (Fig. 1A and E). Using the targeted Lmx1a-β-lactamase reporter ESC line we compared the relative expression of β-lactamase over a 13-day neural induction period. The population of β-lactamase positive cells increased to 42 ± 3% by day nine of neural induction and remained constant up to day 13 (Fig. 2A). Between days 4 and 7, the period within which NS cells have been derived previously, 20–25% of cells expressed β-lactamase but at a lower level than typical expression levels on days 10–13 (Fig. 2B–E shows typical FACs plots of 2 and 10 day NI, respectively). Taken together these data suggest that ESCs exposed to 10 days of NI included a significant proportion of NPs, which had differentiated beyond the NS cell stage.

3.2. NS/NP cells derived after different periods of neural induction possess different characteristics when propagated with EGF and FGF2

Using a method adapted from Conti et al. (2005), we derived neurospheres in non-adherent conditions from cultures obtained from ESCs after 4, 7 or 10 days of NI. Following a period of growth in the presence of EGF and FGF2 we examined gene expression in neurospheres using immunocytochemistry. Neurospheres formed after 10 days (Fig. 3) revealed foci which were immunoreactive for the neural rosette marker, ZO-1, typically surrounded by nestin positive cells (panels A and B). This indicated that the 10-day neurospheres contained NS/NP cells present in rosette form. The neurospheres were positive for musashi-1 throughout the spheres (Fig. 3C) and typically had βIII-tubulin positive cells at the periphery around a core of nestin-positive cells (Fig. 3D). Following plating of neurospheres on laminin, and expansion with EGF and FGF2, neurosphere-derived cells migrated and spread onto the laminin surface. Only the neurospheres derived after 10 days NI gave rise to the structures shown in Fig. 4A, regions rich in nestin-positive neural rosette structures, surrounded by immature neurons. Some cultures, up to 7 days of NI, contained mesodermal cells, as indicated by the presence of the mesodermal marker, Brachyury (not shown); and the presence of spontaneously contracting aggregates, which we suggest may be cardiac myocytes. Cells derived from endoderm were not evident following immunolabelling with anti-α1-fetoprotein (not shown). We concluded that 4 days was an insufficient period to guarantee neural induction. When these cultures were detached, resuspended and grown as monolayers on laminin in the presence of EGF and FGF2, we observed that after two passages cultures derived from 4, 7 and 10-day neurospheres adopted different morphologies. The 4-day NS cells produced a flatter, spindle-shaped morphology, more commonly associated with glial cells (Fig. 4B), while the 7 and 10-day NS/NP cells typically adopted a more bipolar appearance with rounder soma (Fig. 4C and D). We compared the properties of the NS/NP cells derived after the three induction periods by immunocytochemistry. The 4-day NS cells included limited populations of βIII-tubulin positive (4.6 ± 0.7%),

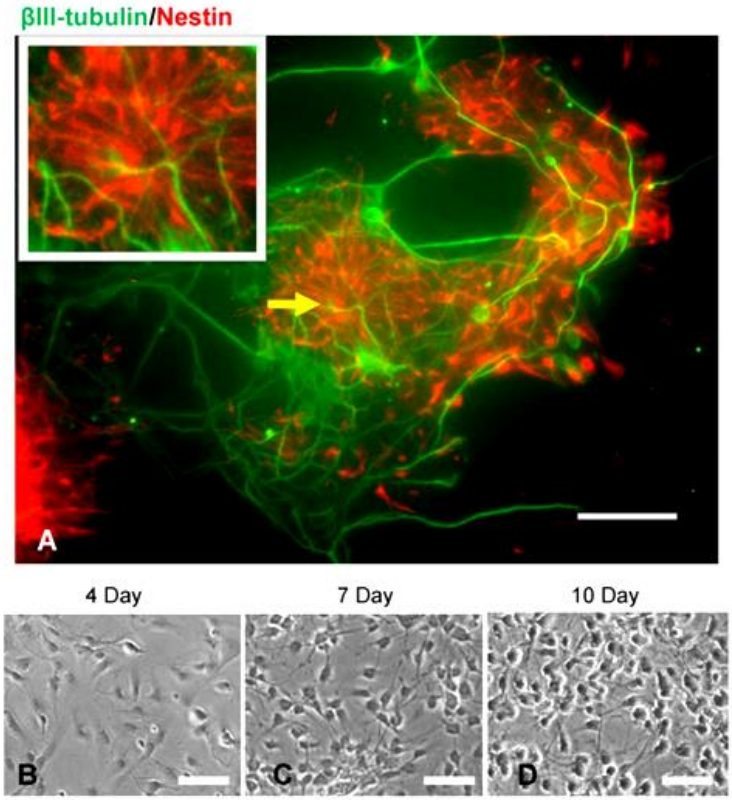

Fig. 4. (A) Immunocytochemistry for βIII-tubulin and nestin in neurospheres formed after 10 days NI, following plating under adherent conditions in the presence of EGF and FGF2, indicated the presence of rosette structures, and the development of neurons at the periphery. (B) Morphological differences between NS/NP cells replated as monolayers from neurospheres derived after 4, 7 or 10 days NI. Four day NS/NPs adopted a flatter and more glial appearance; (C) 7 day NS/NPs and (D) 10 day NS/NPs adopted bipolar or tripolar neural structure with fewer flat cells (scale bars 100 μm).

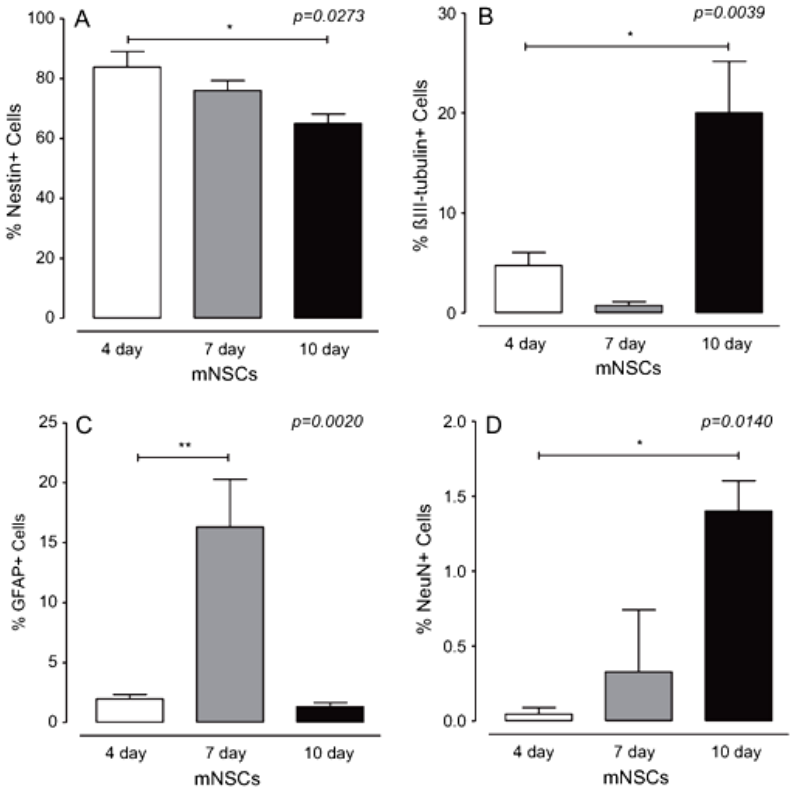

Fig. 5. Percentage of cells immunoreactive for A – nestin, B – βIII-tubulin, C – GFAP and D – NeuN in mNS/NP cultures grown as monolayers in EGF and FGF2, derived from neurospheres originally isolated after 4, 7 or 10 days of neural induction. Nestin levels decreased as NI progressed. By day 10, 20% of cells were βIII-tubulin positive. There was a peak in incidence of GFAP immunoreactivity at day 7. The percentage of more mature NeuN positive neurons was limited, increasing to 1.5% by day 10, indicating that the bulk of the cells were neural progenitors or neuroblasts0. Horizontal bars indicate significant differences between cultures (one-way ANOVA followed by post hoc Dunnett's test; $n = 4$).

GFAP positive (2.0 ± 0.4%) and musashi-1 positive cells (10 ± 1%) and a large population of nestin positive cells (84 ± 6%, Fig. 5), suggesting that most of these cells had differentiated towards an early stage neural phenotype. The 7-day NI cultures also contained few βIII-tubulin cells (0.53 ± 0.2%), but included many more GFAP positive cells (16 ± 4%), most of these cells showed co-localization with vimentin, indicating that they are radial glia (not shown). The majority of the cells were both nestin positive (76 ± 3%) and musashi-1 positive (95 ± 1%, Fig. 5). The 10-day NI cells included a larger population of βIII-tubulin positive neurons (20 ± 5%). Most of the cells were musashi-1 positive (96 ± 1%) and many were still nestin positive (65 ± 3%). Very few GFAP positive cells were present following a 10-day NI (1.3 ± 0.3%, Fig. 5).

3.3. NS/NP cells derived after 10 days of neural induction have the potential to generate multiple neuronal lineages

Since cultures derived after 4-day, 7-day and 10-day NI all included a large proportion of nestin positive cells, we investigated the phenotypic potential of these cultures using three differentiation protocols (1, 2 and 3), each of which has been reported to induce development of particular neural lineages; dopaminergic, serotonergic and cholinergic, respectively (Barberi et al., 2003). Fig. 6 shows that after 16 or 17 days of differentiation using protocols 1, 2 or 3, cultures derived from 4-day and 7-day NI remained predominantly nestin, musashi-1 and GFAP positive, with a small population of βIII-tubulin positive cells (Fig. 6A, B and D) many of which were GABA positive (Fig. 6G). In contrast, cells derived after 10-day NI differentiated into populations containing comparatively few musashi-1, nestin or GFAP positive cells and many βIII-tubulin (Fig. 6B–D). Around 20% of these βIII-tubulin positive cells were also GABA positive (Fig. 6G). Of more significance is the finding that protocol 1 gave rise to a rich population (24%) of tyrosine hydroxylase (TH) positive neurons, as well as 13% 5-HT positive neurons, but only after 10 days of NI. Protocol 2 induced very few TH positive neurons and only 5% 5-HT positive cells (Fig. 6C, E and F). Majority of βIII-tubulin positive cells derived from protocols 1, 2 and 3 were also GABA positive (Fig. 6G). Protocol 3 gave rise to a reduced proportion of β3-tubulin positive cells. No choline acetyltransferase positive neurons were evident following differentiation with any of the protocols. Fig. 7 shows typical images from immunocytochemistry studies after differentiation of 4, 7 and 10-day derived NS/NP cells. When each of the patterning protocols was applied to NS cells derived after 4 days or 7 days of NI, the result was limited expression of βIII-tubulin and a wealth of GFAP, indicating that the majority of the cells had a glial phenotype (Fig. 7A–D). In contrast differentiation of NS/NP cells derived after 10 days produced cultures rich in βIII-tubulin and with regions that were clearly immunopositive for TH or 5-HT (Fig. 7E and F).

4. Discussion

The use of NS/NP cells as a starting point for generating mature neurons offers distinct advantages over ESCs, since the terminal differentiation process is shorter, and the starting population is likely to be considerably more homogenous than the population

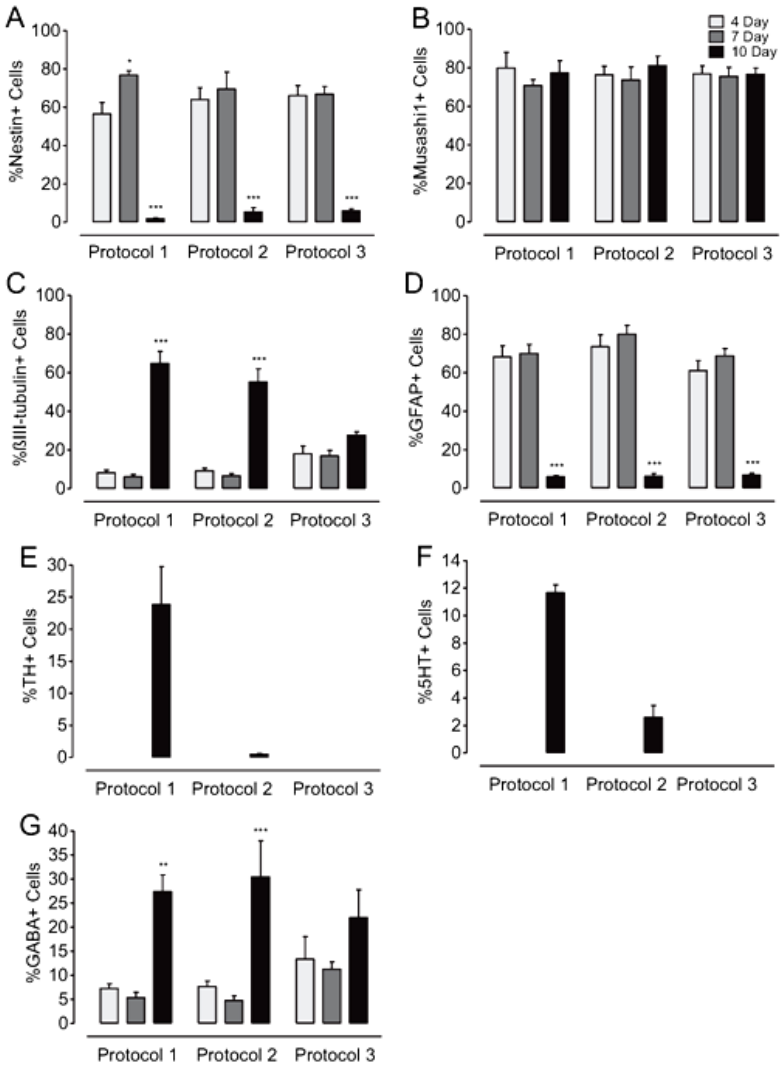

Fig. 6. Three differentiation protocols were used to determine whether dopaminergic, serotonergic neurons could be produced in vitro after removal of maintenance medium, containing EGF and FGF2, from NS/NPs. Samples of NS/NPs derived after 4, 7 or 10 day NI were subjected to the three differentiation protocols and then examined by immunocytochemistry for expression of (A) nestin, (B) musashi1, (C) βIII-tubulin, (D) GFAP, (E) TH, (F) 5-HT and (G) GABA. Protocol 1; 200 ng/ml SHH, 10 ng/ml FGF-2, 100 ng/ml FGF-8 (6 days): protocol 2; 200 ng/ml SHH, 10 ng/ml FGF-2, 100 ng/ml FGF-4 (3 days), then the FGF-4 was replaced with 100 ng/ml FGF-8 (3 days): protocol 3; 500 ng/ml SHH, 10 ng/ml FGF-2, 20 μg/ml RA (4 days), then RA replaced with 100 ng/ml FGF-8 (3 days). All three patterning protocols were followed by incubation in N2B27 medium containing 200 μM L(+)-ascorbic acid and 20 ng/ml BDNF for a further 10 days with media changes being carried every second day. 4 day and 7 day-derived NS cultures resulted in similar outcomes producing 60–80% GFAP positive cells and no TH or 5-HT positive cells. 10-day NS/NPs produced 50–60% neurons using protocols 1 and 2, and protocol 1 led to over 20% TH and over 10% 5-HT positive neurons. 10-day NS/NPs produced less than 10% GFAP positive glial cells whichever of the three protocols was used (statistical analysis was by two-way ANOVA followed by Bonferroni's test; $n = 3$; $^{*}p < 0.05$; $^{***}p < 0.001$).

derived directly from ESCs by NI (Pouton and Haynes, 2007). If populations of NP cells could be stabilised, expanded and stored as frozen stocks they would be an ideal source of neurons for drug discovery and cell therapy. Indeed, defined and committed NPs may be the cells of choice for cell therapy of neurodegenerative diseases such as Parkinson's disease. It may be possible to store frozen stocks of cells which require expansion without the need for further differentiation, which would simplify production of material for clinical use. Propagation of NS cells has been described both from adult brain and also from ESCs (Pollard et al., 2006). The publication of a method for derivation of NS cells from mouse ESCs after 7 days of NI in N2/B27 neurobasal medium was a significant development (Conti et al., 2005), allowing stabilisation of NS cells in EGF and FGF2 for over 100 passages. A recent study investigated the differentiation of NS cell lines after withdrawal of EGF (Spiliotopoulos et al., 2009). The results of differentiation of early and very late passage NS cells were the same, which is encouraging, but the NS cells appeared to be lineage restricted, rather than multipotent, and the neurons produced were predominantly GABAergic.

During neurogenesis *in vivo*, ESCs differentiate into neural epithelial cells, then radial glia, which in turn become neural intermediate progenitor cells, and then neurons (Kriegstein and Alvarez-Buylla, 2009). Similarly, in vitro, ESCs also appear to differentiate into neural epithelial cells, radial glia, and NSCs, which are capable of both symmetrical and asymmetrical cell division. The NSCs then become NP cells, which are only capable of asymmetric division, producing neural precursors, which then

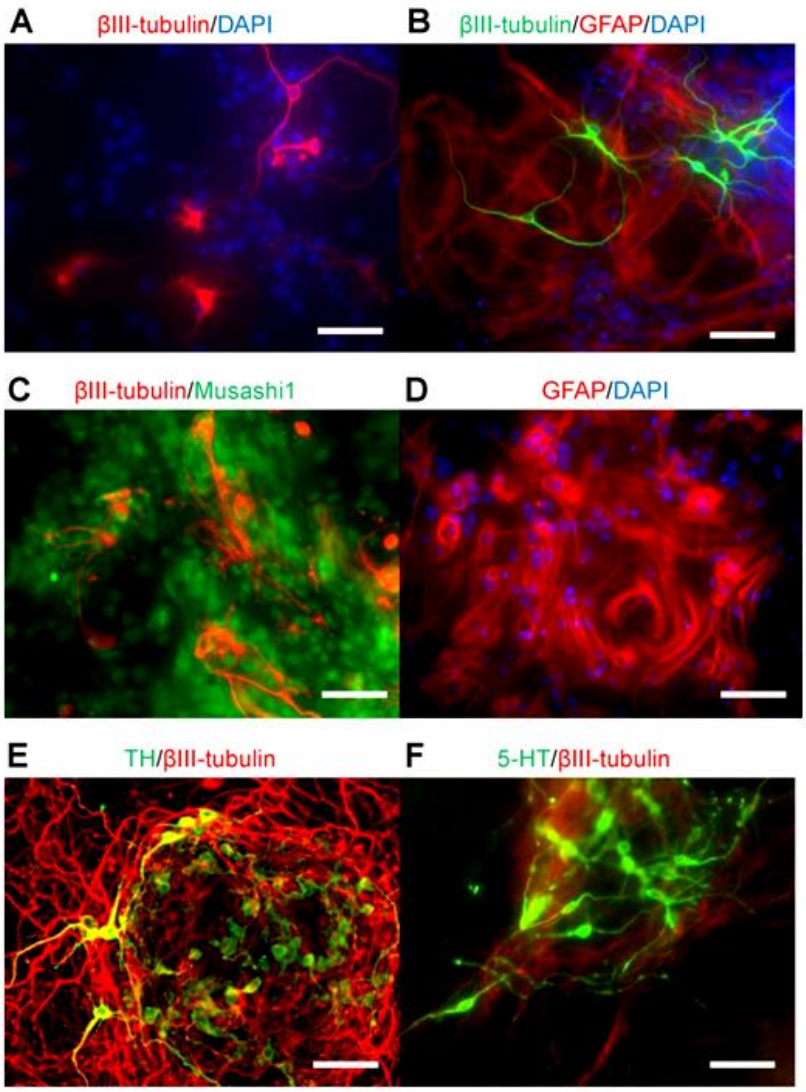

Fig. 7. Immunocytochemistry studies of differentiated cells produced on day 16 using protocols outlined previously (see caption for Fig. 6). Typical differentiated cultures produced using protocol 1 with NS/NP cells derived after 4-days NI (A and B – showing limited βIII-tubulin and many GFAP positive cells), 7 days NI (C and D) or 10 days NI (E and F – showing a wealth of βIII-tubulin and specific tyrosine hydroxylase (TH) and 5-HT immunoreactivity) (scale bar 100 μm).

expand symmetrically and subsequently differentiate into post mitotic neurons (Juliandi et al., 2010). We hypothesised that the propagation of NS cells in EGF and FGF2 may prevent multipotent differentiation after subsequent withdrawal of EGF, and that the length of NI prior to the formation of neurospheres in EGF and FGF2 may influence the subsequent potency of the cultures. We further hypothesised that after longer periods of NI it may be possible to isolate NPs which are committed to dopaminergic or serotonergic phenotypes, which we have observed appear at later stages during in vitro differentiation of ESCs (unpublished data). The published protocol for derivation of NS cells (Conti et al., 2005) calls for 7-days of NI, so we derived neurospheres after 4, 7 or 10 days using a very similar method, that differed only by our use of StemPro neural stem cell culture medium (Invitrogen), rather than NS-A medium (Euroclone), as the base of the medium for neurosphere formation and NS/NP expansion. We chose to derive neurospheres after 10 days NI based on the time course of expression of our Lmx1a reporter. Consistent with this finding we also show that after 10 days of neural induction there is no expression of either Brachyury or α1-fetoprotein, indicating the absence of mesoderm and endodermal lineages. Lmx1a is expressed in the developing nervous system, in the roof plate of the neural tube, the notochord and the otic vesicles (Failli et al., 2002). Lmx1a is known to be essential for development of dopaminergic neurons in the mesodiencephalon (Smidt and Burbach, 2007), and is expressed in mitotic dopaminergic progenitors, precursors and mature dopaminergic neurons (Andersson et al., 2006). Lmx1a works in cooperation with Lmx1b (Lin et al., 2009) to specify dopaminergic development in the floorplate (Ono et al., 2007), and is involved in an autoregulatory loop with Wnt1 (Chung et al., 2009). Forced expression of Lmx1a has been used to enhance the yield of dopaminergic neurons from differentiating cultures of ESCs (Andersson et al., 2006; Friling et al., 2009). In our differentiation experiments, expression of Lmx1a reached a peak at 9–10 days, which indicated that NP cells were present after 10 days of NI. There were clear differences between the cultures transferred to medium containing EGF and FGF2 after different periods of NI, although in all cases, even after only 4 days NI, the bulk of the cells were immunopositive for nestin. On withdrawal of EGF

the 4 and 7-day derived NS cells gave rise to 60–80% GFAP positive cells, indicating glial phenotypes, and less than 20% neurons (at day 7 many of these GFAP positive cells were also immunoreactive to the vimentin, indicating the presence of radial glia). These cells were unable to give rise to dopaminergic or serotonergic neurons. In contrast the cells derived after 10 days of NI already contained some βIII-tubulin positive cells, and were capable of producing up to 60% neurons including 23% dopaminergic neurons (Fig. 6). We considered that the 10-day derived cells were most likely to be a mixture of NP cells and NS cells, given that the cells were not necessarily at the same stage of development throughout each neurosphere.

The neurospheres derived after 10 days of NI in our study clearly included expanded rosettes (Fig. 3) with ZO-1 positive central regions (Elkabetz et al., 2008). Our current hypothesis is that the 10-day cultures contain rosettes which are rich for NPs of specific phenotypes, and that the cultures could potentially be used to derive cultures of specific NPs by picking out individual rosettes or by forming neurospheres with fewer cells. At this stage we do not know whether individual NS/NP cells can be cloned by forming colonies from single cells. Two groups have reported that expansion of neural rosettes, followed by treatment with Shh and FGF8, results in a rich source of dopaminergic neurons (Cho et al., 2008; Perrier et al., 2004). Most research groups have had difficulty producing such high yields of dopaminergic neurons. One explanation may be that these two studies involved fortuitous expansion of cultures from rosettes which were already committed to the dopaminergic phenotype. Indeed it may be that selection of appropriate specified rosettes is the key to enriching cultures for specific neuronal phenotypes.

We used published neural patterning protocols to investigate whether our NS/NP cells were responsive to different growth factors (Barberi et al., 2003). Sonic hedgehog (Shh), and FGF8 have often been used to mimic the neural patterning *in vivo*. Neurogenesis is a complex process involving specific time and concentration-dependent signalling of notch, BMP and Wnt signalling pathways. Wnt1 has an important role in the neural specification of the midbrain and hindbrain (Prakash et al., 2006). Wnt1 activates the dishevelled family of proteins, inhibiting the degradation of β-catenin, which then translocates to the nucleus where it interacts with the TCF/LEF family of transcription factors to induce specific gene expression. During neurogenesis in the floor plate, Wnt1-mediated inhibition of β-catenin degradation modulates Shh expression, and inhibits neurogenesis. Thus, when Shh is present during the early stages, neurogenesis is repressed (Fasano and Studer, 2009). We hypothesise that following a short period of NI, the resultant early NS cells respond to Shh with an inhibition of neurogenesis, rather than patterning. This finding is largely consistent with the findings of Conti et al. (2005) who reported that 7-day NS cells gave rise to 30–40% neurons after EGF withdrawal. This number is greater than those generated from our 7-day NI protocol. However, following withdrawal of EGF, Conti et al. (2005) used FGF to enhance self-renewal and survival, rather than begin patterning with Shh. In our study immediate exposure to Shh following withdrawal of EGF may explain the low number of neurons which developed using our protocol. More importantly our 10-day NS/NP cells did indeed respond to different patterning protocols, although not as rich a population published by Barberi et al. (2003) derived from ES cells (50% TH and 5HT positive cells), we were able to generate 23% dopaminergic neurons with the appropriate patterning protocol (Barberi et al., 2003) (Figs. 6 and 7).

5. Conclusions

Published protocols for differentiation of ESCs to neural phenotypes vary in length of NI, format (monolayer versus aggregate or co-culture) and the timing of additions of differentiation and/or growth factors (Barberi et al., 2003; Kawasaki et al., 2000; Khaira et al., 2009, 2011; Lee et al., 2000; Raye et al., 2007; Wichterle et al., 2002). Often, the length of NI varies considerably and, although many cells derived using such protocols express NS cell markers, such as nestin and musashi-1, the cultures are difficult to characterise in more detail. Our data suggest that although the cultured NS cells from the shorter periods of NI give rise to nestin positive monolayer cultures, such cultures are lineage restricted, possibly as a result of early exposure to EGF and FGF2. 4-day NS cells are not responsive to patterning protocols, and treatment with Shh and FGF8 results in limited differentiation into neurons. Extending the NI period to 10 days prior to the generation of neurospheres in EGF and FGF2, and subsequent expansion as monolayer cultures, enables expansion of NS/NP cells which respond to patterning cues, and are able to generate phenotypically diverse neurons with very small numbers of residual mNS/NP cells and astrocytes.

Acknowledgements

This work was funded in part by the Australian Research Council, Stem Cell Sciences Ltd. (now Stem Cells Inc.), and Pfizer Inc. The Pfizer funding made possible the derivation of the Lmx1a-β-lactamase reporter cell line used in this study. WZ was the recipient of an Australian Research Council APAI PhD Scholarship.

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