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Non-cell autonomous effects of integrin signalling on neurogenesis in the chick embryonic CNS.

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Declaration

I declare that the research described within this thesis is my own work and that this thesis was composed by myself unless otherwise stated.

Neither this thesis nor any part thereof has been submitted for any other degree or professional qualification.

Name: Katherine Long

Signature:

Date:

For my Dad

For his love and encouragement when starting my PhD, he would have been so very proud to see me finish.

This is for you Dad.

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Summary

A fundamental characteristic of neural stem cells (NSCs) is their ability to divide asymmetrically, producing daughter cells of different fates. This requires the regulation of proliferation verses differentiation, which could occur in two different ways; at a cellular or environmental level. Although there is some evidence for each model, little is known about how this regulation occurs. One candidate is the integrin family. Integrins are known to regulate key aspects of stem cell behaviour. The integrin subunits α6 and β1, which heterodimerise into a laminin receptor, are highly expressed within the NSCs of the embryonic ventricular zone and loss of integrin β1 (itgβ1) function within the ventricular zone of the embryonic mouse results in NSC detachment and apoptosis.

The aim of this thesis is to investigate the role of itgβ1 in the regulation of proliferation verses differentiation in the NSC of the chick embryonic neuroepithelium. To test the function of itgβ1 within this system, we are using transfection of a constitutively active (CA*), wild type (WT) and extracellular portion only (EC only) integrin-β1, via electroporation of the chick embryonic CNS, resulting in a patchwork of expression within the NSCs of the midbrain neuroepithelium. This system allows both expressing cells and their non-expressing neighbours to be studied within the same environment. We predicted that if integrins are acting to regulate NSC behaviour via an intrinsic mechanism, only the cells expressing the CA* integrin will alter their behaviour. If the second model is correct and integrins act via an extrinsic mechanism, we predicted that the neighbouring non-expressing cells will also alter their behaviour.

We observed a significant increase in the number of neurons generated upon expression of CA*β1. This increase in neurons was a non-cell autonomous effect; the neurons were GFP and human itgβ1 negative, supporting the second model of extrinsic signals and cell-cell interactions in the regulation of proliferation and differentiation. The increase in neurogenesis was only observed in the midbrain upon CA*β1 expression for 48hrs. A significant increase in mitotic cells was observed 12hrs after electroporation, the earliest time point and by E4 (48hrs) a significant proportion of mitotic cells were abnormally located by 48hrs, resulting in basal mitoses.

Investigation of signalling between cells was carried out using microarray analysis of the two populations of cells, CA*β1 positive and negative. One candidate from the microarray results was the bHLH transcription factor Tal2. Tal2 has previously been shown to be specifically expressed within the midbrain neuroepithelium at the time of electroporation and to play a role in the regulation of neurogenesis.

In summary, this thesis has showed an important role of itgβ1 in the regulation of proliferation and differentiation of NSCs within the chick embryonic neuroepithelium in a non-cell autonomous manner.

Abbreviations:

AJ	Adherens junction
AP	Apical progenitor
APC	Adenomatous polyposis coli
BLBP	Brain lipid-binding protein
BM	Basement membrane
BMP	Bone morphogenic protein
BP	Basal progenitor
BSA	Bovine serum albumin
CNS	Central nervous system
CP	Cortical plate
CSF	Cerebrospinal fluid
DMNT3b	DNA methyltransferase 3β
E#	Embryonic day
EC	Extracellular
ECM	Extracellular matrix
EdU	5-ethynyl-2'-deoxyuridine
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ERK	Extracellular-signal related kinase
ESC	Embryonic stem cell

FACS	Fluorescence-activated cell sorting
FAK	Focal adhesion kinase
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
GFP	Green fluorescent protein
GSC	Germ-line stem cell
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HH#	Hamilton Hamburger stage
HMT	Histone methyltransferase
hrs	Hours
IC	Intracellular
ILK	Integrin linked kinase
INM	Interkinetic nuclear migration
IP	Intermediate progenitor
ISVZ	Inner sub ventricular zone
Itg	Integrin
MAPK	Mitogen activated protein kinase
miR	Micro RNA
NE	Neuroepithelial
NICD	Notch intracellular domain
NSC	Neural stem cell

NPC	Neural precursor cell
OSVZ	Outer sub ventricular zone
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
pFAK	Phosphorylated FAK
PH3	Phosphohistone 3
RA	Retinoic acid
REST	RE1 silencing transcription factor
RGC	Radial glial cell
RNAi	RNA interference
Robo	Roundabout
Shh	Sonic hedgehog
SNP	Short neural precursor
SVZ	Sub ventricular zone
TM	Transmembrane
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labelling
VZ	Ventricular zone
WT	Wild type

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Chapter 1

Introduction.

Neurogenesis has been widely studied in both the embryo and adult. How neural cells are generated and the timing of neurogenesis is well known, along with the key anatomical features of neural stem cells (NSCs) and some of the major signalling pathways involved. One of the fundamental characteristics of NSCs is their ability to divide asymmetrically, producing daughter cells of different fates. This process requires the regulation of proliferation and differentiation. This regulation could occur in two different ways, at a cellular or environmental level. At a cellular level, fate could be determined intrinsically by the regulation of the angle of cell division and segregation of fate determinants within the cell. Fate could also be determined by the signalling environment around the cell. This extrinsic mechanism would determine fate in a more stochastic manner, via signalling and cell-cell interactions post-division. There is evidence to support both mechanisms, but they are not mutually exclusive and may in fact work in combination to regulate proliferation and differentiation.

These two models produce a wide variety of potential candidates for this regulation, including secreted factors, polarity cues and cell surface receptors. One obvious candidate is the integrin family. Integrins are highly expressed on many stem cell types, including NSCs and there is data to support roles in both stem cell adhesion within the niche and control of cell division angle. Integrins are key signalling molecules, able to signal in both directions across the cell membrane. This enables them to signal both inside and outside of the cell, regulating of a large variety of signalling pathways in NSCs as well as their adhesion. Due to their many functions within the cell, integrins could be regulating NSC proliferation and differentiation by either model suggested above, on a cellular or environmental level.

As the aim of this thesis is to investigate the role of integrins in the regulation of proliferation and differentiation of neural stem cells during neurogenesis in the embryo,

the introduction will first summarise neurogenesis and NSC behaviour. I will then give an over-view of integrin structure and function before describing the evidence suggesting integrins may regulate NSC proliferation and differentiation.

The introduction to neurogenesis will briefly outline the characteristics of NSCs and their behaviour before focusing on the steps of neurogenesis relevant to the project; regulation of proliferation and differentiation. To this end, this introduction will focus on the anatomy of neural stem and progenitor cells and their modes of division.

I will then describe the evidence supporting the two models of regulation of proliferation and differentiation and how they might function together. The first model I will focus on is the intrinsic model, the segregation of fate determinants. I will outline the role of polarity in this model as well as the control of the angle of division and mitotic spindle, both of which are required for the positioning and segregation of fate determinants. I will then summarise the evidence supporting this model, including the evidence of fate determinants.

The second model is the extrinsic model, where fate is determined by the signalling environment and interactions a cell receives after it has divided; post-specification via signalling. I will focus on the major signalling pathways already known to regulate neurogenesis, including Notch, Wnt, Shh, FGF, BMP, Robo-Slit and retinoic acid. As with the first model, I will then summarise the evidence supporting the second extrinsic model before outlining the evidence to indicate the two models may act in concert to regulate proliferation and differentiation, including interkinetic nuclear migration and the role of the extracellular matrix.

No matter how proliferation and differentiation are regulated, intrinsic changes within the cell must occur during neurogenesis. The introduction will next focus on these intrinsic changes, including epigenetics, micro-RNA, nuclear receptors, transcription factors and cell cycle length.

The final section of the introduction will focus on the role of integrins in the regulation of differentiation and proliferation. It will outline integrin structure and activation states, inside-out and outside-in signalling as well as cross-talk with other pathways mentioned previously. I will then describe the evidence supporting a role of integrins in the regulation of stem cell behaviour, including their expression, adhesion roles within the niche and

orientation of the mitotic spindle. At the end of this introduction I will then detail the experimental strategy to test our hypothesis that integrins play a role in the regulation of proliferation and differentiation of NSCs in the embryonic CNS.

1.1: NSC characteristics and behaviour.

1.1.1: NSC anatomy.

The walls of the neural tube are a sheet of pseudo-stratified epithelium - the neuroepithelium. There are several well-known characteristics of the neuroepithelial (NE) cells. They have a characteristic bipolar morphology, with a long basal process attached to the pial basement membrane and a shorter apical process attached to the ventricular surface (Figure 1.1a). The cell bodies of these cells remain within the ventricular zone (VZ), the area directly above the ventricular surface which is present throughout neurogenesis, and undergo Interkinetic nuclear migration (INM), migration of the nucleus during the cell cycle (Sauer 1935). It is these characteristics that make NE cells easy to identify, despite the current lack of known markers.

The neuroepithelium is organised into distinct anatomical zones. In the early chick embryo, the majority of the neuroepithelium is the VZ, which contains the proliferating cells (Figure 1.2a). In the mouse embryo, there is a second proliferative zone above the VZ, the sub ventricular zone (SVZ) (Figure 1.2b). The SVZ contains neural progenitor cells (NPCs); daughters of the NSCs that are able to undergo neurogenic divisions. The neurons generated then migrate up into the cortical plate (CP). In the human, the SVZ is split into two cytoarchitecturally distinct zones, the inner and outer SVZ (ISVZ and OSVZ) (Figure 1.2c) (Fietz et al. 2010; Hansen et al. 2010).

1.1.2: Interkinetic Nuclear Migration.

INM was first described by Sauer in 1935: *'mitoses are confined to the region of the lumen not because only nuclei of that region divide, but because a nucleus that is about to divide moves to the region of the lumen to do so'*. Prior to neurogenesis this migration spans the entire neuroepithelium. The cells migrate towards the basal surface to undergo S phase, then migrate towards the apical surface to undergo mitosis (Figure 1.1a). Daughter cells that differentiate will migrate basally, out of the VZ, whereas daughters that remain progenitors remain within the VZ. Later on in neurogenesis, INM is restricted to the

Figure 1.1

progenitors found in the VZ, not in those found in the other more basal proliferative zones. Although much is known about the process of INM, its function is not yet fully understood. Several studies have shown that INM and cell cycle are not regulated by the same mechanisms. Pax6, a transcription factor involved in neural specification, is required to couple the cell cycle to INM. This coupling can be disrupted to show INM and cell cycle are not reliant on each other; blocking cell cycle progression does not block INM and vice versa. Both perturbations do however result in an increase in neurogenesis (Asami et al. 2011; Tamai et al. 2007).

The process of INM suggests that spatial regulation of the cell cycle is important in the regulation of neurogenesis, whether through the availability of cues or signal transduction – the new-born daughter cells are exposed to signalling available at the apical but not basal surface (Murciano et al. 2002). But why do they divide at the apical surface? The apical end foot contains a primary cilium, which protrudes into the ventricle and remains throughout the cell cycle until mitosis. The centrioles associated with the primary cilium will form the centrosomes and mitotic spindle. As the primary cilium is anchored at the ventricular surface, the nucleus must have an apical location to allow the spindle to form (Taverna & Huttner 2010; Schenk et al. 2009). As the neuroepithelium is pseudo-stratified, the cells cannot all have an apical location at the same time, so migrate away after mitosis.

1.1.3: Types of neural progenitors.

NSC/NPCs can be either bipolar, monopolar or nonpolar (Figure 1.1b). Bipolar progenitors are the NE cells and Radial glial cells (RGCs). Both have an apical-basal polarity, a long basal process attached to the pial basement membrane (BM) and a shorter apical process attached to the ventricular surface (Figure 1.1b). Both of these processes are present throughout the cell cycle (Fietz & Huttner 2011). NE cells and RGCs are also referred to as apical progenitors, as they undergo mitosis at the apical surface. NE cells are the earliest of the NSCs, RGCs are generated from NE cells at the onset of neurogenesis. As with NE cells, RGCs have a bipolar morphology, undergo INM and are capable of self-renewing and generating neurons. Prior to neurogenesis, NE cells make up the majority of cells in the neuroepithelium, but during neurogenesis this switches to RGCs. The main differences between the two are the 'glial-like' properties of RGCs, such as expression of glial markers GFAP, BLBP and GLAST (reviewed in Kriegstein & Götz 2003). RGCs also lose some epithelial characteristics, such as replacement of tight junctions with adherens junctions (AJs) and

changes in Extracellular matrix (ECM) expression, such as up-regulation of tenascin-C (Aaku-Saraste et al. 1997; Stoykova et al. 1997). This transition from NE cells to RGCs still occurs *in vitro* when embryonic stem cells (ESCs) are differentiated into neurons, suggesting it is regulated by an intrinsic mechanism programmed into the cells (Nat et al. 2007).

Monopolar progenitors are the OSVZ progenitors and short neural precursors (SNPs), both of which are monopolar at mitosis (Figure 1.1b). OSVZ progenitors were recently identified in the human and ferret (Fietz et al. 2010; Hansen et al. 2010) and are thought to be generated from apical progenitors that delaminate from the ventricular surface, translocate their nucleus to the SVZ and retain their basal process (Fietz & Huttner 2011). In contrast, SNPs only maintain their apical process throughout the cell cycle, retracting their basal process to undergo mitosis at the ventricular surface. Unlike NE cells and RGCs, SNP progeny usually produce post-mitotic neurons from the VZ and are not able to undergo another round of division (Stancik et al. 2010).

Nonpolar progenitors are the basal or intermediate progenitors (BP or IP) and ISVZ progenitors (Figure 1.1b). BPs are generated from APs, which then delaminate from the apical surface and undergo mitosis in an abventricular location. They are nonpolar cells as they retract both their very short apical and basal processes during mitosis and lack apical-basal polarity (Noctor et al. 2008; Attardo et al. 2008). Most of their divisions are symmetric and neurogenic, although there is some evidence that basal progenitors can expand via proliferative divisions. ISVZ progenitors, found in the ferret and human, also retract both processes for mitosis in the ISVZ and are similar to BPs (Fietz et al. 2010; Hansen et al. 2010).

These different types of progenitors show different rates of occurrence depending on the evolutionary class of cortex – lissencephalic verses gyrencephalic (Figure 1.2). The majority of progenitors in the VZ across species are NE cells and RGCs, which dominate in non-mammalian cortices. In lissencephalic mammals there are also SNPs and recent evidence suggests there are basally dividing progenitors, named outer radial glial cells (oRG), which are derived from RGCs and can undergo asymmetric divisions to produce neurons (Wang et al. 2011). These basally dividing progenitors exhibit major differences to the OSVZ progenitors, expressing Tbr2 and generally dividing symmetrically (oRGs are relatively sparse).

Figure 1.2

Gyrencephalic cortices have a greatly expanded SVZ, which can be split into two cytoarchitecturally distinct zones, the ISVZ and OSVZ (Figure 1.2c). This expansion cannot solely come from progenitors dividing apically, due to limitations of space, suggesting it instead occurs from division of OSVZ progenitors (Fietz et al. 2010; Hansen et al. 2010). The NE cells and RGCs of the gyrencephalic cortex resemble those of a lissencephalic cortex. The ISVZ progenitors resemble the BPs present in the mouse; however there appear to be very few, if any, SNPs within a gyrencephalic cortex. It is thought the OSVZ is responsible for the expansion of the cortex, as it is not found in lissencephalic animals. The key characteristic of OSVZ progenitors that enables them to expand the cortex is their similarity to RGCs. They express Pax6, retain their basal process and undergo multiple rounds of asymmetric divisions (Fietz et al. 2010; Hansen et al. 2010). These repeated asymmetric divisions allow expansion of the radial unit, the number of neurons derived from a single RGC (Fietz & Huttner 2011).

At the end of neurogenesis, RGCs switch from neurogenic to gliogenic divisions to generate glia, such as astrocytes, termed gliogenesis (Anthony & Heintz 2008). After this period of gliogenesis, RGCs disappear from the developing cortex, shortly after birth in mammals. NSCs in the adult have glial characteristics and closely resemble astrocytes. These adult NSCs (aNSCs) are derived from embryonic RGCs (Merkle et al. 2004) and are found in the adult SVZ and around the lateral ventricles (Gage 2002), although additional discrete areas of neurogenesis are being discovered, such as the hypothalamus (Migaud et al. 2010).

1.1.4: Different modes of cell division.

To enable the generation of the CNS, NSCs must be able to divide to produce different types of cells at defined times and in different quantities. During the course of neurogenesis, NSCs/NPCs are able to undergo several different types of divisions. Initially, cells divide symmetrically to produce two NE/RGCs, known as a symmetric proliferative division (Figure 1.3a and 1.3b), allowing self-renewal and expansion of the progenitor pool. At the onset of neurogenesis, some cells start to divide asymmetrically, to produce another progenitor cell and a neuron (Figure 1.3a). The relationship between the division angle and daughter cell fate in these asymmetric divisions is extensively studied. Finally, towards the end of neurogenesis, cells undergo symmetrical divisions to produce two neurons, symmetric neurogenic divisions (Figure 1.3a and 1.3b), depleting the progenitor pool. The mode of cell division appears to be a highly regulated process. Cells maintain and regulate

Figure 1.3

their apical-basal polarity, which is coupled to regulation of the angle of the mitotic spindle to ensure correct daughter cell fates. A key feature of asymmetric division vital to the first model (intrinsic regulation within the cell) is the unequal distribution and inheritance of cell fate determinants during mitosis, which critically depends on the establishment of cell polarity in dividing cells. The mode of cell division and the fate of the daughter cells is discussed as part of this first model in the next section.

1.2: Regulation of proliferation and differentiation.

A fundamental characteristic of NSCs is their ability to divide asymmetrically, producing daughter cells with different fates. What determines the fates of the daughter cells, or the mode of cell division, is not yet fully understood. An asymmetric division can occur on two fundamentally different levels, the level of the cell and the level of molecular signalling. This has provided two schools of thought to explain what happens during an asymmetric division; first that there is asymmetric segregation of fate determinants within the cell, creating differential inheritance between the two daughter cells. This is an intrinsic model, with mechanisms based within the cell. The second requires post-division specification, that signalling from the local environment and cell-cell interactions between the two daughter cells confers their fate. These two broad mechanisms are not mutually exclusive and may work in combination to regulate cell fate. The first model occurs at an intrinsic level within the cell and requires fate to be specified prior to completion of cell division. The second model requires post-division specification of fate, in a more stochastic manner. I will examine each model in turn and provide an overview of the evidence for the mechanisms and signalling pathways involved.

1.2.1: Model 1 - Intrinsic mechanism; segregation of fate determinants.

The regulation of cell fate prior to cell division requires the intrinsic segregation of fate determinants. For this to occur the cell must maintain polarity to allow the unequal distribution of its contents in a regulated manner. Once the fate determinants are localised to specific regions of the cell, the angle of division must be precise to ensure their correct inheritance by the daughter cells. To do this the cell would need to control the angle of the mitotic spindle, which would require coupling of the division axis to polarity. As polarity is vital in this model, I will give a brief overview of the polarity of NSCs and its role in

regulating their behaviour, before outlining the evidence supporting this first model of mitotic spindle orientation and segregation of fate determinants.

1.2.1.1: Polarity.

NE and RGCs are bipolar cells with both an apical and basal process; each process has an end foot attached to the ventricular surface and the pial basement membrane respectively (Figure 1.1b). As well as anchoring the cell and enabling INM, the end feet are also sites of signalling, ECM interaction and areas of fate determinant localisation. They are also sites of cell-cell communication, as they contact the neighbouring cells via adherens junctions (AJ).

1.2.1.1 – 1: Apical polarity.

The apical end foot contains the apical zone, a small area of membrane contacting the ventricular surface containing the apical cilia, apical complex, fate determinants and AJs. Each NE cell contains a primary cilia in the apical end foot, which extends into the ventricle to contact the Cerebrospinal fluid (CSF). The cilia can act as a sensor, detecting mechanical movements and signalling molecules, such as Sonic Hedgehog (Shh), and can regulate the cell cycle (Huangfu & Anderson 2005; Quarmby & Parker 2005). Loss of primary cilia in NE cells, via conditional knock-out of functional protein Kif3a, increases brain size by shortening the length of the cell cycle phase G1, allowing progenitors to cycle faster (Wilson et al. 2012). These progenitors express higher levels of Shh pathway components (summarised later and in Figure 1.4) activating Gli and Patched 1, as well as increased cyclinD1 and FGF15. Shortening the length of the cell cycle can be replicated by increasing levels of activating Gli or over-expressing FGF15. In normal development, levels of activating Gli correspond to length of the cell cycle, as high levels of Gli promote a shorter cell cycle length (Wilson et al. 2012). Conditional knock-out of Kif3a in the adult dentate gyrus decreased proliferation and neurogenesis, disrupting expansion of neural progenitors. This was again mediated by Shh signalling and levels of Gli expression (Han et al. 2008).

The apical complex consists of polarity proteins Par3, Par6 and aPKC, which can localise fate determinants to one side of the NSC to allow their differential inheritance during an asymmetric division. Par3 (or mPar3) is a key polarity protein, distributed to the lateral membrane in the apical end feet, where it co-localises on the ventricular surface with junctional protein ZO-1. During mitosis Par3 becomes asymmetrically distributed. If

segregation of Par3 is perturbed, by either knockout or over-expression, progenitors are prevented from dividing asymmetrically (Bultje et al. 2009). Par3 is thought to serve as an intrinsic regulator of Notch signalling via Numb, an inhibitor of Notch activity (summarised later and in Figure 1.4). Over-expression of Par3 up-regulates Notch signalling, promoting proliferation of progenitors, whereas suppression of Par3 down-regulates Notch signalling, promoting neurogenesis (Bultje et al. 2009).

Pals1, another apical complex protein which binds Par3-Par6-aPKC, provides another link between the apical complex and cell fate. The absence of Pals1 results in loss of progenitors, due to their exit from the cell cycle, increasing neurogenesis before the cells undergo rapid cell death. This is in part caused by disruption of the apical complex and AJs as well as loss of primary cilia (Kim et al. 2010).

1.2.1.1 – 2: Basolateral polarity.

NE/RGCs also have a long basal process, with the basal end foot attached to the pial BM. Rakic first described the basal process as a guide for migrating neurons, acting as a scaffold (Rakic 1971). Since then, the basal process has been implicated in many aspects of cell behaviour, from adhesion in the niche to regulation of NSC function. Basal attachment is important for the proliferation and differentiation of progenitors and is maintained throughout mitosis (Noctor et al. 2001; Miyata et al. 2001). The basal end foot is attached to the pial BM via integrins; loss of integrin-β1 results in process detachment and a reduction in RCG and neural progenitor cell (NPC) number due to increased apoptosis (Graus-Porta et al. 2001; Radakovits et al. 2009). Contact with the BM via integrin-β1 has also been shown to be crucial for apical-basal polarity and signalling (Fishell & Kriegstein 2003; Campos et al. 2004).

In contrast to the effect of loss of integrin-β1, disruption of cell attachment to the pial basement membrane by mutation of the nidogen binding site of laminin-γ1 or the absence of α6-integrin or perlecan, has very little effect on progenitor behaviour. There is no effect on RGC proliferation, INM or the orientation of apical cell divisions and neurogenesis. Only neuronal migration and subsequent organisation of the cortex appears to be greatly affected, alongside a small number of ectopically located progenitor cells (Haubst et al. 2006).

Similar to the apical end foot, the basal end foot contains the basal complex, consisting of polarity proteins Lgl/Dlg/Scb (Lethal giant larvae/Discs large/Scribble) and Numb. Deletion of Lgl1 resulted in hyperproliferation of progenitors and mislocalisation of Numb (Klezovitch et al. 2004). Progenitors were unable to segregate Numb, preventing them from dividing asymmetrically and differentiating. They fail to exit the cell cycle and eventually undergo apoptosis (Klezovitch et al. 2004).

1.2.1.1 – 3: Inheritance of polarity.

Both the apical and basal processes are inherited during cell division, differentially so during an asymmetric division. However which daughter cell, the NSC or the daughter fated to become a neuron, inherits which process varies between species. There is some evidence to suggest the basal process is split during symmetric divisions (Kosodo et al. 2008). Splitting occurred in a basal to apical direction and was followed by inheritance of the basal process by one daughter cell (Kosodo et al. 2008) (studied in mouse and chick).

The apical process can also be spit during division and has been shown to be inherited by both daughter cells (Konno et al. 2008). In the mouse both LGN knock-down and Insc over-expression result in the apical process being inherited by only one daughter cell in an asymmetric division (Konno et al. 2008). The daughter cell without the apical process does not extend a new one and differentiates, whereas the daughter retaining the basal process remained a progenitor, despite its abnormal location (Konno et al. 2008).

There is some controversy over whether inheritance of the apical or basal process is indicative of cell fate. Cells inheriting complete processes are able to self-renew, generate BPs and differentiate; suggesting architecture is not the only factor to determine cell fate. In support of this, randomisation of the spindle via LGN perturbation does not affect cell fate, but causes basally located proliferations (Morin et al. 2007). However, evidence from the Zebrafish neural tube contradicts this. Par3, a marker of the apical domain, can be asymmetrically inherited from an oblique or horizontal division. The daughter cell that inherits the apical domain becomes a neuron and the other daughter, which inherited the basal process, extends a new apical process and remains a stem cell (Alexandre et al. 2010). This suggests asymmetric inheritance of a subcellular domain correlates highly with asymmetric daughter cell fate.

This is consistent with later findings in the chick neural tube (Das & Storey 2012) where higher levels of Notch activity are seen in the basal daughter. However, the apical complex has been shown to promote proliferation and self-renewal and Par3 is absent from Tuj1 positive neurons. The apical daughter must therefore lose the apical complex before it can differentiate and the basal daughter must synthesise a new apical complex and process to remain a stem cell (Das & Storey 2012).

1.2.1.1 – 4: Planar cell polarity.

As well as apical-basal polarity, the neuroepithelium has planar cell polarity (PCP), the organisation of the cells within the plane of the epithelium. Various PCP components have been studied in neurogenesis. Knock-out of PCP protein Vangl2 (homologue of Drosophila Strabismus) causes neural progenitors to prematurely exit the cell cycle, depleting the progenitor pool. The NE cells are unable to asymmetrically localise the LGN/NuMA/Pins complex, involved in spindle orientation, increasing neurogenic divisions which have a vertical cleavage plane (Lake & Sokol 2009). Vangl2 is thought to mediate this effect as it acts as a permissive cue to allow asymmetric localisation of the LGN complex (Lake & Sokol 2009).

The PCP pathway has also been shown to orient the mitotic spindle via Wnt pathway components Frizzled and Dishevelled (Dsh) (Summarised later and in Figure 1.4). Dsh can activate the NuMA-dynein complex to mediate spindle orientation, suggesting NuMA acts downstream of the Wnt PCP pathway to orientate cell divisions (Ségalen et al. 2010). Another component of the PCP pathway, Scribble (Scb), part of the Lgl/Dlg/Scb complex, has been shown to regulate spindle orientation in Drosophila neuroblasts and in the zebrafish neural tube (Albertson & Doe 2003; Žigman et al. 2011). However in the zebrafish, Scb is thought to orient the spindle via interaction with cadherins, suggesting that cell-cell interactions may help to orient the spindle alongside polarity.

1.2.1.2 Angle of division.

1.2.1.2 - 1 Mitotic spindle orientation.

Control of mitotic spindle orientation requires the proper assembly and positioning of the spindle and the coupling of the axis of division to cell polarity. Spindle orientation can be set by anchoring the centrosome to the cell cortex, inherited from mother to daughter cell,

or the spindle can form randomly and then rotate to its desired plane. There is evidence to show all three occur in stem cell divisions.

Drosophila neuroblasts undergo repeated rounds of asymmetric divisions to generate a larger apical daughter, the neuroblast, and a smaller basal daughter, the ganglion mother cell (GMC), which undergoes one further round of symmetrical division to generate two neurons. This type of asymmetric division in neuroblasts is generated by the segregation of fate determinants via the apical complex, Par3-Par6-aPKC homologues, which is localised to the cortex via extrinsic signals from the surrounding epithelia. Initially the spindle is not orientated along the asymmetric axis in the neuroblast, but it re-orientates before the start of anaphase (Yamashita & Fuller 2008).

This is in contrast to Drosophila germ stem cells (GSCs), where the mother centrosome is anchored to the cell cortex that contacts the hub cell, the supporting cell in the niche. Here the spindle is set up with the correct orientation, inherited by the daughter cell via inheritance of the mother centrosome (Yamashita et al. 2007). The mother centrosome is consistently inherited by the daughter cell that remains a GSC, linking centrosomes to cell fate. The daughter that retains the mother centrosome remains anchored to the hub cell and is retained in the niche. The other daughter cell loses attachment to the niche, exits and differentiates. The converse is true in Drosophila neuroblasts, where it is the daughter centriole that is retained by the daughter cell which remains a stem cell, meaning the GMC inherits the mother centriole (Januschke et al. 2011). This separation of the centrioles is also tightly correlated with cell fate.

Asymmetric inheritance of the centrosomes has also been linked to cell fate in the mouse VZ. During the peak of neurogenesis, the majority of cell divisions are asymmetric. The mother centriole is preferentially inherited by the daughter retained as a RGC, whereas the daughter centriole is inherited by the daughter fated to undergo neurogenesis (Wang et al. 2009). The older mother centrosome is linked to interacting proteins, such as cenexin, as well as microtubules, but the daughter centrosome is much younger so is yet to acquire the same binding partners. This difference in ability to interact with proteins may be why the mother centriole is preferentially inherited by the self-renewing daughter. Evidence to support this can be seen in loss of mature centriole interacting protein Ninein, disrupting this process, leading to premature depletion of the progenitor pool (Wang et al. 2009).

Inheritance of spindle orientation is dependent on microtubules; transient loss of microtubule stability in *Drosophila* neuroblasts disrupts polarity, allowing the apical complex to form in any location. Once microtubule stability is restored, the new location of the apical complex sets up a new axis of division (Januschke & Gonzalez 2010) suggesting this is regulated in a cell-autonomous manner. Microtubule stability is also required for the inheritance, or memory, of the plane of division and spindle orientation (Januschke & Gonzalez 2010).

Rotation of the mitotic spindle has been observed in the mouse VZ, where increased spindle oscillations were observed prior to an asymmetric division, increasing the time taken to reach anaphase. This suggests symmetrical divisions may be the default mechanism as more signalling and time is required to reorient the spindle for an asymmetric division (Haydar et al. 2003).

1.2.1.2 - 2 Regulation of spindle orientation.

There are many studies into the regulation of spindle orientation and the mechanisms that control it. The Huntington protein (Htt) is localised to the mitotic spindle as it interacts with microtubules and their motor proteins. Depletion of Htt results in dispersion of the motor proteins dynein and NuMA around the spindle poles, instead of being localised to them, modifying spindle angle and promoting neurogenesis (Godin et al. 2010).

The LGN/NuMa/Gα complex interacts with the mitotic spindle. LGN, a G protein regulator leucine-glycine-asparagine repeat protein, has been shown to be necessary for planar spindle orientation in both mouse and chick NE cells (Morin et al. 2007; Konno et al. 2008) where it interacts with NuMA (Nuclear and Mitotic Apparatus protein) and with the α-subunit of G proteins. This complex can regulate spindle orientation by linking the microtubules to the cell cortex. In the chick NE cells, LGN and NuMA have been shown to localise in the lateral belt of the cell cortex during mitosis. Knock-down of this complex results in spindle mis-orienatation and disorganisation of the neuroepithelium (Peyre et al. 2011). Knock-down of LGN alone in mouse NE cells resulted in randomised spindle orientation, generating abnormal basally localised progenitors, but not affecting neurogenesis (Konno et al. 2008). Inscuteable (Insc) links the apical complex to LGN and is known to regulate vertical cleavage plane divisions (thought to be symmetrical, Figure 1.3). Insc over-expression in the NE cells also results in rotation of the spindle and generation of abnormal basal progenitors (Konno et al. 2008). The ability of LGN to bind both NuMA and Insc is thought to be mutually

exclusive. NuMA targets LGN to the mitotic spindles, but Insc can displace NuMA from LGN disrupting the orientation of the spindle (Zhu et al. 2011).

This complex and its function appear to be conserved as the homologues in invertebrates also regulate spindle orientation (Gönczy 2008). LGN is the mammalian homologue of *Drosophila* Pins. As in vertebrates, the apical complex forms a crescent on one side of the cell. However, unlike the lateral localisation in vertebrates which allows for spindle rotation, the apical crescent in *Drosophila* anchors the spindle for a fixed orientation (Peyre et al. 2011). The *Drosophila* homologue of NuMA, Mud, has also been shown to interact with Pins and bind microtubules. In neuroblasts, loss of Mud results in the spindle failing to align correctly, causing symmetrical divisions and over-proliferation of the neural cells (Bowman et al. 2006).

Unlike *Drosophila* neuroblasts, their NE cells normally divide symmetrically and are unable to divide asymmetrically. However, NE cells can divide asymmetrically if their AJs are disrupted. APC, Adenomatous polyposis coli, a component of the Wnt pathway (summarised later and in Figure 1.4) is able to bind to AJs and microtubules. Interactions between APC and the AJs promote spindle orientation for a symmetric division. This prevents the apical complex binding the microtubules and the division being orientated for an asymmetric division, overriding the NE cells ability to divide asymmetrically (Lu et al. 2001).

The apical complex also regulates spindle orientation. Polarity protein Par3 is inherited unequally during an asymmetric division. The Par3 N-terminal contains a microtubule binding domain, which functions to bundle and stabilise microtubules and can be supressed by the C-terminal of Par3, playing a crucial role in the polarisation of neurons (Chen et al. 2013). Deletion of Par3 *in vitro* causes randomisation of spindle orientation. However this is not seen in mammary epithelial cysts, where loss of Par3 shifts spindle orientation to predominantly perpendicular (thought to be asymmetric - Figure 1.3) (Hao et al. 2010). In both cases, deletion of Par3 results in loss of aPKC from the apical surface. Par3 and aPKC usually exclude Pins/LGN from the apical surface via phosphorylation. Without this exclusion, microtubules can associate with Pins around the entire cortex, randomising the spindle angle.

Atypical PKC (aPKC) is another component of the apical complex. In both *Drosophila* and mammalian cells *in vitro* aPKC is required for the apical exclusion of Pins (LGN in mammals)

and correct spindle orientation for a symmetrical division, as deletion of aPKC randomised spindle orientation (Guilgur et al. 2012; Durgan et al. 2011). However in the chick embryo aPKC is not found to regulate spindle orientation or localisation of LGN (Peyre et al. 2011), suggesting regulation of the spindle is not entirely conserved.

1.2.1.3 Fate determinants.

Although the mode of cell division is an important aspect of NSC behaviour, very little is known about the fate determinants that are asymmetrically segregated. Several candidates have been identified in different model systems. In the *Drosophila* NSCs the transcription factor Prospero has been suggested as a fate determinant. Prospero is required for activation of neural differentiation genes and repression of stem cell genes and is only found in a nuclear location in neuroblasts, not in GMCs (Choksi et al. 2006). Loss of Prospero prevented NSCs differentiating, causing them to generate two stem cell daughters (Choksi et al. 2006).

In vertebrates, micro-RNA 124 has been suggested as a candidate fate determinant. It is expressed in the adult SVZ in both the transit-amplifying precursors and the new-born neurons. Blocking micro-RNA 124 function prevented neurogenesis, while its over-expression depleted the NSC pool (Akerblom et al. 2012). In the human Pax6 has also been suggested to act as a fate determinant for neuroepithelial cells (Zhang et al. 2010).

Despite the identification of some fate determinants, how they would be asymmetrically segregated in not clear either. They could be targeted to the apical or basal complexes or be separated just before mitosis. Evidence from *Drosophila* suggests some fate determinants are localised rapidly by phosphorylation. Prospero and its scaffold protein Miranda are initially apically localised in the cell, as Miranda is phosphorylated by aPKC, blocking it from binding to the cell cortex. Prior to mitosis, Miranda is dephosphorylated, allowing both it and Prospero to bind to the basal complex (Sousa-Nunes & Somers 2010), suggesting segregation of fate determinants can be a rapid process which may occur just prior to mitosis.

1.2.1.3 Summary.

It is clear that spindle orientation and polarity appear to be regulated and important during stem cell divisions. Although there are studies that support the role of fate determinants and their unequal segregation, the main evidence against this intrinsic model is found in studies randomising the spindle angle. If this first model was correct, the angle of cell division would correlate to cell fate. It would therefore be expected that randomising spindle angle would randomise cell fate, effecting neurogenesis. However randomising the mitotic spindle in the mouse, by various mutations, had no effect on neurogenesis (Konno et al. 2008; Sakai et al. 2012) and only effected the numbers of basally dividing progenitors produced (Konno et al. 2008; Postiglione et al. 2011). As mitotic spindle orientation is regulated, is may be that the regulation of spindle angle may not be important for the segregation of fate determinants but for the correct location of the daughter cells. Spindle angle could determine the location of the cell due to anchoring via inheritance of the apical or basal processes, or by the positioning of cells within signalling environments – the second model proposed - an extrinsic mechanism.

1.2.2 - Model 2: Extrinsic mechanism - Post-specification via signalling.

The second model proposes that daughter cell fate is specified after cell division. This could occur through signalling by external factors and cell-cell or cell-niche interactions. This model suggests daughter cell fate decisions are more stochastic, the fate of the two similar daughter cells is decided through the signalling environment they are in, not set out prior to cell division. This requires extrinsic signals and cell-cell communication to ensure the two cells adopt different fates during an asymmetric division. I will outline the key extrinsic factors and signalling pathways involved in neurogenesis, their role in regulating NSC proliferation and differentiation and the evidence supporting this model.

1.2.2.1 Extrinsic signalling pathways.

In addition to ECM within the niche, there are several major developmental pathways known to signal to and between NSCs. Many of these have been well studied in neurogenesis and include diffusible signals and those that require cell-cell interactions. I have summarised the key pathways involved and the evidence to support their role in the regulation of NSC behaviour (Figure 1.4). The first pathway I will discuss is Notch signalling,

Figure 1.4

the major cell-cell interacting pathway in neurogenesis that has been extensively studied throughout development.

1.2.2.1 – 1: Notch signalling.

Notch is a large transmembrane receptor that binds to membrane bound ligands Delta, Serrate and Jagged. As both the ligand and receptor are membrane bound, Notch signalling requires cell-cell contact. Ligand binding promotes proteolytic cleavage by γ-secretase and endocytosis of the receptor, creating a soluble fragment, Notch intracellular domain (NICD). NICD is cleaved by γ-secretase upon activation and translocates to the nucleus where it interacts with CBF1, a transcriptional regulator which forms a complex with co-activator Mastermind (Mam) (Figure 1.4) (Fortini 2009). The Notch signalling pathway and its components are highly conserved, found in both *Drosophila* and mouse (de la Pompa et al. 1997).

A major characteristic of Notch signalling is lateral inhibition, a transcriptional feedback that enables two identical cells to adopt different fates. Initially, the two cells express similar levels of both Notch and Delta. Over time, a small stochastic difference alters this balance, which is then amplified by transcription. Notch signalling activates transcription of Enhancer of Split, repressing the activity of the Achaete-scute complex, which in turn regulates Notch and Delta levels. The signal-sending cell (Delta expressing) up-regulates Delta and down-regulates Notch, the signal-receiving cell (Notch expressing) does the opposite (Fortini 2009). This enables a salt and pepper pattern of expression of Notch and Delta within a population of cells.

Notch signalling is known to maintain neural stem cells and inhibit neurogenesis, although it is also a major signalling pathway in development and regulates many types of stem cells, not just neuronal. In the CNS NICD-CBF1 activates target genes such as the Hes family members Hes1 and Hes5, which act to antagonise proneural genes. The Hes transcriptional repressors are normally induced by Notch activation and are able to bind their own promoters, allowing a negative feedback loop. Hes transcription factors can also promote neural gene expression. Hes6 acts a repressor of Hes5, co-operating with proneural genes to promote neurogenesis (Fior & Henrique 2005).

CBF1 activity has been seen only in NSC of the VZ, not in the basal progenitors or SVZ. Notch signalling within the VZ activates CBF1 and promotes stem cell maintenance. Loss of

CBF1 from the VZ NSC converts them to neurogenic progenitors, but forced activation of CBF1 does not revert basal progenitors back to NSC (Mizutani et al. 2007).

Conditional knock-out of CFB1 resulted in complete loss of NSCs and the VZ in both the embryonic and adult telencephalon (Imayoshi et al. 2010). However, in the early postnatal telencephalon loss of CFB1 initially increases the number of dividing cells by expanding the transit-amplifying pool of progenitors, which eventually results in depletion of the progenitor pool (Imayoshi et al. 2010). Further studies in the adult have shown that Notch-1 is required for adult NSC in their active, but not quiescent state. Depletion of Notch-1 signalling causes a selective loss of active NSCs in the adult SVZ but does not affect quiescent NSC numbers, until they become activated on regeneration, both of which result in a reduction in neurogenesis (Basak et al. 2012). Notch activation is also required for the maintenance of quiescent NSCs upon AraC administration (Imayoshi et al. 2010), which depletes the pool of transit-amplifying progenitors. The NSCs and their progenitors are known to interact within the adult SVZ. Epidermal Growth Factor (EGF) and its receptor (EGFR) are expressed on NPCs and exert a non-cell autonomous effect on the Notch activity of their neighbouring NSCs. Over-expression of EGFR reduces proliferation of NSCs in the SVZ by decreasing Notch activity, via the ubiquitination of Notch by Numb (Figure 1.4) (Aguirre et al. 2010).

There are several molecules that act to promote neurogenesis by down-regulating Notch. One of these is neuro-protective gene 7, or Botch. Botch is developmentally expressed in a similar spatial and temporal pattern to Notch-1 and interacts with its extra-cellular domain, regulating its processing (Figure 1.4). Over the course of neurogenesis levels of Botch expression at the cell surface increase as Notch-1 decreases, as more neurons are being generated. As expected, over-expression of Botch increases neurogenesis, in opposition to Notch signalling (Chi et al. 2012).

Numb, another suppressor of Notch signalling, is expressed basally in NE cells. Numb acts by binding to NICD and disrupting its translocation to the nucleus, permitting neuronal differentiation (Wakamatsu et al. 1999; Wai et al. 1999) (Figure 1.4). Asymmetric inheritance of Numb can be used to modulate Notch signalling; Numb becomes localised to one end of the mitotic spindle and is segregated into only one daughter cell. This is also seen with Neuralised, a Drosophila E3 ubiquitin ligase that promotes Delta endocytosis, enhancing its ability to activate Notch (Fortini 2009).

Notch-Delta signalling has also been implicated in the switch from proliferative to neurogenic divisions of NE cells. Delta1 is expressed in progenitors prior to neurogenesis, in the salt and pepper pattern indicative of lateral inhibition. In the chick spinal cord, activation of Notch represses neurogenesis, but inhibition of Notch only arrests proliferation. Delta1 expression however is sufficient to promote neurogenesis and regulates expression of the neurogenic marker Tis21. Mutual Delta-Notch activation is thought to occur in the stem cell pool of the developing spinal cord. The cell autonomous actions of Delta, in part on Tis21, in addition to Delta-Notch lateral inhibition permits the transition from proliferative to neurogenic divisions, leading to neuronal differentiation (Hämmerle & Tejedor 2007).

In the mammalian telencephalon, Delta-like ligands are expressed on post-mitotic neurons and basal progenitors, not in the RGCs (Campos et al. 2001). Mind bomb (Mib), a RING-type E3 ubiquitin ligase that modulates Delta endocytosis, is also expressed in the basal progenitors and neurons. Inactivation of Mib disrupts the apical membrane causing RGCs to lose attachment to both the apical and basal surface, have decreased expression levels of Notch target genes such as Hes and undergo premature differentiation. Mib is expressed in a temporal manner, co-localised with progenitor marker Tbr2 in the VZ and SVZ, but is not expressed in the CP (Yoon et al. 2008). In addition to the lateral inhibition occurring between two neighbouring RGCs via Notch-Delta, there may also be a feedback loop between RGCs and new-born neurons to maintain RGCs in their stem-like state, via other interacting proteins such as Mib.

1.2.2.1 – 2: Wnt signalling.

Wnt signalling, known to mediate many aspects of development, has conflicting roles in neurogenesis. One of these is the regulation, in part, of proliferation of intermediate/basal progenitors before undergoing neurogenesis. In the canonical Wnt signalling pathway, Wnt ligand binds to Frizzled-LRP5/6 receptors at the cell membrane, activating the Dishevelled family proteins which inhibit formation of the axin/GSK-3/APC complex (Figure 1.4). This complex usually promotes degradation of β-catenin. Ligand-mediated inhibition of this complex allows β-catenin to stabilise in the cytoplasm and enter the nucleus to promote gene expression with Lymphoid enhancer-binding factor (LEF) and T-cell factor (Tcf) (Figure 1.4). The conflicting reports of the role of Wnt signalling in neurogenesis suggest its effect may be context dependant. *In vivo*, increased expression of soluble ligand Wnt3a increased

proliferation and neurogenesis, causing the appearance of neural rosettes within the neuroepithelium. Wnt3a was found to expand RGCs but promote early differentiation of intermediate progenitors. This premature differentiation is also observed upon expression of dominant-active LEF and β-catenin, whereas expression of Wnt pathway inhibitor dickkopf 1 (Dkk1) inhibits neurogenesis (Munji et al. 2011).

There is also evidence to suggest canonical Wnt signalling normally promotes neurogenesis in the cortex; Wnt7a and receptors Fz5 and Fz8 are expressed in the VZ. Addition of Wnt7a to NE cell cultures increased neurogenesis via β-catenin and expression of a stabilised β-catenin *in vitro* also promoted neurogenesis, even in the presence of FGF2 (known to prevent differentiation). However, this increase in neurogenesis occurs at the expense of proliferating cells. The same effect is seen upon expression of the stabilised β-catenin *in vivo*, by *in utero* electroporation into the VZ (Hirabayashi et al. 2004). The cells expressing the stable β-catenin undergo increased neurogenesis. This effect on neurogenesis is due to the β-catenin/TCF complex interacting with the promoter of proneural gene Neurogenin1 (Ngn1) to directly regulate its transcription. It is an effect due to canonical Wnt signalling, not β-catenin's role in AJ adhesion.

However, the response to Wnt/β-catenin is different at different time points. Stabilised β-catenin expressed in cortical NE cell cultures from different ages has opposite effects. At E10.5, prior to neurogenesis, expression of the stabilised β-catenin reduced neurogenesis, but its expression promoted differentiation later at E13.5, after the onset of neurogenesis (Hirabayashi et al. 2004). Depletion of β-catenin in the telencephalon medial ganglionic eminence reduces proliferation and promotes premature neurogenesis (Gulacsi & Anderson 2008).

Wnt signalling is stage specific, maintaining patterning prior to neurogenesis, mitogenic during early neurogenesis and promoting differentiation later in neurogenesis. The contradictory evidence for the role of the Wnt pathway in promoting both symmetric proliferative divisions and neurogenesis is due to differential effects on the different sub-types of progenitors.

1.2.2.1 – 3: Other signalling pathways and their interactions with Notch and Wnt.

- 3.1 Shh.

The Hedgehog signalling pathway is important in many aspects of development and neurogenesis. One family member, Sonic Hedgehog (Shh), plays a key role in ventral patterning of the developing CNS, clearly demonstrated by its secretion from the notochord during neural tube patterning. Shh is produced by both the notochord and floor plate, which are both ventral structures within the developing CNS. It is known that Shh is required for induction of the floor plate and differentiation of ventral neurons. Shh is also expressed in the proliferative zones of the developing cortex, where it is thought to regulate the cell cycle. Lack of Shh signalling within these areas reduces neurogenesis and perturbs cortical patterning (Komada et al. 2008).

Shh is a proteolytically cleaved protein. Cleavage produces two secreted proteins, the N terminal (N-Shh) that mediates signalling, and the C terminal (C-Shh) that contains protease activity (Martí & Bovolenta 2002). Shh binds to the Patched (Ptc) membrane receptors, preventing the normal inhibition of smoothened (Smo). Smo is the signalling component of the pathway and is related to G protein receptors. The last steps in the signalling pathway are the transcription factor family Gli (Martí & Bovolenta 2002) (Figure 1.4). Glis can be either transcriptionally activating or repressing depending on upstream Shh signalling.

The well-known role of Shh in CNS development is the neuronal specification of the ventral CNS. Shh is required both short and long range, building a morphogen gradient across the neural tube. This gradient allows the creation of different responses at different threshold concentrations, effecting expression levels of the Pax, Nkx and Dbx family of genes (Martí & Bovolenta 2002). Shh signalling also plays a role in specification of oligodendrocytes, which are generated from either side of the ventral floor plate, and also has a role in the dorsal CNS, being expressed in cortical structures during late development where it is involved in the generation of granule cells (Martí & Bovolenta 2002).

Over-expression of Shh in the mouse spinal cord increases proliferation of progenitors and inhibits differentiation, but only during the period of neurogenesis. Activation of the Shh pathway is not sufficient to promote proliferation after the neurogenic period, suggesting there is a temporal period of competence of NSCs to Shh signalling (Rowitch et al. 1999).

Shh signalling occurs alongside Notch signalling, with evidence to suggest interaction between the two pathways. Deletion of Patched 1 (Ptch1), a negative regulator of Shh, promotes Shh signalling and symmetric proliferative divisions of RGCs, expanding the progenitor pool. Conversely, inactivation of Notch signalling via loss of CBF1 promoted symmetric neurogenic divisions, resulting in premature differentiation of progenitors. The inactivation of Ptch1 modulated Notch downstream targets, up-regulating Blbp and Hes1. Both excess proliferation and up-regulation of Notch targets by Ptch1 can be rescued by inactivation of Notch pathway component CFB1, demonstrating co-operation between the Notch and Shh pathways in proliferation and differentiation (Dave et al. 2011).

Shh also interacts with the Wnt pathway in a temporal and spatial manner. Shh expression is a major characteristic of the ventral floor plate and in the hindbrain, where high Shh levels are maintained, which inhibits neurogenesis. However, in the midbrain, Wnt signalling represses Shh to permit the generation of dopaminergic neurons. Removal of Shh promoted neurogenesis in the hindbrain floor plate, whereas excessively high levels of Shh inhibits both proliferation and neurogenesis in midbrain cells *in vitro* (Joksimovic et al. 2009).

-3.2 FGF.

Fibroblast growth factors (FGFs) and their tyrosine kinase receptors (FGFRs) are part of a complex signalling pathway involved in the regulation of a wide variety of functions in both the embryo and adult. Vertebrates have 22 FGF genes and five FGFR genes, which interact with co-receptors HSPGs. FGFs are also able to interact with non-canonical co-receptors, including ECM molecules such as integrins and NCAMs (Polanska et al. 2009).

FGF2 is expressed within the neuroepithelium and is at its highest levels at the apical surface during early neurogenesis. Loss of FGF2 reduced proliferation of NE cells and later reduced the number of neurons in the cortical layers (Raballo et al. 2000). Injection of FGF2 into the ventricles of E15.5 mice increased the volume of the cortex and neuron number by increasing the rounds of NE cell divisions. Injection of FGF2 antibodies had the opposite effect, reducing cortex volume and neuron number by reducing the proportion of dividing progenitors (Vaccarino et al. 1999). Expansion of FGF8 expression, which is usually anterior in the developing CNS, increases the anterior domain of the neocortex and decreases the posterior domain, with the converse true for reduction of FGF8 expression. Introducing a

second source of FGF8 in the developing cortex duplicates the axis (Fukuchi-Shimogori 2001), providing strong evidence for the role of extrinsic factors influencing neurogenesis.

FGF is another example of one pathway having multiple roles in neurogenesis, effecting both patterning and cortical growth, and like Shh it also interacts with the Notch pathway. Knock-outs of FGFR1 and 2 reduce the volume and area of the cortex by premature neurogenesis, depleting the progenitor pool (Rash et al. 2011). Expression of a dominant negative FGFR also increases neuronal differentiation and RGC cycle exit. Both knock-out of FGFR and expression of the dominant-negative decreased levels of NICD and Notch1, decreasing Hes1 levels. A constitutively active FGFR promotes self-renewal and inhibits differentiation, but to a lesser extent than active Notch (NICD), suggesting FGFR acts upstream of Notch signalling (Rash et al. 2011).

-3.3 BMP.

Bone morphogenetic proteins (BMPs) are a subclass of the TGFβ family, which have multiple functions in both embryonic development and neurogenesis, including neural induction. Within the cortex, BMPs have been shown to negatively regulate cell number in the VZ. Both BMPs and Noggin, an antagonist of BMP signalling, are expressed throughout the developing cortex. BMP2 and 4 are expressed along the ventricular surface. In E13 cortical cells *in vitro*, around the onset of neurogenesis, BMPs inhibit proliferation and promote cell death, whilst moderate doses of BMPs to E16 cortical cells promoted neurogenesis and inhibited gliogeneis. However, high doses of BMP to these cells still reduced cell number (Mabie et al. 1999). If cortical cells are older still, BMPs can now promote the generation of astrocytes. Addition of Noggin to these cultures promoted oligodendrogenesis, inhibiting neurogenesis (Mabie et al. 1999), suggesting BMP signalling regulates the generation of all the major cell types within the embryonic CNS, in a context-dependant manner.

BMP7 is produced in areas adjacent to the cortex and is released into the CSF. Loss of BMP7 decreases the thickness of the cortex by reducing neurogenesis. It also disrupts RGC attachment to the pial BM. This causes a reduction in the number of progenitors, through decreased survival, proliferation and self-renewal, all of which can be rescued by addition of BMP7. Additionally, BMP7 was found to maintain Ngn2 expression in the SVZ. Loss of BMP7 reduced the level of Ngn2 transcript present, suggesting a role in promoting neurogenesis via the CSF (Segklia et al. 2012).

As with Shh and FGF signalling, the BMP pathway also interacts with Notch. In the olfactory epithelium, activating BMP or Notch signalling reduces the thickness of the olfactory nerve. Both activation and inhibition of BMP signalling reduced the number of migrating neurons, which is also observed upon activation of Notch. BMP signalling can generate these two opposing effects as increased BMP levels restrict neuronal movement whereas reduced BMP levels inhibit neuronal differentiation. BMP activation inhibited Notch activity and expression of Noggin, but promoted Hes5 and Delta1 expression. This interaction is not reciprocal as Notch does not appear to affect BMP signalling. (Maier et al. 2011).

-3.4 Robo-Slit.

The Robo-Slit pathway has also been implicated in neurogenesis. The secreted Slit proteins bind to their Roundabout (Robo) receptors, part of the Ig adhesion molecule super-family, to influence adhesion and cytoskeletal dynamics. They were originally discovered as axon guidance cues, but are also involved in many morphogenetic processes (Andrews et al. 2007).

In the mouse Robo-Slit signalling normally promotes self-renewal in VZ progenitors, loss of both Slit and Robo decreases mitotic cells and increases expansion of IPs. However, this does not increase neurogenesis as the cell cycle of IPs lengthens and they fail to retract their apical process due to an increase in N-cadherin. This increase in IPs and decrease in mitotic cells suggests Robo-Slit signalling mediates the mode of cell division. Loss of Robo affected Notch signalling, decreasing the levels of Hes1 expression, suggesting Robo signalling normally modulates transcription of Hes1 (Borrell et al. 2012).

-3.5 Retinoic Acid.

RA treatment of ESCs up-regulates neural genes and RA has also been shown to promote neurogenesis in EGF-responsive stem cells in culture (Siegenthaler et al. 2009; Wohl & Weiss 1998). NE cells within the developing cortex express receptors for Retinoic Acid (RA), RAR and RXR, but not the enzymes required for RA synthesis. RA is instead synthesised and released by the meninges, a process required for early forebrain morphogenesis.

Foxc1 mutants fail to generate cortical meninges, causing defective expansion of the cortex and decreased neurogenesis, despite the Pax6 positive NSC layer being normal. This lack of cortical expansion is due to loss of RA release from the meninges, due to loss of the synthesising enzymes, which normally promotes NSC exit from the cell cycle. The effect of

RA on cell cycle exit and neurogenesis requires the orphan nuclear hormone receptor CoupTF1, which is expressed in a ventral to dorsal gradient. CoupTF1 is up-regulated in Foxc1 mutants and is necessary for RA rescue. Its over-expression can also partially rescue the Foxc1 mutants (Harrison-Uy et al. 2013). Loss of the enzymes required for RA synthesis also leads to the lack of cortical expansion seen in the Foxc1 mutants (Siegenthaler et al. 2009) and both of these mutant phenotypes can be rescued by addition of RA. This data suggests that RA secretion from the meninges acts as a temporal trigger to switch NE cells to neurogenic divisions and as a potent neural differentiation signal.

1.2.3 - Summary of evidence for the two models.

There is evidence to support both the intrinsic and extrinsic models of regulation of NSC proliferation and differentiation. The first model relies on the segregation of fate determinants, very few of which have been identified and little is known about the mechanism of their segregation. However, there is evidence that the angle of cell division is regulated and coupled to polarity, although randomising mitotic spindle orientation did not have any great effect on neurogenesis, but only a mild effect on the generation of basal progenitors (Konno et al. 2008; Postiglione et al. 2011).

The second model of post-division specification is supported by evidence of extrinsic factors regulating daughter cell fate. Extrinsic signals from neighbouring cells and the niche environment are able to promote daughter cell fate in a more stochastic manner than the first model. However, these models are not exclusive of one another and it seems likely that daughter cell fate is regulated by a combination of both models; perhaps segregation of determinants between the daughter cells may differentiate their ability to respond to extrinsic signals, or angle of division may position the daughter cells within a specific area of signalling.

1.2.4 - Co-operation of intrinsic and extrinsic signals?

I have previously summarised the intrinsic and extrinsic models separately, but it is important to realise that these two mechanisms may in fact work in concert with each other to regulate proliferation and differentiation. There is some evidence that extrinsic signals may act to regulate the mode of cell division, such as the role of Robo-Slit signalling

mentioned before, and that some extrinsic signalling pathway mediators are asymmetrically inherited, such as Numb and Neuralised mentioned previously. However, the strongest evidence for co-operation between these two models is seen in INM and the regulation of spindle orientation by ECM.

1.2.4.1 - Interkinetic Nuclear Migration.

INM is a key characteristic of NSCs and is required for neurogenesis. The exact purpose of INM is unknown, but it is clear that it plays a vital role in the regulation of NSC proliferation and differentiation. The major function of INM is to position the nuclei of NSCs at distinct locations during S phase and M phase. There is no evidence to show INM has an effect on progression through the cell cycle, but INM is dependent on cell cycle progression. Pharmacological arrest of the cell cycle in S, G2 or M phase inhibits INM (Ueno et al. 2006). Disrupting INM has also been shown to effect the timing of neural production, causing more early born neurons to be generated at the expense of later born subtypes (Del Bene et al. 2008). One prediction is that INM may position the NSCs in the correct signalling areas to determine daughter cell fate.

INM ensures NSCs divide apically, basally dividing progenitors do not undergo INM, suggesting location of daughter cells is important in cell fate. Mutation of the zebrafish homologue of laminin-γ1 (*tab*) impairs INM and orientation of mitosis, resulting in abnormal basal location of mitoses. The pial BM is also affected, loss of laminin-1 results in a thinner and disorganised BM. The key receptors of the laminins are the integrins and one of their down-stream targets is focal-adhesion kinase (FAK), which becomes phosphorylated upon activation of the pathway (discussed in detail later and in Figure 1.7). Phosphorylated FAK (pFAK) is found at both the apical and basal end feet, but levels in the *tab* mutant are far lower than wild-types. Knock-down of FAK results in a similar phenotype to the *tab* mutant, including mislocalised mitoses. This is due to a migration defect, indicating INM is altered in these mutants causing the cells to divide basally. In both the *tab* and FAK knock-downs, the NE cells retain their apical and basal processes and polarity, but exhibit a randomised spindle angle, which accelerates neurogenesis (Tsuda et al. 2010), suggesting INM is required for the correct fate of the daughter cells.

Myosin II is also required for INM. Addition of myosin II inhibitor blebbistatin impaired the nuclei migration from the apical to basal surface during G1 phase, resulting in an apical shift of S phase nuclei, but no effect on the progression or length of the cell cycle was seen.

Blebbistatin also impaired the basal migration of new-born basal progenitors and increased the number that were neurogenic. This study suggests that the apical to basal phase of INM is controlled by a different mechanism than basal to apical. Myosin II constricts the apical process, forcing the nucleus basally. In the absence of myosin II, this restriction does not occur, allowing the second mechanism to occur, which is most likely dynein driven transport along microtubules (Schenk et al. 2009).

INM positions NSCs apically to divide and disrupting this process greatly perturbs neurogenesis. This suggests the presence of extrinsic cues, located at the apical surface, which the daughter cells must be exposed to. The size of the apical domain of the dividing NSCs may also effect the level of signal they receive. Neurogenic progenitors have a smaller apical domain (Clark et al. 2012) and expansion of the apical domain via Shroom 3 reduces neurogenesis by increasing Notch activity. Loss of either basal complex protein Llgl1 or Shroom 3 activity increases Notch pathway activation, maintaining progenitors and inhibiting neurogenesis. As INM ensures cells undergo mitosis at the apical surface, the relationship between the apical domain and Notch activation could be important in determining cell fate and may require INM to provide signals only during and directly after mitosis (Clark et al. 2012).

A Zebrafish mutant of the motor protein Dynactin-1, *mikre oko* (*mok*), causes disruption of INM, resulting in rapid basal migration and slower apical migration of NE cells. This causes the NE cells to spend less time at the apical surface than those in the wild-type. As Notch signalling is predominantly active at the apical surface, *mok* NE cells are exposed to reduced levels of signalling, exit the cell cycle and prematurely differentiate (Del Bene et al. 2008). Both the Notch receptor, Delta, and the NICD are normally localised in an apical to basal gradient in the Zebrafish neural tube. One function of INM is to expose the cells to this Notch signalling gradient (Del Bene et al. 2008). The premature differentiation phenotype seen in the *mok* mutants can be rescued by activation of the Notch pathway, proving the phenotype is not solely an effect of the dynactin mutant. Normally, NE cells go through the Notch gradient twice in between divisions. Failure of INM creates cells that remain basal for a longer period of time. These cells receive less Notch signalling, making them more likely to differentiate (Del Bene et al. 2008).

This gradient of Notch signalling is also present in both chick and mouse NE cells. In these cells, Notch1 and Delta1 mRNAs fluctuate throughout the cell cycle, being at their lowest

during S phase, correlating with a reduction in Notch activity. Pharmacological blockade of INM results in premature differentiation of NE cells, providing further evidence that INM is required for the temporal control of signalling (Cisneros et al. 2008). If this is the case, does INM regulate NE cells neurogenic state? Notch1 is expressed at the apical surface (Murciano et al. 2002), but is absent in cells undergoing S phase, as are Delta1 and Neurogenin2. NE cells may acquire the ability to express neurogenic genes during G2, as they migrate towards the apical surface. This ability is then repressed during G1, as the cells migrate basally. In this model, INM effectively separates two populations of NE cells dependant on their expression of neurogenic genes (Murciano et al. 2002).

This data supports INM is an example of co-operation of both intrinsic and extrinsic factors regulating proliferation and differentiation. The intrinsic mechanism of migration coupled to cell cycle regulates cell fate by positioning the cell within a certain signalling environment for division, providing the correct cues for either self-renewal or differentiation. The cells that remain as NSCs are moved away from this signalling environment until their next division, while those that differentiate no longer undergo INM. Disrupting INM perturbs this balance, resulting in basal NSCs undergoing differentiation as they no longer receive self-renewing signals found apically.

1.2.4.2 – ECM control of mitotic spindle orientation.

NSCs receive signals from both their environment, the niche, and from neighbouring cells. There are a variety of extrinsic factors that can influence their fate within this environment, including diffusible signals, niche and ECM interactions and cell-cell communication, such as Notch-Delta signalling. Evidence for cell-cell interactions influencing cell fate can be found in *Drosophila* germ-line stem cells (GSCs), where Adenomatous polyposis coli (APC) mutations result in mis-orientated or detached spindles. APC anchors the centrosome to the cadherin in the AJ, which links the GSC to the hub cell, the support cell within the niche. This anchoring of the centrosome by APC ensures an asymmetric division by leaving only one daughter cell attached to the hub cell and therefore retained within the niche (Yamashita et al. 2003). The other daughter cell leaves the niche environment and differentiates.

The ECM within the niche and the proteins that interact with it have long been known to effect neurogenesis. The three micro-domains of AJs are split during asymmetric divisions in the embryonic VZ (Marthiens & ffrench-Constant 2009), allowing both daughter cells to

retain an apical process attached to the ventricular surface directly after mitosis. The adhesion domains of the AJ, containing ZO-1 and N-cadherin, are equally inherited whereas the polarity domain, containing Par3 and aPKC, is differentially inherited upon an asymmetric division. This splitting of AJ domains requires precise and tight control of the cleavage plane, suggesting only a small change of angle is required for symmetric and asymmetric divisions (Figure 1.3), not the previously thought switch from vertical to horizontal cleavage planes. This small change in the angle of division would not place the daughter cells in different locations within the niche, as in *Drosophila* GSCs, suggesting fate is decided by interactions between the cells post-division, whilst they remain attached in the niche.

The niche contains a variety of ECM components, such as the collagens, which also affect neurogenesis. Addition of collagen IV *in vitro* inhibits proliferation and promotes neural differentiation of cortical NE cells (Ali et al. 1998). Many different ECM components are found within the neuroepithelium, but the main receptors for ECM expressed in the NSCs are the integrins. As integrins are the focus of this thesis, their roles in regulating NSC proliferation and differentiation will be discussed in detail at the end of the introduction.

1.3 - Neurogenesis within the cell – Intrinsic signalling pathways.

However cell fate is regulated during neurogenesis, the signals must then be relayed to the nucleus to allow the fate changes to occur. This requires intrinsic signalling pathways to alter or modify gene expression. The major intrinsic pathways and the evidence of their role in neurogenesis are summarised along with other key intrinsic factors, such as regulation of the cell cycle.

1.3.1 Epigenetic mechanisms.

Intrinsic changes can occur via epigenetic mechanisms, which include DNA methylation, histone modifications and non-coding RNAs. Neuronal genes are epigenetically supressed in stem cells which must be removed to permit differentiation. Epigenetics was originally described by Waddington as the '*study of those processes by which genotype gives rise to phenotype*' in 1942, but is now defined as '*changes in the expression or function of genetic elements that are independent of changes to the DNA sequence*' (Jobe et al. 2012).

DNA methylation, well known for its role in long-term gene silencing, such as X-chromosome inactivation and establishment of cell fate (Mohandas et al. 1981; Combes & Whitelaw 2010) is the addition of a methyl group to a cytosine that is in front of a guanine (CpG dinucleotide), a process catalysed by the enzyme DNA methyltransferase (DMNT).

DNA methylation is essential for development, null mutations are embryonic lethal and mutations in related proteins cause severe defects, such as the neurodevelopmental disorder Rett syndrome, caused by a mutation in methyl-CpG binding protein MeCP2 (Bestor 2000). DNA methylation is vital for neurogenesis (Figure 1.5) (Hirabayashi & Gotoh 2010), loss of DNA methyltransferase 3β (DMNT3b) down-regulates neuronal genes, such as Ngn2, and up-regulates glial genes, perturbing neurogenesis (Wu et al. 2010). How is methylation important for neurogenesis? It may regulate the release of repression of genes in a temporal manner. Hes5 is highly methylated at E7.5 in the mouse and completely demethylated by E9.5 (Hitoshi et al. 2011) enabling it to be active at the start of Notch signalling, around E8. If this demethylation does not occur, NSCs fail to form (Hitoshi et al. 2011).

Histone modifications can be either acetylations or methylations, catalysed by histone acetyltransferases (HATs) and histone deacetylases (HDACs) or by histone methyltransferases (HMTs) (Figure 1.5). Acetylation occurs on lysine residues and loosens chromatin, enabling DNA binding proteins and the transcriptional machinery access to the DNA. Methylation of lysine residues can be either activating or silencing depending on the specific location on the histone. Methylation H3K27me3, mediated by Polycomb group proteins, is repressive, whereas Trithorax (Trx) group mediated H3K4me3 activates gene expression (Jobe et al. 2012; Hu et al. 2012).

Chromatin remodelling complexes, such as the Polycomb group proteins (PcG) provide another mechanism of epigenetic control. PcGs are known to mediate repression of lineage-specific genes in ES cells and can form Polycomb repressive complexes (PRC) (Hu et al. 2012).

1.3.2 Micro-RNAs.

Non-coding RNAs include micro-RNA (miRNA) (Figure 1.5), small nucleolar RNA (snoRNA), small interfering RNA (siRNA) and PIWI-interacting RNA (piRNA) (Jobe et al. 2012). miRNAs target 2-8 nucleotide sequences of mRNAs, such short complementation results in a single

Figure 1.5

miRNA being able to interact with many targets; binding to a target results in degradation of the mRNA (Guo et al. 2010). Enzymes Dicer and Drosha are involved in the final stages of miRNA synthesis, disrupting miRNAs by depleting Dicer reduces cortical size and differentiation, causes changes in morphology and increased apoptosis of NE cells (Hu et al. 2012; Volvert et al. 2012). MiRNAs are thought to be involved in radial migration of neurons, as Dicer mutants show impairments in neuronal migration (Volvert et al. 2012), and have been shown to be involved in all stages of neurogenesis, from proliferation of NSCs to axonal path-finding (Volvert et al. 2012) (Figure 1.5).

MiRNA-124 (miR-124) is the most abundant miRNA in the adult brain, where it is up-regulated as transit amplifying cells become neuroblasts and again as they differentiate and leave the cell cycle (Cheng et al. 2009). MiR-124 targets include Jagged-1 and Sox9; over-expression of Sox9 blocks neurogenesis, in contrast to the increase in neuron production upon knock-out of Sox9. Blocking miR-124 delays neurogenesis and up-regulates Sox9 and the expression of mir-124 and Sox9 is spatially exclusive, suggesting miR-124 controls the expression levels of Sox9 to permit neurogenesis in the adult SVZ (Cheng et al. 2009). MiR-124 has also been shown to target basal lamina components lamninin-γ1 and integrin-β1 (Lang & Shi 2012). MiR-9 has also been extensively studied in neurogenesis. It is expressed throughout the neuroepithelium and highly in the VZ (Lang & Shi 2012) where it inhibits expression of nuclear receptor TLX. Over-expression of miR-9 decreases NSC number, inhibiting proliferation and promoting neurogenesis, rescued by TLX expression (Zhao et al. 2009; Lang & Shi 2012).

The epigenetic state is controlled by a network of pathways, of which REST appears to mediate the cross-talk. REST, or element-1 silencing transcription factor/neuron-restrictive silencing factor, is a transcriptional repressor that binds to DNA and co-repressors mSin3 and CoREST, to recruit HDACs and HMTs, acting as a scaffold (Figure 1.5)(Hu et al. 2012). REST acts as a repressor of neuronal genes for non-neuronal lineages, but knock-down of REST prevents NSC formation, suggesting REST has a more complex role in neurogenesis (Jobe et al. 2012). REST interacts with a number of miRNAs, including miR-124 and Dicer, some of which are able to feedback and target REST (Jobe et al. 2012; Lang & Shi 2012). REST binds to the miR-124 locus in non-neuronal cells and progenitors to inhibit miR-124 expression and its neurogenic potential (Ji et al. 2013) (Figure 1.5).

Numerous Dicer knock-outs have shown miRNAs are required to regulate behaviour and generation of distinct populations of progenitors, with later born upper layer neurons more affected (Volvert et al. 2012). MiRNA Let-7b accumulates during neurogenesis and regulates TLX and cyclin D1, inhibiting proliferation and accelerating neurogenesis upon gain of function (Volvert et al. 2012).

1.3.3 Nuclear receptors.

Nuclear receptors are ligand-activated transcription factors with the ability to directly bind DNA, at DNA response elements, and regulate target gene expression (Stergiopoulos & Politis 2012; Eendebak et al. 2011). The 48 nuclear receptors in human and 49 in mouse have varied roles, influencing development, metabolism, morphogenesis and homeostasis (Stergiopoulos & Politis 2012). The family members found in NSCs include Thyroid hormone receptors (TRs), Retinoic acid receptors (RARs and RXRs), Peroxisome proliferator-activated receptors (PPARs) as well as orphan nuclear receptors, which have no known ligand at present, including TLX (homologue of *Drosophila tailless*) (Stergiopoulos & Politis 2012). TLX expression is restricted to the forebrain where it in part regulates the decision to proliferate or differentiate, as loss of TLX decreases progenitor numbers and promotes a faster cell cycle, reducing the size of the cortex due to premature differentiation (Roy et al. 2004).

Nuclear receptors and miRNAs interact to form regulatory pathways (Figure 1.5). TLX has a negative feedback loop with miR-9; miR-9 represses TLX to inhibit proliferation of NSCs and accelerate neurogenesis, which is rescued by restoring TLX function (Zhao et al. 2009). The majority of regulatory loops between nuclear receptors and miRNAs appear to be involved in spatial-temporal dependant functions, such as proliferation and differentiation, coordinating development (Eendebak et al. 2011). TLX is also in a feedback loop with miR-137, which promotes differentiation and inhibits proliferation of NSCs. TLX negatively regulates miR-137 via recruitment of a transcriptional co-repressor LSD1. The TLX target genes include miR-137 and -124, both of which are known regulators of progenitor proliferation (Sun et al. 2010).

1.3.4 Transcription factors.

Pax6 is a transcription factor known to regulate neurogenesis and proliferation. In a mouse lacking functional Pax6, the apical progenitors undergo increased asymmetric divisions,

creating unequal inheritance of the AJ and generating basally dividing progenitors. AJ and Par complex proteins are expressed at lower levels in the cortex of these mice and the basally dividing progenitors exhibit RGC hallmarks, suggesting they have delaminated from the apical surface. Pax6 influences AJ protein expression and therefore adhesion of the apical end foot in RGCs. *In vitro* study of these cells reveals increased levels of asymmetric divisions, thought to be regulated by Spag5, a direct target of Pax6. Spag5 knock-down phenocopies the Pax6 mutants, increasing asymmetric divisions. It also interacts with the spindle machinery, including microtubules and spindle associated proteins such as Aurora A. The basally dividing cells in the Pax6 mutants resemble oRG cells, they show hallmarks of RGCs but divide basally, similar OSVZ cells (Asami et al. 2011).

A major group of transcription factors involved in neurogenesis are the basic helix-loop-helix transcription factors (bHLH), named after the structure of their DNA binding motif. They are a large family including pro-neural NeuroD, Ngn, Mash and non-neural Id and Hes (Ross et al. 2003). The Hes and Id families inhibit neurogenesis and promote the self-renewal of progenitors. Hes 1 and 5, both downstream of Notch, are expressed within the VZ and bind to neural gene loci to repress expression. They can also directly bind to pro-neural proteins, antagonising their activity (Ross et al. 2003). Ids are also expressed within the VZ and inhibit differentiation but through a different mechanism to Hes, Ids bind to E proteins to inhibit pro-neural bHLH that require them for activity, such as Mash and Ngn (Ross et al. 2003). This cross regulation of bHLH factors is a common theme in the mediation of cell fate decisions.

Id proteins act redundantly to enhance proliferation and inhibit differentiation. Due to their redundant nature, several Id genes must be knocked-down to show a phenotype. Loss of three Id genes from NSCs results in premature differentiation and detachment from the niche. Id proteins act on the RAP1-GTPase, promoting RAP1 function in NSCs, RAP1 promotes adhesion to the ECM via integrin signalling (Niola et al. 2012). Id proteins also promote proliferation and cell cycle progression of NSCs. Knock-out of Id1 and 3 causes progenitors to prematurely exit the cell cycle, decreasing neuron number (Miyazono & Miyazawa 2002). Id1 and 3 expression in NE cells is normally induced by BMP2 signalling to inhibit neurogenesis (Miyazono & Miyazawa 2002).

Another family of transcription factors involved in neurogenesis are the Sox family of high mobility group transcription factors. They are expressed throughout neurogenesis, from

ESCs (Sox2) through to differentiated neurons (SoxC – Sox4, Sox11, Sox12) (Bergsland et al. 2011). Sox2 is expressed in the epiblast, ESCs, NE cells and NSCs. It is known to be important in neural potential and is linked to the Shh pathway. Over-expression of Sox3 promotes self-renewal of progenitors and knock-down of Sox3 promotes differentiation, as Sox3 binds to target genes involved in neural differentiation, supressing their activation until Sox11 binds (Bergsland et al. 2011). This was also observed for Sox2, which binds to neural-specific genes in ESCs which are later activated by Sox3, specifying neuronal-lineage programs of gene expression; these genes are pre-selected for activation by the Sox transcription factors (Bergsland et al. 2011).

Sox9 and Sox10 are expressed in RGCs, as well as other glia, and in transit-amplifying precursors in the adult SVZ. A characteristic of NSCs is their ability to form neurospheres in culture, creating an assay for NSC function (Reynolds & Weiss 1996). Cre-mediated recombination allowing stable expression of Sox9 generated neurospheres at a much earlier age than wild-type. *In vivo*, Sox9 expression coincides with the time of *in vitro* neurosphere formation. Conditional loss of Sox9 decreases neurosphere forming ability *in vitro,* disrupts neurogenesis and enlarges ventricles *in vivo*. Sox9 mediates this effect downstream of the Shh pathway. Exposure of NE cells to Shh induces expression of Sox9 and loss of Sox9 activity blocks the neurogenic effects of Shh. Additionally, induction of Sox9 in the absence of Shh is sufficient for NSC induction (Scott et al. 2010).

1.3.5 Cell cycle length.

The cell cycle length of NSC/NPCs is dynamic. Neural progenitors lengthen G1 phase of the cell cycle as neurogenesis progresses. Over-expression of the cdk4/cyclin D1 prevents this lengthening of G1, without affecting any other aspect of the cell cycle, such as INM or cleavage plane. Over-expression prevents neurogenesis, but increases the generation and expansion of basal progenitors, causing a thicker SVZ and larger cortex. RNAi knock-down of cdk4/cyclin D1 has the converse effect, increasing the length of G1 and promoting neurogenesis. There is no expansion of basal progenitors and as with over-expression there was no effect on INM (Lange et al. 2009). A similar effect was seen by lengthening G1 via over-expression of cyclin D1 and E1, which caused expansion of both apical and basal progenitors and a reduction of neurogenesis (Pilaz et al. 2009).

Basal progenitors exhibit a longer G1 than apical progenitors. This could explain the observed increase in G1 over the course of neurogenesis, as more basal progenitors are

generated. Neural progenitors undergoing proliferative divisions undergo a longer S phase than those undergoing neurogenic divisions (Arai et al. 2011). This is thought to be a higher level of quality control, the NSCs that self-renew undergo many rounds of division so take longer to replicate DNA than those generating post-mitotic neurons, to decrease the possibility of errors during replication.

To maintain the correct balance between proliferation and differentiation, the timing of exit from the cell cycle must be tightly regulated. Gde2, a six-transmembrane protein known to be expressed in spinal motor neurons, is expressed in post-mitotic neurons in the developing cortex. Removal of Gde2 prior to neurogenesis delays exit from the cell cycle, increasing progenitor number. This is due to an increase in Notch signalling, both in NICD and Hes5 levels. Gde2 expression in new born neurons may signal to down-regulate Notch signalling in neighbouring progenitors, causing them to differentiate (Rodriguez et al. 2012).

1.4 - The role of integrins in the regulation of proliferation and differentiation of NSCs.

My work has focused on the role of integrins, which are obvious candidates in the regulation of NSC behaviour due to their high expression levels and known functions within the stem cell niche. Previous work has suggested mechanisms by which integrins could contribute to both the intrinsic and extrinsic models mentioned. In this thesis I have set out to determine which of these two roles of integrins is more important, the intrinsic or extrinsic regulation of proliferation and differentiation. Before summarising the experimental approach, I will first review integrin biology – their structure and function, expression within stem cells and the evidence of their roles in regulating stem cell behaviour.

1.4.1 - Integrins.

Integrins are a large family of transmembrane adhesion receptors that bind to ECM. They are the most common family of ECM receptors and are highly conserved throughout evolution. In addition to their role in adhesion, integrins are able to signal bi-directionally across the cell membrane, termed outside-in and inside-out signalling. Extracellular signals can influence intracellular changes, outside-in, and intracellular signals can influence extracellular changes, inside-out (Hynes 2002; Legate et al. 2009).

Figure 1.6

1.4.1.1 - Structure.

Integrins are heterodimeric receptors, consisting of an α and a β subunit. There are 18α and 8β subunits, known to assemble into 24 distinct integrins (Hynes 2002). These can be split into sub-families based on their ligand binding (Figure 1.6); one subfamily recognises the tripeptide sequence RGD, found in fibronectin and vitronectin, another binds to laminins, another to collagens and the final subfamily is leukocyte specific, including integrin-β2 and –β7 (Figure 1.6) (Hynes 2002). Each of the 24 distinct integrins appears to have a non-redundant role, shown by the different phenotypes observed upon knock-out. The severity of the phenotypes varies greatly, from peri-implantation lethality from the loss of integrin-β1 to defects in specific processes, such as angiogenesis, after the loss of integrin-α1 (Hynes 2002).

Electron microscopy revealed both the α and β subunits have a large extracellular domain, over 700 residues, a single α-helix transmembrane (TM) domain and a short cytoplasmic domain, usually around 10-70 residues. Within the extracellular domain the N-terminal consists of a globular region, the ligand binding head domain. The head domain from both the α and β subunits is required for ligand binding. The globular N-terminal head stands on the thin C-terminal legs to make up the rest of the extracellular domain, the C-terminal also connects the transmembrane and cytoplasmic domains (Figure 1.6) (Nermut et al. 1988). The α chain is made of four to five extracellular domains; a β propeller, a thigh and two calf domains and around half of the α chains have an additional I domain. The β leg has 7 domains; a β-I domain, a hybrid domain, four cysteine-rich EGF modules and a tail domain (Campbell & Humphries 2011). The cytoplasmic domains of both the α and β subunits is relatively short and lacks enzymatic activity. The β cytoplasmic tails share a similar homology with conserved motifs that recruit kinases, unlike the α cytoplasmic tails which are highly divergent (Takada et al. 2007).

1.4.1.2 -Activation states.

Integrins are known to occupy three major conformational states; inactive, primed and high affinity. Inactive integrins are thought to adopt a bent conformation, anywhere from a 90^{o} to 135^{o} bend in their extracellular domain, which points the ligand binding head towards the cell surface (Figure 1.6) (Hynes 2002; Askari et al. 2009; Jin et al. 2004). There is some debate as to whether integrins are completely inactive when in their bent conformation, Xiong et al suggest this can be the active form of integrins (Xiong et al. 2003).

Activated integrin is thought to adopt an extended conformation with the head domain now facing away from the cell membrane, this conformation change occurs on ligand binding (Figure 1.6). The degree of extension appears to be ligand and integrin specific (Askari et al. 2009). In general, higher ligand affinity correlates with the greater distance of the head domain from the cell membrane (Mould & Humphries 2004).

There are two models to explain the conformational changes of integrins, the extension/switchblade model and deadbolt model. The extension model requires a conformational change of integrins upon activation from a bent to an upright structure (Luo et al. 2007). The deadbolt model requires a more conservative change around the deadbolt region of the bent integrin (Arnaout et al. 2005). The deadbolt region is a cytoplasmic loop that rotates upon activation to allow access to the TM domain (Xiong et al. 2003). Most evidence points to the extension model of conformational changes upon activation, but both models agree that the TM domain is a key site in integrin activation. The TM domains of α and β interact via a disulphide bridge, maintaining integrin in the bent inactive state. This salt bridge must be disrupted to allow extension and activation of the integrin. During inside-out signalling this is mediated by talin binding to the β cytoplasmic tail, but can also be replicated by point mutations.

Various mutations in the TM and cytoplasmic domains have provided an insight into the link between conformation and activity. Mutations that lock the integrin into its inactive bent state resisted normal inside-out activation, leaving the integrin locked in the inactive state, as extension is required for ligand binding (Zhu et al. 2007). A point mutation within the β1 cytoplasmic tail, D759A, which destroys the salt bridge between the two subunits, did not affect integrin function in the epidermis (Czuchra et al. 2006). However, a charge reversal mutation of the same region, D759R, constitutively activated both integrin-β3 and –β1 (Hughes et al. 1996a; Laursen et al. 2011).

A point mutation within the NPXY motif, that prevents talin binding, locks the integrin in an inactive conformation and is embryonic lethal and severely disrupted the epidermis (Czuchra et al. 2006). However, mutation of the deadbolt region of the β tail did not have any effect on integrin activation (Zhu et al. 2007), suggesting the extension model of activation is the predominate. The conformational state of integrins is highly dynamic and may include intermediate steps. Evidence from point mutations suggests it is regulated by both inside-out and outside-in signalling.

1.4.1.3 - Inside-out signalling.

Integrins are able to regulate their own affinity for a ligand via their activation state. The final step in this activation is the binding of talin, which disrupts the salt bridge between the α and β subunits causing them to separate and the headpiece to extend for ligand binding (Vinogradova et al. 2002; Wegener et al. 2007; Kim et al. 2012). This is inside-out signalling; an intracellular signal activates integrin, allowing it to interact with extracellular ligands (Figure 1.7).

The β cytoplasmic tails lack enzymatic activity but contain a canonical NPXY motif for phospho-tyrosine binding (PTB) domains, which play a major role in downstream signalling. This enables them to bind to proteins with kinase activity, which can also interact with the cytoskeleton. Talin acts as the initial interaction between integrin and the actin cytoskeleton (Pfaff 1998). After talin binding, other proteins can be recruited to reinforce the interaction, such as vinculin and integrin linked kinase (ILK) (Ziegler et al. 2008; Goult et al. 2010). Loss of talin disrupts and inhibits the activation of integrins (Petrich et al. 2007) as does mutation of the talin binding site (Czuchra et al. 2006). More recently, adaptor proteins kindlins were found to be recruited to β tails along with talin and are essential for integrin activation, loss of kindlins is peri-natal lethal and inhibits integrin activation (Montanez et al. 2008). Whereas talin is required to link integrins to the cytoskeleton, kindlins are required for integrin cell surface expression and correct recycling (Margadant et al. 2012).

ILK is also implicated in inside-out signalling, loss of ILK supressed integrin activation (Honda et al. 2009). Although ILK is necessary for integrin activation, it is not sufficient and is unable to rescue activation upon loss of talin (Honda et al. 2009). ILK is also thought to contribute to outside-in signalling, as loss of ILK impairs adhesion and cell spreading (Honda et al. 2009).

Inside-out signalling can also inhibit activation. Inhibitors of β1 cytoplasmic tails can compete with talin binding, such as filamin (Das et al. 2011). They contain the same PTB domain required for the NPXY motif and some of these inhibitors can also promote integrin trafficking and endocytosis, which occurs in the normal recycling of integrins

Figure 1.7

(Pouwels et al. 2012). There are also inhibitors that bind to the α cytoplasmic tail, such as SHARPIN, to inhibit integrin activation by inhibiting recruitment of talin and kindlin. Loss of SHARPIN releases this inhibition and increases β1 activation (Rantala et al. 2011).

1.4.1.4 - Outside-in signalling.

Inside-out signalling promotes attachment to the ECM, but further outside-in signalling is required to strengthen this attachment (Figure 1.7). This is mediated via the clustering of integrins. Initially, this occurs as small adhesions that are unstable, which can stabilise to form dot-like focal adhesions and then larger focal adhesions before finally forming fibrillar like structures (Geiger et al. 2001). This clustering is thought to be in part regulated by Notch and EGFR (Arora et al. 2012) and can act as a core for actin and cytoskeleton dynamics within the cell (Yu et al. 2012). Clustering of integrins can have short term effects on actin polymerisation or long term effects of gene expression and cell survival. Integrins are able to form clusters via interactions with TM domains of other subunits, in a homotypic oligomerisation (Qin et al. 2004).

Activation of integrins results in downstream signalling and due to the lack of enzymatic activity of the cytoplasmic tails, this occurs via the recruitment of protein tyrosine kinases such as FAK and ILK (Giancotti 1999). FAK was one of the first molecules to be identified in integrin signalling. It is a ubiquitously expressed protein that is activated by phosphorylation. Integrin activation of FAK results in its auto-phosphorylation at Y397, leading to association with Src and its subsequent activation (Guan 1997). FAK/Src can then bind to other molecules such as paxillin and tensin and activate MAPK and adaptor protein Crk. FAK can also interact with another kinase, PI3k and has also been linked to phosphorylation of p130Cas, which can then form a complex with Crk and Sos (Vuori et al. 1996).

Integrins can also activate ILK, which can then recruit actin binding protein parvin and PINCH, a Lim domain protein, and kindlin (Harburger & Calderwood 2009). Paxillin, which interacts with both FAK and ILK, is a scaffold protein that links integrins to the Rho family of GTPases (Harburger & Calderwood 2009). The Rho family consists of Cdc42, Rac and Rho. They are required for focal adhesions and actin dynamics, adhesion of integrins to fibronectin signals via the Rho family to organise the cytoskeleton. Cytoskeletal protein paxillin associates with FAK, co-localising to focal adhesions (Clark et al. 1998). Vinculin is another well-known protein to interact with the integrin signalling complex, connecting

integrins to the cytoskeleton for outside-in signalling (Ziegler et al. 2008). Outside-in signalling can also interact with other signalling pathways to effect cell survival and behaviour, such as the G protein signalling pathways (Shen et al. 2012).

1.4.1.5 - Cross-talk with other pathways.

Integrin downstream signalling is able to interact with many pathways to regulate multiple aspects of cell behaviour. Cross-talk between integrins and growth factors can regulate cell survival and proliferation. They can converge on Ras-MEK-MAPK, the major pathway downstream of FAK that is also activated by growth factor signalling (Legate et al. 2009).

Integrin interaction with growth factor receptors can occur at two levels; either at joint downstream signalling molecules or directly between the receptors (ffrench-Constant & Colognato 2004). Integrin-αvβ3 increases phosphorylation of the EGFR and integrin activation can also regulate expression levels of receptors and their localisation (Alam et al. 2007). Equally, growth factor receptors can regulate integrins. Both FGF and TGF-β can influence integrin expression levels and their activation state (ffrench-Constant & Colognato 2004). β1 integrin has also been linked to up-regulation of insulin-like growth factor receptor (IGFR) (Goel et al. 2006).

Outside-in signalling of integrin-β1 has been shown to modulate major signalling pathways in a cell-autonomous manner. For example, integrins activate ILK which modulates GSK3β, part of the canonical Wnt pathway, which in turn modulates Notch signalling (Rallis et al. 2010).

1.4.2 - Integrins and stem cells.

1.4.2.1 Expression.

Integrins are highly expressed on many cell types, including many types of stem cells. Their ability to signal bi-directionally and interact with major pathways involved in development makes them an interesting candidate for regulation of stem cell behaviour.

Integrins are expressed on embryonic stem cells (ESCs), including integrin-β1, and are thought to be vital for their self-renewal. Activation of integrins in ESCs *in vitro* up-regulated stemness-like genes and down-regulated differentiation-like genes (Lee et al. 2010). Human ESCs also express integrin-β1, which is required for their maintenance (Xu et al. 2001).

Integrins can be used as markers of human NSCs. High levels of integrin-β1 or –α6 expression can be used to select for NSCs, these cells are able to generate twice as many neurospheres than low expressing cells. Integrin-β1 is then down-regulated upon differentiation (Hall et al. 2006).

Both integrin-β1 and –α6 are expressed throughout the mouse embryonic VZ, with highest levels adjacent to the ventricular surface, at the onset of neurogenesis (Campos et al. 2004). In the postnatal brain, integrin-β1 is only expressed in the lateral wall of the ventricle. High levels of integrin-β1 expression are also found in neurospheres and cells with the highest levels are able to generate more neurospheres (Campos et al. 2004). Integrins are also required for the activation of MAPK in NSCs, as inhibition or deletion of integrin- β1 decreases MAPK activity (Campos et al. 2004).

1.4.2.2 Adhesion within the niche.

The niche is a specialised microenvironment capable of housing stem cells, and support cells, maintaining self-renewal and allowing exit from the niche for differentiation. It can be localised within a tissue and is not a general property of all cells. There are two main types of niches; stromal niches, as in Drosophila gonads, or epithelial niches, as in the VZ. A stromal niche has a support cell that directly contacts the stem cell. The epithelial niche does not have such a specialised cell type, but ECM is thought to play an important role. Niches can vary greatly in size, from a single cell to many hundreds (Morrison and Spradling, 2008). Integrins are expressed in both niche types and play vital roles in proliferation and differentiation.

Within a stromal niche, such as the *Drosophila* GSC niche in the testis, integrins are required for the anchoring of the hub cells in the correct location. The hub cells are the support cells within the niche and also regulate the spindle orientation of the attached GSCs. In an integrin mutant, the hub cells are mispositioned which causes abnormal spindle orientation in the GSCs (Tanentzapf et al. 2007).

The *Drosophila* ovary contains both a stromal and an epithelial niche, the GSCs and follicular stem cells (FSCs) respectively. The FSCs establish an epithelial niche unique from the GSC stromal niche. Integrins are required to anchor the FSCs to the BM and for their maintenance, FSCs lacking integrin are lost from the niche (O'Reilly et al. 2008a). FSCs may regulate their own niche by secretion of the integrin ligand, laminin. Both integrin and

laminin are required for the anchoring and proliferation of FSCs within the niche (O'Reilly et al. 2008b).

Integrins are also required for adhesion within the niche in mammalian models. High levels of integrin-β1 marks stem cells in the epidermis, but not the transit-amplifying cells (Jensen et al. 1999). Expression of a chimeric integrin protein, containing the cytoplasmic domain of integrin-β1 and the extracellular domain of another unrelated protein, affected adhesion of epidermal stem cells to their niche. The chimeric protein partially inhibited integrin-β1 mediated adhesion of the stem cells causing them to exit the niche, to no longer self-renew and become transit-amplifying cells. Conversely, over-expression of a wild-type integrin-β1 rescues this phenotype and promotes stem cell retention in the niche (Zhu et al. 1999).

The adhesion role of integrins can also be replicated *in vitro*. The loss of integrin-β1 in neurospheres perturbs their adhesion to both laminin and fibronectin *in vitro* but did not affect neurosphere forming potential (Leone et al. 2005). Despite the same number of neurospheres being formed, those without integrin-β1 where smaller in diameter and contained fewer NSCs and more progenitors and neurons. NSCs lacking integrin-β1 proliferate less and show increased levels of cell death and are more sensitive to loss of growth factors EGF and FGF-2 (Leone et al. 2005).

Integrins have been implicated in homing of stem cells to the niche. Haematopoietic stem cells (HSCs) use integrin-α4β1 mediated adhesion to home to the bone marrow and to home to the liver in the embryo (Ellis & Tanentzapf 2010). HSCs up-regulate integrins to allow homing and retention within the niche (Taniguchi Ishikawa et al. 2013).

1.4.2.3 Signalling within the niche: orientation of the mitotic spindle.

The ECM is a major source of instructive signals within the stem cell niche, regulating many aspects of stem cell behaviour (Drago et al. 1991). In the *Drosophila* ovary integrins mediate FSC contact with the basement membrane. This is required for the correct polarisation of FSCs that are not directly contacting a GSC (Fernández-Miñán et al. 2008). Within this niche, integrins are required to maintain the follicular-epithelium, loss of integrin signalling leads to stratification of the epithelium due to abnormal spindle orientation in the wild-type cells directly contacting the mutant cells (Fernández-Miñán et al. 2007). It is integrin signalling, but not adhesion, that is required to maintain the epithelium as the integrin mutant maintains adhesion.

Integrins may also regulate asymmetric division. Deletion of integrin-β1 within the mammary epithelium blocks the ability to regenerate, indicating a loss of functional stem cells. The loss of integrin-β1 randomises the spindle orientation, which is usually perpendicular to the BM (Taddei et al. 2008). Inactivation of integrin-β1, via injection of blocking antibodies into the ventricle of embryonic mice, results in abnormal NSC proliferation, resulting in fewer horizontal divisions (Loulier et al. 2009). This change in division angle does not appear to affect differentiation. The blocking of integrin-β1 also promotes NSC detachment from the ventricular surface, suggesting integrins are required for the retention of the NSCs in the VZ and for correct INM and orientation of division and correct development of the neocortex (Loulier et al. 2009).

Integrins cluster into focal adhesions during interphase, along with actin and microtubules. A mutation in the integrin-β1 cytoplasmic tail, which blocks activation, permits entry into mitosis but blocks formation of the spindle (Reverte et al. 2006). In non-polarised cells *in vitro* integrin mediated adhesion is known to regulate spindle orientation. In these cells, the spindle is usually orientated parallel to the substrate they are attached to. Integrin couples this attachment to the orientation of the spindle, via microtubules and EB1, a microtubule plus-end tracking protein which stabilises microtubules (Toyoshima & Nishida 2007).

Further evidence for the role of integrins in orientation of the spindle is seen in the *Drosophila* ovary. A mutation in itgβ1 (mys) resulted in mis-orienatation of the spindle despite normal location of polarity cues such as Par3 (Fernández-Miñán et al. 2007). In adult *Drosophila* intestinal stem cells (ISCs), integrins interact with Par3 to direct asymmetrical divisions. During an asymmetric division, the Par complex is localised to the apical daughter cell and loss of the Par complex decreases differentiation. Integrins, found at the membrane of the ISC contacting the BM, regulate ISC divisions as loss of integrin increases the number of proliferating cells. Orientation of cell division in ISCs involved a combination of intrinsic Par and extrinsic integrin signals, linking adhesion and polarity. One model would be that adhesion provides information to localise the Par complex for asymmetric segregation. This allows segregation of fate determinants, such as aPKC, part of the Par complex, which regulates Notch pathway activation. Increased aPKC activity enhances Notch signalling in the daughter cell, inducing differentiation (Goulas et al. 2012).

The role of integrins in spindle orientation is further supported by the effect of its downstream targets, such as ILK. ILK is a serine-threonine kinase that forms two cytoskeletal pools, one that binds to actin at focal adhesions and one that binds to tubulin at centrosomes. Several other proteins are known to form similar pools as ILK, such as the signal transducing protein Paxillin. The two pools interact; ILK could relay signals from adhesion pathways to the spindle via the interaction between the pools, suggesting integrin signalling plays a role in organising microtubules (Fielding et al. 2008).

1.5 - Experimental strategy.

Integrins are highly expressed within stem cells and play many roles in regulating their behaviour. As well as data supporting a role for anchoring stem cells within the niche, integrins ability to signal bi-directionally, alongside evidence of interaction with major developmental pathways and regulation of stem cell divisions, make them an obvious candidate for the regulation of proliferation and differentiation.

As discussed earlier, integrins could mediate this regulation on two levels, intrinsically or extrinsically. The intrinsic model requires the segregation of fate determinants prior to division whilst the second model requires post-division specification via signalling. To test which of the two models is most important, we are using transfection of a constitutively active human integrin-β1 (CA*β1) with the D753R mutation (Hughes et al. 1996; Laursen et al. 2011), a wild type human integrin-β1 (WTβ1), a human integrin-β1 that lacks the intracellular domain (EC only) and an empty vector. Transfection will be via *in ovo* electroporation of the chick embryonic CNS, resulting in a patchwork of expression within the NSCs of the midbrain neuroepithelium. Expression of these human integrin-β1 (itgβ1) proteins allows perturbation of integrin signalling within the cell without loss of adhesion, which has been previously shown to cause NSC detachment and apoptosis (Radakovits et al. 2009 and unpublished RNAi data from our lab).

This system allows both the expressing cells and their non-expressing neighbours to be studied within the same environment. We predict that if integrins are acting to regulate NSC behaviour via an intrinsic mechanism, only the cells expressing the constitutively active integrin will alter their behaviour. If the second model is correct and integrins act via an extrinsic mechanism, we predict that the neighbouring non-expressing cells will also alter their behaviour.

Chapter 2

Methods.

2.1: Embryo culture.

Fertile hens' eggs were obtained from the Poultry Unit at the Roslin Institute (J line, brown leghorn). Eggs were incubated at 38°C in a humidified incubator for 45-48 hours prior to electroporation. 3ml of albumin was removed from the pointed end of the egg using a syringe before the egg was windowed. Indian ink was used to allow visualisation of embryos at E2 (it was not required at E4). Embryos were injected using an Eppendorf Femtojet and pulled glass capillaries filled with plasmids at 1µg/µl concentration (in the ratio of 4:1 Integrin:GFP), the plasmid mix was coloured with Fast Green FCF (Sigma F7252) dye to allow visualisation. The tip of the capillary was inserted into the hindbrain and pushed along the ventricle to reach the midbrain, to avoid any tear damage to the region of interest. The embryos were then electroporated using a Harvard Apparatus ECM 830, with the settings: 15 volts, 5 pulses, 50ms duration, 950ms intervals. Electrodes were always placed in the same orientation: anode in the left hand, cathode in the right. After injection and electroporation, embryos are sealed and returned to a 38°C humidified incubator for 12hrs up to 4 days. Embryos were staged according to their day of incubation and Hamburger and Hamilton classification.

At the end of the experiment, embryos for cryosectioning where fixed in 4% paraformaldehyde (PFA) at 4°C for 1 hour. They were then transferred to 1% PBS and kept at 4°C until transferred to 15% and 30% sucrose, also at 4°C. After sucrose treatment embryos were embedded in OCT (Cell Path) and frozen on dry ice. They were kept at -80°C until sectioned transversely at 20µm thickness, at -20°C and mounted onto Superfrost Plus slides (ThermoScientific). Slides were stored at -20 °C.

Embryos used for whole mount staining were fixed as above and transferred into PBS. They were then dissected to remove and flatten the midbrain. The midbrains were then stained as free-floating sections and mounted onto glass slides.

2.2: FACS.

Embryos used for FACS were collected into PBS on ice. They were then dissected using a fluorescent microscope to remove the GFP positive area. This was then dissociated using acutase (Sigma) at 37^{o}C for 10mins. The cells were spun down at 3000rpm for 5mins and resuspended in MEM (Gibco 31095-029).

Cells were sorted on a FACS Aria II directly into lysis or PCR reaction buffer. Cells were separated into GFP positive and negative popultations. For all experiments, between three and five embryos were pooled. For the qRT-PCR experiments, cells were collected in 100 cell pools. For the microarray experiment, all cells were collected, obtaining an average of 25-30,000.

Cells for cell cycle analysis were incubated with a 1:1 volume of iCyt solution (Sony, AE700570) for 2mins before being analysed on the Fortessa. 10,000 events were recorded from each sample. An outline of the basic FACS strategy used in all experiments can be found in Figure 2.1.

2.3: Microarray.

RNA was extracted using the Qiagen RNAeasy micro-kit, using the manufacturer's instructions. Briefly, cells were collected into lysis buffer RLT when sorted, an equal volume of ethanol is added to promote binding conditions, this is then added to the RNeasy MinElute Spin columns in which RNA binds to the silica membrane, DNA is digested using DNase, then column is then washed using ethanol before RNA elution using RNase-free water.

RNA samples were then sent to ARK Genomics, Roslin, for quality checks and were run on an affymetrix 1.0 chicken chip. All bioinformatics analysis was performed in Partek Genomics Suite software and pathway analysis via the Ingenuity Pathway Analysis software. Bioinformatics analysis is described in detail in chapter 5.

Figure 2.1

2.4: Immunohistochemistry.

Areas for staining were selected for as follows: slides containing cryosections were selected for GFP expression (observed on a widefield microscope). Of the GFP positive slides, those with the highest expression were selected for staining (usually the slides at the beginning and end of the electroporated area were not selected).

Slides or free-floating sections were incubated at room temperature for 30mins with a blocking solution containing 10% normal goat serum and 0.1% Triton-X in PBS. The primary antibody (all primary antibodies can be found in table 1, Figure 2.1) was diluted in the same solution and incubated overnight at 4^{o}C. Slides were washed three times in PBS before Alexa Flour secondary antibodies (488, 568 and 647, all Invitrogen, 1/1000) were incubated for 2hrs at room temperature. After 2 PBS washes, Hoechst diluted in PBS (5µg/ml, Sigma B2261) was incubated for 5mins at room temperature. Slides were then mounted with glass coverslips using Fluoromount-G (Southern Biotech) and stored at 4 ^{o}C. All confocal images were taken using a Leica SPE and analysed in ImageJ, Image Pro and Adobe Photoshop.

Antibodies against nuclear antigens required an additional antigen retrieval step. As this step often reduced detection of the cytoplasmic GFP, staining was carried out as above up until Hoechst staining. The slides were then post-fixed in 8% PFA for 1hr at 4^{o}C. After 3 PBS washes, slides were incubated in sodium citrate buffer (10mM, pH6) for 15mins at 80^{o}C and left to cool to room temperature for 20mins. Slides were then washed in PBS. Staining was then repeated as before, for the nuclear antibody. Negative controls were used without either primary or secondary antibody.

For EdU and TUNEL staining, Invitrogen Click-iT 647 kits were used (Invitrogen, EdU C10340, TUNEL C10247). This staining was performed according to manufacturer's instructions prior to any further staining. EdU was injected into the midbrain ventricle 30mins prior to fixation. All steps were carried out at room temperature unless stated otherwise and slides were protected from light at all times. For EdU staining of cryosections: slides were incubated with 0.1% triton-X in PBS for 20mins, washed twice with 3% BSA in PBS, EdU Click-iT reaction cocktail was added for 30mins (for 500µl: 430µl 1x Click-iT reaction buffer, 20µl Copper Sulfate, 1.2µl Alexa Fluor 647, 50µl Reaction buffer additive) and washed twice with 3% BSA in PBS. For TUNEL staining of cryosections: slides were incubated with 0.25% triton-X for 20mins, washed twice with 3% BSA in PBS, TdT reaction buffer was added for

10mins, TdT reaction cocktail was added for 1 hour at 37°C in a humidified chamber (for 500µl: 470µl TdT reaction buffer, 10µl EdUTP, 20µl TdT enzyme), washed three times with 3% BSA in PBS, Click-iT reaction cocktail was added for 30mins (for 500µl: 487.5µl Click-iT reaction buffer, 12.5µl Click-iT reaction buffer additive) and slides were washed once with 3% BSA. TUNEL control slides were used following kit instructions, using DNase I to generate strand breaks; in brief slides were incubated with 0.25% triton-X for 20mins, washed twice with deionized water, incubated with DNase I solution for 30mins (for 1 slide/100µl: 89µl deionized water, 10µl DNase I buffer, 1µl DNase I) and washed once with deionized water. These control slides were then treated as above for TUNEL staining.

Control slides used for staining were used without either primary or secondary antibody and were stained as above.

2.5: Cell Culture.

DF1 cells, a chick fibroblast line (kindly donated by Mike McGrew, Roslin Institute), were cultured at 39°C in the following media: DMEM (Gibco 41966-029), 1 x Non-essential amino acids (Gibco 11140), 1x PenStrep and 10% Fetal Calf Serum. Cells were passaged at 1/5 every 3-4 days, using TrypLE express (Gibco 12605-010). Cells were plated onto 6 well plates for lipofectamine transfection (Lipofectamine 200 reagent, Invitrogen, 11668-027) and plated onto 8 well chamber slides 24hrs later for staining. Cells were fixed 24hrs after re-plating using 4% PFA at 4°C for 20mins. Slides were then stored in PBS at 4 °C. Immunohistochemistry was performed as with cryosections.

2.6: qRT-PCR.

Cells for qRT-PCR were sorted into 100 cell pools directly into CellsDirect qRT-PCR mix (Invitrogen, 11753-100) containing a primer mix: for each tube of sorted cells, 5µl of CellsDirect 2x reaction mix, 2.5µl of primer mix, 0.2µl SuperScript III, 1.3µl dH_20. The primer mix contained 4µl of each primer and made up with water to 25µl for 10 reactions. This was pre-amplified using a 22 cycle PCR and samples were diluted 1/5 with dH_2O afterwards prior to freezing. Pe-amplification PCR: 50°C for 15mins, 95°C for 2mins, 22 cycles of 95°C

for 15secs and 60°C for 4mins, 4°C holding temperature. Samples were stored at -20°C. PCR was performed in a GS1 G-Storm thermal cycler.

Primers used for qRT-PCR were Qiagen Quantitect Primer Assays for Laminin-beta 1 (Gg_LAMB1_2_SG: QT00618632), Numb (Gg_NUMB_1_SG: QT00591857), Hes5 (Gg_HES5_1_SG: QT00630406), Tubulin-beta 3 (Gg_TUBB3_1_SG: QT00709065) and Collagen 4 alpha 2 (Gg_COL4A2_1_SG: QT00625940). 18S was used as a housekeeping gene (EUK 18S: Applied Biosystems 4352930). The qRT-PCR was performed on a Roche Light Cycler 480 II, using SYBR Green (Qiagen 204245) or Express qPCR Supermix with premixed ROX (Invitrogen 11795-200) and a 40 cycle run. For SYBR Green reactions: per well 0.8µl of primer, 4µ SYBR Green and 1.6µl dH_2O. For Express Supermix reactions: per well 0.4µl primer (18S), 4µl Express Supermix and 2µl dH_2O. The cycle used was a follows: 50°C for 2mins, 95°C for 10mins, 40 cycles of 95°C for 15secs and 60°C for 1min. Standard curves were run for each primer to allow relative quantification of CT values.

2.7: Constructs.

All constructs were previously used in the lab and made by Lisbeth Laursen or Veronique Marthiens (former postdocs) and are detailed in appendix A. All integrin constructs were made by Lisbeth Laursen and are in pCDNA3.1+. Cadherin constructs were made by Veronique Marthiens and are in IRES plasmids. Both cytoplasmic (already in lab) and nuclear GFP (Kindly donated by Federico Calegari, Centre for Regenerative Therapies, Dresden) were used. All plasmids contain a CMV promoter. Integrin constructs include a human WT itgβ1, a CA* itgβ1 which contains a point mutation in the salt bridge between the α and β subunits, allowing it to be in its primed active conformation. This mutation is a charge reversal point mutation – D723R (Hughes et al. 1996b). The final itgβ1 construct is an extracellular only itgβ1 (EC only). It lacks the intra-cellular domain, but still has both the extracellular and transmembrane domain. The cadherin constructs used are a WT mouse N-cadherin-IRES-GFP and a mutant that contains a point mutation in the localisation signal. This causes the cadherin to become mislocalised within the cell.

Retransformations were performed using DH5 α sub-cloning efficiency competent cells (Invitrogen 18265-017). Cells were thawed on ice before being added to 0.5µl of DNA, incubated on ice for 5 mins, heated at 42°C for 40secs, 200µl of SOC media was added and

incubated for 1 hr, before being streaked onto agar plates containing the appropriate concentration of ampicillin, either 50 or 100µg/ml (Sigma A5354-10ML) and incubated at 37^{o}C overnight. To obtain DNA concentrations of 1µg/µl, maxi-preps were performed using the Qiagen High-speed Plasmid Maxi Kit (Qiagen 12663) following manufacturer's instructions, briefly bacteria are lysed (alkaline method) and incubated in a QIAfilter cartridge (contains an Anion-exchange resin, removing SDS precipitates and clearing the bacterial lysate) RNA, protein and low-molecular weight impurities are removed using a medium salt buffer. DNA is bound to the filter within the cartridge, washed and eluted in a high salt buffer, mixed with isopropanol (to precipitate) and incubated in the QIAprecipitator to be desalted and concentrated before the final elution. DNA concentration was measured using a Nanodrop.

2.8: EdU labelling.

For all EdU experiments, embryos were injected and electroporated at E2. At either 12 hours post-electroporation (E2+12hr) or at E4, eggs were unsealed and EdU at 4mg/ml was injected into the ventricular space of the midbrain. As before, needles were placed into the hindbrain to avoid any damage to the midbrain neuroepithelium. Eggs were resealed and incubated at 38^{o}C for 30mins, after which embryos were immediately dissected and fixed in 4% PFA at 4^{o}C.

2.9: Selection of areas of interest.

The area of the sections selected for imaging and counting was the middle of the electroporated area in the sections with the best architecture (judged by Hoechst staining). Counts were not performed on any sections with very few GFP positive cells or on areas of a section with low GFP expression.

Chapter 3

Role of integrin-β1 in the chick embryonic neuroepithelium.

3.1: Introduction.

The chick embryo has been used to study development for centuries and was one of the first embryos to be studied due to its accessibility, ease of incubation and relative speed of development, hatching within 20-21 days. In the 1950s its development was catalogued into stages, providing an atlas of landmarks for use in experimental manipulation, which is still used today as Hamburger-Hamilton stages (HH); with the original paper being republished in the 1990s (Hamburger & Hamilton 1992). One key benefit of using this model is the ability to observe and manipulate early embryogenesis, including live imaging *in ovo* of the earliest stages of development (Kulesa et al. 2010).

The chick embryo has also been used extensively as a model to study neural development and neurogenesis. Due to its relative size and accessibility many different experimental procedures can be used, including grafting of tissues, manipulation of signalling pathways with soaked beads, fate mapping and gene expression by electroporation (Crossley et al. 1996; Storey et al. 1998; Garcia-Lopez et al. 2009; Nakamura & Funahashi 2001; Atkins et al. 2000). Further genetic analysis was enabled after the chicken genome was sequenced in 2004 (Hillier et al. 2004).

Due to these many advantages, the chick embryo was an already established model within the lab for the study of early neurogenesis. In particular, electroporation had been used to perturb gene expression within the midbrain neuroepithelium, using RNAi to knock-down itgβ1. The midbrain was chosen as the area to study NE cells due to its large size, making it easier to target during electroporation and dissection. For these reasons, we decided to continue with this system to further study the role of integrin signalling in the regulation of NSC proliferation and differentiation. As knock-down of itgβ1 results in disruption of

adhesion, causing NE cell process detachment and apoptosis, we are using over-expression of itgβ1 constructs via electroporation to perturb integrin signalling. Three human itgβ1 constructs will be expressed via electroporation, a WTβ1, an EC only which lacks the intracellular domain and is therefore unable to promote signalling, and a CA*β1 that is locked in the primed conformation, resulting in increased signalling levels (Hughes et al. 1996b; Laursen et al. 2011). These three constructs, along with an empty vector control, will allow perturbations of signalling at different levels without loss of adhesion. As electroporation of the chick midbrain had already been established in the lab as a system to study neurogenesis, the first aim of this chapter is to validate the expression and function of the human integrin constructs within chicken cells. The second is to study the effect of over expressing the human itgβ1 constructs via *in ovo* electroporation within the midbrain at the onset of neurogenesis.

3.2: Materials and Methods.

3.2.1: Embryo culture.

In this chapter all embryos were incubated for 45-8 hours prior to electroporation with the integrin constructs. They were then sealed and incubated for a further 12 hours, 1 day, 2 days or 4 days before fixation. Embryos electroporated with the N-cadherin constructs were incubated for 4 days prior to electroporation, sealed and incubated for a further 2 days before fixation. Embryos were electroporated on one side of the midbrain or the anterior neural tube, leaving the opposite side as an internal control.

3.2.2: Immunohistochemistry.

The primary antibodies used in this chapter were: anti-GFP (1/500, Abcam ab13970), anti-human integrin beta-1 (1/100, Millipore MAB1981), anti-Tuj1 (1/500Covance, MMS-435P and abcam ab18207), anti-pFAK pY397 (1/100, Invitrogen 44624G).

3.2.3: Cell culture.

A chick fibroblast cell line, DF1 (kindly donated by Mike McGrew, Roslin) was transfected with either an empty vector control or one of the human itgβ1 constructs using lipofectamine (Invitrogen) in 6 well plates. Cells were replated 24hrs later onto 8 well

chamber slides and fixed a further 24hrs later. The chamber slides were stained as above and images were quantified in ImageJ.

3.3: Results.

3.3.1: Validation of the human integrin-β1 constructs within chick cells.

To validate the expression of the human itgβ1 constructs, a chick embryonic fibroblast cell line was used, DF1 cells. Three human itgβ1 constructs were used, a WT, EC only and CA*β1 (Figure 3.1a) to allow perturbations of integrin signalling without causing loss of adhesion and apoptosis. All three human itgβ1 proteins were detected by immunohistochemistry on the surface of DF1 cells using a human itgβ1 specific antibody (Figure 3.1b). To determine if the constructs had any functional effect, phosphorylation of FAK at Y397 can be measured. FAK Y397 is a specific site of auto-phosphorylation resulting from integrin activation and is commonly used as a readout of integrin activity (Guan 2010; Guan 1997). A specific antibody to pFAK Y397 was used and pixel integrated density of pFAK Y397 staining was measured using ImageJ to allow quantification (Figure 3.1b). Compared to the non-transfected control, neither the empty vector, EC only nor WTβ1 had any effect on pFAK pixel integrated density. Expression of the CA*β1 showed a significant increase in pFAK pixel density in comparison to the non-transfected control and expression of the other constructs (Figure 3.1c). This suggests that the human CA*β1 is able to interact with its binding partners and ligands within the chick cells to promote signalling, validating its function within chicken cells. The empty vector, WT and EC only β1 did not appear to have any effect on signalling as pFAK Y397 levels were not affected compared to the non-transfected control, suggesting they do not have any adverse effects in chick cells.

3.3.2: Validation of *in ovo* electroporation.

As the constructs were found to be functional in a chick cell line, the next step was to validate their expression after electroporation within the midbrain neuroepithelium. To allow identification of electroporated cells without antibody staining, the constructs were co-electroporated with a cytoplasmic GFP. This also enabled easy visualisation of NE cell morphology (Figure 3.2b). Embryos were electroporated at E2 (HH10-12), around the onset of neurogenesis, and fixed 48hrs later. As DNA carries a weak negative charge, it is drawn towards the anode during electroporation, leaving the cathode side of the embryo as an internal control (Figure 3.2a). After electroporation, the electroporated cells are

Figure 3.1

Figure 3.2

easily identified by their cytoplasmic GFP expression. This also reveals their characteristic anatomy, including their bipolar morphology (Figure 3.2b), has been maintained.

Electroporation does not target all cells within the neuroepithelium, leaving neighbouring non-expressing cells adjacent to the GFP/human itgβ1 cells. All of the human itgβ1 constructs were expressed within the NE cells and could be detected by human specific antibody staining, which did not show any staining in the empty vector control (Figure 3.2c). Expression of the human itgβ1 also showed a high level of co-expression with GFP for all three constructs (Figure 3.2c). Overall, these results suggest all three human itgβ1 constructs are highly co-expressed with GFP within the midbrain neuroepithelium following electroporation and their expression does not appear to have any major adverse effects on NE cell survival and morphology.

3.3.3: Expression of CA*β1 results in an increase in neurogenesis.

After validation of the expression of the constructs *in ovo*, the effect of perturbing itgβ1 signalling within NE cells could be examined. In order to quantify any effect on the proliferation and differentiation of NE cells, the number of nuclei, GFP positive cells and neurons were counted (neurons were identified by Tuj1 staining). Tuj1 positive neurons were counted using confocal images in ImageJ, using compressed Z-stacks of Tuj1 staining alone alongside compressed Z-stacks of Tuj1 and nuclei staining composites (Figure 3.3). Tuj1 staining was used instead of a nuclear stain to allow greater identification of GFP positive neurons. Expression of the empty vector, EC only and WTβ1 did not affect the number of Tuj1 positive neurons generated, the total number of nuclei or GFP positive cells (Figure 3.4). However, expression of the CA*β1 significantly increased the total number of nuclei and Tuj1 positive neurons, without any effect on the number or proportion of GFP positive cells (Figure 3.4).

Interestingly, expression of the CA*β1 generated a range of phenotypes, with some embryos having a slightly thicker neuroepithelium with increased neurogenesis, whilst others had a more striking phenotype, resulting in a bulbous neuroepithelium protruding into the ventricle, but still showing increased neurogenesis (Figure 3.5). This range of phenotypes does not appear to be an effect of different levels of CA*β1 expression as both extremes show consistent levels of human itgβ1 co-expression with GFP (Figure 3.2c).

Figure 3.3a

Figure 3.3b

Figure 3.3c

Figure 3.4a

Figure 3.4b

Figure 3.5

One notable and surprising result was that none of the neurons found in any of the embryos expressing the CA*β1 were GFP or human itgβ1 positive (Figure 3.5). As mentioned previously, electroporation is not highly efficient; within the neuroepithelium around 40-50% of the cells are GFP positive. The remaining cells are GFP negative, non-electroporated cells, which neighbour the positive cells throughout the area electroporated. The lack of GFP expressing neurons suggests the increased neurons are generated from the neighbouring non-expressing cells. If the neurons are arising from these cells, the increase in neurogenesis would be a non-cell autonomous effect of CA*β1 expression.

3.3.4: Validation of the non-cell autonomous effect.

GFP negative neurons could be generated a variety of ways, including loss of GFP expression upon differentiation. In order to establish if the increase in neurogenesis is a non-cell autonomous effect, we next analysed a range of time points. The increase in neurogenesis was observed at E4 (HH22-23), 48hrs after electroporation. To ensure neurons had not been generated from GFP positive cells that had diluted or turned off their GFP expression, earlier ages were checked; 12hrs after electroporation (E2+12hrs) and 24hrs after electroporation at E3 (HH18-20). The increase in neuron number was not observed at either E2+12hrs or at E3 (Figure 3.6a and b). Additionally, none of the neurons that were present were GFP positive at either age (Figure 3.6a and b), suggesting the increased neurons observed at E4 are not generated from GFP positive cells that later lose their GFP expression.

A later age was also checked, E6 (HH28-29), 4 days after electroporation, to see if the increase in neurogenesis is transient or sustained and if GFP positive cells are later able to generate neurons. The increase in neurogenesis observed at E4 was sustained (Figure 3.6d) with a significant increase in neuron number in the embryos expressing the CA*β1. Unlike all other ages examined, by E6 some of these neurons are now GFP positive (Figure 3.7a), proving that Tuj1 positive cells can be generated from GFP positive cells.

3.3.5: Increased neurogenesis is only observed in the midbrain upon expression of CA*β1.

As not all regions of the neuroepithelium are alike, it was next important to establish if this phenotype was specific to the midbrain or a general role of itgβ1 in all NE cells. In order to

Figure 3.6

Figure 3.7

experiments. In contrast to the midbrain, electroporation of all four constructs within the do this, the constructs were electroporated in another area of neuroepithelium, the anterior neural tube. The empty vector and the 3 human itgβ1 constructs were electroporated at E2 and embryos were fixed 48hrs later, as with the midbrain anterior neural tube resulted in the generation of GFP positive neurons by E4 (Figure 3.7c). Unlike the midbrain, no effect of expression of CA*β1 was observed on either the number of nuclei or the number of Tuj1 positive neurons (Figure 3.8a). These results suggest the effect of CA*β1 is not general to all NE cells.

As the effect appears to be at least partly specific to the midbrain, it was next important to investigate if this effect is specific to perturbation of itgβ1 signalling, or due to a general disruption of adhesion molecules. In order to do this, another adhesion molecule also implicated in neurogenesis would need to be disrupted. One candidate was N-cadherin, a cell-cell adhesion molecule known to play a role in the regulation of NE cell behaviour and neurogenesis. N-cadherin constructs were available within the lab, a WT and mutated N-cadherin, which disrupts the endocytosis of the protein, disrupting its localisation within the cell and normal sites of cell-cell adhesion (resulting in a loss of endocytosis and increased/abnormal expression). Electroporation of these constructs was used to act as a control; to confirm if the increased neurogenesis is due to itgβ1 signalling not a general disruption of adhesion signals. The two N-cadherin constructs were electroporated into the midbrain at both E2 and E4, this later age of electroporation was used as N-cadherin has been implicated in later stages of neurogenesis, when NE cells divide asymmetrically (Lu et al. 2001). Embryos were fixed after 48hrs, at either E4 or E6 and at both ages no increase in neurogenesis observed (Figure 3.8b and c). This would suggest that the effect on neurogenesis in the midbrain is not due to a general disruption in adhesion molecules, but may be specific to perturbation of itgβ1 signalling within the NE cells of the developing midbrain. However, unlike expression of the itgβ1 constructs, GFP positive neurons were observed with expression of both the WT and mutant cadherin (Figure 3.7b). These results suggest that the increased generation of GFP negative neurons observed with expression of the CA*β1 is specific to perturbation of itgβ1 signalling within the neuroepithelium of the midbrain.

Figure 3.8

3.4: Discussion.

There are four main findings from this first results chapter. The expression of the CA*β1 significantly increases the number of nuclei and Tuj1 positive neurons, whereas expression of the WTβ1 and EC only had no effect. This phenotype is only present in the midbrain neuroepithelium upon expression of CA*β1 and expression of CA*β1 creates two phenotype variations; one with a slight thickening of the neuroepithelium and the other with neuroepithelium expanding into the ventricular space. This increase in neurogenesis appears to be a non-cell autonomous effect, as the neurons are GFP negative. These findings will be discussed in detail later; first I will focus on the function of the human itgβ1 constructs within chick cells.

In order to investigate itgβ1 signalling without disrupting adhesion, we are using a human WT, CA* and EC only itgβ1. The WTβ1 did not appear to have any adverse effect and the CA*β1 was found to be functional, increasing pFAK Y397 levels. The EC only β1, lacking the IC domain, is expressed on the cell surface and is still capable of binding to its heterodimer α6, due to its intact TM domain. However it is unable to signal as it lacks the intracellular domain, the main site of signalling in the heterodimer. This truncated itgβ1 has previously been shown to be unable to induce clustering of cytoplasmic downstream signalling proteins, such as FAK (Lewis & Schwartz 1995), preventing it from signalling. However, the functional effect of an itgβ1 lacking the cytoplasmic domain is debated in the literature; some authors do not find any dominant negative function (Mastrangelo et al. 1999; Retta et al. 1998) while others find this truncated itgβ1 does act as a dominant negative (Lee et al. 2006), although the authors do not show the level of function obtained. Other studies have also shown that the cytoplasmic domain is required to generate a dominant negative itgβ1 (Retta et al. 1998), suggesting the EC only β1 we are using will not have any dominant negative effect on integrin signalling. Consistent with this, the expression of the EC only in chick cells does not appear to have any effect on pFAK Y397 levels when compared with controls. This makes the EC only a good control, as it allows separation of increased surface levels of itgβ1 from perturbations of signalling. This allows three different perturbations; EC only that cannot signal, WTβ1 that can signal if activated and the CA*β1 that increases the level of signalling.

As knock-down of itgβ1 results in process detachment and apoptosis, a dominant negative could be used to reduce signalling levels without affecting adhesion. One example of a

well-established dominant negative is the fusion of the EC and TM domain of the interleukin 2 receptor fused to the cytoplasmic domain of itgβ1 (IL2R-β1), which has been previously used in the lab and elsewhere (Smilenov et al. 1994; Relvas et al. 2001; Câmara et al. 2009). This fusion protein enables the clustering of cytoplasmic downstream signalling proteins, sequestering them from endogenous itgβ1 and reducing signalling levels. A dominant negative itgβ1 fusion protein like this could also be used within this system to investigate the effect of reducing integrin signalling within these cells. However this may be complicated due to the high level of endogenous itgβ1 and rapid rate of cell division (a dominant negative should be expressed at the same level or higher than the endogenous protein, the plasmid will be diluted upon division). It is further complicated by the ability of the integrin family to compensate and by the short time periods of these experiments, which may not be sufficient for full function of a dominant negative.

3.4.1: Increase in neurogenesis.

The increase in neurogenesis observed upon expression of the CA*β1 could either be a real and sustained increase in neuronal number, or premature differentiation of cells. If the effect was merely a premature differentiation of cells, the increase in neurogenesis would not be sustained. The number of NSC/NE cells would decrease, as they would be exiting the cell cycle and differentiating too early, resulting in depletion of the progenitor pool. This would result in a final reduction in both cell and neuronal number. However, this is not what we have observed. Instead we see an increase in nuclei numbers alongside neuronal numbers, both of which are sustained at E6 (E6 nuclei count in Appendix D). The increase in cell numbers with the increase in neurons suggests the progenitor cells are dividing to produce the increased neurons. These results suggest that the increase in neurogenesis is a true effect and not due to premature differentiation of progenitors.

3.4.2: Different effects of expression of CA*β1.

Expression of the CA*β1 resulted in a significant increase in the numbers of both nuclei and neurons, without a change in the number or proportion of GFP positive cells. This phenotype was found in a variety of intensities. Some embryos only showed a moderate increase in nuclei alongside the increase in neurogenesis, resulting in a slight increase in thickness of the neuroepithelium. Others showed a much more obvious increase in nuclei, resulting in a neuroepithelium protruding into the ventricle. The proportion of GFP positive cells within these embryos remains relatively consistent, suggesting the range in

phenotypes is not due to a range in CA*β1 expression. There are many possible explanations for this difference in phenotypes. The polarity of the neuroepithelium could be altered, resulting in the second more obvious increase in cell numbers (this will be discussed in detail in the next chapter). It could also be due to the slight differences in the area of midbrain electroporated. The midbrain contains a complex spatial set of signals, including arcs of gene expression (Sanders et al. 2002). It may be that the electroporation occurs in different arcs of signals, creating the different responses observed. Two of the genes that form these arcs of expression are Wnt and Notch, both known to be involved in proliferation and neurogenesis and previously suggested to interact together with integrins in development (Rallis et al. 2010). The effect of these signals on the midbrain phenotype could be assessed by perturbations of these pathways.

Although every effort is made to ensure embryos are injected and electroporated at the same age, there are some small variations between embryo development. This may also be a factor in the variation of phenotype, as neurogenesis and signalling within the midbrain is also temporal. The cells may have slightly different competencies to respond to signals depending on the exact developmental stage of the embryo.

3.4.3: Neurons are GFP negative.

One surprising finding was that the increased neurons observed upon expression of the CA*β1 are not GFP positive, suggesting they are generated from the neighbouring negative cells differentiating. However, there are other possible reasons for why the neurons are GFP negative. As the GFP and itgβ1 constructs are electroporated as plasmids, they are not integrated into the DNA. The plasmid will therefore become diluted upon cell division, especially in cells that undergo repeated rounds of cell division in a short amount of time. It may also be possible that the process of differentiation may inhibit the expression of GFP/human itgβ1, resulting in a loss of GFP expression in the neurons. To confirm the neurons are from the negative cells and have not diluted or lost GFP, we next analysed neuron number and existence of GFP positive cells at different ages, both earlier and later than E4. The earlier ages, E2+12hrs and E3, were analysed in order to confirm neurons were not generated from GFP positive cells and then lost GFP expression. As mentioned earlier, at both E2+12hrs and E3 there was no significant increase in neurogenesis or nuclei numbers in embryos expressing CA*β1. Any neurons that were present were GFP negative,

suggesting the increase in neurons does not come from GFP positive cells that later lose their GFP expression.

A later age, E6, was also analysed to confirm if GFP positive cells were capable of differentiating into neurons and if neurons were able to retain GFP expression. Analysis of this later time point also confirmed the increase in neurogenesis is not transient, as the increase in neurons is sustained in the CA*β1 embryos. Some of these neurons are now GFP positive, proving GFP positive cells are able to differentiate into neurons and neurons are able to express the GFP. These GFP positive neurons also contribute to the significant increase in neurogenesis seen at this age; the number of GFP negative neurons alone is no longer significantly higher than in the other conditions. As no effect on neurogenesis was observed at the two earliest time points, the generation of neurons must occur between E3 and E4 and is sustained for the following 48hrs. Initially this increase in neurogenesis is primarily from the GFP negative cells, but after an additional 48hrs the GFP positive cells now contribute to the increased neuron number. This would suggest that the increase in neurogenesis observed at E4 is a non-cell autonomous effect of CA*β1 expression.

3.4.4: Effect is midbrain specific.

In order to examine if these findings are relevant to all areas of the neuroepithelium, the itgβ1 constructs were electroporated into another region of NE cells, the anterior neural tube, which is commonly used for the study of NE cells and neurogenesis. The constructs were electroporated at E2 and embryos were fixed 48hrs later, as with the midbrain experiments. Unlike the midbrain, GFP positive neurons were observed when all four constructs were expressed in the anterior neural tube. Surprisingly and in contrast to the midbrain results, no increase in neurogenesis was observed with expression of the CA*β1 in the anterior neural tube at this time point. This suggests the effect of constitutively activating itgβ1 is not generalised to all NE cells but may be specific to the midbrain neuroepithelium.

The neural tube and midbrain contain differences in signalling gradients and pathways at this stage in neurogenesis that may be creating these opposing phenotypes. As discussed previously, the midbrain contains various spatial and temporal patterns of signalling, which are different from those found in the neural tube, to allow generation of the different neuronal subtypes found in each region (Sanders et al. 2002; Garcia-Lopez et al. 2009). These gradients of signalling factors have been shown to be sufficient to instruct

progenitors to generate a specific type of neuron at a specific time (Suter et al. 2007). It may be that integrin signalling requires a certain threshold of neurogenic/proliferative signals in order to increase neurogenesis which is not present in the neural tube. If this was the case, addition of these signalling gradients to expression of CA*β1 in any region of NE cells should induce the increased neurogenesis.

Another difference between the neural tube and midbrain is the period of neurogenesis. Although RGCs across all areas of the developing CNS are able to generate neurons, their ability to do so is temporally and spatially dynamic (Anthony et al. 2004). Brain lipid-binding protein (BLBP), exclusively expressed in NE cells and astrocytes, is found in a heterogeneous expression pattern in NE cells early in neurogenesis. BLBP expression correlates with the onset of neurogenesis within the developing cortex and CNS, occurring in a spatiotemporal pattern. Midbrain and spinal cord BLBP expression, and therefore neurogenesis, occur at different times (Anthony et al. 2004). The differences observed upon expression of CA*β1 within the midbrain and spinal cord/neural tube may reflect the developmental differences between the two. Electroporation of the spinal cord/neural tube at a different age, to match the neurogenic capacity of the E2 midbrain, may show a different phenotype.

3.5 Summary.

The aim of this chapter was to validate the expression and function of human itgβ1 constructs within chick cells and then investigate any effect of altering itgβ1 signalling within the midbrain NE cells. Expression of the empty vector, WTβ1 and EC only had no effect, whereas the CA*β1 promoted an increase in nuclei and in neurogenesis. This appeared to be a non-cell autonomous effect, which was validated by the study of CA*β1 expression at different ages, in different areas of neuroepithelium and with the expression of N-cadherin constructs. This confirmed the increased neurons observed upon expression of CA*β1 in the midbrain are generated by the neighbouring, non-electroporated cells; a non-cell autonomous effect.

Chapter 4

Investigation of the non-cell autonomous effect of CA*β1 on neurogenesis.

4.1: Introduction.

In the previous chapter we observed the expression of the CA*β1 significantly increased neurogenesis specifically in the E4 midbrain neuroepithelium. We also observed that the expression of the empty vector, EC only and WTβ1 appeared to have no effect on overall cell number, GFP positive cells or number of neurons. We also previously established that the increased neurons were GFP negative at E4, suggesting the effect of the CA*β1 is non-cell autonomous. This was investigated at different time points and this non-cell autonomous increase in neuron number is thought to occur within a 24hr period, E3-4. After E4 this increase in neurons now occurs in both the GFP positive and negative cells. The aim of this chapter is to investigate this non-cell autonomous effect observed at E4 and to identify how these neurons are generated from the GFP negative cells.

It is first important to confirm the increase in neurogenesis is due to an increase in cell number, opposed to premature differentiation, by examining cell division. The increase in nuclei and neurons seen in the CA*β1 expressing embryos at E4 could be generated in several ways. An increase in cell number could be the result of increased cell divisions, which could be due to a faster cell cycle or the generation of more NE cells able to divide. An increase in cell number could also be due to a decrease in cell death; apoptosis is known to be important in many aspects of development. Both cell division and cell death will be examined in this chapter to identify how the increase in cell number and GFP negative neurons is occurring. The increase in neuron number could also arise from changes in division type, symmetric verses asymmetric, leading to changes in the levels of proliferation

and differentiation. This process may be influenced by itgβ1 signalling and if this was the case, there might be very few changes observed in cell division and cell cycle length.

Secondly we will establish if the architecture and anatomy of the neuroepithelium has been disrupted, which could result in abnormal NE cells behaviour and increased neurogenesis. A fundamental characteristic of NE cells is their bipolar morphology and INM. In this chapter we will investigate if expression of the CA*β1 has any effect on the polarity of the NE cells and INM, by examining expression of apical markers and location of cells in the S and M phases of the cell cycle. We will also examine the number and proportion of GFP positive and negative apical end feet attached to the ventricular surface, to determine if the CA*β1 is affecting adhesion of these cells.

4.2: Methods.

4.2.1: Embryo culture.

In this chapter all embryos were incubated for 45-8 hours prior to electroporation with the integrin constructs. They were then sealed and incubated for a further 12 hours, 1 day, 2 days or 6 days before fixation. For EdU analysis of cell cycle, embryos were injected with 4ug/ml of EdU 30mins prior to fixation.

4.2.2: Immunohistochemistry.

The primary antibodies used in this chapter were: anti-GFP (1/500, Abcam ab13970), Par3 (1/100, Millipore 07-330), aPKC (1/200, Santa Cruz sc-208), Pax6 (1/100, abcam ab5790) and PH3 (1/100, Millipore 06-570). For PH3 staining, prior to staining, cryosections were treated with sodium citrate at 80^{o}c for 15mins. For EdU and Tunel staining, Invitrogen Click-iT kits were used prior to staining according to manufacturer's instructions (Invitrogen, EdU C10340, Tunel C10247), both were Alexa Fluor 647. Phallodin was added with the secondary antibody for whole mount end feet staining (1/40, Invitrogen A12380).

4.2.3: FACS.

For cell cycle analysis by FACS, embryos electroporated at E2 were dissected at E4 using a fluorescent microscope to remove only the GFP area of the midbrain. This was then dissociated in acutase and resuspended in MEM. Sony i-Cyt DAPI solution was added to the

live cells for 2 mins prior to running on the Fortessa. Cell cycle analysis was performed on the total cell population and then split into GFP positive and negative cells.

4.3: Results.

4.3.1: Effect on cell division.

To confirm expression of CA*β1 promotes a real increase in neurogenesis, not premature differentiation, we investigated the effect of CA*β1 expression on NE cell divisions, a fundamental characteristic of these cells that is highly regulated. To assess any changes in cell division, cells in S phase and M phase were examined using EdU and PH3 respectively (Figure 4.1 and 4.2). At E4, when the increase in neurogenesis is first observed, the total percentage of EdU positive cells was not significantly different in any of the conditions when compared to controls. Neither was the percentage of GFP positive cells that are also EdU positive or GFP negative cells that are EdU positive (Figure 4.1). However, if the increase in neurons and nuclei observed at E4 has come from an increase in cell division, these changes may have occurred prior to the effect in neurogenesis observed at E4. To examine this, EdU was quantified in embryos fixed 12hrs after electroporation. As with the older embryos, no effect was observed in the percentage of total EdU positive cells with expression of any of the constructs compared to controls (Figure 4.1). The same was also observed for the percentage of GFP positive cells that were EdU positive at this age (Figure4. 1). These results would suggest that there are no significant effects on the number of cells in S phase in the CA*β1 embryos. Additionally, cells in S phase were observed in the basal region of the neuroepithelium, consistent with normal INM.

To further investigate changes in cell division, PH3 was used to quantify the number of cells in mitosis. As with EdU, PH3 cells were quantified at both E4 and E2+12hrs to examine changes prior to and during the increase in neurogenesis (Figure 4.2). At the 12hr time point, prior to the increased neurogenesis, there was a significant increase in the total percentage of PH3 positive cells in the CA*β1 embryos when compared to the internal control. This increase was not seen when the percentage of PH3 positive cells was broken down into GFP positive and negative, suggesting both populations of cells contribute to the significant increase in mitotic cells at this time point (Figure 4.2a).

Figure 4.1

Figure 4.2

Later, at E4 when the increased neurogenesis is observed, this increase in mitotic cells is no longer observed (Figure 4.2b). Instead, there are significant changes in the location of the PH3 positive cells not observed previously at E2+12hrs. In the internal control, empty vector, EC only and WTβ1 there are significantly more apical PH3 cells than basal (Figure 4.3b). This is also true for all conditions at the E2+12hr time point (Figure 4.3a). Expression of the CA*β1 at the E4 time point did not result in a significant difference between the percentage of apical and basal PH3 cells. Instead there is a significant decrease in the percentage of apical PH3 cells and a significant increase in basal PH3 cells when compared to control (Figure 4.3b). As with the increase in PH3 cells at E2+12hrs, these results are no longer significant if broken down into GFP positive verses negative cells, suggesting both populations of cells contribute to the effect. These results suggest the expression of the CA*β1 initially increases cells undergoing mitosis in both the GFP positive and negative cell populations. After this initial increase in mitosis, the cells appear to undergo mitosis in a more basal location during the increase in neurogenesis.

4.3.2: Cell death.

The changes in cell number observed after expression of CA*β1 could also be affected by levels of apoptosis and cell survival after electroporation. In order to investigate this, TUNEL staining was used to assess the levels of cell death. The percentage of total TUNEL positive cells at E4 was not altered in any of the conditions (Figure 4.4). Neither was the proportion of GFP positive cells that were TUNEL positive at E4 (Figure 4.4). TUNEL positive cells were also examined at a later stage, E6 after the initial increase in neurogenesis. This later stage was used to ensure the extra and abnormally located divisions observed at E2+12hrs and E4 did not later result in cell death. The results at E6 were consistent with the results at E4, the total percentage of TUNEL positive cells and the percentage of GFP positive TUNEL positive cells were not significantly affected by expression of any of the constructs when compared to controls (Figure 4.4). This data shows that expression of the itgβ1 constructs did not have any significant effect on the levels of cell death and the increase in neurogenesis is not arising from an increase in cell survival.

4.3.3: Cell cycle.

NE cells are known to alter their cell cycle length during neurogenesis and alterations in cell cycle length could lead to changes in neurogenesis. As there are some differences in PH3 levels and location, we next examined cell cycle dynamics of these cells using FACS analysis.

Figure 4.3

Figure 4.4

Length of cell cycle phases can also be measured using cumulative BrdU/EdU labelling over a period of time longer than S phase, alongside PH3 staining for mitotic cells, to allow quantification of time taken for a cell to undergo S phase and M phase. Unfortunately, due to complications with repeated administration of EdU and embryo survival, we were unable to generate reliable data from this experiment. Instead, we used FACS analysis, which allowed quantification of the proportion of cells in the different stages of the cells cycle. Cells are divided into G1/0, S and G2/M phases of the cell cycle depending on the quantity of DNA detected via DAPI staining. This technique also enables quantification of the population of cells as a whole as well as the breakdown into GFP positive and negative cells (Figure 4.5).

In all conditions, there are differences between the proportions of cells in the different stages of the cell cycle between the GFP positive and negative cells. This may be due to the electroporation preferentially targeting cells within a certain stage of the cell cycle. Both the empty vector and EC only cells show a very similar proportion of cells in each stage of the cell cycle in the GFP positive and negative populations. The WT and CA*β1 cells are similar to each other but show differences when compared to the empty vector and EC only. Both the WT and CA*β1 GFP positive cells appear to have more cells in G1 phase and less cells in S phase than the empty vector and EC only GFP positive cells. The WT and CA*β1 GFP negative cells appear to have the opposite, with fewer cells in G1 and more cells in S phase than the empty vector and EC only GFP negative cells. Although these results look interesting, the differences observed are not statistically significant. These results suggest there may be subtle differences between the GFP positive and negative cell populations and expression of the different constructs, which may contribute to changes in neurogenesis.

4.3.4: The anatomy of the neuroepithelium expressing the CA*β1.

A major characteristic of NE cells is their anatomy, which is highly regulated and specific to each type of progenitor (described in detail in the introduction). Perturbing adhesion molecule signalling within NE cells may lead to disruptions in neuroepithelium characteristics and anatomy. To asses if the non-cell autonomous increase in neurogenesis was due to changes in NE cell anatomy, apical markers Par3 and aPKC were used. In none of the conditions, including the two variations of neuroepithelium thickness upon CA*β1 expression, were the apical markers expressed at different levels or locations (Figure 4.6a

Figure 4.5

Figure 4.6

and b). These results suggest that NE cell apical polarity is normal in CA*β1 expressing cells.

Next we examined the attachment of NE cell apical end feet to the ventricular surface, a key characteristic of NE cells. Recent work in chick neuroepithelium revealed the fate of NE cells that divide asymmetrically to produce a daughter cell with an apical process and another with a basal process. The daughter with the apical process undergoes neuronal differentiation and loses its apical process, whereas the daughter with the basal process self-renews and reattaches an apical process (Das & Storey 2012). Perturbing itgβ1 signalling within NE cells may affect their adhesion to the ventricular surface; increased signalling may promote preferential adhesion of GFP positive end feet to the ventricular surface when compared to GFP negative end feet, altering the proportion of cells that retain attachment and are able to self-renew. This could be responsible for the non-cell autonomous increase in neurogenesis. As we observe an increase in GFP negative neurons, we predict that the GFP positive cells may be able to retain or reattach their apical process, therefore self-renewing, while the GFP negative cells lose or are unable to reattach their apical process, therefore differentiating.

Attachment of the apical process to the ventricular surface is known to involve integrins, injecting itgβ1 blocking antibodies into the ventricles of E15.5 mice resulted in apical process detachment (Loulier et al. 2009) as did RNAi against itgβ1 electroporated into chick NE cells (unpublished data from the lab). Expression of the CA*β1 may therefore be perturbing apical process adhesion and attachment to the ventricular surface. To determine if expression of the CA*β1 is promoting differential adhesion between the positive and negative cells, the end feet of the cells attached to the ventricular surface were counted using whole mount sections of the midbrain. Embryos were electroporated and fixed as normal. These fixed embryos were then dissected to remove the entire midbrain, which was flattened and stained with Phallodin to reveal the end feet along the entire ventricular surface (Figure 4.6c). No difference was seen in the total number of end feet attached to the ventricular surface, or the proportion of GFP positive verses negative end feet attached to the ventricular surface (Figure 4.6d and e). These results suggest that CA*β1 does not affect attachment of apical end feet to the ventricular surface or apical end feet size, as the same numbers of end feet were observed within a given area.

4.4: Discussion.

In the previous chapter we identified a non-cell autonomous increase in neurogenesis upon expression of CA*β1. It was established that these neurons are generated from the non-expressing neighbouring cells (GFP negative) and not from the GFP positive cells. The main findings of this chapter were firstly that expression of CA*β1 promoted an increase in mitotic cells which become more basally located and secondly that polarity and apical end feet attachment did not appear to be affected by expression of CA*β1.

4.4.1: Increase in mitosis.

To confirm the increase in neurogenesis is due to an increase in cell numbers and not premature differentiation we examined cell division markers. Expression of the CA*β1 significantly increased the level of mitotic cells 12hrs after electroporation, providing a source for the increased cells that are generated by E4. This increase in mitoses was not sustained at the later stage of E4, but there was a significant increase in the number of cells undergoing mitosis in an abventricular location. Due to INM, the majority of cells in the VZ undergo mitosis at the ventricular surface. In the mouse and human developing cortex, cells that undergo mitosis in an abventricular location are often basal progenitors (discussed further in the introduction). These cells act as neural progenitors and undergo division in the SVZ to produce two neurons. Basal progenitors are generated from apical progenitors that have delaminated from the ventricular surface, retracting their processes to become non-polar, such as oRGCs, IPs and Transit amplifying cells (TAPs). Previous work in the lab showed an increase in abventricular mitoses after injection of an itgβ1 blocking antibody into the E15 mouse ventricle alongside apical and basal process detachment (Loulier et al. 2009). The abventricular mitoses observed upon CA*β1 expression may be due to the generation of basal progenitors, not normally found in the chick neuroepithelium. Further investigation of process detachment and the behaviour of these cells would be required to confirm this.

Integrins are known to be expressed in the regions containing basal progenitors in the neocortex. In the human and ferret neocortex, the SVZ is split into the ISVZ and OSVZ, containing different types of basal progenitors. The OSVZ progenitors, thought to be responsible for cortical expansion in humans, require integrins to expand (Fietz et al. 2010) and ECM genes, including integrins, are highly up-regulated in this area (Fietz et al. 2012). These gene sets were found to be more important in the SVZ of the human than the mouse,

indicating increased ECM signalling may be required for the greater capacity of the human SVZ progenitors to self-renew (Fietz et al. 2012). The expression of CA*β1 may be affecting the level of ECM signalling and interactions within the environment, promoting the generation of abventricular dividing cells/basal progenitors. The cells with increased itgβ1 signalling (GFP positive) may be able to self-renew as progenitors, therefore not differentiating, while the GFP negative cells may not be able to self-renew as basal progenitors, so differentiate and increase neuron numbers.

If this were the case, it would provide an explanation for why only GFP negative neurons are generated when both the GFP positive and negative cells contribute to the increase in mitosis and abventricular location. As both these populations showed changes in mitoses, there must be an additional signal to promote neurogenesis only in the GFP negative cells. There are several mechanisms by which this signalling could occur (Figure 4.7). The increased level of integrin signalling in the positive cells may promote their self-renewal and alter the composition of the ECM environment. This may in turn promote the increased division of the negative cells. As these cells have normal levels of integrin signalling, they are unable to self-renew as basal progenitors so undergo differentiation.

Another mechanism for the non-cell autonomous increase in neurogenesis could be increased integrin signalling promotes lateral inhibition; integrin signalling in the positive cell promotes a higher level of a cell surface signal that binds to a receptor in the negative cell and promotes its differentiation, e.g. Notch-Delta signalling. Another possible mechanism could be that integrin signalling triggers an auto-feedback loop within the positive cell to proliferate and self-renew, whilst releasing a signal that triggers a feedback loop for differentiation in the negative cell. As integrin signalling is complex and interacts with many pathways, it is also likely to be a combination of these mechanisms that leads to the non-cell autonomous increase in neurogenesis.

4.4.2: Cell cycle.

Another issue raised by these results is the contrast between the lack of change in EdU levels and the increased PH3 levels. This could be due to a cohort of cells leaving the cell cycle or due to changes in the length of the cell cycle. NE cells are known to alter the length of G1 and S phase prior to neurogenesis, as discussed in the introduction along with

Figure 4.7

evidence to show altering the length of cell cycle phases can lead to neurogenesis (Arai et al. 2011; Lange et al. 2009). NE cells shorten S phase and lengthen G1 prior to neurogenesis. If more cells were undergoing neurogenesis, the overall number of cells in S phase may not be significantly altered; however you might expect to see more cells in G1.

To establish the proportion of cells within each stage of the cell cycle, FACS analysis was used which revealed the proportion of cells in each stage of the cell cycle was different in positive verses negative. This may be due to an electroporation bias towards dividing cells (Pilaz et al. 2009). Although there are some small but interesting changes between conditions, these results are not statistically significant. This would suggest that any changes occurring in the cell cycle at E4 are very subtle. It is likely that there are no statistically significant changes by E4 as both the GFP positive and negative cells generate neurons by E6. To further investigate the role of cell cycle length in this phenotype, analysis should be continued to include earlier time points, to identify differences that lead to the increase in GFP negative neurons, as well as further investigation of the length of the various cell cycle phases. As mentioned in the results, cumulative BrdU/EdU labelling alongside PH3 staining can be used to quantify length of cell cycle phases, but this was not successful. Another experiment using EdU/IdU and BrdU double pulse labelling can also be used to quantify the length of S phase and may reduce some of the difficulties of the longer cumulative labelling experiment.

4.4.3: Anatomy of the neuroepithelium.

As an increase in integrin signalling may affect cell polarity, it was therefore important to establish the anatomy of the neuroepithelium is preserved in the CA*β1 expressing embryos. Polarity proteins Par3 and aPKC are part of the apical complex and have been implicated in the regulation of NE cell behaviour (Costa et al. 2008; Sabherwal et al. 2009; Bultje et al. 2009) (the role of the apical complex is discussed further in the introduction). Integrins have been shown to interact with Par3 and aPKC via Numb (Nishimura & Kaibuchi 2007), where aPKC regulates Numb binding to integrins for endocytosis. In endothelial cells loss of itgβ1 reduced Par3 levels (Zovein et al. 2010) and in intestinal stem cells integrins have been shown to induce asymmetric segregation of Par3 in daughter cells, resulting in an asymmetric division (Goulas et al. 2012). As integrins have been shown to interact with Par3 and aPKC, which have known roles in NE cell division and differentiation, we used these markers to asses if CA*β1 had altered cell polarity. In all of the conditions examined,

both Par3 and aPKC were normally localised to the apical membrane of NE cells, in both GFP positive and negative cells. These preliminary results suggest NE cells are able to establish polarity as normal, but further investigation would be needed to confirm overall cell polarity is not affected, such as examination of basal-complex proteins and additional polarity markers (including AJs and tight junctions); e.g. LGN, Lgl, cadherin, β-catenin and Par6 (Doe 2008).

Another test of neuroepithelium anatomy was to study the ventricular end feet. Apical process attachment is essential for normal NE cell behaviour and inheritance of the apical process has been linked to cell fate (Das & Storey 2012). As mentioned previously, integrins are known to be involved in apical process attachment, consistent with knock-down of itgβ1 in the mouse embryonic VZ NE cells (Radakovits et al. 2009; Loulier et al. 2009). One possible effect of increasing itgβ1 signalling could be an increase in adhesion of apical end feet to the ventricular surface. If this increase only occurred in the GFP positive cells, this might provide an explanation for the non-cell autonomous effect on neurogenesis. If the CA*β1 expressing cells are more adhesive, after a cell division, both their daughter cells would be likely to retain attachment to the ventricular surface and self-renew, as they would be able to reattach their apical processes faster and more efficiently (Figure 4.8a). The GFP negative cells would have a far lower level of adhesion (due to a lower level of integrin signalling) and would be competing against the highly adhesive GFP positive cells. After a division the GFP negative apical end feet may not be able to out-compete the GFP positive end feet to enable attachment to the ventricular surface. This would result in detachment of the GFP negative cells, leading to their differentiation.

Examination of the CA*β1 positive and negative end feet showed no differential adhesion, suggesting the expression of CA*β1 does not provide preferential attachment of the apical process to the ventricular surface. This also suggests the generation of basal progenitors is not due to perturbations of adhesion and attachment, but perhaps due to an increase in cell numbers within a given space, creating competition for attachment to the ventricular surface (Figure 4.8c). Overall, this preliminary data on the polarity and end feet of NE cells indicates expression of CA*β1 is not affecting NE cell anatomy to create the non-cell autonomous increase in neurogenesis. Instead, the generation of basal –type progenitors and the increase in GFP negative neurons may require signalling and cell-cell interactions between the GFP positive and negative cell populations, discussed in the following chapter.

Figure 4.8

4.5: Summary.

In the previous chapter we established expression of the CA*β1 resulted in a non-cell autonomous increase in neurogenesis. In this chapter we investigated this phenotype to identify how these neurons were being generated from the GFP negative cells. As examination of the anatomy of the neuroepithelium did not find any changes, NE cell division and survival were investigated. The increased neurogenesis is not due to a reduction in cell death, but more likely due to the increased mitotic cells present at E2+12hrs. However, the increase in mitosis and the later abventricular location of mitotic cells occurs in both the GFP positive and negative cells. These results suggest that the non-cell autonomous increase in neurogenesis requires additional signalling both within and between cells and is not purely an effect on NE cells anatomy or division.

Chapter 5

Investigation of cell-cell signalling.

5.1: Introduction.

In the previous chapters we established that the expression of CA*β1 results in a non-cell autonomous increase in neurogenesis and that this effect appears to be specific to activation of integrin signalling within the midbrain neuroepithelium. Investigation of the anatomy of the electroporated neuroepithelium showed no changes that would result in abnormal cell behaviour. There was also no observed effect on cell survival in the CA*β1 embryos. In order to establish how these extra neurons were generated we examined cell division and the cell cycle. There was a significant increase in mitotic cells in the CA*β1 embryos at the earliest time point which later led to abnormal location of these cells. Although this effect may provide a source for the increased cell numbers, it was present in both the GFP positive and negative populations. We next investigated the proportion of cells within the different stages of the cell cycle and although there were some interesting differences between the GFP positive and negative populations, they were not significant. Overall these subtle changes in cell division could not solely account for the non-cell autonomous increase in neurogenesis. We predict that the non-cell autonomous effect of CA*β1 on neurogenesis requires signalling between the positive and negative cells.

This non-cell autonomous increase in neurogenesis could occur in many ways and through numerous mechanisms, due to the wide range of interactions between integrin signalling and other pathways. The expression of CA*β1 could promote expressing cells to self-renew and inhibit their differentiation, whilst signalling to neighbouring cells to undergo neurogenesis. It is also possible that expression of CA*β1 specifically promotes signalling, either cell-cell signalling or a secreted factor, that promotes differentiation in neighbouring cells. The graded levels of integrin activity between the two populations of cells may also

have an effect in neurogenesis; GFP positive cells with be high activity and GFP negative cells will be low activity.

The aim of this chapter is to investigate this signalling using microarray analysis. In order to investigate this, a broad approach was needed to identify potential candidates. For this reason we used FACS to separate the two populations of neighbouring cells, GFP positive and negative, for microarray analysis. This would allow investigation of signalling changes both within the GFP positive and negative cells and provide candidates for cell-cell interactions or gene expression changes to promote the non-cell autonomous increase in neurogenesis.

5.2: Methods.

5.2.1: Embryo culture.

For the microarray, three to five embryos, aged E4 (2 days post-electroporation), were dissected to remove the GFP positive area of the midbrain. The midbrain was then dissociated in acutase and resuspended in MEM. For qRT-PCR, embryos were dissected in the same way as above, but were sorted into 100 cell pools of GFP positive and negative cells. Cells were collected directly into qRT-PCR mix (Invitrogen, CellsDirect 11753-100).

5.2.2: FACS.

Cells were sorted on a FACS Aria to collect all GFP positive and negative cells, for the EC only, WT and CA*β1 conditions (Figure 5.1a). Cells were collected in lysis buffer and RNA was extracted using a Qiagen RNA easy micro-kit. RNA was then sent to ARK Genomics, Roslin, for QC checks and run on an Affymetrix chick 1.0 chip. For qRT-PCR experiments, embryos were prepared in the same way but sorted into 100 cell pools directly into CellsDirect PCR mix.

5.2.3: Bioinformatics.

Bioinformatics was performed using the Partek Genomics Suite software. Pathway analysis was performed using Ingenuity Pathway Analysis software. Principle Component Analysis was performed with the help of Alison Downing (ARK Genomics, Roslin).

5.2.4: qRT-PCR.

Prior to qRT-PCR, cDNA was generated and amplified using the Invitrogen CellsDirect Kit. It was then used with Qiagen quantitect primer assays and SYBR Green for qRT-PCR, using a Roche Lightcycler.

5.2.5: Immunohistochemistry.

The primary antibodies used in this chapter were: anti-GFP (1/500, Abcam ab13970) and anti-Tal2 (1/100, Santa Cruz sc-46267).

5.3: Results.

5.3.1: FACS separation of cells and microarray analysis.

In order to investigate the signalling and gene expression in the GFP positive and negative populations of cells, they first had to be separated. To do this, the GFP positive area of the midbrain was dissected and dissociated to allow FACS sorting of the GFP positive and negative cells (Figure 5.1a). Due to the relatively low number of cells obtained from a single embryo, between three to five embryos were pooled for each condition: WTβ1, CA*β1 and EC only. Each pool of embryos was separated into GFP positive and negative cells, creating six samples for microarray analysis. The RNA was extracted from the samples and sent to ARK Genomics at Roslin for quality checks and preparation before the Affymetrix microarray was performed.

All bioinformatics for the samples was performed by myself using the Partek Genomics Suite software. Initially it was important to check the quality of the samples and data sets. One of the quality checks uses hybridization metrics. Four E. coli derived labelled molecules, BioB<BioC<BioD<Cre used in increasing concentrations, are spiked into the labelled samples prior to hybridization. In all samples, these molecules should appear in the same order of concentration after the microarray. These hybridization spikes can be displayed in a line graph (Figure 5.1b) and are in their order of increasing concentrations in each sample. The hybridization metrics for all six samples show a good level of consistency and passed this quality check.

To visualise any differences between the transcriptomes of the six samples, prior to further analysis, principle component analysis (PCA) can used (Figure 5.1c), which enables

Figure 5.1

visualisation of clusters for the entire transcriptome analysed for each sample (PCA was performed with the help of Alison Downing, ARK Genomics). The PCA for these samples shows the CA*β1 GFP positive and negative cells and the EC only positive cells are the most similar. This highlights that only subtle changes between the transcriptomes of the CA*β1 positive and negative cells may cause the non-cell autonomous increase in neurogenesis.

To investigate this further, an anova was the performed on the microarray data to generate a list of fold change between the samples, principally between the positive and negative cells within each condition, secondly between the different conditions. The genes showing a 2 fold change in expression levels can be clustered and displayed on a heat map (Figure 5.2). This heat map was generated comparing the CA*β1 GFP positive and negative samples. The fold change was also included for the four other samples, WTβ1 and EC only GFP positive and negative cells. There are some clear differences between the CA*β1 GFP positive and negative cells and some of these differentially expressed genes are not expressed in the same pattern in the WTβ1 and EC only samples.

Before candidates were selected for further investigation, the microarray results were validated using qRT-PCR. The GFP positive and negative cells were separated using the same FACS strategy as the microarray, except cells were collected from single embryos (dissociated GFP area of midbrain as before) into 100 cell pools. This allowed a large number of biological replicates to be used, whilst providing enough RNA for the qRT-PCR. A number of genes were selected for validation, including Notch pathway genes Hes5 and Numb, neuronal marker Tubb3 (Tuj1/tubulin beta 3) and integrin ligands laminin-β1 (Lamb1) and Collagen 4 alpha 2 (col4a2). These genes were selected as some show up-regulation, others down-regulation and one a mix of up and down-regulation in the different samples. The GFP positive and negative cells were compared, as with the microarray data to generate the fold change in expression. The fold change for the five genes mentioned are consistent in both the microarray and qRT-PCR results (Figure 5.3), validating their expression levels.

In addition to the analysis above, pathway analysis was performed using the Ingenuity Pathway Analysis software. The top five pathways that show the highest fold change for each sample, again comparing GFP positive to negative, are shown in Figure 5.4, along with the top five cellular pathways. The CA*β1 sample pathways include development, cell movement and cell-cell signalling. This is in contrast to the WTβ1 and EC only, where post-

Figure 5.2

Figure 5.3

Figure 5.4

translational modifications, morphology and growth are some of the top pathways indicated to be involved. Within the cellular mechanisms, cell movement, development and proliferation are indicated in all samples. The WTβ1 and EC only also have cell survival indicated as a potential mechanism highlighted from the array results, whereas the CA*β1 sample has cell to cell signalling and cellular organisation indicated. This would suggest that the majority of the genes showing a fold change in expression levels are involved in similar pathways across all of the samples, but the CA*β1 sample contains fold change in more genes involved in cell-cell signalling and the assembly and organisation of cellular structure. This would be consistent with an increase in itgβ1 signalling, as one of the downstream effects involves pathways that organise the cytoskeleton.

5.3.2: Up and down-regulated genes in the CA*β1 expressing cells.

Once the analysis of the samples was complete, the top up and down-regulated genes, identified via fold change between the GFP positive and negative cells, for each construct were identified from the anova results. The genes of most interest are only up or down-regulated in the CA*β1 cells, as these are most likely to be involved in the non-cell autonomous increase in neurogenesis. The top 10 genes only or down-regulated in the CA*β1 are shown in Figure 5.5. Fold change is again measured for GFP positive cells compared to GFP negative cells. A positive number indicates up regulation in the GFP positive cell/down regulation in the negative cell and vice versa for a negative number. The list of genes only up-regulated in the CA*β1 cells include genes linked to immunity, the ribosomal gene encoding part of the 60s subunit, histone proteins and an intermediate filament protein. The list of genes only down-regulated in the CA*β1 cells includes micro-RNAs, a centromere protein involved in the assembly of the kinetochore and a prefoldin subunit required for normal neuronal development (Lee et al. 2011). The micro-RNAs listed have a wide range of targets (Appendix C) including genes involved in neuronal differentiation and key developmental pathways, such as BMP antagonists.

One of the genes only up-regulated in the CA*β1 positive verse negative cells was Tal2, T-cell acute lymphoma 2. Although originally identified in lymphoma, Tal2 was later shown to be important for midbrain development and neurogenesis and early on in neural development is specifically expressed within the midbrain. Previous studies in the mouse have shown it to be required for normal midbrain development, possibly in a non-cell

Figure 5.5

autonomous manner (Bucher et al. 2000). This highlighted Tal2 as a good candidate for further investigation.

5.3.3: Validation of Tal2 expression.

As Tal2 was up-regulated in the CA*β1 GFP positive cells compared to GFP negative cells, it was important to compare its expression levels to the other samples, to identify if Tal2 was up-regulated in the positive cells or down-regulated in the negative cells. The CA*β1 cells were compared to the remaining four samples via anova analysis. Tal2 was found to be up-regulated in the CA*β1 GFP positive cells when compared to any sample (Figure 5.6b) and was not up-regulated in any other comparisons. This confirms that Tal2 is up-regulated in the CA*β1 positive cells opposed to down-regulated in the negative cells.

It was now important to validate these results with detection of protein levels within the neuroepithelium. To do this we used immunohistochemistry to identify cells expressing Tal2 (Figure 5.6a). In order to asses if Tal2 was nuclear in the GFP positive cells, a nuclear GFP was used instead of the cytoplasmic GFP used previously. This would also allow easier identification of GFP positive Tal2 positive cells. The total percentage of Tal2 positive cells was not affected by expression of any of the constructs (Figure 5.6c). However, the percentage of Tal2 positive cells that were also GFP positive was significantly higher in the CA*β1 embryos (Figure 5.6). There was around a two fold increase in the percentage of GFP positive cells expressing Tal2 in the CA*β1 embryos, consistent with the level of fold change observed in the microarray results. These results suggest that the increase in Tal2 expression observed in the microarray are consistent with expression levels observed in the embryos. Due to the previous studies of Tal2 within the midbrain and its specificity to the midbrain, these results indicate Tal2 is a strong candidate for the non-cell autonomous effect of CA*β1 expression on neurogenesis.

5.3.4: ECM gene expression.

As discussed in the previous chapter, ECM genes are known to be highly expressed in regions of the human neocortex containing proliferative basal progenitors (OSVZ). Within our microarray data, there were several ECM related genes that showed a large fold change between the CA*β1 positive and negative cells (Figure 5.7a and appendix E). As ECM has been implicated in the generation of basal progenitors and we have observed an increase in

Figure 5.6

Figure 5.7 page 1

Figure 5.7 page 2

abventricular mitoses in CA*β1 embryos (see previous chapter), we further investigated the changes in these genes across the three conditions. Data from the initial comparisons between positive and negative cells only identifies a fold change between cells, not in which population of cells the level of gene expression has changed. In order to do this, we carried out a second round of comparisons to enable identification of the up or down-regulation of these ECM related genes. When compared to the GFP positive and negative cells in the other samples, these genes were found to be slightly down-regulated in the CA*β1 positive cells and highly up-regulated in the CA*β1 negative cells (Figure 5.7b and appendix E).

5.4: Discussion.

In the previous chapter we investigated changes in cell division that would enable the increased neurogenesis upon expression of CA*β1. The changes observed in mitotic cells were found in both GFP positive and negative cells and were not sufficient to explain the increase in GFP negative neurons observed at E4. This would suggest that there must be further signalling occurring, either between cells or within their environment, to generate neurons specifically from the GFP negative cells.

In order to further investigate the signalling between the positive and negative cells, a broad approach was used. Using the FACS strategy in chapter 4, cells were separated into the positive and negative populations for microarray analysis. This was done using the GFP positive and negative cells for only WTβ1, CA*β1 and EC only as an initial experiment, pooling embryos. Ideally, the microarray would be repeated with additional biological replicates and the empty vector control to allow statistical analysis of the results, but as this technique has not been used in the lab before, we decided to conduct this initial experiment with a quarter of the number of samples. To try to overcome the issues of a lack of statistics to validate the results, we focused only on genes that were only up or down-regulated in the CA*β1 cells and not in the WTβ1 and EC only samples. The microarray results were also validated by qRT-PCR, using the same FACS strategy but with collection of only 100 cells from a single embryo, allowing a greater number of biological replicates. Further to this, any result we chose to follow up would be validated with antibody staining.

Of the genes that were up-regulated only in the CA*β1 positive cells, Tal2 was selected for further investigation. It was one of the top three genes that was only up-regulated in the CA*β1 positive cells and further comparisons across all the samples confirmed Tal2 was up-regulated only in the CA*β1 positive cells. To confirm increased Tal2 expression in the CA*β1 positive cells, antibody staining was used. These results showed a significant increase in the percentage of Tal2 positive cells that are GFP positive in the embryos electroporated with CA*β1. This increase was around two fold, consistent with the fold change observed in the microarray. Overall, this highlighted Tal2 as a strong candidate to investigate.

5.4.1: Known roles of Tal2 in neurogenesis.

Tal2, T-cell acute lymphoma, is a basic helix-loop-helix (bHLH) transcription factor. It was originally discovered to be associated with a complex of bHLH factors associated with T-cell acute lymphoblastic leukaemia, known as the T-ALL complex. Tal2 was found to share a high homology with the other factors in this complex, Tal1 and Lyl1 (Baer, 1993; Xia et al., 1991; Xia et al 1994). The T-ALL complex was originally identified at chromosomal breakpoints in T-cell leukaemia (Bernard et al. 1991) and Tal2 has been shown to be activated by a translocation defect in patients (7;9)(q34;q32) (Xia et al. 1994).

Normal expression was first observed in the adult testis (Xia et al. 1991) and Tal2 expression was later identified in the embryonic brain: in the diencephalon, mesencephalon and metencephalon from E12.5 in mouse (Mori et al. 1999). Within the mesencephalon, Tal2 expression was found in cells within the VZ and intermediate zone and the authors suggest it may have a function in neuronal differentiation (Mori et al. 1999). Tal2 was later shown to be necessary for normal brain development in the mouse (Bucher et al. 2000). A null mutation of Tal2 in mice showed no signs of haematopoiesis defects as expected from its role in lymphoma. However, the null mutation of Tal2 results in post-natal lethality due to progressive hydrocephalus and dysgenesis of the midbrain (Bucher et al. 2000). Tal2 expression was detected within the mesencephalon in E10.5 mice, but no differences were detected in the midbrain during embryogenesis in the null mutants. By P2 subtle differences were observed which were obvious by P16, including enlarged lateral ventricles and reduction of the superficial gray layers (Bucher et al. 2000). The authors suggest the discrepancy between time of Tal2 expression and observed phenotype could be explained by a non-cell autonomous effect, Tal2 positive cells may be required to signal to

neighbouring cells for differentiation. It could alternatively be due to the progeny of Tal2 null cells inheriting a defect that later effects their behaviour, or Tal2 may interact with another gene that leads to this phenotype (Bucher et al. 2000).

Tal2 expression has also been observed in the Zebrafish CNS, specifically in the lateral floor plate. Expression is initially detected at the 13 somite stage, in the anterior neural tube and brain and spread throughout the entire spinal cord later in development (Pinheiro, 2004; Schäfer, 2007). As with the mouse, Tal2 expression was found in the developing midbrain, which increases later in development but is not present in the adult. The Tal2 expressing cells are located above the ventricular surface, roughly by a cell diameter and are found in a mosaic pattern (Pinheiro et al. 2004). This is similar to the pattern of Tal2 positive cells we have observed (Figure 5.6a), in particular the very low numbers of Tal2 cells located at the ventricular surface; the majority of Tal2 positive cells are located in the basal region of the VZ, but not located along the pial basement membrane suggesting these cells are not post-mitotic neurons . In the zebrafish, the later expression of Tal2 within the spinal cord is located to the lateral floor plate, where its expression is again patchy and is most likely dependant on Shh (Pinheiro et al. 2004). The authors suggest these cells are not motor neurons, but an unidentified population of progenitors.

A later study identified these Tal2 positive cells are a different population than their Tal2 negative neighbours within the lateral floor plate (Schäfer et al. 2007). The authors suggest the Tal2 positive cells are neuronal progenitors, that go on in part to generate interneurons, while the neighbouring negative cells are foxa2 positive and are suggested to be non-neuronal cells (Schäfer et al. 2007). These two neighbouring populations of cells interact via Notch signalling and are able to respond differently to Shh, providing evidence of Tal2 positive and negative cells interacting and having different responses to key signalling pathways. This again has similarities to our results showing a mosaic expression of Tal2 alongside mosaic expression of CA*β1, creating a positive and negative population. These two populations of cells are behaving differently, creating the non-cell autonomous increase in neurogenesis. The Tal2 and CA*β1 positive and negative populations could be acting as those in the zebrafish spinal cord, interacting via cell-cell signalling to promote differential responses to key signalling pathways.

5.4.2: ECM gene expression changes.

In the previous chapter we highlighted the known expression of integrins and ECM within the OSVZ of the human and ferret cortex, thought to be required for the capacity of the OSVZ progenitors to self-renew before differentiating and greatly expand the cortex (Fietz et al. 2010; Fietz et al. 2012). Our microarray results suggest that ECM genes are slightly down-regulated in the CA*β1 expressing cells and highly up-regulated in the CA*β1 negative cells, indicating that integrin activation promotes a change in the ECM in neighbouring cells and therefore the extracellular environment. However, these results have not yet been validated, so are only preliminary. Further investigation using qRT-PCR and antibody staining would be required to confirm these changes in ECM. Once validated, altering the levels of these ECM molecules could be used to test their role in the non-cell autonomous effect on neurogenesis and the production of basally dividing cells.

These preliminary results are another example of a non-cell autonomous effect of expression of CA*β1. As ECM is thought to be critical for OSVZ progenitor behaviour, this change in ECM caused by expression of CA*β1 may result in the basal progenitor-type behaviour observed in chapter 4; basal mitoses, increase in neurogenesis and expansion of cell numbers. The ECM genes are slightly down-regulated in the CA*β1 positive cells, suggesting that integrin activation may act as a feedback mechanism for ECM levels within the environment, or that down-regulation of these genes promotes self-renewal of these cells. As these genes are highly up-regulated in the CA*β1 negative cells, there must be a signal between the GFP positive and negative cells to promote this change in expression, which has not yet been identified, but could potentially involve the pathways interacting with Tal2.

Increased levels of ECM interactions and adhesion have been suggested to promote self-renewal of basal progenitors in the OSVZ (Fietz et al. 2012). However these progenitors are also able to undergo neurogenesis and are responsible for the generation of a large number of neurons, allowing expansion of the cortex. This indicates that cells must be able to respond to the levels of ECM and decide when to self-renew or differentiate. Integrin signalling may be required for this decision. From our results, we would predict that high levels of ECM gene expression allow the production of proliferative basal progenitors, but integrin signalling is required for the interactions between these cells and their EC environment (Figure 5.8). High levels of integrin activity would promote cell attachment to

Figure 5.8

this environment and self-renewal of progenitors, whilst lower levels would promote cell detachment from this environment and subsequent differentiation. This could also explain the non-cell autonomous effect in neurogenesis, as the GFP positive cells have a high level of integrin activation whilst the GFP negative cells are effectively low activation level cells, promoting differential responses to the same environment. Further investigation of the role of integrin activation in regulating ECM matrix and the effect of ECM composition on NSCs would be required to identify if this is creating the phenotypes observed or is a consequence of increased integrin signalling.

5.5 Summary.

In the previous chapters we established the expression of CA*β1 results in a non-cell autonomous increase in neurogenesis. Although some changes in mitotic cell number and location were observed, both the GFP positive and negative cells contributed to these changes, which were therefore were not sufficient to explain the generation solely of GFP negative neurons. Interactions and signalling between these two populations must be occurring to promote differentiation in one and not the other. In order to investigate this further, microarray analysis was performed on the GFP positive and negative cells. From this analysis, Tal2 was highlighted as a strong candidate molecule to investigate. The up-regulation of Tal2 in the GFP positive cells was confirmed via antibody staining, validating the results. Tal2 is already known to be important in midbrain neurogenesis and is expressed specifically in the midbrain early in neurogenesis, before expression spreads throughout the CNS. In studies in both the midbrain and spinal cord, cells expressing Tal2 have been suggested to interact with neighbouring non-expressing cells and may affect their behaviour.

Chapter 6

Conclusions and future perspectives

The aim of this thesis was to investigate the role of integrins in the regulation of proliferation and differentiation of NSCs within the embryonic CNS. This regulation is thought to occur in two ways, on an environmental or a cellular level. On a cellular level, fate of NSCs could be determined by the segregation of fate determinants and regulation of the angle of division. On an environmental level fate would be determined in a more stochastic manner, via signalling and cell-cell interactions post-division. As mentioned before, these two models are not mutually exclusive and could act in combination to regulate NSC proliferation and differentiation. We used *in ovo* electroporation to express a WTβ1, a CA*β1 and EC only itgβ1 to create a mixed population of expressing and non-expressing cells within the same environment to allow the study of the intrinsic verses extrinsic models.

This thesis has identified expression of a CA*β1 promotes a non-cell autonomous increase in neurogenesis within the neuroepithelium of the developing midbrain. Investigation of neuroepithelial anatomy, survival and cell division dynamics indicated signalling and interactions between cells was responsible for this effect. Microarray analysis revealed a strong candidate, bHLH transcription factor Tal2. Tal2 was selected due to its known function within the midbrain, as discussed in the previous chapter. If Tal2 expression is responsible for this phenotype, how does expression of CA*β1 increase its expression?

Downstream of itgβ1 outside-in signalling is the Ras-raf-MEK-ERK pathway, known to effect cell proliferation, differentiation and gene expression. Itgβ1 activation activates FAK causing its phosphorylation at Y397. This in turn can bind to and activate signalling molecules such as Src or Shc, which in turn lead to the activation of ERK, part of the MAPK

family. Similar to other bHLH factors, both Tal1 and Tal2 have an ERK phosphorylation site (Cheng et al. 1993; Xia et al. 1994). ERK phosphorylation of neurogenic bHLH factor neurogenin1 may regulate its transcriptional activity; increasing ERK activity leads to neurogenenin1 phosphorylation and its subsequent ability to activate transcription and neurogenesis (Cundiff et al. 2009). Another bHLH factor, NeuroD, is also phosphorylated by the MEK-ERK pathway, increasing its transcriptional activity and promoting nuclear localisation (Petersen et al. 2002). The phosphorylation of bHLH factors by ERK allows signalling from the extracellular environment to influence their activity and ERK phosphorylation of Tal2 could be mediated by itgβ1 activation, generating a model of CA*β1 expression promoting Tal2 activation via ERK phosphorylation (Figure 6.1a).

If this model is correct, Tal2 must then go on to affect neurogenesis. Little is known about Tal2 target genes in the chick, but four genes known to be regulated by Tal2 in humans are listed in Figure 6.1b. This list was generated using Integrated Transcription Factor Platform (http://itfp.biosino.org/itfp/index.jsp) online tool. Two of these genes, MAPK12 and RNF123 are known to play a role in proliferation and differentiation and neurogenesis. MAPK12 is one of the three p38 MAPKs involved in proliferation during development and cancer (Bradham & McClay 2006) and RNF123, an E3 ubiquitin ligase, is implicated in hippocampal neurogenesis (Glahn et al. 2012). As the targets of Tal2 are not known in chick or mouse, it is likely that it in fact targets a much wider range of genes, including more required for neurogenesis.

To find out if Tal2 may be affecting expression of genes up or down-regulated in the microarray data, the online software TransFind (http://transfind.sys-bio.net/index.php/home.html) can be used to predict likely transcription factors that target the list of genes. For the genes up-regulated in the CA*β1 positive cells, only one transcription factor was predicted to bind to a significant number of genes, E12 (Figure 6.1c). E12 is part of the E2A gene, whose other product in E47. The E proteins are a family of bHLH factors known to heterodimerise to bHLH factors. Tal2, like the other members of the T-ALL complex, dimerises with E proteins. It has been found to form heterodimers with E47 and E12 (E2A gene products) to interact and bind to DNA (Xia et al. 1994). This dimerization is required for DNA binding. A family of HLH proteins, the Id proteins, which negatively regulate bHLH factors by heterodimerising with them and preventing DNA binding, have known roles in neurogenesis (Niola et al. 2012; Jung et al. 2010), but the

Figure 6.1

authors did not find an association of Tal2 with Id proteins (Xia et al. 1994). No transcription factors were predicted to bind to a significant number of genes in the list of genes only up-regulated in the CA*β1 cells, suggesting E12 may not up-regulate Tal2. However, E12 and E47 may be regulating gene expression with Tal2, suggesting a possible mechanism of Tal2 to affect the cell behaviour.

A transcription factor was predicted to bind to the genes that were down-regulated in the CA*β1 cells, TF II (Figure 6.1c). TF II is a general transcription factor that is critical to development, known to be expressed in neural precursors in the E8 mouse (Fijalkowska et al. 2010) and its targets include itgα8 and the collagens. A transcription factor was also predicted to bind to a significant number of genes only down-regulated in the CA*β1 cells, NRF-2/GABP, known to regulate cell cycle and protein synthesis genes (Rosmarin et al. 2004).

Although these factors suggest Tal2 may be influencing gene expression within these cells in concert with E12/47, these are only predictions. In order to discover the function of Tal2 within these cells, we will next investigate if Tal2 normally activates or inhibits transcription of its target genes. This can be done using expression of a WT Tal2 and two fusion proteins, Tal2-VP16 fusion, which promotes activation of transcription and a Tal2-EnR fusion (Tal2-engrailed) which promotes inhibition of transcription. The VP16 fusion contains the activation domain of a herpes viral protein, virion protein 16 and the EnR fusion contains the repression domain of the Engrailed protein. Not only would this allow identification of the effect of Tal2 on transcription, but it would also allow investigation of the effect of CA*β1 and Tal2 on neurogenesis. If Tal2 is downstream of the non-cell autonomous effect of expression of CA*β1, expression of a fusion protein, whichever has the opposite effect of WT Tal2, should block the non-cell autonomous effect of CA*β1, while expression of the remaining fusion protein should mimic the phenotype without CA*β1 expression. If Tal2 is found to function downstream of itgβ1 as predicted, investigation of its binding partners and target genes could help to elucidate its currently unknown function in neurogenesis.

In conclusion, this thesis has identified a non-cell autonomous effect of itgβ1 activation on neurogenesis within the chick embryonic CNS. Investigation of the signalling involved has highlighted Tal2 as a strong candidate molecule downstream of itgβ1 signalling. Although already known to be important for correct neuronal development in a number of species, its exact role is not yet understood, but it has been suggested to have a non-cell

autonomous effect and be able to interact with neighbouring populations of cells. Further investigation of Tal2 function within these cells is predicted to reveal a role in neurogenesis, which may lead to a better understanding of its role in this process. Overall, the results of this thesis indicate an important role of integrins in the regulation of proliferation and differentiation of NSCs within the embryonic CNS.

Appendices

Appendix A

Constructs

1. Integrin-β1 constructs

All integrin constructs used were made by a former postdoc Lisbeth Laursen (Laursen et al. 2011) and inserted into pcDNA3.1(-).

The CA*β1 contained a point mutation, D723R charge reversal mutation within the intracellular domain of itgβ1, the region that forms the salt bridge with the α subunit, preventing its formation, locking the integrin in its primed conformational state (Hughes et al. 1996b). The EC only itgβ1 contains only the extracellular and transmembrane domain. Full length WT itgβ1 was also used. All constructs were sequenced prior to use.

2. N-cadherin constructs

The N-cadherin constructs were made by a former postdoc Veronique Marthiens and were inserted into pIRES2-EGFP vectors. All constructs were sequenced prior to use.

NcadWT/pIRES2-EGFP: Sequence of mouse NcadWT (2720pb) inserted between the BglII and EcoRI sites of the vector pIRES2-EGFP (5.3kb).

Mutant NcadAA/pIRES2-EGFP: Sequence of mouse Ncad containing a diAla motif instead of a diLeu motif at positions 758-759 (GCTGCA instead of CTTTTA) inserted between the BglII and EcoRI sites of the vector pIRES2-EGFP.

Appendix B

Fluorescence-Activated Cell Sorting (FACS)

Gating for GFP/PI sorts

Forward scatter and side scatter were used to identify intact cells and exclude dead cells and debris (Figure A). Single cells were identified using forward scatter pulse height and width to exclude doublets/clumps (Figure B). Propidium Iodide (PI) was used as a dead cell stain to exclude any dying cells from analysis (Figure C). Samples were then sorted into GFP positive and negative cells, GFP was detected using the Blue laser and 525-50 band pass filter (Figure D and E). Details of samples and cell numbers can be seen in the legend (Figure F).

Gating for Cell Cycle analysis

Forward scatter and side scatter were used to identify intact cells and exclude dead cells and debris (Figure A). Single cells were identified using forward scatter pulse height and width to exclude doublets/clumps (Figure B). As exclusion of doublets in vital for correct cell cycle analysis, DAPI was used to further gate for singlets, using the UV laser and 450/50 band pass filter (Figure C). Cells were then identified by their level of GFP expression, using the same lasers/filters and gates as above. (Figure D and E). DAPI area histograms were then used to for cell cycle analysis, for the total singlets (Figure F), GFP positive cells (Figure G) and GFP negative cells (Figure H). Details of samples and cell numbers can be seen in the legend (Figure I).

Figure A

Figure B

Appendix C

Micro-RNA targets

Mir-1705

Target Detail	Target Rank	Target Score	miRNA Name	Gene Symbol	Gene Description
Details	1	92	gga-miR-1705	IPO8	importin 8
Details	2	86	gga-miR-1705	DUT	deoxyuridine triphosphatase
Details	3	85	gga-miR-1705	HIPK1	homeodomain interacting protein kinase 1
Details	4	83	gga-miR-1705	LOC423008	protein-L-isoaspartate (D-aspartate) O-methyltransferase-like
Details	5	82	gga-miR-1705	ABHD13	abhydrolase domain containing 13
Details	6	81	gga-miR-1705	MAPK8	mitogen-activated protein kinase 8
Details	7	80	gga-miR-1705	RNF219	ring finger protein 219

Details	8	79	gga-miR-1705	MEIG1	meiosis expressed gene 1 homolog (mouse)
Details	9	79	gga-miR-1705	OLA1	Obg-like ATPase 1
Details	10	79	gga-miR-1705	BRMS1L	breast cancer metastasis-suppressor 1-like
Details	11	79	gga-miR-1705	PCF11	PCF11, cleavage and polyadenylation factor subunit, homolog (S. cerevisiae)
Details	12	79	gga-miR-1705	LOC769589	similar to meiosis expressed gene 1 homolog (mouse)
Details	13	78	gga-miR-1705	TRDMT1	tRNA aspartic acid methyltransferase 1
Details	14	76	gga-miR-1705	UNC119B	unc-119 homolog B (C. elegans)
Details	15	75	gga-miR-1705	NDFIP2	Nedd4 family interacting protein 2
Details	16	73	gga-miR-1705	CCP110	centriolar coiled coil protein 110kDa
Details	17	72	gga-miR-1705	TNRC6A	trinucleotide repeat containing 6A
Details	18	72	gga-miR-1705	CCDC91	coiled-coil domain containing 91
Details	19	72	gga-miR-1705	ELL2	elongation factor, RNA polymerase II, 2
Details	20	71	gga-miR-1705	RAP1B	RAP1B, member of RAS oncogene family
Details	21	71	gga-miR-1705	TBC1D1	TBC1 (tre-2/USP6, BUB2, cdc16) domain family, member 1
Details	22	71	gga-miR-1705	CARS	cysteinyl-tRNA synthetase
Details	23	71	gga-miR-1705	GORASP1	golgi reassembly stacking protein 1, 65kDa
Details	24	71	gga-miR-1705	AFF4	AF4/FMR2 family, member 4

Details	25	71	gga-miR-1705	DNAJA2	DnaJ (Hsp40) homolog, subfamily A, member 2
Details	26	71	gga-miR-1705	ZCRB1	zinc finger CCHC-type and RNA binding motif 1
Details	27	70	gga-miR-1705	RPIA	ribose 5-phosphate isomerase A
Details	28	69	gga-miR-1705	AIMP1	aminoacyl tRNA synthetase complex-interacting multifunctional protein 1
Details	29	69	gga-miR-1705	CMAS	cytidine monophosphate N-acetylneuraminic acid synthetase
Details	30	69	gga-miR-1705	NME1	non-metastatic cells 1, protein (NM23A) expressed in
Details	31	69	gga-miR-1705	RPAP2	RNA polymerase II associated protein 2
Details	32	68	gga-miR-1705	LGALSL	lectin, galactoside-binding-like
Details	33	68	gga-miR-1705	ARF4	ADP-ribosylation factor 4
Details	34	68	gga-miR-1705	ABCC4	ATP-binding cassette, sub-family C (CFTR/MRP), member 4
Details	35	68	gga-miR-1705	LOC422353	similar to synaptotagmin-like protein 2-a delta 2S-II
Details	36	66	gga-miR-1705	LIN9	lin-9 homolog (C. elegans)
Details	37	66	gga-miR-1705	KTI12	KTI12 homolog, chromatin associated (S. cerevisiae)
Details	38	66	gga-miR-1705	PELI1	pellino homolog 1 (Drosophila)
Details	39	66	gga-miR-1705	RAD52	RAD52 homolog (S. cerevisiae)
Details	40	65	gga-miR-1705	C1R	complement component 1, r subcomponent

Details	41	65	gga-miR-1705	GLCCI1	glucocorticoid induced transcript 1
Details	42	65	gga-miR-1705	DDX3X	DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, X-linked
Details	43	65	gga-miR-1705	SMAD7B	TGF-beta signal pathway antagonist Smad7
Details	44	63	gga-miR-1705	SCUBE2	signal peptide, CUB domain, EGF-like 2
Details	45	63	gga-miR-1705	SPATS2L	spermatogenesis associated, serine-rich 2-like
Details	46	63	gga-miR-1705	MEOX2	mesenchyme homeobox 2
Details	47	63	gga-miR-1705	F2RL1	coagulation factor II (thrombin) receptor-like 1
Details	48	63	gga-miR-1705	SRPX	sushi-repeat containing protein, X-linked
Details	49	62	gga-miR-1705	DENND1A	DENN/MADD domain containing 1A
Details	50	62	gga-miR-1705	IL12RB2	interleukin 12 receptor, beta 2
Details	51	61	gga-miR-1705	VPS24	vacuolar protein sorting 24 homolog (S. cerevisiae)
Details	52	61	gga-miR-1705	SFT2D2	SFT2 domain containing 2
Details	53	60	gga-miR-1705	GBGT1	globoside alpha-1,3-N-acetylgalactosaminyltransferase 1
Details	54	60	gga-miR-1705	SECISBP2	SECIS binding protein 2
Details	55	59	gga-miR-1705	PTPRK	protein tyrosine phosphatase, receptor type, K
Details	56	59	gga-miR-1705	KIAA1958	KIAA1958
Details	57	58	gga-miR-1705	ROBO1	roundabout, axon guidance receptor, homolog 1 (Drosophila)

Details	58	58	gga-miR-1705	CXCL14	chemokine (C-X-C motif) ligand 14
Details	59	58	gga-miR-1705	LPCAT1	lysophosphatidylcholine acyltransferase 1
Details	60	58	gga-miR-1705	BAALC	brain and acute leukemia, cytoplasmic
Details	61	57	gga-miR-1705	DCTN4	dynactin 4 (p62)
Details	62	57	gga-miR-1705	GHITM	growth hormone inducible transmembrane protein
Details	63	57	gga-miR-1705	CASK	calcium/calmodulin-dependent serine protein kinase (MAGUK family)
Details	64	57	gga-miR-1705	ING3	inhibitor of growth family, member 3
Details	65	57	gga-miR-1705	PRKCB	protein kinase C, beta
Details	66	56	gga-miR-1705	C2H9orf152	chromosome 2 open reading frame, human C9orf152
Details	67	56	gga-miR-1705	BCKDHB	branched chain keto acid dehydrogenase E1, beta polypeptide
Details	68	56	gga-miR-1705	ITGA9	integrin, alpha 9
Details	69	56	gga-miR-1705	THOC7	THO complex 7 homolog (Drosophila)
Details	70	56	gga-miR-1705	MSN	moesin
Details	71	56	gga-miR-1705	RNF166	ring finger protein 166
Details	72	56	gga-miR-1705	C1H12orf59	chromosome 1 open reading frame, human C12orf59
Details	73	56	gga-miR-1705	ITPKA	inositol 1,4,5-trisphosphate 3-kinase A
Details	74	55	gga-miR-1705	DNAJB9	DnaJ (Hsp40) homolog, subfamily B, member 9

Details	75	55	gga-miR-1705	LOC420793	uncharacterized LOC420793
Details	76	55	gga-miR-1705	ARSJ	arylsulfatase family, member J
Details	77	55	gga-miR-1705	CERK	ceramide kinase
Details	78	55	gga-miR-1705	IQCD	IQ motif containing D
Details	79	55	gga-miR-1705	DCAF10	DDB1 and CUL4 associated factor 10
Details	80	55	gga-miR-1705	ANKRD16	ankyrin repeat domain 16
Details	81	54	gga-miR-1705	YOD1	YOD1 OTU deubiquinating enzyme 1 homolog (S. cerevisiae)
Details	82	54	gga-miR-1705	AR	androgen receptor
Details	83	54	gga-miR-1705	TRIM59	tripartite motif containing 59
Details	84	54	gga-miR-1705	LPIN1	lipin 1
Details	85	54	gga-miR-1705	EPB41L2	erythrocyte membrane protein band 4.1-like 2
Details	86	53	gga-miR-1705	GNOT1	Gnot1 homeodomain protein
Details	87	53	gga-miR-1705	IFT122	intraflagellar transport 122 homolog (Chlamydomonas)
Details	88	53	gga-miR-1705	SLC35B3	solute carrier family 35, member B3
Details	89	52	gga-miR-1705	TP53BP2	tumor protein p53 binding protein, 2
Details	90	52	gga-miR-1705	TMEM120B	transmembrane protein 120B
Details	91	52	gga-miR-1705	ZDHHC15	zinc finger, DHHC-type containing 15

Details	92	52	gga-miR-1705	APCDD1	adenomatosis polyposis coli down-regulated 1
Details	93	52	gga-miR-1705	LOC768494	similar to Chromosome 9 open reading frame 40
Details	94	52	gga-miR-1705	GMFB	glia maturation factor, beta
Details	95	51	gga-miR-1705	SHISA2	shisa homolog 2 (Xenopus laevis)
Details	96	51	gga-miR-1705	LACTB2	lactamase, beta 2
Details	97	51	gga-miR-1705	EIF2S1	eukaryotic translation initiation factor 2, subunit 1 alpha, 35kDa
Details	98	51	gga-miR-1705	WDR51B	WD repeat domain 51B
Details	99	51	gga-miR-1705	LMF1	lipase maturation factor 1
Details	100	51	gga-miR-1705	VAPA	VAMP (vesicle-associated membrane protein)-associated protein A, 33kDa
Details	101	51	gga-miR-1705	PPPDE2	PPPDE peptidase domain containing 2
Details	102	51	gga-miR-1705	CDC42BPB	CDC42 binding protein kinase beta (DMPK-like)
Details	103	51	gga-miR-1705	CUL5	cullin 5
Details	104	50	gga-miR-1705	MMP3	matrix metallopeptidase 3 (stromelysin 1, progelatinase)
Details	105	50	gga-miR-1705	TP73	tumor protein p73
Details	106	50	gga-miR-1705	KIAA1033	KIAA1033
Details	107	50	gga-miR-1705	MIER1	mesoderm induction early response 1 homolog (Xenopus laevis)

Details	108	50	gga-miR-1705	ANGPTL5	angiopoietin-like 5
Details	109	50	gga-miR-1705	TMEM18	transmembrane protein 18
Details	110	50	gga-miR-1705	SMAD7	SMAD family member 7

Mir-1653

Target Detail	Target Rank	Target Score	miRNA Name	Gene Symbol	Gene Description
Details	1	89	gga-miR-1653	C21H1orf174	chromosome 21 open reading frame, human C1orf174
Details	2	86	gga-miR-1653	FBXW5	F-box and WD repeat domain containing 5
Details	3	80	gga-miR-1653	RBKS	ribokinase
Details	4	79	gga-miR-1653	TOM1L2	target of myb1-like 2 (chicken)
Details	5	78	gga-miR-1653	USPL1	ubiquitin specific peptidase like 1
Details	6	77	gga-miR-1653	ZFAT	zinc finger and AT hook domain containing
Details	7	77	gga-miR-1653	LOC417345	uncharacterized LOC417345
Details	8	75	gga-miR-1653	SYT17	synaptotagmin XVII

Details	9	75	gga-miR-1653	KLHL20	kelch-like 20 (Drosophila)
Details	10	75	gga-miR-1653	IFIH1	interferon induced with helicase C domain 1
Details	11	75	gga-miR-1653	SCAF11	SR-related CTD-associated factor 11
Details	12	75	gga-miR-1653	SLC9A3R2	solute carrier family 9 (sodium/hydrogen exchanger), member 3 regulator 2
Details	13	73	gga-miR-1653	NEIL3	nei endonuclease VIII-like 3 (E. coli)
Details	14	72	gga-miR-1653	ARVCF	armadillo repeat gene deleted in velocardiofacial syndrome
Details	15	72	gga-miR-1653	UBE2V2	ubiquitin-conjugating enzyme E2 variant 2
Details	16	72	gga-miR-1653	NAPB	N-ethylmaleimide-sensitive factor attachment protein, beta
Details	17	72	gga-miR-1653	LOC426910	similar to Scale keratin (S-ker) (sKer)
Details	18	72	gga-miR-1653	LOC420300	similar to stem cell antigen 2
Details	19	71	gga-miR-1653	SLC37A4	solute carrier family 37 (glucose-6-phosphate transporter), member 4
Details	20	71	gga-miR-1653	TXLNB	taxilin beta
Details	21	71	gga-miR-1653	KIAA0355	KIAA0355
Details	22	71	gga-miR-1653	RAP1B	RAP1B, member of RAS oncogene family
Details	23	70	gga-miR-1653	UTS2	urotensin 2

Details	24	70	gga-miR-1653	PCF11	PCF11, cleavage and polyadenylation factor subunit, homolog (S. cerevisiae)
Details	25	69	gga-miR-1653	DCX	doublecortin
Details	26	69	gga-miR-1653	GXYLT1	glucoside xylosyltransferase 1
Details	27	68	gga-miR-1653	ASAH1	N-acylsphingosine amidohydrolase (acid ceramidase) 1
Details	28	68	gga-miR-1653	SUZ12	suppressor of zeste 12 homolog (Drosophila)
Details	29	67	gga-miR-1653	MAP7D3	MAP7 domain containing 3
Details	30	67	gga-miR-1653	MAVS	mitochondrial antiviral signaling protein
Details	31	66	gga-miR-1653	MOCS1	molybdenum cofactor synthesis 1
Details	32	66	gga-miR-1653	NT5C3	5'-nucleotidase, cytosolic III
Details	33	65	gga-miR-1653	DUS2L	dihydrouridine synthase 2-like, SMM1 homolog (S. cerevisiae)
Details	34	65	gga-miR-1653	LOC422372	uncharacterized LOC422372
Details	35	65	gga-miR-1653	MKL1	megakaryoblastic leukemia (translocation) 1
Details	36	64	gga-miR-1653	TXNL1	thioredoxin-like 1
Details	37	63	gga-miR-1653	ZNF652	zinc finger protein 652
Details	38	63	gga-miR-1653	USE1	unconventional SNARE in the ER 1 homolog (S. cerevisiae)
Details	39	62	gga-miR-1653	ZRANB1	zinc finger, RAN-binding domain containing 1

Details	40	62	gga-miR-1653	DRG2	developmentally regulated GTP binding protein 2
Details	41	62	gga-miR-1653	BRD3	bromodomain containing 3
Details	42	61	gga-miR-1653	DSTN	destrin (actin depolymerizing factor)
Details	43	61	gga-miR-1653	EIF5A2	eukaryotic translation initiation factor 5A2
Details	44	61	gga-miR-1653	MYST2	MYST histone acetyltransferase 2
Details	45	61	gga-miR-1653	PDPR	pyruvate dehydrogenase phosphatase regulatory subunit
Details	46	60	gga-miR-1653	AIDA	axin interactor, dorsalization associated
Details	47	60	gga-miR-1653	LOC770098	uncharacterized LOC770098
Details	48	60	gga-miR-1653	KDM4B	lysine (K)-specific demethylase 4B
Details	49	60	gga-miR-1653	TTL	tubulin tyrosine ligase
Details	50	60	gga-miR-1653	CSTF1	cleavage stimulation factor, 3' pre-RNA, subunit 1, 50kDa
Details	51	59	gga-miR-1653	ARL13B	ADP-ribosylation factor-like 13B
Details	52	59	gga-miR-1653	CCNB2	cyclin B2
Details	53	59	gga-miR-1653	OTUD4	OTU domain containing 4
Details	54	58	gga-miR-1653	EMB	embigin homolog (mouse)
Details	55	58	gga-miR-1653	AGAP1	ArfGAP with GTPase domain, ankyrin repeat and PH domain 1

Details	56	58	gga-miR-1653	DEAF1	deformed epidermal autoregulatory factor 1 (Drosophila)
Details	57	58	gga-miR-1653	LOC768701	uncharacterized protein KIAA1671 homolog
Details	58	57	gga-miR-1653	NMRAL1	NmrA-like family domain containing 1
Details	59	57	gga-miR-1653	ATM	ataxia telangiectasia mutated
Details	60	57	gga-miR-1653	RPL35A	ribosomal protein L35a
Details	61	57	gga-miR-1653	BTBD11	BTB (POZ) domain containing 11
Details	62	57	gga-miR-1653	STK24	serine/threonine kinase 24
Details	63	56	gga-miR-1653	SLC35E1	solute carrier family 35, member E1
Details	64	56	gga-miR-1653	DIABLO	diablo, IAP-binding mitochondrial protein
Details	65	56	gga-miR-1653	CZH5orf28	chromosome Z open reading frame, human C5orf28
Details	66	56	gga-miR-1653	NFKB1	nuclear factor of kappa light polypeptide gene enhancer in B-cells 1
Details	67	56	gga-miR-1653	CLPTM1L	CLPTM1-like
Details	68	55	gga-miR-1653	RBM5	RNA binding motif protein 5
Details	69	55	gga-miR-1653	CDH8	cadherin 8, type 2
Details	70	55	gga-miR-1653	WNT11	wingless-type MMTV integration site family, member 11
Details	71	55	gga-miR-1653	HSPA4L	heat shock 70kDa protein 4-like

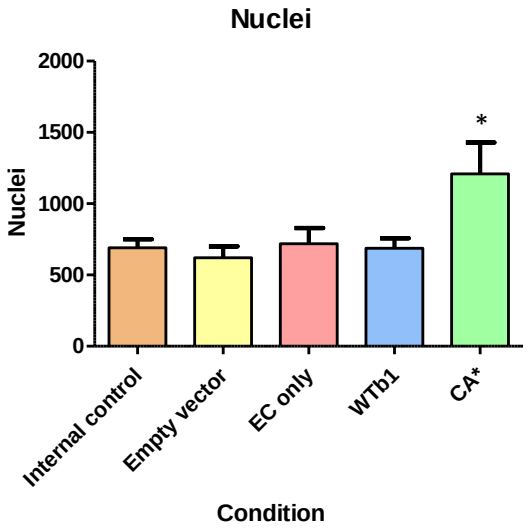
Details	72	54	gga-miR-1653	PEX16	peroxisomal biogenesis factor 16
Details	73	54	gga-miR-1653	LOC776088	uncharacterized LOC776088
Details	74	54	gga-miR-1653	KNG1	kininogen 1
Details	75	54	gga-miR-1653	SH3GLB1	SH3-domain GRB2-like endophilin B1
Details	76	54	gga-miR-1653	LOC424919	similar to photolyase
Details	77	53	gga-miR-1653	SETD5	SET domain containing 5
Details	78	53	gga-miR-1653	HTT	huntingtin
Details	79	53	gga-miR-1653	ASB12	ankyrin repeat and SOCS box containing 12
Details	80	53	gga-miR-1653	CRYM	crystallin, mu
Details	81	53	gga-miR-1653	SMARCD1	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily d, member 1
Details	82	52	gga-miR-1653	SERPINH1	serpin peptidase inhibitor, clade H (heat shock protein 47), member 1, (collagen binding protein 1)
Details	83	52	gga-miR-1653	SCAF4	SR-related CTD-associated factor 4
Details	84	52	gga-miR-1653	LRRC41	leucine rich repeat containing 41
Details	85	52	gga-miR-1653	RBFOX1	RNA binding protein, fox-1 homolog (C. elegans) 1
Details	86	51	gga-miR-1653	LOC396216	mature cMGF

Details	87	51	gga-miR-1653	C6H10orf57	chromosome 6 open reading frame, human C10orf57
Details	88	51	gga-miR-1653	PRKAA1	protein kinase, AMP-activated, alpha 1 catalytic subunit
Details	89	51	gga-miR-1653	SPATS2	spermatogenesis associated, serine-rich 2
Details	90	51	gga-miR-1653	ARFGAP1	ADP-ribosylation factor GTPase activating protein 1
Details	91	50	gga-miR-1653	CX3CL1	chemokine (C-X3-C motif) ligand 1
Details	92	50	gga-miR-1653	RAB26	RAB26, member RAS oncogene family
Details	93	50	gga-miR-1653	MLLT1	myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, Drosophila); translocated to, 1
Details	94	50	gga-miR-1653	TSPAN14	tetraspanin 14
Details	95	50	gga-miR-1653	IP6K3	inositol hexakisphosphate kinase 3
Details	96	50	gga-miR-1653	SPECC1L	sperm antigen with calponin homology and coiled-coil domains 1-like

Appendix D

Cell counts.

E2-6 Nuclei counts, $p<0.05$.



Appendix E

ECM gene expression data.

Figure A

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