Examining the Fundamental Biology of Primitive Neural Stem Cells

by

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Definitive (d) neural stem cells (dNSCs) are multipotent, self-renewing, neurally committed stem cells. More recently, a novel primitive (p)NSC was identified in the early postnatal brain that persists into adulthood. pNSCs are upstream of dNSCs in the neural lineage and are self-renewing with the capacity to generate neural cells. pNSCs, unlike dNSCs, express the pluripotency genes \textit{Oct4}, and \textit{Nanog} and are able to incorporate into the developing blastocyst. Based on these findings we hypothesized that pNSCs are pluripotent cells able to generate cells outside the neuroectodermal lineage. To test this hypothesis, we utilized well established pluripotency assays \textit{in vitro} embryoid body assay and the \textit{in vivo} teratoma formation assay. When placed in these assays pNSCs revealed no pluripotentiality, demonstrating consistent neural commitment and no differentiation into mesoderm or endoderm lineages. In addition, pNSCs expressed the epithelial marker \textit{Krt18} indicating that their origin may be from an embryonic time before neurulation.
Acknowledgments

I am sitting here looking back at the past three years in awe of the support I have been fortunate enough to receive from many people in my life throughout my journey as a graduate student. I always thought writing the ‘Acknowledgments’ section was the easiest and the most straightforward part of my thesis. Yet, no amount of descriptive writing can pay due tribute to the people who have helped me along the way.

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I have learned so much from my experience during this MSc and I am grateful for this afforded opportunity. This is only the beginning.
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<th>Description</th>
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<tbody>
<tr>
<td>Afp</td>
<td>alpha-feto protein</td>
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<tr>
<td>ALK</td>
<td>activin-like kinases</td>
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<tr>
<td>BLBP</td>
<td>brain-lipid-binding protein</td>
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<tr>
<td>BMPs</td>
<td>bone morphogenic proteins</td>
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<tr>
<td>DCX</td>
<td>doublecortin</td>
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<tr>
<td>DG</td>
<td>dentate gyrus</td>
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<tr>
<td>DIA</td>
<td>differentiation inhibitory activity</td>
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<tr>
<td>Dlx</td>
<td>distal-less</td>
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<tr>
<td>DNE</td>
<td>dentate neuroepithelium</td>
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<tr>
<td>dNSC</td>
<td>definitive neural stem cell</td>
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<tr>
<td>EB</td>
<td>embryoid body</td>
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<tr>
<td>EC</td>
<td>embryonal carcinoma</td>
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<tr>
<td>EFH</td>
<td>EGF, FGF &amp; Heparin</td>
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<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>ESC</td>
<td>embryonic stem cell</td>
</tr>
<tr>
<td>FGF2</td>
<td>fibroblast growth factor 2</td>
</tr>
<tr>
<td>Flk1</td>
<td>fetal liver kinase-1</td>
</tr>
<tr>
<td>GFAP</td>
<td>glial fibrillary acidic protein</td>
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<tr>
<td>GLAST</td>
<td>astrocyte specific glutamate transporter</td>
</tr>
<tr>
<td>Term</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>GP130</td>
<td>glycoprotein130</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein coupled receptor</td>
</tr>
<tr>
<td>GSK3β</td>
<td>glycogen synthase kinase 3β</td>
</tr>
<tr>
<td>HD</td>
<td>homeodomain protein</td>
</tr>
<tr>
<td>Hnf3β</td>
<td>hepatocyte nuclear factor 3-beta</td>
</tr>
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<td>Hnf4</td>
<td>hepatocyte nuclear factor 4</td>
</tr>
<tr>
<td>HSC</td>
<td>hematopoietic stem cell</td>
</tr>
<tr>
<td>ICM</td>
<td>inner cell mass</td>
</tr>
<tr>
<td>IL-6</td>
<td>interleukine-6</td>
</tr>
<tr>
<td>iPSC</td>
<td>induced pluripotent stem cell</td>
</tr>
<tr>
<td>JAK/STAT</td>
<td>Janus kinase/signal transducers and activators of transcription</td>
</tr>
<tr>
<td>KO</td>
<td>Knockout</td>
</tr>
<tr>
<td>LEF/TCFs</td>
<td>lymphoid enhancer factor/T cell factor proteins</td>
</tr>
<tr>
<td>LIF</td>
<td>Leukemia Inhibitory Factor</td>
</tr>
<tr>
<td>LIF-R</td>
<td>Leukemia Inhibitory Factor Receptor</td>
</tr>
<tr>
<td>LRP</td>
<td>Lipoprotein receptor-related protein</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>Mbd3</td>
<td>methyl DNA-binding protein 3</td>
</tr>
<tr>
<td>MEFs</td>
<td>mouse embryonic fibroblasts</td>
</tr>
<tr>
<td>mESCs</td>
<td>mouse embryonic stem cells</td>
</tr>
</tbody>
</table>
NECs  neuroepithelial cells
NPCs  neural precursor cells
NSA  neurosphere assay
NSCs  neural stem cells
OB  olfactory bulb
PI3K  phosphoinositide 3-kinase
PIP2  phosphatidylinositol 4,5-bisphosphate
PIP3  Phosphatidylinositol (3,4,5)-trisphosphate
PKB  protein kinase B
pNSC  primative neural stem cell
POU  pit-onc-unc
PRCS  polycomb repressive complexes
pSTAT3  phosphorylated STAT3
RGCs  radial glial cells
RMS  rostral migratory stream
SEZ  subependymal zone
SGZ  subgranular zone
SOCS3  suppressor of cytokine signaling 3
Sox2  sex determining region y-box 2
STAT3  signal transducer and activator of transcription 3
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Definition</th>
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<tbody>
<tr>
<td>TCF</td>
<td>t cell factor</td>
</tr>
<tr>
<td>TGFβ</td>
<td>transforming growth factor beta</td>
</tr>
<tr>
<td>TGFβRs</td>
<td>TGFβ receptors</td>
</tr>
<tr>
<td>UPP</td>
<td>Ubiquitin–Proteasome Pathway (UPP)</td>
</tr>
<tr>
<td>Vegfr-2</td>
<td>vascular endothelial growth factor receptor 2</td>
</tr>
<tr>
<td>VZ</td>
<td>ventricular zone</td>
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Chapter 1

1 General Introduction

1.1 Stem Cells

The term ‘stem cell’ (originally ‘Stammzelle’) was first coined by the German scientist Ernst Haeckel who used ‘stem cell’ to describe the predecessor unicellular organism from which multicellular organisms had evolved (work done by ‘Haeckel, 1868’ as reviewed by Vasuri et al., 2014). This phylogeny-based definition was later changed when Canadian scientists James Till and Ernest McCulloch performed pioneering experiments that led to the discovery of ‘stem cells’ in the hematopoietic system. Their collective works demonstrated that there are single cells capable of self-renewal and differentiation into blood cells comprising the lymphoid and myeloid systems (Becker et al., 1963; Siminovich et al., 1963; Wu et al., 1968). Stem cells are now widely regarded as distinct cell populations possessing two cardinal properties. First, they must be able to self-renew and second, they must be able to give rise to progeny that will differentiate into distinct cell types.

Stem cells can also be characterized by their potentiality. Totipotent stem cells can give rise to all embryonic and extra-embryonic tissues. Examples include the zygote and cells found in the early blastomere (up to the 8-cell stage) (Sadler, 2011). Pluripotent stem cells can give rise to all of the cell types that make up an embryo, but cannot give rise to extra-embryonic tissues such as the placenta. Pluripotent stem cells comprise the inner cell mass (ICM) of the developing blastocyst. Individual cells within the ICM of the developing blastocyst generate cells in all three germ layers and therefore contribute to the development of the embryo proper. Another example of pluripotent cells are induced pluripotent stem cells (iPSCs), differentiated somatic cells reprogrammed back to a pluripotent state where they are then capable of generating cells from all three germ (Takahashi and Yamanaka, 2006). Multipotent stem cells are more limited in their differentiation potential and generally give rise to cells that comprise their tissue of origin. Multipotent stem cells are found within developing and mature tissues. For example, in the adult mammalian brain, neural stem cells are found in the subependymal lining of the walls of the lateral ventricles and have the capacity to give rise to cells within the central nervous system: neurons, astrocytes, and oligodendrocytes (Reynolds & Weiss, 1992; Morshead et al., 1994;
Doetsch et al., 1997). Another example of a multipotent cell source are muscle stem cells, also known as satellite cells. These cells have been shown to be key players in the maintenance of skeletal muscles under physiological and injury conditions (as reviewed by Yuasa et al., 2017). Bipotential and unipotential stem cells have more limited differentiation capacity and include hepatic oval cells in the liver that give rise to hepatocytes and bile duct cells (Oh et al., 2002), and unipotent stem cells such as germline stem cells which give rise to gametes (as reviewed by Yuan and Yamashita, 2010).

1.2 Mouse Embryonic Stem Cells

Pluripotent cells comprising the ICM of the blastocyst are known as embryonic stem cells. Mouse embryonic stem cells (mESCs) were originally isolated in 1981 (Evans & Kaufman, 1981). Without knowing the specifics of the appropriate culture conditions, Martin used conditioned media from previously established PSA-1 embryonal carcinoma cells to successfully culture ICM cells and generate colonies that were able to be passaged (Martin, 1981). Martin demonstrated the importance of using mouse embryonic fibroblasts (MEFs) to ensure that mESCs remained undifferentiated in *in vitro* cultures. Indeed, in the absence of feeder cells, mESCs began to differentiate (Smith and Hooper, 1983). The use of feeder cells to culture ICM-derived ESCs was later replaced as the maintenance of the undifferentiated ESCs was achieved through the use of soluble polypeptide factor, known as differentiation inhibitory activity (DIA), secreted by a Buffalo rat liver cells (Smith and Hooper, 1987). It was later discovered that DIA and the cytokine Leukemia Inhibitory Factor (LIF) are biochemically identical and that LIF is also able to suppress differentiation in early ESC cultures (Smith et al., 1988; Williams et al., 1988). LIF is a member of the interleukine-6 (IL6) family which is involved in an array of biological activities. The pleiotropic effects of LIF have been shown via both *in vitro* and animal knockout (KO) models. Indeed, LIF is involved in implantation of developing embryos (Stewart et al., 1992), in the maintenance of hematopoietic stem cell (HSC) pool (Escary et al., 1993), and in muscle regeneration (Kurek et al., 1997), to name a few. LIF promotes mESC self-renewal, thus maintaining a population of undifferentiated cells.

1.3 Regulators of Pluripotency

One of the goals of stem cell biology is to harness their potential for the purpose of regenerative medicine. Insight into the fundamental biology of stem cells for the purpose of developing cell
replacement strategies requires an understanding of the factors that regulate stem cell behaviour. Herein we will discuss a number of regulators of pluripotency such as signaling pathways and transcription/epigenetic factors.

1.3.1 Pluripotency Signaling Pathways

Signaling pathways involved in pluripotency can be broadly divided into the following categories: Wnt/β-catenin, JAK/STAT, JAK/PI3K, and TGFβ signaling.

1.3.1.1 JAK Signaling pathway

1.3.1.1.1 JAK/STAT

The Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway is known as a pleiotropic signaling pathway involved in a multitude of developmental processes in both mammalian and non-mammalian species. In mammals, the pathway can be activated by a variety of signaling molecules such as cytokines and growth factors. The activation of this pathway has been shown to modulate events such as proliferation, differentiation, cell migration and apoptosis. (Rawlings et al., 2004)

All members of the interleukine-6 (IL6) cytokine family, including LIF, (Boeuf et al., 1997; Niwa et al., 1998) can activate signal transducer and activator of transcription 3 (STAT3). Once LIF binds to its respective cell-surface receptor, LIF-receptor (LIF-R) (Gearing et al., 1991), this complex recruits the transmembrane receptor glycoprotein130 (GP130) (Hibi et al., 1990) and combine to form a LIF-R/GP130 heterodimer. The resultant dimer recruits and phosphorylates Janus kinase (JAK) which subsequently phosphorylates STAT3. The phosphorylated STAT3 (pSTAT3) homodimerizes and the complex translocates to the nucleus. The translocation allows for the regulation of transcription of Oct4, Sox2, and Nanog, the central genes involved in self-renewal, proliferation, and undifferentiation in ESCs (Matsuda et al., 1999). Interestingly, the GP130 ligand is secreted by mESCs themselves, providing another means of the activation of GP130 receptor which can result in partial STAT3 pathway activation. This is believed to provide mESCs with a buffer against spontaneous differentiation should exogenous LIF levels be temporarily lowered (Davey et al., 2007). Repression of the STAT3-pathway, either endogenously by potent JAK/STAT inhibitors such as suppressor of cytokine signaling 3 (SOCS3) (Starr el al., 1997; Naka et al., 1997) or via exogenous blockers leads to loss of self-
renewal, and spontaneous differentiation of mESCs primarily into endodermal and ectodermal cell types (Boeuf et al., 1997; Niwa et al., 1998). Matsuda and colleagues have shown the ability of mESCs to self-renew by constitutive STAT3 activation, in the absence of LIF, further elucidating the importance of STAT3 in maintenance of self-renewal and pluripotency (Matsuda et al., 1999). Given the mESC culturing conditions and components that promote differentiation (such as the serum found in most ESC culturing media), the balance between self-renewal and differentiation is dependent on the balance of STAT3 activation as well as other STAT-independent pathways (Figure 1B).

1.3.1.1.2 JAK/PI3K

With respect to maintaining pluripotency, JAK activation also recruits and activates phosphoinositide 3-kinase (PI3K). The activated PI3K facilitates the phosphorylation of phosphatidylinositol 4,5-bisphosphate (PIP$_2$) to Phosphatidylinositol (3,4,5)-trisphosphate (PIP$_3$). PIP$_3$ in turn acts to activate Akt, also known as protein kinase B (PKB), which is a serine/threonine protein kinase. The role of Akt is two-folds. First, the activated Akt can now translocate to the nucleus and regulate transcription of pluripotency genes. Second, it acts to inhibit glycogen synthase kinase 3ß activity (explained in more detail in the next section) (Yin et al., 2014) (Figure 1B).

1.3.1.2 Wnt/ß-Catenin Signaling Pathway

The contribution of Wnt signaling is seen across developmental processes from anterior-posterior specification of the body axis (Clevers, 2006; van Amerongen and Nusse, 2009;), to playing a role in lineage progression promoting mesoderm and endoderm differentiation (Bakre et al., 2007; Aubert et al., 2002; Haegele et al., 2003). Wnt signaling has also been shown to play a role in regulating pluripotency. In studies done with STAT3-null mESCs, the culturing and propagation of mESCs was accomplished with the use of two small molecules, CHIR99021 (a glycogen synthase kinase 3ß inhibitor, GSK3ß inhibitor), and PD0325901 (a mitogen-activated protein kinase inhibitor, MAPK inhibitor). Together these two inhibitors are known as ‘2i’ factors. Activation of MAPK results in downregulation of pluripotency genes such as Nanog (Hamazaki et al., 2006), while GSK3ß is involved in terminating the Wnt/ß-catenin signaling pathway. In the absence of Wnt signaling, the Axin/APC/GSK3ß complex, also known as ß-catenin degradation complex, acts to phosphorylate ß-catenin (primarily done by GSK3ß)
(Archbold et al., 2012). The phosphorylated cytosolic β-catenin is then targeted for protein degradation by the Ubiquitin–Proteasome Pathway (UPP) and is unable to move to the nucleus and upregulate gene expressions important for pluripotency. To trigger downstream signaling cascades, Wnt ligands such as Wnt3a (Singla et al., 2006) act via binding with the G-protein coupled receptor (GPCR) Frizzled and associated co-receptors (LRP5/6) (He et al., 2004). The ligand/receptor interaction results in the inactivation of Axin/APC/GSK3β complex. The stable β-catenin can now associate with lymphoid enhancer factor/T cell factor proteins (LEF/TCFs) family transcription factors (Arce et al., 2006; Archbold et al., 2011; Clevers, 2006) and lead to gene activation important for activation of pluripotency master regulators of pluripotency (Oct4, Sox2, and Nanog) pluripotency. It is thought that LEF/TCFs transcription factors act as both transcriptional repressors and activators of pluripotency genes, in the absence and presence of Wnt activity, respectively (van de Wetering et al., 1996; Daniels and Weis, 2005). Some mESC culturing protocols will combine LIF and 2i to achieve pluripotent, self-renewing, undifferentiated cultures, which attests to the importance of Wnt signaling to maintain pluripotency (Figure 1A).

The aforementioned Wnt signaling pathway and the accompanying figure describes the canonical Wnt signaling pathway. The role of canonical pathway signalling in promoting ‘stemness’ and pluripotency culminated in the translocation of β-catenin to the nucleus and LEF/TCF complex activation.

The binding and activation of Frizzled receptors by Wnt ligands can also result in the activation the non-canonical pathway. This pathway serves a multitude of roles dependent on the intracellular effectors that are activated. The activation of GTPase Rho (Rho and Rock activation), is responsible for cytoskeletal remodelling and cell polarity (Herman, 2002). This pathway is not implicated in the mechanism of pluripotency. The ‘Wnt-calcium’ pathway which results in the activity of two main downstream effectors: the nuclear factor of activated T cells (NFAT), and the CaMKII-TAK1-NLK mediated NLK activation resulting in the transcription factor NFAT translocating into the nucleus and leading to transcription of differentiation-specific genes. NFAT has been implicated in many developmental processes including cardiac development as well as T-cell differentiation (Schulz and Yutzey, 2004; Macian, 2005). The activation of NLK via phosphorylation of TCF and subsequent prevention of the β-catenin-
LEF/TCF complex binding DNA inhibits the ability of the canonical pathway to activate pluripotency-related transcription. Hence, the CaMKII-TAK1-NLK pathways acts in tandem with the Wnt/β-catenin pathway (Ishitani et al., 2003; Sugimura and Li, 2010; Fan et al., 2017).

Hence, while canonical Wnt signaling is the predominant force in modulating pluripotency, there are contributions from the non-canonical pathway that can modulate the cellular response.

1.3.1.3 TGFβ/Smad Signaling Pathway

Another signaling pathway implicated in maintenance of ESCs in an undifferentiated state is the transforming growth factor beta (TGFβ) signaling pathway. TGFβ signaling can be broken down to a) ligands, b) receptors, and c) Smads. TGFβ superfamily ligands can be separated to two main branches; bone morphogenic proteins (BMPs) and TGFβs. BMP ligands include different isoforms of the protein while TGFβ ligands include members include, but are not limited to TGFβs (TGFβ1,2,3), Activins (Actvin A, B, C), and Nodal. TGFβ receptors (TGFβRs) can be categorized into three groups: TGFβRI (also known as Activin-Like Kinases – ALK), TGFβRII, and TGFβRIII, with type I and II possessing intracellular serine/threonine kinase activity (Massague, 1992). Smads can be divided into 3 major groups: receptor regulated Smads (R-Smads), co-Smads, and inhibitory Smads (I-Smads). R-Smads include Smad1, Smad2, Smad3, Smad5, and Smad8. Smad4 is the only co-Smad. Smad6 and Smad7 are I-Smads (Dennler et al., 2002). With respect to the role of this pathway in maintaining pluripotency, TGFβ/Activin/Nodal and BMP4 will bind to ALK4/5/7 and ALK2/3/6, respectively. The resulting downstream signaling is the phosphorylation of R-Smads. Smads 2/3 and Smads1/5/8 will be phosphorylated by ALK4/5/7 and ALK2/3/6, respectively. These phosphorylated R-Smads, via association with co-Smad Smad4, will translocate to the nucleus where they will upregulate pluripotency gene transcription. It is noteworthy that although BMP4 → ALK2/3/6 → Smad1/5/8 pathway acts to upregulate pluripotency in mESCs, it has been shown to downregulate such expression in hESCs (Park, 2011) (Figure 1C).
Figure 1. Signaling pathways in mESC pluripotency. A) Wnt signaling. This pathway is initiated via binding of Wnt proteins (such as Wnt3a) to the Frizzled receptor (blue) and the accompanying co-receptor LRP5/6 (orange). The activation of the receptor results in the inhibition of APC/Axin/GSK3ß complex which results in the lack of phosphorylation of ß-catenin. As the phosphorylated ß-catenin is targeted and degraded by the Ubiquitin-Proteasome Pathway (UPP), the lack of phosphorylation results in the stable ß-catenin (as denoted by the empty phosphorylation site) translocating into the nucleus, associated with LEF/TFCs, and
affecting gene transcription that cause self-renewal. B) JAK signaling. After the binding of LIF and/or GP130 to the LIFR, the activated receptors dimerize and recruit JAK which in turn phosphorylates and activates STAT3. The phosphorylated STAT3 then translocates to the nucleus and upregulates pluripotency core network of Oct4, Nanog, and Sox2. JAK signaling can also activate PI3K which in turn will activate Akt via PIP3. Akt plays a dual role of inhibiting GSK3β activity, as well as translocating to the nucleus to upregulate pluripotency master regulators. JAK inhibitors such as SOC3 will prevent the downstream STAT3 and PI3K activation C) TGFβ signaling. Two different categories of TGFβ superfamily ligands either bind to ALK4/5/7 or ALK2/3/6. The activation of these receptors results in the activation of R-Smads. Phosphorylated R-Smads translocate to the nucleus via associated with the co-Smad (Smad4) and upregulate pluripotency genes.

Abbreviations: GSK3β, glycogen synthase kinase 3β; LEF/TFCs lymphoid enhancer factor/T cell factor cells; LIF, leukemia inhibitory factor; TGFβ, transforming growth factor β; PIP2, phosphatidylinositol 4,5-bisphosphate; PIP3, Phosphatidylinositol (3,4,5)-trisphosphate.

1.3.2 Pluripotency Transcription Factors
The observation that a specific gene expression profile is consistent with pluripotency was originally established in studies where pluripotency was induced via nuclear transfer (Campbell et al., 1996; Wakayama et al., 1998; Byrne at al., 2007) and cell fusion (Cowan et al., 2005; Yu et al., 2006). The work demonstrating that reprogramming a somatic cell to a pluripotent state can be accomplished through by the expression of only a few genes, coined “master-regulators” of pluripotency, comes from the work of Takahashi and Yamanaka (2006). They used retroviruses that demonstrated that the expression of 4 genes was sufficient to induce pluripotency in somatic cells. These ‘Yamanaka factors’ were Oct4, Sox2, Klf4, and c-Myc and in the original studies, the expression of these genes converted mouse embryonic fibroblasts to “induced pluripotent stem cells” (iPSCs) (Takahashi and Yamanaka, 2006). Since then it has been shown that achieving a pluripotent state can be accomplished by the combinatorial use of Oct4, Sox2, Nanog, and Lin28 (Yu et al., 2007). In mESCs, OCT4, SOX2 and NANOG are major regulatory transcription factors. The complexes formed by this trio enable the modulation of target genes; thus, they are able to activate their own expression as well as other pluripotency genes and also suppress the expression of developmental regulators (important for differentiation). This suppression is accomplished by the signaling cascade between these transcription factors and epigenetic
modulators such as polycomb repressive complexes (PRCs). PRCs are brought to the developmental regulators’ loci, and act to suppress their expression (Lee et al., 2006; Chamberlain, et al., 2008).

Further studies in the field of reprogramming demonstrated that expression of Oct4 alone is sufficient for inducing pluripotency in adult neural stem cells, a population that has endogenous expression of Sox2, c-Myc, and Klf4 (Kim et al., 2009). Although there are many genes involved in self-renewal and pluripotency, Oct4, Sox2, and Nanog are the key players.

1.3.2.1 Oct4

OCT4 is a member of the POU (Pit-Oct-Unc) transcription factor family (Scholer et al., 1990). Structurally, OCT4 protein has three domains: 1) a central POU (Pit-Oct-Unc) domain involved in DNA binding, 2) an N-terminal domain involved in transactivation, and 3) a C-terminal domain involved in cell type-specific transactivation. OCT4 controls the expression of downstream targeted genes by binding an octameric sequence motif of an AGTCAAAT consensus sequence (Herr and Cleary, 1995). The Oct4 locus contains proximal enhancer, a distal enhancer, and a TATA-less proximal promoter. Uniquely, the proximal and distal enhancers have temporal functions at different stages in development; proximal enhancer activity is seen during epiblast formation, while distal enhancer is activated in the ICM, as well as in cultured mESCs (Yeom et al., 1996). Oct4 has been shown to be necessary for development as Oct4-null transgenics are embryonic lethal (Nichols et al., 1998).

In mESCs, maintenance of pluripotency requires a precise level of Oct4 expression. Different studies have shown that both under-expression and over-expression of Oct4 results in differentiation and loss of self-renewal in induced cultures. Decreasing expression of Oct4 by one-half results in mESCs being driven towards a trophoectodermal fate while overexpression (by less than two fold) drives the lineage towards mesoderm and endoderm cell fates (Niwa et al., 2000). The tight regulation of Oct4 expression is maintained in part, through epigenetic modifications. Undifferentiated mESCs have hypo-methylated Oct4 loci and as mESCs begin to differentiate, the regions within these loci become hyper-methylated (which can result from the synergistic workings of methyl-transferases Cdk2ap1 and methyl DNA-binding protein 3 (Mbd3) (Deshpandeh et al., 2009)). Oct4 expression is also modulated at the transcriptional level.
Different members of the orphan nuclear receptor family regulate Oct4 expression. For example, Nr3b2 upregulates the Oct4 promoter activity in mESCs leading to the proper levels of Oct4 expression in mESCs to maintain self-renewal (Zhang et al., 2008).

OCT4 can act to increase the expression of other pluripotency and self-renewal associated factors including the transcription factors SOX2 and NANOG. Indeed, one of the most well-established co-regulators of Oct4 is Sox2. Target genes’ enhancers contain the Oct-Sox elements which allows for the formation of the OCT4-SOX2 complex that leads to an upregulation of both Oct4 and Sox2 in an autoregulatory, positive feedback manner (Chew et al., 2005). Oct4 expression similarly results in upregulation of other pluripotency genes such as Nanog Utf1, and Zfp206 (Rodda et al., 2005). However, since OCT4, SOX2, and NANOG have similar binding sites in their target genes to promote pluripotency, they are thought as the main transcriptional regulators in pluripotent cells (Boyer et al., 2005; Kashyap et al., 2009; Chambers and Tomlinson, 2009).

1.3.2.2 Sox2
SOX2 (sex determining region Y-box 2), is a member of SoxB1 transcription family (Sinclair et al., 1990). SOX2 has a highly conserved DNA binding domain known as the high mobility group (HMG). This transcription factor is necessary for achieving and maintaining pluripotency (Gubbay et al., 1990) and it is also required for neural induction (Wegner and Stolt, 2005). During murine embryogenesis, and similar to Oct4, Sox2 is temporally expressed. It is first expressed in the zygote and as the embryo develops, its expression becomes restricted to the ICM. Deletion of Sox2 in the zygote results in embryonic lethality (Avilion et al., 2003). Expression of Sox2 is required to maintain mESCs in a pluripotent state in vitro as reduction of Sox2 levels (by either siRNA or knockout methods) results in cell differentiation towards a trophoectoderm fate (Keramari et al., 2010). Interestingly, over-expression of Oct4 in Sox2-deficient mESCs can rescue this phenotype and revert the cells back to a pluripotent state. These studies suggest that the role of Sox2 is to regulate the amount of Oct4 expression to ensure pluripotency (Masui et al., 2007; Fong et al., 2008).

1.3.2.3 Nanog
Nanog is essential for embryonic development and pluripotency maintenance (Rossant and Tam, 2009) and overexpression of Nanog substitutes for LIF to successfully maintain undifferentiated
mESCs in culture (Chambers et al., 2003; Mitsui et al., 2003). The mechanism by which NANOG can enhance mESC self-renewal is through its action as a homeodomain protein (HD). As a HD protein, NANOG forms functional dimers via its tryptophan-rich domains (Wang et al., 2008; Mullin et al., 2008). Co-immunoprecipitation studies to better understand the NANOG interactome have shown that its pluripotency partners (such as OCT4) are preferentially associated with the dimeric form as opposed to the monomeric form. The importance of the dimeric form was later elucidated by comparing the roles of tethered monomeric vs dimeric NANOG in mESC pluripotency; monomers are insufficient in maintaining pluripotency of mESCs in the absence of LIF while dimers are capable of such maintenance (Mullin et al., 2008). Although NANOG’s downstream targets overlap those of OCT4’s, NANOG works to maintain a pluripotent ground state, a state of basal proliferation and freedom from any prior epigenetic restriction (Wray et al., 2010). As a result, mESCs are able to activate the cascade of transcriptional circuitry required for pluripotency (Takahashi and Yamanaka, 2006; Silva et al., 2008; Sridharan et al., 2009).

The role of Oct4, Sox2, and Nanog is further elucidated by their description as a ‘pioneer’ proteins. Pioneer proteins are a special category of factors that are able to access DNA sites in condensed chromatin when other factors are unable to do so. Pioneer factors’ access to DNA creates a more easily accessed path to specific DNA sites for other proteins such as histone modifiers (histone acetyl/methyl transferases for example), and transcription factors, allowing for transcriptional activation and revealing their importance in cell programming and directing cell fates. Hence, Oct4, Sox2 and Nanog not only act as transcription factors, but they also act as pioneer factors to create a more permissive nuclear environment for other transcription factors to modulate pluripotency-related expression (Zaret and Carroll, 2011; Iwafuchi-Doi and Zaret, 2014; King and Klose, 2017).

Nanog and Oct4 have both been implicated in the regulation of pluripotency during the pre-implantation epiblast and derivative embryonic stem cells as well as post-implantation development. Although the precise mechanism of the loss of pluripotency is not well established, it is known that the process of post-implantation embryonic development is coincident with reduced DNA accessibility of Oct4 and Nanog regulatory regions, resulting in reduced expression (Osorno et al., 2012). Osorno and colleagues demonstrated that although Nanog is
necessary for the acquisition of the ‘ground state’ pluripotency in mESCs (Silva et al., 2009), its expression in post-implantation embryos is not required for the maintenance of pluripotency. On the other hand, Oct4 expression is necessary for the maintenance of pluripotency in the post-implant embryos and lack of Oct4 expression results in a transient state where pluripotency is lost. Notably, a pluripotent state can be acquired upon re-expression of Oct4.

1.4 Neural Stem Cells (NSCs)

Until the early 1960’s the persisting central dogma in neurobiology was that neurogenesis occurred only during embryonic development and did not occur in the adult CNS. In the 1960s, Altman demonstrated hippocampal and olfactory bulb neurogenesis using autoradiographic labelling (Altman and Das, 1965; Altman, 1969). From the detection of newly formed neurons, to the initial discovery of neural stem cells in the adult murine brain (Reynolds and Weiss, 1992), the characterization of the neural stem cell lineage has expanded substantially in the last 25 years.

1.4.1 NSCs – From Development to Adulthood

1.4.1.1 Embryonic Neural Stem Cells

During embryogenesis, the nervous system arises from neuroectoderm. Neuroepithelial cells (NECs) of the neural tube, that is formed during neurulation, undergo cell division near the ventricular (apical) side (closest to the fluid-filled lumen of the neural tube). These cell divisions are the basis for the formation of the ventricular zone (VZ). NECs undergo symmetric division to expand their population and divide asymmetrically to give rise to radial glial cells (RGCs) (Kosodo et al., 2004). The NECs and their progeny, RGCs, are thought to be the earliest form of embryonic NSCs. RGCs are morphologically defined by their bipolar process extension with an apical process extending to the surface of the lumen and a basal process extending to the surface of the developing brain. The RGC basal processes provide a scaffold for migration of newly born neurons to appropriate cortical layers. RGCs have been shown to be the main source of new neuronal and glial cells in the developing embryonic brain (Malatesta et al., 2003; Miyata et al., 2001; Noctor et al., 2001; Tamamaki et al., 2001).
1.4.1.2 Postnatal Neural Stem Cells
In the postnatal CNS, NSCs reside in two different anatomical niches: in the periventricular region lining of the ventricular system, known as the subependymal zone (SEZ), and in the dentate gyrus (DG) of hippocampus in the subgranular zone (SGZ). The SEZ is seen as the continuation and thinning of the embryonic VZ, while DG is the result of maturation of the embryonic dentate neuroepithelium (DNE) whereby granular cells are generated (Altman and Bayer, 1990; Frotscher et al., 2007; Fujita, 1962; Fujita, 1963; Li and Pleasure, 2007). Once established, the granular cell layer is condensed, and restricted to the SGZ. (Altman and Bayer, 1990; Li and Pleasure, 2007). Although the existence of NSCs in the postnatal CNS is well-accepted, less well established is the relationship between embryonic RGCs and postnatal NSCs. One view is that given the similarity of RGCs to NSCs, they are the same population of cells at different developmental stages. (Malatesa et al., 2003; Götz and Huttner, 2005). In contrast to NECs, RGCs and [later on] NSCs express several markers such as the calcium-binding protein S100β, astrocyte-specific markers glutamate transporter (GLAST) and glial fibrillary acidic protein (GFAP), vimentin and brain-lipid-binding protein (BLBP). While there are a number of similarities between RGCs and NSCs in terms of their expression profile, there still exists some controversy in the literature regarding their origin. However, the restriction of these populations to the same anatomical locations (assuming the existing relationship between embryonic VZ and postnatal SEZ) indicates a linear lineage relationship; NECs give rise to RGCs which in turn become NSCs in the postnatal brain.

1.4.1.3 Adult Neural Stem Cells
Cells from both the SEZ and SGZ contribute to neurogenesis in the adult animal. Under baseline conditions, both SEZ and SGZ-derived NSCs divide asymmetrically and give rise to intermediary progenitors, known as transit amplifying cells (from SEZ) and intermediate progenitor cells (from SGZ). Both of these intermediate cell populations undergo further division and give rise to more committed progeny. The differentiation profile of NSC-derived progeny depends on which neurogenic niche the cell came from. It is well documented that adult SEZ-derived progenitors contribute to olfactory bulb (OB) neurogenesis via migration to the OB through the rostral migratory stream (RMS) and result in the generation of new GABAergic, glutamatergic and dopaminergic interneurons which integrate in the olfactory bulb (OB) more specifically becoming the granule and perigolmerular cells of the OB (Merkle et al.}
2004; Scheffler et al. 2005; Lledo et al. 2008; Brill et al. 2009). The newly generated interneurons are important for proper odor discrimination (Mouret et al. 2009; Kageyama et al. 2012). In addition to neurogenesis in the adult brain, SEZ-derived NSCs can give rise to oligodendrocyte progenitors (Menn et al. 2006). Within the SGZ, neurogenesis results in the generation of granule cells of hippocampus (Cameron et al. 1993; Kempermann et al. 2004). As the main gateway of hippocampal inputs, the DG is implicated in spatial learning, memory, and psychiatric disorders (Noonan et al. 2010; Aimone et al. 2011; Sahay et al. 2011; Snyder et al. 2011; Petrik et al. 2012).

1.4.2 Neural Stem Cell Lineage

Although it appears that NECs are the initial source of NSCs that emerge during embryonic stages, debate remains about how the acquisition of NSC identity takes place. Equating the acquisition of NSC identity with the acquisition of neural fate, there are two prevailing theories. One theory, stemming from the studies done in xenopus suggests that neural fate is acquired via instructive cues (Spemann and Mangold, 1923; Bachiller et al., 2000). The other theory defines neural fate acquisition to be the default pathway in the absence of instructive cues (Smukler et al., 2006; Hitoshi et al., 2004; Tropepe et al., 2001). Evidence for the neural default theory comes from the studies done on mESCs. mESCs readily give rise to neural phenotypes in basic medium, in the absence of exogenous factors (Smukler et al., 2006). Smukler and colleagues observed the same propensity of mESCs to give rise to neural phenotypes when mESCs were cultured in phosphate buffered saline (PBS), after 24 in saline buffer, the majority of mESCs died but, all the cells that survived expressed neural cell markers (such as βIII tubulin, Nestin, and Sox1). This demonstrated that mESCs do not require extrinsic cues and can default towards a neural fate. This also excluded the possibility that factors present in the undefined serum used in mESC culturing was directing the differentiation towards the neural lineage. Tropepe and colleagues inquired about the ability for ESCs’ to acquire a NSC identity using chemically defined medium. Their findings revealed the presence of an intermediary population of NSCs, termed primitive neural stem cells (pNSCs). Thus, the proposed neural stem cell lineage is as follows: pNSCs emerge prior to the other, already mentioned NSCs. Henceforth, the population of NSCs that were originally characterized (Reynold and Weiss,1992) and have been well characterized will be referred to as definitive neural stem cells (dNSCs).
1.4.2.1 Primitive neural stem cells (pNSCs)

pNSCs were first identified as a novel cell type in the neural lineage by Tropepe et al., in 2001 (Tropepe et al., 2001). The pioneering work that led to their detection and derivation was first accomplished using factors FGF2 and LIF both in vitro (from R1 mESCs) and from dissections as early as embryonic day 5.5 (E5.5) (Hitoshi et al., 2004). pNSCs were originally thought to be a transient population that bridged the link between ESCs and dNSCs which are isolated later in embryogenesis (E8.5). However, pNSCs have recently been documented to persist throughout development and into adulthood (Sachewsky et al., 2014; Reeve et al., 2015). pNSCs are LIF responsive and express low levels of Oct4 at all ages of isolation (Figure 2A). In addition, pNSCs in culture and have shown that they are able to give rise to all neural cell types (neurons, oligodendrocytes, and astrocytes) (Figure 2B). Sachewsky and colleagues demonstrated the expression of Oct4 in pNSCs both in vitro and in vivo using Oct4-GFP mice and showing exceedingly rare GFP expression in the periventricular region and with cell sorting techniques immediately after microdissection of the periventricular region (AMNIS) (Figure 2C and 2D) (Sachewsky et al., 2014). Cultured pNSCs, in addition to Oct4, were shown to express the pluripotency markers Nanog, Klf4, and c-myc using PCR (Figure 2F) (Sachewsky et al., 2014).

Hitoshi and colleagues first theorized a NSC hierarchy in which embryonic pNSCs are upstream of dNSCs (Hitoshi et al., 2004). This relationship was demonstrated by Sachewsky et al. using a number of techniques. First, the in vitro colony forming assay, adult-derived colonies cultured in LIF can be passaged to EGF/FGF2-containing medium and give rise to dNSC colonies. However, dNSCs cannot be passaged into pNSC conditions. Additionally, selectively targeting the dNSC population through the use of transgenic mice and drug administration demonstrates that there is no permanent loss of NSCs following ablation as dNSCs always re-emerge 10 days after complete ablation indicating a role for the Oct4-positive, Gfap-negative pNSC population in replacing the downstream Gfap-positive dNSC pool.

1.4.2.2 Definitive neural stem cells (dNSCs)

The term ‘dNSCs’ refers to NSCs during development and adulthood, that are FGF2 and/or EGF-dependent. dNSCs can be isolated from E8.5 (Tropepe et al., 1999). The FGF2 dependent population is downstream of LIF-responsive embryonic pNSCs (Tropepe et al., 2001; Hitoshi et al., 2004) and gives rise to epidermal growth factor (EGF)-dependent dNSCs (Tropepe et al.,
that express the intermediate filament protein glial fibrillary acidic protein (GFAP). The presence of GFAP-positive dNSCs can be detected after embryonic day 16.5 and continues into adulthood (Morshead et al., 2003; Garcia et al., 2004).

### 1.4.2.3 Identifying NSCs

Identifying NSCs and their progeny is accomplished through the use of a panel of markers. The intermediate filament protein, glial fibrillary acidic protein (GFAP) is one of the most well-established markers used to identify dNSCs. However, as the name suggests, it is also expressed in astrocytes (Doetsch et al., 1997; Morshead et al., 2003). Studies done by Doetch and colleagues (1997) demonstrated the expression of GFAP-positive cells in the SEZ. dNSCs also express Nestin (Lagace et al., 2007), Sox2 (Episkopou, 2005), and Id1 (Nam and Benezra, 2005) which are regarded as markers for undifferentiated NSCs. The transit amplifying cells, downstream of dNSCs express similar markers to NSCs. These intermediary progenitors express Nestin (Lendahl et al., Doetsch et al., 2002), Sox2, in addition to Distal-less (Dlx) (Doetsch et al., 2002). The more committed progenitors (neuroblasts) express Nestin (Mignone et al., 2004), Mash1 (Parras et al., 2004), Doublecortin (Dcx) (Koizumi et al., 2006) and PSA-NCAM (Doetsch et al., 2002). The expression of Nestin declines the more differentiated the cells become, with NSCs expressing the highest relative levels and neuroblasts expressing the least. Although the above markers provide us with a better understanding of NSC expression profile, this by no means is an exhaustive list.

### 1.4.2.4 Comparison of pNSCs and dNSCs

There are several key differences between pNSCs and dNSCs. pNSCs are cultured in the presence of LIF, while dNSCs are cultured in the presence of EGF and FGF. pNSCs are also much more infrequent than dNSCs in the postnatal/adult brain with the estimation of 100-fold difference in stem cell number. pNSC colonies tend to be smaller and more adhesive than dNSCs (Sachewsky et al., 2014). In vivo we have demonstrated pNSCs to have longer cell cycle time than dNSCs (3-6 months for pNSCs and 2-3 weeks for dNSCs). Moreover, pNSCs and dNSCs can be differentially regulated by factors such as C-kit and ErbB2 inhibitors. Application of the inhibitor cocktail to NSC cultures results in higher numbers of generated pNSC colonies and lower numbers of generated dNSC colonies. (Morshead et al., 1998; Reeve, 2015; Reeve et al., 2015). These cell types also differ in terms of their neural differentiation capacity. Both pNSCs
and dNSCs give rise to all neural cell types, yet, there is a notable difference in the relative proportion of each neural subtype generated; adult-derived dNSCs give rise to primarily astrocytes, followed by neurons, and oligodendrocytes, while pNSCs give rise to the three populations at equal frequencies in vitro. In addition, adult-derived pNSCs are able to contribute to the ICM of a developing blastocyst using morula aggregations, at a very low frequency (2.5%), while the dNSC counterparts do not contribute to the ICM of developing blastocysts (Figure 2G) (Sachewsky et al., 2014). There is also exclusive expression of Oct4, Nanog, and β-catenin in pNSCs, with none detected in dNSCs. The pluripotency genes Oct4 and Nanog are expressed at much lower levels in pNSCs when compared to mESCs. qPCR analysis demonstrated that pNSC colonies have a lower expression of neurally-committed markers Mash1 and Sox1. Incorporation into the morula, expression of pluripotency gene, and lower expression of certain NSC markers points to the possibility that pNSCs are a less neurally committed cell type (Sachewsky et al., 2014; Reeves et al., 2015). The lineage relationship is presented in figure 3.
Figure 2. Characterization of pNSCs. A) pNSCs can be isolated from murine brain from early embryonic animals to old age animals. pNSCs exist in highest numbers in early postnatal

Taken from Sachowsky et al. 2014 (modified)
animals (PND0-PND7). B) Differentiated adult-derived pNSCs demonstrate neural commitment in vitro as shown by the presence of neurons (βIII tubulin), oligodendrocytes (O4), and astrocytes (GFAP). Scale bar = 50µm C) sections derived from Oct4-GFP mice reveal Oct4+ (green) cells in the periventricular region (DAPI, blue). Higher magnification insets provided (blood vessels, red) scale bar = 20µm. D) AMNIS Imaging Flow Cytometry reveals the presence of Oct4+ LIFR+ live cells and Oct4+/ LIFR+/ GFAP- fixed cells from Oct4-GFP mice. E) RT-qPCR analysis on cortical and periventricular regions demonstrates the detection of Oct4 transcript in exclusively the periventricular region. F) RT-qPCR analysis demonstrate the expression of Oct4, Nanog, Klf4, and C-myc in adult derived pNSC cultures. G) Morula aggregation studies revealed the ability of ESCs and adult derived pNSCs (green) to integrate into the ICM of developing blastocysts 24 hours post-incubation. scale bar = 50µm.

Figure taken and modified from Stem Cell Reports, Vol. 2, N. Sachewsky, R. Leeder, W. Xu, K. L. Rose, F. Yu, D. van der Kooy, and C. M. Morshead, Primitive Neural Stem Cells in the Adult Mammalian Brain Give Rise to GFAP-Expressing Neural Stem Cells, pp. 810-824. Copyright 2014, with permission from Authors.
**Figure 3. The Murine Neural Stem Cell Lineage.** The combination of the recent findings by both Tropepe (2001) and Sachewsky (2014) regarding the NSC lineage provides the following lineage relationship. As shown in schematic A, pNSCs are isolated starting from E5.5. Embryonic pNSCs are GFAP-, Oct4+, and are responsive to LIF. Embryonic pNSCs give rise to FGF-responsive, GFAP- definitive neural stem cells which are seen at E8.5. GFAP+, EGF and FGF responsive dNSCs are found in late embryonic development and persist in the postnatal and mature CNS. Schematic B demonstrate the persistence of both pNSC and dNSCs populations in the mature, adult murine brains.

1.4.3 *In vitro* Neural Stem Cell Assays

In vitro colony forming assays are commonly used to study stem cells and their progeny. Such assays help to demonstrate the cardinal stem cell properties of self-renewal and potency in either adherent or free-floating clonally-derived clusters of cells. There are two well established cell culture assays to grow neural stem cells and their progeny; the neurosphere assay (NSA), and the adherent colony formation assay.
Neural stem cells and their progeny, together termed neural precursor cells (NPCs), can be cultured using the simple and robust NSA. When tissue from the periventricular region is dissociated and plated as single cells in defined media, individual clonally-derived free-floating colonies termed “neurospheres” are formed after 7-10 days in vitro (Reynolds and Weiss, 1996; Chiasson et al., 1999; Gritti et al., 2002). dNSC derived neurospheres grow in serum free media in the presence of EGF and FGF2 while pNSC cultures are grown in serum free media with LIF. Individual neurospheres from primary dissections can be dissociated and re-plated as single cells in appropriate culture conditions (process known as passaging) that can give rise to secondary neurospheres to demonstrate the property of self-renewal. Neural stem cells can also be cultured as adherent monolayers. For the adherent colony formation assay, cells from similar microdissections of the periventricular region are plated at clonal density on an adherent substrate such as support cells (ie. mouse embryonic fibroblasts - MEFs) or gelatin. A schematic of the in vitro neural stem cells assay is presented in figure 4.

Figure 4. Neural Stem Cell Culturing Assay. Neural stem cells can be cultured using colony-forming assays. Both the neurosphere assay, and the adherent colony formation assays are represented by the schematic. After the microdissection of the periventricular region neural stem
and progenitor cells (NPCs) and niche cells are plated at clonal density in the presence of specific growth factors. Individual NSCs will proliferate to form 1° colonies that can be broken down to single cells and passaged to give rise to 2° colonies, confirming the self-renewal capacities of NSCs. NSC-derived colonies can be passaged indefinitely. Individual colonies can be differentiated on an adhesive substrate in the presence of serum to give rise to all three neural cell types, confirming NSC multipotentiality. Population-specific growth factors are exclusively used to culture for the distinct populations of NSCs; EGF, FGF, and heparin (EFH) are used to culture dNSCs, and LIF is used to culture pNSCs.

1.5 Pluripotency Assays
The development of both in vitro and in vivo assays has allowed for the characterization of stem cell potency. To assess pluripotency, two well-established assays are commonly used; the in vitro Embryoid Body (EB) assay and the in vivo teratoma formation assay.

1.5.1 In vitro Embryoid Body Assay
While mESCs can be maintained for long-term culture as undifferentiated colonies, they can also be driven to differentiate into various germ cell lineages (Keller, 1995; Smith, 2001). Three in vitro methods of controlled ESC differentiation have been developed to systemically facilitate differentiation of ESCs in an ordered (as opposed to spontaneous) manner. The three in vitro methods of inducing ESC differentiation include (i) monolayer culturing on specific matrices or in differentiation media (Fico et al., 2008; Takahashi & Yamanaka, 2006), (ii) co-culturing of ESCs with support cells (i.e. stromal cells) (Nakano et al., 1994) , and (ii) the formation of ESC-derived aggregates known as embryoid bodies (EBs) (Doetschman et al., 1985; Keller, 1995). The first two differentiation assays provide a 2D environment for ESCs and are best suited to study the interaction of the cells with their substrate, while the EB assay allows for a 3D microenvironment and enables one to assess the role of the interaction of cells with respect to each other.

An EB is an aggregate of pluripotent cells and serves as a strong indicator that a population of cells has the ability to differentiate into the three germ layers (Doetschman et al., 1985). There are two classic ways that EBs are formed and they include: hanging drops and spontaneous aggregation, also known as ‘static suspension cultures’. These culturing techniques were adopted
from an in vitro differentiation method used for a non-ESC line, embryonic carcinoma (EC) cells (Martin & Evans, 1975) and later they were used to test pluripotency in mESCs (Doetschman et al., 1985; Keller, 1995). The hanging drop method asks for generation of cell suspension drops while the spontaneous aggregation method allows for aggregation through the use of a high cell density microenvironment (>1x10^6 cells per mL) and by placing this single cell suspension in non-adherent culture plates. After 2 days, both methods yield aggregates. The EB medium is the un-supplemented mESC medium; the removal of LIF allows for the process of differentiation to occur through the inactivity of JAK pathway.

The first step in the EB assay is aggregate formation. The self-assembly of cells into an aggregate is mediated by cell adhesion molecules of which E-cadherin is one of the major players (Dang et al., 2004). It has been shown that E-cadherin null (E-cadherin –/–) mESCs are unable to form aggregates. Larue and colleagues performed E-Cadherin rescue experiments where they showed the ability of cadherin –/– mESCs to form aggregates via introduction of E-cadherin. Further, E-cadherin plays a role in mesoderm differentiation (by its downregulation of Brachyury). Larue et al. demonstrated the robust expression of Brachyury in E-cadherin –/– mESCs, while there were no Brachyury expression in E-cadherin +/+ or +/- (Larue et al., 1996). This mutually exclusive expression profile of Brachyury and E-cadherin is congruent with prior findings that E-cadherin becomes downregulated in the embryo prior to primitive streak formation (Vestweber and Kemler, 1984; Damjanov et al., 1986; Butz and Larue, 1995), which is also marked by emergence of Brachyury expression, (Herrmann, 1991). In addition, expression of E-cadherin synergistically promotes endoderm differentiation in conjunction with hepatotropic factors. Dasgupta and colleagues demonstrated that EBs derived from E-Cadherin-expressing ESCs (CE-ES) were able upregulate markers of early (alpha-fetoprotein-AFP), mid (Albumin), and late (glucose-6-phosphate) hepatocyte differentiation. On the other hand, ESCs with lower E-Cadherin levels, known as E-Cadherin-deficient ESCs (CD-ES) expressed the hepatic markers at much lower levels (Dasgupta et al., 2005). Their study demonstrated the role of E-Cadherin not only as a cell adhesion molecule but as a key signal transducer in hepatospecific differentiation.

The size of an aggregate (i.e. an EB) is primarily determined by the starting cell number placed in the EB assay. A number of groups have demonstrated that using physical means of size
restriction can enhance the consistency of the assay, and can drive the aggregates towards a specific lineage. For example, limiting aggregate size by growing EB’s in agarose hydrogel microcapsules (Dang et al., 2004) or in microwell chips with preset diameters (Nakazawa et al., 2013) can limit the proliferation and differentiation of cells within the developing EB’s. Nakazawa and colleagues demonstrated that EBs generated smaller microwell chips (400µm diameter) showed a slower decrease in undifferentiated markers when compared to EBs generated in larger microwell chips (1000µm). This was coincident with larger microwell chips’ higher differentiation rate into mesoderm and endoderm lineages (as demonstrated by higher Flk1, alphaMHC, Nkx2.5, Afp, and Ttr expressions) (Nakazawa et al., 2013).

One of the proposed benefits of the EB assay is that the differentiation of cells within the aggregate recapitulates developmental events. For example, following aggregation, there is emergence of markers of primitive endoderm in the EBs (Maurer et al., 2008), followed by further differentiation into parietal and visceral endoderm (Li et al., 2001), and formation of a cystic cavity within the EB, similar to blastocoele formation in the developing embryo (Murray et al., 2000). As the differentiation of aggregates continues, more differentiated, germ-layer specific markers emerge (Keller, 1995). Expression of endodermal markers such as Hnf3β, Gata4, and Sox17, mesodermal markers such as Flk1 and Brachyury, and ectodermal markers such as Sox1, Nestin, and Keratins have been observed within the EBs (Keller, 1995; Itskovitz-Eldor, 2000). Once the EBs have formed and differentiated, the next step is to identify the various cell types generated within each EB.

1.5.1.1 Germ layer-specific Markers used in the EB Assay
In order to determine germ layer-specific markers in the EB assay, markers identifying the derivatives of specific germ layers are used. Ectoderm gives rise to the nervous system (neuroectoderm) as well as hair, nails and skin. Endoderm gives rise to the inner lining of most of the gastrointestinal tract, the respiratory tract, the tract, as well as some internal organs and mesoderm gives rise to muscle derivatives (smooth muscle, cardiac, and skeletal), connective tissue, and endothelium of blood vessels (Sadler, 2011). The markers used to identify cells in the EB assay can vary depending on the length of differentiation within the EB with early lineage markers be used in EB’s during their formation.

Ectoderm
*Nestin* (Fauzi et al, 2012; Mogi et al, 2009) and *Sox1* (ten Berge urinary et al., 2008) are the most commonly used ectodermal markers. NESTIN is an intermediate filament protein with a protein expression pattern that is exclusive to neurally committed cells. Nestin persists as a marker of neural stem and progenitor cells and is essential for self-renewal of NSCs (Park et al., 2010). Neural tube in *Nestin*–/– early embryos (E9 – E11.5) demonstrated increased apoptosis in the neuroepithelium. In addition, culturing NSCs straight from embryonic dissections demonstrated slower cell cycle and higher cell death in vitro (Park et al., 2010). *Sox1* is a member of the SOX family that is involved in neural induction. *Sox1* expression coincides the emergence of NECs and its expression is seen in NSCs and their progeny (Pevny et al., 1998). Cytokeratins (more commonly referred to as keratins) are markers of ectodermal lineage. Unlike Nestin and Sox1, keratin expression is seen in non-neural epithelial cells (Maurer et al., 2008).

**Endoderm**

Endoderm markers include α-feto protein (*Afp*) (Abe et al, 1996; Sajini et al, 2012), hepatocyte nuclear factor 3-beta (*Hnf3β*), hepatocyte nuclear factor 4 (*Hnf4*), and *Sox17* (Abe et al, 1996, Mogi et al, 2009). The *Afp* gene codes for one of the major plasma proteins produced by yolk sac during embryonic development and is considered an early hepatocyte marker (Qin et al., 2009). *Hnf3β* and *Hnf4* code for transcription factors and are indicators of visceral endoderm during embryogenesis. *Hnf3β* is also involved in early endodermal differentiation in mESCs. Overexpression of *Hnf3β* in mESCs induced the expression of endodermal genes cystic fibrosis transmembrane conductance regulator (*CFTR*) and albumin (Levinson-Dushnik, 1997). *Hnf4* is involved is important in proper gastrulation (Chen et al., 1994). *Sox17* has been shown to facilitate mESC differentiation into both definitive and primitive endoderm in vitro (Qu et al., 2008).

**Mesoderm**

*Brachyury* (*T*), *Flk1*, and *Gata4* are mesoderm markers that have been utilized in the embryoid body assay (Mogi et al, 2009, Fauzi et al, 2012, Qin et al., 2009). Transcription factor Brachyury is considered one of the earliest markers of the mesoderm lineage. It is first expressed during primitive streak formation and plays a role in upregulating important hematopoietic and muscle fate regulators (Lolas et al., 2014). Fetal liver kinase-1 (*Flk1*), also known as vascular endothelial growth factor receptor 2 (*Vegfr-2*), is used as a more differentiated mesodermal marker because of its involvement in vasculogenesis and angiogenesis (Liang et al., 2001). *Gata4* is a mesoderm/endoderm marker and is important in regulating cardiac development (more
specifically heart tube formation). In addition, *Gata4* plays a role in ventral morphogenesis in embryos. *Gata4* /− embryos portrayed defects in lateral-ventral folding (Kuo et al., 1997). In addition, *Gata4* plays a role in myocardiocyte differentiation (Heineke et al., 2007). Since *Gata4* has been shown to be upstream of *Hnf3β* and *Hnf4* (markers of endoderm differentiation) (Fujikura et al., 2002), *Gata4* is referred to as a mesendodermal marker. The summary of the markers discussed are presented in Table 1.

<table>
<thead>
<tr>
<th>Germ Layers</th>
<th>Markers</th>
<th>Function/Role</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ectoderm</td>
<td>Nestin</td>
<td>Intermediate filament (IF) involved in NSC self-renewal. Early marker of NSC.</td>
<td>Park et al., 2010</td>
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<tr>
<td></td>
<td>Sox1</td>
<td>One of the earliest markers expressed in neurally committed cells. Present in NSCs and progeny</td>
<td>Pevny et al., 1998</td>
</tr>
<tr>
<td></td>
<td>Keratins</td>
<td>IF involved in the cytoskeletal stability of epithelial cells</td>
<td>Maurer et al., 2008</td>
</tr>
<tr>
<td>Endoderm</td>
<td>Afp</td>
<td>Codes for major plasma protein secreted by the embryonic yolk sac and later the fetal liver</td>
<td>Abe et al, 1996; Sajini et al, 2012</td>
</tr>
<tr>
<td></td>
<td>Hnf3β</td>
<td>Codes for transcription factor (TF), indicator of visceral endoderm formation. Overexpression of this gene in mESC cell lines results in a much higher propensity for endodermal differentiation</td>
<td>Levinson-Dushnik, 1997</td>
</tr>
<tr>
<td></td>
<td>Hnf4</td>
<td>Codes for TF, important for visceral endoderm formation. Essential for proper gastrulation</td>
<td>Chen et al., 1994</td>
</tr>
<tr>
<td></td>
<td>Sox17</td>
<td>TF that facilitates definitive and primitive endoderm differentiation <em>in vitro</em>. Developmentally, it is expressed in extraembryonic endoderm as well as definitive endoderm.</td>
<td>Qu et al., 2008; Shimoda et al., 2007</td>
</tr>
<tr>
<td></td>
<td>Brachyury</td>
<td>Marker of primitive streak formation, necessary for the development of mesoderm. It is also involved in regulating hematopoietic and muscle fate.</td>
<td>Lolas et al., 2014</td>
</tr>
<tr>
<td>Mesoderm</td>
<td>Flk1</td>
<td>Codes for a receptor that is involved in vasculogenesis and angiogenesis</td>
<td>Liang et al., 2001</td>
</tr>
<tr>
<td></td>
<td>Gata4 (mesendoderm)</td>
<td>Codes for a TF that directs cardiac development as well as regulating lateral-ventral folding of the embryo. Due to its regulation of endodermal markers (being upstream of Hnf3β and Hnf4), it is also a marker of endoderm.</td>
<td>Kuo et al., 1997; Heineke et al., 2007; Fujikura et al., 2002</td>
</tr>
</tbody>
</table>

Table 1: Germ layer-specific markers discussed

1.5.2 *In vivo* Teratoma Formation Assay

The teratoma assay has been used to assess the risk of tumour formation by pre-clinical cells of interest, and it is considered a gold-standard for assessing pluripotency. A teratoma is a tumour
that contains cells derived from more than one germ layer. When pluripotent cell sources such as mESCs and iPSCs are introduced into immunodeficient mice, they have the ability to form a teratomas (Thomson et al., 1998; Takahashi and Yamanaka, 2006; Kooreman and Wu, 2010). The assay depends on a number of factors such as the immunocompetency of the host, the cell type, and the delivery method (Dressel et al., 2008). Studies have shown that the frequency of teratoma formation varies depending on whether the host is immuno-competent, immuno-suppressed, or immune-deficient. Dressel and colleagues have demonstrated that mESCs transplanted into immunocompetent animals form teratomas at lower frequencies when compared to immunosuppressed (via the use of immunosuppressant drug CsA) and immunodeficient mice (Dressel et al., 2008). Hence, to provide the cell source of interest with the best chance of survival, immunedeficient mice are the choice most conducive to teratoma formation as the immune system will not play a role in rejecting the transplanted cells.

Cell type has been shown to be of importance since several studies show that different pluripotent cell populations form teratomas at different frequencies. For instance, Gutierrez-Aranda and colleagues demonstrated that iPSCs produce teratomas at a faster rate and frequency than ESCs (Gutierrez-Aranda et al., 2010). Genetic and epigenetic differences are thought to underlie differences observed in teratoma formation (Laurent et al., 2011).

A number of transplantation sites have also been used for teratoma formation including the sub-renal capsule, subcutaneal, myocardial, intra-muscular, intra-hepatic, and the brain (Prokhorova et al., 2009; Dressel et al., 2010; Nelakanti et al., 2016). The least invasive sites are often the first utilized for teratoma studies.

A schematic of the embryoid body assay and the teratoma formation is presented in figure 5.
Figure 5. Schematic of *in vitro* and *in vivo* pluripotency assays. A) The embryoid body assay. The hanging drop method allowed for the gravity-assisted aggregation of pluripotent cells. The aggregates that are formed, referred to as EBs, will differentiate and give rise to progenies within all three germ layers. B) The teratoma formation assay requires the injection of pluripotent cells into a host animal (preferably an immunodeficient host). The cells will form a mass known as a teratoma. Within the teratoma there will be cells from all three embryonic germ layers.
Chapter 2

2  Rationale, Hypothesis and Objective

2.1  Rationale
We have demonstrated characteristics that are found in pluripotent cells such as the expression of genes known to be involved in pluripotency (Sox2, Klf4, Nanog, and Oct4) and their responsiveness to LIF. Perhaps most compelling is the finding that pNSCs can contribute to the developing blastocyst in morula aggregation studies. While studies to date have focused on the neural lineage capacity of pNSCs that are isolated from the central nervous system, none have explored the pluripotentiality of pNSCs. As such, we propose to examine the non-neural commitment of pNSCs.

2.2  Hypothesis
We hypothesize that pNSCs have the capacity to give rise to non-neural cells thereby revealing their pluripotentiality.

2.3  Research Objectives
We will test our hypothesis with the following objectives:

1. To evaluate pNSC pluripotentiality using the in vitro embryoid body assay. The embryoid body (EB) technique provides a 3D microenvironment where aggregates of pluripotent cells, such as embryonic stem cells or induced pluripotent stem cells, demonstrate their ability to differentiate into the three primitive germ layers (ie. endoderm, mesoderm, and ectoderm) and their progeny that can be validated using PCR, immunocytochemistry and cell culture. We will utilize this well-established paradigm to generate primitive NSC-derived embryoid bodies and examine their lineage potential.

2. To Evaluate pNSC pluripotentiality using the *in vivo* teratoma formation assay. Another well-established assay for pluripotency is the teratoma assay. This involves the injection of pluripotent cells subcutaneously into adult mice and examining their potential to proliferate and form multilineage colonies in an *in vivo* environment. We will inject cells and wait before removing
the tumour and examine the pluripotent capacity of the tumour forming cells derived from this *in vivo* niche.
Chapter 3

3 Materials and Methods

3.1 Mice

CD1 pregnant mice were purchased from Charles River. In a pathogen free facility, a dam was housed in a single cage with a red rectangular/dome house, one nestlet, with food and water available ad libitum. Mice from postnatal day 4 (PND4) until postnatal day 10 (PND10) (males and females) were sacrificed and used for both free floating and adherent colony formation assays. For *in vitro* and *in vivo* mixing experiments, transgenic YFP mice, constitutively expressing YFP under the CAG promoter, were bred in animal facility. (129-Tg(CAG-EYFP)7AC5Nagy/J) YFP reporter mice were sacrificed from PND4-10 and used for both free floating and adherent colony formation assays prior to the mixing experiments. For the teratoma formation assay, to circumvent any transplant rejection of the xenograft, adult immunocompromised SCID/Beige (CB17.Cg-PrkdcscidLystbg-J/Crl) mice were used. A set of SCID/Beige breeding pair was purchased from Charles River and the animals were bred in house in department of comparative medicine (DCM) at the University of Toronto. All procedures were performed in accordance with institutional guidelines and approved by the Experimental Animal Committee at the University of Toronto.

3.2 Neural Stem Cell Culturing Assays

In PND4-10 animals neural stem/progenitors, collectively called neural precursor cells (NPCs) were micro-dissected from the periventricular region of the brain as previously described (Chiasson et al., 1999). The tissues were broken down to single cells by mechanical trituration (25-35 times). Definitive neural stem cells (dNSCs) were cultured by plating single cells at clonal density (10 cells/μl) (Coles-Takabe et al., 2008) in T75 culturing flasks (VWR Scientific) with serum-free medium (SFM) containing 1% penicillin/streptomycin (Invitrogen) and supplemented with EGF (20 ng/mL, Sigma-Aldrich), bFGF (10 ng/mL, Sigma-Aldrich) and heparin (7.35 ng/mL, Sigma-Aldrich). Primitive neural stem cells (pNSCs) were cultured by plating single cells at the density of 40 cells/μl on 0.1% gelatin-coated (EmbryoMax - Cat# ES-006-B) 100mm cell culture dishes (Thermo Scientific™ Nunc™ Cell Culture/Petri Dish – Cat#
1256599) with serum-containing medium and supplemented with LIF (1000U/mL). Colonies were taken 7 days later, broken down to single cells and used for various assays.

### 3.3 Embryonic Stem Cell Culturing

R1 mouse embryonic stem cell lines were used in the following studies. Frozen R1 mESCs vials were kindly given to us by van der Kooy and Zandstra labs. The vials were promptly thawed after being taken from liquid nitrogen and diluted in DMEM plus 15% FBS ESC medium. mESCs were then spun down and re-suspended in LIF-supplemented ESC medium (1000U/mL). mESCs were passaged onto inactivated mouse embryonic fibroblasts (MEFs) feeder layers for the first 2 passages and were then passaged onto 0.1% gelatin-coated plates for subsequent passages. mESCs used for the experiments were between 1 to 5 passages on gelatin.

### 3.4 Embryoid Body Assay

#### 3.4.1 Embryoid Body Assay – Basic

mESCs, pNSCs, and dNSCs were collected into respective tubes, spun down, and washed with un-supplemented serum-containing ESC medium (herein referred to as EB medium). All samples were chemically digested using TrypLE (Gibco – Cat#12604-013) for 5 minutes in 37°C, 5% CO₂ incubators, followed by inactivation, followed by mechanical trituration to produce single cell suspensions. Total number of cells within each sample was estimated using hemocytometer counts and single cell suspensions with concentration of 1000 cells/µl were made with pre-warmed EB medium.

Using a multichannel pipette, rows of 30µl drops were produced on the inner surface of the inverted lid of petri dishes. 15mL of 1X PBS was transferred to each petri dish to avoid potential evaporation of the generated drops. The petri dishes were carefully placed in the incubator for 48 hours. After the two days of ‘hanging’ period, the drops were carefully transferred over to fresh EB medium in 24-well ultralow attachment plates using a P200 pipette. The cultures were then placed into the incubator undisturbed for 7 days. After the seven days of ‘free floating growth’ period, the aggregates were transferred over to 0.1% gelatin coated 24-well tissue culture plates. After the aggregates adhered to the adhesive substrate (0.1% gelatin), the EB medium was changed every other day for 3 days.
3.4.2 Embryoid Body Assay – *in vitro* Mixing
pNSC and dNSC cultures were derived from transgenic YFP pups, constitutively expressing yellow fluorescent protein under the CMV promoter. The embryoid body assay was attempted by mixing 15,000 ESCs with 15,000 pNSCs or dNSCs.

3.4.3 Embryoid Body Assay – Conditioned Media
The embryoid body assay for mESCs was started 2 days prior to the embryoid body assays for pNSCs and dNSCs. The 48-hour head-start allowed for the generation of EB conditioned media (EB-CM) to be used on the NSC-derived aggregates. After day 2 ESC-derived aggregates were transferred to ultralow attachment plates, CM was collected every 48 hours by collecting the supernatant after mESC-derived aggregates were given sufficient time to settle at the bottom of 15mL falcon tubes, filtered via 0.22 µm syringe filter (Millipore GP – Cat#SLGP033RS), and supplemented with fresh EB medium (2 parts EB-CM, 1 part fresh medium) to provide sufficient glucose and L-glutamate levels and avoid potential cell starvation in pNSC-derived and dNSC-derived cultures. The CM was then pre-heated and pre-gassed in 37°C, 5% CO\(_2\) incubators 30 minutes prior to being placed onto pNSC-derived and dNSC-derived aggregates.

3.5 Teratoma Formation Assay

3.5.1 Teratoma Formation Assay – Basic
R1 mESCs, pNSCs and dNSCs isolated and cultured from CD1 pups were collected, counted. 1,000,000 cells were re-suspended in 200µl of 4°C 30% matrigel (in 1X sterile dPBS). SCID/Beige mice were placed under anaesthesia and 1,000,000 cells per each group were injected into the subcutaneous space spanning the hind leg of the animal. Each animal was a host to two injections of cells. The animals were sacrificed from 3-4 weeks post-injection.

3.5.2 Teratoma Formation Assay – *in vivo* Mixing
500,000 mESCs were combined with 500,000 YFP+ pup-derived pNSCs or dNSCs. The 1,000,000 ESC-NSC mixed population were then re-suspended in 200µl of 4°C 30% matrigel (in 1X sterile dPBS). SCID/Beige mice were placed under anaesthesia and 1,000,000 cells per each group were injected into the subcutaneous space spanning the hind leg of the animal. Each animal was a host to two injections of cells. The animals were sacrificed 3-4 weeks post injection.
3.6 Immunostaining

Prior to any immunostaining, all samples were hydrated for 5 minutes in 1X PBS. For intracellular antigens, samples were permeabilized using 0.3% triton in 1X PBS for 20 minutes, followed by 3, 5-minute, washes in 1X PBS, and then blocked with blocking solution containing 5% NGS and 1% BSA for 1 hour at room temperature. Samples were incubated with primary antibodies overnight at 4°C in 1X PBS. The following day samples were washed in 1X PBS (3, 5-minute washes) and incubated with the appropriate secondary antibody for 1-2 hours at room temperature. Samples were stained with nuclear stain Hoechst 33233. Plates were kept hydrated in 1X PBS and were wrapped with parafilm to avoid excessive evaporation, while slides mounted by Mowial (made in-house) and cover slipped for long terms storage. The list of primary and secondary antibodies in Tables 2 and 3.

3.6.1 Hybrid Embryoid Body Aggregates

Mixed mESC-NSC aggregate samples were fixed with 4°C 4% paraformaldehyde for 20 minutes. Samples that needed to be sectioned were cryoprotected in 30% sucrose for 36 hours prior to cryosectioning (at 20µm thickness) and sections were adhered to Superfrost Plus glass slides.

3.6.2 Teratomas

Animals were sacrificed with an overdose of Avertin. Teratomas were excised and placed in 4°C 4% PFA for 6 hours. Samples were then transferred to 30% sucrose for 36 hours prior to cryosectioning (at 20µm thickness) and sections were adhered to Superfrost Plus glass slides.

3.7 Microscopy

To image the stained samples, either a Zeiss Observer D1 inverted, or a Zeiss Spinning Disk Confocal were used to visualize immunofluorescence. Images were acquired at 20x objective using AxioVision and Zen microscopy softwares for Observer D1 inverted and Spinning Disk Confocal microscopes, respectively. For reported differentiation profiles, cells were counted within the field of view in 3 separate representative sections per biological sample. Percentages of differentiated cells were calculated to the total numbers of cells in each field of view.
3.8 RT-qPCR

Aggregates made from the embryoid body assay were collected into Buffer RL (Norgen Biotek) with β-mercaptoethanol. ESC-derived samples were processed according to the manufacturer’s directions using Total RNA Purification Kit (Norgen Biotek – Cat#17200), pNSC-derived and dNSC-derived samples were promptly processed according to manufacturer’s directions using Single Cell RNA Purification Kit (Norgen Biotek – Cat# 51800). cDNA synthesis was carried out with iScript gDNA Clear cDNA Synthesis Kit (Bio-Rad – Cat# 1725034). RT-qPCR reactions were prepared according to the manufacturer’s directions using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad – Cat# 172-5270). RT-qPCR was carried out on Bio-Rad CFX384 Touch Real-Time PCR System (Bio-Rad). Cycling conditions consisted of polymerase activation and DNA denaturation (3 min at 98°C), followed by 40 cycles of 10 s at 95°C and 30 s at 60°C. All reactions were concluded by incubation at 65°C and increasing the temperature (at 0.5°C increments) to 95°C for melting-curve analysis. Bio-Rad SYBR Green Assays: Nestin (dMmuEG5084781), Sox1 (dMmuEG5072088), βIII tubulin (qMmuCID0018119), Olig2 (qMmuCED0003760), Gfap (qMmuCID0020163), Keratin18 (qMmuCED0003446), Flk1 (dMmuEG5073201), T (dMmuEG5079250), Gata4 (qMmuCID0006478), Afp (dMmuEG5084946), Hnf3β (dMmuEG5068477), Hnf4a (qMmuCID0026950), Sox17 (qMmuCID0022827), Oct4 (dMmuEG5066663), Nanog (dMmuEG5072880), Gapdh (dMmuEG5193528), β-actin (dMmuEG5193531).
### Table 2. List of Primary antibodies

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<th>Antibody</th>
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<th>Product Code</th>
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<th>Species</th>
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### Table 3. List of Secondary antibodies

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<td>Alexa Fluor - Goat anti-</td>
<td>1:400</td>
<td>488/568/647</td>
<td>Life Technologies</td>
</tr>
<tr>
<td>mouse IgG</td>
<td></td>
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<tr>
<td>Alexa Fluor - Goat anti-</td>
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<tr>
<td>Rabbit Poly</td>
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Chapter 4

4 Results

4.1 pNSCs form aggregates in the basic embryoid body assay

Morula aggregation studies done by Sachewsky et al (2014) revealed that pNSCs isolated from the adult forebrain could integrate into developing blastocysts. This finding supported the hypothesis that pNSCs may have the potential to contribute to multiple germ layers. To explore this potential we used the embryoid body (EB) assay. We predicted that if pNSCs have pluripotent capacity, they would generate embryoid bodies that contained progeny from all three embryonic germ layers (ectoderm, mesoderm, and endoderm). We employed this assay using three distinct cell populations; mESCs (positive control), early postnatal pNSCs, and early postnatal dNSCs (negative control). pNSCs are exceedingly rare hence we used pNSCs derived from the early postnatal brain for our studies as this is the time when pNSCs are most abundant. Sachewsky et al. (2014) demonstrated that early postnatal animals (PND0-PND7) can yield between 40-60 pNSC-derived colonies which is >20 fold more than the numbers of pNSCs colonies from adult mice (~ 3 pNSC-derived colonies/brain). Hence, we isolated pNSCs from PND4-10 animals (Figure 6).
Figure 6. The Basic Embryoid Body Assay Experimental Paradigm. Single cell suspensions were generated from primary dissections of the germinal zone of the early postnatal brain or from the mESC R1 cell line. Thirty µl drops containing 30,000 cells were placed on inside of petri dish lids and the lids were then inverted. After 2 days gravity-induced aggregation of single cells results in generation of aggregates within each drops. The drops were transferred to ultra-low attachments plates and were cultured for 7 days. By day 9 the aggregates were transferred to gelatin-coated plates for 3 days. Aggregates were examined at days 2, 9, and 12.
Single cells from all 3 groups were plated in EB conditions (Figure 7A) and the numbers and size of the aggregates was examined at 2 (d2) and 9 days (d9) post-plating (Figure 7B-C). At d2, we observed the presence of aggregates in all three populations. We found that mESCs generated 19.58±1.97 aggregates (positive control, herein referred to as EBs) per drop, while pNSCs and dNSCs generated 4.96±0.99 and 33.33±3.43 aggregates, respectively. By d9, the numbers of EBs and pNSC-aggregates was not significantly different; however, the numbers of dNSC-derived aggregates was significantly decreased (Figure 7C). The size of the individual aggregates was significantly different between groups at d2 with EBs measuring 130.44±6.44 µm in diameter, pNSC-derived aggregates (210.90±25.64 µm diameter), and dNSC-derived aggregates (59.03±6.87 µm diameter). By d9, EBs were 2.9-fold larger (379.17±43.17 µm) compared to d2 EBs while d9 pNSC-derived aggregates and d9 dNSC-derived aggregates did not change in size (243.17±24.02 µm and 66.34±8.20 µm; pNSC and dNSC aggregates, respectively).

At d9, EBs and aggregates were transferred to adherent, gelatin-coated plates and were cultured for an additional 3 days. EBs and aggregates adhered to the gelatin-coated wells. Cells migrated away from the centre of the EB colony however this was not observed in pNSC-derived/dNSC-derived aggregates (Figure 7Aiv). Taken together, these findings reveal that the survival/proliferation and cell-cell adhesion are different depending on the starting population of cells.
Figure 7. pNSCs and dNSCs generate colonies in the EB assay  
A) Aggregates were generated from the three populations, mESCs, pNSCs, and dNSCs. Bright-field images
are shown for i) starting populations, ii) d2 iii) d9 and iv) d12 *in vitro*. B) dNSCs generate significantly more d2 aggregates (33.33±3.43) per drop than mESCs (19.58±1.97) and pNSCs (4.96±0.98). The number of generated aggregates significantly declines from d2 to d9 dNSC-derived aggregates while remaining consistent in EBs and pNSC-derived aggregates. C) The size of aggregates is significantly increased from d2 to d9 in EBs (mESC cohort). No significant difference in size was observed in the pNSC and dNSC aggregates. Scale bars in bright field images = 50µm. n=3. Two-tailed T-Test analysis was performed in aggregate number and aggregate size analysis. *p ≤ 0.05.

4.2 pNSC-derived aggregates express neural markers in the embryoid body assay

We predicted that if pNSCs are pluripotent, there would be expression of non-neural lineage markers within the generated aggregates. To test our prediction, we examined the expression profile of the EBs and pNSC-derived/dNSC-derived aggregates using RT-qPCR. As predicted, EBs (positive control) showed expression of the ectoderm markers *Nestin* and *Sox1* (Figure 8A). There were no significant changes in *Nestin* levels while *Sox1* levels significantly declined from d2 to d12 (p=0.036). EBs also expressed the mesodermal marker Brachyury (*T*) (Figure 8B) and the endoderm marker, alpha-feto protein –*Afp* (Figure 8C. The EB differentiation towards ectoderm, mesoderm, and endoderm was coincident with a significant loss in the expression of pluripotency markers *Nanog* and *Oct4* from d0-d12, indicating the differentiation is taking place at the cost of pluripotency (Figure 8D). In contrast to the EB expression, pNSC-and dNSC aggregates did not express the mesoderm and the endoderm markers and only expressed ectoderm markers *Nestin* and *Sox1*. *Nestin* and *Sox1* levels were significantly reduced in d12 pNSC-derived aggregates when compared with the starting population (d0 pNSCs) (p <0.0001), while they were significantly increased in d12 dNSC-derived aggregates when compared with the starting population (d0 dNSCs) (p=0.0273 for *Nestin* and p= 0.0006 for *Sox1*).

We further examined the differentiation profile of EBs using standard immunohistochemistry. We found expression of ectoderm (βIII tubulin, neuronal marker), mesoderm (heavy-chain cardiac myosin, HCCM), and endoderm (transcription factor HNF3β) in d12 EBs (Figure 8E –i-iii). d12 EBs possessed contractile units, indicative of cardiomyocyte differentiation (data not shown).
Figure 8. pNSCs and dNSCs demonstrate neural commitment in the EB assay. RT-qPCR analysis of EBs and pNSC-d/dNSC-d aggregates. Expression levels are relative to d0 time-points within each cohort normalized to the housekeeping gene Gapdh. mESCs can give rise to all germ layers as shown by their expression of neural genes Nestin and Sox1 (ectoderm), Brachyury (T) (mesoderm), Afp (endoderm). The pNSC and dNSC cohorts only express neural genes throughout the EB assay. E) IHC of d12 ESC-derived aggregates indicates the presence of germ layer specific markers. One-way ANOVA, n = 3 (30 mice per n for pNSC and dNSC cohorts; 3
different passages for the R1 mESC cohort). Statistical significances (#) and (*) shown compare d12 time-points to d0 and d2 time points within each cohort, respectively. Straight lines in figures B/C/D indicate no detection. *p ≤ 0.05. Scale bars =100µm. HCCM = Heavy Chain Cardiac Myosin.

4.3 pNSCs co-cultured with mESCs remain neurally committed

We next asked if we could reveal the pluripotency of pNSCs by placing cells in culture conditions that support the generation of all three germ layers. We considered that the co-culture would permit cell-cell contact mediated signaling as well as exposure to factors released by mESCs that may be important for revealing pNSC pluripotency. We used in vitro co-culture experiments, placing pNSCs and dNSCs in an mESCs environment which would permit cell-cell contact mediated signaling as well as exposure to factors released by mESCs that may be important for revealing pNSC pluripotency. pNSCs and dNSCs were derived from constitutive YFP mice (129-Tg(CAG-EYFP)7AC5Nagy/J) to distinguish between mESCs (negative for YFP signal). The in vitro mESC-pNSCs and mESC-dNSCs mixed populations were put through the EB assay and were collected for analysis at d9, a time-point when EBs express endoderm and mesoderm derivatives. We observed YFP+ cells in both mESC-pNSC and mESC-dNSC co-cultures (Figure 9). Observing the d2 hybrid aggregates using combined bright-field/fluorescence microscopy revealed a differential distribution of pNSCs and dNSCs with the mESCs in the co-cultures with pNSCs detected within the mass of the aggregates, surrounded by mESCs versus dNSCs which were predominantly found on the periphery of the aggregates.

To determine whether the YFP+ cells gave rise to non-neural progeny, the mixed aggregates were collected, fixed and sectioned on day 9. We performed immunohistochemistry looking for co-expression of YFP with neural and non-neural markers. YFP+ dNSCs and pNSC expressed the neural markers βIII tubulin (neuronal marker) and GFAP (astrocyte marker) within the mixed colonies (Figure 9Bi-ii). We also looked at the endoderm marker HNF3β (endoderm) as this was expressed in the d9 EBs. HNF3β was expressed in mESCs only (YFP-negative) (25.39±3.05%) but was never co-localized in YFP-positive cells (Figure 9Biii).
Figure 9. Co-cultures of pNSCs with mESCs is not sufficient to induce pluripotency. Co-culturing YFP+ NSCs and YFP- mESCs resulted in hybrid aggregates. A) *in vitro* mixing schematic and bright-field/fluorescence images of d2 co-culture derived aggregates. Bi-ii) pNSCs and dNSCs (YFP+) present in the hybrid aggregates express GFAP and βIII tubulin at d9. Biii) NSCs do not express the endodermal HNF3β. Scale bars = 50µm in low power and 10µm in higher magnification insets in B. Orange arrows indicate colocalization of YFP with lineage marker. Yellow arrowheads indicate YFP+ cells alone and orange arrows indicate colocalization of YFP+ cells with the marker of interest.
4.4 pNSC differentiation profile does not change in the presence of mESC-derived conditioned media in the embryoid body assay

One of the limitations to the co-culture assay was that we could not perform PCR on the YFP-positive pNSC and dNSC populations due to the limited numbers of cells that were found in the mixed aggregates (data not shown). Moreover, we were concerned that cells isolated may include mESCs which may give false positives. To avoid contamination and increase the numbers of pNSC-derived and dNSC-derived aggregates to enable RNA isolation and PCR analysis, we exposed pNSC and dNSCs to conditioned media (CM) from EBs. EB-CM was collected every 48 hours, up to 12 days and used to generate pNSC- and dNSC-derived aggregates in the EB assay (Figure 10).
Figure 10. Conditioned media experimental paradigm. EB-derived conditioned media (EB-CM) was collected every two days, filtered and supplemented with fresh medium and placed in pNSC/dNSC-EB cultures. Cultures were analyzed at d0, d2, d9, and d12. Control cultures consisted of pNSC and dNSCs in EB condition in the absence of EB-CM.
RT-qPCR analysis demonstrated the expression of the three germ-layer markers in EB cultures from which CM was generated. The level of expression is shown as ∆Cq. As predicted, Nestin and Sox1 was expressed through EB formation (Figure 11C). The neuronal marker βIII tubulin was expressed in d12 EBs however the glial markers Olig2 (Oligodendrocytes), and Gfap (astrocytes) were not expressed at any time examined. EBs also expressed the ectoderm/epithelial marker Keratin 18 (Krt18) and mesoderm markers Flk1 and Gata4 (Figure 11C,D). Endoderm markers Hnf4a, Sox17, Afp, and Hnf3β were detected in d12 EBs (Figure 11E). The differentiation of EBs towards the different germ layers was concurrent with the downregulation of pluripotency markers Oct4 and Nanog (Figure 11F).
Figure 11. EBs are pluripotent. EBs used to generate the CM upregulate the expression of germ layer markers and downregulate the expression of pluripotency markers. A) Summary chart
of gene expression. d12 EBs express markers of all three germ layers. B) Representative RT-qPCR amplification plots of d12 EBs. Housekeeping gene (Gapdh) (red), ectoderm (Krt18) (green), mesoderm (Flk1) (blue), and endoderm (Hnf3β) (orange). C-F) Germ layer/pluripotency-specific gene expression of EBs. Data shown as ΔCq with respect to housekeeping gene Gapdh. One-way ANOVA, n = 3 independent cultures of R1 mESCs. *p ≤ 0.05. UD = undetected.

Having demonstrated the ability of the EBs to give rise to all three germ layers, we inquired about the effects of EB-CM on proliferation/cell survival as well as the differentiation profile of pNSC-derived and dNSC-derived aggregates. We examined aggregate size in d9 pNSC-derived and dNSC-derived groups generated in the presence or absence of EB-CM. We found that EB-CM does not result in any significant aggregate size changes, indicating the EB-CM does not affect proliferation of the aggregates (Figure 12A-B). pNSCs expressed the ectodermal markers Krt18, Nestin, Sox1, βIII tubulin, Gfap, and Olig2 at d0. The presence of EB-CM did not change the expression of any of the neural markers at any time point examined (Figure 12 C, D). We detected expression of Nanog in pNSCs (d0), consistent with previous findings (Sachewsky et al., 2014; Reeve et al., 2015) and the expression of Nanog was not detectable at later time-points suggesting that pluripotency was not maintained with continued time in culture.
Figure 12. Conditioned Media derived from differentiating EBs does not alter pNSC survival/proliferation or differentiation profile. A-B) The presence or the absence of CM has no significant effect on the size of pNSC or dNSC d9 aggregates. C, D) RT-qPCR summary
indicates pNSCs express ectoderm, but no mesoderm or endoderm-specific markers, in the presence or absence of CM. n = 3 (30 animals per n). Two-tailed t-test analysis was performed in aggregate size; one-way ANOVA for RT-qPCR ∆Cq comparison. *p ≤ 0.05. Scale bar = 100µm. n.s.=not significant. UD=undetected.

Control dNSC-derived aggregates and EB-CM exposed dNSC-derived aggregates expressed the markers Nestin, Sox1 and the more differentiated neural cell markers, βIII tubulin, Gfap, and Olig2. They did not express Krt18, mesoderm (Flk1, Gata4), endoderm (Hnf4a, Sox17, Afp, Hnf3β), or pluripotency markers (Oct4, Nanog). EB-CM did not affect the differentiation profile of dNSC-derived aggregates with the exception of Olig2 expression in d12 dNSC-derived aggregates in which EB-CM exposed d12 dNSC-derived aggregates expressed higher levels of the oligodendroglial differentiation. These findings reveal that pNSCs and dNSCs behave more similarly to one another than to mESCs in the EB assay (Figure 13).
Figure 13. EB-CM does not change the differentiation profile of dNSCs. (A-B) dNSCs express ectodermal genes *Nestin*, *Sox1*, βIII tubulin, *Gfap*, and *Olig2* but do not express ectoderm marker *Krt18*, mesoderm, endoderm, and pluripotency genes. *Olig2* expression was the only differentially regulated expression in d12 dNSC-derived aggregates. d12 dNSC-derived aggregates exposed to EB-CM had a significantly higher expression of *Olig2* than d12 dNSC-derived aggregates that were not exposed to the EB-CM. *Nestin* expression significantly declines
over time in the dNSC cohort (as shown by higher ΔCq value). βIII tubulin and Gfap were significantly upregulated in the dNSC-derived aggregates over time (as shown by smaller ΔCq values). $n = 3$ (30 animals per n for pNSC and dNSC cohorts; 3 different passages for the R1 mESC cohort). One-way ANOVA for RT-qPCR ΔCq comparison. *$p \leq 0.05$.

4.5 pNSCs do not form teratomas in vivo in the absence of mESCs

We next assessed the pluripotentiality of pNSCs using the well-established in vivo teratoma formation assay (Prokhorova et al., 2009; Dressel et al., 2010; Nelakanti et al., 2016). We performed subcutaneous injections of mESCs, YFP expressing pNSCs or dNSCs ($1 \times 10^6$ cells/injection) bilaterally into immunocompromised SCID/Beige adult mice. By 3 weeks we observed palpable teratomas in 100% of mESC inoculated mice (4 teratomas formed from 4 inoculations performed). Mice that received YFP-positive pNSC ($n=5$) or dNSC injections ($n=2$), did not result in palpable teratoma formation due to the lower proliferative potential of NSCs, even at 7 weeks post-inoculations. The area of the transplant was removed from the inoculated mice was sectioned and no YFP-positive cells were observed suggesting that pNSCs and dNSCs did not survive in the host-tissue (Figure 14A).

In the next series of experiments, we performed a mixing experiment, similar to what was performed in vitro, by mixing pNSCs or dNSCs with mESCs prior to transplantation in vivo, thereby permitting the cell-cell contact and exposure to factors that may reveal the pluripotency of NSCs. YFP-positive pNSCs or dNSCs were mixed 1:1 with YFP-negative mESCs. Mice were sacrificed at 3 and 4 weeks post-injection. Similar to what was observed with mESC transplants only, 100% of the inoculated mice had palpable tumours (Figure 14B-C). There was no significant difference between the mass and size of teratomas with 4-week mESC-pNSC teratomas weighing $2.24 \pm 0.27g$ and being $1.61 \pm 0.15cm$ along the longest axis, and 4-week mESC-dNSC teratomas weighing $1.54 \pm 0.32g$ and being $1.24 \pm 0.22cm$. 
Figure 14. pNSCs are present in tumour when combined with mESCs in vivo. A) no teratomas were generated when pNSCs and dNSCs were solely introduced into the host animal. B) in vivo mixing schematic. pNSCs and dNSCs were combined with mESCs prior to subcutaneous injection resulted in teratoma formation at 100% frequency. Images of excised mESC-pNSC and mESC-dNSC teratomas at 4 weeks post injection. C) summary of groups and the sample size in the teratoma formation assay D) summary of immunohistochemistry analysis performed on ESC-pNSC and ESC-dNSC samples.
We observed the presence of YFP+ cells in both mESC-pNSC and mESC-dNSC teratomas as seen in Figure 15. Notably, the pNSC-ESC teratomas contained significantly more YFP+ cells than dNSC-mESC teratomas. Often YFP-positive dNSCs were restricted in a specific part of the teratomas while pNSCs were more evenly distributed throughout the mass.

Immunohistochemistry data revealed co-labelling of YFP-positive pNSCs and dNSCs with neural markers only. YFP-positive pNSCs and dNSCs are positive for GFAP (astrocytes) (Figure 15Ai-ii), OLIG2 (oligodendroglial lineage) (Figure 15Aiii-iv), and NeuN (neurons) (Figure 15v-vi). pNSCs and dNSCs within teratomas were significantly different in terms of their neural differentiation with 3.23±0.76% (n=3) of YFP+ pNSCs versus 38.46% (n=2) of YFP-positive dNSCs expressing GFAP+. There was a two-fold increase in the percent of OLIG2+ cells derived from pNSCs compared to dNSCs in the mixed teratomas (42.92±3.17% versus 20.71±7.71%; pNSCs and dNSCs, respectively). Very few NeuN+/YFP+ cells were observed in the teratomas, regardless of the transplanted cell type. No YFP-positive cells co-labelled with the endoderm marker, smooth muscle actin (alphaSMA) or mesoderm marker CD31 (endothelial cell marker) (Figure 15vii-x). YFP-negative mESCs present in the teratomas expressed neural markers GFAP, OLIG2, NeuN, as well as non-neural markers AlphaSMA, and CD31. Hence, taken together, the data support the conclusion that pNSCs are more similar to neurally committed dNSCs than pluripotent mESCs.
Figure 15. pNSCs and dNSCs within teratomas are neurally committed. YFP+ pNSCs and dNSCs present within the teratomas are positive for neural markers GFAP, OLIG2, and NeuN. No colocalization of the YFP signal with mesoderm marker CD31 and endoderm marker Alpha-SMA.
SMA was observed. Red arrows indicate the marker of interest colocalizing with YFP-negative mESCs, orange arrows indicate the expression of the marker of interest by the YFP-positive NSCs, yellow arrowheads indicate the YFP-positive NSCs that are not positive for the particular marker. Scale bar =50µm.
Chapter 5

5 Discussion

In this study, we further explored the potential of early postnatal primitive neural stem cells in different variations of: (1) the \textit{in vitro} embryoid body assay, (2) the \textit{in vivo} teratoma formation assay. We have shown that pNSCs have a high capacity of neural and ectodermal commitment and despite the expression of pluripotency markers Oct4 and Nanog do not to give rise to progenies in other germ layers (mesoderm and endoderm). We have arguably given pNSCs some of the best conditions to reveal any pluripotential capacity they may have. Our results indicate that the differentiation capacity of pNSCs more closely mirrors that of dNSCs as opposed to mESCs. In addition, we have shown the expression of cytoskeletal keratin, \textit{Krt18}, in pNSCs. This is the first study to assess NSC potential using well known, robust pluripotency assays to reveal that not only can multipotent NSCs survive and persist in these assay, they maintain their neural commitment throughout the experiments.

5.1 Characterization of pNSC behaviour \textit{in vitro}

5.1.1 Embryoid Body Assay – Basic

Our first objective in this study was to examine how pNSCs respond to the \textit{in vitro} pluripotency assay. Our first analysis involving the embryoid body was a holistic analysis of the differentiation profile of pNSCs. The aim behind this experiment was to see whether pNSCs had the ability to endogenously differentiate into/give rise to non-neural progeny given the basic embryoid body culturing conditions, without the assistance of exogenous factors. mESC behaviour in the EB assay was our gauge for how a pluripotent cell source should behave in such assay. In the mESC cohort, at d2 we observed the formation of mESC-derived aggregates, herein referred to as EBs. As shown by the significant difference in the diameter of d2 and d9 EBs, the d2 EBs were able to utilize the next 7 days in the assay to proliferate in culture. Lastly, when introduced to an adhesive substrate to further promote differentiation, d9 EBs were able to adhere to the gelatin coating and migrate out from the centre of the EB’s dense core to the peripheries. When we looked at the expression profile of EBs, we observed the expression of markers from all three embryonic germ layers.
pNSC and dNSC behaviour in the EB assay more closely mirrored one another. In the both NSC cohorts, at d2 we observed the formation of aggregates from single cells. Quantifying the number of generated aggregates per drop shows that dNSCs form the highest number of aggregates, followed by ESCs and pNSCs when put through the embryoid body assay. This may be surprising at first; however, when we look at d2 aggregate size, the significantly smaller size of d2 dNSC-derived aggregates in comparison to d2 EBs and pNSC-derived aggregates. In addition, looking at the generated number of aggregates per drop, there was a significant decrease in the number of dNSC-derived aggregates from d2 to d9, indicating cell death of dNSCs within the aggregates in response to EB culturing conditions; this decline was not observed in EBs or pNSC-derived aggregates. The smaller d2 dNSC-derived aggregates as well as fewer d9 dNSC-derived aggregates could be due to lack/downregulation of cell surface adhesion requirements (such as cadherins, catenins, and integrins) which results in the mechanical instability of a larger aggregate. In fact, Karpowicz and colleagues have demonstrated the differential expression of cadherins in mESCs, pNSCs, and dNSCs, demonstrating the lack of expression of E-Cadherin in dNSCs (Karpowicz et al., 2007). As mentioned earlier, E-Cadherin has been shown to play a crucial role in EB formation and differentiation; thus, it is not surprising that this population is not thriving in EB conditions.

Another observation that we made was that in contrast to d2 EBs, d2 dNSC-derived and pNSC-derived aggregates did not change in size after 7 days in culture. It is apparent that NSCs are not responding to the EB assay culturing conditions and there are two possible explanations for this phenomenon:

a) *The EB culture medium is causing NSCs to cease proliferating.*

   Neural stem cell culturing protocols calls for dNSCs to be cultured in serum free media in the presence of EGF, FGF, and heparin prior to the EB assay. It has been shown that dNSCs proliferate in response to EGF and FGF (Reynold & Weiss, 1996; Gritti et al., 1996; and Tropepe et al., 1999). Hence, it is plausible that the absence of these mitogens in the EB medium has resulted in the cessation of dNSC proliferation. As for pNSCs, since they were cultured in serum-containing media in the presence of LIF, and given the role of LIF in maintaining self-renewal and proliferation, it is also possible that the lack of LIF in the EB resulted in the cessation of pNSC proliferation. In addition, due to the presence of serum in the EB medium, it is possible that both pNSCs and dNSCs are differentiating as a result, at the expense of proliferation.
b) The EB culture medium is causing NSC cell death which is offsetting NSC proliferation

The increase in the size of EBs over time could be interpreted as the balance in cell proliferation and cell death is tipped more towards proliferation. The EB size is increasing due to higher cell number in d9 versus d2 which can logically only be caused when the rate of cell proliferation is greater than the rate of cell death. However, the lack of change in size of d2 and d9 pNSC-derived and dNSC-derived aggregates indicates that NSC survival is being compromised. The difference between this explanation and the one presented in a is that the absence of the proper growth factors in the EB medium not only impedes proliferation, but is resulting in cell death. Another piece of evidence that would support this explanation is the behaviour of d12 pNSC-derived and dNSC-derived aggregates. Although these d12 NSC-derived aggregates are able to stick down to the gelatin coating, they maintain their spherical, globular shape and we don’t see the spreading out of cells that we see in d12 EBs. The fact that a few cells in the d12 aggregates migrate out to the periphery could be because 1) both pNSCs and dNSCs find the microenvironment within the aggregate a more supportive environment, conducive to cell survival, via the secretion of autocrine and paracrine factors, or 2) a substantial portion of cells within the aggregate are either dead or dying.

Furthermore, our first characterization of the differentiation profile of pNSC-derived and dNSC-derived aggregates revealed the expression of Nestin and Sox1 in these cohorts, indicating a neural lineage commitment. This indicated that pNSCs may require exogenous signaling to reveal their pluripotent potential.

5.1.2 Embryoid Body Assay – in vitro Mixing

In the next step, we looked at the effects of mESC presence on the pNSCs and dNSCs in the EB assay. The aggregate formation was accomplished by combining 15,000 mESCs with 15,000 YFP+ pNSCs or dNSCs prior to drop formation. Having detected the expression of YFP signal at d2 combined aggregates, we allowed the EB assay to proceed until d9. The reasoning behind this was to give pNSCs some time to be exposed to the now overwhelming mESC environment; the environment being both the factors released by mESCs as they are undergoing differentiation as well as any cell-cell contact-mediated effects that may be at play. The constitutive YFP tag allowed us to analyze pNSC differentiation profile via the use of IHC. The caveat in this process is however the limited number of samples and the non-exhaustive nature of
immunofluorescence; we were able to stain for a limited number of markers using this method and it is possible our findings are not reflecting the full differentiation breadth. Here, we observed the neural commitment of pNSCs and dNSCs by the expression of βIII tubulin and GFAP in YFP+ cells. Wanting to have a more comprehensive idea of what the pNSC differentiation profile in these co-cultures are, the next step in our experiments were to utilize florescence-assisted cell sorting (FACS) to separate out the YFP+ populations from the YFP- population and perform RT-qPCR. However, this proved challenging in that we were not able to isolate RNA with good integrity from pNSCs or dNSCs as the isolated YFP+ populations were both very few in number, and very easily killed by the process of FACS. This prompted us to seek other means of experimentation where cell survival and cell quantity were not the rate-limiting steps.

5.1.3 Embryoid Body Assay – Conditioned Media
The premise behind the conditioned media was to expose the differentiating pNSC-derived and dNSC-derived aggregates to the exogenous, yet naturally occurring factors involved in EB differentiation. We first noticed that the presence of EB-CM had no effect on neither d9 pNSC-derived nor d9 dNSC-derived aggregate size. This may suggest that the factors present in the EB-CM do not affect proliferation or cell survival within the aggregates. The CM experiments allowed us to do a more comprehensive screening of the expression profile using RT-qPCR on the aggregates. Before looking at the effects of EB-CM on pNSC-derived and dNSC-derived aggregates expression profile, we looked at the expression profile of EBs from which the CM was generated. Seeing the ability of mESCs to differentiate to a variety of markers from each germ layer made us certain about the presence of signalling molecules in the CM we had used on NSC-derived aggregates. pNSCs demonstrated neural commitment and the presence of CM did not have any detectable effects on their differentiation profile. The same was observed for dNSCs. We have attributed the above phenomenon to the following potential explanations:

a) Factors present in the CM are not in sufficient concentration to affect change

Like many biological cell signaling cascades, insufficient extracellular environmental cues will result in the lack of intracellular response. In order to ensure the EB-CM was not lacking in media components important for cell survival (namely glucose and L-glutamate), we combined the EB-CM with fresh medium which could have resulted in the dilution of the factors below sufficient concentrations. A potential future step would be to keep track of the
level of cytokines present in the EB-CM via western blots or ELISAs which would allow for more consistency of the assay.

b) Factors present in the EB-CM are present in sufficient concentrations; however, the 12-day nature of the embryoid body assay is neither sufficient, nor representative of an in vivo niche setting

Although 12 DIV may be enough for R1 mESCs to give rise to a variety of cell types from all three different germ layers, the same conditions may not be sufficient for pNSCs to reveal such potential if they do in fact possess it. Another piece of evidence supporting this claim is the trends noticed in pNSC-derived aggregates expression. Although pNSC-derived aggregates express neural genes in all time-points, the relative expression of the neural gene declines over time in the assay. One explanation for this would be the downregulation of neural genes at the expense of upregulation other potentially non-neural genes. However, 12 DIV may not be enough to detect the upregulation of the other genes. In order to tackle this caveat in our in vitro experiments, we continued our studies in an in vivo test of pluripotency, the teratoma formation assay.

5.2 Characterization of pNSC behaviour in vivo

Our final step in the characterization of pNSCs was the use of teratoma formation assay. If pNSCs have pluripotential capacity they would be able to form teratomas containing cells from all three germ layers. Our first set of experiments involved the injection of each population (mESCs, pNSCs, and dNSCs) on their own and assess whether a teratoma was formed or not. mESCs formed teratomas at 100% frequency, while no teratomas were generated from NSCs (n=0/5 for pNSCs and n=0/2 for dNSCs). In the absence of a teratoma, and ensuring there are no YFP-positive cells present at the site of the injection, we hypothesized that although pNSCs are unable to form teratomas, given the right environment they may have non-neural lineage contributions. They are not able to be the cells inducing a teratoma; however, they may give rise to non-neural progeny. Hence, we combined 500,000 YFP+ pNSCs or dNSCs with 500,000 unlabelled mESCs. The premise behind these sets of experiments was to provide a supportive in vivo niche that would give pNSCs more time to respond to environmental cues present, thus allowing them to reveal their true potential. The in vivo mixing experiments yielded, with 100% frequency, teratomas within which both pNSCs and dNSCs were present. Our first observation was the apparent difference in the number of YFP+ pNSCs when compared to YFP+ dNSCs.
pNSCs were much more abundant than dNSCs in these teratomas. This difference can be attributed to higher cell survival of pNSCs due to the expression of cadherin that are more closely related to those expressed by mESCs. Both mESCs and pNSCs express high levels of E-Cadherin and P-Cadherin while dNSCs exclusively express high levels of N-Cadherin (Karpowicz et al., 2007). Hence, with respect to NSC survival in teratomas, the difference in Cadherin expression can lead to the differential survival we observed in pNSCs and dNSCs. Last but not least, IHC data demonstrates the expression of neural cell markers GFAP, NeuN, and OLIG2 in the YFP+ populations, once again indicating the propensity of pNSCs to give rise to neural progeny. YFP+ pNSCs/dNSCs did not give rise to any mesodermal (CD31+) or endodermal (alpha-SMA+) progeny in the teratomas.

5.3 Exclusive expression of Krt18 in pNSCs

One interesting finding was the expression of epithelial marker Keratin 18 (Krt18) in the pNSCs and not in dNSCs. Our reasoning behind choosing Krt18 as one of the markers we looked was due to its position with respect to the embryonic germ layers. Krt18 is a marker of epithelium which belongs to the ectoderm and not neuroectoderm. If pNSCs do have a wider differentiation potential than dNSCs, and yet not fully pluripotent, choosing a surface ectoderm marker would have allowed us to pick out this distinction. However, surprisingly we have detected the expression of Keratin18 in the starting population of pNSCs, prior to the EB assay. The detection of an epithelial marker coexisting with the demonstrated neural commitment of pNSCs indicates that pNSCs can give rise to subpopulation of progenies from neural and epithelial fates. Given that the embryonic patterning required for epithelial fate is distinct from that required for neural fate, these findings point to pNSCs being from an earlier, more primitive stage than dNSCs.

5.4 Proposing a new neural lineage

pNSCs can first be isolated from the mouse embryo at E5.5, a time in the embryonic development prior to both gastrulation (E6.5) and neurulation (E8) while dNSCs can be isolated at E8.5. The culmination of previous and current pNSCs has enticed us to propose a new neural lineage in which pNSCs are more closely related to the embryonic radial glial cells (RGCs) and perhaps neuroepithelial cells (NECs). Although expression of pluripotency genes Oct4 and Nanog has never been addressed in neither RGCs nor NECs, what we know about these
populations does not directly negate the possibility of their close associations with pNSCs. In rodents, NECs and RGCs do not express \textit{Gfap}, similar to what has been reported for pNSCs. Are pNSCs a subtype of NECs/RGCs? or do pNSCs lie upstream of NECs/RGCs?

5.5 Future Directions

Thus far our studies indicate that pNSC potential is predominantly restricted to the neural lineage and that these cells display characteristics more consistent with multipotency rather than pluripotency.

One aspect of our studies that is the logical progression of our experiments is to perform the teratoma formation assays, followed by enzymatic/mechanical dissociation of tissues to be FAC sorted. The YFP+ sorted cells will then be analyzed using RT-qPCR to give us a much wider and better understanding of pNSC differentiation potential. The caveat of IHC studies done in the teratomas remains the same in that IHC studies can rarely provide a comprehensive and exhaustive characterization.

One aspect of our studies that we believe could be enhanced is the culturing conditions in which pNSCs are cultured. One explanation that can be used to explain why pNSCs do not portray pluripotent characteristics is the very low expression of pluripotency genes \textit{Oct4} and \textit{Nanog}.

Sachewsky and colleagues showed \textit{Oct4} expression (mRNA levels) in cultured adult pNSCs to be approximately 5% of mESCs (Sachewsky et al., 2014). However, two questions remain:

1. does each individual cell within a pNSC colony express \textit{Oct4} at levels that are 5% of mESCs, or, are 5 cells within a 100-cell pNSC colony express \textit{Oct4} at level that are similar to mESCs? This question has never been addressed.

2. Another point that remains is \textit{in vitro} conditions used to culture pNSCs. Currently there are 3 culture conditions in which our labs have reported culturing pNSCs. The first method is very similar to culturing of dNSCs, via the neurosphere assay. pNSCs are cultured in serum-free media (SFM) in the presence of LIF. The second method is culturing of pNSCs in serum-containing medium, mESC medium, in the presence of LIF and on support mouse embryonic fibroblast cells. The third method (and the method used in our studies) is the culturing of pNSCs in serum-containing medium on gelatin-coated plates. Although all three methods utilize the use of LIF, it is not scientifically sound to make the assumption that pNSC colonies generated from these three methods are essentially the same population. The presence/absence of serum in the media and the
presence/absence of support cells may play important roles in pNSC differentiation. For instance, in a hypothetical scenario, if we find that pNSCs cultured on support cells have higher Oct4 expression than pNSCs cultured on gelatin-coated plates that could imply that pNSCs cultured on gelatin-coated plates downregulate Nanog and Oct4 expression and as a result may differentiate more quickly to neural cell types.

It is worthwhile to better characterize the specific culturing conditions required to for pNSC maintenance.

A potential caveat of our studies done was that we used all the cells within a pNSC colony for both the in vitro and in vivo studies presented. Going back to the earlier argument presented about pNSC culturing, having an overwhelming differentiated population may hinder the capability of Oct4+/Nanog+ cells within the pNSC colony. The idea that the population of a pNSC colony is not fully homogenous and that there are differentiated cell types within the colony could partially explain why Sachewsky and colleagues observed low frequency of incorporation of pNSCs into the growing blastocyst (2.5%) in their morula aggregate studies (Sachewsky et al., 2014) and also why we did not see any indication that pNSC have any non-neural lineage differentiation capacity, aside from Krt18 expression. To improve our chances to observe the unveiling of pNSC pluripotent potential, we recommend the use Oct4-GFP transgenic mice where GFP is expressed under the activity of Oct4 promoter. Culturing pNSCs from these mice, followed by FAC sorting will leave us with a population of pNSCs, dissected and cultured from postnatal animals that homogenously express Oct4. This method will circumvent the presence of any ‘contaminating’ differentiated cell types that would affect the differentiation of Oct4+ cells towards a neural lineage. This purer population of pNSCs can then be used to perform the in vitro and in vivo studies done in our studies to see whether there is a difference in the results. If there are no differences in our results after ‘purifying’ our pNSC cultures, this could suggest that the expression level of Oct4 and Nanog (whether at the transcript or the protein level) are below the threshold needed to reach a pluripotent state. As noted, pluripotency requires the tight regulation of the core transcriptional regulators including Oct4 and Nanog. To study whether the endogenous expression levels of Oct4 and Nanog are too low to effect change, a proposed study would be to overexpress Oct4, Nanog, or both in pNSCs. Looking at the behaviour of induced pNSCs in different pluripotency assays (embryoid body,
teratoma formation, and chimera formation) would allow us to study whether pNSC pluripotency is even possible and if so, determine what minimal required expression is required.

Herein we have assumed that the expression of Oct4 and Nanog in pNSCs were serving as pluripotency inducers and regulators. However, we must also consider the possibility that the presence of these genes in pNSCs can serve an alternative role in pNSCs. Oct4 and Nanog can also be serving as pioneer factors allowing other factors (such as transcription factors) to access chromatin for efficiently. Accordingly, it is possible that that Oct4 and Nanog serve to assist in the upregulation of neural genes in pNSCs. A way to test this hypothesis would be to compare the binding sites of OCT4 and NANOG in pNSCs and a pluripotent source (ESCs) using chromatin immunoprecipitation (Chip assay). If NANOG and OCT4 co-immunoprecipitated with complexes that are important for neural differentiation as opposed to pluripotency, this would indicate a different role of Oct4 and Nanog in pNSCs than ESCs.

Last but not least, to answer to the question regarding pNSCs in the neural lineage we propose looking at RGC-specific marker RC2 (Misson, 1998) in our pNSC cultures. One seemingly counterintuitive piece of information is the ability to culture pNSCs at E5.5 before neurulation or even gastrulation has taken place. NECs emerge at the onset of neurulation, and later give rise to RGCs. To better understand and reconcile these co-existing facts, we propose looking at whole embryos, aging from E5 – E10 and performing immunohistochemistry to understand whether there is the emergence of an OCT4+/NANOG+/RC2+/GFAP- population prior to the unset of neurulation in vivo.

**Conclusion**

The in vitro embryoid body assay reveals that although pNSCs are able to generate aggregates that physically resemble that of mESC-derived EBs, they are nevertheless neurally committed. In addition, by exposing pNSCs to mESC influence via both the in vitro mixing and conditioned media (CM), pNSCs maintain their commitment to the neural lineage. Interestingly, we show that early postnatal pNSCs express Keratin18 (Krt18), an epithelial marker. Finally, the in vivo teratoma formation assay reveals that although pNSCs are unable to generate teratomas when introduced subcutaneously, they are present within a tumour when combined with mESCs where
they give rise to markers of the neural lineage. Here we show that pNSCs demonstrate neural commitments and portray multipotent characteristics.
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