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Special Focus: Stem Cells

Transcription factors and neural stem cell self-renewal, growth and differentiation

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Abbreviations: CBF-1, notch effector C-promoter binding factor-1; CNS, central nervous system; EGFP, enhanced green fluorescent protein; ESC, embryonic stem cell; HSC, hematopoietic stem cell; ID, inhibitor of DNA binding; KO, knock-out; NICD, notch intracellular domain; NSC, neural stem cell; NP, neural progenitor; OPC, oligodendrocyte precursor cell; Sh, short hairpin; Shh, sonic hedgehog; SVZ, subventricular zone; TFs, transcription factors; TCF/LEF, T-cell factor/lymphoid enhancer factor; TLX, human homologue of the drosophilia tailess gene

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The central nervous system (CNS) is a large network of interconnecting and intercommunicating cells that form functional circuits. Disease and injury of the CNS are prominent features of the healthcare landscape. There is an urgent unmet need to generate therapeutic solutions for CNS disease/injury. To increase our understanding of the CNS we need to generate cellular models that are experimentally tractable. Neural stem cells (NSCs), cells that generate the CNS during embryonic development, have been identified and propagated in vitro. To develop NSCs as a cellular model for the CNS we need to understand more about their genetics and cell biology. In particular, we need to define the mechanisms of self-renewal, proliferation and differentiation—i.e. NSC behavior. The analysis of pluripotency of embryonic stem cells through mapping regulatory networks of transcription factors has proven to be a powerful approach to understanding embryonic development. Here, we discuss the role of transcription factors in NSC behavior.

Introduction

The most complex organ of the body, the central nervous system (CNS), comprising the brain and the spinal cord, is composed of three distinct cell types; astrocytes, oligodendrocytes and neurons. These cells help form a complex network of connections that facilitate electro-chemical signaling with the neuron taking center stage. There are approximately 100 billion neurons in the brain and a typical neuron has about 1,000 to 10,000 synapses (that is, it communicates with 1,000–10,000 other neurons, muscle cells, glands, etc). Thus the brain is made-up of trillions of connections. This complexity is further enhanced by the fact that there are many different neuronal cell-types specialized to have particular morphology, connectivity and work with distinct neuromodulators and neurotransmitters.

Diseases of the brain affect millions of people worldwide and are becoming increasingly prominent as the population ages. CNS diseases where neural stem cells (NSCs) could be useful as cellular models or to provide therapeutic solutions include; Alzheimer, Parkinson, stroke, Huntington, Lou Gehrig (ALS) and the devastating disease of childhood—Batten disease. In fact, NSCs are already in clinical trials for the treatment of stroke and of Batten's disease.[1] [Neurospheres contain both NSCs and Neural Progenitors (NPs). Since there are no definitive markers for NSCs or NPs the two populations cannot be separated. The difference between NSCs and NPs is that the latter has limited replication abilities will not passage and is likely to be uni or bipotent].

If we are to tackle the complexity of the CNS and generate solutions for CNS disease states it is essential that we generate therapeutic cellular models. The discovery of NSCs of embryonic and adult CNS[2,3] has opened up the possibility to develop cellular models of the CNS. NSCs are multipotent cells that can be defined simply as cells that have the ability to self-renew and generate the major cell types of the CNS. The NSC characteristic of self-renewal has been linked to cancer and it is important to investigate this link further. The role of NSC in cancer is supported by the work of Singh et al.[4] where they isolated CD133 positive cells from human tumors. The CD133 positive cells gave rise to tumors in vivo in NOD-SCID mice, were serially transplantable and phenocopied the patients' original tumor.[4] Thus, the NSC property of self-renewal is important to understand from a brain

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cancer point of view and may provide targets for anti-cancer drugs. NSCs can be cultured for extended periods of times and this allows the generation of large numbers of specific cell-types such as dopaminergic neurons. Transcription factors (TFs) play prominent roles in developmental processes and have provided excellent tools to understand stem cell-lineage specification. The generation of specific cell-types from NSCs will be important to model neural development and disease states.

TFs and Pluripotency

Several independent studies have sought to define 'stemness' by attempting to identify a set of conserved genes that govern key regulatory pathways of stem cells.[5,6] In an effort to elucidate a common transcriptional profile attributable to 'stemness', two independent studies used hematopoietic stem cells (HSCs), embryonic stem cells (ESCs) and NSCs to perform a genome wide gene expression microarray analysis. Consistent in both reports is that there is a subset of six genes that are common to ESC, HSC and NSC. However, when a third dataset was analyzed only a single gene remained in the common pool.[7] Thus it seems that the concept of 'stemness' via gene profiling is rather vague. A more successful approach to understanding 'stemness' has been the identification of proteins, and in particular TFs, that play crucial roles in pluripotency. Work over the last ten years has revealed that the TFs, OCT4, SOX2 and NANOG, play dominant roles in the maintenance of pluripotency. In mouse embryos lacking Oct4, the pluripotent inner cell mass fail to develop and thus cannot survive past the blastocyst stage.[8] Sox2-null embryos show defective epiblasts and die immediately after implantation.[9] Similarly, the lack of Nanog resulted in embryos failing to develop an epiblast.[10] Thus, these three factors appear to be critical for embryonic development. Indeed, genome-wide studies in both mouse[11,12] and human ESCs[13] revealed that these factors co-occupy and share a substantial portion of target genes that form a characteristic network that maintains cellular pluripotency. Furthermore, the ability of these three TFs to reprogram somatic cells[14,15] is compelling evidence of their role as key regulators of pluripotency.

Central to the maintenance of the pluripotent network is a tight balance of the levels of the TFs. While self-renewal and an ESC state are preserved by overexpressing NANOG, ESCs that are depleted of NANOG are driven towards endodermal lineages.[10,12] Similarly the role of OCT4 as a gatekeeper in the decision between pluripotency and lineage specification[8,16] can be predicted by its concentration. Its expression is high in undifferentiated ESCs, and decreases during differentiation.[7] Precise levels of OCT4 are required for the maintenance of pluripotent ESCs as reduction of OCT4 expression to 50% or less induces trophectodermal differentiation, while overexpression causes differentiation to primitive endoderm and mesoderm.[18-20] As OCT4 is able to cooperate with SOX2,[9] and is involved in the reciprocal regulation of each other's expression[21] to mediate for instance NANOG activity,[22] tweaking the levels of SOX2 also skews the transcriptional network inadvertently. Essentially, elevating the levels of SOX2 decreases expression of its own gene and inhibits SOX2:OCT4 targets like Oct4 and Nanog.[23] In addition, eliciting small increases in SOX2 protein via an inducible system triggers the differentiation of ESCs that gives rise to cell types that exhibits neuroectoderm, mesoderm and trophectoderm markers.[24] Reducing the level of SOX2 in contrast, promote the differentiation of ESCs into trophectoderm-like cells.[21] Thus, these data suggest that a precise level of SOX2 and OCT4 is important to maintain the pluripotent state.

The success of the regulatory network-TFs-pluripotency approach to understanding ESCs suggests that a similar approach applied to NSCs may give insight to the biology of these cells. Important questions that could be addressed include; (1) which TFs are essential for NSC self-renewal, proliferation and differentiation? (2) can this information be used to define markers for NSCs? and (3) are TFs that control the NSC self-renewal potential anti-cancer targets? Although TFs clearly play important roles in stem cell behavior it is important to realize that TFs are part of an intricate network of cell signaling pathways that respond to cell-cell contact, growth factors and cytokines released in autocrine and paracrine fashion. Before discussing specific TFs and their role in NSC behavior we start the review by highlighting three important cell signaling pathways of NSCs; Wnt, Notch and Sonic hedgehog (Shh).

Three Major Cell Signaling Pathways of NSCs

The presence of Wnt/β-catenin pathway within the subventricular zone (SVZ) suggests a role for β-catenin in neural development.[25] β-Catenin is a central and essential component of the canonical Wnt signaling pathway that functions by activating TCF/LEF TFs.[26-28] Conditional mutation of β-catenin results in elimination of the cells at the midhindbrain boundary,[29] decreases in the overall size of the nervous system and the neuronal precursor population.[30] On the other hand, continuous expression of β-catenin resulted in marked generalized hypercellularity of the brain.[31] In NSCs cultures, the addition of Wnt protein caused an increase in survival of NSCs and more efficient colony initiation.[25] However, depending on the stage of development Wnt/β-catenin pathway switches its role into triggering neuronal differentiation.[32] β-catenin and its downstream partners (TCF/LEF) control the balance between progenitor expansion and differentiation.[27,30] It has been proposed that β-catenin alone stimulates neuronal differentiation, whereas β-catenin along with Fgf2 inhibits neuronal differentiation.[33]

Notch signaling has also been implicated in regulating the balance between neuronal differentiation and progenitor expansion.[34,35] The bHLH genes Hes1 and Hes5 which are essential effectors of Notch signaling encode transcriptional repressors and regulate the maintenance of cells in the undifferentiated state and repress neuronal differentiation.[36,37] Embryonic NSCs change their characters over time from Hes-independent neuroepithelial cells, transitory Hes-dependent neuroepithelial cells to Hes-dependent radial glial cells.[36] Hes-related bHLH genes, Hesr1 and Hesr2 are also expressed by NSCs and NPs in the embryonic brains and act as Notch signaling effectors. Hesr1/2 regulates NSC maintenance, possibly in conjunction with HES proteins.[38] Notch signaling seems to be an important signaling pathway in distinguishing stem cells from more limited

Table 1 **TFs and NSC self-renewal**

Transcription factors	Comments
HES1 and **HES5** (bHLH)	Decreased self-renewal by secondary sphere formation.[37,147] *Hes1*$^{-/-}$ and *Hes5*$^{-/-}$ mice exhibit premature exit for differentiation.[36,48,147]
CBF-1 (CSL)	CBFE-EGFPhi cells generate large primary and secondary neurospheres in comparison to CBFRE-EGFPlo/neg cells.[39]
SOX2 (HMG)	Sox2 positive cells contain self renewing properties in vitro.[54,148] Enlarged ventricles in the deficiency and conditional inactivation of Sox2 in mice.[52,54]
HMGA2 (HMGA)	Hmga2 promotes the self renewal of fetal and young-adult stem cells.[64] *Hmga2*$^{-/-}$ mice shows defects in neural stem cell frequency. Hmga2 is regulated by the expression of let-7 microRNA in old adult mice.[64]
BMI-1 (PC)	*Bmi1*$^{-/-}$ neurospheres are smaller than wild-type.[66,83,149] Smaller cerebellum and thinner molecular and cellular layers (reduced expansion of cells).[66,83,149]
GLI-2, GLI-3 (ZFP)	*Gli-2*$^{-/-}$, *Gli-3*$^{-/-}$ cortical cells are unable to passage more than four times and do not retain multipotency.[46] The mutant mice display reduced cortical architecture.

The table lists TFs followed by the protein family they belong to. Comments in the right hand column highlight information that supports the role of these TFs in NSC self-renewal. bHLH, basic helix-loop-helix; CSL-, HMG, high mobility group; PC, Polycomb; ZFP, zinc finger proteins.

progenitors in a variety of tissues. Knockdown of the canonical Notch effector C-promoter binding factor 1 (CBF1/RBP-J) promotes the conversion of NSCs to NPs, whereas activation of CBF1 is insufficient to convert NPs back to NSCs.[39] The results from conditionally ablated transcription factor RBP-J indicated that the RBP-J-mediated signaling might inhibit the differentiation of NSCs into NPs.[40] Mammalian Musashi-1 augments Notch signaling through the translational repression of its target mRNA, mNumb, thereby contributing to the maintenance of NSCs/NPs.[41]

Shh-Gli signaling is another key pathway that is involved in nervous system development by modulating precursor proliferation in different regions of the brain like neocortex, cerebellum and tectum. Shh has also been implicated in cell proliferation and growth of the late embryonic and postnatal dorsal brain.[42-44] Gli-1 expression in Nestin positive NSCs/NPs increases precursor and clonogenic stem cell number in vivo and in vitro.[45] E18.5 cortical tissue deficient in Gli-2 or Gli-3 the downstream mediators of Shh showed reduced primary and secondary neurosphere formation.[46] Gli-2-specific shRNA in NSCs in vivo and in vitro inhibited cell proliferation and the expression of Sox2 and other NSC markers, including Hes1, Hes5, Notch1, CD133 and Bmi-1.[47] Taken together, it appears that Wnt, Notch and Shh signaling pathways play essential roles in the maintenance of NSCs.

NSC Self-Renewal

One of the defining features ascribed to NSCs, and stem cells in general, is the ability to self-renew; to generate duplicate multipotent copies of themselves. At the surface the concept of self-renewal seems straightforward. However, to assay self-renewal is more complicated. The most widely used assay for NSC self-renewal is carried out in vitro by measuring neurosphere formation through passaging of cultures. The number of multipotent neurospheres generated during passaging is taken as a reflection of the self-renewal activity. Table 1 lists the main transcription factors involved in self-renewal.

HES1/5. Mice that lack both Hes1$^{-/-}$/Hes5$^{-/-}$ or mis-expression studies suggest a role for HES1/5 in self-renewal of NSCs.[36,48] The secondary sphere forming capability is reduced in telencephalic cells that lack HES1 and HES5.

CBF-1. Notch signaling has been linked to the stem cell state as mentioned above. The NICD-CBF1 complex generated upon Notch activation targets Hes1/5 genes. Mizutani et al. 2007,[39] have analyzed the role of CBF-1 further. Knockdown of CBF-1 induced neurogenesis. Mizutani et al. 2007,[39] then generated CBF-1 promoter fusions linked to EGFP and identified two discreet populations of cells in vivo, EGFP^{hi} and EGFP^{lo}. On isolating these two populations by using CD133 selection they found that the EGFP^{hi} cells formed greater numbers of multipotent neurospheres and had 3.5–3.8-fold greater expression of Hes1/5. In contrast, EGFP^{lo} cells had higher expression of Mash1. They propose that Notch signaling may allow NSCs to be distinguished from NPs.

SOX2. SOX TFs with a high-mobility-group (HMG) DNA binding domain have been shown to have homologous roles in specification and maintenance of NP identity in the CNS and the peripheral nervous system. The SOXB1 factors (Sox1, Sox2 and Sox3) which are transcriptional activators are co-expressed in the proliferating NSCs/NPs of embryonic and adult CNS.[49-51] A reduction in SoxB1 levels leads to precocious neural differentiation and to the depletion of the progenitor pool, whereas misexpression of SoxB1 family members can block neuronal differentiation and maintain the progenitor population.[49,52-54] SOXB1 transcriptional factors antagonize the neuronal differentiation that is induced by the bHLH proneural proteins MASH1 and the NGNs[49,55] and proneural proteins can directly bind and inhibit SOXB1 protein function. Proneural factors also upregulate Sox21 (SoxB2 group) expression which represses Sox1-3 activity inducing downregulation of progenitor markers, cell cycle exit and neuronal differentiation.[56] Thus, the balance of SOXB1 and proneural activity determines the activation of neurogenesis.

The *Sox2* enhancer, termed *Sox2* regulatory region 2 (SRR2), that is specific to ESCs also functions in NSCs/NPs and drives strong expression in these cells. Chromatin immunoprecipitation assays reveal interactions of class III POU proteins, such as BRN1 and BRN2 with SOX2 at SRR2 in NSCs/NPs.[57,58] POUIII transcriptional factors BRN1, BRN2, BRN4 and OCT6 are widely expressed in the developing CNS with extensive regional overlap.[59-61] In the ventricular zone of the embryonic spinal cord nestin expression

is seen in the regions co-expressing SOXB1 and BRN2 proteins. Group B1 and group C SOX proteins interact with POUIII TFs and activate the nestin neural enhancer.[62] However, a switch in POU TFs from BRN1/2 to BRN3a occurs in post-mitotic cells.[63]

HMGA2. High mobility group A2 (HMGA2) is a chromatin associated protein that potentiates the activity of TFs. In a recent analysis HMGA2 was found to be expressed at high levels in fetal cells and declined with age.[64] Hmga2 KO mice show reduced stem cell numbers throughout the CNS. Nishino et al. 2008,[64] derived a self-renewal index (secondary neurosphere numbers/primary neurosphere numbers) for neurosphere formation and used this to compare KO mice with wild-type controls. HMGA2 KO reduced self-renewal of NSCs by 70% and this could be reversed by expression of Hmga2 in the KO cells. The HMGA2 KO neurospheres were multipotent but much smaller than wild-type controls.

BMI-1. A polycomb family transcriptional repressor, BMI-1 has also been shown to be required for the maintenance of NSCs/NPs.[65,66] Bmi-1 knockout studies have shown progressive postnatal growth retardation and neurological defects.[66] shRNA mediated Bmi-1 reduction causes defects in embryonic and adult NCSs cell maintenance.[65] BMI-1 maintains NSCs by repressing the cyclin-dependent kinase inhibitors, $p16^{Ink4a}$ and $p19^{Arf}$ as well as p21-Rb pathway.[65]

Gli-2/3. Cortical mutant cells from *Gli-2*, *Gli-3* KO mice fail to form both primary and secondary neurospheres.[46] The GLI pathway regulates expression of several NSCs/NPs markers such as Sox2, Hes1, Hes5, Notch1, Bmi-1 and CD-133.[47] This novel circuit of TFs is important for self-renewal of the NSCs cells from embryonic CNS.

TLX. The orphan nuclear receptor TLX has been shown to maintain adult NSCs in an undifferentiated proliferative state. In vivo, TLX mutant mice show a loss of cell proliferation and reduced nestin labeling in the neurogenic areas of the adult brain and in vitro, TLX null cells fail to proliferate.[67] One mechanism by which TLX regulates maintenance of NSCs is by recruiting histone deacetylases to its downstream targets to repress their expression.[68]

Table 2 **TFs and NSCs proliferation**

Transcription factors	Comments
SOXB1 (SOX1, 2 and 3) (HMG)	All Sox null mutants showed defect in brain development.[52,69,70] SOX1 overexpression expands progenitor pool moderately in vivo.[71] Sox2 and 3 expression correlate with proliferating cells in vivo and in vitro[53,73] Conditional Sox2 mutants have less proliferating cells in vivo.[54]
GLI-1 (ZFP)	shRNA knockdown inhibits proliferation in vitro.[78] Overexpression in vivo resulted in enlarged brain and expanded precursor pool.[45]
GLI-2 and **GLI-3** (ZFP)	Null mutants die at birth and show reduced SVZ/VZ volume.[75,76] Mutant NSCs showed reduced proliferation in culture.[46] Truncated/sh-Gli2 inhibit cell proliferation in vivo and in vitro.[47]
HES1 and **HES5** (bHLH)	Neurospheres from *Hes1*$^{-/-}$–*Hes5*$^{-/-}$ telenchephalon were significantly smaller than the wild type.[37] Anti-sense knockdown of Hes1 resulted in less BrdU incorporation in neurosphere culture.[81] In vitro overexpression of Hes1 induces proliferation.[82] However, overexpression of Hes1 and 5 in vivo inhibits neurogenesis without expanding the precursor pool.[37]
ID2 (bHLH)	*ID2*$^{-/-}$ NSCs proliferate slower and form smaller neurospheres in culture.[143]
ID4 (bHLH)	Absence of ID4 compromises the proliferation of NSCs in the ventricular zone.[144]
OLIG2 (bHLH)	Expressed in proliferating NSCs in culture. Null mutant cells showed reduced proliferation in culture.[145]
BMI-1 (PC)	Null mutant haves less proliferating NSCs/NPs in vivo.[66] Mutant cells form smaller neurospheres in culture.[146]

The table lists TFs followed by the protein family they belong to. Comments in the right hand column highlight information that supports the role of these TFs in NSC proliferation. bHLH, basic helix-loop-helix; HMG, high mobility group; PC, Polycomb; ZFP, zinc finger proteins.

NSC Growth

NSC/NP growth is regulated at two levels by TFs. The first is at the level of the cell cycle. The second is at the level of early differentiation. The main methods used to measure NSC growth are, (1) neurosphere size, (2) rates of BrdU incorporation and (3) number of cells in vivo in particular CNS locations.

SoxB1. The SoxB1 genes are thought to be critical in maintaining the NSC state. The main mechanism seems to be through inhibition of differentiation. Some evidence exists to support a role for these genes in proliferation. All SoxB1 null mutants have defects in brain development.[52,69,70] SOX1 overexpression induces expansion of the NP pool in vivo followed by neuronal differentiation.[71] But in vitro overexpression of SOX1 promotes neural differentiation.[72] Sox2 expression correlates with proliferating NSCs/NPs in vivo and in vitro.[53] Sox2 conditional KO mutants have less proliferating cells in vivo and form less primary neurospheres in culture.[54] However, subsequent passaging and differentiation of mutant cells were unaffected. Sox3 has also been shown to express in proliferating cells in vivo and in vitro.[73] Whether Sox2 and 3 directly control proliferation is not clear. Table 2 lists the main TFs involved in proliferation.

GLI family. The Gli family of TFs, consisting of Gli-1, 2 and 3 are the main mediators of the Hedgehog signaling pathway which is well known to regulate NSC proliferation and self-renewal.[74] Both Gli-2 and Gli-3 null mutants die at birth.[75,76] They displayed a much reduced SVZ/VZ as well as cortex. In vitro culture of NSCs from these mutants showed greatly reduced cell proliferation and neurosphere formation.[46] Gli-1 null mutant on the other hand appeared normal,[77] and NSCs derived from these mice form multipotent neurospheres that can be maintained over multiple passages.[78] However, knockdown of Gli-1 with shRNA impaired proliferation and neurosphere formation. In addition, overexpression of Gli-1 in vivo resulted in enlarged brains and expanded precursor pools.[45] The authors also showed using an inducible Gli-1 expression system that the number of neurospheres formed

Table 3 **TFs and repression of NSC differentiation**

Transcription factors	Comments
HES1/5 (bHLH)	*Hes*$^{-/-}$ embryos prematurely differentiate into neurons.[36]
HESR1/2 (bHLH)	Misexpression of Hesr1 and Hesr2 by electroporation in mouse brain at embryonic day 13.5 transiently maintains neural precursor cells.[38]
ID4 (HLH)	Premature differentiation of *ID4*$^{-/-}$ cortical stem cells.[144]
SOX1/2/3 (SOXB1) (HMG)	A reduction in SoxB1 levels leads to precocious neural differentiation whereas misexpression of SoxB1 family members can block neural differentiation.[49,52-54]
REST (ZFP)	Inhibition of Rest in hippocampal NSCs leads to activation of neuronal specific genes.[150]
POU3F2/F3 (ZFP)	Interact with SOX2 and control expression of NSC/NPs genes like nestin, BFAB.[57,151]

The table lists TFs followed by the protein family they belong to. Comments in the right hand column highlight information that supports the role of these TFs in repression of NSC differentiation. bHLH, basic helix-loop-helix; HMG, high mobility group; ZFP, zinc finger proteins.

correlated with the level of Gli-1 expression with higher levels giving rise to more neurospheres.

Gli-2 has been reported as a novel regulator of Sox2 expression, which is essential for the maintenance of NSCs. Besides, neocortical cells from Gli-2 mutant mice showed compromised neurosphere forming abilities.[46] Primary cultures of E18.5 Gli3 mutant neocortices in full NSC media yielded transiently forming clumps that rapidly degenerated, whereas NSC cultures from wild-type siblings formed stable neurospheres.[46]

HES1 and HES5. TFs of the basic-helix-loop-helix (bHLH)1 family play important roles in regulation of neurogenesis in the CNS. Hes1 is expressed at high levels in the VZ of the developing CNS. Persistent expression of Hes1 severely perturbs differentiation of NPs in the CNS. NPs infected with Hesl-transducing retrovirus stayed in the VZ/SVZ or the ependymal layer and did not differentiate into neurons or glial cells.[79] Hes5, a neural-specific factor, shows a similar expression pattern in the developing CNS to that of Hes1. Hes5 is expressed at high levels throughout the VZ of the developing CNS, but the level decreases as neural differentiation proceeds.[80] Thus, HES1 and HES5 encode transcriptional repressors and maintain the number and status of undifferentiated NSCs and NPs cells in the developing CNS.[36]

When the NSCs exit the cell cycle in the VZ of the neural tube, these cells express Notch ligands (Delta and Jagged) on the cell surface and activate the Notch of neighboring progenitor cells. In the Notch-activated cells, intracellular domain (ICD) is released and forms a complex with the DNA binding protein RBP-J in the nucleus. This complex induces Hes1 and Hes5 expression. Hes1 and Hes5 repress both the expression and activity of Mash1, Math3 and Ngn2 by binding to their promoter and recruiting the corepressor TLE/Grg, and then neuronal differentiation is inhibited.[35]

There are also some evidence for involvement of Hes1 and 5 in NSC proliferation. In vitro, NSCs from *Hes1*$^{-/-}$ *and Hes5*$^{-/-}$ mutants form fewer and smaller neurospheres compared to wild type.[37] In addition, anti-sense knockdown of Hes1 resulted in less BrdU incorporation in human neurosphere cultures.[81] In vitro overexpression of Hes1 in granule neuron precursor induced proliferation.[82] However, in vivo overexpression of both HES1 and 5 inhibited neurogenesis without expanding the NP pool.[37] Thus, the role of Hes genes may be to maintain precursor cells in a proliferation competent state rather than regulating their cell cycle directly.

BMI-1. NSCs depend increasingly on BMI-1 for proliferation as development proceeds from embryonic through adult stages.[65] BMI-1 promotes NSC self-renewal, maintenance and development in the nervous system by repressing the p16^{Ink4a} and p19^{Arf} senescence pathways. Deletion of Ink4a and Arf from BMI-1 knockout mice partially rescued NSC self-renewal and NSC frequency.[83] However, using lentiviral-delivered shRNAs in vitro and in vivo, Fasano et al. 2007,[65] found no evidence of an increase in either p16^{Ink4a} or p19^{Arf} at any developmental stage 48 hours after reduction of Bmi-1. Instead, the cell cycle inhibitor p21/Cip1 was rapidly upregulated. In support of their role in proliferation, stable expression of Bmi-1 in human HSCs promotes long term in vitro expansion of these cells.[84] Expression of BMI-1 in astrocytes has been shown to convert these terminally differentiated cells to NSC-like cells that were able to proliferate and form multipotent neurospheres that self-renewal.[85]

HESR1 and HESR2. Hes-related bHLH genes, termed Hesr genes (also known as Hey, HERP, HRT, CHF and gridlock) have been identified as immediate transcriptional targets in Notch signaling.[86] HES and HESR proteins differ primarily in that Hes proteins contain a conserved proline residue in the basic region, while Hesr proteins do not. Hers1 and Hers2 are expressed by NSCs in developing brain. It has recently been reported that HESR1 and HESR2 negatively regulate neuronal bHLH genes and promote maintenance of NSCs in the developing brain.[38] Iso T, et al.[87] revealed that HESR and HES proteins exert synergistic effects by forming heterodimers. Thus, it is possible that HESR and HES cooperatively regulate maintenance of NSCs. Table 3 lists the main TFs involved in repression of differentiation.

REST. REI silencing transcription factor (REST) or neuron restrictive silencer factor (NRSF) is expressed throughout early development where it regulates a large network of neuronal genes.[88] REST had been implicated in the transcriptional networks that regulate ESC pluripotency, as the Rest gene is a target of Oct4, Sox2 and Nanog binding.[12] However, REST appears to have quite distinct transcription networks in NSCs compared to ESC.[89] REST is able to both silence and repress neuronal genes in embryonic hippocampal NSCs by creating a chromatin environment that contains both repressive local epigenetic signature (characterized by low levels of histones H4 and H3K9 acetylation and elevated dimethylation of H3K9) and H3K4 methylation.[88]

NSC Differentiation

NSC differentiation can be seen as a two step process, where committed progenitors are first formed (early differentiation)

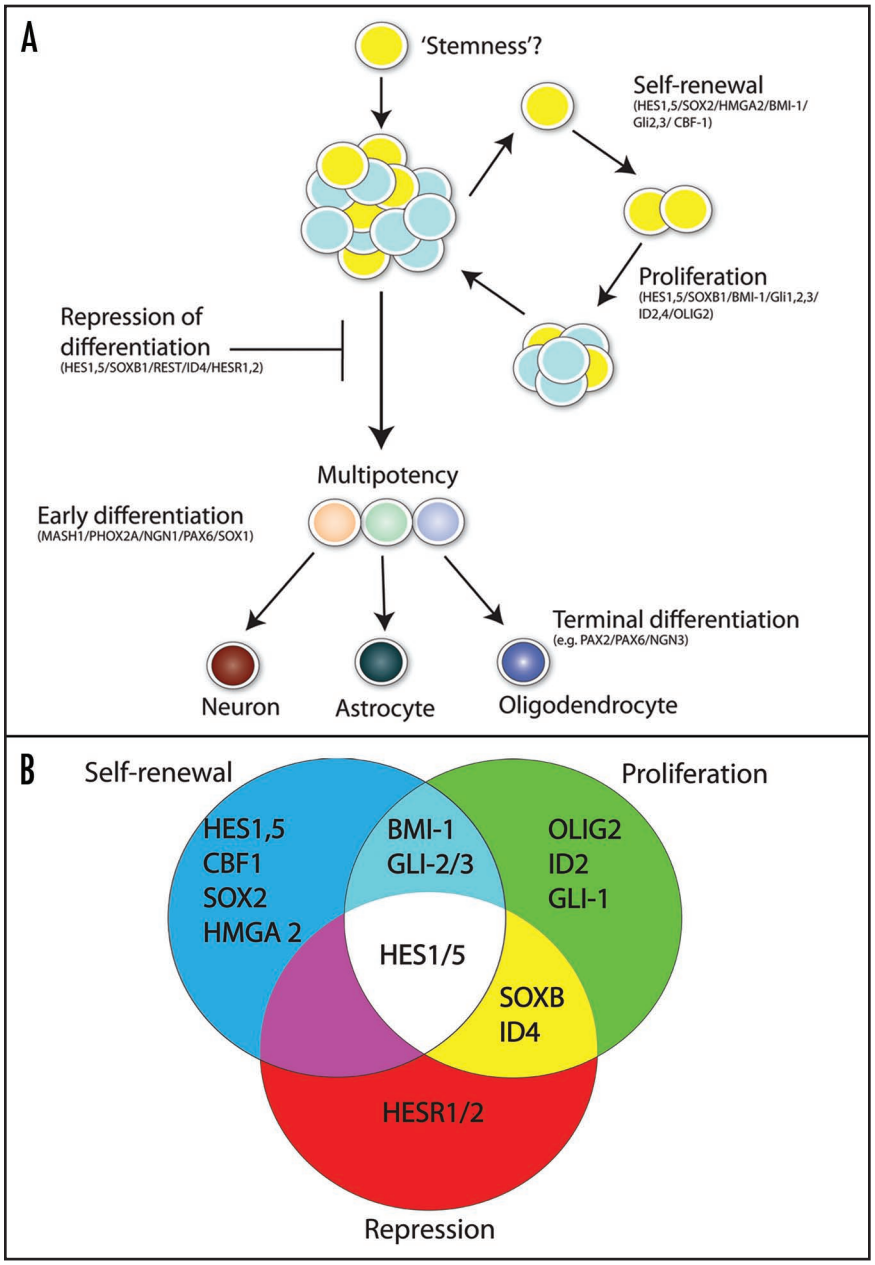

Figure 1. (A) TFs and NSC behavior. The figure outlines NSC behavior and the TFs involved in each step. The starting cell (yellow) that gives rise to neurospheres is likely to be a NSC. What defines 'stemness' in the context of the NSC is currently unknown. The NSC undergoing 'self-renewal' generates a copy of itself. The next phase is proliferation where NSCs generate NPs (blue) and a neurosphere forms. A number of TFs are implicated in repression of differentiation of these NPs keeping the neurosphere growing. If differentiation signals are imposed the NSCs/NPs leave the cell cycle and form committed progenitors (light brown, green and blue) which then go on to form terminally differentiated cells; neurons, astrocytes and oligodendendrocytes (dark brown, green and blue). The TFs implicated in each of the steps are placed in brackets. For further information on TFs see Tables 1–4 and text. (B) Overlapping functions of TFs. The three processes that are involved in neurosphere formation are presented as circles in a Venn diagram. Self-renewal—blue, proliferation—green and repression of differentiation—red. Overlap in circles shows TFs that have more than one function.

followed by the generation of neurons, astrocytes and oligodendrocytes (terminal differentiation). Here we focus on TFs involved in early differentiation (Fig. 1). A large number of factors have been identified that promote terminal differentiation of committed progenitors and these are mentioned in Table 4 but not discussed further here. The generation of committed progenitors depends on the interplay of factors contributing to lineage initiation and specification, lineage commitment, cell cycle exit and feedback loops inhibiting expression of neural stem/progenitor-related TFs.

MASH1. NSCs/NPs express high levels of bHLH factors that besides forming a network to maintain cells in their undifferentiated state, can repress proneural genes. The inhibitory bHLH HES1 protein inhibits the transcriptional activity of proneural gene mammalian achete-scute homologue (MASH1); indirectly by binding to promoter sequences recognized by MASH1, and directly by heterodimerizing with MASH1 such that it cannot heterodimerize with E47 transcription factor to activate other proneural genes.[90] In fact, mouse embryonic NSCs overexpressing HES1 failed to differentiate into neuron and glial cells[79] while loss of HES1 in the olfactory epithelium increased both the level of MASH1 and MASH1-positive NSCs in the olfactory placode.[91]

While HES1 negatively regulates the function of MASH1 in neuronal differentiation, NUMB2 and NUMB4 increase MASH1 expression, with concurrent expression of Delta1 and Tuj1.[92] This induction occurs only when the levels of proneural NUMB2 and 4 was in a 2-molar excess of NUMB1. In addition, the binding of myocyte enhancer factor 2C (MEF2C) and Ca^{2+}/calmodulin-dependent kinase II, induced by apoptosis signal-regulating kinase 1 (ASK1), on the MASH1 promoter can also upregulate the expression of MASH1.[93] Overexpression of MASH1 in neural crest stem cells induces morphological differentiation and expression of neuronal markers.[94] This effect was in part exerted by its ability to upregulate the expression of the paired homeodomain transcription factor PHOX2a, which in turn induces the expression of cyclin-dependent kinase inhibitor p27 (Kip1) to coordinate cell cycle exit.[95] Besides activating PHOX2a, MASH1 was shown to regulate the expression of Neurogenin-1 (NGN1) and subsequently NeuroD, in the olfactory neuron progenitors.[96] Brief overexpression of NGN1 and NeuroD in *Xenopus laevis* ectodermal explants revealed a spectrum of neural genes regulated by these proneural bHLH TFs.[97] Such genes include Math3, HEN1, Dll1, Elavl3, Gadd45g, MyT1 and Hes-6.

Table 4 **TFs and NSCs differentiation**

(A) TFs involved in neurogenesis

Transcription factors	Comments
PITX3 (HOM) DA neurons	Potentiates Nurr1 in specifying for the phenotype.[152]
FOXA1/A2 (WH) DA neurons	Specification of phenotype by regulating Ngn2 expression; regulates Nurr1, engrailed 1, aromatic l-amino acid decarboxylase and tyrosine hydroxylase during development.[142]
NGN2 (bHLH) DA neurons	Differentiation of Nurr1-positive DA neurons from NSCs.[153]
L3/LHX8 (HOM) Cholinergic neurons	Development or maintenance of cholinergic neurons in the basal forebrain.[154]
GATA2 (ZFP) GABAergic neurons	Activate genes specific to the GABAergic neurons subtype in the midbrain. Absence leads to differentiation into glutaminergic neurons.[155]
PAX2 (PB) GABAergic neurons	Specification of interneurons in the dorsal horn.[156]
NGN2 (bHLH) GABAergic neurons	Region-specific functions: Increased GABA neurons in the hindbrain; decreased GABA neurons in the forebrain.[157]
MASH1 (bHLH) GABAergic neurons	Region-specific functions: Promotes GABA neurons in the forebrain; decreased GABA neurons in the hindbrain.[157]
PTF1a (bHLH) GABAergic neurons	Favors GABAergic over glutaminergic neurons.[168]
GATA2 (ZFP) Serotonergic neurons	Development of serotonergic neurons in the rhombomere region[155] and organization of serotonergic dorsal raphe cells.[159]
EAGLE (Eg) (ZFP) Serotonergic neurons	Regulates serotonergic neuron development by regulating the expression of serotonin transporter.[160]
PET-1 (ETS) Serotonergic neurons	Differentiation and maintenance of cells in hindbrain raphe nuclei.[161]
LMX1b (HOM) Serotonergic neurons	Serotonergic neuron specification[162] and maintenance.[163]

(B) TFs involved in astrogliagenesis

Transcription factors	Comments
OLIG2 (bHLH)	Expressed in immature astrocytes; deletion leads to loss of astrocytes in the cerebral white matter[164] but its nuclear export is required for astrocytes differentiation.[165,166]
Stem cell leukaemia (SCL) (bHLH)	Astrocyte specification in ventral neural tube.[167]
PAX6 (HOM)	Promotes maturation.[168]
MCP-1-induced protein (MCPIP) (Novel)	Overexpression increases GFAP expression and astrocytic morphology.[169]
NF1A (NF)	Differentiation of astrocytes precursors.[170,171]
STAT3 (ZFP)	Astrocyte differentiation.[106]
CSL (ZFP)	Activates GFAP promoter.[106]

(C) TFs involved in oligodendrogliagenesis

Transcription factors	Comments
SOX4 (HMG)	Present in OPCs, inhibit myelination.[173,174]
SOX5/6 (HMG)	Present in OPCs, inhibit Sox9 and Sox10.[175]
SOX8 (HMG)	Oligodendrocyte specification, together with Sox9.[176]
SOX9 (HMG)	Contribute to glial[177] and oligodendrocyte specification, together with Sox8.[176]
SOX10 (HMG)	Terminal differentiation, activating myelinating genes.[178,179]
SOX11 (HMG)	Present in OPCs.[173]
SOX17 (HMG)	Oligodendrocyte differentiation.[180]
MASH1 (bHLH)	OPC specification.[107,181]
NGN3 (bHLH)	Oligodendrocyte development, possibly maturation and myelination.[107]
OLIG1 (bHLH)	Oligodendrocyte specification[188] with controversial role in maturation.[184,185]
OLIG2 (bHLH)	Oligodendrocyte maturation[186] cooperates with Nkx2.2.[192]
HES5 (bHLH)	Inhibits OPC differentiation.[188,189]
ID2/4 (HLH)	Inhibits OPC differentiation.[188-190]
KROX24 (ZFP)	Maintenance of OPCs, and during immediate stage of differentiation.[191]
MyT1 (ZFP)	OPC proliferation and differentiation.[192]
ZFP488 (ZFP)	Oligodendrocyte differentiation, cooperating with OLIG2.[73]

Table 4 **TFs and NSCs differentiation (continued)**

TST1/OCT6/SCIP/BRN1/2 (ZFP)	Downregulated in early phases of oligodendrocyte development.[193]
YINYANG 1 (YY1) (ZFP)	Oligodendrocyte differentiation[194] by repressing Tcf4 & ID4.[111]
ATF5 (ZFP)	Expressed in OPCs, inhibit differentiation.[196]
NKX2.2 (HOM)	Oligodendrocyte maturation.[197]
NKX6.2 (HOM)	Expressed in myelinating oligodendrocytes.[198]
HOXB4/A2 (HOM)	Expressed throughout oligodendrocyte development.[199]
MSX1 (HOM)	Overexpression in mouse neurospheres promoted oligodendrogenesis.[200]

The table lists TFs followed by the protein family they belong in. Comments in the right hand column highlight information that supports the role of these TFs in NSC differentiation. HOM, homedomain; WH, winged helix; bHLH, basic helix-loop-helix; HMG, high mobility group; ZFP, zinc finger proteins. PB-, ETS, ETS domain. In (A) neuron type is given in brackets.

SOX1. SOX1 affects neurogenesis. Kan et al. reported that SOX1 binds to the promoter of HES1 and suppresses its expression, disrupts cell cycle by preventing cells from entering the G_2 phase, and directly drives the promoter activity of NGN1.[72] SOX1 also suppresses β-catenin-mediated TCF/LEF signaling by binding to β-catenin itself. Thus, SOX1 promotes neurogenesis through multiple independent pathways.

PAX6. GATA-2,[98] and PAX6,[99] promote differentiation by inducing the transcription of negative regulators of cell cycle. These proneural factors can inhibit the expression of TFs that maintain the NSC state. PAX6 can induce the expression of NGN2,[99] which then downregulates the expression of SOX1-3.[49] HES6.2 is upregulated by NGN1/2 and synergizes with these proneural factors to promote neuronal differentiation by repressing HES5 and inhibiting downstream Notch effectors.[100] Proneural TFs can also inhibit gliogenesis. Examples of such TFs include NGN1, functioning by sequestering the CREB binding protein (CBP)—mothers against decapentaplegic homolog 1 (SMAD1) transcription complex from promoters of astrocyte differentiation genes and by inhibiting signal transducer and activator of transcription (STAT)-3 which would otherwise induce astroglial lineage.[101]

Despite a variety of TFs known to play a role in neuronal lineage initiation and specification, much less is known about astroglial differentiation. Various signaling molecules can induce the differentiation of NSCs towards the astroglial lineage. Such factors include cytokines belonging to the interleukin (IL)-6 family, those of the activin/Bone Morphogenetic Protein (BMP) family, ciliary neurotrophic factor, and recently Nogo-66. These ligands signal through various pathways which converge in the nucleus to activate STAT3 and SMAD1,[102-104] to induce the transcriptional expression of glial proteins such as glial fibrillary acidic protein.[105] In addition, Notch signaling activates the GFAP promoter via the CSL DNA-binding protein.[106]

Interestingly, MASH1 is expressed in oligodendrocyte progenitors and might have a role in specifying the differentiation of oligodendrocytes from immature glial cells.[107] Other oligodendrocyte-specifying TFs include bHLH proteins OLIG1 and OLIG2 that are activated by Shh[108] and bFGF signals.[109] OLIG2 regulates the development of oligodendrocytes by enabling the differentiation of NG2-positive synantocytes into the oligodendroglial lineage.[110]

MicroRNA

Post-transcriptional gene regulators, such as microRNAs, are likely to be important for controlling the balance between self-renewal and differentiation in NSCs. MicroRNAs, a family of small (~22 nucleotides long), non-coding RNAs similar to the siRNAs involved in RNA silencing, have been shown to play important roles in diverse processes including apoptosis, fat metabolism, cancer, major signaling pathways, tissue morphogenesis and development.

MicroRNAs originate from stem-loop precursors in the genome. Transcription produces primary microRNA transcripts (pri-microRNAs), which are then cleaved by the nuclear RNase III enzyme Drosha to release precursor microRNAs.[111] After Drosha processing, pre-microRNAs are exported out of the nucleus by the nuclear transport receptor Exportin-5, in a process requiring the hydrolysis of GTP to GDP.[112,113] Pre-microRNAs are next cleaved by the cytoplasmic RNase III enzyme Dicer to produce ~22 nucleotide microRNA duplexes.[114,115] After Dicer processing, one strand of the microRNA duplex is usually degraded while the other persists as a mature microRNA.[116] The strand that has a less thermodynamically stable 5' end is thought to be incorporated into effector complexes called microRNA-containing RNA-induced silencing complexes (miRISCs).[117,118] These miRISCs recognize and bind to target mRNAs to modulate their expression.

MicroRNAs modulate target expression in two different ways: by directing transcript degradation or inhibiting translation.[119] In plants and very rarely in animals, microRNAs bind to highly complementary microRNA binding sites in target mRNAs to guide sequence-specific cleavage. This process is similar to RNA interference.[120] In animals, microRNAs bind to partially complementary microRNA binding sites and repress translation. This repression is achieved by interfering with translation or by guiding degradation processes that are initiated by mRNA deadenylation and decapping.[121]

A number of microRNAs exhibit distinct spatial and temporal expression patterns during development.[122-124] Additionally, some microRNA expression patterns show species conservation, e.g., miR-1 in muscles, miR-124 in the CNS and miR-10 in anterior-posterior patterning.[123] These observations indicate that

microRNAs may be involved in the specification and maintenance of tissue identity and other facets of development.

About 70% of the microRNAs identified by 2005 were expressed in mammalian brains, suggesting possible roles of these microRNAs in neural function.[124-126] Studies in invertebrate model systems have identified lsy-6, the first microRNA found to play a role in neuronal patterning,[127] and miR-9a, which ensures the generation of the precise number of neuronal precursor cells during development.[128] In vertebrate models, the restoration of a single microRNA (miR-430) in zebrafish modified to prevent production of endogenous microRNAs ameliorated deficits in neuroectodermal development and neuronal differentiation.[129]

In addition to being important regulators of vertebrate CNS development,[125,126,129] microRNAs also play key roles during neural differentiation in vitro.[125,130] During neural differentiation, Smirnova et al.[130] demonstrated that the most highly expressed microRNAs in adult brain, miR-124 and miR-128, were preferentially expressed in neurons; miR-23 was restricted to astrocytes; miR-26 and miR-29 had stronger expression in astrocytes than neurons; and miR-9 and miR-125 were fairly evenly distributed.[130] Overexpression of miR-124, miR-128 and miR-9 in NPs decreased astrocyte differentiation, whereas inhibition of miR-9 alone or in combination with miR-124 led to reduced neurogenesis.[131]

Studies which show that Dicer-deficient mice lacking mature microRNAs die at embryonic day 7.5 and lack multipotent stem cells support a role for microRNAs in stem cell self-renewal.[132,133] Indeed, Rybak et al. demonstrate that microRNAs let-7 and mir-125 and the pluripotency factor Lin-28 participate in an autoregulatory circuit that controls microRNA processing during neural stem cell commitment.[134] Changes in expression of let-7 and one of its known targets, the transcriptional regulator HMGA2, during aging may contribute to the decline in NSC function.[64] In human glioma neurosphere cultures, miR-128 has been shown to specifically block glioma self-renewal via post-transcriptional regulation of the NSC self-renewal factor Bmi-1.[135]

Laminin γ1 and integrin β1, which are highly expressed in NSCs/NPs cells and repressed upon neuronal differentiation, were recently identified as targets of miR-124.[136] MiR-124 also regulates the small C-terminal domain phosphatase 1 (SCP1), a phosphatase implicated in neural development, further supporting its role in neurogenesis.[137] Furthermore, let-7 has been shown to target Hunchback, a gene which regulates the temporal identity of neuroblasts.[138-140] The identification of these and other microRNA targets in the NSCs will help us to better understand the role of microRNAs in regulating of neural stem cell self-renewal and differentiation.[141]

Conclusion

Taking the success of linking TFs to pluripotency we have attempted in this review to examine the data that links TFs to NSC behavior (Fig. 1). TFs have been found to affect NSC self-renewal, proliferation, repression of differentiation and differentiation (early and late). Some of the TFs involved in these behaviors have overlapping functions (Fig. 1B). For example, BMI-1 and GLI-2/3 have roles in self-renewal and proliferation. Interestingly, HES1 and 5 are the only factors that play a role in all three pre-differentiation steps (Fig. 1B). Further identification of TFs and their roles will help elucidate the regulatory networks that control NSC behavior and this information will be crucial for the use of NSCs as cellular models of development and disease.

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