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Stem Cells: Defining and Reprogramming a Neural Lineage

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ABSTRACT

Stem Cells: Defining and Reprogramming a Neural Lineage

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Stem cells show great promise as cellular replacement and drug delivery therapies for a number of neurological disorders. This dissertation examines embryonic and adult types of stem cells, their plasticity, and mechanisms governing their development (Chapters 1-5). Our studies illustrate that leukemia inhibitory factor (LIF) signaling and bone morphogenetic protein (BMP) signaling are key regulators of neural stem cell (NSC) maintenance with opposing functions (Chapter 2). These signaling pathways generate discrete astroglial cell types previously thought to be identical, affect the size of the NSC pool, and control fate commitment during development. Further, we demonstrate that NSCs exist in the adult hippocampus (Chapter 3). BMP inhibition with noggin expands the adult NSC pool both in culture and *in vivo*. This expansion will serve as a useful paradigm to investigate factors regulating NSCs and the functional role of hippocampal neurogenesis. Finally, we use nuclear transfer to address the role of the donor cell epigenome in reprogramming (Chapter 4). We find within a neural lineage that reprogramming occurs less frequently in more differentiated cells. These results are important for the understanding of epigenetics and the production of autologous stem cells for potential therapy. Future studies, both planned and prospective, are discussed (Chapter 5) with emphasis placed on nuclear reprogramming.

A TRIBUTE:

This work is dedicated to all whom are dear to me, whose numerous sacrifices provided many opportunities for subsequent generations-including my own. May they continue to live through their shared history, their lessons taught, and may their spirit reach out to others.

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ABBREVIATIONS

aNPC: adult neural precursor cell

BDNF: brain-derived neurotrophic factor

bHLH: basic helix-loop-helix transcription factors

BLBP: brain lipid binding protein

BMP: bone morphogenetic protein

BMPR1a, -1b, II: bone morphogenetic protein receptor subtype-1a, -1b, -II

BrdU: bromodeoxyuridine

BSA: bovine serum albumin

CNTF: ciliary neurotrophic factor

DG: dentate gyrus

Dmnt: DNA acetyltransferase

E: embryonic day (x)

EGF: epidermal growth factor

eNPC: embryonic neural precursor cell

ENS: enteric nervous system

FACS: Fluorescence Activated Cell Sorting

FGF: fibroblast growth factor

GABA: γ-aminobutyric acid

GCL: granule cell layer

GLAST: astrocyte-specific glutamate transporter

GFAP: glial fibrillary acidic protein

GFP: green fluorescent protein

H: hilus

Hdac: histone deacetylase

Hes: hairy enhancer of split transcription factors

Hesr: Hes-related genes

Hip: hippocampus

ICM: inner cell mass

Id: inhibitor of differentiation transcription factors

INM: interkinetic nuclear migration

JAK: Janus kinase (not John A. Kessler)

LIF: leukemia inhibitory factor

LIFR: leukemia inhibitory factor receptor

LTP: long-term potentiation

MGE: medial ganglionic eminance

ML: molecular layer

n: number of replicates

NB: NSE-BMP4

Ngn: neurogenin

NEP: neuroepithelium precursors

NF: pan-neurofilament

NF1-A/B/C/X: Nuclear Factor 1 family of transcription factors

NICD: Notch intracellular domain

NN: NSE-noggin

Nog: noggin

NPC: neural precursor cell

npSMAD: nuclear phospho-SMAD 1/5/8

NSC: neural stem cell

NSE: neuron specific enolase

NT: nuclear transfer

ntES: nuclear transfer-derived embryonic stem cells

NT3: neurotrophic factor-3

OB: olfactory bulb

OG2: Pou5f1-gfp transgenic mice

OL: oligodendrocyte

p: passage

PGC: primordial germ cell

PGD: pre-implantation genetic diagnosis

PN: postnatal day (x)

PSA-NCAM: polysialic acid-neural cell adhesion molecule

RG: radial glia

RNAi: ribonucleic acid interference

SCNT: somatic cell nuclear transfer

SEM: standard error measurement

SGZ: subgranular zone

SMAD: Sma Mad related proteins

STAT: Signal Transducer and Activator of Transcription

SVZ: zone

TE: trophectoderm

TSC: trophoblast stem cells

VZ: ventricular zone

WT: wild type

ZGA: zygotic genome activation

TABLE OF CONTENTS

TABLE LEGEND

FIGURE LEGEND

CHAPTER 1: GENERAL INTRODUCTION

Neural Development: a stem cell continuum

Mammalian development normally begins with fertilization. The fusion of egg and sperm produce a zygote that is capable of generating every cell in the embryo proper and all the support tissue of the extraembryonic lineage. Since the zygote also self-renews (and can generate more than one embryo), these cells are considered totipotent stem cells. As development proceeds, cells gradually lose the ability to commit to particular lineages. This first commitment separates cells that can still generate all the cells in the embryo from cells that only generate the extraembryonic tissue. This separation is easily identifiable at the blastocyst stage where cells in the inner cell mass (ICM) generate the fetus and cells in the trophectoderm (TE) generate the extraembryonic lineage (Wang and Dey, 2006). Though the first differentiation is distinguishable at the blastocyst stage, it likely occurs much earlier. How much earlier is currently unknown (Hansis, 2006). ICM cells that can self-renew are considered to be pluripotent since they can generate several lineages, whereas TE cells that self-renew are multipotent because they generate many cell types within one lineage. Interestingly, stem cells from both tissues can be harvested and grown in culture for hundreds of passages. Cells from the ICM produce embryonic stem cells (ESC) and cells in the TE create trophoblast stem cells (TSC). However, whether these in vitro cells are the equivalent of their in vivo counterparts is debatable (Rossant, 2001). It is also currently unclear how many cells in the ICM or TE are capable of generating ESC or TSC lines.

After implantation, gastrulation occurs to separate the fetus into endoderm, mesoderm, and ectoderm. The nervous system is then specified out of the ectoderm and separated from the epidermis. This immature tissue is known as neuroectoderm (De Robertis and Kuroda, 2004; Stern, 2005). Once definitive neuroectoderm exists, these cells are generally considered restricted to the neural lineage (multipotential). Cells that can self-renew are known as embryonic neural stem cells (eNSC). ENSC undergo dramatic changes that accompany the different demands of building a central nervous system. During neurulation, the neural tube closes and organizes into rostral-caudal (R-C) and anterior-posterior (A-P) axes (Chizhikov and Millen, 2004; Lupo et al., 2006). From this reorganization the forebrain, midbrain, and hindbrain form. During this time, the neuroepithelium looks layered ('pseudostratified'). Neuroepithelial precursor cells (NEP) show typical epithelial features and are highly polarized along their apical–basal axis, as is obvious from the organization of their plasma membrane (Huttner and Brand, 1997). NEPs undergo symmetric self-renewal to generate two daughter eNSCs (McConnell, 1995; Rakic, 1995). In this process, the nuclei of neuroepithelial cells migrate up and down the apical–basal axis during the cell cycle, a process known as interkinetic nuclear migration (INM). The cells then divide upon reaching the apical surface (Chenn and McConnell, 1995). Further, tight junctions and adherences junctions are present at the most apical end of the lateral plasma membrane and are necessary for NEP self-renewal (Cappello et al., 2006; Zhadanov et al., 1999).

Before the onset of neurogenesis, the neuroepithelium transforms into a tissue with numerous cell layers. The apical layer that lines the ventricle and contains most of the eNSC cell bodies is referred to as the ventricular zone (Gotz and Huttner, 2005). With this switch, NEP

downregulate certain epithelial features - notably tight junctions yet not adherens junctions (Aaku-Saraste et al., 1996). Concomitantly, glial hallmarks appear and the neuroepithelial cells give rise to a distinct, but related, cell type: radial glial (RG) cells. These cells exhibit both residual neuroepithelial as well as astroglial properties (Kriegstein and Gotz, 2003). Radial glia notably maintains INM, but instead of nuclear migration progressing to the surface, RG nuclei do not leave the VZ. Rather, nuclei undergo mitosis at the apical surface of the VZ and migrate basally for S phase of the cell cycle. RG also share cytoskeletal features with NEP, such as having processes that span the apical to basal surfaces and the intermediate filament proteins nestin and RC2. In contrast, glial properties arise in RG including glycogen granules (Gadisseux and Evrard, 1985), the astrocyte-specific glutamate transporter (GLAST), Ca^{2+}-binding protein S100, vimentin, and brain-lipid-binding protein (BLBP). These characteristics appear in most VZ cells during, but not before, neurogenesis (Kriegstein and Gotz, 2003). Radial glia serve as neuronal progenitors in all regions of the CNS, acting in a spatiotemporal pattern (Anthony et al., 2004; Noctor et al., 2001; Noctor et al., 2004), but also may constitute a heterogeneous cell population (Calegari et al., 2005). Interestingly, this change of eNSCs towards glial identity maintains in NSC into adult and throughout lifetime, at least in a subpopulation of RG (Alvarez-Buylla and Lim, 2004; Doetsch et al., 1999a; Tramontin et al., 2003).

As the primary neurogenic period ends during mid- to late-gestation, RG become biased towards astrocytic differentiation. The molecular mechanisms for this change are largely unknown, but are discussed in Chapters 2 and 5. Gliogenesis then occurs primarily in the neonatal period where RGs have been observed to lose their apical process, leave the VZ/SVZ and, differentiate into astrocytes (Schmechel and Rakic, 1979b; Voigt, 1989). In contrast, some

RG in the middle ganglionic eminance (MGE) lose their basal process and retain contact with the lateral ventricle. These cells are then maintained as NSC into adulthood Permanently labeling radial glia during postnatal development demonstrates that NSCs are maintained into the adult, generate glia (astrocytes and oligodendrocyte), ependymal cells, and participate in adult neurogenesis in the olfactory bulb (OB) and hippocampus dentate gyrus (DG) (Ganat et al., 2006; Merkle et al., 2004; Spassky et al., 2005). During this neonatal period, ependymal cell generation along the ventricular wall pushes NSC into the second germinal zone, the subventricular zone (SVZ). These cells remain in contact with the ventricle, but gain more glial characteristics such as expression of glial acidic fibrillary protein (GFAP) and slightly increased cellular ramification. These GFAP-expressing NSCs then persist throughout adulthood within the SVZ along the lateral ventricular wall (Doetsch et al., 1999a). Since these cells contain many properties similar to mature astrocytes elsewhere in the adult brain, it is currently unclear whether all astrocytes can act as NSC, or whether only GFAP-expressing cells in specialized niches possess the characteristics of NSC. Further, the molecular mechanisms regulating astrocyte commitment versus NSC maintenance during postnatal periods are largely unknown. *In Chapter 2 it is demonstrated that late embryonic NSCs acquire glial characteristics, but are maintained as NSC in response to leukemia inhibitory factor (LIF) signaling. Meanwhile, bone morphogenetic protein (BMP) signaling promotes differentiation into a mature astrocytic fate and loss of precursor characteristics both in vitro and in vivo.*

Adult neurogenesis is restricted to the SGZ of the hippocampal DG and the SVZ adjacent to the lateral ventricle (Altman and Das, 1965; Kaplan and Hinds, 1977). Although GFAP

expressing NSCs in the SVZ have been well characterized, the identity of cells contributing to neurogenesis in the hippocampus remains controversial (Doetsch et al., 1999a; Doetsch et al., 1997; Garcia et al., 2004; Seri et al., 2001). Currently, these cells are proposed to be neurogenic progenitor cells, but not NSC because they lack the ability to self-renew (Bull and Bartlett, 2005; Seaberg and van der Kooy, 2002). In Chapter 2 we found that neural precursor cells endogenously produce BMPs which promote neural precursor exit from cell cycle (Bonaguidi et al., 2005). We therefore asked whether prior attempts to demonstrate a hippocampal stem cell are confounded by the endogenous production of BMPs. *In Chapter 3, we demonstrate that hippocampal cells cultured in the presence of the BMP inhibitor noggin are able to self-renew and to generate neurons, and that noggin maintains the hippocampal stem cell niche in vivo.*

Nuclear Reprogramming

During development, cell type is distinguished by orderly selection of genes either expressed or switched off. The ability to acquire and inherit gene-expression patterns efficiently is crucial to the individual history of cell differentiation. In most cases, this 'molecular memory' occurs without alterations or deletion of any DNA sequences, but rather by epigenetic mechanisms including: DNA methylation, histone methylation and acetylation, phosphorylation, and RNAi silencing (Pennisi, 2001). Traditionally, epigenesis is a one-way continuum beginning with a totipotent zygote that becomes more restricted during fetal lineage commitment, and terminating with committed cell types and lineage restricted progenitor pools. Currently, it is accepted that de-differentiation or reversal of the continuum can occur (Reik and Dean, 2002), and it is

sometimes mediated via cell fusion (Blau et al., 1985; Cowan et al., 2005; Weimann et al., 2003; Ying et al., 2002). However, transdifferentiation, or the ability to generate cells of different germ layers remains controversial within mammalian systems. For example, numerous studies have postulated that hematopoietic stem cells can differentiate into neural and epithelial derivatives or neural stem cells can undergo differentiation towards the hematopoietic lineage. Meanwhile, other studies have refuted those claims (Alvarez-Dolado et al., 2003; Brazelton et al., 2000; Cogle et al., 2004; Harris et al., 2004; Mezey et al., 2000; Nygren et al., 2004; Wagers et al., 2002). While the possibility of transdifferentiation currently remains unclear, all studies agree that the probability of transdifferentiation is low.

Epigenetic reprogramming occurs naturally in the primordial germ cell (PGC) and zygote. In the mouse genital ridge, a rapid and possibly active genome-wide demethylation occurs in both male and female (embryonic day) E10.5-E11.5 PGCs. This results in the erasure of parental-specific gene expression known as imprints (Kato et al., 1999a; Obata et al., 1998; Tada et al., 1998). PGC-derived pluripotent embryonic germ (EG) cells can also erase imprints (Tada et al., 1998). In female PGCs, the inactive X chromosome is reactivated (Tam et al., 1994). This mechanistically undefined event erases any aberrant epigenetic modifications to prevent the inheritance of epimutations (Morgan et al., 1999). Following imprinting in the germ line, the parental genomes exhibit epigenetic asymmetry at fertilization. During 0–5 hours post fertilization, parental chromosomes in the zygote can interact directly with maternally inherited cytoplasmic factors in the oocyte until pronuclear membrane formation. The paternal chromatin is sequentially remodeled, silenced, and ultimately compacted with protamines (Steger, 1999). It

then undergoes marked active demethylation, while the maternal genome undergoes further passive *de novo* methylation (Mayer et al., 2000; Monk et al., 1987). Demethylation of the paternal genome may be essential to make it compatible for early activation of the embryonic genome, or may have accompanied evolution of developmental asymmetry between parental genomes (Reik et al., 2001). Although a number of maternally inherited oocyte cytoplasmic factors have the potential to modify the paternal epigenetic states, epigenetic reprogramming mechanisms in the zygote remain unknown.

Epigenetic reprogramming, or de-differentiation, can also be induced by transplantation of a somatic nucleus into the oocyte. This process restores totipotency or pluripotency to the somatic nucleus (Munsie et al., 2000; Solter, 2000; Wakayama et al., 2001). However, the overall clone survival rate is extremely low and those that do survive are largely abnormal. Common anomalies in the adult include respiratory distress, increased birth weight, and major cardiovascular abnormalities known collectively as 'large offspring syndrome' (Young and Fairburn, 2000). Imprinted genes in the adult are largely abnormal (Humpherys et al., 2001), but can be normal (Inoue et al., 2002) pending the nucleus donor cell type. Likewise, DNA methylation is altered (2 to 5 misexpressed loci per 1,000), but tolerated (Yanagimachi, 2002). Overall gene expression from cloned neonatal liver and placenta can differ by 4% though the animals appear grossly normal (Humpherys et al., 2002). Epigenetic marks can be faithfully removed and reestablished on either X chromosome (Eggan et al., 2000); yet both male and female mice can be produced from the same male ES donor (Eggan et al., 2002). Therefore, epigenetic alterations in "successfully" cloned animals are common, but tolerated. Overall,

clone abnormalities along with the failure to transmit those abnormalities to their offspring (Tamashiro et al., 2002) suggest that developmental failure of NT embryos results from the inability to "reprogram" the epigenetic profile of the somatic donor nucleus back to that of a fertilized zygote (Gurdon, 1999).

Recent analysis of developing clones is beginning to reveal reprogramming errors during early development. Individually assessed cloned bovine embryos show active but not passive demethylation activity (Bourc'his et al., 2001; Dean et al., 2001). Precocious *de novo* methylation is observed in bovine nuclei beginning at the 4-cell stage instead of the 8-16-cell stage (Kang et al., 2001) and subsequent pre-implantation development methylation patterns are mosaic (Kang et al., 2002). Genes critical for implantation and post-implantation development (IL6, FGF4, and FGFr2) also show abnormal expression in cloned bovine embryos (Daniels et al., 2000). In mouse clones, genes essential for pre-implantation development are improperly regulated. For example, Pou5f1 (OCT4) is expressed temporally correct, but spatially incorrect. The altered expression patterns have been correlated with development and ability to derive embryonic stem cell (ESC) lines. Additionally, a number of other essential embryonic genes and epigenetic modifiers have altered levels in mouse clones. These include the SRY homeobox protein Sox2, Pou5f1-related gene Dppa3 [PGC7/Stella, (Li et al., 2005)] the major translation initiation factor in 2-cell embryos eIF-1A, histone deacetylase 1 [hdac1, (Inoue et al., 2006)] DNA methyltransferase 1 [Dmnt1, (Chung et al., 2003)], and nuclear organization factor nucleoplasmin 2 (Tamada et al., 2006). Finally, multiple imprinted genes (Mann et al., 2003) and genome-wide transcription (Inoue et al., 2006) are perturbed even in morphologically normal

cloned embryos. In all, these observations suggest that reprogramming errors occur early in development and that some epigenetic information may be lost. However, it is currently not understood how these errors affect cloned embryo development. *Studies described in Chapter 4 seek to correlate clone pre-implantation development and ESC derivation with proper expression of embryonic genes and downregulated expression of donor nucleus genes.* Using different donor cells from a defined neural lineage may reveal a role for how 'molecular memory' influences reprogramming and leads to more efficient derivation of autologous stem cells for therapeutic purposes.

CHAPTER 2: LIF AND BMP SIGNALING GENERATE SEPARATE AND DISTINCT TYPES OF GFAP-EXPRESSING CELLS

Abstract:

Bone morphogenetic protein (BMP) and leukemia inhibitory factor (LIF) signaling both promote the differentiation of neural stem/progenitor cells into glial fibrillary acidic protein (GFAP) immunoreactive cells. This study compares the cellular and molecular characteristics, and the potentiality, of $GFAP^+$ cells generated by these different signaling pathways. Treatment of cultured embryonic subventricular zone (SVZ) progenitor cells with LIF generates $GFAP^+$ cells that have a bipolar/tripolar morphology, remain in cell cycle, contain progenitor cell markers and demonstrate self-renewal with enhanced neurogenesis - characteristics that are typical of adult SVZ and subgranular zone (SGZ) stem cells/astrocytes. By contrast, BMP-induced $GFAP^+$ cells are stellate, exit the cell cycle, and lack progenitor traits and self-renewal - characteristics that are typical of astrocytes in the non-neurogenic adult cortex. In vivo, transgenic overexpression of BMP4 increases the number of $GFAP^+$ astrocytes but depletes the $GFAP^+$ progenitor cell pool, whereas transgenic inhibition of BMP signaling increases the size of the $GFAP^+$ progenitor cell pool but reduces the overall numbers of astrocytes. We conclude that LIF and BMP signaling generate different astrocytic cell types, and propose that these cells are, respectively, adult progenitor cells and mature astrocytes.

Introduction:

Neural stem/progenitor cells (NSCs) in the early embryonic ventricular zone (VZ) do not express GFAP, the classical astrocytic marker, but, by late embryonic development, NSCs do begin to express it (Imura et al., 2003). In the adult brain, $GFAP^+$ neurogenic progenitors exist in the subventricular zone (SVZ) of the lateral ventricle (Altman, 1969; Doetsch et al., 1999a) and the subgranular zone (SGZ) (Altman and Das, 1965; Seri et al., 2001) of the hippocampal dentate gyrus. Thus, some $GFAP^+$ cells in the postnatal brain have stem cell potential (Imura et al., 2003; Laywell et al., 2000; Morshead et al., 2003), and a morphologically distinct subpopulation of $GFAP^+$ cells is the predominant source of constitutive adult neurogenesis (Garcia et al., 2004). However, most GFAP-expressing cells in the generative zones, as well as GFAP-expressing astrocytes outside of these zones, do not act as progenitors in the normal adult brain (Laywell et al., 2000). It is currently unclear how these mature astrocytes are derived, and the lineage relationships between $GFAP^+$ progenitors and mature astrocytes are not well defined.

NSCs express GFAP in response to several signaling molecules, including the leukemia inhibitory factor (LIF)/ciliary neurotrophic factor (CNTF) and BMP families (Gross et al., 1996; Johe et al., 1996). Canonically, LIF/CNTF activates the JAK/STAT pathways, whereas BMPs signal primarily through SMAD pathways. Nevertheless, their signaling pathways have points of convergence in the regulation of GFAP, leading to suggestions that these cytokine families activate astrogliogenesis through the same mechanisms (Nakashima et al., 1999a; Sun et al., 2001). However, whereas BMP signaling promotes the generation of astrocytes from SVZ forebrain stem cells both in vitro and in vivo (Gomes et al., 2003; Gross et al., 1996), LIF

signaling inhibits the restriction of early embryonic forebrain stem cells to a glial lineage and helps to maintain a stem cell phenotype (Shimazaki et al., 2001). Furthermore, BMP2 treatment of progenitor cells cultured from LIFR$^{-/-}$ animals induces astrogliogenesis (Koblar et al., 1998), indicating that signaling from this receptor is not necessary for the generation of astrocytes. It is currently unclear whether LIF and BMP signaling generate GFAP-expressing cells with similar characteristics and developmental potential. We therefore used a combined in vitro and in vivo approach to compare the properties of GFAP-expressing cells that are generated in response to LIF versus BMP signaling. Our findings suggest that LIF signaling induces GFAP^{+} progenitor cells, whereas BMP signaling promotes a mature astrocyte phenotype that lacks stem/progenitor cell potential.

Materials and Methods:

Animals. The generation of NSE-BMP4 and NSE-Noggin transgenic mice is described elsewhere (Gomes et al., 2003; Guha et al., 2004). Timed-pregnant CD1 mice were obtained from Charles River (Wilmington, MA).

Immunochemistry. Postnatal day 15 (P15) brains were fresh-frozen on dry ice, cut into 10 μm coronal sections, fixed in 4% paraformadehyde (PFA), and blocked with 10% goat serum for 1 hour. Primary antibodies diluted in PBS containing 1% BSA and 0.25% Triton X-100 were applied overnight at 4°C. Antibodies were as follows: Ki67 (rabbit polyclonal, 1:1000; Novocastra), GFAP (mouse IgG1 or rabbit polyclonal, 1:400; Sigma), vimentin (mouse IgM, 1:4;

Developmental Studies Hybridoma Bank). Primary antibodies were visualized with mouse or rabbit Cy2- or Cy3-conjugated secondary antibodies (Jackson Laboratories). Nuclei were counterstained with Hoechst 33342 (Sigma). Cells were counted in the dentate SGZ (a two-nucleus-wide band below the apparent border between the GCL and the hilus and inner third of the GCL) and ML (superior/dorsal to the GCL), and normalized to the area analyzed in mm^2 (Kempermann et al., 2003).

Bromodeoxyuridine (BrdU) labeling. BrdU (10 mM) was added to differentiating neural cells on day 6, and processed on day 7 after 16 hours. Cells were fixed with 4% PFA and processed with 2N HCl for 45 minutes, then 0.1 M Borax (pH 8.5) for 15 minutes before immunochemistry.

Immunochemistry of cultures. Prior to PFA fixation, 15 μl/ml O4 (mouse IgM, Chemicon) and 5 μg/ml LeX/CD15 (mouse IgM, clone MMA; BD Biosciences) were added to cells for 30 minutes at 4°C. Fixed coverslips were blocked with serum for 45 minutes and incubated with primary antibodies at room temperature for 2-3 hours. Antibodies were as follows: BrdU (mouse IgG2a, 1:1000; clone BU-1, Chemicon), ßIII-tubulin (mouse IgG2b, 1:400; Sigma), SOX1 (rabbit polyclonal, 1:1000; a kind gift from Dr Hisato Kondoh, Osaka University). Primary antibodies were visualized with Alexa 647-(infrared) Alexa 555/594-(red); Alexa 488-(green) and Alexa 350-(blue) conjugated secondary antibodies (Molecular Probes). Cells were counted in seven alternate fields of each coverslip and verified in a minimum of three independent experiments.

Generation of progenitor cell neurospheres and differentiation cultures. The ganglionic eminences of E18.5 mice were dissociated and grown in serum-free medium (SFM) with EGF (20 ng/ml, human recombinant, Biosource) for 7 days, as previously described, to generate neurospheres (Mehler et al., 2000; Zhu et al., 1999). Primary spheres were grown for 3-4 days in vitro (DIV), and then passaged by dissociating with 0.25% trypsin (Invitrogen) for 2 minutes followed by incubation with a soybean trypsin inhibitor (Sigma), a 5-minute spin, and repeated trituration. Secondary spheres were grown for an additional 3-4 DIV and used for subsequent studies. For differentiation studies, neurospheres were dissociated and plated at a density of $1x10^4$ cells/cm^2 onto poly-D-lysine-coated (PDL, Sigma, 20 μg/ml for >1 hour) coverslips within 24-well culture plates, and then grown for 7 DIV in SFM plus 2 ng/ml EGF and 250 ng/ml Noggin (R&D Systems), 20 ng/ml LIF (Chemicon) or LIF+Noggin, or 20 ng/ml BMP4 (R&D Systems). Cells were re-fed on day 3.

Retrovirus production and neurosphere infection. The EGFP-N1 cassette (Clontech) was cloned into the *Bgl*II and *Bst*BI sites on the pLXRN retrovirus shuttle vector (Clontech) and replaced the G418r cassette. The rat 1.9 kb GFAP promoter (Sun et al., 2001) was partially digested and inserted into the *Hpa*I and *Bgl*II shuttle sites, which excised the P_{RSV}. Virus was packaged by co-transfecting the shuttle (rGFAPp-EGFP or control P_{RSV}-EGFP) with VSVG into GP2-293 cells (Clontech), using Lipofectamine 2000 (Invitrogen). Supernatant collected on days 2, 3 and 6 was concentrated 1000x and stored at -80°C until use. Secondary neurospheres were passaged, infected with 15 μl virus in 10 ml medium the day following dissociation, cultured and passaged once more before plating for differentiation.

Fluorescent-activated cell sorting (FACS) and neurosphere-forming assay. Plated cells were harvested, resuspended at a density of $1x10^6$ cells/ml in SFM, and sorted on the basis of forward-side scatter and GFP expression at 1000 events/second. Sorted cells were plated at a density of 1000 cells/well into non-adherent 96-well plates containing SFM plus 20 ng/ml EGF, or 2 ng/ml EGF plus 20 ng/ml LIF where denoted. Cell survival was >85% by Trypan Blue exclusion analysis. The numbers of free-floating spheres were counted at day 7 in a minimum of three independent experiments.

RT-QPCR (reverse transcriptase-quantitative polymerase chain reaction). Plated cells were treated with cytokines for 20 hours before harvesting RNA using RNeasy, according to the manufacturer's protocol (Qiagen). Reverse transcriptase (RT) was performed using Thermoscript (Invitrogen), and QPCR using Platinum SYBR Green (Stratagene). Specificity of the PCR reaction was confirmed by running PCR products on a 2% agarose gel. Two replicates were run for each cDNA sample with the test and control primers. An amplification plot showing cycle number versus the change in fluorescent intensity was generated by the Sequence Detector program (Applied Biosystems).

Results:

LIF and BMP both promote GFAP expression by cultured NSCs, but induce different morphologies.

Embryonic NSCs proliferate in vitro in the presence of FGF2 and/or EGF to form floating cell clusters termed neurospheres (Reynolds and Weiss, 1996). We prepared E18.5 EGF-responsive neurosphere cultures to investigate the mechanisms generating different populations of $GFAP^+$ cells. BMP4 is expressed endogenously (Fig. 1A) by neurosphere cells; BMP7 and BMP2 are also produced but at lower levels (Gross et al., 1996; Nakashima et al., 1999b). Endogenous BMPs regulate GFAP expression by these cells, and treatment with the BMP inhibitor noggin decreases GFAP transcripts fourfold (Fig. 1B, C). Thus, study of the effects of LIF signaling independent of BMP signaling requires the inhibition of endogenous BMPs. In fact, treatment with noggin decreases GFAP transcript levels twofold in the presence of LIF relative to LIF alone (Fig. 1B). Treatment with LIF increases levels of GFAP transcripts 16-fold, whereas treatment with BMP4 increases them more than 32-fold (Fig. 1B). Changes in the number of cells that express GFAP parallel the changes in levels of GFAP transcripts (Fig. 1G). Treatment with noggin significantly reduces the number of $GFAP^+$ cells ($P<0.05$). Conversely, LIF treatment increases the number of $GFAP^+$ cells ($P<0.05$), and BMP4 treatment results in an even larger increase, such that more than 90% of BMP4-treated cells express GFAP ($P<0.001$).

Although treatment with LIF or BMP4 each increases the number of cells expressing GFAP, the morphologies of those cells differ drastically (Fig. 1D-F, H-J). Treatment with LIF alone promotes a mixture of elongated bipolar/tripolar and stellate morphologies (Fig. 1E). However, when noggin is included to inhibit endogenous BMP signaling, LIF treatment promotes the bipolar/tripolar morphology at the expense of a stellate one (Fig. 1F, I). By contrast, treatment with BMP4 leads to cells with a stellate morphology characteristic of some

mature astrocytes (Fig. 1D, H). $GFAP^+$ cells generated after BMP4 treatment had an average of six major processes per cell, whereas $GFAP^+$ cells generated in the presence of LIF and noggin have an average of slightly more than two major processes per cell (Fig. 1J).

BMP4 but not LIF prompts exit of $GFAP^+$ cells from cell cycle

To determine whether $GFAP^+$ cells generated by BMP4 and LIF signaling differ with respect to their ability to enter the S-phase of the cell cycle, the cells were labeled with a long (16 hour) pulse of BrdU on day 6 of the 7-day differentiation protocol. In control cultures, a small number of $GFAP^+$ cells were labeled with BrdU (18.9±2.6%, Fig. 2A, F). Treatment with BMP4 decreased BrdU incorporation to an almost quiescent state [2.6±0.4%, $P<0.01$; Fig. 2E,F; (Gross et al., 1996)]. Conversely, noggin inhibition of endogenous BMPs increased BrdU incorporation by 130% (44.0±4.0%, $P<0.01$; Fig. 2B, F). LIF alone did not significantly alter proliferation (29.1±1.8%; Fig. 2C, F). However, LIF treatment in the presence of noggin increased BrdU incorporation by 63% compared with noggin alone, and approximately 70% of the $GFAP^+$ cells incorporated BrdU under these conditions (70.2±4.9%, $P<0.01$; Fig. 2D,F). BMP4-treated cells did not incorporate BrdU even after a prolonged 2-day pulse on days 9-10 of differentiation, indicating that the cells had not simply paused in G0. BMP4 treatment did not alter cell survival either (see Table 1 for clonal analysis).

LIF increases, whereas BMP4 decreases, neural precursor markers in $GFAP^+$ cells

The foregoing observations suggested that the $GFAP^+$ cells generated by LIF signaling might represent stem/progenitor cells, whereas the quiescent $GFAP^+$ cells generated by BMP signaling

might represent more differentiated astrocytes. We therefore compared these populations of cells with respect to the expression of neural stem/progenitor cell markers. We first examined the SRY transcription factor SOX1, which is expressed by both early and adult progenitor cells, but not by astrocytes (Bylund et al., 2003). SOX1 was expressed by just 7.0±1.1% of the control $GFAP^+$ cells (Fig. 3A, F). However noggin, LIF and LIF plus noggin all increased the number of $SOX1^+GFAP^+$ precursors by approximately 200% (Fig. 3B-D, F; $P<0.005$). Conversely, BMP4 decreased the number of $SOX1^+GFAP^+$ precursors by 43% (Fig. 3E, F; $P<0.05$). The glycoprotein LeX (CD15/SSEA1) is also expressed by neural stem/progenitor cells (Capela and Temple, 2002; Kim and Morshead, 2003). In control cultures, 11.7±1.5% of $GFAP^+$ cells expressed LeX (Fig. 3G, L). Inhibition of endogenous BMP by noggin did not alter LeX expression by $GFAP^+$ cells (Fig. 3H, L). However, LIF treatment increased the number of LeX^+GFAP^+ cells by 81% (20.2±2.2%, $P<0.005$; Fig. 3I, L), and LIF plus noggin increased LeX^+GFAP^+ cells by 127% (25.1±2.0%, $P<0.005$; Fig. 3J, L). By contrast, BMP4 treatment decreased the number of LeX^+GFAP^+ cells by 92% (1.0±0.8%, $P<0.005$; Fig. 3K, L), and virtually no LeX^+ cells were present in the BMP4-treated cultures. Additionally, the same pattern of findings was observed with a third neural progenitor marker, the intermediate filament vimentin.

LIF promotes, whereas BMP4 inhibits, a $GFAP^+$ multipotential stem/progenitor cell fate

As LIF and BMP appear to induce discrete $GFAP^+$ cell populations, we hypothesized that those cells would display different potentialities. To directly test whether the $GFAP^+$ cells induced by cytokines could behave as stem cells, we used the well-characterized rat GFAP promoter

(rGFAPp) (Sun et al., 2001; Takizawa et al., 2001), FACS, and neurosphere-generating assays to select, purify and assess stem cell activity in the $GFAP^+$ cell populations. Neural stem cell activity is defined as the ability to form self-renewing neurospheres and to generate multiple neural lineages: neurons, astrocytes and oligodendrocytes. A retroviral vector was used to introduce the rGFAPp driving eGFP into expanding neurospheres. After passage, the neural stem cells were plated for differentiation, with or without cytokines, for 5 days. To determine whether eGFP expression faithfully mirrored GFAP expression, the cells were examined by immunocytochemistry for GFAP, the neuronal marker ßIII-tubulin (TuJ1), and the oligodendrocyte marker O4, to identify which cell types displayed GFP fluorescence. EGFP fluorescence was exhibited by 29.4±3.3% of the $GFAP^+$ cells, indicating infection efficiency. Importantly, 99.7±0.3% of the GFP cells were also $GFAP^+$ positive, demonstrating that the virus faithfully selects $GFAP^+$ cells. Also, eGFP did not localize to any neurons or oligodendrocytes (Fig. 4A, B). Furthermore, the cells expressing eGFP displayed morphologies similar to those observed after GFAP staining of control and cytokine-treated cultures (Fig. 4C-G). After 5 days of cytokine treatment, $GFAP^+$ cells were sorted on the basis of forward-side (FS) scatter and eGFP levels. Dead cells were omitted by FS scatter and $eGFP^+$ cells were clearly discernable (Fig. 4H-I, Fig. 5A). Sort purity was 99.2±0.7%, and cell survival was 85.0±1.2% as measured by Trypan Blue exclusion.

After 7 days in the neurosphere-forming assay, 2.0±0.1% of the $GFAP^+$ cells that were not treated with cytokines formed neurospheres (Fig. 5B, G). BMP4 treatment drastically reduced the ability of the cells to form neurospheres (0.0±0.1%, $P<0.01$; Fig. 5F, G), while

inhibition of BMP (noggin) increased the number of GFAP$^+$-generated neurospheres by approximately 66% (3.4±0.1%, P<0.05; Fig. 5C, G). LIF treatment not only increased the number of GFAP$^+$-generated neurospheres by approximately 58% (3.3±0.1%, P<0.05; Fig. 5D, G), but also increased neurosphere size. LIF treatment plus noggin increased neurosphere numbers by approximately 47% compared with controls and further increased neurosphere size, but did not enhance neurosphere numbers relative to LIF alone (3.0±0.1%, P<0.05; Fig. 5E,G). These data demonstrate that LIF-generated cells have an enhanced ability to form neurospheres, whereas BMP-generated GFAP$^+$ cells rarely form neurospheres under these conditions. To determine whether the neurosphere cells actually had stem cell potential, single neurospheres were plated for differentiation in the absence of cytokines. Neurons, astrocytes and oligodendrocytes emerged from spheres generated under all conditions Fig. 5H), except for treatment with BMP4 (where neurospheres rarely formed), demonstrating that the neurosphere cells are multipotent. Hence, LIF promotes, whereas BMP4 inhibits, a GFAP$^+$ multipotential stem cell fate.

LIF increases neuron production from GFAP-expressing cells.

To determine whether exposure to LIF alters the potential of GFAP$^+$ cells, control or cytokine-treated GFAP$^+$-derived neurospheres (derived from the FACS-sorted cells) were dissociated and plated for 4-5 days of differentiation. The resultant cells were processed for ßIII-tubulin to determine neuron numbers. Control spheres produced a small number of neurons (3.8±2.5%; Fig. 5I, M). BMP inhibition (noggin) did not significantly change the number of neurons generated,

although there was a trend towards an increase (6.8±1.5%; Fig. 5J, M). However, LIF increased neuron numbers by 90% (11.0±1.1%, $P<0.005$; Fig. 5K, M). Moreover, LIF plus noggin further increased neurogenesis by 55% relative to LIF alone (17.1±2.8%, $P<0.005$; Fig. 5L, M). These observations demonstrate that LIF-induced GFAP$^+$ cells have an increased ability to produce neurons that is further enhanced by suppressing BMP signaling.

LIF maintains GFAP-expressing multipotent progenitors for prolonged periods of time

We then investigated whether LIF could maintain the GFAP$^+$ progenitor cell state for prolonged periods of time. Because high mitogen levels may reprogram more committed cells to exhibit progenitor characteristics that they might otherwise not display (Kondo and Raff, 2000; Anderson, 2001), neural progenitors were plated with only low (1-2 ng/ml) levels of EGF with LIF, and were passaged repeatedly. Neural cells could be propagated this way as a monolayer for at least 10 passages and for several months (Fig. 6A). By contrast, in the absence of LIF, the cells became progressively sparser with attempted passaging and could only be passaged a few times. We then examined whether GFAP-expressing cells generated in the presence of LIF could still proliferate (incorporate BrdU) and express the immunocytochemical characteristics of progenitor cells at the higher passages. In the LIF-treated cultures, GFAP-expressing cells at passage 7 (p7) still incorporated BrdU (2-hour pulse) and expressed the progenitor marker LeX (Fig. 6B). LIF-treated cultures were also able to generate neurons at high passages (>p7), whereas nearby GFAP-expressing cells remained in cell cycle, as assayed by Ki67 expression (Fig. 6C). To directly assess the self-renewal and multipotentiality of GFAP$^+$ cells maintained by LIF, higher

passage (>p7) cells were infected with the rGFAPp-EGFP retrovirus, selected by FACS, plated in a neurosphere-forming assay (2 ng/ml EGF plus 20 ng/ml LIF) and subsequent spheres plated for differentiation. GFAP-expressing cells maintained by LIF were able to self-renew at high passages, as evidenced by neurosphere formation, and they retained multipotentiality and, specifically, the ability to produce neurons (Fig. 6D-E). Thus, LIF maintains GFAP-expressing cells as multipotential stem/progenitor cells for prolonged periods of time, independent of exposure to high levels of other mitogens.

BMPs regulate the morphology of GFAP-expressing cells in vivo, and are necessary and sufficient for cell-cycle exit.

In vivo, GFAP-expressing astrocytes are derived from radial glia during the postnatal period (Schmechel and Rakic, 1979a; Voigt, 1989). Most of these cells lose progenitor function, but radial glia also give rise to $GFAP^+$ adult stem cells in the SVZ and hippocampus (Eckenhoff and Rakic, 1984; Merkle et al., 2004; Rickmann et al., 1987; Voigt, 1989). To test the hypothesis that BMPs developmentally regulate the maturation of $GFAP^+$ cells in vivo, we generated transgenic animals that overexpress either BMP4 or its antagonist noggin under the control of the neuron-specific enolase (NSE) promoter (Gomes et al., 2003; Guha et al., 2004). Transgene expression begins before gliogenesis at embryonic day 16 (E16), peaks postnatally, and persists into adult life (Gomes et al., 2003). Therefore, these animals serve as an excellent model to study the development of GFAP-expressing cells. BMP4 overexpressing animals have an increased number of $GFAP^+$ cells in the brain, whereas the noggin overexpressing animals conversely have

significantly reduced numbers of $GFAP^+$ cells [Fig. 7 and (Gomes et al., 2003)]. Similarly, BMP4 overexpressing animals have increased numbers of S100ß-expressing cells, whereas numbers of these cells are significantly reduced in the noggin transgenic animals [Fig. 7 and (Gomes et al., 2003)]. In the adult brain, $GFAP^+$ progenitor cells in neurogenic regions including the hippocampal SGZ remain in cell cycle (Garcia et al., 2004; Seri et al., 2001). As the NSE transgene is expressed at the highest levels in the hippocampus (Gomes et al., 2003), we examined the effects of BMP signaling on the proliferation of $GFAP^+$ cells in the SGZ using Ki67 and GFAP double labeling. Overexpression of BMP4 depleted the SGZ of $Ki67^+GFAP^+$ cells, whereas inhibition of BMP signaling by noggin significantly increased the number of these cells (Fig. 8). Specifically, at P15, 60.6±3.8 GFAP-expressing cells/mm^2 (in 10 μm sections) remained in cell cycle in the SGZ of wild-type animals (Fig. 8B, L). Inhibition of BMP signaling significantly increased the number of $Ki67^+GFAP^+$ cells by nearly 100% (118.4±2.4 cells/mm^2, $P<0.03$; Fig. 8A, L). Conversely, overexpression of BMP4 reduced the number of these cells by 92% (10.4±1.3 cells/mm^2, $P<0.03$; Fig. 8C, L). High magnification confocal images demonstrated the co-localization of Ki67 and GFAP in the SGZ (Fig. 8D, Fig.9A-C). To determine whether BMP signaling is necessary for promoting the cell-cycle exit of $GFAP^+$ cells, we investigated co-labeling with Ki67 in the hippocampal molecular layer (ML), an area that does not normally contain proliferative $GFAP^+$ cells in the adult (Garcia et al., 2004). We found only rare $Ki67^+GFAP^+$ cells in the ML in P15 wild-type mice (6.2±0.3 cells/mm^2; Fig. 8F, L), and BMP4 overexpression almost completely depleted this small population of cells (1.1±0.2 cells/mm^2, $P<0.04$; Fig. 8G, L). However, noggin overexpression markedly increased the number of cycling $GFAP^+$ cells remaining in the ML (27.4±1.5 cells/mm^2, $P<0.01$; Fig. 8F,H,L),

demonstrating that BMP is necessary for the normal exit of GFAP$^+$ cells from the cell cycle in this region.

Because GFAP-expressing progenitors in the adult neurogenic SVZ and SGZ contain fewer processes than do multipolar astrocytes in non-neurogenic regions (Garcia et al., 2004), we compared the morphology of GFAP$^+$ cells in the hippocampal SGZ and ML regions in control and transgenic animals (Fig. 8I-M). GFAP$^+$ cells in the ML of wild-type animals exhibited highly branched stellate morphologies (4.1±0.1 branches/cell, Fig. 8J, M), and overexpression of BMP4 resulted in further ramification of astrocytic processes (5.5±0.1 branches/cell, $P<0.05$; Fig. 8K, M). Conversely, noggin overexpression in the ML prevented the formation of the stellate morphology, and GFAP$^+$ cells in the ML of these animals maintained the elongated morphology typical of GFAP$^+$ cells in the SGZ (2.4±0.1 branches/cell, $P<0.01$; Fig. 8E,H-I,M). Therefore, BMPs regulate the mature morphology of GFAP$^+$ cells in the non-neurogenic hippocampus ML. Notably, the morphology and proliferative state of GFAP$^+$ cells in the SGZ of wild-type and noggin animals, and in the ML in noggin animals, are similar to that of the LIF-generated GFAP$^+$ cells in vitro (Fig. 8A,B,D,E,J; Fig. 1F; Fig. 2C,F).

BMPs regulate the maturation of GFAP-expressing cells in vivo

The foregoing observations suggested that BMPs regulate the maturation of GFAP-expressing cells in vivo by promoting cell-cycle exit and increasing process ramification. Consequently, we further investigated whether BMP signaling regulates the maturation of GFAP$^+$ progenitor cells into astrocytes in vivo by examining the expression of progenitor cell markers in the hippocampal SGZ and ML. Because LeX staining was difficult to quantitate in vivo, we instead

used the progenitor marker vimentin, in addition to SOX1 (Garcia et al., 2004; Seri et al., 2001). The wild-type SGZ contained 32.5±0.7 cells/mm^2 that were GFAP$^+$SOX1$^+$ and 157.6±0.2 cells/mm^2 that were GFAP$^+$vimentin$^+$ (Fig. 10B,I-J). In noggin overexpressing animals, the number of GFAP$^+$ progenitors was significantly increased, as assayed both by SOX1 (83.9±4.6 cells/mm^2, P<0.02, Fig. 10A,D,I) and by vimentin (209.8±4.4 cells/mm^2, P<0.03; Fig. 10J, Fig.11A-F). By contrast, BMP4 overexpression in the developing SGZ reduced the number of GFAP$^+$ progenitors, as assessed by SOX1 (18.2±1.5 cells/mm^2, P<0.02; Fig. 10C, I) and vimentin (101.1±1.2 cells/mm^2, P<0.03, Fig. 10J, Fig. 11G-I). Thus, BMP signaling promotes the loss of progenitor markers, as well as a quiescent state in GFAP$^+$ cells in neurogenic areas. To determine whether BMPs are necessary for the maturation of GFAP$^+$ cells in non-neurogenic regions, we analyzed SOX1 and vimentin expression in the ML of the noggin-overexpressing animals. GFAP$^+$ cells in the ML rarely co-express SOX1 or vimentin in wild-type mice (SOX1, 3.9±0.2 cells/mm^2; vimentin, 10.5±3.1 cells/mm^2; Fig. 10F, I, J), or in BMP4 overexpressing mice (SOX1, 3.0±0.2 cells/mm^2; vimentin, 8.7±5.0 cells/mm^2; Fig. 10G, I, J, Fig. 12D-I). However, BMP inhibition (noggin overexpression) in the ML increased the number of GFAP$^+$ cells maintaining progenitor cell markers, suggesting that BMP signaling is necessary for astrocyte maturation in this region (SOX1, 7.3±0.2 cells/mm^2, P<0.05; vimentin, 37.9±5.1 cells/mm^2, P<0.01; Fig. 10E,H-J, Fig. 12A-C). Thus, BMP signaling in vivo causes GFAP$^+$ cells to exit the cell cycle and lose progenitor markers in normally neurogenic regions, similar to the observations in vitro. Conversely, inhibiting BMP signaling in non-neurogenic regions in vivo prevents GFAP$^+$ cells from maturing, as assessed by exit from the cell cycle and the loss of

progenitor markers that typically occur in this region (Bylund et al., 2003; Hutchins and Casagrande, 1989).

Discussion:

We show here that LIF and BMP signaling results in the commitment of NSCs to distinct GFAP$^+$ cell populations that differ with respect to morphology, proliferation, gene expression and developmental potential (Table 2). LIF signaling generates proliferative, bipolar/tripolar GFAP$^+$ cells with stem/progenitor cell properties, whereas BMP signaling generates stellate, GFAP$^+$ astrocytes that lack stem/progenitor cell potential. Using transgenic animals that overexpress either BMP4 or noggin, we confirm that BMP signaling in vivo is both necessary and sufficient to induce mature GFAP$^+$ astrocytes, whereas the inhibition of BMP signaling promotes a proliferative, GFAP$^+$ progenitor phenotype characteristic of astrocytes in the adult SGZ.

LIF signaling generates GFAP-expressing progenitors

Numerous previous studies have demonstrated that LIF/CNTF signaling induces GFAP expression by cultured embryonic NSCs (Bonni et al., 1997; Johe et al., 1996; Morrow et al., 2001; Rajan and McKay, 1998; Song and Ghosh, 2004; Sun et al., 2001). The GFAP$^+$ cells induced in these studies had a partially ramified morphology, and no effect on proliferation was noted (Bonni et al., 1997). Our findings regarding the effects of LIF differ from these numerous previous studies, because we performed our experiments in the absence of BMP signaling. Cultured neural stem/progenitor cells produce BMPs endogenously [Fig. 1, (Gross et al., 1996;

Nakashima et al., 1999b)] and this alters the phenotype adopted by the cells. By including the BMP inhibitor noggin along with LIF, we were able to more precisely define the role of LIF signaling. We found that LIF signaling not only induces the expression GFAP, but also promotes re-entry into the cell cycle and maintenance of the progenitor cell traits (Figs. 1-4, 6).

Substantial evidence has been generated previously indicating that LIF signaling exerts effects on re-entry into cell cycle and on the maintenance of stem/progenitor cell traits. Null mutation of the LIF receptor (LIFR) decreases the number of progenitors derived from E14 brain in vitro, and a reduction in the levels of gp130 decreases progenitor cell re-entry into cell cycle in vivo (Hatta et al., 2002; Shimazaki et al., 2001). Conversely, LIF injection in vivo or treatment of E14 progenitors in vitro increases re-entry into cell cycle (Hatta et al., 2002; Pitman et al., 2004). In the adult, $LIFR^{+/-}$ mice show a loss of EGF-responsive progenitors derived from the SVZ, whereas CNTF injection increases the number of multipotential adult progenitors in vivo and in vitro at the expense of glial-restricted cells (Shimazaki et al., 2001). These observations are fully concordant with our finding that LIF signaling promotes a stem/progenitor cell state. In most neurosphere cultures, only about 2% of the cells are typically `stem' cells that have the capacity to self-renew; the remainders are multipotent progenitor cells. This same principle probably applies to the $GFAP^{+}$ cells in the LIF plus noggin conditions. These cells are definitely not terminally differentiated astrocytes, as they proliferate, express progenitor cell markers, and display multipotentiality. In fact, when LIF-generated $GFAP^{+}$ cells were subsequently treated with BMP4, they adopted the morphology and characteristics of BMP4-generated astrocytes.

These findings are consistent with a hypothesis that LIF signaling generates a $GFAP^+$ progenitor cell that differentiates into an astrocyte under the influence of BMP signaling (Fig. 13).

Mechanisms for astrocyte generation

Astrocyte generation has been postulated to occur through the convergence of LIF/CNTF and BMP signaling. The transcriptional co-activator p300 bridges the LIF and BMP signaling targets STAT3 and SMAD1, and mediates their cooperative effects on GFAP expression (Nakashima et al., 1999a). Our studies do not address the question of whether BMP signaling can promote astrogliogenesis without some prior activation of STAT signaling. BMP2 treatment of progenitor cells cultured from $LIFR^{-/-}$ animals induces astrogliogenesis, (Koblar et al., 1998) indicating that the LIFR is not required, but it is possible that STAT signaling is activated by cytokines that do not utilize the LIFR such as EGF (Shuai et al., 1993; Zhong et al., 1994). In this regard, it is noteworthy that BMP signaling does not promote astroglial differentiation of early embryonic neural stem cells, but rather promotes neuronal differentiation (Mabie et al., 1999). This may reflect both the presence of high levels of neurogenin1/2 (NGN1/2) that sequester the CBP-SMAD1 transcription complex away from astrocyte differentiation genes (Sun et al., 2001), and the absence of signaling from the EGFR (Eagleson et al., 1996; Gross et al., 1996; Zhu et al., 1999). EGFR is associated with a switch in bias of the cells from neurogenesis to gliogenesis (Burrows et al., 1997), and EGFR regulates the ability of stem cells to interpret LIF, but not BMP, as a $GFAP^+$-inducing agent (Viti et al., 2003). EGFR expression is upregulated in neural stem cells between E13 and E16 in mice (Burrows et al., 1997), and GFAP expression in response to LIF and BMP can first be observed at approximately E14.5 (Eagleson et al., 1996;

Gross et al., 1996). FGF2 signaling also primes neural stem cells for gliogenesis, at least in part, by the removal of histone methylation at the STAT-binding site on the rat GFAP promoter (Song and Ghosh, 2004). This may reflect an FGF2-mediated increase of EGFR expression (Lillien and Raphael, 2000). Concurrent with increased EGFR expression levels, there is a decrease in the expression of genes that inhibit gliogenesis (*Ngn1/2*) (Sun et al., 2001), and an increase in the expression of other putative proglial genes, such as hairy-enhancer of split 1/5 (*Hes1/5*) and Hes-related genes 1 and 2 (Takizawa et al., 2003). Thus, the ability of neural stem cells to interpret BMPs as pro-astrocytic differentiation factors depends upon the status of other signaling pathways and the intrinsic regulation in the cell, but may not depend upon the LIF/CNTF-mediated conversion of $GFAP^-$ NSCs into $GFAP^+$ progenitor cells (Fig. 13).

BMP4 signaling generates mature astrocytes

The ability of noggin to prevent the maturation of $GFAP^+$ progenitor cells into astrocytes in vivo indicates that BMP signaling normally regulates astrocytic lineage commitment. However, BMP4 and other BMPs are abundantly expressed throughout the nervous system (Furuta et al., 1997; Mehler et al., 1997). How then is the progenitor cell phenotype maintained in the adult brain? Noggin is normally expressed in the SVZ and SGZ of adult animals, and helps to maintain a niche for adult neurogenesis (Chmielnicki et al., 2004; Lim et al., 2000). Furthermore, antisense noggin reduces proliferation in the adult dentate gyrus (Fan et al., 2004). These observations are consistent with our findings that noggin preserves the $GFAP^+$ progenitor cell phenotype and prevents the astrocytic differentiation of these cells. Thus, noggin not only maintains the proliferation of cells within the niche, but more generally maintains a multipotent

progenitor cell phenotype by inhibiting BMP-directed differentiation. Our data further suggest that the number of $GFAP^+$ progenitor cells is inversely proportional to the amount of BMP signaling in the developing hippocampus. Because noggin is expressed in the anterior subiculum in neonates, and in the dentate gyrus from one week of age into adulthood (Fan et al., 2003), it was not clear whether noggin overexpression in this area would have much effect. However, we found that noggin overexpression markedly increased the number of $GFAP^+$ progenitor cells in the SGZ, indicating that the levels of endogenous noggin expression are insufficient to fully inhibit BMP signaling in this area. The almost complete depletion of $GFAP^+$ progenitor cells from the SGZ of BMP-overproducing animals highlights the essential role played by BMP inhibitors such as noggin in maintaining the progenitor cell phenotype.

Mature astrocytes and adult progenitors are separate cell populations

The molecular characterization of GFAP and the relatively limited number of cell types that express the protein led to its use as a surrogate marker for the astrocyte phenotype. The lack of an unambiguous biochemical marker has complicated the precise definition of astrocyte identity and of the astrocytic lineage (Gotz and Steindler, 2003; Kimelberg, 2004). Although it is clear that some $GFAP^+$ cells in the adult brain have stem cell potential (Doetsch et al., 1999b; Garcia et al., 2004; Imura et al., 2003; Morshead et al., 2003; Seri et al., 2004), only a morphologically distinct subpopulation of $GFAP^+$ cells produce new neurons (Garcia et al., 2004). This has led to terms such as radial astrocyte and horizontal astrocyte, which are based on morphological criteria in vivo, to help to distinguish the unique subsets of $GFAP^+$ cells in the brain that display progenitor cell traits (Seri et al., 2004). The lineage relationship between adult progenitor cells and other

astrocytes has been unclear. Our findings suggest a lineage relationship in the rodent brain in which $GFAP^+$ progenitors generate mature astrocytes in response to BMP signaling (Fig. 13), and, further, that these represent distinct and separable cell types. It may therefore be inappropriate to continue to use the same term - astrocyte - for these disparate cell types, particularly as $GFAP^+$ progenitors cells also generate other lineages in the normal adult brain (Garcia et al., 2004). It might be more accurate to use terms such as radial progenitor or horizontal progenitor, and to reserve the use of the term `astrocyte' for more terminally differentiated phenotypes, as these cells differ in their morphology, molecular characteristics and potentiality (Morest and Silver, 2003).

CHAPTER 3: NOGGIN REVEALS THE PRESENCE OF NEURAL STEM CELLS IN THE ADULT HIPPOCAMPUS

Abstract:

Adult hippocampal precursor cells fail to self-renew in culture suggesting that the adult hippocampus contains neurogenic progenitor cells but not neural stem cells (NSCs). However we demonstrate here that hippocampal precursor cells cultured in the presence of the BMP inhibitor noggin are able to self-renew and to generate neurons. We further establish that noggin is expressed in the adult SGZ in vivo and this limits BMP signaling in proliferative cells as evidenced by reduced levels of nuclear phospho-SMAD1/5/8. Transgenic overexpression of noggin in the SGZ increased all precursor cell populations, but proportionally increased the $GFAP^+$ NSC population at the expense of transient amplifying and migratory precursor cells. This suggests that noggin acts primarily to maintain the $GFAP^+$ NSCs, which in turn generate transient amplifying and migratory precursor cells. Thus the adult hippocampus contains a population of NSCs, and BMP inhibition by noggin maintains the hippocampal stem cell niche.

Introduction:

Neurogenesis persists in the adult brain in the subgranular zone (SGZ) of the hippocampal dentate gyrus (DG) as well as in the subventricular zone (SVZ) adjacent to the lateral ventricle (Altman and Das, 1965; Kaplan and Hinds, 1977). Neuron turnover in the granule cell layer

(GCL) of the adult DG maintains normal hippocampal function, is correlated with hippocampus-dependent learning, and may be involved in depression and epilepsy (Gould et al., 1999; Parent et al., 1997; Raber et al., 2004; Santarelli et al., 2003; Shors et al., 2001; van Praag et al., 2002). Although glial fibrillary acidic protein (GFAP)-expressing stem cells in the SVZ have been well characterized, the identity of cells contributing to neurogenesis in the hippocampus remains controversial (Doetsch et al., 1999a; Doetsch et al., 1997; Garcia et al., 2004; Seri et al., 2001). Early studies hypothesized that self-renewing multipotential cells persist in adult hippocampus (Bull and Bartlett, 2005; Seaberg and van der Kooy, 2002). However, because self-renewing cells cannot be isolated from the adult hippocampus, more recent studies have suggested that the adult hippocampus contains GFAP-expressing neurogenic progenitor cells (NPCs) but not stem cells (NSCs) (Bull and Bartlett, 2005; Seaberg and van der Kooy, 2002).

Neural stem and progenitor cells reside in microenvironmental niches that are modified by environmental stimuli (Hagg, 2005; Ma et al., 2005). For example, bone morphogenetic proteins (BMPs) are negative regulators of the adult SVZ and juvenile SGZ neurogenic niches (Bonaguidi et al., 2005; Lim et al., 2000). BMPs exert their biological effects by binding to type I (BMPRIa and BMPRIb) and type II (BMPRII) receptor subunits that are organized with minor modifications of the prototypical TGFβ subclass of serine-threonine kinase receptors (For review, see (Derynck and Zhang, 2003). Binding of BMPs to their receptors leads to phosphorylation of SMAD1, SMAD5, and SMAD8 (SMAD1/5/8), which translocate to the nucleus and activate transcription. BMP actions are regulated *in vivo* by proteins such as noggin,

follistatin, chordin, and neurogenesin that antagonize BMP signaling by directly binding BMPs and blocking ligand activity.

In previous studies we found that neural precursor cells endogenously produce BMPs which promote neural precursor exit from cell cycle (Bonaguidi et al., 2005). This suggested that prior attempts to demonstrate a hippocampal stem cell might have been confounded by the endogenous production of BMPs. Here, we demonstrate that hippocampal cells cultured in the presence of the BMP inhibitor noggin are able to self-renew and to generate neurons, and that noggin regulates precursor cell proliferation in the hippocampus in vivo. We conclude that the adult hippocampus contains a population of NSCs, and that BMP inhibition by noggin maintains the hippocampal stem cell niche.

Materials and Methods:

Animals. The generation of the NSE-Noggin transgenic mice is described elsewhere (Guha et al., 2004). FVB and C57/BL6 male mice were purchased from Jackson Laboratories. Adult mice were used between 2 and 4 months of age. All mice were housed in a facility with a 14 hr light/10 hr dark cycle and allowed free access to food and water. Experiments were conducted according to protocols approved by IACUC and Northwestern CCM.

BrdU. BrdU (Sigma) was given via four i.p. injections of 50 mg/kg daily every 3 hrs for 3 consecutive days followed by cardiac perfusion 3 hrs after the final injection (Cao et al., 2004).

Immunochemistry of tissue sections. Adult mice were perfused with saline, then 4% paraformadehyde (PFA). Brains were harvested, post-fixed in 4% PFA, dehydrated with 30% sucrose in PBS, and embedded into OCT. 10 μm saggital sections were processed for antigen retrieval using 10 mM sodium citrate, pH 7.1 at 95°C for 20 min and cooled for 30 min (p-SMAD experiments). Sections were blocked with 10% goat serum for 1 hour. Primary antibodies diluted in PBS containing 1% BSA and 0.25% Triton X-100 were applied overnight at 4°C. Antibodies used include BrdU (mouse IgG2a 1:1000; Chemicon), GFAP (mouse IgG1, 1:400; Sigma), PSA-NCAM (mouse IgM, 1:500; Chemicon), phospho-SMAD1/5/8 (rabbit, 1:100 Cell Signaling Technologies), noggin (rat, 3 μm/ml, Regeneron), and green fluorescent protein (rabbit, 1:500, Molecular Probes). Primary antibodies were visualized with appropriate mouse or rabbit Alexa-Fluor-350/488/594/647 secondary antibodies (Molecular Probes). Nuclei were counterstained with Hoechst 33342 (Sigma). Sections stained for BrdU were pretreated with 2N HCl for 30 min and neutralized with Borax, pH 8.5, for 10 min before blocking. Cells were visualized using confocal laser microscopy (LSM 510 META, Zeiss) and counted as previously described (Bonaguidi et al., 2005).

Primary neurosphere cultures. Adult neurospheres were isolated according to published methodologies (Bull and Bartlett, 2005). Adult (2-4 months of age, 3-4 per group) male C57/BL6 mice were killed by cervical dislocation and their brains immediately removed. A 2-mm-thick coronal slice was cut between 1.22 and 2.70 mm, relative to bregma, according to an

adult mouse brain atlas (Paxinos and Franklin, 2001). Hippocampal and posterior SVZ (pSVZ) areas were dissected (see Fig. 1) from the caudal side and carefully trimmed of all white matter. Tissue samples were minced with scissors and enzymatically digested with 0.1% trypsin-EDTA (Sigma) for 7 min at 37°C. The digestion was quenched with 0.014% w/v trypsin inhibitor (type I-S from soybean; Sigma) in Hank's Buffered Saline Solution (HBSS). After centrifugation, the pellet was resuspended in 1ml serum-free medium (SFM) and mechanically triturated until smooth. The cells were filtered through a 70 μm cell sieve (BD Biosciences), centrifuged, resuspended in 100 μl SFM, and viable cells were counted on a hemocytometer using trypan blue (Sigma). The cells were plated at a density of 7000 cells/cm^2 in non-vacuum treated 24-well plates (BD Biosciences) with 1ml SFM per well. SFM consisted of DMEM:F12 medium, supplemented with N2, B27, 100 U/ml penicillin/streptomycin, glutamine (all from Gibco), and 2μg/ml heparin (Sigma). The following growth factors were also included: 20 ng/ml human recombinant EGF, 10 ng/ml mouse recombinant FGF-2 (both from BD Biosciences), and where denoted 250 ng/ml Noggin (R&D Systems). Primary hippocampal cells were incubated for 8 d, and pLV cells for 7 d, in humidified 5% CO2 to permit primary neurosphere formation. The neurospheres were then counted and collected for either passaging or immunocytochemistry.

Neurosphere passaging and differentiation. Neurospheres were dissociated as previously described (Bonaguidi et al., 2005). Spheres were harvested after 4-5 days, cells were counted and plated for self-renewal at a density of 1x10^4 cells/cm^2 in SFM plus EGF, FGF, and where denoted noggin. The cell number per sphere was calculated by dividing the cell number by sphere number. Secondary sphere formation was performed in 96-well plates as previously

described (Bonaguidi 2005). Spheres were plated for differentiation at passage 11. For passage 11 expansion, cells were plated at low density ($1x10^3$ cells/cm^2) in EGF, FGF, and noggin. Once cells had grown for 5 days, spheres were plated in SFM (without heparin) onto poly-D-lysine-coated (PDL, Sigma, 20 μg/ml for >1 hour) coverslips within 24-well culture plates, and allowed to differentiate until flattened and adherent for 5–7 d in humidified 5% CO2. This was achieved by plating with 1 ng/ml FGF for the first day and removal of FGF thereafter. In some cases, replacing media contained 20 ng/ml BMP4 (R&D Systems). The differentiated neurospheres were then fixed with 4% formaldehyde (Sigma) in 0.1 M PBS at room temperature for 15 min. After washing with PBS, cells were double stained for the neuronal pan-neurofilament (NF) and the 'astrocytic' marker GFAP with Hoechst counter stain (see below).

Immunochemistry of cultures. Primary neurospheres were processed in suspension. Spheres were fixed with 4% formaldehyde for 30min at room temperature, blocked with 10% goat serum for 1 hr, and primary antibodies applied overnight at 4°C. Antibodies were used at the same concentrations as described above with the addition of NF (rabbit, 1:4 Zymed). Primary antibodies were visualized using Alexa-488 and -594 secondary antibodies (Molecular Probes) applied for 5 hrs at room temperature. Adherent cultures were processed and all images acquired as previously described (Bonaguidi et al., 2005).

Morphometric and statistical evaluation. P-SMAD quantitation was performed using MetaMorph software version 6.3r5 (Molecular Devices). Confocal z-stacks acquired at 100x were flattened into 2.5 μm optical sections using LSM 510 Image Examiner (Zeiss) and imported into MetaMorph. Individual cells were traced in the GCL and SGZ and measured for signal

intensity. Average intensity in GCL cells was normalized to 1. For *in vivo* proliferation studies, Hoechst, BrdU, GFAP, and PSA-NCAM were acquired using 4-channel confocal microscopy. GFAP and PSA-NCAM were pseudo-colored red for easier visualization using Image Examiner software. All images were imported into Photoshop CS (Adobe) to prepare figures. Statistical evaluations for 2-group tests (unpaired, 2-tailed, student's t-test) and 3-group (ANOVA, with Bonferroni post-hoc test) were performed using Prism software (GraphPad).

Results:

Noggin allows for self-renewal of cells from the hippocampus.

Adult precursors proliferate in vitro in the presence of EGF and/or FGF to form floating cell clusters termed neurospheres (Gritti et al., 1996; Morshead et al., 1994; Reynolds and Weiss, 1992). Neural stem cells (NSC) are distinguishable from neural progenitor cells (NPCs) by the ability to self-renew, i.e. expand continually (Bull and Bartlett, 2005). GFAP-expressing cells from the anterior SVZ (aSVZ) and posterior SVZ (pSVZ), but not the hippocampus, give rise to neurospheres that continually expand (Bull and Bartlett, 2005; Gritti et al., 1999). We prepared adult EGF and FGF-responsive neurosphere cultures to investigate the effects of BMP inhibition on hippocampal and pSVZ cell self-renewal. Cells were isolated from the dorsal part of the hippocampus (Hip) or the lateral wall of the lateral ventricle (pSVZ) and cultured through multiple passages (Fig. 1A). As previously reported (Bull and Bartlett, 2005), cells isolated from the hippocampus failed to expand when cultured in EGF and FGF alone (Fig. 14B,C). However, noggin addition allowed hippocampal cultures to expand for 10 passages, the longest time

studied, with more than a 1000-fold increase in cell number (Fig. 14B, D). SVZ-derived neurospheres cultured without noggin expanded 7 times faster than noggin-treated hippocampal neurospheres (pSVZ: 16.9x, Hip: 2.35x), but noggin addition to pSVZ cultures did not further increase the expansion in cell numbers (Fig. 14B). Since the number of cells after passaging depends upon both cell self-renewal and proliferation, we counted the number of spheres as well as the number of cells in each sphere to define effects on the two processes. Noggin addition to primary hippocampal neurospheres had no effect on either the number of spheres that formed (0.04±0.01% vs. 0.05±0.02%; Fig. 15A) or the number of cells per sphere (206±72 vs. 257±171; Fig. 15B). However addition of noggin to secondary hippocampal neurospheres significantly increased both sphere number (0.45±0.08% vs. 1.23±0.11%, P=0.0072; Fig. 15C) and cells per sphere (193±74 vs. 1135±269, P=0.025; Fig. 15D). By contrast, addition of noggin to either primary or secondary pSVZ neurospheres had no effect on sphere number or on the number of cells per sphere (Figure 15). These findings indicate that BMP inhibition promotes both self-renewal and proliferation of hippocampal precursor cells *in vitro* but does not alter self-renewal or proliferation of cultured pSVZ precursor cells.

The presence of phosphorylated-SMAD1/5/8 in the nucleus (np-SMAD) is an indicator of BMP signaling within a cell. We therefore examined the cellular localization of np-SMAD in primary hippocampal and SVZ neurospheres to determine whether endogenous BMP signaling occurs in the cultured cells. Hippocampal neurospheres had strong np-SMAD staining in most cells, which was reduced by culturing in the presence of noggin (Fig. 16A-B). In contrast,

neurospheres from the pSVZ did not have strong np-SMAD staining (Fig. 16C) but rather displayed strong p-SMAD staining in the cytoplasm of some cells (Fig. 16D). Hence, BMP signaling occurs in hippocampal precursors but not pSVZ precursors *in vitro*.

BMP signaling inhibits multipotential differentiation of hippocampal precursors.

Neural stem cells possess the ability to generate both neurons and glia. However hippocampal precursors cultured in the presence of EGF and FGF alone generate only glia, and inclusion of additional factors such as BDNF is necessary to facilitate neuronal differentiation *in vitro* (Bull and Bartlett, 2005). Since we found that endogenous BMP signaling is responsible for limiting self-renewal by cultured hippocampal precursors, we asked whether it is also responsible for limiting neuronal lineage commitment. After 10 passages in the presence of noggin, hippocampal neurospheres were differentiated as intact spheres for 5-7 days. The majority of neurospheres (67±5%; Fig17. A, B, D) contained both neurons (neurofilament immunoreactive cells) and astrocytes (GFAP immunoreactive cells). Addition of BMP4 during differentiation significantly reduced neuronal differentiation (13±3%, P=0.010; Fig. 17C, D). Thus, noggin inhibition of BMP signaling maintains multipotentiality of hippocampal precursors as well as their ability to self-renew.

Noggin inhibits BMP signaling in SGZ precursor cells *in vivo*.

We next determined whether noggin is expressed in the SGZ *in vivo*, and whether altering levels of noggin expression influence hippocampal neurogenesis. To alter levels of noggin expression we utilized transgenic mice in which the neuron specific enolase promoter drives expression of noggin and a GFP cassette (NSE-Noggin) (Guha et al 2004; Bonaguidi et al, 2005). Transgene expression begins in the hippocampus of these animals when the GCL differentiates during postnatal development (Bonaguidi et al., 2005; Guha et al., 2004; Li and Pleasure, 2005). In wild-type (WT) mice, noggin is expressed in the adult GCL, SGZ, and hilus (Fig. 18A-C) indicating that it is appropriately localized to be involved in regulation of hippocampal neurogenesis. The distribution of noggin is similar in the NSE-Noggin mice, but levels are substantially increased (Fig. 18D-F).

Neurogenesis in the adult SGZ begins with activation of quiescent $GFAP^+$ precursors that give rise to a rapidly dividing progenitor pool (tAP: transient amplifying progenitors). Amplifying progenitors commit to PSA-$NCAM^+$ migratory precursors that differentiate into immature neurons, mature in the granule cell layer (GCL), and integrate into the hippocampus circuitry (for review see (Ma et al., 2005). We next sought to determine the relationship between proliferation and BMP signaling in the SGZ by examining BrdU incorporation and its relationship to np-SMAD staining. $BrdU^-$ cells in the GCL display strong np-SMAD staining whereas $BrdU^+$ cells, and specifically $BrdU^+GFAP^+$ cells, demonstrate reduced np-SMAD

($BrdU^-$: 1.00±0.02, $BrdU^+$: 0.71±0.01, $BrdU^+GFAP^+$: 0.58±0.04 Fig. 18G-K). Thus, reduced BMP signaling is associated with proliferation in the hippocampal DG *in vivo*.

To specifically test whether the level of BMP signaling determines the rate of cell proliferation in the SGZ precursor populations, we examined BrdU incorporation and its relationship to np-SMAD staining in NSE-noggin mice that overexpress noggin in the hippocampus (Fig. 19D-F). This approach allowed examination of proliferation of all of the major SGZ precursor populations: $GFAP^+$ precursors ($BrdU^+GFAP^+$), transient amplifying cells ($BrdU^+$, $GFAP^-$ and PSA-$NCAM^-$), and proliferative migratory precursors cells ($BrdU^+$PSA-$NCAM^+$) (Ming and Song, 2005). Noggin overexpression significantly increased the number of overall proliferative precursors (481±18 vs. 778±61 cells/mm^2, P=0.007; Fig. 19A-B,E), proliferative stem cells (104±4 vs. 229±28 cells/mm^2, P=0.010; Fig. 19A-B,E), proliferative transient amplifying progenitor cells (271±12 vs. 381±15 cells/mm^2, P=0.004; Fig. 19A-E), and proliferative migratory precursor cells (107±6 vs. 168±23 cells/mm^2, P=0.048; Fig. 19C-E). The increase in the number of $BrdU^+$ cells in each precursor population could reflect either enhanced proliferation of each cell type or increased proliferation of the $GFAP^+$ stem cells that then generated increased numbers of the other precursor proliferative pools. To distinguish between these two possibilities, we examined the proportion of proliferative cells in each precursor population. Noggin did not change the proportion of proliferative migratory precursors (22.1±1.2% vs. 23.1±0.8%, P=0.382; Fig. 19F). However, the proliferative $GFAP^+$ stem cell fraction in the transgenic animals was significantly increased (21.5±0.2% vs.

29.2±1.5%, P=0.006; Fig. 6F) at the expense of transient amplifying cells (56.4±1.4% vs. 47.6±1.4%, P=0.010; Fig. 19F). These results indicate that the effects of noggin on proliferation in the SGZ occur primarily on the $GFAP^+$ stem cells that then, in turn, generate more proliferative transient amplifying and migratory precursor cells. More generally our observations indicate that the rate of neurogenesis in the hippocampus is regulated by the level of BMP signaling in a $GFAP^+$ stem cell population.

Discussion:

This work demonstrates that the adult hippocampus contains a stem cell population, and that noggin inhibition of BMP signaling regulates hippocampal stem cell proliferation and ultimately neurogenesis. We demonstrate that BMP signaling occurs endogenously in neurospheres derived from hippocampal precursors and that blocking BMP signaling with noggin is sufficient to foster hippocampal cell self-renewal, expansion, and multipotentiality *in vitro*. This study directly recapitulates the finding by Bull & Bartlett (2005) that hippocampal precursors do not self-renew when cultured in EGF and FGF alone, and both studies support similar observations by Seaberg and van der Kooy (2002). Some earlier studies suggested that hippocampal stem cells can be expanded in mitogen alone or mitogen plus serum (Gage et al., 1995; Palmer et al., 1997). However, serum contains BMPs, and BMPs and serum each prevent expansion of hippocampal but not SVZ stem cells, so it seems likely that the earlier studies included cells from the adjacent pSVZ.

Unlike hippocampal stem cells, pSVZ precursor cells grown as neurospheres do not exhibit much endogenous BMP signaling, and noggin addition does not significantly alter their self-renewal or expansion. Further, pSVZ precursors expand at a more rapid rate than the self-renewing hippocampal precursors *in vitro*. The initial decline in hippocampal precursors likely reflects the coarse hippocampal dissection compared to the fine pSVZ dissection since the primary cultures begins with both precursors and more committed cells. Moreover, the reasons for the differences in expansion rate are unclear, but may result from differences in the niches occupied by the cells *in vivo*. Indeed, this study demonstrates that niche components are critical for interpreting data regarding cell identity. As the roles of extrinsic factors in regulating self-renewal, proliferation, fate commitment, and differentiation are elucidated, *in vitro* assays can better reflect their *in vivo* counterparts. This is particularly relevant for studying neuronal differentiation from adult hippocampal precursors. *In vivo*, SGZ precursors preferentially commit to neurons instead of glia. Instead, *in vitro* hippocampal precursors preferentially differentiate into astrocytes (Bull and Bartlett, 2005; Seaberg and van der Kooy, 2002). We demonstrated that endogenous BMP signaling is responsible, at least in part, for the bias of cultured hippocampal stem cells towards astrocytic differentiation since addition of noggin enhanced neuronal lineage commitment. However glial differentiation predominated even in the presence of noggin, indicating that other factors are responsible for the differences in the behavior of the stem cells in vivo and in vitro. While EGF and FGF may reprogram precursors *in vitro* (Anderson, 2001; Doetsch et al., 2002), it is also likely that critical niche components that promote neurogenesis and/or inhibit gliogenesis are missing. Such factors have only been

partially defined but include positive regulators of neurogenesis including BDNF, Wnt3, and Ca^{2+}-signaling (Bull and Bartlett, 2005; Deisseroth et al., 2004; Lie et al., 2005; Louissaint et al., 2002) and inhibitors of negative regulators such as noggin and neurogensin (Lim et al., 2000; Ueki et al., 2003).

Noggin and BMP inhibition are critical components of the adult neurogenic niche *in vivo.* Noggin not only promotes neuronal commitment by SVZ stem cells (Lim et al., 2000), but also increases proliferation and precursor number in the SGZ (Bonaguidi et al., 2005; Fan et al., 2004). We demonstrate in this study that in the adult SGZ, this effect occurs on the stem cells themselves, which in turn increase the proliferative pools of transient amplifying progenitors and migratory precursors. It seems likely that noggin promotes self-renewal by proliferative cells in the SGZ in vivo as it does *in vitro*, but it is also possible that noggin increases the activation rate of quiescent stem cells. Several other regulators of adult precursor proliferation have been identified (for review, see (Hagg, 2005; Lehmann et al., 2005) that help modulate the rate of neurogenesis in the hippocampus. However the profound effects of noggin overexpression in the hippocampus (this study and Bonaguidi et al, 2005) suggest that BMP signaling is a key regulator of the rate of neurogenesis. It is therefore a good candidate for molecular interventions to define the functional roles of adult neurogenesis in the hippocampus. Since neurogenesis is correlated with hippocampus-dependent learning, and may be involved in depression and epilepsy (Gould et al., 1999; Parent et al., 1997; Raber et al., 2004; Santarelli et al., 2003; Shors et al., 2001; van Praag et al., 2002), pharmacologic manipulation of BMP signaling in the hippocampus could potentially exert profound effects on hippocampal function and behavior.

CHAPTER 4: DIFFERENTIATED CELLS ARE LESS EFFICIENT THAN PROGENITOR CELLS FOR CLONING BY SOMATIC CELL NUCLEAR TRANSFER

Abstract:

During somatic cell nuclear transplantation (SCNT) a mature differentiated state is reprogrammed back to an uncommitted totipotent or pluripotent state. Though reprogramming does occur, this process is extremely inefficient for currently unknown reasons. One major factor is the commitment state of the donor cell. Conflicting reports have suggested that as differentiation hierarchy progresses, reprogramming efficiency increases or decreases pending the lineage studied. We have recently defined a neural lineage where embryonic neural precursor cells (eNPC) are committed to an adult precursor cell (aNPC) state via LIF signaling/BMP inhibition and to a mature astrocyte state via BMP signaling. We therefore used these cells as nuclear donors to test that hypothesis. We find that as cells become more committed, reprogramming efficiency declines.

Introduction:

The remarkable ability to remodel chromatin normally occurs during fertilization. Oocytes can also reprogram somatic cell chromatin via nuclear transplantation (NT), but at an inefficient rate. Development of NT-derived blastocysts into embryonic stem cells (ESC) or cloned animals is heavily influenced by the differentiation state of the donor genome (Kato 2000). In murine

models, the efficiency of ES cell derivation from NT-derived blastocysts is the worst when using well-defined differentiated donor cells, such as lymphocytes (Hochedlinger and Jaenisch, 2002) and neurons (Eggan et al., 2004; Li et al., 2004), slightly better for fibroblast or neural stem cell (NSC) populations (Blelloch et al., 2006; Wakayama et al., 2005), and best for ESC and their tumorigenic counterpart, embryonal carcinoma cells (Blelloch et al., 2004; Wakayama et al., 2001). Further, development after NT is the most efficient using blastomeres from earlier development (Ono and Kono, 2006). However, recent reports using neural and hematopoeitic lineages propose that cloning efficiency increases with differentiation. Neural progenitor cells (NPCs) were less likely than their progeny to foster NT-derived embryo and fetal development (Mizutani et al., 2006). Also, a well-defined hematopoietic lineage demonstrated that as differentiation hierarchy progressed from adult hematopoietic stem cells to progenitor cells to granulocytes, development after NT increased (Sung et al., 2006). As a result, it is currently unclear how the donor epigenetic state affects reprogramming.

The low efficiency of development following NT is, in part, due to faulty global reprogramming, leading to defects in early embryonic gene expression and aberrant expression of donor cell genes (Gao et al., 2003a; Humpherys et al., 2002). During normal development, early embryos undergo a well-orchestrated series of DNA methylation and histone modification changes that are believed to play an important role in establishing a chromatin state permissive to early embryonic gene expression. In contrast, NT-derived embryos typically show abnormal patterns of DNA methylation and histone modifications, which are consistent with the donor epigenome (Bourc'his et al., 2001; Dean et al., 2001; Kang et al., 2001; Kang et al., 2002; Santos

et al., 2003; Sung et al., 2006). In particular, *Pou5f1 (Oct3/4)*, a gene essential for pre-implantation lineage specification and the production of ES cells, failed to be re-expressed in a large number of somatic clones (Boiani et al., 2002; Bortvin et al., 2003). This error is likely due to improper demethylation of the Pou5f1 promoter (Hattori et al., 2004; Simonsson and Gurdon, 2004). Therefore, Pou5f1 serves as an important indicator of proper reprogramming.

These data suggest that cellular differentiation influences the epigenetic state of the donor cell nucleus, and affects the efficiency at which an enucleated oocyte can reprogram a donor cell into a pluripotent ESC fate. We tested a defined neural lineage to address whether progressive differentiation positively or negatively influences reprogramming. Pluripotent ESC commit through multiple stages to a multipotential embryonic neural precursor cell (eNPC) state. These cells are then maintained, but further committed into the adult as adult neural precursor cells (aNPC). Finally, eNPC differentiate into mature astrocytes (along with neurons and oligodendrocytes) (Bonaguidi et al., 2005). We demonstrate that NT-derived embryos from eNPC donors develop with higher efficiency than those derived from astrocytes. However, NT-derived blastocysts and primary outgrowths from eNPC donors possess variability among Pou5f1 expression and donor-specific traits indicating incomplete reprogramming. These data suggest that more differentiated neural cells are harder to reprogram, and that complete reprogramming is difficult to achieve.

Methods and Materials:

Animals. B6 (C57BL/6J), D2 (DBA/2), B6D2F1 (C57BL/6J x DBA/2), and OG2 (Pou5f1 promoter driving EGFP on B6;CBA background (Szabo et al., 2002) mice were purchased from Jackson Laboratories. Time-pregnant CD1 females were purchased from Charles River Laboratories. All experimental animal procedures were approved by the Institutional Animal Care and Use Committees of Northwestern University.

ES cell lines and culture conditions. The ES cell line R1 (Nagy et al., 1993), derived from 129/Sv x 129/Sv-CP, was provided by Dr. Rex Chisholm at passage 18. The monoclonal red fluorescent protein (mRFP) cassette (gift from G. Smith) was placed in the pcDNA3.1 expression plasmid (Invitrogen) under the control of a CMV promoter and electroporated into R1 ESC produce stable cells lines (Otero et al., 2004). Cells in these experiments ranged from approximately passage 25 to 35. R1 ES cells were cultured on 0.1% gelatin in Dulbecco modified Eagle medium (Gibco) supplemented with 20% heat-inactivated fetal calf serum (Hyclone), 1000 U/ml of leukemia inhibitory factor (Chemicon), 2 mM L-glutamine, 1% minimum nonessential amino acid solution (Gibco), and 1% ß-mercaptoethanol (Sigma). Embryonic bodies were derived as previously published (Otero et al., 2004). Embryonic neural precursor cells (eNPC) were harvested from postnatal (PN) day 0-2 and grown as previously described (Bonaguidi et al., 2005). ENPC from OG2 mice were genotyped using primers to EGFP (Jackson Labs). Adult neural stem cells (aNPC) and astrocytes were produced as previously described (Bonaguidi et al., 2005). Briefly, neurospheres were dissociated and plated

on PDL for 7 days. 20 ng/ml LIF and 250 ng/ml noggin committed cells to aNPC and 20 ng/ml BMP4 differentiated precursors into astrocytes.

Donor cell preparation. One night before nuclear transfer (NT) experiments, the serum concentration on ESC was reduced from 20% to 5% to achieve 80-90% confluency the following day irrespective of ESC line used. Neurospheres were split the morning before NT experiments to produce spheres of 4-16 cells. For NT, cell types: ESC, eNPC, aNPC, and astrocytes were dissociated with 0.25% trypsin, quenched with serum or trypsin inhibitor, centrifuged, and resuspended in 10% polyvinyl pyrrolidone (PVP) (for ESC) or 3% PVP (eNPC, aNPC, astrocytes) in H-CZB. Cells were put on ice until loaded on the manipulation table.

Nuclear Transfer. Nuclear transfer was performed according to the method reported in (Wakayama et al., 1998) with a slight modification (Gao et al., 2003b). Female BDF1 mice (8–14 weeks old) were superovulated by sequential injection of 7.5 international units (IU) of pregnant mare serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG). Oocytes were collected in M2 medium 13–14 h after the administration of hCG. Cumulus cells were removed from the oocytes by treatment with 0.1 mg/ml hyaluronidase. After a brief culture in CZB medium, groups of 15-25 oocytes were transferred to H-CZB medium containing 2.5 µg/ml cytochalasin B (CB). The spindle chromosome complex (SCC) was removed using a pipette with an inner diameter of 8–10µm assisted by piezo-drill pulses. For nuclear transfer using ESC as nuclear donors, a small cell (<10 µm) was drawn in and out of the injection pipette until its plasma membrane was broken and was then injected into enucleated oocytes. For the neural

cells, live cells with a diameter of 7–9 μm were selected. The reconstructed oocytes were cultured in CZB medium for 1–3 h before activation treatment. The reconstructed oocytes were activated in calcium-free CZB medium containing 10 mM strontium and 5 μg/ml CB for 6 hr. Cloned embryos were then cultured in CZB (Gao et al., 2003b) for 4-5 days. The embryos developed to the morula or blastocyst stage and were plated for primary outgrowth formation (ES derivation) or processed for immunofluorescence. Parthenotes were created by activating oocytes in a similar manner.

ES cell line derivation. Derivation of the Pou5f1-1 ESC line followed the methodology of Bryja and Bonilla et al. with modifications (Bryja et al., 2006). To parallel *in vitro* conditions for cloned embryo culture, fertilized zygotes were collected 30 hrs post-hCG from B6 females mated with OG2 males and placed into CZB media. After culturing pre-implantation embryos for 4 days, blastocysts (Blasts) with a green inner-cell mass (ICM) were transferred to 4-well plates (BD) containing mouse embryonic fibroblasts (MEFs) in serum replacement media (20% SR). This media had the same composition as R1 ES media, but used knockout DMEM and knockout serum replacement (Gibco) instead of DMEM and fetal calf serum, respectively. Blasts attached, hatched, generated primary outgrowths, and were allowed to grow for 6 days. Primary outgrowths were dissociated enzymatically (2.5% Trypsin) and mechanically (fire-polished pipette) and re-plated on fresh MEFs in 20% FCS medium. This media was the same as 20% SR, but contained fetal calf serum. The following day, the media was changed to 20% SR, and colonies grew for 4-5 days. Continued passaging was performed by dissociating cells with 0.25% trypsin, plating on MEFs in 20% FCS for 1 day, followed by growth on MEFS in 20% SR

for 1-3 days. Pou5f1-1 ESC were expanded to 10cm dishes and frozen at passages 6-8. MEFs were obtained from embryonic day 13.5 (E13.5) mouse embryos as described before (Hogan et al., 1986) and were used up to passage 2. MEF feeders were mitotically inactivated by treatment with mitomycin C for 2 hours (10 μg/ml, Roche) or overnight (1 μg/ml). After mitomycin C inactivation, cells were washed with phosphate-buffered saline (PBS), trypsinized and seeded (75,000 cells/cm^2) into gel-coated plates. MEFs were incubated in 20% FCS for 2-3hrs before adding ESC.

Immunochemistry of Embryonic Stem Cells and Cloned Embryos. Cloned embryos had the zona pellucida removed with Tyrode's Acidic Solution (Sigma) for 30 sec. They were fixed with 2% paraformadehyde in PBS for 30 min. After washing three times in a blocking solution of PBS containing 10% normal donkey serum (NDS), 0.1% Triton X-100, cloned embryos were stored at 4°C until processed. ESCs were plated on gel-coated coverslips. Cells were fixed with 4% formaldehyde in PBS for 15 min. After washing 2 times in PBS, cells were blocked with NDS for 30 min. Primary antibodies diluted in PBS containing 1% BSA and 0.25% Triton X-100 were applied overnight at 4°C. Antibodies used included OCT3/4 (goat polyclonal, 1:100, Santa Cruz), Nestin (mouse IgG1, 1:500; BD Pharmingen), Nucleostemin (goat polyclonal, 1:200, R&D), and green fluorescent protein (rabbit, 1:500, Molecular Probes). Primary antibodies were visualized with appropriate mouse, rabbit, or goat Alexa-Fluor-488 or -594 secondary antibodies (Molecular Probes). Nuclei were counterstained with Hoechst 33342 (Sigma). Labeled embryos and cells were viewed using a Zeiss Axiovert microscope as previously described (Bonaguidi et al., 2005).

Results:

Cloning efficiency with eNPC and astrocyte donors

We compared the cloning efficiency for pre-implantation development *in vitro* using eNPCs and astrocytes as donor cells for SCNT. We isolated eNPCs from small neurospheres and astrocytes from BMP4-differentiated eNPCs. These cell populations constitute multipotential and unipotential steps, respectively, within a neural lineage that is well characterized according to differentially expressed genes (Fig. 20). Only viable cells with healthy, smooth and intact membranes were selected as nuclear donor cells for SCNT. Pre-implantation development of cloned embryos reconstructed with different neural cells is shown in Table 3. Embryos reconstructed with eNPC donors develop to at least the blastocyst stage, while astrocyte-derived embryo development ceases at the 8-cell stage. The SCNT cloning efficiency with eNPC to the blastocyst stage is 14.1% and to the morula stage are 28.1% of activated reconstructed oocytes (Table 3, Fig. 21A). To further address reprogramming, we analyzed gene expression of eNPC-derived morulae and blastocysts 5 days after SCNT by immunofluorescence for the inner cell mass (ICM) marker Oct4 and eNPC marker Nestin. Control parthenotes at the expanded blast stage possess nuclear Oct4 in a couple cells; meanwhile hatched parthenotes have an increase in the number of $Oct4^{+}$ cells suggesting ICM expansion. These embryos lack significant nestin staining (Fig. 21C). Meanwhile, SCNT embryos from eNPC donors do not hatch with regularity and vary from morula to expanded blast stages. While the expanded blast is largely similar to the parthenote control at the same stage, some early blasts lack nuclear Oct4 and morula express

nestin (Fig. 21B). These results suggest that embryos derived from eNPC donors have a wide range of phenotypes. In some cases, reprogramming occurs properly, while others either fail to turn off the donor epigenome or turn on the embryonic epigenome.

Nuclear Transfer with donors from Pou5f1-gfp mice

Oct4 is essential for ICM lineage specification, the production/maintenance of ESC, and serves as a good indicator of reprogramming (Boiani et al., 2005; Nichols et al., 1998; Niwa et al., 2000; Niwa et al., 2005). We therefore used the Pou5f1-gfp (OG2) mouse as a source of cells for SCNT and ESC derivation. In this system, pre-implantation embryos and ESC fluoresce green (Fig. 22A-B) and can be analyzed using time-lapse video recording without destroying the cells. We therefore created a new ES line to serve as a low-passage donor cell source and as a positive control for ESC derivation rates. This ESC line, Pou5f1-1, expresses Oct4 and nucleostemin, markers of ESC, and presents normal karyotype (Fig. 22C-E). The line forms embryoid bodies (EB) and downregulates gfp expression upon differentiation (Fig. 22F). Thus, the Pou5f1-1 line exhibits ESC characteristics. Since eNPC, but not astrocyte, donors for SCNT allow development to the blastocyst stage, we isolated eNPC from OG2 animals to use for SCNT. Surprisingly, the SCNT cloning efficiency from OG2 donors to the blastocyst stage is 1.6% compared to 14.1% with CD1 donors, both using eNPC (Table 4). The OG2 genetic background is the inbred strain C57/Bl6 (B6), while the CD1 donors are an outbred strain. The genetic background influences pre-implantation development after SCNT with inbred strains fairing worst (Eggan 2001). Therefore, we mated OG2 mice to DBA/2 mice to create B6D2F1 hybrids,

which have been shown to foster better development. ENPC derived from this background do not promote significantly greater development after SCNT than on the B6, with a 3.1% blastocyst formation rate (Table 4). Moreover, morulae and blastocysts express gfp ectopically (Fig. 4A-B). Some blastocysts are able to generate primary outgrowths for ESC formation, but downregulate gfp expression (Fig. 23C). To further demonstrate we could generate cloned blastocysts with proper morphology from donor-labeled cells, we used R1 ESC with mRFP driven by a CMV promoter (R1-mRFP). Using R1-mRFP donors for SCNT, highly variable development still occurs, but expanded blastocysts with proper morphology and red fluorescence can be produced (Fig. 23D-E). Hence, reprogramming using donors from OG2 mice is less efficient than from CD1 counterparts.

Discussion:

The results described in this paper show that the differentiation status of the donor cell nucleus can strongly influence the efficiency of deriving blastocysts, but that this rate may not accurately reflect overall genetic reprogramming. Cloned morulae and blastocysts from eNPC donors exhibit a wide range of molecular phenotypes from apparently normal blastocyst characteristics to aberrantly low expression of pre-implantation genes or high expression of donor cell genes. These molecular traits may more accurately represent the true variability and inefficiency that results from somatic cells and the NT process. This inefficiency is even greater for cells from OG2 donors for currently unknown reasons. Besides epigenetic profiling (Boiani et al., 2002; Bortvin et al., 2003; Humpherys et al., 2002; Humpherys et al., 2001; Santos et al., 2003),

reprogramming is functionally measured using (a) the rate of blastocyst formation, (b) the fraction of survival to birth or to adulthood after implantation of totipotent NT-derived embryos, and (c) the frequency of pluripotent ES cell derivation from cloned blastocysts in culture. These different assays, the technical demands of performing NT, and the lack of standardization make it difficult to compare studies amongst groups. Therefore, only published reports that investigated donor cells within a non-germ cell lineage will be considered below.

Pre-implantation development rates have been compared for hematopoietic and neural lineages. Long-term hematopoietic stem cells (LT-HSC) provide less efficient rates than do short-term HSC (ST-HSC), hematopoietic progenitor cells (HPC) and differentiated granulocytes, respectively (Sung et al., 2006). One study with neural progenitor cells (NPC) and differentiated neural cells observed the same, that more committed cells yielded better development after NT (Mizutani et al., 2006). This contrasts with other reports that found the opposite [This study and (Yamazaki et al., 2001)]. These differences are not likely influenced by genetic background since both studies (Mizutani et al., 2006) used B6D2F1 donor cells. One possibility for the differences, though, is the age of the donor cells. Yamazaki et al. used progenitors from earlier neural development as donors (E15.5-E17.5) than in the study by Mitzutani et al. (PN0-3) and this study (E18.5-PN3). However, differences in the age of donor cells likely are not a strong contributor to observed phenotypes since this study and Mitzutani et. al used similar age donors. Another proposed reason for the differences among groups is the donor cell-cycle stage. Cells in S-phase do not foster development after NT and rapidly dividing cells, such as neural progenitors, are more likely to be in S-phase (Campbell et al., 1996). This

aspect again does not accurately reflect observations amongst these studies because (a) HSC are quiescent cells and (b) progenitors in the Mitzutani et al study are less likely to be in S-phase than this study. Finally, the cellular niche (culture environment) effects NT-derived embryo development rates, but all three studies used the same medium (Boiani et al., 2005; Chung et al., 2002). Hence, reasons for differences in pre-implantation rates are currently unclear and other functional tests are necessary to infer differences in reprogramming.

Development of implanted clones to term is the functional test of totipotency. The reconstructed oocyte is forced to generate every cell in the epiblast proper and extraembryonic lineage. Indeed, the test is so rigid that few NT-derived embryos survive to term. As a result, numbers are too low to make meaningful numerical comparisons. For example, the only study to directly compare donors within a somatic lineage is Mitzutani et al. NPCs and differentiated neural cells produce pups at less than a 1% rate. Pups from NPCs survived, while the two pups from differentiated cells did not. Therefore, the authors concluded that NPCs are more easily reprogrammed (Mizutani et al., 2006). As an alternative, the functional test of ESC-formation rates may be more plausible to demonstrate pluripotency (Blelloch et al., 2006; Eggan et al., 2001; Wakayama et al., 2005). Further, ntES are currently proposed as functionally equivalent to ESC derived via fertilization (Brambrink et al., 2006; Wakayama et al., 2006b). However, currently no single study has directly compared pluripotent formation rates for a lineage. Rather, only when comparing two studies by different investigators do NSC form ESC at a higher rate than neurons (Blelloch et al., 2006; Eggan et al., 2004). Future studies designed to directly test embryos created by NT from defined lineages for molecular characterization, ESC formation, or

even mid-gestation development (Yamazaki et al., 2001) may provide insight into the efficiency of reprogramming somatic donor cells. Then again, it is conceivable that differences in reprogramming less and more differentiated cells may not exist in murine models (Boiani et al., 2005) as they do in amphibian models (Gurdon et al., 2003).

CHAPTER 5: GENERAL DISCUSSION

Regulation of Astrocyte Commitment

Fate determination genes promoting neuronal and oligodendrogial (OL) are well understood. For example, bHLH (basic-helix-loop-helix) genes of the Mash, Math, and neurogenin (Ngn) families actively promote neuronal commitment. Meanwhile, bHLH genes of the Olig family actively promote OL commitment [for review see (Kageyama et al., 2005)]. However, the role of repressor-type bHLH genes in astrocyte commitment is unclear. The hairy and enhancer of split (Hes), Hes-related (Hesr), and inhibitor of differentiation (Id) families prevent commitment to neuronal and OL fates. For instance, Hes1 antagonizes Mash1 by two different mechanisms: repressing the expression at the transcriptional level and inhibiting the activity at the protein–protein interaction level (Chen et al., 1997; Sasai et al., 1992). Also, ID1 and ID3 inhibit Mash1 and Ngn to block neuronal commitment, while ID2 and ID4 inhibit Olig1 and Olig2 to block OL commitment (Nakashima et al., 2001; Samanta and Kessler, 2004; Vinals et al., 2004). Still, these inhibitors do not necessarily actively promote astrocyte fate, but instead can maintain a precursor state. Importantly, neural stem cells through most of embryonic development and into adult have glial characteristics.

The proposed discrepancy between NPC maintenance and promotion of glial commitment is exemplified by the Notch and LIF/CNTF signaling pathways. Activation of the receptor Notch induces signaling both dependent and independent of the Hes family: Hes1,

Hes3, Hes5, Hesr1 and Hesr2 (Iso et al., 2003). Notch1, Notch3, and Notch signaling have been found to increase NPC numbers and decrease both neuronal and glial commitment (Androutsellis-Theotokis et al., 2006; Hitoshi et al., 2002). Also, Hes1 gain of function increases NPC numbers and decreases differentiation (Ishibashi et al., 1994). Loss-of-function studies also support a role for Hes family members in progenitor maintenance and neuronal suppression both in vivo (Baek et al., 2006; Hatakeyama et al., 2004; Ishibashi et al., 1995; Sakamoto et al., 2003) and in vitro (Ohtsuka et al., 1999). Meanwhile, Notch signaling can also promote glial identity. Notch1 promotes a radial glia phenotype in the cortex (Chambers et al., 2001; Gaiano et al., 2000) and expression of Hes1 or Hes5 promotes Mueller glia commitment in the retina (Furukawa et al., 2000; Hojo et al., 2000). Additionally, loss of Hes1/Hes3/Hes5 function differentiates cortical radial glia into neurons (Hatakeyama et al., 2004). Further, Notch1 and Notch3 induce GFAP expression (Tanigaki et al., 2001), presumably through the direct interaction between the Notch intracellular domain (NICD) and human GFAP promoter (Ge et al., 2002). Interestingly, in all the studies Notch signaling or its effectors either (a) induce glia that retain the ability to generate neurons (b) induce GFAP expression without demonstrating astrocyte differentiation, or (c) increase colony size or NPC maintenance. Therefore, it is likely that Notch signaling promotes both progenitor maintenance and glial identity.

We demonstrated that LIF signaling induces a $GFAP^+$ stem cell that maintains the ability to self-renew and generate neurons [Chapter 2, (Bonaguidi et al., 2005)]. Canonically, LIF signals through STATS, specifically STAT3, to promote GFAP expression and glial identity (Bonni et al., 1997). Currently, LIF and STAT3 have not been shown to promote cell cycle exit

and mature astrocyte differentiation. In fact, we found that LIF signaling in the absence of BMP signaling increases proliferation in addition to promoting a NPC state (Bonaguidi et al., 2005). Intriguingly, cross talk exists between the LIF and Notch pathways. For example, Hes1 and Hes5 proteins bind to STAT3 directly, thereby inducing phosphorylation and activation of STAT3 by recruitment of JAK2. Hes proteins may therefore function as non-receptor scaffold proteins that allow JAK2 to phosphorylate STAT3 with enhanced effectiveness. Further, the ability of Hes5 to increase GFAP and induce a radial glia phenotype is dependent upon STAT3 (Kamakura et al., 2004). Thus, Notch signaling is mediated in part by LIF signaling at the level of STAT3. It is therefore possible that both Notch and LIF signaling maintain a NSC state throughout development into adulthood, which progressively gains glial characteristics. In support of this theory, LIF or Notch 1 overexpression in the aSVZ promotes a quiescent stem cell fate in the adult at the expense of generating progeny (Bauer and Patterson, 2006; Gaiano et al., 2000).

In contrast, BMP signaling promotes a mature astrocyte fate defined by cell-cycle exit, loss of NPC characteristics, and the inability to self-renew or to generate neurons. This is the first distinction made between two signaling cascades regarding glial identity and progenitor maintenance. Both cascades induce glial identity, but LIF signaling promotes maintenance of the NSC pool, while BMP signaling depletes it (Bonaguidi et al., 2005). It also serves as a paradigm for investigating active inducers of astroglial commitment. For example, BMP signals through Smads to activate gene transcription. In addition, BMPs induce the ID family of bHLH repressors. Id4 and to a lesser extent Id2 inhibit Olig1/2 to promote astrogliogenesis from NPCs

(Samanta and Kessler, 2004). It is therefore currently unknown whether the Ids or Smads are also capable of actively promoting an astrocyte fate. Also, performing differential gene profiling experiments for targets of LIF and BMP signaling should provide candidates genes that actively induce an astrocyte fate. These studies are currently ongoing in the Kessler Lab.

Recently, the first astroglial inducing genes have been characterized. The Nuclear Factor I (NFI) family of site-specific DNA-binding proteins (also known as CTF or CCAAT box transcription factor) functions both in viral DNA replication and in the regulation of gene expression. The family has four different genes (NFI-A, B, C, and X) that generate multiple isoforms by alternative RNA splicing (Gronostajski, 2000). NFIA and NF1B seem to have two roles in coordinating the transition from neurogenesis to gliogenesis in the spinal cord: (1) a permissive requirement for the inhibition of neurogenesis to maintain an undifferentiated NPC pool, and (2) the instructive promotion of a glial differentiation program (Deneen et al., 2006). During neurogenic periods, NF1A reduction by RNAi promotes loss of NPCs and commitment to the neuronal lineage. Interestingly, misexpression of Hes5 or of Notch ICD is sufficient to rescue this NFIA-RNAi phenotype, as well as to rescue the expression of progenitor markers. Importantly, however, misexpression of Notch effectors does not rescue the requirement of NFIA for induction of GLAST (glial identity). Later in development, misexpression of NFIA or NFIB is sufficient to accelerate GFAP expression in NPCs by several days and foster migration away from the VZ. In contrast, NF1A depletion reduced GFAP expression without changing the number of astrocytes (as measured by the expression of NFIB and NFIA, respectively). Further, Olig2 antagonizes the astrocyte inducing effects of NF1A (Deneen et al., 2006). Many questions

result from this study. Are NF1A/B involved in gliogenesis elsewhere in the CNS beyond the spinal cord? Does NF1A/B mediate Notch, LIF, or BMP signaling? How are the pathway effectors involved? Since NFI-binding sites have been found in the GFAP promoter (Cebolla and Vallejo, 2006) do these signaling pathway show convergence? Are the pro-glial effects of NF1A/B reversible? These questions may help to define the mechanisms regulating the conversion from glial NSC to mature astrocytes and whether this is reversible.

BMP Signaling in Adult Hippocampal Neurogenesis and Functional Correlates

Adult neurogenesis is the complicated process by which new neurons are specified from uncommitted progenitor cells and terminates with functional integration into a preexisting circuitry. This process occurs in the SVZ aligning the lateral ventricular wall and SGZ of the dentate gyrus (DG) in the hippocampus. Neurogenesis in these regions is categorized into steps including progenitor proliferation, lineage commitment, migration, survival/maturation, and functional integration (for review, see Ming and Song '05). Each step can be regulated by numerous identified factors including growth factors, morphogens, chemokines, neurotransmitters, hormones, and neurotrophic factors (for review, see Haag '05). In particular, BMP signaling may be involved in different steps of neurogenesis. We demonstrated in Chapter 3 that BMP signaling regulates progenitor proliferation. Specifically, noggin increases the GFAP-expressing stem/progenitor cell proliferation that in turn increases overall NPC number (Fig. 19). BMP signaling also plays a role in lineage commitment by promoting astrocyte and not neuronal differentiation. This fate can be ameliorated by noggin administration (Lim 2000).

Intriguingly, BMP signaling is present in the majority of GCL cells as identified by p-Smad1/5/8 and staining intensity increases upon stage of differentiation (Fig. 18). Therefore, BMP signaling may be involved in neuron subtype specification and/or neuron survival/maturation. For example, in the enteric nervous system (ENS) development BMP4 promotes the development of Trk-C expressing neurons and their dependence on neurotrophin-3 for survival. When noggin is misexpressed, Trk-C neurons are decreased in number while other neuron pools are increased due to a lack of apoptosis (Chalazonitis et al., 2004). Similarly, misexpression of noggin in the skin increases the number of neurons in the trigeminal and dorsal root ganglia by preventing apoptosis. Strikingly, the density of innervations in the skin is dramatically increased without a change in the levels of neurotrophins (Guha 2004). Since many developmental mechanisms are conserved in adult neurogenesis (Lledo et al., 2006)) and BMP signaling is evident in immature ($BrdU^+PSA\text{-}NCAM^+$) and mature ($NeuN^+$) neurons, BMP signaling may regulate neuronal subtype and promote dependency on neurotrophins in the adult DG. In further support of this hypothesis, noggin and brain derived neurotrophic factor (BDNF) synergistically act to increase GABAergic medium spiny neuron production and survival in the adult caudate putamen (Chmielnicki '04). Thus, BMP signaling may have effects on neuron survival/maturation independent of its role in precursor proliferation and fate decisions.

In the DG, the network primarily determines cell survival of newborn neurons. In this system, granular neurons mediate synaptic transmission from glutamatergic inputs in the entorhinal cortex and GABAergic inputs in local interneurons through mossy fiber outputs onto

the CA3 region pyramidal cells. It has been shown that a compensatory network is capable of pattern recognition without the inclusion of a Hebbian synapse (Cromme and Dammasch, 1989). If left undisturbed, its structural dynamics develop to a stable state in which all neurons attain their homoeostatic range; if disturbed by an external afference, it re-adjusts all synaptic weights until balance is again reached (Butz et al., 2006). In the case of neurogenesis, the two main systemic influences on neuronal survival are the number of neurons born from NPCs and the input to them from the entorhinal cortex and local interneurons (Lehmann et al., 2005). As such, it is not surprising that neurotransmitters have profound effects on neuron survival and NPC proliferation [for reviews, see (Ge et al., 2007; Hagg, 2005)]. Also, any environment that challenges the system with novelty increases neuron survival (Kempermann, 2002). These include enriched rearing and housing, spatial learning tests, and mossy fiber stimulation (Gould et al., 1999; Kempermann et al., 1997; Nilsson et al., 1999).

What then is the purpose for adult neurogenesis? From a system level, new neurons buffer the system from too much input, or foster avoidance of catastrophic interference (Wiskott et al., 2006). For example, neurogenesis increases in response to temporal lobe epilepsy and strong Perforant Pathway input. This is due to a dramatic amplification of NPC and neurons are rapidly added to the GCL (Parent et al., 1997). However, over time this response is unsustainable and leads to wide spread cell death (Magloczky et al., 2000)}. Therefore, neurogenesis may be able to act as a compensatory mechanism for responding to environmental stimuli.

Also, little doubt exists that cell proliferation in the DG at least contributes to or is even necessary for some hippocampal memory functions (Monje et al., 2002; Raber et al., 2004; Shors et al., 2001). New neurons integrate into the DG, which has the role of pattern separation, instead of memory storage as the CA3 possesses. Many hypotheses exist regarding how the hippocampal circuit functions in memory formation, storage and retrieval, but the idea that the dentate gyrus provides distinct codes to the network via the granule cells' mossy fibers has been one of the least controversial (Hasselmo and Wyble, 1997; Treves and Rolls, 1994). This function seems computationally inevitable, due to its highly divergent input structure (200,000 entorhinal cortex cells project to >1 million dentate gyrus granule cells in the rat) and the sparse, powerful mossy fiber projection to the CA3 (Amaral et al., 1990; Henze et al., 2002). Sparse activity in the dentate gyrus following exposure to spatial environments has been observed experimentally with both implanted electrodes (Jung and McNaughton, 1993) and in an immediate-early gene study (Chawla et al., 2005), Further, mounting evidence proposes that immature granule cells are more 'excitable', with a stronger propensity for long-term potentiation (LTP) than fully mature neurons, suggests that these cells may have a unique role in the processing of the dentate gyrus circuit (Schmidt-Hieber et al., 2004; van Praag et al., 2002). Thus, new neurons in the DG are properly placed and possess competitive advantages to dramatically alter hippocampal circuitry and hence learning and memory. As discussed earlier, BMP signaling regulates multiple aspects to influence the number of new neurons. Current studies in the Kessler Lab suggest that BMP overexpression decreases neurogenesis and results in poorer performance in memory tasks. Conversely, noggin overexpression may increases neurogenesis resulting in better performance. Hence, BMP signaling may act as a negative

regulator of learning and memory. Given the potentially pleiotropic regulation BMP signaling has in the dentate gyrus, mechanisms regulating these effects on memory remain unknown.

Nuclear Reprogramming

We had four primary findings in Chapter 4. (a) Reconstructed embryos using SCNT with NPC donors develop at a more efficient rate than BMP-differentiated astrocytes donors. (b) Reconstructed embryos using OG2 eNPC donors develop less efficiently than from CD1 eNPC donors. (c) New ESCs have been developed from zygotic OG2 embryos. (d) Primary outgrowths were generated from OG2 eNPC donor cells. These observations begin to address the questions of if and how the donor cell plasticity affects epigenome reprogramming. However, more NT studies are required to strengthen current observations. First, we need to perform NT using aNPC donor cells and evaluate cloning efficiency rates to the blastocyst stage. ANPC are derived from eNSC using LIF and noggin. These cells possess progenitor characteristics and can be further differentiated into astrocytes by BMP signaling (Bonaguidi et al., 2005). Therefore, we hypothesize that embryos prepared from aNSC donors will exhibit a developmental efficiency at rates between those observed using eNSC and astrocyte donors. It is also conceivable that reconstructed embryos from aNSC donors yield a developmental rate similar or better than, eNSC donors. ANPC exhibit similar progenitor characteristics as eNPC including nestin, vimentin, SOX1, and LeX expression; cell cycle progression, and multipotentiality maintenance (Bonaguidi et al., 2005). Additionally, aNPC possess a slower cell cycle length than eNPC suggesting that fewer cells would be in S-phase. Thus, aNPC have similar progenitor

characteristics as eNPC and fewer cells incapable of promoting development after NT (Campbell et al., 1996). The relative importance of those factors in promoting reconstructed embryo development is currently unclear.

As discussed in Chapter 4, pre-implantation development rates alone is not a functional indication of reprogramming. ESC derivation efficiency or post-implantation development rates are also necessary to demonstrate functional reprogramming. Since pluripotency is more attainable than totipotency, we will focus on ESC derivation. We have demonstrated the technical ability to generate ESC from zygotic OG2 embryos and primary outgrowths from OG2 embryos reconstructed with eNSC nuclei (c, d). These were prepared using embryos at the blastocyst stage. We performed basic characterization of the Pou5f1-1 ESC line to demonstrate that the line possessed molecular and functional features of other ESC lines. Yet, we have not currently demonstrated that this line is pluripotent. This will be preformed while evaluating ntES lines we derive.

Many technological advances have been made recently in ESC derivation. These primarily include media modifications and manipulations of LIF signaling and TGFβ-superfamily signaling (Blelloch et al., 2006; Bryja et al., 2006; Chen et al., 2006; Ogawa et al., 2004; Ogawa et al., 2007; Wakayama et al., 2006a; Ying et al., 2003). While these niche changes increase the efficiency of deriving ESC from blastocysts or morulae, ESC can now be derived from earlier blastomeres. For instance, a single blastomere from 8-cell embryos can be biopsied and co-cultured with already existing ESC to derive a new line. This procedure is

largely inefficient - 4% and 2% of biopsied blastomere create ESC in mice and humans, respectively - and have not currently been applied to derive ntES lines (Chung et al., 2006; Klimanskaya et al., 2006). Another recent report demonstrated efficient derivation of multiple ESC lines using single blastomeres from 2-cell, 4-cell, and 8-cell embryos. In this case, ESC derivation efficiency decreased as development progressed (2-cell: 69%, 4-cell: 40%, and 8-cell: 16%, (Wakayama et al., 2006a)). Importantly, using whole blastocysts as a positive control, ESC derivation rates are at best 88%. Intriguingly, ESC derivation rates were lower using mice carrying a gfp transgene (Wakayama et al., 2006a). We also observe lower pre-implantation development rates after NT using donor from the OG2 mice. The technical advances in ESC derivation allow for multiple questions to be addressed. Are ESC lines developed isolated from different developmental stages molecularly and functionally equivalent? How does isolation affect directed differentiation? What are the molecular mechanisms regulating ESC derivation? Is this procedure applicable for producing ntES lines? We intend as a second experiment to derive ntES lines from blastocysts and investigate whether ntES lines can be created from 4-cell embryos. This would allow examination of NT-derived embryos from astrocyte donors for ES derivation, since they do not currently progress to the blastocyst stage. It also fosters evaluation of potential differences among donor cell types.

A third experiment to test how donor cell plasticity affects epigenome reprogramming involves culturing reconstructed embryos in media formulated for the donor cell. Such culturing can dramatically increase development efficiency after SCNT. For example, 2.0% and 5.5% of embryos reconstructed with primary myoblast donors progress to the blastocyst stage in CZB

and KSOM embryonic media. Meanwhile, culturing reconstructed embryos in the myoblast media F10:DMEM results in 43.4% efficiency to the blastocyst stage (Gao et al., 2003a). Further analysis showed that the myoblast-derived embryos expressed the glucose transporter-4 (GLUT) instead of GLUT1 (myoblasts express GLUT4, while zygotic embryos express GLUT1). This suggested that the interaction between the embryo and its niche (culture media) is essential for embryo survival and reprogramming (Gao et al., 2003a; Gao et al., 2003c). In addition, embryo culture media can increase ESC derivation efficiency while simultaneously decreasing post-implantation development. For instance, using cumulus derived blastocysts the ESC formation rate increases from 8% in CZB media to 39% in αMEM media. Conversely, postnatal development to E10.5 decreases from 26% in CZB to 9% in αMEM (Boiani et al., 2005). Further, pre-implantation culture media can cause differential effects on gene imprinting epigenetics that contribute to epigenetic anomalies later in development (Doherty et al., 2000; Lane and Gardner, 1994). Thus, culturing reconstructed embryos in media formulated for growth of the donor cells may allow for further reprogramming to occur and enhance ESC derivation. Growing embryos reconstructed from neural cells in DMEM:F12 supplemented with BSA, glutamine, and pyruvate may result in better pre-implantation development and ESC derivation. Of particular interest is whether differences will result among donor cell types: eNPC, aNPC, and astrocytes, especially since NT embryos using eNSC donors develop more efficiently than from astrocyte donors when cultured in CZB. Such culturing may also enhance ntES derivation rates from reconstructed blastocysts or 4-cell embryos.

A fourth experiment involves examination of NT-derived embryo molecular characteristics and correlating correct epigenetic reprogramming with pre-implantation development rates and ntES derivation efficiency. These characteristics involve gene expression specific to donor cells, expression specific to embryos, and chromatin remodeling proteins. Within the specified neural lineage, numerous genes are differentially expressed (Chapters 2 and 4). In addition, microarray studies to perform gene profiling are currently underway. Then, the donor cells will be completely defined transcriptionally. Preliminary studies suggest some of these genes are expressed in reconstructed embryos with eNPC donors (Chapter 4), similar to other studies (Gao et al., 2003a; Ng and Gurdon, 2005). A more detailed study is currently necessary to determine how donor cell type influences epigenetic memory.

Many genes essential to pre-implantation development have also been recently described. These include transcription factors and epigenetic modifiers. Nanog, Sox2, and Spalt-like gene 4 Sall4 are necessary for pre-implantation embryo development and inner cell mass (ICM) specification (Avilion et al., 2003; Chambers et al., 2003; Elling et al., 2006; Mitsui et al., 2003; Zhang et al., 2006), while Cdx2 is essential for trophectoderm (TE) specification (Niwa et al., 2005). Interestingly, a related Spalt-like gene, Sall3, is hypermethylated in placenta found in all cloned mice (Ohgane et al., 2004). None of these genes has yet been correlated with development after NT. Additionally, maternal-effect genes are necessary for embryo development. For example, embryos deficient in the SWI/SNF-related complex catalytic subunit Brg1, maternal antigen that embryos require (Mater), or RAD6-related gene mHR6A undergo 2-cell block, which is common for embryos reconstructed via SCNT (Bultman et al., 2006; Roest

et al., 2004; Tong et al., 2000). Specifically, Brg1 is the first known regulator of zygotic genome activation (ZGA) (Bultman et al., 2006). Also, heterochromatin organizers are essential for pre-implantation development. Chromatin assembly factor 1 (CAF-1) mutants arrest during compaction and display disorganized heterochromatin normally observed in 2- to 4-cell embryo (Houlard et al., 2006). Thus, many genes involved in normal embryo development are now available as markers of reprogramming. These traits may play an essential role towards understanding the mechanisms regulating nuclear reprogramming and accelerate stem cell derivation for allogenic therapy.

Table 1: Clonal analysis of BMP4 effects on astrocyte differentiation

	Control	BMP4
Total progeny	124	102
GFAP+	27 (22%)	98 (96%)
BrdU+	34 (27%)	1 (1%)
Death	7 (6%)	3 (3%)

Clonal analysis was performed on 100 P1 cells plated individually in Terasaki wells under control conditions (EGF 10 ng/ml), and on 100 cells plated in the presence of BMP4 (10 ng/ml BMP4 + EGF 10 ng/ml). Cells were pulsed with BrdU for 12 hours to assay proliferation, live/dead staining was performed to assess cell death, and GFAP immunocytochemistry was performed on day 7 to assay astrocyte differentiation. BMP treatment prompted exit from the cell cycle (lack of BrdU incorporation) without altering cell survival, and 96% of the cells expressed GFAP. Thus, BMP4 acts as an instructive rather than stochastic/selective cue to promote astrocyte differentiation.

Table 2: Comparison of GFAP-expressing cells generated in response to LIF versus BMP4

Phenotype	BMP4	LIF
GFAP expression	+++	+
Stellate morphology	+	-
Cell cycle exit	+	-
LeX expression	-	+
Sox1 expression	-	+
Vimentin expression	-	+
Neurosphere formation (self-renewal)	-	+
Neuron production by progeny	-	+

LIF and BMP4 induce discrete $GFAP^+$ cell populations from embryonic stem cells, when endogenous BMPs are taken into account. LIF-induced $GFAP^+$ cells have a bipolar/tripolar, morphology, re-enter the cell-cycle, express proteins characteristic of progenitors, and function as multipotential stem/progenitor cells capable of self-renewal and the generation of neuronal progeny. By contrast, BMP-induced $GFAP^+$ cells have a mature astrocytic phenotype as demonstrated by stellate $GFAP^+$ morphology, exit from the cell cycle, loss of stem/progenitor markers and the inability to self-renew.

Table 3: eNPC donors foster better development than astrocyte donors.

Cell type-CD1	Active	2-cell	4-cell	8-cell	Morula	Blastocyst
eNPC	64	57	44	41	18 (28.1%)	9 (14.1%)
Parthenote	116	114	113	113	111(95.7%)	108 (93.1%)
Astrocyte	35	22	8	2	0 (0.0%)	0 (0.0%)
Parthenote	44	42	41	41	41 (93.2%)	41 (93.2%)

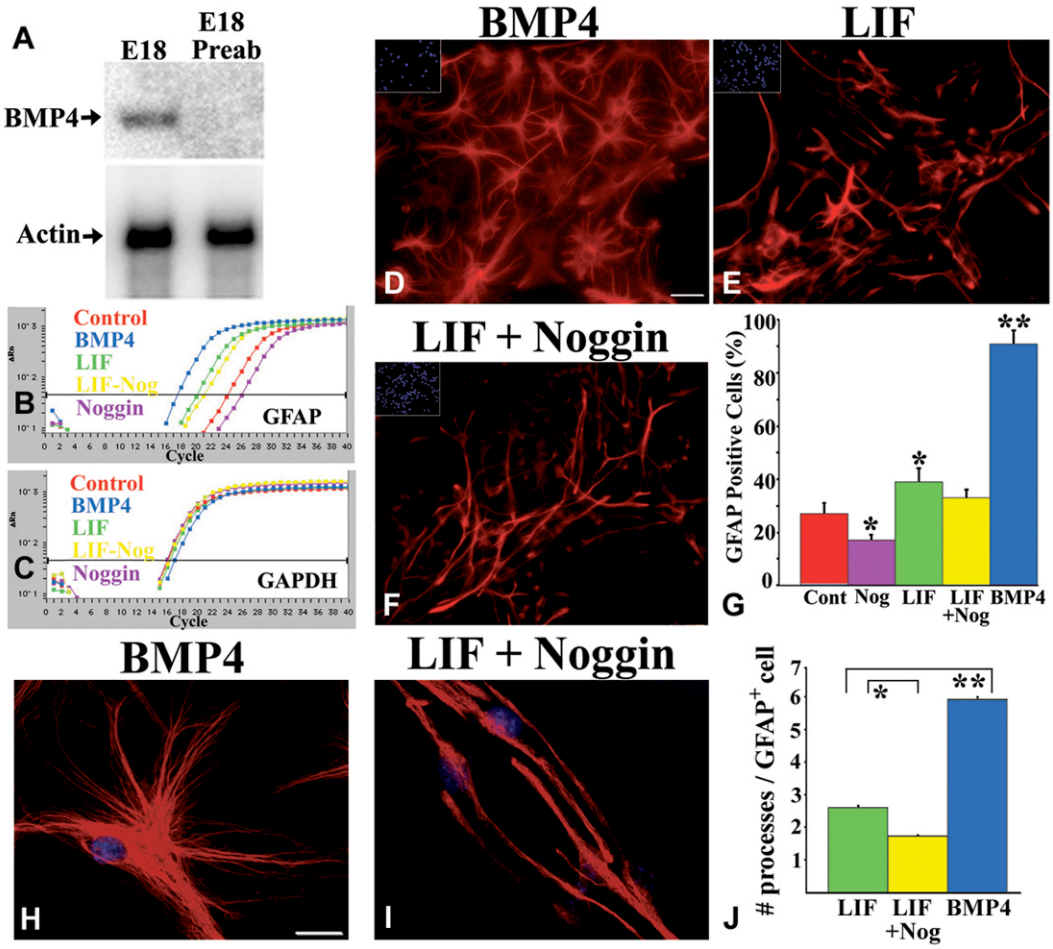
ENPC and astrocytes were used as donor cells for NT, activated, and cultured for 4 days to assess pre-implantation development rates. Parthenotes were created by activating oocytes.

Table 4: OG2 eNPC donors foster worse development than CD1 neurosphere donors

Cell type: (CD1)	Active	2-cell	4-cell	8-cell	Morula	Blastocyst
eNPC	64	57	44	41	18 (28.1%)	9 (14.1%)
Parthenote	116	114	113	113	111(95.7%)	108 (93.1%)
Cell type: (OG2:B6;CBA)						
eNPC	64	52	32	22	12 (18.8%)	1 (1.6%)
Parthenote	45	44	44	44	44 (97.8%)	41 (91.1%)
Cell type (OG2:B6D2F1)						
eNPC	32	23	10	6	5 (16.0%)	1 (3.0%)
Parthenote	49	48	48	48	48 (98.2%)	43 (88.2%)

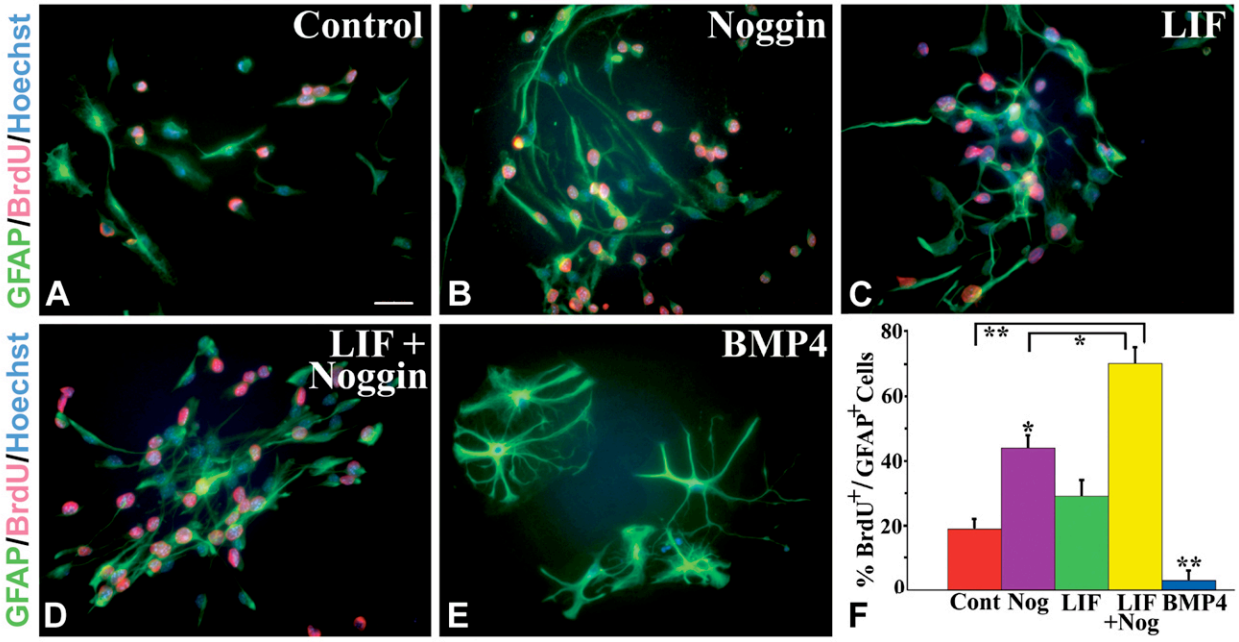
ENPC from CD1 background, carrying the Pou5f1-gfp (OG2) transgene from B6; CBA background, and carrying the OG2 transgene from B6D2F1 background were used as donor cells for NT. After activation and 4-day culture, pre-implantation development rates were assessed. Parthenotes were created by activating oocytes.

Figure 1: LIF and BMP both increase GFAP expression but promote different morphologies



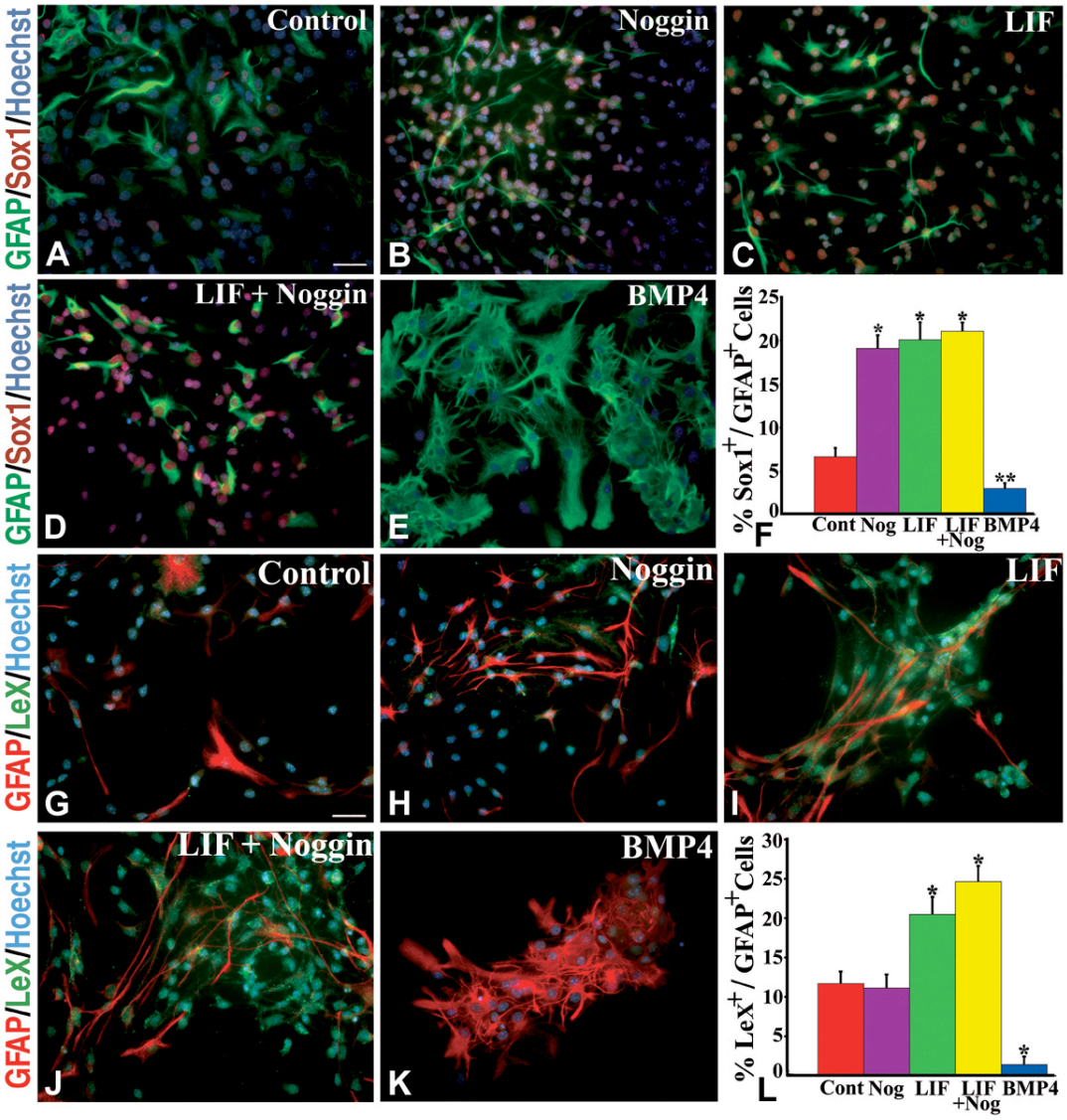
(A) Western analysis demonstrates that endogenous BMP4 is present in E18.5 EGF-responsive neurosphere cultures. Preabsorption of the antibody with BMP4 (preabsorb) eliminates the band. (B, C) Quantitative PCR analysis illustrates that noggin inhibition of endogenous BMP decreases GFAP levels, whereas exogenous LIF and BMP4 increase GFAP levels in dissociated neurospheres plated for a 20-hour differentiation. Note that BMP treatment produces the highest level of GFAP. (D-F, H, I) Immunofluorescence reveals that 7-day LIF and BMP4 treatments promote different GFAP-expressing cell morphologies. Red, GFAP; blue insets, Hoechst nuclear counter stains. (D, H, J) BMP4 treatment produces a stellate morphology with an increased process number. (E, J) LIF treatment alone induces a mixture of elongated bipolar/tripolar and stellate morphologies. (F, I, J) In the presence of noggin, LIF promotes the bipolar/tripolar morphology and reduces process number. (G) Quantitation of GFAP immunofluorescent cells. Noggin treatment significantly reduces the number of $GFAP^+$ cells. Conversely, LIF, and LIF plus noggin, treatment increases the number of $GFAP^+$ cells and BMP4 treatment results in an even larger increase. (G) $^{*}P<0.05$, $^{**}P<0.001$; (J) $^{*}P<0.01$, $^{**}P<0.001$ ANOVA. Error bars are ±s.e.m. Scale bars: 20 μm in D-F; 10 μm in H, I.

Figure 2: BMP4 prompts, whereas LIF reduces, exit of GFAP$^+$ cells from the cell cycle



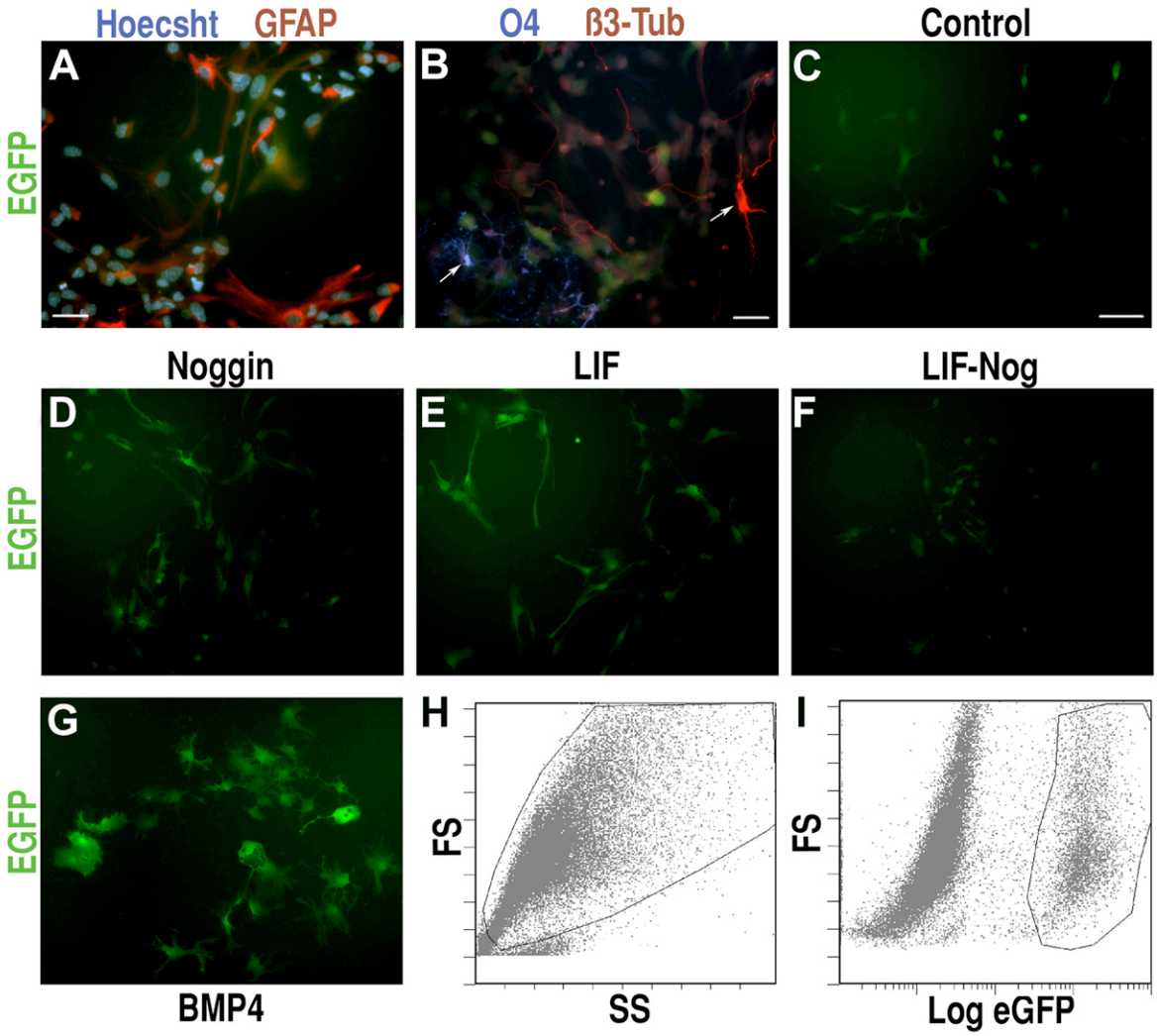
Neurosphere cells were plated and allowed to differentiate with or without cytokines for 6 days, followed by 16 hours of labeling with BrdU. (A, F) Only a small percentage of GFAP$^+$ cells (green) incorporate BrdU (red). (B, F) Treatment with noggin to inhibit endogenous BMP signaling enhances BrdU incorporation. (C, D, F) Treatment with LIF alone does not significantly alter BrdU incorporation. However, LIF treatment in the presence of noggin greatly increases BrdU incorporation compared with treatment with noggin alone. (E, F) BMP4 treatment virtually abolishes BrdU incorporation. (F) Quantitation of the percentage of GFAP$^+$ cells that incorporate BrdU. $^*P<0.01$, $^{**}P<0.001$ ANOVA. Scale bars: 20 μm in A-E.

Figure 3: LIF increases, whereas BMP4 decreases, neural precursor markers in GFAP⁺ cells



Neurosphere cells were plated and allowed to differentiate with or without cytokines for 7 days before immunocytochemical examination. (A, F) Few differentiated GFAP⁺ cells express the progenitor marker SOX1. (B-D, F) Treatment with LIF, noggin, and LIF plus noggin all increase the percentage of SOX1⁺GFAP⁺ precursors. (E, F) Conversely, BMP4 treatment decreases the percentage of SOX1⁺GFAP⁺ precursors. (G, L) Similarly, few differentiated GFAP⁺ cells express the neural stem cell marker LeX. (H, L) Treatment with noggin does not alter LeX expression on GFAP⁺ cells. (I, J, L) LIF treatment with or without noggin increases the number of LeX⁺GFAP⁺ cells. (K, L) By contrast, BMP4 treatment decreases the number of LeX⁺GFAP⁺ cells. Thus LIF and BMP have opposite effects on stem/progenitor cell markers in GFAP⁺ cells. $^{*}P<0.05$, $^{**}P<0.005$ ANOVA. Scale bars: 20 μm in A-F, H-K.

Figure 4: The rGFAPp-eGFP retrovirus and FACS faithfully select GFAP$^+$ cells



Neurospheres were infected with the rGFAPp-eGFP retrovirus and plated for differentiation for 7 days, with or without cytokines. (A, B) Differentiating cells were processed for immunocytochemistry. eGFP (green) driven by the 1.9 kb rat GFAP promoter is expressed by oligodendrocytes, as indicated by the arrows (B; red, bIII-Tubulin, neurons; blue, O4, oligodendrocytes). (C-G) Live GFP fluorescent imaging to monitor the cell morphologies of the selected cells (green indicates eGFP-expressing cells). Untreated (control) cells had a mixture of morphologies. Treatment with BMP4 induced a stellate morphology, whereas LIF treatment induced a bipolar/tripolar morphology. Thus, findings with the GFP-selected cells recapitulated the findings from GFAP immunocytochemistry of cultured neurosphere cells exposed to cytokines (see Fig. 1). (H, I) FACS analysis for eGFP expression purifies GFAP-expressing cells. Dead cells are excluded by the gates in the forward-side scatter plot (H). GFP-expressing cells are selected according to high fluorescence. Scale bars: 20 mm for A, B; 50 mm for C-G.

Figure 5: LIF promotes, whereas BMP4 inhibits, a GFAP-expressing multipotential stem cell fate and neuron production

(A) Experimental paradigm for the cytokine induction, rGFAP promoter and FACS selection, and stem cell analysis of $GFAP^+$ cells. Neurospheres were expanded, infected with the rGFAPp-EGFP retrovirus, and further expanded, plated for 5 days to differentiate with or without cytokines and then sorted on the basis of eGFP expression (green). Sorted cells were plated in a neurosphere-forming assay to assess self-renewal and subsequent spheres were plated for differentiation to assess the ability to form neurons (red), astrocytes (purple) and oligodendrocytes (blue). (B, G) In the neurosphere-forming assay, 2% of control $GFAP^+$ cells self-renew (green indicates cells that are GFP positive). (C-E, G) LIF and noggin treatments each increase the percentage of $GFAP^+$ cells that form neurospheres (green). LIF treatment also increases neurosphere size, which is further increased by additional BMP inhibition. (F, G) BMP4 treatment prevents the self-renewal of $GFAP^+$ cells. (H) A single GFP-positive neurosphere plated for differentiation gives rise to oligodendrocytes (O4, blue), neurons (ß-tubulin, red) and astrocytes (GFAP, pink), demonstrating multipotentiality. (I-M) GFP-expressing neurosphere populations were dissociated to assess neuronal progeny. (I, M) GFAP-expressing (GFP positive, green) cells produce few neurons (red). (J, M) Noggin treatment alone does not significantly change neuron production. (K, M) LIF treatment significantly increases the number of neurons generated. (L, M) LIF treatment with BMP inhibition further increases neuron production. (G) $^{*}P<0.05$, $^{**}P<0.01$; (M) $^{*}P<0.01$, $^{**}P<0.005$ ANOVA. Scale bars: 100 μm in B-F; 20 μm in H-L.

Figure 5: LIF promotes, whereas BMP4 inhibits, a GFAP-expressing multipotential stem cell fate and neuron production

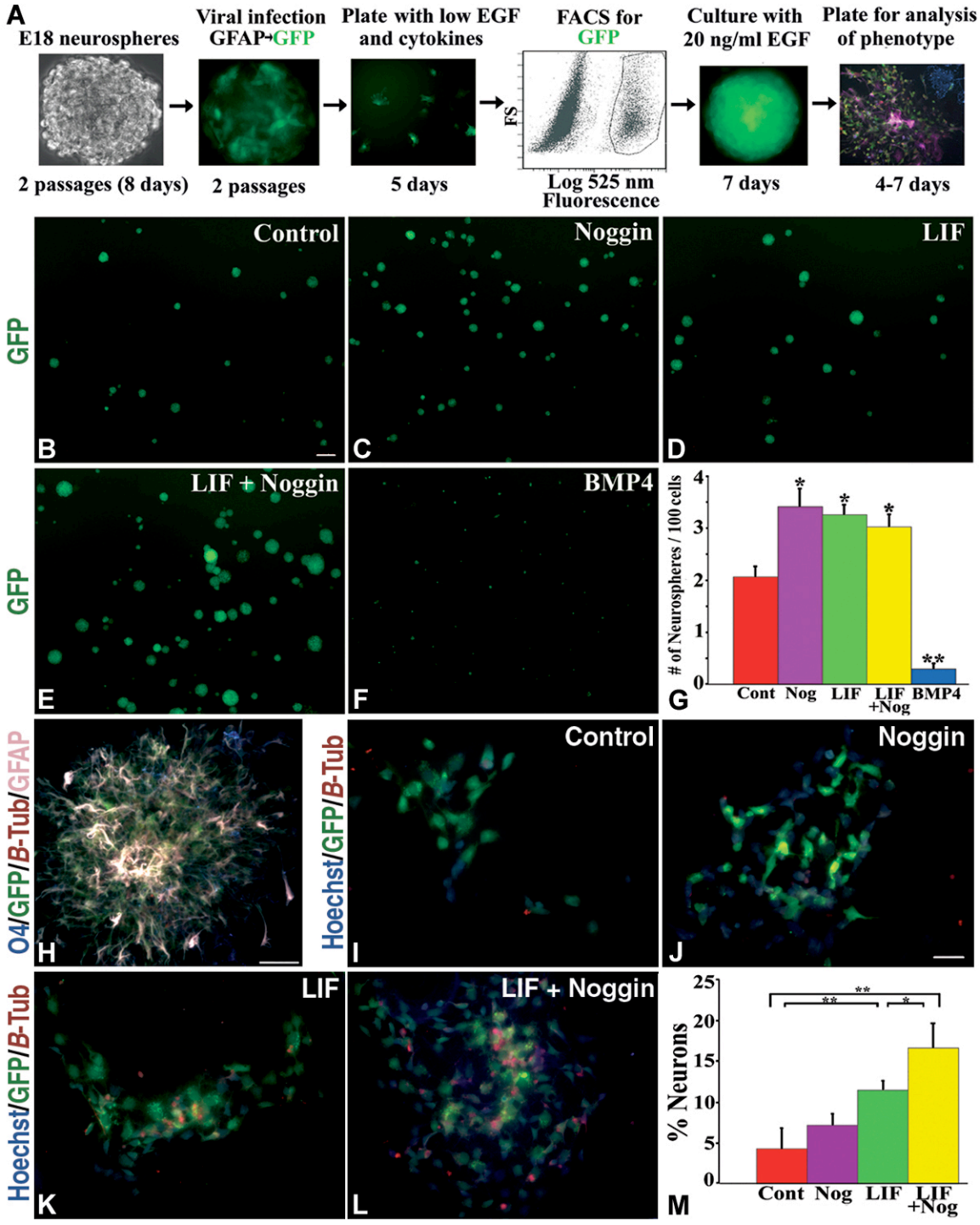
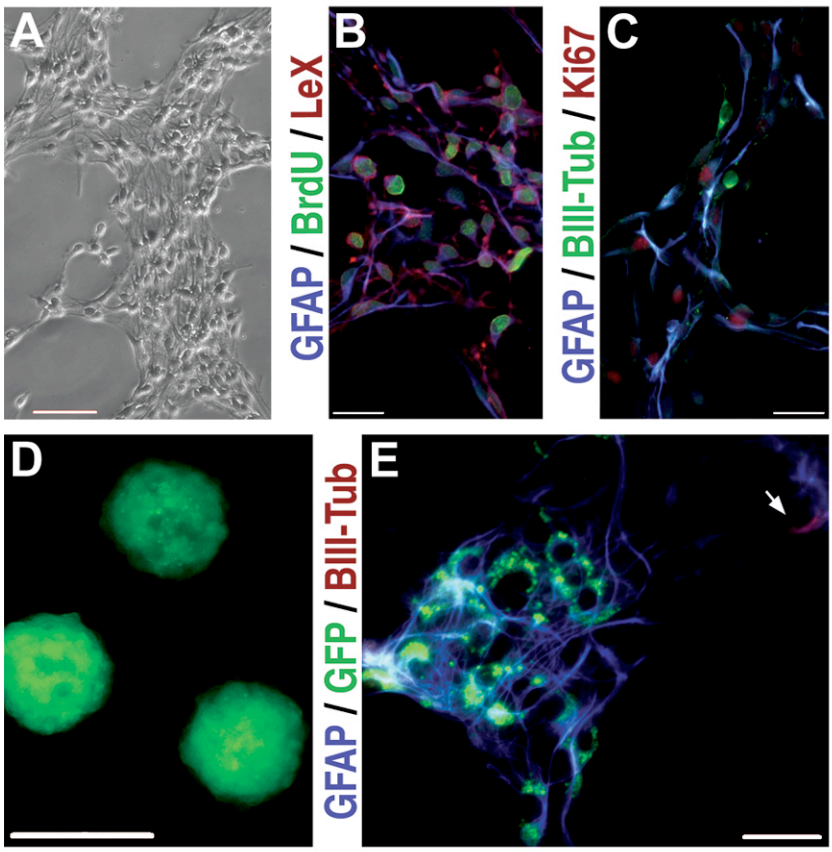
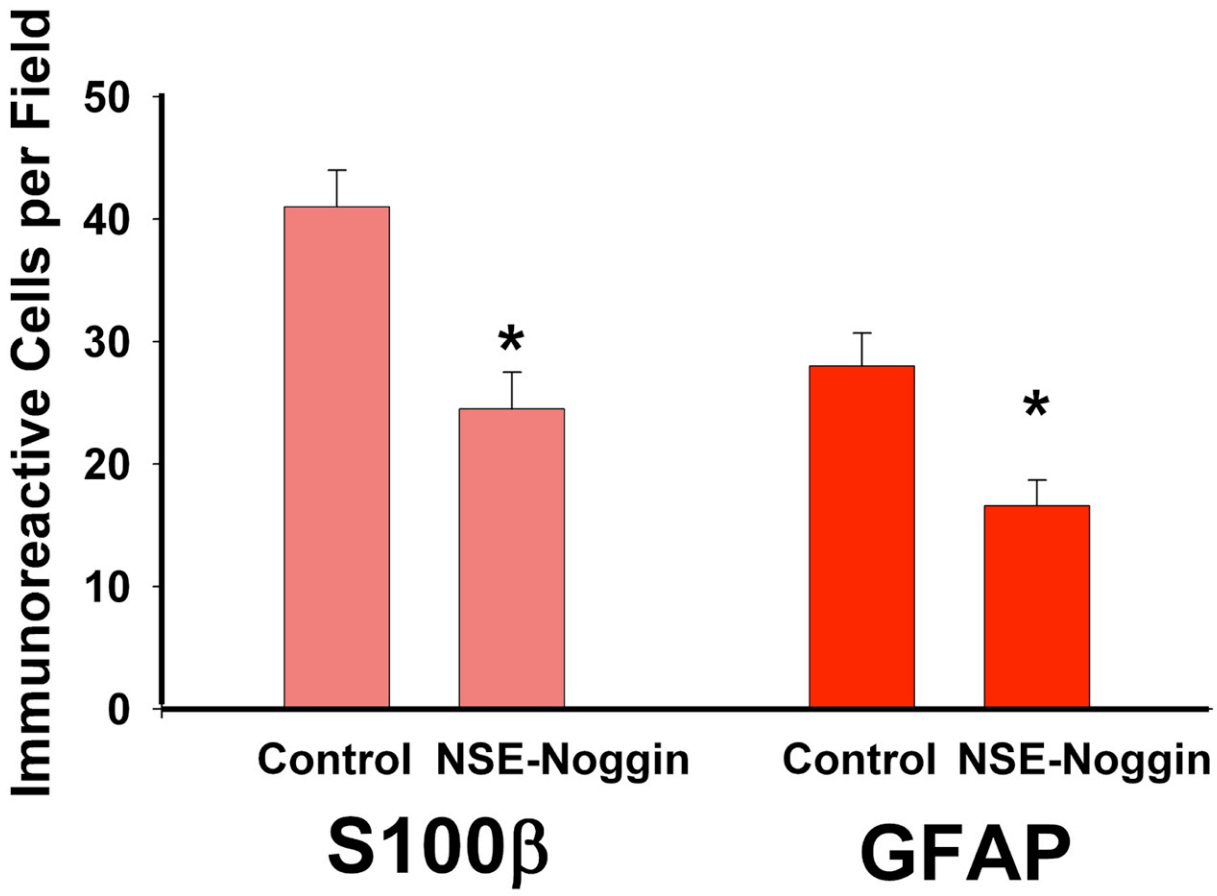


Figure 6: LIF maintains GFAP-expressing multipotent progenitors



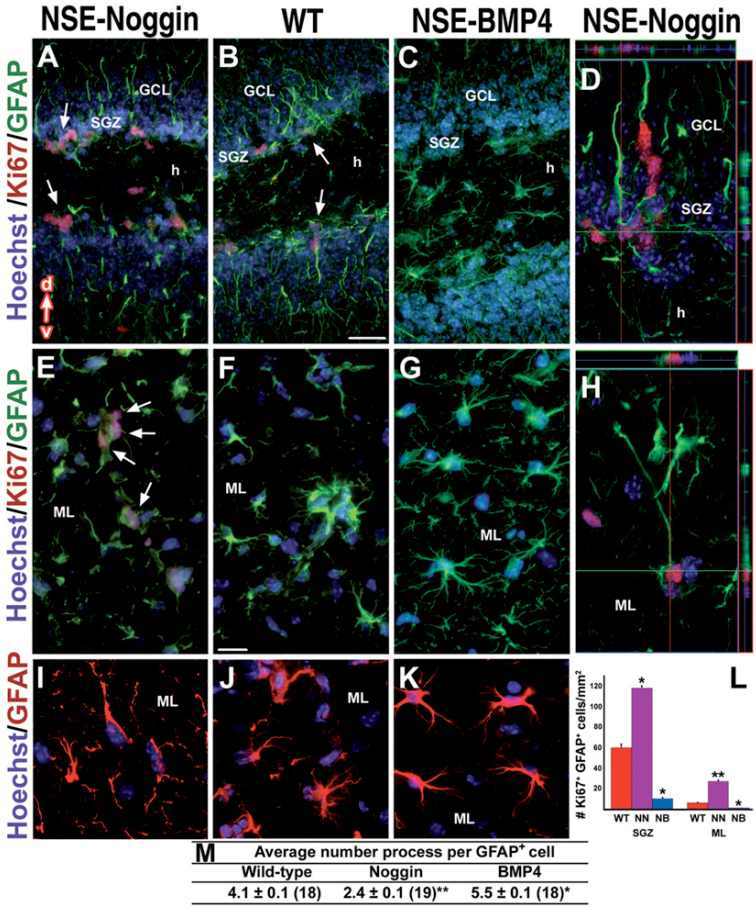
(A) LIF and low (1-2 ng/ml) EGF propagate neural progenitor cells as a monolayer for at least 10 passages. (B) At higher passages (p7 is shown), GFAP-expressing cells proliferate, as shown by BrdU incorporation (green, 2-hour pulse), and exhibit the progenitor marker LeX (red). (C) Higher passage cultures are still able to generate neurons (green), whereas nearby GFAP-expressing cells remain in cell cycle, as demonstrated by Ki67 expression (red). (D, E) To directly test the ability of GFAP-expressing cells for self-renewal and multipotentiality, higher passage cells were infected with the rGFAPp-EGFP retrovirus, selected by FACS, plated in a neurosphere-forming assay to assess self-renewal (in the absence of high mitogen), and subsequent spheres plated for differentiation to assess the ability to form neurons (red) and astrocytes (blue). (D) GFAP-expressing cells are able to self-renew at high passages, as shown by neurosphere formation (green, GFP). (E) Spheres formed from high passage GFAP-expressing cells retain the ability to produce neurons (red; blue indicates astrocytes; green, GFP). Scale bars: 20 μm in A-C, E; 100 μm in D.

Figure 7: Noggin decreases astrocyte markers in the postnatal hippocampus



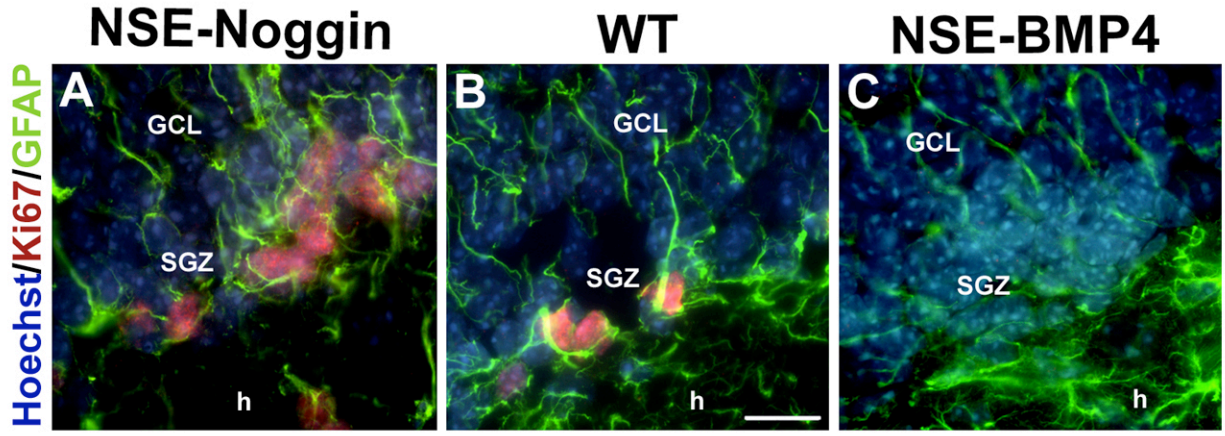
Noggin was overexpressed in transgenic animals under the control of the neuron-specific enolase (NSE) promoter and processed for immunocytochemistry at postnatal day 15 (P15). Cell counts for the astrocyte markers GFAP and S100b were acquired at high magnification throughout the hippocampus. Noggin overexpression significantly decreased the total number of $GFAP^+$ and $S100b^+$ cells compared with wild-type littermates. *P< 0.01, Student's t-test

Figure 8: BMPs regulate morphology and are necessary and sufficient for the cell-cycle exit of GFAP-expressing cells in vivo



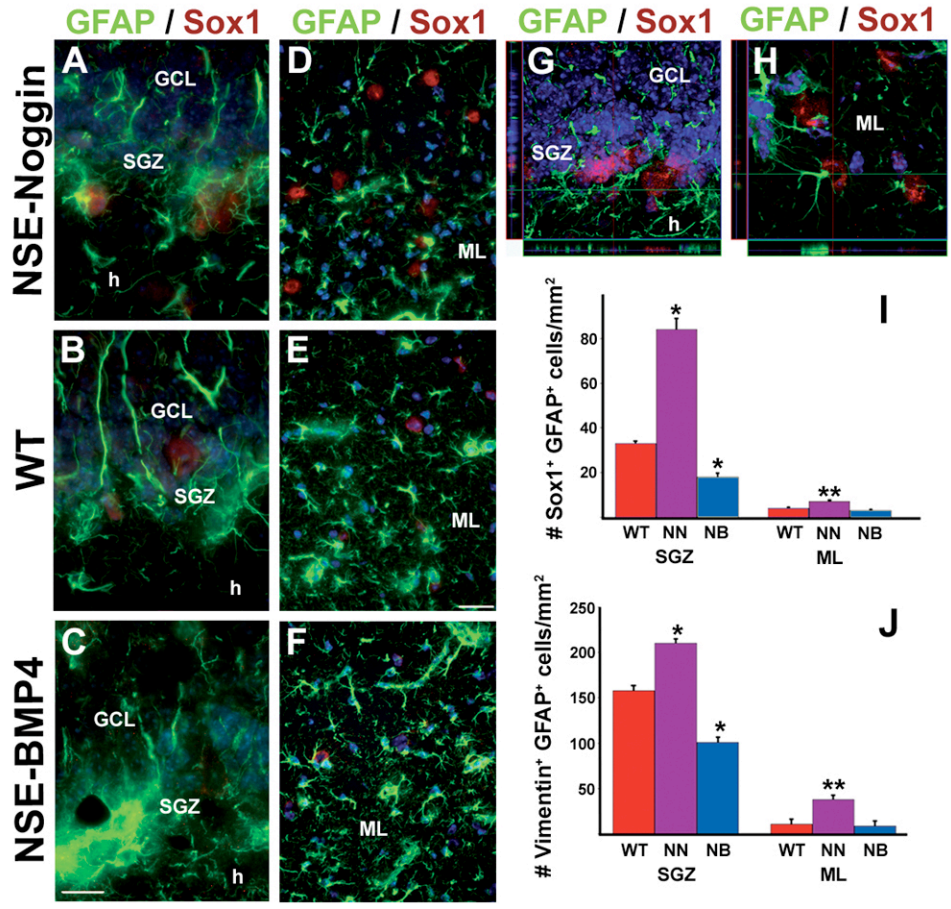
Transgenic animals overexpressing BMP4 or noggin were analyzed at PN15. (A-K) Immunofluorescence for GFAP and Ki67 in the hippocampus SGZ (A-D) and ML (E-K). (L) Plot of $Ki67^{+}GFAP^{+}$ cells in the SGZ ($^{*}P<0.03$) and the ML ($^{*}P<0.01$, $^{**}P<0.04$). (M) Average number of processes per $GFAP^{+}$ cell. Numbers in parentheses indicate the number of cells analyzed. $^{*}P<0.01$, $^{**}P<0.001$ ANOVA. (A, B, L) GFAP-expressing cells in the SGZ remain in cell cycle and are increased in number when noggin is overexpressed. Arrows indicate clusters of $Ki67^{+}$ cells. (C, L) Overexpression of BMP4 in the SGZ promotes cell-cycle exit of $GFAP^{+}$ cells. (D) High magnification confocal image demonstrating co-localization of Ki67 and GFAP in the SGZ of noggin mice. (F, G, L) Few GFAP-expressing cells in the ML remain in cell cycle in wild-type or BMP4-overexpressing mice. (E, L) BMP inhibition in the ML maintains $GFAP^{+}$ cells in cell cycle. Arrows (E) indicate $Ki67^{+}GFAP^{+}$ cells. (H) High magnification confocal image demonstrating co-localization of Ki67 and GFAP in the ML of noggin mice. (J, K, M) $GFAP^{+}$ cells in the ML exhibit branched morphologies that become further ramified with BMP4 overexpression. (I, M) Noggin overexpression in the ML prevents the formation of stellate morphology, and these cells resemble the thin elongated $GFAP^{+}$ cells in the SGZ (H, D). GCL, granule cell layer; SGZ, subgranular zone; h, hilus; ML, molecular layer; WT, wild type; NN, NSE-Noggin; NB, NSE-BMP4. Scale bar in B: 20 µm for A-C, E-G; 10 µm for D, H-K.

Figure 9: BMP signaling promotes exit of GFAP-expressing cells from the cell cycle in vivo



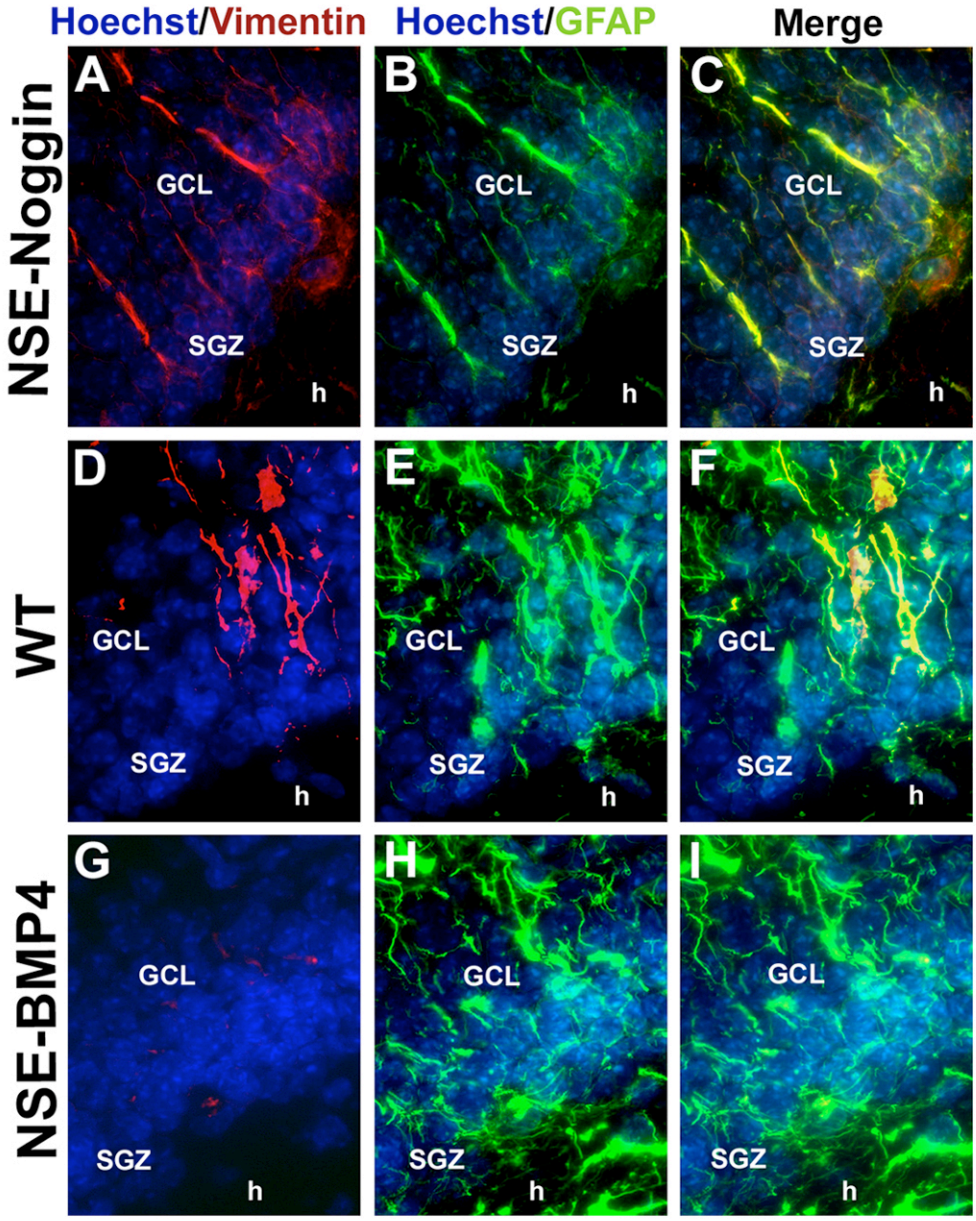
High magnification images demonstrate co-localization of Ki67 and GFAP in the postnatal SGZ. BMP overexpression decreases the number of $GFAP^+$ cells that incorporate Ki67 (C), whereas BMP inhibition with noggin increases Ki67 incorporation into $GFAP^+$ cells (A) compared with wild-type controls (B). SGZ, subgranular zone; GCL, granule cell layer; h, hilus; WT, wild type.

Figure 10: BMPs regulate the maturation of GFAP-expressing cells in vivo



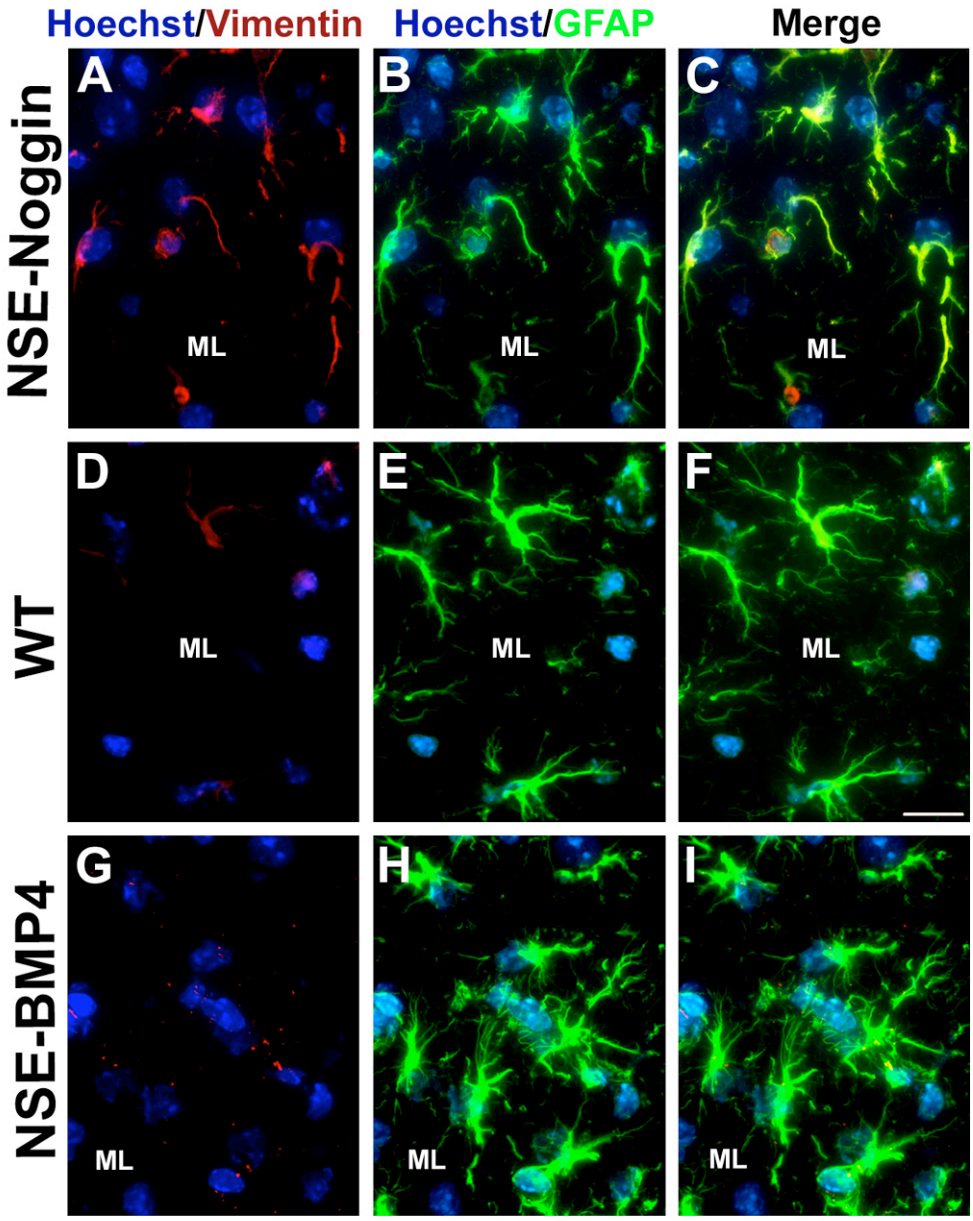
(A-H) GFAP (green), and SOX1 or vimentin (red) in the hippocampus SGZ (A-C,G) and ML (D-F,H) in P15 wild-type (B,E), NSE-noggin (A,D,G,H) and NSE-BMP4 (C,F) animals. Cells were counterstained with Hoechst (blue). (I, J) Number of SOX1-(I) and vimentin-(J) expressing GFAP$^+$ cells. (A, B, D, I, J) GFAP-expressing cells in the SGZ remain as progenitors and increase in number when noggin is overexpressed, as assessed by SOX1 and vimentin co-labeling. (C, I, J) Overexpression of BMP4 in the SGZ promotes the loss of progenitor markers in GFAP$^+$ cells. (D) Confocal image demonstrating co-localization of SOX1 and GFAP in the SGZ of noggin mice. (F, G, I, J) GFAP-expressing cells in the ML rarely co-express SOX1 or vimentin in wild-type or overexpressed BMP4 mice. (E, I, J) BMP inhibition in the ML increases progenitor markers in GFAP$^+$ cells. (H) Confocal image demonstrating co-localization of SOX1 and GFAP in the ML of noggin mice. (I) $^*P<0.02$, $^{**}P<0.05$; (J) $^*P<0.03$, $^{**}P<0.01$ ANOVA. Scale bar: in C, 10 µm for A-C, G, H; in E, 20 µm for D-F.

Figure 11: BMP signaling decreases vimentin expression in GFAP$^+$ cells in vivo



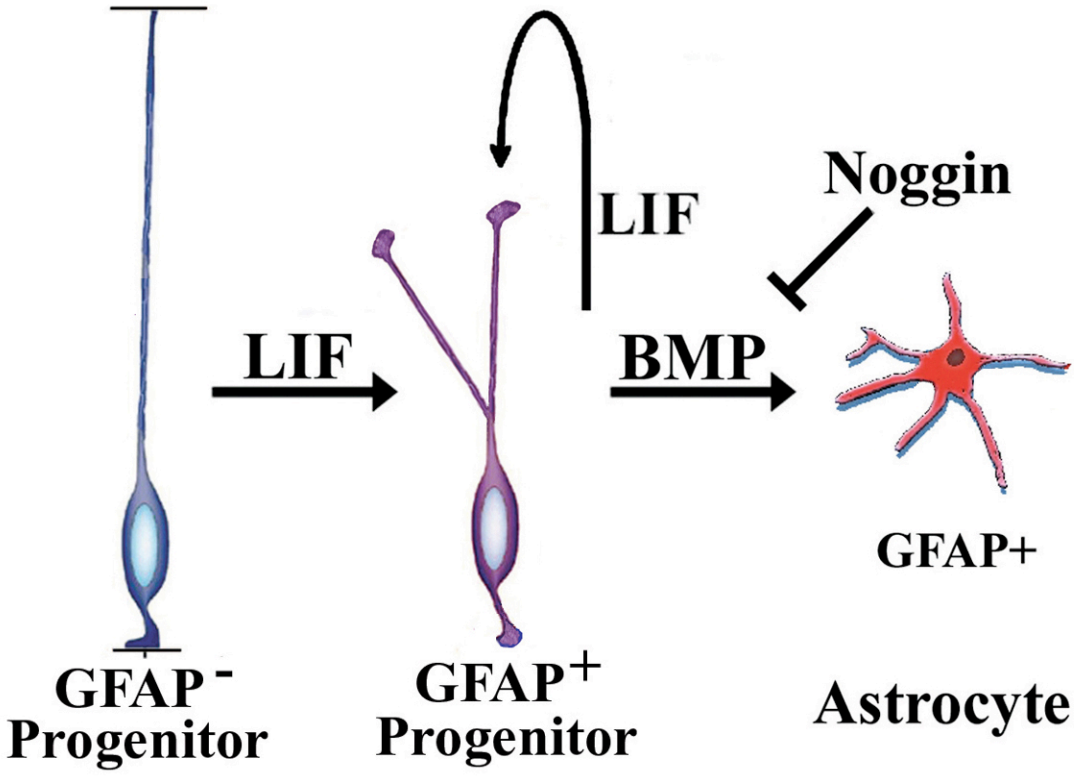
Neural progenitor status was investigated in wild-type, NSE-noggin and NSE-BMP4 animals by processing P15 brains for immunofluorescence for GFAP (green) and for the neural progenitor marker, vimentin (red) in the hippocampus SGZ. (A-F) Some GFAP-expressing cells in the SGZ express vimentin and that number is increased when noggin is overexpressed. (H-I) Overexpression of BMP4 promotes the loss of vimentin in GFAP$^+$ cells, demonstrating that BMP inhibition is necessary in this region to maintain progenitor cell traits. Blue color indicates Hoechst nuclear counterstain. GCL, granule cell layer; SGZ, subgranular zone; h, hilus; WT, wild type. Scale bar: 10 mm for A-I.

Figure 12: BMP inhibition maintains vimentin expression in GFAP$^+$ cells in vivo



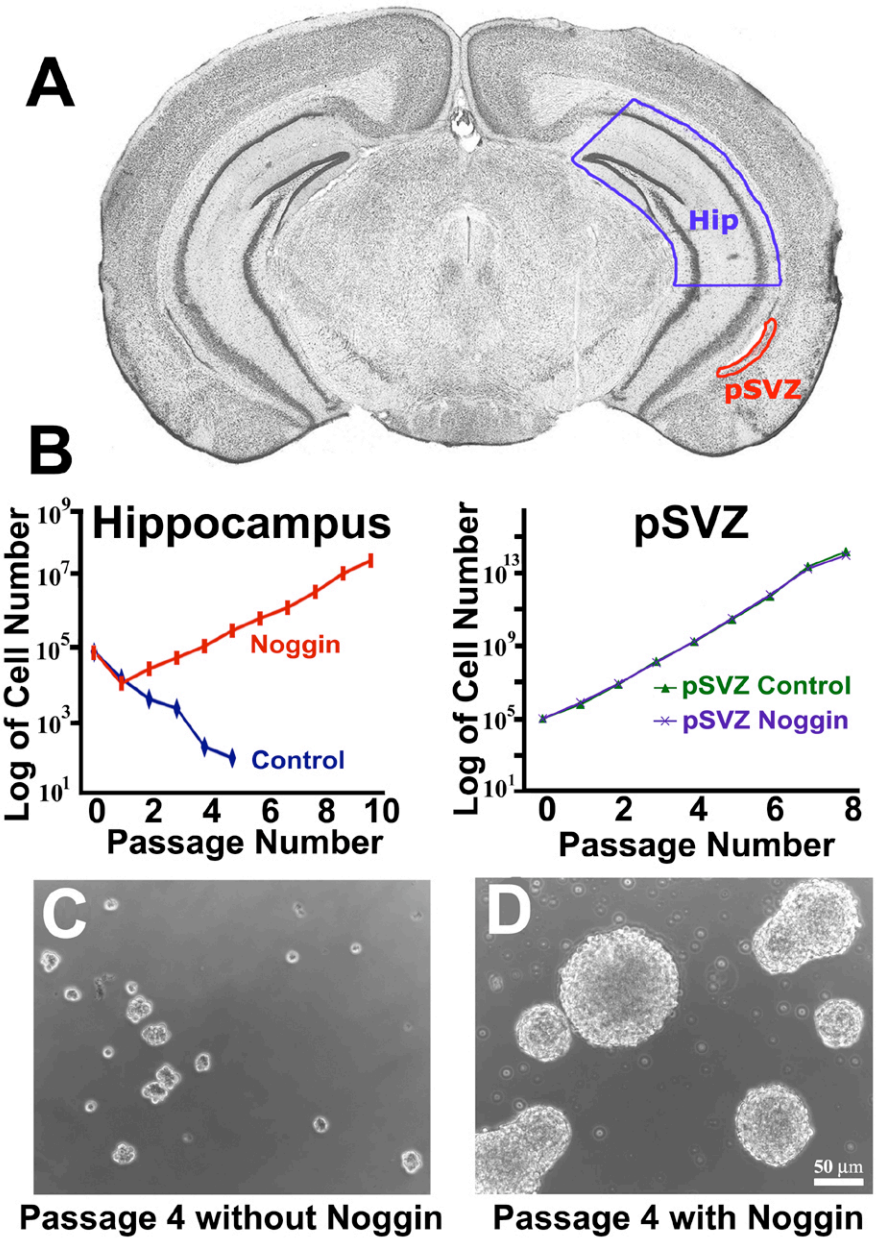
Neural progenitor status was investigated in wild-type, NSE-noggin and NSE-BMP4 animals by processing P15 brains for immunofluorescence for GFAP (green) and a neural progenitor marker, vimentin (red), in the hippocampus ML. (D-I) GFAP-expressing cells in the ML rarely co-express vimentin in wild-type or BMP4-overexpressing mice. (A-C) BMP inhibition in the ML increases vimentin expression in GFAP$^+$ cells, demonstrating that BMP is necessary for astrocyte maturation in this region. Blue color indicates Hoechst nuclear counterstain. ML, molecular layer; WT, wild type. Scale bar: 10 mm for A-I.

Figure 13: Model for the affects of LIF and BMP on astrocyte differentiation



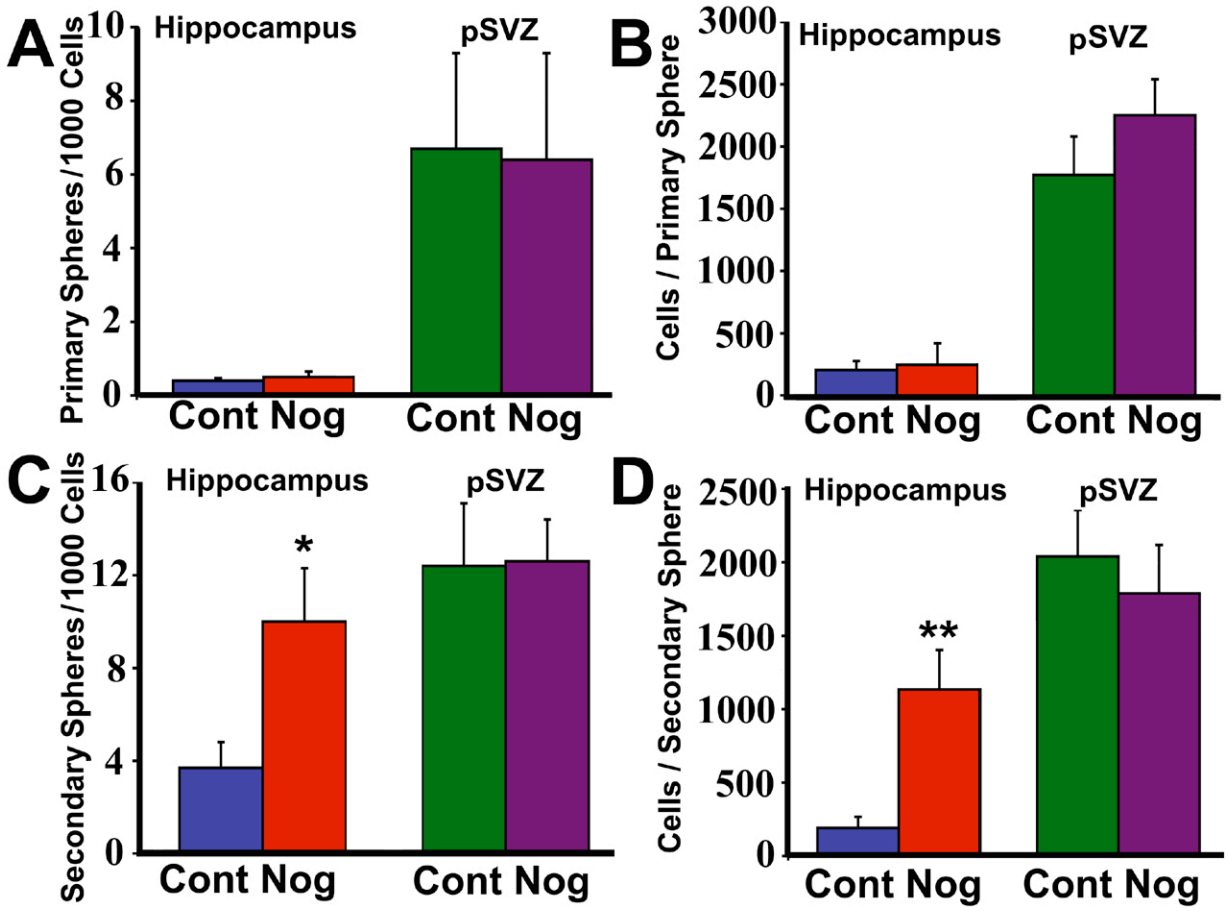
Stem cells in the developing rodent brain are initially $GFAP^-$, but later express GFAP during the postnatal and adult periods. LIF fosters maintenance of stem/progenitor trait throughout the continuum, but converts $GFAP^-$ cells into $GFAP^+$ ones. By contrast, BMPs induce exit from cell cycle and the loss of stem/progenitor cell traits, resulting in what we have termed a mature astrocyte. Inhibition of endogenous BMPs is required to prevent maturation of adult stem cells into the mature astrocytic phenotype.

Figure 14: Noggin allows for hippocampal cell self-renewal



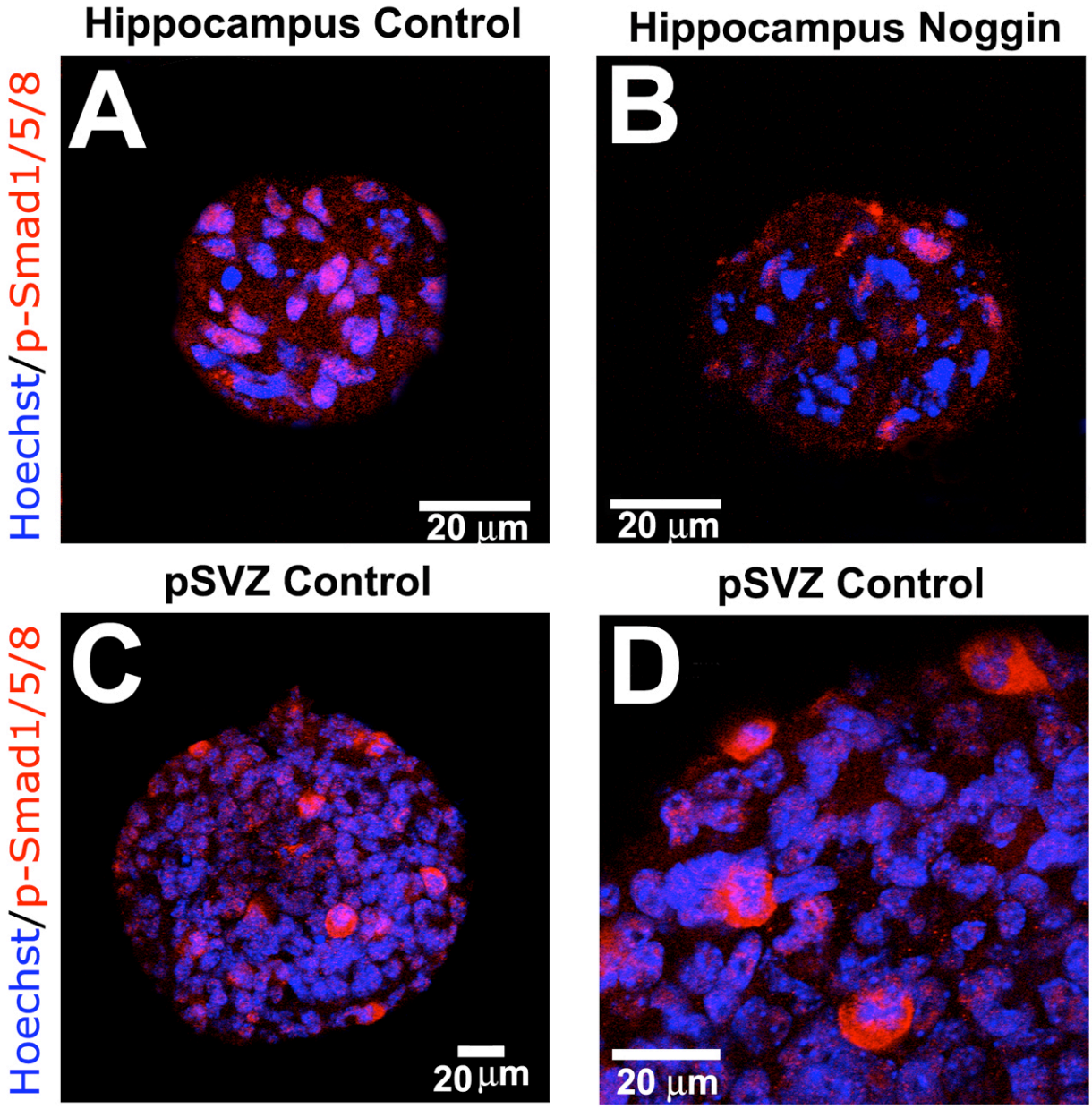
(A) 2 mm thick coronal sections were prepared from adult brain and placed caudal side up. The dorsal hippocampus (Hip) was isolated followed by the posterior subventricular zone (pSVZ) region. (B, C) Neurosphere cultures derived from the hippocampus fail to expand cell numbers in the presence of EGF and FGF through multiple passaging. (B, D) However hippocampal cultures grown with noggin addition expand for over 10 passages. (B) Neurospheres derived from pSVZ expand at a faster rate than do hippocampal neurospheres with noggin addition. Noggin addition to pSVZ cultures does not affect expansion cell numbers. Hip (n=3). pSVZ (n=4). Panel (A) is modified from Paxinos and Franklin (2001), with permission.

Figure 15: Noggin increases the frequency and cell number of secondary hippocampal neurospheres



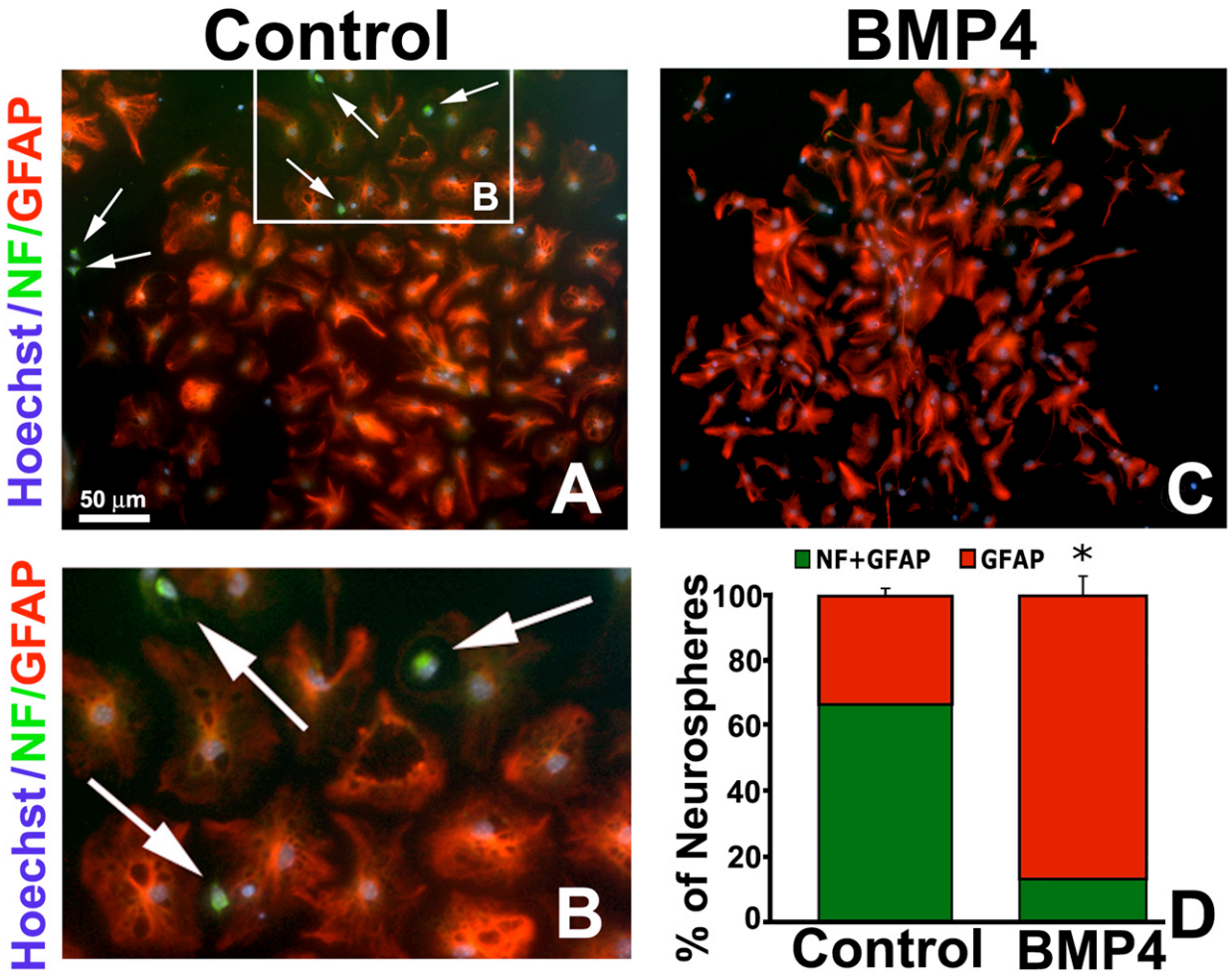
(A, B) Primary spheres derived from the pSVZ are more numerous and contain more cells than do spheres derived from the Hip. Noggin does not affect the number of primary neurospheres nor the number of cells per primary neurosphere derived from either the pSVZ or Hip. (C, D) Noggin addition significantly increases the number of secondary hippocampal neurospheres as well as the number of cells per neurosphere. However noggin did not affect either the number of secondary pSVZ neurospheres or the number of cells per pSVZ neurosphere. Error bars represent mean ± SEM, Hip (n=4). pSVZ (n=4). *P< 0.01, **P< 0.03 by unpaired Student's t-test.

Figure 16: Strong BMP signaling occurs in hippocampal progenitors, but not pSVZ progenitors, *in vitro*



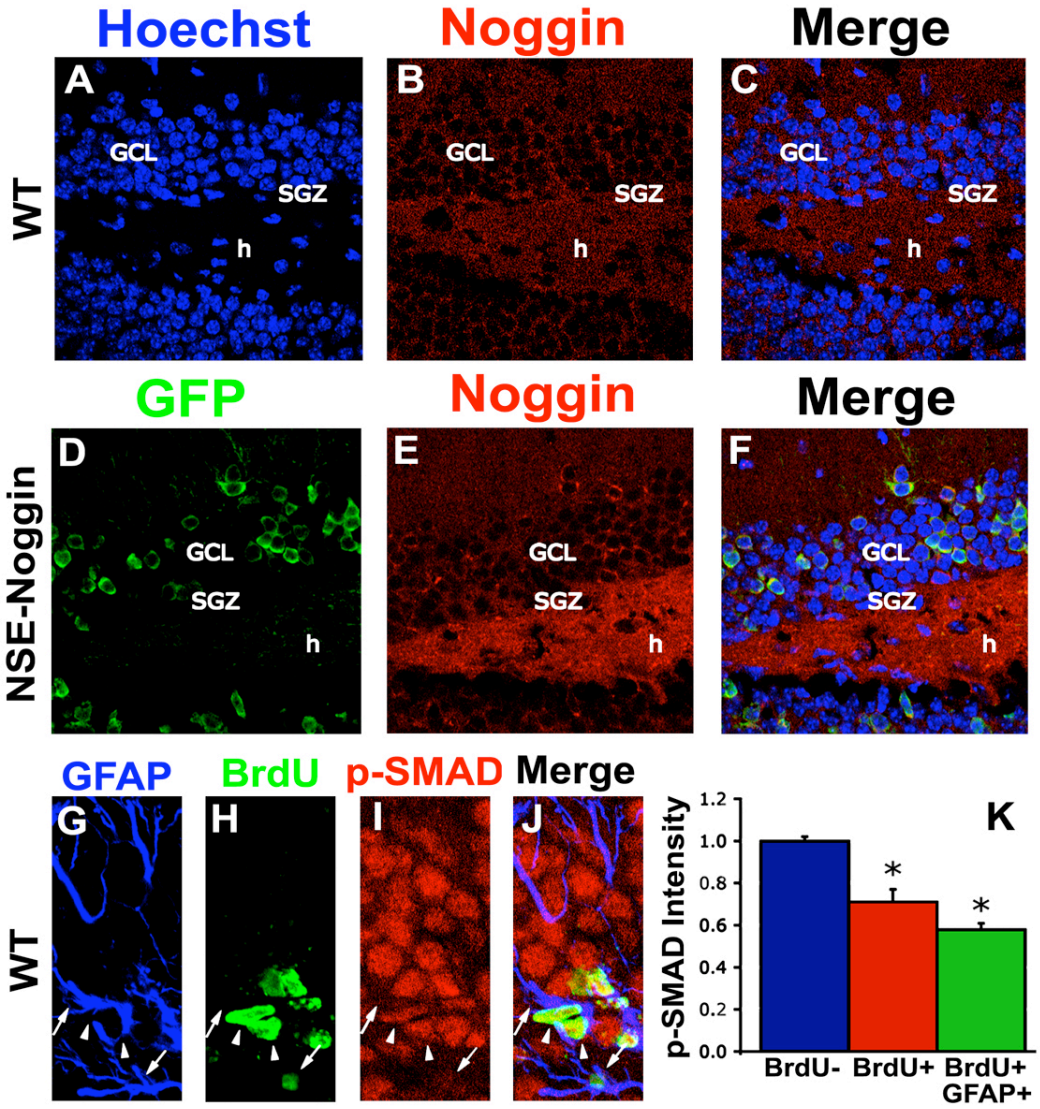
(A) Primary hippocampal neurospheres grown in EGF and FGF for 8 days exhibit prominent nuclear phospho-SMAD1/5/8 (np-SMAD). (B) Noggin addition reduces np-SMAD staining in hippocampal neurospheres. (C) pSVZ primary neurospheres do not display strong np-SMAD staining. (D) pSVZ spheres do have p-SMAD staining in the cytoplasm of some cells.

Figure 17: BMPs inhibit adult hippocampal multipotential differentiation



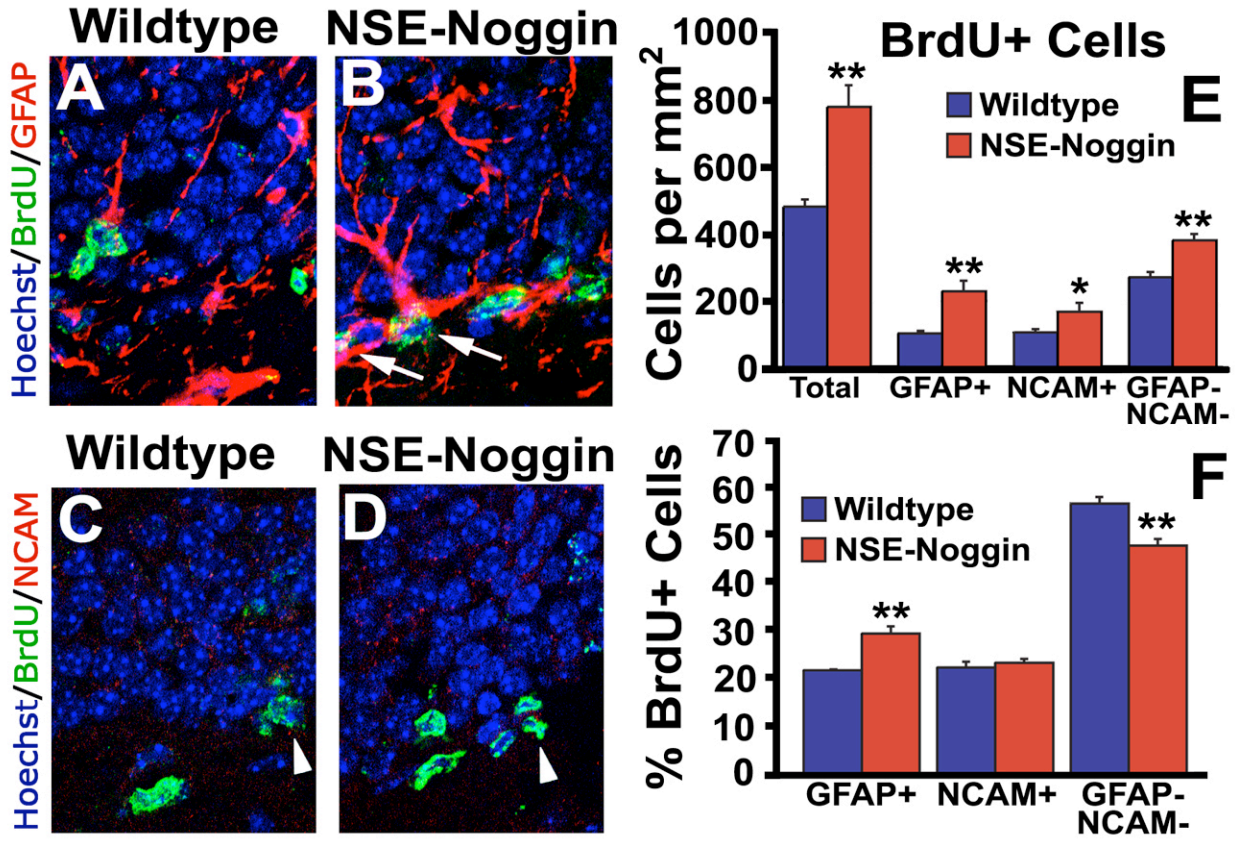
Passage 11 neurospheres grown chronically in EGF, FGF, and noggin were plated for differentiation with or without BMP addition for 5-7 days. (A, B) Hippocampal neurospheres grown with noggin differentiate into both neurons (pan-neurofilament (NF) fluorescence) and glia (glial fibrillary acidic protein (GFAP) immunofluorescence). Arrows point to NF-positive cells. (C) BMP addition during differentiation inhibits neuronal differentiation and promotes more glia-only spheres. Error bars represent mean ± SEM of three experiments. *P=0.01 by unpaired Student's t-test.

Figure 18: Noggin is expressed in the adult hippocampus and inhibits BMP signaling in SGZ precursor cells in vivo



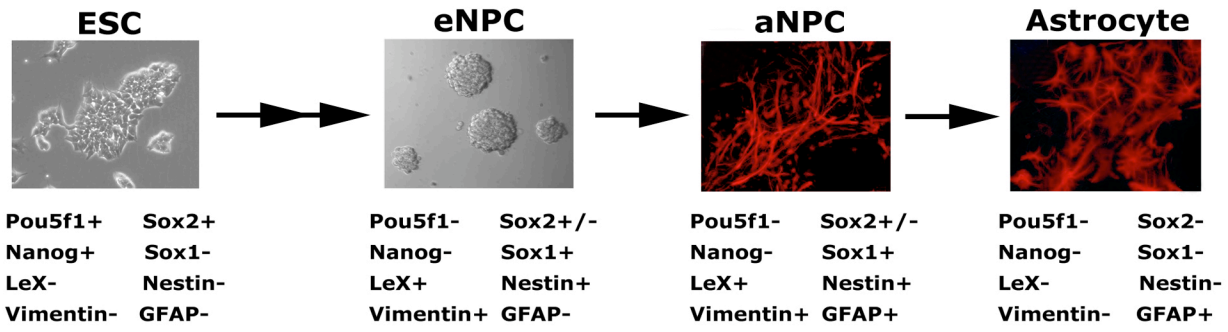
(A-C) Noggin is expressed in the SGZ, GCL, and hilus of the adult dentate gyrus (D-F) Transgenic animals were created in which the neuron specific enolase (NSE) promoter drives expression of noggin and a GFP cassette. Cells in the GCL express GFP and noggin levels are enhanced in the GCL, SGZ, and hilus. (G-K) BMP signaling was assessed by phospho-SMAD 1/5/8 immunofluorescence. P-SMAD is decreased in proliferating cells in the SGZ and specifically within $GFAP^+BrdU^+$ cells. Arrows point to $GFAP^+$ cells, arrowheads to $PSA\text{-}NCAM^+$ $BrdU^+$ cells. WT: FVB wild-type; GCL: granule cell layer; SGZ: subgranular zone; h: hilus; p-SMAD: phosho-SMAD1/5/8. Error bars represent mean ± SEM of at least 20 cells from 3 separate wild-type mice. *Differs from the $BrdU^-$ group at $p< 0.01$ by ANOVA with the Bonferroni post hoc test.

Figure 19: Noggin increases adult SGZ stem/progenitor cell proliferation



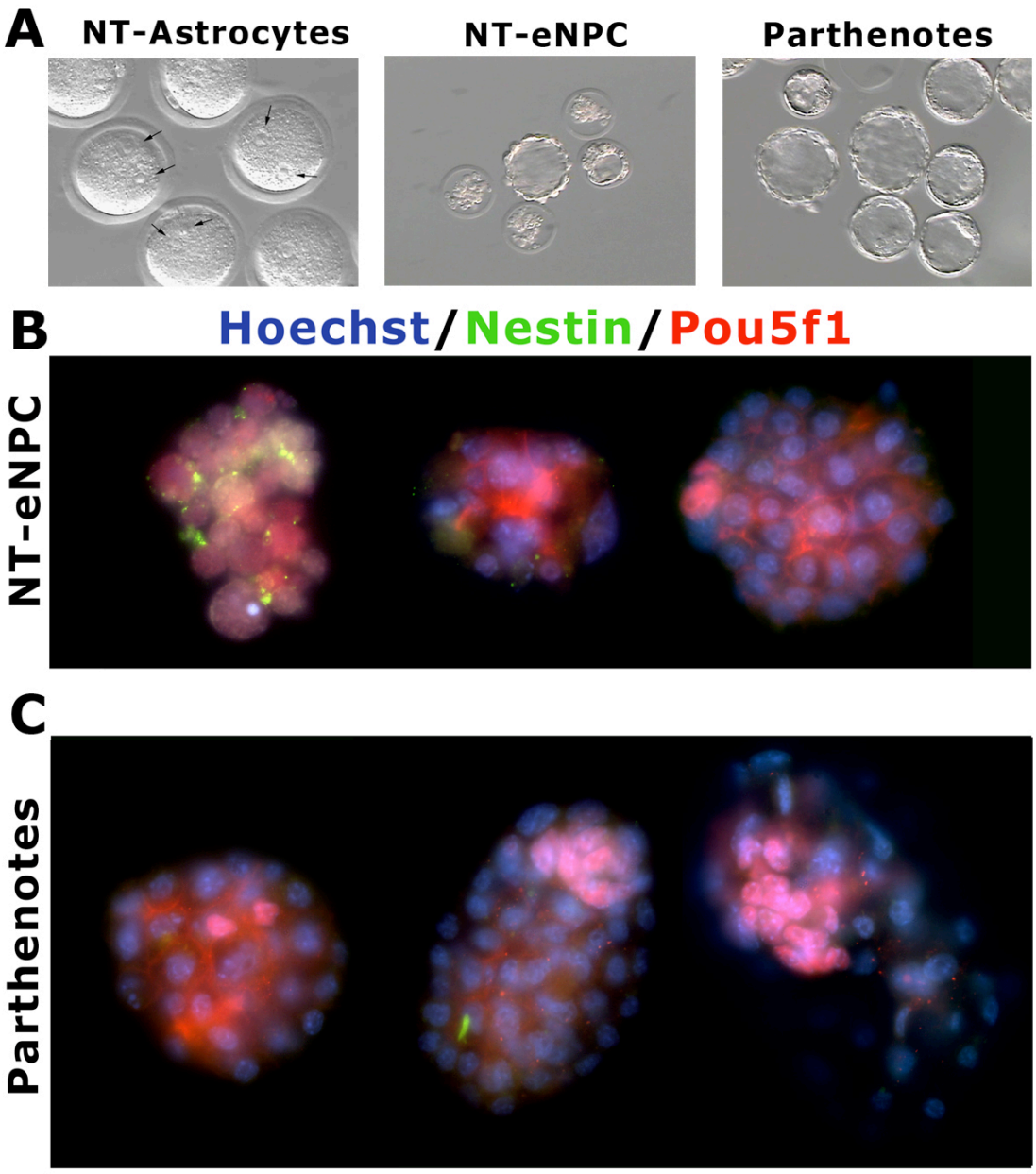
Noggin was overexpressed under the control of the NSE promoter. (A-E) BrdU labeling and immunofluorescence reveals an increase in multiple precursor populations: GFAP$^+$ stem cells, PSA-NCAM$^+$ migratory precursors, and GFAP$^-$ PSA-NCAM$^-$ transient amplifying progenitors. (F) Although all three precursor populations are expanded, the proportion of GFAP$^+$ stem cells is increased at the expense of transient amplifying progenitors. Arrows point to GFAP$^+$ BrdU$^+$ cells, arrowheads to PSA-NCAM$^+$ BrdU$^+$ cells. NCAM: PSA-NCAM. Error bars represent mean ± SEM of three experiments *P<0.05; **P≤0.01 by unpaired Student's t-test

Figure 20: Defining a neural lineage



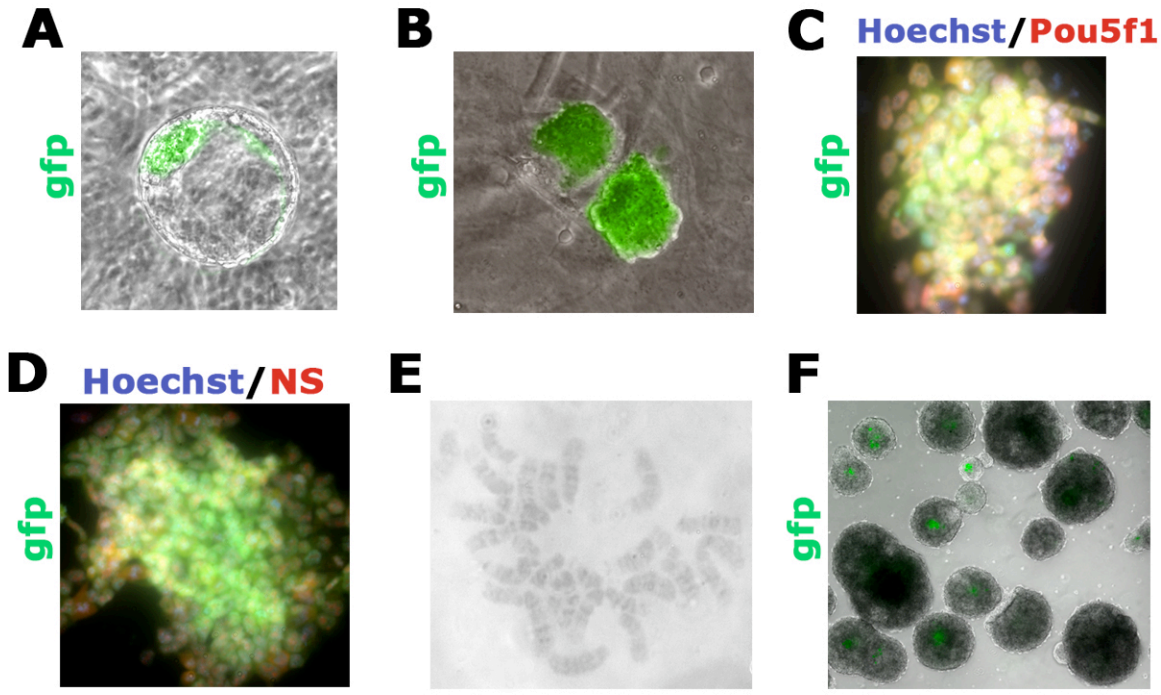
ESCs are pluripotent rapidly dividing cells characterized by Pou5f1 and Nanog expression. ESC commit into eNPC via EGF and FGF2 signaling. ENPC are multipotential rapidly dividing cells that express Sox1, Nestin, and commit to aNPC via LIF signaling and BMP inhibition. ANPC are also multipotential and rapidly dividing, but have glial characteristics (GFAP) and are biased towards astrocyte differentiation. They differentiate into quiescent stellate astrocytes via BMP signaling and lose precursor characteristics. ESC, embryonic stem cell; eNPC, embryonic neural precursor cell; aNPC, adult neural precursor cell.

Figure 21: Characteristics of morulae and blastocysts derived from eNPC donors via SCNT



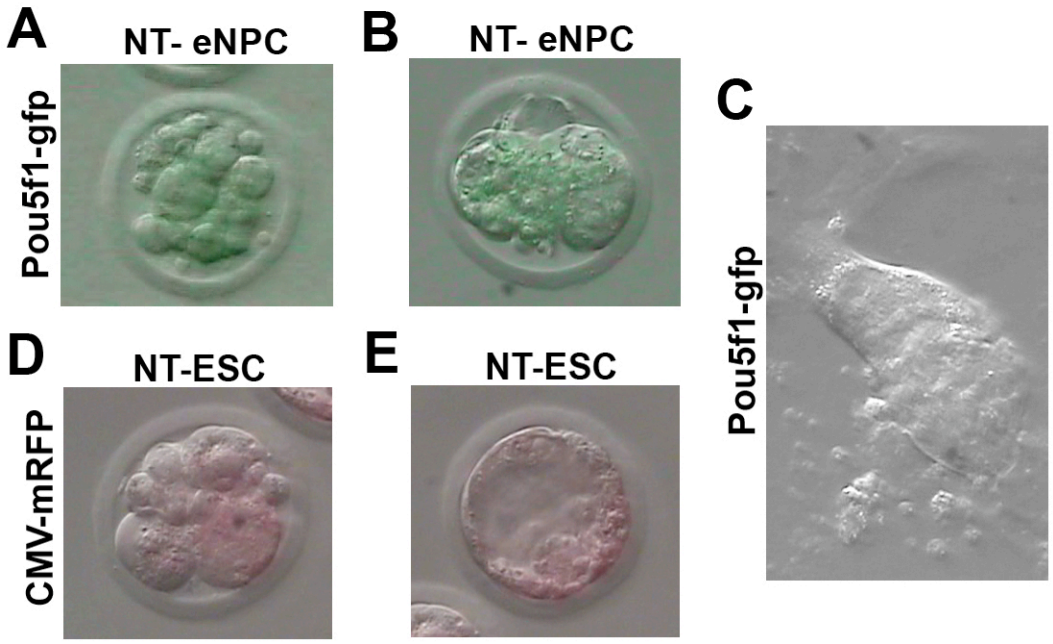
(A) eNPC and astrocytes generate activated oocytes via NT, but only eNPC donors generate blastocysts. (B-C) eNPC-derived morulae and blastocysts exhibit variable phenotypes from proper Pou5f1 and Nestin expression to improper expression of embryonic or donor traits compared to parthenote controls. Arrows point to pronuclei. eNPC, embryonic neural precursor cell; NT, nuclear transfer derived.

Figure 22: Derivation and characterization of the Pou5f1-1 ESC line



(A) Blastocysts were cultured from the zygote stage and placed on MEF feeder layers. The inner cell mass is green indicative of gfp driven by the Pou5f1 promoter. (B) ESC outgrowths were harvested and split for passaging. Colonies retain gfp expression – shown is passage 1. (C-D) ESC plated on glass coverslips were processed by immunofluorescence for markers of ESC: Pou5f1 and Nucleostemin (NS). (E) Karyotype analysis. (F) ESCs are able to form embryoid bodies and lose gfp expression by 5 days.

Figure 23: Analysis of embryos derived from tagged donor cells



(A-B) Embryos reconstructed with eNPC OG2 donor cells develop to the morula and early blastocyst stage and express gfp. (C) The blastocyst was plated for ESC derivation, formed a primary outgrowth, and lost gfp expression. (D-E) Embryos reconstructed with ESC donors tagged with an rfp construct develop to the morula and late expanded blastocyst stage and express rfp. ESC, embryonic stem cell; eNPC, embryonic neural precursor cell; NT, nuclear transfer derived.

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APPENDIX A: MOUSE CLONING DATA SUMMARY

A.1 Adult Donors

Donor Cell	Stage	2-cell	Morula	Blast	Term	Media	Technique	Ref
Cumulus	G0	47	10	6	NA	CZB	Injection	(Chung et al., 2002)
Cumulus	G0	85	31	13	NA	CZBG^	Injection	(Chung et al., 2002)
Cumulus	G0	90	61	37	NA	CZB-G	Injection	(Chung et al., 2002)
Cumulus	G0	NA	53	NA	0.7	CZB-G	Injection	(Wakayama and Yanagimachi, 2001)
Cumulus	G0	79	41	NA	0.5	CZB-G	Electrofusion	(Ogura et al., 2000b)
Cumulus	G0	68	30	NA	NA	CZB-G	Injection	(Eggan et al., 2000)
Cumulus	G0	89	19	5	NA	CZB-G	Conventional	(Heindryckx et al., 2001)
Cumulus	G0	84	48	20	NA	CZBG > KSOM	Injection	(Chung et al., 2002)
Cumulus	G0	36	6	6	NA	KSOM	Injection	(Chung et al., 2002)
Cumulus	G0	NA	56	38	NA	KSOM	Injection	(Kim et al., 2002)
Cumulus	G0	92	37	19	0.5	KSOM	Conventional	(Rybouchkin et al., 2002)
Cumulus	G0	92	44	28	NA	KSOM > G2	Conventional	(Heindryckx et al., 2001)
Cumulus	G0	85	46	38	NA	WM	Injection	(Chung et al., 2002)
Cumulus	G0	93	44	35	NA	WM > KSOM	Injection	(Chung et al., 2002)
Cumulus	G0	81	11	3	NA	G1>G2	Injection first	(Munsie et al., 2000)
Cumulus	G0	87	23	14	NA	G1>G2	Conventional	(Heindryckx et al., 2001)
Cumulus	G0	68	22	8	NA	M16	Mod. Conv.	(Zhou et al., 2000)
Cumulus	G0	77	30*	9*	0.3	M16	Injection	(Boiani et al., 2002)
Cumulus	G0	78	NA	19	0	M16	Sendai fusion serial NT	(Kato et al., 1999b)
Follicular Epithelial	G0	82	NA	28	0	M16	Sendai fusion serial NT	(Kato et al., 1999a)
Sertoli	NA	NA	40	NA	0	CZB-G	Injection	(Wakayama et al., 1998)
Sertoli (P3-5)	NA	59	37	NA	0.9	CZB-G	Injection	(Ogura et al., 2000a)
Sertoli (P8-10)	NA	58	22	NA	0.2	CZB-G	Injection	(Ogura et al., 2000a)
Sertoli (culture)	G0?	68	49	NA	0.9	CZB-G	Injection	(Ogura et al., 2000a)
Fibroblast	NA	NA	54	NA	0.6	CZB-G	Injection	(Wakayama and Yanagimachi, 1999)
Fibroblast	NA	89	54	NA	NA	CZB-G	Injection	(Eggan et al., 2000)
Macrophage	NA	NA	31	NA	0	CZB-G	Injection	(Wakayama and Yanagimachi, 2001)
Spleen	NA	NA	22	NA	0	CZB-G	Injection	(Wakayama and Yanagimachi, 2001)
Thymus	NA	NA	3	NA	0	CZB-G	Injection	(Wakayama and Yanagimachi, 2001)

Donor Cell	Stage	2-cell	Morula	Blast	Term	Media	Technique	Ref
B cell	NA	78	16	4.7	10**	CZB-G	Injection	(Hochedlinger and Jaenisch, 2002)
T cell	NA	89	20	6.5	1**	CZB-G	Injection	(Hochedlinger and Jaenisch, 2002)
T cell	NA	98	12	NA	0	CZB-G	Injection	(Inoue et al., 2005)
NKT	NA	75	74	NA	0.2	CZB-G	Injection	(Inoue et al., 2005)
LT-HSC	NA	87	6	NA	0.3	CZB-G	Injection	(Inoue et al., 2006)
Brain	NA	NA	22	NA	0	CZB-G	Injection	(Wakayama et al., 1998)
Cortical neuron	G0	93	2	NA	0	CZB-G	Injection	(Osada et al., 2002)
Purkinje neuron	G0	83	0	NA	0	CZB-G	Injection	(Osada et al., 2002)
Myoblast	NA	96	NA	2	NA	CZB-G	Injection	(Gao et al., 2003a)
Myoblast	NA	96	NA	0	NA	WM	Injection	(Gao et al., 2003a)
Myoblast	NA	85	NA	25	NA	DMEM > WM	Injection	(Gao et al., 2003a)
Myoblast	NA	91	NA	43	NA	DMEM	Injection	(Gao et al., 2003a)

Data represents embryo survival as % of activated embryos (pronuclei formation), except
* % of 2-cell development
** % term survival of blastocysts transferred to surrogate mother, reconstructed from NT-derived ES cells
^ Activation without DMSO present

A.2 Fetal Donors

Donor Cell	Stage	2-cell	Morula	Blast	Term	Media	Technique	Ref
fibroblast (E14)	NA	64	23	NA	0	CZB-G	Injection	(Ogura et al., 2000a)
fibroblast (E15)	M	NA	37	NA	0	CZB-G	Sendai fusion	(Ono et al., 2001a)
fibroblast (E15)	M	NA	31	NA	0.9	CZB-G	Sendai fusion Serial NT	(Ono et al., 2001a)
Neural (E16 CP+MZ)	NA	NA	29	NA	1.1	CZB-G	Injection	(Yamazaki et al., 2001)
Neural (E16 VZ+IZ)	NA	NA	38	NA	4.7	CZB-G	Injection	(Yamazaki et al., 2001)
Ovary (E13-15)	NA	NA	59	NA	2.6	CZB-G	Injection	(Wakayama and Yanagimachi, 2001)
Testis (E13-15)	NA	NA	57	NA	1.1	CZB-G	Injection	(Wakayama and Yanagimachi, 2001)
PGC(E13-16)	NA	NA	65	NA	0	CZB-G	Injection	(Lee et al., 2002)
PGC(E13-16)	NA	83	81*	56*	0	M16	Injection	(Boiani et al., 2002)

Data represents embryo survival as % of activated embryos (pronuclei formation).

A.3 Embryonic donors

Donor Cell	Background	Stage	2-cell	Morula	Blast	Term	Media	Technique	Ref
E14 ES p33	129/Ola	G0	NA	13	NA	0	CZB-G	Injection	(Wakayama et al., 1999)
E14 ES p33	129/Ola	G1?	NA	16	NA	0.8	CZB-G	Injection	(Wakayama et al., 1999)
E14 ES p33	129/Ola	G2/M?	NA	35	NA	0.5	CZB-G	Injection	(Wakayama et al., 1999)
J1 ES p11	129/Sv	G1?	NA	18	NA	2.8	CZB-G	Injection	(Eggan et al., 2000)
J1 ES p35	129/Sv	G1?	NA	25	NA	0	CZB-G	Injection	(Eggan et al., 2000)
V26.2 ES	C57BL/6	G1?	NA	24	NA	1.2	CZB-G	Injection	(Eggan et al., 2001)
V39.7 ES	BALB/c	G1?	NA	28	NA	0	CZB-G	Injection	(Eggan et al., 2001)
V6.5 ES	C57BL/6 x 129Sv	G1?	NA	21	NA	4.7	CZB-G	Injection	(Eggan et al., 2001)
129B6 ES	129/Sv x C57BL/6	G1?	NA	27	NA	4.5	CZB-G	Injection	(Eggan et al., 2001)
R1 ES p32	129/SvJ x 129/Sv	G0	NA	32	NA	3.6	CZB-G	Injection	(Wakayama et al., 1999)
R1 ES p19	129/SvJ x 129/Sv	G1?	76	54	NA	2.8	CZB-G	Injection	(Gao et al., 2002)
R1 ES p19	129/SvJ x 129/Sv	G1	50	19	13	NA	M16	Mod. Conv.	(Zhou et al., 2001)
R1 ES p32	129/SvJ x 129/Sv	G1?	NA	38	NA	3	CZB-G	Injection	(Wakayama et al., 1999)
R1 ES p19	129/SvJ x 129/Sv	G2	66	16	6	NA	M16	Mod. Conv.	(Zhou et al., 2001)
R1 ES p32	129/SvJ x 129/Sv	M?	NA	24	NA	2	CZB-G	Injection	(Wakayama et al., 1999)
R1 ES p19	129/SvJ x 129/Sv	M	95	69	55	2	M16	Mod. Conv.	(Zhou et al., 2001)
R1 ES p25	129/SvJ x 129/Sv	M	96	75	59	0	M16	Mod. Conv.	(Zhou et al., 2001)
NARA5 p10	C57BL/6 x C3H	M	78	46	NA	0.5	M16	Sendai fusion	(Amano et al., 2001)
NARA5 p10	C57BL/6 x C3H	M	90	81	NA	1.5	M16	Sendai fusion Serial NT	(Amano et al., 2001)
TT2 ES	C57BL/6 x CBA	M	99	NA	70	3.2	CZB-G	Sendai fusion	(Ono et al., 2001b)
TT2 ES	C57BL/6 x CBA	M	99	NA	51	3.7	CZB-G	Sendai fusion Serial NT	(Ono et al., 2001b)
Trophecto-derm	C57BL/6 x CBA	M	81	62	NA	2	M16	Sendai fusion Serial NT	(Tsunoda and Kato, 1998)
8-cell	C57BL/6 x CBA	G1	93	NA	46	8.3	M16	Electrofusion	(Cheong et al., 1993)
4-cell	C57BL/6 x CBA	G1	100	NA	71	14.3	M16	Electrofusion	(Cheong et al., 1993)
4-cell	C57BL/6 x CBA	M	100	87	83	35.7	CZB > CZB-G	Sendai fusion Serial NT	(Kwon and Kono, 1996)

Donor Cell	Background	Stage	2-cell	Morula	Blast	Term	Media	Technique	Ref
2-cell	C57BL/6 x CBA	G1	97	83	78	21.7	M16	Electrofusion	(Cheong et al., 1993)
2-cell	C57BL/6 x CBA	S	38	0	0	0	M16	Electrofusion	(Cheong et al., 1993)
2-cell	C57BL/6 x CBA	G2	78	33	21	NA	M16	Electrofusion	(Cheong et al., 1993)

Data represents embryo survival as % of activated embryos (pronuclei formation).

A.4 Direct Lineage Donor Comparison

Donor Cell	Stage	2-cell	Morula	Blast	Term	Media	Technique	Ref
LT-HSC	NA	83	4.1	NA	NA	KSOM	Injection	(Sung et al., 2006)
ST-HSC	NA	86	7.9	NA	NA	KSOM	Injection	(Sung et al., 2006)
HPC	NA	89	10.6	NA	NA	KSOM	Injection	(Sung et al., 2006)
granulocyte	NA	71	34.5	NA	0.3	KSOM	Injection	(Sung et al., 2006)
NPC	NA	72	7.1	NA	0.3	CZB-G	Injection	(Mizutani et al., 2006)
NPC committed	NA	61	28.8	NA	0.8	CZB-G	Injection	(Mizutani et al., 2006)
Neural (E16 CP+MZ)	NA	NA	29	NA	1.1	CZB-G	Injection	(Yamazaki et al., 2001)
Neural (E16 VZ+IZ)	NA	NA	38	NA	4.7	CZB-G	Injection	(Yamazaki et al., 2001)

Data represents embryo survival as % of activated embryos (pronuclei formation).

APPENDIX B: MOUSE NT-ESC DERIVATION EFFICIENCIES

Donor Cell	Cell line (background)	Morula	Blast	ESC lines	ESC rate	ESC rate (active)	Ref
ESC line	V6.5 (B6x129)	NA	NA	2	50	3.1	(Eggan et al., 2001)
NSC lines	NS5 (129)	NA	5.4	5	100	5.4	(Blelloch et al., 2006)
NSC lines	NSV6.5 (B6x129)	NA	10.9	3	60	6.5	(Blelloch et al., 2006)
NSC lines	Cor 1-5 (MF1)	NA	5.1	6	50	2.6	(Blelloch et al., 2006)
Olfactory Neurons	WT (129 mix)	NA	13.4	5	7.6	1.0	(Eggan et al., 2004)
Olfactory Neurons	OMP-GFP	NA	12.4	6	16	1.9	(Li et al., 2004)
Olfactory Neurons	OMP-Cre x Z/EG	NA	8.4	7	28	2.4	(Li et al., 2004)
Olfactory Neurons	M71-Cre x Z/EG	NA	10.9	3	25	2.7	(Li et al., 2004)
Lymphocytes	WT (B6D2F1)	16.3	3.7	1	4.8	0.2	(Hochedlinger and Jaenisch, 2002)
Lymphocytes	WT (B6 x129)	20.3	6.5	1	5.0	0.3	(Hochedlinger and Jaenisch, 2002)
NKT	CD1+ (B6x129)	73.9	NA	6	NA	4.1	(Inoue et al., 2005)
LT-HSC	LKS,CD34- (B6D2F1)	5.9	NA	2	15	NA	(Inoue et al., 2006)
Fibroblasts (TT)	M (129)	NA	47.7	1	2.4	1.1	(Wakayama et al., 2001)
Fibroblasts (TT)	M (B6)	NA	41.9	4	4.5	1.9	(Wakayama et al., 2001)
Fibroblasts (TT)	F (B6)	NA	47.2	5	6.7	3.1	(Wakayama et al., 2001)
Fibroblasts (TT)	M (B6x129)	NA	29.7	7	13	3.8	(Wakayama et al., 2001)
Fibroblasts (TT)	M Tg-EGFP	NA	22.4	1	5.3	1.2	(Wakayama et al., 2001)
Fibroblasts (TT)	WT (B6 x129)	NA	12.6	3	25	3.2	(Blelloch et al., 2006)
Fibroblasts (TT)	WT (129)	NA	17.5	4	22	3.9	(Blelloch et al., 2006)
Fibroblasts (TT)	WT (inbred)	14.5	NA	7	18	2.5	(Wakayama et al., 2005)
Fibroblasts (TT)	WT (hybrid)	15.1	NA	18	24	3.6	(Wakayama et al., 2005)
Cumulus	WT (129)	NA	29.5	1	7.7	2.3	(Wakayama et al., 2001)
Cumulus	WT (B6D2F1)	NA	43.8	9	16	6.9	(Wakayama et al., 2001)
Cumulus	Tg-EGFP	NA	42.4	7	14	5.9	(Wakayama et al., 2001)
Cumulus	WT (B6D2F1)	45.8	NA	13	38	NA	(Inoue et al., 2006)
Cumulus	WT (inbred)	33.8	NA	8	9.1	3.1	(Wakayama et al., 2005)
Cumulus	WT (hybrid)	50.0	NA	42	19	9.5	(Wakayama et al., 2005)
Cumulus	WT (B6D2F1)	NA	26	5	8.2	2.1	(Boiani et al., 2005)
Cumulus	WT (B6D2F1)	NA	52*	33	39.3	20.4	(Boiani et al., 2005)
Sertoli	Tg (hybrid)	30.6	NA	4	26	8.2	(Wakayama et al., 2005)

Data represents embryo survival as % of activated embryos (pronuclei formation).
ESC rate = number of ESC lines / morula or blasts
ESC rate (active) = number of ESC lines / activated embryos
*Culture in α-MEM media, instead of CZB-G (or KSOM)