APPLICATION OF ADULT STEM/PROGENITOR CELLS IN THERAPEUTIC TREATMENT OF CEREBELLAR DEGENERATIVE DISEASES

By

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To my Mom and Dad for their unconditional love and support that I will forever be thankful for.

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LIST OF ABBREVIATIONS

AAV	Adeno-associated virus
bFGF	basic fibroblast growth factor
β-gal	β-galactosidase
BMDCs	Bone marrow-derived cells
BPE	bovine pituitary extract
CMV	cytomegalovirus
CNS	Central Nervous System
DMEM	Dulbecco's Modified Eagle Medium
EBs	embryoid bodies
EGF	Epidermal growth factor
EGFR-PTK	Epidermal growth factor receptor protein tyrosine kinase
ES cells	embryonic stem cells
ESNPs	embryonic stem cell-derived neural precursor
FACS	fluorescent-activated cell sorting
FBS	Fetal bovine serum
FISH	fluorescence in situ hybridization
Floxed	lox-P flanked
GFAP	glial fibrillary acidic protein
GFP	Green fluorescent protein
GIRK	G-protein-coupled, inward rectifying potassium
H & E	Hematoxylin and eosion
HSCs	Hematopoietic stem cells
LPO	laminin-, poly-L-ornithine
MASCs	Multipotent astrocytic stem cells

MATH 1	mouse atonal homolog 1
NSCs	Neural stem cells
PBS	Phosphate Buffered Saline
Pcd	Purkinje cell degeneration
RMS	Rostral migratory stream
SCA 1	Spinocerebellar Ataxia 1
SEZ	Subependymal zone
SSC	Sodium Chloride Sodium Citrate
SSEA 1	stage-specific embryonic antigen-1
SVZ	subventricular zone

Abstract of Dissertation Presented to the Graduate School
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Trauma/injury or neurodegenerative diseases within the cerebellum can give rise to ataxia, or incoordination of movements, that affects a large population worldwide and currently has no treatments available. Use of adult stem/progenitor cells as a source of transplantable donor cellular agents is a reasonable strategy to replace or repair the degenerated or at-risk neuronal populations to restore homeostasis within the cerebellum. Two stem/progenitor cell types that show the most promise to reconstitute lost neural tissue are neural stem/progenitor cells (NSCs) derived from the CNS and hematopoietic stem cells (HSCs) found within bone marrow. NSCs possess restricted developmental potential and give rise to tissue-specific progeny within the neural lineage while HSCs have been shown to retain broader differentiation potential that extends to atypical lineages outside of the blood system, including Purkinje neurons of the cerebellum, through cell-cell fusion. Experimental paradigms were created that take advantage of the variation within the differentiation potential and mechanisms utilized by NSCs and HSCs to replace or repair the multiple complex cerebellar cell types impacted by trauma/injury or diseases.

NSCs were used in homotopic transplantation into weaver mutant mice for replacement of cerebellar neurons. Donor cells were found to survive, migrate, and apparently initiate

differentiation but no impressive region-specific identities were adopted by the donor cells despite earlier studies that suggested the potential of these cells to respond to *in vivo* cues when placed in a permissive/instructive milieu. HSCs, on the other hand, were used as delivery vehicles to transfer neuroprotective genes/factors into the degenerating Purkinje neurons of a transgenic mouse model of Spinocerebellar Ataxia 1 (SCA1). Genetically modified HSCs were transplanted into Sca1 mice and the recipient cerebella were examined for donor-derived heterokaryons and for expression of the neuroprotective genes for proof of principle of using stem cell fusion and gene therapy to treat a neurological disorder. Together, the present study describes a thematic research approach in the establishment of novel therapeutic strategies for ataxia by using adult stem/progenitor cells in rescuing at-risk neuronal populations, with either direct cell replacement or repair through providing new genetic material, in well-characterized animal models of cerebellar ataxia.

CHAPTER 1
INTRODUCTION TO ADULT STEM/PROGENITOR CELLS

Adult Stem Cell Diversity and Therapeutic Potential

Stem cells can be isolated from many adult tissues at various developmental stages and be used for cell-based therapies for repair and restoration of homeostasis. Sites harboring these tissue-specific stem cells include blood, skin, intestine, gonad, brain, breast, liver, muscle, lung, and kidney (Nystul and Spradling, 2006; Vats et al., 2005; Hombach-Klonisch et al., 2008). Studies show that stem cells possess the ability to differentiate into cell types found within the tissue of origin, and may be suited for replacement of damaged tissue within the same region. Degeneration of neural tissue in the central nervous system (CNS) can lead to many devastating disorders including Alzheimer's disease, Parkinson's disease, Huntington's disease, and Spinocerebellar Ataxias, just to name a few. Many of these neurodegenerative diseases involve the loss of specific neuronal populations and the challenge lies in the development and the generation of needed cell types *in vitro* followed by heterologous, or even better, autologous transplant into the damaged area. For neurodegenerative disorders, transplantation of neural stem cells (NSCs) into a damaged CNS region would be the most straightforward and plausible approach for generating new neural tissue. NSCs have already demonstrated the ability to develop into lineage appropriate cell types and they can be maintained and expanded in culture for transplantation into transgenic and other animal models of neurodegenerative disorders. However, they have not been shown to have the ability to generate all the different types of neuronal and glial cells, including Purkinje neurons that selectively undergo degeneration in many movement disorders, and it currently does not seem to be possible to have stem cells treat the entire range of CNS disorders. Aside from potency, other important factors to consider include accessibility, and the robustness of the stem cell resource. NSCs reside in brain regions

that are hard to access without disturbing the parenchyma and they also exist in small numbers, hence stem cells derived from other regions/sources should be considered for use in CNS repair in addition to NSCs. Recent studies have shown that some adult stem cells possess broader differentiation potential than previously thought, and they have the ability to generate cell types outside the tissue of origin. Bone marrow derived cells (BMDCs), in particular, have been reported to give rise to atypical lineages of liver, intestine, heart, skeletal muscle cells and Purkinje neurons (Ferrari et al., 1998; Gussoni et al., 1999; Jackson et al., 1999; Petersen et al., 1999; Lagasse et al., 2000; Krause et al., 2001; Orlic et al., 2001; Priller et al., 2001; Alvarez-Dolado et al., 2003; Weimann et al., 2003), through mechanisms that likely comprise both trans-differentiation and cell-cell fusion. Thus BMDCs represent another possible candidate for replacement or protection therapies in neurodegenerative disorders, especially those involving atrophy of Purkinje neurons since that is the only neuronal population within the CNS that BMDCs have been shown to turn into through cell-cell fusion. In contrast to NSCs, BMDCs are better characterized stem cells, are easily accessible, and bone marrow transplants are less invasive than intracranial injections for future clinical applications.

The complexity of the CNS requires that multiple approaches are needed to repair, replace, or protect damaged neural tissue, so both types of adult stem cells mentioned above should be considered for complimentary therapeutic approaches. Moreover, other modalities involving gene therapy or drug administration might also prove beneficial in conjunction with cell-based therapy, considering that more than one factor often contribute to neurodegeneration. The work presented here possesses an underlying theme that exploits the potential of using two different types of adult stem cells, under different conditions, to replace degenerating neuronal populations. This study also focuses on the repair of the cerebellum because this is a region

affected in numerous movement disorders, but there are currently very little to no effective treatments available to remedy the loss of this important CNS structure. In addition, there are well-characterized cerebellar mutant and transgenic mouse models available for testing new cell and molecular therapies. Methods include direct transplantation of NSCs into compromised cerebella for examination of transplant survival and functional integration of donor cells, as well as transplantation of BMDCs in combination with gene therapy for a less invasive bone marrow transplant approach. The intended goal is to derive cell-based strategies with different donor populations and different delivery systems to achieve novel therapeutic paradigms for the rescue of at-risk neuronal populations within the degenerating cerebellum.

Overview of Neural Stem/Progenitor Cells as Donor Population

Most neurons within the CNS of higher vertebrates are postmitotic and non-proliferative. However, recent studies show that persistent neurogenesis occurs in restricted and discrete neuropoietic zones throughout postnatal and adult life (Steindler and Pincus, 2002). Specifically, robust and persistent neurogenesis is found within two regions of the CNS of rodents and humans: the subependymal zone (SEZ) lining the lateral ventricles of the forebrain (Lois and Alvarez-Buylla, 1994; Curtis et al., 2007) and the subgranular layer in the dentate gyrus of the hippocampus (Kirschenbaum et al., 1994; Kuhn et al., 1996; Eriksson et al., 1998; Gould et al., 1999; Kornack and Rakic, 1999). NSCs are mostly concentrated in the SEZ of the forebrain since this is a vestigial remnant of the embryonic germinal zone that displays constitutive proliferation. Cells within this neurogenic zone continuously generate populations of neuroblasts that migrate a long distance from the lateral ventricles to the olfactory bulb via the rostral migratory stream (RMS) to develop into mature interneurons (Luschkin 1993; Lois and Alvarez-Buylla, 1994). Studies from Doetsch et al. (1997) showed that the cytoarchitecture of the SEZ consists of three basic cell types that represent the astrocytes (often referred to as “B” cells) that

give rise to highly proliferative precursor cells ("C"cells), which then become progressively restricted in developmental potentials before dividing into migrating neuroblasts ("A" cells). Only the astrocytes showed NSC attributes with the characteristic mitotic quiescence and the ability to give rise to progenitors, including cells of neuronal phenotype (Doetsch et al., 1999). In addition, these astrotypic NSCs can form multipotent proliferative clones, or neurospheres, that give rise to the three major classes of neural cells: neurons, astrocytes, and oligodendrocytes *in vitro* (Kukekov et al., 1999; Kukekov et al., 1997; Reynolds and Weiss, 1992; Richards et al., 1992). Work from our laboratory (Laywell et al., 2000) further confirmed that astrocytes isolated from the SEZ of the developing brain up to the end of the second postnatal week are multipotent stem cells, which we refer to as multipotent astrocytic stem cells (MASCs) that are capable of forming neurospheres and give rise to both neurons and glia. These MASCs, with attributes of neural stem/progenitor cells, thus represent potential donor population for cell replacement strategies.

Aside from the SEZ, another region with transient neurogenesis is the postnatal cerebellar cortex. This is a region of active proliferation during early development with newly generated granule cells forming the transient external granule cell layer (Hatten et al., 1997). However, this has not been a site widely reported as a source of multipotent neurospheres under the standard culture conditions that combine removal of cell-cell/substrate contact with serum-free medium containing growth factors. Under a novel culture paradigm from our laboratory (Laywell, et al., 2005), neurosphere-like cell clusters can be generated from serum-, and growth factor-dependent conditions under higher density and demonstrated to be multipotent with the ability to form all three primary CNS cell types as well. Others were also able to isolate stem cell-like populations from murine cerebellum that can form neurospheres under clonogenic

conditions and give rise to region appropriate cerebellar cell types including parvalbumin-expression interneurons, GABA-ergic neurons, and glutaminergic neurons both *in vitro* and *in vivo* (Lee et al., 2004; Klein et al., 2004). In addition, cerebellar-derived MASCs possess the ability to survive, migrate, and differentiate when transplanted into the SVZ of normal adult mice (Zheng et al., 2006), and represent another possible candidate for cell replacement strategies. Even though in the study by Zheng and colleagues, only cell types with mostly glial morphology and a small population of olfactory interneurons were generated from the transplant into the SVZ of wildtype mice, it is possible that the cerebellar MASCs could have enhanced responses to endogenous cues present within the damaged or degenerated cerebellum following homotopic transplantation. In the current study, changes of transplant location from SVZ to cerebellum and the host environment from healthy adult mice into postnatal mutant pups may provide additional guidance and/or foster interactions that result in an increase in the generation of region-specific neuronal populations within the cerebellum.

Overview of Bone Marrow Cells as a Donor Population

Bone marrow cells that harbor hematopoietic stem cells (HSCs) are pluripotent and highly proliferative, with the ability to generate up to an estimated 10^{13} mature blood cells in the normal adult lifespan, and capable of self-regeneration that ensures sufficient hematopoiesis over the lifetime (Szilvassy, 2003). In addition to generating and maintaining all the lymphoid and myeloid cells needed for blood, bone marrow, spleen, and thymus systems, BMDCs have also demonstrated the ability to differentiate into cells of nonhematopoietic tissues including liver, intestine, heart and skeletal muscle cells (Krause et al., 2001; Orlic et al., 2001; Lagasse et al., 2000; Gussoni et al., 1999; Jackson et al., 1999; Petersen et al., 1999; Ferrari et al., 1998). Furthermore, there is evidence, albeit controversial, showing that BMDCs have the potential of turning into neuronal cell types both *in vitro* and *in vivo*. One of the first reports used lethally

irradiated adult mice as recipient of GFP^+ bone marrow cells transplanted through intravascular injection and found GFP^+ cells expressing neuron specific genes within the olfactory bulb (Brazelton et al., 2000). Another similar study took the approach of injecting bone marrow cells into neonatal PU.1 knockout mice that normally lacked macrophages, neutrophils, mast cells, osteoclasts, and B and T cells at birth, in order to obtain an estimation of the amount of donor derived cells that are present within the CNS. The authors found approximately 2.3 to 4.6% of bone marrow derived cells within the brain, but only a small population of 0.3 to 2.3 % expressed neuronal marker NeuN (Mezey et al., 2000). Even though the rodent evidence for trans-differentiation is not so strong (Deng et al., 2006), data showing BMDCs giving rise to neurons and glia following transplants in humans (Cogle et al., 2004) is rather provocative. Despite such encouraging results, few of BMDCs bearing neuronal antigenic profile also displayed morphological characteristics of neurons (Brazelton et al., 2000; Mezey et al., 2000), and it was not clear how long these BMDCs persisted in the brain or what mechanism was behind the observed plasticity. Following a long term bone marrow transplant into lethally irradiated adult mice, Priller and colleagues (2001) reported neogenesis of functional bone marrow-derived Purkinje neurons within the cerebellum and believed that trans-differentiation was the driving force behind the observed lineage switch. However, upon closer examination, *in vitro* culture of stem cells with different lineages revealed hybrid cells bearing abnormal number of chromosomes that most likely result from cell-cell fusion (Terada et al., 2002; Ying et al., 2002). In these two reports, co-culture of ES cells with either bone marrow cells or neural stem cells yielded hybrid populations that expressed both donor cell markers and ES cell-like properties that could have led to the conclusion that trans-differentiation was involved if DNA content of the cells was not analyzed. Other *in vivo* studies have also reached similar

conclusions based on bone marrow transplants using sex mismatched donor cells and close examination of donor-derived Purkinje neurons for the presence of extra nuclei (Weimann et al., 2003a; Weimann et al., 2003b). One in-depth study utilized the Cre/lox recombination system to further show that bone marrow transplants using BMDCs from mice expressing Cre recombinase into transgenic animals with floxed LacZ reporter genes generated cells with β-galactosidase activity, which is possible only as a result of cell-cell fusion (Alvarez-Dolado et al., 2003).

These findings, together, suggest that controlled cell-cell fusion is a mechanism that can be exploited to expand the developmental scope of adult stem cells. This would be especially beneficial for complex cell types such as Purkinje neurons in the CNS that are vulnerable in many neurodegenerative disorders but lack evidence for *de novo* genesis after birth. Specifically, induced plasticity may be exploited for novel therapeutic strategies involving the use of cell fusion to deliver modified genomes for reconstitution, or better said, neuroprotection, of degenerated or at-risk neuronal populations. One disorder with specific Purkinje neuron atrophy that might benefit from this system is spinocerebellar ataxia 1 which is a gain of function polyglutamine repeat disease that currently has no effective treatments available. The cause underlying the neuronal cell death has not yet been elucidated, but evidence shows that the degeneration of Purkinje cells may be due to protein misfolding and impaired protein clearance as a result of the toxic gain of function caused by glutamine expansion (Orr and Zoghbi, 2007; Orr and Zoghbi, 2001; Zoghbi and Orr, 2000). The current study sets out to test the concept of using genetically modified BMDCs as vehicles to deliver potentially neuroprotective genes or factors into degenerating neurons for restoration of homeostatic balance within the host system as a novel therapeutic treatment.

CHAPTER 2
MATERIALS AND METHODS

Supplier Information

Abcam (Cambrige, MA), Amersham (Piscataway, NJ), Atlanta Biologicals (Norcross, Ga), Aves Labs, Inc (Tigard, OR), Becton-Dickinson/BD Biosciences (San Jose, CA), Bio-Rad (Hercules, CA), Carl Zeiss Microimaging Inc (Thornwood, NY), Cemines (Golden, Co), Chemicon (Temecula, CA), Corning Inc (Corning, NY), DAKO (Carpintera, CA), Developmental Studies Hybridoma Bank (Iowa City, IA), Fisher Scientific (Pittsburgh, PA), GE Healthcare Life Sciences (Piscataway, NJ), Invitrogen (Carlsbad, CA), Jackson Labs (West Grove, PA), Leica (Bannockburn, IL), Midsci (St. Louis, MO), Millipore (Billerica, MA), New England Biolabs (Ipswich MA), Open Biosystems (Huntsville, AL), Promega (Madison, WI), Qiagen (Valencia, CA), R&D Systems (Minneapolis, MN), Santa Cruz Biotechnology (Santa Cruz, CA), Sigma Aldrich (St. Louis, MO), Stratagene (La Jolla, CA), Thermo Fisher Scientific (Huntsville, AL), Vector Laboratories (Burlingame, CA).

Strains of Mice

- Beta-Actin-GFP mice: STOCK Tg(CAG-EGFP)D4Nagy/J (stock #003116, The Jackson Laboratory, Bar Harbor, ME)
- C57/BL6J (stock #000664, The Jackson Laboratory)
- Cre recombinase mice: STOCK Tg(hCMV-cre)140Sau/J (stock #002471, The Jackson Laboratory)
- Floxed ROSA 26 mice: B6.129S4-Gt(ROSA)26Sor ^{tm1Sor}/J (stock #003474, The Jackson Laboratory)
- ROSA 26 mice: B6;129S-Gt(ROSA)26Sor/J (stock #002073, The Jackson Laboratory)
- Weaver mice: B6CBACaAw-J/A-Kcnj6^{wv}/J (stock #000247, The Jackson Laboratory)
- UBC-GFP mice: C57BL/6-Tg(UBC-GFP)30S^{cha}/J (stock #004353, The Jackson Laboratory)

- Spinocerebellar Ataxia 1 158Q knock-in mice (kind gift from Dr. H.Y. Zoghbi)

Antibody List

Primary Antibodies

- Allophycocyanin (APC)-CD117 (c-Kit) (rat monoclonal, 1:200, BD)
- Ataxin-1 (rabbit, 1:1000, gift from H.Y. Zoghbi Lab)
- β-III-tubulin/Tuj1 (mouse monoclonal, 1:1000, Promega)
- Calbindin (mouse monoclonal, 1:2000, Sigma)
- CD11b (rabbit polyclonal, 1:10, Abcam; mouse polyclonal, 1:100, BD Biosciences)
- C-Myc (rabbit polyclonal, 1:100, Santa Cruz Biotechnology; rabbit polyclonal, 1:50, Sigma)
- CNPase (mouse monoclonal, 1:250, Chemicon)
- Gamma-aminobutyric acid (A) Receptor alpha 6 subunit ($GABA_A\alpha6$) (rabbit, 1:500, Chemicon)
- GABA (rabbit, 1:1000, Sigma)
- Glutamate (rabbit, 1:500, Sigma)
- Glial fibrillary acidic protein (GFAP) (rabbit, 1:10, Thermo Fisher; mouse, 1:10, Thermo Fisher; mouse, 1:500, Chemicon; mouse, 1:1000, Dako)
- Green Fluorescent protein (GFP) (rabbit polyclonal, 1:1000, Millipore; chicken polyclonal 1:1000, Aves Labs; chicken 1:500, Abcam)
- Lis1 (N-19) (goat polyclonal 0.5-2ug/ml, Santa Cruz Biotechnology)
- Mouse Atonal Homolog-1 (MATH-1) (rabbit, 1:10, Developmental Studies Hybridoma Bank)
- NeuN (mouse monoclonal, 1:1000, Chemicon)
- O4 (mouse monoclonal IgM, 1:150, Chemicon)
- PE CD3e (hamster, 1:10, BD)
- PE CD4 (rat, 1:10, BD)

- PE CD8a (rat, 1:10, BD)
- PE-Cy7 Sca-1 (Ly-6A/E) (rat , 1:200, BD)
- PE Ly-6G (Gr-1) (rat, 1:10, BD)
- R-Phycoerythrin (R-PE)-CD5 (Ly-1) (rat monoclonal, 1:10, BD)
- R-Phycoerythrin (R-PE) CD11b (rat monoclonal, 1:10, BD)
- R-Phycoerythrin (R-PE)-B220/CD45R (rat monoclonal, 1:10, BD)
- R-Phycoerythrin (R-PE)-TER-119 (Ly-76) (rat monoclonal, 1:10, BD)
- RU49/ZFP-38 (rabbit, 1:1000, CeMines)
- S-100β (rabbit, 1:400, Sigma)
- Stage-Specific Embryonic Antigen-1 (SSEA-1) (mouse, 1:200, Abcam)

Secondary Antibodies

- Cy3 goat anti mouse IgG (1:300-1:500, Jackson Labs)
- Cy3 goat anti mouse IgM (1:300, Jackson Labs)
- Fluorescein-labeled goat anti chicken IgY (1:1000, Aves Labs)
- Horseradish peroxidase-conjugated anti rabbit IgG (1:500, Sigma)
- Oregon Green goat anti mouse IgG (1:1000, Invitrogen)
- Oregon Green goat anti rabbit IgG (1:1000, Invitrogen)

Methods

Generation of Astrocyte Monolayer, Neurospheres, and tau-Embryonic-Derived Neural Precursors (τESNPs)

MASCs: Astrocyte monolayers were derived from cerebella of neonatal (P1-P8) transgenic mice constitutively expressing GFP (strain #003116, Jackson Laboratory, Bar Harbor, MI, USA). Following decapitation, cerebella were removed for dissociation into single-cell suspensions as previously described (Laywell, 2000; Laywell, 2005). Briefly, cerebellar tissue was isolated and minced with a razor blade before incubation in trypsin for 5 minutes in 37^{o} water bath. Cells were triturated serially with 5ml pippets followed by glass Pasteur pippets three times or until single-cell suspension is achieved. After being pelleted and washed several

times in medium, cells were cultured in standard T75 tissue culture flasks with growth medium consisting of Dulbecco's Modified Eagle Medium with F12 supplements (DMEM/F12, Invitrogen) containing N-2 supplement (Invitrogen) and 20μg/mL bovine pituitary extract (BPE, Invitrogen), 5% fetal bovine serum (FBS, Atlanta Biologicals), 20ng/mL epidermal growth factor (EGF, Sigma), and 10ng/mL basic fibroblast growth factor (bFGF, Sigma). Astrocyte monolayers were expanded and passaged up to a maximum of four times before being collected for transplantation.

ESNPs: Cells were derived from the J1 ES cell line carrying the EGFP cDNA knock-in at the *tau* gene and collected at stage IV of a four-step culture protocol as previously described (Goetz et al., 2006). Briefly, cells were expanded on mitomysin C-inhibited embryonic fibroblasts and gelatin in step I, followed by induction of embryoid bodies (EBs) in step II, and attachment of the EBs to laminin-, poly-L-ornithine (LPO)-coated surfaces to derive neural precursor cells in step III. Cells were transferred to another LPO-coated surface in stage IV and cultured with bFGF and passaged once before dissociation into single-cell suspensions for transplantation.

Transplantation in Weaver Mice

Both populations of donor cells were characterized prior to transplantation as previously described (Laywell et al., 2000; Laywell et al., 2005; Goetz et al., 2006). For transplants, MASCs or ESNPs were collected, triturated into single cell suspensions and resuspended at a concentration of $5x10^4$ or $1x10^5$ cells/μl in serum-free DMEM/N-2 medium as described above. Postnatal day 1- 8 weaver (strain B6CBACaAw-J/A-Kcnj6^{wv}/J, Jackson Laboratory) mouse pups, including homozygous (*wv*/*wv*), heterozygous (*wv*/+), or control wildtype (+/+) littermates, were first cryo-anesthetized before unilaterally injected with 1μl of the cell suspension into the

right hemisphere of the cerebellum using a Hamilton syringe with 25s gauge needle. A total of 23 mice received MASCs and 28 received ESNPs transplants within the cerebellar cortex.

Immunohistochemistry of Weaver Transplants

Following transplantation of MASCs or τESNPs into the cerebella of weaver mice, animals were sacrificed at one, two, three, four, or five weeks post transplantation and transcardially perfused with 4% paraformaldehyde in 0.1M phosphate buffered saline (PBS, pH=7.4). Brains were then removed, postfixed overnight in perfusate, and sectioned through the coronal plane at a thickness of 30μm using a vibratome or 20μm on a frozen microtome. Sections were incubated in PBS + 0.1% triton (PBSt), 10% FBS, and the following primary antibodies overnight at 4^{o}C: rabbit GFP (1:1000, Invitrogen), chicken GFP (1:1000, Aves), mouse β-III tubulin (1:1000, Promega), mouse NeuN (1:1000, Chemicon), rabbit GFAP (1:10, Thermo Fisher), rabbit glutamate (1:500, Sigma), rabbit RU49/ZFP-38 (1:1000, CeMines), rabbit gamma-aminobutyric acid(A) Receptor alpha 6 subunit ($GABA_A\alpha6$) (1:500, Chemicon), rabbit MATH-1 (mouse atonal homolog 1) (1:200, Chemicon), and mouse stage-specific embryonic antigen-1 (SSEA-1) (1:200, Abcam). The next day, sections were washed three times in PBSt and incubated with the appropriate secondary fluorescent antibodies at 1:300.

Generation of MASCs and Neurospheres for *in Vitro* Characterization

Astrocyte monolayers were derived from both cerebellum and subependymal zone (SEZ) of early postnatal (postnatal day 1-8) C57/BL6 mice, or constitutively expressing Green Fluorescent Protein (GFP) +/+ transgenic mice (Jackson Laboratory). Female and male mice were decapitated, and their cerebella and/or SEZ were removed minced with a razor blade and placed into DMEM/F12 medium with N2 supplements (N2 media) containing 1X antibiotics (100X, Invitrogen) for 15 min. Following aspiration of the antibiotic N2 solution, 4 ml of

trypsin (0.25%, Atlanta Biologicals) was added to the cells for 5 min in 37^{o}C water bath. Cells were washed off with N2 media and trituated with 5ml pippet followed by glass Pasteur pippet until single cell suspension is achieved. Tissue remained in chunks were allowed to settled to the bottom and only the portion containing a single cell suspension is collected. This process was repeated 2-3 times until homogenous cell suspensions were achieved. Cells were cultured in standard T75 tissue culture flasks with growth medium consisting of Dulbecco's Modified Eagle Medium with F12 supplements containing N2 supplement, 5% fetal bovine serum (FBS, Atlanta Biologicals), 20ng/mL epidermal growth factor (EGF, Sigma), and 10ng/mL basic fibroblast growth factor (bFGF, Sigma). After 1-2 days in culture, cells that did not attach to the culture flasks were collected to generate neurospheres. Cells were trypsinized, centrifuged, and triturated into a single-cell suspensions before a secondary culture was initiated. After counting with a hemacytometer, cells were resuspended in growth medium and aliquoted into ultra low attachment polystyrene 6-well plates (Corning) at densities ranging from $1x10^3$ to$1x10^5$ cells/cm2. Cultures were supplemented with growth factors every second day and neurospheres became apparent within 3-5 days. Generation of neurospheres from PU.1 mice differs only in that whole brains were extracted from embryonic day 15 (E15) of PU.1 +/-, +/+, and -/- littermates since the PU.1 knockout mouse is embryonic lethal. Each brain was processed individually to avoid cross-contamination of cells. Brains were dissociated into single cell suspensions and neurospheres were generated from the floating cells of primary cultures as described above.

Immunocytochemistry of MASC Monolayers and Neurospheres in Culture

Confluent astrocyte monolayers were trypsinized, pelleted, and resuspended in DMEM/F12 containing N2 supplements and 5% FBS. Cells were plated onto glass coverslips that had been sequentially coated with poly-L-ornithine (10µg/ml, Sigma) and laminin (5µg/ml,

Sigma). Neurospheres were induced to differentiate by plating the spheres on coated glass coverslips in a drop of N2 medium containing 5% FBS. Three to five days after plating, cells were fixed with 4% paraformaldehyde in PBS for 20 min and processed for immunofluorescence. Cells on coverslips were incubated with 500µl of PBSt containing 10% FBS and primary antibodies against the following antigens: glial fibrillary acidic protein (GFAP) (1:10 Thermo Fisher); β-III tubulin (1:1000 Promega); and CD11b (1:100 BD PharMingen), at 4^{o}C overnight. Following three washes in PBSt, 500µl of PBSt containing 10% FBS and 1:500 of appropriate secondary antibodies was added for one hour at room temperature.

Chromosome Painting

Astrocytes and neurospheres were processed for fluorescence *in situ* hybridization (FISH) following combined GFAP and β-III tubulin immunolabeling and DAPI nuclear counterstaining. Mouse X (FITC-conjugated) and Y (cy3-conjugated) chromosome probes (Open Biosystems) were used as described (Laywell et al., 2005) to analyze cell ploidy. Briefly, after cells were air dried at room temperature overnight, they were incubated for 15 min in a 3:1 mixture of methanol and acetic acid, digested with pepsin (1mg/ml in 0.01N HCl, Sigma), immersed in formaldehyde (1% in PBS, Sigma), and hybridized overnight with chromosome paints for 5 min at 74^{o}C, followed by 16 hours at 37^{o}C. Following hybridization, cells were washed first in 1:1 formamide:2xSSC (Sodium Chloride Sodium Citrate), then 2xSSC, and 4xSSC with 0.1% NP40 at 46^{o}C. Cells were mounted with Vectashield mounting media containing DAPI (Vector Laboratories) and glass coverslips (Fisher Scientific).

Qualitative and Quantitative Analysis of Cells

Since the chromosome painting protocol abolishes the fluorescence signal of immunolabeled cells, the phenotypic characterization of cells containing abnormal numbers of

sex chromosomes was achieved by matching immunolabeled and fluorescence in situ hybridization processed cells on the basis of an extensively photodocumented DAPI nuclear staining pattern. The nuclear pattern of GFAP/β-III tubulin immunolabeled cells was photodocumented with 5X, 10X, 40X and 63X objectives before and after the FISH procedure so individual double labeled cells could be aligned with the correct orientation. For quantification, 16 random fields on the coverslips were photographed at 40X magnification. The number of diploid and aneuploid cells was recorded and divided by the total cell number to calculate the percentage of putatively fused cells.

Detection of LacZ expression through X-gal Staining

SEZ and cerebellum from transgenic mice expressing Cre recombinase (Jackson Laboratory, stock #002471), gt(Rosa)26Sor tm1Sor/J (Jackson Laboratory, stock #003474) for the floxed stop cassette in front of the LacZ gene, and gt(Rosa)26Sor/J (Jackson Laboratory, stock #002073) as control for ubiquitous expression of LacZ, were isolated and cultured separately as astrocyte monolayers (as described above). First passage female Cre cells were co-cultured with first passage male gt(Rosa)26Sor ^{tm1Sor}/J cells at a 1:1 ratio. This male/female mix of cells provided an additional control to this study because β-gal+ cells should reveal a chromosomal pattern of XXXY with fusion between cre recombinase-expressing cells and the cells harboring the LacZ gene. Polyclonal neurospheres were generated from both transgenic mice under higher density culture conditions and without methocellulose, and attached onto poly-L-ornithine/laminin coated coverslips. Cells were fixed in 4% paraformaldehyde for 10 min, then incubated in prewarmed X-gal stock solution (100ml of 0.1M PBS, 5mM potassium ferricyanide, 5mM potassium ferrocyanide, 40mg magnesium chloride, 20 µl NP40 and 10mg sodium deoxycholate supplemented with 1mg/ml x-gal in Dimethyl Sulfoxide (DMSO), Sigma) at 37^{o}C until a blue reaction product became apparent.

Generation of Recombinant scAAV 7 Plasmids

Glutathione S-transferase, theta 2 class (Gstt2), poly(rC) binding protein 3 (Pcbp3), and DnaJ (Hsp40) homolog, subfamily B, member 4 (DNAJ) cDNA clones were obtained from Invitrogen. Lissencephaly 1/Platelet-activating factor acetylhydrolase, isoform 1b, beta1 subunit (Pafah1b1) cDNA was obtained by RT-PCR using the following primer set: AATGGTGCTGTCCCAGAG (forward primer) and AATCAACGGCACTCCCAC (reverse primer). All cDNAs were subcloned into the adeno-associated virus (AAV) proviral plasmid. Plasmids were named as follow: pTR2-CB-Gstt2, pTR2-CB-Pcbp3, pTR2-CB-DNAJ, and pTR2-CB- Pafah1b1. In order to distinguish the expression of endogenous genes and their corresponding recombinant genes, a c-Myc and polyhistidine (His) tags were engineered into the C-terminus of the proteins. Plasmid specific forward primer (GCAACGTGCTGGTTATTGTGC) and the following reverse primers were used to remove the stop codons from the cDNAs:

Gstt2 (TCAGACTCTAGAAGGAATCCTAGCAATTCG),

Pcbp3 (CGTCCAATTATGCTCTAGATAAGATCATTGGGAAT),

DNAJ (GCAAGGTTCTTCTCTCTAGAGGAGGCAGGGAG), and

Pafah1b1 (ATTCGCCCTTATTCTAGAACGGCACTCCCAC).

New plasmids were named as follow: pTR2-CB-Gstt2-cmyc/his, pTR2-CB-Pcbp3-cmyc/his, pTR2-CB-DNAJ-cmyc/his, and pTR2-CB- Pafah1b1-cmyc/his. Double-stranded AAV (dsAAV) or self-complementary AAV (scAAV) proviral vector cassettes were generously provided by Dr. A. Srivastava since scAAV shows higher transduction efficiency when compare to unmodified AAV due to bypass of the single stranded viral transcription (Wang et al., 2003). The dsAAV proviral plasmid was modified through removal of the EGFP sequence by first digesting with AgeI and SacI, and inserted with the following linker:

(CCGGTACGCGTTCTAGAAAGCTTGATATCCCTGCAGGGCGGCCGCGCTAGCGAGCT) containing multiple cloning sites (MCS) to facilitate the subcloning of cDNAs. Each of the four proviral plasmids generated contains a cytomegalovirus (CMV) immediate early enhancer and chicken β-actin promoter upstream of a simian virus 40 (SV40) early splice donor/splice acceptor site, one of the modifier genes (Table 5-1), and the SV40 polyadenylation sequence. All four double-stranded, self-complementary AAV7 vectors (scAAV7-DnaJb4-c-Myc, scAAV7-GSTT2-c-Myc, scAAV7-Lis1-c-Myc, and scAAV7-Pcbp3-c-Myc) were packaged individually, and a 5th plasmid of scAAV7-GFP for direct and fluorescent reporter activity was also produced, using the calcium phosphate precipitation method. Briefly, human embryonic kidney (HEK) 293T cells were plated in 15-cm-diameter plates and cultured until 70-80% confluent. Cells were cotransfected with 15 μg of the proviral plasmid, 45 μg of pAdeno-helper plasmid, and 15 μg of AAV-helper pRC7 plasmid for supply of the *rep* and *cap* genes and other necessary helper functions in *trans*. Following 60-72 hr transfection, cells were collected and lysed through three cycles of free-thaw treatments. Vectors were purified through the Benzonase treatment, iodixanol step gradient centrifugation, and HiTrap Q HP columns (GE Healthcare Life Sciences). Titers or genome numbers of the viral vectors were determined through DNA slot-blot analysis.

Western Blot

For determination of transgene expression, all four of the plasmids were tested for expression after cloning into the proviral vector plasmid. Four ug of each plasmid was used to transfect 90-95% confluent HEK T cells (in 6-well plate) using Lipofectamine 2000 according to protocol and collected after 72hr. For collection of transfected cells, 1ml of dPBS was put into each well and cells were scraped off with cell scrapers and spun down for 3min. at approx.

3000g. RIPA lysis buffer (250ul, UpState Chemicals/Chemicon) was used to lyse each sample of cells, followed by addition of a mixture of 950ul of lamille buffer combined with 50ul of 2-mercaptoethanol into the lysates in 2:1 ratio (i.e. 50ul of cells in lysis buffer and 100ul of the mixture) for each sample, and boiled in hot water for approx. 5 min. Lysates were loaded into the precast Tris-HCl gel (Bio-rad) and ran for 45-1hr in running buffer. The gel was transferred to a nitrocellulose membrane for 30 min in cold transfer buffer and then placed into blocking solution (5% dry milk into 50ml PBSt) for an hour at room temperature followed by primary antibody (anti-cMyc, Sigma) incubation overnight at 4^{o}C. After washing in PBSt two times, the membrane was incubated with horseradish peroxidase-conjugated anti-rabbit secondary antibody for 30 min at room temperature. Protein was visualized by an enhanced chemiluminescence (ECL) plus kit (GE Healthcare) detection system following the manufacturer's instructions.

Murine Bone Marrow Isolation and Sorting for Sca1^{+}, c-Kit^{+}, Lin^{-} Populations

Donor cells were harvested from 8-12 week-old male transgenic GFP^{+} mice (strain #C57BL/6-Tg(UBC-GFP)30Scha/J, stock #004353) purchased from the Jackson Laboratory. Bone marrow was flushed out of the femurs and tibias of donor GFP^{+} mice with a 30G needle and a 3cc syringe with PBS and into a dish containing PBS. Bone marrow was dissociated into single cell suspension using a 25G needle. Cells were filtered through a nylon filter, triturated with 5ml pippets, and counted for a final volume of $2x10^{6}$ cells/100µl in PBS with 10% FBS. Primary antibodies were added for 30 min at 4^{o}C at 1:200 of APC-conjugated rat CD117/c-kit, and PE-Cy7-conjugated rat Sca-1/Ly-6 (stem cell antigen), 1:10 PE-conjugated Lineage (Lin) cocktail (rat CD4, CD5, CD8a, CD11b, B220, Gr-1, Ter-119, and hamster CD3e) (BD Biosciences). GFP^{+}, Sca-1^{+}, c-kit^{+}, Lin^{-} (SKL) cells were sorted into Iscove's modified Dulbecco's medium (IMDM) (Gibco) using a fluorescent-activated cell sorter.

Recombinant AAV7 Transduction of HSCs and Bone Marrow Transplants

SKL cells were infected with an rAAV 7 vector at 1 x 10^5 viral particles/cell for two hours at 37^{o}C in serum-free IMDM and swirled every 15 min for even distribution. Cells were washed with PBS and used directly for transplantation. Recipient mice were 8-12 week-old female spinocerebellar ataxia 1 mice (Watase et al., 2001; kind gift from Dr. H.Y. Zoghbi) lethally irradiated 48 hrs prior to transplantation with one dose of 950 cGy from a cesium-137 source. 5x10^3 vector transduced SKL cells, along with a 9,000 Sca-1$^-$, c-kit$^-$, Lin$^+$ radio-protective dose suspended in 150µl of total volume in PBS, were injected into the right retro-orbital sinus of lethally irradiated mice. Mice were provided with water treated with Baytril antibiotics for two weeks post transplantation. Information on the total number and genotype of the mice that received bone marrow transplant is provided in Table 5-2.

Bone Marrow Culture

Whole bone marrow from both UBC-GFP and C57/BL6 mice were isolated as described above and cultured *in vitro* to test for expression of AAV7 plasmids. Following trituration into single cell suspension, BMDCs were separated into 2x10^6/ml of culture media and placed into a 6-well flat bottom culture plate (Midsci) containing a total of 2mls/well (4x$10^6$of cells/well). The culture media consisted of IMDM with 20ng of IL 3 (R&D Systems), 20ng of IL 6 (R & D Systems), 50ng of stem cell factor (SCF) (R& D Systems), 2mM Glutamax, and 100U of 10,000U/ml, 10mg/ml penicillin-streptomycin (Invitrogen) in 1ml of media. UBC-GFP BMDCs served as fluorescent control cells while C57/BL6 cells received 100 MOI of scAAV7-GFP or one of the plasmids containing the modifier gene along with the c-Myc/his reporter tag. Cells were cultured in 37^{o}C incubator during and after viral infection. Transduction efficiency was determined by direct visualization of GFP expression following 72 hrs in culture or through FACS analysis of GFP and intracellular c-Myc expression.

Flow Cytometric Analysis of Transgene Expression within Peripheral Blood

Peripheral blood obtained by tail vein puncture was examined at one month post transplantation and at the end of the survival period for multilineage and GFP expression analysis. Nucleated cells (buffy coat) were separated through ficoll density gradient centrifugation and examined for expression of GFP along with B220 for presence of B lymphocytes, CD11b for macrophages, or CD4 for T lymphocytes by FACS. Data were evaluated using the Cellquest^{TM} software.

Immunohistochemical Analysis of BMDCs in Cerebellum Following Bone Marrow Transplants

Female heterozygous Sca1 mice were sacrificed between 12 wk and 40 wk post transplantation and perfused transcardially with 4% paraformaldehyde in 0.1M PBS. Brains were removed, postfixed overnight in perfusate, and transferred to 30% sucrose for another 24 hrs before sectioning through the sagittal plane at a thickness of 14μm using a frozen microtome (Leica). Sections were processed as follows for the different antibodies:

Calbindin and CD11b: Sections were incubated in PBS supplemented with 10% FBS and 0.1% Triton X-100. Primary antibodies of GFP (chicken polyclonal 1:1000, Aves Labs) and Calbindin (mouse monoclonal 1:2000, Sigma), or GFP and CD11b (mouse polyclonal 1:100, BD Biosciences) were added for overnight at 4^{o}C. After washing the sections three times in PBSt, Cy3 goat anti-mouse (1:500, Jackson Labs) and FITC goat anti-chicken (1:1000, Aves Labs) were applied for 2 hours at room temperature.

Ataxin-1: Procedures were modified from Skinner et al. (1997). Briefly, sections were mounted on plus-charge slides and microwaved in 0.01M urea for antigen retrieval. Series of peroxidase, serum, avidin, and biotin blocks (Vector Laboratories) followed according to Vectastain manufacturer's instructions, and ataxin-1 11NQ (rabbit, kind gift from Dr. HY Zoghbi) at a

dilution of 1:1000 was added for 48 hours at 4oC. Biotinylated anti-rabbit (goat, 1:150) was added for 30 min and followed by DAB development using Elite ABC reagent kit (Vector Laboratories) and DAB kit (Vector Laboratories). GFP (chicken polyclonal 1:500, Abcam) was added overnight at 4^{o}C for dual staining. Alexa fluor 488 anti-chicken (donkey, 1:500, Invitrogen) was used the next day and incubated for 45 min at room temperature.

c-Myc: Sections were mounted on plus-charge slides and air dried overnight. Slides were subjected to high heat retrieval in Dako target retrieval solution (pH9, Dako) for 20 min and cooled slowly in room temperature for another 20 min. Primary antibodies of c-Myc (rabbit polyclonal 1:100, Santa Cruz Biotechnology) and GFP (chicken polyclonal 1:500, Abcam) were added overnight at 4^{o}C. Alexa Fluor 594 anti-rabbit (donkey, 1:500, Invitrogen) and Alexa Fluor 488 anti-chicken (donkey, 1:500, Invitrogen) were then applied for 45 min at room temperature the next day.

Fluorescent *in Situ* Hybridization Analysis of GFP^{+} Purkinje Heterokaryons

Sagittal sections of cerebella post bone marrow transplants were processed for GFP and Calbindin labeling using standard immunohistochemistry procedures as described above. The nuclei were counterstained with DAPI and the staining patterns were extensively photodocumented with 10X, 20X, and 40X objectives to relocate the exact position of the GFP^{+} Purkinje neurons following FISH when the fluorescent signals were abolished. Mouse X (FITC-conjugated) and Y (cy3-conjugated) chromosome probes (Open Biosystems) were used to detect the presence of donor derived Y chromosome. Briefly, after slides were photodocumented for immunofluorescent expressions, they were air dried at room temperature overnight. Slides were next incubated for 30 min in 0.2N HCl, retrieved in 1M NaSCN in 85^{o}C for 30 min, followed by digestion in pepsin diluted in prewarmed 0.9% NaCl (pH 2.), and hybridized with chromosome probes for 10 min at 62^{o}C, followed by 48 hours of further hybridization at 37^{o}C. Cells were

washed first in 1:1 formamide:2X SSC (Sodium Chloride Sodium Citrate), then 2X SSC and 4X SSC with 0.1% NP40 at 46^{o}C following hybridization. Sections were covered with mounting media containing DAPI (Vector Laboratories) and glass coverslips (Fisher Scientific).

CHAPTER 3
TRANSPLANTATION OF EMBRYONIC AND ADULT NEURAL STEM CELLS IN THE GRANULOPRIVAL CEREBELLUM OF THE WEAVER MUTANT MOUSE

Introduction

Restoring tissue integrity and function following injury or neurological disease remains challenging due to the limited regenerative potential of the central nervous system (CNS). Recent advances in therapeutic strategies include enhancing recruitment of newly generated endogenous neurons to a lesioned or degenerating area and transplantation of exogenously generated stem/progenitor cells to replace at-risk or lost cells (Daniela et al., 2007; Steindler and Pincus, 2002; Rossi and Cattaneo, 2002). Neural stem/progenitor cell transplantation has been studied extensively with cells isolated and expanded from neurogenic regions within the CNS: the subependymal zone (SEZ) (Lois and Alvarez-Buylla, 1994) or the dentate gyrus of the hippocampus (Kaplan and Hinds, 1977; Kirschenbaum et al., 1994; Kuhn et al., 1996; Kornack and Rakic, 1999). Another region that has not been widely viewed as a source of multipotent cells, but nonetheless retains transiently active cell proliferation, is the postnatal cerebellum. The cerebellar cortex continues to generate granule cells and forms the transient external granule cell layer up to postnatal day 15 (Hatten et al., 1997), and we and other groups have shown that cells derived from this area are capable of forming multipotent proliferative clones, or neurospheres, with the ability to form all three primary CNS cell types: neurons, astrocytes, and oligodendrocytes (Laywell et al., 2000; Laywell et al., 2005; Lee et al., 2005; Klein et al., 2005). These cerebellar-derived neurogenic cells are glial fibrillary acidic protein (GFAP)-expressing astrocytes similar to the stem cells derived from the SEZ areas surrounding the lateral ventricle, and we refer to both the forebrain and cerebellar clonogenic cells as multipotent astrocytic stem/progenitor cells (MASCs). MASCs represent potential therapeutic candidates for replacement and repair following cell loss in the CNS resulting from injury or disease as they

have the ability to respond to intrinsic environmental cues by anatomically integrating into a host brain and differentiating into neurons and astrocytes when transplanted into the lateral ventricles of normal adult mice (Zheng et al., 2006). In these previous studies, engraftment in the hindbrain was less robust following intraventricular transplantation, possibly due to the extensive distance and other factors that could hinder the migration and integration of the MASCs.

Enhanced green fluorescent protein (EGFP)-expressing ESNPs (embryonic stem cell-derived neural precursor) have also been well characterized by our lab and others, with previous studies showing the ability of these cells to acquire multiple neuronal phenotypes and to functionally integrate into the developing brain both *in vitro* and *in vivo* (Goetz et al., 2006; Wernig et al, 2004; Benninger et al., 2003; Brüstle et al., 1997; Brüstle et al., 1999; Okabe et al., 1996). Following transplantation into the lateral ventricle, a large percentage of the ESNPs were found to differentiate into glutamatergic neurons despite the failure to acquire region-specific identities, and they might be predisposed to respond to intrinsic cues when placed in an optimal environment.

The present study was designed as a comparative analysis to examine the potential of somatic tissue-derived MASCs versus embryonic stem cell-derived neurons to potentially thrive within a CNS environment that might be conducive for such integration using the neurological mutant mouse model, weaver (gene symbol *wv*). Weaver mice have been well characterized for cerebellar development studies due to the histopathological hallmark of severe granule cell loss, leading to a "granuloprival" cerebellum, that results in reduced brain size (Sidman et al., 1965; Rakic and Sidman, 1973; Smeyne and Goldowitz, 1989) as well as deficits within the Purkinje cell population (Eisenman et al., 1998; Herrup and Trenk, 1987; Smeyne and Goldowitz, 1990; Bayer et al., 1996), dopaminergic neurons in the substantia nigra (Triarhou et al., 1988; Roffler-

Tarlov et al., 1996), and the deep cerebellar nuclei (Bayer et al., 1996; Maricich et al., 1997; Martí et al., 2001). Clinical and pathological features are similar to patients suffering from cerebellar ataxia, including instability of gait and tremor of the extremities. These symptoms can be attributed to a single base pair mutation in the gene coding for a G-protein-coupled, inward rectifying potassium channel of the GIRK2 family (Patil et al., 1995) that is expressed in all cell groups suffering defects in weaver but also expressed in regions where no damage has occurred or been detected to date (Schein et al., 1998). The severe depletion of the granule interneurons makes weaver mouse an attractive model for studying and providing comparison of these two different donor populations because it has been shown that the MASC default neuron generation program is interneurons (Laywell et al., 2000; Zheng et al., 2006; Scheffler et al., 2005) while the ESNPs have been shown to differentiate largely into glutamatergic neurons (Wernig et al., 2004).

There have been many previous transplantation studies in which fetal or embryonic cerebellar cells were transplanted into other neurological mutant mouse models such as the Purkinje cell degeneration (*pcd*) mutant (Triarhou et al., 1996; Triarhou et al., 1986; Triarhou et al., 1987; Sotelo and Alvarado-Mallart, 1986, 1987, 1988), including the use of an immortalized neural progenitor cell line (Snyder et al., 1992) for functional recovery or restoration of molecular homeostasis (Snyder et al, 1995; Rosario et al., 1997; Li et al., 2006), as well as many other studies (Rossi and Cattaneo; 2002) attempting to replace at-risk neuronal populations, but there has been a paucity of successful cell integration findings leading up to the *in vivo* bioassay tested here. That is, here we use a well-characterized cerebellar neurological mutant mouse with defined cell loss that occurs gradually beginning in early postnatal life, as a host for two completely different neural stem/progenitor cell populations. One of the cells studies here, MASCs, represents a potential indigenous source of cerebellar granule interneurons (Laywell et

al., 2000; Laywell et al., 2005; Lee et al., 2005; Klein et al., 2005), and the other, ESNPs, is believed to be amongst the most potent of stem cells capable of generating numerous types of neural cells (Goetz et al., 2006; Wernig et al, 2004; Benninger et al., 2003; Zhang et al., 2001; Brüstle et al., 1997; Brüstle et al., 1999; Okabe et al., 1996). Thus the current study provides insights into the developmental potential, cell fate choice and differentiation of both MASCs and ESNPs within an injured host CNS environment, and for attempting cell replacement following intra-parenchymal transplantation during a peak period of cell loss.

Cerebellar-Derived MASCs Show Extensive Migration but Limited Differentiation Following Intracerebellar Transplantation in Postnatal Weaver Mice

Astrocytes derived from postnatal mouse cerebella have been shown to harbor stem-like characteristics through expression of stem cell markers such as nestin, and their ability to give rise to neurons, astrocytes, and oligodendrocytes when cultured in neurosphere-like conditions (Fig 3-1, B and C). When cultured as monolayers, MASCs are pure astrocyte populations that are greater than 95% immunopositive for GFAP with a few CD11b positive microglia mixed in (Fig 3-1A). Cells were collected from astrocyte monolayers and directly injected into the right hemisphere of the cerebella of *wv*/+, *wv*/*wv*, and +/+ littermates between postnatal day 1-8, with 23 mice injected with MASCs and 28 with ESNPs. Donor cells are distinguished from the host tissue through their expression of GFP, and analysis of cell survival, migration, and integration were done following survival periods ranging from one week to five weeks post transplantation. As early as one week post transplantation, cells can be seen to migrate away from the site of injection and to settle in all three primary cerebellar layers - molecular, Purkinje, and granule cell layers (Fig 3-2 A, B) -with predilection towards the white matter. No specific migration pattern was observed and the age at which the mice received the donor cells did not seem to have any major impact based on the range chosen for this study. The majority of cells that survived and

showed active migration seemed to adopt an astrocyte-like morphology with numerous, fine processes, but only some were found to be immunopositive for the immature glial marker GFAP (Fig 3-2 D). While a small population of MASCs also expressed the neuronal marker β-III tubulin (Fig 3-2 C), most cells remained immature without antigenic expression for mature neuronal cell markers such as NeuN or for cerebellar specific markers such as MATH-1, RU49, and $GABA_A\alpha6$. Overall, transplanted cells survived and migrated in all three host environments consisting of +/+, *wv*/+, and *wv/wv* cerebella, but cells found to express the antigenic profiles mentioned above seemed to be more prevalent (15 out of 23 animals) in the *wv*/+ and *wv/wv* transplants as opposed to the wildtype littermates (8 out of 23).

Embryonic Stem-Cell Derived Neural Precursors Exhibit Multiple Neuronal Morphologies and Phenotypes upon Transplantation within the Postnatal Weaver Cerebellum

ESNPs carry the EGFP reporter gene behind the tau promoter and EGFP fluorescence was shown to be restricted to neuronal progeny through *in vitro* characterization (Wernig et al., 2004). Specifically, after one day in culture, GFP positive cells were found to be immunopositive for β-III tubulin (Fig 3-3 B) but did not colocalize with GFAP-expressing cells (Fig 3-3 A). The same trend was observed after 4 days in culture where greater than 95% of the cells became both GFP and β-III tubulin immunopositive (Fig 3-3 C). As seen with the MASCs, ESNPs exhibited the ability to survive, migrate, and differentiate post- transplantation in the weaver mouse model. However, while ESNPs seemed to show a greater affinity towards each other and had the tendency to re-aggregate into clusters and remained at the site of injection, cells remained viable and appeared to exhibit the same survival trend as the MASCs. Small groups of cells did have the ability to migrate and were capable of moving away from the injection site into all three cerebellar layers once out of the cell clusters (Fig 3-4 A). The appearance of these cells were also vastly different from the MASCs, with more varied cell

morphologies with neuronal characteristics, including long processes and small cell bodies (Fig 3-4 B, C). For example, bipolar cells resembling young migratory neurons, and cells with more complex, somatic-neuritic morphologies including ramified processes that gave rise to thin varicose axons were observed. Immunohistochemical analysis showed that the majority of ESNPs expressed both neuronal cell markers β-III tubulin (Fig 3-4 D) and NeuN (Fig 3-4 E), but still seemed to lack region-specific gene/marker expression. However, a small population of ESNPs was immunolabeled for the excitatory neurotransmitter glutamate (Fig 3-4 F) which is expressed exclusively by granule cells within the cerebellum. Donor cells exhibiting the antigenic profile mentioned above were found in 20 *wv*/+ and *wv/wv* animals and in only eight +/+ littermates out of 28 successful transplants.

Embryonic Stem-Cell Derived Neural Precursor Transplantation Gives Rise to Neoplasia

ESNPs were dissociated into single cell suspensions at the time of transplantation but retained the ability to re-aggregate into small to medium sized cell clusters within the host environment as mentioned above. More often than not, these clusters remained in place and did not seem to invade the host tissue. However, in four out of 51 animals with cells successfully transplanted inside the cerebellum, neoplastic-like formations were found four weeks post-transplantation within the injected hemisphere while the contralateral hemisphere was unaffected (Fig 3-5 A). This was observed only in animals injected with ESNPs but not with the MASCs transplants, and the EGFP positive cells could be found bordering the transformed host tissue or in the center of the cell mass within the cerebellum (Fig 3-5 D, E). In one case, a solid tumor-like sphere was formed and believed to have its own source of blood and nutrient supply, being that it was separated from the rest of the underlying parenchyma. In other cases, the host tissue seemed to undergo the process of transformation which caused the injected hemisphere to swell and appear enlarged when compared to the contralateral control side (arrow, Fig 3-5 A). Many

cells within the tumor-like structures were immunopositive for β-III tubulin (Fig 3-5 D, E) and SSEA-1 (inset, Fig 3-5 D) during the early stages of transformation. Hematoxylin and eosin (H & E) staining showed at least one tumor to be a teratoma at its end stage, with the presence of cells containing lineages outside of the CNS, such as skeletal muscle fibers (Fig 3-5 B) and hair follicles (Fig 3-5 C). Overall, three *wv*/+ and one +/+ mice out of 28 transplants had neoplastic formations that appeared to be teratomas. It is noteworthy that these neoplasias occurred despite what appeared to be homogeneous ESNP starting cultures that served as sources for these weaver cerebellar transplants.

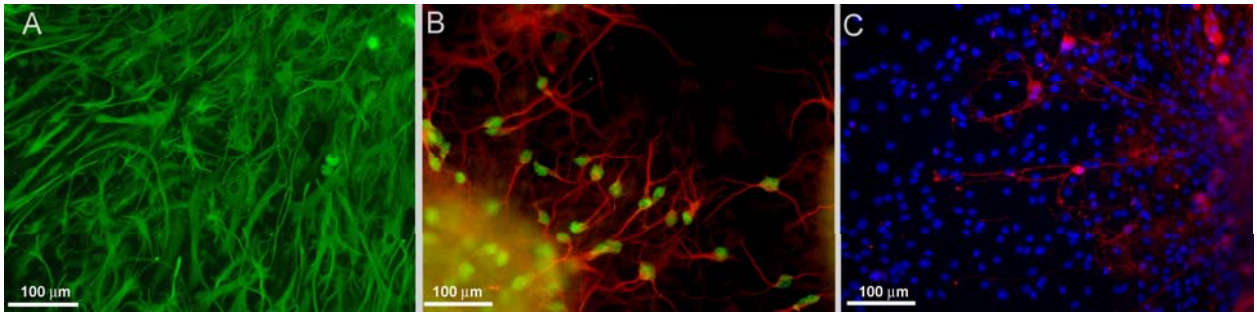


Figure 3-1. Cerebellar-derived MASCs have the ability to differentiate into neurons, astrocytes, and oligodendrocytes in vitro. A) MASCs derived from neonatal cerebellum and cultured as monolayers contain >95% GFAP-expressing astrocytes (green). B) When cultured as neurospheres, MASCs are capable of differentiating into neurons expressing neuronal markers including β-III tubulin (red) and NeuN (green), and in C) oligodendrocytes expressing markers such as CNPase (red) in C. (blue=DAPI)

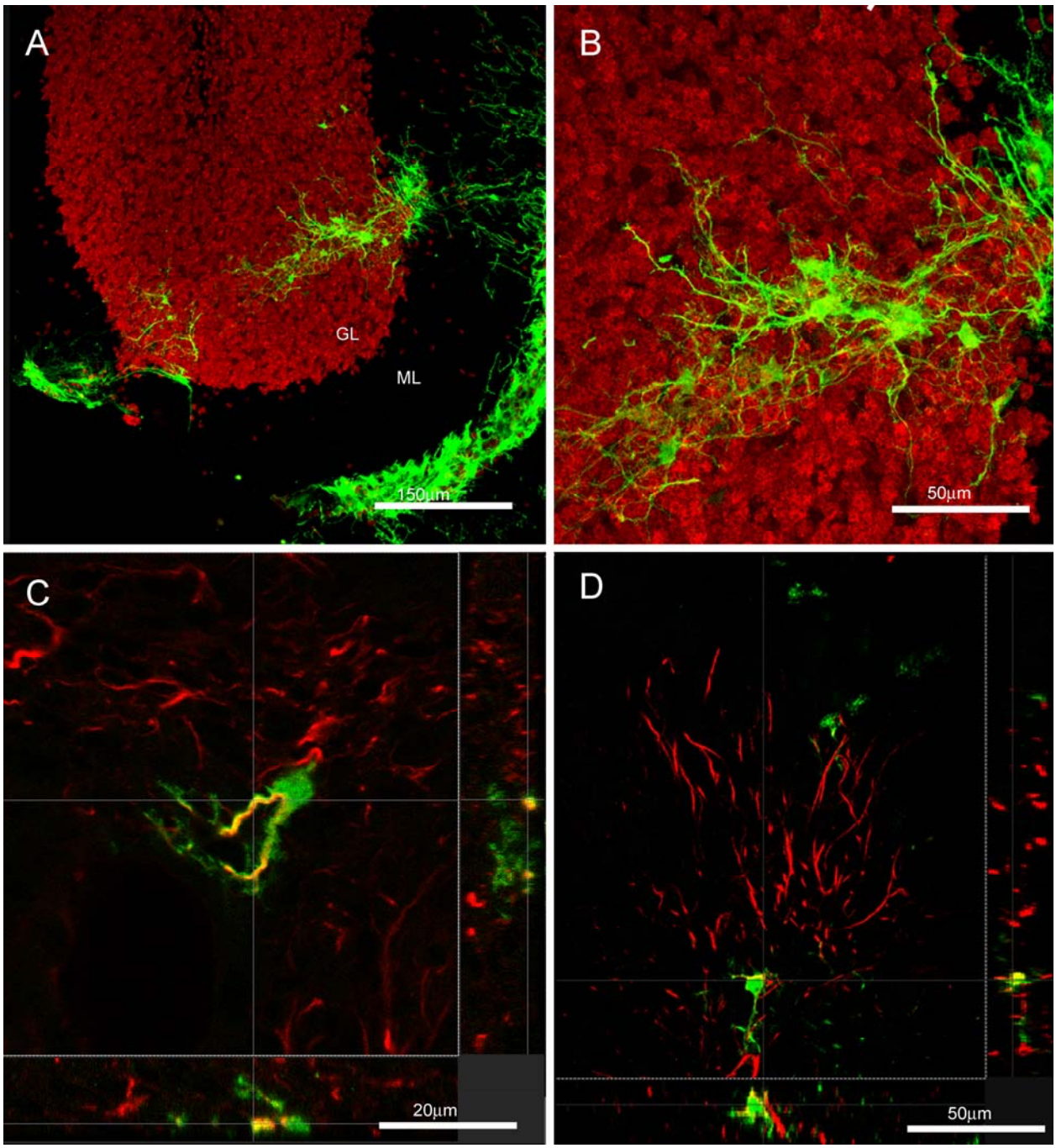


Figure 3-2. Cerebellar-derived MASCs are able to survive, migrate, and differentiate upon transplantation into the weaver cerebellum. A) Confocal images show donor-derived GFP+ , cerebellar-derived MASCs have survived and migrated away from the injection site (on top of the molecular layer) within the cerebellum. B) Higher magnification micrograph of the same field as in A shows extended arborizations of the grafted cells with astrocyte-like morphologies within the granule cell layer. C) A small population of the GFP+ donor cells display neuronal phenotypes through expression of the immature neuronal marker β-III tubulin (red), D) a subpopulation of MASCs also are immunopositive for the astrocyte marker GFAP (red). (GL=granule layer, ML=molecular layer)

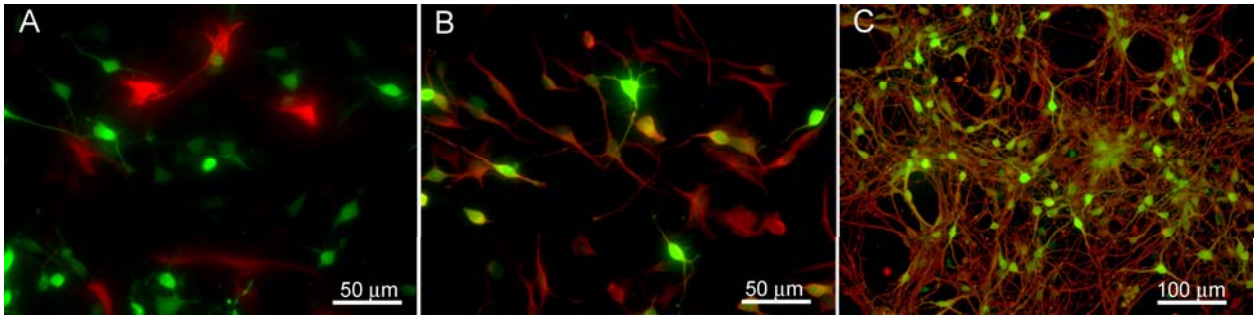


Figure 3-3. Tau-EGFP-ESNP cells express neuronal fate in culture. A) Following one day in culture, GFP+ ESNPs are not immunopositive for GFAP (red), B) but do colocolize with neuronal marker β-III tubulin (red) showing commitment onto the neuronal lineage. C) After four days in culture, ESNP have proliferated into dense cell population and more than 95% of the ESNPs are immunolabeled for both GFP and β-III tubulin (red).

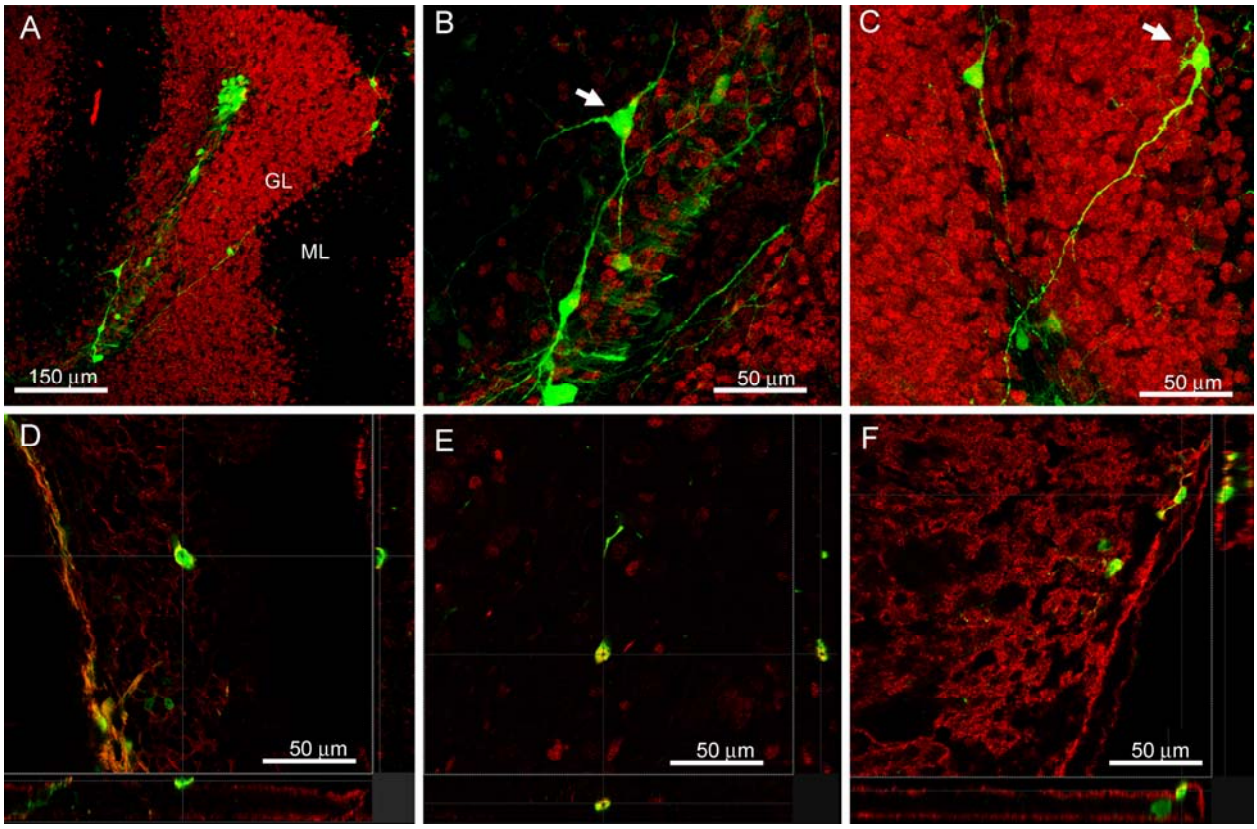


Figure 3-4. Grafted ESNPs have the ability to survive, migrate, and differentiate into mature phenotypes following transplantation into the weaver mouse model. A) Confocal microscopy shows that GFP+ ESNPs (green) can re-aggregate into cell clusters but retain the ability to migrate away from the aggregation. B) Higher magnification image show that some of these cells display mature neuronal phenotypes with bifurcated axons (arrow), or C) possess ramified processes with long, thin, and varicosed axon (arrow). D) A small population of the donor cells express the pan neuronal marker β-III tubulin (red), E) ESNPs also differentiate into cells immunopositive for the mature neuronal marker NeuN (red), and F) neurotransmitter

glutamate (red) which is expressed exclusively by the granuel cells within the cerebellum. (GL=granule layer, ML=molecular layer)

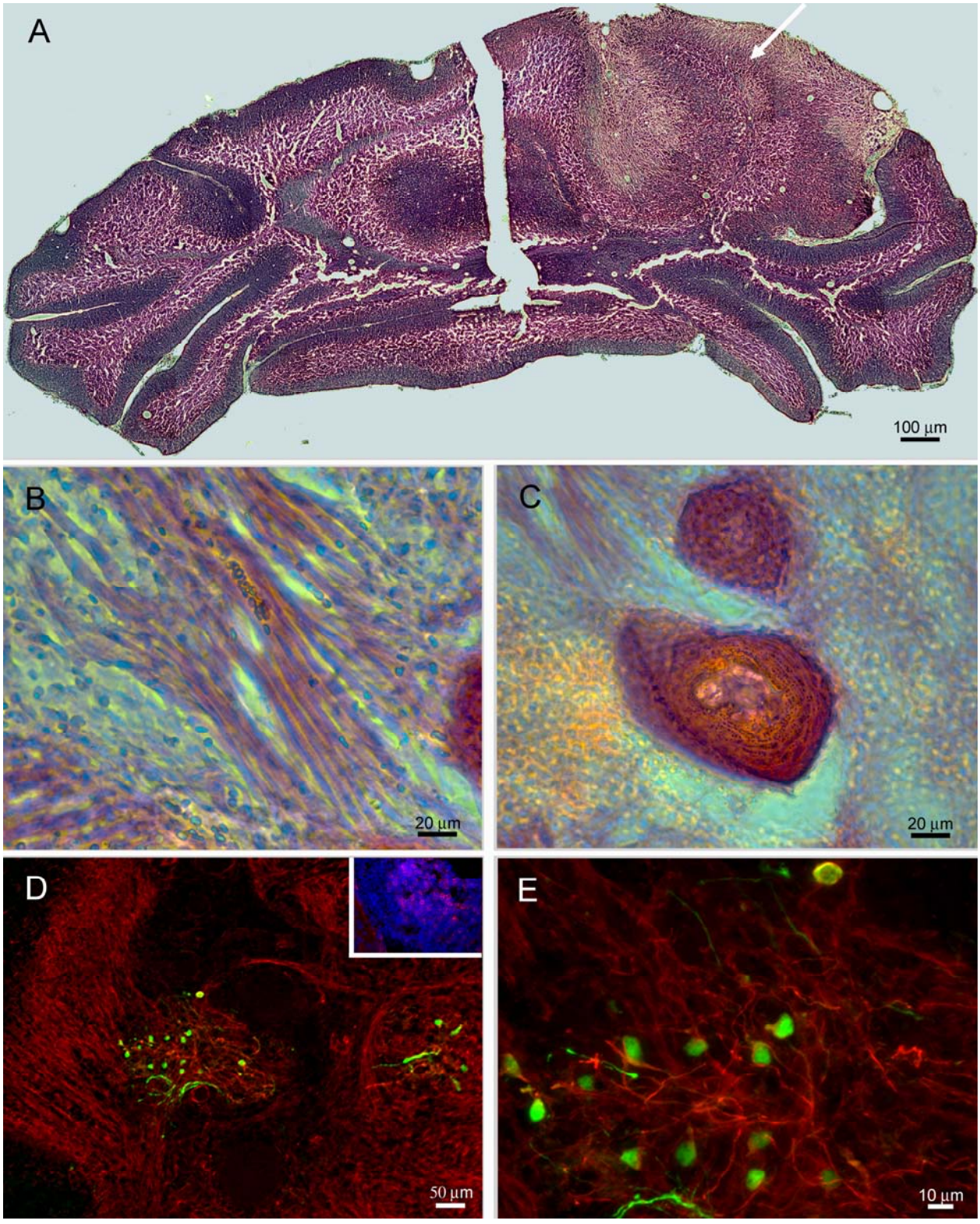


Figure 3-5. Embryonic stem cell-derived neural precursors are capable of transforming host tissue and give rise to tumor-like spheres. A) Montage of images from H& E staining shows the transplanted hemisphere being disrupted by a protruding cell mass (arrow) within the parenchyma but not on the contralateral side. B) Non-neural lineages can be found within the teratoma-like neoplasia inside the cerebellum such as hair follicles, and in (C) skeletal muscle fibers. D) GFP^+ ESNPs (green) can be seen within the transformed tissue and immunopositive for the marker SSEA-1 (red, inset). E) Higher magnification of GFP^+ ESNPs inside the neoplasia show they also express β-III tubulin as with the starting ESNP population used for transplantation.

CHAPTER 4
FUSION OF NEURAL STEM CELLS IN CULTURE

Introduction

Stem cells have been widely recognized for their therapeutic potential as a source of specific cell types needed for tissue, including central nervous system (CNS) reconstitution following injury or disease. Among the various types of stem cells, embryonic stem (ES) cells have been perceived to hold the most promise while adult stem cells are generally thought to be more restricted in terms of developmental potential. However, such views were called into question after it was shown that adult hematopoietic stem cells are capable of crossing lineage boundaries by differentiating into liver, intestine, heart and skeletal muscle cells (Ferrari, et al., 1998, Gussoni, et al., 1999, Jackson, et al., 1999, Krause, et al., 2001, Lagasse, et al., 2000, Orlic, et al., 2001, Petersen, et al., 1999) while neural stem cells might be able to adopt a hematopoietic fate *in vivo* (Bjornson, et al., 1999) . Such unexpected stem cell plasticity generated great excitement in light of the ethical controversies associated with the study and use of ES cells, but it also raised questions regarding the mechanistic nature of such developmental phenotypic fluidity (Laywell, et al., 2005)**.** Lineage switching was thought to result from the process of "trans-differentiation", but it also could involve the recently discovered phenomenon of stem cell fusion, of the type first demonstrated *in vitro* by Terada et al. and Ying et al. (2002). In these two studies, co-culture of ES cells with either bone marrow derived cells or neural stem cells yielded hybrid populations that expressed both the donor cell markers and ES cell-like properties that could have led to the conclusion that trans-differentiation was involved. However, upon closer examination, the hybrid cells were shown to be polyploid, suggesting that the hybrid phenotype resulted from cell fusion.

In the present study, we examined the possibility that cell fusion is a naturally occurring event not only between two different stem cells types, as previously demonstrated, but also between cells of the same lineage including young neurons and astrocytes found within neurosphere cultures of differentiating neural stem cells. We show that multipotent neurogenic astrocytes derived from either the SVZ or the cerebellar cortex of postnatal mice can contain abnormal sex chromosome counts when examined using FISH. We employed a method based on Cre/lox recombination, a technique that has been extensively utilized for conditionally turning on or off gene expression, to detect cell fusion between two mouse lines. Astrocytes harvested from SVZ or cerebellum of mice ubiquitously expressing Cre recombinase, under the control of a cytomegalovirus (CMV) promoter, were co-cultured with cells obtained from the conditional Cre reporter mouse line gt(Rosa)26Sor ^{tm1Sor}/J. This reporter mouse contains the LacZ reporter gene that is expressed only after the Cre-mediated excision of an upstream lox-P flanked (floxed) stop cassette, and cell fusion is detected through the expression of β-galactosidase.

The breakdown of physical barriers between cells could be attributed to a number of factors, including the presence of highly fusogenic macrophages that have been known to contribute to the formation of multinucleated giant cells (Terada, et al., 2002). We therefore looked at the potential role of CNS macrophages, e.g. microglia, in cell fusion. In addition, microglia are derived from the myeloid lineage which has been implicated in recent literature to possess the ability to fuse with CNS neurons (Alvarez-Dolado, et al., 2003). Cultured astrocytes were studied for ploidy using combined immunocharacterization with CD11b, a surface marker strongly expressed by macrophages, and FISH sex chromosome counts; in order to rule out the downregulation of microglia-associated surface proteins after a fusion event, we also examined

microglia-free cultures derived from a transgenic mouse lacking the transcription factor PU.1, which is needed to produce myeloid progenitors (Scott, et al., 1994).

In all, the experiments performed here should add considerable insight into the plasticity of CNS stem/progenitor cells. Whether or not fusion is prevalent in SVZ- or cerebellar-derived neurosphere cultures, an understanding of such phenomena is required before exploiting CNS stem/progenitor cells as therapeutic reagents.

Astrocyte Monolayers Contain Cells with Aneuploid Sex Chromosomes

Astrocyte monolayers derived from SVZ have been shown to display neural stem cell attributes, as they can give rise to multipotent neurospheres (Laywell, et al., 2000). A typical astrocyte monolayer derived from the SVZ is shown to be immunolabeled for the astrocyte-specific intermediate filament protein, GFAP (Fig. 4-1 A). Similar cultures derived from both male and female mice were processed for FISH using specific, labeled probes against mouse X and Y chromosomes. The X chromosome probe is conjugated to FITC while the Y chromosome probe is conjugated to cy3 which provided red and green signals that can be seen clearly within each nucleus. While most cells display a normal diploid state, a number of cells harbor extra sex chromosomes (Fig. 4-1 B). To determine whether or not cells containing abnormal numbers of chromosomes are the same cells with GFAP expression, neurospheres were plated onto poly-L-ornithine/laminin coated glass coverslips and cells were allowed to differentiate and to migrate out from the spheres for 3-5 days. In Fig. 4-1 C and D, three cells are visible that robustly express GFAP, and the same three cells were recognized based on nuclear patterns. While two of the cells are seen to contain the normal number of sex chromosomes with one X and one Y each, the third cell clearly possesses two X chromosomes and one Y chromosome (Fig. 4-1 D, asterisk). Such examples indicate that the cultured astrocytes may acquire an abnormal number of chromosomes through cell fusion. It is possible that cell fusion is induced due to close

proximity of the cells within high density cell cultures, but quantification of the number of aneuploid cells found within both high and low density culture conditions suggest otherwise. Using 40X magnification and 16 random field samples for each coverslip, 539 cells out of 3717 total number of cells (14.5%) surveyed were aneuploid in non-confluent culture conditions while 1621 cells out of 9991 cells counted (16.2%) were aneuploid in confluent conditions. This further indicates that spontaneous cell fusion might take place even in the absence of selective pressures such as space limitation using the current culture paradigm.

Analysis of Cell-Cell Fusion Using a Cre/lox Recombination System

To test for cell fusion and to assess the possibility of post-fusion chromosomal resolution (Wang, et al., 2003), we utilized a Cre/lox recombination system. Cells from sex mismatched Cre-recombinase expressing mice and the gt(Rosa)26Sor ^{tm1Sor}/J mouse line with a floxed stop cassette upstream of the LacZ gene, were first cultured as astrocyte monolayers individually for one passage, and then combined together as co-cultures at a 1:1 ratio. These co-cultured cells, along with individual cultures of gt(Rosa)26Sor/J and Cre-expressing cells as positive and negative controls respectively, were then stained for the LacZ gene product, β-galactosidase (β-gal). β-gal expression is detected only if Cre recombinase is present in the nucleus of a gt(Rosa)26Sor ^{tm1Sor}/J cell and successfully cleaves the floxed stop cassette, indicating fusion among one or more cells in the culture. A small number of cells that migrated out from neurospheres were found to be β-gal positive, as indicated by blue X-gal reaction product (Fig. 4-2). The vast majority of cells, however, do not express detectable β-galactosidase, indicating that there is a low rate of cell fusion occurring in this culture paradigm.

Cells Immunopositive for CD11b Retain a Diploid State

Spontaneous cell fusion is a process that could be attributed to the involvement of microglia, resident CNS macrophages that are prevalent within primary cell cultures. Using a combination of antibodies against CD11b-a part of the CD11b/CD18 heterodimer (Mac-1) expressed by macrophages in mice-, β-III tubulin, a neuron-specific intermediate filament protein, and GFAP (for reference to these phenotypic markers, see Laywell, et al, 2000) differentiating spheres generated from astrocyte monolayers derived from SVZ and cerebellum of postnatal day 1-10 C57/BL6 mice can be seen to contain neurons, astrocytes, and microglia (Fig. 4-3 A). It is plausible that cells containing abnormal numbers of sex chromosomes are microglia that have engulfed nearby astrocytes, so immunolabeling with CD11b and *in situ* hybridization were needed to find out whether or not cells possessing microglial antigenic profiles are the same cells found to contain aneuploid nuclei. In Fig. 4-3 B, a group of six cells can be seen that are immunopositive for CD11b, but all were observed to be diploid by FISH with X and Y sex chromosome probes (Fig. 4-3 C). Overall, only 5 out of 65 cells that were identified to be microglia were found to contain more than two chromosomes. These data suggest that microglia are not the instigators of cell fusion leading to aneuploidy in this culture paradigm.

Cultures Derived From PU.1 Knock-Out Mice Contain Aneuploid Cells

To further confirm the lack of a role for microglia in cell fusion, neurospheres were generated from a mouse line that lacks PU.1, a myeloid specific Ets family transcription factor essential for the formation of macrophages and other myeloid progenitors (Scott, et al., 1994, Walton, et al., 2000). As expected, immunolabeling with anti-CD11b revealed the absence of microglia in spheres derived from the knockout mice (Fig. 4-4 C). In contrast, wildtype littermates revealed a robust microglial presence (Fig. 4-4 A). FISH analysis of sex

chromosomes, however, showed the ratio of aberrant chromosome counts to be comparable between the mutant and wildtype cultures (Fig. 4-4 B, D), strongly suggesting that microglial participation is not necessary for chromosome aneuploidy to occur.

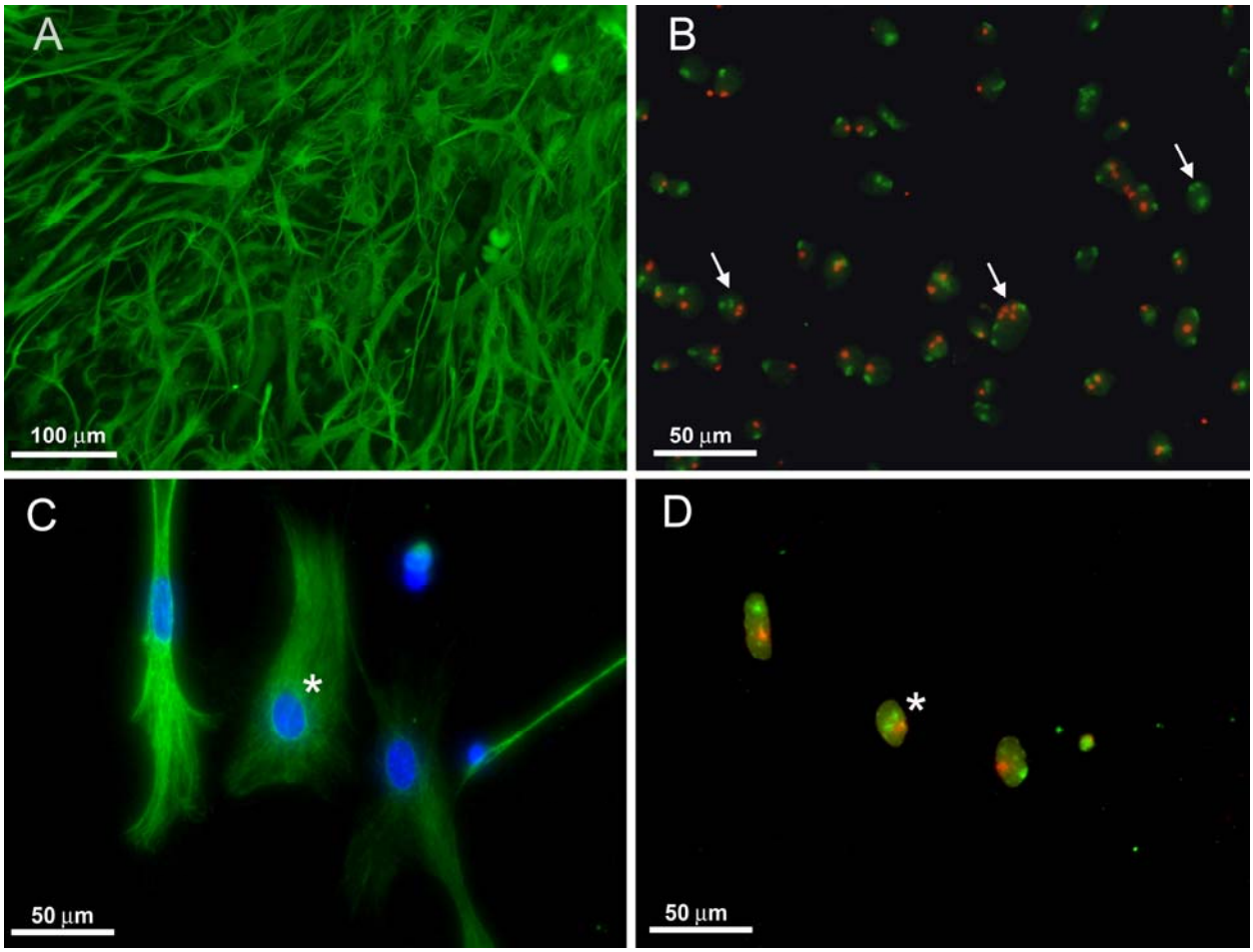


Figure 4-1. Astrocyte monolayers contain cells with polyploid sex chromosomes. A) an astrocyte monolayer derived from the SVZ is shown to consist mostly of cells immunopositive for GFAP (green). B) chromosome painting specific for the mouse X-chromosome (green) and Y-chromosome (red) reveals cells with abnormal chromosome counts (arrows) within the astrocyte monolayer culture. C) high magnification photomicrograph shows a group of cells immunopositive for GFAP (green) before chromosome painting, and D) the same group of cells as seen in C are shown after chromosome painting. Asterisks indicate corresponding cell that is immunolabeled for GFAP (C) and contains 3 sex chromosomes (D).

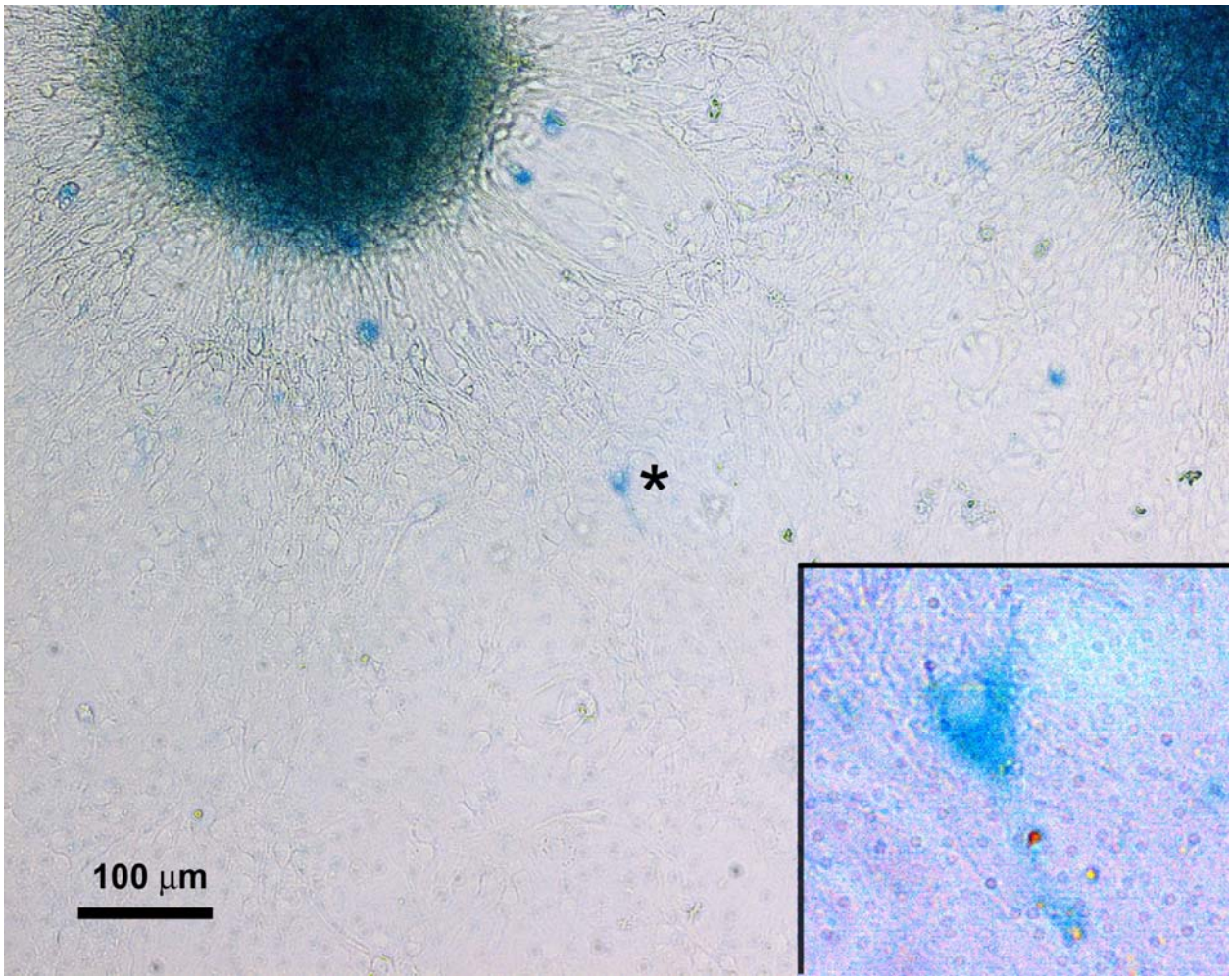


Figure 4-2. Cre/lox recombination system shows neurospheres derived from co-cultures of 2 different mouse lines contain cells positive for β-gal expression, indicating occurrence of fusion. The neurosphere is blue as a result of presumed X-gal retention due to thickness of the sphere. Asterisk shows an example of β-gal positive cell following X-gal staining. The same cell is seen at higher magnification in insert.

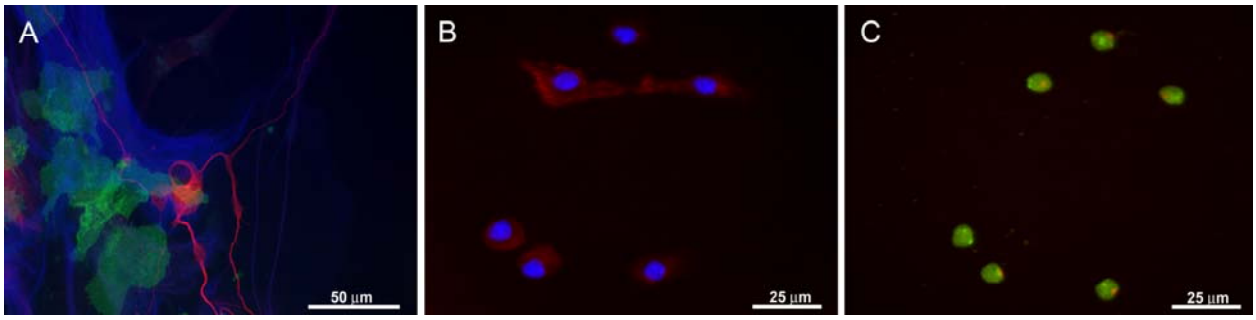


Figure 4-3. Cells immunopositive for the microglial marker, CD11b, retain diploid states. A) an example of a cerebellar-derived neurosphere shows the presence of microglia, neurons, and astrocytes within the culture after immunolabeling for CD11b (green), β-III tubulin (red), and GFAP (blue). B) a high magnification photomicrograph shows cells immunopositive for CD11b (red) before chromosome painting. C) same group of cells are seen after chromosome painting, and shown to be diploid.

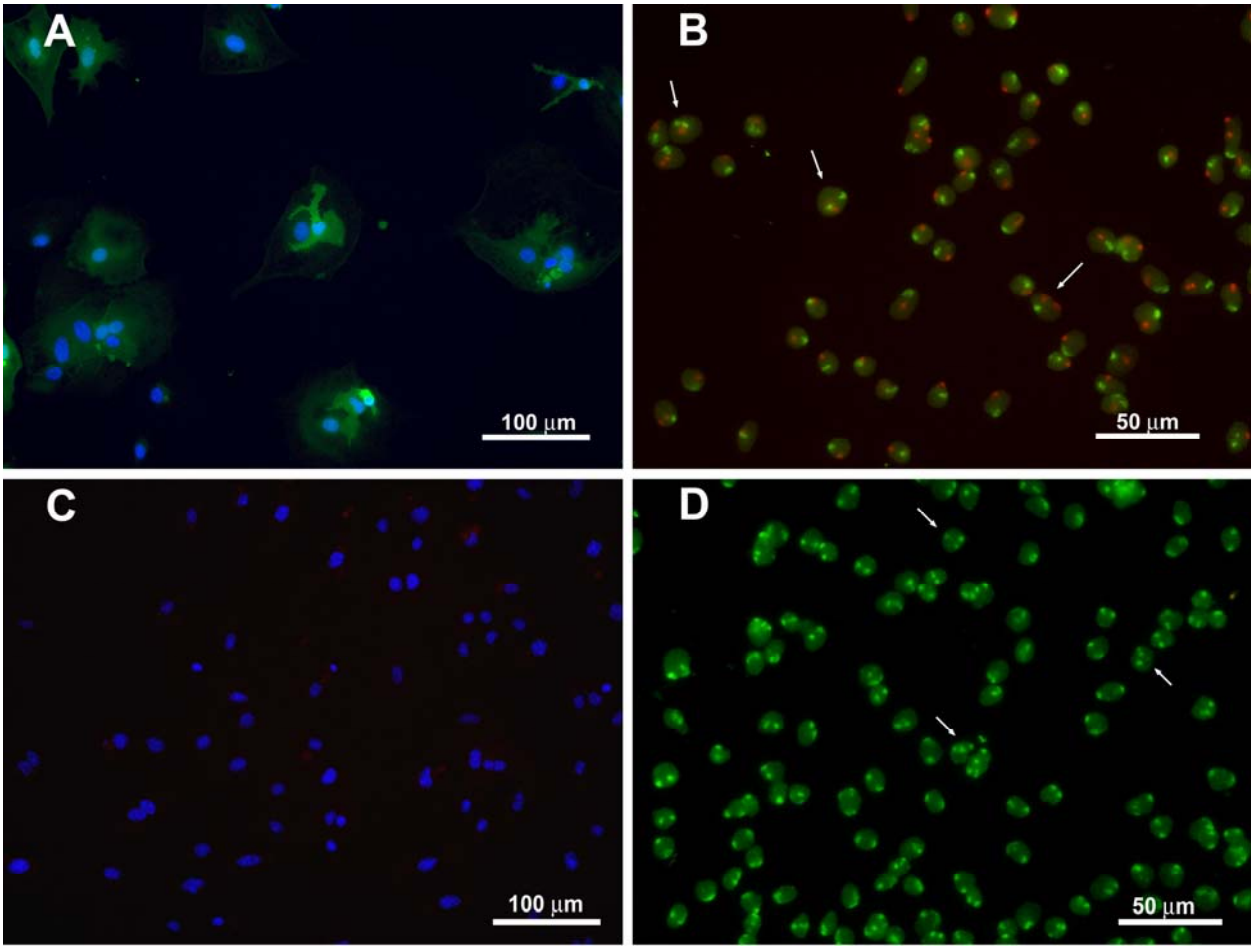


Figure 4-4. Neurospheres derived from PU.1 wildtype and mutant mice were immunolabeled with CD11b and counterstained with DAPI. A) are found within the wildtype culture (green), B) Chromosome painting reveals that the cultures contain cells that are aneuploid (arrows). C) no microglia are found in the PU.1 mutant culture (red), but

in D) chromosome painting reveals that the culture contained approximately the same percentage of cells that are aneuploid (arrows) as in wildtype culture seen in B.

CHAPTER 5
A PROOF OF PRINCIPLE FOR COMBINING STEM CELL FUSION AND GENE THERAPY AS TREATMENT FOR SPINOCEREBELLAR ATAXIA 1

Introduction

Studies have shown that adult stem/progenitor cells possess broad differentiation potential that can be attributed to cell-cell fusion in addition to the widely accepted process of trans-differentiation (Wang et al., 2003; Terada et al., 2002; Ying et al., 2002; Chen et al., 2006). Bone marrow derived cells (BMDCs) in particular show evidence of *in vivo* fusion with somatic cells to become atypical lineages of liver, intestine, heart, skeletal muscle and Purkinje neurons (Alvarez-Dolado et al., 2003; Weimann et al., 2003b; Krause et al., 2001; Orlic et al., 2001; Lagasse et al., 2000; Gussoni et al., 1999; Jackson et al., 1999; Petersen et al., 1999; Ferrari et al., 1998). These findings suggest that targeted cell fusion is a mechanism that can be exploited to expand the developmental scope of adult stem cells. Neural stem cells, for instance, have garnered much research interest to be used as donor populations for replacement of at-risk cell types within many of the neurodegenerative diseases, but have not shown the capability of differentiating into all the cell types within the complex central nervous system (CNS) as shown in findings from chapter 3 of this study. The list of neuronal types that is difficult to generate include specialized Purkinje neurons within the cerebellum that are vulnerable in many movement disorders, and it would be useful to derive neural tissue through different means. Induced plasticity can be exploited for novel therapeutic strategies such as repair or rescue of dying neurons by providing new genetic materials into the degenerating neuronal populations through heterotypic cell fusion between BMDCs and brain cells. One disorder with specific Purkinje neuron atrophy that might benefit from this system is spinocerebellar ataxia 1 (SCA1), which is a gain of function polyglutamine repeat disease that currently has no effective treatments available (Orr and Zoghbi, 2007; Orr and Zoghbi, 2001; Zoghbi and Orr, 2000).

Specifically, SCA1 is an autosomal dominant disorder caused by expanded glutamine repeats in the ataxin-1 protein. Despite wide expression of the mutant ataxin-1 within the CNS and throughout the rest of the body, selective cell death is predominant within the cerebellar Purkinje neurons as well as neurons within the brain stem and the spinal cord, leading to progressive ataxia, dysarthria, dysmetria, nystagmus, and general motor deterioration (Zoghbi and Orr, 1995). The mechanism underlying the pathogenesis has not yet been elucidated, but evidence shows that the degeneration of Purkinje cells may be due to protein misfolding and/or impaired protein clearance as a result of the toxic gain of function caused by the glutamine expansion (Cummings et al., 1998; Cummings et al., 1999; Skinner et al., 2001). Overexpression of chaperones have shown beneficial effects on the disease progression (Cummings et al, 2001), and other genes with the potential to modify or suppress SCA1 neuropathology have also been identified through a genetic screen using a *Drosophila* SCA1 model (Fernandez-Funez et al., 2000). The current study set out to test the concept of using BMDCs as non-invasive delivery vehicles to distribute potential neuroprotective or disease-modifying genes and factors, through cellular fusion, into the degenerating Purkinje neurons of a Sca1 knock-in mouse carrying 154 polyglutamine repeats (Watase et al., 2002) as a novel therapeutic treatment (Fig 5-1). The BMDCs are first genetically modified by adeno-associated virus (AAV) which is a nonpathogenic human parvovirus that has shown great potential for use in gene transfer and gene therapy (Srivastava, 2005; Berns et al., 1996; Muzycaka et al. 1992). AAV serotype 2 (AAV2) in particular has the ability to transduce a wide range of cells and tissues *in vitro* and *in vivo* (Snyder et al., 1997; Xiao et al., 1996; Flotte et al., 1993) and is currently in phase I/II clinical studies for an array of diseases (Snyder et al., 2005; Flotte et al., 2004; Kay et al., 2000; Flotte et al., 1996). Other serotypes with slight variation in structure and function have since been

developed and AAV serotype 7 was found to be capable of transducing Sca-1^+, c-kit^+, Lin^- bone marrow cells/hematopoietic stem cells (HSCs) most efficiently (Maina et al., 2008). In addition, recent improvement in the vector design has allowed the use of a double stranded vector, or self-complementary vector (scAAV), in this study which increases the transduction efficiency through bypassing the need for viral second-strand DNA synthesis (Maina et al., 2008; Wu et al., 2007; Wang et al., 2003). We demonstrate that the transgenes carried by the BMDCs/HSCs are stably expressed within the cerebellum and exert potentially attenuating effects through observations on the nuclear inclusions associated with the disease. This is proof of concept that a combination of cell-cell fusion and gene therapy may be a potential regimen for restoring the homeostatic balance within neurodegenerative diseases.

Adeno-Associated Viral Plasmid Design and Expression of the Transgenes in HEK 293T Cells

Recent advances show that the combination of using AAV serotype 7 capsid and a double-stranded recombinant AAV genome give rise to viral vectors with high transduction efficiency on Sca-1+, c-kit+, Lin- (SKL) population of bone marrow cells (Maina et al., 2008; Han et al., 2008). Therefore, transgenes of interest were packaged into scAAV7 vectors for maximal transduction of the SKL cells which have been shown to be hematopoietic stem cells (Krause et al., 2001; Osawa et al, 1996; Spangrude et al., 1988; Muller-Sieburg et al., 1986). Selection of the transgenes with potential neuroprotective effects was based on a previous study by Fernandez-Funez and colleagues (2000) in which four genes with attenuating effects on the SCA1 manifestations were identified in a genetic screen using a SCA1 Drosophila model. Murine homologs of the four modifier genes were determined to be DnaJ subfamily B member 4 protein (DnaJb4), Lisencephaly-1(Lis1), glutathione S-transferase theta 2 class (Gstt2), and poly(rc)-binding protein 3 (pcbp3), with functions associated with chaperone activity, neuronal

migration, cellular detoxification, and mRNA stabilization respectively (Table 5-1). Recombinant AAV genomes were constructed through the removal of the AAV coding region flanked by two mutated AAV inverted terminal repeats (ITRs) and replaced with genes of interest along with the c-Myc/his reporter sequence for detection (Fig 5-2 A). Expression of transgenes following cloning into the proviral expression cassette was tested through western blot probed against the c-Myc/his tag or against the specific Lis1 gene within the plasmid (Fig 5-2 B). rKiK3 was used as positive control for c-Myc specificity since that was the original plasmid in which the cMyc/his reporter sequence was obtained from (Raisler et al., 2002), and all four genes were expressed and detected with the correct protein size. For detection of viral transduction, whole bone marrow was cultured in 37^{o}C *in vitro* and expression of GFP through infection with scAAV7-GFP viral vector can be directly visualized through fluorescent microscopy following 72 hours in culture. Approximately 40% of the cells appeared to express GFP. GFP and intracellular c-Myc expression can also be detected through FACS, but at a much lower percentage of 2-4% as a result of the difficulty in detecting intracellular signal and the damaging effect of processing cultured bone marrow cells through the cell sorter.

Donor Labeled Purkinje Heterokaryons are Binucleated and Possess Y Chromosomes

Following isolation of the GFP$^{+,}$ Sca-1^{+}, c-kit^{+}, Lin^{-} HSCs from wildtype male mice through FACS, bone marrow cells were infected with one of the four recombinant scAAV7 vectors. Genetically modified HSCs were immediately transplanted, through retro-orbital sinus injection, into heterozygous Sca1 females that received whole-body irradiation 48 hrs prior to the procedure (Fig 5-1). FACS analysis for GFP expression confirmed that all transplanted mice showed robust peripheral blood reconstitution (60%-100% of GFP^{+} cells) at the end of the varied survival periods. Donor labeled GFP^{+} Purkinje neurons (Fig 5-3 A), which refers to heterokaryons that resulted from cell-cell fusion between BMDCs/HSCs and Purkinje neurons

from here on, all expressed the Purkinje cell region specific marker Calbindin (Fig 5-3 B). These Purkinje heterokaryons were detected in the cerebellum starting around 24 weeks post transplantation as previously reported (Weimann et al., 2003b; Priller et al., 2001). At the beginning of the bone marrow transplant studies, β-Actin-GFP mice (stock #003116, Jackson Laboratory) were used as donor animals in which the bone marrow were derived from. FACs analysis of the BMDCs revealed weak expression of the GFP (<50%) and as a consequence, the number of fused events between BMDCs and Purkinje neurons was low. Therefore, in the second half of the bone marrow experiments, a different donor population was derived from UBC-GFP mice that had approximately 99%-100% robust expression of GFP. The number of fused, GFP^+ heterokaryons increased and was more easily detected. 12 out of 18 mice receiving UBC-GFP donor HSCs had fused Purkinje neurons within the cerebellum and an average of 10 Purkinje neurons was found in each animal. In comparison, only four out of 24 mice receiving β-Actin-GFP bone marrow were found to harbor GFP^+ heterokaryons, and less than five GFP^+ Purkinje neurons were found in each animal. Table 5-2 documents the total number of animals receiving the bone marrow transplant, their genotype, as well as which viral vector was used to transduced the BMDCs.

The fusion events occurred between healthy Purkinje cells with full dendritic arbors (Fig 5-3 A) as well as between degenerating/atrophied Purkinje cells that had smaller cell bodies and altered dendritic arborizations. GFP^+ Purkinje neurons were further analyzed in serial laser confocal optical sections, and shown to be binucleated (Fig 5-3 C, arrows) with two nuclei that were distinctively different in size and morphology as expected from heterotypic cell fusions. To further confirm that the GFP^+ Purkinje neurons resulted from fusion between sex-mismatched donor bone marrow cells and host Purkinje neurons, fluorescent *in situ* hybridization (FISH)

analysis was carried out to show the presence of a Y chromosome within one of the two nuclei found in the GFP^+ Purkinje neurons within cerebella of female recipient mice. Nuclear patterns, based on DAPI staining before the FISH procedure (Fig 5-3 D, E; arrow and arrow head), were used to relocate the two nuclei (Fig 5-3 F, arrow and arrow head) within the fused cell after the GFP fluorescence was abolished due to the FISH procedures. Fluorescence-conjugated chromosome probes identified the X chromosomes in green and the Y chromosomes in red (Fig 5-3 F). In the inset of figure 5-3, F, one of the two nuclei (arrow head) was clearly shown to contain the cy3-labeled Y chromosome, which indicated that the GFP^+ Purkinje neuron was involved in a fusion event with the HSC and not a product of trans-differentiation.

Donor Labeled GFP^+ Cells Express Modifier Genes *in Vivo* with Possible Effects on Nuclear Inclusions

Another prevalent donor derived cell population observed within the recipient cerebella was GFP^+, $CD11b^+$ microglia (Fig 5-4 A, B). A small number, approximately 20-30%, of the GFP^+ microglia expressed the reporter gene c-Myc (Fig 5-4 C, D) tethered to the modifier genes, which indicated that the viral genome has stably integrated and expressed within the host environment. C-Myc expressions were also found in GFP^+ Purkinje heterokaryons (Fig 5-4 E, F) which further validated the concept of using bone marrow cells to deliver genes or factors missing within the at-risk neuronal population in SCA1. As expected, c-Myc expressions were observed in the cytoplasm (Fig 5-4 D, F) of the GFP^+ cells, which corresponds with the cellular localizations of the four modifier genes. In addition to the CNS, c-Myc expressions were also detected in the spleens of the transplanted mice, which further confirmed integration and expression of the viral genomes.

The transgenes used in this study were found through examination of retinal phenotypes in the *Drosophila* model of SCA1 where the pathology was directed to the eye (Fernandez-Funez et

al., 2000). In the current murine model, nuclear inclusions that have been considered to be one of the hallmark neuropathologies of trinucleotide diseases (Servadio et al. 1995) were found in the Purkinje neurons and used as a measurement of the potential attenuating effects of the modifier genes. The nuclear inclusions were immunopositive for ubiquitin and the SCA1 gene product, ataxin-1. The ataxin-1 positive inclusions varied in size and number within the Purkinje neurons (Fig 5-5 A, B). Within the cerebellum of this Sca1 mouse model, some Purkinje cells had a single, large nuclear inclusion (Fig 5-5 A, B; arrow heads) while a smaller number were observed to contain two inclusions (Fig 5-5 B, arrow), or multiple inclusions that were much smaller in size (Fig 5-5 A, asterisk). Similar findings were reported by Skinner and colleagues (1997) in another Sca1 study where mutant ataxin-1 carrying long glutamine repeats localized within the nuclei of Purkinje neurons as a single, large inclusion, in contrast from the wildtype ataxin-1 overexpressed *in vitro* which appeared as smaller, multiple inclusions. We showed the fused GFP^+ Purkinje heterokaryons did not contain the single, dense nuclear inclusions found most prevalent in the Sca1 cerebellum, but instead possessed smaller inclusions (Fig 5-5 C, D; arrows) which suggested possible modifying effects of the genes overexpressed through the HSCs.

Bone Marrow Derived Cells Can Fuse to Other Cell Types within the Cerebellum

Previous reports (Alvarez-Dolado et al., 2003; Weimann et al., 2003b; Johansson et al., 2008; Mangrassi et al., 2007) found that only Purkinje neurons and microglia were the only bone marrow-associated, labeled cells found within the CNS following *in vivo* transplantation. Interestingly, a small number of the cells observed within the current paradigm did not appear to be either Purkinje neurons or microglia, but instead exhibited morphological characteristics resembling interneurons within the molecular layer (Fig 5-6 A-C, green) based on morphological assessments. Another donor-labeled, GFP^+ cell type we observed possessed all of the attributes

of unipolar protoplasmic astrocytes similar in morphology to Bergmann glial cells (Fig 5-6 D) which are closely associated with Purkinje neurons. These cells had somata located near the cell bodies of the Purkinje neurons, and radial fibers that extend toward the pial surface. In addition, these cells were immunopositive for glial fibrillary acidic protein (GFAP) (Fig 5-6 E, F) as well as S100β and found in the correct spatial orientation as Bergmann glia within the cerebellar Purkinje and molecular layers. The interneuron cell types presented in Fig 5-6 (A-C) were found in different transplanted mice and were extremely rare in occurrence (n=1 in three separate animals). However, more than ten putative Bergmann glial cells (Fig 5-6 D-F) were observed in at least four mice throughout different areas of the cerebellum, and thus are not such rare events.

Table 5-1. Modifier Genes Packaged into scAAV7 Vectors

Gene name	Functions
DnaJb4	Chaperon protein, Heat shock protein 40 homolog
Lis 1 (Lissencephaly 1 protein)	alias Platelet-activating factor acetylhydrolase 1B alpha subunit (pafah1b1), Nuclear migration, neuronal differentiation.
Gstt2 (Glutathione S-transferase theta class 2)	Glutathione transferase, cellular detoxification involvement
Pcbp3 (poly(rC) binding protein 3)	Nucleic acid binding protein involved in mRNA stabilization, translational activation and/or silencing.

Four murine homologs to the genes found to have suppressive/modifying effects on the disease progression of a *Drosophila* SCA1 model. Each gene is inserted into a separate AAV proviral expression cassette for a total of four scAAV7 vectors.

Table 5-2. Bone Marrow Transplant Summary

Donor Mice	Modifier Gene	# of Recipients	Donor Cell Type	# of Purkinje Heterokaryons	# of non-Purkinje GFP^+ Cell types
β-Actin GFP	DnaJb4	15 (+/-) 3 (wt)	Whole BMDCs	4	0
	Gstt2	3 (+/-)	HSCs	2	0
	Lis 1	3 (+/-) 1 (wt)	HSCs	0	0
	Pcbp3	0	N/A	N/A	N/A
UBC-GFP	DnaJb4	9 (+/-) 1 (wt)	HSCs	> 10	>10
	Gstt2	0	N/A	N/A	N/A
	Lis 1	0	N/A	N/A	N/A
	Pcbp3	6 (+/-) 2 (wt)	HSCs	>10	>10
β-Actin GFP	No virus	3 (+/-) 1 (wt)	Whole BMDCs	ND	ND

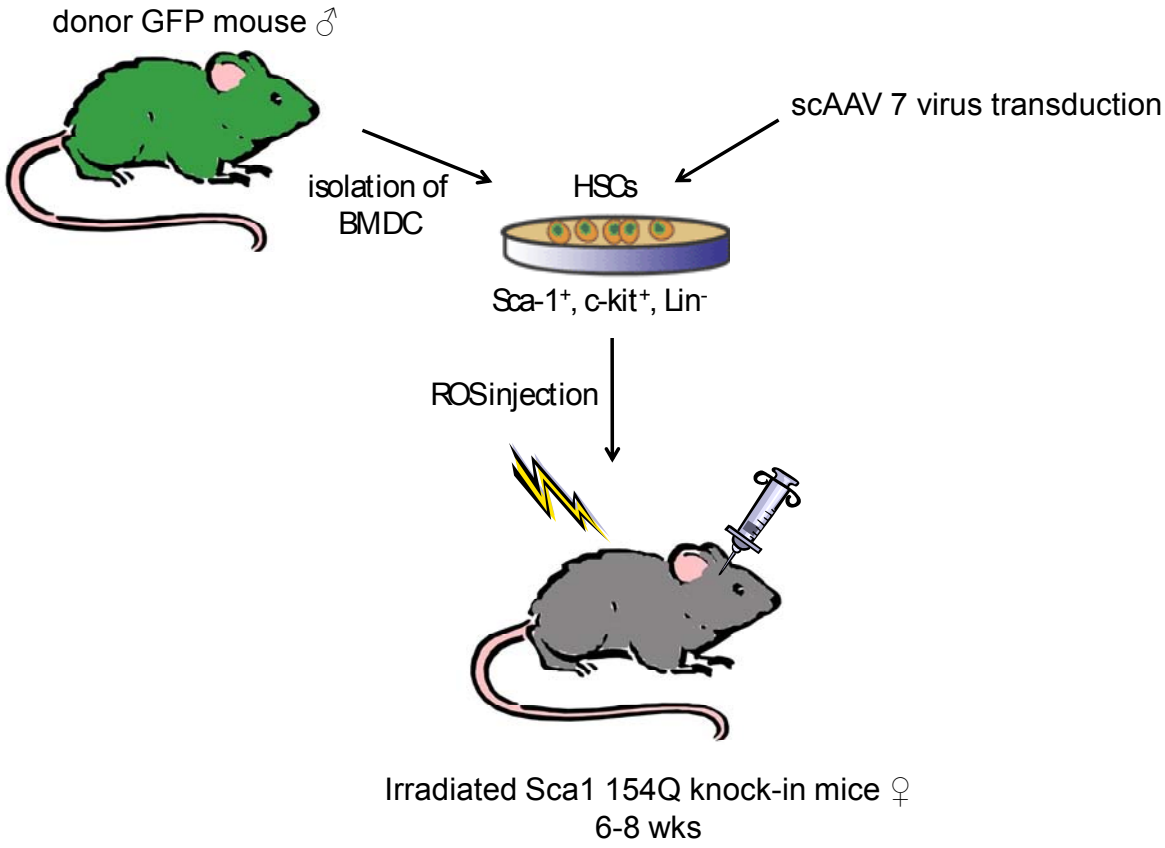
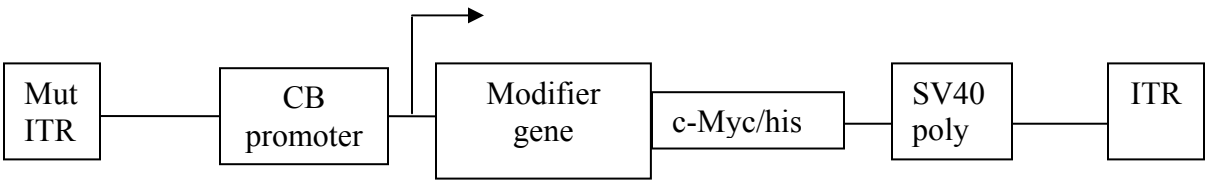


Figure 5-1. Schematic representation of the experimental paradigm. GFP^+ hematopoietic stem cells (HSCs), immunopositive for Sca-1 and c-kit but negative for lineage cocktail, are isolated from male GFP transgenic mice through FACS. HSCs were next transduced with one of the scAAV7 vectors at an MOI of 100 and transplanted into irradiated female Sca1 heterozygous recipients through retro-orbital injection.

A.



B.

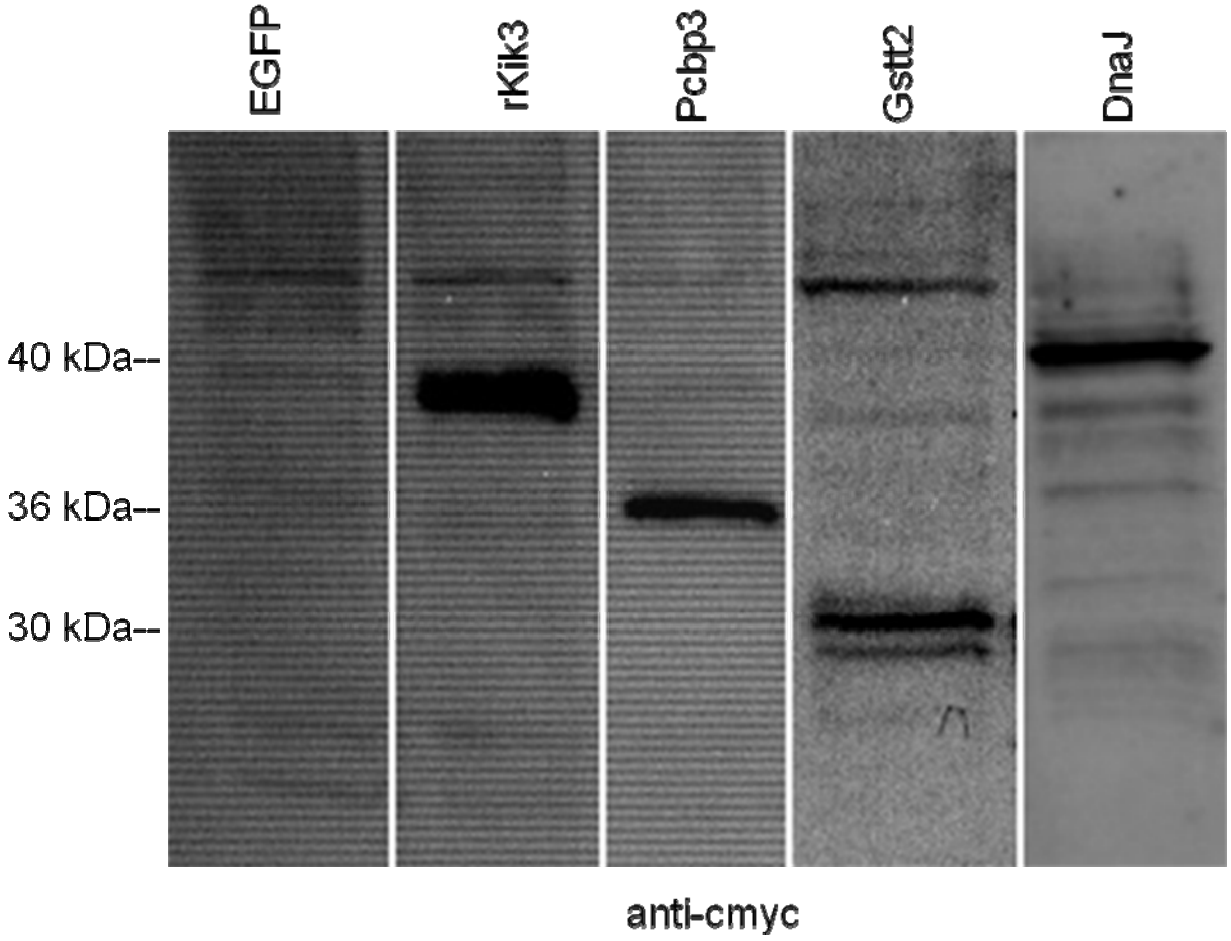


Figure 5-2. Plasmid design and proviral cassette expression in HEK 293T cells. A) Each of the four double stranded AAV7 viral vector contains the inverted terminal repeats (ITR) at both ends, the CMV enhancer and chicken β-actin promoter (CB promoter), cDNA of the modifier gene tethered to a c-Myc/his tag, and the SV40 polyadenylation site. B) Western blot analysis of the proviral cassette expression *in vitro* probed against the reporter tag, c-Myc/his. The eGFP construct does not contain the c-Myc/his reporter tag and served as the negative control for unspecific bands. rKiK3 is the original construct from which the c-Myc/his tag was obtained and shows a robust band that corresponds with the KiK3 gene. For the pcbp3 construct, the protein was expressed at the correct size of 36 kDa, for Gstt2 was 30 kDa, and for Dnajb4 was 40 kDa.

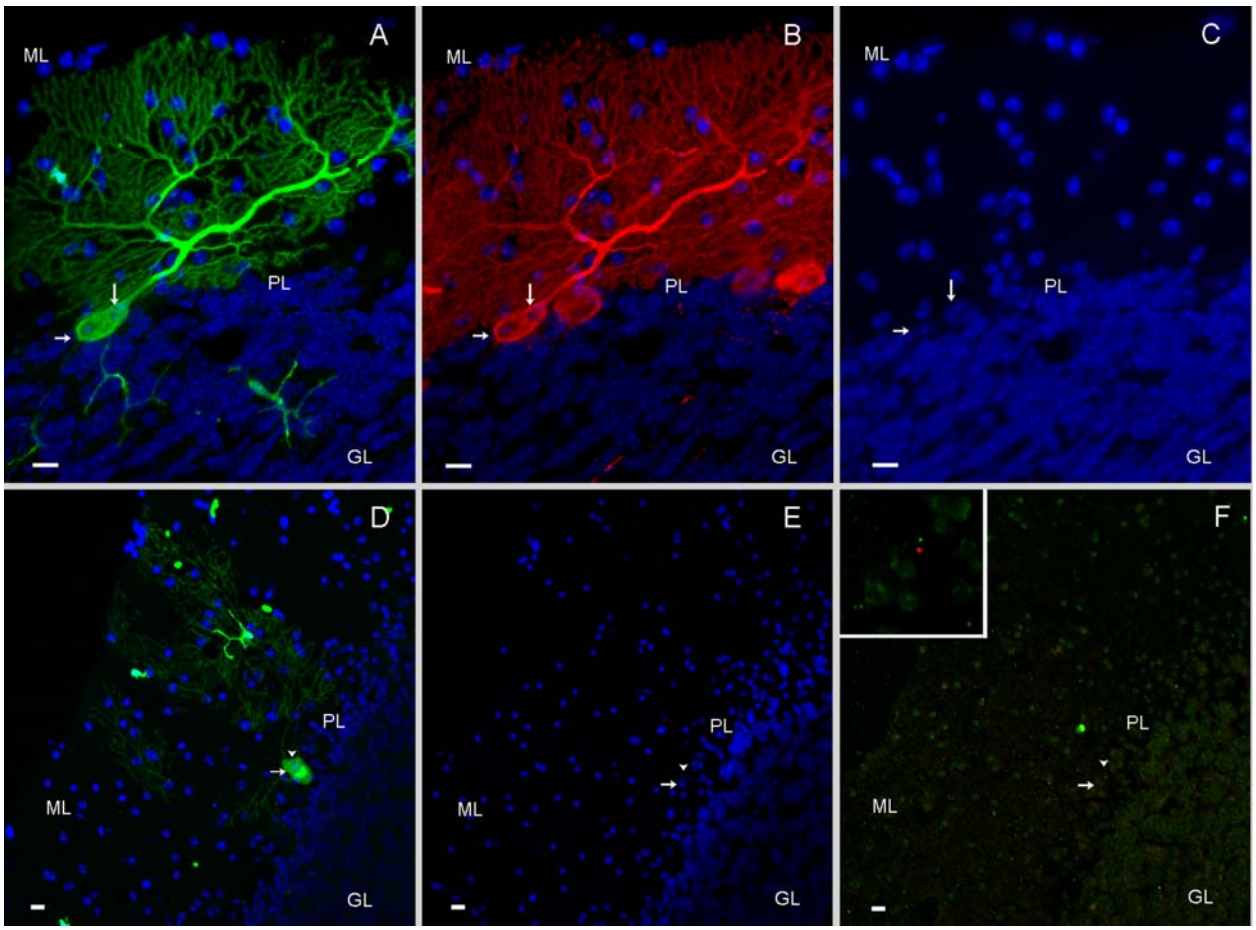


Figure 5-3. Fused Purkinje heterokaryons are binucleated and have Y chromosomes in female recipient mice. A) An example of a GFP^+ Purkinje neuron (green) derived from GFP^+ male HSC. B) The same GFP^+ Purkinje neuron also express Purkinje cell marker Calbindin (red). C) DAPI staining (blue) reveals that the fused Purkinje neuron contains morphologically distinct nuclei of different size (arrows). D) Another binucleated GFP^+ Purkinje neuron (green, arrow and arrow head) contains one Y chromosome in one of the two nuclei (arrow head) shown before (E) and after (F) FISH analysis. The cy3-labeled Y chromosome (F, inset) is detected amongst FITC-labeled X chromosomes (green) in the female recipient cerebellum. (scale bar=10μm, GL=inner granule layer, PL=Purkinje layer, ML=molecular layer.)

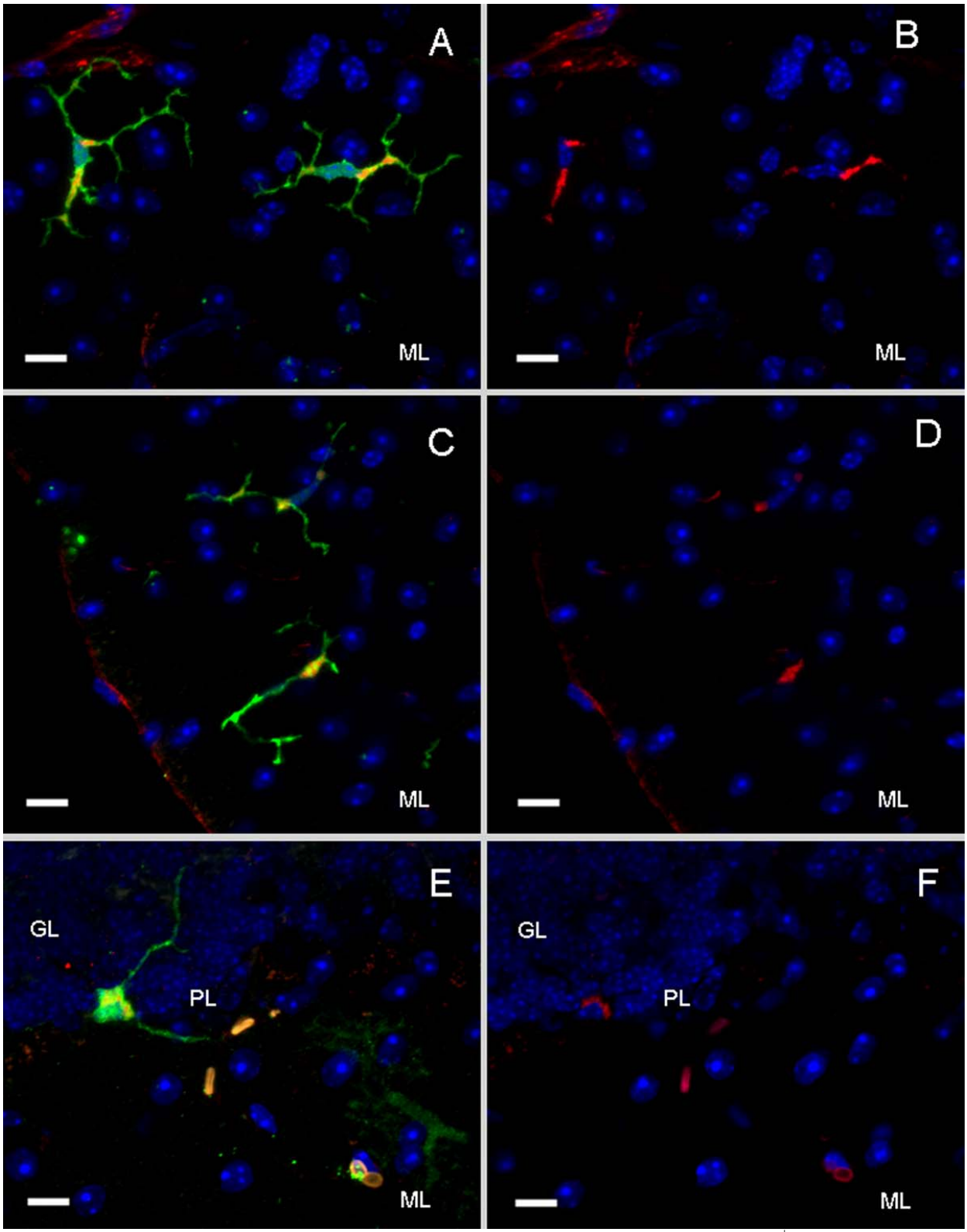


Figure 5-4. Donor labeled cells express c-Myc/his reporter tag. A) Some GFP^{+} cell type found in recipient cerebella have the morphology of microglia (green) and are co-localized, as

shown in B), for the surface marker CD11b (red). C) 20-30% of the microglia (green) are shown in D) to express c-Myc (red) in the cytoplasmic regions where all four of the modifier genes are localized. E) Fused, GFP^{+} Purkinje neurons (green) are also immunopositive for c-Myc (red) in F. (scale bar=10μm, GL=inner granule layer, PL=Purkinje layer, ML=molecular layer. DAPI=blue)

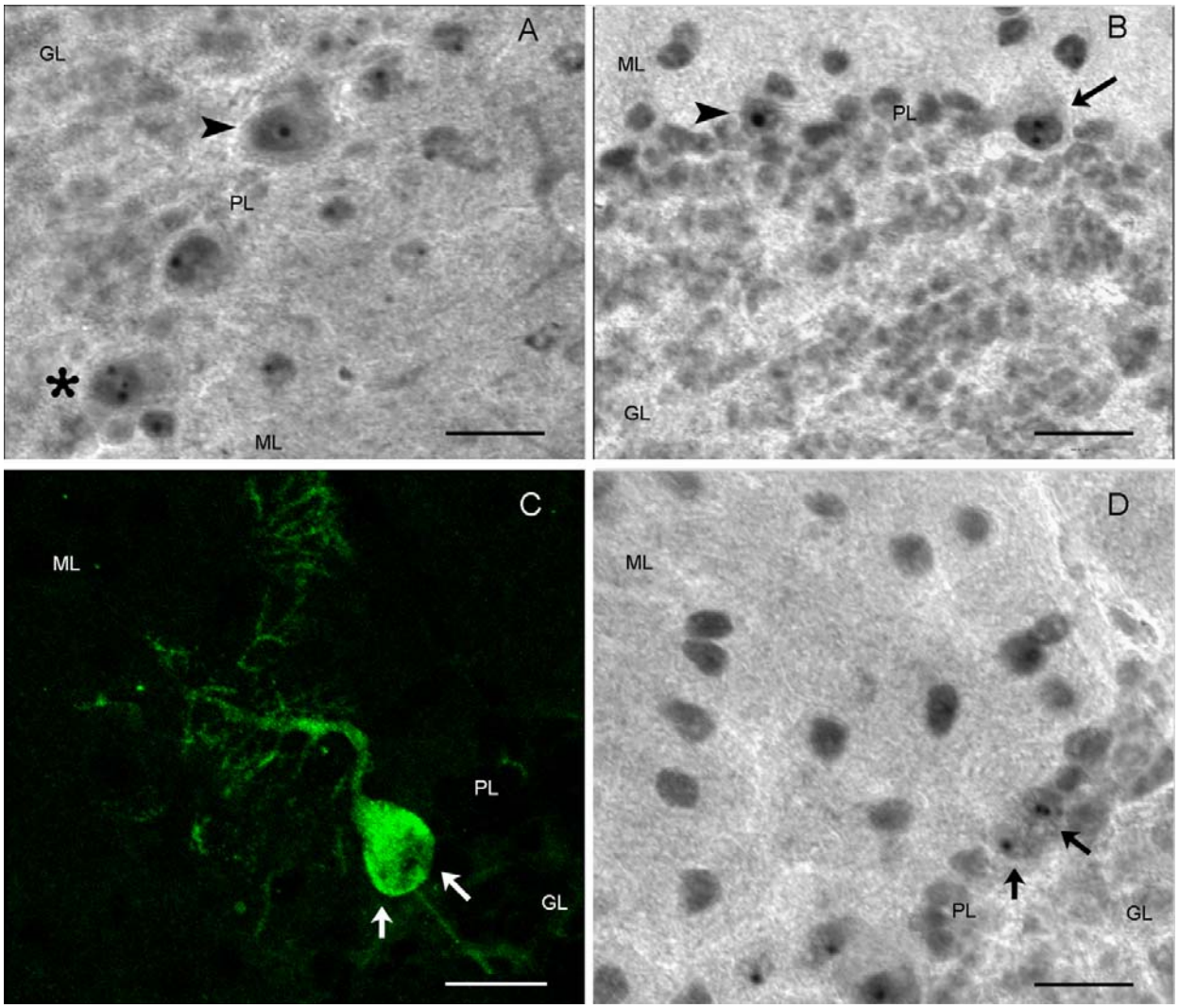


Figure 5-5. Nuclear inclusions vary in size and appear to be smaller in fused Purkinje neurons. A) and B) are examples of the ataxin-1^{+} nuclear inclusions found in the heterozygous Sca1 mice. Most common are Purkinje neurons with a single large inclusion (arrow heads) but a small number of cells are found to contain two (arrow) or multiple (asterisks) smaller-sized inclusions. C) GFP^{+} Purkinje neurons (green) also contain axaxin-1^{+} nuclear inclusions but D) they are the small-sized variety (arrows) and not the typical large single inclusions seen in non-fused Purkinje cells in A) and B). (scale bar=20μm)

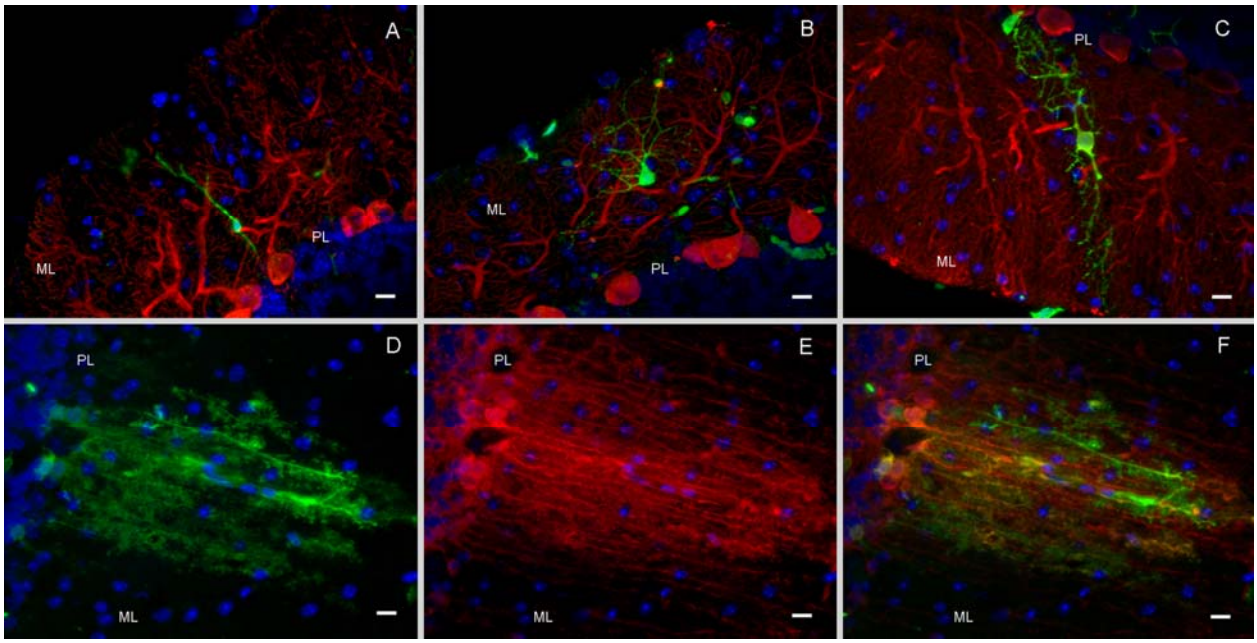


Figure 5-6. Bone marrow cells give rise to multiple neuronal cell types within the cerebellum. In addition to fusion involving Purkinje neurons and differentiation into microglia, GFP^+ cells resembling interneurons (A, B, and C, green=GFP, red=Calbindin) were found within the molecular layer. Specifically, cells in A), B) and C) resemble interneurons, possibly stellate neurons of the molecular layers. None of the GFP^+ cells (green) resembling interneurons are co-labeled with Calbindin (red). D) Another cell type found in multiple transplant recipients was GFP^+ unipolar astrocyte (green) that resembles Bergmann glia that are closely associated with Purkinje neurons, structurally and functionally. E) These cells are immunopositive for glial fibrillary astrocytic protein (GFAP) (red) typical of Bermann glia, and F) shows merged image of GFP and GFAP (yellow). (scale bar=10μm, PL=Purkinje layer, ML=molecular layer. DAPI=blue)

CHAPTER 6
DISCUSSION AND CONCLUSIONS

The potential for stem/progenitor cells to become a source of transplantable donor cells for cell-based neuroprotective and/or replacement therapies has been a focus of many recent studies, especially in the CNS where most cells lack inherent regenerative capabilities. A great deal of these studies has focused on the use of embryonic stem (ES) cells and adult neural stem cells derived from SEZ in particular, to directly replace the degenerated neuronal populations within diseases or trauma/injury. It is a reasonable strategy that has shown great promise, but many challenges remain in terms of finding alternative sources of stem cells that would not provoke ethical concerns, retain pluripotency, and at the same time can be safely integrated within a host system. Part of the rationale for current study was to determine the potential successful transplantability of another source of neural stem/progenitor cells derived from the cerebellum to differentiate into the principle neuronal types lost in cerebellum. However, despite best efforts, many reports have already shown that adult neural stem cells do not possess the ability to differentiate into every type of neuronal cell found within the complex CNS, including the highly complex cerebellar Purkinje neurons that are vulnerable in many movement disorders. Therefore, in conjunction with studies on neural stem cells, adult stem cells derived from other parts of the body, such as the bone marrow, are also being investigated for their potential to rescue at-risk neuronal populations that complement the first part of this study that looked at both ES and adult brain-derived stem/progenitor cells for cell replacement in a cerebellar mutant mouse. Observations that adult stem cells derived from various developmental periods and body areas are able to give rise to cells of another lineage through trans-differentiation have been rare. Instead it was found that a primary mechanism through which cells take on identities outside of their typical lineages is through cell-cell fusion, which was reported by our lab and others to

occur spontaneously *in vitro* and *in vivo* between stem cell types and between stem cells and somatic cells. Specifically, bone marrow cells possess the ability to fuse with Purkinje neurons, and this fusogenic property could be further developed and refined as a therapeutic approach to rescue the dying neurons since direct replacement is not yet a possibility. Thus, this dissertation describes a thematic research approach that exploits the potential of using adult stem cells in rescuing at-risk neuronal populations, with either direct cell replacement or repair through providing new genetic material, in well-characterized cerebellar transgenic and mutant animal models.

Comparative Analysis of Cerebellar-derived MASCs and ESNPs in Transplantation

In the first part of my studies where cerebellar-derived MASCs were used as a donor population in a homotopic transplantation paradigm within the weaver mutant mouse cerebellum, their differentiation capabilities were compared to an established embryonic cell line that has been shown to generate multiple types of neurons that can integrate within host CNS environments. Results show that both donor stem/progenitor cell populations, derived from either a postnatal neurogenic zone or an embryonic cell line, have the ability to survive, migrate, and initiate differentiation into neuronal phenotypes within the granuloprival weaver mouse cerebellum. However, neither of these donor populations adopted impressive region-specific identities, particularly neurons of the granule, Purkinje, or molecular layers, despite earlier studies that suggested the potential of these stem/progenitor cells to respond to in vivo cues when placed in a permissive/instructive environment. Specifically, cerebellar- or lateral ventricle SEZ-derived MASCs were previously observed to enter into the rostral migratory stream after transplantation into the SEZ and lateral ventricles, and found to migrate into the olfactory bulb where a small population differentiated into appropriate olfactory interneurons (Zheng et al., 2006). This suggests that the spatially and temporally restricted population of astrocytes, the

MASCs, retain the ability to respond to *in vivo* cues provided by the host system. The low percentage of transplants that generated olfactory interneurons reported by Zheng et al. (2006) was explained by the possibility that cerebellar MASCs were not optimally primed to respond to cues related to olfactory neurogenesis, hence it was expected that homotypic transplantation into the mutant cerebella would prompt more impressive neural differentiation and engraftment, should the MASCs retain a transcriptional profile capable of responding to specific cerebellar neurogensis cues. Klein et al. (2005) showed that cerebellar-derived neurospheres gave rise to both GABA-expressing and glutamate-expressing interneurons resembling Lugaro cells and granule cells, respectively, following transplantation into wildtype P4 cerebella, but they differentiated into glial cells when transplanted into the forebrain, demonstrating that those cerebellar-derived cells were capable of retaining intrinsic regional attributes in an uninjured CNS environment. Furthermore, it has been shown that *in vivo* neurogenesis can increase in response to injury or disease (Jin et al., 2001; Jankovski et al., 1998; Gould et al., 1997) in the SEZ and hippocampus, which would suggest that the weaver mouse should be a suitable model for inducing cellular replacement with its discrete and localized pattern of granule neuron degeneration. However, in the present study, the majority of the cells retained glial-like or undifferentiated stem/progenitor cell morphologies, and they lacked expression of region specific transcription factors, with only a small number of cells differentiating into immature neurons or astrocytes. This is in keeping with many previous studies that showed engraftment of neural progenitor cells into metabolic disease models (Lee et al., 2007; Snyder et al., 1995) or neurological mutants (Li et al., 2006; Rosario et al., 1997) which seem to exert corrective, therapeutic effects on the at-risk neuronal population, but did not show the acquisition of region specific phenotypes through appropriate expression of transcriptional factors or morphological

profiles, e.g. parallel fiber axonal arbors typical for granule cells or the dense and elaborate dendritic trees characteristic of Purkinje neurons. One explanation is that the MASCs studied here were able to survive and home towards the degenerating areas but they did not undergo appropriate, terminal phenotypic differentiation due to a downregulation of receptors or transcription or other morphogenetic factors during the *in vitro* culturing process prior to transplantation; an alternative explanation could be there is a lack of requisite fate choice and differentiation signals within the abnormal weaver cerebellum which may result from a severe homeostatic imbalance within the injured host environment that could be non-conducive for complete functional integration. The weaver mutation is widely believed to be intrinsic to the affected granule neurons, but earlier reports (Gao et al., 1993; Gao et al., 1992) have shown that some mutant granule cells transplanted into a wildtype cerebellum are capable of differentiating and migrating normally which suggests the weaver gene might act non-autonomously and could hinder host-donor cell interactions. However, a small population of granule cells does survive and persist normally under the conditions present in the weaver cerebellum and it might be these same cells that were found to be able to differentiate properly within a wildtype cerebellum in the observations reported by Gao and colleagues. Furthermore, data presented here seem to suggest that the cellular deficits present within some of the *wv*/+ or *wv*/*wv* brains may promote a slightly enhanced donor cell response towards acquisition of neuronal phenotypy. Hence it is likely that a fine balance exists between an impaired host environment that is amenable to cell replacement versus one that may be too toxic for immediate (and within the timeframes, albeit rather protracted here) and desired repair.

Regarding ESNPs, they have been shown to acquire complex morphologies and adopted excitatory neurotransmitter phenotypes both *in vivo* and *in vitro* (Goetz et al., 2006; Wernig et al,

2004; Benninger et al., 2003; Brüstle et al., 1999; Brüstle et al., 1997; Okabe et al., 1996), and they were expected to generate more neuronal phenotypes since they have been induced into a dominant neuronal lineage prior to transplantation. Morphologically, these donor cells did appear to be more mature than those derived from cerebellar MASCs, exhibiting extensive numbers of varicose processes and round somata. Some of them also express mature neuronal markers including NeuN and the neurotransmitter glutamate not seen with the MASC population. This suggests that inherent differences within differentiation potential do exist between these two neural stem/progenitor cell populations. Despite the more mature antigenic and morphological profiles, ESNPs still did not appear to significantly commit to regional differentiation identities as indicated by the lack of cerebellar-specific transcriptional factor expressions for MATH-1, $GABA_A\alpha6$, and RU49. This embryonic cell line-derived donor population was primed for neuronal lineage and expected to be more responsive to the neurogenic cues within the host environment, so a lack of regional commitment suggests that successful engraftment is highly dependent upon the interplay between the host environment and the donor populations, and that the context of the injury conditions can be specific and crucial for the promotion of preferential cell replacement.

Another phenomenon observed here was the aggressive nature of the neoplastic formations within a small number of ESNP transplants. Previous studies reported that donor cell clusters of varied sizes with teratoma appearances were found when ESNPs were transplanted into the SEZ, but they did not seem to disrupt the parenchyma nor was there evidence of infiltration by non-neural donor cells (Wernig et al., 2006). In contrast, our study shows that host tissue surrounding the GFP^+ neoplastic formation was transformed and adjoined with the neoplasm as confirmed by H & E staining. The difference could be attributed to particular

locations of the injection sites, but it does not preclude the possibility of ESNPs giving rise to neoplastic formations. In comparison, previous studies have shown similar invasive sphere-like structures forming within ventricular walls of animals intraventricularly transplanted with cerebellar MASCs, but in these experiments no changes were detected in the surrounding tissues (Zheng et al., 2002). These same donor cells did not give rise to neoplastic formations in any of the current transplants when injected directly into the cerebellum. This suggests, once again, that differences exist between the proliferation and differentiation potential of the two different stem/progenitor cell populations studied here, and that more studies are obviously needed to uncover ways to coax stem/progenitor cells into appropriate patterns of cellular differentiation before neural stem/progenitor cells can be considered for therapeutic applications within neurologically compromised patients. This part of the present study presents an important *in vivo* bioassay, within a presumptive cell-replacement-supportive neurological mutant mouse brain, that utilizes two of the most disparate and plastic neural stem/progenitor cell populations that have always been assumed to be the most promising candidates for neuro-cellular replacement therapies.

Homotypic Cell-Cell Fusion *in Vitro*

This next part of the study looked at another mechanism besides differentiation utilized by stem cells to derive new identities, and established the foundation in which this property can be used to expand the developmental scope of adult stem cells. Chapter 4 showed evidence that cells within neural stem/progenitor cell cultures harbored excessive numbers of sex chromosomes, supporting the notion that cell-cell fusion can occur. Cellullar fusion has been known for decades to be a fundamental and important phenomenon during the development and functioning of multicellular organisms, but more recently it has been found to be a mechanism potentially responsible for so-called trans-differentiation ability and plasticity exhibited by adult

stem cells (Terada et al., 2002; Ying et al., 2002; Chen and Olson, 2005). The data presented suggest that spontaneous cell fusion might be induced under the selective pressure of tissue culture conditions utilized in the current paradigm. However, other factors, including the proximity of the cells, space limitation, fusogens (Chen and Olson, 2005; Jahn et al., 2003; Weber et al., 1998), or presence of other known fusion causative agents such as macrophages may also be implicated. In our study, the percentage of aneuploid cells was comparable between confluent and non-confluent astrocyte monolayers, implying that fusion does not simply result from high cell density conditions. One could hypothesize that microglia, due to their demonstrated ability to fuse with osteoclasts, giant cells, and hepatocytes (Camargo et al., 2003; Willenbring et al., 2004), might be the catalytic agents of cell fusion seen in our CNS neurosphere cultures. Indeed, the acquisition of extra chromosomes could be a microglia-mediated phenomenon, but the observation that most cells immunopositive for CD11b retained a diploid state suggests that this interpretation is unlikely. Nevertheless, CD11b antigen expression could be downregulated in microglia after a fusion event, leading to an undercount of polyploid CD11b cells. However, this is not a feasible explanation since evidence shows that PU.1 +/+ and PU.1 -/- microglial-free cultures contained similar levels of aneuploid cells. This suggests that, at least under the present culture conditions, cell fusion is not restricted to hematopoietic and embryonic stem cells, and may be a byproduct of conditions within cell culture or endogenous tissue environments. Cells grown under conditions described here thus acquired extra chromosomes through mechanisms independent of microglial participation. Of course, there are alternative explanations to cell fusion, including the possibility that chromosomes simply failed to separate properly and could be the basis for the observed aneuploidy. However, this would only explain those examples with even numbers of excess

chromosomes, but not those with odd numbers such as the two X and one Y documented in Fig. 4-1D (asterisk). Results from the Cre/lox recombination studies also support the notion of cell fusion, but the number of β-gal$^+$ cells is lower than the proportion of cells observed to contain abnormal chromosome numbers. This suggests that different scenarios including the failure of chromosomes to separate, or the presence of active cell division at the time of cell attachment, should be taken into consideration along with cell-cell fusion.

Occurrence of neural cell fusion *in vitro* suggests that similar events may occur *in vivo*. Alvarez-Dolado, et al. (2003) presented evidence for *in vivo* fusion of bone marrow-derived cells with cardiomyocytes, hepatocytes, and Purkinje neurons. There have been questions raised about a lack of plasticity of adult stem cells (Dor and Melton, 2004; Wagers et al., 2002), but the relatively low rates of cell fusion observed both *in vitro* and *in vivo* does not support cell fusion as the only mechanism underlying adult neural stem cell multipotency. Since an understanding of mechanisms underlying cell fusion between stem/progenitor cells and other somatic cells could ultimately provide insights into directed fusion and induced plasticity amongst different populations of immature and mature cells, additional studies are needed to resolve precise fusogenic factors and events in culture paradigms as exploited here. One could even envision novel therapeutic strategies for human diseases whereby targeted cell fusion is used to deliver modified genomes and factors that ultimately rescue at-risk populations of cells following injury or degenerative disease. One possibility would be to use bone marrow-derived cells to deliver and release an enzyme, e.g. acid sphingomyelinase, in an animal model of the storage disease Niemann-pick type A to rescue a degenerating Purkinje cell population (Bae et al., 2005), or the use of bone marrow-derived cells to deliver chaperones or other housekeeping genes compromised within the at-risk Purkinje neurons in a trinucleotide repeat disorder, e.g.

spinocerebellar ataxia 1 as presented in chapter 5. Such an approach would exploit cell fusion to expand the developmental and therapeutic scope of adult stem cells. This would be especially beneficial for specialized cell types such as Purkinje neurons that are at risk in many neurodegenerative disorders and lack the ability to be generated after birth (Weimann et al., 2003a; Weimann et al., 2003b), but are nonetheless susceptible to fusogenic events (Alvarez-Dolado et al., 2003; new data presented here).

Bone Marrow Derived Cells Deliver Potentially Neuroprotective Genes to Purkinje Neurons through Heterotypic Cell-Cell Fusion

Developmental plasticity within somatic adult stem cells appeared more extensive than expected when a series of studies presented data that cells derived from one organ can give rise to cell types of unrelated lineages (Ferrari et al., 1998; Gussoni et al., 1999; Jackson et al., 1999; Petersen et al., 1999; Lagasse et al., 2000; Krause et al., 2001; Orlic et al., 2001). Most believed that the observed phenomenon was due to the process of trans-differentiation, but *in vitro*, co-culture studies between embryonic stem cells and either BMDCs or neural stem cells, and data from our laboratory (discussed in chapter 4), showed that donor populations took on the identity of the host cells through the method of cellular fusion instead. Together, the findings suggest that cell-cell fusion is an additional mechanism that can be exploited to expand the role of adult stem cells in therapeutic treatments. This last part of my dissertation study is a proof of concept that heterotypic cell fusion can be utilized as a rescue strategy in which HSCs from bone marrow are used to provide neuroprotective genes/factors for injured or degenerating cells, such as specialized Purkinje neurons, that currently do not have other repair mechanisms available. We show that Purkinje neurons possessing two nuclei and donor-derived Y chromosomes are found in the recipient animals approximately six months post bone marrow transplant, but that the number of fused cells remains low as previously reported (Priller et al., 2001; Weimann et al.,

2003b; Alvarez-Dolado et al., 2003). However, this does not indicate fusion events to be artifactual rare occurrences that are random and sporadic as some have come to believe (Wagers et al. 2002). Recent studies by Johansson et al. (2008) showed that formation of heterokaryons increased 10-100 fold in chronic inflammation conditions, while Nygren et al. (2008) further demonstrated that fusion could be inhibited through treatment with the anti-inflammation drug prednisolone, suggesting that cell fusions are indeed biologically significant events that may provide a protective role in the face of trauma or in the case of neurodegenerative diseases that have been associated with components of inflammation (Singec & Snyder, 2008). Given these new findings, it is possible that the rate of fusion is slowed down in the current experimental paradigm due to the administration of antibiotics to the experimental mice for two weeks after bone marrow transplant which could have reduced both the irradiation-induced inflammation as well as the general inflammation component that may be associated with neurodegeneration. Future studies should be focused on the search for small molecule compounds that would promote low levels of inflammatory response without overwhelming the immune system, which could ultimately induce higher percentages of cell-cell fusion.

We also showed that the expression of neuroprotective genes is detected both *in vitro,* following culture of the bone marrow cells for 72 hrs, and *in vivo* 24-32 weeks post bone marrow transplant through the detection of the reporter c-Myc; this importantly demonstrates the stable transduction of the BMDCs/HSCs using the novel scAAV7 vectors. The percentage of transduced cells is approximately 20-30% with the use of a large vector-to-cell ratio of 100,000 to 1, which is in agreement with previous reports. Low transduction efficiency of AAV vectors can be attributed to the failed intracellular trafficking from the cytoplasm into the nucleus and the subsequent degradation by the host cell proteasome machinery. Phosphorylation by the

epidermal growth factor receptor protein tyrosine kinase (EGFR-PTK) at the tyrosine residues of the vector capsid was found to specifically impair the nuclear transport of the vectors (Zhong et al., 2007) and generation of vectors containing point mutations within the tyrosine residue increased transduction efficiency at lower doses (Zhong et al., 2008). The expression of the neuroprotective genes can therefore be increased through the use of the mutated vector for future improvement on the transduction efficiency of the current system.

For evaluation of the potentially attenuating effects of the transgenes used in this study, ataxin-1^{+} nuclear inclusions were examined. It has been postulated that these nuclear aggregations found in many neurodegenerative disorders are derived from expanded polyglutamine repeats that confer toxic gain of function effects through alteration of the protein conformation which consequently disrupts normal association with the nuclear matrix or other proteins that accumulate in cell bodies (Skinner et al. 1997; Cummings et al., 1998). It has been shown that overexpression of mutant ataxin-1 *in vitro* forms large, single inclusions while overexpression of the wildtype ataxin-1 *in vitro* forms smaller, multiple inclusions, which was also observed in the Purkinje neurons fused with the genetically modified HSCs under the current *in vivo* model. While general observations on the behavior of the heterozygous Sca1 mice do not lead to obvious improvements in their physical condition or behavior for most of the recipient mice, a few of the mice receiving HSCs harboring the pcbp3 gene subjectively seemed more active and in better health than their control littermates (this of course could result from a variety of our experimental manipulation). Histological assessment of their cerebella, however, revealed twice as many fused Purkinje neurons, suggesting that the cell-cell fusion and/or this particular modifier gene may impart therapeutic effects. Even though each modifier gene was introduced *in vivo* one at a time in the current study, it might be possible that a synergistic effect

may be seen if the genes were co-expressed in combination or all at once. This will be an important point for future studies.

Aside from the successful proof-of-principle demonstration of a system potentially useful for the repair of at-risk Purkinje neurons through a novel delivery of new genome, another unexpected observation was the fusion seen with other cerebellar cell types not reported in previous studies (Alvarez-Dolado et al., 2003; Weimann et al., 2003b; Johansson et al., 2008; Magrassi et al., 2007). While a few of the molecular layer interneurons that we observed may be "random occurrences", GFP^+ unipolar astrocytes with radial fibers ensheathe Purkinje neuron somata and dendrites, resembling Bergmann glial cells, were consistently observed throughout the cerebellum in more than one recipient mouse. Bergmann glia have intimate associations with Purkinje neurons both structurally and functionally (Bellamy, 2006; Yamada et al., 2000), and have been implicated to play a role in neurodegeneration through impaired glutamate transport in the spinocerebellar ataxias (Custer et al., 2006; Vig et al., 2006). Previous studies have shown that damage within the cerebellum increased the number of fused cells (Bae et al., 2005; Magrassi et al., 2007) and it is possible that selective pressure within the cerebellum, and also potential impairment within the Bergmann glia, play a role in cell-cell fusion mechanism that is not yet completely understood. Another possibility is that Bergmann glia are in fact a putative type of cerebellar stem cell (Alcock et al., 2007; Sottile et al., 2006), and *in vitro* studies have demonstrated the propensity of different stem cell types to fuse in culture (Terada et al., 2002; Ying et al., 2002; Chen et al., 2006).

Another advantage of the current rescue paradigm is in the delivery method: bone marrow transplants have been in clinical use for a long time and are less invasive as compared to direct injection into the brain, which could cause damage to the parenchyma during the process. Even

though whole-body irradiation was used in the current experimental paradigm to allow reconstitution of the donor blood cells, previous findings in which parabiosis was used to create chimaerism between two mice showed that peripheral blood reconstitution was above 50% and that irradiation was unnecessary to induce fusion (Johansson et al., 2008). In addition, Magrassi and colleagues (2007) showed that mice receiving chemical treatments with Treosulfan and Fludarabine, and mice receiving irradiation, achieved similar levels of circulating fluorescent cells and thus showed that irradiation is not a prerequisite for fusion to occur.

Reports of aneuploid Purkinje neurons being found within aged rats and mice also have been around for a long time (Del Monte, 2006; Mann et al., 1978; Mares et al., 1973; Lapham, 1968) and it is therefore reasonable to propose that they are the predominant fusogenic-capable cell type within CNS capable of forming heterokaryon with BMDCs. Specific and focused cell fusion offers tremendous therapeutic potential in terms of rescuing highly specialized Purkinje neurons that are the sole neuronal output within the cerebellar cortex and they are the target of many neurodegenerative diseases. We show here that the use of genetically modified BMDCs/HSCs to rescue degenerated Purkinje neurons can provide a non-invasive delivery of therapeutic factors that could not be achieved using traditional viral gene therapy or cell replacement therapy alone. It also ensures that the cells receiving transgenes would be the population at-risk instead of global cell/tissue exposure to the CNS. Combining cell replacement regimes with viral vector directed gene therapy to achieve a novel therapeutic paradigm can further expand the scope in which adult stem/progenitor cells may be an efficacious alternative used to rescue at-risk cellular populations associated with neurological diseases.

Conclusions

Data presented in this final dissertation reveal the potential of adult stem/progenitor cells to be used in therapeutic treatment for movement disorders involving ataxia. Through the

application of different genetic, molecular, and cellular approaches, our understanding of the developmental scope of adult stem cells was considerably widened. More research is clearly needed to determine better ways of generating specific neuronal populations needed for particular CNS deficits, and also to better understand the interplay between donor populations and the host environment for achieving optimal integration. Regarding the use of adult stem cells as a delivery vehicle for neuroprotective agents, more work is needed on the specific mechanisms underlying cell fusion, so it can be better controlled and targeted to the particular cell population in need. The present study embarked on novel approaches for the potential treatment of ataxia, and further expands the scope in which adult stem/progenitor cells may be used to rescue at-risk populations within animal models of neurological disease.

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BIOGRAPHICAL SKETCH

Kwang-Lu Amy Chen was born in 1978 in Taipei, Taiwan. She graduated valedictorian from Warren County High School in Front Royal, Virginia in 1996. Following graduation, she attended Duke University in Durham, North Carolina and obtained a B.S. degree in biology with minors in chemistry and psychology in 2000. Amy then started working in a small biotech start-up company, Cogent Neuroscience Inc., as an assistant research scientist in Durham, North Carolina. For two years, her work included the development and optimization of the *in vitro* rat model of Huntington's Disease as a platform to screen for therapeutic small molecule compounds for future clinical trials. She also worked six months at a Howard Hughes Medical Institute laboratory under the direction of Dr. Bryan Cullen at the Duke Medical Center before entering the Interdisciplinary Program for Biomedical Sciences leading to the degree for doctor of philosophy at the University of Florida. During her graduate training, Amy has published first author papers for her contribution to the process of stem cell fusion *in vitro* and to elucidating the fate of neural stem cells following transplantation into mutant and transgenic mouse models. She has also presented work at several international conferences including the 9th International Conference on Neural Transplantation and Repair in 2005, International Society for Stem Cell Research in 2005 and 2007, Annual Conference for the Society for Neuroscience in 2005 and 2008, and the 59th Annual Symposium on Cancer Research: Stem Cells in Cancer and Regenerative Medicine in 2006.