Characterization and Therapeutic Potential of Human Amniotic Fluid Cells in Mediating Neuroprotection

Anna Jezierski

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Department of Cellular and Molecular Medicine
Faculty of Medicine
University of Ottawa

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Human amniotic fluid cells form functional gap junctions with cortical cells

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General Abstract

Brain injury, either surgically induced or as a result of trauma or stroke, is one of the leading causes of death and disability worldwide. Since transplantable stem cell sources are showing a great deal of promise and are actively being pursued to provide neuroprotection post-injury, in this body of work, we set out to characterize and examine the therapeutic potential of amniotic fluid derived (AF) cells as a potential cell source for cell-based therapies in mediating neuroprotection post-injury. Despite their heterogeneity, we found that AF cells are mainly epithelial in origin and express various genes involved in stem cell maintenance and neural commitment. A very small subset of AF cells also express pluripotency markers OCT4a, SOX2 and NANOG, which can be enriched for by single cell cloning. SOX2 positive clones have the capacity to give rise to a neuronal phenotype, in neural induction conditions, which can be used to examine the neural differentiation capabilities of AF cells. Subsequently, we examined the ability of AF cells to mediate a neuroprotective effect in a surgically induced brain injury model through gap junctional-mediated direct cell-cell communication and as a vehicle for GDNF delivery post-injury. AF cells express high levels of CX43 and are able to establish functional gap junctional intercellular communication (GJIC) with cortical astrocytes. We report an induction of Cx43 expression in astrocytes following injury and demonstrate, for the first time, CX43 expression at the interface between implanted AF cells and host astrocytes. In an effort to boost host endogenous neuroprotective mechanisms post-injury, via neurotrophic factor delivery, we engineered AF cells to secrete GDNF (AF-GDNF). GDNF pre-treatment significantly increased AF cell and cortical neuron survival rates following exposure to hydrogen peroxide. AF-GDNF cells, seeded on polyglycolic acid (PGA) scaffolds, survived longer in serum-free conditions and

continued to secrete GDNF post-implantation activating the MAPK/ERK signaling pathway in host neural cells in the peri-lesion area. Despite some promising trends, we did not observe significant behavioural improvements following AF-GDNF/PGA implantation nor reduced lesion volume during the 7 day time-frame. In conclusion, through GJIC with cortical astrocytes and delivery of exogenous neurotrophic factors, AF cells hold great promise in mediating neuroprotection post-injury.

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Contributions of Co-Authors

All technical work and preparations of the thesis and manuscripts were carried out by the thesis author, except as otherwise specified. I have been very fortunate to collaborate with the following people. Fluorescence activated cell sorting was carried out by the Stem Core Laboratories at the Ottawa Hospital Research Institute by Paul Oleynik. Amniotic fluid samples were provided by Dr. Andrée Gruslin and Bogdan Zurakowski performed all animal surgeries. Dr. Fred Gage (Salk Institute, La Jolle, CA, US) provided the SOX2 promoter construct, Dr. Bernard Massie (NRC, Montréal, QC, Canada) provided the pTet07CSII-CMV-DsRed lentivector and Dr. Kursad Turksen (Sprott Centre for Stem Cell Research, Ottawa Hospital Research Institute, Ottawa, ON, Canada) provided that HaCaT cell line. Dr. Abdellah Ajji provided the PGA scaffolds in Chapter 4. Dr. Kerry Rennie setup the beam-walk and cylinder tasks to measure behavioural improvements following injury and contributed to Figure 7-9 in Chapter 3 and Figure 7b and 11b in Chapter 4. Julie Haukenfrers provided technical assistance and contributed to Figure 2c-d, 3a, and 9c in Chapter 4. Roger Tremblay performed Western blots corresponding to Figures 4b-c and 8c-d in Chapter 2, Figure 1b in Chapter 3 and Figure 1d in Chapter 4. Maria Ribecco-Lutkiewicz provided assistance with viral vector construction, production and infection and contributed to Figure 9b-c in Chapter 2.

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List of Abbreviations

293SF: 293SF-PacLV
ACTB: β –Actin
AF-DsRed: AF cells expressing DsRed
AF-GDNF: AF cells secreting GDNF
AF: amniotic fluid
AF26: amniotic fluid cells at 26 weeks of gestation
AFS cells: amniotic fluid stem cells
ANOVA: analysis of variance
BDNF: brain derived neurotrophic factor
bp: base pair
C-KIT: CD117-the receptor for stem cell factor
cDNA: complementary deoxyribonucleic acid
CFDA: carboxyfluorescein diacetate
CM: conditioned media
CNS: central nervous system
CNTF: ciliary neurotrophic factor
CX: connexin
CX26: connexin 26
CX43: connexin 43
CXCL12: chemokine (C-X-C motif) ligand 12
DiI: octadecyl(C18)-indocarbocyanine
DLL: delta like ligand
DMEM: dulbecco's modified eagle medium
DNA: deoxyribonucleic acid
DOX: doxycycline
DsRed: discosoma red fluorescent protein
ECM: extracellular matrix
EGFP: enhanced green fluorescence protein
ELISA: enzyme-linked immunosorbent assay
ERK: extracellular signal-regulated kinase
ES cells: Embryonic stem cells
EVA: polyethylene-co-vinylacetate
FACS: fluorescence activated cell sorting
FBS: fetal bovine serum
FDA: food and drug administration
GDNF: glial cell derived neurotrophic factor
GFAP: glial fibrillary acidic protein
GFL: GDNF family of ligands
GFP: green fluorescence protein
GFR-α1: GDNF family receptor alpha
GJAI: gap junction alpha-1 protein
GJIC: gap junctional intercellular communication
GRO: growth related oncogene

H_2O_2: hydrogen peroxide
HLA-DR: human leukocyte antigen-D related
HES1: hairy and enhancer of split 1
hMito: human specific mitochondrial antibody
hNuc: human specific nuclear antigen
HRP: horseradish peroxidase
IBA1: ionized calcium-binding adapter molecule 1
ICC: immunocytochemistry
IL-1β: interleukin-1β
IL-8: interleukin 8
iPS cells: induced pluripotent stem cells
IRES: internal ribosome entry site
JAG: jagged
K8: keratin 8
Ki67: proliferation-related Ki-67 antigen
MAP2: microtubule-associated protein 2
MAPK: mitogen-activated protein kinase
MCAO: middle cerebral artery occlusion
MCP: monocyte chemotactic protein
L: ladder
miR: miRNAs
mRNA: messenger RNA
NES: nestin
NeuN: neuronal nuclei
NFL: neurofilament light chain
NGF: nerve growth factor
NICD: notch intracellular domain
NMDA: N-methyl-D-aspartate
NSC: neural stem cell
NSE: neuron specific enolase
NT-3: neurotrophin-3
NT2/D1: NTERA-2 clone D1
OCT4a: POU homeodomain transcription factor
PBS: phosphate-buffered saline
PCR: polymerase chain reaction
PD: Parkinson's Disease
PEG: polyethylene glycol
PGA: polyglycolic acid
PI: propidium iodide
PLA: polylactic acid
PLL: poly-L-lysine
PT: post-test
QPCR: quantitative polymerase chain reaction
RBP-J: recombinant signal binding protein immunoglobulin kappa J
RNA: ribonucleic acid

RT-PCR: Reverse transcription polymerase chain reaction
RT: reverse-transcription
SDF-1: stromal cell-derived factor 1
SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel
SEM: standard error of mean
SGZ: subgranular zones
SOX2: SRY (sex determining region Y)-box 2
SVZ: subventricular zone
TBI: traumatic brain injury
TBS: tris-buffered saline
TBST: tris-buffered saline with Tween20
TGF-β: transforming growth factor beta
TGF: transforming growth factor
VEGF: vascular endothelial growth factor
WB: western blot

Chapter 1 - General Introduction

1.1 Brain injury

Brain injury, mostly as a result of traumatic brain injury (TBI) or stroke, is one of the leading causes of death and disability in North America [1-4]. It is estimated that 1.3 million Canadians are living with acquired brain injury and struggling with long term cognitive, emotional, sensory and motor deficits [5]. Disability prevalence is similar in the United States where it is estimated that nearly 5.3 million Americans are living with the effects of TBI and another 1.1 million with the effects of stroke [6]. In addition to the incidents of TBI and stroke, close to 800,000 patients underwent neurosurgical procedures in the United States in 2008 [7]. This figure encompasses emergent, life-preserving surgeries, as well as planned procedures, which in some cases can result in brain damage and neurobehavioral functional deficits [7]. For instance, such damage can occur during the excision of brain tumors or the removal of epileptic foci. In addition, neurosurgical operations also have the potential to cause unavoidable healthy brain tissue damage as a result of application of pressure, tissue stretching, hemorrhage, and the use of electrocautery [7].

Brain injury, whether surgically induced or as a result of trauma or stroke, can cause irreversible loss of tissue and function by disrupting cortical integrity and functionality. Specifically, brain injury can result in cellular dysfunction, neuronal death and the loss of neural connections [8]. The extensive loss of cells following brain injury contributes substantially to functional impairments and despite considerable progress, pharmacological interventions do not yet effectively remedy sensory and motor deficits. Current treatment methods in clinical practice primarily aim to reduce intracranial pressure in an effort to minimize brain damage caused by swelling. However, these therapies have a modest effect

on acute brain damage or subsequent cell death pathways, and as such do not ameliorate functional impairments nor provide sustained efforts to promote repair or regeneration [9].

1.2 Mechanism of brain injury

The pathophysiological events following brain injury are associated with primary injury, induced via direct insult to the brain and secondary injury, initiated minutes after trauma and lasting for weeks, months or even years [10]. Injury to the brain tissue initially results in pan-necrosis (the loss of many cellular elements) within the injured core accompanied by the loss of selectively vulnerable cell types in the surrounding areas due to edema, ischemia, excitotoxicity, increase in free radicals and altered gene expression [11-13]. More specifically, the excessive release and/or inadequate uptake of glutamate, following primary injury, results in the overstimulation of glutamate receptors. In particular, activation of the N-methyl-D-aspartate (NMDA) receptor allows excessive entry of calcium into the cell, which activates several calcium-sensitive enzymes, including proteases and phospholipases, which directly damage cellular proteins and lipids and is the major mechanism for neuronal death post-injury [14]. Prolonged rise in extracellular calcium levels stimulates the formation of reactive oxygen species, which also damage DNA and impair the integrity of cell and organelle membranes by initiating lipid peroxidation. Collectively, calcium overload and production of free radicals, results in an increase in the permeability of the inner and outer mitochondrial membrane causing the release of cytochrome c into the cytoplasm. Cytochrome c initiates an apoptotic cell death program, culminating in the activation of the apoptosis factor Caspase3, which cleaves a number of key cellular proteins, leading to the death of neural cells surrounding the initial injury [15]. Among the surviving

neurons, many synaptic connections are lost after injury, compromising the integrity of neuronal circuits, which support sensory, motor or cognitive functions [16, 17]. As a result, repairing this type of damage is not a trivial feat, owing to the diversity of cell types and the complexity of the circuitry involved [18]. Nevertheless, due to its delayed nature, the cell death that accompanies secondary brain injury is a major target for therapeutic intervention.

Both primary and secondary injury initiates an inflammatory response, with infiltration of immune cells into the brain. In addition, injury to the brain activates residing astrocytes and microglia to release various pro-inflammatory cytokines [19, 20]. Included among these cytokines are Tumor Necrosis Factor-α (TNF-α) and Interleukin-1β (IL-1β), which mediate inflammation, recruit reactive astrocytes to the injury site and initiate a glial response (astrogliosis), which acutely acts to sequester and clean debris at the injury site [20]. Astrogliosis is fundamental for both limiting the areas of damage through the formation of a glial scar and for the post-insult remodeling and recovery of neural function [21]. Cellular components of the glial scar include reactive astrocytes, which help buffer excess glutamate and secrete neurotrophic factors, and activated microglia, which along with monocyte-derived macrophages, clear out dead tissue. Although the glial scar forms an effective barrier protecting the healthy portion of the brain from accumulated pro-inflammatory cytokines, oxidative compounds and cellular debris at the injury site, it also limits the capacity for axonal regeneration and migration of neuroblasts into the injury site [22, 23]. This evolution of the brain injury response exemplifies the dynamic interplay between events promoting repair and regeneration competing with mechanisms of damage and inflammation (Figure 1). Accordingly, stimulation of neuroprotective and regenerative

potentials within the injured brain will require one or more of the following processes: cellular replacement, delivery of neurotrophic factors, promotion of axonal guidance and modulation of the host immune response [24]. In an effort to repair the brain, at least two sources for neuronal replacement can be envisioned: stimulation of endogenous neurogenesis and implantation of cells with neuroregenerative and/or neuroprotective potential.

1.3 Stimulation of endogenous neurogenesis

To restore the integrity and function of the brain following injury, both formation of new neurons via neurogenesis and the restoration of lost neural connections are necessary. Following brain injury, a limited degree of neurogenesis has been observed [25-29]. This endogenous neurogenic response occurs in the subventricular and the subgranular zones (SVZ and SGZ; respectively) [30]. Although neurons are post-mitotic, the induction of neurogenesis resides in a subpopulation of proliferating neural stem and progenitor cells [31-35]. Following damage to the brain, the neuroblasts generated in the SVZ and SGZ migrate to the damaged region and differentiate to neurons or glia to re-establish functional connectivity with the rest of the brain. However, the level of neurogenesis and successful migration to the injury site is insufficient to effectively restore lost neural tissues and promote functional recovery [36]. This is largely attributed to the extensive loss of structurally supportive cerebral parenchyma, formation of a glial scar at the injury site and the cytotoxic environment that ensues post-injury [37]. Thus, while the adult brain is capable of supporting the integration of newly born neurons into pre-existing circuitry, endogenous

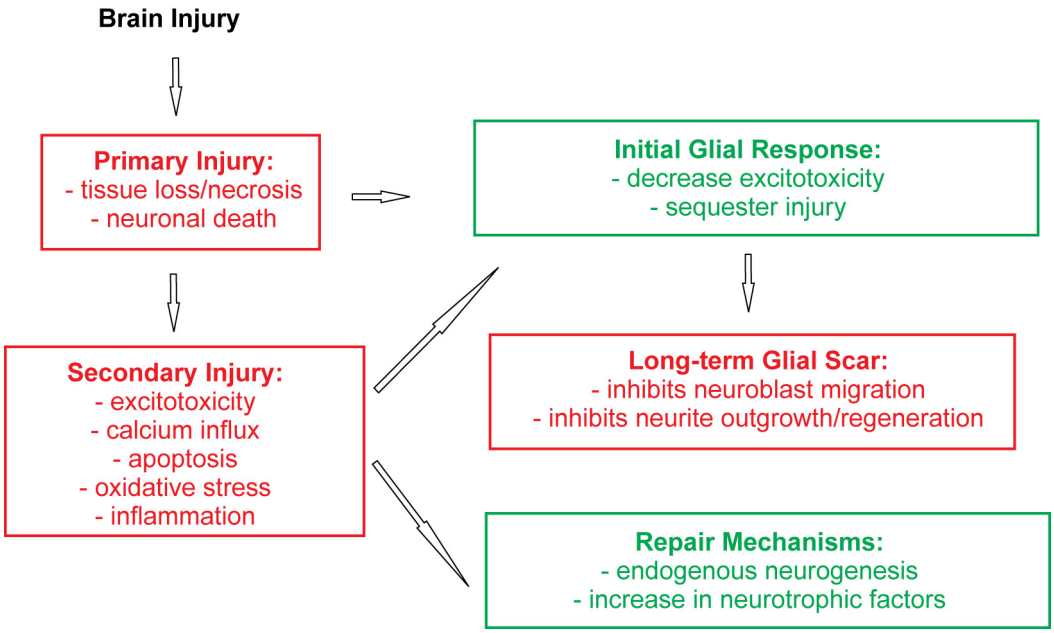

Figure 1. Schematic of events following brain injury
Mechanisms of damage and inhibition or regeneration (red boxes) occur alongside permissive and reparative mechanisms (green box). A better understanding of the multidimensional cascade of secondary brain injury can aid in the development of more effective clinical treatments for brain injury.

neurogenesis does not appear to occur on a scale that is sufficient to replace the substantial portion of cells lost due to injury [38].

1.4 Implantation of stem cells with neuroregenerative and/or neuroprotective properties

Due to the failure of the brain to fully recover through endogenous mechanisms, novel strategies that promote neuroprotection and neuroregeneration require further investigation. Recent advances in the stem cell field have boosted efforts to explore the therapeutic potentials of cell-based therapies to repair brain injury (Reviewed in [39-42]. The concept of cellular replacement following transplantation has been based on the assumption that neurological dysfunction, due to injury, may be improved through the introduction of new cells that can replace lost neurons (neuroregeneration) or deliver trophic support to surviving cells to enhance survival rates, plasticity and functional repair (neuroprotection) [43]. During the past decade, several stem and progenitor cell types and cell lines from various sources have been assessed for their potential to improve functional and behavioural outcomes after transplantation into clinically relevant models of brain injury, as a rationale for cell-based strategies to promote brain repair [43]. In brief, several groups have transplanted embryonic stem cells [44], human fetal neural progenitor cells [45-47] or bone marrow-derived stem cells [48, 49] into brain injured animals and demonstrated that the cells engrafted into the injured brain can survive and in part, aid behavioural dysfunction and promote repair. Although these studies highlight the potential therapeutic role of stem cells in brain injury, the detailed mechanism by which the implanted cells mediate their effects remains elusive. However, it many cases the transplanted cells did not differentiate

into neurons nor functionally integrate into the neuronal network. Thus, it has been proposed that the mechanism underlying the functional improvements observed in these animal studies was mediated, in part, due to the release of protective neurotrophic factors from implanted cells [50, 51] or through their modification of the local microenvironment to enhance endogenous neuroprotective and neuroregenerative mechanism; thus preserving existing cells and neuronal circuitry from further damage [43].

In cell replacement therapies, the ability of implanted cells to be able to terminally differentiate into the correct neuronal subtype, integrate and recapitulate the complex neurocircuitry, in addition to being resistant to the hostile environment that causes death to endogenous neurons, is a considerable challenge. As a result, the goal for specific neuronal cell replacement and functional integration is complex and Phase I clinical trials with stem cells testing this approach are under investigation [52]. In light of these findings, neuroprotection, through production and/or delivery of neurotrophic factors to the injured tissue, is emerging as a more plausible approach to cell-based therapies and may potentially provide a clinical benefit equally meaningful to cell replacement.

1.5 Neurotrophic Factors

Recent advances in the understanding of molecular pathways and their physiological roles have demonstrated that neurotrophic factors play an important role in the development, maintenance and regeneration of the nervous system [53]. Neurotrophic factors have also been shown to be potent neuroprotective agents and their discovery and characterization

have been instrumental in understanding the mechanisms in the development, plasticity and repair of the nervous system [54]. For instance, they serve as neuroprotectant molecules against cytotoxic cell damage by upregulating calcium buffering proteins, antioxidant enzymes and anti-apoptotic factors [55]. In response to injury, many trophic factors and their receptors have been shown to increase in concentration, suggesting an endogenous neuroprotective response [56]. Accordingly, many studies have attempted to increase neuroregeneration and repair at the injury site with exogenously supplied neurotrophic factors such as nerve growth factor (NGF), brain derived neurotrophic factor (BDNF) and glial cell derived neurotrophic factor (GDNF) [30]. Based on the success of these studies, neurotrophic factors have become attractive candidates as therapeutic agents in many clinical conditions such as brain injury and chronic neurodegenerative diseases. To date, strategies using neurotrophic factors focus on preventing the progressive loss of neurons, maintaining neuronal connections and function and inducing additional regenerative responses in neurons such as axonal sprouting.

Among the different neurotrophic factors, GDNF has received considerable attention as a potent neurotrophic factor [57]. The GDNF family of ligands (GFL) is distantly related to the transforming growth factor (TGF) superfamily and includes four structurally related trophic factors: GDNF, neurturin, artemin and persephin [58]. GDNF binds to a multi-subunit receptor system comprised of GDNF receptor alpha (GFR-α1) and Ret subunits, which function as ligand binding and signaling components; respectively, and trigger many intracellular signaling pathways (Figure 2). For instance, the Mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) signaling pathways has been

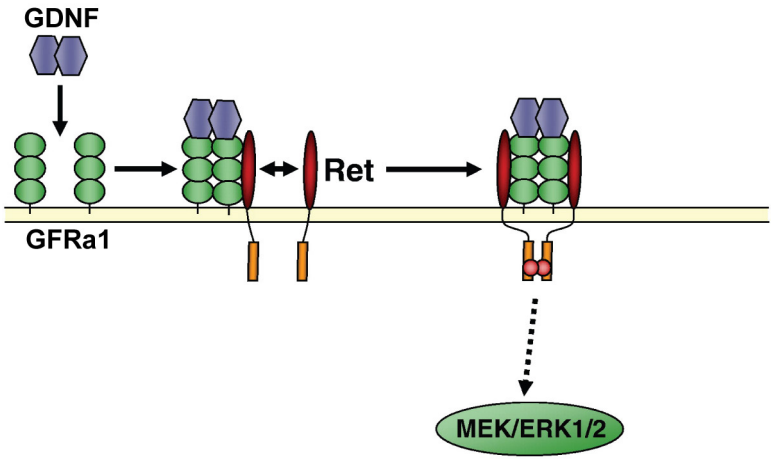

Figure 2. GFRα1 and Ret-mediated GDNF signaling pathways
Binding of GDNF to GPI-anchored GFRα1 increases the affinity of GFRα1 for Ret, inducing its recruitment and dimerization. Ret is activated by autophosphorylation of tyrosine residues shown in red, leading to the activation of (in green) the MAPK/ERK1/2 signaling pathway (adapted from [59]).

shown to be involved in GDNF-induced neuronal survival, differentiation and neurite outgrowth [60]. GDNF has also been shown to promote differentiation and migration of cortical GABAergic neurons [61], promote progenitor proliferation in the adult hippocampus and substantia nigra [62] as well as promoting regeneration of injury-primed sensory neurons in the lesioned spinal cord [63]. Notably, the neuroprotective effects of GDNF have been shown in different experimental models of brain injury, by local administration using viral vectors encoding the GDNF gene and by transplantation of GDNF-expressing cells (reviewed in [64]). In these experiments, GDNF has been shown to be neuroprotective resulting in reduced neuronal death and infarct volumes as well as enhanced functional and behavioual improvements.

In particular, the role of GDNF in Parkinson's Disease (PD) has been extensively studied and has consistently demonstrated both neuroprotective and neuroregenerative effects [65]. In brief, GDNF delivery to the striatum or the substantia nigra protects dopaminergic neurons against subsequent toxin-induced injury and rescues previously damaged neurons, promoting recovery of motor function (Reviewed in [65]). In fact, based on the successful results of animal experiments, a clinical trial using GDNF began for human PD patients. Although GDNF delivery also showed to have some trophic effects and restore motor function in Phase I trials [66-68], Amgen, the company holding the patent on GDNF therapy for PD, brought the clinical trial to an end due to adverse effects and lack of therapeutic efficacy in subsequent Phase II trials [69]. A contributing limitation to the success of the clinical trials was effective delivery of GDNF to the central nervous system (CNS) due to its inability to cross the blood-brain barrier [70, 71]. In fact, delivering neurotrophic factors to

the brain has proven to be a non-trivial matter and in the case of GDNF, direct intracerebroventricular and intraputamenal infusion of the protein resulted in poor distribution and bioavailability in PD patients. Alternatives to overcome this limitation, ranging from fusion with viral proteins to conjugation with antibodies, appear to provide a more efficient way of delivering GDNF to the CNS. In fact, picking up where Amgen left off, Amsterdam Molecular Therapeutics obtained a license from Amgen to use their GDNF gene in conjunction with their adeno-associated virus gene therapy technology to potentially improve delivery and distribution [72]; however, inadequate transgene expression at the injection site hindered the success of this approach. Currently, Medgenesis has resumed clinical trials for GDNF using convection-enhanced delivery [73], a minimally invasive technique using microcathethers to achieve more precise and homogenous GDNF distribution throughout the putamen in PD patients, while minimizing point-source accumulation toxicity [74].

Due to the challenges surrounding GDNF delivery to the brain, transplantation of neurotrophic factor-expressing cells is an emerging strategy to provide continuous delivery of a specific neurotrophic factor to boost the endogenous repair mechanisms. For instance, Emborg *et al.* [75] found that human neural progenitor cells, engineered to secrete GDNF, were able to survive transplantation, produced GDNF for three months and demonstrated increased functional recovery in test animals. Although transplantable stem cell sources are showing a great deal of promise and are aggressively being pursued to provide neuroprotection and potentially restore neurological function after brain injury [42, 76], the first challenge to their use clinically is to define the most appropriate stem cell source.

1.6 Stem cell sources for cell-based therapy in the injured brain

Stem cells, depending on their origin, are divided into two main groups: embryonic and adult stem cells. Embryonic stem (ES) cells are pluripotent cells derived from the inner cell mass of a blastocyst [77, 78]. Due to their plasticity and unlimited capacity for self-renewal, ES cells have been pursued as an invaluable cell source for cell-based therapies. However, ES cell utility in the clinic is hindered by safety concerns regarding their tendency to form tumors when injected in their undifferentiated or partially differentiated state *in vivo* [79] as well as the ethical considerations concerning their derivation from human embryos [80, 81]. On the other hand, adult stem cell sources reside in their respective niches in adult tissues in which they function to maintain their multipotency [82]. Accordingly, adult stem cells are constantly renewing and in case of damage, repopulating the tissues in which they reside [83]. Although their differentiation potential and proliferative capacities are limited compared to ES cells, the derivation of adult stem cells can be performed in an autologous setting and does not involve the destruction of human embryos [84]; however, their reduced proliferative potential renders the production of sufficient numbers of cells for use in cell-based therapies difficult.

In an effort to overcome the limitations associated with ES and adult stem cells, novel human stem cell sources are being actively pursued for use in cell-based therapies. In recent years, induced pluripotent stem cells (iPS) have been generated by reprogramming human somatic cells through viral transduction with defined transcription factors (Oct4, Sox2, Klf-4 and c-Myc) and accordingly represent a patient-specific pluripotent stem cell source [85]. iPS cells are very similar to ES cells; they exhibit similar morphology and growth

properties, express ES cell markers and have been shown to differentiate into the derivatives of all three embryonic germ layers. However, despite major advances in iPS technology, the reprogrammed cells are vulnerable to genomic instability [86, 87] and often retain the epigenetic memory of the donor tissue, which skews their differentiation potential [88]. In addition to the limitations of ES cell, these factors limit the safety and efficacy of iPS cells for therapeutic applications [89].

1.7 Amniotic fluid cells as a source for cell-based therapy in the injured brain

Due to the drawbacks associated with ES, adult and iPS stem cells, effort towards finding an alternative source of human cells for cell-based therapies has shifted towards examining the potential of cells derived from fetal tissues. Fetal cells, although more lineage committed than ES cells, show higher proliferation and differentiation capacities in comparison to adult progenitors and do not form teratomas *in vivo*. In fact, different multipotent progenitors have been isolated from fetal tissue; namely hematopoietic, mesenchymal, neural and lung progenitors [90-94]. However, the collection of fetal tissue during gestation is associated with high risk for the fetus and mother [95], limiting its therapeutic application; thus, attempts are currently underway to obtain stem cells from extra-embryonic sources, such as amniotic fluid (AF), umbilical cord and the placenta. Since amniotic fluid (AF) is commonly collected for routine diagnostic testing and is a widely accessible source of fetal cells, prompted an interest in examining the possibility that AF might contain multipotent fetal-derived cells [96]. The AF is a protective and nourishing liquid that surrounds the fetus during development and is composed mainly of electrolytes, proteins, carbohydrates, amino acids, lipids, enzymes and hormones [97-99]. In addition, fluid secretions from the fetus into

the AF contribute a variety of fetal cells, resulting in a heterogeneous population of cells derived from fetal skin, gastrointestinal, respiratory and urinary tracts, as well as the amniotic membrane [100]. AF cells display a broad range of morphologies and growth characteristics, varying with gestational age and fetal development [100]. Viable, adherent cells from AF are highly proliferative and are classified into three main groups: epithelioid-like cells, amniotic fluid specific cells and fibroblast-like cells [100, 101]. Within this heterogeneous cellular pool, a subpopulation of AF-derived cells was shown to express OCT4 [102], a nuclear transcription factor that plays a role in maintaining ES cells differentiation potential and capacity for self-renewal [103], suggesting that AF may contain a subset of multipotent stem cells. This multipotentiality of AF cells was confirmed through the isolation of AF-derived mesenchymal stem cells, which were able to differentiate towards adipogenic, osteogenic and chondrogenic lineages under specific culture conditions [104, 105]. More recently, there is evidence that AF also contains stem cell sub-population(s), termed human amniotic fluid stem (AFS) cells, that are broadly multipotent (Figure 3) [106]. Given their heterogeneity, these AFS cells are isolated based on c-Kit (CD117-the receptor for stem cell factor [107]) expression, clonally expanded and have been shown to harbour the potential to differentiate towards the three embryonic germ layers and do not form teratomas *in vivo* [106]. Since the identification of AFS cells, several other studies have confirmed the multilineage differentiation potential of clonally-derived AF cells [105, 108, 109]. However, it's important to note that the assessment of differentiation potential of these AF cells has largely relied on marker expression and further investigation is required to demonstrate that AF cell-derived differentiation progeny is capable of acquiring functional characteristics of the desired cell type. Nevertheless, an important

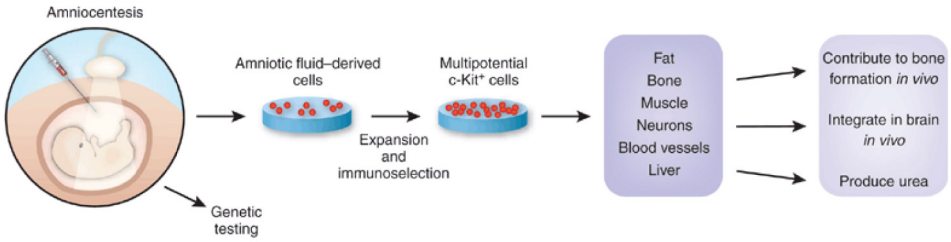

Figure 3. Multipotent potential of AF cells
Multipotent AF–derived cells are isolated by collecting a heterogenous population of cells through amniocentesis and enriching for cells that express C-KIT by Fluoresence Activated Cell Sorting. Under appropriate inducing conditions, the C-KIT^{+} cells can be differentiated into derivatives of all three primary germ layers (adapted from [110]).

feature of AF cells is that they can be reliably and consistently isolated from the AF as well as easily purified and expanded in order to produce appropriate cell number for clinical use. They also have the added benefit of being cryopreservable in a cell bank that will facilitate elective clinical administration to the patient in the future.

1.8 Application of AF cells for neuroprotection and brain repair

There are two general ways by which transplantation of AF cells might contribute to brain repair following injury: cellular replacement and neuroprotection via trophic factor delivery and direct cell-to-cell communication with host cells via gap junctions (discussed in Section 1.9). For AF cells to serve as direct cell replacements for lost or damaged cells following injury, requires that the cells terminally differentiate and acquire functional properties of the desired cell types and integrate into the existing neurocircuitry. Although AF cells have been reported to acquire neuronal morphology and express neuronal markers under neural induction conditions [106, 111-115], there is speculation over whether or not these cells are indeed capable of differentiating into functional neurons [116]. To date, there is little evidence to support that AF-derived cells can be used to directly replace lost or damaged neurons in the injured brain. Nevertheless, the few studies in which AF cells have been transplanted into the brain or peripheral nervous system, after various types of injury, have met with some encouraging results [117-121]. For instance, AF cells have been shown to improve memory and sensory/motor functions following cerebral ischemia induced by middle cerebral artery occlusion (MCAO) in mice as soon as 4 days after cell injection [120]. In a rat model of PD, implantation of AF cells into the rat striatum prevented the degeneration of nigrostriatal dopaminergic neurons, when administered prior to the

neurotoxin 6-OHDA [117]. Subsequent studies showed that injection of AF cells into the lateral ventricle had a similar effect despite the finding that the majority of the implanted cells did not exhibit a dopaminergic phenotype [121, 122]. Since clear evidence was lacking in these studies to suggest that AF cells had differentiated into mature neurons, capable of integrating into the host circuitry to restore function, it remains unclear whether replacement of lost cells is the underlying mechanism for the improvements observed post-transplantation in the injured animals. In fact, as previously described, the field of regenerative medicine has shifted focus to the ability of transplanted cells to provide neuroprotection and stimulate endogenous repair mechanisms of damaged nervous tissue through the production/delivery of trophic factors [123-128]. Such paracrine mechanisms have also been postulated to explain some of the positive effects of other stem cell types in animal models of organ/tissue injury [126, 129, 130]. As such, AF cells may provide support for surviving host cells through the release of trophic factors, offer protection from the toxic environment surrounding the injury and/or stimulate endogenous repair mechanisms [123, 126, 131]. To date, the mechanisms by which damaged neural tissue may benefit from AF cell transplantation have yet to be defined; however, AF cells have been shown to express and secrete a number of factors that could potentially support neuroprotective and/or reparative functions [73, 132]. Studies in which conditioned media (CM), rather than AF cells, have been used in injured tissues supports the notion that secreted factors mediate, at least in part, the beneficial effects of the transplanted cells. For instance, AF-CM was shown to re-instate blood flow in a hindlimb ischemia model likely owing to the presence of pro-angiogenic factors and cytokines: vascular endothelial growth factor (VEGF), stromal cell-derived factor 1 (SDF-1), chemokine (C-X-C motif) ligand 12 (CXCL12), TGF-β and

(interleukin 8) IL-8, present within the CM [73, 133]. Enhanced angiogenesis is also beneficial in animal models of stroke [134], traumatic brain injury [135] and nerve injury [136]. AF-CM was also shown to stimulate other endogenous repair mechanisms, such as proliferation of dermal fibroblasts near the injury site in an excision wound model and recruitment of endothelial progenitors to ischemic skin flap [73, 132]. In addition, RT-PCR analysis of AF cells has shown that they express a number of neurotrophic factors, such as BDNF, NGF, ciliary neurotrophic factor (CNTF) and neurotrophin-3 (NT-3) [118]. Since neurotrophic factors have been shown to be neuroprotective in brain injury models, it is likely that some of the beneficial effects of AF cells may be explained, in part, through the secretion of trophic factors. Other paracrine mechanisms, such as immunomodulation through the secretion of immune-modulating cytokines (such as IL-6, growth related oncogene (GRO) and monocyte chemotactic protein (MCP)) [137, 138] and creation of a supportive milieu [139] for regeneration might also contribute to the ability of AF-derived cells to limit damage and/or stimulate repair of injured tissues. In addition to their inherent ability to mediate protective and reparative functions, AF cells can also be engineered to deliver specific neurotrophic factors to enhance the protection and repair of damaged tissue during the acute and chronic phase following injury. For instance, AF cells engineered to secrete GDNF have been shown to ameliorate motor deficits in rats subjected to sciatic nerve injury [140]. Similarly, delivery of AF cells engineered to secrete GDNF and BDNF to the ischemic rat brain was shown to enhance recovery and reduce infarct size [141, 142].

1.9 Intercellular communication between grafted cells and the host

Although it has been suggested that AF cells exert beneficial effects in brain injury [120], there is speculation over whether or not these cells are capable of integrating into the brain and developing functional connectivity with recipient cells to support regenerative and neuroprotective capabilities. The success of this strategy depends, in part, on the degree of intercellular communication between grafted cells and the host neural circuitry and the re-establishment of functional networks in the CNS has been one of the underlying goals of stem-cell mediated therapies [143]. In fact, an essential step in the functional integration of grafted cells into the host neural circuitry, even before mature electrochemical synaptic communication, is cell-cell coupling via gap junctions that modulates neuronal network activity [143]. This integration is partially dependent on the formation of gap junctional intercellular communication (GJIC), which is considered to be an indispensable mechanism for the propagation of information among cells in the CNS. This essential pathway is composed of two juxtaposed, membrane bound connexon hemichannels, each composed of six connexin (Cx) subunits which are joined to bridge the cytoplasm of two neighbouring cells (Figure 4) [144]. Several studies provide evidence that connexin levels in the brain increase after brain injury [145-147] and that the function of hemichannels is of key importance [148]. This consolidation allows the transfer of small ions, nutrients, metabolites, second messengers and more recently miRNAs [144, 149] and may mediate both the spread of cytotoxic signals between cells [150] and allow intercellular passage of neuroprotective factors [151]. For instance, the extent of GJIC among astrocytes and their role in spatial buffering of ions (potassium and calcium), long-range signaling and exchange of small permeating molecules (glutamate, ATP and glucose) within the astroglial

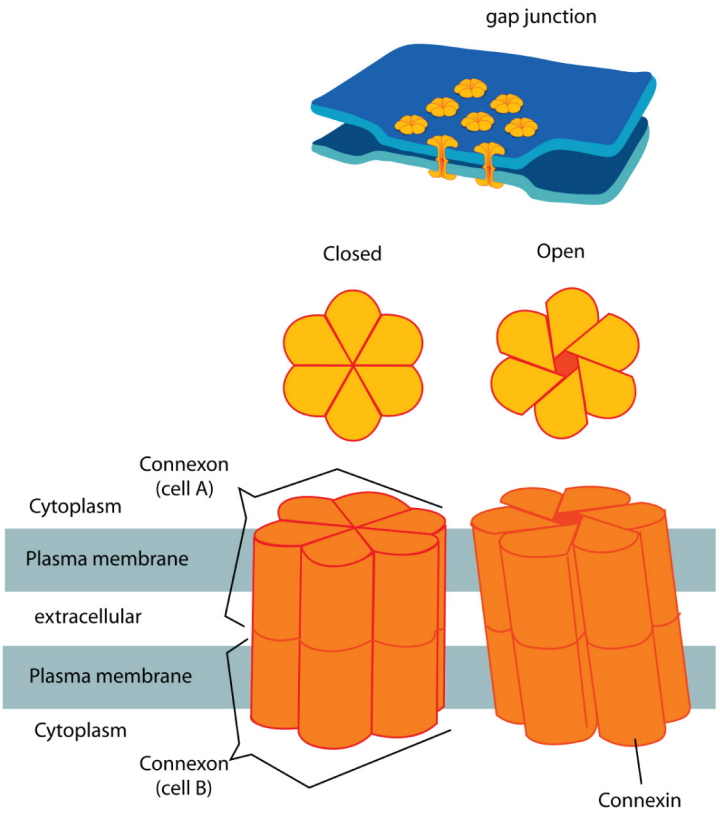

Figure 4. Connexin-mediated gap junctional intercellular communication
Gap junctions are composed of two juxtaposed, membrane bound connexon hemichannels, each composed of six connexin subunits, which are joined to bridge the cytoplasm of two neighbouring cells. This consolidation allows for the transfer of small ions, nutrients, metabolites and secondary messengers (adapted from [152]).

syncytium has been proposed to mediate a neuroprotective effect [151]. GJIC between graft and host cells underlies many of the early cellular interactions and has been shown to play a central role in the rescue of damaged host cells after brain injury [143]. More specifically, gap junction formation and function have been shown to be pivotal for ensuring host cell well-being by inhibiting neuronal death and suppressing gliosis [143]. In fact, suppression of GJIC was accordingly associated with a significant decrease in the host rescue effects attributed to neural stem cell (NSC) engraftment [143]. As such, in addition to diffusible factors secreted by NSC [153], direct intercellular communication between exogenous NSCs and host cells has been proposed as an additional mechanism by which stem cells exert a homeostatic and/or protective effect on damaged host cells [143]. Therefore, a better understanding of the interactive processes by which grafted cells integrate into host neural tissue may provide insights into the interplay between donor and recipient cells that can be exploited to enhance cell-based therapies in the future.

1.10 Biocompatible polymer scaffolds for neuroimplantation

The objective of brain tissue engineering is to repair, replace and regenerate tissue at the damaged site in order to re-establish functionality at both the cellular and organ level. Despite the promise of cell-based therapies for brain injury, many of the transplantation studies failed to correlate cell grafting and motor sensory improvement likely owing to poor cell survival following transplantation. Even upon the successful delivery and survival of injected cells, due to the loss of vast amount of cerebral parenchyma and an inhospitable injured core, the newly delivered cell implants were unable to integrate into the neural circuitry and maintain the support required for growth and vascularization resulting in

massive cell death [154]. Accordingly, even the most capable cells require a template to guide restructuring and biomaterial scaffolds are likely to reinforce the success of cell-based therapies by providing a microenvironment that facilitates the survival, proliferation, differentiation and connectivity of transplanted and/or endogenous cells [154]. Hence, to promote repair in the injured brain a combination of the right neurotrophic factors, cell source and template is required for successful neuroregeneration.

Currently, an array of biomaterials are being explored as safe and effective cellular supports or scaffolds for neuroreparative therapies [155]. Damage to the brain produces immediate and delayed cell death leading to the formation of a lesion cavity and glial scar. Placing a biomaterial scaffold into the damaged cavity may provide structural support to the surrounding brain tissue to minimize secondary cellular degeneration and allow cells from the adjacent parenchyma to infiltrate and potentially promote local tissue regeneration [156]. As such, scaffolds can function as a substrate to guide endogenous cellular organization and infiltration, axon regeneration and neurite growth and then degrade, alleviating concerns over long-term biocompatibility [157-160]. In fact, an ideal scaffold would recapitulate many of the features of the native extracellular matrix (ECM) in target tissue. Scaffolds can also serve as delivery platforms for transplanted cells and be engineered to enhance cellular attachment, growth and differentiation and potentially facilitate connectivity between graft and host cells. Implantable scaffolds have been used to treat a variety of issues associated with brain injury and disease, including replacing tissue lost, delivering drugs to help treat neurological diseases as well as serving as coatings for brain-implanted devices to limit inflammation [161]. However, each of these applications poses challenges unique to that

purpose. For example, scaffolds designed as treatments for brain injury should be tailored to fit the cavity size, allow for adequate cell infiltration with eventual degradation while minimizing cell death and inflammation by employing biocompatible materials to prevent further glial scarring [162]. In order to support cell-based therapies, the scaffold should also be highly porous with an interconnected architecture, a controlled size, shape and alignment of pores to facilitate oxygen, nutrient and waste transfer as well as rapid vascularization to enhance cell graft survival, integration and function.

Biocompatible, biodegradable polymers, from both synthetic and naturally derived sources, have been widely used as scaffold materials [155]. Electrospun synthetic polymers can be prepared with a high degree of control over their structure creating highly porous meshes of ultrafine fibres that resemble the ECM topography. The ability to mimic the ECM structural organization is an important consideration in the rational design of a cell-responsive scaffold platform upon which additional functionalities can be incorporated such as protein coatings or chemical conjugation of specific signaling molecules [163]. Accordingly, synthetic polymers have many advantages for use as scaffolds. They can be tailored to produce a wide range of mechanical properties and degradation rates and their known composition can be designed to minimize the immune response. Commonly used synthetic polymers include: Polyethylene glycol (PEG), Polyethylene-co-vinylacetate (EVA) and Polyglycolic acid (PGA) and Polylactic acid (PLA), among others. PGA, a synthetic biodegradable polymer [158], is an attractive scaffold for cell implantation into the brain. In addition to being approved by Health Canada and the Food and Drug Administration (FDA), PGA is easily shaped (i.e. tailored to fit into the cavity created by injury) and demonstrates optimal

mechanical strength, biocompatibility and biodegradability [164]. PGA polymers degrade at different rates over time via hydrolysis and enzymatic degradation from host cells. Hydrolysis of PGA hydrolyzes higher molecular weight polymers into monomers of glycolate, which is a normal metabolic product of saccharide metabolism in human cells [162, 165]. Furthermore, PGA has superiority over naturally derived polymers, such as agarose, nitrocellulose, collagen, fibrin and fibronectin [166] which are derived from biological (plant or animal) tissues, lack homogeneity and mechanical strength and can stimulate a severe immune response [158]. Growing evidence suggests that PGA-based scaffolds are safe and effective for neuroregenerative therapies in the brain [154, 162, 165]. In fact, PGA scaffolds have been shown to mitigate secondary injury caused by inflammation and also impede astroglial scar and cavity formation [154]. Furthermore, the injured brain has been shown to interact reciprocally with NSCs supported by a PGA scaffold to reconstitute lost tissue following stroke [154]. Preliminary results from our laboratory also demonstrate that PGA polymer scaffolds permit optimal adhesion, survival and differentiation of mouse neural cells in addition to providing a platform for the delivery of cytokines, growth factors and neurotrophic factors [167].

1.11 Rationale and Hypothesis

AF cells have attracted a great deal of attention as an alternative cell source to ES and adult stem cells for regenerative medicine. Since AF contains a mixture of cells shed from the developing fetus, incorporation of AF cells into cell-based therapies is hindered by their heterogeneity. Standards for cell-based therapies require defined cell populations that satisfy criteria for safety and efficacy. Thus a better understanding of the complexity of cells within

the AF and variation among gestation samples from different donors is imperative. We hypothesized that AF cell composition differs with gestational age, which potentially affects their utility for certain applications in cell-based therapies. Accordingly, we set out to examine the molecular components involved in self-renewal, neural commitment and differentiation of AF cells obtained at different gestational ages from different donors and to generate single cell clones for subsequent studies.

The usage of stem cells is a promising strategy for mediating neuroprotective effects in the injured brain. However, the success of this approach relies, in part, on proper intercellular communication between graft and host cells, which plays a central role in the rescue of damaged cells after brain injury. GJIC between grafted NSCs and brain cells appears to be an essential participant in the neuroprotective effect associated with NSC engraftment, particularly at the gap junction interface. Accordingly, we hypothesized that the formation of GJIC between AF and host cells would result in cell-cell based communication and potentially aid in mediating a neuroprotective effect post-implantation. These findings will help shed light as to whether AF cells are capable of integrating into the brain and developing functional connectivity with the host tissue, which can be explored in future studies to examine the neuroprotective capabilities of AF cells.

In this body of work, we have used a surgically induced brain injury model to mimic the incidental damage inflicted on the brain during neurosurgical procedures. In this model, as in those of TBI and stroke, brain injury results in neuronal loss and disruption of the brain parenchyma. Therefore, successful cell-based therapies in the injured brain will rely on several mechanisms among which neuroprotection and expression of neurotrophic factors

are key components to enhance endogenous protective mechanisms of surviving cells. The neuroprotective role of GDNF has been well-characterized following brain injury and exogenous delivery to the injured brain has been shown to promote endogenous repair mechanisms and enhance the success of cell-based therapies [168]. Since GDNF delivery to the brain is hindered by its inability to penetrate the blood-brain barrier, we hypothesized that seeding AF cells, engineered to secreted GDNF (AF-GDNF), on PGA scaffolds would provide an effective, direct method to delivery GDNF locally to the injury site, boost host neuroprotective mechanisms and ameliorate behavioural deficits. Given the vast loss of cerebral parenchyma following injury, we rationalized that seeding AF-GDNF cells on PGA scaffolds would enhance cell attachment and overall graft survival post-implantation through trophic factor support. These results will provide useful information for the future application of AF cells in cell-based therapies for brain injury.

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Chapter 2 - Probing stemness and neural commitment in human amniotic fluid cells

Probing stemness and neural commitment in human amniotic fluid cells

Anna Jezierski[1,2], Andrée Gruslin[2,3] , Roger Tremblay[1], Dao Ly[1] , Cathie Smith[1], Kursad Turksen[2,4], Marianna Sikorska[1], and Mahmud Bani-Yaghoub[1,2]

[1] Neurogenesis and Brain Repair, Neurobiology Program, Institute for Biological Sciences, National Research Council Canada, Ottawa, Canada.

[2] Department of Cellular and Molecular Medicine, Faculty of Medicine, University of Ottawa, Ottawa, Canada

[3] Department of Obstetrics and Gynecology, Faculty of Medicine, University of Ottawa, Ottawa, Canada

[4] Sprott Centre for Stem Cell Research, Ottawa Hospital Research Institute, Ottawa, Canada

Abstract

Recently, human amniotic fluid (AF) cells have attracted a great deal of attention as an alternative cell source for transplantation and tissue engineering. AF contains a variety of cell types derived from fetal tissues, of which a small percentage is believed to represent stem cell sub-population(s). In contrast to human embryonic stem (ES) cells, AF cells are not subject to extensive legal or ethical considerations; nor are they limited by the lineage commitment characteristics of adult stem cells. However, to become therapeutically valuable, better protocols for the isolation of AF stem cell sub-populations need to be developed. This study was designed to examine the molecular components involved in self-renewal, neural commitment and differentiation of AF cells obtained at different gestational ages. Our results showed that, although morphologically heterogeneous, AF cells derived from early gestational periods ubiquitously expressed *KERATIN 8* (K8), suggesting that the majority of these cells may have an epithelial origin. In addition, AF cells expressed various components of NOTCH signaling (ligands, receptors and target genes), a pathway involved in stem cell maintenance, determination and differentiation. A sub-population of K8 positive cells (< 10%) co-expressed NESTIN, a marker detected in the neuroepithelium, neural stem cells and neural progenitors. Throughout the gestational periods, a much smaller AF cell sub-population (< 1%) expressed pluripotency markers, *OCT4a*, *NANOG* and *SOX2,* from which SOX2 positive AF cells could be isolated through single cell cloning. The SOX2 expressing AF clones had the capacity to give rise to a neuronal phenotype in culture, expressing neuronal markers such as MAP2, NFL and NSE. Taken together, our findings demonstrated the presence of fetal cells with stem cell characteristics in the amniotic fluid, highlighting the need for further research on their biology and clinical applications.

Introduction

Stem cells can be derived from a variety of tissues during embryonic and fetal development as well as in adulthood; and hence may potentially be exploited for both tissue engineering and cell transplantation [1]. Pluripotent stem cells, derived from human embryonic stem (ES) cells, have the ability to give rise to all three embryonic germ layers, while maintaining their ability to proliferate indefinitely in culture. However, their application for therapy remains closely associated with ethical concerns related to both their origin and teratogenic potential [1-5]. Similarly, adult stem cells can be obtained from a number of tissues, but they generally display low proliferation rates and lineage restrictions [6-9]. Consequently, ongoing efforts continue to search for more practical sources of stem cells in humans.

Recent studies have shown that human amniotic fluid (AF) contains a small sub-population of stem cells, enriched based on C-KIT expression, with self-renewal and differentiation potential [10]. However, despite the wide and well-established use of AF in routine prenatal diagnosis, the properties and origin of AF derived stem cells is poorly understood. It is believed that AF cells originate from several fetal tissues, including skin and the lining of the digestive, respiratory and urinary systems [11-13]. In addition, the nature and characteristics of AF cells may vary with gestational age. Thus, we have used several samples from the second and third trimesters of gestation to provide a comprehensive molecular characterization of AF cells, with regard to the genes involved in stem cell maintenance, neural fate commitment and differentiation, with an underlying focus on *KERATIN* expression and NOTCH signaling.

The evolutionarily conserved NOTCH signaling pathway plays diverse roles in cell-fate specification during embryogenesis and in adult tissues [14]. NOTCH comprises a family of highly conserved receptors, whose activation is induced by their specific ligands, DELTA-LIKE (DLL) and JAGGED (JAG), through cell–cell interactions [14]. Once activated, the NOTCH intracellular domain (NICD) is cleaved by γ-secretase, which leads to translocation of the NICD into the nucleus. Subsequently, NICD is associated with the transcription factor recombination signal binding protein for immunoglobulin kappa J region (RBP-J) to generate a transactivation complex, which initiates transcription of target genes such as the *hairy/enhancer of split* (*HES*) transcriptional repressors. Of particular interest is the fact that NOTCH signaling regulates stem cells in many different settings, including the skin and nervous system, among others (reviewed in [15]). During fetal skin development, the epidermis changes from a simple epithelium covered by periderm to stratified epithelium and finally to keratinized epidermis [16, 17], suggesting that epithelial cells likely contribute significantly to the cellular composition of the AF. During development, different compartments of the epidermis containing stem cell sub-populations are established that are maintained into adulthood [18]. Since NOTCH signaling has been implicated in both epithelial and epidermal stem cells [18, 19], we sought to determine whether we could identify the expression of keratin filament proteins in AF culture. We examined the expression of keratin intermediate filament protein, KERATIN 8 (K8), a marker of simple epithelia [20-22], since it is the first keratin to appear in embryogenesis, as early as in pre-implantation embryos [23] and has been shown to be expressed in undifferentiated human ES cells [24, 25] and mouse epiblast stem cells [25, 26]. Lastly, we determined whether AF cells express the conventional markers of pluripotency: OCT4a, SOX2 and NANOG, which

could be exploited as a method of enriching for stem cell sub-populations by clonal analysis. A better understanding of the cellular and molecular profile of AF cells will provide a basis for the generation of novel lineages of primary human cell lines that are suitable for cell-based therapies.

Materials and Methods

Cell Culture

Samples of amniotic fluid were obtained from the Ottawa Hospital (Ottawa, Ontario, Canada) following routine amniocentesis carried out on pregnant women at 15 to 35 (AF15 - AF35) weeks of gestation. All the procedures were performed following the guidelines established by the Ottawa Hospital and National Research Council Canada Research Ethics Boards; a written consent was obtained from each woman to use the amniotic fluid for both genetic analysis and research purposes. Genetic analysis revealed normal karyotypes and the pregnancies resulted in successful births. Shortly after amniocentesis, amniotic fluids were diluted with Phosphate Buffered Saline (PBS) and spun at 200 *x g* for 5 mins; pellets were re-suspended in the expansion media composed of Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen) supplemented with 20% inactivated fetal bovine serum (FBS, Hyclone). The cells isolated from each AF sample were plated in a 10 cm culture dish (Corning) and expanded. Cells were passaged by trypsinization (0.25% Trypsin, 0.1% EDTA, Wisent) and expanded serially with a split ratio of 1:3 for 5 -10 passages at 70% confluency and used for cellular and molecular analyses. AF cells were maintained in a humidified incubator under 5% CO_2 at 37°C. The viability of AF cells was routinely checked with trypan blue and cell survival dye, carboxyfluorescein diacetate (CFDA). NT2/D1 (NT2) embryonal carcinoma (EC) cells (ATCC) were seeded at a density of 2 x 10^6 cells in HG-DMEM (Invitrogen) and 10% FBS (Hyclone) replenished every 2 days [27]. Human SH-SY5Y cells (ATCC) and E13 embryonic cortical cultures were prepared, as previously described [28, 29].

To generate AF-derived single cell clones, a single cell suspension was prepared by gentle trypsinization and individual AF cells were deposited, by Fluorescence Activated Cell Sorting (FACS), with a cell density of 1 cell per well of a 96-well flat bottom plate (Nunc) in 100 µl of expansion medium. Once the cultures became 70% confluent, clones were sub-cultured first into 24-well plates and then, 6-well plates (Nunc) and eventually into 10 cm culture plates (Corning), using the above-mentioned conditions (Figure 8A). The clonal AF cells, were thereafter expanded serially with a split ratio of 1:3 and cultured in DMEM containing 0.5% FBS and N2 supplement for up to one week to generate neurons.

RNA Extraction and RT/QPCR

Total RNA was extracted from cell, using TriReagent (Molecular Research Centre), as per manufacturer's instructions. The RNA was quantified with NanoDrop (Thermo Fisher Scientific), with a final A_{260}/A_{280} ratio of 1.9-2.0. One microgram of total RNA was reverse transcribed, using Quantitect Reverse Transcriptase (Qiagen) for cDNA preparation. A no reverse transcriptase control was performed in parallel. PCR amplifications were carried out on a BIORAD DYNA Cycler with a 25 µl volume containing: 1X PCR Reaction Buffer, 200 µM dNTP mixture, 1.5 mM $MgCl_2$, 0.05 units *Taq* polymerase, 5 µM sense and antisense oligonucleotide primers and 10 ng of cDNA. The PCR program consisted of a denaturing step for 3 mins at 94°C, followed by 30 secs at 94°C, 30 secs at 56-70°C and 30 secs at 72°C for 40 cycles. The PCR final extension period was 5 mins at 72°C. The reaction products were separated on a 2% agarose gel containing 0.2 µg/ml of ethidium bromide and visualized on a UV transilluminator. The gel images were captured with a FluorChem 8900 Imager (Alpha Innotech) and the amplicon size was determined by comparison with a 1 kb

Plus DNA Ladder (Invitrogen). *β-ACTIN* (*ACTB*), NT2 and SH-SY5Y cells were used as a normalizing housekeeping gene and positive controls for RT/QPCR expression analysis, respectively. Quantitative PCR (qPCR) was performed using Fast SYBR Green Master Mix (Bio-Rad) on 7500 Fast Real-Time PCR System (Applied Biosystems). Approximately, 10 ng of cDNA was used per individual reaction with primer concentrations of 5 μM. Quantitative PCR amplifications were performed using the following conditions: Initial denaturation at 94°C, 20 secs and 40 cycles at 94°C, 5 secs; 60°C, 30 secs. All data was normalized to ACTB, using the ΔΔCT method. To identify the expression of self-renewal, neural commitment and differentiation genes, specific primers were designed using publicly available Primer3 software [30]

Table 1. Sequence and annealing temperatures of primers for RT/QPCR

Designation	Sequence (5'-3')	Annealing Temp. (°C)	Amplicon Size (bp)
Self-renewal			
SOX2 - F	GGAGCTTTGCAGGAAGTTTG	60	460
SOX2 - R	GGAAAGTTGGGATCGAACAA		
q*SOX2* - F	CACTGCCCCTCTCACACATG	60	81
q*SOX2* - R	TCCCATTTCCCTCGTTTTTCT		
OCT4a - F	CTTCGGATTTCGCCTTCTCG	60	412
OCT4a - R	TGCAGAGCTTTGATGTCCTG		
q*OCT4a* - F	CTGAGGGCGAAGCAGGAGTC	60	170
q*OCT4a* - R	CTTGGCAAATTGCTCGAGTT		
*OCT4*b - F	CTTGCTGCAGAAGTGGGTGGAGGAA	70	169
OCT4b - R	CTGCAGTGTGGGTTTCGGCA		
q*OCT4b* - F	GCTCGAGAAGGATGTGGTCC	60	81
q*OCT4b* - R	CGTTGTGCATAGTCGCTGCT		
NANOG - F	GACTGAGCTGGTTGCCTCAT	56	276
NANOG - R	TTTCTTCAGGCCCACAAATC		
q*NANOG* - F	GCAGAAGGCCTCAGCACCTA	60	81
q*NANOG* - R	AGGTTCCCAGTCGGGTTCA		
Notch Signaling Pathway			
NOTCH1 - F	CACCCAGAACTGCGTGCA	58	726
NOTCH1 - R	GGCAGTCAAAGCCGTCGA		
NOTCH2 - F	GCTGATGCTGCCAAGCGT	58	475
NOTCH2 - R	CCGGGGAAGACGATCCAT		
DLL1 - F	GTCGACTCCTTCAGTCTGCC	60	413
DLL1 - R	CAGATCGGCTCTGTGCAGTA		
DLL3 - F	GACCCTCAGCGCTACCTTTT	60	249
DLL3 - R	TACATCTTCAGGGCGATTCC		
DLL4 - F	ACCGACCTCTCCACAGACAC	60	484
DLL4 - R	CGCTGATATCCGACACTCTG		
HES1 - F	AAAATGCCAGCTGATATAATGGAG	60	314
HES1 - R	GGTCTGTGCTCAGCGCAGCCGTCA		
HES5 - F	CAGAGTCCCTGCCGTTTTAG	60	265
HES5 - R	GAGTTCGGCCTTCACAAAAG		
JAG1 - F	ACACACCTGAAGGGGTGCGGTATA	60	608
JAG1 - R	AGGGCTGCAGTCATTGGTATTCTGA		
JAG2 - F	TGAACGGGTACCAGTGTGTG	60	275
JAG2 - R	TCTTGCCACCAAAGTCATCA		
Neural Markers			
NES - F	GCGTTGGAACAGAGGTTGGA	58	327
NES - R	TGGGAGCAAAGATCCAAGAC		
PAX6 - F	CAATCAAAACGTGTCCAACG	60	431

PAX6 - R	TGGTATTCTCTCCCCCTCCT		
ASCL1 - F	GTCGAGTACATCCGCGCGCTG	60	220
ASCL1 - R	AGAACCAGTTGGTGAAGTCGA		
CD133 - F	CAGTCTGACCAGCGTGAAAA	60	200
CD133 - R	GGCCATCCAAATCTGTCCTA		
NSE - F'	GAGCGGGCAGTGGAAGAAAAGG	56	292
NSE - F'	GTCCGGCAAAGCGAGCTTCAT		
NFL - F"	TCCTACTACACCAGCCATGT	56	284
NFL - R"	TCCCCAGCACCTTCAACTTT		
MAP2 - F	TCAGAGGCAATGACCTTACC	56	321
MAP2 - R	GTGGTAGGCTCTTGGTCTTT		
β-ACTIN - F	TCACCCACACTGTGCCCATCTACGA	60	295
β-ACTIN - R	CAGCGGAACCGCTCATTGCCAATGG		

*** *q*** designates primers used for QPCR
' designates primers from Van Buren *et al*., Clin Can Res 2007: 13(16) 4704-4712; **"** primers from Lee *et al*., Plos One 2009: 4(5) e5586,

Antibodies

The following antibodies were used in this study: SOX2 (1:300, ICC, in house), OCT3/4 (1:200, WB, Santa Cruz), CD133 (1:200, WB, Santa Cruz), C-KIT (1:50, ICC, Santa Cruz), β-ACTIN (1:5000, WB, Sigma), PAX6 (1:1000, WB, ICC, Santa Cruz), JAG1 (1:100 ICC, Santa Cruz), RBP-J (1:100, ICC, Cosmo Bio Co., LTD.), MAP2a+2b (1:200, ICC, Sigma), Hoechst (1:1000, ICC), NOTCH1 (1:100, ICC, Upstate Biotech), NFL (1:200, ICC, Chemicon), Keratin 8 (1:2, Developmental Studies Hybridoma Bank), horseradish peroxidase (HRP) anti-rabbit and anti-mouse (1:5000, WB, Jackson Immunoresearch Laboratories) and immunocytochemistry fluorescence-conjugated (Alexa Fluor 488 anti-rabbit, goat or rat) secondary antibodies were used at a dilution of 1:500 (Molecular Probes, Invitrogen).

Protein Extraction and Western Blot Analysis

Cells were washed with ice-cold TBS (25 mM Tris-HCL, pH 7.5, 150 mM NaCl) and lysed directly in the tissue culture plate on ice, in ice-cold lysis buffer (25 mM Tris-HCL, pH 7.6, 150 mM NaCl, 1% Triton-X, 1% Na.Deoxycholate) containing 1 mM phenyl-methylsulfonyl fluoride (PMSF) and a protease inhibitor cocktail (Roche Diagnostics), as previously described [29]. Cell lysates were incubated on ice for 30 mins and clarified by centrifugation at 16 000 *x g* at 4°C for 15 mins. Total protein concentrations were determined, using the Bio-Rad Protein Assay (Bio-Rad). The supernatant aliquots containing equal amounts of total protein (40 µg) were diluted 1:2 with Laemmli sample buffer (Bio-Rad) for a total loading volume of 20 µl, run on a 12% SDS-PAGE (90 V for approximately 3 hrs and transferred to a nitrocellulose membrane (Amersham), using a wet transfer apparatus (Bio-Rad) at 20 V overnight at 4°C. Membranes were washed once with TBST (TBS containing 0.05% Tween-20, Sigma) and blocked with 5% skim milk in TBST for 2 hrs at room temperature. The membrane was incubated with primary antibodies (see the Antibodies section for specifics) overnight at 4°C with gentle rocking. B-ACTIN (ACTB) (Bio-Rad, 1:5000) was used as a loading control. Membranes were washed five times for 5 mins per wash in TBST. All secondary antibodies, anti-rabbit and anti-mouse IgG-HRP conjugates (Bio-Rad, 1:5000), were diluted in 5% skim milk and the membranes were incubated for 1 hr at room temperature. Membranes were washed five times for five minutes per wash in TBST and consequently detected by the enhanced chemiluminescence system (New England Nuclear). The ECL signal was analyzed by FluorChem 8900 (Alpha Innotech). Rainbow markers (Amersham) were used to determine the protein sizes.

Immunocytochemistry

Cells were rinsed three times with PBS, fixed with 65% ethanol containing 0.15 M NaCl for 20 mins and blocked with Protein Block Serum Free (Dako) for 30 mins. Primary antibodies were diluted in Antibody Diluent (Dako) and cells were incubated with the primary antibody for 1 hr at room temperature. Following washes with PBS, cells were incubated with the secondary antibody for 1 hr, washed and covered with the Vectashield mounting medium (Vector Laboratories). The immunoreactivity was examined under an Axiovert 200 M fluorescence microscope (Zeiss). Pictures were taken with AxioCam and Axiovision 4.7 (Zeiss) and processed with Adobe PhotoShop (Adobe Systems Incorporated). SOX2, OCT4a or KERATIN positive cells were quantified against the total number of Hoechst positive cells, using at least 15 random images at 20X magnification. The majority of images shown are representative of AF26, unless otherwise specified.

DNA transfection and virus production

The promoter reporter SOX2 construct (pAP2-PrSox2-EGFP) was generated by sub-cloning the SmaI-BamHI full-length *Sox2* promoter fragment (-5255 to +279 CDS), derived from the p-PrSox2-EGFGP-IRES-PURO plasmid (a generous gift from Dr. Fred Gage, Salk Institute, La Jolla, CA, USA), into the pAP2-EGFP retroviral vector [31]. Briefly, 293 GPG cells [32] were plated the night before on a 10 cm dish in 5 ml DMEM supplemented with 10% inactivated FBS, 1 μg/ml tetracycline (Sigma), 2 μg/ml puromycin (Sigma) and 300 μg/ml G418 (Gibco). Twenty micrograms of the pAP2-PrSox2-EGFP was diluted in 500 μl of serum free medium (DMEM) and incubated at room temperature for 7 mins with 30 μl of Lipofectamine2000 (Invitrogen) also previously diluted into 500 μl of DMEM. The DNA-

Lipofectamine complex was added drop-wise to 293 GPG cells at 70% confluency. Medium was replaced 7 hrs post-transfection and the transfection efficiency was confirmed based on GFP expression 24 hrs later, using fluorescence microscopy (Zeiss). Virus conditioned medium was collected and replenished daily for a span of three days. The virus-conditioned medium was filtered through a 45 μm low protein binding filter (Millipore) and concentrated, using an Ultra Centrifugal Device (Ambion) [29], according to manufacturer's instructions. The concentrated virus was either used directly to infect AF cells or stored at -80°C. AF cells were seeded the night before and infected at 70% confluence three times within 24 hrs, with 200 μl of concentrated virus and 8 μg/ml of polybrene in 1 ml of DMEM media. Three days following infection, the medium was replaced with DMEM, 0.5% FBS and N2 supplement to induce neuronal differentiation. In parallel experiments, the pAP2-PrSox2-EGFP was also directly electroporated into AF cells, using the Neon Transfection System (Invitrogen). Briefly, 1 μg of pAP2-PrSox2-EGFP was diluted to a total volume of 10 μl Re-suspension Buffer E containing 1.0 X 10^5 dissociated AF cells. The Neon pipette containing the cell suspension and plasmid was electroporated inside the Neon 10 μl tip, using the following conditions: Voltage: 1350 V, Pulse Width: 15 ms and Pulse Number: 3. Following electroporation, the cells were seeded into a well of a 24-well plate containing 500 μl of DMEM + 20% FBS. Three days following transfection, the medium was replaced to induce neuronal differentiation.

Flow Cytometry Analysis

AF cells were washed with PBS, trypsinized and resuspended in 2 ml of cold 2% FBS/PBS. Dissociated single cells were stained with C-KIT antibody (Santa Cruz sc-5535) for 30 mins

at 4°C. Following the incubation period, the cells were washed twice with cold 2% FBS/PBS and incubated for 30 mins at 4°C with a secondary phycoerythrin (PE)-conjugated antibody. The cells were subsequently washed, resuspended in 2 ml of cold 2% FBS/PBS, filtered through a 70 μm filter and analyzed using a MoFlo Cell Sorter (Beckman Coulter).

Karyotypic Analysis

For karyotypic integrity of clonal AF-F5 cells, the cells were incubated for 4 hrs in medium containing 0.1 μg/ml Karyomax Colcemid solution (Invitrogen), harvested by trypsinization and spun at 175 *x g* for 5 mins at room temperature. The cells were incubated for 20 mins at room temperature in 10 ml of hypotonic solution (0.075 M KCl) and fixed with fresh methanol/acetic acid fixative (3:1 vol/vol). The fixed cell suspension was dropped onto a clean glass slide. The slide was subsequently incubated with Hoechst for 10 mins and mounted with Vectashield (Vector). Pictures of the metaphase spreads were taken with a 40X objective (Axiovert Zeiss).

Statistical Analysis

Results are analyzed using a one-way analysis of variance (ANOVA), followed by Newman-Keuls post-hoc test. Results are expressed as mean + standard error of mean (SEM) and considered significant at $p < 0.05$.

Results

Early AF cells express abundant levels of KERATIN 8

Based on their morphology and growth characteristics, AF cells can be classified into three cell types: epithelial-like, amniotic fluid-specific and fibroblast-like [33]. Since the composition and cellular and molecular properties of AF cells may vary with gestational age, we examined AF-derived cells ranging from 15 to 35 weeks of gestation. The cells isolated from the AF were plated in culture-grade dishes to reproducibly establish primary cultures. Ki67, a well-characterized cell growth marker, that excludes the resting (G_0) phase, was used along with Hoechst staining to show proliferation (Figure 1A) and mitogenic configuration (Figure 1B, arrowheads); respectively. Furthermore, cell survival dye, CFDA, was used to ensure that AF cells maintain a viable and metabolically active state throughout culturing (Figure 1C). The doubling time of the established primary cultures was 21.5 hrs, with approximately 500 doublings observed.

Given that fetal epithelial cells contribute largely to the composition of AF during gestation, we sought to investigate whether K8, a key marker of simple epithelia and early embryonic stages [23], is expressed in AF cells. Immunocytochemistry showed fibrillary cytoplasmic staining for K8 in AF cells (Figure 2A, bottom panel). Furthermore, K8 positive cells were abundant (over 97%) in early gestation periods (AF 16 and 26); whereas, they were significantly decreased (approximately 75% reduction) at later gestational periods (AF28 and AF35; Figure 2B), suggesting that the majority of earlier gestation cells in the AF are epithelial in origin. This notion is further established by expression of *K7*, *K18* and *K19* in AF cells (Supplementary Figure 1).

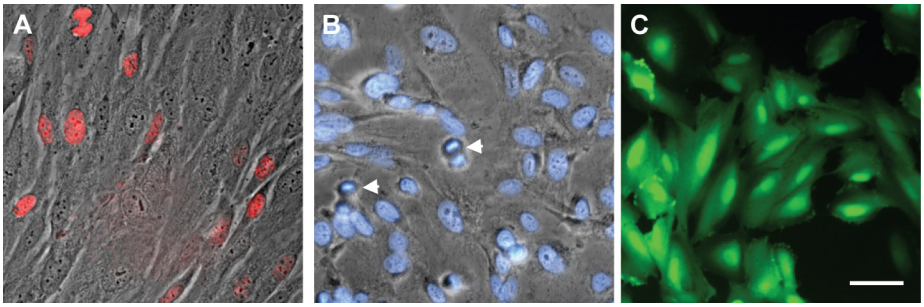

Figure 1. The proliferative activity of AF cells
A. AF cells readily expressed Ki-67 (red), a marker commonly used for proliferation analysis. Ki-67 was detected at both interphase (arrowheads) and mitotic (arrow) stages. **B**. Similarly, Hoechst (blue) staining demonstrated proliferation in AF culture. Several mitotic figures (arrowheads) were present in any random field of view. **C**. Cell survival dye, CFDA (green), reveals that AF cells are viable and metabolically active throughout culturing. Scale bar: 50 μm.

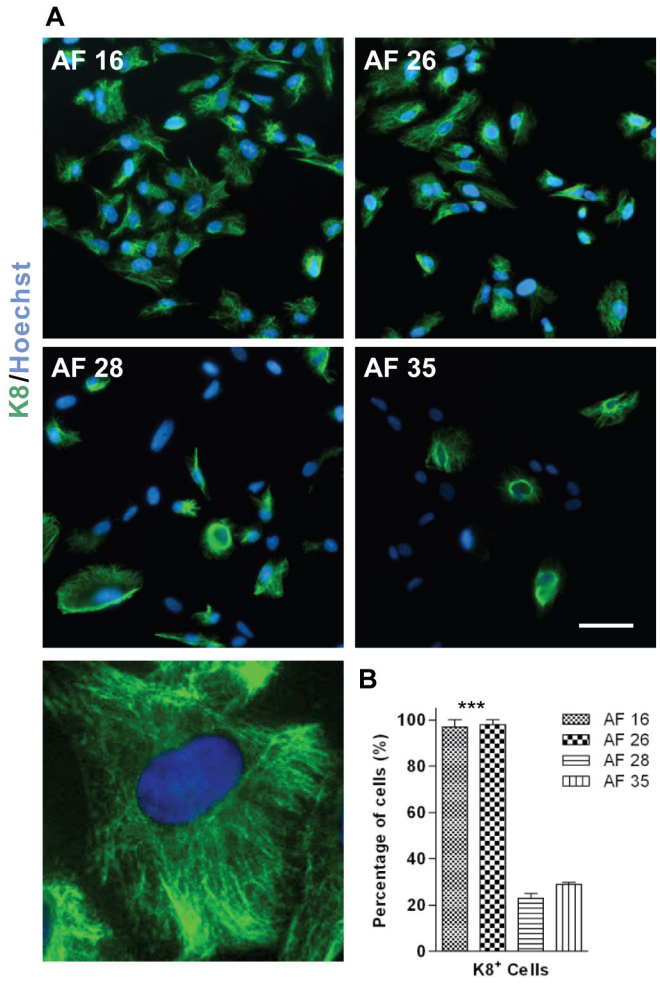

Figure 2. Expression of KERATIN 8 in AF cells at different gestational periods
A. Immunocytochemistry of AF cells from 16, 26, 28 and 35 weeks of gestation (AF16 – AF35) showed significantly higher KERATIN 8 (K8, green) positive cells in AF16 and AF26 cultures compared with AF28 and AF35 cultures. Hoechst (blue) staining was used to determine the total number of cells. **B**. Statistical analysis confirmed that more than 95% of AF16 and AF26 cells were $K8^+$; whereas, there was a significant reduction in expression of K8 in AF28 and AF35 cultures. Data (mean + SEM, n=3, *** $p<0.0001$ vs AF28/35) was obtained from 15 independent fields of view. Scale bar: 50 µm.

AF cells contain components of the Notch signaling pathway

Since NOTCH signaling has been shown to play multiple roles in the regulation of epidermal development [34] and since several NOTCH receptors (NOTCH1-NOTCH4), ligands (DELTA-LIKE (DLL1 and DLL4) and JAGGED (JAG1 and JAG2) and effectors (HES1 and HES5, Figure 3A), are expressed in the developing skin [35, 36], we examined these components of the NOTCH signaling pathway to gain a better understanding of its critical role in AF cells. Our RT-PCR analysis detected multiple NOTCH receptor and ligand transcripts expressed in AF samples ranging from 15 to 35 weeks of gestation (Figure 3B). *NOTCH1*, *NOTCH2* and *JAG1* were all ubiquitously expressed and *HES1* appears to be the effector gene uniformly expressed in all gestation samples examined, by comparison to *HES5*. Immunocytochemistry further highlighted the ubiquitous expression of NOTCH1, JAG1 and the presence of DLL1 and DLL4 in sub-populations of AF cells (Figure 3C). Ubiquitous RBP-J expression, a key mediator of NOTCH signaling, was also observed in the AF cultures (Figure 3C). When associated with some NOTCH proteins, RBP-J acts as a transcriptional activator that activates transcription of NOTCH target genes, such as *HES1*. The presence of the various components of NOTCH signaling suggests that AF cells can be used as a human cell model to study complex signaling pathways.

AF cells express genes involved in neural cell fate lineage

During fetal development, expression of NOTCH1 or its downstream regulators, such as HES1, inhibits neuronal differentiation and results in the maintenance of a progenitor state, as a precursor to neural differentiation. Hence, we used RT-PCR to examine the

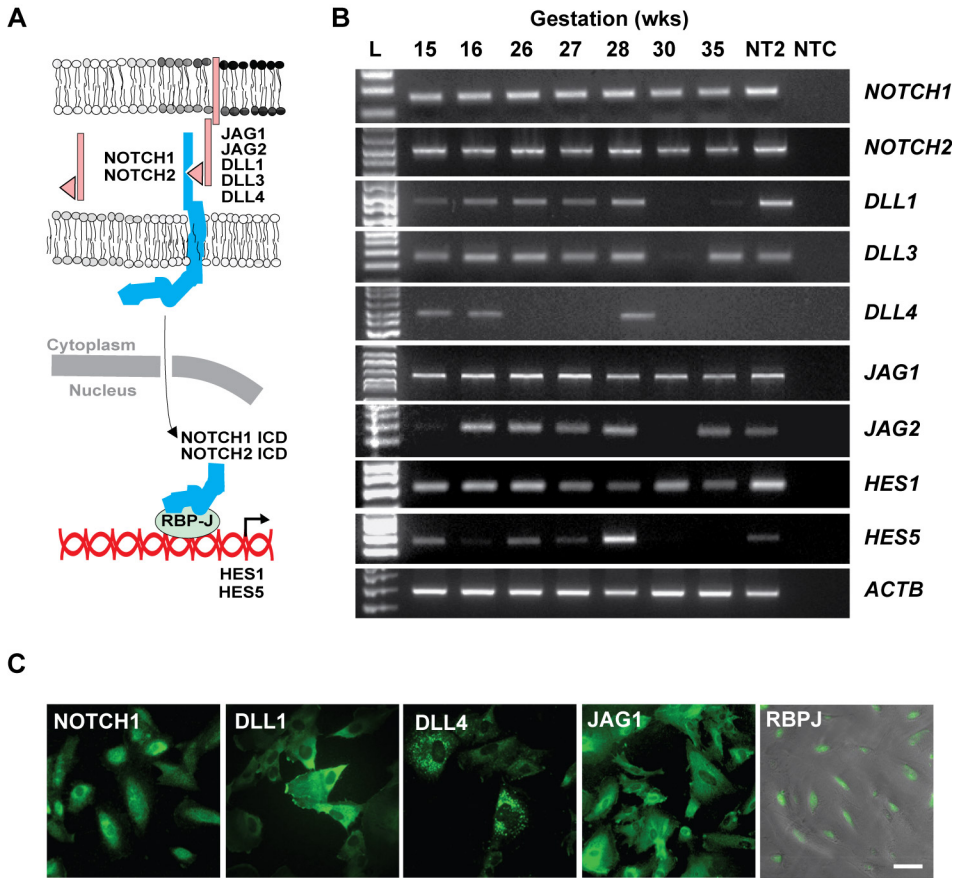

Figure 3. Characterization of NOTCH signaling in AF cells

A. Schematic diagram of the NOTCH signaling pathway. Upon binding of NOTCH ligands (JAG1, JAG2 and DLL1, DLL3, DLL4), NOTCH receptors (NOTCH1 and NOTCH2) are cleaved and the NOTCH intracellular domain (NOTCH1/2 ICD) translocates to the nucleus to associate with an RBP-J transcription factor to regulate gene expression of target genes, *HES1* or *HES5*. **B**. RT-PCR analysis of the mRNA harvested from AF cells at 15 to 35 weeks (wks) of gestation for genes involved in the NOTCH signaling pathway. AF cells displayed a ubiquitous expression of *NOTCH1* and *NOTCH2, JAG1* and *HES1,* regardless of their gestational age. The other ligands (*DLL1, DLL3 and JAG2)* and *HES5* appeared to be present only in some of the samples. *β-ACTIN (ACTB)* and undifferentiated human NT2 cells were used as internal and positive controls, respectively. NTC, No Template Control. **C**. Representative immunocytochemistry further confirming the presence of NOTCH1, DLL1, DLL4, JAG1 and RBP-J in AF cells. Scale bar: 50 µm.

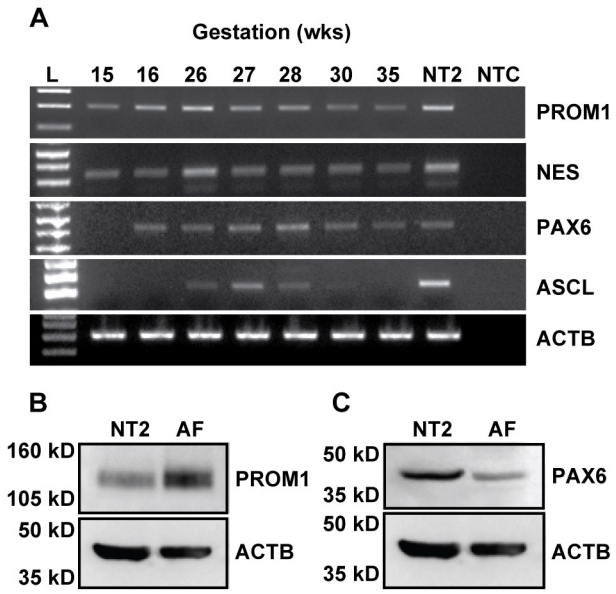

Figure 4. Expression of neural markers in AF cells

A. RT-PCR analysis of mRNA harvested from AF cells at 15 to 35 weeks (wks) of gestation for early neural markers. AF cells expressed low levels of *PROM1 (CD133)*, *NESTIN (NES), PAX6* and *ASCL (MASH)*. *β-ACTIN (ACTB)* and undifferentiated human NT2 cells were used as internal and positive controls, respectively. NTC, No Template Control. **B-C**. Western blot analyses confirmed that both PROM1 and PAX6 proteins were expressed in AF cells. NT2 cells and ACTB were used as positive and internal controls, respectively.

expression of common early neural markers. Our data showed that AF cells ubiquitously express low levels of reported early neural genes: *NESTIN* (*NES*), *PAX6*, *ASCL* (*MASH-1*), along with *PROMININ-1* (*PROM1,* also known as *CD133*) [37] (Figure 4A). Western blotting further confirmed that AF cells express PROM1 and PAX6 (Figure 4B-4C). These findings suggest that a distinct cell population, within the amniotic fluid cultures, may harbour progenitor cells with the potential to differentiate along the neural lineage, under the appropriate conditions. Since both RT-PCR and Western blot rely on cell lysates from the heterogeneous cell population, we further determined more specifically what percentage of K8 positive cells were also positive for NES expression, a marker of neuroepithelial stem cells (Figure 5A-5C). Our double staining data showed that NES is co-expressed with K8 in approximately 10% of the AF cells in early gestational periods (Figure 5D). The subcellular analysis of K8 and NES confirmed distinct localization of these intermediate filaments within the cytoplasm (Figure 5C).

Small sub-populations of AF cells express pluripotency markers

Although NESTIN has been used as a marker of neuroepithelial and neural stem cells, it has also been shown to be expressed in early progenitor cells in other tissues such as limbal epithelia and the bulge region of the hair follicle [38-40]. Therefore, we employed a number of pluripotency markers, *SOX2, OCT4* and *NANOG*, to determine whether AF cells express the genes known to be critical for self-renewal of embryonic stem cells. We observed very low transcript levels of *SOX2* and *OCT4a* expression by QPCR (Figure 6A), which fell outside of the linear range, reflecting the small subset of AF cells positive for nuclear SOX2 (approximately 1%) and OCT4a expression, using immunocytochemistry (Figure 6B).

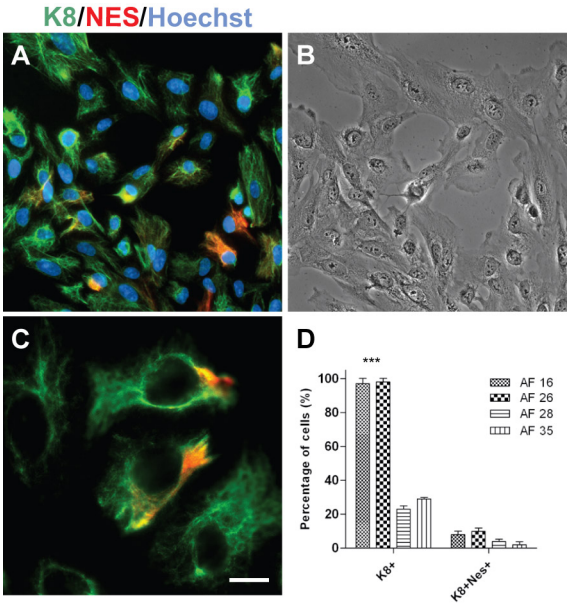

Figure 5. Co-expression of NESTIN and KERATIN 8 in AF cells
A. Representative immunocytochemistry of AF cells from 26 weeks of gestation showing that a sub-population of K8 positive cells (green) also expressed NESTIN (red) with Hoechst nuclear counterstain (blue). **B**. The corresponding phase contrast image of A. C. Higher magnification detailing co-expression of K8 and NES immunofluorescence (yellow) in AF cells. **D**. Statistical analysis showed that there was a significant difference in the percentage of cells positive for KERATIN 8 ($K8^+$) and both KERATIN 8 and NESTIN ($K8^+NES^+$) at different gestational ages (AF16 – AF35). Data (mean + SEM, n=3, ** $p<0.0001$ vs $K8^+$ AF28/35) were obtained from 15 independent fields of view. Scale bar: 50 μm.

Notably, based on a recent publication by Liedtke *et al*. [41], we discriminated the expression of *OCT4* by its two known isoforms: *OCT4a* and *OCT4b*, only the former of which is ascribed as a pluripotency marker [41]. To distinguish *OCT4a* from *OCT4b*, RT/QPCR primers for *OCT4a* were designed to flank Exon1 and Exon2, whereas those for *OCT4b* were designed to flank Exon3 and Exon4 (lacking Exon1). Functionally, the two OCT4 human isoforms show different expression patterns and differ in their ability to confer self-renewal [42, 43]. In accordance, we used an OCT4 antibody (SC-5279), which recognizes a single epitope localized at amino acid 1-134 of the OCT4 protein, which excludes the concomitant recognition of the OCT4b isoform [41]. Approximately 0.1% of AF cells expressed OCT4a (Figure 6B). Similar to the levels observed for *SOX2* and *OCT4a,* we found very low *NANOG* expression by QPCR analysis (Figure 6A). NT2 cells were positive for the expression of all genes examined, characteristic of an embryonal carcinoma cell line. Although very few AF cells express *SOX2*, *OCT4a* and *NANOG*, we were able to enrich for the expression of these genes by purifying a population of cells that expressed the cell surface antigen C-KIT (CD117), the receptor for stem cell factor (SCF) [44], using fluorescence activated cell sorting (FACS), as previously reported [10]. A total of 1.05% ± 0.3% of AF cells were C-KIT positive, resulting in approximately a 1-3 fold increase in gene expression of the *SOX2*, *OCT4a* and *NANOG,* compared to C-KIT negative cells (see Supplementary Figure 2A-D).

SOX2 expressing AF cells have the capacity to differentiate into neuronal cells

Since AF cultures contain a few cells that express *SOX2*, we infected AF cells with pAP2-PrSox2-EGFP, a retroviral vector that contains the SOX2 promoter upstream of the EGFP

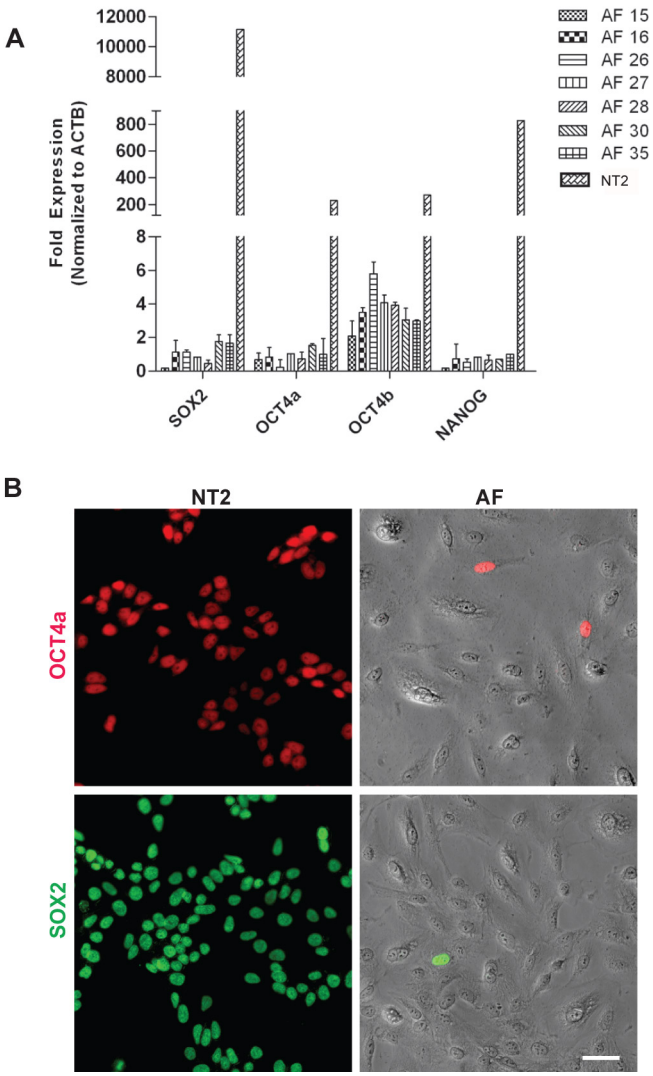

Figure 6. Presence of pluripotent stem cell markers in a small subset of AF cells
A. QPCR analysis of AF cells ranging between 15 to 35 weeks of gestation (AF15 – AF35) revealed low levels of transcripts for *SOX2*, *OCT4a, OCT4b* and *NANOG*. Fold expression levels were normalized against *β-ACTIN* (*ACTB*) (mean + SEM, n=3). Undifferentiated NT2 cells were used as positive control. **B**. Immunocytochemistry confirmed that only a small number of AF cells express SOX2 and OCT4, compared to undifferentiated NT2 cells. Scale bar: 50 µm.

reporter gene (Supplementary Figure 3A) to identify promoter activity and hence SOX2 expression. Following retroviral infection, we monitored the fate of a sub-population of SOX2-EGFP positive cells, mainly found in clusters (Supplementary Figure 3B). Since SOX2 expression is found in neural stem cells, each cluster was carefully marked after which cultures were treated with DMEM, 0.5% FBS and N2 supplement for up to one week to induce neuronal differentiation. Neuronal morphology was monitored within each cluster, using live fluoresence and Hoffman modulation contrast imaging, followed by MAP2 immunocytochemistry (Figure 7E-7F). In contrast, such a sub-population of cells was not observed in either uninfected control cultures (Figure 7A-7B) or those infected with EGFP retrovirus (Figure 7C-7D). Further analysis by both RT-PCR and immunocytochemistry revealed that these SOX2-positive clusters were also positive for other neuronal specific markers, neurofilament light chain (NFL)/NF68 and neuron-specific enolase (NSE) (Figure 7G and 7I, also see Supplementary Figure 3C).

Since AF26 cells displayed a high proliferative potential, with a doubling time of 21.5 hrs, expressed many of the markers involved in stem cell maintenance, neural commitment and differentiation, we used AF26 cells to generate single cell derived clones to enrich for SOX2 expression (Figure 8A). We characterized the top three clones AF-C2, AF-F5 and C-KIT positive AF-C12 based on the expression of *SOX2*, *OCT4a*, *NANOG* and *NESTIN* (Figure 8B). AF-F5 and C-KIT positive AF-C12 clones expressed *SOX2*, *NANOG* and *NESTIN* to a higher level that AF-C2 clones. Given the heterogeneity of AF cells, the generation of a homogenous cell population, through single cell cloning, is important in the interpretation of molecular analyses and functional tests. Specifically, of the SOX2 expressing clones, AF-F5

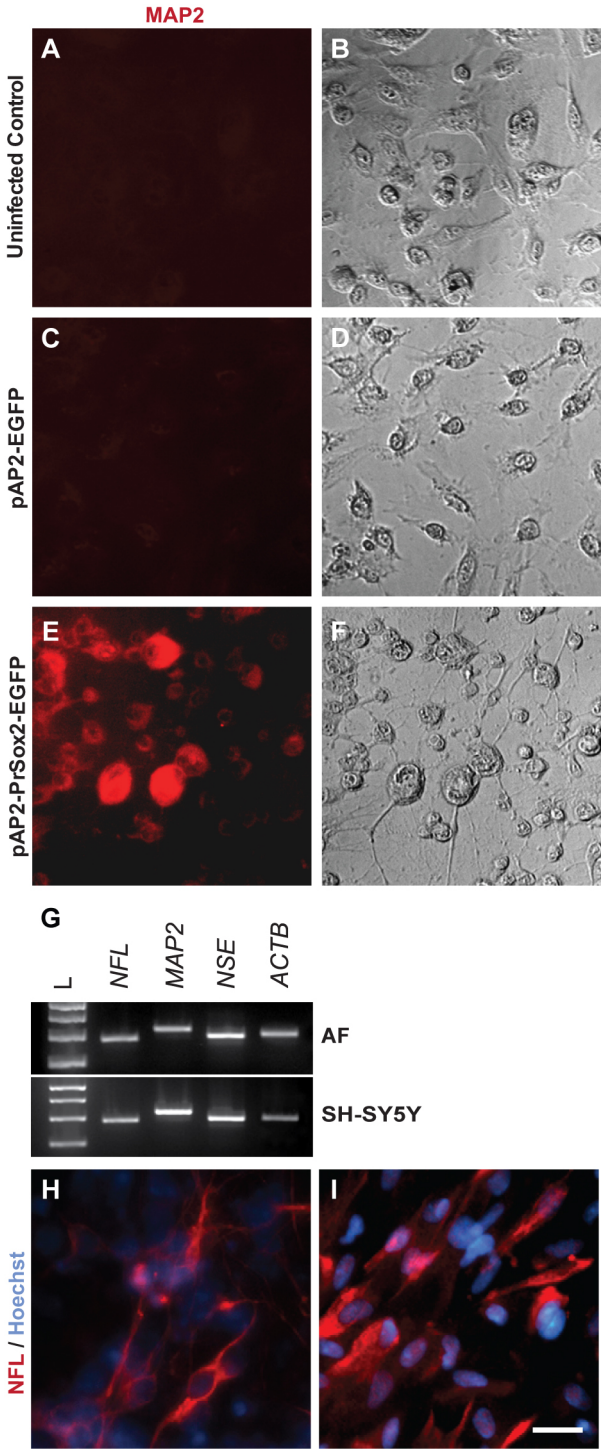
MAP2
A
B
Uninfected Control
C
D
pAP2-EGFP
E
F
pAP2-PrSox2-EGFP
G
L
NFL
MAP2
NSE
ACTB
AF
SH-SY5Y
H
I
NFL / Hoechst

Figure 7. Neuronal differentiation of SOX2 positive AF cells
A and **C**. Immunocytochemistry showed no MAP2 positive cells in either uninfected (A) or pAP2-EGFP infected (C) AF cultures. **E**. Clusters of AF cells infected with pAP2-PrSox2-EGFP differentiated into MAP2 positive neuronal cells. **B**, **D** and **F**. Phase contrast images of A, C and E; respectively. Only cells in panels E/F displayed neuronal morphological features. **G**. RT-PCR analysis of the prSox2-EGFP AF26 cells in neuronal induction media revealed the presence of transcripts for *NFL*, *MAP2* and *NSE*. SH-SY5Y cells and *ACTB* were used as positive and internal controls; respectively. **H**, **I**. Immunocytochemistry confirmed the expression of NFL (red) in mouse embryonic cortical cultures (H) and AF infected cultures (I) with Hoechst nuclear counterstain (blue). Scale bar: 50 μm.

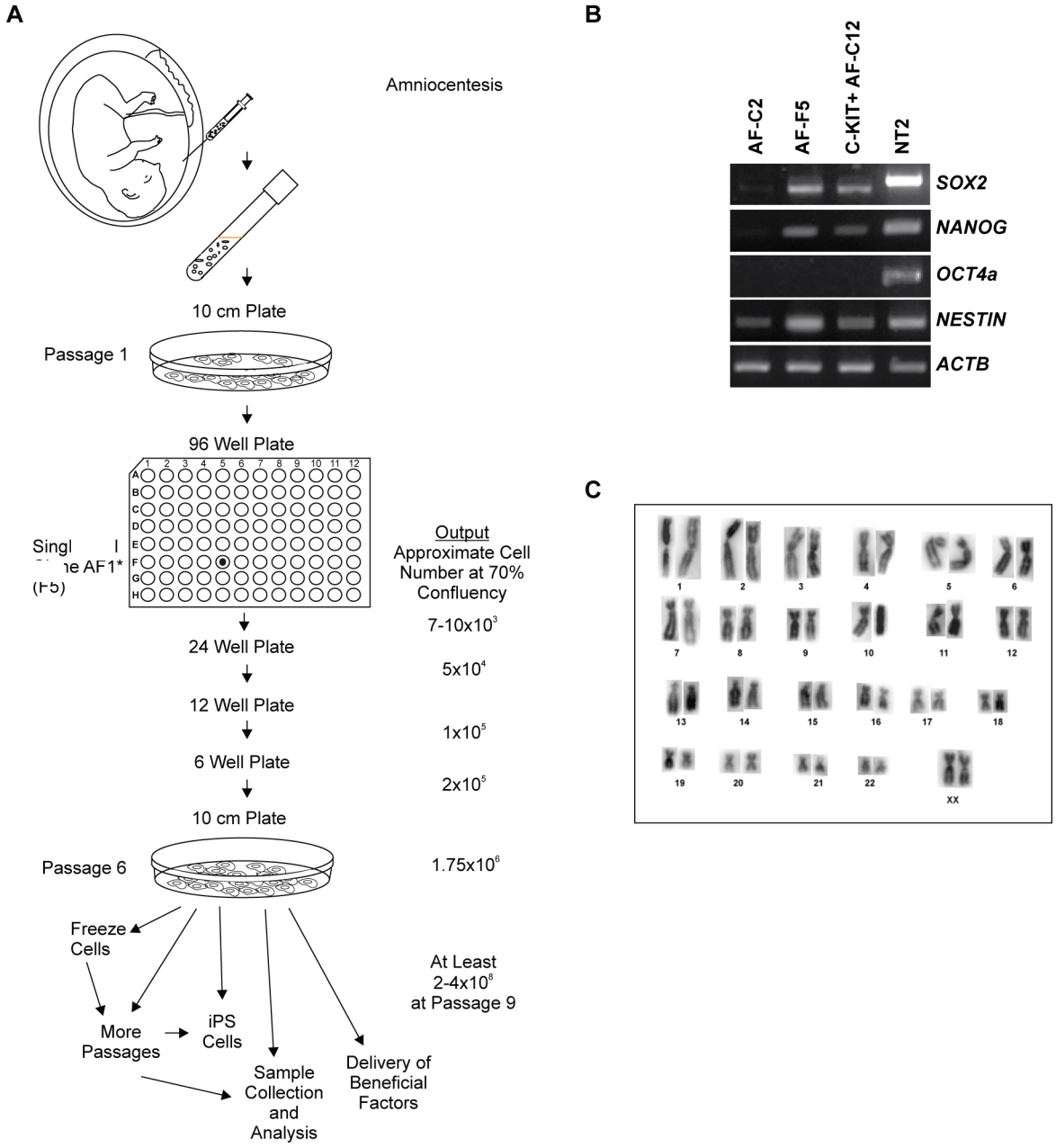

Figure 8. Generation and karyotypic integrity of AF derived single cell clones
A. AF cells were harvested from donors and expanded in culture in 10 cm plates. For the generation of single cell clones, a single cell suspension was prepared and individual AF cells were deposited, by Fluorescence Activated Cell Sorting (FACS), with a cell density of one cell per well of a 96-well plate. Resulting clones were sub-cultured first into 24-well plates and thereafter expanded serially to 10 cm plates and subsequently used for a number of cellular and molecular purposes. **B**. RT-PCR analysis of the top three clones: AF-C2, AF-F5 and C-KIT positive AF-C12 for the expression of *SOX2*, *OCT4a*, *NANOG* and *NESTIN*. **C**. AF-F5 clones were shown to preserve their karyotypic integrity after multiple passages in culture.

cells were able to survive multiple freeze/thaw cycles and SOX2 expression was found in approximately 70% of the cells, in contrast to the cells found in the heterogenic culture (Figure 9A-9B). The SOX2 expression in the clones was validated by Western blotting and immunocytochemistry (Figure 9C-9D) and represents a clonal cell line that can be used to examine the neural differentiation potential of SOX2 positive AF cells for future cell-based therapies. Accordingly, we used AF-F5 cells in subsequent studies in Chapter 2 and 3 to evaluate the neuroprotective potential of AF cells in the injured brain. Importantly, AF-F5 clones were able to preserve their karyotypic integrity after multiple passages (500 doublings) and freeze/thaw cycles in culture (Figure 8C).

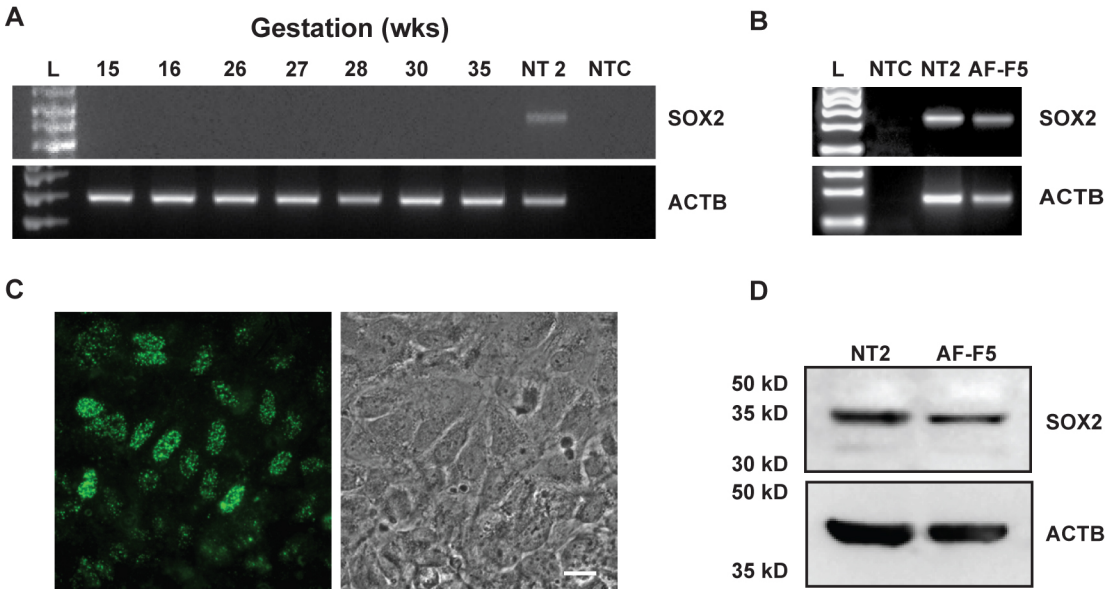

Figure 9. SOX2 enriched AF single cell clones
A. SOX2 was not detected in AF cells, using conventional RT-PCR analysis. **B**. In contrast, SOX2 was readily detected in the single cell clones obtained from AF-F5 (AF) cells. *β-ACTIN (ACTB)* and undifferentiated human NT2 cells were used as internal and positive controls, respectively. NTC, No Template Control. **C**. Immunocytochemistry verified the expression of SOX2 protein in the same sub-population. Right panel: Phase contrast image of the SOX2 positive AF clone. **D**. Similarly, western blot analysis demonstrated the presence of SOX2 in the AF single cell clone. Undifferentiated NT2 cells: positive control. ACTB: loading control. Scale bar: 25 μm.

Discussion

Human AF cells harbour a high proliferative capacity, without forming teratomas *in vivo* [45], a typical problem associated with human ES cells. By examining their growth phase, we found that AF cells have a doubling time (21.5 hrs) similar to that of AF membrane epithelial cells (18 -24 hrs, depending on passage number [46]) and shorter than that observed for human ES cells (32 hrs [47]) and bone marrow-derived mesenchymal stromal cells (36-72 hrs [48]). However, a major underlying issue remains surrounding their heterogeneity, based on AF composition, which varies with gestation and hence the source of putative stem cell sub-populations remains unknown [49, 50]. This necessitates the generation of clonally-derived AF cell populations for future studies. Despite the heterogeneity of AF-derived cells, we found that the majority of the cells obtained from earlier gestations (AF16-26) were positive for K7, K8, K18 and K19. The presence of these early type I (K18 and K19) and type II (K8 and K7) heteropolymeric keratin pairs suggests that AF cells mostly resemble simple epithelial cells [20-22]. The epithelium includes continuous sheets of tightly linked cells that constitute the surfaces (such as epidermis and corneal epithelium) and linings (such as the digestive, respiratory and uro-genital epithelia) of the body [16, 19], and are readily shed into the AF by the developing fetus [49]. Interestingly, the expression of K8 and K18 pair has been recently shown to be characteristic of the epithelial nature of undifferentiated human ES cells and epiblast stem cells [24, 25]. Thus, since K8 is preferentially expressed in early gestational periods, putative stem cell sub-populations found within the native AF may originate either from the fetal skin, urothelium [51, 52] or cells lining the lungs and oral-nasal cavities of the fetus [49]. Indeed, the majority of cells derived from the skin at the time of amniocentesis (16-17 weeks of

gestation) are periderm cells [52] and both K8 and K18 are found in the periderm cells of the developing skin [16, 53]. During embryonic and fetal development, the epidermis changes from a simple epithelium, covered by the periderm, to stratified epithelium and finally to the keratinized epidermis [16]. The change from fetal periderm to definitive epiderm occurs at about 21 weeks of gestation [52]. At this time, the periderm cells have shed and the five cell layers typical of adult epidermis are present [16]. Interestingly, early epidermal cells also express high levels of K8 during development [54]. The subsequent keratinization of the fetal skin, including follicular and interfollicular keratinization, occurs around 22-25 weeks of gestation [49], which would explain why fewer K8 cells are present in the later gestational periods. Although the cells found within the AF have been categorized into three main types: epithelial (E-type), fibroblast-like (F-Type) and amniotic fluids (AF-Type) cells [51, 52], previous studies have demonstrated that keratin staining is restricted to mainly epithelioid (Type-E) and some amniotic fluid (Type- AF) type cells; fibroblast-like (Type-F) cells have been shown to be negative for keratin suggesting a more mesenchymal origin [55]. Our results certainly support these findings.

Since NOTCH signaling is one of the most important pathways implicated in both epithelial and epidermal stem cells (reviewed in [18, 19]), we investigated the expression of multiple components of NOTCH signaling in AF cells. Our results showed that various components of the NOTCH signaling pathway were expressed in AF cells. Among them, we did find that *NOTCH1*, *NOTCH2* and *JAG1* were ubiquitously expressed in all samples and that *HES1* represents the primary target of NOTCH-RBP-J signaling in AF cells. Interestingly, *HES1* also represents one of the main target genes in the skin epidermis [34]. In fact, multiple

NOTCH receptors are expressed in the skin and when all canonical NOTCH signaling is ablated in embryonic epidermis, proliferation is reduced and differentiation is impaired, suggesting that NOTCH signaling also acts as a switch directing epidermal stem cells to initiate differentiation [34]. However, NOTCH signaling is complex and depends on the epidermal compartment [56], and since the AF contains a mixture of cell types from various fetal tissues, including cells partially or fully committed to a particular fate, the exact role of NOTCH signaling in AF cells remains elusive. Since NOTCH is important in all tissues and signaling through NOTCH ligands may involve many responses in cells from different systems, a more homogenous population of AF cells would certainly aid in better defining the role of NOTCH during fetal development.

Similarly, it remains to be determined whether NOTCH signaling is involved in neural commitment of AF cells. AF cells expressed low levels of neural markers, *NESTIN* and *PAX6*, with trace expression of *ASCL*. *PROM1,* a proposed marker of progenitor cells, was ubiquitously expressed in all gestational periods examined. In particular, PROM1 expression has recently been proposed as a means of isolating neural progenitor cells from human ES cells [37]. However, it is important to note that PROM1 negative cells have also been shown to give rise to clonogenic neural stem cells [57], bringing into question the redundancy of PROM1 marker expression. Hence, in addition to PROM1, we also examined NESTIN, a marker used in a number of stem and progenitor cell types, such as neural stem and progenitors cells as well as in cells of human limbal epithelia [40] and the bulge region of the hair follicle [38, 39], the latter of which may also contain resident epithelial stem cells [58]. Interestingly, the NESTIN-positive cells in the bulge region were shown to be able to

differentiate into neuronal cells, when cultivated under clonal conditions [38] and NESTIN expression was shown to co-localize with K5, K8 and K15 in the hair follicle bulge cells, outer-root sheath cells and basal cells of the sebaceous glands [39]. Similarly, we also observed NESTIN co-expression in K8 positive AF cells (approximately 10%), suggesting that the K8-NESTIN positive cells may represent a potential progenitor cell population within fetal epithelial cells. In fact, NESTIN positive cells were also found in the basal layer of the epidermis [59], which further corroborates our findings. Our results suggest that NESTIN expression defines a sub-population of epithelial-like AF cells whose potential as a putative stem cell population is currently under investigation.

Initial efforts have focused on the use of cell surface antigen C-KIT for the isolation of stem cell sub-populations. In fact, clonal AF stem cells, originating from a sub-population of C-KIT (receptor for stem cell factor) positive cells, have been shown to give rise to cell lineages inclusive of all three germ layers [10] – a hallmark which confers a greater advantage over most known adult stem cells sources. Despite these promising results, it is still unclear as to the origin of C-KIT derived AF stem cells, which is further complicated by the developmental distribution of C-KIT expression. This receptor protein is present in human ES cells [60], primordial germ cells, hematopoietic stem cells and some somatic cells. Multipotent C-KIT positive cells isolated from the dermis can acquire neural, hepatic and renal properties [61], and those of the bone marrow have been differentiated into bone, cartilage, fat, neural and pancreatic cells [48], making it challenging to truly define the origin of C-KIT positive AF cells. Therefore, a more specific marker for the isolation of AF stem cell sub-populations is required. To resolve this issue, we determined the degree to

which AF cells express the hallmarks of pluripotency: *OCT4a*, *SOX2* and *NANOG*. Although our data showed that all three genes were enriched 1-3 fold in C-KIT positive cells, it is possible that other important factors are also enriched by C-KIT sorting [62] that could contribute to stemness. In addition, the low number of OCT4 and SOX2 expressing cells prior to cloning can be an explanation of why no detectable levels of the transcript or protein were found, as also recently reported by Li *et al.* [45]. This is not surprising since when ES cells are triggered to differentiate, the expression of *OCT4a* and *SOX2* is down-regulated, confirming that these genes are required for self-renewal and maintenance of pluripotency. Indeed, elevated levels of *SOX2*, *OCT4a* and *NANOG* expression were recently shown in induced Pluripotent Stem (iPS) cells generated from AF cells via ectopic expression of four human factors: OCT4/SOX2/KLF4/C-MYC [45].

Infection of AF cultures with a SOX2 promoter EGFP retroviral vector identified small clusters of SOX2 positive cells, which were differentiated into NFL, NSE and MAP2 positive cells, markers which are expressed in neurons. NFL and MAP2 provide structural support to cell shape and facilitate the transport of particles and organelles within the cytoplasm [63]. NSE (also known as enolase gamma subunit) is a glycolytic isoenzyme, which is expressed in the central and peripheral nervous systems. Together, the expression of these proteins was found in sporadic clusters of AF cells, very similar to the pattern and distribution observed for SOX2 positive cells. Supporting our observations, the expression of SOX2 in the brain has been shown to be restricted to neural stem and progenitor cells, glial precursors and proliferating astrocytes [29]. Notably, SOX2 has been shown to employ BRN2 as a partner (rather than OCT4) for targeting *NES* expression in neural stem and

progenitor cells [64, 65] and seems to function upstream of *C-KIT* in ES cells, based on recent microarray and chromatic immunoprecipitation data [66]. Whether, SOX2 serves the same function in AF cells remains to be elucidated.

Since single cell assays, performed by limiting dilution (a direct measure of clonality), are an essential and rigorous approach to examine the cell fate of any given stem cell, we employed this method to enrich for AF clones that maintained SOX2 expression following multiple passages and freeze/ thaw cycles. SOX2 expression was validated with RT-PCR, Western blotting and immunocytochemistry in two of the three clones generated. Given the heterogeneity of AF cells and the ability to isolate clonal sub-populations, the characterization and standardization of AF cells is crucial before AF cell-based therapies can be translated to future clinical applications. In support of this goal, we have generated clonal cell lines of AF cells based on single cell cloning protocols as well as SOX2 and C-KIT expression for subsequent studies. This is the first report, to our knowledge, describing the NOTCH signaling pathway in AF cells and showing that pure SOX2 positive clones can be enriched from AF culture and used for neuronal induction. Our data is in agreement with others reporting the presence of neural-derived AF cells [10, 67].

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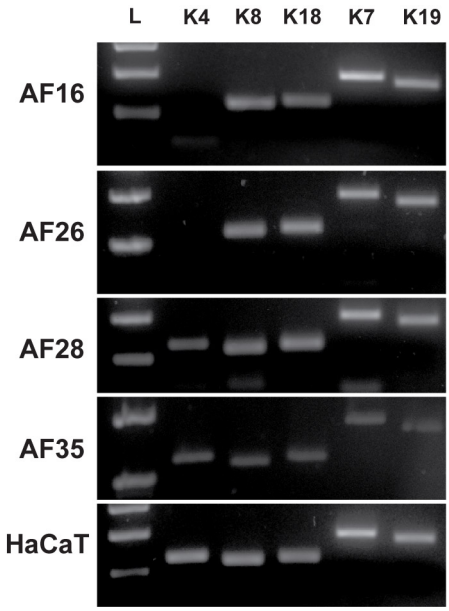
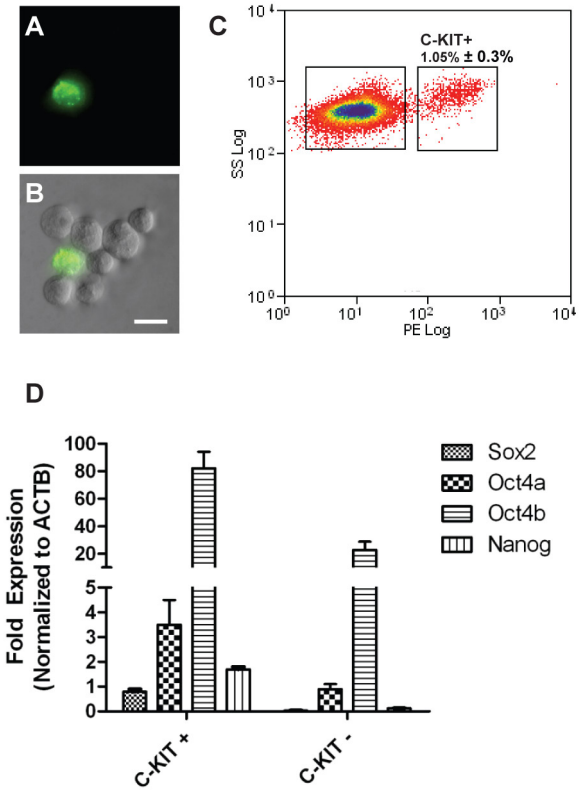
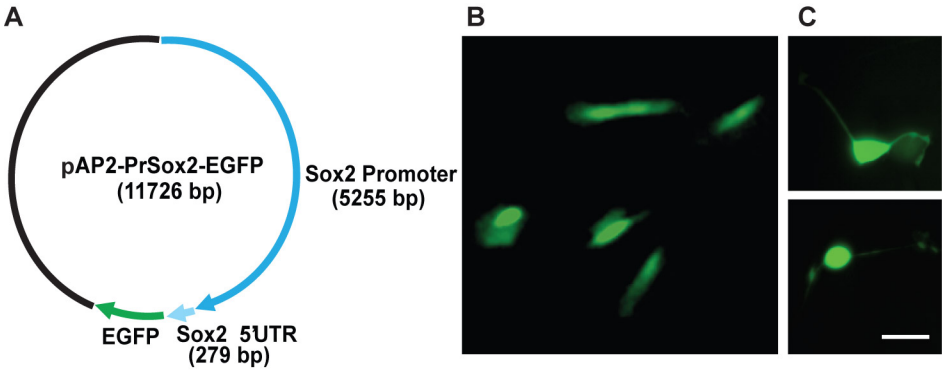


Supplementary Figure 1. Summary of *KERATIN* expression in AF cells
RT-PCR analysis for *KERATIN* expression of AF cells from 16, 26, 28 and 35 weeks of gestation (AF16 – AF35). Expression of *Keratin* (*K*) 8, 18, 7 and 19 was highest in early gestation periods (AF16-AF28) and decreased in AF35 cells. K4 expression was only found in AF28-AF30 cultures. HaCaT cells were used as a positive control.



Supplementary Figure 2. Expression of pluripotent stem cell markers in the C-KIT sorted AF cells

A-B. Immunofluorescence of dissociated AF cells stained with C-KIT (green) antibody prior to sorting for C-KIT positive cells (C-KIT^{+}) by Fluorescence Activated Cell Sorting (FACS). B, merged immunofluorescence and phase contrast images. **C**. Flow cytometry gating for C-KIT^{+} AF cell population. **D**. QPCR analysis of mRNA harvested from C-KIT positive (C-KIT^{+}) and negative (C-KIT^{-}) AF cells directly following sorting showed enrichment for the expression of *SOX2*, *OCT4a/b* and *NANOG*. Fold expression levels were normalized to *β-ACTIN* (*ACTB*). Scale bar: 50 μm.



Supplementary Figure 3. Identification of SOX2 positive cells in AF cultures, using SOX2 promoter-EGFP retrovirus

A. The AP2 retroviral vector containing SOX2 promoter-EGFP was used to infect AF cell cultures. **B-C**. The SOX2 expressing AF cells were identified based on EGFP expression (green) prior (B) and after (C) differentiation into neurons. Scale bar: 50 μm

Chapter 3 - Human amniotic fluid cells form functional gap junctions with cortical cells

Human amniotic fluid cells form functional gap junctions with cortical cells

Anna Jezierski[1,2], Kerry Rennie[1], Roger Tremblay[1], Bogdan Zurakowski[1], Andrée Gruslin[2,3], Marianna Sikorska[1] and Mahmud Bani-Yaghoub[1,2]

[1] Neurogenesis and Brain Repair, Neurobiology Program, Institute for Biological Sciences, National Research Council Canada, Ottawa, Canada.

[2] Department of Cellular and Molecular Medicine, Faculty of Medicine, University of Ottawa, Ottawa, Canada

[3] Department of Obstetrics and Gynecology, Faculty of Medicine, University of Ottawa, Ottawa, Canada

Abstract

The usage of stem cells is a promising strategy for the repair of damaged tissue in the injured brain. Recently, amniotic fluid (AF) cells have received a lot of attention as an alternative source of stem cells for cell-based therapies. However, the success of this approach relies significantly on proper interactions between graft and host tissue. In particular, the re-establishment of functional brain networks requires formation of gap junctions, as a key step to provide sufficient intercellular communication. In this study, we show that AF cells express high levels of CX43 (GJA1) and are able to establish functional gap junctions with cortical cultures. Furthermore, we report an induction of Cx43 expression in astrocytes following injury to the mouse motor cortex and demonstrate for the first time CX43 expression at the interface between implanted AF cells and host astrocytes. These findings suggest that CX43-mediated intercellular communication between AF cells and cortical astrocytes may enhance neuroprotective capabilities by mediating modulatory, homeostatic and protective factors in the injured brain and hence warrant further investigation.

Introduction

Recent advances in regenerative medicine have boosted efforts to explore the therapeutic potentials of stem cells to repair damaged tissue in the injured brain (Reviewed in [1-4]). In particular, the transplantation of embryonic stem cells [5], fetal neural stem or progenitor cells [6-8] or bone marrow-derived stem cells [9, 10] into the injured brain has been explored extensively. However, human embryonic stem (ES) cells and fetal neural stem cells (NSCs) are subject to ethical considerations and the risk of tumor development, whereas adult neural stem cells have limited proliferation capabilities and lineage restriction. Therefore, other stem cell sources, such as human amniotic fluid (AF) cells [11-13] are being considered for therapeutic applications. There is evidence that AF contains stem cell sub-population(s) [14] isolated based on C-KIT (CD117-the receptor for stem cell factor [15]) expression, which share some of the characteristics of embryonic and adult stem cells [14]. For instance, several reports have shown that AF cells can differentiate along the adipogenic and osteogenic [16-18], myogenic [19, 20] and endothelial [21] pathways. Furthermore, AF cells have been shown to harbour the potential for neurogenic differentiation, using different induction protocols [14, 18, 22-25]; however, the proof that these cells can differentiate into functional neurons remains elusive [26, 27].

Nonetheless, the versatility of AF-derived cells for therapeutic applications has been investigated in various animal injury models in the central and peripheral nervous system [14, 28-32]. Although it has been suggested that AF-derived cells exert beneficial effects on the ischemic brain to an extent comparable with the neuroprotective effect of embryonic neural progenitor cells [32], it remains to be determined whether or not these cells are

capable of integrating into the brain and developing functional connectivity with the host tissue to support neuroregenerative and protective capabilities. The success of this strategy depends on the formation of a rapid and efficient intercellular communication between grafted AF cells and the host tissue followed by the re-establishment of functional networks. In fact, a recent report by Jäderstad *et al*. [33] clearly shows that an essential step in the functional integration of grafted NSCs, even before mature electrochemical synaptic communication, is cell-cell coupling via gap junctions. This integration is, at least in part, dependent on the formation of gap junctional intercellular communication (GJIC), which is considered to be an indispensable mechanism for the propagation of information among cells in the central nervous system (CNS). Gap junctions are composed of two juxtaposed, membrane bound connexon hemichannels, each composed of six connexin subunits, which are joined to bridge the cytoplasm of two neighbouring cells [34, 35]. This consolidation allows the transfer of small ions and molecules, nutrients, metabolites, second messengers and more recently miRNAs [34, 36]. Intercellular communication between graft and host cells underlies many of the early cellular interactions post implantation, which provides insights into the interplay between grafted and host cells in mediating the transfer of therapeutic factors or buffer harmful ion concentrations post-injury. More specifically, connexin-associated gap junction formation and function has been proposed to be pivotal for ensuring host cell well-being and potentially mediating a neuroprotective effect [37]. In fact, NSC-mediated rescue of damaged host neurons did not occur when gap junction formation was suppressed by pharmacological and/or RNA-inhibition strategies [37].

Although AF cells have been previously transplanted into several tissues, including the brain, currently there is no information on gap junctions in these cells and whether they form a means of intercellular communication with the host tissue. Therefore, a better understanding of the interactive processes by which AF cells integrate into host neural tissue may provide insights into the interplay between donor and recipient. In this study, we examine the expression of connexins in AF cells at the RNA and protein levels, using *in vitro* and *in vivo* techniques. In addition, we determine whether AF cells can form functional gap junctions with other AF cells as well as with cortical cells.

Materials and Methods

Cell Culture

Human amniotic fluid (AF) cells were obtained from the Ottawa Hospital, General campus (Ottawa, Ontario, Canada), following amniocentesis in women at 15 to 35 weeks of gestation (AF15 - AF35). The study was approved by the Ottawa Hospital and National Council Canada-Research Ethics Boards and a written informed consent was obtained from each donor. AF cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) supplemented with 20% Fetal Bovine Serum (FBS, Hyclone) and maintained at 37 °C and 5% CO_2 (as described in [38]). AF cells were passaged at 70% confluency every 2-3 days, using 0.05% Trypsin/EDTA (Invitrogen) as a 1:3 split ratio.

To generate AF-derived single cell clones [38], a single cell suspension was prepared by gentle trypsinization and individual AF cells were deposited one cell per well of a 96 well plate, containing 100 µl of DMEM + 20% FBS, using a MoFlo cell sorter (BeckmanCoulter). Once the cultures became 70% confluent, clones were sub-cultured first into 24-well plates, followed by 6-well plates (Nunc) and eventually into 10 cm culture plates (Corning), using the above-mentioned conditions. To purify C-KIT positive AF AF-C-KIT$^+$) cells from AF cultures, dissociated single AF cells were stained with C-KIT antibody (Santa Cruz, sc-5535) for 30 mins at 4°C, as previously described [14]. Following the incubation period, the cells were washed twice with cold 2% FBS in PBS and incubated for 30 mins at 4°C with a secondary phycoerythrin (PE)-conjugated antibody. The cells were subsequently washed, resuspended in 2 ml of cold 2% FBS in PBS, filtered through a 70 µm filter and analyzed using a MoFlo Cell Sorter (Beckman Coulter). The clonal (AF-F5) and

C-KIT positive AF cells were thereafter expanded serially with a split ratio of 1:3 and cultured in DMEM containing 20% FBS to establish the AF-F5 single cell-derived clonal line and C-KIT positive AF cell population.

Mouse cortical progenitors were isolated from the embryonic day 13 (E13) ventricular zone, plated onto poly-L-lysine (PLL)-coated coverslips (9×10^5 living cells/ml) in DMEM + 10% FBS and examined within 24 hrs after plating, as previously described [39]. Cortical neurons were generated from neural progenitors by reducing the serum concentration (i.e. 0.5% FBS) during the first 24 hrs, followed by treatment with DMEM + N2 supplement to limit the generation of glial cells. Medium was replenished every 48 hrs for 7 days. Astroglial cultures were generated from E13 neural progenitors (0.5 - 5×10^5 living cells/ml) cultured in DMEM + 10% FBS. Medium was replenished every 48 hrs for 3 weeks and cells were passaged several times to eliminate neurons in the cultures [39]. To generate mixed cultures of cortical neurons and astrocytes, E13 neural progenitors were maintained in DMEM + 10% FBS for 2 weeks without passaging. NT2-D1 progenitor cells (ATCC) were cultured in DMEM (Invitrogen) media supplemented with 10% FBS (Hyclone). Pure cultures of NT2-derived neurons (NT2-N) were prepared as previously described [40]. HaCaT cells were a generous gift from Dr. Kursad Turksen (Sprott Centre for Stem Cell Research, Ottawa, ON) and were cultured in DMEM (Invitrogen) supplemented with 10% FBS (Hyclone) and split every 2 days.

RNA Extraction and RT-PCR

Total RNA was extracted from cells, using TriReagent (Molecular Research Centre), as previously described [38]. Total RNA was quantified with NanoDrop (Thermo Fisher

Scientific) and 1 µg was reverse transcribed using Quantitect Reverse Transcriptase (Qiagen). RT-PCR amplifications were carried out using iQ Supermix (Bio-Rad) in a 20 µl volume containing, 5 µM sense and antisense primers (Table 1) and 15 ng of cDNA. The PCR program consisted of a denaturing step for 3 mins at 94°C, followed by 30 secs at 94°C, 30 secs at 55-58°C and 30 secs at 72°C for 40 cycles. The final PCR extension period was 5 mins at 72°C. PCR products and 1kb ladder (Invitrogen) were separated on a 2% Ethidium Bromide agarose gel and the images were captured with FluorChem 8900 Imager (Alpha Innotech). The amplicon size was confirmed by comparison with the ladder (Invitrogen). Expected amplicon sizes are shown in Table 1. *B-ACTIN* (*ACTB*) was used as a normalizing gene. NT2/D1, HaCaT and NT2-neurons (NT2-N) were used as positive control.

Table 1. Sequence and annealing temperatures for RT-PCR

Designation	Sequence (5'-3')	Annealing Temp. (ºC)	Amplicon size (bp)	Ref.
CX26-F	CTGCAGCTGATCTTCGTGTC	55	308	[18]
CX26-R	AAGCAGTCCACAGTGTTG			
CX30-F	GCTACCTGCTGCTGAAAGTG	58	326	[41]
CX30-R	CGTTGTGTATGAATGGAGCA			
CX32-F	GACAGGTTTGTACACCTTGC	58	500	[42]
CX32-R	CGTCGCACTTGACCAGCCGC			
CX36-F	AACGCCGCTACTCTACAGTCTTCC	55	268	[20]
CX36-R	GATGCCTTCCTGCCTTCTGAGCTT			
CX37-F	GTTGCTGGACCAGGTCCAGG	58	416	[41]
CX37-R	GGATGCGCAGGCGACCATCT			
CX40-F	GTACACAAGCACTCGACCGT	58	509	[41]
CX40-R	GCAGGGTGGTCAGGAAGATT			
CX43-F	CAATCACTTGGCGTGACTTC	58	408	[41]
CX43-R	GTTTGGGCAACCTTGAGTTC			
CX45-F	GGAGCTTCCTGACTCGCCTGC	58	467	[41]
CX45-R	CGGCCATCATGCTTAGGTTT			
GAPDH-F	CATGACCACAGTCCATGCCATCACT	58	461	
GAPDH-R	TGAGGTCCACCACCCTGTTGCTGTA			

Antibodies

The following antibodies were used in this study: β-ACTIN (1:5000, WB, Sigma), Cx43 (1:500, ICC; WB, 1:4000, ICC, Sigma), Cx26 (1:100, Zymed), GFAP (1:200, ICC, NeoMarkers), GFAP (1:200, ICC, DAKO), Golgin-97 (1:200, ICC, Molecular Probes), MAP2 (1:200, ICC, Sigma), Human nuclear antigen (1:100, ICC, Antibodies-online), Human Mitochondrial Marker (MTCO2) (1:50, ICC, ABcam), C-KIT antibody (1:100, FACS, Santa Cruz sc-5535) and fluorescence-conjugated secondary antibodies (Alexa Fluor 488 anti-rabbit or mouse, Rhodamine anti-mouse and Alexa 647 anti-mouse, 1:500, Molecular Probes). Hoechst (1:1000, Sigma) was used to stain nuclei.

Western Blotting

Cells were washed with cold TBS and lysed directly in the culture plate using ice-cold lysis buffer (25mM Tris-HCL, pH 7.6, 150mM NaCl, 1% Triton-X, 1% Na.Deoxycholate), containing a protease inhibitor cocktail (Roche). Cell lysates were incubated for 30 mins on ice and clarified by centrifugation at 20 000 x *g* at 4°C for 20 mins. Protein samples (40 µg) and a molecular weight rainbow marker (Amersham) were electrophoresed on a 10% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) and transferred to a nitrocellulose membrane (Amersham), using a wet transfer apparatus (Bio-Rad) at 20 V overnight at 4°C. The membranes were incubated in TBS containing 5% non-fat milk with 0.1% Tween-20 (Sigma) for 1 hr at room temperature to block non-specific binding and then incubated in primary antibodies overnight at 4°C. The membranes were then washed three times for 10 mins with TBS containing 0.1% Tween-20 and incubated with a peroxidase conjugated secondary antibody for 1 hr at room temperature. Immunoreactivity was visualized, using

chemiluminescent substrate (New England Nuclear) and captured by FluorChem 8900 (Alpha Innotech).

Immunocytochemistry

Cells were grown on coverslips, washed with PBS and fixed with 65% ethanol containing 0.15 M NaCl for 20 mins [38]. For staining with human nuclear antigen, cells were fixed with 3% paraformaldehyde for 5 mins, washed twice with PBS and permeabilized for 20 mins in 0.2% Triton-X in PBS (pH 7.0). Following fixation, the coverslips were blocked with Serum Free Protein Block (Dako) for 30 mins and incubated for 1 hr at room temperature with the primary antibody. Following three subsequent washes (5 mins each) in PBS, the coverslips were incubated with a fluorescence-conjugated secondary antibody for 1 hr, washed and counter-stained with Hoechst (Sigma). The coverslips were mounted, using Vectashield mounting medium (Vector Laboratories) and immunoreactivity was examined under an Axiovert 200 M fluorescence microscope (Zeiss) and a confocal microscope (Olympus). For GFAP, Cx43 and Hoechst staining in sections containing implanted AF-DsRed cells, Alexa 488 and Alexa 647 were used as the secondary antibodies to visualize Cx43 and GFAP, respectively. In addition, different filter sets were used for Hoechst (excitation 365 nm, emission 420 nm) and AF-DsRed cells (excitation 546 nm, emission 575 nm). A separate image was acquired for each fluorophore (Hoechst, Alexa 488, DsRed and Alexa 647), using a laser scanning confocal microscope (Olympus Fluoview with BX61 microscope), and the four images were superimposed, using Adobe Photoshop. In the sequential quadruple staining of AF cells with Cx43, GFAP, human nuclear antigen (hNuc) and Hoechst, cells were first stained with Cx43 and hNuc antibody, washed and pre-

incubated with a Mouse Ig Blocking Reagent (Vector Laboratories) to reduce undesired binding of the subsequent antibody staining for GFAP detection. The cells were then counterstained with Hoechst.

Dye Coupling

Dye coupling experiments were performed to evaluate the functionality of gap junctions between individual AF cells and other AF cells in culture or mouse neural progenitors, neurons and astrocytes, as previously described [40, 43]. In brief, AF cells were preloaded with two dyes: 0.1% 1-1' dioctadecyl-3,3,3,3-tetramethylindocarbocyanine percholate (DiI, Invitrogen) and 0.1% calcein-acetoxymethyl ester (Calcein AM, Invitrogen) for 20 mins at 37°C. DiI, a lipophilic dye that binds to cell membranes and is not transferred to adjacent cells, was used to label donor cells. Calcein AM is a membrane permeant form and is taken up by donor cells and hydrolyzed to Calcein (MW=623) by cellular esterases. After cleavage, Calcein was readily transferred to adjacent receiving cells through gap junction channels. The DiI and Calcein loaded cells were washed with isotonic glucose solution to remove excessive dye and dissociated into a single cell suspension, following incubation in Trypsin-EDTA (Invitrogen) for 2 mins. Preloaded single AF cells were plated onto cultures of AF or mouse cortical neural progenitors, neurons or astrocytes and dye coupling was evaluated after 4 hrs. The level of coupling was determined by counting Calcein positive, DiI negative cells coupled to a Calcein positive, DiI positive cell and the data was presented as mean + SEM.

Scratch Wound Injury

Scratch wound procedure was performed as previously described [44]. Briefly, scratch wound injury was applied to confluent cortical cultures grown on coverslips, using a $21^{1/2}$G needle (0.8 mm diameter) to create a wound. The cells were washed three times with culture media, fixed with 65% ETOH + 0.15 M NaCl without injury, or 15 mins, 24 hrs, 48 hrs or 72 hrs post-injury. In some experiments, AF cells were plated onto cortical cultures immediately following injury and the co-cultures were fixed at the above-mentioned time points. The labeling of AF cells with GFP was performed, as previously described [38].

Surgically Induced Brain Injury Model

In this study, we used an *in vivo* model to study brain injury caused by neurosurgical procedures in the motor cortex. Brain injury studies were approved by the Animal Care Committee at the National Research Council Canada. Briefly, 6 week old C57 black mice (C57Bl/6, Charles River) weighing about 25 g were anesthetized with isoflurane (Aerrane, Baxter) and placed into equal groups: injury or no injury with cell injection or implant. Prior to injury, mice were placed in a stereotaxic frame and a midline incision was made in the skin to expose the skull. The bone overlying the motor cortex was removed with a dental drill following mapping, using specific stereotaxic coordinates (from "AP -0.25 mm to -1.0 mm, Lat +0.7 mm", to "AP +1.25 mm to +3.0 mm, Lat +2.4 mm") with respect to Bregma (0 mm), as previously described [45]. Injury to the left motor cortex was performed using a sterile graduated needle to remove neural tissue to a depth of 1 mm (Supplementary Figure 1). The injury site was sealed with bone wax, covered with topical anesthetic (0.50% Marcaine Bupivacaine Hydrochloride, Sigma) and the skin was sutured.

For transplantation studies, AF cells engineered to express DsRed driven from a CMV promoter by lentiviral infection (Tet07-CMV-DsRed) were injected into three sites within the motor cortex (1 x 10^5 cells in 2 µl of PBS per injection), using a 10 µl Hamilton syringe, controlled by an infusion pump at a constant speed (0.5 µl/min) over 4 mins. The syringe was held in place for 5 mins and then gradually withdrawn. After the procedure, bone wax was used to seal the injection sites and the skin was sutured. The animals were allowed to recover in their cages, sacrificed after 12 days and the brains were perfused, removed and fixed with 4% paraformaldehyde in PB overnight, washed twice with PB and transferred to 30% sucrose in PB for 2 days. The brains were frozen in O.C.T. compound and sectioned into 8 µm slices (Leica CM 1950). Prior to staining, the sections were thawed at room temperature for 15 mins, washed three times (5 mins each) in PBS and immunostained, as described earlier. Injected AF cells were identified based on DsRed expression (AF-DsRed).

Results

AF cells predominantly express Connexin 43 (CX43)

To establish a profile of connexin expression in AF cells, we performed RT-PCR to examine the expression of connexins commonly found in the brain (*CX26, CX30, CX32, CX36, CX37, CX40, CX43* and *CX45)*. Our results show that AF cells ubiquitously expressed *CX43* (*GJA1*) and *CX45* (*GJA7*) at all gestation periods examined (AF15 – AF35) (Figure 1A). The expression of *CX30*, *CX32*, *CX36*, *CX37* or *CX40* was not detected in any of the gestational periods (data not shown), whereas *CX26* (*GJB2*) RNA was expressed in a few of the gestation periods (Figure 1A) and CX26 protein was only found in a small subset of AF cells in culture (Figure 1C, g-h). Of the connexins expressed, CX43 was the most abundant protein in AF cells, as determined by Western blotting and immunocytochemisty (Figure 1B; 1C, a-f). Similar to other connexins, CX43 is assembled into connexons in the *trans*-Golgi network and transported to the cell membrane where adjacent hemichannels, on apposed cells, dock to form gap junction plaques [46]. Indeed, we found an intracellular pool of CX43 in the perinuclear Golgi apparatus, as confirmed by *trans*-Golgi network membrane protein Golgin-97 positive staining in AF cells (Figure 1C, a-b). Subsequently, CX43 is translocated from the Golgi apparatus to the cell membrane (Figure 1C, c-d) and this dynamic process leads to the formation of discrete gap junctions between adjacent cells, as observed by distinct punctate staining at the cell-cell boundaries between individual AF cells (Figure 1C, e-f). This characteristic was not observed for CX26 in AF cells where protein expression was confined to the perinuclear region (Figure 1C, g-h). In order to examine the functionality of gap junctions, we pre-loaded individual donor AF cells with two dyes (DiI and Calcein) [40, 43] and plated them onto confluent cultures of AF cells.

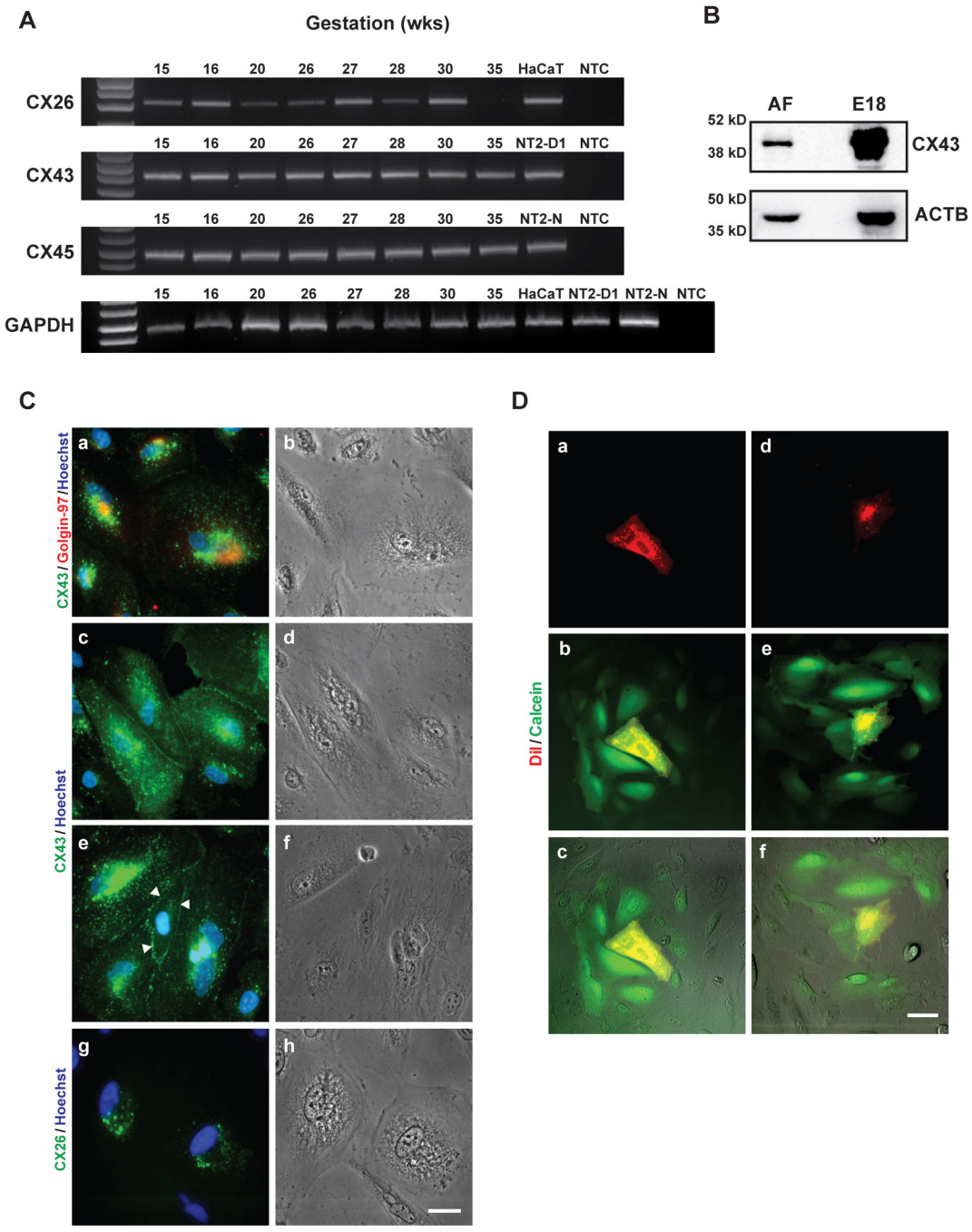

A
Gestation (wks)
15 16 20 26 27 28 30 35 HaCaT NTC
CX26
15 16 20 26 27 28 30 35 NT2-D1 NTC
CX43
15 16 20 26 27 28 30 35 NT2-N NTC
CX45
15 16 20 26 27 28 30 35 HaCaT NT2-D1 NT2-N NTC
GAPDH
B
AF
E18
52 kD
38 kD
CX43
50 kD
35 kD
ACTB
C
CX43 / Golgin-97 / Hoechst
CX43 / Hoechst
CX26 / Hoechst
a
b
c
d
e
f
g
h
D
DiI / Calcein
a
b
c
d
e
f

Figure 1. Expression of Connexins in human amniotic fluid cells
A. RT-PCR analysis of Connexin (*CX*) expression in AF cells at 15 to 35 weeks (wks) gestation. AF cells expressed *CX26*, *CX43*, *CX45* in some/all gestation periods examined. *GAPDH* transcript and human HaCaT, NT2-D1 and NT2-N cells were used as internal and positive controls, respectively. NTC, No Template Control. HaCaT, Human keratinocyte cell line; NT2-D1, (NTera-2) human teratocarcinoma cell line and NT2-derived neurons (NT2-N). **B**. Western blot analyses confirmed the expression of CX43 protein in AF26 (top panel) cells. Embryonic day 18 (E18) mouse brain and β-ACTIN (ACTB) were used as positive and internal controls, respectively. **C**. Immunocytochemistry further verified the presence of CX43 and CX26 proteins in AF cell cultures. CX43 expression (green) was detected as punctuate staining in the perinuclear region and the cell membrane. CX43 appeared to be associated with Golgi Complex in the perinuclear region, as determined by Golgin-97 (red) and CX43 double staining (a). The punctuate staining pattern (a, c, e) demonstrated the dynamic translocation of CX43 protein from Golgi Complex (a) to the cell membrane (e, arrowheads). Unlike CX43, CX26 protein expression appeared limited to the perinuclear region (g). Nuclei were stained with Hoechst (blue). Panels b, d, f and h represent the corresponding phase contrast images of a, c, e and g; respectively. **D**. Dye coupling assessment in AF cells. AF donor cells (AF26) were pre-loaded with DiI (red) and Calcein (green) and plated as single cell suspensions onto confluent monolayers of receiving AF cells. Calcein transferred from the donor AF cell to adjacent receiving cells, indicating the formation of functional gap junctions between individual AF cells within 4 hrs. Scale bar: 25μm (C), 50 μm (D).

Dye coupling, consistent with the presence of functional gap junctions, was scored by Calcein transfer from labeled donor AF cells to recipient AF cells 4 hrs post-plating. Coupling was observed in an average of 9 ±1.06 SEM Calcein positive recipient cells coupled to one DiI positive AF cell (Figure 1D, a-f).

CX43 expression in C-KIT positive and single cell-derived clonal AF cell populations

Given the heterogeneity of AF cells [38], we used two protocols to establish a more homogenous cell population based on single cell cloning [38] and C-KIT expression [14], as previously reported. We generated single cell-derived clonal AF (AF-F5, described in Chapter 2) and C-KIT positive AF (AF-C-KIT, Figure 2A) cell populations and found a similar RNA expression profile for *CX43* and *CX45* (Figure 2B) and protein expression for CX43 (Figure 2C, a-d). Dye transfer experiments further confirmed the functionality of gap junctions formed in AF-C-KIT (Figure 2D, a-c) and AF-F5 (Figure 2D, d-f) cultures. Since standards for cell-based therapies require defined cell populations, henceforth, single cell derived AF-F5 cells were used in all subsequent experiments herein.

Intercellular communication between AF cells and cortical cultures

CX43 is considered to be the most ubiquitously expressed member of the connexin family in the mammalian brain and during brain development, specifically in neural progenitor cells and astrocytes [47]. Hence, we sought to determine whether AF cells could form functional gap junctions in co-cultures with cortical cells; in particular, with cortical progenitors and astrocytes which are known to express high levels of Cx43 [47] (Figure 3A-B and C-F;

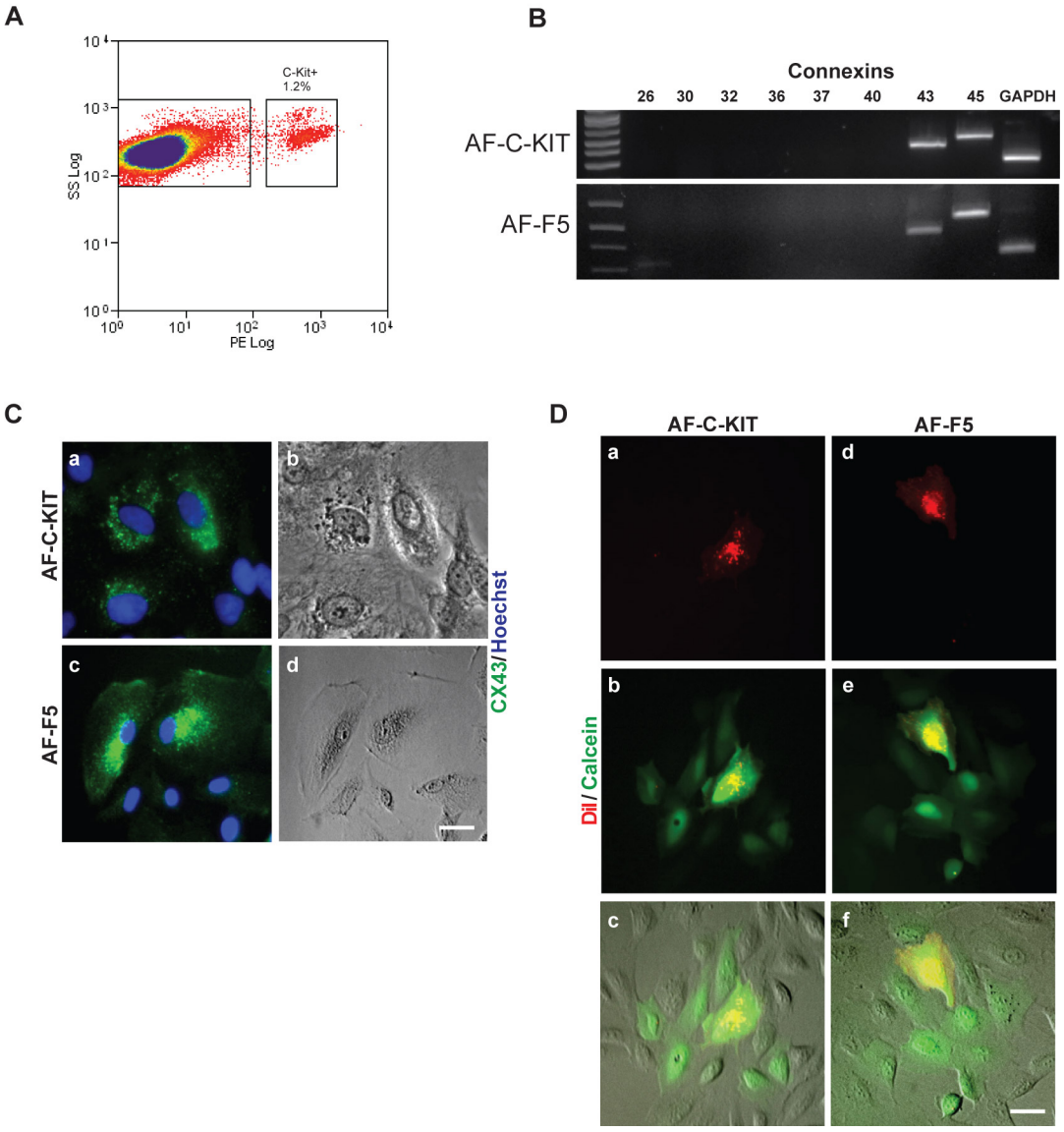


Figure 2. Connexin expression in C-KIT positive and single cell-derived AF clones
A. C-KIT positive AF cells were obtained by Fluorescence Activated Cell Sorting (FACS). **B**. RT-PCR analysis of Connexin (*CX*) expression in C-KIT positive (AF C-KIT) and single cell derived (AF-F5) AF clones. *GAPDH* transcript was used as an internal control. **C**. Immunocytochemistry confirmed the expression of CX43 (green) in AF C-KIT and AF-F5 cultures. Hoechst was used as a counter-stain (blue). (b) and (d) represent the corresponding phase contrast images. **D**. AF donor cells were pre-loaded with DiI (red) and Calcein (green) and plated as single cells on cultures of AF cells. Calcein transferred from the donor AF cell to neighbouring cells, confirming the formation of functional gap junctions among AF C-KIT cells as well as AF-F5 cells within 4 hrs. Scale bar: 25 µm (C), 50 µm (D).

respectively). Indeed, when AF cells were seeded on cortical cultures, CX43 was detected at the cell-cell boundary between AF cells and GFAP positive cortical astrocytes (Figure 4A-C, arrows). In parallel co-cultures, AF cells were distinguished from mouse cortical cells, using a human specific nuclear antigen (hNuc) (Figure 4D-I) or in some instances with a human specific mitochondrial antigen (hMito) (see Figure 6). Quadruple staining confirmed that the cortical cells, which formed gap junctions with AF cells, were GFAP positive astrocytes (Figure 4G-I). Interestingly, when AF cells were cultured alone, CX43 expression was predominantly cytoplasmic and perinuclear (Figure 1C, a-d); however, AF cells co-cultured with cortical cultures showed more membrane-bound CX43 staining between adjacent cortical astrocytes (Figure 4C,F,I, arrows). Even in the presence of cortical neurons, the majority of CX43 expression was observed at the boundary between AF cells and GFAP positive astrocytes (Figure 5A-B, arrowheads); whereas a negligible amount of Cx43 protein was observed between AF cells and neurons at the interface of neurites and the AF cell membrane (Figure 5A-B, arrows). Although the staining results suggest that AF cells form CX43 mediated gap junction with target cells, we performed dye transfer experiments (as outlined earlier) to confirm functional gap junction formation and intercellular communication between AF cells and cortical cultures. Coupling was observed in an average 15 ±5.21 SEM Calcein positive cortical astrocytes coupled to a single Calcein positive, DiI positive donor AF cell after 4 hrs (Figure 5C-E). Approximately 20% of seeded donor cells were able to transfer Calcein to cortical astrocytes. Of note, in earlier cortical cultures (2 days *in vitro*), AF cells were able to establish functional communication with cortical progenitor cells, which also express high levels of Cx43 (Supplementary Figure 1);

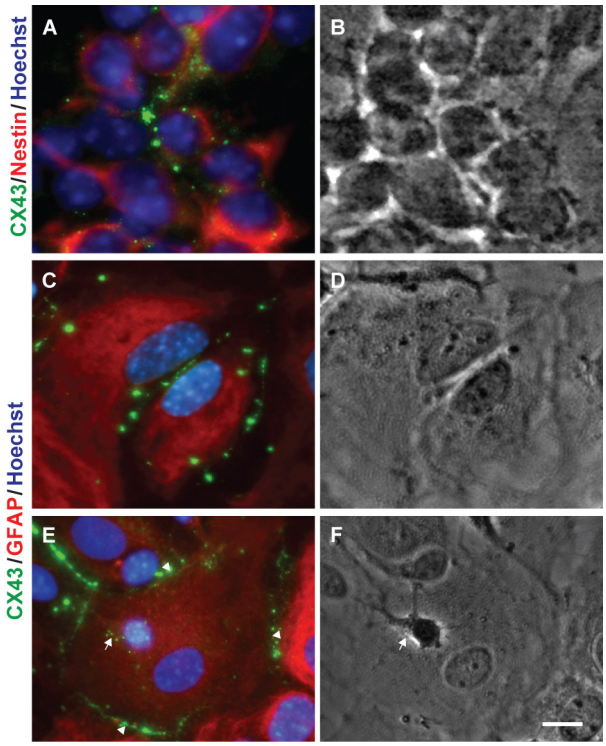


Figure 3. Cx43 expression in mouse cortical cells
A. Immunocytochemistry showed that Cx43 (green) is expressed in Nestin (red) positive cortical progenitors. **C**. Similarly, abundant levels of Cx43 protein were detected at cell-cell boundaries in cortical astrocytes. **E**. Only a limited amount of Cx43 was detected in immature neurons (arrow), whereas astrocytes maintained high levels of Cx43 expression. **B**, **D** and **F** are the corresponding phase contrast images of A, C and E, respectively. Nuclei were stained with Hoechst (blue). Scale bar: 10 μm.

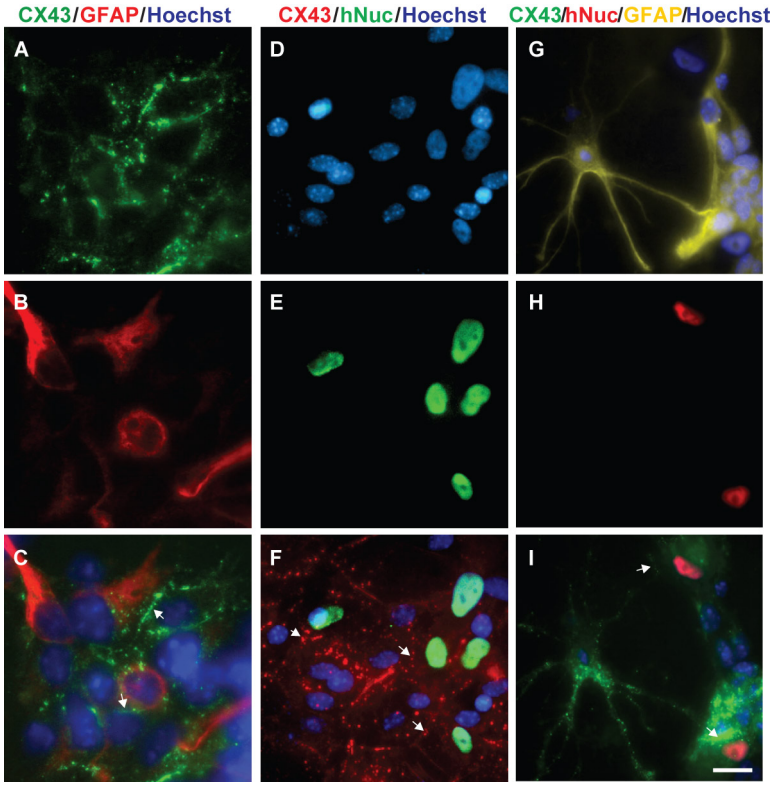


Figure 4. AF cells form gap junctions with cortical astrocytes *in vitro*
Immunocytochemistry showed that AF cells established gap junctions with cortical astrocytes. **A-C**. CX43 (green) was expressed at the boundary between AF cells and GFAP (red) positive cortical astrocytes. **D-F**. In parallel experiments, AF cells stained with an antibody against human specific nuclear antigen (hNuc, green), showed discrete, punctuate CX43 (red) staining at the cellular boundary with cortical cells (arrows). **G-I**. To determine the identity of cortical cells, similar cultures were stained with GFAP (yellow), hNuc (red) and Cx43 (green). Hoechst (blue) was used as a counter-stain. Scale bar: 8 µm (A-C), 15 µm (D-F), 20 µm (G-H).

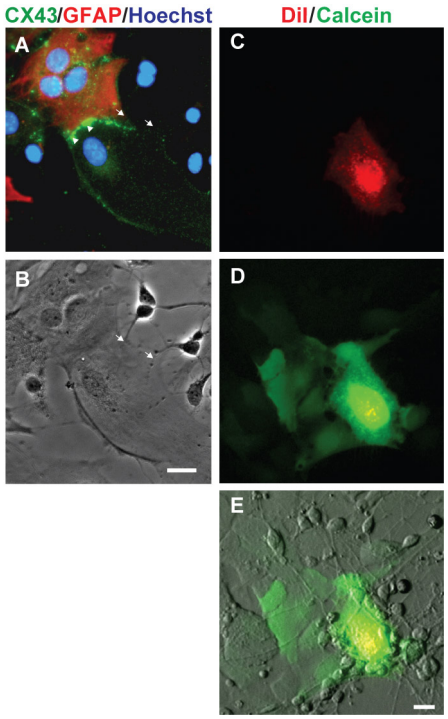


Figure 5. AF cells selectively establish intercellular communication with cortical astrocytes

A-B. Co-cultures of AF cells with cortical cells. Immunostaining showed abundant levels of Cx43 protein (green) at the junction between AF cells and cortical astrocytes (GFAP (red), see arrowheads), whereas, there was only a limited amount of staining detected at the boundary with neurons (arrows). **C-E**. AF cells were pre-loaded with DiI (red) and Calcein (green) and plated as a single cell suspension on cortical cultures to examine coupling. AF cells readily established functional gap junctional communication with astrocytes within 4 hrs, as indicated by Calcein transfer, a phenomenon not observed between AF cells and neurons. Scale bar: 10 μm.

however, functional communication was largely observed between AF cells and cortical astrocytes in later cultures (3 weeks; Figure 5C-E). We found very little functional communication between AF cells and mature neurons (data not shown). In support of this observation, Cx43 protein expression has been known to decrease significantly following neuronal differentiation [40, 48].

Cx43 expression following injury

As a response to brain injury, astrocytes proliferate and infiltrate the damaged region in an effort to preserve neural tissue and restrict inflammation [49]. Since Cx43 is the main protein expressed in both astrocytes and AF cells and results in functional gap junctional intercellular communication, we examined the interaction of AF cells with host cells in both *in vitro* and *in vivo* brain injury models. More specifically, we used an *in vitro* scratch wound model using cortical cultures, a well characterized model to investigate the astrocytic response to mechanical injury [50], as well as in an *in vivo* surgically induced brain injury model targeting the primary motor cortex [51]. Cortical astrocytes express abundant levels of Cx43 (Figure 6A, B) and following scratch-induced injury, migrate to mediate wound closure (Supplementary Figure 2). Although astrocytes maintain Cx43 expression following injury, they did not demonstrate the capacity to repair the wound within the first 24 hrs post-injury (Figure 6C, D). Glial processes were shown to protrude into the injured region within 48 hrs with astrocytes eventually filling the gap approximately 72 hrs post-injury (Supplementary Figure 2). In contrast, AF cells seeded onto injured cortical cultures, 15 mins after scratch injury, were able to adhere to the denuded area within 24 hrs and mediate wound closure 48 hrs post-injury (Figure 6E-F, arrows). In order to determine whether

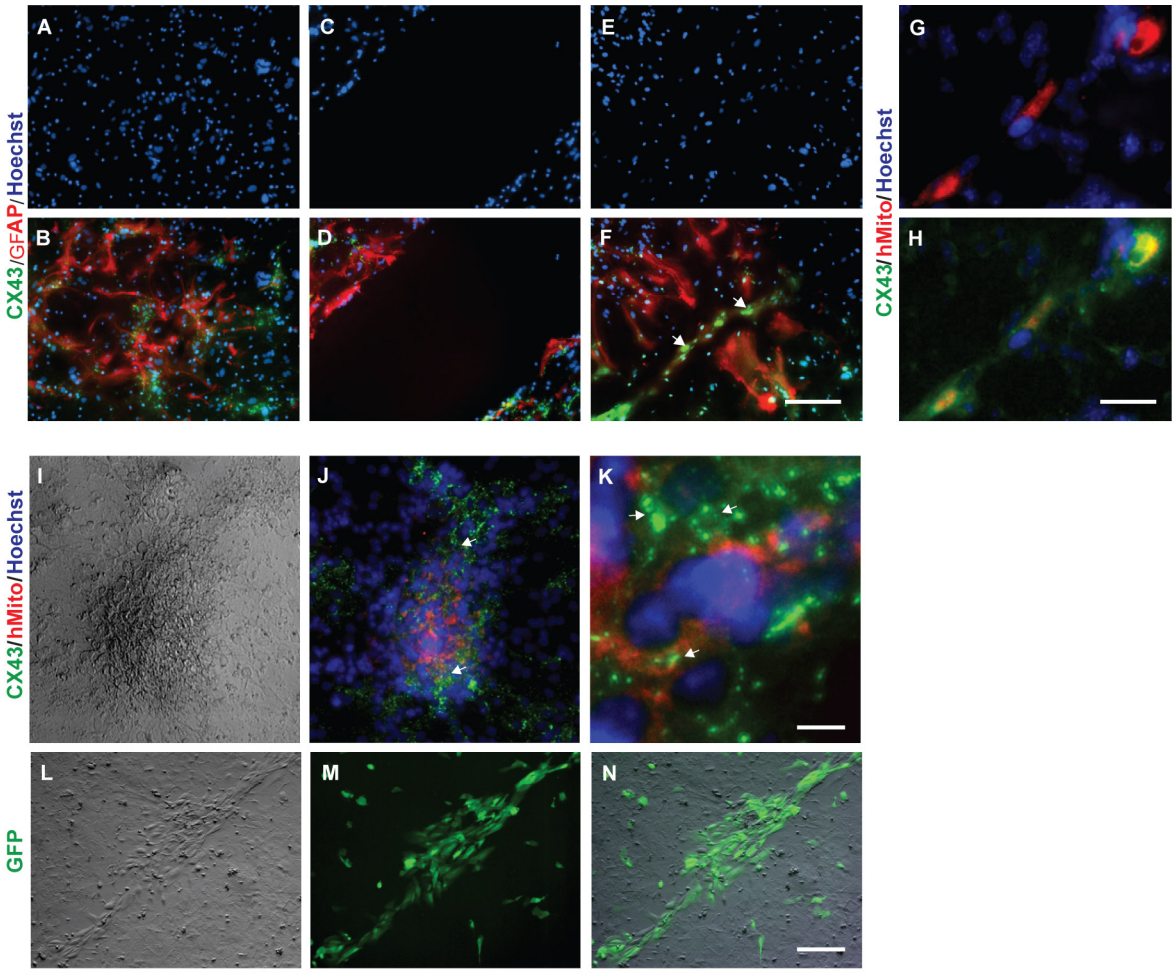


Figure 6. AF cells repair the scratch-induced wound injury in cortical cultures
A-B. A low magnification of confluent cortical cultures stained with Hoechst (A and B, blue), GFAP (B, red) and Cx43 (B, green). **C-D**. Scratch injury was applied to parallel cortical cultures and stained with the same markers 24 hrs later. GFAP positive astrocytes were seen at the scratch border (D), without repairing the wound. **E-F**. In contrast, when AF cells were seeded following scratch, they filled the injury site (F, arrows), maintained CX43 expression (F, green) and facilitated wound repair within 48 hrs. **G-K**. To further confirm AF cells located in the injury site, separate cultures were stained with human mitochondrial marker (hMito, red), CX43 (green) and Hoechst counter stain (blue). Cx43 was readily expressed at the boundary between AF cells and cortical cells (K). **L-N**. Live assays, using GFP tagged AF cells, were also used to confirm wound repair after scratch injury in cortical cultures. Scale bar: 150 µm (A-F), 35 µm (G-H), 90 µm (I,J), 10 µm (K), 80 µm (L-N).

seeded AF cells were able to establish connectivity with cortical astrocytes, via Cx43-mediated gap junction formation, we stained AF cells with a human specific mitochondrial antibody (hMito) and examined CX43 expression between AF and cortical cells (Figure 6G-K). CX43 was readily expressed at the boundary between AF cells and cortical astrocytes (Figure 6K). Complementary to these experiments, we performed a live assay by seeding GFP-tagged AF cells following injury and confirmed that AF cells readily filled the injury site, mediating wound closure (Figure 6L-N).

Using a mouse model of brain injury, the motor cortex was injured, as previously described [45, 51], resulting in a cavity that forms as a result of tissue loss and cell death (Figure 7B,D,F and Supplementary Figure 3) compared to sham (uninjured) brains (Figure 7A,C,E). Compared to the organized architecture of neurons and astrocytes in the control brain (Figure 7E), the damaged cortex showed significant neuronal loss, a disarray of neurite extensions and a significant infiltration of astrocytes to the injured area (Figure 7F). Since astrocytes exhibit a high degree of coupling through gap junctions, composed mainly of Cx43 [53], we examined the immunohistochemical distribution of Cx43 in the injured brain. Indeed, compared to the uninjured brain (Figure 8A,C), abundant levels of Cx43 were detected at the perimeter of the injury site (Figure 8B,D). Areas of intense Cx43 puncta were specifically observed in astrocytes within close proximity to the injury site (Figure 8F-G) compared to shams (Figure 8E). The up-regulation of Cx43 enhances GJIC in the brain post-injury. To determine whether gap junctions form between AF and cortical cells *in vivo*, and hence hold translational-relevance, we injected AF cells labeled with DsRed (AF-DsRed) into the injured motor cortex (Figure 9A-B). Immunohistological analysis revealed

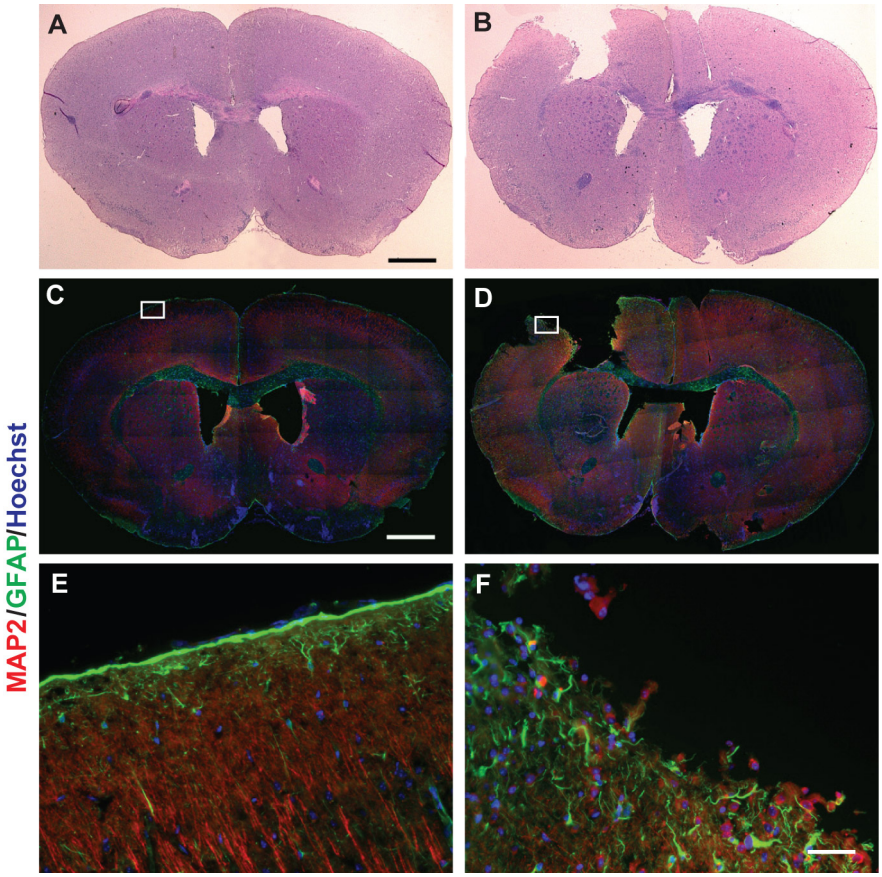


Figure 7. Cellular architecture in normal and injured motor cortices
A-B. Hematoxylin and eosin (H&E) staining of coronal sections from mice subjected to sham surgery (A) and motor cortex injury (B). **C-D**. Mosaic confocal images of adjacent sections show MAP2 (red) positive neurons and GFAP (green) astrocytes in sham (C) and injured (D) brains. **E-F**. Higher magnifications of the insets in C and D. In contrast to the well-organized architecture in the control motor cortex (E), there was a significant loss in the number of neurons, associated with an increase in astrogliosis in the injured cortex. Hoechst (blue) was used to label the nuclei. Scale bars: 1000 µm (A-D), 100 µm (E-F).

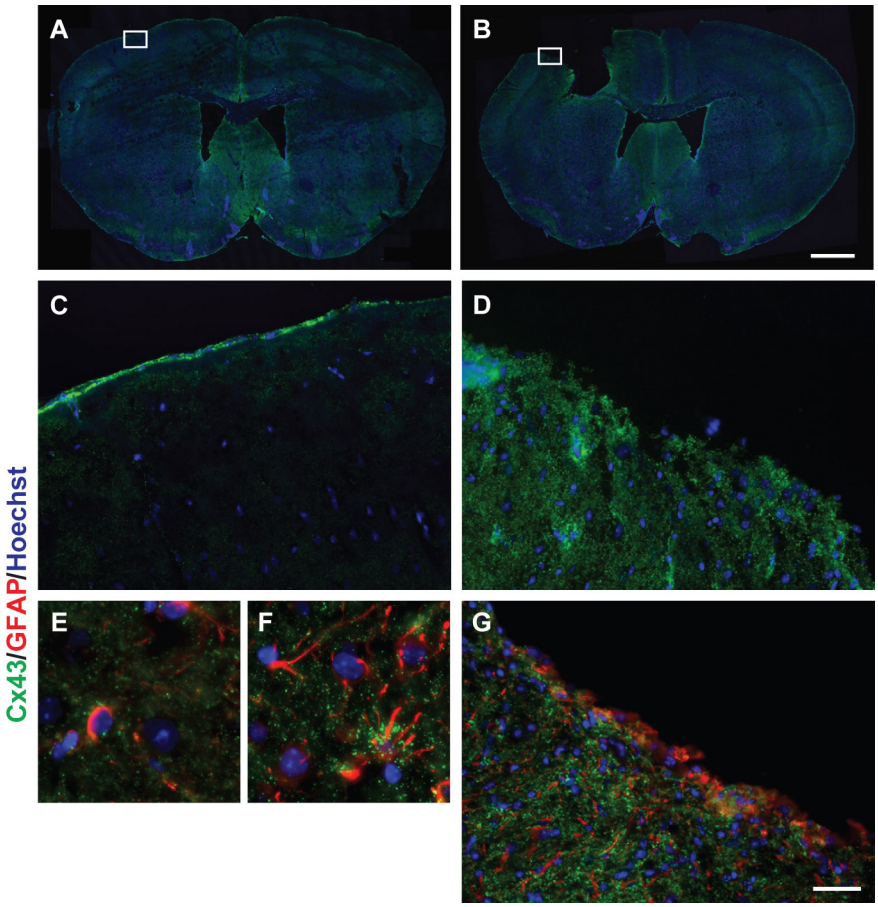


Figure 8. Cx43 expression in the control and injured motor cortices
Immunohistochemistry of Cx43 (green) in the brains of mice subjected to sham surgery (A,C,E) and motor cortex lesion (B, D, F,G). **A-B**: The stitched confocal images show the expression of Cx43 in the control (A) and injured (B) brains. **C-D**. Higher magnification images of the areas of motor cortex indicated by insets in A and B are shown in C (sham) and D (lesion). Increased Cx43 staining was observed in the cortex adjacent to the injury site compared to sham brains. **E-G**. Double-labeling with GFAP (red) and Cx43 (green) indicated that Cx43 was expressed mainly in astrocytes (red) and more abundant in the injured cortex (F,G), compared to sham cortex (E). Nuclei were stained with Hoechst (blue). Scale bar: 1000 µm (A-B), 100µm (C,D,G); 10 µm (E,F).

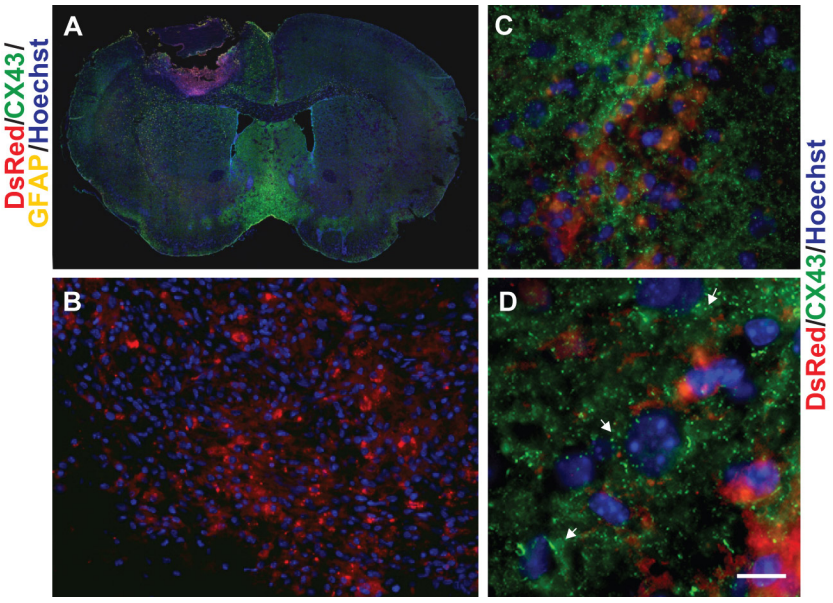


Figure 9. Gap junctions between AF cells and astrocytes following cell implantation into the injured motor cortex

A. Stitched confocal image of mouse brain after being subjected to motor cortex injury and receiving AF-DsRed (red) cell injection. Immunocytochemistry shows the distribution of Cx43 protein (green) at the interface between AF-DsRed cells and injured host tissue. **B**. A higher magnification of the implant confirmed that AF-DsRed cells could be easily traced in the injured brain. **C-D**. Intense Cx43 expression (green) was observed at the site of implantation. More specifically, distinct Cx43 immunostaining was detected at the junction between AF-DsRed (red) and cortical cells (see arrows). Hoechst was used as a counter stain (blue). Scale bars: 1 000 µm (A), 100 µm (B), 50 µm (C), 7 µm (D).

abundant Cx43 expression in the implanted area surrounding AF-DsRed cells (Figure 9C-D). At 12 days post injection, the majority of AF-DsRed cells were located within the injury site and needle tracks (Figure 9C), accompanied by CX43 expression surrounding the injection site. In fact, Cx43 expression was observed at the junction of AF-DsRed and neighbouring cells, as seen at higher magnification (Figure 9D). Although the long-term outcome of AF cell implantation into the motor cortex injury model has not been examined, abundant Cx43 expression between cortical astrocytes and AF cells suggests a means for the establishment of GJIC following AF cell engraftment.

Discussion

Functional graft integration following injury in the CNS is limited, in part, by the potential absence of intercellular communication between grafted cells and host tissue. Thus, it is expected that enhancement of connexin-mediated intercellular gap junction formation would result in improved cell-cell communication between host and graft cells, as a prelude to developing functional connectivity with host cells to support neuroregenerative and protective capabilities. Since AF cells have attracted a great deal of attention as an alternative cell source for cell-based therapies, we examined their potential to form gap junctions and found that AF cells express abundant levels of CX43. Using well-established methods to isolate homogenous C-KIT and single cell-derived AF cell clones, we demonstrated that CX43 plays an important role in intercellular communication among AF cells. These results are in agreement with the expression of CX43 in other stem cell populations such as ES [54], NT2/D1 [43] and P19 [55] cells. In fact, CX43 is the most prevalent connexin protein in vertebrates (see [56] for review), expressed in at least 34 tissues and 46 cell types [57, 58], where it plays a critical role in coordinating tissue functions and cellular homeostasis.

In the brain, Cx43 is highly expressed in the developing cortex and maintains its expression in cortical astrocytes throughout adulthood [56, 59, 60]. The presence of active gap junctions in astrocytes allows the regulation of glucose and oxygen delivery to neurons for their energetic and metabolic needs [61, 62]. For instance, Cx43 mediates the transfer of lactate from astrocytes to neurons as energy substrates and facilitates the synthesis of neurotransmitters for synaptic activity [62, 63]. Similarly, the delivery of glucose and

oxygen from the blood to the brain is regulated through an astrocytic network, which is dependent on Cx43 [62]. Changes in both spatial and temporal CX43 protein expression are seen following various types of CNS pathologies such as ischemia, neurodegenerative disorders and traumatic injury [64]. These events result in severe disturbances in cellular ion homeostasis, energy depletion, oxidative stress and the initiation of cascading events leading to necrosis and/or apoptosis resulting in functional impairments. Following brain injury, an infiltration of Cx43 positive reactive astrocytes is readily observed in the injured core [60]. The induction of CX43 expression is a substantial factor in the astroglial response and may mediate protection by buffering neurons from excitotoxicity and hypoxic depolarization by taking up extracellular glutamate and potassium, which might enhance the survival of neighbouring neurons by protecting against elevated excitotoxic and oxidative stress [33, 65, 66]. Consistent with these observations, we observed an increase in Cx43 expression in cortical astrocytes at the injury site and hence the number of gap junction plaques formed at the interface between neighbouring cells. The temporal pattern of CX43 expression post-injury suggests a window of opportunity, which may facilitate the formation of Cx43 mediated gap junctions with AF cells implanted in close proximity to the injury site. These gap junctions, which are established rapidly, may permit AF cells to directly influence host network activity, through an exchange of ions and molecules, resulting in the buffering and maintenance of homeostasis following injury. Several molecules are gap junction permeable including small ions (Na^+, K^+, Ca^{2+}, H^+, CL^-), second messengers (cAMP), amino acids (glycine, glutamate) and metabolites (glucose, adenosine, ATP) [67-69]. Hence, GJIC may permit AF cells to participate in endogenous ion homeostasis and influence inter and intracellular calcium alterations and extracellular potassium and glutamate uptake, which

can buffer the excitotoxic response following injury and potentially enhance the survival and function of neuronal populations. Accordingly, increased Cx43 expression post-injury has been shown to mediate a neuroprotective effect [70] further supported by the observation that Cx43-null mice demonstrate increased apoptosis and lesion volume post-injury [71, 72].

Cx43-mediated GJIC between grafted NSCs and brain cells appears to be an essential participant in the neuroprotective effect associated with NSC engraftment, particularly at the connexin-associated gap junction interface [73]. Utilizing NSCs grafted into an *ex vivo* model system for striatal tissue, Jäderstad *et al.* [73] found that Cx43 expression transiently peaked in host cells following traumatic stimulation, suggesting a window of opportunity for NSCs to establish gap junctions with the host tissue and potentially mediate neuroprotection. It has been proposed that grafted NSCs can protect impaired host cells post-injury through paracrine support by secreted trophic factors as well as buffering of harmful levels of ions and molecules and induction of beneficial calcium signaling patterns, the latter of which is dependent on GJIC [33, 73-75]. In fact, both *in vitro* and *in vivo* beneficial effects of NSC transplants were abrogated when Cx43 gap junction formation or function was suppressed by pharmacologic, and/or RNA-inhibition strategies, supporting a role for gap junctional coupling in some modulatory, homeostatic and protective actions on the host microenvironment [33, 73]. Nevertheless, the direct mechanisms underlying how gap junction coupling facilitates NSCs' beneficial effects *in vivo* remains to be determined. Since AF cells express high levels of CX43 and form functional gap junctions with astrocytes, they have the capacity to mimic a similar connexin-mediated neuroprotective effect during this critical time frame post-injury. In fact, preventing damaged cells from

dying has emerged as one of the possible benefits of stem cell transplantation through neurotrophic support [1, 2, 25] and more recently through the described direct cell-cell communication via gap junctions [33, 73]. In fact, cell-cell coupling has been regarded as an early form of communication that precedes and acts as a template to establish subsequent electrochemical synapses [73]. In this instance, implanted AF cells expressing CX43 may form gap junctional coupling with astrocytes to possibly preserve neurons at the injury site. In support of this view, the role of astrocytes in early stages of neuroprotection is gaining more recognition and initial AF cell-astrocyte interactions may play an important role during the early stages in graft-host interactions [37]. This notion is further substantiated by findings *in vitro,* which confirm that astrocytic Cx43 gap junctions and hemichannels remain functionally open following injury, which is accompanied by changes in both spatial and temporal Cx43 expression, following various models of CNS injury (Reviewed in [70]). The enhanced subcellular translocation of CX43 from the perinuclear compartment to the membrane boundary between AF cells and cortical astrocytes can facilitate CX43 mediated GJIC *in vivo*, help buffer pathological stimuli as well as serve as a platform to deliver beneficial factors through direct communication. In fact, another implication of AF cell-host GJIC is coupled to recent findings where Cx43 gap junctions have been shown to allow cell-to-cell transfer of short interfering RNA [69] and microRNAs [36], potentially allowing grafted cells to directly inhibit gene expression in adjacent host cells. Gap junctional coupling between endogenous and host cells thereby can represent a new approach for the delivery of regulatory factors with potential for gene therapy applications [76-78]. Interestingly, short-term hypoxic preconditioning of NSCs has been shown to increase Cx43 expression and improve subsequent graft and host communication, which makes

preconditioning an attractive strategy for future engraftment studies to facilitate GJIC post implantation [79]. Lastly, CX43 has also been observed at the borders of AF and cardiac cells, following transplantation into the heart [80], further supporting the application of AF cells in regenerative medicine through the formation of functional gap junctions *in vivo*.

Gap junctions provide an essential means for direct cellular communication between grafted and host cells post implantation. Several molecules such as second messengers, metabolites, ions, amnio acids and even regulatory genetic material are gap junction-permeant and may influence host homeostasis post-injury. Identifying which trans-cellularly passed molecules are beneficial post-injury is crucial in developing novel therapeutic strategies to alleviate neuronal damage and/or promote endogenous neuroprotection post-injury. Although we have shown that AF cells can form functional GJIC with cortical astrocytes *in vitro*, their ability to so *in vivo* and to a scale sufficient enough to mediate a neuroprotective effects remains to investigated. Since the exogenous delivery of neurotrophic factors has been shown to mediate neuroprotection in host tissue post-injury, in Chapter 4 we set out to use AF cells, engineered to express glial cell derived neurotrophic factor (GDNF), as an alternative approach to enhance endogenous neuroprotective mechanisms following implantation into the injured motor cortex.

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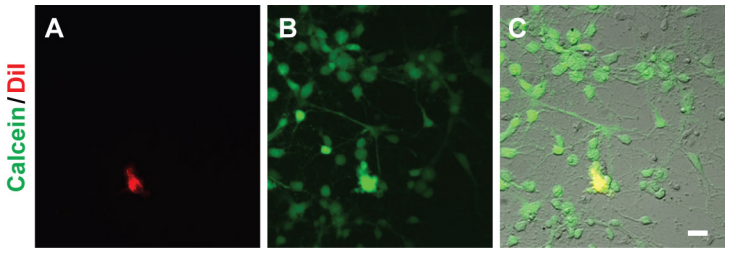
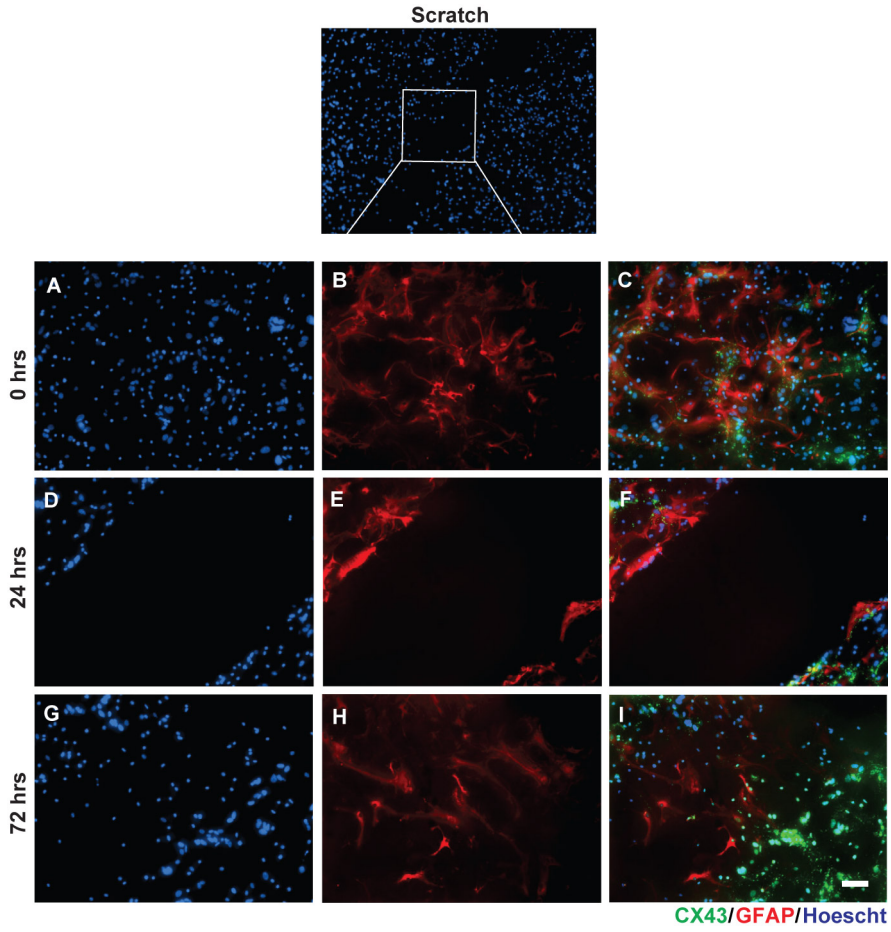
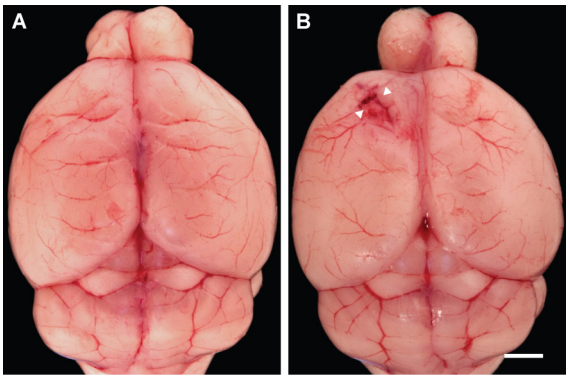


Supplementary Figure 1. AF cells form gap junctions with neural progenitors *in vitro* **A-C**. AF cells were pre-loaded with DiI (red) and Calcein (green) and plated as a single cell suspension on neural progenitor cultures to examine coupling. AF cells readily established functional gap junctional communication with neural progenitors within 4 hrs, as indicated by Calcein transfer, a phenomenon not observed between AF cells and mature neurons. Scale bar: 50 μm.



Supplementary Figure 2. Scratch induced injury in cortical cultures
A low magnification of confluent cortical cultures stained with Hoechst (blue) showing a scratch injury. **A-B**. Non-scratched cortical cultures stained for GFAP (red), Cx43 (green) and Hoechst (blue). **D-F**. Scratch injury was applied to parallel cortical cultures and stained with the same markers 24 hrs later. GFAP positive astrocytes were seen at the scratch border (E). **G-I**. Glial processes were shown to protrude into the injured region within 48 hrs with astrocytes eventually filling the gap approximately 72 hrs post-injury. Scale bar: 150 μm.



Supplementary Figure 3. Motor cortex injury
Whole un-injured (A) and injured (B) brains from non-perfused mice. Arrowheads indicate injury in the left motor cortex after removing the neural tissue at a depth of 1 mm. Scale bar: 1.6 mm.

Chapter 4 - Neuroprotective effects of GDNF-expressing human amniotic fluid cells

Neuroprotective effects of GDNF-expressing human amniotic fluid cells

Anna Jezierski[1,2], Kerry Rennie[1], Bogdan Zurakowski[1], Maria Ribecco-Lutkiewicz[1], Julie Haukenfrers[1], Abdellah Ajji[3], Andrée Gruslin[2,4], Marianna Sikorska[1], and Mahmud Bani-Yaghoub[1,2]

[1] Neurogenesis and Brain Repair, Neurobiology Program, Institute for Biological Sciences, National Research Council Canada, Ottawa, Canada.

[2] Department of Cellular and Molecular Medicine, Faculty of Medicine, University of Ottawa, Ottawa, Canada

[3] Department of Chemical Engineering, Polytechnique Montréal, Montréal, Québec

[4] Department of Obstetrics and Gynecology, Faculty of Medicine, University of Ottawa, Ottawa, Canada

Abstract

Brain injury can result in severe impairments due to the irreversible loss of tissue and function by disrupting cortical integrity and functionality. Accordingly, brain injury continues to be one of the leading causes of disability worldwide. Despite decades of research, there is currently no pharmacologically effective treatment for preventing neuronal loss and repairing the brain. Neurotrophic factors, such as Glial cell derived neurotrophic factor (GDNF), have been shown to have a potent neuroprotective effect in experimental models of brain injury; however, penetrance of the blood brain barrier and delivery to the vulnerable cells remains a major obstacle in the brain. As a result, novel therapeutic approaches, such as cell-based therapies, are being actively pursued to repair tissue damage and restore neurological function after injury. In this study, we examined the neuroprotective potential of amniotic fluid (AF) cells, engineered to secrete GDNF (AF-GDNF), in a surgically induced model of brain injury. Our results show that GDNF pre-treatment of AF cells and cortical neurons, 2 hrs prior to exposure of hydrogen peroxide, significantly increases cell survival rates compared to untreated cells. Since improving the efficacy of cell transplantation depends on enhanced graft cell survival, we investigated whether AF-GDNF cells seeded on polyglycolic acid (PGA) scaffolds could enhance graft survival following implantation into the lesion cavity. Encouragingly, the AF-GDNF/PGA implants survived longer in serum-free conditions compared to control cells and continued to secrete GDNF post-implantation into the injured motor cortex. GDNF delivery in the acute period following injury was sufficient to activate the MAPK/ERK signaling pathway in host neural cells in the peri-lesion area, potentially boosting endogenous neuroprotective pathways. Despite some promising trends, we did not observe significant behavioural

improvements following AF-GDNF/PGA implantation on the beam-walk and cylinder tasks or reduced lesion volume during the 7 day timeframe. In this study, we demonstrate that AF cells represent a new potential human cell source for the delivery of GDNF to the injured brain in mediating a neuroprotective effect.

Introduction

Brain injury, whether surgically induced or as a result of trauma or stroke, causes cellular dysfunction, neuronal death and the loss of neural connections in the acute period following injury resulting in functional impairments [1]. Despite considerable progress, pharmacological interventions do not yet effectively prevent cell death, minimize behavioural deficits or enhance recovery. Hence, there is growing interest in novel therapeutic approaches, such as cell-based therapies, to repair tissue damage and restore neurological function following brain injury [2, 3]. Stem cells have been proposed as a powerful tool in the treatment of several human diseases, both for their ability to represent a source of new cells to replace those lost due to tissue injuries or degenerative diseases and for their ability to produce trophic factors able to minimize damage and promote recovery. In fact, recent advances in the stem cell field have boosted efforts to explore the therapeutic potential of stem cells to enhance the repair of tissues following brain injury (Reviewed in [3-6]). However, ultimately the most appropriate cell source for cell-based therapies in the injured brain will depend on several key factors including accessibility, scalability, safety, efficacy and ethical considerations. To this end, embryonic stem (ES) cells are subject to ethical considerations due to their derivation and the added risk of tumorigenicity; whereas, adult stem cells are hindered by reduced proliferative rates limiting their numbers for use in transplantation studies. Therefore, other stem cell sources, such as fetal human amniotic fluid (AF) cells are being actively pursued as a novel source of therapeutic cells. AF cells are routinely obtained by amniocentesis and have been used traditionally as a diagnostic tool for genetic anomalies. As such, these cells are readily available, easily procured and expanded in culture and not subject to the ethical issues or *in vivo* teratoma formation that is

commonly associated with the use of ES and induced pluripotent stem cells. More importantly, sub-populations of AF cells, with stem cell characteristics, have been isolated based on C-KIT expression and shown to harbour the potential to differentiate towards the three embryonic germ layers [7]. These characteristics, along with their low antigenicity [8-11], have made AF cells a promising alternative source of cells for use in tissue engineering and cell-based therapies. In recent years, AF cells have been studied in a variety of experimental models of injury and disease [12-14] and their application for nervous system repair has met with some success [15-23].

Neuroprotection, through the production or delivery of neurotrophic factors, to stimulate an endogenous neuroprotective response in the injured brain is a promising application of cell-based therapies. Neurotrophic factors promote neuronal survival through receptor-mediated processes and are thought to be involved in nervous system development, maintenance and response to injury [24, 25]. In particular, Glial cell line-derived neurotrophic factor (GDNF), has received considerable attention as a potent neurotrophic factor on a wide spectrum of neuronal populations in different experimental models of brain injury [26]. As a member of the transforming growth factor (TGF)-β superfamily, GDNF acts through a unique multicomponent receptor system in which GDNF family receptor alpha 1 (GFR-α1) provides the ligand-binding component and the transmembrane Ret tyrosine kinase functions as the signal transduction element activating several intracellular signaling cascades [27]. Specifically, the Mitogen-activated protein kinase (MAPK)/Extracellular signal-regulated kinase (ERK) signaling pathway has been shown to be involved in GDNF-induced neuronal survival, differentiation and neurite outgrowth (reviewed in [28]).

GDNF was initially identified as a neurotrophic factor required for the survival of dopaminergic neurons [29] and has been since used extensively in a number of human clinical trials for the treatment of Parkinson's Disease (PD) [30-35]. In addition to its described role in PD, GDNF has also been shown to promote differentiation and migration of cortical GABAergic neurons [36], promote progenitor proliferation in the adult hippocampus and substantia nigra [37] and reduce cerebral infarction and neuronal damage induced by ischemic injury following middle cerebral artery occlusion (MCAO) [38]. Accordingly, in an effort to enhance the neuroprotective potential of cell-based therapies, stem cells have been genetically engineered to secrete neurotrophic factors as biodelivery vehicles to enhance the protection of damaged tissues through genetic modification. The neuroprotective effect of exogenously delivered GDNF has been demonstrated in different experimental models of focal and global brain ischemia, by local administration of the neurotrophic factor, using viral vectors carrying the GDNF gene, and by transplantation of GDNF-expressing cells (reviewed in [26]). For instance, delivery of GDNF through transplantation of human fetal and immortalized C17.2 neural progenitor cells, engineered to secrete GDNF, has been shown to improve cognitive function and enhance survival and neuronal differentiation in traumatic brain injury models [39, 40]. In addition, GDNF-expressing bone marrow derived stem cells and human neural stem cells (NSCs) have been shown to provide neuroprotection for rats with intracerebral hemorrhage and stroke [41]. More recently, AF cells have also been engineered to secrete GDNF and shown to ameliorate motor deficits and reduce infarct size in rats subjected to sciatic nerve crush injury and MCAO, respectively [20, 42, 43].

Despite the promise of cell-based therapies for brain injury, the poor survival of engrafted cells, due to the loss of vast amount of parenchyma, formation of a cavity and an inhospitable injured core, is a major limitation in cell transplantation studies [44]. Hence, combining cells with a synthetic polymer scaffold delivery system, implanted at the target site, provides a promising strategy for increasing cell viability and localized neurotrophic factor delivery [45, 46]. Scaffolds provide a three-dimensional lattice that can be engineered to support cell attachment and survival *in vitro* as well as serving as a temporary extracellular matrix after transplantation *in vivo* [45]. Furthermore, damage to the brain produces immediate and delayed cell death leading to the formation of a lesion cavity. It is known that the lack of regeneration in the brain might arise from the complex cellular and molecular environment in the damaged area and the lack of structural continuity, which makes cellular adhesion impossible [46]. Placing a scaffold into the damaged area or cavity may additionally provide support for the surrounding brain tissue, function as a substrate for cell growth, neurite formation and allow cell infiltration (reviewed in [47]) to promote repair after brain injury. In fact, implantation of a scaffold into a cortical cavity in rats decreased cell death, secondary cell loss and lesion growth and supported neural cell growth [48]. Biocompatible, biodegradable polymers, from both synthetic and naturally occurring sources, have been used as scaffold materials [47]. In the central nervous system (CNS), the most widely used synthetic scaffolds are poly(α-hydroxy esters), such as polyglycolide (PGA), polylactide (PLA) and polylactide-*co*-glycolide (PLGA) [47]. Specifically, PGA is a synthetic, biodegradable polymer that is an attractive template for cell transplantation into the brain [46, 49, 50]. In addition to being approved by the Food and Drug Administration (FDA) [51, 52], PGA is easily shaped (i.e. tailored to fit into the cavity created by injury)

and can be modified for optimal mechanical strength, biocompatibility and biodegradability [53]. Importantly, PGA scaffolds have been shown to provide a matrix to support NSCs *in vivo*, allow diffusion of nutrients to implanted cells, become vascularized and eventually degrade alleviating concerns of long-term biocompatibility [49, 50, 54-56]. Encouragingly, the injured brain has been shown to interact reciprocally with mouse NSCs supported by a PGA scaffold to reconstitute lost tissue following stroke [44]. Furthermore, results from our laboratory demonstrate that PGA polymer scaffolds permit optimal adhesion, survival and differentiation of mouse neural cells in addition to providing a platform for the delivery of cytokines, growth factors and neurotrophic factors [57]. Hence, in this study, we examined the virally mediated gene delivery of GDNF from implanted AF cells, seeded on PGA scaffolds, in mediating neuroprotection in a surgically induced model of brain injury which allows us to simulate the cortical and parenchymal damage resulting in significant behavioural deficits that are observed following neurosurgical procedures [58].

Materials and Methods

Cell Culture

Human amniotic fluid (AF) cells were obtained from the Ottawa Hospital (Ottawa, Ontario, Canada), following amniocentesis in women at 15 to 35 weeks of gestation (AF15- AF35). The study was approved by the Ottawa Hospital and National Council Canada-Research Ethics Boards and a written informed consent was obtained from each donor. Single cell derived AF-clones (AF-F5) were cultured in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) supplemented with 20% Fetal Bovine Serum (FBS, Hyclone) and maintained at 37°C and 5% CO_2 (as previously described [59]). AF-derived single cell clones (AF-F5), henceforth referred to as AF cells, were used in this study and derived as previously described [60]. AF cells were passaged at 70% confluency every 2–3 days, using 0.05% Trypsin/EDTA (Invitrogen) as a 1:3 split ratio.

Mouse cortical progenitors were isolated from the Embryonic day 13 (E13) ventricular zone, plated onto poly-L-lysine (PLL)-coated coverslips (9×10^5 living cells/ml) in DMEM + 10% FBS and examined within 24 hrs after plating, as previously described [61]. Cortical neurons were generated from neural progenitors by reducing the serum concentration (i.e., 0.5% FBS) during the first 24 hrs, followed by treatment with DMEM + N2 supplement (Invitrogen) to limit the generation of glial cells. Medium was replenished every 48 hrs for 7 days [61].

RNA Extraction and RT-PCR

Total RNA was extracted from cells, using TriReagent (Molecular Research Centre), as previously described [59]. Total RNA was quantified with NanoDrop (Thermo Fisher Scientific) and 1 μg was reverse transcribed (RT) using Quantitect Reverse Transcriptase (Qiagen). Quantitative PCR (QPCR) was performed using Fast SYBR Green Master Mix (Bio-Rad) on 7500 Fast Real-Time PCR System (Applied Biosystems). Approximately, 10 ng of cDNA was used per individual reaction with primer concentrations of 5 μM. Quantitative PCR amplifications were performed using the following conditions: Initial denaturation at 94°C, 20 secs and 40 cycles at 94°C, 5 secs; 60°C, 30 secs. All data was normalized to ACTB, using the ΔΔCT method and specific primers were designed using publicly available Primer3 software [62].

Table 1. Sequence and annealing temperatures of primers for QPCR

Designation	Sequence (5'-3')	Annealing Temp. (°C)	Amplicon Size (bp)
mGDNF-F	TCGGCCGAGACAATGTATGA	60	79
mGDNF-R	CAACATGCCTGGCCTACTTTG	60	
mGFRα1-F	CCAGCGGGAACTCCTTTGT	60	79
mGFRα1-R	GCCCTGTAGCAGTTCTTCAACA	60	
mGFRα2-F	CACCACCTGCACATCTATCCA	60	79
mGFRα2-R	GAGCTCTGTGAAACACATGCTTAAC	60	
mGFRα3-F	CAGACCCACTGTCATCCTATGGA	60	75
mGFRα3-R	CAGGTATGCCCGCAGACAT	60	
mGFRα4-F	GGCAGAAACAGTCCTTGTTTTGT	60	75
mGFRα4-R	GGAGAGCCAGGGCAGTGA	60	
mc-Ret-F	TGACCATGGGTGACCTCATCT	60	77
mc-Ret-R	TACAAGCTTCATTTCTGCCAAGTACT	60	
mACTB-F	GCTCTGGCTCCTAGCACCAT	60	75
mACTB-R	GCCACCGATCCACACAGAGT	60	

Generation of GDNF lentivector (pTet07CSII-CMV-GDNF-DsRed)

A third generation lentiviral vector (pTet07CSII-CMV-DsRed, a generous gift from Dr. Bernard Massie, NRC, Montreal, QC) and a commercially available plasmid containing the full-length cDNA sequence of human GDNF (pCR-BluntII-TOPO-GDNF, Invitrogen) were used to clone a constitutively active GDNF lentivector (pTet07CSII-CMV-GDNF-DsRed). Briefly, the full-length GDNF fragment was cut from the pCR-BluntII-TOPO-GDNF plasmid with endonucleases XhoI and BamHI (New England Biolabs) and sub-cloned into pTet07CSII-CMV-DsRed lentivector, which was linearized with the same endonucleases for directional cloning (Supplementary Figure 1). The GDNF DNA fragment was ligated upstream of an Internal Ribosome Entry Site (IRES) and the gene encoding Discosoma red fluorescent protein (DsRed), using T4 DNA ligase (New England Biolabs). The resulting vector plasmid, pTet07CSII-CMV-GDNF-DsRed, represents a third generation lentivector with the transgenes GDNF and DsRed separated by an IRES under the control of a single Cytomegalovirus (CMV) promoter. Sequence analysis confirmed that there were no mutations in the pTet07CSII-CMV-GDNF-DsRed; the pTet07-CMV- DsRed backbone vector was used as a control. The plasmids were prepared and expanded from transformed *Escherichia coli* DH5α chemi-competent cultures, using Qiagen MaxiPrep kits (Qiagen), as per manufacturer's instructions.

Transfection and lentiviral production

The human embryonic kidney packaging cell line HEK293SF-PacLV (293SF) was used to produce the third generation GDNF lentivirus [63]. This cell line constitutively expresses the lentiviral proteins gag/pol, while expressing rev and VSV-G under the control of the

transcriptional regulators Cumate (50 μg/ml, Sigma) and Doxycycline (Dox, 1 μg/ml, Sigma) [63]. Briefly, 293SF cells were plated the night before in a 10 cm culture dish in FreeStyle EX media (Invitrogen). Fifteen micrograms of the pTet07CSII-CMV-GDNF-DsRed or pTet07CSII-CMV-DsRed control vector was diluted in 300 μl of serum free medium (DMEM) and incubated at room temperature for 20 mins with 15 μl of Lipofectamine2000 (Invitrogen), also previously diluted into 300 μl of DMEM. The DNA-Lipofectamine complex was added drop-wise to 293SF cells at 70% confluency. Medium was replaced 7 hrs post-transfection and the transfection efficiency was estimated based on DsRed expression 24 hrs later, using fluorescence microscopy (Zeiss). The following day, the medium was replaced with fresh DMEM medium supplemented with 1 μg/ml Dox and 50 μg/ml Cumate for the induction of the rev and VSV-G. The virus conditioned medium was collected and replenished daily for a span of 3 days, filtered through a 0.45 μm low protein binding filter (Millipore) and concentrated using Lenti-X Concentrator (Clontech), as previously described [64]. The concentrated virus was either used directly to infect AF cells (Figure 1A) or stored at −80°C in 10% FBS + DMEM for future use. Following infection, cells were enriched by Fluorescence Activated Cell Sorting (FACS) sorting based on DsRed expression (Figure 1B) using a MoFlo Cell Sorter (Beckman Coulter, StemCore Laboratories).

GDNF ELISA

Conditioned media from cultures of GDNF-secreting AF cells (AF-GDNF-DsRed) and control AF-DsRed cells were collected at various time points post-infection. GDNF protein levels were determined using a GDNF ELISA kit, according to the manufacturer's

instructions (Promega). In brief, Maxisorp 96-well flat-bottomed ELISA plates (Nunc) were coated with anti-GDNF monoclonal antibody diluted in carbonate coating buffer (pH 8.2) and incubated overnight at 4ºC. Plates were then incubated in blocking solution for 1 hr at room temperature. GDNF standards ranging from 0 to 1000 pg/ml and undiluted samples were added and incubated at room temperature for 6 hrs with shaking and washed with TBST. The captured GDNF was incubated with a polyclonal antibody specific to GDNF overnight at 4ºC. After washing, the amount of bound GDNF was detected by a specific horseradish-peroxidase conjugated antibody following a 2 hr incubation, with shaking, at room temperature. Plates were then washed, and incubated with 100 µl of enzyme substrate (TMB solution) for 15 mins at room temperature. The enzyme reaction was stopped by adding 100 µl of 1N HCl (Sigma) per well and the absorbance at 450 nm was recorded on a SpectraMAX (Molecular Devices) microplate reader. GDNF levels were calculated from the standard curve in the linear range. To determine *in vivo* GDNF secretion from AF-GDNF seeded scaffolds, cell and brain tissue extracts were prepared using a GDNF lysis buffer (137 mM NaCl, 20 mM Tris (pH 8.0), 1% NP40, 10% glycerol), containing a protease inhibitor cocktail [63] and processed as described.

Cytotoxicity Assay

AF cells were exposed to media alone or varying concentrations of GDNF (1.5 and 10 ng/ml; R&D Systems) and following a 2 hr pre-treatment, hydrogen peroxide (H_2O_2) at 200 µM was added to the cultures for 18 hrs. Surviving, adherent cells were scored based on Carboxyfluorescein Diacetate (CFDA, Invitrogen) fluorescence (a cell-permeant esterase substrate that can serve as a viability probe) and the data were expressed as percent

surviving cells. Cellular fluorescence was quantitated using a CytoFluor (Millipore) fluorescence measurement system with excitation filter 480/20 nm and emission filter 530/25 nm. GDNF secreting cells (3 x 10^5 cells; AF-GDNF-DsRed) were seeded onto 0.4 μM pore size Transwell filters (Costar) in 12-well tissue culture plates in DMEM and co-cultured with recipient cortical neurons allowing the transfer of secreted GDNF into the culture media (Figure 3B). Following 24 hrs co-culture, H_2O_2 was added at 50-100 μM overnight and the cortical neuron viability was assessed as described. The activation of Caspase3 was assessed in live cells in H_2O_2 – treated cultures using the CellEvent Caspase3/7 green detection reagent (Invitrogen), as per manufacturer's instructions. Briefly, the Caspase 3/7 Detection Reagent was added to cell cultures, incubated for 30 mins and apoptotic cells with activated Caspase-3/7 were visualized by bright green cells under an Axiovert 200 M fluorescence microscope (Zeiss). The fluorescence emission of the dye when bound to DNA is ~530 nm.

Antibodies

The following antibodies were used in this study: β-ACTIN (1:5000, WB, Sigma), GDNF (1:1000, WB, Santa Cruz), ERK (1:000, WB, Cell Signaling), pERK (1:1000, WB, Cell Signaling), NeuN (1:5000, WB; 1:500 ICC, Abcam), GFAP (1:200, ICC, DAKO), IBA1 (1:2500, ICC, Wako) and fluorescence-conjugated secondary antibodies (Alexa Fluor 488 anti-rabbit or mouse, Rhodamine anti-mouse, 1:500, Molecular Probes). Hoechst (1:1000, Sigma) was used to stain nuclei.

Western Blotting

Animals were perfused with cold saline, the brains were removed and the tissue surrounding the injured region was removed using a scalpel. The excised tissue was washed with cold TBS and lysed using ice-cold lysis buffer (25 mM Tris-HCL, pH 7.6, 150mM NaCl, 1% Triton-X, 1% Na.Deoxycholate), containing a protease inhibitor cocktail [63] and phosStop phosphatase inhibitor cocktail [63] specifically for ERK blots (Sigma). Cell lysates were incubated for 30 mins on ice and clarified by centrifugation at 20 000 *x g* at 4°C for 20 mins. Protein samples (40 µg) and a molecular weight rainbow marker (Amersham) were electrophoresed on a 10% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) and transferred to a nitrocellulose membrane (Amersham), using a wet transfer apparatus (Bio-Rad) at 20V overnight at 4°C. The membranes were incubated in TBS containing 5% non-fat milk or 5% BSA (Bovine serum albumin (Sigma), for Erk blots) with 0.1% Tween-20 (Sigma) for 1 hr at room temperature to block non-specific binding and then incubated in primary antibodies overnight at 4°C. The membranes were then washed three times for 10 mins with TBS containing 0.1% Tween-20 and incubated with a peroxidase conjugated secondary antibody for 1 hr at room temperature. All secondary antibodies, anti-rabbit and anti-mouse IgG-HRP conjugates (Bio-Rad, 1:5000), were diluted in 5% non-fat milk or BSA and the membranes were incubated for 1 hr at room temperature. Immunoreactivity was visualized, using chemiluminescent substrate (New England Nuclear) and captured by FluorChem 8900 (Alpha Innotech). Fold-change in pERK levels between test groups was performed using densitometry analysis using Image J software (NIH).

Immunohistochemistry

Mice were sacrificed 1 day following the last behavioural assessment, perfused with saline and then fixed in 10% neutral buffered formalin. Mouse brains were post-fixed overnight, washed twice in PB (0.1 M phosphate buffer, pH 7.4) and then infiltrated with 30% sucrose in PB at 4°C overnight or until they sank. The brains were then embedded in OCT compound (Fisher) and stored at -80°C. Coronal sections at a thickness of 8 μM or 40 μM were cut with a cryostat (Leica CM 1950), mounted on glass slides (Fisher) and stored at -80°C until use. Thick 40 μM floating sections were collected in PB, mounted and dried on glass slides and stored at room temperature till use. Frozen sections were thawed at room temperature for 15 mins and then placed in PBS for 15 mins. The sections were then blocked for 30 mins in 5% goat serum + 0.25% Triton-X in PBS, incubated with primary antibodies for 1 hr at room temperature and then washed 3 times with PBS. The sections were then incubated with species-specific secondary antibodies for 1 hr at room temperature and following subsequent washes the sections were counterstained with Hoechst (Sigma) for 5 mins at room temperature and mounted using Vectashield mounting medium (Vector Laboratories). Immunoreactivity was examined under an Axiovert 200 M fluorescence microscope (Zeiss) and a confocal microscope (Olympus). For cell counts, the peri-lesion area analyzed was offset from the lesion boundary by 140 μM to avoid introducing error by edge irregularities and 2000 μM from the midline to ensure the same region was analyzed in all animals, irrespective of lesion size (Figure 11A, square insert). Five random images were captured with the confocal microscope within a defined region, lateral to the lesion edge and midway through the cortical depth. Cell counts were acquired by placing the same size box in five random regions within the pre-defined area for each section analyzed. To quantify

astrocyte and microglial infiltration, the number of GFAP and IBA1 positive cells, satisfying the criteria for size and staining intensity, were automatically counted using the Northern Eclipse image analysis software (Empix).

For cresyl violet staining, serial coronal sections 320 μM apart (-0.70 mm to 2.22 mm from Bregma (0 mm)) were cut and mounted on glass slides. The sections were stained with 0.1% cresyl violet solution (Sigma) for 10 mins, coverslipped with permount mounting medium (Fisher) and used for the evaluation of loss of hemispheric tissue due to injury, as described.

Polyglycolic Acid (PGA) Scaffolds

The PGA implants were designed and manufactured by Dr. Abdellah Ajji (Polytechnique Montreal, Montreal, QC). PGA is a synthetic aliphatic polyester that was synthesized from chemical moieties by electrospinning procedures to generate a porous, nanofibrous PGA mesh (Patent Publication No. EP2448605 A1). The PGA fibres have a mean fibre diameter of approximately 50 μm and comprise a network of randomly oriented fibres (Figure 5B). The electrospun PGA sheets were cut into pieces of 2 mm x 1 mm in size, tailored to the cavity post-surgery and engineered to degrade approximately 13 - 15 weeks once implanted into the brain (unpublished data) – a timeframe that is compatible with neuroregeneration strategies. Prior to cell seeding and transplantation, the PGA scaffolds were sterilized with 50 μg/ml Penicillin and Streptomycin (Sigma) for 30 mins inside a well of a 96-well plate, washed twice in PBS and soaked overnight in DMEM. The sterile PGA scaffolds were coated with poly-L-lysine (Sigma) for 2 hrs at room temperature to facilitate AF cell adhesion. The poly-L-lysine was subsequently removed and the scaffolds were left to air-dry

for 30 mins. AF cells were seeded at a density of 3×10^5 cells/well onto the poly-L-lysine coated PGA scaffolds and cultured with regular replacement of culture medium every two days. Cell adhesion and viability were assessed by CFDA and Propidium iodide (PI) staining, as previously described.

Surgically Induced Motor Cortex Brain Injury

In this study, we used an *in vivo* model to study brain injury caused by neurosurgical procedures in the motor cortex. This surgically induced motor cortex brain injury model allows us to simulate the cortical and parenchymal damage resulting in significant behavioural deficits that are observed following neurosurgical procedures [58]. Brain injury studies were approved by the Animal Care Committee at the National Research Council Canada. A schematic timeline of experimental procedures is outlined in Figure 9A. Briefly, 6 week old C57Bl/6 (Charles River) weighing approximately 25 – 30 g were anesthetized using isoflurane (Aerrane, Baxter). Prior to injury, the mice were placed in a stereotaxic frame and the skin was opened to expose the motor cortex injury site. The bone was removed with a dental drill following mapping, using specific stereotaxic coordinates (from "(Anterior-Posterior) AP -0.25 mm to -1.0 mm, Lateral (Lat) +0.7 mm", to "AP +1.25 mm to +3.0 mm, Lat +2.4 mm") with respect to Bregma (0 mm), as previously described [65]. Injury to the left motor cortex was performed using a sterile graduated needle to remove neural tissue to a depth of 1 mm. The scaffold containing 300 000 cells was placed inside the injury site, sealed with bone wax, covered with topical anesthetic (Marcaine Bupivacaine Hydrochloride 0.50%, Sigma) and the skin was sutured. The animals were allowed to recover in their cages until behavioural testing at days 3, 5 and 7 post-surgery. The animals

were sacrificed 8 days after surgery to examine neuroprotection in the injury site. The brains were perfused, and fixed with 10% neutral buffered formalin, washed twice with PB and transferred to 30% sucrose in PB for 2 days. The brains were embedded in OCT compound, frozen and sectioned into 8 μM or 40 μM slices using a cryostat (Leica CM 1950), for staining and volume measurements; respectively. Prior to staining, the sections were thawed at room temperature for 15 mins, washed three times (5 mins each) in PBS and immunostained, as described earlier.

3D Brain Injury Rendering

Serial images from the mouse brain atlas of Paxinos and Watson [66] spanning from Bregma +4.28 to -5.34 mm were aligned using Reconstruct software (http://synapses.bu.edu). On each image, the entire brain as well as the primary motor cortex (M1), were outlined. Serial 8 μM sections, stained with 0.1% cresyl violet, from the brain of a mouse sacrificed three hours after motor cortex injury were used to outline the lesioned area on each corresponding image from the atlas. A 3-dimensional (3D) rendering of the brain, showing the location of the motor cortex and the actual lesion, was generated from the traced outlines using a Boissonnat surface reconstruction algorithm.

Beam-Walk behavior task

Forelimb and hindlimb coordination was examined using the foot fault test on the balance beam. The beam-walk task involves scoring foot faults (slips) during traverse of an elevated beam following injury. The apparatus consisted of a round beam (100 cm in length), which was elevated off the floor by two support stands. One of the stands also supported an

enclosed goal box. Each mouse received a minimum of 3 days of pre-surgical training on the beam task and the latency to traverse the beam was recorded. Mice that did not readily cross the beam (latencies greater than 20 secs) were given additional days of training before surgery or were removed from the study. All mice were performing at a similar level prior to surgery. During each post-surgical trial, the mice traversed the elevated beam and entered the goal box. Each mouse was given four consecutive trials on the beam daily. For each trial, the mouse was placed at the start and allowed to traverse the beam and was given 30 secs inside the goal box between trials. For the first two trials on the beam, the right side of each animal was recorded, and for the last two trials on the beam, the left side of each animal was recorded. Following injury, the animals were assessed with three trials for their ability to cross the balance beam at three time intervals (3, 5 and 7 days post-injury). The total number of left and right forelimb and hindlimb faults were scored by watching video recordings of each trial in slow motion. To be considered a fault, the mouse's forelimb or hindlimb had to fall completely off the beam. Analysis was performed blinded to the treatment group.

Cylinder behaviour task

The cylinder behaviour task was used to measure forelimb preference during vertical exploration to evaluate impairment after injury. The apparatus consisted of a clear plexiglass cylinder sitting on a sheet of clear plexiglass, elevated approximately two feet off the floor. The cylindrical shape of the chamber encourages vertical exploration of the walls with the forelimbs. Under the plexiglass sheet, a mirror was angled at 45° and a video camera was pointed at the mirror so that the mouse's paws could be clearly seen. Each mouse was placed inside the cylinder for 10 mins and allowed to explore and the entire session was recorded.

The mice were given one session prior to surgery to establish the baseline, and then were tested at 3, 5 and 7 days post-surgery. The videos were analyzed frame-by-frame and incidences of right and left forelimb use for weight-bearing exploratory behaviours were recorded. For each instance of vertical exploration of the cylinder wall, it was determined whether the mouse placed the left or right paw first, whether the paws were placed simultaneously on the wall or whether only a single paw was used. The preference for usage of the ipsilateral forelimb during exploration following injury using the following formula, as previously described [67].

Percent ipsilateral forelimb preference = [Ipsilateral / Ipsilateral + Contralateral]*100

Analysis was performed blinded to the treatment group.

Volume of tissue loss

Mice were sacrificed 1 day following the last functional assessment, the brains were frozen and thick coronal sections (40 μM) taken every 320 μM were stained with cresyl violet (Sigma). Following staining, digital images of the sections were acquired using a stereomicroscope equipped with a digital camera. For each section, the contour was drawn around the remaining ipsilateral hemisphere and the entire contralateral hemisphere using Image J software [68]. The area of each hemisphere for each section was obtained, added and multiplied by the distance (0.04 mm) between successive sections in the set to obtain total cortical volume (mm^3), as previously described [69]. The percent lesion was indirectly measured by subtracting the remaining area in the ipsilateral hemisphere from the total intact area of the contralateral hemisphere and then expressed as a percentage by the following

formula [70]: Percent Lesion = [Contralateral – Ipsilateral /Contralateral]*100

Statistical Analysis

Results are analyzed using a one-way or two-way analysis of variance (ANOVA), followed by Newman-Keuls and Bonferroni post-hoc test; respectively, where appropriate. For the beam-walk and cylinder data, Levene's test was conducted to evaluate the assumption of homogeneity of error variance between the treatment groups. In cases where Levene's statistic was significant, the raw data were subjected to square root transformations to reduce the error variability prior to conducting any further statistical tests. Repeated measures ANOVAs with treatment (sham, injury, scaffold, AF-DsRed, and AF-GDNF) as the between-subjects variable and post-surgical interval as the within-subjects variable were carried out. If the overall ANOVA was significant, post-hoc Dunnett's tests with injury alone as the comparison group were conducted to determine which groups differed significantly. In cases where there was a significant interaction between treatment group and post-surgical interval, subsequent one-way ANOVAs with post-hoc Dunnett's tests were performed at each post-surgical time point to determine which groups differed significantly at each time point. Correlations between beam-walk performance at each post-surgical time point and lesion volumes were calculated using Pearson's product moment correlation. Outcome measures were evaluated by observers blinded to the experimental conditions. Results are expressed as mean ± standard error of mean (SEM) and considered significant at $p < 0.05$.

Results

Genetically engineering AF cells to constitutively secrete GDNF

The lentiviral-based delivery system was engineered to produce a recombinant GDNF protein to be subsequently delivered to the injured brain, as proof of principle of GDNF-mediated neuroprotection *in vitro* and *in vivo*. To generate the GDNF lentivector, the GDNF sequence was inserted into the pTet07CSII-MCS-DsRed lentivector backbone, downstream of a constitutively active CMV promoter and upstream of IRES-DsRed (Supplementary Figure 1), to achieve high expression levels and to allow for monitoring of transfection and infection efficiency. Using DsRed as a reporter, we successfully achieved a minimum of 75% infection rate of AF cells with pTet07CSII-CMV-GDNF-DsRed (AF-GNDF) and pTet07CSII-CMV-DsRed (AF-DsRed) cells (Figure 1A), which were further enriched by FACS sorting into pure AF-GDNF and AF-DsRed (backbone control) populations (Figure 1B). Since AF cells do not express endogenous GDNF protein, Western blotting and ELISA analysis confirmed the virally-driven expression of GDNF in infected AF cells and the concomitant secretion of GDNF into the culture media, respectively (Figure 1C-D). The media collected from virally-transduced AF-GDNF cells contained high levels of secreted GDNF (1.0 ng/ml), comparable to the levels detected in AF-GDNF cell lysates (1.2 ng/ml; Figure 1D), in contrast to the undetectable levels in the media collected from AF-DsRed and non-transduced AF cultures (0 ng/ml; data not shown). Hence, GDNF expression in AF cells is purely driven from the lentivector and the virally-infected cultures consistently secrete GDNF for at least 3 weeks post-infection, in growth media conditions (Figure 1E). These results show that AF-GDNF cells can be cultured stably *in vitro* and have the long-term capacity to secrete high levels of GDNF protein. Constitutive GDNF expression was not

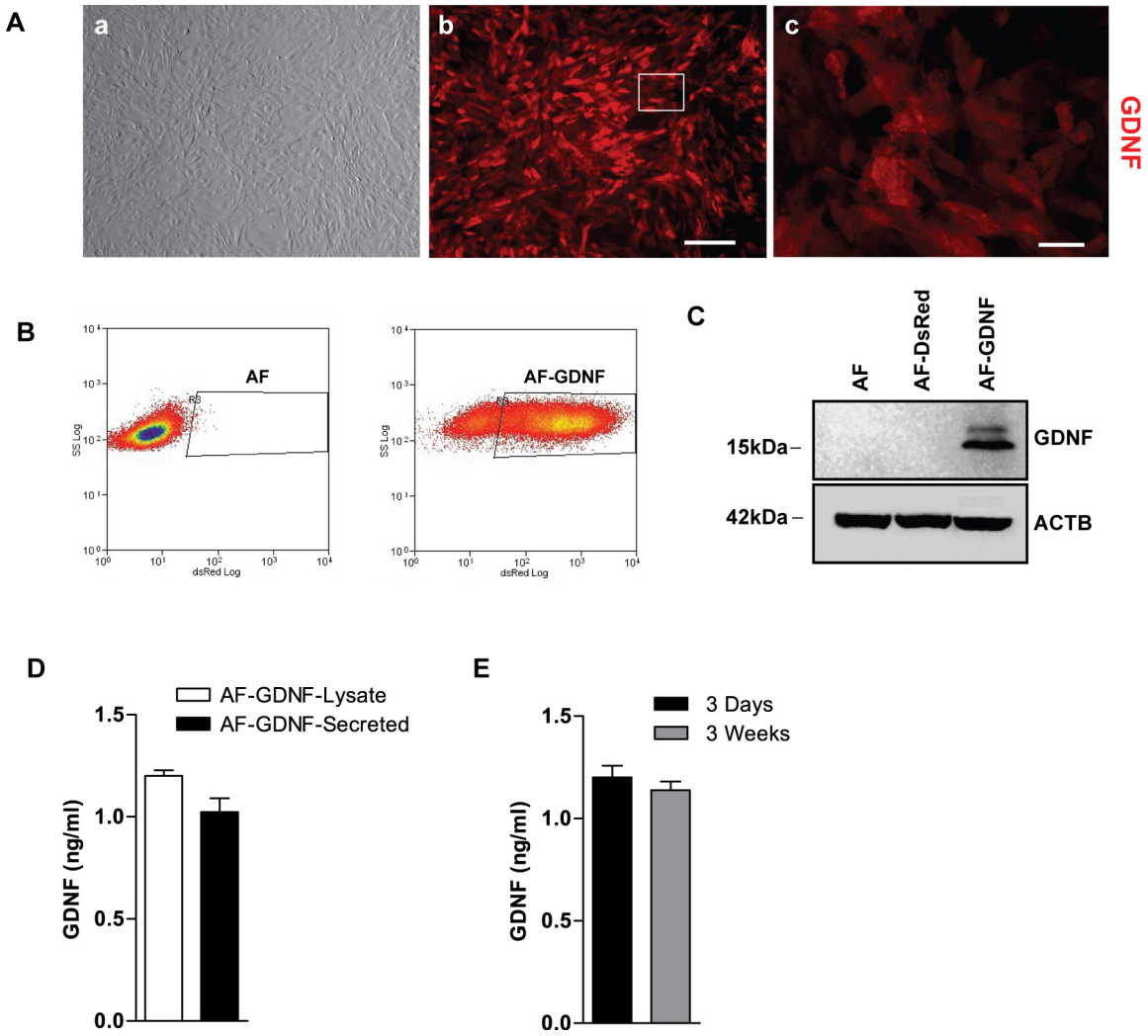


Figure 1. GDNF lentivector design and infection of AF cells
A. (a-b) AF cells were infected with pTet07CSII-CMV-GDNF-DsRed (AF-GDNF, Red) and GDNF-DsRed expression was confirmed by fluorescence microscopy. (c) Higher magnification of inset in (b). **B**. Infected AF-GDNF cells were enriched by fluorescence activated cell sorting (FACS), based on DsRed expression. **C**. Western blot analyses confirmed expression of GDNF protein in AF-GDNF cells, but not in pTet07CSII-CMV-DsRed (AF-DsRed) and non-infected AF cells. β-actin (ACTB) was used as an internal control. **D**. ELISA confirmed GDNF secretion into culture media and expression in cell lysates from AF-GDNF cells compared to undetectable levels in AF-DsRed and non-transduced cultures (data not shown). **E**. ELISA analysis confirmed that AF-GDNF cells consistently secrete GDNF up to 3 weeks post-infection (mean + SEM, n=3). Scale bar: 150 µm (b) and 50 µm (c).

toxic to transduced AF cells in culture nor did it commit the cells towards any particular phenotype when compared to AF-DsRed and non-transduced cultures (data not shown).

Neuroprotective effect of GDNF during cytotoxicity in vitro

GDNF has been shown to have a potent protective effect against neuronal damage induced by brain injury and excitotoxic damage [26]. Hence, to examine the protective role of GDNF *in vitro*, AF cells were exposed to hydrogen peroxide (H_2O_2), which induces neuronal cell death that proceeds via an apoptotic pathway [71]. In culture, hydrogen peroxide significantly reduced AF cell viability, in a dose-dependent manner, compared to untreated cells (Supplementary Figure 2). Treatment of AF cells for 18 hrs with 200 μM H_2O_2 resulted in approximately 50% cell death (Figure 2A and Supplementary Figure 2). Using a live cell *in vitro* Caspase3 detection assay, we confirmed that the cell death observed following hydrogen peroxide treatment was mediated, in part, via the activation of Caspase3 resulting in cell shrinkage and membrane blebbing (Figure 2B) accompanied by DNA fragmentation, evident as early as 3 hrs post-treatment (Figure 2C). Furthermore, we examined the effects of hydrogen peroxide treatment on cortical neuron cultures. Western blotting analysis, following exposure of cortical neurons to increasing concentration of hydrogen peroxide (50 and 100 μM H_2O_2), resulted in an increase in Caspase3 and a decrease in NeuN protein expression, indicative of neuronal death 18 hrs following treatment (Figure 2D). We also observed a decrease in phosphorylated (pErk) and total Erk levels, a pro-survival pathway in neurons (Figure 2D). To test the effect of GDNF on hydrogen peroxide-induced cytotoxicity in AF cells, cultures were exposed to medium alone or pre-treated with varying therapeutic concentrations of recombinant GDNF (1.5 and 10 ng/ml) for 2 hrs prior to addition of

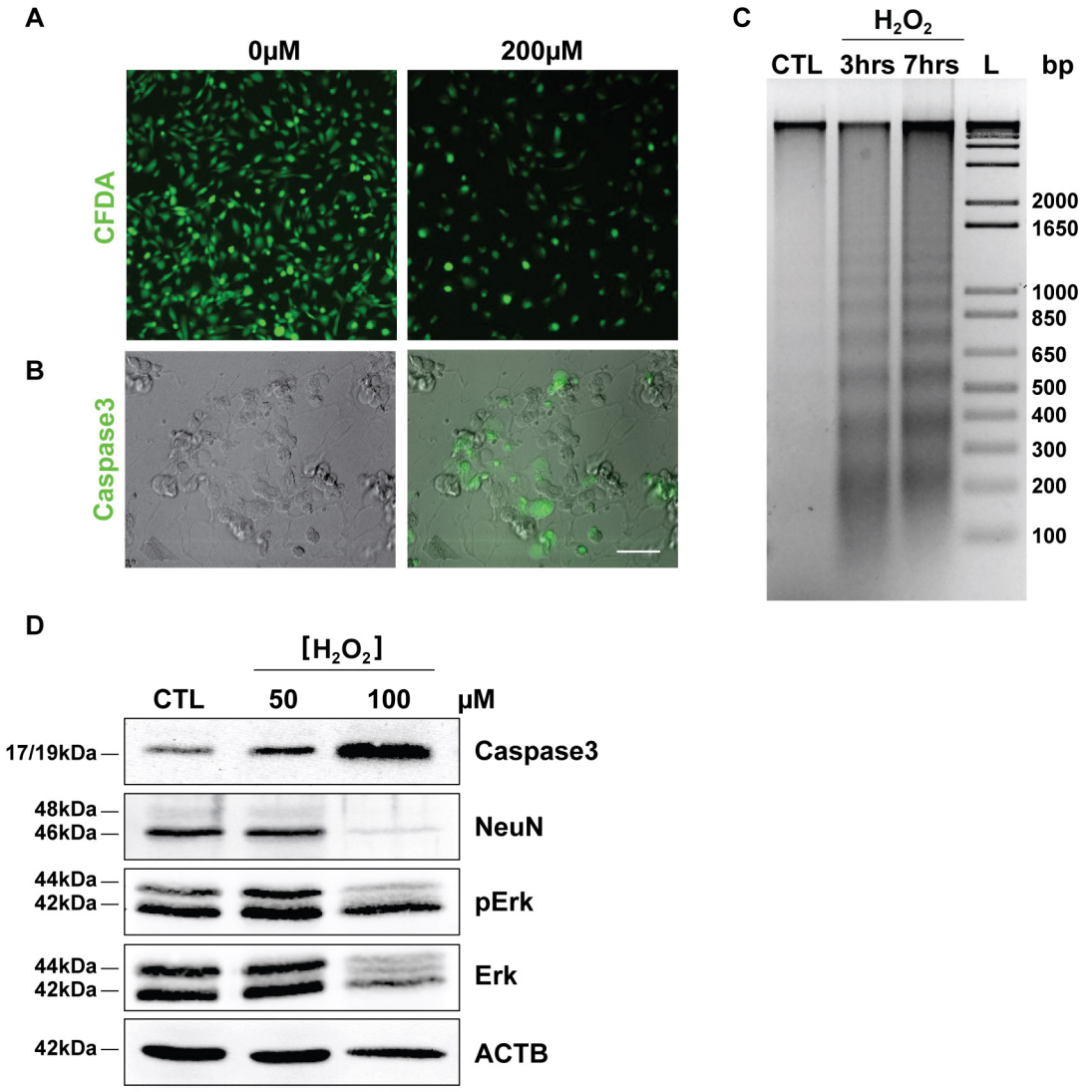


Figure 2. Hydrogen peroxide induces cell death in AF and cortical neurons
A. Cell viability was assessed using CFDA dye (green) following treatment with H_2O_2, which resulted in approximately 50% cell death 18 hrs after treatment. **B**. Cell death observed following H_2O_2 treatment was mediated, in part, via the activation of Caspase3 (green) resulting in cell shrinkage and membrane blebbing and **C**. DNA fragmentation seen as early as 3 hrs after treatment. CTL: control cells not exposed to H_2O_2; bp: base pairs; L: 1Kb DNA ladder. **D**. Western blotting analyses of cortical neurons treated with H_2O_2 indicate a decrease in pErk and NeuN and increase in Caspase3 protein levels. B-actin (ACTB) was used as a loading control. Scale bar: 50 μm.

200 μM H_2O_2 for 18 hrs. GDNF pre-treatment markedly decreased AF cell susceptibility to hydrogen peroxide *in vitro* at concentrations as low as 1.5 ng/ml (Figure 3A); although higher GDNF concentrations of 100 ng/ml appeared to be toxic *in vitro*, resulting in increased cell death (data not shown). To determine whether GDNF secreted from virally-infected AF cells was able to confer the same level of protection on cortical neurons *in vitro*, we plated AF-GDNF cells onto Transwell filters containing 0.4 μM pores allowing the transfer of secreted GDNF to recipient cortical neurons in co-culture, as illustrated in Figure 3B. ELISA experiments confirmed GDNF protein levels of 1.6 ng/ml inside the shared co-culture media (Figure 3C), a comparable concentration to the protective dosage of 1.5 ng/ml of recombinant GDNF (Figure 3A) that had no long-term toxicity in culture (Figure 1E). Cortical neurons were co-cultured with the Transwell inserts containing AF-GDNF or AF-DsRed cells for 18 hrs prior to the addition of 100 μM hydrogen peroxide, a concentration resulting in approximately 50% cell death of cortical neurons *in vitro*. As shown in Figure 3D, a significantly higher number of surviving cortical neurons were observed in the AF-GDNF than AF-DsRed co-cultures compared to hydrogen peroxide treatment alone. This enhanced survival of GDNF-treated cortical neurons, in response to a toxic concentration of hydrogen peroxide, suggests that GDNF secreted from AF cells is capable of protecting cortical neurons *in vitro*.

Expression of endogenous GDNF and its signaling components following brain injury

It is well established that expression of GDNF is often induced after brain injury and previous studies have shown that the biological effects of GDNF are mediated by GFRα1 and c-Ret [72, 73]. Hence, prior to our transplantation studies, we performed QPCR analysis

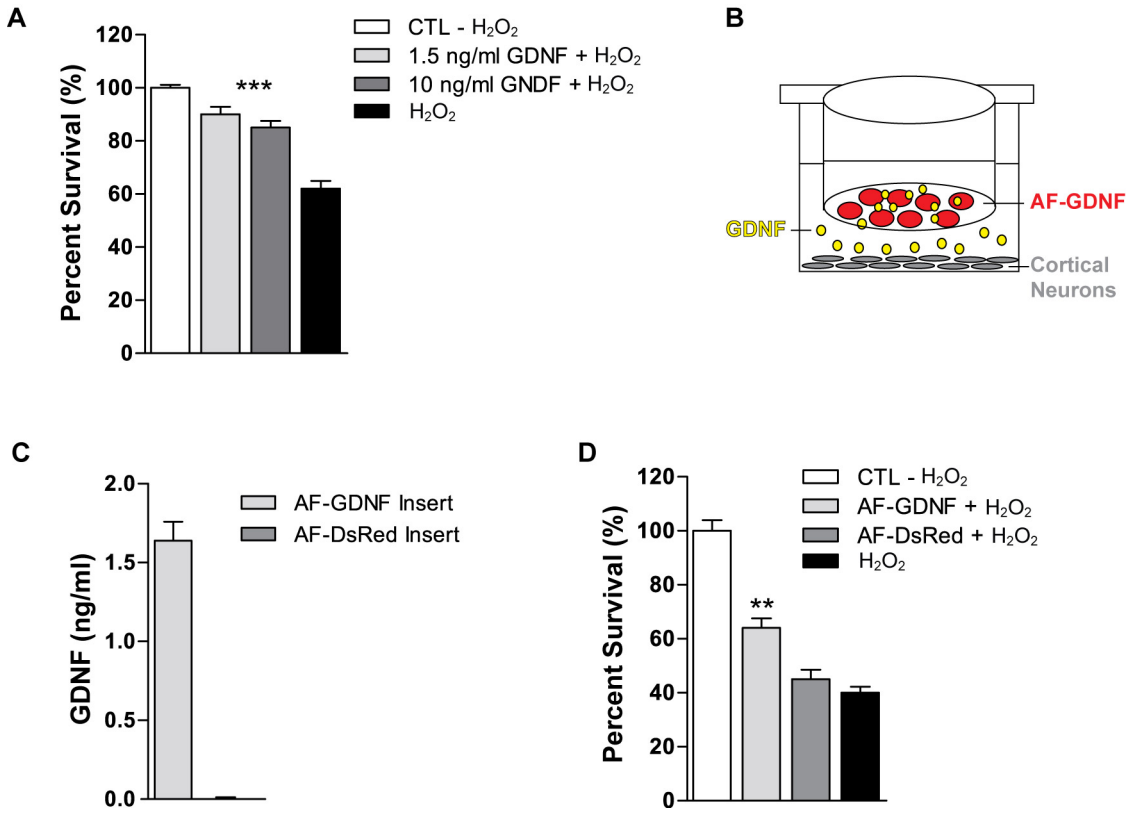


Figure 3. GDNF pre-treatment reduces hydrogen peroxide-mediated cell death
A. Pre-treatment of AF cells with GDNF for 2 hrs, prior to addition of H_2O_2, significantly reduced cell death compared to H_2O_2 treated cells (mean + SEM, n=3; *** $p<0.001$ vs H_2O_2). **B**. Schematic of GDNF-secreting AF cells (AF-GDNF) seeded in Transwell filters co-cultured with cortical neurons as recipient cells. **C.** ELISA confirming AF-GDNF cells in Transwell cultures actively secrete GDNF compared to undetectable levels in AF-DsRed cells (data not shown). **D**. Cell death was decreased in cortical neurons exposed to GDNF secreted from AF-GDNF cells in Transwells following addition of H_2O_2 (mean + SEM, n=3, ** $p<0.01$ vs H_2O_2). Percent survival was scored based on CFDA assay. CTL: control cells not exposed to H_2O_2.

at different time points post-injury (3 hrs, 3 and 7 days) to determine the expression of endogenous GDNF, c-Ret and the GDNF receptor family (GFRα1, 2, 3 and 4) in the peri-lesion area of the left motor cortex following injury (Figure 4A – yellow). QPCR analysis revealed an upregulation of GDNF, GFRα1 and c-Ret expression, relative to sham animals at all time points (Figure 4 B-D); whereas, GFRα2 and 3 were not expressed within the time frame examined (data not shown). Although GFRα4 was initially upregulated at 3 hrs post-injury, its expression decreased at later time points (Figure 4E). Hence, the concomitant injury-induced expression of GDNF and GFRα1, in the peri-lesion cortex, suggests that GDNF signaling through c-Ret and GFRα1 may represent an endogenous neuroprotective response that can be stimulated following injury supporting a role for exogenous GDNF delivery at the lesion site.

Biocompatible polymer scaffolds

Currently, engraftment of implanted cells in the brain is hindered by the vast amount of parenchyma that is lost post-injury, thereby limiting the ability of cells to survive post-implantation. Since recent studies have shown that cells seeded on 3D synthetic, biocompatible polymers have a higher survival rate post-implantation, rather than cells alone [74, 75], we used PGA scaffolds to deliver AF-GDNF cells into the lesion site following injury (Figure 5A). The electrospun PGA scaffolds used in this study have a mean fibre diameter of approximately 50 μm and comprise a network of randomly oriented fibres creating a highly porous mesh of nanofibres which provides a higher surface area for cell attachment (Figure 5B). The scaffolds were cut to 2 mm x 1 mm in size, dimensions tailored to fit the cavity post-surgery, and coated with poly-L-lysine to facilitate cell adhesion [76].

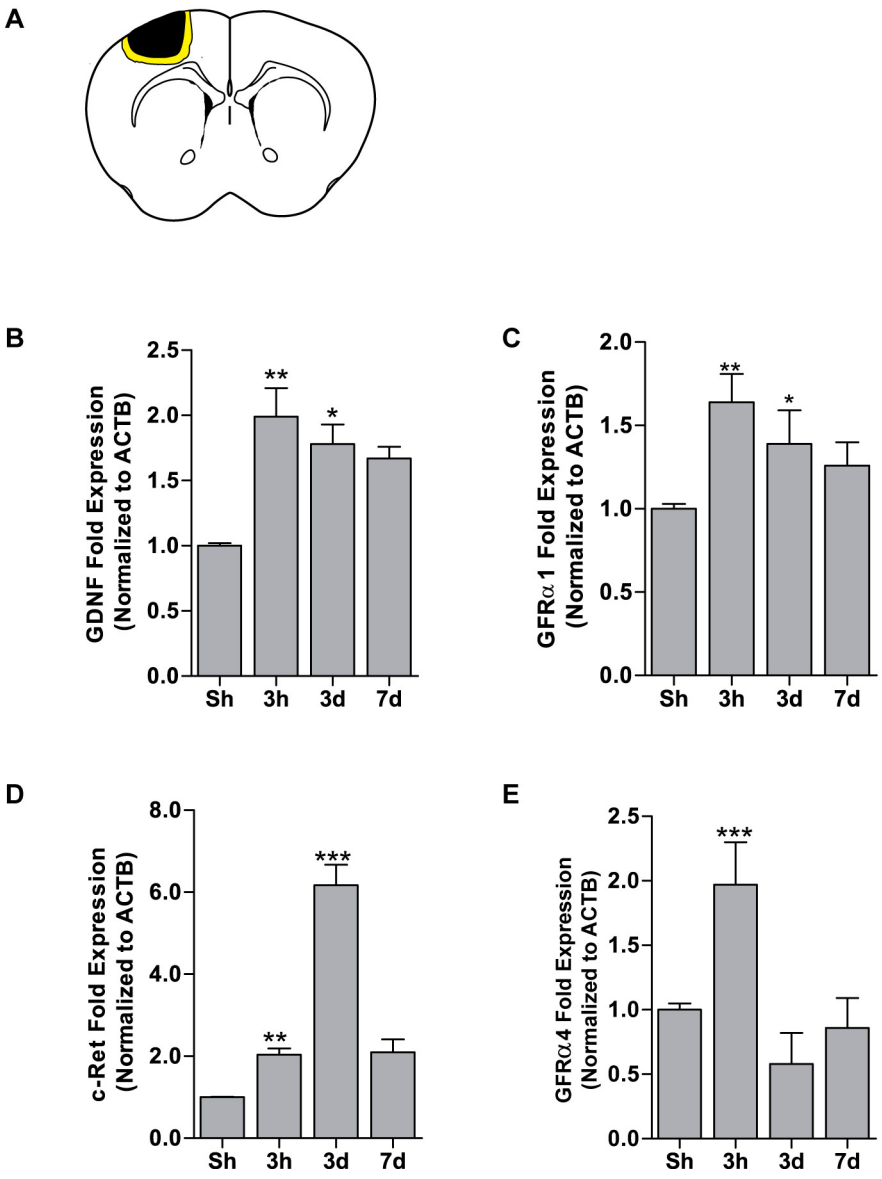


Figure 4. Expression of GDNF signaling components post-injury
A. Schematic representation outlining tissue sample collection from the peri-lesion area (yellow) surrounding the implantation site (black)**. B-E.** QPCR analysis of GDNF, GFRα1, GFRα4 and c-Ret expression in brain tissue samples surrounding the peri-lesion area in the motor cortex at 3 hrs, 3 days and 7 days (3h, 3d and 7d) post injury relative to sham (sh) animals (mean + SEM, n=3; * $p<0.05$, ** $p<0.01$, *** $p<0.01$ vs sham).

Lastly, the PGA scaffolds were engineered to degrade approximately 13 - 15 weeks once implanted into the brain (unpublished data, data not shown) – a timeframe that is compatible with neuroregenerative strategies.

The interaction between AF cells and the PGA scaffold is crucial for the functionality of the graft to ensure cell survival and sustained delivery of GDNF post-implantation. To test the ability of PGA scaffolds to support AF cell attachment and survival *in vitro,* we seeded AF cells directly onto PGA scaffolds placed in tissue culture plates in growth media. Two week post-seeding, the scaffold and AF cell co-cultures were stained with CFDA and PI to examine cell survival and death, respectively. The majority of CFDA positive AF cells readily attached onto the nanofibres, penetrated the 3D-PGA scaffold (Figure 5C, a-b) and grew throughout the highly porous fibrous PGA mesh (Figure 5C, c). The lack of PI staining observed during the entire culture period suggests a lack of toxicity. Based on these promising results, we seeded 3 x 10^5 AF-GDNF or AF-DsRed cells onto the PGA scaffolds in serum free media for subsequent implantation into the injured brain cavity. The AF-GDNF or AF-DsRed / PGA implants were monitored *in vitro* to parallel *in vivo* implantation studies. *In vitro*, media was collected from the wells containing either AF-GDNF or AF-DsRed / PGA implants for 9 days, which reflected the length of time the scaffolds were implanted in the injured brain. During this timeframe, the AF-GDNF and AF-DsRed cells readily attached to the PGA scaffold (Figure 6A, a-b) and ELISA analysis confirmed GDNF protein secretion from AF-GDNF/PGA cultures, compared to undetectable level in AF-DsRed / PGA control media (Figure 6B). The decrease in GDNF levels over time was paralleled by a gradual reduction in the number of cells due to serum free culture conditions.

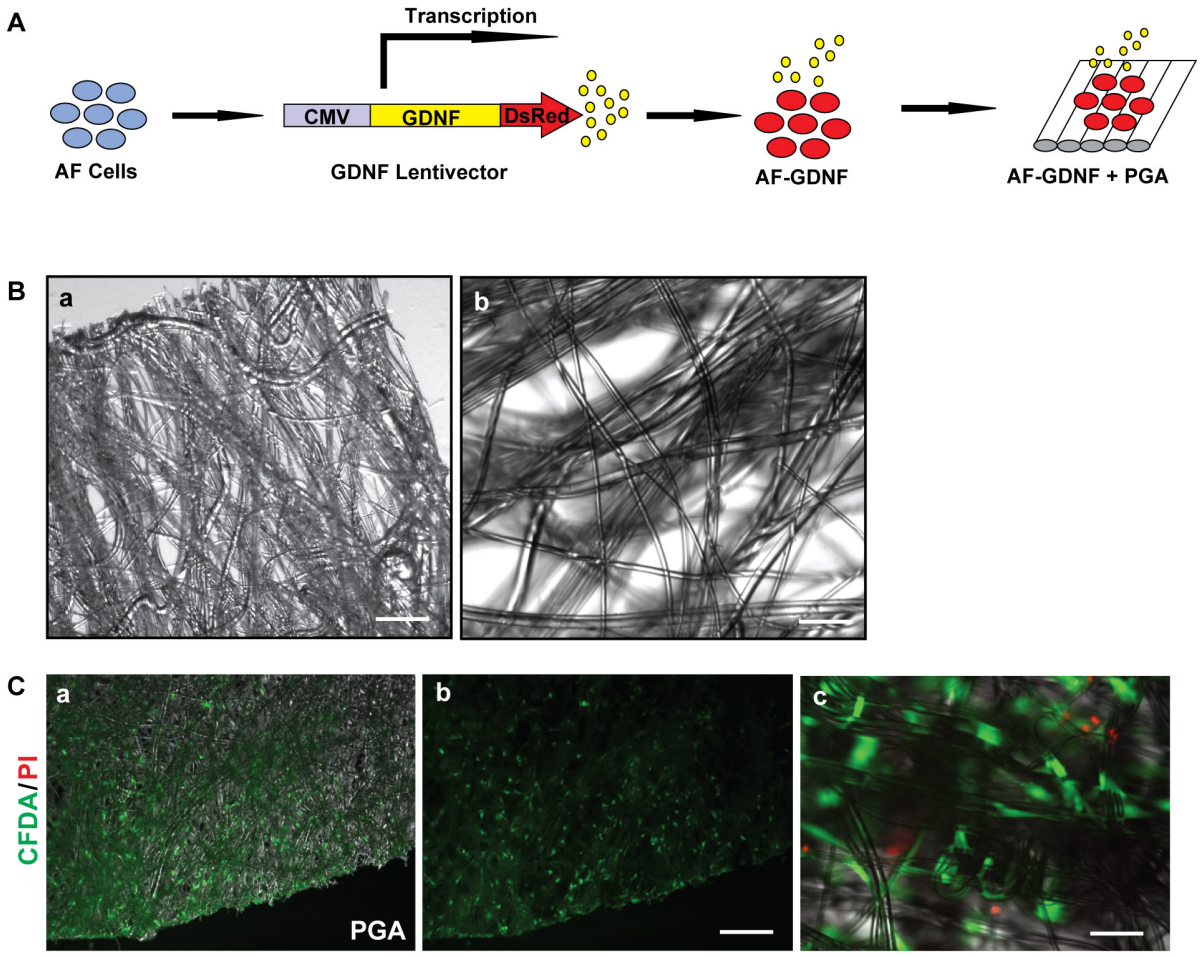


Figure 5. Survival of AF cells on PGA scaffolds
A. Schematic outlining the approach for the generation of GDNF-secreting AF cells seeded on polyglycolic acid (PGA) polymers**. B.** (a) Phase contrast image of randomly oriented nanofibres within the PGA mesh. (b) higher magnification of individual nanofibres in (a). **C**. (a-c) AF cells seeded on PGA scaffolds readily attached and impregnated the PGA scaffold. Cell survival and death assays with CFDA (green) and PI (red) dye revealed that the majority of seeded AF cells were alive with very few dead cells observed 2 weeks post-seeding. Higher magnification in (c) shows AF cells (CFDA, green) attached to individual PGA fibres. Scale bar: 150 µm (B(a), C(b)) and 50 µm (B(b), C(c)).

Interestingly, at day 7 in culture, AF-GDNF cells showed greater survival on the scaffolds (Figure 6A, c, e) compared to AF-DsRed cells based on sustained DsRed expression (Figure 6A, d). Hence, the ability of the PGA scaffolds to support AF cell attachment, survival and GDNF secretion attests to PGA's suitability as a substrate for AF-GDNF/AF-DsRed transplantation *in vivo*. Furthermore, the enhanced survival of GDNF expressing AF cells is promising for the generation of effective cell lines for local delivery of neurotrophic factors and for sustained survival and function of the graft *in vivo*.

Surgery and characterization of motor cortex injury model

In this study, we used a surgically induced brain injury model to reproducibly mimic the incidental damage inflicted on the brain during neurosurgical procedures [58, 77]. Such damage can occur during excision of brain tumors or removal of epileptic foci resulting in loss of brain tissue and neuronal death. Surgically induced unilateral injury to the left motor cortex was performed using a sterile graduated needle to remove neural tissue to a depth of 1 mm using specific stereotaxic coordinates (from "AP -0.25 mm to -1.0 mm, Lat +0.7 mm", to "AP +1.25 mm to +3.0 mm, Lat +2.4 mm") with respect to Bregma (0 mm), as previously described [65]. This surgically induced motor cortex brain injury model allows us to simulate the cortical and parenchymal damage resulting in significant behavioural deficits that are observed following certain neurosurgical procedures. The actual lesioned volume and average extent of injury was mapped to the mouse brain atlas (Supplementary Figure 3A). The injury produced a cavity in the dorsal cortex that extended from the cortical surface to the deeper cortical layers, but not the underlying corpus callosum. To confirm accuracy in our surgical parameters, a 3D reconstruction of the injury mapped the actual

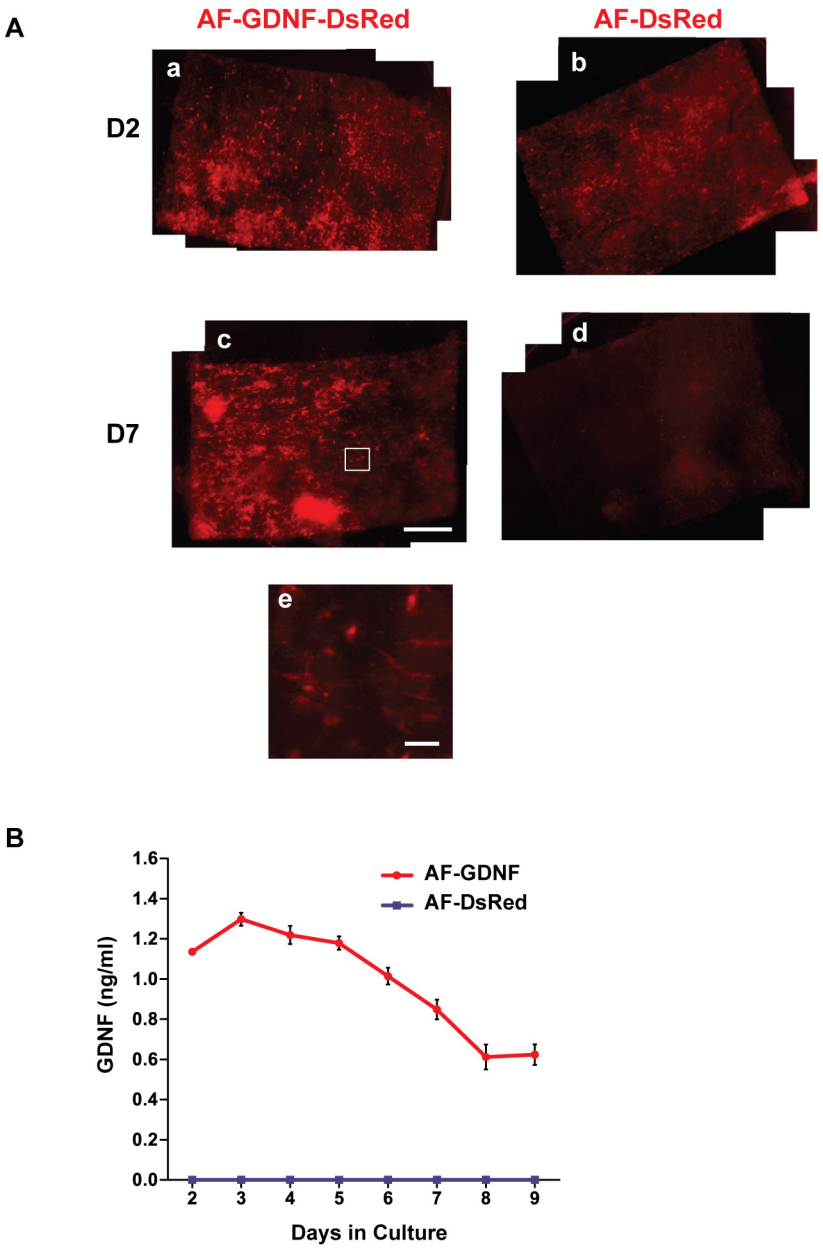


Figure 6. AF-GDNF seeded scaffold cultures
A. Following infection (a,c) AF-GDNF and (b,d) AF-DsRed cells were seeded on PGA scaffolds for a total of 9 days in culture in serum free media. (e) Higher magnification of the inset in (c) shows AF-GDNF cells on the PGA scaffold. **B**. Culture media were collected daily from AF-GDNF and AF-DsRed seeded scaffolds to examine levels of GDNF secreted into the media (mean ± SEM). Scale bar: 150 µm (a-d) and 20 µm (e).

lesioned region, relative to the mouse brain atlas coordinates, for the primary motor cortex. The actual lesion was shown to overlap the motor cortex region, as expected based on the stereotaxic coordinates (Supplementary Figure 3B). Unilateral injury to the left motor cortex, due to tissue removal, results in significant damage and loss of cortical cells producing a cavity (Figure 7A, a; arrowheads) that expands over time due to secondary injury mechanisms in the peri-lesion area; the contralateral hemisphere remains grossly intact as do sham brains (Figure 7A, b). One week following injury, the motor cortex is characterized by neuronal loss as shown by decreased NeuN positive cells (Figure 7B, a), damage of neurite extensions and organization evidenced by MAP2 staining (Figure 7B, c) and gliosis observed by infiltrating GFAP positive astrocytes in the peri-lesion area (Figure 7B, e); compared to sham operated brains (Figure 7B, b,d,f; respectively).

Evaluation of GDNF secretion from AF-GDNF cells in vivo

The AF-GDNF and AF-DsRed / PGA implants were maintained in serum free culture for 2 days prior to being transplanted into the lesioned cavity. To confirm that AF-GDNF cells seeded on the PGA scaffolds continued to secrete GDNF into the surrounding host tissue post-implantation, we collected brain samples from the peri-lesion area surrounding the implantation site (Figure 4A, yellow), and the contralesional motor cortex, for ELISA analysis at 3 and 7 days post-implantation. Significantly higher amounts of GDNF (up to 1.8 ng/ml) were detected in the peri-lesion area of the motor cortex surrounding the AF-GDNF/PGA implants, compared to the other test groups (Figure 8A) and the contralateral side (Supplementary Figure 4) at 3 days post-implantation. Increased GDNF levels were sustained through to day 7 post-implantation in AF-GDNF animals (0.4 ng/ml, Figure 8A),

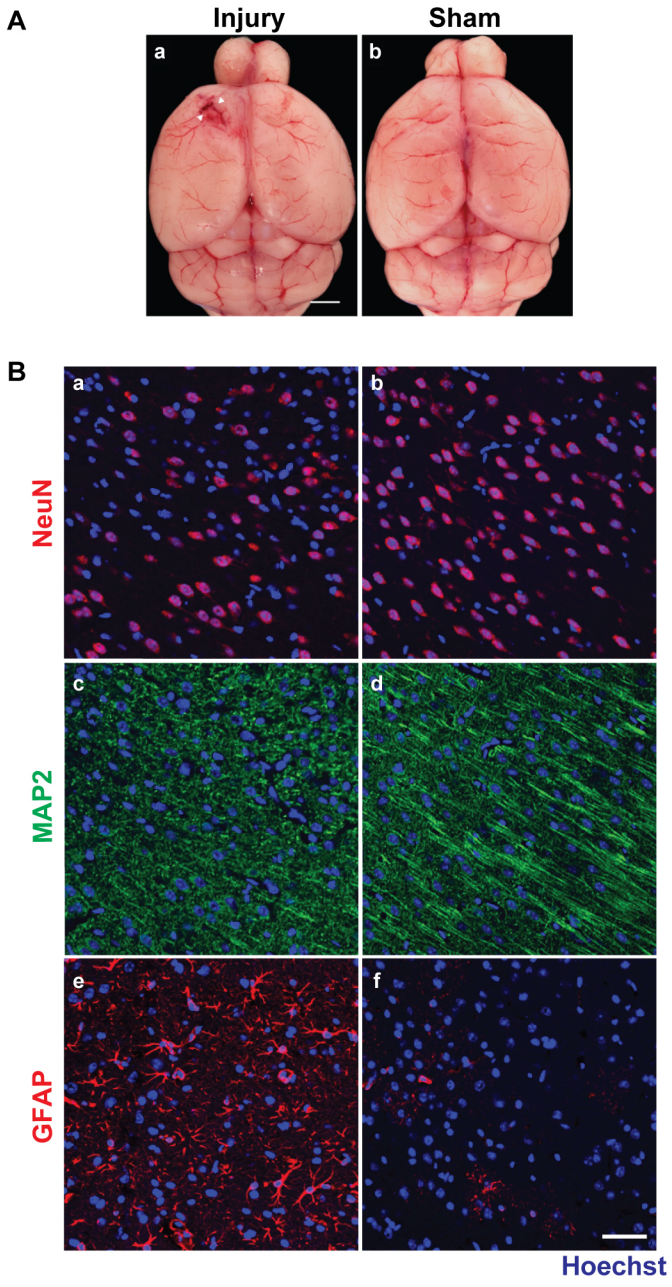


Figure 7. Lesion site and cellular architecture of sham and injured motor cortices
A. Images of whole brain showing lesion site and resulting cavity in the left motor cortex of (a) injured mice compared to (b) sham. Arrowheads indicate lesion boundaries. **B**. The peri-lesion site is characterized by neuronal loss as shown by (a) decreased NeuN (red) positive cells, (c) damage of neurite extensions and organization (MAP2, green) and (e) astroglial infiltration (GFAP, red) compared to the well organized architecture in sham brains (b,d,f respectively). Hoechst (blue) was used to label nuclei. Scale bar: 1.6 mm (A) and 80 µm (B).

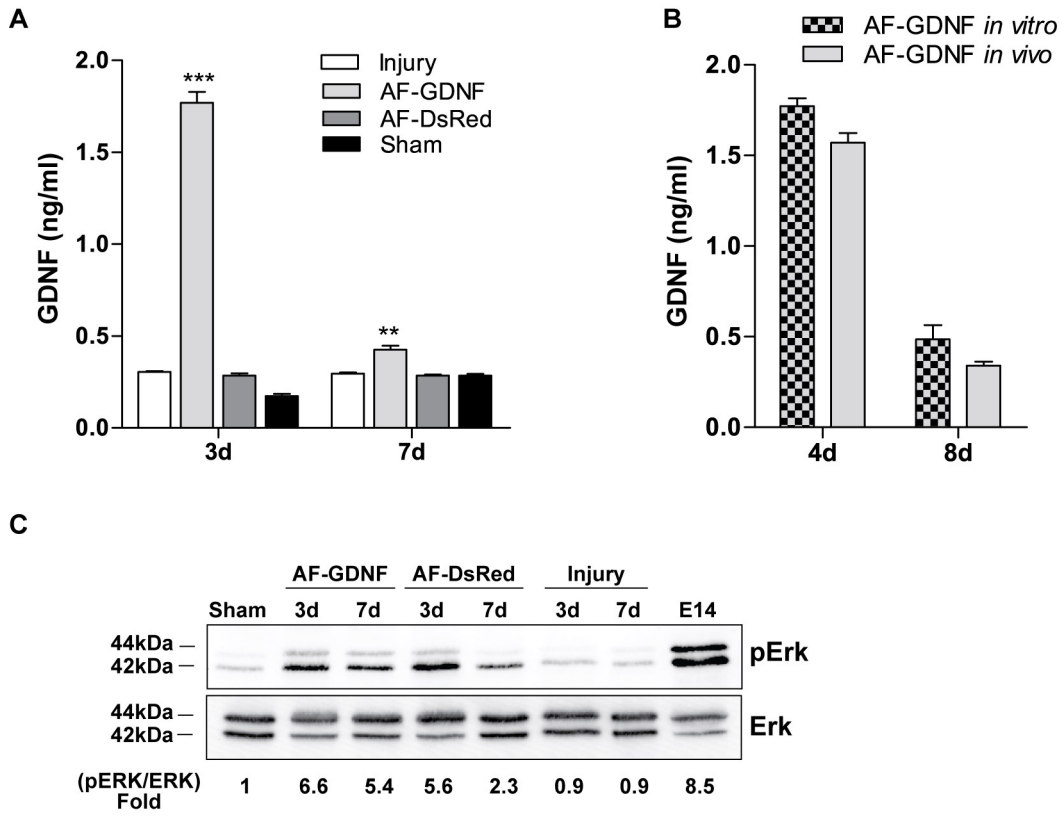


Figure 8. *In vivo* GDNF expression following transplantation
A. ELISA quantitation of the amount of GDNF protein secreted in brain tissue surrounding the peri-lesion area at 3 and 7 days (d) post-injury. There was a significant increase in GDNF in the brain samples from mice receiving the AF-GDNF implants compared to the other test groups (mean + SEM, n=3; *** $p<0.001$, ** $p<0.01$ vs injury). **B**. AF-GDNF/PGA implants were removed 3 and 7 days post-surgery and the levels of GDNF secretion were measured by ELISA at 4 and 8 days, following 24 hrs in serum free culture. GDNF protein secretion levels were very similar to those observed in *in vitro* cultures. **C**. The ability of GDNF to activate the MAPK/ERK pathway was assessed by Western blotting of ERK phosporylation (pErk) *in vivo* at 3 and 7 days post-transplantation of AF-GDNF and AF-DsRed / PGA implants. Increased pErk was observed for AF-GDNF cells at day 3 and 7 compared to a transient increase for AF-DsRed cells at day 3. Embryonic day 14 (E14) and total Erk levels were used as positive and internal controls.

which suggests that the grafted cells continue to produce and secrete GDNF at the implantation site. Since endogenous GDNF is expressed following injury (Figure 4B), the amount of GDNF being secreted from the AF-GDNF/PGA implants can be more accurately assessed by measuring GDNF secretion levels exclusively from the AF-GDNF/PGA implants. Hence, the AF-GDNF/PGA implants were removed from the brain post-implantation and placed in tissue culture plates containing DMEM media for subsequent ELISA analysis at day 4 and 8 *ex vivo* (one day following removal from the brain). *Ex vivo* AF-GDNF/PGA implants continued to secrete GDNF in culture to comparable levels observed *in vivo* (1.59 ng/ml at day 4 and 0.34 ng/ml at day 8; Figure 8B), which suggests that the majority of the GDNF detected in the peri-lesion area was derived from AF-GDNF/PGA implants. Interestingly, the *in vivo* levels were very comparable to secretion levels detected in parallel AF-GDNF/PGA *in vitro* cultures (Figure 8B), which is indicative of no apparent toxicity to the cells in the acute period post-implantation or due to the PGA scaffold *in vivo*. No evidence of AF-GDNF or AF-DsRed cell migration into host brain was observed at 7 days post-implantation (data not shown) suggesting that the implanted cells remained confined to the PGA scaffold. The sustained GDNF production and secretion from AF-GDNF/PGA implants *in vivo*, comparable to *in vitro* levels, is a strong indication that the AF-GDNF/PGA graft remains viable post-implantation. As a result, AF-GDNF cells can serve as a source of GDNF that can act in an autocrine and paracrine fashion exerting a protective effect on the grafted cells as well as the host tissue, respectively.

Biological activity of GDNF secreted by AF-GDNF/PGA implants

Recent reports provide evidence that GDNF exerts its neuroprotective effects through the activation of the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) signaling pathway [78, 79]. In the context of brain injury, the neuroprotective effect of GDNF has been shown to involve the activation of ERK signaling in cortical neurons and astrocytes [78]. Hence, we examined the biological activity of GDNF secreted by the AF-GDNF/PGA implants *in vivo*. Indeed, we observed a consistent 6.6 and 5.4 fold increase in the phosphorylation level of ERK1/2 in the peri-lesion area surrounding AF-GDNF/PGA implants, sustained from 3 to 7 days post-transplantation, respectively, compared to sham and injury alone animals (Figure 8C). A transient increase in ERK1/2 phosphorylation was also observed for AF-DsRed/PGA implants only at day 3, likely owing to the secretion of cytokines and growth factors from the AF cells, such as VEGF [23], that could potentially activate ERK signaling in surrounding neural cells. These results demonstrate that exogenously delivered GDNF post-injury can activate the ERK signaling pathway in surrounding cortical cells.

Assessment of motor recovery following implantation

An injury to the left motor cortex is associated with both cortical and subcortical damage and produces sensory/motor deficits. Unilateral injury in the left motor cortex results in behavioural asymmetries in the use of contralateral hindlimb and forelimbs; accordingly, behavioural deficits and recovery of function were assessed using the beam-walk and cylinder tests. A timeline of experimental procedures is outlined in Figure 9A. These tests

were applied to determine whether GDNF delivery to the injured site could ameliorate behavioural deficits and enhance recovery.

Animals with unilateral injury in the brain exhibit deficits in forelimb and hindlimb function contralateral to the injury [80]. For the beam-walk task, a fault was recorded whenever the forelimb or hindlimb slipped off the beam (Figure 9B hindlimb fault, 9C forelimb fault, 9D no fault). Animals were scored at three time-points: 3 days post-surgery (post-test 1), 5 days post-surgery (post-test 2) and 7 days post-surgery (post-test 3). Total contralateral faults were scored for forelimb and hindlimb in each animal group and the results are summarized in Figure 9E-G. At post-test 1, the average number of contralateral faults committed by the test groups was similar and significantly higher compared to non-injured shams (Figure 9E). The amount of contralateral faults decreased from post-test 1 to post-test 3 among all injured groups. Although AF-GDNF animals had the fewest number of faults at post-test 3, this difference was not statistically significant (Figure 9G). The trend towards greater improvement in AF-GDNF animals over time may have reached statistical significance if the mice had been tested at longer post-surgical intervals. We did not observe any group differences in latencies for the animals to traverse the beam (data not shown).

Animals with unilateral injury to the brain typically develop a preference for using the forelimb ipsilateral to the injury for exploration preferring not to use the affected, contralateral forelimb [81]. The use of forelimbs for weight bearing movements was analyzed by looking at “right paw first” contacts as a percentage of the total number of instances in which a paw made contact with the cylinder wall (Figure 10A). This asymmetry

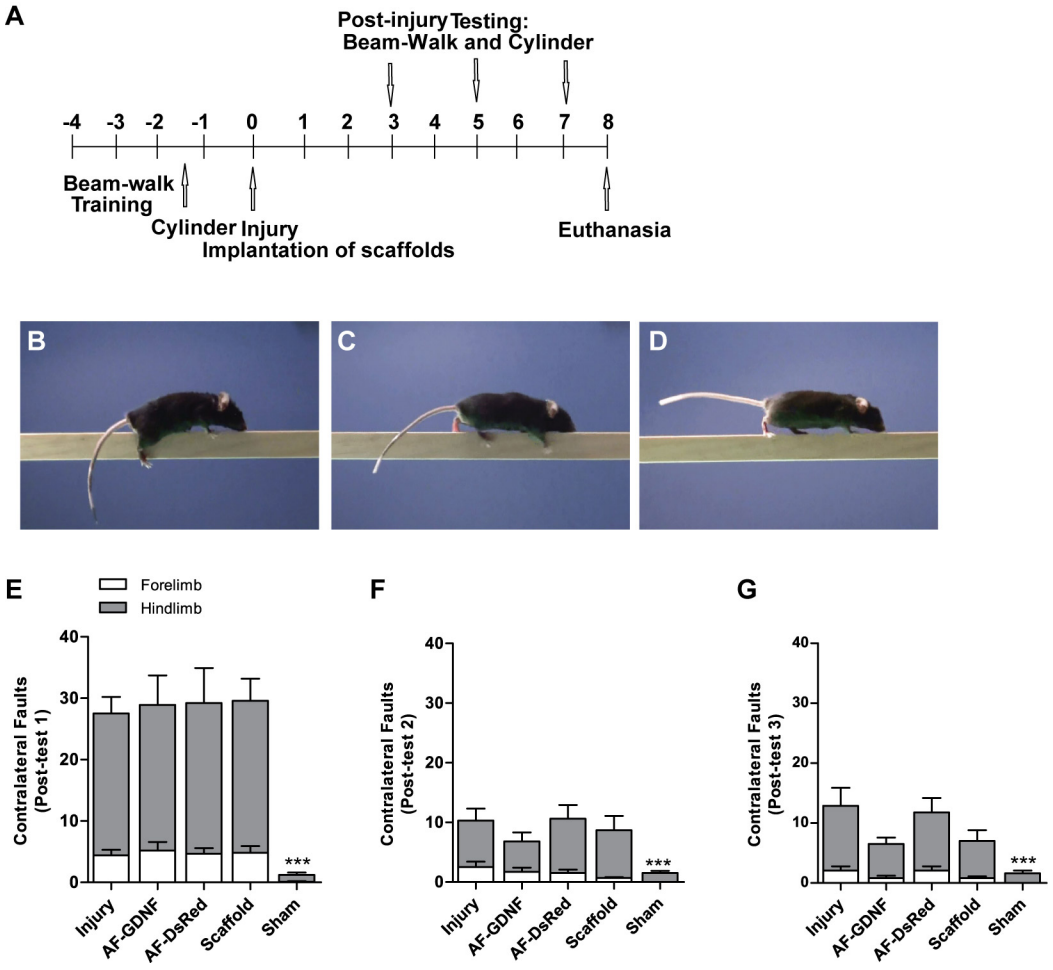
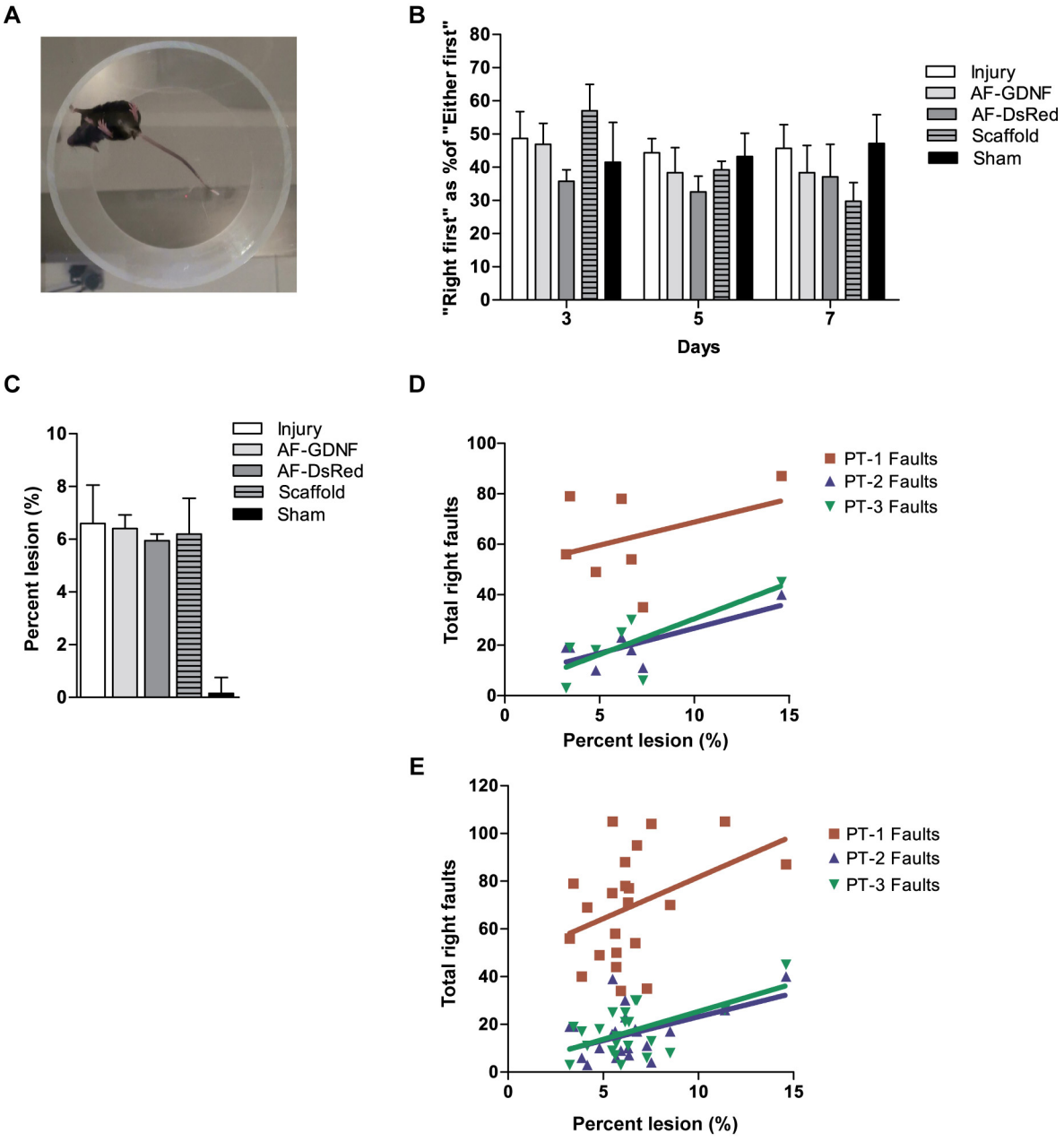


Figure 9. Effect of GDNF on forelimb and hindlimb faults during beam-walk
A. Schematic of timeline of experimental procedures for behavioural studies. Following injury, the animals were tested on the beam walk and the number of foot faults was scored by whether the **B**. hindlimb or **C**. forelimb paw slipped off the beam compared to **D**. sham animals (no faults). Animals were scored at three post-test intervals: 3 (PT-1), 5 (PT-2) and 7 (PT-3) days post-surgery. Total contralateral hindlimb faults were scored and summarized in **E-G**, respectively**,** for each post-test (mean + SEM, n=8; *** $p<0.001$ vs injury). Neither the total contralateral hindlimb nor forelimb faults were significantly decreased in AF-GDNF mice compared to injury alone.


A
B
"Right first" as %of "Either first"
Days
Injury
AF-GDNF
AF-DsRed
Scaffold
Sham
C
Percent lesion (%)
D
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Percent lesion (%)
PT-1 Faults
PT-2 Faults
PT-3 Faults
E
Total right faults
Percent lesion (%)
PT-1 Faults
PT-2 Faults
PT-3 Faults

Figure 10. Effect of GDNF on forelimb use and percent tissue loss
A. Following injury, the cylinder behaviour task was used to measure forelimb preference during vertical exploration at 3, 5 and 7 days post-surgery. The results of the cylinder task are summarized in **B**. presented as "right first" contacts as a percentage of total number of contacts in which either the left or right paw was placed on the cylinder wall. AF-GDNF animals did not show a preference for ipsilateral forelimb use. **C.** Tissue loss following injury was calculated as the volume of the injured hemisphere as a percent of the intact hemisphere for each test group at 8 days post-surgery (mean + SEM, n=8). The percentage of left cortical tissue loss was similar for each test group with no significant decrease observed in AF-GDNF animals**.** Correlation analysis between lesion volume and observed behavioural deficits was performed for each post-test (PT) interval at 3 (PT-1), 5 (PT-2) and 7 (PT-3) days for **D**. injured animals alone and **E**. collectively for all groups. A strong positive correlation was observed between the lesion volume and the observed total number of contralateral faults in our injury model at PT-2 and PT-3 in both injured animals alone (n=8; PT-2 R^2=0.58, PT-3 R^2=0.58) and all test groups (n=8; PT-2 R^2=0.23, PT-3 R^2=0.33).

index was calculated for the pre-surgical baseline and at each post-test interval (3, 5 and 7 days). As seen in Figure 10B, the asymmetry index was near 50% for injury-alone animals (as for shams) suggesting that the mice did not shown an increased reliance on the non-affected paw and thereby we were unable to detect any effect of GDNF delivery using this task.

Volume of Tissue Loss

The results of the behavioural assessment were complemented by measuring the total amount of brain tissue in each hemisphere to determine the amount of tissue loss following injury, as a readout, to determine whether GDNF delivery to the injury site results in the protection of surrounding tissue. Only intact brain tissue was measured and did not include the volume taken up by the PGA scaffold. The percentage of cortical tissue loss due to injury was not significantly different in animals receiving AF-GDNF and AF-DsRed / PGA implants compared to injury and PGA scaffold alone animals, with an average tissue loss of approximately 6% among the test groups (Figure 10C). These results suggest that within the time frame examined, GDNF delivery to the injury site did not significantly reduce tissue loss due to secondary injury.

In order to confirm that our behavioural read-out (beam-walk faults) is a good indicator of the degree of tissue loss in the brain, we performed a correlation analysis between the number of contralateral faults and the amount of tissue loss in the injury alone group. We found that the correlation between lesion volume and beam-walk performance at post-test 1, although positive, was not significant likely owing to a high degree of variability among

animals at this post-surgical recovery interval. However, there was a significantly strong positive correlation between the lesion volume and the observed total number of right hindlimb faults in our injury model at post-test 2 and post-test 3 after injury. (i.e the larger the lesion volume, the more severe the contralateral behavioural deficit; Figure 10D). In fact, the lesion size accounts for approximately 70% of the variability in hindlimb behavioural performance at post-test 3, which suggests that the chosen behavioural task is a good readout for the amount of tissue damage in the brain. Based on this observation in the injury alone animals, we then performed the correlation analysis across all the lesioned groups in the study and found that the positive correlation between lesion volume and behavioural deficits remained (Figure 10E). Our correlation analysis demonstrates a linear relationship between lesion volume and the degree of behavioural deficits observed, which suggests that in this surgically induced brain injury model reliable functional data can be produced that can provide additional information for the evaluation of potential neuroprotective agents, despite our lack of behavioural improvement following GDNF delivery during the time-frame examined.

Evaluation of neuroprotective effect in injured brains

Since scaffolds containing cells, engineered to secrete specific neurotrophic factors, at the injury site have been considered as possible treatments to achieve successful neuroprotection [82, 83], we examined the effect of AF-GDNF/PGA implants on neuronal survival and gliosis in the peri-lesion area following injury. In addition to neuronal loss, injury to the brain leads to tissue disruption resulting in the release of molecules from injured and dead cells; which elicit microglial and astrocyte activation [84, 85]. Hence, we used cell counts to

quantify neuronal cells, as well as microglial and astrocyte infiltration (gliosis) in the peri-lesion area to determine whether we had improved neuronal survival and reduced gliosis following delivery of GDNF to the injured site. A defined peri-lesion area, lateral to the lesion edge and midway through the cortical depth was analyzed (Figure 11A, square inset). Cells in this peri-lesion area, surrounding the injury site, are most vulnerable to secondary injury that evolves in response to the insult and represent the most appropriate candidates to evaluate neuroprotection. The number of surviving neurons was scored by counting the number of nuclei positive for NeuN, a neuronal specific nuclear protein, within the defined peri-lesion area. Activated microglia and infiltration of reactive astrocytes were scored in a similar manner by evaluating the number of IBA1 and GFAP positive cells, respectively. Figure 11B-G summarizes the confocal immunofluorescence in the peri-lesion area for NeuN, GFAP and IBA1 and the cell counts in injury, AF-GDNF and sham mice. We found the number of NeuN positive cells to be decreased in the peri-lesion area for all injured mice compared to the corresponding cortical region in sham mice; however, GDNF did not decrease the loss of NeuN cells in AF-GDNF mice compared to injury alone (Figure 11B-C, data not shown). Following injury, activated microglia and astrocytes infiltrate the injured core, peaking at 7 days post-injury [86]. Indeed, we found a significant increase in both microglia and astrocyte infiltration into the peri-lesion area in injury and AF-GDNF mice compared to shams, but no difference between injury and AF-GDNF, AF-DsRed and Scaffold alone mice (Figure 11D-G, data not shown). There was no detectable activation of gliosis in the contralateral cortex (data not shown). Furthermore, we observed very few TUNEL and Caspase3 positive cells in the peri-lesion area among the injured test groups at 7 days post-injury (data not shown). In summary, GDNF delivery did not subdue gliosis in

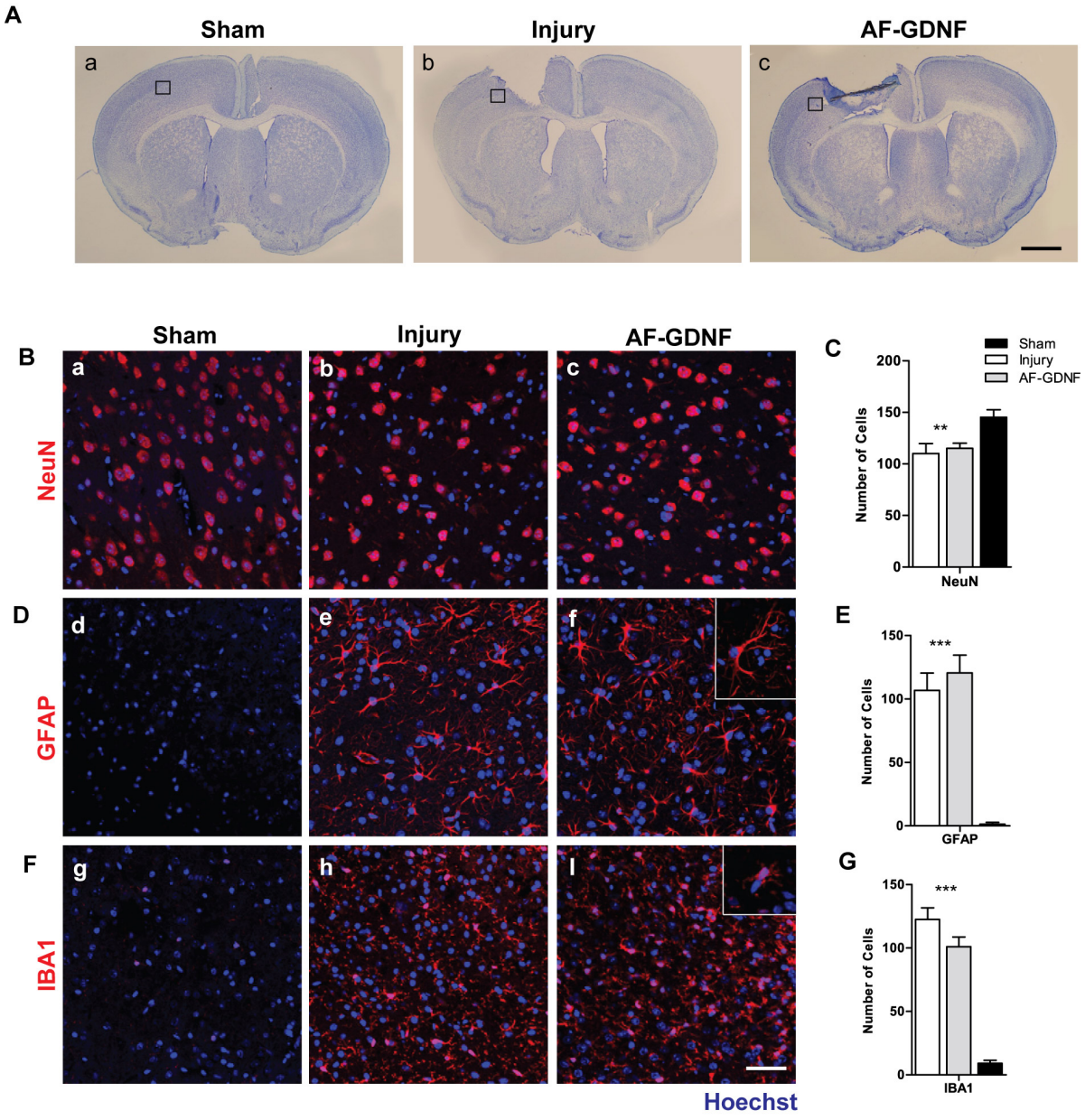

Figure 11. Histological analysis of neuroprotective effect in injured brains
A. Cresyl violet staining of coronal sections of (a) sham, (b) injury and (c) AF-GDNF mice showing relationship of the peri-lesion area (square) examined in **B,D,F** relative to the injured cavity. Confocal immunofluorescence staining for **B**. neurons (NeuN, red), **C**. astrocytes (GFAP, red) and **D**. microglia (IBA1, red) in the peri-lesion area. Insets represent a higher magnification of astrocytic and microglial morphology. **B,D, F** Compared to (a,d,g) sham animals, the peri-lesion area in the (b,e,h) injury and (c,f,i) AF-GDNF animals contain fewer numbers of neurons (e,f) and increased gliosis by infiltrating astrocytes and microglia (b,c,h,i). Hoechst (blue) was used to label nuclei. **C,E,G**. Quantification of neurons and astrocyte and microglial activation based on cell counts in the peri-lesion area (inset in **A;** mean + SEM, n=8; *** $p<0.001$, ** $p<0.01$ vs sham).

the peri-lesion area, as evidenced by a failure to reduce the number of infiltrating microglia or astrocytes, nor did we find an increase in NeuN positive cells.

Discussion

Brain injury, either surgically induced or as a result of stroke or trauma, not only results in considerable disability and mortality, but is also a major cause of functional impairments severely affecting the quality of life of an individual [87]. Despite decades of research and development, there is currently no pharmacologically effective treatment available for preventing neuronal loss or repairing the brain after injury, beyond promoting the circulation of the blood supply to the damaged area and reducing brain swelling in the immediate post-injury phase [88-90]. Current management strategies for brain injury are mainly focused on neuroprotection, such as preventing tissue loss with pharmacological agents, but these still demonstrate poor long-term outcomes [91, 92]. Fortunately, increasing evidence suggests that cell-based transplantation therapies may be a promising therapeutic strategy for protecting host cells from secondary injury and alleviating functional impairments following brain injury [93]. Secondary brain injury is caused by a combination of inflammatory, cytotoxic and apoptotic processes that proceed the immediate injury resulting in cell death [94]. Several lines of evidence suggest that neurotrophic factors play an important role in the cellular events that ensue following brain injury and neurotrophic factor delivery to the lesion site can protect against neural cell death and help re-establish neuronal networks by stimulating the sprouting of injured neurons [95]. The spatial and temporal balance between neuronal survival and death depends, in part, on the overall level and duration of neurotrophic factors present and the type of receptors that are expressed post-injury [96].

GDNF is a potent neurotrophic factor that plays an important role in the survival of several neural populations in the CNS by increasing the production of anti-apoptotic proteins and cell survival factors, while reducing the production of pro-apoptotic proteins [97-101]. In our surgically induced brain injury model, we found that GDNF, GFRα1 and its signaling moiety c-Ret were upregulated immediately after injury *in vivo* which provides a rationale for delivering GDNF as a therapeutic agent in the early stages post-injury to stimulate endogenous neuroprotective mechanisms. Accordingly, we employed lentivirally-infected AF cells expressing GDNF (AF-GDNF) to evaluate the neuroprotective potential of exogenously supplied GDNF following unilateral injury to the left motor cortex. This virally-mediated gene delivery approach has been explored in many animal models of brain injury and neurodegenerative diseases [102-105]. Pre-treatment with GDNF by intracerebroventricular or intraparenchymal injection [106-108], topical application on cortical surfaces [38, 109-112], delivery using viral vectors [113-117] and transplantation of GDNF-expressing cells [39, 118-122] prior to injury reduced cerebral infarct volume, delayed neuronal death and improved behavioural deficits. Specifically, the neuroprotective effects of GDNF administration prevented the activation of Caspase3 [38, 110, 112, 115, 123-125] and enhanced neurite outgrowth [125] suggesting that GDNF may inhibit apoptotic pathways as well as be essential for the formation of appropriate neuronal circuits post-injury. To confirm whether GDNF pre-treatment modulates neuronal outcome after injury, we studied the effects of GDNF on mouse cortical neuron cultures exposed to hydrogen peroxide induced cell death. Consistent with previous findings [26], GDNF pre-treatment from AF-GDNF cells significantly protected both AF cells and cortical neurons from hydrogen peroxide mediated cell death. Although we did not investigate the direct

mechanism by which GDNF exerted its protective effects *in vitro*, it is very likely that it may have acted through its ability to prevent apoptosis by preventing Caspase3 activation. In fact, prevention of apoptosis has been proposed as the mechanism of GDNF-induced neuroprotection in dopaminergic neurons [97] and animal models of ischemia [26, 116, 124]. Furthermore, we found that AF-GDNF/PGA implants survived longer in serum-free conditions compared to the AF-DsRed/PGA implants. This suggests that constitutive GDNF secretion from AF cells may potentially improve the survival of the transplanted cells. Our observations support previous studies that have shown that even short-term pre-treatment of cellular grafts with neurotrophic factors, for a limited period of time, promotes graft survival and functional integration *in vivo* [126-128]. Since poor cell viability is a major limitation in cell-based therapies [128, 129], the increased survival of the AF-GDNF/PGA implant graft is a promising strategy for improving clinical efficacy of cell transplantation therapies. In fact, many strategies have been pursued to improve graft survival *in vivo* [130-134] of which protection of donor cells or modulation of the host tissue environment has proven to be most promising [135]. Hence, by genetically modifying AF cells to secrete GDNF, AF-GDNF/PGA secretion of GDNF *in vivo*, can then act in an autocrine and paracrine fashion to confer protection of transplanted AF cells while potentially protecting host neural cells from secondary injury, respectively. In fact, following pre-treatment with GDNF, a recent study has shown that the therapeutic potential of AF cells is enhanced and likely mediated through local paracrine action, owing to the increased secretion of cytokines and growth factors including IL-6, VEGF, SDF-1 and IGF-1 which were detected in GDNF-treated AF cell conditioned media [23]. These factors are known to exert neuroprotective effects in the brain [136-139] and can work in concert with GDNF to stimulate an endogenous

neuroprotective response. In fact, the favourable outcomes following AF cell transplantation in the nervous system have been attributed to the endogenous trophic factors secreted by AF cells [19, 140] which may aid in limiting further damage and/or stimulate repair of injured tissues by modulating the immune response [141, 142], decreasing glial scar formation and rendering the peri-lesion environment less toxic to surrounding cells [10, 143]. Such paracrine mechanisms have also been postulated to explain some of the positive effects of other stem cell sources in animal models of organ/tissue injury [144-146]. Although GDNF pre-treatment strategies are not a feasible clinical approach, increasing the survival of grafted cells and hence GDNF delivery can aid in promoting host endogenous neuroprotective mechanisms following transplantation.

Despite the growing evidence suggesting that increasing the supply of neurotrophic factors to the injured brain can be a potent way to protect host cells and restore neural function, delivery to the brain has proven to be quite difficult. The presence of the blood-brain barrier makes it impossible for large proteins and complex compounds to cross from the blood into the brain. Undeniably, the major limitation to the success of GDNF in PD clinical trials has been the effective delivery of GDNF to the CNS due to its inability to cross the blood-brain barrier, peripheral side-effects following systemic administration and difficulty in targeting the appropriate cells in the brain [147, 148]. Hence, clinical trials have used local intracerebralventricular and intraputamenal injections for precise delivery of GDNF to the target areas [30-34]. Nevertheless, to date, the majority of these clinical trials for directed GDNF delivery in PD patients have shown a lack of therapeutic benefit largely due to poor delivery, distribution and bioavailability with the majority of GDNF being confined to the

immediate vicinity of the catheter tip [34, 149]. Currently, efforts are underway to improve GDNF delivery, distribution and dosing in target tissues to increase bioavailability and efficacy in PD [35]. However, in the case of brain injury models, which often result in the formation of an infarct or lesion cavity, injecting GDNF directly into the cavity site is futile. To circumvent this delivery issue, we used PGA scaffolds to deliver GDNF-secreting AF cells locally into the cavity, an approach which could potentially reduce systemic side effects and increase the therapeutic effectiveness by ensuring the virally encoded GDNF is delivered to the target region. This is particularly a promising approach in a surgically induced brain injury model in which tumor or tissue excision leaves a cavity, which is often filled with an absorbable hemostat (an oxidized regenerated cellulose product manufactured by Johnson & Johnson) to provide hemostasis. However, these commercially available hemostats do not facilitate neuroregeneration. Accordingly, implantation of AF-GDNF/PGA implants, at the time of surgery, can ensure the efficient delivery of the right factor in a clinically relevant time frame (when the neurosurgical intervention is carried out) to potentially improve functional recovery.

An important advantage of PGA scaffolds is that they are easily shaped to fit the injured cavity and are biodegradable via hydrolysis into monomers of glycolate, a normal metabolic product of saccharide metabolism in human cells [150, 151], which are non-toxic to donor or host cells. By providing a temporary and artificial extracellular matrix [152], we found that PGA scaffolds were able to support AF-GDNF cell attachment, survival and GDNF secretion *in vitro* and *in vivo*. Although we observed a progressive decrease in GDNF levels from AF-GDNF/PGA implants over time, in parallel with the gradual reduction of cell

numbers due to serum-deprivation *in vitro* and hostile *in vivo* conditions, the GDNF delivered in the acute time frame was sufficient to activate the MAPK/ERK signaling pathway in host cells which has been shown to promote neuronal survival [153]. AF-GDNF mediated increase in ERK phosphorylation in host tissue suggests that cortical neurons and astrocytes respond to exogenous GDNF levels by activating the MAPK/ERK signaling cascade. The MAPK/ERK pathway has been shown to be involved in GDNF-induced neuronal survival and potentially responsible, in part, for mediating its neuroprotective effect [28, 78]. Although ERK signaling is multifaceted in modulating neuronal survival [154], it has been shown that ERK directly phosphorylates pro-Caspase9, which inhibits Caspase9 processing and subsequent Caspase3 activation, thereby blocking the caspase cascade during apoptosis [155]. Interestingly, we also observed a transient AF-DsRed mediated increase in Erk phosphorylation at 3 days post-implantation. This finding suggests that even in the absence of GDNF secretion, trophic factors released from AF cells are able to activate the MAPK/ERK pathway in host cells. More specifically, VEGF, a factor that is secreted in AF-conditioned media [23] has been shown to protect cortical neurons following traumatic brain injury (TBI) by activating the ERK pathway [136]. Thus, in the absence of genetic modification, AF cells secrete a number of factors that could potentially enhance endogenous regeneration and neuroprotection [156-158], the mechanism by which remains elusive.

In the current study, we assessed the behavioural motor deficits in an animal model of surgically induced brain injury to evaluate the neuroprotective potential of AF-GDNF/PGA implants. Previous studies have shown that brain injury to the motor cortex results in

significant behavioural deficits contralateral to the injured side, as assessed by the beam-walk and cylinder test. Unilateral brain injury models tend to induce a hemiparesis-like effect, which can cause the rodent to slip to one side, usually contralateral to the injury site and hence has been used to assess motor coordination in lesioned mice; whereas, the cylinder task has proven to be an efficient test of the unilateral deficits in voluntary limb use [159]. Despite some promising trends, we failed to observe a significant functional improvement of AF-GDNF animals compared to the other test groups. A major limitation to our *in vivo* studies has been the rapid, spontaneous recovery of injured animals on the beam-walk task. Although all test group animals exhibited increased contralateral forelimb and hindlimb faults compared to sham animals at 3 days post-injury, by 7 days post-injury, the number of discernable slips had dropped substantially. As a result, the window of opportunity to be able to identify GDNF-mediated functional improvements was significantly limited. This could be attributed to the notion that animals that sustain injury resulting in functional impairments can learn to cope using compensatory motor strategies during behavioural analysis, potentially masking any effect of the treatment. For instance, at every successive beam-walk post-test, the animals compensate by shifting reliance towards the non-injured paw and through tail deviations towards the impaired limb to provide balance for leaning on the non-impaired limb – as such, these compensatory behaviours mask the true deficit making it hard to assess whether the recovery is due to GDNF treatment or motor learning (rehabilitation) effects. The use of a tapered ledged beam, which provides a step support so that the animals are not induced to compensate using alternative motor strategies, could potentially aid in scoring the deficits more easily. Although we did not observe an effect of GDNF on functional outcomes, we cannot exclude the possibility

that a beneficial effect of GDNF might be detected in other behavioural tasks [160, 161]. Moreover, we failed to identify any increased reliance on the ipsilateral paw using the cylinder task to measure forelimb preference during vertical exploration in injured animals. Since the cylinder task measures gross aspects of motor function, based on our observations, a task that more accurately analyses finer aspects of voluntary motor control, such as the staircase task, would likely be more sensitive. In the staircase task, animals are allowed to access food pellets by reaching down on either side of a raised plinth located along the center of a corridor in order to grasp and retrieve food pellets located on the steps of a baited descending staircase. This task provides a measurement of skilled paw use where independent forelimb reaching and grasping abilities can be quantitatively assessed, as a measure of fine motor control [162, 163].

In this study, we examined behavioural deficits for a period of 7 days to determine the neuroprotective effect of GDNF post-injury. Although the trend towards greater improvement in GDNF animals at post-test 3 on the beam-walk task did not reach statistical significance, it is possible that testing the mice at a longer post-surgical interval may have revealed a significant effect of GDNF, as observed by Minnich *et al.* [164]. Following infection, we achieved GDNF secretion levels within the therapeutic dosage of 1.5 ng/ml from AF-GDNF/PGA implants, similar to the neuroprotective dosage of recombinant GDNF used *in vitro* and that reported by Minnich *et al.* [164]. Although GDNF secretion levels were high at 3 days post-transplantation, the gradual decrease in GDNF levels over time suggests that the lack of behavioural improvement may be due to the decrease in GDNF expression. In fact, the neuroprotective effect of GDNF has been shown to be dose

dependent [35]. For instance, using adenoviral delivery of GDNF, Schmeer *et al.* demonstrated a dose-dependent rescue of axotomized rat ganglion cells where a higher dosage of GDNF increased the number of surviving cells [165]. This dose-dependent relationship may also be relevant to behaviour recovery. Accordingly, had GDNF expression been sustained at the therapeutic dosage during the entire 7 day period, behavioural recovery may have been enhanced and this remains to be investigated. However, it is important to note that higher GDNF concentrations can also be associated with neuronal and synaptic toxicity [35] and hence an appropriate dosing regime will need to be clearly defined in future studies. By using a constitutively active GDNF lentivector and transplanting the AF-GDNF/PGA implants directly into the injured cavity, we aimed to deliver a low therapeutic concentration that was non-toxic and decreased over time to boost endogenous repair mechanisms while circumventing any long-term side-effects of sustained GDNF expression.

Since AF-GDNF/PGA implants demonstrated a significant neuroprotective effect *in vitro* but not enhancement of behavioural recovery post-injury *in vivo*, we examined the injured cortex to assess neuronal loss and gliosis as determinants of a subdued secondary injury response. In this study, we have used a surgically induced model of brain injury to mimic the damage inflicted on the brain during neurosurgical procedures, such as excision of brain tumors or removal of epileptic foci, as previously described [57, 58, 60, 65, 77]. Similar to TBI and stroke, the injury site is characterized by an infiltration of astrocytes, invasion of activated microglia, formation of a glial scar, apoptosis of neurons and an expanding lesion volume over time [77, 166]. Cresyl violet staining and volumetric analysis showed that the average cortical tissue loss of 6% among injured animals was similar for all test groups at 7

days post-injury. This evolution of the lesion was accompanied by neuronal loss and damage to the cell bodies and neurite extensions, accompanied by an infiltration of activated microglia and reactive astrocytes (gliosis) in the peri-lesion area, a natural response to injury [84, 167]. This observed neuronal loss and gliosis was similar between test groups and the lack of increased gliosis following transplantation of the PGA and AF-GDNF/PGA implants in the peri-lesion area suggests that the PGA itself (or its hydrolysis) did not elicit an enhanced inflammatory response, a property previously described for PGA [44]. Lastly, we observed very few TUNEL or Caspase3 positive cells in the peri-lesion area at 7 days post-injury. Future studies should examine Caspase3 activity at early time points since activated Caspase3 has been shown to appear at 6-72 hours following TBI [168].

In conclusion, we demonstrate that AF cells represent a new potential human cell source for the delivery of neurotrophic factors to the injured brain. Although GDNF was neuroprotective *in vitro* and enhanced survival of grafted AF cells, it did not significantly decrease behavioural deficits or lesion volume *in vivo*. In this study, we only focused on the short-term neuroprotective outcome of GDNF delivery in the acute injury phase; however, the long-term neuroprotective/neuroregenerative effect of the AF-GDNF/PGA implants will need to be examined in the future and is critical for evaluating the fate of the implanted AF-GDNF cells as well as assessing safety and long-term efficacy *in vivo*. Nonetheless, this study examines the envisioned use of AF cells following brain injury in an effort to boost host endogenous protection mechanisms through neurotrophic factor delivery. Implantation of PGA scaffolds, following surgical brain trauma, seems suitable for those patients with

lesions that present a poor chance of cavity recovery and may itself provide an improved microenvironment for promoting functional recovery.

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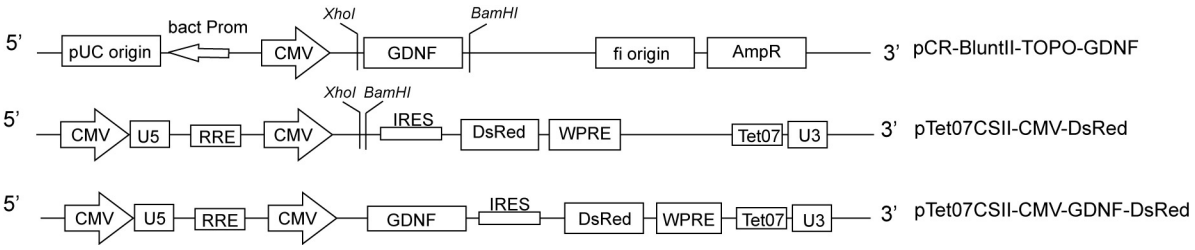
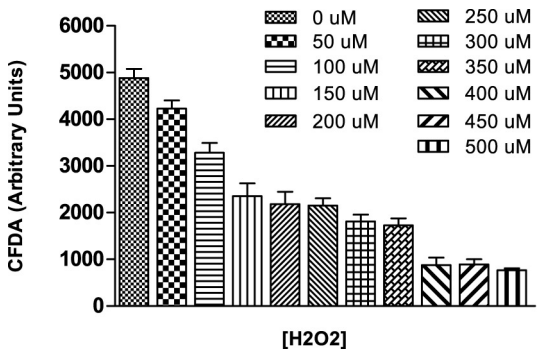
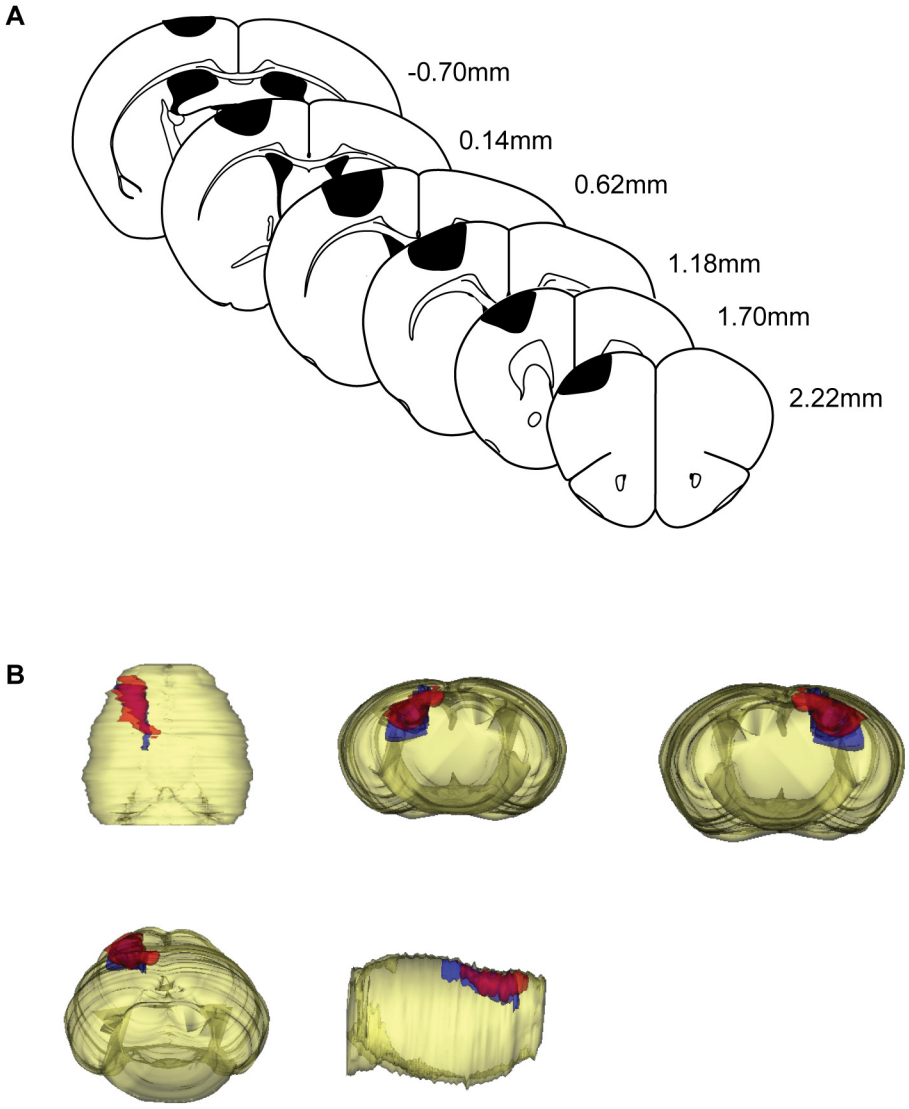


Supplementary Figure 1. Construction of pTet07CSII-CMV-GDNF-DsRed vector
A. To construct the pTet07CSII-CMV-GDNF-DsRed, the sequence for recombinant GDNF was cut from the pCR-BluntII-TOPT-GDNF plasmid and inserted into the lentivector pTet07CSII-CMV-DsRed immediately upstream of the sequence encoding IRES-DsRed.

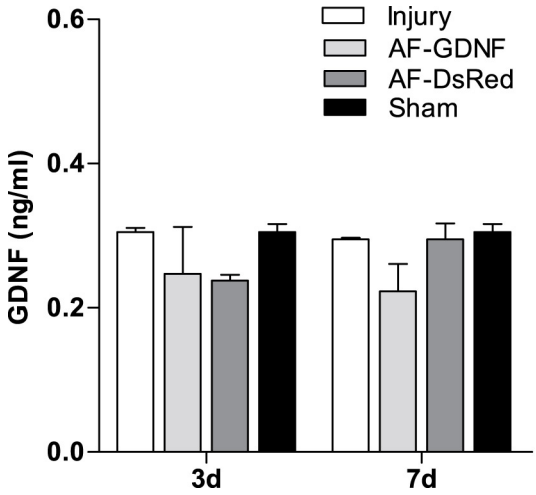


Supplementary Figure 2. AF cells susceptibility to hydrogen peroxide mediated cell death

A. H_2O_2 treatment decreases AF cell viability in a dose-dependent manner compared to untreated cells. Surviving adherent cells were scored based on CFDA fluorescence (arbitrary units). A concentration of 200 μm H_2O_2 was used from subsequent H_2O_2 – mediated cytotoxicity assays for AF cells (mean + SEM, n=3).



Supplementary Figure 3. Injured area within the left motor cortex
A. Schematic representation of the location and average extent of lesion one week post injury (ranging from 2.22 mm to -0.70 mm from Bregma (0 mm)). **B**. The injured region (Red/Orange) was mapped according to the mouse brain atlas (motor cortex shown in Blue) and 3D rendition shows that the actual injury overlaps well with the location of primary motor cortex (Pink/Magenta).



Supplementary Figure 4. *In vivo* GDNF expression in right motor cortex
ELISA quantitation of the amount of GDNF protein secreted in the right motor cortex, contralateral to the injury, at 3 and 7 days (d) post-injury. There was no significant difference in GDNF levels among the test groups (mean + SEM, n=3).

Chapter 5 - General Discussion

5.1 Overview of findings

Cell-based therapies have been extensively explored as an approach for the treatment of several human diseases, due to their ability to represent a new cell source for cell replacement therapies and produce trophic factors able to minimize damage and promote recovery in injured tissues. In recent years, amniotic fluid (AF) cells have emerged as an alternative source of stem cells for cell-based therapies. Accordingly, this body of work has focused on characterizing AF cells (Chapter 2) and examining their therapeutic potential in mediating a neuroprotective effect in the injured brain through direct cell-cell communication via gap junctions (Chapter 3) and trophic factor delivery (Chapter 4).

Despite the wide and well-established use of AF cells in routine prenatal diagnosis, the properties and origins of AF-derived cells in poorly understood. AF cells originate from several fetal tissues including the skin and the lining of the digestive, respiratory and urinary systems [1, 2] giving rise to a heterogeneous cell population that differs greatly based on gestational age. This heterogeneity significantly hinders the application of AF cells for future cell-based therapies and a better understanding of the inherent variation among amniocentesis samples, from different donors, is instrumental before these cells can be used for cell-based therapies. In Chapter 2, we used AF samples from different donors, originating from the second and third trimesters of gestation (15-35 weeks), to provide a comprehensive molecular characterization of AF cells with regard to genes involved in stem cell maintenance, neural commitment and differentiation. Despite their heterogeneity, we found that a large percentage of AF cells were largely epithelial in origin, likely derived from the different layers of the developing skin and cells lining the urothelium, lungs and

oral-nasal cavities of the fetus based on the KERATIN expression profile. We observed that AF cells expressed various components of the NOTCH signaling pathway as well as early neural markers, namely NESTIN, CD133 and PAX6. We also found that a very small number of cells (<1%) expressed the pluripotency markers OCT4a, SOX2 and NANOG, which can be enriched for by single cell cloning to generate clonal cell lines. Clusters of SOX2-expressing AF cells were able to give rise to neuronal-like morphology expressing makers such as MAP2, NFL and NSE following treatment with neural induction media. Hence, our findings demonstrate the presence of fetal cells with stem cell characteristics in the AF. The fact that AF cells with potential stem cell properties are found at different gestational ages, displaying different gene expression profiles, necessitates the need for proper identification and characterization of AF-cell derived clones for future studies.

In an effort to pursue the therapeutic potential of AF cells in our subsequent studies, we generated single cell clones from AF cells, derived at 26 weeks of gestation. The generation of a homogenous cell population is important in the interpretation of molecular analysis and functional tests to ensure that our results did not reflect the additive effects of one or more cell types. Since the usage of stem cells is a promising strategy for the repair of damaged tissue in the injured brain, we used our clonally-derived AF (AF-F5) cells in Chapter 3 and Chapter 4 to examine the therapeutic potential of AF cells in mediating neuroprotection. In Chapter 3, we sought out to examine whether AF cells could form functional gap junctions with host cortical cells to establish gap junctional intercellular communication (GJIC). Although AF cells have been previously transplanted into the brain [3-5], there is no information on gap junctions in these cells and their capacity to establish a means of

intercellular communication with the host tissue. In this study, we have shown that AF cells express high levels of CX43 and are able to establish Cx43 mediated functional gap junctions with cortical astrocytes. Furthermore, we report an induction of Cx43 expression in astrocytes following injury to the mouse motor cortex and demonstrate for the first time CX43 expression at the interface between implanted AF cells and host astrocytes. Since GJIC between grafted and host cells is proposed to be an essential participant in the neuroprotective effect associated with stem cell engraftment [6, 7], CX43-expressing AF cells could potentially play a role in the rescue of damaged cells after brain injury and promote neuroprotection.

Recent advances in the understanding of molecular pathways and their physiological roles have demonstrated that neurotrophic factors play an important role in the development, maintenance and regeneration of the CNS [8]. Specifically, GDNF has received a lot attention as a potent neurotrophic factor in experimental models of brain injury. Accordingly, in Chapter 4, we examined the neuroprotective potential of AF cells, engineered to secrete GDNF (AF-GDNF), in a surgically induced model of brain injury. Our results show that GDNF pre-treatment of AF cells and cortical neurons, 2 hrs prior to exposure of hydrogen peroxide, significantly increased cell survival rates compared to untreated cells. Encouragingly, the AF-GDNF cells seeded on PGA implants (AF-GDNF/PGA) survived longer in serum-free conditions compared to control cells and continued to secrete GDNF post-implantation into the injured motor cortex. The increased survival of AF-GDNF/PGA implants is a promising strategy for improving clinical efficacy of cell transplantation therapies, particularly in brain injury models when the cells are

introduced into a cytotoxic environment. Resultantly, many transplanted cells do not survive and undergo apoptosis, thereby limiting their neurotrophic factor delivery and endogenous paracrine actions. However, due to the increased survival of AF-GDNF cells, GDNF delivery in the acute period following injury was sufficient to activate the MAPK/ERK signaling pathway in host cells in the peri-lesion region, potentially boosting endogenous neuroprotective pathways. Despite some promising trends, we did not observe significant behavioural improvements following AF-GDNF/PGA implantation on the beam-walk and cylinder tasks or reduced lesion volume during the 7 day time-frame. Nevertheless, in this study we demonstrate that AF cells represent a new potential human cell source for the delivery of GDNF to the injured brain in an effort to mediate a neuroprotective effect.

5.2 Future directions

The reported neural potential of AF cells has stimulated an interest in developing AF cell-based therapies for treating damaged neural tissue. Similar to our studies, AF cells have repeatedly been reported to acquire a neuronal morphology and express neuronal markers such as β-III tubulin, NeuN, MAP2, NFL and NSE *in vitro* under neural induction conditions [4, 9-14]. Nevertheless, these observations do not prove the neuronal differentiation and functionality of AF cells. To be regarded as functional neurons, AF cells must form synapses capable of maintaining a stable resting potential and generating action potentials with characteristic changes in membrane voltage when stimulated. Furthermore, electrophysiological recording and electron microscope examination of pre- and post-synaptic specializations are required to confirm the neuronal properties of AF cell differentiated neurons [15, 16]. To date, the generation of action potentials by neuronally

differentiated AF cells has not been demonstrated and clear evidence that AF cells can form functional synapses, after neural induction, is lacking [17]. Hence, the ability of AF cells to differentiate into mature functional neurons requires further investigation and the use of AF cells for cell replacement therapies *in vivo* is still very much in its infancy.

Given the heterogeneity of AF cells, the isolation of specific cell types through clonal methods requires a detailed phenotypic and molecular analysis to standardize AF cell research [18]. Furthermore, it is important to ascertain which sub-populations of AF cells might be more suitable for use in neural applications. For instance, Arnhold *et al* reported enhanced neuronal differentiation capacity of C-KIT$^-$ compared to C-KIT$^+$ AF cells [19], although these differences have not yet been examined *in vivo*. In our studies, we showed that SOX2$^+$ AF cells acquire a neuronal phenotype in neural induction conditions and potentially represent an AF cell sub-population primed for neuronal differentiation. SOX2 expression in the brain has been found in neural stem cell (NSC) populations, which are self-renewing multipotent cells that can differentiate towards neurons, astrocytes and oligodendrocytes; the main cell types in the adult central nervous system [20]. Accordingly, we have generated a SOX2$^+$ clonal AF cell line to examine the neural differentiation potential of SOX2$^+$ AF cells *in vitro* and *in vivo* compared to other clonal AF cells (including AF-F5 and C-KIT$^+$ AF-C12 clones). In future studies, we hope to ascertain whether SOX2$^+$ AF cells harbor the same differentiation potential compared to NSC and potentially represent a suitable starting cell population for cell-replacement therapies for brain injury. Furthermore, the question of whether AF cells should be pre-differentiated towards a neuronal fate or a specific neuronal subtype *in vitro,* prior to implantation,

requires further investigation. In order to do so, a better understanding of the culture conditions needed to direct AF cells towards a specific neuronal subtypes will be necessary. Accordingly, the generation of clonal AF cell lines will provide new tools to evaluate the therapeutic potential of AF cells.

On the other hand, AF cells have been shown to exert beneficial effects post-implantation independent of neuronal differentiation or integration into the host circuitry [5, 21, 22]. Based on these observations, neuronal differentiation of AF cells may not be required to achieve positive outcomes. In fact, research thus far supports this notion as transplantation of AF cells has been shown to improve behavioural outcomes after peripheral nerve injury and brain ischemia without evidence of cell replacement [5, 23, 24]. In fact, it is becoming more clear that grafted AF cells may exert a trophic effect on the host microenvironment by secreting neurotrophic and mitogenic factors [25] or through direct cell-cell communication with host cells via gap junction [6, 7]. Such paracrine mechanisms have been postulated to explain some of the positive effects of other stem cell sources in animal models of organ/tissue injury (reviewed in [26]). However, the mechanisms by which AF cells can provide neuroprotection or ameliorate behavioural outcomes post-injury requires further investigation. Specifically, identifying which factors are secreted by AF cells, and under which conditions, will greatly enhance our understanding of their neuroprotective characteristics *in vivo*. Furthermore, functional communication between grafted and host cells plays a pivotal role in delivering therapeutic agents to the damaged tissues and buffering harmful ion concentrations post-injury. Although we have shown that AF cells can form functional gap junctions with cortical astrocytes *in vitro*, it remains to be determined

whether AF cells are capable of integrating into the brain and developing functional connectivity with the host tissue *in vivo* and whether this phenomenon occurs to a scale sufficient enough to confer neuroprotective effects.

Since the realization that the beneficial effects of stem cell transplantation may be due to the localized release of trophic factors and not attributed (in part or entirely) to stem cell differentiation or replacement, research efforts have focused on modulating the paracrine actions of stem cells to enhance their therapeutic efficacy. A number of different approaches, primarily focused on genetic manipulation and *in vitro* preconditioning, have been examined to increase stem cell survival upon transplantation (thereby increasing the total amount of secreted trophic factors and prolonging the duration of secretion) [26, 27]. Accordingly, we have engineered AF cells to secrete GDNF (AF-GDNF) and examined their neuroprotective capabilities following implantation on PGA scaffolds into a surgically induced brain injury model. Despite promising protective results following hydrogen peroxide exposure and enhanced graft survival of AF-GDNF cells *in vitro,* we failed to observe significant behavioural improvements of AF-GDNF/PGA implants *in vivo*. Although we examined whether AF-GDNF/PGA implants could preserve endogenous neuronal cell populations from further cell death 7 days post-injury, the long-term effects of AF-GDNF/PGA implants on endogenous neuroprotective mechanisms and functional outcomes will need to be examined in future studies. Furthermore, it remains of high importance to closely monitor the fate of the implanted cells as well as evaluate the potential paracrine actions of both AF-GDNF and AF-DsRed cells in mediating a neuroprotective effect post-injury, in conjunction with GDNF delivery. These paracrine actions may exert

beneficial effects on host neural cells within the peri-lesion and/or the host microenvironment resulting in long-term functional improvements that can only be assessed at later post-surgical intervals (1-2 months). By elucidating the mechanisms behind AF cell paracrine actions will provide valuable insight into the biology and therapeutic application of AF cells. Furthermore, long-term studies are also crucial in assessing safety, efficacy and the immune status of AF cell transplantation *in vivo*. Specifically, AF cells have received considerable attention for their utility in cell-based therapies based on their immune-privileged status [28] and observed low immunogenicity and greater survival in xenogeneic and allogeneic hosts [4, 29-33]. Characterization of AF cell surface markers revealed low expression of major histocompatibility (MHC) Class I and lack of MHC Class II or co-stimulatory molecules CD80, CD86 and CD40 [34]. AF cells have also been shown to express very low levels of human leukocyte antigen-D related (HLA-DR) and not stimulate $CD8^+$ T cell infiltration post-implantation [35]. Nevertheless, further research is required in order to understand the immunological properties of AF-derived cells in order to enhance graft survival and mitigate functional recovery. Lastly, in addition to providing a scaffold for the delivery of AF-GDNF cells, examining the long-term effects of PGA implants in the injured brain remains to be investigated. Specifically, examining the ability of PGA scaffolds to provide support to surrounding brain tissue, function as a substrate to promote host neural cell growth, axon regeneration and neurite formation will be fundamental in evaluating the neuroregenerative potential of PGA scaffolds in brain injury models. Future clinical applications will rely on the ability of biomaterials to be effective for mediating structural repair of the brain following injury and promote functional recovery.

5.3 Biomedical implications

AF cells have a long history of use in prenatal diagnostic applications, including karyotyping and other genetic and molecular tests to detect fetal abnormalities. In brief, AF cells provide clinically important information about the fetus and accordingly can be used to generate specific cell lines *ex vivo* to study specific chromosomal aberrations [36, 37]. In medical genetics, the future developments of new therapeutic strategies directly depend on a better understanding of the mechanisms by which naturally occurring genetic variation contribute to disease. Furthermore, given that AF cells are readily accessible, pose little to no ethical concerns and do not form teratomas *in vivo*, they have also been investigated as a promising alternative source of cells for tissue engineering and cell-based therapies [38]. As these cells are collected before birth, they may be of potential value for autologous cell treatments, either *in utero* or post-natally, of defects diagnosed during gestation [39]. Since AF cells can be cryopreserved in a cell bank, which will facilitate elective clinical administration to the patient in the future, there is a need for extensive research to establish the putative clinical use of AF cells in humans. The relatively low immunogenicity of AF cells makes them a source of interest for allogeneic transplantation and the first private amniotic fluid cell bank in the US was opened by Biocell Center in October 2009 in Medford, Massachusetts [40], prompting the need to establish national and international registries of cell lines derived from AF in order to make them available to researchers worldwide. This strategy will facilitate the development of guidelines for the derivation and characterization of new cell lines from the heterogeneous AF cell pool and allow researchers to choose the most appropriate cell line for a particular application, hopefully leading to more rapid development of effective cell-based therapies.

Enhancement of neurotrophic function in the injured brain may delay neuronal death, positively influence neuronal growth and differentiation and potentially enhance functional recovery. Accordingly, delivery of neurotrophic factors post-injury continues to be a treatment option with vast clinical potential, particularly if pharmacological compounds with a clinically relevant route of administration can be developed. Despite the vast amount of research that has shown preclinical efficacy in experimental models of brain injury, regrettably, all multi-center clinical trials have failed to demonstrate robust improvements in the outcomes following brain injury and to date, the current standard of care for brain injury patients consists solely of supportive and rehabilitative measures [41, 42]. Although the reasons for the lack of translational bench-to-bedside is multifaceted, further evaluation of the neuroprotective effects of GDNF in appropriate, clinically-relevant, experimental models of brain injury [43] is paramount to improve treatment paradigms in the clinic. This is clearly illustrated in the ongoing clinical trials for GDNF treatment in Parkinson's Disease (PD) patients [44]. Since the first clinical trial initiated in 1996 by Amgen [45], the majority of clinical trials to date for directed GDNF delivery in PD patients have shown a lack of therapeutic benefit largely due to poor delivery, distribution and bioavailability in the brain. As such, optimizing GDNF delivery in target tissue is a critical parameter for achieving efficacy in clinical trials. To date, there is currently insufficient data with regards to dosing, mode of delivery, therapeutic time-frame and possible side-effects of GDNF delivery following brain injury and exploring cell-based therapies in combination with biomaterials holds great promise in advancing GDNF-mediated therapy to improve functional outcomes. Biomaterials can enable and augment the targeted delivery of drugs and neurotrophic factors and facilitate the implantation of cellular grafts into the brain to mediate neuroprotection

[46]. Recent advances in biomaterial technology has led to the development of encapsulation of trophic factors within the scaffold, which itself acts as a vehicle for controlled delivery of specific factors to the local cell microenvironment, paving the way for widespread use in a variety of biomedical applications [46]. Collectively, the accessibility of AF cells, combined with well-characterized brain injury models and biomaterials, enables thorough preclinical investigation to guide future clinical trials for optimal cell-based treatment strategies following brain injury.

5.4 Conclusions

AF cells are emerging as a new powerful tool for basic research as well as for the establishment of new cell-based therapies. The ability to generate clonal AF derived cell lines, which harbour a high proliferative potential, has stimulated interest in characterizing and evaluating the therapeutic potential of AF cells. Through GJIC with cortical astrocytes and delivery of exogenous neurotrophic factors, such as GDNF, AF cells hold great promise in developing neuroprotective therapies following brain injury.

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