Progranulin is expressed within motor neurons, promotes neuronal cell survival, and is secreted at least in part via the constitutive secretory pathway

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ABSTRACT

Progranulin is a secreted high molecular weight growth factor. While inappropriate over-expression of the progranulin gene has been associated with many cancers, haploinsufficiency leads to atrophy of the frontotemporal lobes and development of a form of dementia (FTLD-U). We have demonstrated that progranulin is abundantly expressed in mouse motor neurons *in vivo* and primary cultures *in vitro*. The cellular location of progranulin was examined using confocal microscopy. Progranulin was localised to organelles using immunocytochemistry and by examining the intracellular fate of exogenously expressed fluorescently-labelled progranulin. Taken together these studies revealed progranulin to be located within compartments of the secretory pathway. Stable transfection of the human *progranulin* gene into the NSC-34 cell line stimulates the appearance of dendritic structures and provides protection against apoptosis. Control cells, while expressing basal levels of progranulin do not survive in serum free conditions. This work highlights the importance of progranulin as a neuroprotective growth factor and may represent a therapeutic target for neurodegenerative diseases including FTLD-U and ALS.

RÉSUMÉ

La progranuline est un facteur de croissance sécrété de haut poids moléculaire. Tandis qu' une sur-expression inappropriée du gène codant la progranuline est observé dans de nombreux cancers de différentes origines, l'haploinsuffisance, quant à elle, est responsable de l'atrophie des lobes fronto-temporaux et du développement d'une forme de démence (FTLD-U). Nous avons démontré que la progranuline est exprimée abondamment dans les neurones moteurs murins in vivo et in vitro dans un modèle de culture primaire. La localisation subcellulaire de la progranuline a été examinée en microscopie confocale par immunofluorescence indirecte et confirmé par l'étude de la distribution subcellulaire d'une progranuline marquée a la eGFP. La progranuline se distribue ainsi le long des voies de secrétions. La surexpression de gène humain de la progranuline dans les cellules NSC34 stimule l'apparition de structures dendritiques et protège contre la mort cellulaire induite par la carence en sérum du milieu tandis que les cellules ne surexprimant pas la progranuline demeurent sensibles à ce stimulus. En conclusion, ce travail souligne l'importance de la progranuline comme facteur de croissance neuroprotecteur et peut représenter une cible thérapeutique de choix pour les maladies neurodégénératives.

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CHAPTER III- RESULTS

ABBREVIATIONS

ACTH: adrenocorticotropic hormone

AD: Alzheimer's disease

ALS: amyotrophic lateral sclerosis

AMV: avian myeloblastosis virus

APS: ammonium persulfate

AraC: cytosine ß-D-arabinofuranoside

ATCC: American Type Culture Collection

BDNF: brain derived neurotrophic factor

BFA: brefeldin A

BrdU: bromodeoxyuridine

BSA: bovine serum albumin

CHMP2B: chromatin modifying protein 2B

CNS: central nervous system

CSP: constitutive secretory pathway

DAPI: 4',6-diamidino-2-phenylindole

ddH_2O: deionized distilled H_2O

DMEM: Dulbecco's modified eagle medium

DNA: deoxyribonucleic acid

E13: embryonic day 13

ECL: enhanced chemiluminesence

ECM: extracelullar matrix

EDTA: ethylenediaminetetraacetic acid

eGFP: enhanced green fluorescent protein

fALS: familial amyotrophic lateral sclerosis

FBS: fetal bovine serum

FGF: fibroblast growth factor

FITC: fluorescein isothiocyanate

FTD: frontotemporal dementia

FTLD: frontotemporal lobar degeneration

FTLD-U: frontotemporal lobar degeneration with ubiquitin positive inclusions

GFP: green fluorescent protein

GRN: granulin

HeNe1: helium/neon 1 laser

hPgrn: human progranulin

HSP: heat shock protein

5-HT: 5-hydroxytryptamine (serotonin)

IBIRI: ubiquitin-immunoreactive inclusions

IGF-1: insulin growth factor-1

IL-1: interleukin-1

kb: kilo base pairs

kDa: kiloDaltons

LB: lysogeny broth

LBLI: lewy-body like inclusions

LSM 510: laser scanning microscope 510

MAPT: microtubule-associated protein tau

MND: motor neuron disease

mRNA: messenger ribonucleic acid

MV: microvesicle

MVB: multivesicular bodies

NCI: neuronal cytoplasmic inclusions

NP-40: nonyl phenoxypolyethoxyethanol-40, Tergitol-type-40

ºC: degree Celsius

PBS: phosphate buffer solution

PBST: phosphate buffer solution with 0.5% Tween 20

PCDGF: prostate cancer-derived growth factor

PCR: polymerase chain reaction

PFA: paraformaldehyde

PGRN: progranulin

PGRN: progranulin gene

PM: plasma membrane

PMN: primary motor neurons

PNFA: progressive nonfluent aphasia

POMC: proopiomelanocortin

PPA: primary progressive aphasia

PPV: plasmalemmal precursor vesicle

RIPA: radio immuno precipitation assay

RNA: ribonucleic acid

Rpm: revolutions per minute

RSP: regulated secretory pathway

RT-PCR: real time polymerized chain reaction

sALS: sporadic amyotrophic lateral sclerosis

SD: semantic dementia

SDS-PAGE: sodium dodecyl sulphate-polyacrylamide gel electrophoresis

SLPI: serine leukocyte protease inhibitor

SOD: superoxide dismutase

TBST: tris buffer solution with 0.5% Tween 20

TDP43: TAR DNA binding protein 13

TGFe: epithelial transforming growth factor

TGN: trans-Golgi network

TUNEL: dUTP nick end labeling

UV: ultraviolet

VCP: valosin-containing protein

VEGF: vascular endothelial growth factor

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CHAPTER I

LITERATURE REVIEW

Introduction:

In an era where a short life expectancy in developed countries is a thing of the past, aging has become a very popular focus for scientific research. While it has provided resolution for many illnesses that would otherwise have been fatal, scientific research has not been able to unravel one problem that has haunted the aging population since ancient times: neurodegenerative disorders. Among the numerous types of neurodegenerative diseases, three in particular have received special attention: Parkinson's disease (PD), Alzheimer's disease (AD) and Amyotrophic lateral sclerosis (ALS). A condition that has received less public attention, that is, however, just as clinically relevant in the aging population, is frontotemporal lobar degeneration (FTLD).

Aside from a small number of neural stem cells that are created daily, cells of the brain and spinal cord are not readily regenerated *en masse*; thus, excessive damage can be devastating. The degenerative diseases of the central nervous system (CNS) encompass a heterogeneous group of disorders characterized by spontaneous, progressive degeneration of neurons in a specific region or system in the brain, spinal cord or both. These diseases may be sporadic or familial and show considerable clinical variability; there is often overlap of phenotypic features that may make it hard to distinguish one type from another. They are informally divided into two groups according to phenotypic effects. These categories are not mutually exclusive: one group comprises conditions causing problems with movement (e.g. PD and ALS); and the second consists of conditions affecting memory and conditions related to dementia (e.g. AD and FTLD).

Neurodegenerative diseases cause tremendous difficulties for families as well as for patients themselves. Loss of memory or cognition leads to poor judgement in the workplace and home, often leading to loss of income, financial devastation and legal

difficulties (Levy et al., 1996). With FTLD in particular, loss of insight is very common, which makes it very hard to reason with patients. Patients often become socially withdrawn, seeming to have lost concern for themselves and others, which can cause tension with friends and family (Levy et al., 1996).

Unfortunately, most neurodegenerative diseases have unknown etiology, and thus, very few therapeutic options are available to patients. As there are currently no preventive or therapeutic approaches that address cognition, patients with FTLD are treated only for their superficial symptoms, such as depression, carbohydrate cravings and management of dis-inhibition, instead of the underlying causes (Mendez, 2009). Although new drug therapies for clinical trials have been initiated (Mendez, 2009), eventual treatment is still in the unforeseen future. We are still in the initial stage where it is crucial to follow any leads that may ultimately uncover a link to the reasons why FTLD and other neurodegenerative diseases are occurring.

Lately, genetic studies have been very useful in suggesting potential underlying factors of disease; however, how these factors intertwine to bring about neurodegeneration is not yet clear. For FTLD, pedigree analysis has uncovered the involvement of 2 prominent genes, both located on chromosome 17: MAPT and PGRN. MAPT, a gene encoding for microtubule-associated tau protein, has been implicated in many tauopathies, as well as familial FTLD (Ghidoni et al., 2006). Mutations in progranulin (PGRN) were recently characterized in familial FTLD patients, but have since become a very interesting topic of research, since PGRN has also been linked to carcinogenesis in numerous tumour types (Cheung et al., 2004; Davidson et al., 2004; Donald et al., 2001; Jones et al., 2003; Jones et al., 2006; Liau et al., 2000; Matsumura et al., 2006; Monami et al., 2006; Pan et al., 2004; Serrero and Ioffe, 2003; Wang et al.,

2003). Since mutations in PGRN in FTLD result in a decrease in the normal physiological levels of PGRN, it is possible that a perfect balance of PGRN levels is necessary to maintain homeostasis: an excess may lead to carcinogenesis through increased cell survival, while too little at least in the context of neuron survival leaves cells unprotected and vulnerable to cell death.

PGRN is a secreted glycoprotein that promotes mitosis, survival, and migration in many cell types (Ong and Bateman, 2003). Although it has received much attention due to it's relation to FTLD, it has yet to be well characterized in the CNS. Given the uncertainty of whether or not PGRN contributes to motor neuron function and survival, we sought first to investigate the expression of PGRN in the rodent spinal cord, as well as in several motor neuron model systems, and then to investigate its possible biological activities using an immortalized motor neuron cell model.

1.1 Neurodegenerative Disorders

1.1.1 Frontotemporal Lobar Degeneration (FTLD)

Following Alzheimer's disease and Parkinson's disease, frontotemporal lobar degeneration (FTLD) is the third most common neurodegenerative dementia (Cruts and Van Broeckhoven, 2008). Clinically, it accounts for 5-10% of all dementia and comprises between 10-20% of dementia seen in patients with a disease onset before the age of 65 (Eriksen and Mackenzie, 2008). There are two major subclasses of FTLD, which then further divide into smaller categories, encompassing a variety of clinical presentations. The two major forms of FTLD are the frontotemporal dementias (FTDs) and the primary progressive aphasias (PPAs) (Cruts and Van Broeckhoven, 2008). While FTDs are characterized by behavioural, social conduct and personality changes, PPAs present with language related problems, either in the form of progressive nonfluent aphasias (PNFAs), where patients have difficulty expressing language, or semantic dementias (SDs), where patients have difficulties understanding the meaning of words. Typically, in the FTLD population, 57% of patients have FTD, 24% show symptoms of PNFA, and 19% have SD (Cruts and Van Broeckhoven, 2008). Among the total population of FTLD patients, 14% of patients show Parkinson-like symptoms, including rigidity, bradykinesia or postural instability, and between 4-17% of patients develop ALS (Cruts and Van Broeckhoven, 2008).

FTLD has a highly variable age of onset; patients have presented with symptoms between 33 and 80 years of age, however the majority of cases appear between 60 to 69 years of age. The prevalence of FTLD is approximately 10-20 in 100,000 people, and disease duration can vary between 1 and 20 years after diagnosis. In the advanced stages

of disease, memory loss can occur, and in most cases, patients die from sudden and unexplained reasons (Cruts and Van Broeckhoven, 2008).

Indications of FTLD often include symmetric or asymmetric atrophy (including neuronal loss, astrocytic gliosis, and spongiosis) of the frontal and/or temporal lobes of the brain (Cruts and Van Broeckhoven, 2008). Cases are frequently characterized by accumulation of insoluble hyperphosphorylated tau protein in neurons and glia (Eriksen and Mackenzie, 2008), but the most common clinical presentation includes neuronal cytoplasmic inclusions (NCI) within the superficial layers of the neocortex and the hippocampal dentate granule cells (Eriksen and Mackenzie, 2008). In cases where neurons are tau and α-synuclein negative, but positive for ubiquitin, the condition is termed FTLD-U (Eriksen and Mackenzie, 2008). Most commonly, the brain regions affected in FTLD are those responsible for controlling behaviour, planning, personality, emotions, and reasoning, as well as some areas that control speech and movement (Cruts and Van Broeckhoven, 2008).

FTLD has a strong genetic component. For the FTD sub-category of patients in particular, 30-50% of patients show some genetic component, often involving autosomal dominant mutations (approximately 80% of familial cases). To date, many mutations have been uncovered, spanning numerous human chromosomal regions including 3p, 9q, 9p, 17q (Eriksen and Mackenzie, 2008). In particular, four genes have been identified as crucial factors in disease pathology: *MAPT*, *PGRN*, *VCP* (valosin containing protein), and *CHMP2B* (charged multivesicular body protein 2B) (Cruts and Van Broeckhoven, 2008). Due to the complexity of genetic contributions, only mutations in *PGRN* will be discussed in this thesis.

1.1.2 **FTLD and PGRN**

The association of *PGRN* with FTLD was first described in 2006, when two papers were published in *Nature* that showed that mutations of the PGRN gene on chromosome 17 were the underlying cause of the disease (Baker et al., 2006; Cruts et al., 2006). Less than 3 years later, 127 mutations in *PGRN* have been discovered, of which 66 are pathogenic (39 have unclear pathogenic nature, 22 are non-pathogenic) (Cruts, 2009). *PGRN* mutations are present in many familial cases of FTLD-U, but are also apparent in sporadic cases of FTLD-U. It is estimated that approximately 5-11.2% of all FTLD patients carry *PGRN* mutations, (making *PGRN* mutations as common as *MAPT* mutations (Eriksen and Mackenzie, 2008)), and that approximately 12.8-25.6% of familial FTLD cases are caused by *PGRN* mutations (Cruts and Van Broeckhoven, 2008). Unlike *MAPT* mutations that appear to result in a toxic gain of function that leads to FTLD-U (Rademakers and Hutton, 2007), mutations in *PGRN* appear to cause FTD-U through a loss of function mechanism.

The pathogenic mutations with the most devastating consequences to date are those that result from pre-mature termination codons (Eriksen and Mackenzie, 2008), thus creating a functionally null allele, leading to haploinsufficiency. While most of these null alleles are the result of nonsense mutations, a few missense mutations that cause either low PGRN expression or secretion have been shown to either lead to or are a major risk factor for FTLD-U (Bronner et al., 2007; Mukherjee et al., 2006; Mukherjee et al., 2008; Shankaran et al., 2008; Van der Zee et al., 2007). To date, there have not been any reported incidences of truncated PGRN proteins being synthesised suggesting that mutations are not causing a dominant negative effect (Eriksen and Mackenzie, 2008). The

consensus view is that almost all mutant *PGRN* transcripts are undergoing nonsense-mediated mRNA decay (NMD), which ultimately results in a 30-35% reduction in PGRN protein levels (Eriksen and Mackenzie, 2008).

Phenotypically, patients with PGRN mutations present with behavioural abnormalities, language problems and Parkinsonism (Eriksen and Mackenzie, 2008). Some patients with PGRN mutations show pathological AD (Behrens et al., 2007), other present with tau, and alpha-synuclein pathology (Leverenz et al., 2007). In all cases, there is much variability with disease onset, rapidity of disease progression and clinical presentation; there is even variability within families carrying the same mutation (Eriksen and Mackenzie, 2008). This large variability suggests that environmental factors, as well as other genes and genetic mechanisms, play a considerable role in disease expression (Eriksen and Mackenzie, 2008). Patients with PGRN mutations have a mean age of FTLD onset of 59 ± 7 years, an average disease duration of 3-12 years and a mean age of death of 65 ± 8 years (Eriksen and Mackenzie, 2008).

The most prominent neuropathology associated with *PGRN* mutations involves tau-negative, ubiquitin reactive neuronal inclusions (Baker et al., 2006; Cruts et al., 2006; Gijselinck et al., 2008). PGRN has been identified in activated microglia surrounding plaques in AD (Baker et al., 2006), however PGRN has not been found within FTLD-U inclusions (Eriksen and Mackenzie, 2008). Patients with familial FTLD-U with PGRN mutations have insoluble inclusions that lack progranulin, suggesting that the mutated gene product is not aggregating in these cases. These inclusions, however, do contain transactivation response DNA binding protein of M_r43 kD (TDP-43) (Neumann et al., 2006). TDP-43 mutations have been recently characterized in fALS and sALS, thus

creating an interesting link between PGRN, TDP-43, FTLD-U and ALS. Many laboratories are now investigating the relationship between TDP-43 and PGRN.

It has recently been shown that TDP-43 translocates from the nucleus to the cytoplasm in axotomized motor neurons (Moisse et al., 2009) and while the expression of PGRN decreases in these neurons, it is increased in surrounding microglia. However, these changes were not found to be pathological (Moisse et al., 2009). One group of researchers has reported that PGRN is implicated in the regulating the proteolytic cleavage of TDP-43 (Zhang et al., 2007), since the depletion of PGRN in H4 gliomas resulted in the activation of caspase-3 and the accumulation of cleaved TDP-43 . This would suggest a functional relationship between the loss of PGRN and mobilization of TDP-43; however, this conclusion has been challenged by other investigators, who found no such effect on TDP-43 (Dormann et al., 2009; Shankaran et al., 2008).

1.1.3 Amyotrophic Lateral Sclerosis (ALS)

The motor neurons of the brain and spinal cord are targets of injury in a fairly large number of sporadic and hereditary neurodegenerative disorders, as well as in certain infections (e.g., poliomyelitis) and some autoimmune diseases (Kumar et al., 2003). The most common of these primary neurodegenerative disorders affecting motor neurons is amyotrophic lateral sclerosis (ALS), also known as Lou Gehrig's disease or motor neuron disease (MND) (Okouchi et al., 2007). ALS is a degenerative disorder involving the upper and lower motor neurons of the pyramidal system, resulting in progressive muscle weakness, atrophy and spasticity (Kumar et al., 2003). The causes and pathogenesis of most cases of ALS remain unknown (Pasinelli and Brown, 2007), despite extensive

analysis of potential environmental, toxic, infectious and immunologic factors (Kumar et al., 2003).

While most cases of ALS are sporadic, familial cases (which are usually inherited in an autosomal dominant fashion (Okouchi et al., 2007)) account for about 5% to 10% of cases (Okouchi et al., 2007), often inherited with age-dependent penetrance of about 96% by the age of 70 (Al-Chalabi and Brown, 2005). About 20% of these familial cases are associated with mutations in the gene coding for the enzyme Zn/Cu superoxide dismutase (SOD1) (Okouchi et al., 2007), located on the long arm of chromosome 21 (Almer and Robberecht, 2006).

Pedigrees with familial ALS comprise the most informative group for gene-searching experiments (Okouchi et al., 2007). Using linkage studies, the initial discovery of mutations in SOD1 and other loci were found (Okouchi et al., 2007). These mutations were a crucial discovery, as they have led to the generation of transgenic mouse models and *in vitro* models of fALS (Almer and Robberecht, 2006), which have provided great insight into molecular mechanisms of disease progression. As a result of these models, many candidate genes have been studied as possible modifiers or susceptibility factors in both fALS and sALS (Al-Chalabi and Brown, 2005; Pasinelli and Brown, 2007).

1.1.4 ALS and PGRN

In situ hybridization experiments previously performed in our laboratory reveal that motor neurons express a high level of *PGRN* (Figure 1), suggesting a significant role for PGRN in the biology of the motor neuron; however, the significance of PGRN in healthy and diseased motor neurons is still unclear.

Recently Irwin et al. investigated PGRN expression in post-mortem ALS patients and demonstrated an increase in PGRN in degenerating long motor tracts in both the brain stem and spinal cord (Irwin et al., 2008). Their principal finding was that PGRN was increased in regions of neuronal cell loss, but was not increased in the frontal cortex, hippocampus or cerebellum of ALS cases versus control tissue sections. There was a marked increase in dystrophic neurons though upper and lower motor neuron expression was variable. PGRN was seen in both neurons and activated microglia. It should be noted that they also saw expression of PGRN in lower motor neurons of the spinal cord in the control samples. Their work strongly complimented the work of *Malaspina et al*, who, in 2001, detected high levels (a 400 fold increase) of PGRN mRNA in spinal cords from ALS patients ((Mackenzie, 2007a; Malaspina et al., 2001). This 400 fold increase corresponded directly to expression within the motor neurons and glia involved in cell death (Malaspina et al., 2001). Interestingly, TDP43 expression was present in both ALS and control samples; however, the expression was not as pronounced as that of PGRN (Irwin et al., 2008).

PGRN expression is also increased in the lumbar cord motor neurons from mouse models of *superoxide dismutase 1* (*SOD1*) familial ALS (Ferraiuolo et al., 2007; Lobsinger et al., 2007). *PGRN* was one of only 21 genes with dysregulated motor neuron expression in $SOD1^{G37R}$ and $SOD1^{G85R}$ mice at the onset of the disease, as judged by weight loss but before the detection of overt neurological symptoms (Lobsinger et al., 2007). The *PGRN* gene was also up-regulated in SOD^{G93A} mice, but late in the disease progression (Ferraiuolo et al., 2007). In contrast, a decrease in PGRN expression was noted in the NSC-34 cell line engineered to express SOD^{G93A} (Kirby et al., 2002).

It has been suggested that the *PGRN* gene may be a modulator of disease progression in ALS since a correlation between genetic variations in *PGRN* and the age of onset or survival of patients with ALS has been reported (Sleegers et al., 2008). However, other investigators have yet to confirm this observation (Pickering-Brown et al., 2008a; Schymick et al., 2007b). The degeneration of motor neurons in ALS is often accompanied by the presence of ubiquitinated inclusions containing TDP-43 (Neumann et al., 2006; Tan et al., 2007) similar to those seen in PGRN-dependent FTLD-U. In some patients, FTLD may be accompanied by motor neuron disease (Mackenzie, 2007b; Strong, 2001). This suggests a possible link between PGRN and ALS. However, mutations in *PGRN* are infrequent in patients with ALS-FTLD, and it is uncertain whether or not they are pathogenic (Schymick et al., 2007a; Spina et al., 2007). Furthermore, most patients with FTLD-U due to *PGRN* mutations present limited evidence of accompanying neuromuscular defects (Le Ber et al., 2007; Kelley et al., 2007; Mackenzie, 2007a; Pickering-Brown et al., 2008b).

1.2 Progranulin (PGRN)

1.2.1 Overview of Progranulin (PGRN)

Progranulin (PGRN) is a mitogenic growth factor found in many tissues and cell types of the body (Daniel et al., 2000). PGRN has many alternative names, including pro-epithelin, granulin-epithelin precursor, acrogranin and PC cell-derived growth factor (PCDGF), as a result of being identified by many different groups studying various aspects of the biology of this growth factor (Bateman and Bennett, 1998). Progranulin is by a single gene in humans and rodents (chromosomes 17 and 11, respectively) (Eriksen

and Mackenzie, 2008); however, in other species, including the zebrafish (*Danio rerio*), several progranulin homologues exist (Cadieux et al., 2005).

The human form of the progranulin gene consists of an 8.0kb genomic sequence located on chromosome 17q21.32 (Bhandari et al., 1992; Cruts and Van Broeckhoven, 2008; Eriksen and Mackenzie, 2008). It is composed of 13 exons, with a Kozac consensus sequence for initiation of translation located within exon 2 (Cruts and Van Broeckhoven, 2008). This gene encodes for 7 and one half repeats of the granulin (GRN) motif; the GRN half-domain, designated paragranulin, is found amino-terminal to the multiple repeats that are designated GRNs A-G (Cruts and Van Broeckhoven, 2008; He and Bateman, 2003). Each GRN-encoding domain spans two exons, the C-terminal and N-terminal of each granulin domain are located within consecutive exons (Eriksen and Mackenzie, 2008). Each GRN domain is separated by linker sequences of various lengths (Eriksen and Mackenzie, 2008).

The full length PGRN protein consists of 593 amino acids, and has a predicted molecular weight of 68.5 kDa (Eriksen and Mackenzie, 2008). Mature PGRN, however, is N-glycosylated and, as a result, the final secreted product has a mass of between 88kDa (Cruts and Van Broeckhoven, 2008) and 90kDa (Eriksen and Mackenzie, 2008) as estimated by SDS-PAGE electrophoresis. PGRN can be cleaved by extracelullar proteases, such as elastase, into GRN peptides with sizes of approximately 6kDa (Cruts and Van Broeckhoven, 2008) to 25kDa (Eriksen and Mackenzie, 2008). The nascent PRGN protein bears an amino-terminal signal sequence that prompts transport through the secretory pathway (Cruts and Van Broeckhoven, 2008).

The first GRN peptides to be identified were GRN A, B, C and D and paragranulin in 1990 (Bateman et al., 1990), isolated from human inflammatory

leukocytes. GRN F has been identified in human urine (Sparro et al., 1997). Fish homologue homologues, GRNS, 1,2 and 3 were purified from extracts of the carp hematopoietic tissues (Belcourt et al., 1993).

The GRN peptides are characterized by a novel $X_{2-3}\underline{C}X_{5-6}\underline{C}X_5\underline{CC}X_8\underline{CC}X_6\underline{CC}X_5\underline{CC}X_1\underline{C}X_{5-6}\underline{C}X_2$ sequence, linked by six disulfide bridges (Hrabal et al., 1996). These cysteine residues are needed for stable folding into four stacked β-hairpins (Hrabal et al., 1996). Each hairpin is connected to the next via two parallel disulfide bonds (Hrabal et al., 1996).

1.2.2 Overview of known PGRN biological functions

While the signalling pathways associated with PGRN in neurons remain unknown, in non-neuronal cell types, it activates growth factor-related signal transduction pathways including the phosphorylation of shc, p44/42 mitogen-activated protein kinase, phosphatidylinositol 3-kinase, protein kinase B/AKT, and the $p70^{S6}$ kinase (He et al., 2002; Lu and Serrero, 2001; Zanocco-Marani et al., 1999). Due to these kinase activations, PGRN plays a significant role during carcinogenesis in numerous tumour types (Cheung et al., 2004; Davidson et al., 2004; Donald et al., 2001; Jones et al., 2003; Jones et al., 2006; Liau et al., 2000; Matsumura et al., 2006; Monami et al., 2006; Pan et al., 2004; Serrero and Ioffe, 2003; Wang et al., 2003). PGRN has also been described in wound repair and inflammation (He et al., 2003; Ong et al., 2006; Wang et al., 2003; Zhu et al., 2002), as well as having an important role in early embryonic development (Cadieux et al., 2005; Diaz-Cueto et al., 2000; Qin et al., 2005). The ability of PGRN to regulate critical proliferative, survival and motility signals in a diverse range of non-neuronal cell types suggests that it may support similar functions in nerve cells.

To date, there have not been any reports of a *bona fide* PGRN receptor, but several studies have identified PGRN binding partners (Bai et al., 2009; Gonzalez et al., 2003; Zhu et al., 2002). PGRN has been shown to interact with perlecan, a heparan sulfate proteoglycan found in the basement membrane and cell surfaces of cells (Gonzalez et al., 2003); perlecan has been shown to bind several growth factors and receptors (Whitelock et al., 2008), for example VEGF and FGFs (Ornitz, 2000; Robinson et al., 2006), leading to both pro-angiogenic and anti-angiogenic effects (Whitelock et al., 2008). In endochondral bone, PGRN associates with ADAMTS-7, a metalloprotease important for degradation of extracelullar matrix proteins found in cartilage (Bai et al., 2009). PGRN has been shown to stimulate chrondrocyte maturation and induce bone growth, while addition of ADAMTS-7 completely abolished this mitotic action (Bai et al., 2009). ADAMTS-7 was also shown to be capable of converting PGRN into its smaller peptide fragments; thus, ADAMTS-7 has been suggested to be a PGRN-convertase that negatively regulates chondrocyte hypertrophy and bone growth by binding to PGRN and reducing its activity (Bai et al., 2009).

In models of wound healing, serine leukocyte protease inhibitor (SLPI) directly binds to PGRN via its C-terminal domain, as confirmed by yeast two-hybrid experiments (Zhu et al., 2002). *Zhu et al* proposed that SLPI and PGRN are able to form a complex *in vivo*, and, through this complex, can inhibit the cleavage of PGRN by elastase. Furthermore, they suggest that the SLPI-PGRN complex can inhibit elastase-induced digestion of other proteins, such as fibronectin, collagen type 1 and vitronectin.

There are two proposed mechanisms for how SLPI can block the action of elastase: by binding to PGRN or by binding to elastase (Zhu et al., 2002). SLPI binds within the linker regions of PGRN, which is also where the elastase cleavage sites are

found. It has been postulated that elastase may not be the only convertase responsible for the cleavage of PGRN, and that the binding of SLPI to the linker regions may protect PGRN from similar cleavage (Zhu et al., 2002).

While sharing similar structure, individual GRNs have been proposed to have contrasting physiologic function (Van Damme et al., 2008; He and Bateman, 2003; Zhu et al., 2002). While PGRN appears to have both mitotic and anti-inflammatory properties, GRN B appears to boost inflammation and inhibit cell proliferation in epithelial cells (Zhu et al., 2002). Elevated GRN A has been noted in the serum of prostate cancer patients; however, further studies are required to investigate it's role and function in relation to the cancer (Deftos and Abrahamsson, 1998). Therefore, the amount of PGRN cleavage (and subsequently the amount of SLPI and proteinases) in a particular cell type may act as a biological switch between the two physiological states.

1.2.3 PGRN in the nervous system

PGRN is synthesised in neurons in many brain regions including the cerebral cortex, in the Purkinje cells of the cerebellum, and in the hippocampus (Daniel et al., 2000). In addition, it is widely distributed in the developing central nervous system and the dorsal root and sympathetic ganglia within the peripheral nervous system (Daniel et al., 2003). The roles of PGRN in normal neuronal function and development, in either the central or peripheral nervous systems are poorly understood. It is known, however, that PGRN contributes to normal brain development, since it regulates the male-specific differentiation of the neonatal hypothalamus (Suzuki et al., 2000a; Suzuki et al., 1998). Moreover, in culture, PGRN stimulates the proliferation of PC12 cells (Daniel et al., 2000), as well as the estrogen-dependent growth of hippocampal neurospheres (Chiba et

al., 2007) and may be neurotrophic for cortical and motor neurons (Van Damme et al., 2008).

The first GRN peptide to be investigated with respect to the survival of neurons was GRN E. Van Damme *et al*. investigated the effect of both GRN E and full length PGRN on neurite outgrowth, observing changes in both motor and cortical neuron cultures (Van Damme et al., 2008). Using neuronal cultures raised in serum-free medium to eliminate the effect of other growth factors, they plated motor neurons from 14-day-old rat embryo ventral spinal cord at low density. When GRN E was added on day 1, they observed a neuroprotective effect against cell death in a dose dependent manner. The real trophic effect was visible after one week of treatment, as by this time all of the control cells had died. At their maximum dose of GRN E (100ng/ml), they saw a 64% increase in survival on day 2 and a 101% increase by day 6. They saw similar results in cortical neuron cultures (41.4% increase), and thus concluded that GRN E is neurotrophic. Running the same experiments with PGRN (100ng/ml) instead of GRN E, they found a 38.5% increase in survival in motor neurons and a 22% increase for cortical neurons. They also tested the effect of GRN E and PGRN on neurite extension length and found that, while neither GRN E nor PGRN had any effect on cell soma size, both increased maximal neurite length. They saw a larger effect on neurite length with PGRN treatment relative to GRN E, supporting speculation that the GRN peptides have different biological functions.

Some of the GRNs have pro-inflammatory effects (Ahmed et al., 2007; Eriksen and Mackenzie, 2008), and are expressed within ameboid microglia (Bigio, 2008). To date, PGRN has been located to several loci in the mouse CNS: within microglia, and neurons in the superficial neocortex, hippocampal granular layer and cerebellar Purkinje

layer (Bigio, 2008). In humans, similar expression patterns are observed: antibodies directed against PGRN show expression within cortical neurons and in activated microglia (Baker et al., 2006; Bigio, 2008).

1.2.4 PGRN Knock-out Studies

In 2007, a transgenic PGRN knock-out mouse was created by *Kasayuga et al* (Kayasuga et al., 2007). They sought to investigate the role of PGRN in sexual differentiation of rodent brains, as they had previously reported that *PGRN* gene expression could be induced by androgens in the rat neonatal hypothalamus (Suzuki et al., 1998). Their model was created to elucidate putative physiological functions of PGRN in the brain, and their studies primarily investigated differences in phenotype regarding sexual dimorphisms, such as anxiety, aggression and sexual behaviour. The development of PGRN deficient mice in itself was an important discovery: the offspring of PGRN deficient mice were fertile and there was no significant difference in litter size or the number of weaned pups when compared to the control PGRN positive mice. This is surprising since it has been shown that PGRN is expressed both in sperm acrosomes (Baba et al., 1993) and oocytes (Suzuki et al., 2000b), as well as having been shown to be critical for in the development of embryos *in vitro* and implantation *in vivo* (Diaz-Cueto et al., 2000). An embryonic lethal phenotype had been expected (Kayasuga et al., 2007).

Nevertheless, PGRN deficient mice showed attenuation of sexual behaviour (the incidence of ejaculation was decreased) and increased aggression (hypothesized to be a result of increased anxiety rather than an increase in male sexual behaviour). *Kayasuga et al* proposed that these changes were a result of alteration of the serotonergic system, particularly that the response system to androgens may be underdeveloped (Kayasuga et

al., 2007). They noted decreased levels of serotonin (5-HT1A) mRNA in the hippocampus of PGRN null mice, and that the phenotype of $PGRN^{-/-}$ mice was very similar to estrogen-receptor-ß knock-out mice, thus ultimately implicating PGRN as a modulator of sex steroids on the serotonergic system (Kayasuga et al., 2007).

Another study investigating the behaviour of PGRN null mice has recently shown sexual differences in expression of anxiety not seen in PGRN knockout mice, but are present in wild-type mice (Chiba et al., 2009). Castration of both groups of mice and re-introduction of testosterone after puberty did not affect anxiety (Chiba et al., 2009), suggesting that PGRN plays a role in the development of anxiety before puberty, particularly in a brain region called the Locus Cereleus (LC). Typically, females have larger LCs than do their male counterparts (Chiba et al., 2009; Chiba et al., 2007; Guillamon et al., 1988). While there were no changes in morphology with respect to sex in PGRN null mice, morphology of the LC in both genotypes differed, as PGRN null mice showed an increase in volume and in number of cells in this region (Chiba et al., 2009). As the LC is a conversion locus for most of the noradrenergic neurons in the brain, this implicates PGRN in the organization of the adrenergic systems that regulate anxiety in a sex-dependent fashion.

This PGRN knockout model also has clinical relevance, since there have been cases of FTD patients reporting anxiety, anger and heightened aggression (Kayasuga et al., 2007; Mendez et al., 2006). Drugs that increase serotonin activity, such as selective serotonin reuptake inhibitors (SSRIs) and 5-HT receptor agonists (Huey et al., 2006; Ikeda et al., 2004; Kayasuga et al., 2007; Lebert et al., 2004), have shown effectiveness in reducing these symptoms. Moreover, a decrease in 5-HT receptors has been described in brains of FTD patients (Kayasuga et al., 2007; Procter et al., 1999). These findings

suggest a possible role for PGRN in serotonin pathway modulation; however levels of 5-HT receptors in FTD patients specifically with PGRN mutations as compared to the many other genetic causes of FTD has yet to be described.

1.3 Growth Factors and Neuroprotection

Growth factors, in particular vascular endothelial growth factor (VEGF) (Wang et al., 2007) and insulin-like growth factor-1 (IGF-1) (Kaspar et al., 2003), have shown promise as neuroprotective agents in murine models of familial ALS. When mice are deficient for the hypoxia-response elements of the VEGF promoter, and post-transcriptional modification to VEGF are altered, the mice develop an adult-onset motor neuron disease very similar to ALS (Lu et al., 2007). Like that found for the PGRN gene, genetic mutations that alter final VEGF protein products lead to neurodegenerative phenotypes. IGFs have been implicated in development, but are also present in differentiated neurons that no longer divide (Bence-Hanulec et al., 2000). IGFs play a role in the mature nervous system, which seems to include functional maintenance and survival of neurons (Neff et al., 1993).

Many other growth or neurotrophic factors, including hepatocyte growth factor (HGF) and glial cell line derived neurotrophic factor (GDNF), have been implicated in neuronal survival (Henderson et al., 1994; Kadoyama et al., 2007). Fibroblast growth factor 2 (FGF-2) is also involved in the development and differentiation of various cell types, including cells of the nervous system (Schiera et al., 2007). FGF-2 plays a role in hippocampal neurogenesis (Palmer et al., 1999), and both *in vitro* and *in vivo* studies have demonstrated its neurotrophic properties (Schiera et al., 2007). Findings showing that

other growth factors are implicated in neuronal survival and potential therapies for ALS would suggest that if PGRN is neurotrophic for motor neurons, it could also have therapeutic potential in neurodegenerative diseases.

1.4 Secretion of Peptides

1.4.1 Overview of the peptide processing

Neuropeptides are biologically active peptides, composed of 30-40 amino acids, existing either in a linear or ring conformation (Hook et al., 2008). They are derived from longer precursor proteins, called proneuropeptides or prohormones (Hook et al., 2008). These neuropeptides are present within the precursor as distinct segments. Precursor proteins are capable of encoding one or multiple copies of the biologically active neuropeptides (Hook et al., 2008). Interestingly, precursor proteins can undergo different processing depending on individual tissue regions to generate different protein products (Hook et al., 2008). Proteolytic cleavage of the precursor usually occurs at pairs of residues (Lys-Arg, Lys-Lys, Arg-Arg, or Arg-Lys), monobasic Arg residues, or multiple basic residues that flank the neuropeptide (Hook et al., 2008). The amino acid sequences of neuropeptides may differ between species, and two or more isoforms may be present within a single species. These isoforms arise primarily through alternative splicing or post-transcriptional or post-translational modification (Nakamura et al., 1995).

Neuropeptides are typically found in the brain; however, some are distributed widely throughout the central nervous system (Joels, 2000). They tend to be synthesized by specific neurons and localized to specific nerve tracts, which argues for definitive roles

within the nervous system (Joels, 2000). Neuromodulators may also originate from non-neuronal sources (Seifert et al., 1996).

It is speculated that many neuropeptides act as neuromodulators; in contrast to neurotransmitters, neuromodulatory substances are considered to exert slower, but more prolonged, neurotrophic actions (Koh and Hille, 1997). These actions are believed to enhance or inhibit (and therefore modulate) the response of neurons to neurotransmitters (Joels, 2000).

The journey of a peptide destined for secretion begins at the level of the ribosome, the structure responsible for translation of the mRNA into corresponding sequence of amino acids that yields the mature protein. These ribosomes are classically bound to the endoplasmic reticulum (ER); however, free ribosomes (ribosomes free-floating in the cytoplasm) have been also been implicated in the translation of secretory peptides (Oda and Ito, 1981). Proteins that carry a distinct signal sequence in their N-terminal domain are recognized by a signal-recognition particle (SRP), which binds the signal sequence and the ribosome (Johnson, 1997). This targets the complex to the ER in a GTP-dependent process (Johnson, 1997). Proteins are often translocated into the ER as they are being synthesized (Blázquez and Shennan, 2000). Once in the lumen of the ER, proteins are stripped of their signal peptides by signal peptidases, and undergo several early modifications, such as protein folding (formation of disulfide linkages), assembly into multimeric species and primary glycosylation (Blázquez and Shennan, 2000). It is the role of the ER to make sure that proteins achieve their correct tertiary and quaternary structures required for proper biological activity (Blázquez and Shennan, 2000).

Unless proteins contain an ER-retention sequence (often in the form of a KDEL sequence at the carboxyl-terminus), they will be ferried through to the Golgi apparatus via

vesicular transport. They are transported from the ER to the Golgi in vesicular-tubular clusters (VTC), although the precise mechanism by which this occurs is still not fully understood (Hong, 1998). The transport of vesicles from the ER is achieved through either COPI or COP II coat protein-coated vesicles, which transport the vesicles to the most proximal Golgi compartment, the *cis*-Golgi (Blázquez and Shennan, 2000). From the *cis*-Golgi, the proteins are carried via vesicle flow from one Golgi stack to the next until they reach the *trans*-Golgi network (TGN). Once at the TGN, further sorting will occur to separate the proteins destined for the plasma membrane from those destined for lysosomes (Blázquez and Shennan, 2000).

There are two primary secretory pathways that endocrine cells use to shuttle proteins to their final destinations at the plasma membrane (PM), or ultimately get released out of the cell via exocytosis: the regulated secretory pathway (RSP) and the constitutive secretory pathway (CSP). Alternatively, peptides may exit the cell via fusion of multivesicular bodies (MVBs) with the PM (Keller et al., 2006), releasing vesicles known as exosomes (Schiera et al., 2007).

1.4.2 Vesicles: secretory granules versus microvesicles versus exosomes

Many types of vesicles have been described that release cell contents into the extracelullar space. Neurotransmitters are commonly packaged into synaptic vesicles, commonly released via the RSP at neuronal synapses. Secretory granules typically contain hormones (for example insulin and glucagon), and secrete them at the PM via exocytosis (Moriyama et al., 2000). Secretory granules can also store monoamines, as well as prohormones while they are converted by proteolysis to their mature form (Moriyama et al., 2000). Common markers for secretory granules are the chromogranins

(Moriyama et al., 2000). Similar to secretory granules, microvesicles (MVs; also called synaptic-like microvesicles) are also acidic in nature. MVs tend to be large, vary in size (100-1000nm), and are released by active processes involving RNA and protein synthesis (Schiera et al., 2007).

Exosomes, which are much smaller in size (60-90nm), are released from multivesicular bodies (MVBs), and are derived from the endosomal system (Keller et al., 2006). Exosomes typically do not contain nuclear, mitochondrial or ER proteins, but rather proteins commonly found in the cell cytosol or at the PM (Keller et al., 2006; Mears et al., 2004). Most cells of haematopoietic lineage are capable of releasing exosomes (Thery et al., 2001). Exosomes are also present in serum and ascites fluid, which has potential use in diagnosis and biomarker analysis (Keller et al., 2006).

1.4.3 Regulated Secretory Pathway (RSP)

The RSP is activity dependent: vesicles are released upon stimulation of the cells, requiring involvement of secondary messengers such as calcium or cyclic AMP (Blázquez and Shennan, 2000; Park and Loh, 2008). In RSP, peptides are synthesized in the cell body, and then transported to the secretion sites at the PM. At the level of the cell body, peptide hormones are packed into electron-dense peptidergic vesicles in the TGN, and subsequently transported along a microtubule-based transport system (Park and Loh, 2008), bringing them to their final destination at the release site. When they approach their destination, peptidergic vesicles are passed on to cortical actin filaments. Often the secretion sites are some distance from the site of synthesis (for example at nerve terminals, which are located an axon distance away from the cell body). Some of the

vesicles will remain in the actin cortex as a reserve pool, while another portion of the vesicles are transferred to the plasma membrane (Park and Loh, 2008). These granules can be stored in the reserve pools for hours, or days until induction to fuse with the PM (Blázquez and Shennan, 2000). Of the population that is sent to the plasma membrane, a fraction will be docked and primed for release. This subpopulation forms the "readily releasable" pool of peptidergic vesicles; the portion of vesicles that are ready for immediate fusion with the plasma membrane and consequent expulsion of their inner contents into the extracellular environment upon stimulation of the cell. After these "readily releasable" vesicles are depleted, the reserve pool is used to replenish their numbers and get the cell ready for subsequent stimulus (Park and Loh, 2008). The primary purpose of the RSP is to keep secretion low in the absence of stimulus, and to permit a massive, immediate increase in release of stored material at specified times (Blázquez and Shennan, 2000).

1.4.4 Constitutive Secretory Pathway (CSP)

In contrast to the RSP, the CSP is not stimulation-dependent: small vesicles are continuously released from the cells. Like the RSP, vesicles are packaged with peptide hormones, neurotrophins or neuropeptides in the TGN; however, in the CSP, vesicles are transported directly to the plasma membrane without creating any reserve pool (Park and Loh, 2008). As proteins are not stored, these vesicles are not electron-dense (Blázquez and Shennan, 2000). There are two options for transport to the plasma membrane: vesicles can be directly sent from the TGN to the PM, or they can pass to the PM via an intermediate compartment, called the intermediate endosomal compartments (early/late endosome and/or recycling compartments) (Park and Loh, 2008). Unlike RSP, CSP is not

driven by cell stimulation, but rather by the biosynthetic rate of secretory proteins at the endoplasmic reticulum (Park and Loh, 2008). Thus, secretion is thought to occur rapidly after synthesis of the protein (Blázquez and Shennan, 2000). Importantly, increased secretion of proteins via the CSP is not achieved through stimulation of a second messenger system, but solely through increased protein synthesis (Blázquez and Shennan, 2000). The CSP is responsible primarily for the shuttling of membrane proteins (such as receptors) to the PM, as well as the release of secretory proteins for maintenance of cell survival, differentiation and growth, and is therefore thought to be present in all cells (Blázquez and Shennan, 2000).

1.5 Hypothesis and rationale

1.5.1 Hypothesis

Considering the strong genetic evidence implicating progranulin in neurodegenerative diseases, we hypothesize that progranulin is neuroprotective against stress. We further hypothesize that progranulin is secreted via the constitutive pathway, potentially localized in microvesicles, playing an important role in the maintenance and/or survival of neurons.

1.5.2 Rationale

Previous work in our lab using *in situ* hybridization (Figure 1) showed progranulin mRNA localized within many neuronal subpopulations, and perhaps glia, in the brain stem, cervical spinal cord, and dissociated spinal cord-DRG cultures. Our lab also created a stably transfected line of NSC34 cells over-expressing progranulin. Validation of the cell line was achieved through both RT-PCR and Western blot analysis, to insure the

appropriate expression of vector alone and PGRN-containing plasmids (Figure 2A and B). Preliminary experiments with this new cell line uncovered interesting results: the cells were capable of living in serum-free medium for 52 days (Figure 3), while vector-only and untransfected cells scarcely lived beyond a week. This prompted further study to investigate the effects of overexpressing PGRN in neuronal cells.

Previous reports have also suggested that PGRN has an effect on neurite projection (Van Damme et al., 2008). Preliminary experiments in our lab have shown that NSC-34 cells produce pronounced neuronal extensions when grown on an extracellular matrix containing recombinant PGRN protein (Figure 4). This suggests that PGRN may have an effect on membrane synthesis at the level of the growth cone in neuronal cells. This process of plasmalemmal expansion (expansion of the plasma membrane) has been shown to involve exocytosis complexes and is thought to be mediated by growth factors (Pfenninger, 2009), though particular growth factors have yet to be defined. As this process is distinct from neurotransmitter release (Pfenninger, 2009), it is very possible that PGRN is acting at the growth cone to increase the formation of axons and dendrites via membrane synthesis.

To date, there have not been any reports about the subcellular localization of PGRN in neurons. Furthermore, the secretion of PGRN in the CNS has not been well characterized. These are important missing elements in further understanding the biochemistry of this important protein. Based on the new genetic information concerning PGRN mutations with respect to FTLD, and perhaps even a link to ALS, any general information about PGRN will be very useful in uncovering its involvement in neurodegenerative diseases, and subsequently, in developing future therapeutic treatments.

We therefore sought to characterize the subcellular location of PGRN within mice spinal cord, and further investigate its intracellular location using a neuronal model system, the spinal cord-DRG culture. We then used an immortalized cell line, the NSC-34 motor neuron-like cells, to investigate localization and secretion of PGRN in the CNS. While further experiments are still needed, we have also performed preliminary studies to investigate the method of secretion of PGRN, whether via the regulatory or constitutive pathway.

CHAPTER II

MATERIALS AND METHODS

2.1: Chemicals

2.1.1: Cell Culture Material and Antibodies

NSC-34 cells were grown in Dulbecco's Modified Eagle Medium (DMEM, 1X) containing 10% fetal bovine serum (FBS) and 10μg/ml gentamicin, in 75cm^2 vented flasks from Sarstedt. When cells reached 70% confluency, they were passaged using 0.5% trypsin-EDTA (Gibco). Cell culture materials were purchased from Invitrogen (Burlington, Ontario, Canada).

Cytosine ß-D-arabinofuranoside (AraC; Sigma) was reconstituted in water for a stock solution of 50mg/ml. To induce cell differentiation of NSC-34 cells via inhibition of DNA synthesis, AraC was added to cell media at a concentration of 1:500, 2 days following plating of the cells. Drug was removed after 48 hours (next medium replenishing). Brefeldin A, a fungal metabolite used to inhibit transport from the ER to the Golgi apparatus, was purchased from New England BioLabs and used at concentrations of 2.5μg/ml for 30 minutes. All antibodies used in the following experiments are listed in Table 1.

2.2: Analysis of Progranulin Expression

2.2.1: Paraffin-Embedded Section Preparation

Spinal cords were obtained from pregnant CD1 mice. Spinal cords were collected and fixed in 4% paraformaldehyde in PBS for 1 hour, cut into cervical, thoracic, lumbar and sacral sections, then all were fixed overnight at the Neuropathology Department of

the Montreal Neurological Institute. Each section was subsequently processed for paraffin sectioning. 4 μm slices were cut in the coronal plane and mounted onto positively charged slides (Fisher Scientific). Sections were dried in the oven overnight, then deparaffinized and rehydrated the next day (two 2 minute incubations in Citrisolv, a 2 minute incubation in 100% ethanol, a 2 minute incubation in 95% ethanol, a 2 minute incubation in 75% ethanol, a 2 minute incubation in 50% ethanol, rinsed in large volumes of water, washed in large volume PBS). Antigen retrieval was performed using a high pH buffer (Tris-EDTA with 0.05% Tween, pH 9.0), placed in a pressure cooker for 10 minutes. After cooling, the sections were placed in a 3% hydrogen peroxide solution for 10 minutes to block endogenous peroxidase activity. Sections were rinsed thoroughly in PBS and placed in blocking buffer (10% (w/v) horse serum, 5% bovine serum albumin (BSA), 0.3% Triton X-100 in PBS) for 1 hour. Sections were then processed by the immunofluorescence protocol outlined below.

2.2.2: Spinal cord-DRG cultures Preparation

Dissociated primary motor neurons cultures were graciously provided by Heather D. Durham's laboratory at the Montreal Neurological Institute. These cultures were taken from embryonic day 13 (E13) mice, plated on either 25mm or 14mm coverslips (Electron Microscopy Sciences), and grown for 4 to 7 weeks after dissociation (Roy et al., 1998). Cultures were fixed within the original plates using 4% PFA for 10 minutes, and incubated with permeabilization buffer (PBST with 0.2% Triton X-100) for 2 minutes, the cultures were post-fixed for 1 minute with 4%PFA, followed by incubation in blocking buffer (PBS with 5% (w/v) horse serum (Hyclone)) for one hour. Coverslips were then processed by the immunofluorescence protocol outlined below.

2.2.3: NSC-34 Cell Preparation

The NSC-34 cell-line were cultured on German glass coverslips in DMEM with 10% fetal bovine serum. For subcellular localization of PGRN, cells were treated with AraC in order to induce differentiation of the rounded, more neuroblastoma-like cells (see section 2.1 for drug information). Cells were fixed in 4%PFA, rinsed twice with PBST, and incubated with permeabilization buffer (PBST with 0.2% Triton X-100) for 20 minutes. After being washed three times with PBST, the cultures were post-fixed for 10 minutes with 4%PFA, followed by extensive washing. Fixed cells were incubated in PBST with 0.5% (w/v) membrane blocking reagent (GE Healthcare) for one hour, and were then processed by the immunofluorescence protocol outlined below.

2.2.4: Immunofluorescence Protocol

Spinal cord sections, and coverslips with either spinal cord-DRG cultures or NSC-34 cells were prepared according to the protocols outlines above, then transferred to fresh blocking buffer containing the primary antibodies (see table for primary antibody concentrations as well as the corresponding secondary antibodies used in conjunction). Incubation with the primary antibody continued overnight at 4ºC. Samples were washed three times 5 minutes in PBS (pH 7.4), and then incubated with the appropriate secondary antibodies in fresh blocking buffer for 45 minutes at room temperature. Samples were washed three times 5 minutes in PBS. Some samples were additionally counterstained using 300nM DAPI in PBS for 5-10 minutes at room temperature in the dark. Cultures were washed three times with PBST, once with ddH_2O, and then mounted onto slides using Immu-mount (Thermo Fisher), or fluorescent mounting medium (Dako).

Fluorescence was visualized by confocal microscopy (LSM 510), using argon and HeNe1 lasers. Images were processed with Zen software. Identification of primary motor neurons within the heterogeneous culture was based upon SMI32 immunoreactivity and cell body size (Roy et al., 1998).

To control for nonspecific binding of the mouse progranulin antibody, antigen-competition was carried out by pre-adsorbing the antibody at 1:500 dilution with 300ng/mL and 400ng/mL recombinant mouse PGRN (Alexis Biochemicals). The mixture was incubated for 24 hr at 4°C and following centrifugation, the supernatant was collected and used as a primary antibody, as described above.

2.3: Cloning Experiment:

2.3.1: Polymerase Chain Reaction (PCR)

Human *PGRN* DNA was amplified using a MiniPrep kit from Roche. DNA was quantified using a spectrophotometer at 260nm absorbance. PCR primers were designed to include digestion sites for restriction enzymes. For pEGF-N1-hpgrn, forward primer 5' C GAA TTC GAA TTC ACC ATG TGG ACC CTG GTG AGC 3' and reverse primer 5' GAC GTC GAC CCC AGC AGC TGT CTC 3' were designed for EcoR1 and Sal1 digestion respectively.

PCR was carried out under the following conditions: 1µl DNA, 2µl each primer, 2µl $MgSO_4$, 2µl dNTPs, 5µl 10X Thermostable buffer, 0.25µl Deep Vent enzyme and 39.75µl sterile ddH_2O. Control samples contained 1µl sterile ddH_2O in place of the DNA template. PCR cycle: 94°C for 10 minutes, then 40 cycles of 94°C 1 min, 62°C 1 min,

72°C 1 min, followed by 10 minutes at 72°C. When cycle was finished, PCR block was kept at 4°C until samples were run on 1% agarose electrophoresis gel.

2.3.2: Vector Digest

1µg plasmid was used in the restriction digest. Along with this template, 42µl of sterile ddH_2O, 5 units of EcoR1 enzyme, 5 units of Sal1 enzyme, 5µl 10X NEBuffer and 0.5µl 100X bovine serum albumin (BSA) was mixed gently on ice. For control samples, template single stranded DNA was replaced by sterile ddH_2O. The reaction was allowed to proceed for 2 hours at 37ºC. Enzymes were then heat inactivated by incubation at 65ºC for 20 minutes. Vector digests were run on 1% agarose electrophoresis gel containing ethidium bromide alongside PCR products. Photos were taken of the bands under ultraviolet (UV) light. Bands were subsequently cut out from the gel and purified according to the protocol outlined below.

2.3.3: DNA Digest

For each PCR DNA product, 1µg single stranded *hPGRN* DNA was used in the restriction digest. Along with this template, 35µl of sterile ddH_2O, 0.5µl EcoR1 enzyme, 0.5µl Sal1 enzyme, 5µl 10X NEBuffer and 0.5µl 100X bovine serum albumin (BSA) was mixed gently on ice. For control samples, template single stranded DNA was replaced by sterile ddH_2O. The reaction was allowed to proceed for 2 hours at 37ºC. Enzymes were then heat inactivated by incubation at 65ºC for 20 minutes. The digested DNA inserts were purified according to the protocol outlined below.

2.3.4: Vector/DNA Purification

Purification protocol was adapted from Quiagen purification kit.

STEP 1: Vector purification: vector was cut directly out of 1% agarose gel with sterile razor blade and placed into a 1.5ml microcentrifuge tube. The tube was weighed, and 3 volumes of Buffer QG per 1 volume of gel slice was added. The tube was incubated at 50ºC for 10 minutes with samples being vortexed every 2-3 minutes to aid with solubilization. 1 gel volume equivalent of isopropanol was added to the sample, and the tube was inverted several times to mix. Proceed to Step 2.

DNA purification: 500µl of Buffer QG was added directly to the DNA digest tube. Proceed to Step 2.

STEP 2 for both vector and DNA:

The sample was applied to the miniElute column and was then centrifuged for 1 minute. The flow through was discarded and the MiniElute column was placed back in the same collection tube. 500µl of Buffer QG was added to the spin column, then centrifuged for 1 minute. The flow through was disregarded. To wash, 750µl of Buffer PE was added to the MiniElute column; it was then centrifuged for 1 minute. The flow through was disregarded, the spin column was then centrifuged an additional 1 minute at 13000rpm. The MiniElute column was placed into a clean 1.5ml microcentrifuge tube. To elute DNA, 10µl of sterile ddH_2O was added to the center of the membrane. The column was left standing for 1 minute, then centrifuged for 1 minute. Samples were either used immediately or stored at -80ºC for future use.

2.3.5: Ligation of Vector and Insert

Ligation mixtures were created as a 3:1 ratio, vector size to insert size respectively. As the vectors were 4.7 kb and the DNA insert is 1.8kb, a 3:1 ratio was already in place. Thus, 50ng of insert and 50ng of linearized vector were mixed together with ddH_2O for a total volume of 17µl and chilled on ice. 2µl of 10X T4 DNA Ligase Buffer (BioLabs) was added to the mixture and kept on ice. 1µl of T4 DNA Ligase (BioLabs) was added and tube was incubated at 20ºC for 20 minutes. Product was either chilled on ice for immediate use or stored at -80ºC for future use.

2.3.6: Transformation

Competent cells (E. Coli, aliquots of 50µl, Invitrogen) were thawed on ice. 10µl of the chilled ligation mixture was added to the cells and mixed gently by pipetting up and down. Cells were incubated on ice for 30 minutes, then heat shocked at 42ºC for 2 minutes, followed by chilling on ice for 5 minutes. 950µl LB broth (at room temperature) was added to the cells, which were subsequently incubated at 37ºC for 1 hour. Cells were centrifuged at 12000rpm for 30 seconds. 100µl of the pellet (plus media) was spread onto a kanamycin coated LB plated Petri dishes and grown overnight in a 37ºC incubator.

2.3.7: Confirmation of Ligation

All of the following was performed in a sterile environment, with sterile equipment and materials. Petri dishes were removed from 37ºC incubator and individual colonies were identified. Colonies were picked off individually and placed into individual

wells of a 96 well plate. 5µl of sterile LB broth was added to each of the 96 wells. PCR was performed on each of the individual colonies of bacteria, using 4µl of each as the template DNA. The same PCR conditions were used as for the original PCR of the *hPGRN* insert. PCR products were run on a 1% agarose gel, along with PCR products of *hPGRN* as a positive control for insert size. The pEGFP-C2-hPGRN vector ligation step was not successful, thus was left to complete at a later time. Experiments were carried out using the pEGFP-N1-hPGRN plasmid only.

200µlof LB medium was added to each remaining 1µl bacterial, along with 1:1000 kanamycin. The 96-well plate was sent back to the incubator until after the PCR reaction was complete. These stocks were used for the vector amplification steps below.

2.3.8: Plasmid Amplification and Extraction from Bacteria

100µl of bacterial colonies with the correct band size for the *hPGRN* insert were grown overnight at 37ºC in tissue culture tubes for plasmid amplification. Extraction of the vectors from the bacteria was performed using the MiniPrep kit from Roche.

2.4: Experiment s using pEGFP-hPgrn Plasmids

2.4.1: Transfection

NSC-34 cells were grown on 25mm German glass coverslips in 6 well plates. At 70% confluency, cells were washed twice in serum-free OPTIMEM in preparation for transfection. Serum-free, antibiotic-free OPTIMEM and Lipofectamine were combined according to manufacturers instructions. For each coverslip, 4µg of pEGFP-N1-hPGRN, or control empty vector pEGFP-N1, was added to the appropriate volume of OPTIMEM-

lipofectamine solution and mixtures were incubated for 30 minutes prior to addition to well containing 1.5ml of serum-free DMEM. Cells were incubated with the transfection mixture for 4 hours, after which the mixture was replaced with DMEM containing 10% FBS and gentamicin. It should be noted that only one clone of the pEGFP-N1-hPGRN plasmid was experimentally tested.

2.4.2: Immunocytochemistry

Transfected cells were either treated with brefeldin A (BFA) or directly fixed in 4% PFA 48 hours post-transfection. NSC-34 cells to be used to analyze Golgi apparatus involvement in protein transit were treated with 2.5ug/ml BFA for 30 minutes at 37˚C. Cells (BFA treated or untreated) were fixed for 10 minutes with 4% PFA, followed by 1 minute treatment with NP-40 (1X), and 2 minute post-fix with 4% PFA. Cells were then either placed in blocking buffer (5% horse serum in PBS) for further analysis with double-labeling, or directly washed in PBS for initial confirmation of successful transfection. Cells were mounted with Dako mounting medium onto positively charged slides and sealed with clear nail polish. Slides were analyzed using an LSM 510 confocal microscope, using Zen software for image processing.

2.4.3: Quantification of hPRGN -eGFP secretion in NSC-34 Cells

Quantification of hPGRN-eGFP was accomplished through measuring fluorescence of cellular medium with a SpectraMaz M5 fluorimeter (Molecular Devices). Medium for plate reader: 1ml and 2ml increments of medium from each well was collected and spun down to remove any possible cell debris. DMEM collected from untransfected cells (containing 10% FBS and gentamicin) was used as a blank against the

samples. Blanks and samples were placed in wells of 24-well plates, each sample present in triplicate. Plates were read and analyzed according to the SoftMax Pro software using 485nm as excitation, and 505nm to 535nm as the emission parameters for eGFP. Results were expressed as means ± standard deviations, and compared using an unpaired t-test in Sigma Plot (SYSTAT Software Inc.).

2.4.4: Blocking hPGRN-eGFP Secretion with Brefeldin A

Ideal drug treatment was first carried out with non-transfected NSC-34 cells and found to be 2.5µg/ml for significant effect without toxicity. Thus, pEGFP-N1-hPgrn, empty vector and untransfected control NSC-34 cells were treated with 2.5µg/ml BFA for 30 minutes, then primed for either calcium-stimulation experiments or immunofluorescence according to the protocol outlined above. Fixed cells were incubated with antibodies either against the trans-Golgi network (GM130) overnight, then washed thoroughly, followed by subsequent incubation with the appropriate secondary antibodies. After further washing, cell were mounted and viewed by confocal microscopy. Refer to the antibody table for all antibody working dilutions.

2.4.5: Sensitivity to Cytosolic Calcium

48 hours post-transfection, different concentrations of $CaCl_2$ were added to each well: 0.05mM, 0.1mM. 0.5mM, 1.0mM, 3.0mM to test the effect of calcium on secretion. After 90 minutes of incubation with the calcium, medium from each well was transferred to separate vials and 1X solution of protease inhibitors was added to each vial. Vials were spun down to remove any cell debris, then aliquots of 1ml were placed into 24 well plates

and analyzed with an M5 fluorimeter. Calcium sensitivity results were expressed as means ± standard deviations, and compared using an unpaired t-test in Sigma Plot (SYSTAT Software Inc.).

2.5 Progranulin Over-Expression:

2.5.1: NSC-34 Transfection with human *PGRN*

Dr. Suneil Malik was responsible for the generation of a stably transfected cell line. NSC-34 cells were transfected with human *progranulin* (pcDNA-Pgrn) or empty vector (pcDNA) using Lipofectamine (Invitrogen) and selected with G418 for one month according to manufacturer's instructions.

2.6: Survival Assays

2.6.1: Cell Lines and Culture Conditions

The NSC-34 cell line (generous gift from Dr. Neil Cashman) was maintained in DMEM with 10% fetal bovine serum unless otherwise stated (Cashman et al., 1991). For stable transfections NSC-34 cells were transfected with human progranulin (pcDNA-Pgrn) or empty vector (pcDNA) using Lipofectamine (Invitrogen) and selected with G418 for one month according to the manufacturer's instructions. Serum deprivation assays were carried out in 6 well plates using 200,000 cells/well and cultured in 4ml of RPMI (with glutamine) for 3, 6, 9, 12 and 15 days without the addition or exchange of fresh medium. For each time point the average cell number was determined over 6 visual fields per well at 10X magnification using an Olympus phase-contrast microscope. For long

term cultures NSC-34 cells were plated at a density of 200,000/well in 6-well plates and maintained in serum free RPMI medium. Fresh medium was provided every 10 days and 10X magnification photos taken at 20 and 57 days using an Olympus phase-contrast microscope. Survival results were expressed as means ± standard deviations, and compared using an unpaired t-test in Sigma Plot (SYSTAT Software Inc.).

2.6.2: BrdU Proliferation Assay

NSC-34 cells were plated on German glass, photo-etched Coverslips (Electron Microscopy Sciences) in 6 well plates at 200,000/well and cultured in 4ml of RPMI (with glutamine) for six days. 12 hours prior to fixation/processing, BrdU labeling solution was added to each well at a concentration of 10μM (Roche Applied Sciences). At the time of fixation, cells were washed three times in PBS to remove excess unincorporated BrdU, then fixed using 4% PFA/PBS for 20 minutes. After being rinsed three times in PBST, cells were incubated in permeabilization buffer (0.2% Triton X-100 in PBST) for 20 minutes. Cell were subsequently post-fixed for 10 minutes with 4% PFA/PBS. After being rinsed three times with PBST, the cells were placed in 0.1M sodium borate pH 8.5 for 2 minutes at room temperature. The cultures were incubated in PBST with 0.5% (w/v) membrane blocking reagent (GE Healthcare) for one hour followed by the addition of anti-BrdU Alexa-488 (1:200, Invitrogen) for 45 minutes in blocking buffer at room temperature. After rinsing three times in PBST, cells were counterstained with 300nm DAPI for 5 minutes in the dark. Cells were then rinsed twice with PBST, once with ddH_2O and then mounted onto slides using Immu-mount (Thermo Fisher). Fluorescence was visualized with an Axioskop2 microscope equipped with appropriate filters and total

cells (DAPI) versus proliferating cells (Alexa-488) were counted manually by visual inspection. Proliferation results were expressed as means ± standard deviations, and compared using an unpaired t-test in Sigma Plot (SYSTAT Software Inc.).

2.6.3: Apoptosis TUNEL Assay

NSC-34 cells were plated on German glass, photo-etched Coverslips (Electron Microscopy sciences) in 6 well plates at 200,000/well and cultured in 4ml of RPMI (with glutamine) for six days. At time of fixation, cells were washed twice in PBS, then fixed using 4% PFA/PBS for 20 minutes. After being rinsed three times in PBST, cells were incubated in permeabilization buffer (0.2% Triton X-100 in PBST) for 20 minutes. Cells were subsequently post-fixed for 10 minutes with 4% PFA/PBS. After being washed extensively with PBST, they were stored at 4^{o}C in sterile PBS. At time of processing, cells were rinsed once with PBS, and then overlaid with reaction solution from the Fluorescein *In Situ* Death Detection Kit (Roche Applied Science), as directed by manufacturer's instructions. Cells were incubated at 37^{o}C for 1 hour, and then rinsed twice with PBST at room temperature in the dark. After rinsing three times in PBST, cells were counterstained with 300nm 4',6-diamidino-2-phenylindole (DAPI) for 5 minutes in the dark. Cells were then rinsed twice with PBST, once with ddH_2O and then mounted onto slides using Immu-mount (Thermo Fisher). Fluorescence was visualized with an Axioskop2 microscope equipped with appropriate filters and total cells (DAPI) versus apoptotic cells (FITC) were counted manually by visual inspection. Apoptosis results were expressed as means ± standard deviations, and compared using an unpaired t-test in Sigma Plot (SYSTAT Software Inc.).

Antibody	Titre	Source	Secondary
Mouse Pgrn	1:500	R&D Systems	Donkey-Anti-Sheep (Alexa Fluor 488 or 594)
Tdp-43	1:100	Proteintech	Donkey-Anti-Rabbit (Alexa Fluor 488)
Cytochrome C	1:400	Pharmingen	Donkey-Anti-Mouse (Alexa Fluor 488 or 594)
Calreticulin	1:400	StressGen	Donkey-Anti-Rabbit (Alexa Fluor 488 or 594)
Gm130	1:300	Pharmingen	Donkey-Anti-Mouse (Alexa Fluor 488 or 594)
Chromogranin A	1:100	RDI	Donkey-Anti-Rabbit (Alexa Fluor 488)
Synaptophysin	1:200	Sigma	Donkey-Anti-Rabbit (Alexa Fluor 488)
Snap-25	1:1000	Sigma	Donkey-Anti-Rabbit (Alexa Fluor 488)
Gfap	1:400	Proteintech	Donkey-Anti-Rabbit (Alexa Fluor 488)
Cd11b	1:100	BioLegends	No Secondary Required
Smi32	1:1000	Sternberger	Donkey-Anti-Mouse (Alexa Fluor 488)
Additional fluorescence reagents used			
Phalloidin	1:200	Invitrogen	
Lysotracker™	50 nM	Invitrogen	

Table 1. Antibodies and reagents used for immunofluorescence. This table demonstrates the concentrations used for each primary antibody, its source, as well as its corresponding secondary antibody. Each secondary antibody was used at a concentration of 1:200. Additional reagents used for fluorescent labeling are also noted.

CHAPTER III

RESULTS

3.1. Motor neurons of the murine spinal cord express PGRN

The expression pattern of PGRN has been described in the adult brain, but not in the remainder of the nervous system. We investigated the expression of PGRN protein in normal mouse spinal cord. Expression of PGRN at the protein level was demonstrated by the presence of PGRN-immunoreactive protein within motor neurons in cross-sections of lumbar spinal cord. Figure 5 shows co-labelling of large neurons in the ventral cord immunoreactive with both anti-PGRN and the neurofilament marker SMI32, which strongly labels motor neurons (Roy et al., 1998). The expression of PGRN protein was prominent in motor neurons labelled with SMI-32 in primary from spinal cord-DRG cultures (Figure 6a) and was also present in CD11b positive microglia in these cultures (Figure 6c). PGRN was not present in astrocytes (Figure 6b) as demonstrated by non-overlap with glial fibrillary acidic protein (GFAP). This was not unexpected since these cells are derived from a separate lineage from microglia. Microglia are derived from haematopoietic stem cells (Eglitis and Mezey, 1997), while astrocytes have separate astrocyte precursor cells (Mi and Barres, 1999).

3.2. Subcellular localization of PGRN within spinal cord-DRG cultured motor neurons and NSC-34 immortalized motor neuron cells is complex

Confocal immunofluorescence microscopy was used to localize the subcellular distribution of PGRN within primary motor neurons. Specificity of the immunoreactivity was validated by antigen competition using murine PGRN (Figure 7A). PGRN is primarily found in the cell body showing a punctate distribution within the cytoplasm; however, PGRN expression was also prominent within the axons of neurons. PGRN was

not observed within the nucleus nor was it present within mitochondria as defined by TDP43 and Cytochrome C immunofluorescence, respectively (Figure 7B-a,b). Interestingly, PGRN did not colocalize with calreticulin, a calcium binding protein that facilitates transit of correctly folded proteins and is a marker the endoplasmic reticulum (ER) (Figure 8a). PGRN expression was also absent from the trans-Golgi network (TGN) as demonstrated by the marker GM130 (Figure 8b). This finding was most surprising, since pre-PGRN carries a signal peptide to direct passage through the secretory pathway, and also undergoes glycosylation and formation of disulfide bonds, both of which are most likely to occur within the ER and TGN. PGRN did not colocalize with another secreted peptide, chromogranin A, which is presumed to be located within dense core granules (Figure 9a); however the size of PGRN granules does appear to be of relative dimension similar to the chromogranin A-containing vesicles. PGRN did appear to have some colocalization with synaptophysin (Figure 9d), a marker for synaptic vesicles. Some very minor colocalization with lysosomes was noted using the Lysotracker dye (Figure 9b). Antibody to PGRN did not colocalize with SNAP-25, a marker for neurotransmitter vesicle docking and release sites (Figure 9c).

Further studies confirmed that the subcellular distribution of PGRN in NSC-34 cells was comparable to that of the primary motor neurons (Figure 10), including labelling of NSC-34 cells with SMI-32 which is consistent with motor neuron-like properties (Figure 10a).

3.3. Subcellular localization of progranulin-enhanced Green Fluorescent Protein (PGRN-eGFP) in NSC 34 cells

Due to the lack of colocalization with the TGN and other markers of the secretory pathway, as well as the shortage of commercial PGRN antibodies available at the time, we developed a green fluorescent protein (GFP)-tagged PGRN construct to further investigate PGRN subcellular localization (Figure 11 and 3.12). This construct, consisting of an enhanced GFP (eGFP) tag fused to the C-terminus of the human *PGRN* gene (named pEGFP-N1-hPGRN), was transiently transfected into NSC-34 cells. 48 hours after transfection, confocal microscopy of the cells demonstrated successful transfection, and a granule-like appearance that was very different from the control empty eGFP vector-transfected cells (empty eGFP vector expression was predominantly nuclear, Figure 13d). The pEGFP-N1-hPGRN expressing cells demonstrated clear colocalization of PGRN with the TGN (Figure 13b); however, little or no colocalization with the ER was seen (Figure 13a). PGRN was clearly distinct from the mitochondria (Figure 13c). It is possible that the discrepancy seen between the eGFP-tagged progranulin and that of the PGRN is the epitope recognized by the antibody itself. The antibody to PGRN was raised against the recombinant mouse PGRN. It is unclear whether it would detect an immature form of the protein (the conformation of PGRN before glycosylation and formation of disulfide bridges is complete).

3.4 PGRN-eGFP is secreted by NSC-34 cells

The process of secretion of PGRN in neuronal cells has not yet been defined. Using the pEGFP-N1-hPGRN plasmid, NSC-34 cells were transfected to examine

whether or not these motor neuron-like cells are capable of secreting PGRN. Using the GFP tag and a fluorimeter that picks up emission wavelengths between 505 and 530nm, it was confirmed that PGRN is present within the media, thus suggesting secretion, although the quantity of eGFP fluorescence was quite small. Samples of the medium were taken at 48 and 72 hours post-transfection. Only a 5-10% difference was seen in the transfected cells versus the control untransfected cells (Figure 14), suggesting either basal rates of secretion, or low transfection efficiency. Under the confocal microscope, we were able to conclude that the transfection efficiency was around 80% (data not shown), thus supporting a basal level of secretion, possibly under the regulated secretory pathway.

Using the Golgi inhibitor brefeldin A (BFA), it was possible to halt secretion and keep PGRN from passing through the TGN. Confocal microscopy with the fluorescently labeled PGRN shows the effect of BFA on PGRN, which no longer co-localizes with the Golgi (Figure 15). Put together, these results suggest that NSC-34 cells do secrete PGRN, although the exact mechanism of secretion requires further study.

3.5 Over-expression of PGRN in NSC-34 cells promotes a neuron-like morphology.

The cell line NSC-34 was established by fusing embryonic spinal cord cells with neuroblastoma and in the differentiated state, these cells are reported to exhibit motor neuronal properties (Cashman et al., 1992; Durham et al., 1993). They are frequently used to investigate neuroprotective processes and to model motor neuron degeneration *in vitro* (Chi et al., 2007; Gal et al., 2007; Guo and Bhat, 2007; Kanekura et al., 2005; Kirby et al., 2005; Maystadt et al., 2007; Zou et al., 2007). In culture most NSC-34 cells exhibit a

rounded and undifferentiated morphology; however, serum-deprivation stimulates many of the cells to undergo differentiation and to extend neurite-like projections (Durham et al., 1993; Eggett et al., 2000). Serum-deprivation is also associated with significant apoptosis of the non-differentiated cells (Eggett et al., 2000).

NSC-34 cells that express human PGRN were established by transfection and selected for stable incorporation of the human PGRN gene. Previous work had demonstrated that human PGRN is mitogenic for mouse embryonic fibroblasts (Zanocco-Marani et al., 1999). Untransfected NSC-34 cells, NSC-34 transfected with empty PCDNA3 vector (NSC-34/vector), and NSC-34 cells that were transfected with human *PGRN* cDNA subcloned into pcDNA3 (NSC-34/PGRN) continued to express equivalent levels of murine PGRN mRNA, while only the NSC-34/PGRN cells expressed the human PGRN mRNA (Figure 2). Over-expression of PGRN was associated with morphological changes, such as flattening of cell shape and more pronounced neurite-like extensions (Figure 16). In NSC-34/PGRN cells, PGRN punctate immunoreactivity was found throughout the cell body and within the projections (Figure 16).

3.6 PGRN over-expression promotes cell survival in NSC-34

The effect of PGRN expression on cell survival was investigated by subjecting cell cultures to prolonged periods of serum deprivation. Between 12 and 15 days following the removal of serum, cell number significantly decreased in NSC-34/vector cultures, while cultures of NSC-34/PGRN cells showed no such change (Figure 17A). By 6 days in serum-free culture medium there was no statistically significant difference in cellular proliferation between the NSC-34/vector and NSC-34/PGRN cells as

demonstrated by BrdU incorporation (Figure 17B); however, there was a significant increase in TUNEL-positive (i.e. apoptosing) cells in the NSC-34/vector cultures versus the NSC-34/PGRN cultures (Figure 17C).

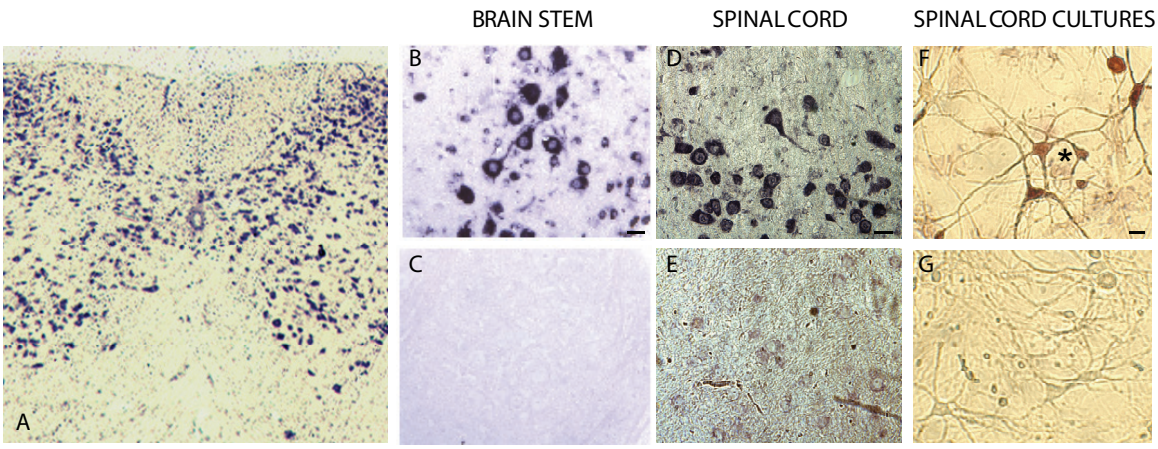


Figure 1. PGRN is expressed in the mouse central nervous system, both in vivo and in vitro. In situ experiments show gene expression pattern of murine PGRN in brain (B, C); cervical spinal cord (A, D, E) and primary cultures of dissociated spinal cord-DRG (F, G). In situ hybridization, to detect PGRN mRNA in saggital section of pontine grey matter in saggital section (A) and cross-section of cervical spinal cord (D, E). The majority of neurons throughout the grey matter of the spinal cord express PGRN as well as ependymal cells and possibly microglial cells (A). Note in particular the robust expression of PGRN mRNA in large motor neurons in panels B, D. Panels C and E illustrate the hybridization signal observed with the sense control applied to serial sections to those shown in panels B and D, respectively. (F) Motor neurons (asterisk) as well as other neuronal subtypes in dissociated spinal cord-DRG cultures, express PGRN; (G) equivalent sense control. Scale bar (panels B-G) represents 20μm. Original magnification of panel A was 10X.

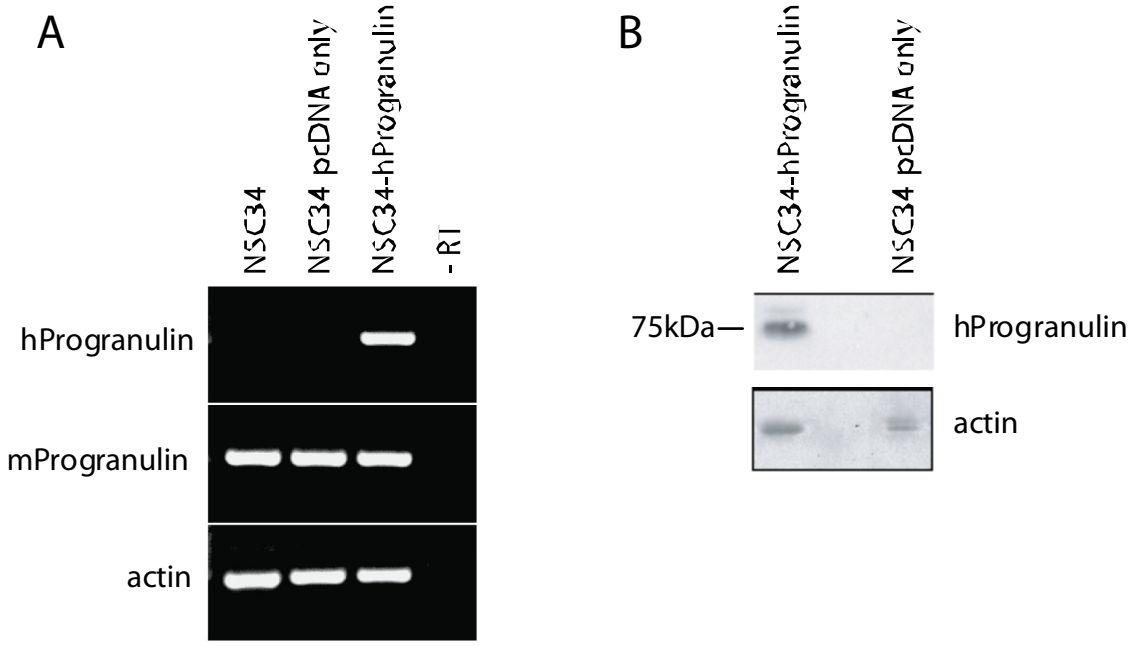


Figure 2. **Validation of stable transfectants**. The motor neuron-neuroblastoma hybrid cell line, NSC-34, was transfected with empty pcDNA vector or pcDNA-hPGRN and selected for drug resistance over a three-week period. (A) Species-specific PGRN primers were used to confirm the stable integration of hPGRN. (B) Western Blot analysis confirmed presence of hPGRN in stable transfectants and not in the vector only control cells.

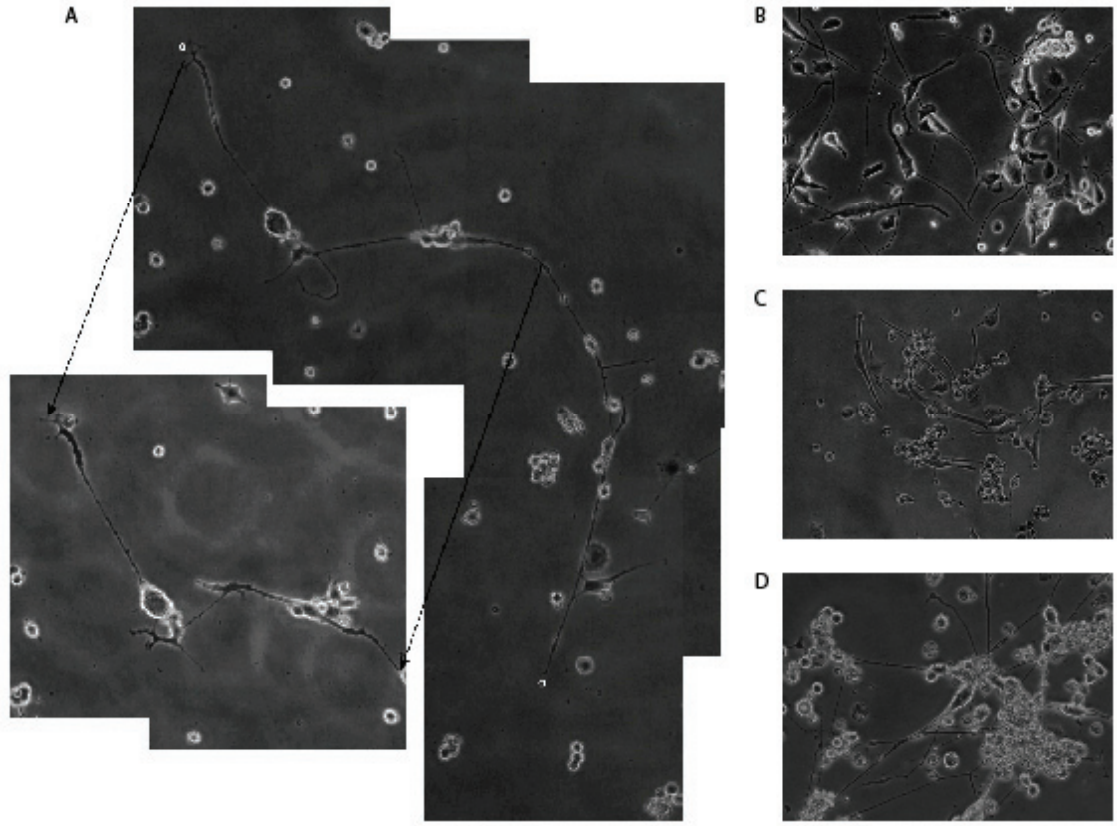


Figure 3. PGRN is a sufficient trophic stimulus to maintain prolonged survival of NSC-34 cells in serum-free medium for 52 days. NSC-34 cells stably transfected with pcDNA3/PGRN and grown in serum-free medium (A) for 20 days, the cut-out box illustrating the same cells photographed 3 hr later showing continued active extension and retraction of processes; (B) for 51 days, and (C, D) for 67 days in serum free medium. All NSC-34/vector control cells died before day 20 (not shown). Images were taken at an original magnification of 15X.

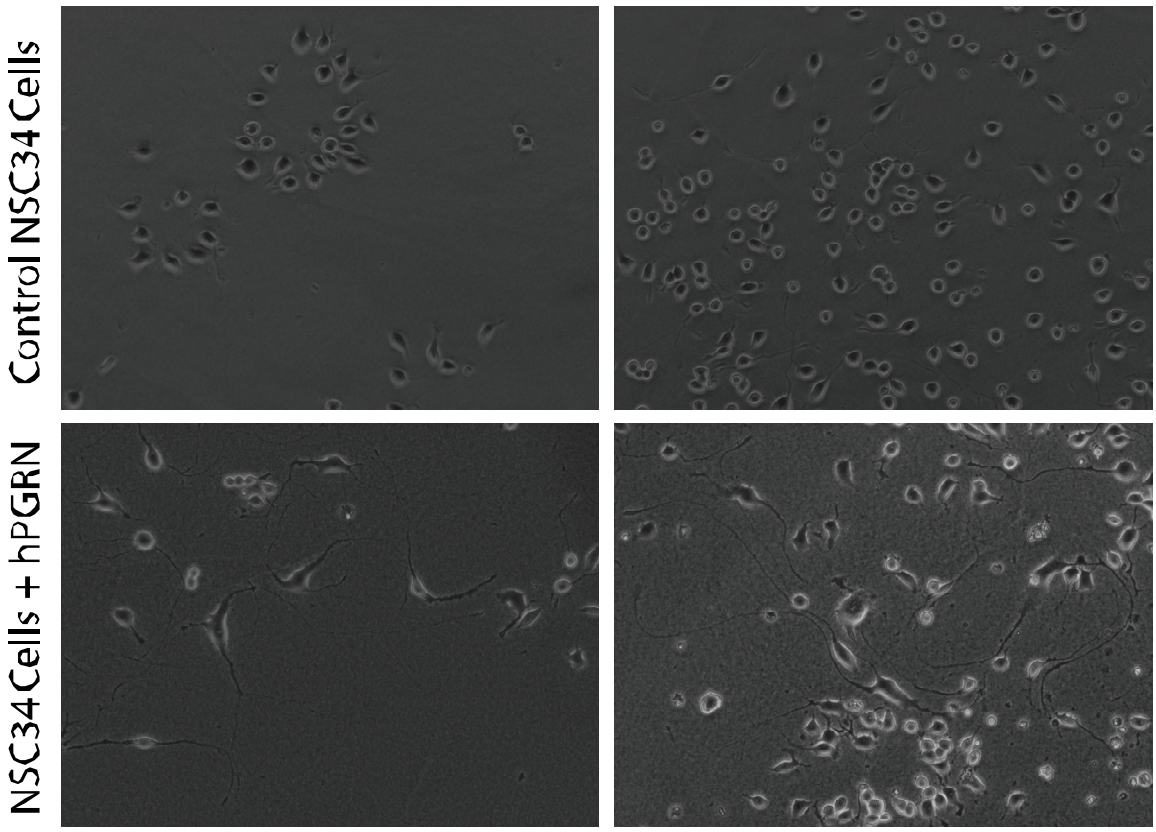


Figure 4. PGRN increases neurite outgrowth in NSC-34 cells. When cells were grown on nitrocellulose plated with recombinant human PGRN (lower panels), dramatic neuronal extensions were observed compared to control cells (top panels). Cells grown on PGRN also seemed to have a much greater proliferation rate than control cells, but this proliferation was not quantified. Images were taken at 15X magnification.

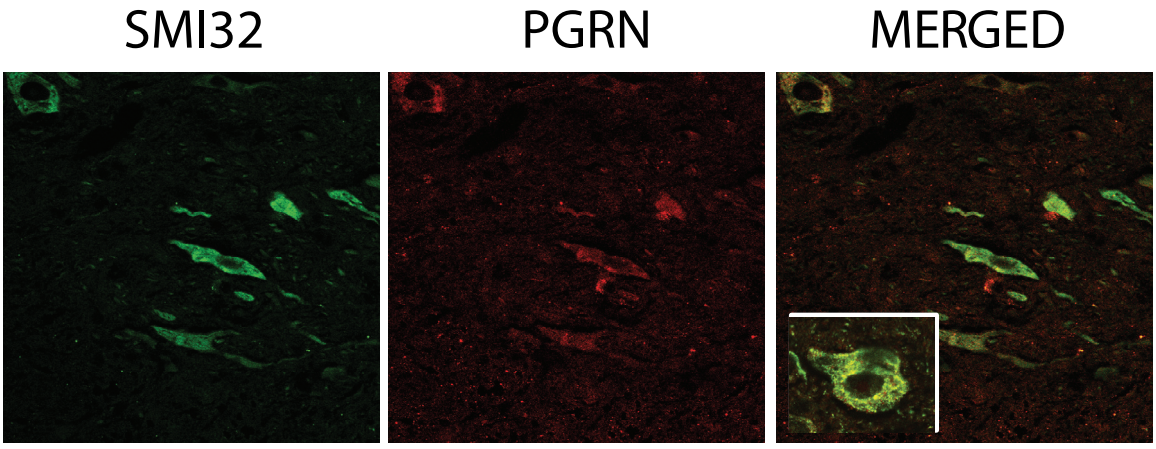


Figure 5. PGRN is localized within motor neurons of the mouse lumbar spinal cord. Progranulin is localized within motor neurons of the mouse spinal cord. Labelling of paraffin-fixed cross-sections of murine spinal cord with SMI32 marker against hypo-phosphorylated neurofilaments, and anti-PGRN. Merged channels at high magnification (40X) are shown in the right-most panel. Hatched boxes show neurons at 63X magnification.

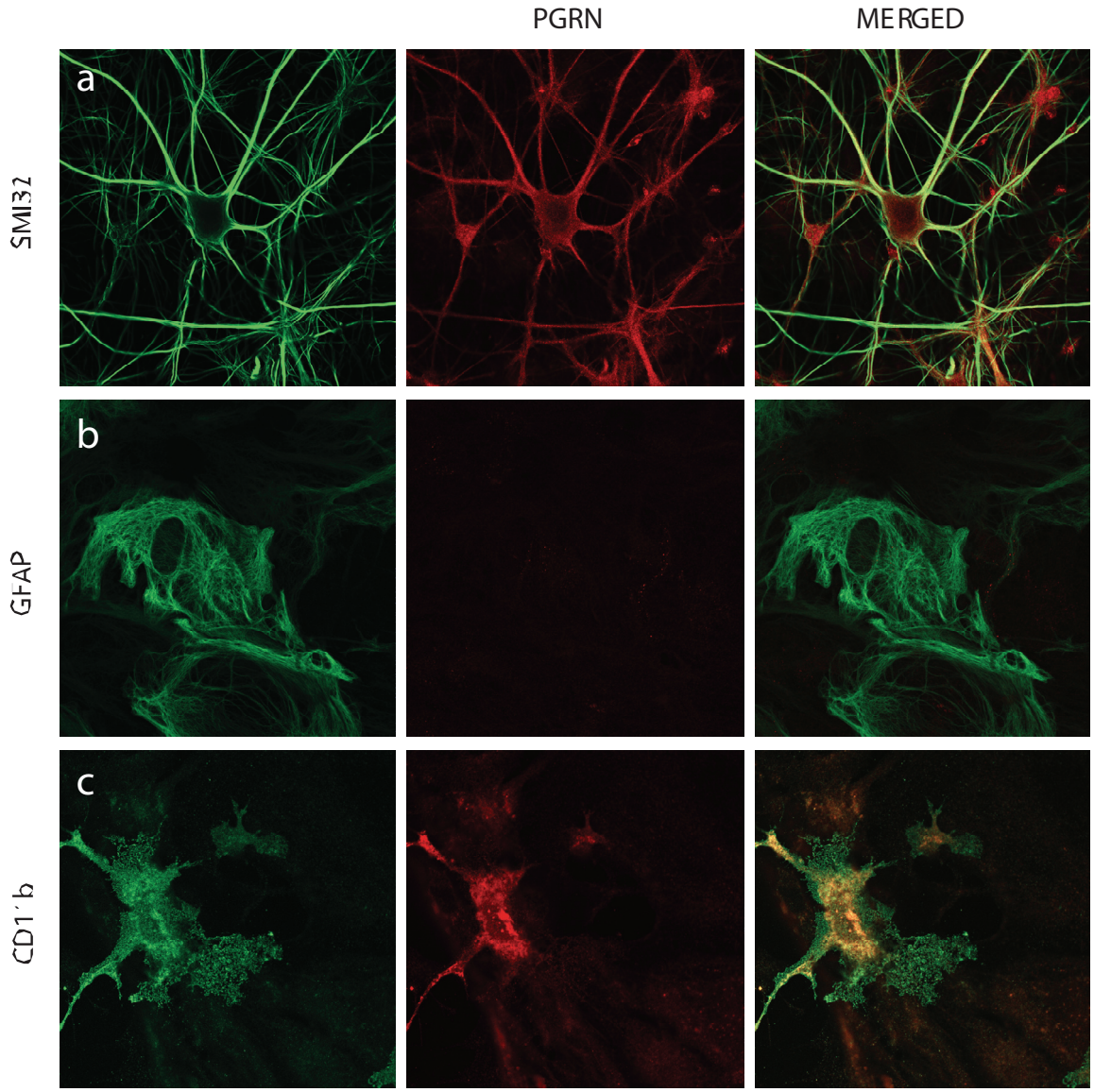


Figure 6. PGRN is expressed within dissociated spinal cord-DRG cultures. Confocal Images taken of dissociated spinal cord cultures. PGRN (red) is very clearly expressed within motor neurons (a), labelled with SMI32 (green). PGRN is also expressed by microglia, as demonstrated by colocalization between PGRN and CD11b (c). Astrocytes, however, do not express PGRN, as demonstrated in (b).

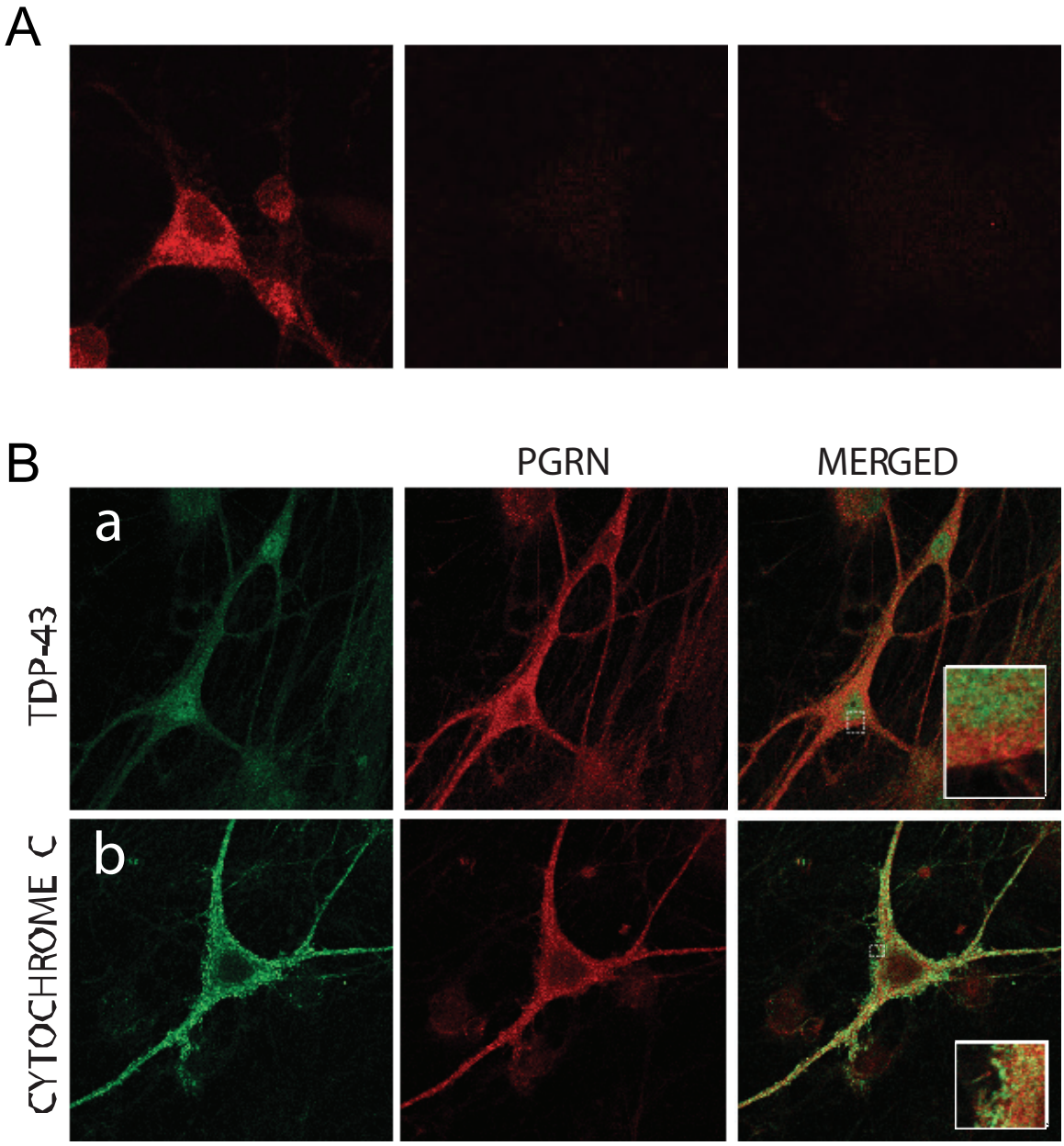


Figure 7. PGRN does not colocalize with nuclear or mitochondrial markers. (A) Motor neuron labeled with antibody to mouse PGRN (left hand image) is attenuated by antigen-competition with 300 ng recombinant mouse PGRN (middle and right hand images). When anti-PRGN was pre-absorbed with 400 ng of mouse recombinant PGRN, no signal was observed in the primary motor neurons (not shown). Shown are confocal images taken at 100X. (B) Progranulin is not distributed in nuclei or mitochondria, organelles that are not part of the secretory pathway. Immunolabelling of motor neurons in dissociated spinal cord-DRG cultures with anti-TDP-43 (a) and anti-cytochrome C (b) and anti-PGRN (middle column). Merged images (right column) show no colocalization of TDP-43 or cytochrome C with endogenous mouse PGRN. Confocal images were captured at 63X magnification, hatched boxes represent 3-5X zoom.

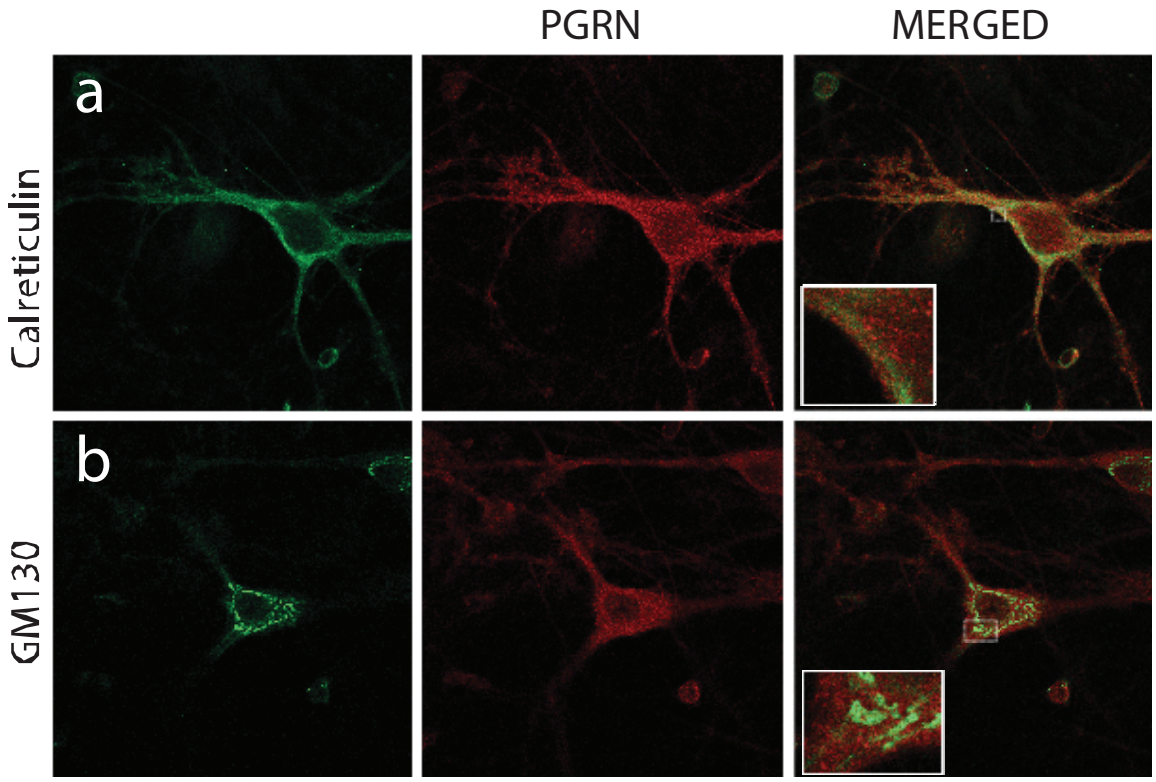


Figure 8. PGRN antibody does not colocalize with the ER or Golgi. Expression of PGRN and markers of the secretory system. Immunolabelling of motor neurons in dissociated spinal cord-DRG cultures with anti-Calreticulin (a), anti-GM130 (b), and anti-PGRN (middle column). Confocal images were captured at 63X magnification, hatched boxes represent 3-5X zoom.

Figure 9. PGRN does not colocalize with chromogranin A or SNAP-25, but has potential overlap with lysosomes and synaptophysin. Immunolabelling of motor neurons in dissociated spinal cord-DRG cultures with Chromogranin A (a), LysoTracker (b), SNAP-25 (c), Synaptophysin (d), and anti-PGRN (middle column). Merged images (right column) show no colocalization with any markers of the secretory pathway, except perhaps synaptophysin and a tiny population of lysosomes. Confocal images were captured at 63X magnification, hatched boxes represent 3-5X zoom.

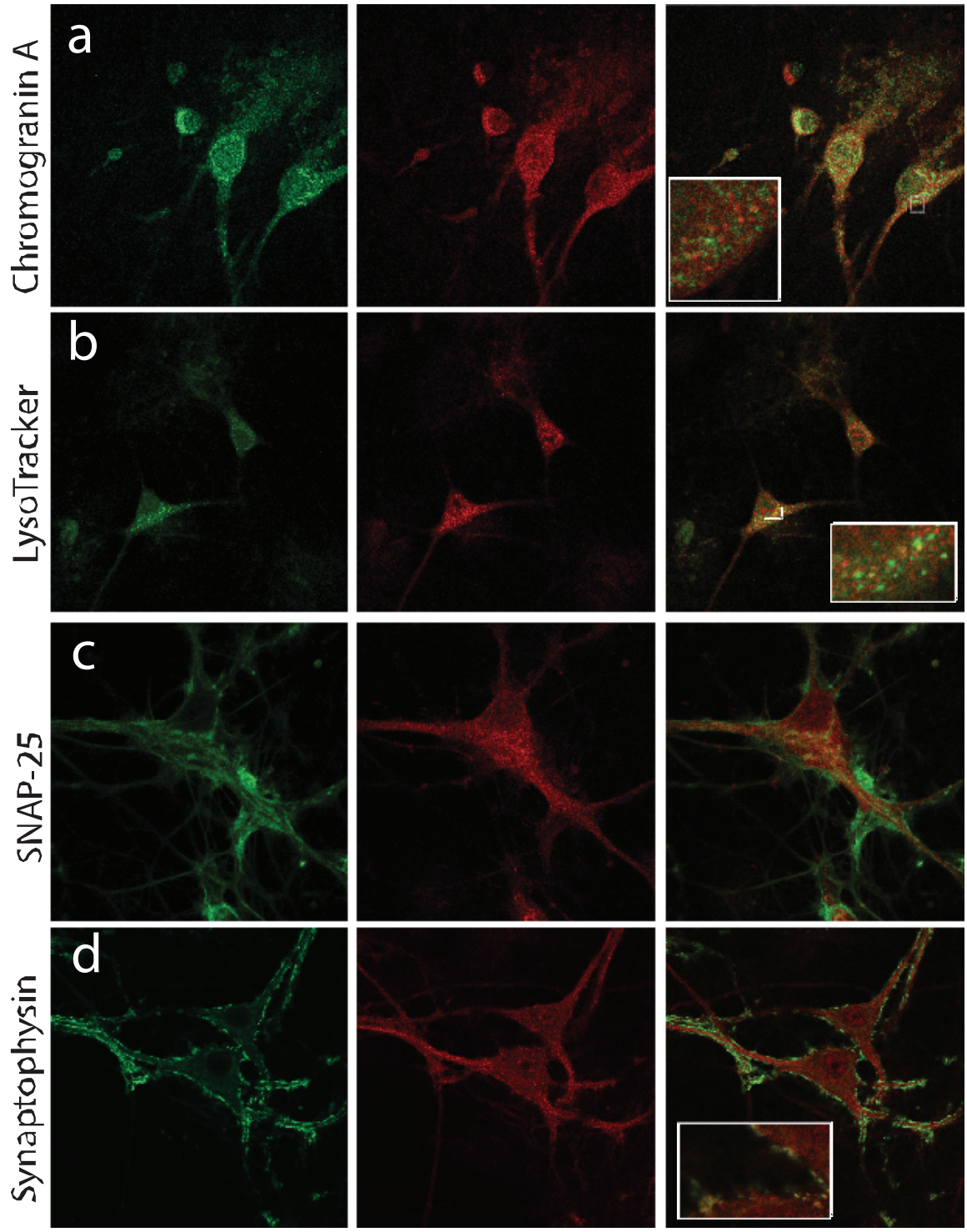
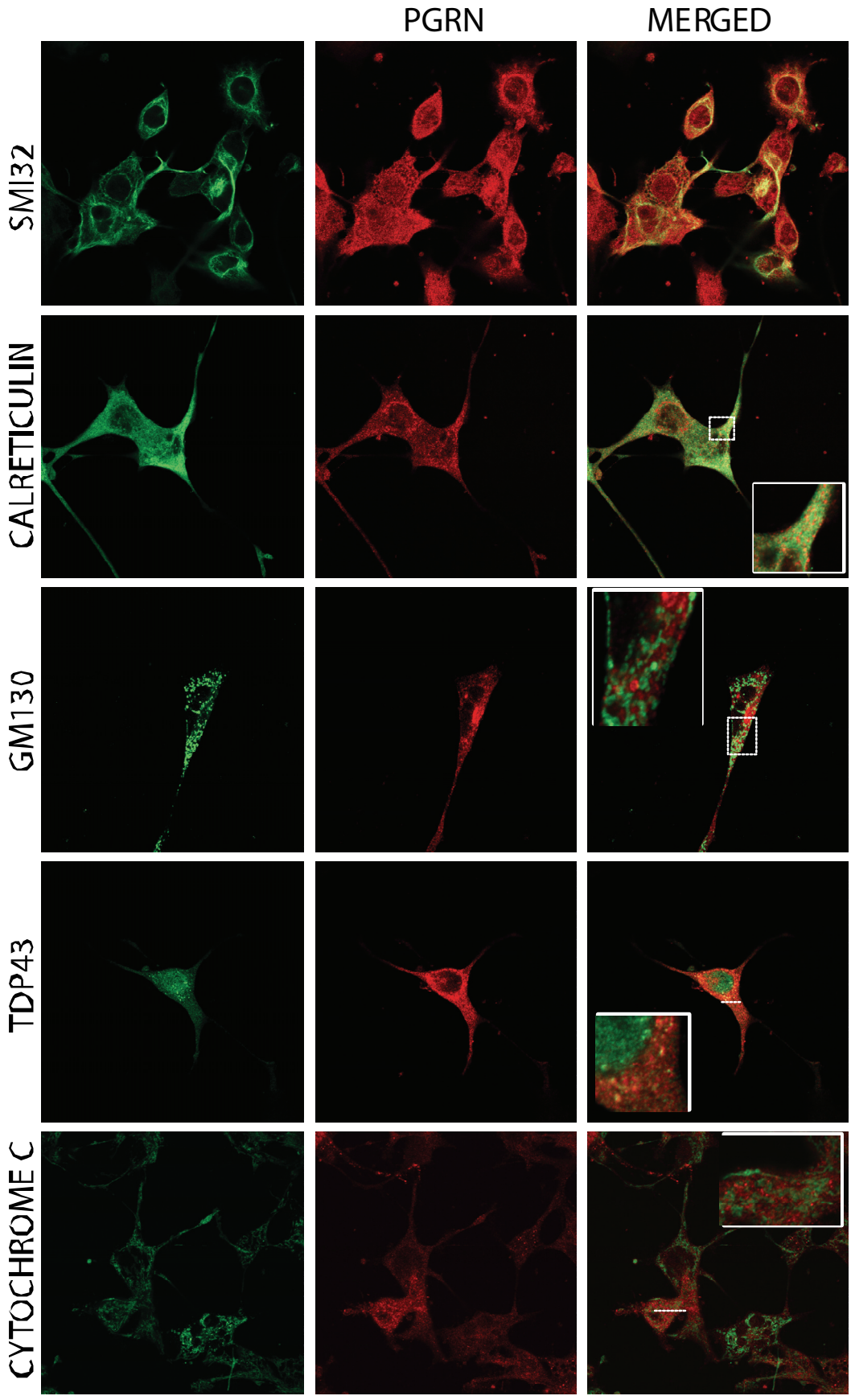

a
Chromogranin A
b
LysoTracker
c
SNAP-25
d
Synaptophysin

Figure 10. Colocalization of PGRN with subcellular markers reveals similar patterns in NSC-34 as with spinal cord-DRG cultures. Detection of neurofilaments labeled with SMI32 (a) reinforces neuronal properties of NSC-34 cell line. Similar to the spinal cord cultures, PGRN does not colocalize wih the ER, as shown by a lack of overlap with calreticulin (b), the trans-Golgi network, as labelled with GM130 (c), TDP-43 (d) or cytochrome C (e), the mitochondrial marker. Confocal images were captured at 63X, while hatched boxes reveal cells at 63X with an added 3X maginification.


PGRN
MERGED
SMI32
CALRETICULIN
GM130
TDP43
CYTOCHROME C

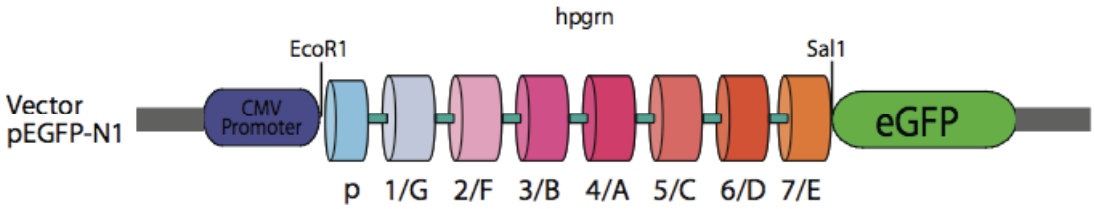


Figure 11. pEGFP-N1-hPGRN cloning construct. The human progranulin gene (hpgrn) was cloned into the pEGFP-N1 plasmid using EcoR1 and Sal1 restriction enzyme digestion sites. This construct leads to the formation of an eGFP-tagged human progranulin protein upon transfection. Because the eGFP molecule is fused to the C-terminus of the PGRN protein, it does not affect its N-terminal signal sequence required for entry into the endoplasmic reticulum.

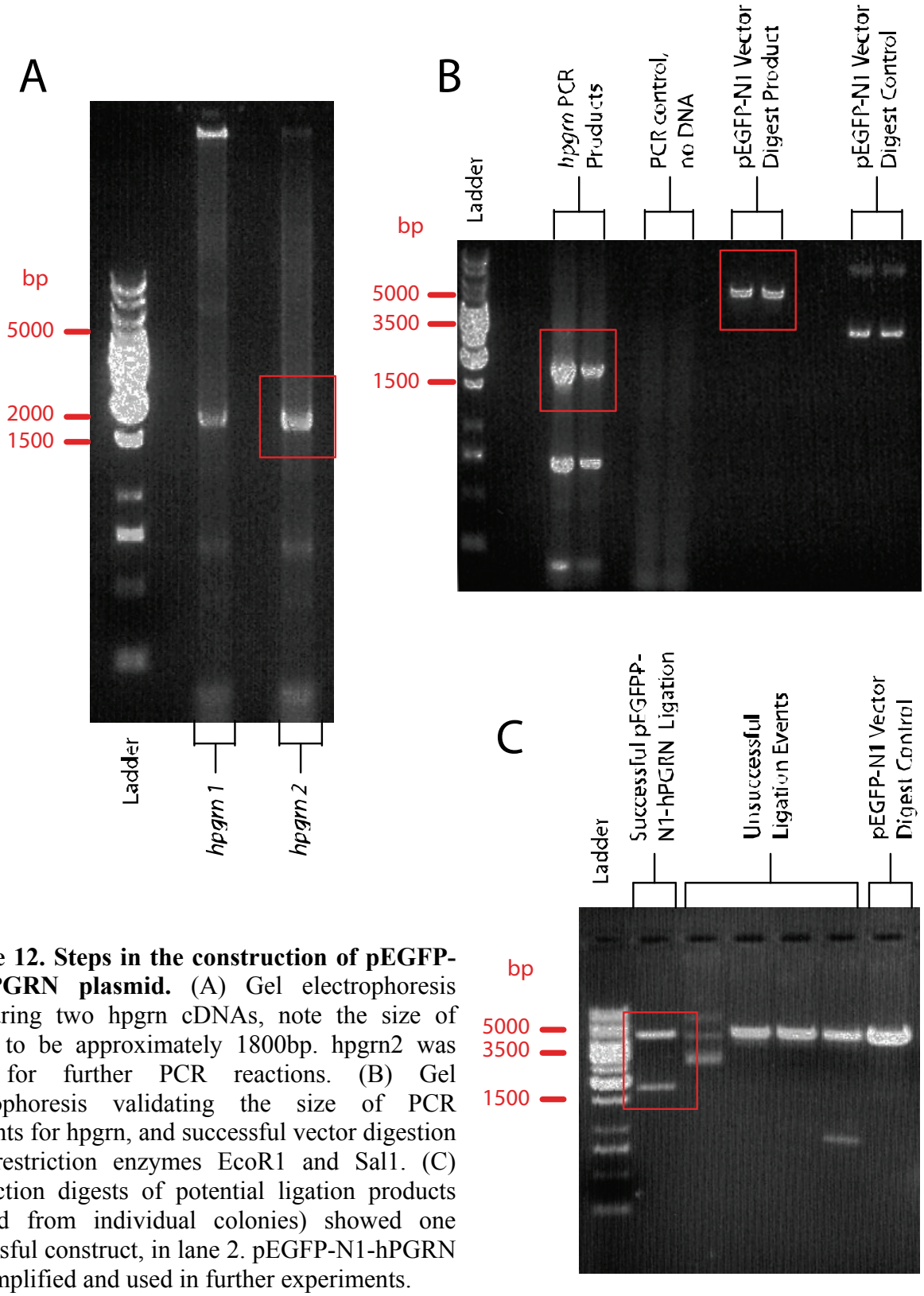
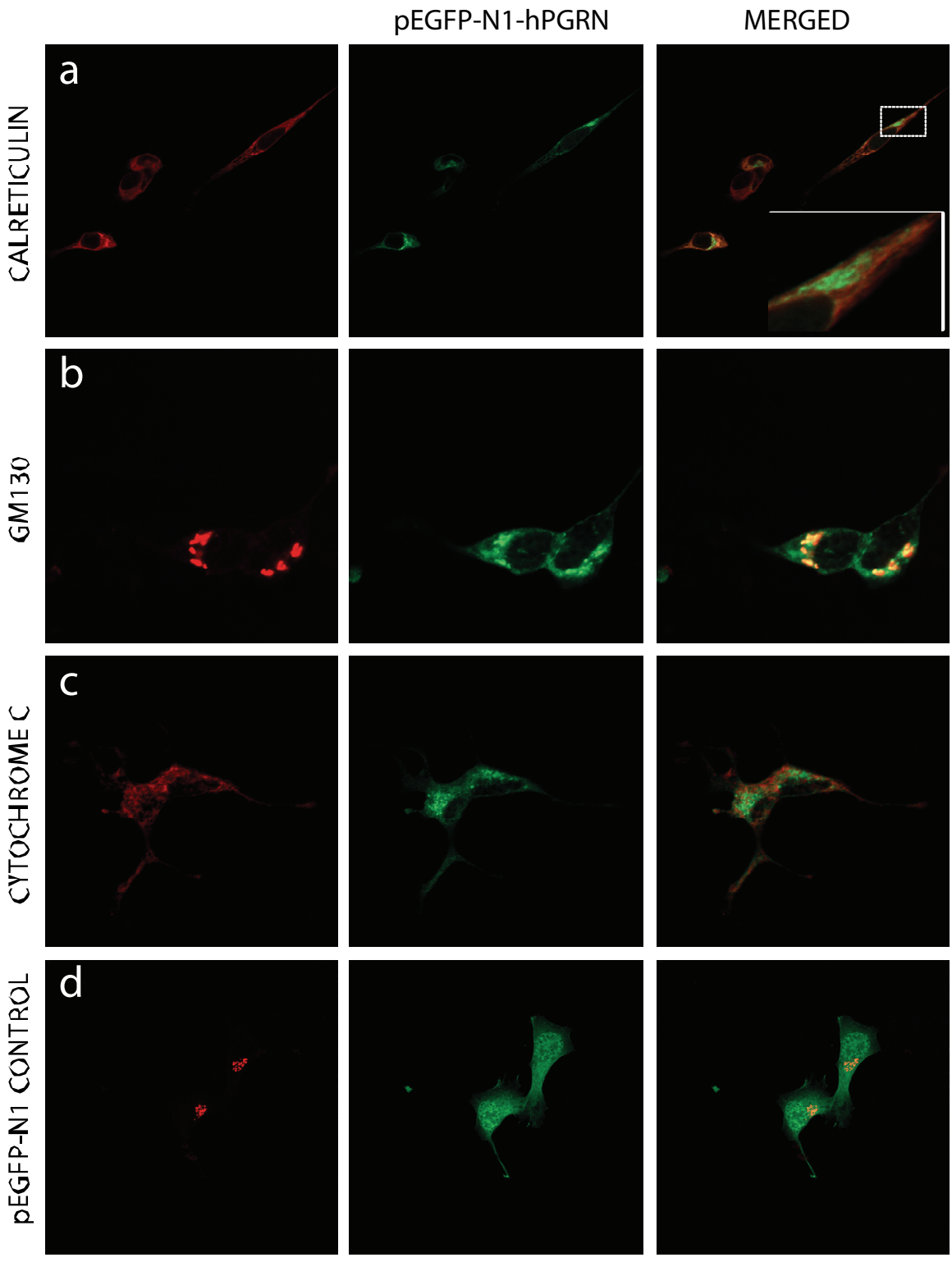


Figure 12. Steps in the construction of pEGFP-N1-hPGRN plasmid. (A) Gel electrophoresis comparing two hpgrn cDNAs, note the size of hpgrn to be approximately 1800bp. hpgrn2 was used for further PCR reactions. (B) Gel electrophoresis validating the size of PCR reactants for hpgrn, and successful vector digestion with restriction enzymes EcoR1 and Sal1. (C) Restriction digests of potential ligation products (picked from individual colonies) showed one successful construct, in lane 2. pEGFP-N1-hPGRN was amplified and used in further experiments.

Figure 13. Transfection of pEGFP-N1-hPGRN plasmid shows granular morphology with particular PGRN localization in the Golgi apparatus. While PGRN does appear to show some colocalization with the ER marker calreticulin (a), the overlap seen with PGRN and the golgi marker GM130 (b) is very prominent. There is no overlap between mitochondria (c) and PGRN. Panel (d) reveals that the pEGFP-N1 vector only transfection, reveals primarily a nuclear GFP signal, and a very different subcellular distribution to that of PGRN, which appears distinctly granular in panels (a, b and c). Confocal images were taken at 63X, while hatched boxes reveal images at 63X with an added 3X zoom.


pEGFP-N1-hPGRN
MERGED
a
b
c
d
CALRETICULIN
GM130
CYTOCHROME C
pEGFP-N1 CONTROL

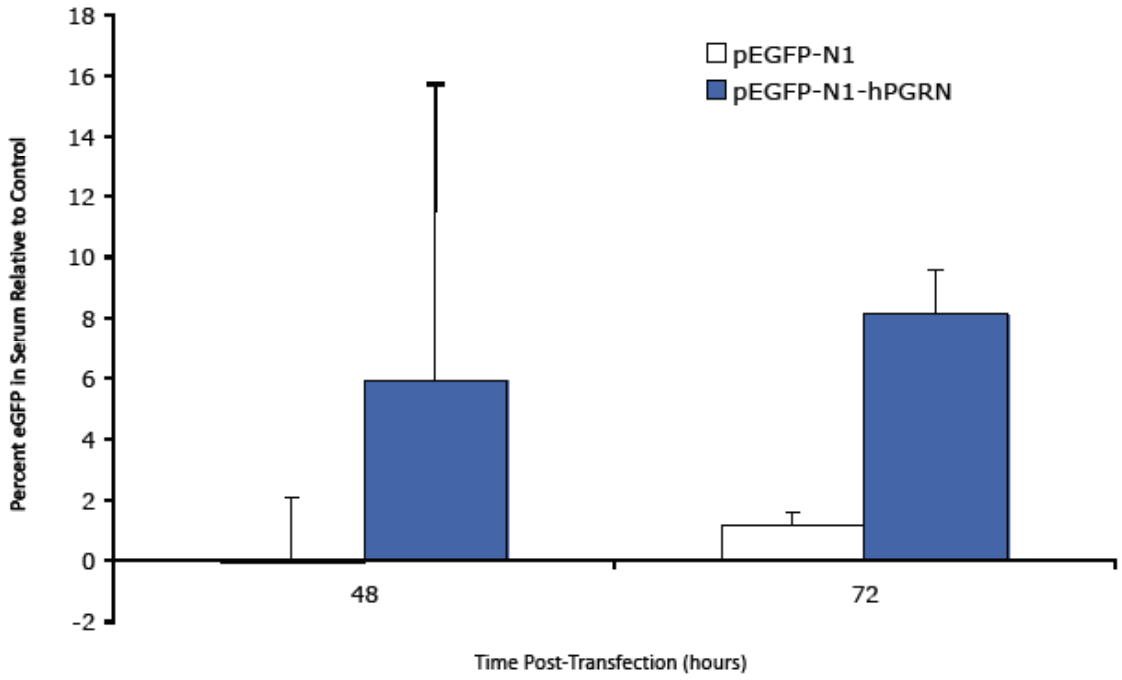


Figure 14. **Secretion of eGFP into the cell medium by NSC-34 cells.** A fluorimeter was used to quantify the amount of eGFP in the medium of NSC-34 cells transfected with the pEGFP-N1-hPGRN vector construct. After 48 hours, there was a 5.9% increase of eGFP signal in the medium relative to untransfected control cells, and an 8.1% increase after 72 hours. NSC-34 cells transfected with pEGFP-N1 vector only plasmids did not contain any eGFP in the medium after 48 hours, however after 72 hours, there was a 1.19% increase relative to control, possibly due to recycling/turnover of cellular contents. Error bars represent standard deviations (SDs), each using a minimum of 6 determinations.

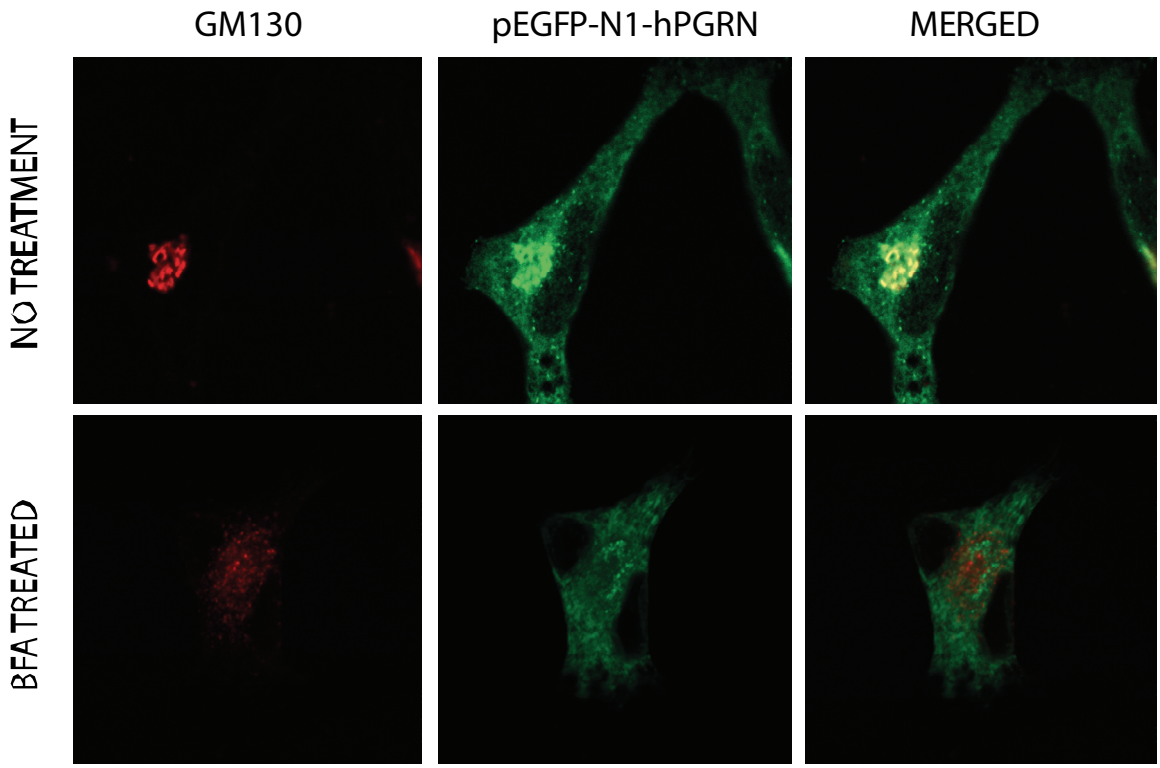


Figure 15. Treatment of NSC-34 cells with Golgi inhibitor Brefeldin A further implicates PGRN in the secretory pathway. Addition of 2.5ug/ml BFA successfully disrupted the Golgi apparatus, as observed by its dispersed and fractionated appearance in the left (red) lower panel (demonstrated by the GM130 marker). When compared to the upper panel, PGRN no longer colocalizes within the Golgi, apparently being sequestered in the ER as the secretory pathway is inhibited. Images were taken by confocal microscopy at 63X with an additional 2x zoom.

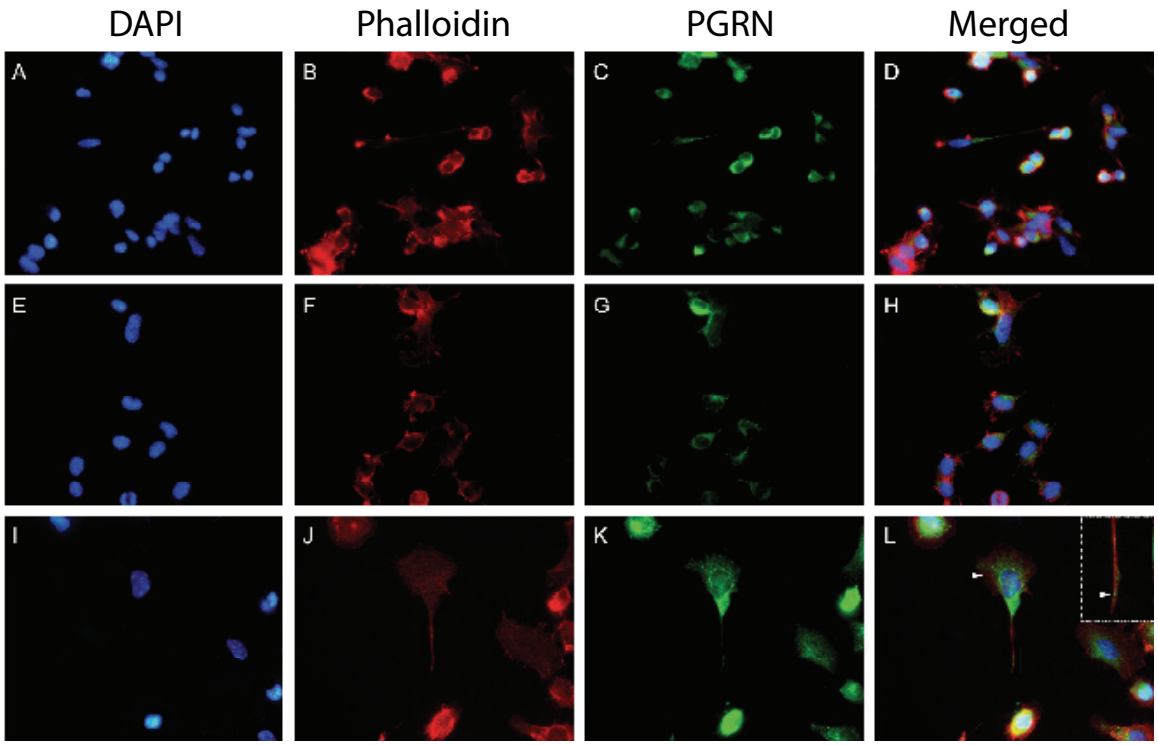
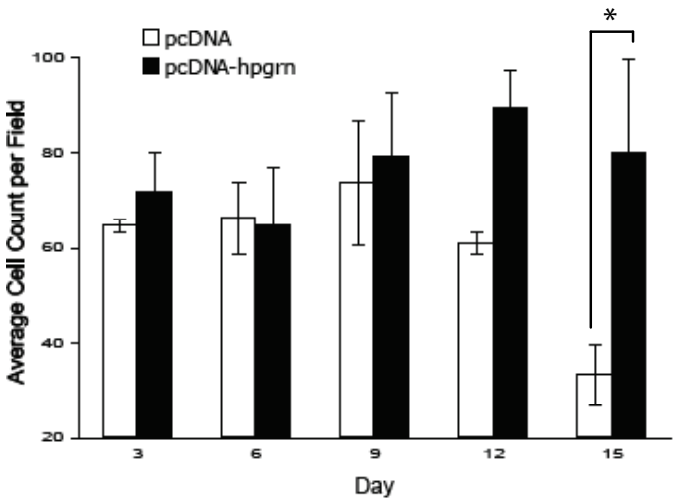


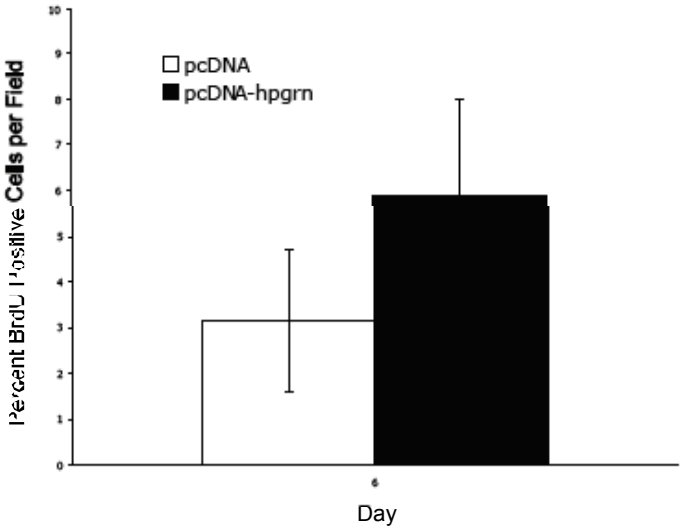
Figure 16. PGRN induces a change in cell morphology. Untransfected NSC-34 cells (A-D), transfected with pcDNA3 vector only (E-H) or transfected with pcDNA3-hPGRN (I-L). Micrographs showing the distribution of DAPI (A, E, I), F-actin (B, F, J), hPGRN (C, G, K) and merged images (D, H, L). PGRN over-expression promotes more extensive cytoskeletal extensions (hatched box) and is localized within presumptive secretory vesicles (arrowheads).

Figure 17. PGRN is neurotrophic for neurons by decreasing apoptosis, and not by increasing proliferation. Stable vector only transfectants (pcDNA; white) and cells stably over-expressing hPGRNs (pcDNA-PGRN; black) were cultured in serum-free RPMI medium. (A) Average cell counts per field (at 10x magnification) were determined at three-day intervals by phase-contrast microscopy for fifteen days. NSC-34 cells that over-expressed hPGRN demonstrated increased survival as compared to controls (Asterisks denote $P<0.005$). (B) Cell proliferation assay based on 12 hr BrdU incorporation following 6 days culture in serum-free medium. Over-expression of hPGRN during serum deprivation did not significantly increase cell proliferation rates ($P>0.1$). (C) Apoptosis assay based on the TUNEL- labelling method following 6 days in serum-free medium. Over-expression of hPGRN during serum deprivation protected against apoptosis (Asterisks denote $P<0.0001$).

A


pcDNA
pcDNA-hpgrn
Average Cell Count per Field
100
80
60
40
20
3
6
9
12
15
Day
*

B


pcDNA
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Cells per Field
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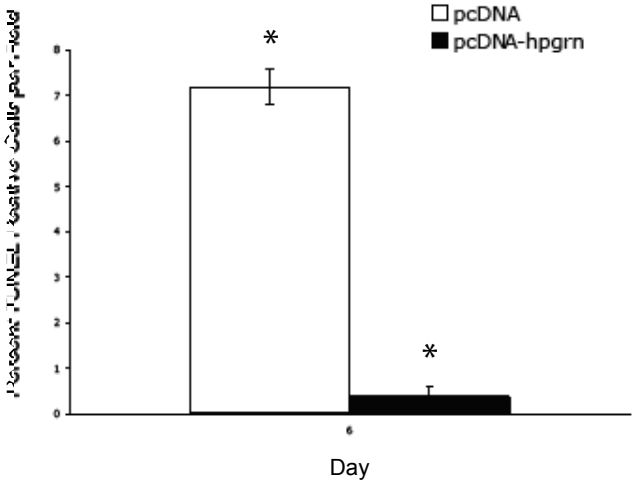
C


pcDNA
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CHAPTER IV

DISCUSSION AND CONCLUSIONS

Mutations of the PGRN gene cause frontotemporal lobar degeneration accompanied by the appearance of ubiquitinated-inclusion bodies (Baker et al., 2006; Cruts et al., 2006). The formation of ubiquitinated inclusions occurs in other neurodegenerative diseases, in particular in ALS (Neumann et al., 2006). While mutations of *PGRN* do not appear to cause ALS (Pickering-Brown et al., 2008a; Schymick et al., 2007b), recent research suggests that PGRN is neurotrophic for spinal cord motor neurons (Van Damme et al., 2008). Here we confirmed that *PGRN* mRNA and protein is expressed in mouse spinal motor neurons, and that spinal cord microglia also express PGRN (Figures 3.1, 3.5 and 3.6). The expression in microglia, which also has been seen in the brains of Alzheimer's patients (Baker et al., 2006), is of interest, given that microgliosis accompanies motor neuron degeneration. Recent evidence demonstrates that in peripheral inflammation PGRN inhibits the activity of the proinflammatory cytokine tumour necrosis factor-alpha (Kessenbrock et al., 2008; Zhu et al., 2002), and it is possible that PGRN may also regulate inflammatory processes in the spinal cord.

Cells of different origins handle PGRN in different ways. Whereas epithelial cells appear to secrete PGRN constitutively, innate immune cells including neutrophils process this growth factor to yield 6kDa granulin peptides that are stored within granules (Bateman et al., 1990). Similarly, PGRN is stored within the acrosomes of guinea pig spermatozoa (Anakwe and Gerton, 1990), which are vesicular storage structures. In addition, yeast-two hybrid studies have identified PGRN as a potential partner for nuclear proteins such as cyclin-T (Hoque et al., 2003), adding a further level of complexity to the localization and roles of PGRN.

The subcellular localization and fate of PGRN in neurons has not been previously defined. PGRN appears to be a strong candidate for entry into the ER/Golgi pathways since the PGRN gene encodes a signal sequence for co-translational entry into the ER, it is rich in disulfide bridges and N-glycosylation sites both of which form during transit through the ER and Golgi compartments. However, it has been suggested that in nerve cells PGRN may localize with either mitochondria or endosomal-lysosomal-like structures (NIH Symposium on Progranulin and the TDP-43 Proteinopathies), which, if correct, has profound implications with respect to the mechanism of action of PGRN. It was therefore important to clarify the sub-cellular compartmentalization of PGRN in nerve cells.

Punctate PGRN immunofluorescence was observed within the cell bodies and in the axons of primary motor neurons in culture, but not in their nuclei (Figure 7B-a). We were unable to detect any co-localization of PGRN with mitochondria in primary motor neurons in culture (Figure 7B-b). However, we were also unable to detect PGRN in the ER or Golgi apparatus (Figure 8). The absence of PGRN immunoreactivity in the ER/Golgi system, despite the structural arguments that suggest it would be likely to enter the secretory pathway, may reflect a genuine dissociation of localization. Given the structural features of mature PGRN this may also be a misleading result due to, for example, low protein concentration in the ER/Golgi or an inability of the antibody to react with immature protein as it transits through the ER/Golgi processing pathways. We therefore employed an alternate strategy to examine the localization of PGRN within neuronal cells. NSC-34 motor neuron-like cells were transfected with a PGRN-eGFP fusion protein or with an eGFP control alone. The eGFP control exhibited green

immunofluorescence that concentrated mostly in the nucleus (Figure 13d). In contrast PGRN-eGFP exhibited a punctuate fluorescence in the cell body and axons, but not the nucleus, in a pattern that was also observed for endogenous PGRN. The PGRN-eGFP fusion protein co-localized with GM130, a marker for the trans-Golgi apparatus (Figure 13b), but not with a mitochondrial marker (Figure 13c). We conclude, therefore that neuronal PGRN is not widely associated with mitochondria but enters the ER/Golgi secretory pathway.

There are three major exit pathways from the Golgi apparatus; namely, to the lysosome, to the regulated secretory pathway, or to the constitutive secretory pathway (Dannies, 1999). Vesicles that shuttle proteins between the three pathways are different in their characteristics. Exosomes are typically used to shuttle proteins from the lysosomes or endosomal compartments to the plasma membrane (Keller et al., 2006), while secretory granules (also called dense core secretory granules) and microvesicles generally transport proteins through the regulated and constitutive pathways, respectively (Moriyama et al., 2000; Schiera et al., 2007). There are also vesicles involved in neurotransmitter release, called synaptic vesicles, which are typically subject to regulated secretion (Hook et al., 2008). Another type of vesicle, the plasmalemmal precursor vesicles (PPVs), are clusters of smaller pleotropic vesicles of approximately 150nm in diameter that accumulate at the plasma membrane of neurons and are involved in the membrane expansion that occurs at neuronal growth cones (Pfenninger, 2009).

The vesicle-like PGRN structures in the motor neurons did not colocalize with chromogranin A (Figure 9a), which is located within dense core secretory granules of endocrine cells and shuttled through the regulatory pathway (Day and Gorr, 2003). Chromogranin A has been investigated as a player in the biogenesis of secretory vesicles,

however these studies have led to many speculations as to function and few conclusions. It is proposed that chromogranin A may bind or co-aggregate with the cargo within secretory granules (Day and Gorr, 2003; Gorr et al., 1992), and that this is prompted by the acidic milieu of secretory vesicles (Day and Gorr, 2003). It has also been suggested that if secretory vesicles lack chromogranin A, they are shuttled through the endosomal secretory pathway and undergo constitutive secretion (Arvan and Castle, 1998), thus suggesting a role for chromogranin A in the sorting of secretory proteins within particular vesicle subtypes (Day and Gorr, 2003; Gorr et al., 1989; Huttner and Natori, 1995). Dense core secretory granules vary in size, but typically have a diameter between 80-200nm (Kim et al., 2001). The lack of colocalization between PGRN and chromogranin A would suggest that PGRN is not located within the same dense core secretory granules, and is likely to be found within a different vesicle subtype.

We detected only limited colocalization of PGRN with lysosomes, suggesting that this organelle is at most only a minor destination for PGRN (Figure 9b). Presence of PGRN within lysosomes would suggest that it is being packaged into exosomes and, subsequently either degraded or released via exocytosis. Contents of exosomes are consistently used as biomarkers of pathology, as they are found within serum and ascites fluid of tumour patients (Keller et al., 2006), and can be measured under both healthy and non-physiological conditions. Interestingly, decreased levels of PGRN have been found within the serum of patients with mutations in the *PGRN* gene (Sleegers et al., 2009), and have been shown to be a reliable biomarker for early detection of FTLD. This might suggest that PGRN is located within exosomes, however due to our limited co-localization evidence, it is likely that the bulk of PGRN is located within another vesicle subtype. Also, the typical size of exosomes ranges between 30-100nm in diameter.

PGRN-containing vesicles appear to be of slightly larger dimensions, as demonstrated by electron microscopy in neutrophils and macrophages cells (Lin et al., 2007).

PGRN is unlikely to be secreted primarily from synaptic junctions since it did not co-localize with the SNAP-25 marker for neurotransmitter vesicle docking and release sites (Figure 9c). Also, the lack of sensitivity to extracelullar calcium with respect to regulation of secretion would suggest localization to the constitutive secretory pathway, which is not the typical pathway taken by neurotransmitter-containing vesicles.

We did, however, see evidence of some co-localization between PGRN and synaptophysin (Figure 9d). Synaptophysin, a synaptic vesicle integral membrane glycoprotein, is a known biomarker for a vesicle subtype called microvesicles (MVs) (Moriyama et al., 2000). These vesicles are distinct from secretory granules, and are widely distributed within endocrine cells (Moriyama et al., 2000). Studies have demonstrated the presence of MVs in neurons, which exist as vesicles of approximately 100 to 1000nm in diameter (Schiera et al., 2007). A recent study reported that PGRN-containing vesicles were identified by electron microscopy to be contained within pale to optically dense vesicles on the order of 200-500nm in diameter (Lin et al., 2007). Other studies have reported the presence of growth factors VEGF and FGF-2 within similar sized MV, which are released by neurons into the extracelullar space by way of vesicle shedding (Schiera et al., 2007). More research is required to investigate the relationship between synaptophysin and PGRN, given that, at the resolution available, we cannot exclude the possibility that co-localization of these proteins is due to a non-specific overlay effect. Also, only one clone of the PGRN-eGFP plasmid was tested experimentally, which introduces another potential caveat. Following further characterization and validation, the NSC-34 PGRN-eGFP system, in conjunction with

confocal microscopy of primary motor neuron cultures, will provide a novel system with which to investigate in greater detail the regulation and mechanism of PGRN secretion in neurons.

We used the NSC-34 cell line to investigate the effects of PGRN upon cell growth and survival. PGRN elicited a change in the appearance of the NSC-34 cells, leading to a more flattened cell shape and more prominent neuritic extensions. Serum deprivation was employed as an apoptotic challenge. Upon prolonged incubation in serum-free medium the number of NSC-34/vector cells declined, becoming statistically significantly different from NSC-34/PGRN between days 12 and 15. This was due to reduction in apoptosis in NSC-34/PGRN cells, as the number of TUNEL-positive cells was significantly lower in cultures of NSC-34/PGRN cells compared to cultures of NSC-34/vector cells as early as day 6. The percentage of BrdU cells was not significantly changed at early stages of serum-depletion, suggesting that PGRN is cytoprotective rather than proliferative in NSC-34 cells in the absence of serum. In non-neuronal cells, such as dermal fibroblasts, PGRN is strongly protective against acidosis (Guerra et al., 2007), suggesting that it may play a widespread role in protecting cells against metabolic shocks in their microenvironment.

PGRN may promote neurite extension in cortical neurons (Van Damme et al., 2008). When SLPI is added in conjunction with PGRN, effect of PGRN on neurite outgrowth is inhibited (Van Damme et al., 2008), thus SLPI may inhibit neurotrophic properties of PGRN or might scavenge PGRN and, consequently, keep it away from whatever it needs to interact with for neuroprotection (Van Damme et al., 2008). This suggests that PGRN needs to be cleaved to result in neuroprotection, which, through our experiments cannot be confirmed nor supported. The addition of exogenous PGRN,

however, had greater effect on neurite extension than addition of the GRN E peptide alone (Van Damme et al., 2008), suggesting that the precursor protein is playing a major role in neurite outgrowth, either in it's full length form, or through the interplay of all it's constituent GRN peptides. Growth of NSC-34 cells on nitrocellulose-sequestered recombinant, full length PGRN strongly suggests that the precursor form is important for neurite projection (Figure 4). In the studies involving long-term serum-deprived NSC-34/PGRN cultures that showed increase in length of neurite projections (Figure 3), however, we cannot exclude the possibility that these pronounced extensions may be due to improved overall neuronal health rather than the direct stimulation of neurite outgrowth.

The mechanisms involved in neurite growth have been subject of speculation since 1953 (Pfenninger, 2009). It is a complex process, involving synthesis of membrane proteins and transport to the growth cones, either in distal axons of dendrites (Pfenninger, 2009). Membrane proteins can be synthesized either at the level of the perikaryon (cell body of the neuron) or in the distal processes, as the ER and Golgi apparatus can extend into the larger dendrites of neurons (Pfenninger, 2009). The bulk of the protein synthesis, however, occurs in the perikaryon and is transported to the growing neurites via plasmalemmal precursor vesicles (PPVs). These PPVs are thought to pass through the regulated secretory pathway, budding from the TGN in vesicles of approximately 150nm in diameter (Pfenninger, 2009). In *de novo* growth of axons, exocytosis of PPVs does not occur only at growth cones, but also along the growing axon (Hazuka et al., 1990). Little is known about the fusion of these PPVs to the plasma membrane; however, it is hypothesized that it involves the SNARE complex proteins, syntaxin and synaptosomal-associated proteins (SNAPs) (Pfenninger, 2009). Interestingly, SNAP-25 deficient mice

do not have problems with neurite extension (Washbourne et al., 2002). It has since been demonstrated that the mechanism of membrane expansion occurs in the growth cone of the axon, involves SNARE complexes that differ from neurotransmitter release, and is regulated by growth factors (Washbourne et al., 2002). In our experiments, we investigated colocalization of PGRN with SNAP-25, but did not look at the interplay of PGRN with other members of the SNAP family of proteins. If neurite extension does occur via a process separate from neurotransmitter release, it would be of interest to investigate the colocalization of PGRN with other SNAP proteins, or members of the syntaxin family involved in neurite outgrowth.

PGRN is not the only growth factor with reported neurotrophic properties; the neuroprotective effect of VEGF has been tested both *in vitro* and *in vivo* (Lambrechts and Carmeliet, 2006). Motor neurons express VEGF receptors, as do neighbouring non-neuronal cells (Bogaert et al., 2006), which implicates VEGF as a possible regulator of both cell types (Lambrechts and Carmeliet, 2006). Administration of recombinant VEGF has been shown to increase the integrity of neuromuscular junctions (Bogaert et al., 2006), as well as reduce astrogliosis in SOD1 mice (Bogaert et al., 2006). VEGF also has been shown to protect motor neurons from AMPA receptor-mediated excitotoxicity (Tovar-y-Romo et al., 2007), which has been implicated in spinal cord degeneration (Tovar-y-Romo et al., 2007). ALS patients themselves have lower levels of VEGF in their cerebrospinal fluid (CSF) when compared to healthy individuals and neurological control patients (Devos et al., 2004). *In vitro* experiments show promising results as intramuscular injections of VEGF by way of viral-vectors into the SOD1 mouse model of ALS resulted in slower onset of paralysis as well as increased survival (Azzouz et al., 2004). Interestingly, PGRN was found to stimulate VEGF expression in MCF-7 breast

cancer cells, where the two factors may act together to promote metastasis and angiogenesis in human breast cancer (Pan et al., 2004). They may act in a similar coordinated manner in exerting neuroprotective properties.

IGFs are also capable of enhancing the outgrowth of motor neurons (Kaspar et al., 2003). It has also been reported that IGF-1 can reduce motor neuron death, delay the onset of motor deficits and increase the lifespan of SOD1 mice (Dagvajantsan et al., 2008). One clinical trial reported decreased disease progression in ALS patients when they received high doses of IGF-1 (Lai et al., 1997), however another clinical trial reported no benefit (Borasio et al., 1998). IGF-1 has also been shown to be involved in the regulation of plasmalemmal expansion at growth cones via the PI3K- AKT pathway (Laurino et al., 2005).

The similarities between other growth factors, especially VEGF and IGF, and PGRN would suggest a promising role for PGRN in future therapeutic interventions for neurodegenerative diseases. PGRN seems capable of reproducing many of the same neurotrophic effects as VEGF and IGF, including both protection from stress and possible involvement in plasmalemmal expansion. Even the secretion of PGRN could follow the path taken by these growth factors, as both VEGF and FGF-2 are secreted at least in part by microvesicles via membrane shedding. Further experiments are certainly needed to confirm the PGRN vesicle subtype, which will help to elucidate the mechanism of action of this important growth factor.

Conclusions

Primary motor neuron cultures and the NSC-34 cell line provide useful models in which to investigate the cell biology, function and mode of action of PGRN in neurons. We have demonstrated that PGRN is highly expressed in normal spinal cord neurons, and that it enters the ER/Golgi secretory pathway. We have demonstrated that PGRN is secreted, at least in part, via the constitutive secretory pathway and may be localized within microvesicles. We have also demonstrated that over-expression of PGRN in NSC-34 cells promotes a neuron-like morphology, as well as promotes cell survival, while not significantly increasing cell proliferation. This work supports the hypothesis that PGRN is neurotrophic.

CHAPTER V

ADDITONAL RESULTS & FUTURE DIRECTIONS

5.1 Effect of calcium and BFA on PGRN Secretion

Defining secretion to one particular pathway is very complex. Although PGRN may be located within vesicles that are typically exocytosed by constitutive secretion, this does not rule out the possibility that PGRN is still being processed via the regulated pathway. One way to investigate this is to stimulate secondary messengers that are involved in the RSP by modifying the calcium concentration in the extracelullar medium. During regulated secretion, when a cell is stimulated (which is often associated with a rise in cytosolic calcium), the F-actin network associated with the apical membrane depolarizes, allowing fusion of secretory vesicles with the PM (Quinn et al., 2007). Calcium-regulated exocytosis has, by definition, a characteristic graded response to calcium, which is subject to many factors, including the heterogeneity in individual vesicle's sensitivity to calcium (Blank et al., 1998). Vesicle fusion has been reported in synaptosomal fractions of rat brain tissue at extracelullar calcium levels as low as 0.1nM-10μM (Trikash et al., 2004).

Considering the lack of information regarding the nature of PGRN-containing vesicles, our investigation of the response of these vesicles to cytosolic calcium is very preliminary and requires further attention. We observed that the addition of calcium to the medium in small increments (on the scale of 0.05mM to 3.0mM) had no significant effect on PGRN secretion (Figure 18A). The effect of BFA, a fungal metabolite used to inhibit transport from the ER to the Golgi apparatus, was also used to investigate PGRN secretion with and without the addition of calcium. BFA reduced the amount of secretion of PGRN, but not by a significant amount, and addition of calcium did not rectify this loss of secretion (Figure 18B). This would suggest that PGRN is not found within reserve vesicles, which could fuse to the PM under the influence of cytosolic calcium, even if the

production of more PGRN was inhibited. Cells subjected to calcium treatment were observed under confocal microscopy, and did not appear to have any differences in PGRN vesicle distribution (data not shown). Under the influence of calcium, if PGRN were secreted via the regulatory pathway, it would be reasonable to expect to find a higher proportion of PGRN-containing vesicles located near the apical membrane (Quinn et al., 2007). This would suggest that PGRN secretion is not under the control of regulated secretion, and is thus secreted by way of the constitutive pathway. This experiment should be repeated, however, with the addition of potassium, in order to simulate an action potential, which could also trigger the fusion of vesicles to the PM under the regulated secretory pathway (Jaskiw et al., 2008).

Alternatively, constitutive secretion can also be upregulated. One example of this is the secretion of VEGF, which typically follows the constitutive pathway, but can be induced via cytokines (Dolecki and Connolly, 1991) and low oxygen tension (Brogi et al., 1994). Future experiments will investigate the secretion of PGRN under the influence of external factors in the hope of further characterizing the secretion pattern of PGRN.

The eGFP-tagged PGRN will also be very useful in the search for binding partners and putative PGRN receptors. If it is possible to mass produce a PGRN-eGFP protein with correct protein folding, it may be possible to track this protein after exogenous addition to the extracelullar medium, to see whether or not this protein is internalized by neuronal cells.

Ultimately, future direction will include electron microscopy studies to investigate the properties of PGRN-containing vesicles directly in motor neurons and other non-neuronal cells of the nervous system.

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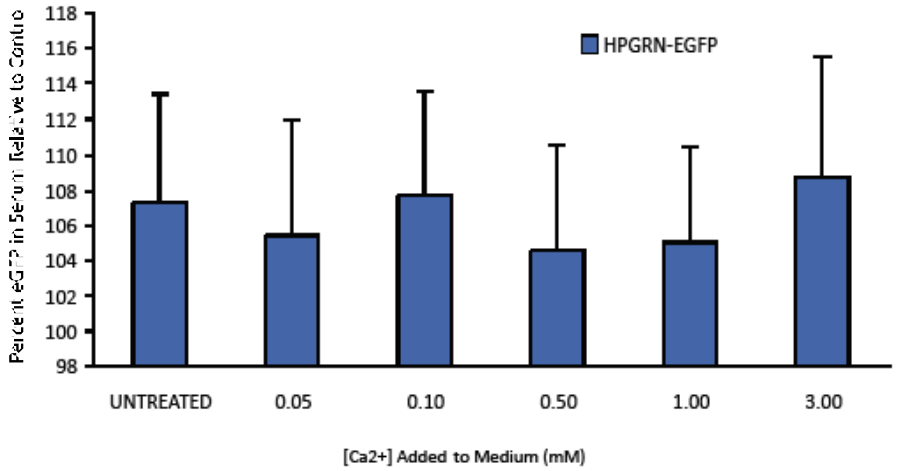

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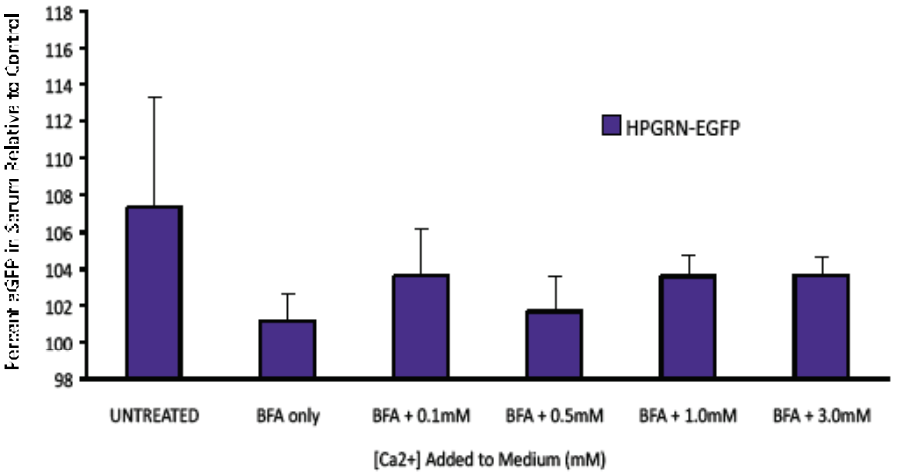

Figure 18. Effect of calcium and BFA on PGRN secretion. NSC-34 cells were transfected with the hPGRN-eGFP construct, and 48 hours post-transfection, incremental amounts of calcium were added to the cell medium to study its effect on secretion. In (A), only calcium was added to the cells, while in (B), cells were treated with BFA 30 minutes prior to addition of calcium. In both cases, cells were compared to comtrol untransfected cells that also received the same calcium, or calcium plus BFA treatment. None of the treatments resulted in significant differences.

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APPENDIX:

AUTHORS CONTRIBUTIONS:

Progranulin is expressed within motor neurons, promotes neuronal cell survival, and is secreted at least in part via the constitutive secretory pathway.

Cara Lise Marie Ryan[1], David C. Baranowski[1*], Babykumari P. Chitramuthu[1], Suneil Malik[1], Zhi Li[1], Mingju Cao[1], Sandra Minotti[2], Heather D. Durham[2], Hugh P.J. Bennett[1] and Andrew Bateman[1].

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1. Cara Lise Marie Ryan: I undertook the NSC-34 cell survival, TUNEL and BrdU analyses, and localization of progranulin in spinal cord sections and NSC-34 cells. I performed the subcellular localization of progranulin in primary spinal cord motor neurons by confocal microscopy, as well as the design, cloning, preparation and transfection of the peGFP-N1-hPGRN construct. I also performed all immunocytochemistry and secretion assays using the peGFP-N1-hPGRN construct.

2. Dr. David Baranowski helped me with the survival assay experiments as well as was responsible for statistical analysis of the data. He was also responsible for the RT-PCR validation of the NSC-34/PGRN overexpressing cell line seen in Figure 2 and trained me in many laboratory procedures, including basic microscopy.

3. Dr. Babykumari Chitramuthu carried out the experiments involving growing NSC-34 cell on a recombinant progranulin-containing extracellular matrix seen in Figure 4.

4. Dr. Suneil Malik established the NSC-34/PGRN cells and performed the Western blot in Figure 2 to validate the presence of PGRN in the NSC-34/PGRN cell line.

5. Dr. Zhi Li prepared and characterised the recombinant human progranulin used in Babykumari's experiments.

6. Dr. Ming Cao performed the *in situ* hybridizations seen in Figure 1.

7. Sandra Minotti and Dr. Heather D. Durham provided the primary spinal cord-DRG cultures and advised on several aspects of the work. Dr. Durham is also my co-supervisor for my master's project.

8. Dr. Andrew Bateman undertook the long-term proliferation experiments with NSC-34/PGRN seen in Figure 3. He was also involved in the overall design of the study.

9. Dr. Hugh P.J. Bennett is my master's supervisor and was involved in the overall design of this project and was a great help with the editing of this thesis.

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September 13, 2007

The McGill University Animal Care Committee certifies that
Cara Ryan has successfully completed a
Mouse Methodology Workshop on ***August 29, 2007***.

The training included the following procedures:

- ✔ Handling and restraint
- ✔ Gavage (tube feeding)
- ✔ Blood collection: saphenous
- ✔ Injections: subcutaneous, intramuscular, intraperitoneal
- ✔ Determination of anaesthetic depth
- ✔ Euthanasia by cervical dislocation

Certification is valid for 5 years, starting on the date of the workshop.

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(Confirmation of training can be obtained by request to the above email address)

Note: Trainee must keep this certificate as other institutions may request it as evidence of training

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August 7, 2007

The McGill University Animal Care Committee certifies that

Cara Ryan has successfully completed the

Advanced Level
of the
Theory Training Course on Animal Use for Research and Teaching
on
August 6, 2007.

The training includes the following topics:

- **Basic Level:** Regulations & Procedures, Ethics, Basic Animal Care, Occupational Health & Safety
- **Advanced Level:** Anesthesia, Analgesia, Euthanasia, Categories, Influencing Factors and Environmental Enrichment

Please note that this certificate does NOT include practical training, which is obtained by successfully completing an Animal Methodology Workshop where another certificate is issued.

Certification is valid for 5 years, starting on the date indicated above.

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Note: Trainee must keep this certificate as other institutions may request it as evidence of training

McGill University
Environmental Health and Safety

THIS IS TO CERTIFY THAT

Cara Ryan

Division of Experimental & Investigative Medicine

SUCCESSFULLY COMPLETED CORE TRAINING IN

Workplace Hazardous Materials Information System (W.H.M.I.S.)

ON

Tuesday, October 30, 2007

Pietro Gasparrin
Environmental Health & Safety Office

Wayne Wood
Manager, Environmental Health & Safety

Valid Until Saturday, October 30, 2010

THE RESEARCH INSTITUTE OF THE McGILL UNIVERSITY HEALTH CENTRE

ENVIRONMENTAL HEALTH AND SAFETY CERTIFICATE

This is to certify that ***Cara Ryan*** from the Endocrine Laboratories, McGill University successfully completed training in ***Biosafety*** on ***February 18, 2008***.

Pubalee Bera

Pubalee Bera, Environmental Health & Safety Officer

Jean-Marie Chavannes, Technical Support Manager

Employee's signature

Laboratory supervisor's signature
Dr. Hugh Bennett

Certification is valid until February 18, 2011