

IFRS1 for the study of myelination

Immortalized Schwann cells IFRS1 as a new strategic tool for the study of myelination and demyelination

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Abstract

Spontaneously immortalized Schwann cell lines were established from long-term cultures of adult Fischer rat peripheral nerves. One of them, termed as *immortalized Fischer rat Schwann cells 1* (IFRS1), has been shown to retain distinct Schwann cell phenotypes, such as spindle-shaped morphology with expression of glial cell markers (S100, glial fibrillary acidic protein and p75 low affinity neurotrophin receptor) and transcription factors (Krox20, Oct6 and SOX10), synthesis and secretion of neurotrophic factors and cytokines, and fundamental ability to myelinate neurites in cocultures with adult rat dorsal root ganglion neurons and nerve growth factor-primed PC12 cells. Consequently, IFRS1 cells and their cocultures with neuronal cells can be beneficial tools for exploring the precise mechanisms of myelination as a result of neuron-Schwann cell interactions and the pathogenesis of demyelinating neuropathies.

Keywords: *immortalized Schwann cells; coculture; myelination; demyelination*

Note* These authors contributed equally to this work.

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1. Introduction

Schwann cells, the glial cells in the peripheral nervous system (PNS), play key roles in development, differentiation, physiologic homeostasis, and axonal regeneration and remyelination after injury. They provide trophic support for the growth and maintenance of neurons and ensheath their axons in either a myelinating or an unmyelinating form (Kidd *et al.*, 2015). Upon peripheral nerve injury, Schwann cells lose axonal contact and change their phenotype in favor of axonal regeneration and functional repair. The 'activated' Schwann cells migrate toward the site distal to the injury where they participate in the process of Wallerian degeneration by eliminating axonal and myelin debris in concert with macrophages. They proliferate to form the bands of Büngner as guideposts for regenerating neurites, secrete various neurotrophic and chemotactic factors to help direct neurites toward the target tissues and protect injured neurons from cell death, and finally remyelinate neurites (Susuki, 2014; Ferguson & Smith, 2015). Schwann cell abnormalities are closely associated with the development and progression of peripheral neuropathies

(Midroni & Bilbao, 1995; Scherer, 1997; Dalakas, 2015).

Culture of Schwann cells and their coculture with neuronal cells have been utilized to investigate the mechanisms for myelination and demyelination in the PNS (Rosenbluth & Moon, 2003; Felitsyn *et al.*, 2007; Syed *et al.*, 2010). In most of the previous studies, however, Schwann cells and neurons were obtained from embryonic or neonatal animals. Because it is recognized that some biological properties of these cells change with maturation and aging (Horie *et al.*, 1990; Mirsky *et al.*, 2001), culture systems of adult animal neurons and/or Schwann cells appear to mimic peripheral nerve degeneration and regeneration better than those of embryonic or neonatal animal cells. Although the primary culture of adult rodent Schwann cells has been established (Suzuki *et al.*, 1999; Kaewkhaw *et al.*, 2012), it needs a time-consuming process to obtain good yields of Schwann cells and sufficiently eliminate fibroblasts and other cells from the connective tissue-enriched mature peripheral nerves. Schwann cell lines established from adult animal tissues and tumor cells possess high proliferative activity, and are easy to handle for molecular and biochemical analyses. However, the characteristics of these cell

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lines differ to some extent from those of the original Schwann cells, and few of them have been proved to be capable of myelinating neurites in coculture with neuronal cells (De Vries & Boullerne, 2010).

In our recent study (Sango *et al.*, 2011a), spontaneously immortalized Schwann cell lines were established from long-term cultures of adult Fischer 344 rat dorsal root ganglia (DRG) and peripheral nerves. One of these cell lines, designated *immortalized Fischer rat Schwann cells 1* (IFRS1), has been shown to retain characteristic features of Schwann cells as illustrated in the following sections, thereby being a useful tool for exploring the mechanisms of peripheral nerve degeneration and regeneration.

2. Characterization of IFRS1 cells

2.1 Establishment of IFRS1 cells

Primary and long-term cultures of Schwann cells from DRG and peripheral nerves from adult Fischer 344 rats were maintained in serum-free medium with N2 supplement, 40 ng/mL neuregulin (NRG)-1 β and 5 μ M forskolin, and were passaged once every 4-6 weeks, during which neurons and fibroblasts ceased to grow in the cultures. After 4-5 months in

culture, spontaneously emerging Schwann cell colonies were isolated and further expanded. One of these cell lines were designated as IFRS1 and further characterized.

2.2. Expression of glial cell markers and neurotrophic factors

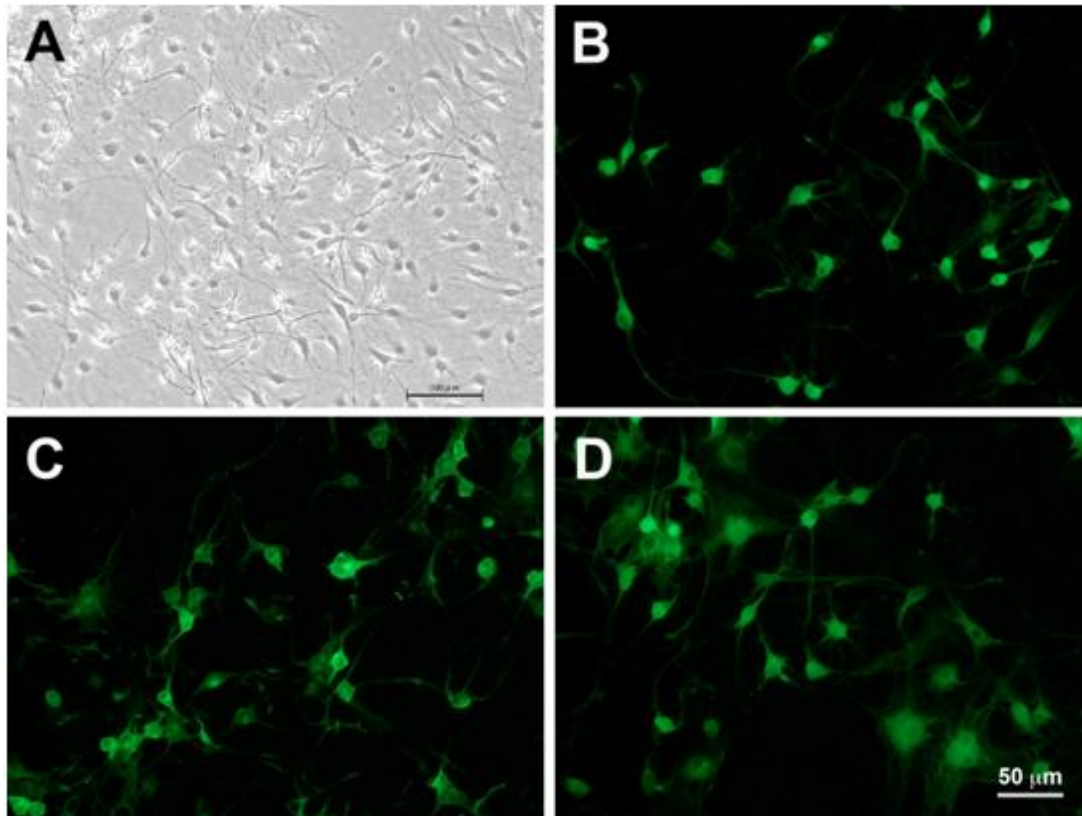
IFRS1 cells displayed spindle-shaped morphology under phase-contrast microscopy with intense immunoreactivity for glial cell markers, such as S100 protein, glial fibrillary acidic protein (GFAP) and p75 low affinity neurotrophic factor receptor (p75^{NTR}) (**Fig.1**). RT-PCR analysis revealed the mRNA expression of neurotrophic factors (nerve growth factor (NGF), glial cell line-derived neurotrophic factor (GDNF) and ciliary neurotrophic factor (CNTF)), cell adhesion molecules (L1, neural cell adhesion molecule (NCAM) and N-cadherin), transcription factors (Krox20, Oct6 and SOX10), myelin proteins (myelin protein zero (MPZ), peripheral myelin protein 22 (PMP22) and myelin-associated glycoprotein (MAG)) and NRG-1 receptors (ErbB2 and ErbB3) in IFRS1 cells (Sango *et al.*, 2011a). These molecules are also expressed in primary cultured Schwann

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cells and *immortalized mouse Schwann cells 32* (IMS32) (Watabe *et al.*, 1995, 2003).

[Figure 1] IFRS1 cells showed distinct

Schwann cell phenotypes such as spindle-shaped morphology (A) as well as immunoreactivity for S100 (B), p75^{NTR} (C) and GFAP (D).



2.3. Synthesis and secretion of neurotrophic molecules

Conditioned medium (CM) obtained from IFRS1 cells promoted the survival and neurite outgrowth of adult rat DRG neurons in a concentration-dependent manner (10%<20%<100%) (Fig.2), suggesting that IFRS1 cells secrete neurotrophic molecules required for neuron survival and axonal regeneration after injury. The neurite-outgrowth

promoting activities of CM at 20% in medium were comparable to those of NGF at 50 ng/mL, whereas cotreatment with anti-NGF neutralizing antibody attenuated the activity of NGF, but not CM. In addition, NGF had no significant effects on the viability of DRG neurons (Sango *et al.*, 2011a). These findings indicate that neurotrophic and neuroprotective molecules other than NGF released from IFRS1 cells play a major role in the sustained viability of

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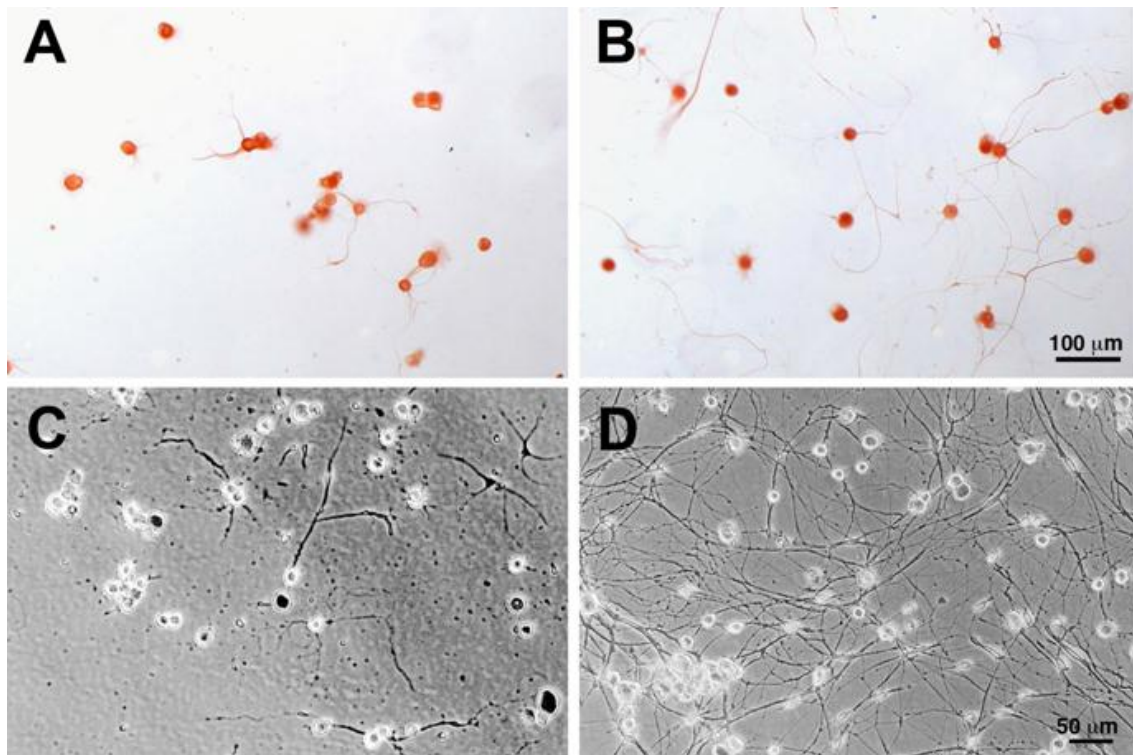
neurons. In our previous studies, members of the interleukin-6 cytokine family, such as CNTF, leukemia inhibitory factor, oncostatin M and cardiotrophin-1 promoted both survival and neurite outgrowth of DRG neurons (Sango *et al.*, 2008, 2014). It seems likely that IFRS1 cells produce and secrete these molecules, however, we have not obtained the direct evidence to prove this.

Protein expression of galectin-1 (GAL-1), a member of a family of β -galactoside-binding animal lectins, was detected in IFRS1 cells and their culture supernatant (Sango *et al.*, 2012b; Takaku *et al.*, 2013). Despite lacking a signal leading peptide, GAL-1 is subject to externalization by a non-classical secretory pathway (Sango *et al.*, 2004). Following externalization, some of the galectin molecules associate with surface or extracellular matrix glycoconjugates where lectin activity is stabilized, while the others free from glycoconjugate ligands are rapidly oxidized in the non-

reducing extracellular environment. The growing evidence suggests that both reduced and oxidized forms of GAL-1 are involved in the process of Wallerian degeneration and subsequent axonal regeneration with functional recovery (Camby *et al.*, 2006), and GAL-1 in the oxidized form lacks lectin activity but could promote axonal regeneration and Schwann cell migration as a cytokine-like molecule (Takaku *et al.*, 2016).

[Figure 2] Conditioned medium (CM) obtained from IFRS1 cells promoted survival and neurite outgrowth of cultured adult rat DRG neurons. For the neurite outgrowth assay, DRG neurons were maintained in serum-free medium (control; **A**) or CM (**B**) for 2 days and were immunostained with anti- β III tubulin antibody. For the survival assay, neurons were maintained in serum-free medium (control; **C**) or CM (**D**) for 7 days and dead neurons were detected by positive trypan blue staining.

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2.4. Myelin formation in coculture with neuronal cells

One of the most important and fundamental properties of Schwann cells is to myelinate axons during the development and regeneration of PNS. Our previous attempts to show myelin structure in coculture with IMS32 cells and adult mouse DRG neurons or PC12 cells resulted in failure. The high proliferative activity of IMS32 cells might impede stable neuron-Schwann cell interactions, which usually take 4-6 weeks to form the myelin sheath (Sango *et al.*, 2011b). In contrast to IMS32 cells, exogenous application of growth

stimulants such as forskolin and NRG-1 β is required for the proliferation and passage of IFRS1 cells. Namely, the absence of these molecules suppressed excess proliferation of IFRS1 cells and made it possible to maintain their coculture with adult rat DRG neurons (Sango *et al.*, 2011a) and NGF-primed PC12 cells (Sango *et al.*, 2012a).

2.4.1. Adult rat DRG neurons

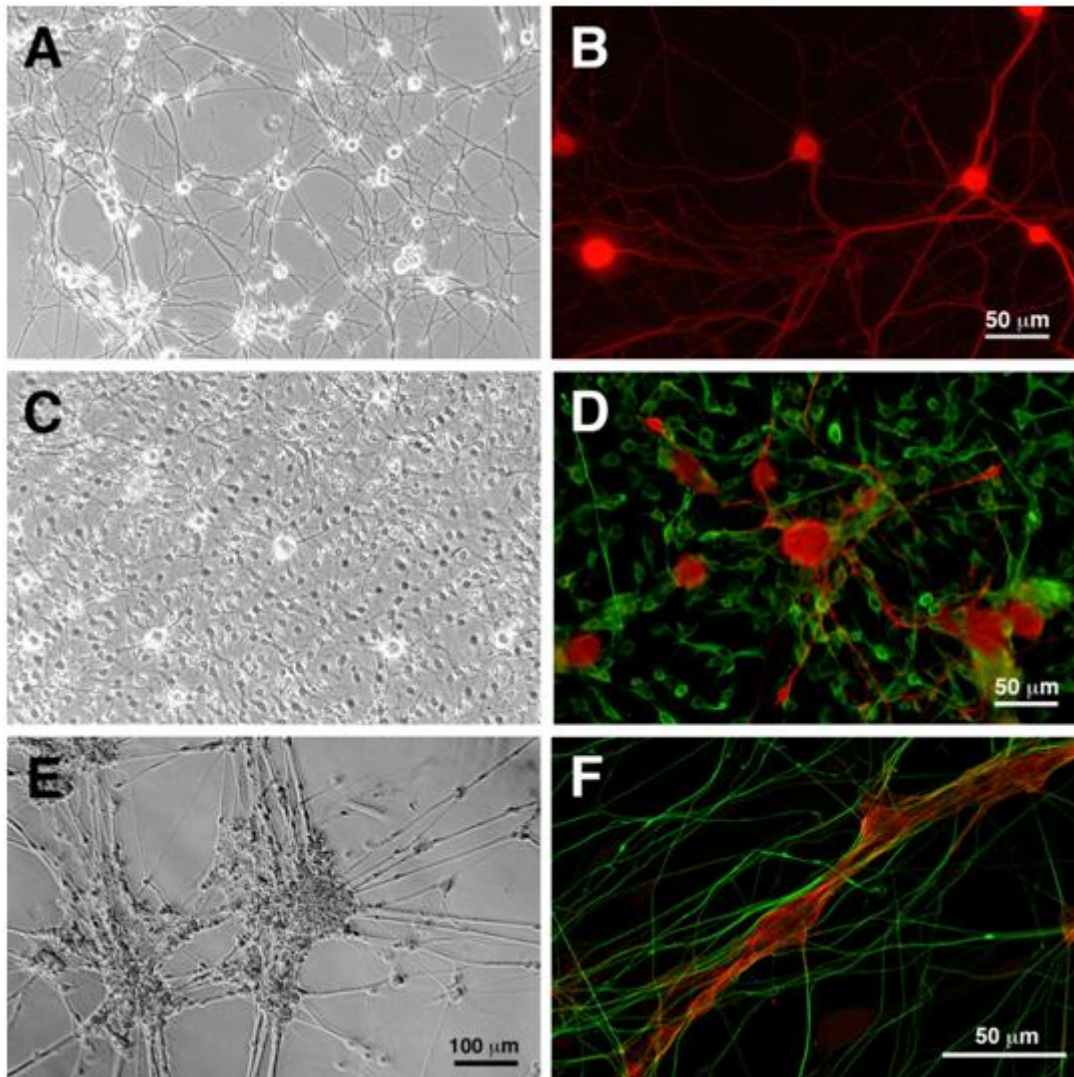
DRGs dissected from 12-week-old rats were mechanically and enzymatically dissociated, and subjected to density gradient centrifugation with 30% Percoll to eliminate myelin sheath. This

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procedure resulted in a high yield and purity of neurons, with a small number of non-neuronal cells (Sango *et al.*, 2008, 2014). The cells were then seeded on type I collagen-coated wells and maintained in serum-free medium with N2 supplement, NGF (10 µg/mL), GDNF (10 µg/mL) and CNTF (10 µg/mL) for a week. Under these conditions, vigorous neurite outgrowth from the neurons was observed (**Fig.3A, B**). Subsequently, IFRS1 cells were cocultured with DRG neurons (**Fig.3C, D**) and maintained in serum-free medium with B27 supplement, ascorbic acid (50 µg/mL) and GDNF (10 µg/mL) for up to 4 weeks (**Fig.3E**). Myelin formation was illustrated by double immunofluorescence staining with antibodies against MPZ and βIII tubulin (**Fig.3F**), and further confirmed by Sudan black B staining and electron microscopy.

[Figure 3] Myelination in coculture of DRG neurons and IFRS1 cells. After 7 days of primary culture, we observed vigorous neurite outgrowth from DRG neurons (phase; **A**) and immunocytochemistry with anti-βIII tubulin antibody; **B**). After 3 days of coculture, DRG neurons and IFRS1 cells were observed under a phase-contrast microscope (**C**) and were immunostained for neurofilament (red) and vimentin (green; **D**). After 28 days of coculture, DRG neurons had aggregated to form clusters of neuronal cell bodies with a network of neuritic processes, and some Schwann cells were tightly attached to the neurites (**E**). Myelin formation was illustrated by immunocytochemistry with antibodies to MPZ (red) and βIII tubulin (green; **F**).

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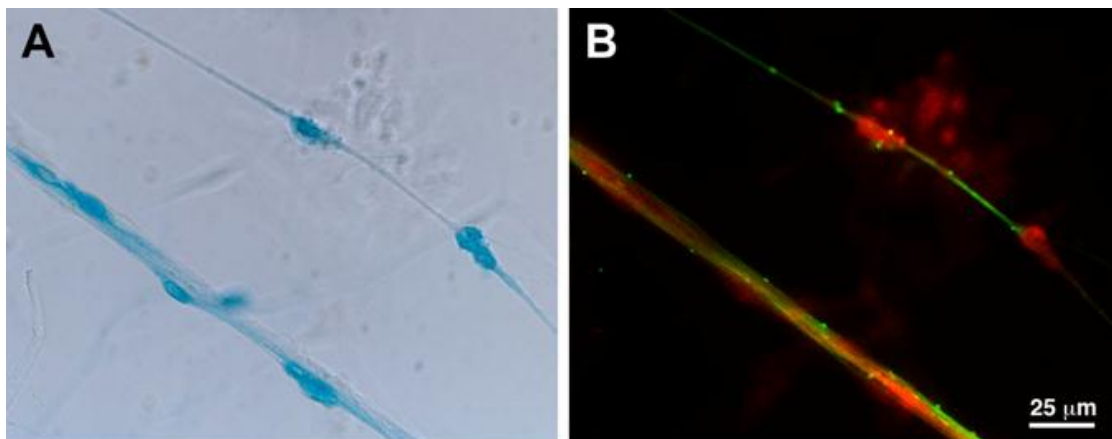
Because both DRG neurons and IFRS1 cells are derived from adult animals, this coculture system appears to mimic peripheral nerve regeneration and remyelination better than those with immature animal cells. In addition, IFRS1 cells can be stably and effectively used in coculture. Despite these advantages, the primary culture of DRG neurons contains a small number of non-neuronal cells, including Schwann

cells, and it cannot be denied that primary cultured Schwann cells might myelinate neurites in the coculture system. To distinguish IFRS1 cells from the primary cultured Schwann cells, β -galactosidase gene was inserted into IFRS1 cells. These LacZ-labeled IFRS1 cells, but not the primary cultured Schwann cells, were stained blue by 5-Bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal). The findings from X-Gal staining and

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following immunocytochemistry indicate that IFRS1 cells are capable of myelinating neurites in coculture with DRG neurons (**Fig.4**).

[Figure 4] After 28 days of coculture with DRG neurons and LacZ-labeled IFRS1 cells, myelin formation was illustrated by X-Gal staining (**A**) combined with immunocytochemistry with antibodies to MPZ (red) and β III tubulin (green; **B**).



2.4.2. NGF-primed PC12 cells

If the protocol for stable coculture systems with IFRS1 cells and pure neuronal cell lines is established, the problems arising from the primary culture as described above will be avoided. Then, we conducted cocultures of the neural crest-derived pheochromocytoma cell line PC12 and IFRS1. Since NGF has been shown to promote neurite outgrowth from PC12 cells (Greene & Tischler, 1976), the PC12-IFRS1 coculture was maintained in serum-free medium with B27 supplement, ascorbic acid (50 μ g/mL) and NGF (10 μ g/mL). However,

this protocol did not work, largely due to the excess proliferation of PC12 cells. B27 supplement contains various nutrients and anti-oxidants, which might trigger the proliferation of PC12 cells even under serum-free culture conditions. Replacement of B27 with N2 supplement, which contains only five nutrients (insulin, transferrin, selenium, putrescine and progesterone) and no anti-oxidants, partially prevented the overgrowth of PC12 cells but resulted in massive IFRS1 cell death by 3 weeks of coculture. Our trial-and-error experience helped generate possible solutions to these problems, and finally the following protocol was

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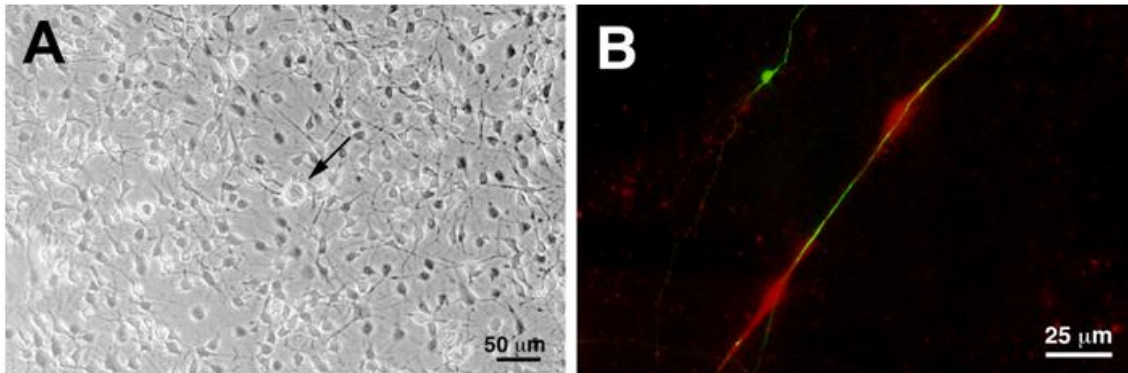
established.

1. PC12 cells were seeded on type I collagen-coated wells at low density ($3 \times 10^2/\text{cm}^2$) and maintained in serum-free medium with N2 supplement, ascorbic acid (50 $\mu\text{g}/\text{mL}$) and NGF (50 ng/mL) for 7 days. Exposure of PC12 cells to the NGF-rich condition with minimum nutrients suppressed their proliferation and accelerated neurite extension.
2. When IFRS1 cells were cocultured with NGF-primed PC12 cells, the cell density ratio of PC12 cells to IFRS1 cells was adjusted from 1:100 to 1:200. Incubation of the coculture in serum-containing medium for the initial 2 days was needed for the differentiation of IFRS1 cells from round to spindle-shaped morphology (**Fig.5A**).
3. The coculture was then maintained in serum-free medium with B27 supplement, ascorbic acid (50 $\mu\text{g}/\text{mL}$), NGF (10 ng/mL) and recombinant NRG-1 type III (sensory and motor neuron-derived factor (SMDF), 25

ng/mL). SMDF has been recognized as a potent inducer of myelination in coculture of primary cultured Schwann cells with DRG neurons or superior cervical ganglion neurons (Syed *et al.*, 2010). After 3 weeks of coculture under this condition, PC12 cells aggregated to form small clusters of cell bodies with a neurite network. These morphological changes indicate stable neuron-Schwann cell interactions. After 4 weeks of coculture, myelin formation was illustrated by immunocytochemistry (**Fig.5B**), and further onfirmed by Sudan black B staining and electron microscopy.

[Figure 5] Myelination in coculture of NGF-primed PC12 cells and IFRS1 cells. After 3 days of coculture, PC12 cells (arrows) and IFRS1 cells were observed under a phase-contrast microscope (**A**). After 28 days of coculture, myelin formation was illustrated by immunocytochemistry with antibodies to MPZ (red) and β III tubulin (green; **B**).

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The PC12–IFRS1 coculture model has the following advantages relative to the previous models with primary cultured neurons and/or Schwann cells. First, it can be prepared at the researchers' convenience without the time-consuming processes of the primary culture. Secondly, it is composed of pure neuronal and Schwann cell lines and does not contain other cells derived from primary cultures. Thirdly, it is free of ethical problems that may arise from sacrificing experimental animals. Lastly, it can be prepared and maintained by routine culture techniques without genetic manipulation of cells. Despite these advantages, close attention should be paid to avoid overgrowth and/or death of the cocultured cells for each experiment. It seems plausible that genetic manipulation or use of DNA alkylating agents is more effective and convenient to suppress the proliferative activity of PC12 cells than adjusting their cell density at seeding.

2.4.3. NSC-34 cells

In addition to the coculture systems described above, our current investigation focuses on the establishment of the coculture models of IFRS1 cells and NSC-34 motor neuron-like cells that were produced by fusion of motor neuron-enriched embryonic mouse spinal cord cells with mouse neuroblastoma cells (Cashman *et al.*, 1992). NSC-34 cells possess the properties of motor neurons, such as expression of choline acetyltransferase and neurotrophin receptors (p75^{NTR} and TrkB), acetylcholine synthesis and release, and generation of action potentials, and NSC-34-IFRS1 coculture models would be more suitable than previous models (Gingras *et al.*, 2008) to study motor neuron diseases (*e.g.* amyotrophic lateral sclerosis, Charcot–Marie–Tooth disease and immune-mediated demyelinating neuropathies).

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3. *In vitro* models of drug-induced demyelinating disorders

Demyelination in the PNS can be caused primarily by Schwann cell or myelin defects and secondarily by axonal degeneration. Some of the demyelinating neuropathies result from metabolic disorders of Schwann cells, such as inherited and drug-induced lysosomal storage disorders, leprosy and cytomegalovirus-associated neuropathy (Midroni & Bilbao, 1995).

Amiodarone hydrochloride (AMD), an anti-arrhythmic agent prescribed for patients with atrial fibrillation and ventricular arrhythmias, has a number of adverse effects, including peripheral neuropathy (Orr & Ahlskog, 2009). AMD and its metabolites (desethylamiodarone and bis-desethylamiodarone) are likely to inhibit lysosomal phospholipases and induce accumulation of phospholipids and other substances in the lysosomes of Schwann cells, thereby being a cause of schwannopathy and myelinopathy; however, the precise mechanisms of AMD neurotoxicity remain largely unclear. In our recent study, the viability and biochemical properties of IFRS1 cells and the myelination in DRG-IFRS1 and PC12-IFRS1 coculture systems were affected by AMD. Treatment of IFRS1

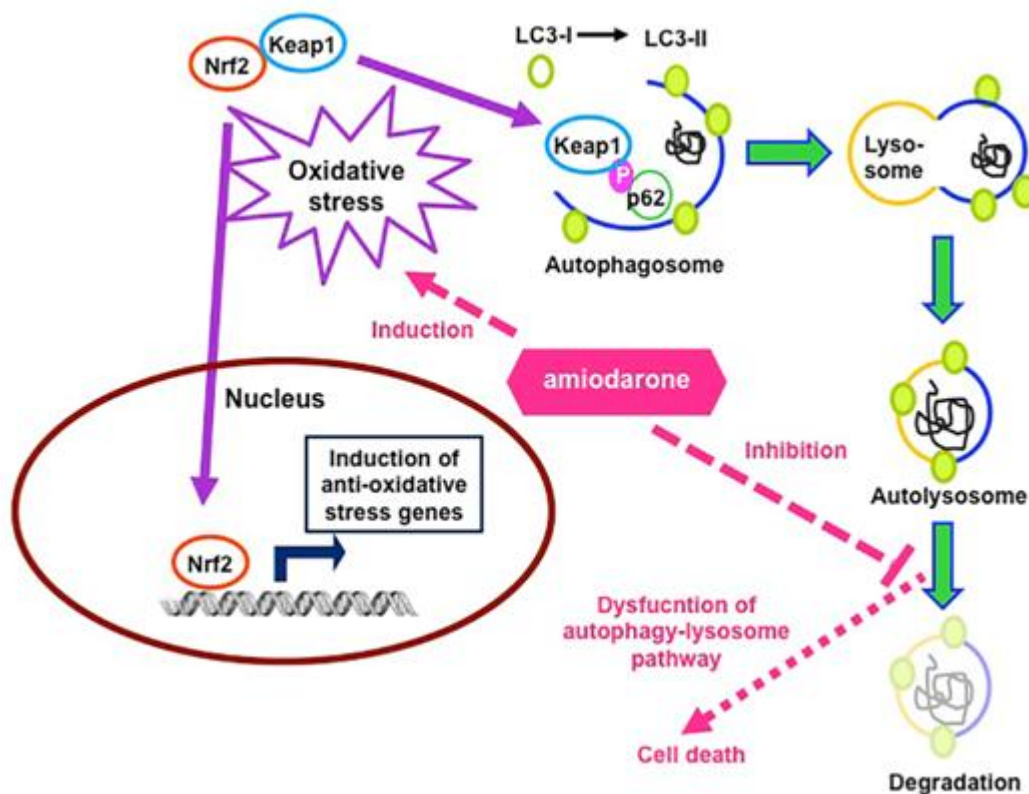
cells with AMD (1, 5, and 10 μM) induced dose- and time-dependent cell death, intracellular storage of phospholipids, upregulation of the expression of a lysosomal marker LIMP2 and GM_2 and GM_3 gangliosides, and oxidative stress (enhanced Nrf2 DNA binding activity and decreased GSH/GSSG ratios). It also induced the upregulation of the expression of autophagy markers LC3-II and p62, with phosphorylation of p62. A possible connection between oxidative stress and autophagy in AMD toxicity was further investigated. Under normal conditions, Nrf2 is negatively regulated by interaction with the ubiquitin ligase adaptor Keap1 in the cytoplasm. Under exposure to AMD, however, Nrf2 seems to be released from Keap1 and translocated to nuclei where it binds to antioxidant response elements in promoters to upregulate the expression of anti-oxidative stress genes (Ma, 2013). On the other hand, AMD-induced upregulation and phosphorylation of p62 is likely to enhance p62's binding affinity for Keap1 (Ichimura *et al.*, 2013). The Keap1-phosphorylated p62 complexes accelerate the formation of autophagosomes, which fuse with lysosomes to form autolysosomes, and finally autolysosomes are degraded by

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proteases (Hung *et al.*, 2013). However, the diminished activities of lysosomal enzymes under exposure to the higher concentration of AMD might impair the degradation of autolysosomes (**Fig.6**). Furthermore, treatment of the cocultures with AMD induced detachment of IFRS1 cells from neurite networks in a time- and dose-dependent manner (Niimi *et al.*, 2016). These findings suggest that AMD-induced lysosomal storage

accompanied by enhanced oxidative stress and impaired autophagy-lysosome pathway in Schwann cells might be a cause of demyelination.

[Figure 6] Involvement of oxidative stress and dysfunction of autophagy-lysosome pathway in IFRS1 cells under exposure to amiodarone (AMD). Modified from Graphical Abstract, Niimi *et al.*, European Journal of Neuroscience 2016; 44: 1723-1733.



In addition to AMD, dichloroacetate (Felitsyn *et al.*, 2007), δ -aminolevulinatate (Felitsyn *et al.*, 2008) and tumor necrosis factor- α antagonists (Alshekhlee *et al.*, 2010) have been reported to induce

demyelinating neuropathies. It is expected that IFRS1 cells and the coculture systems will help to elucidate the pathogenesis of these disorders.

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4. Conclusion and future aspects

IFRS1 cells retain characteristic features of Schwann cells as described above, in particular, fundamental ability to myelinate neurites in coculture with neuronal cells. Although growth stimulants such as forskolin and NRG-1 β are needed for the passage of IFRS1 cells, they can be more stably and effectively used for molecular and biochemical analyses as well as coculture studies than primary cultured Schwann cells. DRG neuron-IFRS1 and PC12-IFRS1 coculture systems are valuable tools for exploring neuron-Schwann cell interplay during axonal degeneration and regeneration, as well as pathogenesis of demyelinating neuropathies and novel therapeutic approaches against them. IFRS1 cells and the coculture systems have been employed for the study of axonal regeneration (Takaku *et al.*, 2013, 2016), diabetic neuropathy (Tsukamoto *et al.*, 2015; Takaku *et al.*, 2016) and AMD neurotoxicity (Niimi *et al.*, 2016), and will further be utilized by collaborating with research groups focusing on inherited and immune-mediated

neuropathies. Also, it gives us pleasure that more and more researchers in the PNS field will make good use of IFRS1 cells, which are now commercially available

[[https://www.abmgood.com/Immortalized-Rat-Schwann-Cells-\(IFRS1\)-T0294.html](https://www.abmgood.com/Immortalized-Rat-Schwann-Cells-(IFRS1)-T0294.html)].

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Conflict of interest

All authors declare no conflict of interest in this paper.

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