An in vitro model to study myelin maintenance using small interfering ribonucleic acid in cocultures of neurons and Schwann cells.

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Abstract

The synthesis of the myelin sheath by Schwann cells in the peripheral nervous system is controlled by several transcription factors including Early Growth Response 2 gene (Egr2). This latter factor is also involved in the maintenance of peripheral myelin. The current study was aimed to set up a new method to study the involvement of various genes in peripheral myelin maintenance. Modified self-delivery small interfering RNAs (siRNAs) are used to silence candidate genes *in vitro,* without using transfection reagents. These siRNAs were used on organotypic cocultures of neurons and Schwann cells contained in dorsal root ganglia (DRG). DRG were extracted from embryonic rat. Control non-targeting siRNAs tested did not induce significant demyelination in cocultures. Anti-Egr2 siRNAs down-regulated *in vitro* their target gene expression by 60%. Furthermore, treatment with anti-Egr2 siRNAs resulted in abnormalities of the myelin sheaths in cocultures. The current results are in line with previous findings involving Egr2 in active myelin maintenance, which were obtained using conditional knockout experiments in mice. The results obtained constitute a proof of concept for the use of self-delivery siRNAs to investigate the molecular mechanisms of myelin maintenance *in vitro*.

Key words: Dorsal root ganglion, cell culture, Schwann cells, myelin maintenance, small interfering RNAs.

Résumé

Un modèle *in vitro* pour l'étude de la maintenance myélinique par utilisation de petits acides ribonucléiques interférents (siRNA) sur des cocultures de neurones et de cellules de Schwann.

La synthèse de la gaine de myéline par les cellules de Schwann dans le système nerveux périphérique est contrôlée par plusieurs facteurs de transcription dont Egr2. Ce dernier facteur est aussi impliqué dans la maintenance de la myéline périphérique. L'objectif de la présente étude est de développer une nouvelle technique pour étudier l'implication de divers gènes dans la maintenance de la myéline du système nerveux périphérique. De petits ARN interférents (siARN) modifiés ont été utilisés en auto-administration pour induire *in vitro* l'extinction de l'expression de gènes candidats sans utiliser des réactifs de transfection. Ces siARN ont été utilisés sur des cocultures organotypiques de neurones et de cellules de Schwann contenus dans des ganglions rachidiens postérieurs provenant d'embryons de rat. Les siRNA contrôles, sans cibles n'ont pas entrainé de démyélinisation significative des cocultures. Les siARN anti-Egr2 ont régulé à la baisse l'expression de leur gène cible à 60% *in vitro.* En outre, le traitement avec les siARN anti-Egr2 a provoqué des anomalies des gaines de myéline des cocultures. Ces présents résultats sont en accord avec ceux des études antérieures impliquant Egr2 dans la maintenance active de la myéline, qui ont été obtenus en utilisant des expériences KO (knockout) sur des souris. Les résultats présentés constituent une preuve de concept pour l'utilisation des siARN auto-administrés pour l'étude des mécanismes moléculaires de la maintenance de la myéline *in vitro.*

Mots clés : ganglions rachidiens postérieurs, culture cellulaire, cellules de Schwann, maintenance myélinique, petits ARN interférents.

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1. Introduction

The coexistence of neurons and glial cells is a specific characteristic of all higher vertebrate nervous systems. In peripheral nerves, Schwann cells produce segments of myelin, a multilamellar lipid-rich membrane that is wrapped around axons. The main function of myelin is to serve as an insulator to facilitate signal propagation along axons. The Egr2 transcription factor is a prime regulator of Schwann cells myelination. Nerves from Egr2- knockout mice being hypomyelinated and populated with Schwann cells that fail to exit the cell cycle (Topilko et *al.*, 1994). Mutations in Egr2 are found in patients with congenital hypomyelinating neuropathy, Charcot-Marie-Tooth disease or Dejerine-Sottas syndrome (Warner et a*l.*, 1998; Timmerman et *al.*, 1999). During the last two decades, many molecular effectors of peripheral nerve myelination have been identified (Woodhoo et *al.*, 2009). Egr2 is permanently expressed in myelinating Schwann cells and is down-regulated after axonal injury. This phenomenon provokes elimination of myelin sheath, a process known as Wallerian degeneration (Ghislain and Charnay, 2006). Consequently, Egr2 expression might be required for myelin maintenance. Very little knowledge is available on the maintenance of peripheral myelin (Canu et *al.*, 2009; Bremer et *al.*, 2011).

Therefore, a new method in the current work is set up to study the involvement of genes in peripheral myelin maintenance using small interfering RNAs (siRNAs) to silence target genes *in vitro*. For this purpose, "self-delivery" siRNAs

(Accell siRNA, Dharmacon) is used to silence candidate genes without using transfection reagents. These modified siRNAs were previously shown to be able to down-regulate target genes *in vivo* after direct injection in skin ([Gonzalez-Gonzalez et *al.*, 2010; Lara et *al.*, 2012-]). Another siRNA targeting Krüppel like factor 6 splice variant 1 (KLF6-SV1) was shown to inhibit ovarian cancer development after intraperitoneal injection (DiFeo et *al.*, 2009). In the current study, anti-Egr2 and anti-Dicer siRNAs were used on cocultures of myelinating Schwann cells and neurons extracted in DRG from E17 embryonic rat (*in vitro* model).

2. Materials and methods

2.1. Animal materials

For cocultures of dorsal root ganglia (containing neurons and Schwann cells), embryos of days 15 to 17 (E15-E17) from adult pregnant female Sprague-Dawley rats were used. All rats were obtained from DEPRE breeding center, Saint-Doulchard, France.

Two animals per cage were housed, in a temperature and humidity-controlled room with 12:12 h light/dark cycle. They were fed with standard chow and water *ad libitum*. Adequate measures were taken to minimize pain and discomfort. The animals were housed for at least one week before entering the experiments. All procedures were performed with the approval of the animal ethics committee of the University of Limoges (authorization number15-2013-15).

2.2. Methods

2.2.1. Organotypic DRG culture

- ***Dissection of dorsal root ganglia***

The procedure used for dissection is a modification of the method described by Svenningsen et *al.* (2003) for the preparation of neuron-Schwann cell cocultures. A pregnant, E15 to E17 female Sprague-Dawley rat was anesthetized by inhalation of isoflurane mixed to O_2, which is delivered by an anesthesia system adapted to rats. The uterus with embryos (mean number of embryos/ uterus: 12) was extracted under sterile conditions and placed in 100 mm Petri dish with cold sterile L15 medium. The embryos were removed and transferred into a new dish containing cold L15 medium. The rat was sacrificed by intracardiac injection of 0.5 ml ketamine at 10 mg/ml (PANPHARMA). In each embryo, the spinal cord was removed under a dissecting microscope (Leica MZ 6) and the DRG were cut one by one using microscissors and transferred to an eppendorf tube containing 1 ml cold L15 medium. All embryos extracted from uterus were dissected.

- ***Dissociation and plating***

The dissociation of embryonic Dorsal Roots Ganglia (DRG) was performed using the method described by Sveninngsen et *al.* (2003). All DRG were dissociated in 0.25% trypsin in L-15 medium at 37°C for 15 min. The dissociation was realized mechanically through a plastic Pasteur pipette. The step of DRG dissociation give an important cluster of isolated cells. Afterwards, the cells were pelleted and resuspended in Neurobasal medium (Gibco) containing 2% of B27 (Gibco),0.3% of glutamax (Gibco), 100 ng/ml of nerve growth factor (Beckton-Dickinson) and 1% of penicillin-streptomycin (Invitrogen). This medium was prepared in advance and frozen in aliquots to feed the cultures. The cells were plated on Matrigel (1:3) coated 12-well plates (Thermo Fischer Scientific Nunc A/S) at 1-5.10^4 cells/ ml and incubated at 37°C with 5% CO_2 and 90% humidity. Seven days after plating, 50 µg/ml of ascorbic acid (Sigma) was added to the medium to trigger myelination by the endogenous Schwann cells. Medium was changed every two days.

- ***Treatment of organotypic cultures with self-delivery siRNAs***

Pools of modified self-delivery Accell siRNAs (Thermo scientific Dharmacon) diluted at 1µM in culture medium were used. No ascorbic acid was added to the culture medium during the siRNA treatment to avoid stimulating myelination during the experiment. Each pool of Accell siRNAs contains 4 different short sequences targeting the same mRNA (Table 1). Non-targeting (NT) Accell siRNAs were used as control. The cocultures were treated at 1 µM for each pool of siRNAs (following the manufacturer instructions) for three days, five days and one week, respectively. The medium was changed on day 3 (with new siRNAs added) in the case of a five-day treatment and on day 5 in the case of a one-week treatment.

Table 1. Oligonucleotide primers used for RT-qPCR

Gapdh	Forward primer TGCACCACCAACTGCTTAG
	Reverse primer GGATGCAGGGATGATGTT
Hprt	Forward primer CTCATGGACTGATTATGGACAGGAC
	Reverse primer GCAGGTCAGCAAAGAACTTATAGCC
Egr2	Forward primer CAGTACCCTGGTGCCAGCTG
	Reverse primer TGTGGATCTCTCTGGCACGG
Dicer	Forward primer GAGTCTCTTGCTGGTGCCAT
	Reverse primer CGCCTGTGAGTCTGGAAGC

- ***Relative quantification of mRNA by RT-qPCR in cocultures***

For real-time PCR, total RNA was extracted using the RNeasy mini kit (Qiagen). Extraction was carried out according to the protocol recommended by the kit supplier. The concentration and purity of total RNA were measured using a Nanodrop ND-1000 spectrophotometer (Labtech). The reverse transcription was performed using Quantitect reverse transcription kit (Qiagen) according to the supplier protocol. The quantification was carried out in a Rotor- Gene 6000 apparatus (Corbett Life Science, series software 1.7) in a final volume of 25 µl, with 12.5 µl of 2x Rotor-Gene SYBR Green PCR master mix (Qiagen), 1µM of forward primer and reverse primer, 2 µl of template cDNA (≤100 ng/ reaction), completed with RNease-free water. Quantification was performed according to the supplier recommendations.

Gapdh (glyceraldehyde-3-phosphate dehydrogenase) and Hprt (Hypoxanthine Phosphoribosyl

Transferase) were used as reference genes. The primers used for RT-qPCR are mentioned in table 2.

Table2. Self-delivery siRNA nucleotide sequences

Dicer	Sequence 1 5'CCCCUAUCGUGAAAUUAUG 3'(13546.4 g/mol)
	Sequence 2 5'CCAGGAUCCACAAAACGAA 3' (13514.0 g/mol)
	Sequence 3 5'UGAUCAGCCUCAUCGAUUU 3' (13572.2 g/mol)
	Sequence 4 5'GUCUGAAGCUCGUAUAGGC 3' (13566.6 g/mol)
Egr2	Sequence 1 5'CCAGAAGGCAUCAUCAAUA 3' (13510.8 g/mol)
	Sequence 2 5'CUGACUUGUUUGGUUAUUA 3' (13546.3 g/mol)
	Sequence 3 5' GGUUUAAGUAUGGCUGUAU 3' (13535.2 g/mol)
	Sequence 4 5' CCAGUAACUCUCAGUGGUU 3' (13561.4 g/mol)

- ***Immunostaining of cocultures***

The cocultures were fixed with 4% paraformaldehyde in phosphate-buffer saline (PBS) for 15 min at room temperature. They were rinsed with PBS 3x5 min and permeabilized with 0.5% triton x-100 in PBS for 5 min. The saturation was carried out with 3% bovine serum albumin (BSA) in PBS for 30 min. A mouse monoclonal anti-myelin basic protein (MBP) antibody (clone SMI-94, Covance) diluted at 1:1000 in 1.5% BSA was added and incubated overnight at 4°C. Cells were rinsed with PBS 3x5 min and a secondary goat anti-mouse antibody conjugated to Alexa 488 (Invitrogen) diluted 1:500 was added for 2 hours at room temperature and rinsed 3x5 min. Cultures were treated with Hoechst 33342 (Sigma-Aldrich, France) diluted at 1:5000 in PBS for 5 min; finally rinsed 3x5 min with PBS and examined under an inverted fluorescence microscope (Leica DM IRB).

2.2.2. Statistical analysis

The relative mRNA expression was calculated using the conventional delta-delta Ct method (LivaketSchmittgen 2001). Student t-test was used to analyze the results. GraphPad Prism was used to present the results as graphs with standard error bars. Each value was expressed as mean ± s.e.m (standard error of the mean). Values of $p < 0.05$ were considered as statistically significant.

3. Results

3.1. Effect of self-delivery siRNAs on Egr2 expression *in vitro*

To determine whether self-delivery siRNAs were able to down-regulate Egr2 mRNA in cocultures of DRG neurons and Schwann cells, cultured cells were incubated with Accell siRNAs diluted at 1 µM in culture medium. Control cultures receiving no siRNAs, non-targeting siRNAs and cultures receiving anti-Egr2 siRNAs were treated simultaneously. Quantification of Egr2 expression after treatment with anti-Egr2 siRNAs showed a significant

Decrease (63%) of Egr2 expression relative to GAPDH. By contrast, treatment with non-targeting siRNAs (NT-siRNA) did not induce significant changes in Egr2 mRNA expression (Fig.1).

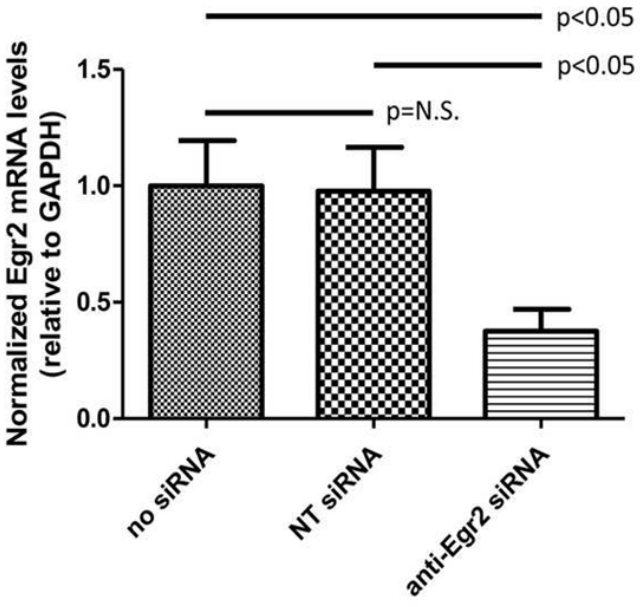

Fig. 1: Real-Time qPCR quantification of Egr2 expression three days after treatment with self-delivery siRNAs.

Using Hprt, another stable reference gene, as an internal control resulted in very similar

results. For this latter treatment, data were not shown.

The level of expression of Egr2 (relative to GAPDH) was normalized to the mean expression level in the absence of siRNAs.

- **Effect of siRNAs targeting Egr2 on myelin maintenance *in vitro***

To detect the effect of anti-Egr2 siRNAs on myelin maintenance *in vitro*, MBP immunostaining on cocultures was performed at various times after siRNA treatment (3 days, 5 days and one week). Cocultures treated with anti-Egr2 siRNAs showed moderate signs of demyelination at day 3, manifesting as an irregular and weaker MBP immunostaining in myelinated fibers (Fig. 2 A-B).

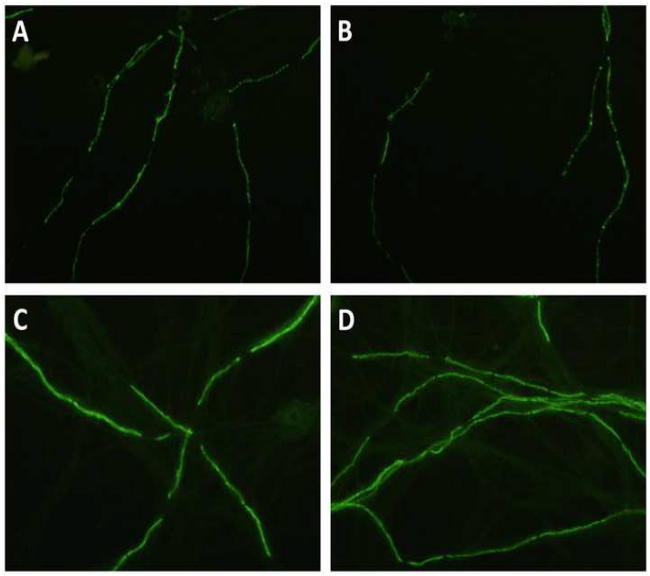

Fig. 2: Effect of siRNAs on myelin sheath in neuron-Schwann cell cocultures: cocultures treated with anti Egr2 siRNAs (A, B) and cocultures treated with non-targeting siRNAs (C, D).

Treatment of cocultures during 5 or 7 days with anti-Egr2 siRNAs did not result in more marked areas of demyelination (data not shown). Treatment with non-targeting siRNAs (Fig. 2 C-D) did not result in any significant demyelination.

In particular, anti-Egr2 siRNA did not induce a diffuse and massive demyelination, as can be observed with some chemical demyelinating agents such as lysophosphatidylcholine [250 ug/ml for 15 hours] (Fig 3A). The myelin sheath control which was not treated with lysophosphatidylcholine did not show signs of demyelination (Fig. 3B).

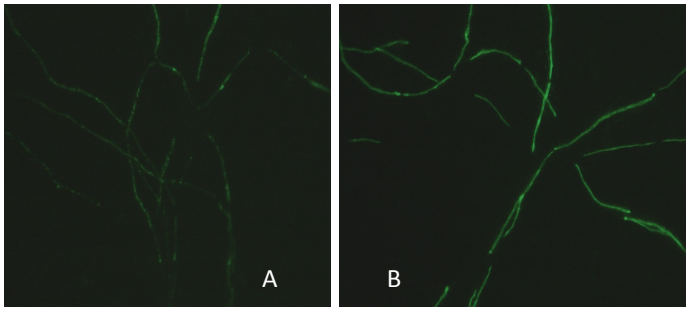

Fig. 3: Effect of lysophosphatidylcholine (LPC) on myelin sheath in neuron-Schwann cell cocultures: cocultures treated with LPC (A) and cocultures which did not treat with LPC (B)

- **Effect of siRNAs targeting Dicer on gene expression and myelin maintenance**

Treatment of neurons and Schwann cells cocultures with anti-Dicer siRNAs was associated with 72% reduction of Dicer mRNA relative to control (Fig. 4).

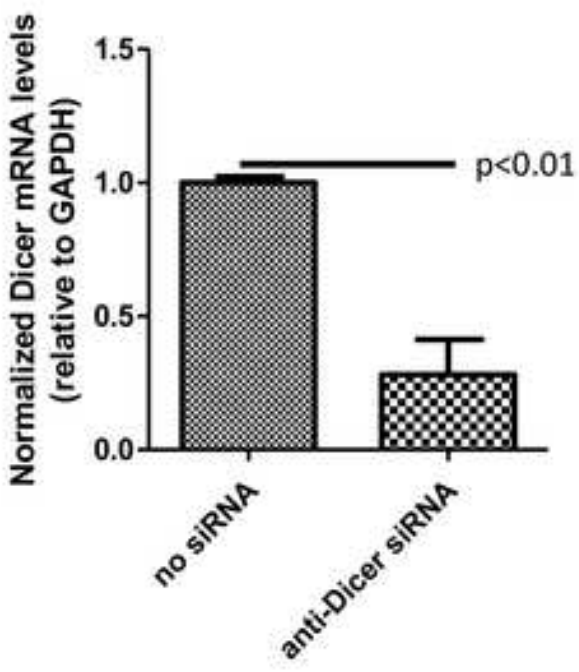

Fig. 4: Quantification of Dicer mRNA expression three days after treatment with self-delivery siRNAs in neuron-Schwann cell cocultures.

Patterns of demyelination were not significantly different from those observed with anti-Egr2 siRNAs. These last results demonstrate that expression of Dicer is necessary for peripheral myelin maintenance *in vitro*.

The level of expression of Dicer mRNA (relative to GAPDH) was normalized to the mean expression level in the absence of siRNAs.

4. Discussion

Egr2 is a key regulator of the peripheral nervous system myelination. It is activated in Schwann cells after axonal contact, before myelination and then drives peripheral nerve myelination (Murphy et *al.*, 1996; Topilko et *al.*, 1997). In the peripheral nerve, Egr2 is expressed in myelinating Schwann cells but remains expressed at high levels throughout life, which led to the hypothesis that Egr2 could also be involved in peripheral myelin maintenance. In experiments using inducible knockout of the Egr2 gene in mouse sciatic nerves (Cre-Lox excision of the Egr2 gene using estrogen receptor-induced Cre expression), Decker et *al.* (2006) showed that focal excision of the Egr2 gene after injection of hydroxytamoxiphen resulted in active demyelination in the injected area. This suggested that Egr2 constant expression in the peripheral nervous system is required for peripheral myelin maintenance. In the absence of Egr2 gene expression, a specific program of dedifferentiation would be engaged, leading to active myelin destruction. One possible mechanism for this active demyelination would be the reactivation of Egr2-silenced genes involved in maintaining the immature stage of Schwann cells (Decker et *al.*, 2006).

The advantage of self-delivery siRNAs was their efficiency for *in vitro* inactivation of target genes. Through this, it has been confirmed that Egr2 silencing results in active demyelination in the absence of non-specific effect of the siRNAs (no significant demyelination after treatment with non-targeting siRNAs). The current study, which uses a completely different technique, confirms the findings of Decker et *al.* (2006), suggesting that peripheral myelin maintenance is a dynamic phenomenon requiring constant expression of Egr2 in the peripheral nervous system.

The siRNA induced demyelination could also constitute a clever model of demyelination, because chemically-induced PNS demyelination also results in significant axonal injury because of non-specific toxic effect of the chemical agents (as is the case with lyso-phosphatidyl-choline).

Finally, the involvement in peripheral myelin maintenance of microRNA-processing enzyme Dicer was tested. It was previously shown that Dicer inactivation results in defective peripheral nerve myelination (Pereira et *al.*, 2010). This led to speculate that the persistent expression of Dicer mRNA in the peripheral nerves (which is mostly attributable to its expression by Schwann cells) could also be necessary for adequate peripheral myelin maintenance. The down-regulation of target mRNA in cell cultures by anti-Dicer siRNAs was showed.

Contrary, with Egr2 which is only expressed by Schwann cells in the peripheral nervous system, it cannot be rule out that anti-Dicer siRNAs could also target Dicer in neurons, which could indirectly contribute to the observed demyelination through perturbed axon-Schwann cell interactions.

A question regarding the relatively moderate demyelinating effect of anti-Egr2 siRNAs in cocultures of DRG neurons and Schwann cells remains. The down-regulation of Egr2 in cocultures of neurons and Schwann cells did not result in a complete demyelination but rather in a partial demyelinating effect, the myelin sheaths appearing as irregular and less intensely stained by anti-MBP antibodies. Over the course of time, new axons continue to develop from cultured neurons and new myelin sheaths are formed, which may represent a protracted myelination process rather than the maintenance of stable myelin sheaths. In addition, the level of inhibition of Egr2 in these cocultures (about two thirds of basal level) could be insufficient to impair myelin maintenance in the absence of "adjuvant" demyelinating agents, such as macrophages which contribute to demyelination in peripheral nerves.

5. Conclusion

The results obtained confirm the central role of Egr2 constant expression in peripheral myelin maintenance and demonstrate that Dicer expression is required for myelin maintenance. These results also constitute a proof of concept for the use of self-delivery siRNAs to investigate the molecular mechanisms of myelin maintenance *in vitro* and suggest that this simple technique could be a versatile means to induce a gene-driven demyelination.

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