

## Lysophospholipid Receptors Are Differentially Expressed in Rat Terminal Schwann Cells, As Revealed by a Single Cell RT-PCR and *In Situ* Hybridization

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Received January 19, 2006; accepted March 3, 2006

Terminal Schwann cells (TSCs) that cover motor neuron terminals, are known to play an important role in maintaining neuromuscular junctions, as well as in the repair process after nerve injury. However, the molecular characteristics of TSCs remain unknown, because of the difficulties in analyzing them due to their paucity. By using our previously reported method of selectively and efficiently collecting TSCs, we have analyzed the difference in expression patterns of lysophospholipid (LPL) receptor genes (*LPA*<sub>1</sub>, *LPA*<sub>2</sub>, *LPA*<sub>3</sub>, *S1P*<sub>1</sub>, *S1P*<sub>2</sub>, *S1P*<sub>3</sub>, *S1P*<sub>4</sub>, and *S1P*<sub>5</sub>) between TSCs and myelinating Schwann cells (MSCs). LPL, which includes lysophosphatidic acid (LPA) and sphingosine 1-phosphate (S1P), is the bioactive lipid that induces a myriad of cellular responses through specific members of G-protein coupled receptors for LPA. It turned out that *LPA*<sub>3</sub> was expressed only in TSCs, whereas *S1P*<sub>1</sub> was expressed in TSCs and skeletal muscle, but not in MSCs. Other types of LPL receptor genes, including *LPA*<sub>1</sub>, *S1P*<sub>2</sub>, *S1P*<sub>3</sub>, *S1P*<sub>4</sub>, were expressed in both types of Schwann cells. None of the LPL receptor gene family showed MSCs-specific expression.

**Key words:** terminal Schwann cell, myelinating Schwann cell, lysophospholipid receptors, single cell RT-PCR, *in situ* hybridization

### I. Introduction

Lysophospholipid (LPL) is a phospholipid signaling molecule and its functions that have been reported up to now include cell proliferation, myogenesis, stress fiber formation, neurite retraction and transient increase of Ca<sup>2+</sup> [1, 2, 7]. The effects of LPL are mediated through distinct G-protein coupled receptors that are encoded by a different gene family. The first lysophosphatidic acid (LPA) receptor gene identified was the “ventricular zone gene-1 (*vzg-1/LPA*<sub>1</sub>)”, which was abundantly expressed in the ventricular zone of the embryonic cerebral cortex [3]. Recent studies demonstrated that LPA is related to the up-regulation of myelin-specific protein, morphological changes, and survival of

Schwann cells (SCs) [22, 24]. Especially, Weiner and Chun demonstrated that *LPA*<sub>1</sub> was expressed by postnatal SCs, and that LPA promotes SC survival via *LPA*<sub>1</sub> activation and a pathway including Gi, PI3K (phosphoinositide-3-kinase) and Akt [23].

In the peripheral nervous system, SCs play important roles in the axonal regeneration following nerve injury. It is well known that there are two types of SCs, myelinating Schwann cells (MSCs) and terminal Schwann cells (TSCs). TSCs are located at neuromuscular junctions (NMJs) and the number of TSCs is usually 2–6 cells at individual NMJs [15]. Following partial denervation, processes of TSCs extend to the neighboring NMJ and induce the axonal regeneration from the neighboring NMJ [16–18]. On the other hand, MSCs extend, migrate, and proliferate in the same axonal tube after nerve injury [19, 20]. These differences in post-injury reaction between TSCs and MSCs may reflect the physiological significance of each type of SCs. However, the analysis of TSCs is difficult due to the paucity

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and the intricacy of collecting TSCs. We have previously devised a novel method of obtaining TSCs at the single cell level and found a TSC-specific gene, *Herp*, by applying PCR-differential display [13].

In order to elucidate the molecular characteristics of TSCs when compared to MSCs, we have studied the expressions of LPL receptors by single cell RT-PCR and *in situ* hybridization.

## II. Materials and Methods

### Animals

Adult Wistar rats (9- to 10-week-old female) were purchased from a breeder (SLC, Japan) 4 days prior to treatment, and maintained in a temperature- and humidity-controlled animal facility with a 12:12-hr light:dark cycle. The studies were approved by the Kyoto Prefectural University of Medicine Institutional Animal Care and Use Committee and all the experiments were carried out in full compliance with the Rules and Regulations for the Experimental Use and Care of Laboratory Animals at Kyoto Prefectural University of Medicine.

### Preparation of total RNA from skeletal muscle and Schwann cells, and of genomic DNA

Total RNAs from soleus muscle and kidney were extracted and purified with RNeasy Mini kit (Qiagen, USA) according to the manufacturer's instruction. Total RNAs from terminal Schwann cells and myelinating Schwann cells were prepared as previously described [13]. Briefly, single terminal Schwann cell was isolated as follows. The soleus muscle was dissected out from the animals after deep anesthesia by intraperitoneal injection of sodium pentobarbital. The muscle was trimmed in a central portion at the entry point of the sciatic nerve branch and embedded in a low-melting temperature agarose gel. Cross sections, 500  $\mu$ m in thickness, of the muscle were prepared with a microslicer. The sections that include the nerve branch were selected for further studies. NMJs were stained with a fluorescent dye, 4-(4-diethylaminostyryl)-*N*-methyl-pyridinium iodide (4-Di-2-ASP, Molecular Probes, USA). After enzymatic treatment for cell dissociation, individual cells were collected carefully with microcapillaries under an epifluorescent stereomicroscope (Nikon, Japan) and transferred into small tubes containing the cell lysis buffer in Strataprep total RNA microprep kit. Myelinating Schwann cells were prepared from the sciatic nerves of adult Wistar rats. Sciatic nerves, free of perineurium and epineurium, were incubated with collagenase and trypsin to obtain dissociated cells. The cells were collected and transferred into the lysis buffer by using microcapillaries under conventional phase contrast microscope. After adding yeast tRNA (50 ng) to the above cell lysates, total RNA from each single cell was purified according to the instruction manual in the kit. Genomic DNA from kidney was purified with DNeasy tissue kit (Qiagen, USA) according to the manufacturer's instruction.

### cDNA synthesis

Total cDNAs from each type of single cell were synthesized and amplified using SMART cDNA synthesis kit (Clontech, USA) as previously described [4, 14]. The optimized number of thermal cycling was set at 18. Under these conditions, each amplified total cDNA fraction was obtained as moderately strong smears ranging from 0.2 to 4.0 kb with a scattering of several bright bands. Gene expression of *Herp* (a marker of terminal Schwann cell), *S-100 $\beta$* , and *GAPDH* in each cDNA fraction was confirmed by PCR.

First strand cDNAs from soleus muscle and kidney were reverse-transcribed from 5  $\mu$ g of DNase I-treated total RNA using random hexamers and Superscript II RNase H<sup>-</sup> (Invitrogen, USA).

### PCR amplification of the genes of LPL receptor family from the cDNAs and genomic DNA

PCR using the amplified cDNAs, the first strand cDNA, and the genomic DNA as templates, was carried out with *ExTaq* DNA polymerase (TaKaRa Bio, Japan).

For the PCR reactions, sets of primers corresponding to each gene of LPA receptor family were used: 5'-CACTAA-CCAATGGCAGTATTTGTC-3' and 5'-CTGGCTTAGGC-CAAACCACATAA-3' (*LPA<sub>1</sub>*), 5'-AGCCTGCTTGCTCTC-CTRCTCAT-3' and 5'-TAAAGGGTGGAGTCCATCAG-3' (*LPA<sub>2</sub>*), 5'-TACATGCTACACACACGGGTGTA-3' and 5'-TAGCGATCTCCTCATGCTCGTA-3' (*LPA<sub>3</sub>*), 5'-GCCTA-AGGACTATGCTGCTGTAA-3' and 5'-GAGTGTGACC-AAGGACAGTCATA-3' (*SIP<sub>1</sub>*), 5'-CGGAGGCACTGAC-TAATCAGATT-3' and 5'-TCCCAGCACTCAGGACACA-GTTA-3' (*SIP<sub>2</sub>*), 5'-AACCACAACCTCCGAGAGATCCAT-3' and 5'-TTGAAGAGGATGGAGCACTCCTT-3' (*SIP<sub>3</sub>*), 5'-TRCTSAASACSGTGCTGATGAT-3' and 5'-CKGCTGC-GGAAGGAGTAGATGA-3' (R=A/G, K=G/T: *SIP<sub>4</sub>*), 5'-CGTGTCTGTGCTTCTGCAA-3' and 5'-CTGCAAAC-TGTTGGAGGAGTCTT-3' (*SIP<sub>5</sub>*).

Each PCR products were size-fractionated with 2% agarose gel electrophoresis followed by visualization with ethidium bromide. 2-log Ladder DNA size marker (New England BioLab, USA) was used as a size marker.

### Sequencing

PCR products were cloned into pCRII TOPO plasmid DNA (Invitrogen, USA). Nucleotide sequence of each product was determined using Big Dye terminator cycle sequencing kit (v1.1) (Applied Biosystems, USA) and PRIZM310 Genetic Analyzer (Applied Biosystems, USA). Homology search was performed with BLAST 2.0 software program for the databases of GenBank, EMBL, DDBJ, and PDB.

### In situ hybridization

Animals were deeply anesthetized with sodium pentobarbital and were transcardially perfused with 4% paraformaldehyde in phosphate buffered saline. Soleus muscles were dissected out and embedded in a low-melting temperature agarose gel. Longitudinal sections of the muscle, 200  $\mu$ m

thick, were prepared with a microslicer.

The PCR-amplified cDNA fragment of rat *LPA<sub>3</sub>* (nt 1661–2111, GenBank NM\_023969) was cloned into pGEM-T Easy plasmid DNA (Promega, USA). Digoxigenin-labeled cRNA probes were obtained by *in vitro* transcription using the plasmid as template. Sense- and antisense-strand probes were synthesized using SP6- and T7 RNA polymerase (Roche Diagnostics, Switzerland), respectively.

*In situ* hybridization was carried out according to the whole mount *in situ* hybridization protocol as described elsewhere [5, 13] using alkaline phosphatase-labeled anti-DIG antibody (Fab)<sub>2</sub> fragment (Roche Diagnostics, Switzerland) and NBT/BCIP.

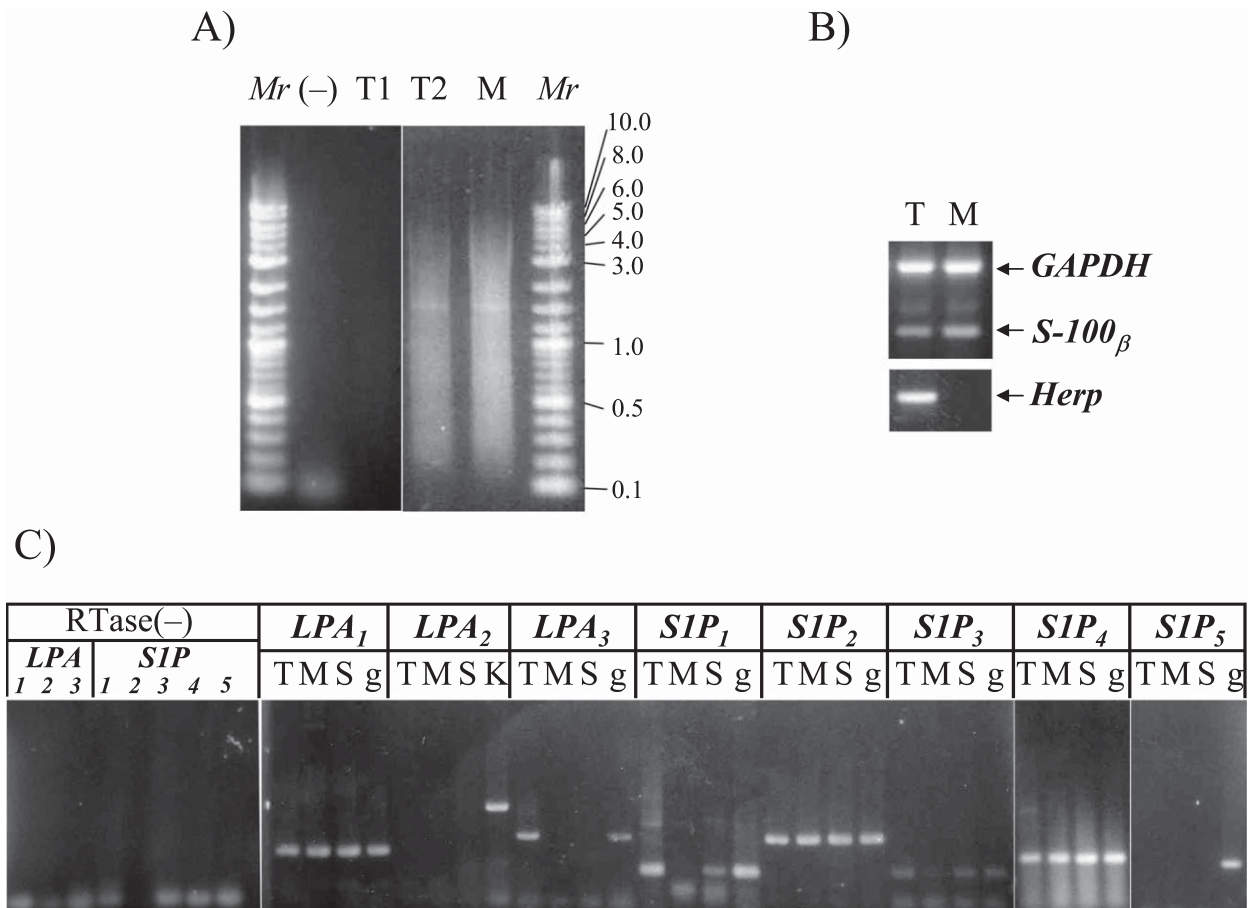
### III. Results

In order to study whether each member of LPL receptor family is expressed in a cell-type-specific manner, we have analyzed the total cDNAs prepared from TSCs, MSCs and

skeletal muscle, respectively, by PCR amplification with each gene-specific primer.

For this purpose, we collected each type of Schwann cells and purified their total RNAs followed by first strand cDNA synthesis and PCR amplification of total cDNAs. As shown in Figure 1A, we obtained total cDNAs fractions from each type of Schwann cell. Some of the amplified cDNAs fractions were insufficient (such as T1 in Fig. 1A). Such total cDNAs fractions were not used for the following analysis. Then, the expression of three genes, including *Herp* (a marker of terminal Schwann cell, as demonstrated in [13]), *S-100β* (a marker of Schwann cell) and *GAPDH* (a housekeeping gene) in each cDNA fractions was confirmed by PCR (as shown in Fig. 1B). The total cDNAs fractions expressing three genes mentioned above were used for analyzing the expression of LPL receptor mRNAs.

We compared the pattern of mRNA expression of LPL receptor family genes between terminal Schwann cells and myelinating Schwann cells. PCR reactions were carried out



**Fig. 1.** (A) Representative results of the size distribution of total cDNAs amplified from terminal Schwann cells and myelinating Schwann cells. T1 is of a failure in amplification for total cDNAs from terminal Schwann cells. T2 shows a successful amplification for total cDNAs from terminal Schwann cells. (-) denotes reverse transcriptase-omitted reaction as a negative control. (B) cDNA amplification of *GAPDH*, *S100β* and *Herp* from each single cell-derived total cDNA fraction. (C) Comparison of each LPL receptor mRNA expression among TSCs, MSCs and skeletal muscles, using each gene-specific primer set. RTase(-) indicates negative controls: results of amplification using each gene-specific primer set and reverse transcriptase-omitted total cDNAs fraction. *Mr*, DNA size marker; T, terminal Schwann cells; M, myelinating Schwann cells; S, skeletal muscles; K, kidney; g, rat kidney genomic DNA.

using each set of the family member gene-specific primers and the total cDNAs prepared as described above. Amplification target sequence of each gene except for *LPA<sub>2</sub>* was included within a single exon of each gene. Rat genomic DNA, therefore, was used as a positive control template for each PCR reaction. For *LPA<sub>2</sub>*, however, rat kidney cDNAs were used as a positive control. Because each primer for *LPA<sub>2</sub>* is located in different exons of the gene, the amplification condition of PCR conducted for cDNA template was not applicable to genomic DNA template. Hence, cDNAs from adult rat kidney where *LPA<sub>2</sub>* mRNA is known to be expressed was used as the positive control template. On the other hand, reverse transcriptase-omitted reaction product as shown in Fig. 1A) was used as a negative control template.

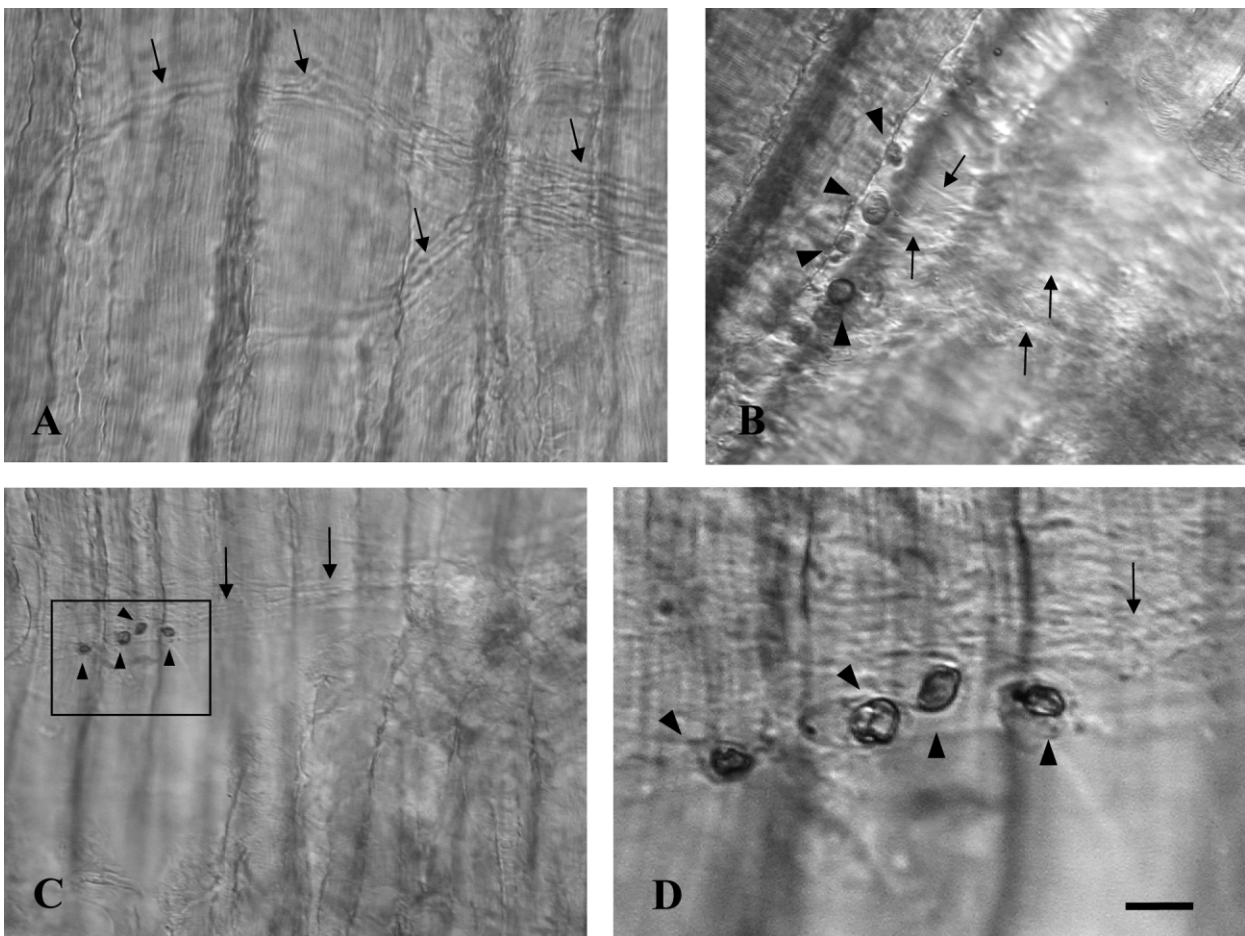
As shown in Figure 1C, *LPA<sub>3</sub>* was expressed only in TSCs, whereas *SIP<sub>1</sub>* was expressed in TSCs and skeletal muscle, but not in MSCs. Neither *LPA<sub>2</sub>* nor *SIP<sub>5</sub>* was detectable in either type of Schwann cell. Other types of LPL receptor genes, including *LPA<sub>1</sub>*, *SIP<sub>2</sub>*, *SIP<sub>3</sub>*, and *SIP<sub>4</sub>*, were expressed in both types of Schwann cells. None of the LPL receptor family genes showed MSC-specific expression.

To confirm whether *LPA<sub>3</sub>* was expressed selectively in TSCs, we have performed *in situ* hybridization in NMJs of adult rat soleus. *In situ* hybridization was carried out for the longitudinal sections of adult rat soleus muscle, according to a whole mount *in situ* hybridization protocol. With antisense probe, strong signals were detected specifically in the cell bodies located on top of motor nerve terminals, but not in nerve terminals themselves, axons, or postsynaptic areas (Fig. 2B, C). No signal was detected using sense probe as a negative control (Fig. 2A).

#### IV. Discussion

The present study demonstrated the specific expression of *LPA<sub>3</sub>* in TSCs present in NMJs of adult rat soleus, with single-cell based RT-PCR as well as *in situ* hybridization. We have also demonstrated by RT-PCR that none of the LPL receptor family genes showed MSCs-specific expression.

In the peripheral nervous system, there are two types of Schwann cells, TSCs and MSCs. Both types of Schwann



**Fig. 2.** *In situ* hybridization in adult rat soleus using *LPA<sub>3</sub>* sense cRNA probe as a negative control (A) and *LPA<sub>3</sub>* antisense cRNA probe (B–D). Cell bodies of terminal Schwann cells on the rat soleus were stained (black arrowheads in B, C, D). Unstained axons were visualized under a differential interference contrast microscope (black arrows). D is a higher magnification image of the area indicated by a rectangle in C. Longitudinal and horizontal stripes were muscle fibers. Bar=50  $\mu$ m (A), 20  $\mu$ m (B), 40  $\mu$ m (C), and 10  $\mu$ m (D).

cells share the same lineage of cell origin, that is, neural crest cells, but behave in a different manner after maturation [8]. MSCs are known to contribute to axonal outgrowth and guidance in the regeneration process following nerve injury [6, 19]. On the other hand, TSCs appear to play pivotal roles in maintaining the structure and function of NMJs as well as in the repair process [6, 21]. Recently, Son and Thompson and Son *et al.* demonstrated that processes of TSCs extend to the neighboring NMJ following partial denervation and the axonal regeneration was observed along the processes of TSCs [16, 18]. The mode of nerve sprouting at NMJ is different from that at the nodes of Ranvier following injury. The regenerating sprouts which emerge from the nodes of Ranvier always extend distally within the basal lamina of MSCs [14]. On the other hand, the terminal nerve sprouts penetrate their own Schwann cell basal lamina tubes and grow toward the neighboring NMJ [17]. These different modes of nerve sprouting may reflect different properties of Schwann cell resources, i.e., TSCs and MSCs. However, there are few studies on the comparative aspects of physiological as well as pathological characteristics of TSCs and MSCs. We previously devised a method of isolating and collecting TSCs [13] and this method enabled us to analyze gene expression in TSCs by RT-PCR. Here, we have applied a similar method to elucidate the expression of LPL receptor family genes in TSCs and compared the expression between TSCs and MSCs.

LPL is a phospholipid signaling molecule that has intercellular signaling pathways and has the broad biological functions such as cell proliferation, myogenesis, stress fiber formation, neurite retraction and increase of  $Ca^{2+}$  [9–12]. The LPL receptor family of G-protein coupled receptors comprises two groups: LPA receptors and sphingosine-1-phosphate (S1P) receptors. LPA receptors consist of  $LPA_1$  (Edg-2/lp<sub>A1</sub>/vzg-1/rec1.3),  $LPA_2$  (Edg-4/lp<sub>A2</sub>) and  $LPA_3$  (Edg-7/lp<sub>A3</sub>), and S1P receptors include  $SIP_1$  (Edg-1/lp<sub>B1</sub>),  $SIP_2$  (Edg-5/lp<sub>B2</sub>/AGR16/H218),  $SIP_3$  (Edg-3/lp<sub>B3</sub>),  $SIP_4$  (Edg-6/lp<sub>B4</sub>), and  $SIP_5$  (Edg-8/lp<sub>B5</sub>/nrg-1). Weiner and Chun studied the expression of LPL receptors in Schwann cells (SC) *in vivo* and *in vitro* using total RNA by Northern blot analysis, and reported that  $LPA_1$  was expressed at high level by SCs *in vivo* and *in vitro*, whereas  $SIP_1$  was only expressed in SCs *in vivo*. Neither  $LPA_2$  nor  $SIP_2$  was detectable, although  $SIP_3$  was expressed in SCs *in vivo* and *in vitro* [23]. On the contrary, we were unable to detect the expression of  $SIP_1$  in isolated MSCs. This difference might be caused by the sampling method, because Weiner and Chun recognized expression of  $SIP_1$  *in vivo*. Considering the fact that MSCs and TSCs behave differently after nerve injury, it would be worth studying changes of expression of each member of LPL gene family after nerve injury. Those studies are currently underway.

In conclusion, we studied the differential expression of LPL receptor gene family in TSCs and MSCs at single cell-based RT-PCR and found that  $LPA_3$  is specifically expressed in TSCs as confirmed by *in situ* hybridization. This differential expression of LPL receptors in TSCs and MSCs may

underlie the behavior of each type of Schwann cells after nerve injury.

## V. Acknowledgments

This work was supported in part by a Grant-in-Aid for Scientific Research (B) (to SO) and by a Grant-in-Aid for Young Scientists (B) (to TY) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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