

## Roles of Layilin in Regulation of Low-Density Lipoprotein Receptor in Malignant Glioma Cells

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(Received for Publication: August 19, 2020)

### Abstract

**Objective:** Layilin is a type I transmembrane protein that possesses a C-type lectin motif in its extracellular domain, and whose functions have not yet been completely understood. Previously, we reported that layilin was highly expressed in malignant glioma cells. In this study, we investigated whether layilin was functionally related to low-density lipoprotein receptor (LDLR) in malignant glioma cells because one of the characteristics of malignant glioma cells is the high expression of LDLR.

**Methods:** Under layilin-knockdown (KD) conditions in A172 cells, a malignant glioma cell line, we measured LDLR mRNA and protein levels by quantitative polymerase chain reaction and western blotting, respectively. Furthermore, we measured LDL uptake in layilin-KD cells by LDL uptake assay.

**Results:** Even though the amounts of mRNA for LDLR were unaffected by layilin-KD, the amounts of LDLR protein were significantly increased by layilin-KD 48 h after transfection with small interfering RNAs for layilin ( $p < 0.05$ ). Accordingly, LDL uptake was increased by layilin-KD ( $p < 0.05$ ).

**Conclusion:** Our data suggest a novel function of layilin, that is, down-regulation of LDLR at the protein level.

### Key words

Layilin, LDL, LDL receptor

### 1. Introduction

Layilin is a type I transmembrane protein that possesses a C-type lectin motif in its extracellular domain<sup>1</sup>. Initially, layilin was reported to function as a cell surface receptor for hyaluronic acid<sup>2</sup>, and its intracellular part was reported to interact with cytoskeletal proteins such as talin, merlin, and radixin<sup>1,3</sup>. However, its functions have not yet been completely understood. Previously, we reported that layilin was more strongly expressed in malignant glioma cells than in normal astrocytes, and it mediated epithelial-mesenchymal transition (EMT)-like change to ac-

quire and promote high invasiveness through SNAIL in malignant glioma cells<sup>4</sup>. In other words, the strong expression of layilin could be one of the characteristics of malignant glioma cells.

Also, another characteristic of malignant glioma cells is the high expression of low-density lipoprotein receptor (LDLR), in that it has been reported that normal glia cells rarely express LDLR, but multiple malignant glioma cell lines strongly express LDLR<sup>5</sup>. Recently, based on the high expression of LDLR, use of LDLR as a therapeutic target has been intensively investigated<sup>5-7</sup>. As is well known, LDLR, a cell surface protein with a molecular weight of 160 k, cap-

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tures serum LDL particles and delivers them into cells<sup>8,9</sup>). Presently, it is unclear why LDLR is highly expressed in malignant glioma cells.

As their characteristics mentioned above, both layilin and LDLR are highly expressed in malignant glioma cells, and we are unaware of any report on the functional relations between these two molecules. Therefore, in this study, we investigated whether layilin affected the amounts of LDLR and LDL uptake in malignant glioma cells.

## 2. Materials and methods

### 2.1 Cell culture

A172 cells of a human malignant glioma cell line (DS Pharma Biomedical Co., Ltd., Osaka, Japan), derived from glioblastoma (WHO grade IV), were cultured in an RPMI1640 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 µg/ml streptomycin (Sigma-Aldrich). The cells were cultured at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>.

### 2.2 Transfection of A172 cells with small interfering RNA (siRNA)

A172 cells were transfected with 200 pmol siRNA per 100-mm dish using lipofectamine<sup>®</sup> RNAi-MAX (Invitrogen, Carlsbad, CA, USA). The two kinds of siRNA for human layilin mRNA (siL-1; nucleotides 1019–1043, AAGCTGCCTTGAATCTGGCCTACAT and siL-2; nucleotides 1564–1598, CACAGAAGGTC TATGAACAAGCTTA in NM\_001258390.1; Invitrogen) were used to avoid off-target effects. As a negative control, A172 cells were similarly transfected with a control siRNA (Stealth RNAi<sup>™</sup> Negative Control Medium GC Duplex, Invitrogen).

### 2.3 RNA extraction and reverse transcription

RNA extraction and the following reverse transcription were performed as described previously<sup>10</sup>. Briefly, total RNA samples extracted from A172 cells using RNeasy<sup>®</sup> (Qiagen, Venlo, The Netherlands) were subjected to reverse transcription using a High Capacity cDNA Reverse Transcription Kits (Life Technologies, Rockville, MD, USA) according to the manufacturers' instructions.

### 2.4 Quantitative PCR (qPCR)

qPCR was performed by an ABI Prism<sup>®</sup> 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). To estimate the amounts of mRNA

for layilin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), a mixture of 2 µg of the total RNA sample-derived cDNA, 300 nM each of forward and reverse primers, and Power SYBR<sup>®</sup> Master Mix (Applied Biosystems) was subjected to qPCR. Nucleotide sequences of the primers are as follows: layilin: 5'-CACAGCCTGCCAGGACCTTTA and 5'-TGCACCGGTCATCATTCCA, and GAPDH: 5'-TGGTATGGTGGAAGGACTCA and 5'-ATGCCAGTGAGCTTCCGTT. To estimate the amounts of mRNA for LDLR, a mixture of 2 µg of the total RNA sample-derived cDNA, a solution of TaqMan<sup>®</sup> Gene Expression Assays (Hs01092524\_m1, Applied Biosystems), and TaqMan<sup>®</sup> Gene Expression Master Mix (Applied Biosystems) was subjected to qPCR. The thermal cycle conditions used were 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 60 sec.

### 2.5 Protein extraction from cultured cells

Cultured cells were collected and washed in phosphate-buffered saline (PBS) and were sonicated in a lysis buffer containing 20 mM Tris-HCl, 250 mM NaCl, 1% NP-40, 1 mM dithiothreitol, and a protease inhibitor cocktail (Roche, Basel, Switzerland). After centrifugation, the collected supernatants were used as whole cell protein samples. Nuclear and cytosolic fractions were prepared with a Nuclear/Cytosolic Fractionation Kit (CELL BIOLABS INC, San Diego, CA, USA).

### 2.6 Western blotting

Western blotting was performed as described previously<sup>10</sup>. The protein samples, which were separated by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), were transferred onto polyvinylidene difluoride membranes. Goat polyclonal antibodies against human layilin (R&D Systems, Inc., Minneapolis, MN, USA), mouse monoclonal antibodies against human LDLR (Santa Cruz Biotechnology, Inc., Dallas, TX, USA), and β-actin (Sigma) were used as primary antibodies. Horseradish peroxidase-conjugated rabbit anti-goat IgG antibodies (Agilent/DAKO, Santa Clara, CA, USA) and anti-mouse IgG antibodies (Cell Signaling Technology Inc., Danvers, MA, USA) were used as secondary antibodies, respectively. The bound antibodies were visualized using ImmunoStar<sup>®</sup> LD (FUJI FILM, Osaka, Japan).

### 2.7 Measurement of LDL uptake

The LDL uptake was assessed by LDL Uptake

Cell-Based Assay Kit (Cayman Chemical, Ann Arbor, MI, USA). Specifically,  $8.0 \times 10^4$  A172 cells, transfected with siL-1, siL-2, or siC in an RPMI 1640 medium containing 10% FBS, were seeded onto 35-mm glass-based dishes (ASAHI GLASS, Tokyo, Japan). At 48 h later, the culture medium was replaced with a serum-free RPMI 1640 medium containing LDL-DyLight™ 550. After 6 h, the A172 cells were fixed in PBS containing 4% paraformaldehyde. Nuclei were stained with DAPI (Wako Pure Chemical Industries, Osaka, Japan). The uptaken LDL-DyLight™ 550 and nuclei were visualized by fluorescence microscopy (BZ-X710, KEYENCE, Jena, Germany). Fluorescent brightness of the LDL-DyLight™ 550 and the number of pixels were measured using a BZ-X Analyzer (KEYENCE).

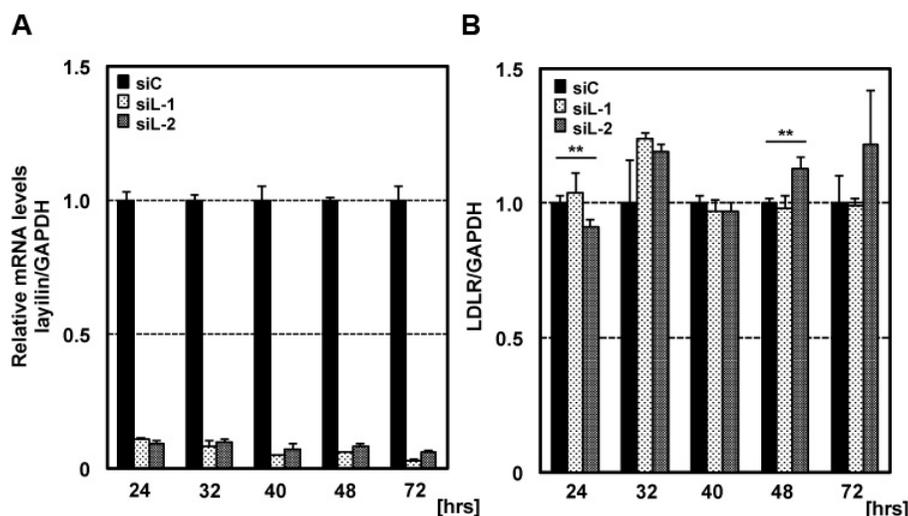
## 2.8 Statistical analysis

Statistically significant differences were calculated with the Student *t*-test. A value of  $p < 0.05$  was considered to be statistically significant.

## 3. Results

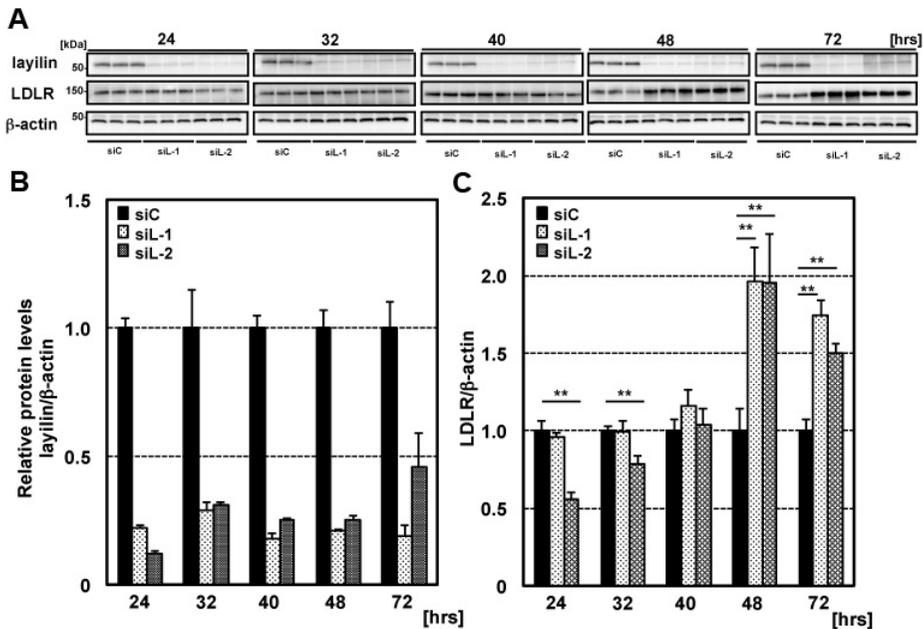
### 3.1 Effects of layilin-KD on the amounts of LDLR mRNA and proteins

To assess the effects of layilin on mRNA and protein levels of LDLR in A172 cells, we prepared layilin-KD A172 cells using two kinds of siRNAs for layilin. After 24, 32, 40, 48, and 72 h, we prepared total RNA and protein samples from the cells. The results showed that the mRNA expression of layilin was completely suppressed by the two siRNAs through the experimental time of 24–72 h after transfection (Fig. 1A). In the measurement of mRNA for LDLR, we found that the amounts of mRNA for LDLR were unaffected by layilin-KD through the experimental time of 24–72 h after transfection (Fig. 1B). Next, our results showed that the protein amounts of layilin were decreased to approximately 1/4 by the two siRNAs through the experimental time of 24–72 h after transfection (Fig. 2A). The protein amounts of LDLR were found to be significantly decreased at 24 and 32 h after transfection with siL-2 but not with siL-1. This decrease may be due to off-target effects. Interestingly, the protein amounts of LDLR were found to be significantly increased by



**Fig. 1.** Effects of layilin-KD on the expression of LDLR in A172 cells

A172 cells were transfected with control siRNA (siC) and two kinds of layilin siRNA (siL-1 and siL-2). RNA extracted from the cells was used for reverse transcription and subsequent qPCR to estimate the amounts of mRNA for layilin, LDLR, and GAPDH. Measured mRNA levels of layilin and LDLR were corrected using those of GAPDH (A and B, respectively). In each panel, the average of the corrected mRNA levels in the samples transfected with siC was defined as 1.0 ( $n=3$  in each condition). Mean values with SD are shown.



**Fig. 2.** Effects of layilin-KD on the amounts of LDLR protein in A172 cells

The protein samples, which were extracted from the whole cells, were subjected to western blotting (A). The intensity of the detected bands was measured by densitometry. The measured intensities of the layilin and LDLR bands were normalized using that of  $\beta$ -actin bands (B and C, respectively). The average of the normalized intensity of the layilin and LDLR bands in the “siC” samples was defined as 1.0.

nearly 2-fold at 48 h after siRNA transfection, and a significant increase was still observed at 72 h after siRNA transfection (Fig. 2B). These data suggest that layilin acts to down-regulate the amounts of LDLR at the protein level but not at the mRNA level.

### 3.2 Effects of layilin-KD on LDL uptake

Generally, the major function of LDLR is uptake of LDL. Therefore, we investigated whether the observed increase in LDLR proteins actually increased the uptake of LDL by the cells. The results showed that the number of moderate and high fluorescence intensity pixels, which indicate uptaken fluorescent LDL molecules, was increased by approximately 3-fold (Fig. 3A, B). These data indicate that layilin is involved in regulating LDL uptake.

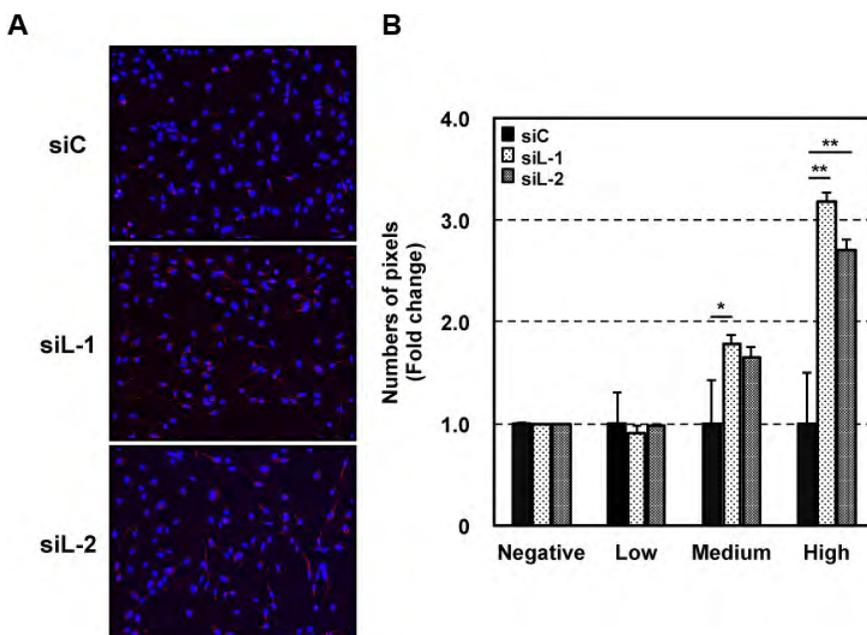
## 4. Discussion

Our findings are summarized as follows: 1) layilin down-regulates LDLR at the protein level but not at the mRNA level; and 2) layilin is involved in regulating LDL uptake.

Regarding the first point, as layilin-KD in-

creased the amounts of LDLR protein, although layilin-KD did not affect LDLR transcription, we speculate that layilin suppresses LDLR protein stability. Generally, the amounts of LDLR are regulated at the rate of LDLR degradation that is promoted by pro-protein convertase subtilisin/kexin type 9 (PCSK9) and inducible degrader of the LDL-receptor (IDOL)<sup>11,12</sup>. Thus, layilin may suppress LDLR protein stability by directly acting on LDLR or by up-regulating the expression levels and activity of PCSK9 and IDOL. This point needs to be clarified in the future.

Also, as multiple malignant glioma cell lines strongly express LDLR, LDLR has attracted attention as a therapeutic target of malignant gliomas<sup>5,11,12</sup>. Notably, LDL-based nanoparticles containing anticancer drugs have been developed. Furthermore, polyester-based nanoparticles with LDLR-binding peptides have been developed as a drug transporter. Drug delivery systems targeting LDLR have great potential for glioma therapy<sup>13-15</sup>. Therefore, an important next step would be to clarify the mechanism by which layilin regulates LDLR proteins in malignant glioma



**Fig. 3.** Effects of layilin-KD on LDL uptake.

First,  $8.0 \times 10^4$  A172 cells transfected with siL-1, siL-2, or siC in an RPMI 1640 medium containing 10% FBS were seeded onto 35-mm glass-based dishes. Then, 48 hours later, the cells were cultured in a serum-free RPMI 1640 medium containing LDL-DyLight<sup>TM</sup> 550 for 6 h. After the cells were fixed in PBS containing 4% paraformaldehyde, nuclei were stained with DAPI. Finally, LDL-DyLight<sup>TM</sup> 550 (red) and the nuclei (blue) were visualized using fluorescence microscopy (A). Fluorescent brightness of LDL-DyLight<sup>TM</sup> 550 and the numbers of the pixels were measured using a BZ-X Analyzer. Fluorescent brightness values of 0–23, 24–100, 101–177, and 178–255 were defined as “Negative”, “Low”, “Medium”, and “High”, respectively (B).

cells.

Regarding the second point, as layilin-KD increased LDL uptake, this suggested that layilin suppressed LDL uptake by down-regulating the amount of functional LDLR. Recently, several studies have been conducted on the roles of LDL in cancer cells. Increased LDLR expression and the consequent increased uptake of LDL cholesterol have been reported in breast cancer cells<sup>16–18</sup>. Furthermore, LDL cholesterol and 27-hydroxycholesterol, a metabolite of cholesterol, have been reported to promote proliferation and migration associated with EMT<sup>16–19</sup>. Similar results focusing on 27-hydroxycholesterol have been reported in glioblastoma cell lines such as U118MG and U251MG<sup>20</sup>. These studies and the present study suggest that layilin may suppress proliferation and migration associated with EMT by the down-regulation of LDLR in malignant glioma. However, we previously reported that layilin was es-

sential for TNF- $\alpha$ -induced EMT of renal tubular epithelium-derived cancer cells<sup>21</sup>. Moreover, we recently reported that layilin induced SNAIL-mediated EMT-like change to enhance the invasive ability of glioblastoma cell lines such as A172 cells<sup>4</sup>. Our series of studies have provided evidence that layilin promotes EMT and invasive ability in cancer cells. Remarkably, although layilin suppresses LDLR protein levels, the down-regulation of LDLR does not seem to suppress LDL-mediated EMT. This point is a new problem raised by the present study. Future studies on the role of LDL/LDLR in layilin-mediated EMT and also the role of layilin in LDL/LDLR-mediated EMT are needed to resolve this problem.

## 5. Conclusion

Our data indicate that layilin down-regulates LDLR at the protein level to suppress LDL uptake.

### Acknowledgments

We thank Ms. Yuka Sawada and Ms. Michiyo Yokoyama for their technical assistance.

### Conflicts of interest

Atsuko Kamijo-Ikemori reports receiving grants from Astellas Pharma Inc., Daiichi Sankyo Company, Limited, Merck & Co., Inc., Mitsubishi Tanabe Pharma Co., Ltd., Otsuka Pharmaceutical Co., Ltd., Pfizer Inc., and Sanwa Kagaku Kenkyusho Co., Ltd., outside the submitted work. Yugo Shibagaki reports receiving grants from Alexion Pharmaceuticals, Inc., Astellas Pharma Inc., Baxter Limited, Bayer Yakuhin, Ltd., Chugai Pharmaceutical Co., Ltd., Kyowa Kirin Co., Ltd., Otsuka Pharmaceutical Co., Ltd., Sumitomo Dainippon Pharma Co., Ltd., Takeda Pharmaceutical Company Limited, and Teijin Pharma Limited, outside the submitted work. Tomohiro Kato reports receiving grants from Astellas Pharma Inc., Chugai Pharmaceutical Co., Ltd., Daiichi Sankyo Company, Limited, Pfizer Inc., Sanofi K.K., and Takeda Pharmaceutical Company Limited, outside the submitted work. Shu Ushimaru, Mitsumi Arito, Atsuhiko Tsutiya, Toshiyuki Sato, Kazuki Omoteyama, Masaaki Sato, Naoya Suematsu, and Manae S. Kurokawa have nothing to disclose.

### References

- 1) Borowsky ML, Hynes RO. Layilin, a novel talin-binding transmembrane protein homologous with C-type lectins, is localized in membrane ruffles. *J Cell Biol* 1998; 143: 429–442.
- 2) Bono P, Rubin K, Higgins JM, et al. Layilin, a novel integral membrane protein, is a hyaluronan receptor. *Mol Biol Cell* 2001; 12: 891–900.
- 3) Bono P, Cordero E, Johnson K, et al. Layilin, a cell surface hyaluronan receptor, interacts with merlin and radixin. *Exp Cell Res* 2005; 308: 177–187.
- 4) Kaji T, Arito M, Tsutiya A, et al. Layilin enhances the invasive ability of malignant glioma cells via SNAI1 signaling. *Brain Res* 2019; 1719: 140–147.
- 5) Maletínská L, Blakely EA, Bjornstad KA, et al. Human glioblastoma cell lines: levels of low-density lipoprotein receptor and low-density lipoprotein receptor-related protein. *Cancer Res* 2000; 60: 2300–2303.
- 6) Ahmad F, Sun Q, Patel D, et al. Cholesterol Metabolism: a Potential therapeutic target in glioma.

- blastoma. *Cancers (Basel)* 2019; 11: 146. doi: 10.3390/cancers11020146.
- 7) Pirmoradi L, Seyfizadeh N, Ghavami S, et al. Targeting cholesterol metabolism in glioblastoma: a new therapeutic approach in cancer therapy. *J Investig Med* 2019; 67: 715–719.
- 8) Sudhof TC, Goldstein JL, Brown MS, et al. The LDL receptor gene: a mosaic of exons shared with different proteins. *Science* 1985; 228: 815–822.
- 9) Brown MS, Goldstein JL. A receptor-mediated pathway for cholesterol homeostasis. *Science* 1986; 232: 34–47.
- 10) Asano K, Arito M, Kurokawa MS, et al. Secretion of inflammatory factors from chondrocytes by layilin signaling. *Biochem Biophys Res Commun* 2014; 452: 85–90.
- 11) McNutt MC, Lagace TA, Horton JD. Catalytic activity is not required for secreted PCSK9 to reduce low density lipoprotein receptors in HepG2 cells. *J Biol Chem* 2007; 282: 20799–20803.
- 12) Zhang CP, Tian Y, Zhang M, et al. IDOL, inducible degrader of low-density lipoprotein receptor, serves as a potential therapeutic target for dyslipidemia. *Med Hypotheses* 2016; 86: 138–142.
- 13) Wang Z, Duan X, Lv Y, et al. Low density lipoprotein receptor (LDLR)-targeted lipid nanoparticles for the delivery of sorafenib and Dihydroartemisinin in liver cancers. *Life Sci* 2019; 239: 117013. doi:10.1016/j.lfs.2019.117013.
- 14) Zhang B, Sun X, Mei H, et al. LDLR-mediated peptide-22-conjugated nanoparticles for dual-targeting therapy of brain glioma. *Biomaterials* 2013; 34: 9171–9182.
- 15) Di Mauro PP, Cascante A, Brugada Vilà P, et al. Peptide-functionalized and high drug loaded novel nanoparticles as dual-targeting drug delivery system for modulated and controlled release of paclitaxel to brain glioma. *Int J Pharm* 2018; 553: 169–185.
- 16) Rotheneder M, Kostner GM. Effects of low- and high-density lipoproteins on the proliferation of human breast cancer cells in vitro: Differences between hormone-dependent and hormone-independent cell lines. *Int J Cancer* 1989; 43: 875–879.
- 17) De Gonzalo-Calvo D, López-Vilaró L, Nasarre L, et al. Intratumor cholesteryl ester accumulation is associated with human breast cancer pro-

- liferation and aggressive potential: a molecular and clinicopathological study. *BMC Cancer* 2015; 15: 460. doi:10.1186/s12885-015-1469-5.
- 18) Gallagher EJ, Zelenko Z, Neel BA, et al. Elevated tumor LDLR expression accelerates LDL cholesterol-mediated breast cancer growth in mouse models of hyperlipidemia. *Oncogene* 2017; 36: 6462–6471.
- 19) Cruz P, Epuñán MJ, Ramírez ME, et al. 27-hydroxycholesterol and the expression of three estrogen-sensitive proteins in MCF7 cells. *Oncol Rep* 2012; 28: 992–998.
- 20) Liu L, Li MY, Xing Y, et al. The oncogenic roles of 27-hydroxycholesterol in glioblastoma. *Oncol Lett* 2019; 18: 3623–3629.
- 21) Adachi T, Arito M, Suematsu N, et al. Roles of layilin in TNF- $\alpha$ -induced epithelial-mesenchymal transformation of renal tubular epithelial cells. *Biochem Biophys Res Commun* 2015; 467: 63–69.