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Constitutive suppression of PRL-3 inhibits invasion and proliferation of gastric cancer cell *in vitro* and *in vivo*

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Abstract

Objective: Overexpression of PRL-3 (phosphatase of regenerating liver-3) has been reported to implicate in tumor progression and metastasis of gastric carcinoma. Here we examined what alterations will occur in the phenotype of gastric cancer cells *in vitro* and *in vivo* when PRL-3 expression was knocked down.

Methods: We constructed small interfering RNA (siRNA) expressing vector which stably interfered PRL-3 expression and transfected into SH101-P4 cells which express the highest *PRL-3* mRNA levels among 13 gastric cancer cell lines. The new SH101-P4 subclones, in which PRL-3 was stably reduced, were established and their *in vitro* growth, motility and abilities of liver metastasis from the injected spleen *in vivo* were analyzed.

Results: PRL-3 knockdown effectively suppressed invasion and growth of SH101-P4 cells *in vitro*. Liver metastasis *in vivo* was significantly decreased when PRL-3 expression was suppressed. The primary tumor size in the injected spleen was tended to be smaller in PRL-3 knockdown clones than in the controls.

These findings suggest that PRL-3 expression may contribute not only to the establishment of metastasis but also to the growth of primary foci of human gastric cancer. Therefore, PRL-3 may be one of the target molecules in the anti-gastric cancer therapy.

Introduction

Although gastric cancer has gradually decreased in prevalence, it still accounts for a large portion of the cancer-related deaths in Japan. Like other cancers, growth and metastatic abilities are main lethal processes which determine the malignancy of the gastric cancer. Protein tyrosine phosphatases (PTPs) play a fundamental role in regulating diverse proteins that participate essentially in every aspect of cellular physiology and pathogenic processes [1]. PRL-1, -2 and -3 represent a novel class of PTP superfamily members that are ubiquitously expressed in various organs and are upregulated in the regenerating liver [2, 3]. Interestingly, overexpression of PRL-1 and PRL-2 has been found to transform mouse fibroblasts and hamster pancreatic epithelial cells in culture and to promote tumor growth in nude mice [2, 3]. Similarly, PRL-3 enhances the growth of human embryonic kidney fibroblasts [4]. In addition to growth ability, overexpression of PRL-3 in Chinese hamster ovary (CHO) cells promotes *in vitro* cell motility and invasion [5]. Several other reports have been published suggesting the involvement of PRL-3 in cell motility not only in the experimental *in vitro* and *in vivo* models but also in clinical human cancers, such as malignant melanoma, breast cancer, pancreatic cancer, ovarian cancer and nasopharyngeal cancer [6-11].

Previously, we have examined gastric and colon cancer tissues and found that the higher levels of *PRL-3* mRNA were correlated with distant metastases [12, 13]. Moreover, in the colon cancer model, we have shown the effect on the *in vivo* metastasis by using the siRNA methods which *PRL-3* mRNA expression was transiently suppressed [13]. Since there were only few reports on the role of PRL-3 in gastric

cancer metastasis *in vitro* and *in vivo*, we decided to conduct this study using gastric cancer cells.

In this report, to evaluate the long-term effect of inhibition of PRL-3 expression on the cancer cell, we constructed new subclones of SH101-P4 gastric cancer cell line, in which *PRL-3* expression was stably reduced by transfecting the siRNA PRL-3 vector. Using PRL-3 knockdown cells, we examined how PRL-3 contributed cell proliferation and invasion ability *in vitro*. Furthermore, the role of PRL-3 in metastasis *in vivo* by using hepatic metastasis model was investigated.

Materials and Methods

Cell Cultures

Thirteen cell lines derived from human gastric carcinoma were used in this study. Eight gastric cancer cell lines of the HSC series (HSC-57, tubular adenocarcinoma; HSC-42, HSC-58 and HSC-59, poorly differentiated adenocarcinoma; HSC-40, HSC-44, HSC-45 and HSC-60, signet ring cell carcinoma) and SH101-P4 (tubular adenocarcinoma) were established by one of the authors (K.Y.) [14, 15]. Three cell lines of the MKN series (MKN-1, adenosquamous cell carcinoma; MKN-7 and MKN-74, tubular adenocarcinoma) were provided by Dr. T. Suzuki (Fukushima Medical University, Fukushima) [16, 17]. The TMK-1 cell line (poorly differentiated adenocarcinoma) was a gift from Dr. W. Yasui (Hiroshima University, Hiroshima) [18]. The cells were maintained in RPMI-1640 (GIBCO Life Technologies, Inc., Grand Island, NY) supplemented with 1 mM L-glutamine, 10% fetal bovine serum (FBS;

GIBCO Life Technologies, Inc.) and 12.5 μg/ml gentamycin (Sigma, St. Louis, MO) under humidified 5% CO_2 in air at 37℃.

RNA Extraction and Quantitative Reverse Transcription-polymerase Chain Reaction (RT-PCR) Analyses

Total RNAs from gastric cancer cell lines (1 x 10^6) were isolated using RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Quantitative RT-PCR was performed with a SYBR Green real-time quantitative RT-PCR assay kit (Qiagen) on RNA extracts obtained from gastric cancer cell lines using an ABI PRISM 7700 Sequence Detection System (ABI, Carlsbad, CA). The primer set used for RT-PCR amplification of the *PRL-3* was as follows: forward, 5'-GGG ACT TCT CAG GTC GTG TC-3'; reverse, 5'-AGC CCC GTA CTT CTT CAG GT-3'. As an internal control, the levels of *β-actin* expression were also analyzed (forward, 5'-CCA CGA AAC TAC CTT CAA CTC C-3'; reverse, 5'-TCA TAC TCC TGC TGC TTG CTG ATC C-3'). A master mix (50 μl) of the following reaction components was prepared to the indicated end concentration: 25 μl of 2 x QuantiTect SYBR Green RT-PCR Master Mix, 10 ng of total RNA, 1 mM of the primer pair, reverse transcriptase, and 0.5 μl of QuantiTect RT Mix. They were mixed and amplified for 40 or 45 cycles with the following regimen: reverse transcription at 50˚C for 30 min; denaturation at 94˚C for 30 sec; annealing at 60˚C for 30 sec; extension at 72˚C for 1 min.

Plasmid Construction and Transfection

Human *PRL-3* cDNA sequence was obtained from NCBI website (Accession No. NM_032611). The siRNA sequence used for PRL-3 gene silencing was designed based on published data as following: sense: 5'- GAT CCG TGA CCT ATG ACA AAA CGC TTC AAG AGA GCG TTT TGT CAT AGG TCA CTT TTT GGA AA -3', antisense: 5'-AGC TTT TCC AAA AAG TGA CCT ATG ACA AAA CGC TCT TTG AAG CGT TTT GTC ATA GGT CAC G-3' [13]. As a negative control, siRNA targeted GFP was used based on the manufacturer's instructions as following: sense: 5'-GAT CCG GTT ATG TAC AGG AAC GCA TTC AAG AGA TGC GTT CCT GTA CAT AAC CTT TTT GGA AA-3', antisense: 5'-GCC AAT AGA TGT CCT TGC GTA AGT TCT CTA CGC AAG GAC ATG TAT TGG AAA AAC CTT TTC GA-3'. These sequences were cloned between *Bam*HI and *Hind*III restriction site in pSilencer 3.1-HI/neo plasmids (Applied Biosystems, Carlsbad, CA) according to the manufacturer's instructions. Breifly, sense siRNA and antisense siRNA oligonucleotides were mixed with annealing solution, and incubated 90 ^{o}C for 3 minutes and 37 ^{o}C for 1 hour to form the hairpin siRNA template. Then, annealed siRNA template insert were ligated into the pSilencer vector using T4 DNA ligase by incubating for 3 hours at room temperature. All constructs were sequenced to confirm that the oligonucleotides were correctly inserted.

Before transfection procedure, SH101-P4 cells were cultured on 24-well plates in RPMI-1640 with 10% FBS until 75% confluence. At first, 1μL of Oligofectamine (Invitrogen, Carlsbad, CA) and 1 μg of the pSilencer containing PRL-3 siRNA sequence or GFP siRNA sequence or vector DNA were mixed and diluted to a final volume of 20 μl and incubated for 15 minutes at room temperature. Then 1 μl of

complexes were added to the each 24-well plate cells and incubated in a CO_2 incubator. For stable transfection, the SH101-P4 pSilencer vectors transfected cell lines were selected with 0.5 mg/ml of G-418 (GIBCO Life Technologies, Inc.) and cloned by limiting dilution.

Cell Invasion Assay

Cell invasion assay was performed using Transwells (6.5 μm in diameter, polycarbonate membrane, 8 μm pore size) coated with extracellular matrix gel obtained from Chemicon (Temecula, CA). Each 1 x 10^5 cells was placed in the upper chamber with 0.5 ml serum-free medium, whereas the lower chamber (24-well plate) was loaded with 1 ml of medium containing 10% FBS. After 48 hours of incubation at 37°C with 5% CO_2, the cells were fixed with 4% paraformaldehyde and then counterstained with hematoxylin. The cells that had migrated into the lower chamber were observed and counted under a light microscope. For each group, the average of quintuplicate samples and their standard error was calculated.

Cell Proliferation Assay

Cell growth and survival of each PRL-3 siRNA vector transfected cells were determined using the Premix WST-1 Cell Proliferation Assay System (Takara Biochemicals, Tokyo, Japan), as described elsewhere [13]. Briefly, 1 x 10^5 of each cells (100 μl/well) maintained in phenol-red-free medium for 24 hours were inoculated into 96-well microtiter plates and cultured at 37°C for 48 hours. After incubation, 10 μl of Premix WST-1 was added to each microculture well, and the plates were incubated for

30 minutes at 37^{o}C, thereafter absorbance was read at 450 nm using a microplate reader. Absorbance in the cells with no treatment was considered to be 100%. For each group, the average of quintuplicate samples and their standard error was calculated.

Hepatic Metastasis Model

Subconfluent cultures of the gastric cancer cells were harvested and washed three times with phosphate buffered saline (PBS). SH101-P4 gastric cancer cells (5 x 10^5 cells in 0.1 ml of PBS) were injected into the spleen of the eight-week-old BALB/cA *Jcl*-nu female mice under each condition (nontreated group, SH101-P4 [n = 5]; vector DNA transfected group, SH101-P4^{vector} [n = 5]; *PRL-3* siRNA vector transfected group, SH101-P4$^{\lambda PRL\text{-}3}$ [n = 5]). After 28 days, mice were sacrificed under anesthesia and the liver and the spleen were taken, fixed with 10% buffered formalin (pH 7.4) then processed for histological examination. Tumor growth was monitored by measuring the number of metastatic foci in 6 sections per liver.

Statistical Analysis

Student's two sided *t*-test was used to compare values of test and control samples. *P*-values less than 0.05 were considered statistically significant. All statistical analyses were performed using StatView software (SAS Institute Inc., Cary, NC).

Results

We first examined the mRNA expression levels of *PRL-3* in 13 gastric cancer cell

lines using quantitative RT-PCR (fig. 1). Among 13 gastric cancer cell lines that expressed *PRL-3* mRNA with various levels, SH101-P4 showed the highest level of *PRL-3* mRNA expression while MKN-7 cells revealed the lowest. There were several reports showing the correlations between overexpression of PRL-3 and cancer metastasis in several cancers [7, 8, 13]. Therefore, we chose SH101-P4 for the following RNA interference studies as a parental cell.

In order to assess the phenotypes of tumor cells in which PRL-3 expression was constitutively suppressed, we established two PRL-3-downregulated clones (SH101-P4$^{\Delta PRL\text{-}3\text{-}1}$ and SH101-P4$^{\Delta PRL\text{-}3\text{-}2}$) and clonal controls (SH101-P4^{vector} and SH101-P4$^{\Delta GFP}$). The *PRL-3* mRNA expression in SH101-P4$^{\Delta PRL\text{-}3\text{-}1}$ and SH101-P4$^{\Delta PRL\text{-}3\text{-}2}$ were reduced by 95% and 50%, respectively, compared with parental SH101-P4. No difference among the parental SH101-P4, SH101-P4^{vector} and SH101-P4$^{\Delta GFP}$ cells was observed in *PRL-3* mRNA expression levels (fig. 2A). This RNA interference mediated effect was specific, as *β-actin* levels did not differ significantly among the treated cells and controls. SH101-P4$^{\Delta PRL\text{-}3\text{-}1}$ subclone was used in the following experiments.

To evaluate the constitutive knockdown effects of PRL-3 expression on gastric cancer cell invasion *in vitro*, we performed Transwell invasion assay. As shown in figure 2B, the average percentage of the cells migrated were as follows: SH101-P4, 100 ± 12.0%; SH101-P4$^{\Delta PRL\text{-}3\text{-}1}$, 23.9 ± 6.5%; SH101-P4^{vector}, 95.0 ± 13.0%; SH101-P4$^{\Delta GFP}$ and 98.5 ± 11.1%. Cell invasion *in vitro* was significantly suppressed in SH101-P4$^{\Delta PRL\text{-}3\text{-}1}$ in comparison with SH101-P4 ($P = 0.0024$).

Next, we attempted to analyze the effect of PRL-3 knockdown on gastric cancer cell growth *in vitro*. The average cell number percentage of the each cell lines after 50

hours of cultivation, supposing the cell number at the beginning as 100%, was 228 ± 29.3%, 216 ± 24.4%, 219 ± 19.5% and 144 ± 23.5% in SH101-P4, SH101-P4^{vector}, SH101-P4$^{\Delta GFP}$ and SH101-P4$^{\Delta PRL\text{-}3\text{-}1}$, respectively (fig. 2C). SH101-P4^{vector} and SH101-P4$^{\Delta GFP}$ showed similar cell proliferation activity compared with the parental SH101-P4 cells as determined by WST-1 assay, while SH101-P4$^{\Delta PRL\text{-}3\text{-}1}$ showed significantly suppressed cell proliferation compared with SH101-P4 ($P = 0.0092$).

Finally, we evaluated the constitutive PRL-3 knockdown effect on metastasis *in vivo* using mice hepatic metastasis model. SH101-P4 and its subclones were injected into the spleen of BALB/cA mice and the numbers of macroscopic metastatic foci in the liver were counted four weeks after tumor inoculation. The mean number of liver metastases for each case was 3.1 ± 2.9 and 2.6 ± 2.4 in SH101-P4^{vector} and SH101-P4, respectively. In contrast, the number of metastatic foci markedly decreased in SH101-P4$^{\Delta PRL\text{-}3\text{-}1}$ (0.7 ± 0.7). Statistical significances between number of liver metastases of SH101-P4$^{\Delta PRL3\text{-}1}$ injected and SH101-P4^{vector} injected spleen ($P = 0.0211$), or between SH101-P4$^{\Delta PRL\text{-}3\text{-}1}$ injected and SH101-P4 injected spleen ($P = 0.0312$) were detected, respectively (fig. 3A). The growths of splenic tumor were evaluated by weighting the whole spleen at the time of sacrifice. The weight of the spleen varied in each animal, and the mean weight was 215 ± 131 mg, 419 ± 301 mg and 559 ± 395 mg in SH101-P4$^{\Delta PRL\text{-}3\text{-}1}$, SH101-P4^{vector} and SH101-P4, respectively (fig. 3B). Although the weights of SH101-P4$^{\Delta PRL\text{-}3\text{-}1}$ injected spleen had tendency to be smaller than controls, no statistical difference was observed (between SH101-P4$^{\Delta PRL\text{-}3\text{-}1}$ and SH101-P4^{vector}, $P = 0.257$; SH101-P4$^{\Delta PRL\text{-}3\text{-}1}$ and SH101-P4, $P = 0.391$).

As shown in figure 3C, each cancer cells formed the white nodules clearly demarcated from normal tissues in the injected spleen as well as in the metastatic liver. In addition, one out of ten SH101-P4 and two out of ten SH101-P4^{vector} injected mice showed the peritoneal dissemination. On the other hand, peritoneal dissemination was not observed in the SH101-P4$^{\Delta PRL\text{-}3\text{-}1}$ injected mice. As shown in figure 3D, the histology of the cancer tissues in primary foci were not distinctively different in PRL-3 knockdown and parental cells.

Discussion

In this study, we examined the *PRL-3* mRNA expression levels in 13 gastric cancer cell lines originating from various histological types. We could not find correlation between the histological types of these cell lines and the expression levels of *PRL-3*. The expression of *PRL-3* in SH101-P4 was distinctively higher than the other cells. Although the reason why SH101-P4 showed the high *PRL-3* levels is remained uncertain, one report showed that only SH101-P4 cells were able to grow in medium without polypeptide growth factor while other HSC-39, HSC-40, HSC-41, HSC-42 and HSC-45 cells required growth factors to be maintained in the culture [19]. PRL-3 when highly expressed, might act as a trigger of another cell proliferation pathway, which is independent known growth factor pathways.

In order to evaluate the effect of PRL-3 on invasion and growth of gastric cancer, we established the SH101-P4$^{\Delta PRL\text{-}3\text{-}1}$ cell in which PRL-3 expression was constantly and semipermanently suppressed by a plasmid vector expressing *PRL-3* specific siRNA

whose sequence was the same as our previous report [13]. PRL-3 knockdown greatly reduced the gastric cancer cell invasion *in vitro*. Because the growth ability was also reduced greatly, results of cell invasion might be influenced. Cell growth of SH101-P4$^{\Delta PRL\text{-}3\text{-}1}$ was reduced by 34% compared with SH101-P4 after 48hours, while cell invasion of SH101-P4$^{\Delta PRL\text{-}3\text{-}1}$ was reduced by 76% compared with SH101-P4 after 48hours. Since the reduction rate of cell invasion was much higher than that of cell proliferation by PRL-3 knockdown, we considered PRL-3 plays the important role in cell invasion ability. The similar results were obtained in our previous study with transient transfection of oligonucleotide siRNA against PRL-3 to DLD-1 colon cancer cells [13]. The effect of PRL-3 on cell motility has been reported not only in gastric and colon cancer, but also in some other types of cancer cells such as human myeloma cell and mouse melanoma cell [20, 21]. Although the mechanism how PRL-3 works upon cell invasion is still undefined, several reports has been published which clarified the PRL-3 target molecules while some of them are known to be involved in cell motility [22]. For example, Peng et al. reported that integrin α1 was directly interacted with PRL-3 using yeast two-hybrid method [22]. They also reported that PRL-3 directly downregulated the phosphorylation level of integrin β1 and upregulated the phosphorylation level of Erk1/2 which involved in cell motility [22]. Wang et al. showed PRL-3 promoted epithelial-mesenchymal transition leading metastatic cancer by downregulating PTEN via PI3K pathways [23]. Recently, Mizuuchi et al. reported the direct involvement of PRL-3 in cell invasion by isolating a molecule, KRT8, which directly dephosphorylated by PRL-3 [24]. Therefore, PRL-3 may regulate cell invasion by modulation of phosphorylation state of known or unknown molecular targets.

On the other hand, the effect of PRL-3 on cell growth is a little controversial. In this study, contribution of PRL-3 not only on cell motility, but also on cell growth *in vitro* and *in vivo* was suggested. Recently, Cai *et al* [25] and Li *et al* [26] reported that the gastric cancer cell proliferation and motility were suppressed when PRL-3 expression was inhibited by micro RNA system. Our result was consistent with these reports. Certainly, cell proliferation and motility are derived from totally different pathways, however, there are some molecules which work on both cell growth and motility. For example, PRL-1, a member of tyrosine phosphatase family, was reported to be involved in both cell growth and motility [4, 6]. Overexpression of PRL-1 lead the high cellular growth while PRL-1 localized primarily in nucleus [4]. PRL-1 was firstly reported to be localized in the nucleus, but Wang et al. [27] demonstrated that PRL-1 moves from endoplasmic reticulum during the interphase to the centrosomes in mitotic phase. PRL-1 in interphase acted as prenylation-dependent manner while PRL-1 in mitotic phase was expected to work on cell division by regulating its spindle dynamics in prenylation-independent manner [27]. In addition, PRL-1 promotes cell motility in prenylation-dependent manner [28]. Since more than 75% of amino acid sequences of PRL-1 and PRL-3 are identical and both contain C-terminal prenylation motif CAAX to be prenylated [2, 29], it is comprehensible that PRL-3 might possess this dual ability, prenylation-dependent and prenylation-independent manner like PRL-1.

In this report, liver metastasis was reduced by about 77% in SH101-P4$^{\Delta PRL\text{-}3\text{-}1}$ whose *PRL-3* mRNA expression was reduced by 95% compared with SH101-P4. The percentage of liver metastasis suppression was lower than that of *PRL-3* mRNA

suppression. Percentage discrepancy between *PRL-3* expression and metastasis formation was reported in another report. Li *et al*. reported the relationship between *PRL-3* expression and peritoneal metastasis in gastric cancer using microRNA [26]. In their report, peritoneal dissemination was reduced by about one third, while *PRL-3* mRNA expression was reduced by more than 80% [26]. These results may indicate that there might be another molecule which acts to form gastric cancer metastasis. However, PRL-3 may contribute to the establishment of metastasis since the effects of PRL-3 knockdown were experimentally significant.

It is likely to take some time before the overall picture of the mechanisms how PRL-3 plays a role in cell growth and motility becomes evident and surely further study will be needed. However, PRL-3 may be one of the candidates for molecular target for anticancer therapy by controlling both tumor growth and tumor metastasis.

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Figure Legends

Fig.1 *PRL-3* mRNA expression in various human gastric cancer cell lines detected by RT-PCR analysis. The levels of *β-actin* mRNA expression were used as internal control. Relative mRNA expression of each cell line was calculated when the expression of MKN-1 was considered as 100%. The mean value of triplicate samples was plotted with bars indicating SE.

Fig. 2 Effect of *PRL-3* small interfering RNA on SH101-P4 gastric cancer cells. A) Introduction of *PRL-3*-small interfering RNA in gastric cancer cells down-regulates endogenous *PRL-3*. Relative *PRL-3* mRNA expression levels of SH101-P4 subclones. The results are shown as the ratio between *PRL-3* and *β-actin*. The *PRL-3* mRNA expression in SH101-P4$^{\Delta PRL\text{-}3\text{-}1}$ and SH101-P4$^{\Delta PRL\text{-}3\text{-}2}$ were reduced by 95% and 50%, respectively, compared with parental SH101-P4. The mean value of triplicate samples was plotted with bars indicating SE. B) PRL-3 knockdown inhibits gastric cancer cell invasion *in vitro*. The number of cell invaded to the lower chamber from the upper chamber was counted after 48 hours of incubation. Parental SH101-P4 cell number was considered as 100% and the ratio of subclonal cell number was calculated. Statistical significances between SH101-P4 and SH101-P4$^{\Delta PRL\text{-}3\text{-}1}$, or SH101-P4 and SH101-P4^{vecter}, or SH101-P4 and SH101-P4$^{\Delta GFP}$ were determined by *t* test. (**P* = 0.0024, ***P* = 0.7590, ****P* = 0.9312). C) Growth of SH101-P4 cell and its subclones. Cell growth was evaluated by WST-1 assay after 48 hours of incubation as described in Materials and

Methods. Statistical significances between SH101-P4 and SH101-P4$^{\Delta GFP}$, or SH101-P4 and SH101-P4^{vecter}, or SH101-P4 and SH101-P4$^{\Delta PRL\text{-}3\text{-}1}$ after 50 hours of incubation were determined by *t* test. (**P* = 0.7361, ***P* = 0.0894, ****P* = 0.0092). The mean values of quintuplicate samples were plotted with bars indicating SE.

Fig. 3 PRL-3 knockdown resulted in the inhibition of liver metastasis *in vivo*. SH101-P4 and its subclones were injected into the spleen of BALB/cA mice. Mice were sacrificed 28 days after the injection. A) The metastatic tumor growth was evaluated by counting the number of metastatic foci in 6 sections per liver. Statistical significances between SH101-P4$^{\Delta PRL\text{-}3\text{-}1}$ and SH101-P4^{vecter}, or SH101-P4$^{\Delta PRL\text{-}3\text{-}1}$ and SH101-P4 were determined by *t* test. (**P* = 0.0211, ***P* = 0.0312). The mean values of quintuplicate samples were plotted with error bars. B) The primary tumor growth was evaluated by weighing the spleen. Statistical significances between SH101-P4$^{\Delta PRL\text{-}3\text{-}1}$ and SH101-P4^{vecter}, or SH101-P4$^{\Delta PRL\text{-}3\text{-}1}$ and SH101-P4 were determined by *t* test (**P* = 0.257, ***P* = 0.391). C) The macroscopic images of the representative primary splenic tumor and metastatic hepatic foci. Arrowheads and white arrows indicate the primary splenic tumor and metastatic hepatic foci occurred in SH101-P4 cell injected mouse. D) The microscopic sections of splenic tumors x20, H&E. Higher magnification (x200) provided in the inset.

Figure 1

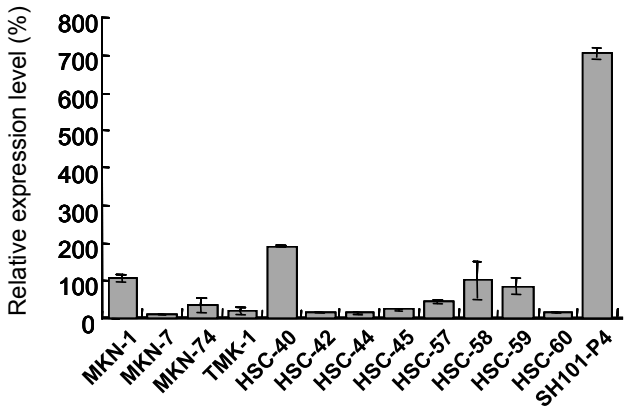
Relative expression level (%)
800
700
600
500
400
300
200
100
0
MKN-1
MKN-7
MKN-74
TMK-1
HSC-40
HSC-42
HSC-44
HSC-45
HSC-57
HSC-58
HSC-59
HSC-60
SH101-P4

Figure 2

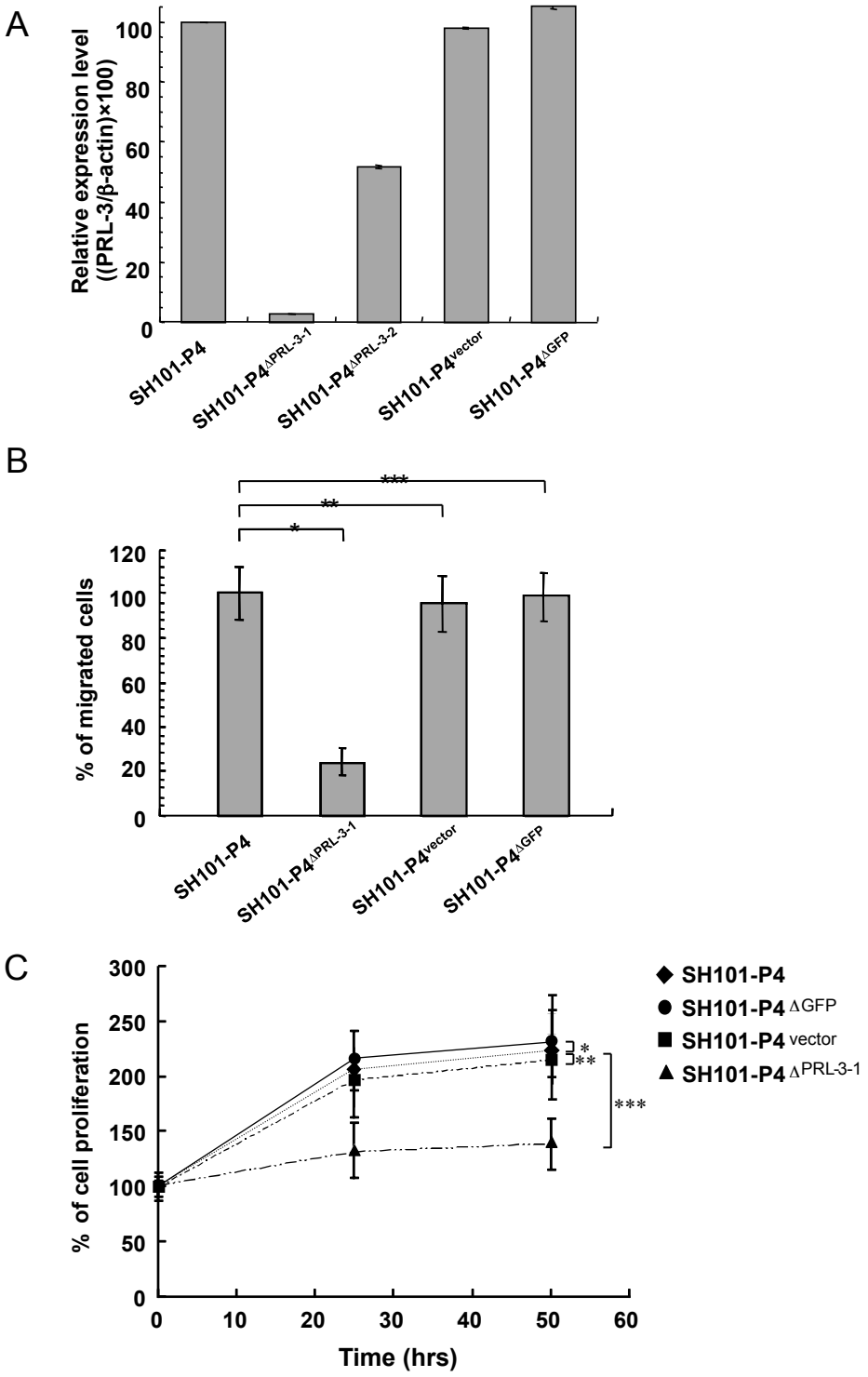

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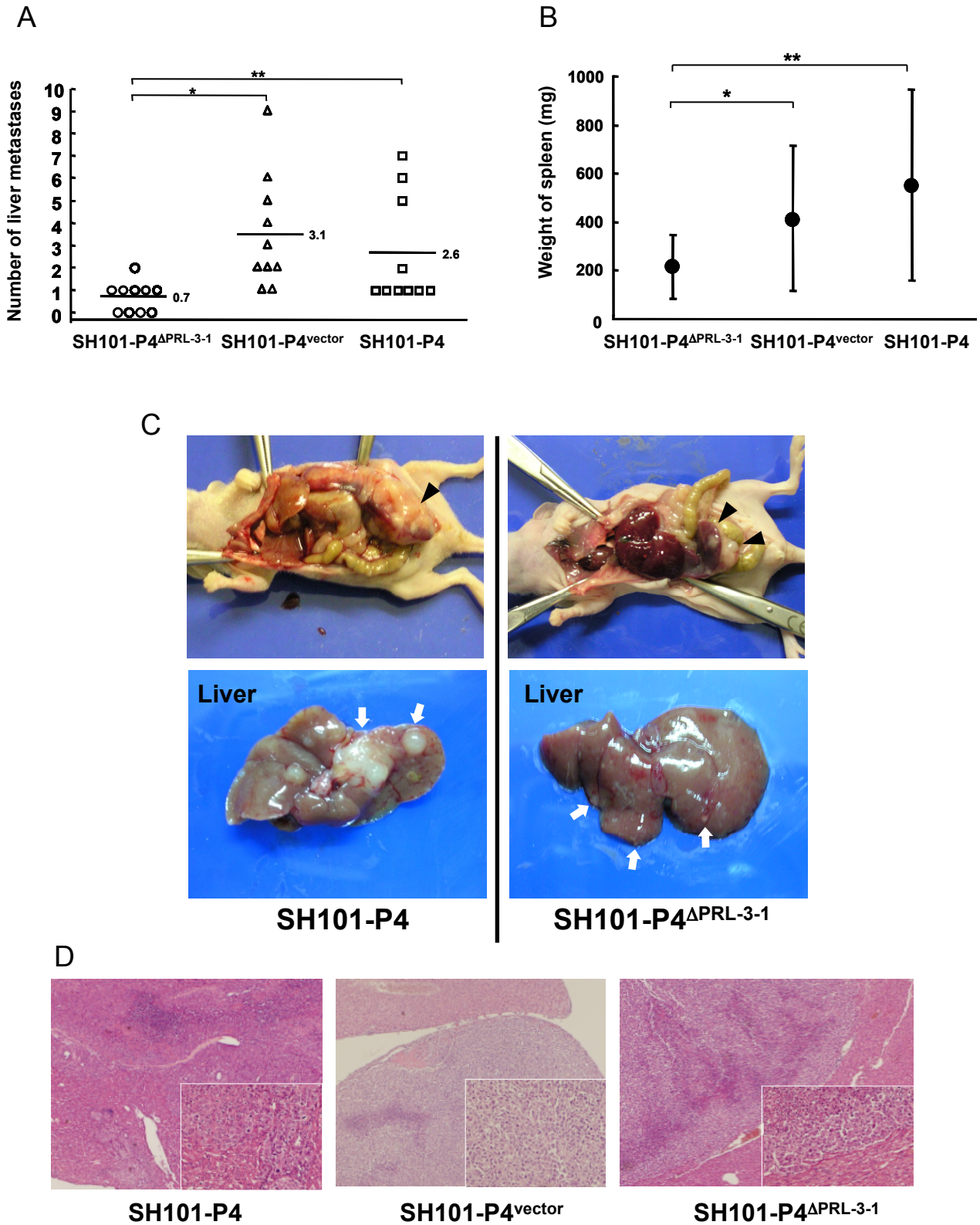
Figure 3


A
Number of liver metastases
10
9
8
7
6
5
4
3
2
1
0
**
*
3.1
2.6
0.7
SH101-P4ΔPRL-3-1
SH101-P4vector
SH101-P4
B
Weight of spleen (mg)
1000
800
600
400
200
0
**
*
SH101-P4ΔPRL-3-1
SH101-P4vector
SH101-P4
C
Liver
Liver
SH101-P4
SH101-P4ΔPRL-3-1
D
SH101-P4
SH101-P4vector
SH101-P4ΔPRL-3-1