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Requirement of phosphatase of regenerating liver-3 for the nucleolar localization of nucleolin during the progression of colorectal carcinoma

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Phosphatase of regenerating liver-3 (PRL-3) is a protein tyrosine phosphatase (PTP) that is frequently overexpressed in liver metastases of colorectal carcinomas (CRCs). The PTP activity of the PRL-3 protein is indispensable for the promotion of distant metastasis of CRC; however, little is known about the effect of PRL-3 on cell growth. In this study, we investigated a novel protein that can connect to PRL-3 to modulate the proliferation of CRC cells. In CRC-derived SW480 cells, transduction of ectopic wild-type PRL-3, but not the C104S catalytic ''dead'' mutant, up-regulated cell proliferation and increased the population of cells at the S and G_2/M phases. Also, inhibition of PTP activity of the PRL-3 protein by treatment with the PRL-3 inhibitor suppressed cell proliferation in a dose-dependent manner as well as *PRL-3* knockdown by RNA interference. Using a comparative study of monodimensional gel electrophoresis of immunoprecipitates from *PRL-3*-transfected SW480 cells and subsequent mass spectrometry analysis, nucleolar-specific protein nucleolin (NCL) was identified as a novel PRL-3-binding protein. We confirmed physiological interaction between PRL-3 and NCL, and found that PRL-3 phosphatase activity was associated with the suppression of the phospho-NCL levels and nucleolar assembly of NCL protein. In CRC cases, nucleolar NCL expression was correlated not only with higher levels of PRL-3 expression but also with frequent incidence of lymph node metastasis and a higher clinicopathologic stage. These findings suggest that NCL is involved in PRL-3-mediated cancer progression/metastasis signaling, which plays an important role in the acceleration of CRC growth. (*Cancer Sci* 2010; 101: 2254–2261)

The *phosphatase of regenerating liver-3* (*PRL-3*) gene encodes a protein tyrosine phosphatase (PTP) that has been of particular interest as a key activator of cancer cell motility and metastasis. By global gene expression profiles of liver metastases of colorectal cancers (CRCs) using serial analysis of gene expression, the *PRL-3* gene transcript has been identified as the only gene that is consistently overexpressed in liver metastases of CRC, whereas it is undetectable in normal colorectal epithelia and shows intermediate expression in advanced primary tumors.[1] Indeed, elevated levels of *PRL-3* mRNA expression have been reported in various human malignancies, including those of the stomach,[2] colorectum,[3] breast[4] and ovary;[5] in all of these cancers, *PRL-3* mRNA elevation is correlated with higher incidence of distant metastasis and/or poorer patient prognosis.

Evidence supporting the biological significance of PRL-3 in cancer progression has been accumulated by several molecular biological approaches. Ectopic transduction of recombinant *PRL-3* increased cancer cell motility and invasiveness in mouse melanoma cells *in vitro* and *in vivo*,[6] and knockdown of *PRL-3* expression by RNA interference abrogated liver metastases of CRC cells in mouse models.[3] Moreover, inactivated *PRL-3* mutations at the catalytic site (D72A and C104S) reduced cell motility, suggesting the significance of PRL-3 PTP activity for the acceleration of cancer cell migration.[6] Also, the molecular mechanism by which PRL-3 accelerates cancer cell migration has been investigated. Previous studies have reported that PRL-3 is involved in the ras homolog (RHO) GTPase signaling pathway to promote motility and invasion,[7] and that the PTP activity of PRL-3 triggers the epithelial–mesenchymal transition of cancer cells by activating the phosphatidylinositol 3-OH kinase signaling pathway as a result of down-modulation of phosphatase and tensin homolog (PTEN) activity.[8] At present, adhesion molecule integrin α1 (ITGA1)[9] and the ezrin/radixin/moesin family member ezrin (EZR)[10] have been identified as PRL-3-binding molecules. Recently, we identified the cytoskeletal intermediate filament keratin 8 (KRT8) as a physiological PRL-3-binding protein by a comparative analysis of phosphorylated protein profiles showing differential expression in wild-type and C104S phosphatase ''dead'' mutant PRL-3-transfected SW480 cells.[11] In addition to the interaction of PRL-3 with KRT8, PRL-3 phosphatase activity is required for dephosphorylation of KRT8 at Ser73 and Ser431, suggesting the possible function of PRL-3 as a ''dual'' phosphatase in the regulation of reorganization and stabilization of keratin filaments.[11] Thus, various research has targeted the significance of PRL-3 as a cancer motility-related phosphatase.

On the other hand, PRL-3 has also been suggested to have an impact on cell growth. Transduction of PRL-3 resulted in enhanced proliferation of melanoma cells,[6] and *PRL-3* knockdown using small-interfering RNA (siRNA) impaired the growth of ovarian cancer cells;[5] however, little is known about the mechanism of PRL-3 in cell growth. In this study, we therefore attempted to find a novel PRL-3-interacting protein that is capable of up-regulating cell proliferation.

Materials and methods

Cell culture, treatment, and gene transfection. Human CRC-derived SW480 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured at 37°C in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) and antibiotics (Invitrogen, Carlsbad, CA, USA). Cells were treated with the PRL-3 inhibitor 1-(2-bromobenzyloxy)-4-bromo-2-benzylidene rhodanine (Sigma, St. Louis, MO, USA), which was dissolved in DMSO. We confirmed that the final concentration of DMSO did not influence the cell viability.[11,12] Wild-type (pGFP-PRL-3) and C104S mutant (pGFP-C104S) *PRL-3* expression vectors were generated.[13] Each plasmid was transfected into SW480 cells using Lipofectamine 2000 (Invitrogen). pGFP vector was used as a negative control.

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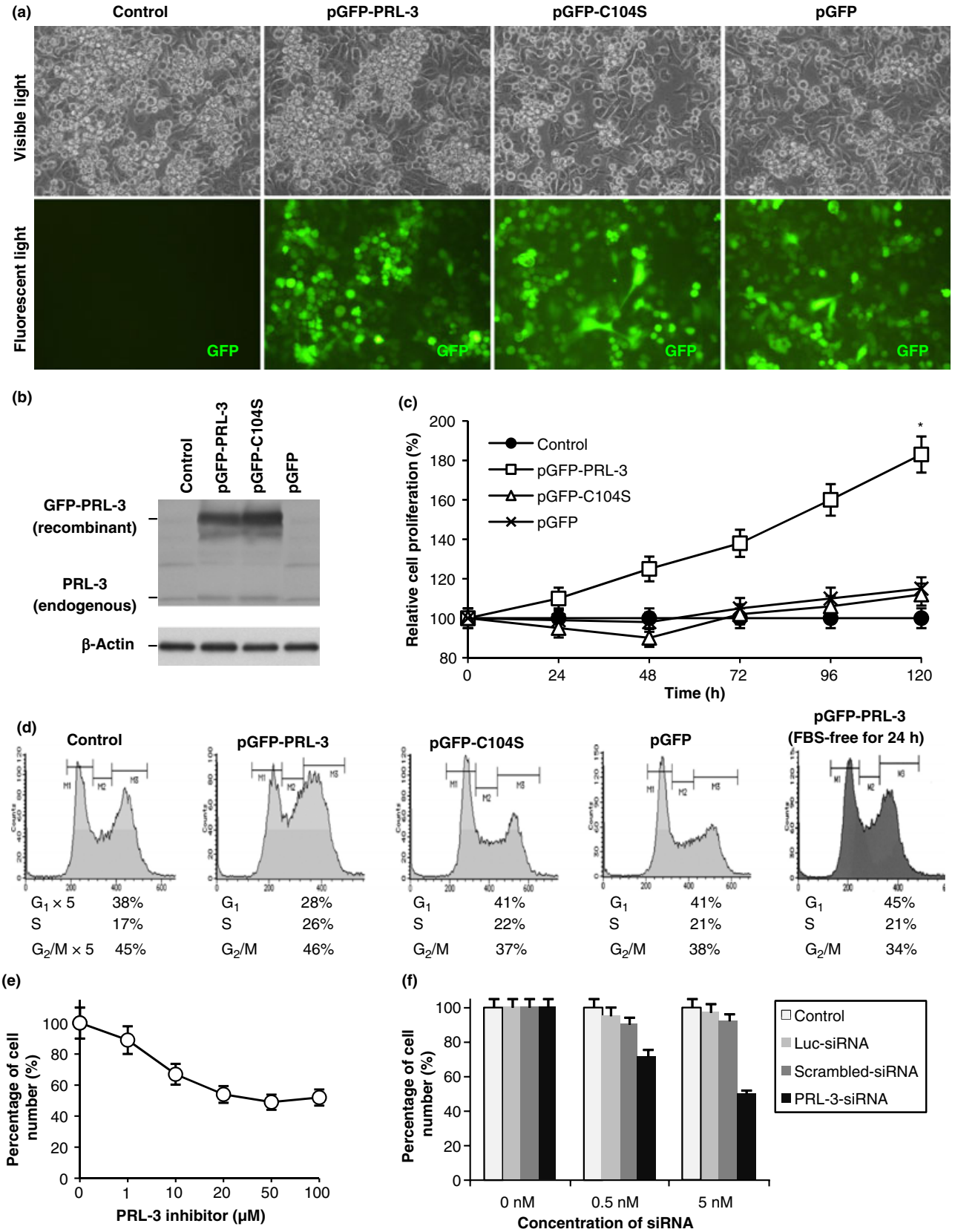

Fig. 1. Protein tyrosine phosphatase (PTP) activity of phosphatase of regenerating liver-3 (PRL-3) was required for the promotion of cell growth in colorectal carcinoma (CRC)-derived SW480 cells. (a) Representative images of gene transfection. (b) Detection of recombinant GFP-PRL-3 and GFP-C104S proteins by immunoblotting (IB). (c) Results of the WST-1 cell proliferation assay. $*P < 0.05$. (d) Percentages of cells in the G_0/G_1, S, and G_2/M phases were analyzed 48 h after gene transfection. (e) Effect of the PRL-3 inhibitor on cell growth. (f) Effect of knockdown of PRL-3 by small-interfering RNA (siRNA) on cell growth. Scrambled-siRNA and Luc-siRNA were used as negative controls.

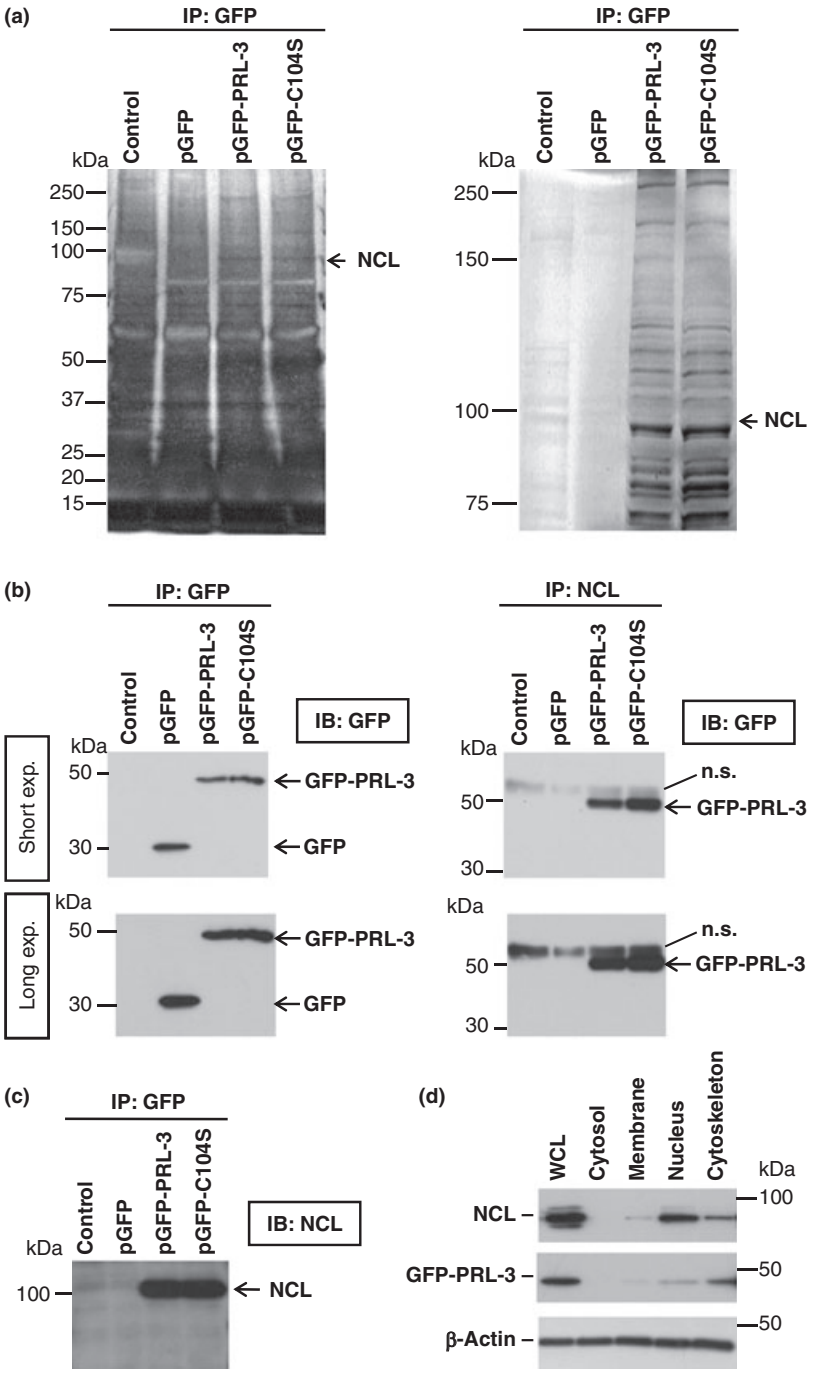

Fig. 2. Physiological interaction between phosphatase of regenerating liver-3 (PRL-3) and nucleolin (NCL). (a) Full image (left) and high resolution image (right) of SDS-polyacrylamide gel electrophoresis. Immunoprecipitation (IP) was performed with anti-GFP antibody, and the gel was visualized with silver staining. (b,c) The interaction between PRL-3 and NCL. (d) Protein fractionation analysis of the pGFP-PRL-3-transfected SW480 cells.

Small-interfering RNA (siRNA) transfection. For the RNA interference analyses, PRL-3-siRNA (5′-GUG ACC UAU GAC AAA ACG CTT-3′ and 5′-GCG UUU UGU CAU AGG UCA CTT-3′) was designed and synthesized based on the coding sequence of human *PRL-3* (NM_032611).[3] Control scrambled-siRNA (5′-ACG CUA UAG CUA GAG CAA CTT-3′ and 5′-GUU GCU CUA GCU AUA GCG TUU-3′) and siRNA targeted Luciferase (Luc-siRNA; 5′-CGU ACG CGG AAU ACU UCG ATT-3′ and 5′-UCG AAG UAU UCC GCG UAC GTT-3′) were also synthesized.[3] SW480 cells were seeded in 100-mm plates (1×10^5 cells/dish) in RPMI-1640 supplemented with 10% FBS. The following day, each siRNA was added to the culture at a final concentration (0–5 nM) in 1.5 mL of RPMI-1640 without antibiotics and FBS in the presence of 0.8% Oligofectamine (Invitrogen).

WST-1 cell proliferation assay and flow cytometric analysis. The effects of pGFP-PRL-3 and pGFP-C104S on cell growth were determined using the Premix WST-1 Cell Proliferation Assay System (Takara Bio, Tokyo, Japan). The tetrazolium salt WST-1 was evaluated for use in a colorimetric assay. Briefly, 1×10^5 cells were inoculated into triplicate wells and maintained in phenol red-free medium, and 48 h after gene transfection, 10 μL of WST-1 was added to each microculture well. The plates were incubated for 30 min at 37°C, after which the absorbance at 450 nm was measured using a microplate reader. The absorbance in the cells without gene transfection was considered to be 100%. The percentages of cells in the G_0/G_1, S and G_2/M phases were analyzed with a FACS Caliber cytometer (BD Biosciences, San Jose, CA, USA). Forty-eight

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hours after gene transfection, cells were collected and fixed, treated with RNase A, and stained with propidium iodide.

Mass spectrometry (MS). Immunoprecipitates with anti-GFP antibody (MBL, Nagoya, Japan) obtained from SW480 cells transfected with pGFP-PRL-3 and pGFP-C104S were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and the gels were stained with a Silver Staining Kit (Wako Pure Chemical, Osaka, Japan). The protein bands were excised from the gels and the excised bands were analyzed in the Pro Phoenix Laboratory (Hiroshima, Japan). Briefly, gel pieces were incubated in 100 mM NH_4CO_3 and dehydrated with acetonitrile. In-gel digestion of the extracted proteins was carried out with 10 μg/mL trypsin in 50 mM NH_4CO_3/5 mM $CaCl_2$ for 16 h. The digested peptides were extracted with a mixture of 5% formic acid/50% acetonitrile. The MS of the resulting peptides was recorded on the MALDI-TOF spectrometer (Waters, Milford, MA, USA) in reflection mode. The resulting peptides were matched with their corresponding proteins by a MASCOT search of the NCBI database.

Immunoblotting (IB) and immunoprecipitation (IP). Cells were lysed in a buffer containing 150 mM NaCl, 10 mM Tris-HCl (pH 7.4), 0.5 mM EDTA, 1% Triton-X, 1% Protease Inhibitor Cocktail (Sigma), and 1% Phosphatase Inhibitor Cocktail (Sigma). The immunoprecipitation matrix (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was incubated with 5 μg of each antibody against GFP, and the lysates were transferred to the complex. Total cell lysates or immunoprecipitates were resolved by SDS-PAGE and transferred onto a PVDF membrane (Millipore, Bedford, MA, USA). The primary antibodies were as follows: anti-PRL-3 (Sigma), anti-nucleolin (NCL; Sigma), anti-phospho-NCL (p-NCL; Novus, Littleton, CO, USA), anti-GFP, and anti-β-actin (Sigma). Horseradish peroxidase (HRP)-conjugated antimouse and -rabbit IgGs (GE Healthcare, Piscataway, NJ, USA) and ExactaCruz Western Blot Reagent (Santa Cruz Biotechnology) were used as secondary antibodies. The specific bindings were visualized using an Enhanced ChemiLuminescence detection system (GE Healthcare). Cellular compartmentalization of SW480 cells was achieved by differential lysis and centrifugation using a ProteoExtract kit (EMD Biosciences, Darmstadt, Germany). Briefly, 1×10^6 cells attached in the tissue culture plate were washed and lysed in Extraction Buffer I. After transferring the supernatant (fraction 1: cytosolic fraction), Extraction Buffer II was added to the dish to obtain the supernatant (fraction 2: membrane fraction). To extract the fraction 3 (nucleic fraction) and fraction 4 (cytoskeleton fraction), Extraction Buffer III and IV were added to the tissue culture plate, respectively.

Immunohistochemistry. Cells were grown on glass coverslips and then fixed with methanol. After washing with PBS, cells were stained with antibodies against GFP, NCL, and p-NCL. Cy3-conjugated antirabbit IgG (GE Healthcare) and Cy2-conjugated antimouse IgG (GE Healthcare) were used as secondary antibodies. The nuclei were stained with DAPI. A total of 68 sporadic human CRCs surgically removed at Kobe University Hospital (Kobe, Japan) were employed. None of these patients received adjuvant chemotherapy or radiotherapy before surgery. Informed consent was obtained from all patients, and the study was approved by the Kobe University Institutional Review Board. Histological examination was performed according to the General Rules for Clinical and Pathological Studies on Cancer of the Colon, Rectum and Anus.[14] A modified version of the immunoglobulin enzyme bridge technique with a Linked Streptavidin-Biotin kit (Dako, Glostrup, Denmark) was used. Primary antibodies against PRL-3 and NCL were applied to sections. Sections were incubated with biotinylated goat antimouse or antirabbit IgG and streptavidin conjugated to HRP. Chromogenic fixation was carried out by immersing the sections in a solution of 3,3′-diaminobenzidine. Sections were counterstained with Mayer's hematoxylin. Normal colorectal epithelial cells and smooth muscle cells of the vessels were used as internal controls. The immunoreactivity of anti-PRL-3 antibody was graded as follows: ++, >30% cancer cells showed *PRL-3* expression exceeding that of the internal controls; +, >90% cancer cells showed no increase in the expression of *PRL-3* compared with the internal controls; and −, immunoreactivity of anti-PRL-3 was undetectable in cancer cells. Also, the expression patterns of nucleolar NCL were evaluated as positive only when the cancer cells expressed higher nucleolar NCL levels than those in normal colorectal epithelial cells. The immunoreactivities of PRL-3 and NCL were evaluated independently by three pathologists (S.S., E.M. and H.Y.).

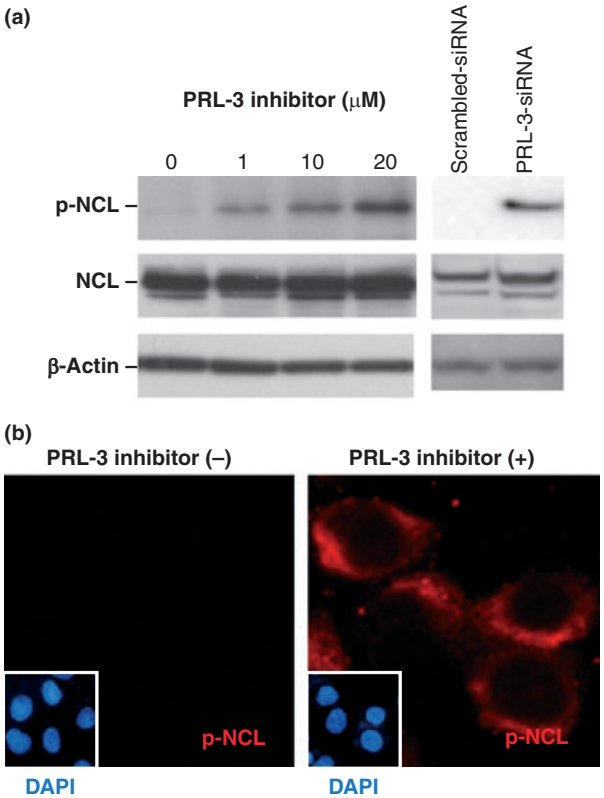

Fig. 3. Effects of the phosphatase of regenerating liver-3 (PRL-3) inhibitor on phospho-nucleolin (p-NCL). Phospho-nucleolin (p-NCL) levels in SW480 cells. (a) SW480 cells were treated with the PRL-3 inhibitor for 48 h. The results were reconfirmed by knockdown of endogenous PRL-3 by PRL-3-siRNA. (b) Cytoplasmic localization of p-NCL.

Statistical analysis. We used the chi-squared-test and Kruskal–Wallis test to evaluate the relationship of NCL expression with clinicopathologic characteristics and the PRL-3 status in CRC tissues. *P*-values <0.05 were considered statistically significant.

Results

PRL-3 phosphatase activity promoted cell growth. To examine the impact of PRL-3 phosphatase on cell growth, we first transduced recombinant wild-type and C104S mutant *PRL-3* expression vectors into CRC-derived SW480 cells. Forty-eight hours after transfection, >80% cells showed mainly cytoplasmic expression of recombinant GFP-PRL-3, GFP-C104S, and GFP proteins (Fig. 1a). Evidence for expression of the constructs was confirmed by IB (Fig. 1b). Although SW480 cells showed a low level of endogenous PRL-3 expression that was almost equivalent to that of the normal colonic epithelium,[3] transduction of

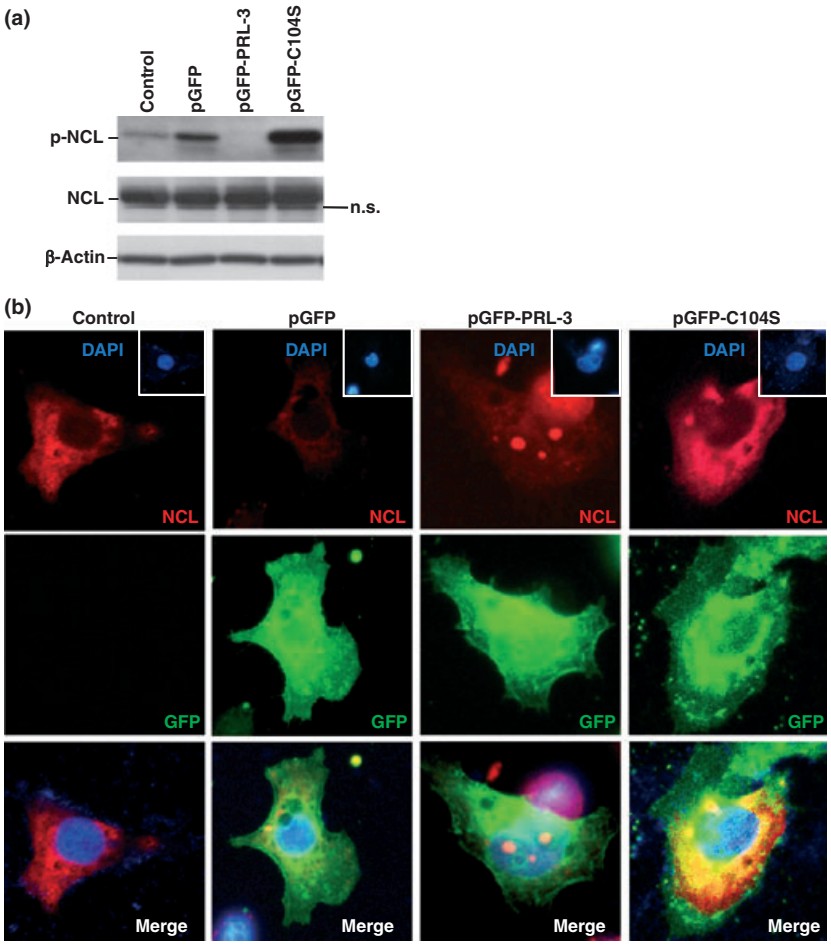

Fig. 4. Requirement of the protein tyrosine phosphatase (PTP) activity of phosphatase of regenerating liver-3 (PRL-3) for nucleolar assembly of nucleolin (NCL). (a) SW480 cells were transfected with pGFP-PRL-3, pGFP-C104S, or pGFP vectors. Forty-eight hours post transfection, the lysates were used for detection of pNCL and NCL proteins. (b) Nucleolar localization of NCL was regulated by PRL-3.

wild-type *PRL-3*, but not the C104S mutant, increased cell proliferation (Fig. 1c). Also, an increased population of cells in the S and G_2/M phases was confirmed in SW480 cells transfected with pGFP-PRL-3, whereas no significant change was detected in cells transfected with pGFP-C104S (Fig. 1d). In the pGFP-PRL-3 transfectant, an increased percentage of cells in G_2/M phase was considered not to indicate either G_2 arrest nor the presence of tetraploid G_1 cells, because treatment with FBS-free RPMI-1640 for 24 h decreased the cells in the G_2/M phase (Fig. 1d). To confirm the impact of PTP activity of the PRL-3 protein on cell growth, we next treated the SW480 cells with the PRL-3 inhibitor for 48 h and detected that treatment with the PRL-3 inhibitor suppressed cell proliferation in a dose-dependent manner (Fig. 1e). As reported in our previous study,[11] 0–100 μM of the PRL-3 inhibitor was not toxic to the SW480 cells examined. Knockdown experiment with PRL-3-siRNA supported the specificity of this PRL-3 inhibitor on the PTP activity of endogenous PRL-3 (Fig. 1f).

Physiological interaction of PRL-3 and NCL. For the identification of the PRL-3-interacting protein, SDS-PAGE was performed to separate immunoprecipitates from total cell lysates of SW480 cells in the presence or absence of transfection with pGFP-PRL-3, pGFP-C104S, and pGFP. Equivalent concentration of each protein lysate was used for IP with anti-GFP antibody overnight. Although no candidate protein was found in the lysates from control SW480 cells and the pGFP transfectants, a clearly different protein pattern was observed in the cells overexpressing wild-type and C104S mutant PRL-3 at 48 h post transfection (Fig. 2a). Among several candidate protein bands, a 90 kDa protein was identified by MS as corresponding to NCL (NCBI GDB accession no. NM_005381). The physiological interaction of PRL-3 with endogenous NCL protein in SW480 cells was confirmed by IP. The signal that indicated interaction between GFP-PRL-3 and NCL in pGFP-C104S-transfected SW480 cells was stronger than that in the pGFP-PRL-3 transfectant (Fig. 2b,c). Phosphatase of regenerating liver-3 (PRL-3) localizes mainly at the cytoskeleton;[11] nevertheless, NCL is known as a major nucleolar phosphoprotein.[15] To uncover the discrepancy between the intracellular localization and interaction of PRL-3 and NCL, we assessed the distribution of these proteins with protein fractionation. In pGFP-PRL-3-transfected SW480 cells, localization of NCL was detected in the nucleus and cytoskeleton, which was similar to the localization pattern of recombinant PRL-3 protein (Fig. 2d). The validity of the anti-NCL antibody used in this study has been reported previously.[16,17]

The PTP activity of PRL-3 protein is required for up-regulation of nucleolar NCL levels. We next investigated the role of PRL-3 PTP activity on the NCL phosphorylation using the PRL-3 inhibitor and recombinant *PRL-3* expression vectors. Treatment with the PRL-3 inhibitor for 48 h increased the p-NCL levels in a dose-dependent manner, whereas no significant change was detected in the NCL levels (Fig. 3a). A similar effect was detected when the cells were transfected PRL-3-siRNA (Fig. 3a). We also confirmed that p-NCL localized in the cytoplasm (Fig. 3b). Furthermore, the wild-type PRL-3 protein fused with GFP also decreased p-NCL levels, but the C104S mutant increased p-NCL levels in comparison with the negative controls; the negative controls consisted of untransfected cells and cells transfected with pGFP, both of which express endogenous PRL-3 (Fig. 4a). Interestingly, the pGFP-PRL-3 transfectant showed nucleolar accumulation of NCL; however, in the

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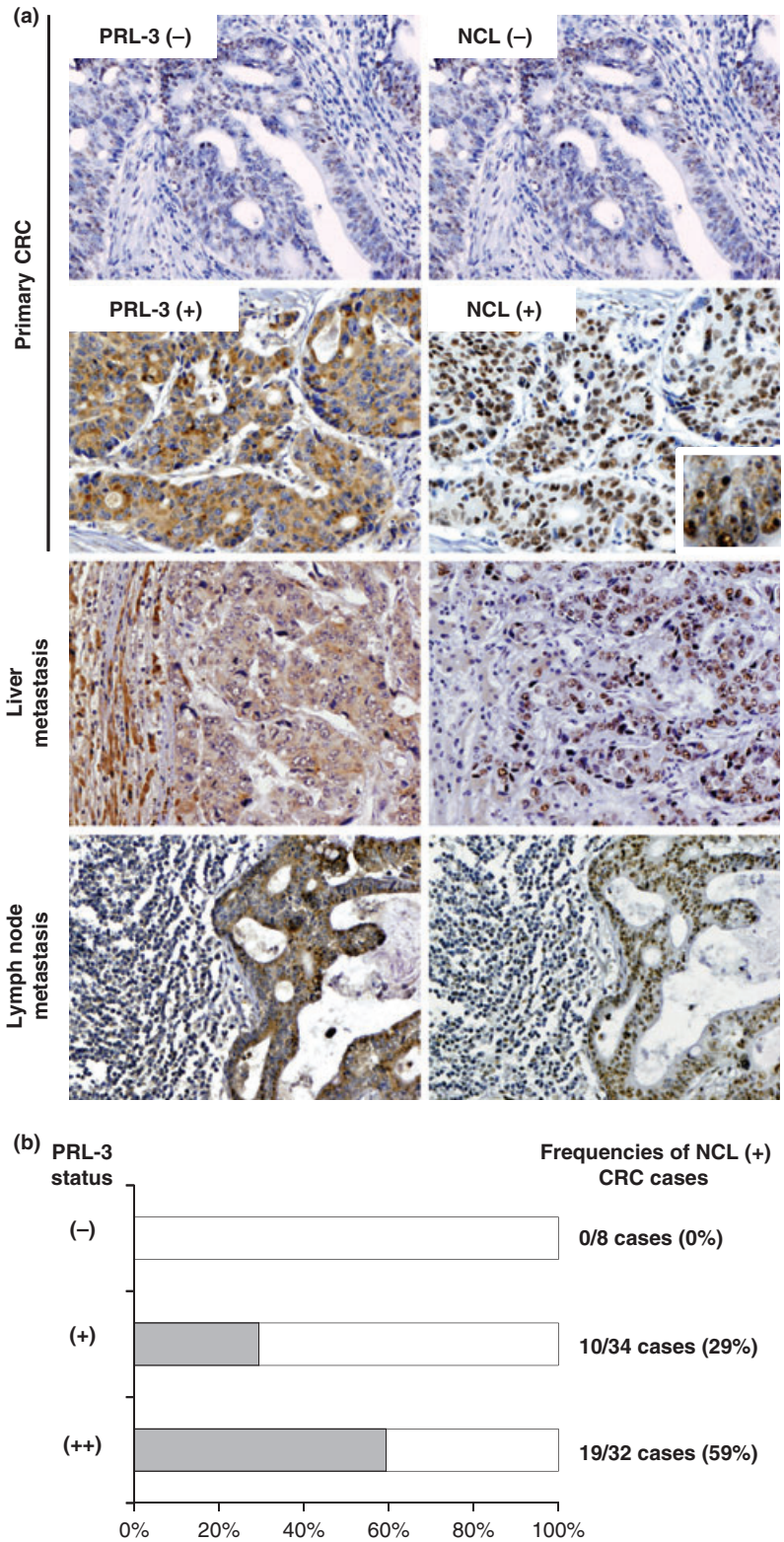

Fig. 5. Nucleolar localization of nucleolin (NCL) was associated with high levels of phosphatase of regenerating liver-3 (PRL-3) expression in CRC tissues. (a) Immunohistochemistry of PRL-3 and NCL expression in primary CRCs and metastatic sites (liver and lymph node). Original magnification, ×200. A representative image of the nucleolar assembly of NCL protein is shown in the inset (×400). (b) Association of nucleolar NCL expression with PRL-3 status in CRC tissues. Percentages of NCL(+) cases in each PRL-3 subtype are shown ($P < 0.001$).

pGFP-C104S transfectant, NCL protein diffusely was distributed in the cytoplasm (Fig. 4b).

Overexpression of PRL-3 was associated with nucleolar NCL expression and CRC progression. The stability of NCL is regulated post-transcriptionally by its phosphorylation by casein kinase 2 (CK2), and phosphorylation enhances NCL as a substrate for a protease.[18] Taken together with the results of our *in vitro* experiments, we hypothesized that PRL-3 PTP activity may be required for nucleolar assembly of NCL protein. We therefore examined the association between the nucleolar NCL expression and the PRL-3 status in CRC tissues (Fig. 5a). As in previous reports,[3,19] high levels of PRL-3 expression in CRCs showed significant correlation with various clinicopathologic parameters, such as depth of invasion ($P = 0.003$), lymphatic ($P = 0.008$) and venous vessel invasion ($P = 0.007$), lymph node metastasis ($P = 0.013$), and clinicopathologic stage ($P = 0.011$; Table S1).

Table 1. Correlation of NCL expression with clinicopathologic findings in CRC

	NCL†	(–) *n* = 39	(+) *n* = 29	*P*-value‡
	n = 68	*n* (%)	*n* (%)	
Ave. age (years)	65.3 ± 13.0	65.9 ± 14.7	64.6 ± 10.6	0.297
Gender				
Male	37	21 (58)	16 (42)	0.915
Female	31	18 (58)	13 (42)	
Location§				
Right	31	20 (65)	11 (35)	0.278
Left	37	19 (51)	18 (49)	
Ave. size (mm)	47.6 ± 23.5	47.2 ± 25.6	48.0 ± 20.9	0.696
Histology¶				
Well	17	11 (65)	6 (35)	0.609
Mod.	48	27 (56)	21 (44)	
Poor	3	1 (33)	2 (67)	
Depth invasion††				
≤MP	19	12 (63)	7 (37)	0.551
>MP	49	27 (55)	22 (45)	
Lymphatic vessel invasion				
(–)	18	12 (67)	6 (33)	0.083
(+)	50	27 (54)	23 (46)	
Venous vessel invasion				
(–)	17	11 (65)	6 (35)	0.07
(+)	51	28 (55)	23 (45)	
Lymph node metastasis				
(–)	33	23 (70)	10 (30)	0.013*
(+)	35	16 (46)	19 (54)	
Clinicopathogic stage‡‡				
I/II	34	24 (71)	10 (29)	0.011*
III/IV	34	15 (44)	19 (56)	

†The degree of nucleolin (NCL) immunoreactivity was evaluated as described in the text; ‡*P*-values <0.05 were considered as statistically significant. *$P < 0.05$; §Right, cecum, ascending and transverse colon; and Left, descending, sigmoid colon and rectum; ¶Abbreviations: Well, well differentiated tubular adenocarcinoma; Mod, moderately differentiated tubular adenocarcinoma; and Poor, poorly differentiated adenocarcinomas. ††≤MP, tumor infiltration within muscularis propria; and >MP, tumor infiltration beyond muscularis propria; ‡‡Clinicopathologic stage was evaluated according to General Rules for Clinical and Pathological Studies on Cancer of the Colon, Rectum and Anus[14] ; CRC, colorectal carcinoma.

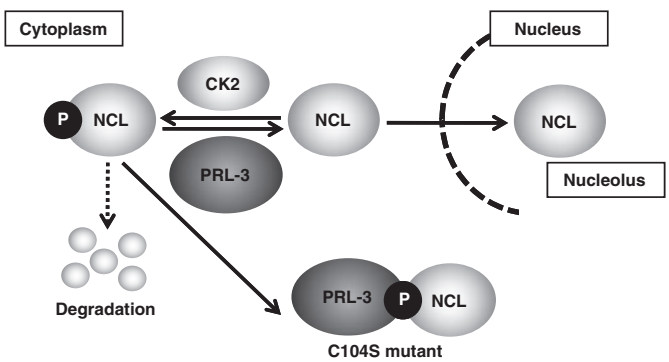

Fig. 6. Proposed model for the regulation of nucleolin (NCL) localization by phosphatase of regenerating liver-3 (PRL-3). Casein kinase 2 (CK2)-mediated phosphorylation promoted the degradation of NCL protein.[18] Phosphatase of regenerating liver-3 (PRL-3) dephosphorylated p-NCL in the cytoplasm, which consequently increased nucleolar assembly of NCL. The C104S mutant PRL-3 physiologically bound to NCL as a substrate-trapping mutant, thereby forming a stable complex with NCL.

Interestingly, the presence of nucleolar NCL had a significant relationship with the PRL-3 status ($P < 0.001$; Fig. 5b). Furthermore, nucleolar localization of NCL was closely associated with an increased incidence of lymph node metastasis ($P = 0.013$) and a higher clinicopathologic stage ($P = 0.011$) during the progression of CRC (Table 1).

Discussion

Phosphorylation and dephosphorylation are major regulatory events, affecting the functional activities of diverse proteins that modulate cellular processes, such as transcriptional regulation, apoptosis, cell cycle progression, protein degradation, and protein trafficking. In this study, we present evidence pointing to NCL as a PRL-3-interacting protein and the necessity of PRL-3 phosphatase activity for NCL assembly in the nucleoli. NCL is one of the major constituents of nucleoli in exponentially growing cells,[20] and its biological functions are involved in various kinds of intracellular signaling, particularly the organization of nucleolar chromatin,[21] packaging of ribosomal RNA,[22] and assembly of ribosomes by shuttling between the nucleus and the cytoplasm.[23] In addition, NCL has been shown to serve as a substrate for CK2 during the interphase of the cell cycle[18] and as a substrate for the cell cycle-regulatory CDC2 kinase during mitosis.[24,25] These phenomena support the possible involvement of NCL in the promotion of mitosis. Moreover, we examined the role of PRL-3 PTP activity in the dephosphorylation of NCL and found that p-NCL was localized in the cytoplasm (Fig. 3b). As reported previously,[18] the dephosphorylated form of NCL is a substrate for CK2 protease, physiologically leading to the proteolytic cleavage of NCL. Taken together with the results that an increased level of NCL expression in the nucleolus was observed in the pGFP-PRL-3 transfectant (Fig. 4), these findings suggest that PRL-3 may play a role in controlling the nucleolar distribution and stabilization of NCL by dephosphorylation in the cytoplasm (Fig. 6).

We have shown physiological binding between recombinant PRL-3 and endogenous NCL by IP, in which binding of the C104S mutant PRL-3 to substrate NCL was much stronger than that of the wild-type PRL-3 (Fig. 2b,c). This may have been caused by the transient nature of the binding between the wild-type phosphatase and its substrate. Because the C104S mutant at the PTP active site is known to impair the dephosphorylation activity without disturbing the binding activity to its substrate,[26] the catalytic ''dead'' PRL-3 protein may act as a substrate-trapping mutant, forming a stable complex with NCL. Recently, we have shown that down-modulation of the cytoskeletal intermediate filament KRT8 by PRL-3 regulates CRC cell motility *in vitro* and *in vivo*, and found identical results for the interaction between recombinant PRL-3 and KRT8.[11] This may also support the reliability and consistency of the results in the present study. Also, in the present study we observed colocalization of recombinant C104S mutant PRL-3 and NCL in the cytoplasm (Fig. 4b), which was presumably caused by steady binding between C104S mutant PRL-3 and NCL.

The biological functions of the PRL-3 have been linked to the progression and metastasis of carcinoma cells.[27] To our knowledge, this is the first report to reveal the PRL-3-interacting protein in cell proliferation. In our previous study, the RNA interference of *PRL-3* mRNA did not influence CRC-derived DLD-1 cell growth;[3] however, the transduction of recombinant PRL-3 up-regulated the proliferation of SW480 cells. The growth-promoting effect of PRL-3 may probably depend on cell type, which may explain how the PRL-3 status did not completely accord with nucleolar NCL localization in human CRC cases (Fig. 5b). Moreover, although Fiordalisi *et al.*[7] have reported little staining of PRL-3 in the nucleus, our identification of a connection between PRL-3 and

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NCL may suggest that the nuclear localization of PRL-3 is due to its binding with this nucleolar protein. Although the function of NCL in the promotion of CRC growth is yet to be elucidated, it has been documented that NCL binds specifically to *B-cell lymphoma-2* adenylate uridylate (AU)-rich element, which functions in regulating the stability of *BCL-2* mRNA in HL-60 leukemia cells.[28] Since BCL-2 functions as an inhibitor of apoptosis, and overexpression of BCL-2 is an important component in the development of human cancers,[29] high BCL-2 expression in CRCs is an obstacle to cancer chemotherapy.[30] Indeed, in the present study, high levels of NCL expression and nucleolar localization of NCL in CRC tissues were closely associated with higher frequency of lymph node metastasis and higher clinicopathologic stage (Table 1). High-level expression of nucleolar NCL protein in surgical and biopsied CRC specimens may provide clinicians useful information not only for identifying occult metastases, but also for initiating adjacent chemotherapy after the surgical treatment of primary tumors. Nucleolin (NCL) may provide a novel therapeutic target for intractable CRC metastasis.

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Disclosure Statement

The authors have no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Correlation of phosphatase of regenerating liver-3 (PRL-3) expression with clinicopathologic findings in colorectal carcinoma (CRC).

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