Identification of the Critical Site of Calponin 1 for Suppression of Ovarian Cancer Properties

TAKAKO YAMANE[1], KAZUO ASANOMA[1], HIROAKI KOBAYASHI[2], GE LIU[3], HIROSHI YAGI[1], TATSUHIRO OHGAMI[1], AKIMASA ICHINOE[1], KENZO SONODA[1], NORIO WAKE[3] and KIYOKO KATO[1]

[1]*Department of Obstetrics and Gynecology, Faculty of Medical Sciences, Kyushu University, Fukuoka, Japan;*
[2]*Department of Obstetrics and Gynecology, Faculty of Medical Sciences, Kagoshima University, Kagoshima, Japan;*
[3]*Research Center for Environment and Developmental Medical Sciences, Kyushu University, Fukuoka, Japan*

Abstract. *Background: Although several studies have demonstrated the tumor suppressive function of CNN1 (calponin 1), no studies have performed a site-specific analysis of CNN1 on tumor cell activities. Materials and Methods: We herein studied the site-specific effects of CNN1 in ovarian cancer cells using full-length CNN1 (fCNN1), three CNN1 repeats (3CNRs), or the first CNN1 repeat (CNR1) expression vectors. Ovarian cancer cells stably expressing each construct were analyzed for in vitro proliferation, cell motility, invasion, and soft agar assays. An in vitro model of pleural dissemination was also established. Results: Cell proliferation, anchorage-independent colony formation, cell motility, and cell invasion were all suppressed in fCNN1, 3CNRs, and CNR1-stably-expressing cells. CNN1 expression in mesothelial cells suppressed cancer cell invasion into a monolayer of mesothelial cells. Conclusion: CNR1 showed similar suppressive effects as fCNN1. Results suggest CNR1 as a potential small synthetic peptide candidate for therapeutic strategies against ovarian cancer.*

Calponin 1 (CNN1) is one of the modulators of actomyosin contraction (1), and earlier studies have demonstrated its involvement in smooth muscle contraction (2). CNN1 is also known as a cardiac and smooth muscle differentiation marker (3-6).

Correspondence to: Dr. Kazuo Asanoma, Department of Obstetrics and Gynecology, Faculty of Medical Sciences, Kyushu University, 3-1-1Maidashi, Higashi-ku, Fukuoka 812-8582, Japan. Tel: +81 926425394, Fax: +81 926425414, e-mail: asanoma@med.kyushu-u.ac.jp

Key Words: Calponin 1 (CNN1), F-actin, cell motility, cell invasion.

Recent studies have revealed CNN1 functions in cancer development (7-11). CNN1-deficient (CNN1–/–) mice exhibited morphological fragility in blood vessels and peritoneum (9). Consistent with these phenotypes, intravenous injection of B16 melanoma cells in CNN1–/– mice led to enhanced lung metastasis, and intraperitoneal injection of B16 cells led to enhanced peritoneal dissemination compared to wild-type mice (9). This peritoneal dissemination in CNN1–/– mice was suppressed by inducing CNN1 expression in peritoneal epithelium by adenovirus vector (12). Suppression of CNN1 expression in tumor vessels has been observed in melanoma, hepatocellular carcinoma, renal cell carcinoma, and colon cancer (13-16), and down-regulation of CNN1 in tumor cells was observed in leiomyosarcoma. Previous studies have demonstrated tumor suppressive effects of CNN1 by induction of CNN1 expression in tumor cells (6, 7, 17). We previously reported tumor suppressive effects of CNN1 on ovarian cancer by transducing its expression in cancer cells and in peritoneal mesothelial cells (8).

Several studies pursued identification of sites in CNN1 critical for its function and found that the actin-binding site (ABS) and the first CNN1 repeat (CNR1) were crucial for actin binding (18-21). Among previous studies, Mino *et al.* reported that the synthetic peptide of ABS bound F-actin with a similar affinity to that of CNR1 (19). Binding of CNN1 to actin has been shown to be modified by CNN1 phosphorylation (1, 2, 11, 17, 22, 23), and Ser-175 and Thr-184 are important phosphorylation sites located in the CNR1 (1, 17, 22). Although the functional importance of CNR1 on actin binding has been studied, no studies have performed site-specific analysis of CNN1 on its tumor suppressive function.

In the present study, we studied the effects of full-length CNN1 (fCNN1) or specific CNN1 domains in ovarian cancer

cells. We also established an *in vitro* model to observe ovarian cancer cell invasion into mesothelial cell monolayers.

Materials and Methods

Cell lines. Two transplantable ovarian cancer cell lines (SHIN-3 and SKOV3i.p.1), a uterine cervical cancer cell line (HeLa), a mesothelial cell line from Chinese hamster peritoneum (CCL-14), and 293A cells were used for this study. A serous adenocarcinoma cell line, SHIN-3, was purchased from Scienstuff Co. Ltd. (Nara, Japan). SKOV3.i.p.1 cells were kindly provided by Dr. Isaiah Fidler (24). CCL-14 and HeLa cells were purchased from the American Type Culture Collection (ATCC) (Manassas, VA, USA). 293A cells were purchased from Invitrogen (Carlsbad, CA, USA).

SHIN-3, SKOV3i.p.1, and CCL-14 cells were cultured in RPMI1640 supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin, 100 U/ml penicillin, at 37°C in 5% CO_2 in air. HeLa and 293A cells were cultured in DMEM. The identities of SHIN-3, SKOV3i.p.1, and CCL-14 cells were confirmed by the Japanese Collection of Research Bioresources (JCRB) Cell Bank using DNA profiling (Short Tandem Repeat, STR) in March 2015.

Plasmids and transfection. The full-length open reading frame of CNN1, fCNN1, was kindly provided by Dr. Shun'ichiro Taniguchi, that were prepared in a pEGFP-C2 vector (Clontech, Palo Alto, CA, USA), as described previously (11). The three CNN1 repeats (3CNRs) or first CNN1 repeat (CNR1) were subcloned by polymerase chain reaction (PCR) and ligated into the pEGFP-C2 vector. The sequences of the constructs were confirmed using an ABI PRISM BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Carlsbad, CA, USA). Four μg of EGFP-fCNN1, -3CNRs, -CNR1 or control expression vector were transfected into SHIN-3, SKOV3i.p.1, CCL-14, and HeLa cells by electroporation. Amaxa Cell Line Nucleofector kit V was used for the electroporation (Lonza, Budapest, Hungary). After 48 h, cells were treated with 500 μg/ml G418 and at least three clones stably expressing each construct were established.

Cell sorting by flow cytometry. GFP-positive cells were sorted by BD FACS Aria SORP, using the FACS Diva Software (BD Bioscience, Franklin Lakes, NJ, USA). Propidium iodide (PI) was used to exclude the dead cells. Cell numbers were counted by hemacytometer or TC20 automated cell counter (Biorad, Hercules, CA, USA).

Immunofluorescence cell staining. HeLa cells transfected with either fCNN1, 3CNRs, or CNR1 expressing vectors were fixed with 4% paraformaldehyde-PBS solution, permeabilized with 1% Triton-X-PBS solution, and stained with diluted rhodamine phalloidin to visualize F-actin (Invitrogen). GFP and phalloidin were observed under a confocal immunofluorescence microscope Zeiss LSM700 system (Carl Zeiss Microscopy GmbH, Jena, Thuringia, Germany).

In vitro cell invasion and motility assay. Cell motility and invasion were evaluated by the transwell chamber assay, as described previously (25). Cell culture inserts with 8-μm pores were covered with matrigel for invasion assay (BD Bioscience). A total of 5.0×10^4 cells cultured under serum starvation overnight, were plated in upper wells without serum. Complete growth medium with 10% fetal bovine serum was placed in the lower wells. After 12 h, the membranes were collected for analysis. The membranes were stained with hematoxylin solution and the penetrated cell numbers were counted.

Soft agar colony formation assay. Cells expressing high levels of EGFP-tagged protein were sorted by FACS Aria and counted, and 1.0×10^4 cells were plated with 0.3% agar upon a 0.5% agar layer. Twenty one days after plating, colony numbers were counted.

In vitro invasion assay through a mesothelial cell monolayer. CCL-14 cells expressing high levels of GFP-tagged protein were sorted by FACS Aria and immobilized by treatment with 10 μg/ml mitomycin C (Sigma-Aldrich, St. Louis, MO, USA) for 24 h. A total of 1.0×10^5 CCL-14 cells were plated on cell culture inserts with 8-μm pores covered with matrigel. After 24 h, a total of 1.0×10^4 of cancer cells were plated on the CCL-14 cell monolayer. The penetrated cell numbers were counted after 24 h of culture.

Western blotting. Cell lysates were separated on 10% SDS-polyacrylamide gels and transferred onto polyvinylidene difluoride membranes (Millipore Billerica, MA, USA). The primary antibodies used were GFP (ab6556, Abcam, Cambridge, UK) and GAPDH (FL-335, Santa Cruz Biotechnology, Santa Cruz, CA, USA). After incubation with horseradish peroxidase-linked secondary antibodies (Promega, Madison, WI, USA), the blots were detected using an enhanced chemiluminescence (ECL) system.

Statistical analysis. Data are represented as the mean±S.D. Assay data were analyzed with the Student's *t*-test. A value of $p<0.05$ was considered significant.

Results

Localization of CNN1 proteins on F-actin fibers. To investigate the site-specific effects of CNN1 on tumor properties, we produced EGFP-tagged vectors that expressed either full-length CNN1 (fCNN1), three CNN1 repeats (3CNRs), or the first CNN1 repeat (CNR1) (Figure 1A). The empty vector with luciferase was used as a control. Expression of all four constructs was confirmed at estimated molecular weights using an anti-GFP antibody (Figure 1B). We first examined the ability of the three CNN1 constructs to localize on F-actin fibers. Because of their large cytoplasmic area, HeLa cells were used for transient transfection and confocal microscopic analysis. Twenty cells for each construct were analyzed and the overlapping ratios of the CNN1 proteins on total F-actin fiber lengths were quantified. The control cells transfected with EGFP-luciferase alone showed 35.7% localization of GFP on the actin fibers (Figure 1C). In contrast, EGFP-fCNN1 transfectants showed remarkable localization of the protein on the actin fibers (73.0%). EGFP-3CNRs and -CNR1 also showed a remarkable localization on the actin fibers compared to the control (68.1% and 66.0%, respectively; Figure 1C). The forced expression of these CNN1 constructs resulted in morphological changes including polymerization of actin fibers in the flattened cytoplasm (Figure 1C).

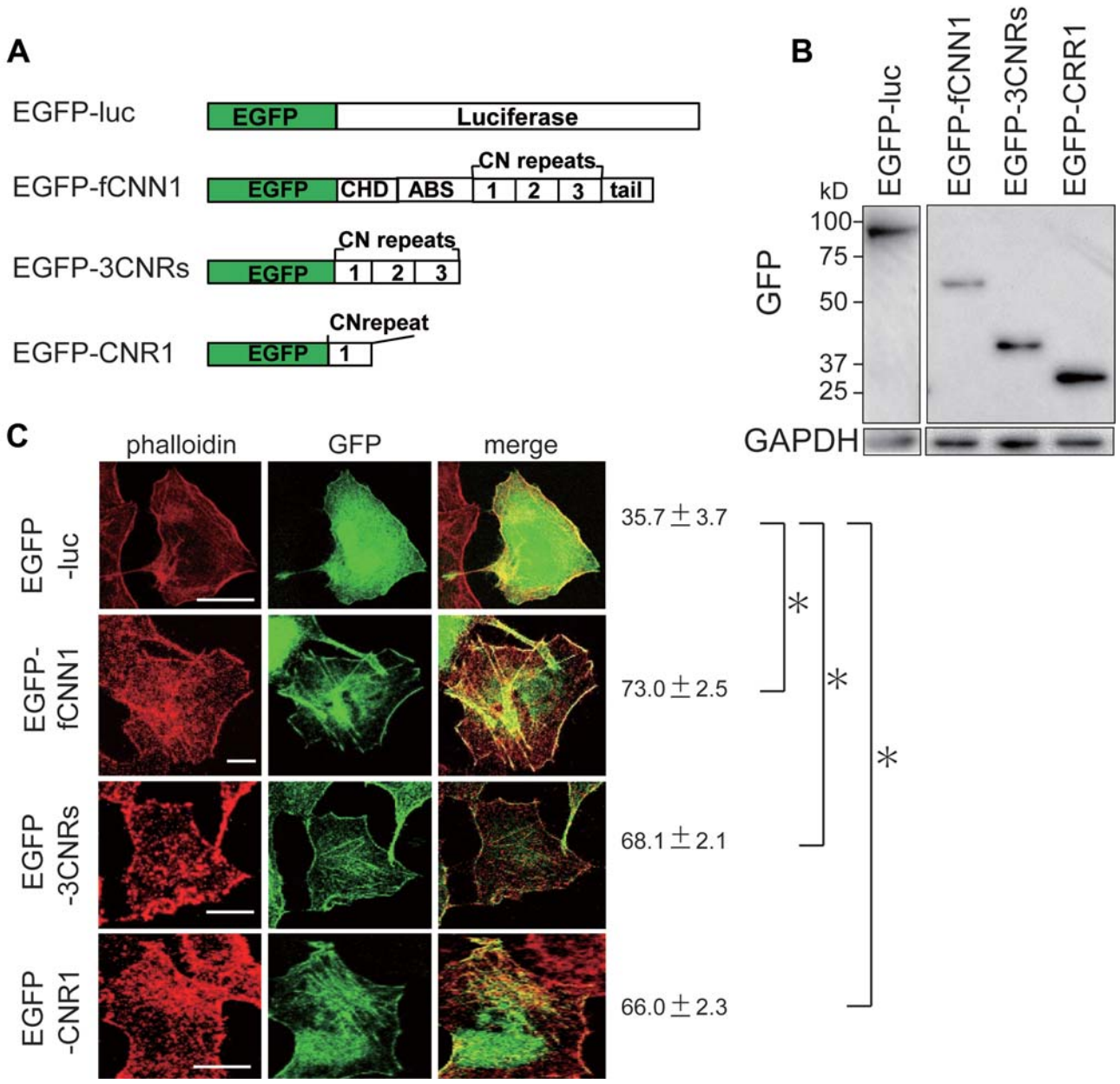

Figure 1. *Production of site-specific CNN1 constructs and their association with F-actin. A. Schematic presentation of EFGP-tagged fCNN1, 3CNRs, CNR1 and control luc constructs. B. Western blotting showing that each construct was successfully expressed in 293A cells. C. Confocal microscopic images of HeLa cells transfected with each construct (green). Rhodamine phalloidin was used to visualize F-actin (red). Right panels shows overlaid images. Co-localized signals are shown yellow. *p<0.05, compared to mock control transfectants.*

Effects of CNN1 expression on tumor cell activities of ovarian cancer cells. Next we examined effects of fCNN1, 3CNRs, and CNR1 expression on tumor cell properties. Ovarian cancer cells SHIN-3 and SKOV3i.p.1 were transfected with each of the three constructs and stable transfectants were obtained. Because the cell clones obtained expressed rather low levels of the gene product, cell sorting was used to obtain cell populations expressing higher levels of the gene products (Figure 2A). GFP-positive cells were sorted by BD FACS Aria and used for the following assays. In comparison to control cells, cell proliferation, anchorage-independent colony formation, cell motility, and cell invasion were suppressed by fCNN1 expression in both SHIN-3 and SKOV3i.p.1 cells (Figure 2B-E). Similar results were

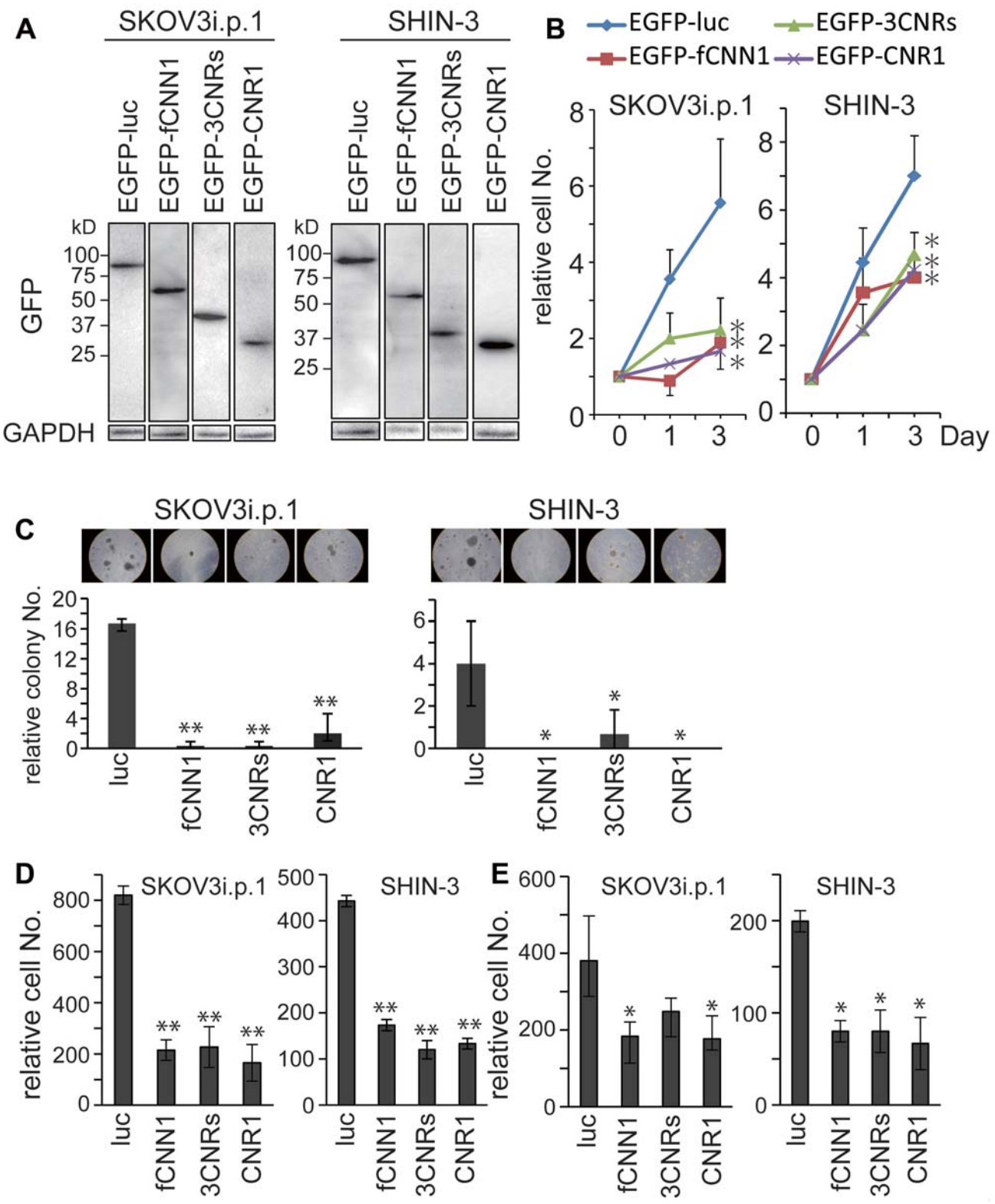

Figure 2. *Tumor properties of SKOV3i.p.1 and SHIN-3 cells expressing CNN1 domain constructs. A. Establishment of stable transfectants of EGFP-tagged fCNN1, 3CNRs, or CNR1-expressing cells in SKOV3i.p.1 and SHIN-3 cells. At least three cell clones for each construct were established and analyzed. Cell proliferation (B), anchorage-independent clonogenicity (C), cell motility (D), and invasion (E) were examined with each transfectant. Columns, mean of triplicate experiments; bars, standard deviations; *p<0.05, **p<0.01, compared to mock-control transfectants.*

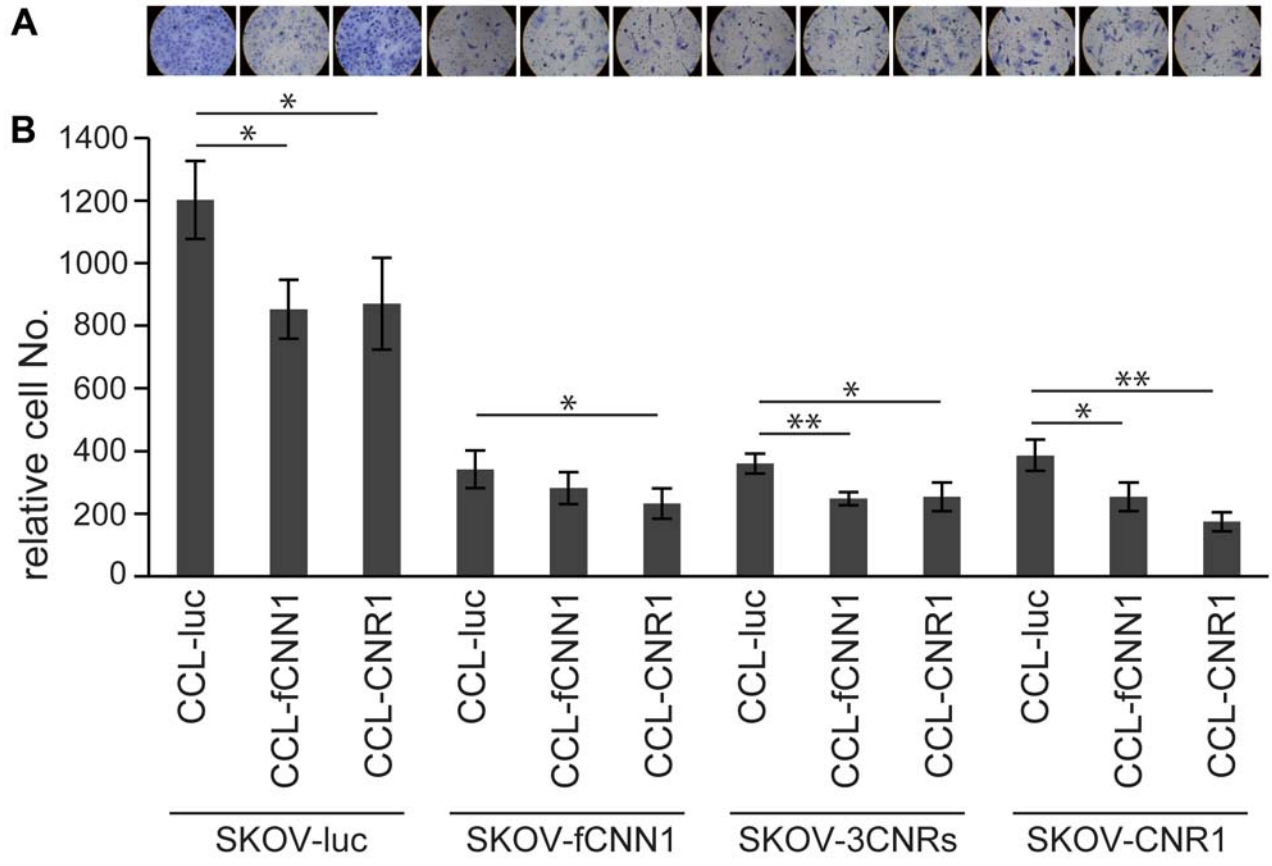

Figure 3. *In vitro invasion assay of SKOV3i.p.1 cell invasion through CCL-14 cell monolayer. SKOV3i.p.1 cells expressing EGFP-tagged fCNN1, 3CNRs, CNR1 or control construct were plated on a monolayer of immobilized CCL-14 cells expressing EGFP-tagged luciferase (mock control), -fCNN1, or -CNR1 construct. A. The penetrated cells on the other side of culture insert membranes were stained with hematoxylin solution. B. Quantified data of Figure 3A. Columns, mean of triplicate experiments; bars, standard deviations; *p<0.05, **p<0.01, compared to mock control transfectants. CCL, CCL-14; SKOV, SKOV3i.p.1.*

obtained by 3CNRs and CNR1 expression in both SHIN-3 and SKOV3i.p.1 cells (Figure 2B-E). These data suggested that CNR1 is sufficient for suppression of tumor cell properties.

Effects of CNN1 expression in mesothelial cells on cell invasion. CNN1 expression in mesothelial cells has been suggested to affect cancer cell implantation in the peritoneum (10). Peritoneal dissemination of cancer cells in CNN1–/– mice was suppressed by inducing CNN1 expression in peritoneal mesothelial cells by adenovirus vector (12). We established an *in vitro* model to analyze tumor cell invasion through a mesothelial cell monolayer. Twenty-four hours of treatment of mesothelial CCL-14 cells with 10 μg/ml mitomycin C completely immobilized the cells. These immobilized CCL-14 cells were plated on matrigel to obtain a confluent monolayer. In comparison to control cells, fCNN1-, 3CNRs-, and CNR1-expressing SKOV3i.p.1 cells showed suppressed cell invasion through the mesothelial cell-matrigel layer. On the other hand, fCNN1 and CNR1 expression, but not 3CNRs, in CCL-14 cells also suppressed cancer cell invasion through the mesothelial cell-matrigel layer (Figure 3A and 3B).

Discussion

CNN1 stabilizes actomyosin and suppresses cell motility (1, 7, 26, 27). On the other hand, CNN1 expression in tumor cells suppresses their proliferation, anchorage-independent clonogenicity, and tumorigenicity in nude mice, as well as their motility and invasion (7, 8, 17). Site-specific analyses revealed that ABS or the CNR1 was found to be critical for actin binding (18-21). Functional importance of CNR1 has been suggested because two well-known phosphorylation sites, Ser-175 and Thr-184, reside in CNR1 (1, 17, 22). Our study demonstrated that CNR1

exhibited similar functions in actin localization and suppression of tumor cell properties as full-length CNN1. CNR1 not only associated with F-actin, but also suppressed cell proliferation, motility, invasion, and anchorage-independent clonogenicity. Further studies are required to explore the relationship between stabilization of actin fibers and suppression of tumor properties by CNN1 expression. However, considering that our smallest peptide fragment, CNR1, exerted remarkable effects, our data might suggest that the stabilization of actin fibers resulting from the association between CNR1 and F-actin may be the mechanistic reason underlying the effects of CNR1 on multiple tumor properties of ovarian cancer cells.

CNN1 expression in mesothelial cells prevented tumor cell implantation on the peritoneum (8, 9, 12). We established an *in vitro* model to study tumor cell invasion through a monolayer of immobilized mesothelial cells. Our results showed that forced expression of CNN1 in mesothelial cells prevented ovarian cell invasion through a constructed mesothelial cell monolayer. CNN1 expression in both cancer cells and mesothelial cells prevented cancer cell invasion to a high degree. This effect of CNN1 on cell invasion was also similarly observed with CNR1.

The above results suggested that CNN1 expression could suppress ovarian cancer development by controlling two different targets, tumor cells themselves and mesothelial cells, as described previously (8, 10). In both strategies, our *in vitro* data suggested that CNR1 exerted similar effects as full-length CNN1. Considering therapeutic strategies using a small synthetic peptide, CNR1 is a potential candidate that may help control ovarian cancer development.

Acknowledgements

The Authors appreciate the technical support from Dr. Tomoka Takao, Ms. Yoko Miyanari, Ms. Yumi Konno, and the Research Support Center, Graduate School of Medical Science, Kyushu University. They also appreciate Dr. Shun'ichiro Taniguchi at Shinshu University for providing us a pEGFP-fCNN1 plasmid. This work was supported by JSPS KAKENHI Grant Number 26462529, 23249075, 23390392.

References

1 Winder SJ and Walsh MP: Calponin: thin filament-linked regulation of smooth muscle contraction. Cell Signal *5*: 677-686, 1993.

2 Winder SJ and Walsh MP: Smooth muscle calponin. Inhibition of actomyosin MgATPase and regulation by phosphorylation. J Biol Chem *265*: 10148-10155, 1990.

3 Miano JM and Olson EN: Expression of the smooth muscle cell calponin gene marks the early cardiac and smooth muscle cell lineages during mouse embryogenesis. J Biol Chem *271*: 7095-7103, 1996.

4 Ya J, Markman MW, Wagenaar GT, Blommaart PJ, Moorman AF and Lamers WH: Expression of the smooth-muscle proteins alpha-smooth-muscle actin and calponin, and of the intermediate filament protein desmin are parameters of cardiomyocyte maturation in the prenatal rat heart. Anat Rec *249*: 495-505, 1997.

5 Samaha FF, Ip HS, Morrisey EE, Seltzer J, Tang Z, Solway J and Parmacek MS: Developmental pattern of expression and genomic organization of the calponin-h1 gene. A contractile smooth muscle cell marker. J Biol Chem *271*: 395-403, 1996.

6 Horiuchi A, Nikaido T, Ya-Li Z, Ito K, Orii A and Fujii S: Heparin inhibits proliferation of myometrial and leiomyomal smooth muscle cells through the induction of alpha-smooth muscle actin, calponin h1 and p27. Mol Hum Reprod *5*: 139-145, 1999.

7 Takeoka M, Ehara T, Sagara J, Hashimoto S and Taniguchi S: Calponin h1 induced a flattened morphology and suppressed the growth of human fibrosarcoma HT1080 cells. Eur J Cancer *38*: 436-442, 2002.

8 Ogura T, Kobayashi H, Ueoka Y, Okugawa K, Kato K, Hirakawa T, Hashimoto S, Taniguchi S, Wake N and Nakano H: Adenovirus-mediated calponin h1 gene therapy directed against peritoneal dissemination of ovarian cancer: bifunctional therapeutic effects on peritoneal cell layer and cancer cells. Clin Cancer Res *12*: 5216-5223, 2006.

9 Taniguchi S, Takeoka M, Ehara T, Hashimoto S, Shibuki H, Yoshimura N, Shigematsu H, Takahashi K and Katsuki M: Structural fragility of blood vessels and peritoneum in calponin h1-deficient mice, resulting in an increase in hematogenous metastasis and peritoneal dissemination of malignant tumor cells. Cancer Res *61*: 7627-7634, 2001.

10 Taniguchi S: Suppression of cancer phenotypes through a multifunctional actin-binding protein, calponin, that attacks cancer cells and simultaneously protects the host from invasion. Cancer Sci *96*: 738-746, 2005.

11 Sugaya A, Takeoka M, Itano N, Taguchi A, Ehara T and Taniguchi S: Calponin h1-S175T point mutation enhances resistance to actin cytoskeleton perturbation in human cancer cells. Anticancer Res *30*: 1071-1078, 2010.

12 Hashimoto S, Takeoka M and Taniguchi S: Suppression of peritoneal dissemination through protecting mesothelial cells from retraction by cancer cells. Int J Cancer *107*: 557-563, 2003.

13 Koganehira Y, Takeoka M, Ehara T, Sasaki K, Murata H, Saida T and Taniguchi S: Reduced expression of actin-binding proteins, h-caldesmon and calponin h1, in the vascular smooth muscle inside melanoma lesions: an adverse prognostic factor for malignant melanoma. Br J Dermatol *148*: 971-980, 2003.

14 Sasaki Y, Yamamura H, Kawakami Y, Yamada T, Hiratsuka M, Kameyama M, Ohigashi H, Ishikawa O, Imaoka S, Ishiguro S and Takahashi K: Expression of smooth muscle calponin in tumor vessels of human hepatocellular carcinoma and its possible association with prognosis. Cancer *94*: 1777-1786, 2002.

15 Islam AH, Ehara T, Kato H, Hayama M, Kobayashi S, Igawa Y and Nishizawa O: Calponin h1 expression in renal tumor vessels: correlations with multiple pathological factors of renal cell carcinoma. J Urol *171*: 1319-1323, 2004.

16 Yanagisawa Y, Takeoka M, Ehara T, Itano N, Miyagawa S and Taniguchi S: Reduction of Calponin h1 expression in human colon cancer blood vessels. Eur J Surg Oncol *34*: 531-537, 2008.

17 Kaneko M, Takeoka M, Oguchi M, Koganehira Y, Murata H, Ehara T, Tozuka M, Saida T and Taniguchi S: Calponin h1 suppresses tumor growth of Src-induced transformed 3Y1 cells in association with a decrease in angiogenesis. Jpn J Cancer Res *93*: 935-943, 2002.
18 Mezgueldi M, Mendre C, Calas B, Kassab R and Fattoum A: Characterization of the regulatory domain of gizzard calponin. Interactions of the 145-163 region with F-actin, calcium-binding proteins, and tropomyosin. J Biol Chem *270*: 8867-8876, 1995.
19 Mino T, Yuasa U, Nakamura F, Naka M and Tanaka T: Two distinct actin-binding sites of smooth muscle calponin. Eur J Biochem *251*: 262-268, 1998.
20 Fattoum A, Roustan C, Smyczynski C, Der Terrossian E and Kassab R: Mapping the microtubule binding regions of calponin. Biochemistry *42*: 1274-1282, 2003.
21 Mezgueldi M, Fattoum A, Derancourt J and Kassab R: Mapping of the functional domains in the amino-terminal region of calponin. J Biol Chem *267*: 15943-15951, 1992.
22 Uyama Y, Imaizumi Y, Watanabe M and Walsh MP: Inhibition by calponin of isometric force in demembranated vascular smooth muscle strips: the critical role of serine-175. Biochem J *319(Pt 2)*: 551-558, 1996.
23 Abouzaglou J, Benistant C, Gimona M, Roustan C, Kassab R and Fattoum A: Tyrosine phosphorylation of calponins. Inhibition of the interaction with F-actin. Eur J Biochem *271*: 2615-2623, 2004.
24 Xu L, Xie K and Fidler IJ: Therapy of human ovarian cancer by transfection with the murine interferon beta gene: role of macrophage-inducible nitric oxide synthase. Hum Gene Ther *9*: 2699-2708, 1998.
25 Li D, Takao T, Tsunematsu R, Morokuma S, Fukushima K, Kobayashi H, Saito T, Furue M, Wake N and Asanoma K: Inhibition of AHR transcription by NF1C is affected by a single-nucleotide polymorphism, and is involved in suppression of human uterine endometrial cancer. Oncogene *32*: 4950-4959, 2013.
26 Kake T, Kimura S, Takahashi K and Maruyama K: Calponin induces actin polymerization at low ionic strength and inhibits depolymerization of actin filaments. Biochem J *312(Pt 2)*: 587-592, 1995.
27 Leinweber B, Tang JX, Stafford WF and Chalovich JM: Calponin interaction with alpha-actinin-actin: evidence for a structural role for calponin. Biophys J *77*: 3208-3217, 1999.

Received August 17, 2015
Revised September 15, 2015
Accepted September 16, 2015