Biochemical and Biophysical Research Communications 404 (2011) 488–493

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Synemin down-regulation in human hepatocellular carcinoma does not destabilize cytoskeletons *in vivo*

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ARTICLE INFO

Article history:
Received 25 November 2010
Available online 6 December 2010

Keywords:
Cytoskeleton
Hepatocellular carcinoma
Intermediate filament
Microfilament
Microtubule
Plectin
Synemin

ABSTRACT

Synemin is a large intermediate filament protein that has been identified in all types of muscle cells. It plays a role in human muscle diseases; however, the role of synemin in tumor cell transformation has rarely been investigated. Because hepatocellular carcinoma cells are morphologically different from normal human hepatocytes, we hypothesized that altered synemin expression and cytoskeletal disorganization might underlie this pleomorphic transformation. To test this hypothesis, we studied synemin expression in hepatocellular carcinoma and liver tissues by immunohistochemistry and immunoblotting. In addition, we analyzed the expression level and organization of all cytoskeletal elements after synemin knock-down in human Chang liver cells. Previously we found that plectin knock-down in human Chang liver cells causes a reduction in cytokeratin 18 expression with effects on intermediate filament disorganization and altered cellular morphology. In this study we also compared the effects of synemin knock-down and plectin knock-down on the cytoskeleton expression and organization. The results revealed that synemin expression was down-regulated in human hepatocellular carcinoma compared with normal liver, which is similar to the plectin expression. Surprisingly, the expression of cytoskeletal elements (cytokeratin 18, actin and tubulin) was not influenced by synemin knock-down in human Chang liver cells. The organization of cytoskeletal networks was also unaltered after synemin knock-down. In conclusion, both plectin and synemin are down-regulated in human hepatocellular carcinoma *in vivo* and transformed human liver cell *in vitro*. However, the mechanism of cell transformation caused by synemin knock-down is different from that of plectin knock-down. Plectin, but not synemin, knock-down provoked liver cell transformation via suppressing cytokeratin 18 expression and disrupting intermediate filament networks. Synemin knock-down did not influence the cytoskeleton expression and organization of human Chang liver cells.

1. Introduction

In animal cells, including human hepatocytes, cellular outline and structural integrity are maintained by the cytoskeleton. The cytoskeleton is composed of three organelles: microtubule (MT), microfilament (MF) and intermediate filament (IF) [1]. MTs are long, hollow cylinders composed of multiple isotypes of α- and β-tubulin with an outer diameter of 25 nm [2]. MFs are two-stranded helical polymers composed of actin with a diameter of 5–9 nm [3]. IFs, with a diameter of 10 nm, are ropelike fibers comprising a large heterogeneous family, including cytokeratin (CK) [4]. In human hepatocytes, the IF polymers are formed by a specific

Abbreviations: MT, microtubule; MF, microfilament; IF, intermediate filament; CK, cytokeratin; IFAP, intermediate filament associated protein; HCC, hepatocellular carcinoma; HRP, horseradish peroxidase; DMEM, Dulbecco's modified eagle medium; FCS, fetal calf serum; siRNA, small interfering RNA; PBS, phosphate buffered saline; FITC, fluoroscein-conjugated isothiocyanate; SDS, sodium dodecyl sulfate; PVDF, polyvinylidene fluoride.

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doi:10.1016/j.bbrc.2010.12.008

CK pair: CK8 (type II, molecular weight 52 kDa) and CK18 (type I, molecular weight 45 kDa) [5].

Proper cross-linking organization among cytoskeleton structures is critical for establishing the internal architecture and overall morphology of cells. Several cross-linking proteins which mediate the interaction between IFs and other cytoskeletal networks have been identified [4]. Among these, plectin is the most versatile. Plectin has binding sites for IF proteins, tubulin and actin [6], and is expressed in a variety of tissues and mammalian cell types [7,8]. Another cross-linking protein, synemin, was initially described as an IF-associated protein (IFAP) because it is co-localized and co-purified with the IF proteins desmin and vimentin [9,10]. Recently synemin has been identified as a member of a large IF protein superfamily that is present in all types of muscle cells in association with desmin- and/or vimentin-containing IFs [11].

Synemin may function to link muscle cell IFs directly to both myofibrillar Z-lines and costameres [12]. It plays a role in human muscle diseases including myofibrillar myopathies [13] and muscular dystrophies [14]. However, the role of synemin in tumor cell transformation has not been investigated very often, and only a few reports on this subject have been published. For example, synemin is present in human reactive and malignant astrocytes and associates with ruffled membranes in astrocytoma cells [15]. Synemin expression is induced in proliferating and malignant biliary epithelial cells [16]. And synemin mRNA was seen to be down-regulated and the synemin protein lost in breast cancer, and aberrant promoter methylation of the synemin gene was associated with early breast cancer relapse [17]. Until now, the role of synemin in human hepatocellular carcinoma (HCC) has been investigated extremely rarely.

Hepatoma cells are morphologically different from normal liver cells. Previously, we hypothesized that the pleomorphism of hepatoma cells was caused by instability and disorganization of the cytoskeleton. Our previous study on modulation of CK18 in human HCC [18] indicated that CK18 is unstable in these cells. We further speculated that the cytoskeletal instability might be related to cytoskeleton-associated proteins such as plectin and/or synemin. We found that plectin was down-regulated in human HCC tissues *in vivo* and in the human hepatoma PLC/PRF/5 cell line *in vitro* [19]. We also found that knock-down of plectin in human Chang liver cells caused a reduction in CK18 expression with effects on IF disorganization and altered cellular morphology. However, plectin deficiency did not influence the expression and organization of MFs and MTs [20].

Previously, by immunohistochemical assay, we found that the expression of synemin was down-regulated in human HCC tissues [21]. This change was accompanied by changes in plectin expression in human HCC, which was also down-regulated. In the present study, we hope to clarify whether synemin deficiency, like that of plectin, can influence the expression and organization of the cytoskeleton in human liver cells. First, we examined the expression of synemin in human HCC tissue by Western blot assay. Second, we used human Chang liver cells as the experimental model to clarify the influence of siRNA-produced synemin deficiency on the expression and organization of the major cytoskeletal elements (MT, MF and IF) and the overall morphology of these cells.

2. Materials and methods

2.1. Tissue samples and antibodies

Freshly surgically resected HCC tissues from three liver cancer patients were studied. All HCC cases were histopathologically diagnosed as grade II. These samples were stored at −80 °C before protein extraction and Western blot analysis. In addition, paraffin blocks from 10 cases of grade II HCC were also obtained. The following commercial primary and secondary antibodies were used for immunohistochemistry, immunoblotting and immunofluorescence assay: anti-CK18, anti-actin and anti-tubulin monoclonal antibodies raised in mouse (Zymed Laboratories, Inc., South San Francisco, California, USA); anti-synemin polyclonal antibody raised in goat (Reglia AB, Co., Stena Center, Goteborg, Sweden); anti-mouse and anti-goat IgG (Jackson Immuno Research Laboratories, Inc., West Grove, Pennsylvania, USA). Anti-mouse and anti-goat IgG was conjugated to biotin for immunohistochemistry, to horseradish peroxidase (HRP) for Western blot analysis and to rhodamine or fluorescein isothiocyanate (FITC) for immunofluorescent staining.

2.2. Immunohistochemistry

Deparaffinized and rehydrated sections were treated with 3% H_2O_2 for 10 min to eliminate endogenous peroxidase activity. Nonspecific binding sites were blocked with bovine serum albumin for 10 min. The sections were incubated with anti-synemin polyclonal antibody (1:50 dilution) for 1 h at room temperature. The biotinylated second antibody (anti-goat IgG) was added and the final signal was expressed by the avidin–biotin peroxidase technique in the presence of H_2O_2 [22]. These sections were evaluated under light microscopy (Olympus BX51, Tokyo, Japan).

2.3. Cell culture

Chang liver cells from normal human liver tissue (obtained from ATCC CCL-13) were maintained in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS), 50 unit/mL penicillin and streptomycin, and 200 mM L-glutamine (purchased from Invitrogen, Carlsbad, California, USA). Cells were grown over 2–4 days at 37 °C in 5% CO_2/95% air atmosphere, and medium was replaced every 2 days. For mRNA studies, cells were sub-cultured in 24-well plates (4×10^3 cells per well); for protein studies, cells were seeded in six-well plates (4×10^4 cells per well) and grown for 24 h before experiments.

2.4. Synemin siRNA treatment of Chang liver cells

Small-interfering RNA (siRNA) oligonucleotides were used to reduce synemin mRNA and protein expression in Chang liver cells. siRNA duplex oligonucleotides (5′-PUCACCUCCAAUAGAUGUGCUU-3′; 5′-PCUAAUCGGCACCUCUCUCUUU-3′; 5′-PCGAGCCGUCAUCUCUGUCGUU-3′; 5′-PCUGAACCGUAGCUCUCUUGUU-3′) were purchased from Dharmacon, Inc. (Lafayette, Colorado, USA). Oligonucleotides were transfected into cells at 50% confluence using Lipofectamine 2000 Reagent (Invitrogen, Carlsbad, California, USA) according to the manufacturer's instructions. Experiments were conducted 72 h after transfection.

2.5. Immunofluorescence

Cells with or without synemin siRNA treatment were grown on 24-well plates (4×10^3 cells per well). Prior to staining, cells were washed with ice-cold phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, and 1.5 mM KH_2PO_4, pH 7.4) and fixed for 20 min with 3.7% paraformaldehyde/PBS at room temperature. After fixation, cells were washed three times with PBS and permeabilized with 0.1% Triton X-100/PBS for 2 min at room temperature. Cells were washed three times in PBS and double-stained for 60 min with primary antibodies (anti-CK18, anti-actin, anti-tubulin and anti-synemin) at room temperature and then washed with PBS again. Cells were then incubated with rhodamine-conjugated anti-mouse IgG and FITC-conjugated anti-goat IgG secondary antibodies for 30 min at room temperature. Finally,

the unbound antibodies were removed by washing twice for 10 min in PBS [23]. The images were evaluated under a fluorescence microscope (Olympus BX51, Tokyo, Japan).

2.6. Cytoskeleton extraction

In general, extractions were performed according to French's method [24]. Unless otherwise indicated, the preparation was conducted at 4 °C. Briefly, the minced specimen first was incubated with CSK buffer (300 mM sucrose, 3 mM $MgCl_2$, 0.5% Triton X-100, 10 mM piperazine-1, 4-bis[2-ethanesulphonicacid], 1.2 mM phenylmethylsulfonyl fluoride, and 0.1 mM iodoacetamide) containing 100 mM NaCl, for 5 min. After three washes, pellets were treated with CSK buffer containing 250 mM $(NH_4)_2SO_4$ for 10 min, and then with the same buffer containing bovine pancreatic DNase I (100 μg/mL) and RNase A (100 μg/mL) for 10 min. Pellets were rinsed with buffer containing DNase I, RNase A, and $(NH_4)_2SO_4$ for 10 min at room temperature. Finally, the preparation was resuspended in CSK buffer, and the concentration was adjusted to 1 mg/mL.

2.7. Western blot analysis

Total protein extracts were prepared in sample buffer containing 0.5 M Tris–HCl, pH 6.8, 10% sodium dodecyl sulfate (SDS), 5% 2-mercaptoethanol and 30% glycerol. Equal amounts of the extracted lysate (10 μg for cell extraction, 40 μg for tissue extraction) were separated by 8% sodium dodecyl sulfate–polyacrylamide gel electrophoresis [25]. After electrophoresis, proteins were transferred to polyvinylidene fluoride (PVDF) membranes using the semi-dry transfer method (Bio-Rad Laboratories, Inc., Hercules, California, USA). Membranes were blocked for one hour at room temperature (or 4 °C overnight) with a blocking buffer containing 5% (w/v) non-fat milk. Membranes were then incubated with monoclonal antibodies overnight at 4 °C. Membranes were incubated with secondary antibodies for 60 min at room temperature and visualized using enhanced chemiluminescence reagent (NEN™ Life Science Products, Boston, Massachusetts, USA).

3. Results

In the paraffin blocks from the 10 HCC cases, the staining pattern of normal liver cells showed uniform strong positive staining for synemin antibody, but the staining of HCC cells showed only weak positive reactions. The expression of synemin in HCCs was significantly reduced compared to that in the pair-wise normal liver cells in all HCC cases examined (Fig. 1A). In order to confirm the above findings, total protein extracted from freshly obtained normal liver and HCC tissues for Western blot assay was processed. This assay showed that synemin expression was down regulated in HCC cases compared to normal liver tissues (Fig. 1B). The Western blot results were compatible with the immunohistochemistry findings.

To analyze the effects of synemin deficiency on the cytoskeletal network of human liver cells, RNA interference was employed to suppress synemin protein expression in human Chang liver cells (Fig. 1C). While non-targeting control RNAi did not affect expression of synemin, synemin levels were markedly reduced in synemin-specific siRNA-treated cells. Quantitative analysis revealed that the expression level of synemin was reduced by 70% (data not shown). In contrast, expression levels of CK18, actin and tubulin were unchanged. For this quantitative analysis, each experiment was repeated at least three times.

To investigate whether synemin is important for organization of the cytoskeleton in these cells, we compared cytoskeletal networks

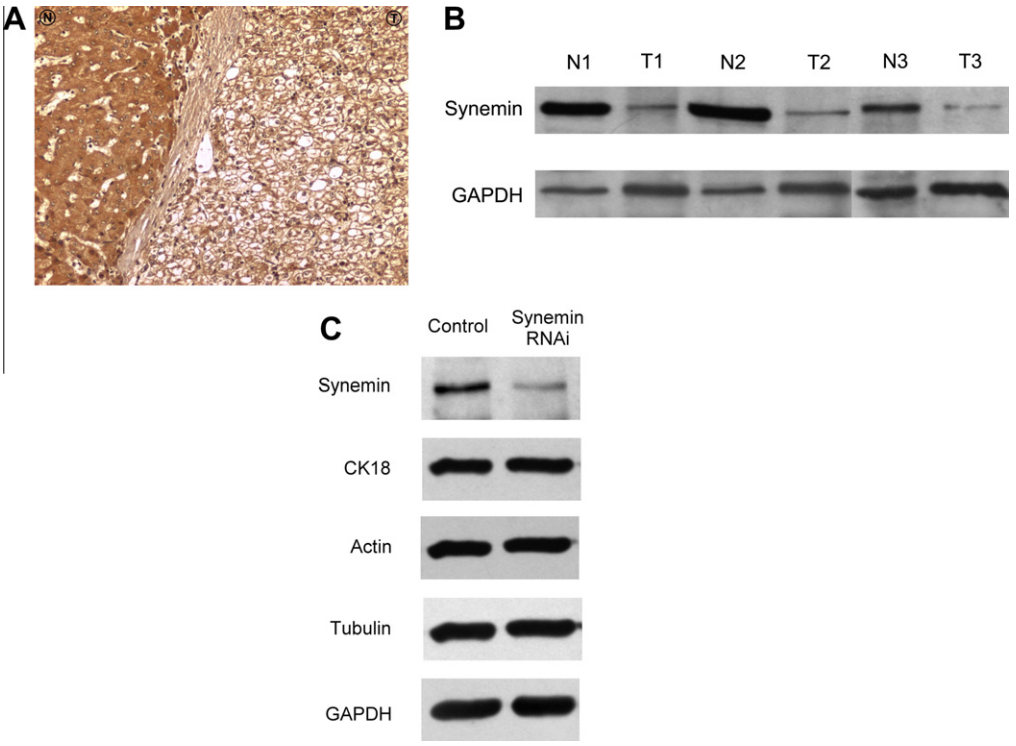

Fig. 1. (A) In a paraffin section from a HCC case, HCC cells (on the right side) show weak staining to anti-synemin antibodies, while pair-wise human normal liver cells (on the left side) from the same specimen were strongly positive. The HCC and non-tumor liver tissues are separated by fibrous connective tissue (200×). (B) Western blot analysis of normal liver and HCC tissues. Total proteins extracted from liver (N1–3) and HCCs (T1–3) tissues were analyzed using monoclonal anti-synemin antibodies. The expression of synemin was decreased in HCC tissues compared to normal liver tissues. (C) Western blot analysis of synemin siRNA treatment of human Chang liver cells. Total protein extracts were separated on 8% SDS–PAGE. Expression level of synemin was down-regulated by synemin siRNA, but expression of CK18, actin and tubulin was unchanged.

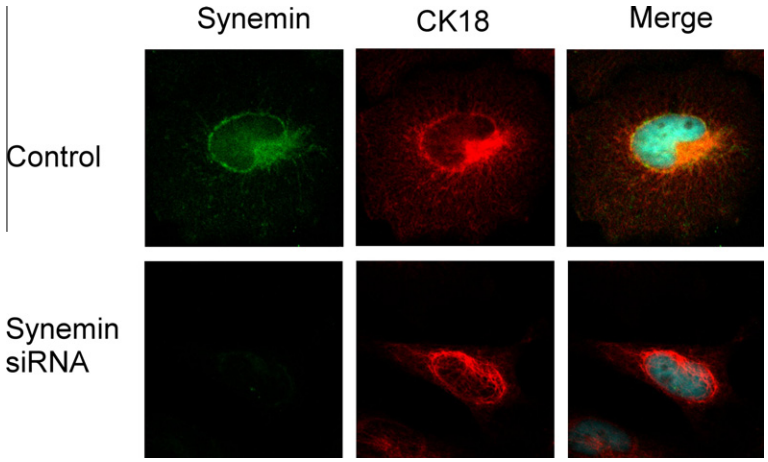

Fig. 2. Immunofluorescence of IF in synemin siRNA-treated Chang liver cells. Upper panels (control) are mock-treated cells (negative siRNA control); lower panels are synemin siRNA-treated cells. *Merge* indicates merged images of synemin and CK18 immunofluorescence.

in control and synemin knock-down cells by immunofluorescence. In control cells, synemin was distributed mainly in dense wooly networks in a perinuclear pattern. Synemin also extended to the cell periphery in delicate filaments or on short, irregularly oriented fibers and dotted lines (Figs. 2–4). In synemin siRNA-treated cells, the general cell shape was changed as a result of synemin suppression. Although synemin immunostaining was dramatically diminished by siRNA, it was not completely eliminated, in contrast to the immunoblot data. This discrepancy could be due to differences in sensitivity of the two techniques, as well as to rapid scattering of the fluorescence signal.

CK18 was present in fine filament networks in the cytoplasm, with the greatest abundance in the perinuclear region, and mesh-like distributions extending toward the cell membrane. The CK18 filaments co-localized with synemin (Fig. 2, merged image). Synemin staining also overlapped with actin staining of control cells. Synemin was distributed diffusely throughout the cytoplasm with some enrichment around the nucleus where MFs were concentrated. Synemin and MF also partially co-localized at the cell periphery where synemin appeared as short filamentous assemblies (Fig. 3). MTs of control cells appeared as long, wavy fibers of uniform thickness that were abundant around the perinuclear region. Synemin co-localized with MT mainly in the perinuclear region but showed little overlap in the cell periphery (Fig. 4). Surprisingly, we did not found obvious differences between control and synemin knock-down cells in the organization of CK18, MF and MT network.

4. Discussion

The roles of cytoskeletal instability in pleomorphism of human HCC cells were investigated in our laboratory. In this study, using immunohistochemistry and immunoblot assay *in vivo*, we found that the expression of synemin was down-regulated in HCC tissues compared to that in pair-wise normal liver cells. *In vitro* study indicated that the morphology of human Chang liver cells changed with siRNA-mediated suppression of synemin. However, Western blot assay showed that synemin knock-down did not influence the expression of CK18, actin and tubulin in these cells. Immunofluorescent observation demonstrated that the organization of cytoskeletal networks in synemin knock-down cells was not altered.

Little is known about synemin expression in human liver and HCC tissues *in vivo*; in this study we examined the expression of synemin in HCC tissues by immunohistochemistry and Western blot assay. We found synemin expression was down-regulated in human HCC compared with that in normal liver. The results were comparable to other research, which reported that synemin expression was low in human liver cells and not expressed in HCC cells [16]. Until now, most studies have reported that synemin is mainly present in muscle cells but rare in liver cells. Our data

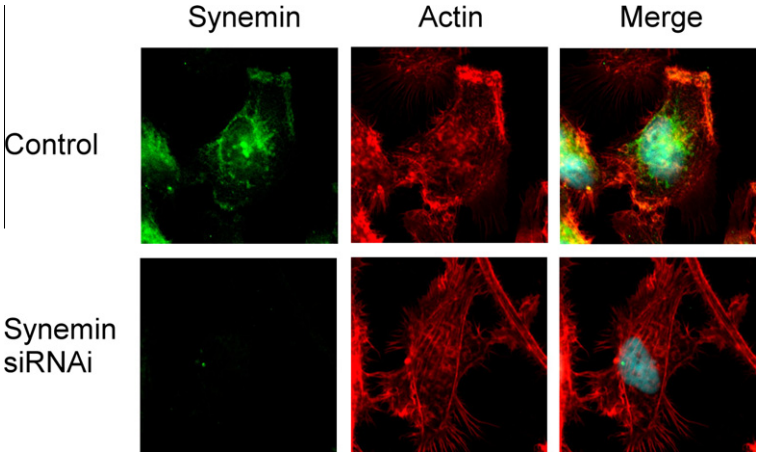

Fig. 3. Immunofluorescence of MF in synemin siRNA-treated cells. Upper panels (control) are mock-treated cells (negative siRNA control); lower panels are synemin siRNA-treated cells. *Merge* indicates merged images of synemin and actin immunofluorescence.

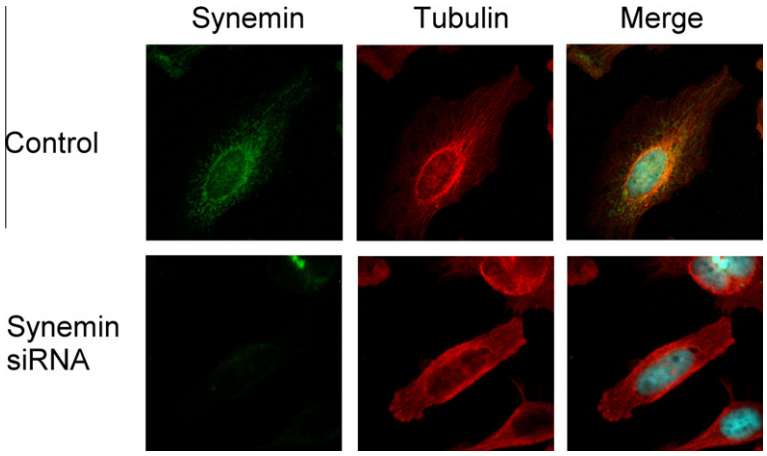

Fig. 4. Immunofluorescence of MT in synemin siRNA-treated cells. Upper panels (Control) are mock-treated cells (negative siRNA control); lower panels are synemin siRNA-treated cells. *Merge* indicates merged images of synemin and tubulin immunofluorescence.

clearly showed that synemin was actually abundant in human liver cells and was down-regulated in human HCC cells.

Because synemin was down-regulated in human HCC, we speculated that deficiency of synemin might cause the transformation of normal liver cells to pleomorphic HCC cells. To clarify this speculation, human Chang liver cells were used as an experimental model to investigate cell transformation due to synemin deficiency. We found that the general cell shape was altered and shrinkage occurred as a result of synemin suppression in synemin siRNA-treated liver cells. Morphological alteration of cells by synemin siRNA has also been found by others, who have reported that the overall cell shape of synemin-silenced astrocytoma cells was changed and the size was smaller than that of controls [26]. These data supported our speculation that synemin deficiency might provoke liver cell transformation. We previously found plectin knock-down in human Chang liver cells resulted in transformation of these cells; in this study, we further established that not only plectin suppression but also synemin suppression could transform the human Chang liver cells.

Our previous study confirmed that plectin knock-down in human Chang liver cells decreased the expression of CK18 and caused the disorganization of cytoskeleton and further result in transformation of these cells [19,27]. We were curious as to whether the effects of synemin knock-down were analogous to plectin. In this study, we were surprised to find that the expression of cytoskeletal elements (CK18, actin and tubulin) was not altered by synemin knock-down in human Chang liver cells. Further immunofluorescent experiments revealed that the organization of cytoskeleton (IF, MF and MT) was also unaffected by synemin knock-down. These results demonstrated that the effect of synemin knock-down on the cytoskeleton was distinct from that of plectin. The effects of synemin suppression on cytoskeletal expression and organization have rarely been investigated until now. Other studies reported that treatment with synemin siRNAs down-regulate the protein levels of synemin, but not those of actin and vimentin in human glioblastoma cells [26]. Knock-down of synemin in HeLa cells did not change the protein levels of α-tubulin; and the filamentous organization of vimentin was also unchanged in these cells [28]. Based on these data, we considered that synemin deficiency might act directly, not via disrupting other cytoskeletal elements, to induce morphological change in liver cells.

The mechanism of synemin knock-down action on the liver cell transformation is still unclear. Although synemin was initially classified as an IFAP [10]; subsequent study discovered that it is not an IFAP, but instead contains the characteristic IF rod domain and is a novel member of the IF protein superfamily [29]. Recently synemin was identified in association with desmin- and/or vimentin and functions as a component of heteropolymeric IFs [12]. According to these reports, we raise a hypothesis here to explain how synemin knock-down can cause liver cell transformation without disturbing the cytoskeletal expression and organization. We consider that synemin is associated with desmin- and/or vimentin but that, unlike plectin, it has no binding site for CK, actin and tubulin. However, the cytoskeleton in human liver parenchyma cells is comprised of CK, actin and tubulin, excluding desmin and vimentin. Therefore, the cross-linking of cytoskeleton networks in human liver cells might be reliant on plectin, but not on synemin. That is, the function of synemin in liver cells would then be more like a cytoskeletal component, not a cross-linking protein. The possible mechanism of synemin deficiency on the liver cell transformation is still being investigated in our laboratory.

In conclusion, we investigated the expression of synemin in human HCC and liver tissues *in vivo* and the effects of synemin knock-down in Chang liver cells *in vitro*. In addition, we compared the outcome of synemin suppression to that of plectin. The results demonstrated that both plectin and synemin are down-regulated in human HCC *in vivo*; and knock-down of synemin and plectin provokes transformation of Chang liver cells *in vitro*. In human liver cells, plectin was mainly a cross-linking protein while the role of synemin was more alike that of a cytoskeletal component. Knock-down of plectin and synemin by siRNA in human Chang liver cells caused different effects. Plectin-deficient liver cells showed CK18 down-regulation and disorganized cytoskeleton networks. In contrast, synemin- deficient cells showed no changes in the expression and organization of the cytoskeleton.

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