Cytoplasmic accumulation of connexin32 expands cancer stem cell population in human HuH7 hepatoma cells by enhancing its self-renewal

Yohei Kawasaki1,2, Yasufumi Omori1, Qingchang Li1,3, Yuji Nishikawa1, Toshiaki Yoshioka1, Masayuki Yoshida1, Kazuo Ishikawa2 and Katsuhiko Enomoto1

1 Department of Molecular and Tumour Pathology, Akita University Graduate School of Medicine, Akita, Japan
2 Department of Otorhinolaryngology, Akita University Graduate School of Medicine, Akita, Japan
3 Department of Pathology, College of Basic Medical Sciences, China Medical University, Shenyang, China

Although the connexin32 (Cx32)-mediated gap junction is abolished in hepatocellular carcinoma (HCC), the expression of cytoplasmic Cx32 tends to increase in correspondence with the grade of malignancy. Establishing a Tet-off expression system in human nonmetastatic HuH7 HCC cells where cytoplasmic Cx32 was overexpressed by doxycycline (Dox) withdrawal, we previously demonstrated that overexpression of cytoplasmic Cx32 made HuH7 cells metastatic in mice. In our study, hypothesizing that the cytoplasmic Cx32-induced metastasis may involve expansion of the cancer stem cell (CSC) population, we examined whether cytoplasmic Cx32 controlled the size of the side population (SP) in HuH7 Tet-off Cx32 cells. Fluorescence-activated cell sorting revealed that SP was expanded in a Dox-free medium compared with a Dox-supplemented one. Although cytoplasmic Cx32 did not block maturation from SP to non-SP, purified SP reconstituted a larger SP fraction in the Dox-free medium than in the Dox-supplemented one. Furthermore, although SP from HuH7 Tet-off mock cells formed a similar number of CSC spheres of a similar size whether with or without Dox, SP from HuH7 Tet-off Cx32 cells developed a greater number of larger CSC spheres in the Dox-free medium than in the Dox-supplemented one. Taken together, these results suggest that accumulation of cytoplasmic Cx32 should enhance self-renewal of CSC to expand the CSC population in HCC.

Connexin proteins are exclusive components of gap junction, through which 2 adjacent cells exchange small (Mr < 1,000) water-soluble molecules directly between cytoplasms of each other. A hexamer of connexin molecules functions as a hemichannel called “connexon,” and the connexons provided by 2 adjacent cells dock with each other in a cell–cell contact area to complete a gap junction channel. At present, the connexin gene family comprises more than 20 members in humans, and their expression patterns vary from tissue to tissue.1,2,3

Gap junctional intercellular communication (GJIC) plays pivotal roles in tissue homeostasis, and its downregulation or disruption causes several human diseases, including peripheral neuropathy, deafness and cataract.4 Moreover, a considerable number of studies have revealed that GJIC suppresses cancer promotion during carcinogenesis and, thus, that GJIC is commonly downregulated in cancerous lesions5,6 with some exceptions.7,8 In the liver, normal hepatocytes express connexin26 (Cx26) and connexin32 (Cx32), both of which form gap junctions at cell–cell contact areas9,10. In hepatocellular carcinoma (HCC), the expression of these 2 connexin proteins is altered differently, i.e., while the expression of Cx26 is abolished, Cx32 remains expressed but is translocated into cytoplasm instead of plasma membrane, resulting in failure of the formation of a gap junction.11,12 Intriguingly, as noted in various human cancers, the amount of connexin proteins that accumulate in cytoplasm tends to increase in correspondence with the grade of malignancy.11,13–17
We previously explored the contribution of such a cytoplasmic Cx32 protein to progression of HCC by using human nonmetastatic HuH7 hepatoma cells in terms of intrahepatic metastasis. In that study, severe combined immunodeficiency (SCID) mice orthotopically xenografted with HuH7 cells developed intrahepatic and peritoneal metastatic foci only when a high level of Cx32 expression was induced in cytoplasm of the grafts, indicating that cytoplasmic accumulation of Cx32 protein could induce a metastatic ability in HuH7 cells.

For the last few years, an avalanche of studies has revisited a classical idea based on the hierarchy model in cancer tissues, and many of them have successfully confirmed that cancer stem cells (CSCs) could be enriched in a distinct population. In this model, a cancer tissue consists of a heterogeneous cell population organized in a hierarchical manner sustained by CSCs at the apex. CSCs have the potential to self-renew extensively as well as that to produce non-CSCs and are considered to be tumor-initiating cells. On the other hand, although non-CSCs account for the absolute majority of the population within a cancer tissue, their proliferation is self-limiting and, thus, non-CSCs fail to develop a tumor.

Flow cytometry and cell sorting

For side population (SP) analysis, trypsinized cell pellets were incubated at 37°C for 90 min with 20 µg/ml Hoechst 33342 (Sigma-Aldrich) in 1% FBS/Hank’s Balanced Salt Solution (HBSS) in the presence or absence of 50 nM verapamil (Sigma-Aldrich), further supplemented with 4 µg/ml Dox for the cells having been maintained in the presence of Dox. After washing with HBSS, the cells were resuspended in 2 µg/ml propidium iodide (PI)/HBSS, filtered through a 40-µm cell strainer (BD Biosciences Discovery Labware, Bedford, MA) and then applied to MoFlo Cell Sorter (Beckman Coulter, Fullerton, CA). Hoechst 33342 was excited with the UV laser at 350 nm, and fluorescence emission was measured with the 405/BP30 (Hoechst blue) and the 570/BP20 (Hoechst red) optical filters. PI labeling was measured with the 630/BP30 filter for elimination of dead cells. The SP fraction was defined as the fraction that was resistant to Hoechst staining and was made undetectable by verapamil treatment.

For analysis of CD133-positive fraction, cell pellets were incubated with allophycocyanin (APC)-conjugated anti-human CD133/2 monoclonal antibody (mAb) clone 293C3 (Miltenyi Biotec, Bergisch Gladbach, Germany) at the dilution of 1:11 in the presence or absence of 4 µg/ml Dox at 4°C for 15 min. After washing with HBSS, the cells were resuspended in 2 µg/ml PI/HBSS, filtered through a 40-µm cell strainer and then applied to MoFlo Cell Sorter. To determine the negative fraction, APC-conjugated mouse IgG2b isotype antibody (Mythlenyi Biotec) was excited with a helium-neon laser at 633 nm, and the fluorescence emission was measured with the 660/BP optical filter.

Sphere formation assay and immunohistochemistry

To avoid adhesion and subsequent maturation to non-CSCs, cells were cultured in a serum-free semisolid medium. A total of $1 \times 10^5$ cells each of the SP and the main population (MP) fractions were seeded in 80 µl of serum-free RPMI1640 medium, the recipe of which was mentioned above, containing 0.33% agar on 100 µl of a solidified serum-free RPMI1640 basal layer containing 0.5% agar in wells of a 96-well plate. Fifty microliters of serum-free RPMI1640 was further poured on the top of each well as a reservoir of fresh medium and growth factor, and this top liquid layer was changed every 3 days. The cells were incubated for 20 days.
in the same atmosphere as that for the monolayer culture. The number of formed spheres was counted, and the dimension of each sphere was analyzed by imagej.

The spheres derived from SP cells were embedded in Tissue-Tek O.C.T. Compound (Sakura Finetek, Tokyo, Japan), frozen and sectioned in a cryostat. The 6-μm cryosections were fixed in pure acetone for 7 min at −20°C and incubated with anti-Cx32 mAb clone CX-2C2 (Invitrogen, Carlsbad, CA) diluted at 1:200. Specific signals were revealed with diaminobenzidine chromogen by using Envision System (DAKO, Carpinteria, CA). Nuclei were stained with Meyer’s hematoxylin.

**Xenograft into mice**

Male SCID mice (C.B-17/lcr-scid/scid) were purchased from CLEA Japan (Tokyo, Japan) and maintained under a specific pathogen-free condition. The mice received humane care and were fed with autoclaved water and chow. The protocol of the animal work was approved by the Animal Research Committee, Akita University, and was in accordance with the Regulation for Animal Experimentation of the University. After cell sorting by fluorescence-activated cell sorting (FACS), each of the SP and the MP fractions was resuspended in HBSS containing 1% FBS and 4 μg/ml Dox at the density of $5 \times 10^5$ cells/ml. Each suspension of SP and MP cells was injected subcutaneously into the right and left flanks of a SCID mouse, respectively, at 200 μl per site. Apart from this experiment, $1 \times 10^5$ unsorted cells each were xenografted into SCID mice. For the group to which Dox was administered, drinking water was supplemented with 2 mg/ml of Dox, given to the mice beginning 1 week before implantation of the cells, and a new bottle was provided every 3 days. The mice were euthanized with CO2 inspiration 12 weeks after implantation, and a new bottle was provided every 3 days. The mice were euthanized with CO2 inspiration 12 weeks after implantation, and autopsies were performed immediately. The developed tumors were fixed in 20% formalin/phosphate-buffered saline (PBS) for hematoxylin-eosin staining.

**Immunoblotting analysis**

Immunoblotting analysis was performed mostly as described previously. Anti-Cx32 polyclonal antibody (pAb) (Sigma-Aldrich) and anticycraldehyde-3-phosphate dehydrogenase (GAPDH) mAb clone 6C5 (HyTest, Turku, Finland) were diluted at 1:500 and 1:10,000, respectively. Horseradish peroxidase (HRP)-conjugated anti-rabbit and anti-mouse IgG antibodies (GE Healthcare Bio-Sciences, Piscataway, NJ) were diluted at 1:2,000 and 1:5,000, respectively. Finally, the protein-antibody complex was visualized with a WEST-one Western Blot Detection System (iNtRON Biotechnology, South Korea) following the protocol provided by the manufacturer.

**Indirect immunofluorescence**

Indirect immunofluorescence was performed as described previously. Anti-Cx32 pAb, anti-Golgi 58K protein mAb clone 58K-9 (Sigma-Aldrich) and anti-mannosidase II mAb clone 53FC3 (Covance, Emeryville, CA) were diluted at 1:400, 1:50 and 1:5,000, respectively. After incubation with HRP-labeled anti-rabbit IgG antibody (KPL, Gaithersburg, MD) and biotin-labeled anti-mouse IgG antibody (KPL), specific signals were revealed by Fluorophore Tyramid Amplification Reagent (Perkin Elmer Life Sciences, Boston, MA) and rhodamine-labeled streptavidin (KPL). Nuclei were stained with diaminobenzidine dihydrochloride (KPL) at a concentration of 0.5 μg/ml.

**Statistical analysis**

Student’s t-test was performed for the estimation of statistical significance. $p$ values are 2 tailed. All experiments were independently repeated at least 3 times except for the tumorigenicity assay of xenografts in mice, which was performed only once.

**Results**

**Inducible overexpression of cytoplasmic Cx32 protein in HuH7 Tet-off Cx32 cells**

To assess the effects of cytoplasmic Cx32 in human HCC-derived HuH7 cells without any necessity to consider clonal diversity, we used HuH7 Tet-off Cx32 cells along with HuH7 Tet-off mock cells as a control, both of which had previously been generated. As shown in Figure 1a, HuH7 Tet-off Cx32 and the mock cells express similar basal levels of endogenous Cx32 protein when cultured in RPMI1640 medium supplemented with 4 μg/ml of Dox. On the other hand, the Dox-free medium induces overexpression of Cx32 protein in HuH7 Tet-off Cx32 cells but not in the mock cells. A densitometric analysis reveals an ~5-fold induction compared with the expression level in an uninduced state (Fig. 1b). In these 2 types of HuH7 cells, as is the nature of HCC-derived cells, neither endogenous nor exogenous Cx32 protein is capable of forming any gap junction channels at a cell–cell contact area, and, instead, fluorescent signals for Cx32 protein are merged with those for Golgi markers such as Golgi 58K protein and mannosidase II (Fig. 1c and Supporting Information Fig. 1), indicating that Cx32 protein is localized in cytoplasm, notably in Golgi apparatuses.

Therefore, cultured in a Dox-free medium, HuH7 Tet-off Cx32 cells mimic a high-grade HCC with respect to the Cx32 expression pattern such as cytoplasmic accumulation of overexpressed Cx32 protein.

**Cytoplasmic accumulation of Cx32 protein expands the SP fraction representing CSCs**

A small subset of cells presenting a highly active efflux of Hoechst 33342 dye is resistant to the dye, as revealed by FACS, and is thus called “the side population (SP)” against “the main population (MP),” which is stained with the dye. It is well known that SP is the fraction into which stem cells in normal tissues are efficiently enriched. As is the case not only with normal tissues, a number of recent studies on various, otherwise not all, malignant tumors have revealed...
Figure 1. (a) Immunoblotting of Cx32 protein expressed in HuH7 Tet-off Cx32 and mock cells. The cells were cultured in the presence or absence of Dox. After incubation for 48 hr, the cells were lysed and subjected to immunoblotting to detect Cx32 protein, and GAPDH as a loading control. (b) Densitometric analysis of 3 independent immunoblottings for Cx32 protein expressed in HuH7 Tet-off Cx32 and mock cells. Error bars represent the SD (n = 3). *p < 0.01. (c) Indirect immunofluorescence of Cx32 protein (left) and Golgi 58K protein (middle) in HuH7 Tet-off Cx32 and mock cells in the presence or absence of Dox. Nuclei were stained with diamidino phenylindole dihydrochloride. Note that signals of both Cx32 and Golgi 58K proteins are colocalized (right). Scale bar, 20 µm.
that the cells from SP but not from MP display a series of phenotypes signifying CSC.\textsuperscript{31–36} Furthermore, Chiba \textit{et al.}\textsuperscript{23} found SP within the cell line HuH7 and proved that SP in HuH7 cells was the exclusive fraction containing CSCs.

Considering SP to be a CSC marker of HuH7 cells, we analyzed the effects of cytoplasmic Cx32 protein on the measuring size of SP with FACS. In an experiment (Fig. 2a), when HuH7 Tet-off Cx32 cells were cultured continuously for 10 days in the presence of 4 \( \mu \)g/ml of Dox, the verapamil-sensitive SP fraction accounted for 0.59% of the whole population. In contrast, the SP fraction in the cells cultured in a Dox-free medium expanded to up to 8.0% of the whole population. Figure 2b shows that, in HuH7 Tet-off Cx32 cells, the proportion of the SP fraction to the whole population is \( \sim 10 \times \) times higher in a Dox-free medium than in a Dox-supplemented one. Such a Dox-dependent alteration of the size of SP is not observed in HuH7 Tet-off mock cells. Therefore, it is concluded that the cell population expressing a higher level of cytoplasmic Cx32 protein contains a larger SP fraction.

To confirm that CSCs of our HuH7 Tet-off Cx32 and mock cells are contained exclusively in the SP fraction, we xenografted SP and MP fractions subcutaneously into a flank of SCID mice after cell sorting and examined tumorigenicity of the grafts (Table 1). As presented in Figure 2c, although \( 1 \times 10^5 \) cells from the SP fraction developed a tumor at each inoculated site in all the mice examined, \( 1 \times 10^5 \) cells from the MP fraction could form no palpable tumors in any mice, indicating that the CSC population resides only in the SP fraction in our HuH7 Tet-off Cx32 and mock cells as well as in parental HuH7 cells.\textsuperscript{23} It should be noted that the SP fraction-derived tumors are histologically identical to tumors raised from unsorted HuH7 Tet-off Cx32 cells (Fig. 2d).

### Cytoplasmic accumulation of Cx32 protein elevates self-renewal rate of SP cells

CSCs have 2 distinct functions as their essential nature. One is to mature to tumor progenitor cells that then produce a great majority of the cells constituting a tumor mass. The other is to self-renew to maintain the tumor-initiating ability.\textsuperscript{23} Cytoplasmic Cx32 protein-induced expansion of SP may be explained by the enhancement of self-renewal of CSCs. We purified SP cells by FACS from HuH7 Tet-off Cx32 cells and the mock cells, which had been maintained in a Dox-supplemented medium, and cultured them in the presence or absence of Dox. Maturation from CSCs to non-CSCs is not avoidable in a monolayer culture. Consistently, although the purified SP cells were inoculated into the culture, the vast majority of the population is occupied by MP cells after a 1-week culture whether with or without Dox (Fig. 3a). However, the SP fraction from HuH7 Tet-off Cx32 cells but not from the mock cells is kept much larger in a Dox-free medium than in a Dox-supplemented one (Figs. 3a and 3b), suggesting that cytoplasmic Cx32 protein might play roles in elevating the self-renewal rate of CSCs.

Several studies have reported that CSCs of HuH7 cells are contained in the CD133-positive fraction.\textsuperscript{37,38} Our pilot experiment showed that more than 80% of the SP fraction in HuH7 Tet-off Cx32 cells are CD133-positive cells in whether the presence or absence of Dox when cultured stably (Supporting Information Fig. 2). We thus evaluated the effects of cytoplasmic Cx32 protein on SP cells by analyzing the size of the CD133-positive fraction after a 1-week culture of purified SP cells in the presence or absence of Dox. As shown in Figures 3c and 3d, the CD133-positive fraction of HuH7 Tet-off Cx32 cells is significantly larger in a Dox-free medium compared with in a Dox-supplemented one.

### Cytoplasmic accumulation of Cx32 protein enhances sphere formation of CSCs

Similarly to stem cells in normal tissues, when CSCs are cultured in a serum-free medium on a nonattachment dish or in a serum-free semisolid medium, they proliferate as sphere-like cellular aggregates and sustain their undifferentiated state without maturing to non-CSCs, resulting in a pure culture of CSCs.\textsuperscript{25,39,40} To define the roles of cytoplasmic Cx32 protein in self-renewal of CSCs, we isolated SP and MP cells separately by FACS and incubated each of them in a serum-free semisolid medium with or without Dox for 20 days. As shown in Figures 4a and 4b, MP cells have almost no capacity for sphere formation, whereas SP cells exhibit an efficient ability to develop numerous large spheres, confirming that the SP fractions of our HuH7 Tet-off Cx32 and the mock cells represent the CSC population. As expected, spheres of SP cells from HuH7 Tet-off Cx32 cells are increased in both number and size in a Dox-free medium compared with in a Dox-supplemented one (Figs. 4b and 4c). On the other hand, SP cells from HuH7 Tet-off mock cells show similar capacities for sphere formation regardless of the presence or absence of Dox (Figs. 4b and 4c). As shown in Figure 4d, Cx32 protein remains localized in cytoplasm of the sphere-forming CSCs and constitutes no gap junction plaques in a cell–cell contact area. These results clearly indicate that cytoplasmic accumulation of Cx32 protein enhances self-renewal of CSCs in HuH7 cells.

### Cytoplasmic accumulation of Cx32 protein is not involved in reverse conversion from non-CSCs to CSCs

Unlike normal tissues where the cellular hierarchy is rigorously organized unidirectionally from stem cells toward terminally differentiated cells, it has been assumed that the cellular hierarchy within tumors should be relatively shallow, allowing non-CSCs to revert to CSCs to a limited extent.\textsuperscript{26} Therefore, cytoplasmic Cx32 protein may enhance not only self-renewal of CSCs but also reverse conversion from non-CSCs to CSCs, contributing in part to expansion of the CSC population. To examine such a possibility, MP cells sorted from HuH7 Tet-off Cx32 and the mock cells were introduced into a 1-week monolayer culture with or without Dox. Interestingly, Figure 5a demonstrates that a small SP fraction has

\textit{Int. J. Cancer: 128, 51–62 (2011) © 2010 UICC}
Figure 2. (a) A representative result of FACS analysis of the SP fraction in HuH7 Tet-off Cx32 and mock cells. The cells were cultured in the presence or absence of Dox for 10 days. The trypsinized cells were stained with Hoechst 33342 dye and applied to the MoFlo cell sorter. The SP fraction was defined as the fraction that was resistant to Hoechst staining and was made undetectable by verapamil treatment. (b) Diagrammatical presentation of 5 independent FACS analyses of the SP fraction. Error bars represent the SD (n = 5). *p < 0.001. (c) Tumorigenicity of SP and MP cells in SCID mice. A total of $1 \times 10^5$ cells each of the SP and MP fractions from HuH7 Tet-off Cx32 cells were subcutaneously implanted into the right and left flanks of SCID mice, respectively. Note that a tumor developed only at the right flank, into which SP cells were injected. (d) Histology of the developed tumors from SP cells (left) and the unsorted cells (right). Hematoxylin-eosin staining. Scale bar, 100 μm.
Indeed arisen from MP cells. There is, however, no significant difference in the size of the SP fraction between the presence and the absence of Dox (Fig. 5b), reflecting that overexpression of cytoplasmic Cx32 protein is not involved in reverse conversion from non-CSCs to CSCs. Although overexpression of Cx32 protein is induced equally in both SP and MP fractions of HuH7 Tet-off Cx32 cells by Dox withdrawal (Fig. 5c), only SP cells are susceptible to cytoplasmic Cx32 protein and enter self-renewal cycles.

A similar finding was also seen in a plating efficiency assay. As presented in Figure 5d, SP cells show a higher plating efficiency in a Dox-free medium than in a Dox-supplemented one, whereas the plating efficiency of MP cells remains pretty low in both media.

Takedogether, it has become apparent that cytoplasmic Cx32 protein forces only SP cells to self-renew extensively, resulting in expansion of the CSC population in HuH7 Tet-off Cx32 cells.

### Discussion

Aberrant localization of Cx32 protein in cytoplasm is rather common in HCCs and is one of the mechanisms underlying downregulation of Cx32-mediated GJIC during HCC development.\(^6,11\) A considerable number of multidisciplinary studies have revealed that expression of cytoplasmic connexin protein tends to increase in correspondence with the grade of malignancy.\(^13,41\) We previously reported that such cytoplasmic accretion tends to increase in correspondence with the grade of malignancy.\(^6,11\) A considerable number of multidisciplinary studies have revealed that expression of cytoplasmic connexin protein tends to increase in correspondence with the grade of malignancy.\(^13,41\)

One of our most intriguing findings is that the SP fraction reappeared in a cell culture into which pure MP cells had been inoculated, suggesting that reverse conversion from non-CSCs to CSCs occurred (Fig. 5a). We initially thought that this was due to an inevitable error of FACS allowing contamination of an extremely small number of SP cells into “pure” MP cells. However, it was confirmed that the performance of our device satisfied the accuracy required for our experiments (Supporting Information Fig. 4). Furthermore, when we cultured pure MP cells derived from several different human cell lines, not all of them showed reverse conversion (data not shown). Therefore, the reverse conversion from non-CSCs to CSCs we observed is real. The...
Figure 3. (a) FACS analysis of the SP fraction in the population reconstituted by purified SP cells. SP cells purified from HuH7 Tet-off Cx32 and mock cells were cultured in the presence or absence of Dox. After incubation for 1 week, the trypsinized cells were stained with Hoechst 33342 dye and applied to the MoFlo cell sorter. (b) Diagrammatic presentation of 4 independent FACS analyses of the SP fraction in the reconstituted population. Error bars represent the SD (n = 4). *p < 0.001. (c) FACS analysis of the CD133-positive fraction in the population reconstituted by purified SP cells. SP cells purified from HuH7 Tet-off Cx32 and mock cells were cultured in a serum-free medium either with or without Dox. After incubation for 1 week, the trypsinized cells were reacted with APC-conjugated anti-human CD133/2 mAb and applied to the MoFlo cell sorter. To determine CD133-negative fraction, nonspecific reaction to APC-conjugated mouse IgG2b isotype antibody was also measured (left panel). (d) Diagrammatic presentation of 5 independent FACS analyses of the CD133-positive fraction in the reconstituted population. Error bars represent the SD (n = 5). *p < 0.001.
Figure 4. CSC sphere formation in serum-free semisolid medium. A total of $1 \times 10^3$ cells each of SP and MP isolated from HuH7 Tet-off Cx32 and mock cells were seeded in a serum-free semisolid medium either with or without Dox. After incubation for 20 days, the number and the size of developed CSC spheres were recorded. (a) A representative result. (b) The number of developed CSC spheres per well in 5 independent experiments. Error bars represent the SD ($n = 5$). *$p < 0.001$. (c) The size of developed CSC spheres. The dimension of each sphere was measured and presented in pixel. Error bars represent the SD ($n = 25$). *$p < 0.001$. (d) Cytoplasmic expression of Cx32 in the spheres derived from SP cells. Scale bar, 20 μm. Proper reactivity of the antibody was confirmed by staining cryosections of a normal mouse liver, in which Cx32 gave punctate signals in a cell–cell contact area (Supporting Information Fig. 3).
Figure 5. (a) FACS analysis of the SP fraction in the population reconstituted by isolated MP cells. MP cells isolated from HuH7 Tet-off Cx32 and mock cells were cultured in the presence or absence of Dox. After incubation for 1 week, the trypsinized cells were stained with Hoechst 33342 dye and applied to the MoFlo cell sorter. (b) Diagrammatic presentation of 5 independent FACS analyses of the SP fraction in the reconstituted population. Error bars represent the SD (n = 5). (c) Immunoblotting of Cx32 protein expressed in each fraction of SP and MP from HuH7 Tet-off Cx32 and mock cells. The cells were cultured in the presence or absence of Dox. Immediately after cell sorting, the cells were lysed and subjected to immunoblotting to detect Cx32 protein, and GAPDH as a loading control. (d) Plating efficiency of SP and MP cells isolated from HuH7 Tet-off Cx32 and mock cells. A total of $1 \times 10^5$ cells each of SP and MP were seeded in a growth medium in the presence or absence of Dox. Three days after incubation, the cells on culture dishes were stained with hematoxylin and counted in 5 areas (1 cm$^2$ each). Each value was converted to that for a 60-mm dish. Error bars represent the SD (n = 3). *p < 0.001.
cellular hierarchy in immortalized cell lines may be much shallower than that in human cancers. Or reverse conversion may be seen only in monolayer culture in vitro and may not be significant in SCID mice in vivo. Consistently, even $2 \times 10^6$ MP cells failed to result in a tumor in SCID mice (data not shown). At any rate, the existence of reverse conversion would not affect the conclusion in our study.

Following up our previous study, this article has provided further evidence for a pathogenic function of cytoplasmic Cx32 protein favorable for progression of HCC. We recognize that several important issues still remain to be elucidated. The molecular mechanism underlying cytoplasmic Cx32-induced expansion of the CSC population is now under investigation. Relocation of Cx32 protein to the plasma membrane should not only restore tumor-suppressive GJIC but also abrogate an oncogenic function of cytoplasmic Cx32 protein. Restoration of normal subcellular sorting of connexin protein may become a novel area of research to pursue from the aspect of cancer treatment.

Acknowledgements

The authors are very grateful to Ms. Fujiko Hirasawa and Mr. Masayuki Sone for their helpful suggestions, Ms. Reiko Ito, Ms. Yuko Doi and Ms. Makiko Kawamata for their technical assistance and Ms. Eriko Kumagai for her secretarial work. This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (Y. Omori).

References

1. Bruzzone R, White TW, Paul DL. Connections with connexins: the molecular basis of direct intercellular signaling. *Eur J Biochem* 1996;238:1–27.
2. Beyer EC, Berthoud VM. The family of connexin genes. In: Harris AL, Locke D, eds. Connexins. New York: Humana Press, 2009, 3–26.
3. Sohl G, Willecke K. An update on connexin genes and their nomenclature in mouse and man. *Cell Commun Adhes* 2003;10:173–80.
4. Anand RJ, Hackam DJ. The role of gap junctions in health and disease. *Cirt Care Med* 2005;33:S535–S538.
5. Leithe E, Sirnes S, Omori Y, Rivedal E. Downregulation of gap junctions in cancer cells. *Crit Rev Oncoig* 2006;12:225–56.
6. Mesnil M, Crespin S, Avanzo JL, Zaidan-Dagli MI. Defective gap junctional intercellular communication in the carcinogenic process. *Biochim Biophys Acta* 2005;1719:125–45.
7. Ito A, Katoh F, Kataoka TR, Okada M, Tsubota N, Asada H, Yoshikawa K, Maeda S, Kitamura Y, Yamashiki H, Nojima H. A role for heterologous gap junctions between melanoma and endothelial cells in metastasis. *J Clin Invest* 2000;105:1189–97.
8. Ito A, Koma Y, Uchino K, Okada T, Ohbayashi C, Tsubota N, Okada M. Increased expression of connexin 26 in the invasive component of lung squamous cell carcinoma: significant correlation with poor prognosis. *Cancer Lett* 2006;234:239–48.
9. Nicholson B, Dermietzel R, Teplow D, Traub O, Willecke K, Revel JP. Two homologous protein components of hepatic gap junctions. *Nature* 1987;329:732–4.
10. Vinken M, Henkens T, De Rop E, Fraczek J, Vanhaecke T, Rogiers V. Biology and pathobiology of gap junctional channels in hepatocytes. *Hepatology* 2008;47:1077–88.
11. Krutovskikh V, Mazzoleni G, Mironov N, Omori Y, Augeon AL, Mesnil M, Berger F, Partensky C, Yamashiki H. Altered homologous and heterologous gap-junctional intercellular communication in primary human liver tumors associated with aberrant protein localization but not gene mutation of connexin 32. *Int J Cancer* 1994;56:87–94.
12. Omori Y, Krutovskikh V, Mironov N, Tsuda H, Yamashiki H. Cx32 gene mutation in a chemically induced rat liver tumour. *Carcinogenesis* 1996;17:2077–80.
13. Inose T, Kato H, Kimura H, Faried A, Tanaka N, Sakai M, Sano A, Sohda M, Nakajima M, Fukai Y, Miyazaki T, Masuda N, et al. Correlation between connexin 26 expression and poor prognosis of esophageal squamous cell carcinoma. *Ann Surg Oncol* 2009;16:1704–10.
14. Jamieson S, Going JJ, D’Arcy R, George WD. Expression of gap junction proteins connexin 26 and connexin 43 in normal human breast and in breast tumours. *J Pathol* 1998;184:37–43.
15. Kanczuga-Koda I, Sulkowski S, Lenczewski A, Koda M, Wincewicz A, Baltaziak M, Sulowska M. Increased expression of connexins 26 and 43 in lymph node metastases of breast cancer. *J Clin Pathol* 2006;59:429–33.
16. Mehta PP, Perez-Stable C, Nadji M, Mian M, Asotra K, Roos BA. Suppression of human prostate cancer cell growth by forced expression of connexin genes. *Dev Genet* 1999;24:91–110.
17. Oyamada M, Krutovskikh VA, Mesnil M, Partensky C, Berger F, Yamashiki H. Aberrant expression of gap junction gene in primary human hepatocellular carcinomas: increased expression of cardiac-type gap junction gene connexin 43. *Mol Carcinog* 1990;3:273–8.
18. Li Q, Omori Y, Nishikawa Y, Yoshioka T, Yamamoto Y, Enomoto K. Cytoplasmic accumulation of connexin32 protein enhances motility and metastatic ability of human hepatoma cells in vitro and in vivo. *Int J Cancer* 2007;121:536–46.
19. Fialkow PJ, Gartler SM, Yoshida A. Clonal origin of chronic myelocytic leukemia in man. *Proc Natl Acad Sci USA* 1967;58:1468–71.
20. Furth J, Kahn MC. The transmission of leukemia of mice with a single cell. *Am J Cancer* 1937;31:276–82.
21. Jordan CT, Guzman ML, Noble M. Cancer stem cells. *N Engl J Med* 2006;355:1253–61.
22. Klonisch T, Wiechec E, Hombach-Klonisch S, Ande SR, Wesselborg S, Schulze-Osthoff K, Los M. Cancer stem cell markers in common cancers—therapeutic implications. *Trends Mol Med* 2008;14:450–60.
23. Chiba T, Kata K, Zheng YW, Yokosuka O, Saisho H, Iwama A, Nakauchi H, Taniguchi H. Side population purified from hepatocellular carcinoma cells harbors cancer stem cell-like properties. *Hepatology* 2006;44:240–51.
24. Nakabayashi Y, Taketa K, Miyano K, Yamane T, Sato J. Growth of human hepatoma cell lines with differentiated functions in chemically defined medium. *Cancer Res* 1982;42:3858–63.
25. Hermann PC, Huber SL, Herrler T, Aicher A, Ellwart JW, Guba M, Bruns CJ, Heesschen C. Distinct populations of cancer stem cells determine tumor growth and metastatic activity in human pancreatic cancer. *Cell Stem Cell* 2007;1:313–23.
26. Mani SA, Guo W, Liao MJ, Eaton EN, Ayyanan A, Zhou AY, Brooks M, Reinhard F, Zhang CC, Shipitsin M, Campbell LL, Polyak K, et al. The epithelial-mesenchymal transition generates cells with properties of stem cells. *Cell* 2008;133:704–15.
27. Gossen M, Bujard H. Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. *Proc Natl Acad Sci USA* 1992;89:5547–51.
28. Falciatori I, Borsellino G, Halissanos N, Boitani C, Corallini S, Battistini L, Bernardi G, Stefanini M, Vicini E. Identification and enrichment of spermatogonial stem cells displaying
side-population phenotype in immature mouse testes. *FASEB J* 2004;18:376–8.
29. Goodell MA, Brose K, Paradis G, Conner AS, Mulligan RC. Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. *J Exp Med* 1996;183:797–806.
30. Shimano K, Satake M, Okaya A, Kitanaka J, Kitanaka N, Takemura M, Sakagami M, Terada N, Tsujimura T. Hepatic oval cells have the side population phenotype defined by expression of ATP-binding cassette transporter ABCG2/BCRP1. *Am J Pathol* 2003;163:3–9.
31. Grichnik JM, Burch JA, Schulteis RD, Shan S, Liu J, Darrow TL, Vervaet CE, Seigler HF. Melanoma, a tumor based on a mutant stem cell? *J Invest Dermatol* 2006;126:142–53.
32. Hirschmann-Jax C, Foster AE, Wulf GG, Schulteis RD, Shan S, Liu J, Darrow TL, Vervaet CE, Seigler HF. Melanoma, a tumor based on a mutant stem cell? *J Invest Dermatol* 2006;126:142–53.
33. Hirschmann-Jax C, Foster AE, Wulf GG, Schulteis RD, Shan S, Liu J, Darrow TL, Vervaet CE, Seigler HF. Melanoma, a tumor based on a mutant stem cell? *J Invest Dermatol* 2006;126:142–53.
34. Moserle L, Indraccolo S, Ghisi M, Frasson C, Fortunato E, Canevari S, Miotti S, Tosello V, Zamarchi R, Corradin A, Minuzzo S, Rossi E, et al. The side population of ovarian cancer cells is a primary target of IFN-alpha antitumor effects. *Cancer Res* 2008;68:5658–68.
35. Stozek PP, Pieretti-Vanmarcke R, Masiakos PT, Dinulescu DM, Connolly D, Foster R, Dombkowski D, Preffer F, Maclaughlin DT, Donahoe PK. Ovarian cancer side population defines cells with stem cell-like characteristics and Mullerian inhibiting substance responsiveness. *Proc Natl Acad Sci USA* 2006;103:11154–9.
36. Wang J, Guo LP, Chen LZ, Zeng YX, Lu SH. Identification of cancer stem cell-like side population cells in human nasopharyngeal carcinoma cell line. *Cancer Res* 2007;67:3716–24.
37. Ma S, Chan KW, Hu L, Lee TK, Wo JY, Ng IO, Zheng BJ, Guan XY. Identification and characterization of tumorigenic liver cancer stem/progenitor cells. *Gastroenterology* 2007;132:2542–56.
38. Suet suga A, Nagaki M, Aoki H, Motohashi T, Kunisada T, Moriwaki H. Characterization of CD133+ hepatocellular carcinoma cells as cancer stem/progenitor cells. *Biochem Biophys Res Commun* 2006;351:820–4.
39. Kondo T. Stem cell-like cancer cells in cancer cell lines. *Cancer Biomark* 2007;3:245–50.
40. Tirino V, Desiderio V, d’Aquino R, De Francesco F, Pirozzi G, Graziano A, Galderisi U, Cavaliere C, De Rosa A, Papaccio G, Giordano A. Detection and characterization of CD133+ cancer stem cells. *PLoS One* 2008;3:e3469.
41. Omori Y, Li Q, Nishikawa Y, Yoshitani K, Takakura Y. Sphere-forming stem-like cell populations with drug resistance in human sarcoma cell lines. *Ann Surg Oncol* 2009;16:3763–74.
42. Fujii H, Honoki K, Tsujiuchi T, Kido A, Yoshitani K, Takakura Y. Sphere-forming stem-like cell populations with drug resistance in human sarcoma cell lines. *Ann Surg Oncol* 2009;16:3763–74.
43. Risbud S, Wiker H, Pantel K. Biological relevance of disseminated tumor cells in cancer patients. *Int J Cancer* 2008;123:991–2006.
44. Hill RP, Perris R. “Destemming” cancer stem cells. *J Natl Cancer Inst* 2007;99:1435–40.
45. Chiba T, Miyagi S, Saraya A, Aoki R, Seki A, Morita Y, Yonemitsu Y, Yokosuka O, Taniguchi H, Nakauchi H, Iwama A. The polycomb gene product BMI1 contributes to the maintenance of tumor-initiating side population cells in hepatocellular carcinoma. *Cancer Res* 2008;68:7742–9.
46. Peng D, Peng C, Li C, Zhou Y, Li M, Ling B, Wei H, Tian Z. Identification and characterization of cancer stem-like cells from primary carcinoma of the cervix uteri. *Oncol Rep* 2009;22:1129–34.
47. Fujii H, Honoki K, Tsujiuchi T, Kido A, Yoshitani K, Takakura Y. Sphere-forming stem-like cell populations with drug resistance in human sarcoma cell lines. *Ann Surg Oncol* 2009;16:3763–74.
48. Hjelmeland AB, Rich JN. The hypoxic microenvironment maintains glioblastoma stem cells and promotes reprogramming towards a cancer stem cell phenotype. *Cell Cycle* 2009;8:3274–84.
49. Saiki Y, Ishimaru S, Mimori K, Takatsuno Y, Nagahara M, Ishii H, Yamada K, Mori M. Comprehensive analysis of the clinical significance of inducing pluripotent stemness-related gene expression in colorectal cancer cells. *Ann Surg Oncol* 2009;16:2638–44.