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# Inversed relationship between CD44 variant and c-Myc due to oxidative stress-induced canonical Wnt activation

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ABSTRACT

Cancer stem-like cells express high amount of CD44 variant8-10 which protects cancer cells from redox stress. We have demonstrated by immunohistochemical analysis and Western blotting, and reverse-transcription polymerase chain reaction, that CD44 variant8-10 and c-Myc tend to show the inversed expression manner in gastric cancer cells. That is attributable to the oxidative stress-induced canonical Wnt activation, and furthermore, the up-regulation of the downstream molecules, one of which is oncogenic c-Myc, is not easily to occur in CD44 variant-positive cancer cells. We have also found out that CD44v8-10 expression is associated with the turn-over of the c-Myc with the experiments using gastric cancer cell lines. This cannot be simply explained by the model of oxidative stress-induced Wnt activation. CD44v8-10-positive cancer cells are enriched at the invasive front. Tumor tissue at the invasive area is considered to be composed of heterogeneous cellular population; dormant cancer stem-like cells with CD44v8-10 $^{high}$/ Fbw7 $^{high}$/ c-Myc $^{low}$ and proliferative cancer stem-like cells with CD44v8-10 $^{high}$/ Fbw7 $^{low}$/ c-Myc $^{high}$.



## 1. Introduction

Oxidative stress is a double-edged sword for cancer cells; excessive redox stress can induce apoptotic death whereas appropriate level of oxidative stress promotes cancer cell proliferation [1,2]. Canonical Wnt signal with β-catenin as a signal transducer plays a crucial role not only in embryonic development but also the maintenance of malignancy, such as unregulated cell proliferation, leading to invasion and metastasis. β-Catenin is phosphorylated by the ubiquitin complex composed of Axin, Glycogen synthase kinase 3β (GSK3β), and APC and undergoes the proteasome-dependent rapid degradation [3,4].

It has recently been shown that redox stress causes the activation of canonical Wnt signaling pathway. Among numerous molecules binding to Dishevelled (Dvl), an adaptor protein of Wnt signaling pathway, nucleoredoxin (NRX) was identified in large quantity [5]. NRX selectively activates canonical Wnt signal depending in response to exogenous oxidative stress in which redox-sensitive Dvl association with NRX inhibits the ubiquitin ligase activity of GSK3β and leads to the stabilization of β-catenin. β-Catenin spared from phosphorylation translocates into the nucleus and binds to T cell factor/lymphoid enhancer factor (TCF/LEF) promoter, thereby up-regulating the expression of Axin-2, CD44, c-Myc, cyclinD1, and β-catenin on its own [3,5].

CD44 is one of the cancer stem-like cell (CSC) markers as well as an adhesion molecule, the ligands of which are osteopontin, hyaluronic acids etc. [6]. Alternative splicing machinery makes diversity in CD44 isoforms; CD44 variant (CD44v) with variable exons inserted between exon 5 and exon 16 (Fig. S1-A) is exclusively expressed in epithelial cells, while CD44 standard (CD44s) is expressed widely in both epithelial and mesenchymal cells [6,7]. Epithelial–mesenchymal transition (EMT) is an important process in which gives epithelial cells enhanced resistance to anoikis (anchorage-dependent cell death), enhanced migratory potential and invasiveness, and further increased production potential of extracellular matrix (ECM) components. EMT occurs during the development, response to inflammation and injury, and last but not least, in malignant neoplasms [8]. EMT induces the shift from CD44v isoform to which includes CD44s, which is devoid of all CD44 variable exons via epithelial splicing regulatory protein 1 (ESRP1) and Twist, one of the master regulators for EMT [7].

We have recently found out that ESRP1-CD44v-xCT (glutamate-cysteine exchange transporter)-glutathione (GSH) axis renders numerous epithelial cancer cells resistant to reactive oxygen species (ROS) [9]. Notably, this axis is maintained by the expression of CD44v8–10, but not by CD44v6, which has been implicated as a cause of enhanced invasive potential and distant metastasis [10,11].

CSCs have been defined as undifferentiated cell population with self-renewal capacity and multi-lineage differentiation potential [12]. However, it is little known what makes CSCs quiescent or proliferative; If stem cells aberrantly proliferate into daughter cells

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to form a differentiated tissue, they will only to be exhausted [13,14]. Previous reports have suggested that oncogenic molecule c-Myc is a candidate molecule to regulate the proliferative plasticity of CSCs [15,16]. In this research, we have focused on the relationship between CD44v, especially CD44v8–10, and c-Myc, and further investigated the biological significance of the inversed relationship between CD44v and c-Myc *in vivo*.

## 2. Materials and methods

### 2.1. Establishment of MKN28-R1 cell line

A cDNA encoding full-length of human CD44v8–10 was introduced into the pRc/CMV expression plasmid (Invitrogen) as previously described in [9]. Parental MKN28 cells were transfected with this vector with the use of the Lipofectamine 2000 (Invitrogen) and FuGENE HD (Roche, Tokyo, Japan) reagents. Cells stably expressing CD44 were obtained and maintained by the selection with 2 mM genecitin (G418).

Details are described in the Supplementary document.

## 3. Results and discussion

### 3.1. CD44 variant prevents oxidative stress from promoting canonical Wnt signaling

Immortalized keratinocyte cell line HaCaT cells were chronologically exposed to redox stress ($H_2O_2$ at the concentration of 100 μM). HaCaT expresses both CD44s and CD44v isoforms. Western blot analysis revealed that CD44v expression dynamically changed, whereas CD44s expression did not under oxidative stress conditions (Fig. 1A). The same phenomenon in which CD44s expression was stable compared to CD44v under redox stress was recognized in semi-quantitative RT-PCR (Fig. S1-C). CD44v and β-catenin tended to show the inversed expression manner. β-Catenin was most up-regulated 6 h after the treatment, when CD44v expression level was significantly decreased (Fig. 1A). It is precisely unknown why c-Myc and cyclinD1 did not present the same expression change (Fig. 1A), although these two molecules are generally accepted as downstream molecules of β-catenin-mediated Wnt signaling pathway [3]. Dysregulated ubiquitin–proteasome degradation of c-Myc was probably attributable to the gradually accumulated and up-regulated c-Myc expression in time-dependent manner [17]. In the same experimental protocol, we performed quantitative RT-PCR to know the change in transcription level of CD44 and β-catenin. The transcription level of CD44 and β-catenin also tended to show the inversed change especially 3–6 h after the treatment with $H_2O_2$ (Fig. 1B).

One of the prostate cancer cell lines, LNCaP cells, which are lack of CD44 expression due to epigenetic silencing [18], showed the abrupt increased expression of β-catenin (Fig. 1C). Besides, p38MAPK is an evolutionary conserved proline-directed serine/threonine protein kinase considered important in mediating stress, inflammatory and immune responses, and cell survival and apoptotic process. In contrast of Fig. 1A, active form of p38MAPK (p-p38MAPK) significantly changed in Fig. 1C, which strongly suggests that CD44v expression attenuated intracellular ROS.

Well-differentiated gastric cancer cell line MKN28 cells are also lack of CD44 expression due to the hypermethylation of the CpG islands of the *CD44* gene [19]. MKN28 cells transfected with the mock vector (MKN28-MOCK) or the vector coding the full length of human CD44v8–10 (MKN28-R1) was established. $H_2O_2$ treatment revealed that CD44v expression attenuated the degree of p-p38MAPK up-regulation (Fig. 1D). Dysregulated ubiquitin–proteasome degradation of c-Myc is considered to be partially responsible for the accumulated and increased expression of c-Myc under the redox stress in the chronological order (Fig. 1D). The difference in the up-regulation of p-p38MAPK of between MKN28-MOCK and -R1 cells implies that CD44v protects cells from redox stress, as we have previously reported [9].

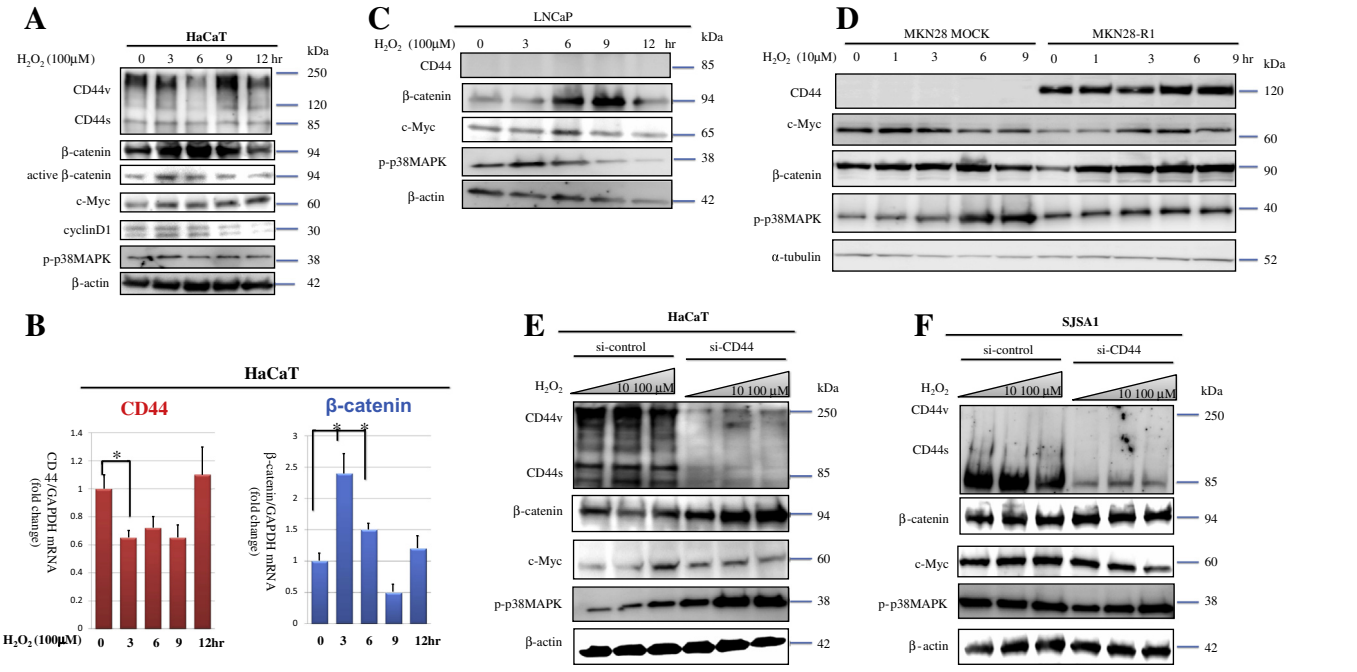


**Fig. 1.** CD44v expression attenuates redox-stress-induced Wnt activation in the negative feedback machinery. (A) Western blot analysis of HaCaT cells exposed to 100 μM $H_2O_2$ for 0, 3, 6, 9 and 12 h. β-Actin is an internal control. (B) Quantitative RT-PCR to detect the transcriptional fold change of CD44 and β-catenin in HaCaT cells exposed to 100 μM $H_2O_2$ for 0, 3, 6, 9 and 12 h. *: $p < 0.01$ (Student's *t* test). (C) Western blot analysis of LNCaP cells exposed to 100 μM $H_2O_2$ for 0, 3, 6, 9 and 12 h. (D) Western blot analysis of MKN28 cells (MKN28 MOCK v.s. MKN28-R1) exposed to 10 μM $H_2O_2$ for 0, 1, 3, 6 and 9 h. (E) Western blot analysis of HaCaT cells treated with si-RNA (si-control or si-CD44) for 48 h and subsequently exposed to 10 or 100 μM $H_2O_2$ for 24 h. (F) Western blot analysis of SJSA1 cells treated with si-RNA (si-control or si-CD44) for 48 h and subsequently exposed to 10 or 100 μM $H_2O_2$ for 24 h.

Si-RNA-mediated depletion of CD44 expression rendered HaCaT cells susceptible to oxidative stress-induced canonical Wnt signal activation in terms of β-catenin expression level. Even in the absence of $H_2O_2$ treatment, both β-catenin and downstream oncogenic molecule c-Myc were up-regulated (Fig. 1E). In contrast, β-catenin expression change under $H_2O_2$ treatment was much the same after the depletion of CD44 as the si-control. Redox stress-induced Wnt signal activation in mesenchymal osteosarcoma cell line SJSA1 cells was not totally influenced regardless of CD44 expression (Fig. 1F).

We have recently shown that CD44v8–10 attenuates oxidative stress via increased synthesis of GSH [9]. Therefore, up-regulated CD44v, but not CD44s, due to canonical Wnt signal activation is expected to prevent oxidative stress from activating β-catenin-mediated Wnt signaling. That is why we have speculated that CD44v8–10 and c-Myc show an inversed expression pattern. To be more precise, CD44v8–10-positive cancer cells do not tend to activate canonical Wnt signal under redox stress by ESRP1-CD44v-xCT-GSH axis. By contrast, CD44v8–10-negative cells are expected easily to activate oxidative stress-induced canonical Wnt activation, thereby up-regulating the expression of c-Myc (Fig. S1-D).

### 3.2. CD44v affects both synthesis and degradation of c-Myc in gastric cancer cells in vitro

Western blot analysis revealed that MKN28-R1 expressed undetectable level of c-Myc and decreased β-catenin expression (Fig. 2A). Fluorescent cellular immunohistochemical analysis (F-IHC) also showed the mutually exclusiveness between CD44v8–10 and c-Myc expression. Of note, c-Myc was expressed heterogeneously in control MKN28 cells independent of cell cycle (Fig. 2B). Poorly-differentiated gastric cancer cell line KATO-III cells show the constitutive activation of canonical Wnt signal transduction due to the amplification of β-catenin-coding gene *CTNNB1* [20]. Si-RNA-mediated CD44 depletion increased the expression of β-catenin and c-Myc in comparison with KATO-III cells treated with si-control (Fig. 2C).

Fbw7 belongs to a member of F-box family proteins, which forms F-box protein (SCF) ubiquitin ligase complex with Skp1 and Cul1. There are three isoforms of Fbw7 (α, β, γ), which is responsible for the ubiquitin–proteasome-dependent degradation of c-Myc [17,21]. Quantitative RT-PCR revealed that MKN28-R1 cells expressed much higher level of CD44 and much lower level of both c-Myc and Fbw7-α and -β isoforms (Fig. 2D), which strongly suggests that the turn-over of both transcription to mRNA and degradation of protein was more rapid in control MKN28 cells than MKN28-R1 cells. Indeed, the treatment with MG132 of MKN28-R1 cells led to the ectopic expression of c-Myc. The degree of c-Myc accumulation in MKN28-MOCK cells treated with MG132 was more significant than that of MKN28-R1 cells (Fig. 2E), which was consistent with the fact that Fbw7 expression was significantly higher in MKN28-MOCK than -R1 cells (Fig. 2D). Furthermore, the treatment with CHX resulted in the rapid decrease of c-Myc expression in MKN28-MOCK cells as compared with MKN28-R1 cells (Fig. 2F), which was consistent with the result that MKN28-MOCK cells highly harbor c-Myc mRNA (Fig. 2D).

Quantitative RT-PCR analysis after si-RNA-mediated CD44 depletion in KATO-III cells revealed that both c-Myc and Fbw7-α and -β isoforms became significant higher as compared with si-control-treated KATO-III cells (Fig. 2G). The treatment with MG132 of KATO-III cells in the absence of CD44 by si-RNA dramatically increased the expression of c-Myc as compared with that of control (Fig. 2H).

These results strongly suggests that the turn-over composed of both the synthesis and stabilization of c-Myc protein was more prompt in CD44-depleted KATO-III cells than in control. Of note,

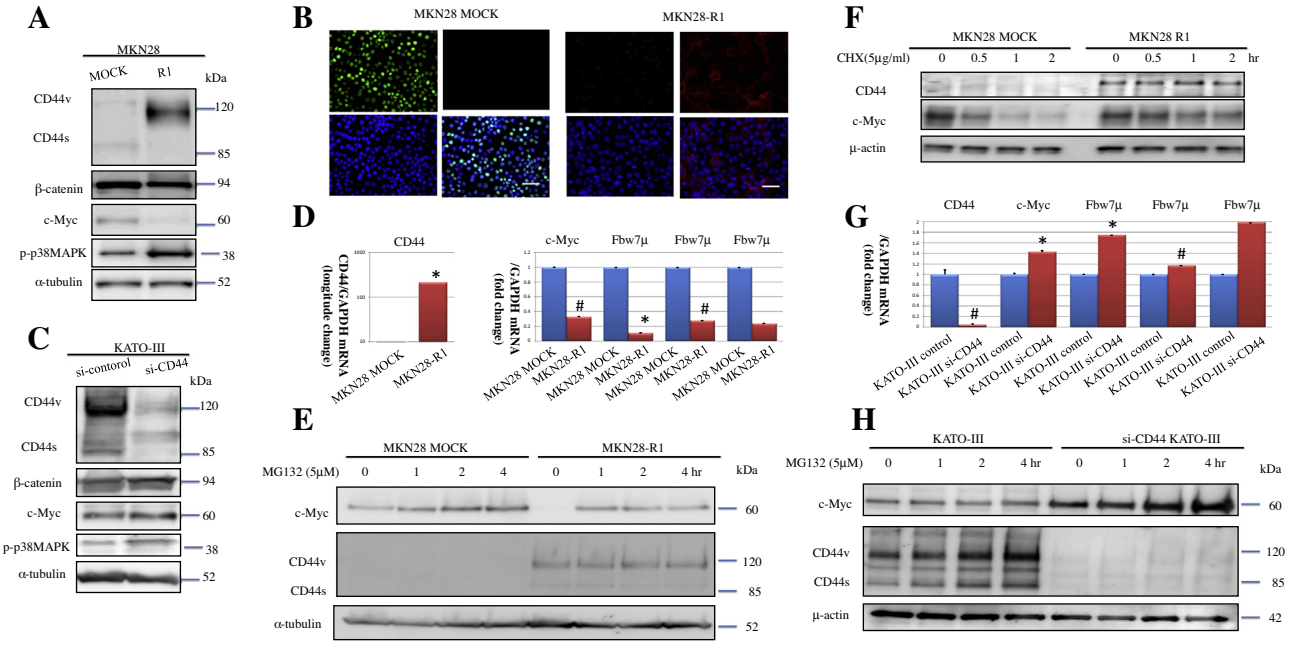


**Fig. 2.** The expression of CD44v and c-Myc is negatively correlated each other *in vitro*. (A) Western blot analysis of MKN28 cells transfected with mock vector or CD44v8–10 coding vector (MKN28 MOCK v.s. MKN28-R1). α-Tubulin was an internal control. (B) Fluorescent cellular immunestaining of MKN28-MOCK and MKN28-R1 with CD44v8–10 in red (Texas Red) and c-Myc in green (Alexa Fluor 488). Nuclei were stained with Hoechst 33342 (blue). (C) Western blot analysis of KATO-III cells after the treatment of si-CD44 for 48 h. (D) Quantitative RT-PCR to detect the transcriptional fold change of CD44, c-Myc, and Fbw7-α, β, γ in MKN28 cells (MKN28 MOCK v.s. MKN28-R1). *: $p < 0.05$, #: $p < 0.005$ (Student's *t* test). (E) Western blot analysis of MKN28 cells (MKN28 MOCK v.s. MKN28-R1) treated with 5 μM MG132 for 0, 1, 2 and 4 h. (F) Western blot analysis of MKN28 cells (MKN28 MOCK v.s. MKN28-R1) treated with 5 μg/ml MG132 for 0, 0.5, 1, and 2 h. (G) Quantitative RT-PCR to detect the transcriptional fold change of CD44, c-Myc, and Fbw7-α, β, γ in KATO-III cells after the treatment of si-CD44 for 48 h. *: $p < 0.05$, #: $p < 0.005$ (Student's *t* test). (H) Western blot analysis of KATO-III cells (after si-control v.s. si-CD44 for 48 h) treated with 5 μM MG132 for 0, 1, 2 and 4 h.

the expression level of Fbw7-γ isoform was not significantly influenced either in MKN28-R1 cells or CD44-transiently depleted KATO-III cells (Fig. 2D and G). Collectively, CD44v expression is likely to inhibit both the transcription of c-Myc and simultaneously the degradation of this onecogenic protein, thereby slowing the turn-over the c-Myc expression in gastric cancer cells *in vitro*. This process cannot be explained by the simple model such as Fig. S1-D.

### 3.3. Inversed expression was recognized between CD44v and c-Myc in vivo

IHC analysis of normal gastric mucosa obtained from 11-week-old male nude mice revealed that the undifferentiated stem-like cells in the bottom of the fundic glands strongly express CD44v8–10, while differentiated mucosal cells no longer expressed CD44v8–10. In contrast, c-Myc is not expressed in the cells at the bottom of the fundic glands, and only recognized at the proliferative mucosal gland cells (Fig. 3A). It has been proposed that one or a few cells in the isthmus continually self-renew and provide transit amplifying cells (TA cells) that migrate bi-directionally, up to the mucosal surface and down to the gland base, as they differentiate into mature cells of the gastric unit composed of 4 kinds of terminally differentiated cells; oxyntic (parietal) cells, zymogenic (chief) cells, surface mucous foveolar (pit) cells, and hormone-secreting enteroendocrine cells [22–24]. Gastric epithelial stem cells express Lgr5, much the same as intestinal stem cells [24,25]. There are several reports that aberrant expression of c-Myc in stem cells or TA cells can lead to the exhaustion of normal hematopoietic stem cells in the bone marrow [15,16], which suggests the importance of fine regulation of plasticity between quiescence and proliferative status of stem cells to maintain the tissue homeostasis.

Simultaneous over-expression of Wnt1, keeping the gastric progenitor cells undifferentiated, and prostaglandin $E_2$ ($PGE_2$), synthesized by cyclooxygenase 2 (COX-2) exclusively in the gastric mucosa using the keratin 19 promoter developed gastric well-differentiated adenocarcinoma [26]. The genetic engineering mice were named as gastric neoplasia mice (GAN mice). We have performed F-IHC of the tumor lesion in GAN mice with the combination of the antibody to CD44v8–10 and to Ki-67, a typical proliferative marker. c-Myc functions as a strong driver for cell cycle [27], so that Ki-67 expression is expected to be positively correlated with that of c-Myc, as previously reported in the perspective of pathological analyses [28,29]. CD44 splicing pattern is heterogeneous in numerous kinds of tumors and changeable depending on its progression [30,31]. Much the same as human well-differentiated adenocarcinomas, CD44v8–10 was heterogeneously expressed in the branched glands in a GAN mouse, and notably, Ki-67 was positive exclusively in CD44v-negative cancer cells (Fig. 3B). GAN mice were mated with CD44 knockout mice and the strain of CD44-null GAN mice were established [9]. Furthermore, F-IHC of tumor tissue of CD44-wild and CD44-null GAN mice was performed with the combination of the antibody to CD44v8–10 and to c-Myc, a typical oncogenic driver molecule. Mutually exclusive expression manner between CD44v8–10 and c-Myc was identified (Fig. 3C). In addition, generality of the inversed expression manner between CD44v8–10 and c-Myc was demonstrated using the xenograft model derived from prostate cancer cell line PC-3 cells (Fig. 3D). Thus, the inversed expression pattern between CD44v8–10 and c-Myc was recognized in gene engineering

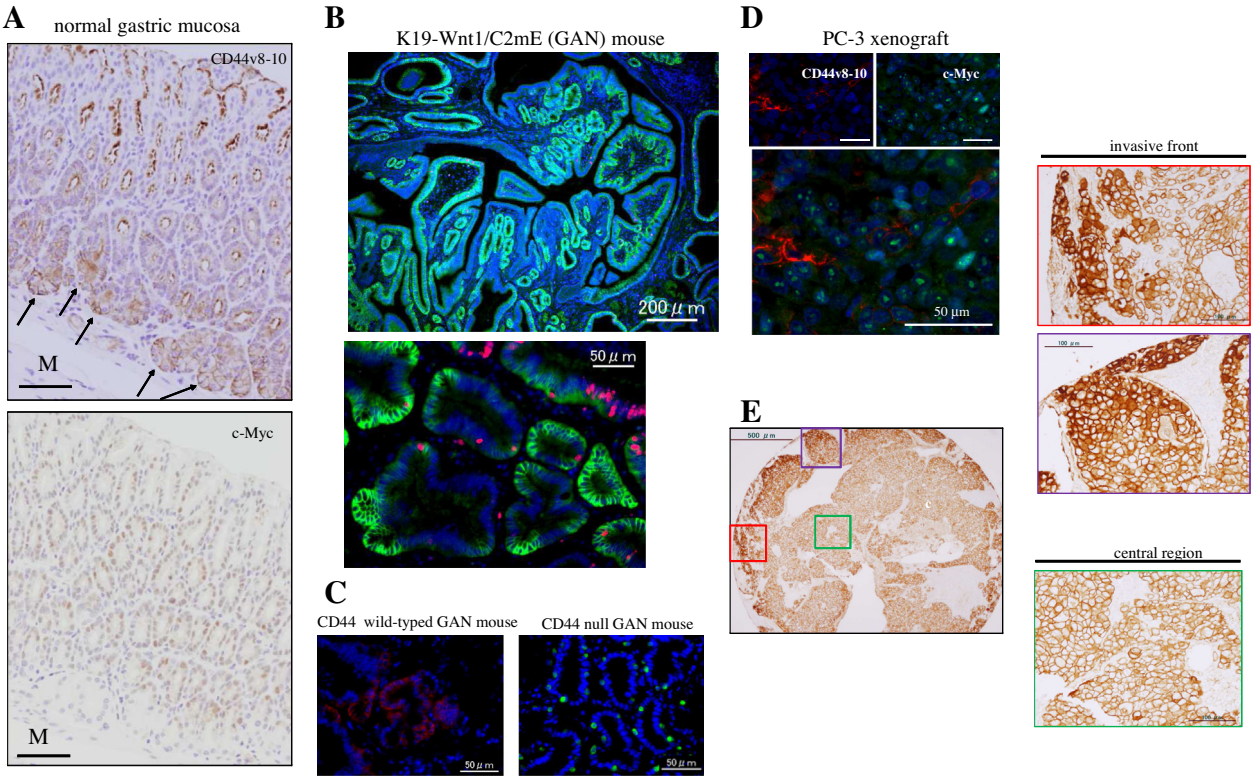


**Fig. 3.** CD44v and c-Myc showed the inversed expression pattern *in vivo*. (A) IHC of normal gastric mucosa of BALB/c nude mice with antibodies to mouse-specific CD44v8–10 or to c-Myc. Arrows indicate the fundic glands. M, sub-mucosal muscle layer. Scale bar, 50 μm. (B) Fluorescent tumor tissue immunostaining of K19-Wnt1/C2mE transgenic (GAN) mouse with CD44v8–10 in green (Alexa Fluor 488) and Ki-67 in red (Texas Red). Nuclei were stained with DAPI (blue). Scale bar, 200 μm (upper) and 50 μm (lower). (C) Fluorescent tumor tissue immunostaining of CD44-wild and CD44 null GAN mice with CD44v8–10 in red (Texas Red) and c-Myc in green (Alexa Fluor 488). Nuclei were stained with DAPI (blue). Scale bar, 50 μm. (D) Fluorescent tissue immunostaining of PC-3 cells-derived xenograft with CD44v8–10 in red (Texas Red) and c-Myc in green (Alexa Fluor 488). Nuclei were stained with DAPI (blue). Scale bar, 50 μm. (E) Tissue immunohistochemistry of metastatic foci in the lymph node of breast infiltrating ductal adenocarcinoma with specific antibody to human CD44v8–10 antibody (IMGENEX (IMH-364), #41, a 48-year female breast cancer patient with lymph node metastasis.). Scale bar, 500 μm (left) and 100 μm (right).

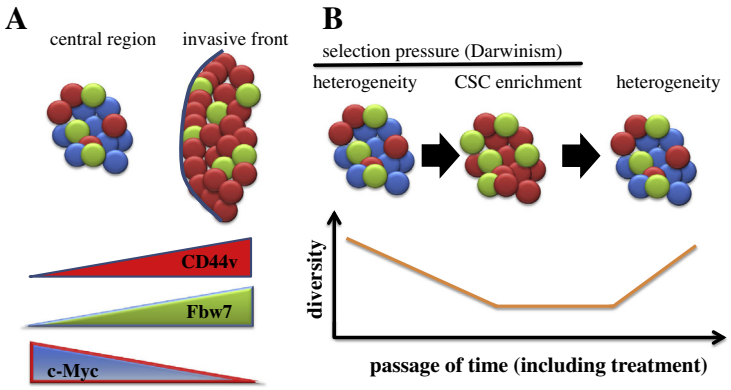


**Fig. 4.** Graphic conclusions to explain the mechanism in which CSC are enriched. (A) Compared to central tumor region, CSCs are significantly enriched at the invasive front in terms of CD44v8–10 expression amount. Fbw7, an ubiquitin E3 ligase to degradate c-Myc, is considered to be highly expressed in the invasive front. Green cells correspond to the dormant CSCs with CD44v8–10$^{high}$/Fbw7$^{high}$/c-Myc$^{low}$. Red cells correspond to proliferative CSCs with CD44v8–10$^{high}$/Fbw7$^{low}$/c-Myc$^{high}$. Blue cells correspond to non-CSCs; precursor or more differentiated cancer cells. (B) Darwinism theory to explain how heterogeneity is transiently lost during the passage of time including the clinical treatment such as chemotherapy. Selection pressure such as hypoxia, chronic inflammation and hypo-growth factors leads to the enrichment of CSCs. After micro-environmental stress decreases, heterogeneity again recovers in the minimal residual disease.

gastric cancer model mice and xenografts derived from cancer cells in other organs.

### *3.4. Enrichment of CSC at the invasive front in terms of "tumor evolutionary theory"*

IHC of human breast cancer metastatic tissue array with a specific antibody to human CD44v8–10 revealed that breast cancer cells at the invasive front highly expressed CD44v8–10 at the cellular membrane than the central region of the metastatic foci in the lymph node (Fig. 3E).

It is widely accepted that EMT expands the CSC population at the invasive front. Epithelial cancer cells no longer express CD44v after EMT, instead become to express CD44s [7]. Paradoxically, however, there have been several reports that CD44-highly expressing cancer cells are enriched at the invasive front with activated canonical Wnt signal [32–34]. To be sure, there is a controversial report that Fbw7 was down-regulated at the invasive front of aggressive breast adenocarcinoma [35], but high Fbw7, the SCF-based E3 ligase subunit, expression prevents the cell cycle progression during G0 and G1-S phase at the invasive front [36].

CSCs enriched at the invasive front are proliferative or quiescent depending on the kind of tumors and the effect of microenvironment, which is consistent with the fact that CD44v8–10-positive breast CSCs exist even after they have underwent EMT. We could not further investigate the expression level of Fbw7 at the invasive front because there is no antibody to Fbw7 available for tissue IHC. There is likely to exist a heterogeneity in Fbw7 expression at the invasive front where CSCs are enriched; both dormant CSCs with CD44v8–10$^{high}$/Fbw7$^{high}$/c-Myc$^{low}$ and proliferative CSCs with CD44v8–10$^{high}$/Fbw7$^{low}$/c-Myc$^{high}$ are expected to heterogeneously exist (Fig. 4A). Micro-environmental stress such as hypoxia, hypo-nutrition, oxidative stress and chronic inflammation leads to the enrichment of CSCs, which have the biological characteristics for capable of survival and proliferation [9,30,37–39]. This Darwinism selection theory is also referred to as "bottle-neck theory" (Fig. 4B).

## 4. Conclusions

We have revealed that CD44v8–10 is negatively correlated with c-Myc expression both *in vitro* and *in vivo*. This inversed relationship is partly attributable to redox stress-enhanced canonical Wnt signaling pathway. It remains to be known, however, how CD44v suppresses both transcription and degradation of oncogenic molecule c-Myc. The plasticity in terms of whether CSCs are dormant or proliferative is expected to be determined by c-Myc degradation dependent on Fbw7 ubiquitin ligase.

## Author contributions

Conceived, designed and performed the experiments, wrote the paper: GJY. Emended the paper: HS.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.12.016.

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