

Human colorectal CD24⁺ cancer stem cells are susceptible to epithelial-mesenchymal transition

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Abstract. Conventional cancer chemotherapy preferentially destroys non-stem cancer cells within a tumor, and a subpopulation of cancer stem cells (CSCs) is more resistant and survives, leading to relapses and metastasis. However, recent studies suggest that CD24 and susceptibility to epithelial-mesenchymal transition (EMT) can serve as markers of CSCs. We report that CD24⁺ cells are susceptible to induction of EMT, a phenotype important for cancer metastasis. We studied the responsiveness of CSC markers to TGF- β , an effective EMT inducer. The data on CD24 demonstrated that CD24⁺ cells are susceptible to EMT, a phenotype important for cancer metastasis in two colorectal cancer cell lines, the CaR-1 and CCK81. CD24⁺ cells expressed Notch 1 in response to exposure to TGF- β in culture and showed higher tumorigenic activity compared to controls. This evidence shows that CD24⁺ cells are susceptible to EMT induction and to cancer progression and is indicative of the candidacy of CD24 as a therapeutic target in CSC.

Introduction

Conventional cancer treatments preferentially destroy non-stem cancer cells within tumors, whereas cancer stem cells (CSCs) are more resistant and survive, which can subsequently cause a relapse, and in some cases, life-threatening metastasis (1,2). Identification of a regulatory mechanism, such as a functional cell surface marker, would be useful for distinguishing CSCs from non-stem cancer cells, which would allow for reduction of the dosage of chemo- and radiotherapy

and maximize tumor targeting (3). Previously, we identified CD13/aminopeptidase as a cell surface marker, which is preferentially expressed in CSCs of gastrointestinal organs. CD13/aminopeptidase has a functional role in the reduction of reactive oxygen species (ROS) in tumor cells by modulating glutathione synthesis, thereby contributing to the survival of CSCs after chemo- and radiotherapy (4). CD13 is also upregulated during the epithelial-mesenchymal transition (EMT), a phenotype important for cancer metastasis (5). Recent studies suggest that susceptibility to EMT can serve as a marker of CSCs (6). Because CSCs are a heterogeneous cell population (7), further research is necessary for identification of CSC markers.

Since the identification of rare CSCs in leukemia (8-10), molecular markers for detection of CSCs have been reported in solid tumors of the head and neck (11), gastrointestinal system (12), colon (13,14), breast (15) and brain (16,17). CD44 (hyaluronic acid receptor) is one of the most commonly studied surface markers, which is expressed by almost all cancer stem cells, and CD24 (heat-stable antigen) is another surface marker expressed in many tumor types (18). Although CD44⁺CD24⁻ cell populations have been identified as CSCs in breast cancer (19), expression of CD133, CD166, CD44, CD29, CD24, Lgr5, and nuclear β -catenin has been suggested to mark the CSC population in the colon (20).

Subsequent studies showed that although it is unclear whether CD133 is a marker of colon CSCs, other cell surface markers, such as epithelial-specific antigen, CD44, CD166, Musashi-1, CD29, CD24, leucine-rich repeat-containing G protein-coupled receptor 5, and aldehyde dehydrogenase 1, have been shown to be promising candidates (21). A recent study on mice narrowed down the targets and demonstrated that CD24 can be used to isolate Lgr5⁺ putative colonic epithelial stem cells; their data suggest that the presence of CD24 expression in normal colonic epithelium may have important implications in the use of colorectal cancer therapies targeting CD24 (22). Here we studied the responsiveness to TGF- β of CSCs carrying various markers (TGF- β is an effective EMT inducer). The data on CD24 demonstrated that CD24⁺ cells are susceptible to EMT induction and are associated with tumorigenesis in mice.

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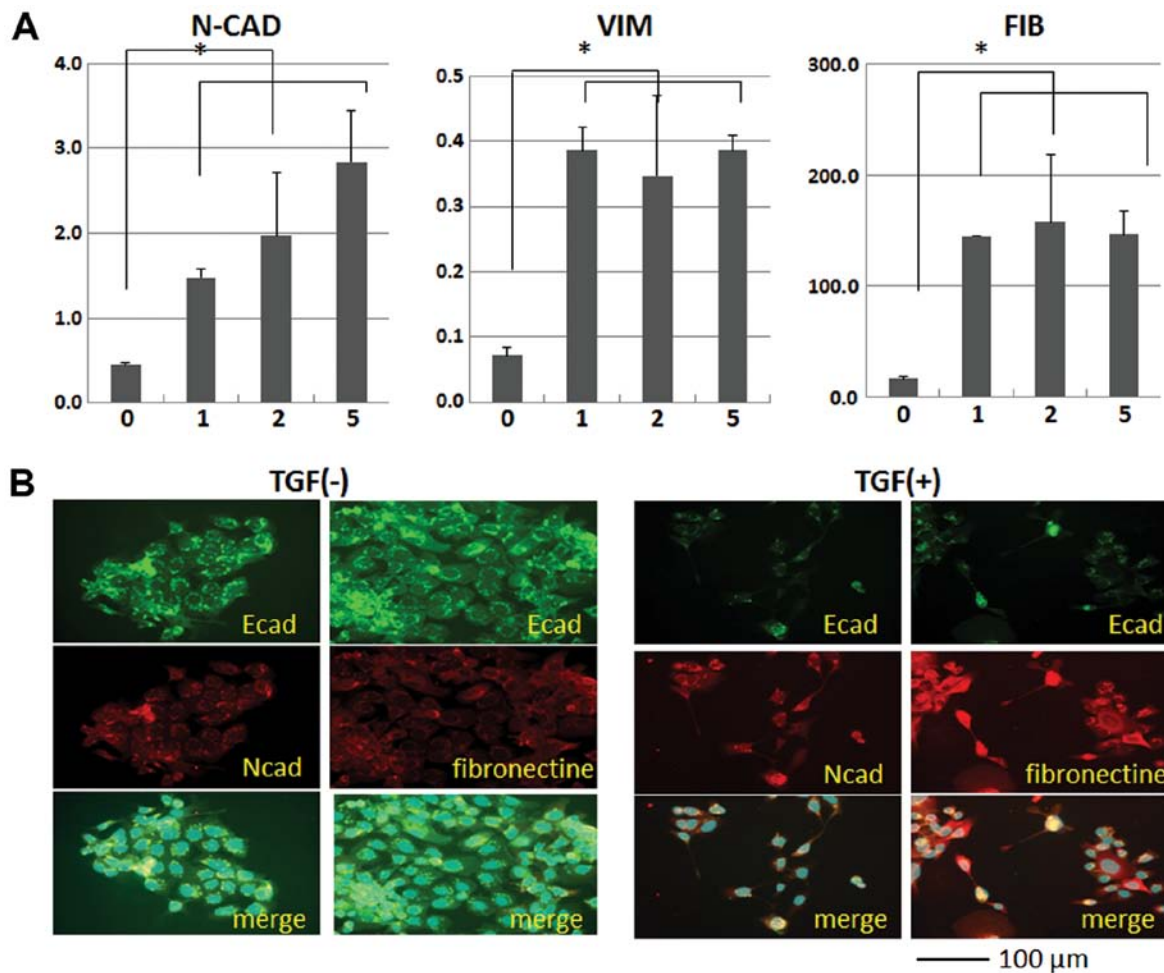


Figure 1. TGF- β stimulates epithelial-mesenchymal transition (EMT) in colorectal cancer cell lines. (A) Quantitative RT-PCR of mRNA of EMT markers, such as N-cadherin, vimentin and fibronectin. (B) The immunohistochemical analysis of EMT markers before and after exposure to TGF- β in culture. Asterisks show statistical significance ($p < 0.05$).

Materials and methods

Cell culture. Human CRC cell lines were obtained from American Type Culture Collection (ATCC) and cultured in minimum essential medium (MEM; Invitrogen, CA, USA) containing 10% fetal bovine serum (FBS; Gibco, CA, USA) at 37°C in a humidified atmosphere containing 5% CO₂. For flow cytometry and cell sorting, an allophycocyanin (APC)-conjugated anti-human CD44 antibody, a fluorescein isothiocyanate (FITC)-conjugated anti-human CD24 antibody, and a phycoerythrin (PE)-conjugated anti-human N-CAD antibody (BD Bioscience, San Jose, CA, USA) were used for characterization of cancer cells. Labeled cells were analyzed on a BD FACS Aria II Cell Sorter System (Becton-Dickinson, Franklin Lakes, NJ, USA), followed by data analysis using the Diva program (Becton-Dickinson), as described previously (4,5).

The expression study. Total RNA was extracted from cells, reverse-transcribed to cDNA, and subjected to PCR analysis using specific primers as described previously (4,5).

Animal experiments. Cells were injected subcutaneously into NOD/SCID mice as described previously (4,5). These mice

were monitored for up to 10 weeks and sacrificed when the tumors reached a maximum diameter of 15 mm. All animal studies were approved by the Animal Experiments Committee of Osaka University.

Statistical analysis. For continuous variables used in an *in vitro* analysis, the data were calculated as mean \pm SD and were analyzed using the Wilcoxon rank test. The relationship between mRNA expression and clinicopathological factors was analyzed using the χ^2 test and Student's *t*-test. Kaplan-Meier survival curves were plotted and compared using the generalized log-rank test. Univariate and multivariate analyses for identification of factors prognostic of overall survival were performed using the Cox proportional hazards regression model. All calculations were performed using the JMP software (SAS Institute, Cary, NC, USA). Differences with a *p*-value of < 0.05 were considered statistically significant.

Results

TGF- β stimulates EMT. To study the EMT mechanism, we cultured the colorectal cancer cell lines CaR-1 and CCK81 in the medium containing TGF- β . The data from quantita-

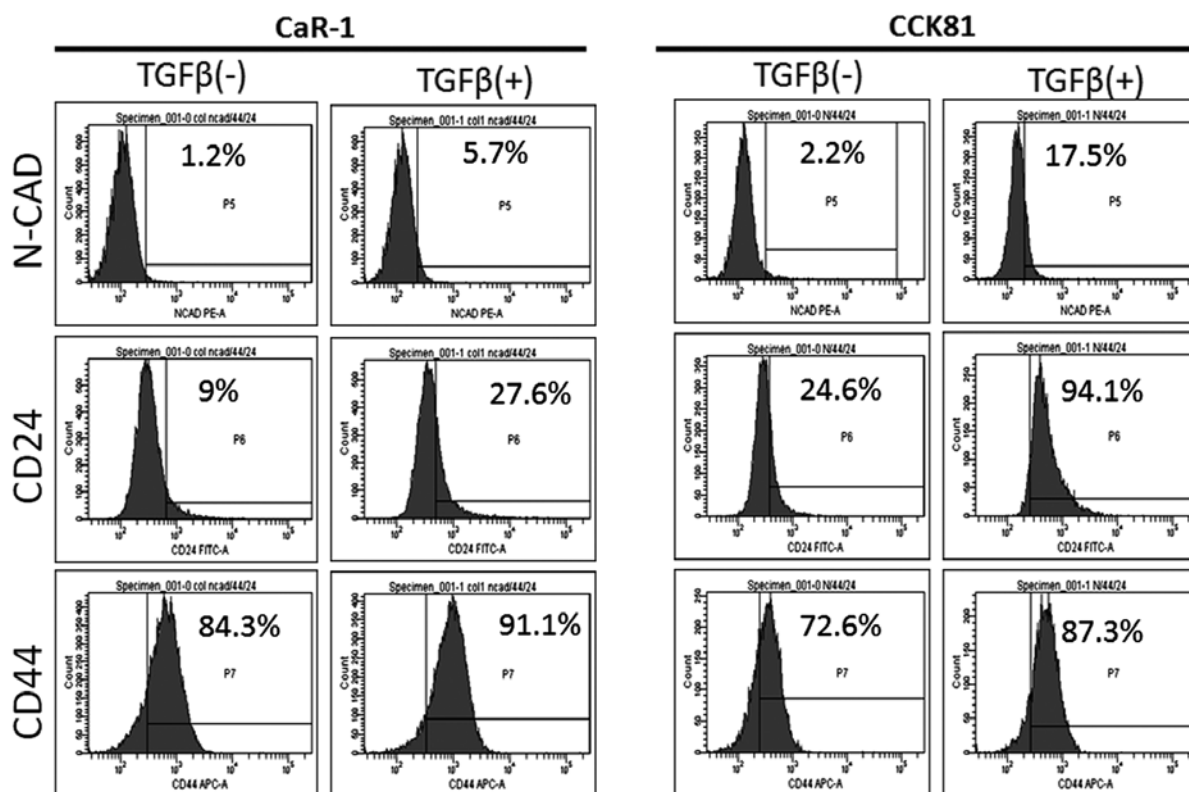


Figure 2. TGF- β increased CD24 expression in colorectal cancer cells. The expression of N-cadherin, CD24, and CD44 was studied after exposure to TGF- β in culture.

tive PCR indicated that expression of EMT markers such as N-cadherin, vimentin, and fibronectin was increased in a dose-dependent manner with TGF- β concentration in the culture medium (Fig. 1A). Immunohistochemical analysis indicated that expression of these genes was increased after exposure to TGF- β (Fig. 1B). The data show that EMT was induced in the cell lines we examined under these conditions.

TGF- β increases CD24 expression in colorectal cancer cells.

To study the effect of TGF- β , we investigated the expression of CSC markers CD44, CD24, and N-cadherin. The data indicated that the expression of these markers was increased after TGF- β exposure; the effect was strong in CD24 compared with the other two markers (Fig. 2). Accordingly, in subsequent experiments, we focused on CD24.

CD24 is enriched in the EMT cells. We were interested in whether CD24⁺ cells are susceptible to TGF- β -induced EMT. To this end, we subjected the colorectal cancer cell lines CaR-1 and CCK81, to fluorescence-activated cell sorting (FACS) and analyzed the results. The data showed that EMT-primed cells, which were marked by the expression of N-cadherin, were enriched in CD24⁺ cells (Fig. 3). The data were consistent between the two cell lines, suggesting that CD24 is a marker of EMT.

CD24 stem markers. We wanted to identify the molecules expressed in CD24⁺ cells, which could be associated with cancer stemness. We analyzed the expression of several

markers, including c-kit, Bmi1, SCF, and Notch 1 (Fig. 4). Then we assessed the effect of adding TGF- β (EMT inducer) to cell culture medium on each separate cell population by FACS sorting. The data on Notch 1 expression indicated that CD24⁺ cells are likely to be CSCs. CD24⁺ cells responded to TGF- β , and this effect was more appreciable in N-cadherin⁺ cells (Fig. 5), suggesting that CD24⁺ cells are prone to EMT, and CD24⁺ N-cadherin⁺ cells are more sensitive to TGF- β than are CD24⁺ N-cadherin⁻ cells. The data showed that the expression of Notch 1 correlated with expression of CD24, suggesting that there was a link between the CD24⁺ CSCs and the Notch 1 pathway.

CD24⁺ cells show high tumorigenic activity. To assess the tumorigenic potential of CD24⁺ cells, we injected the cancer cells into immunocompetent NOD/SCID mice subcutaneously. The results showed that CD24⁺ cancer cells had a higher tumorigenic potential compared with CD24⁻ cells with respect to both tumor frequency and tumor size (Fig. 6). These data suggest that in our experimental model, CD24⁺ cells drive tumorigenicity, which is one of the characteristics of CSCs.

Discussion

Previous studies pointed to the candidacy of CD24 as a CSC marker in colorectal cancer (20,21). The present study shows that CD24⁺ colonic cancer cells increase in number after exposure to TGF- β in culture, compared with N-CAD⁺ and CD44⁺ cells, suggesting that CD24⁺ cells are susceptible to

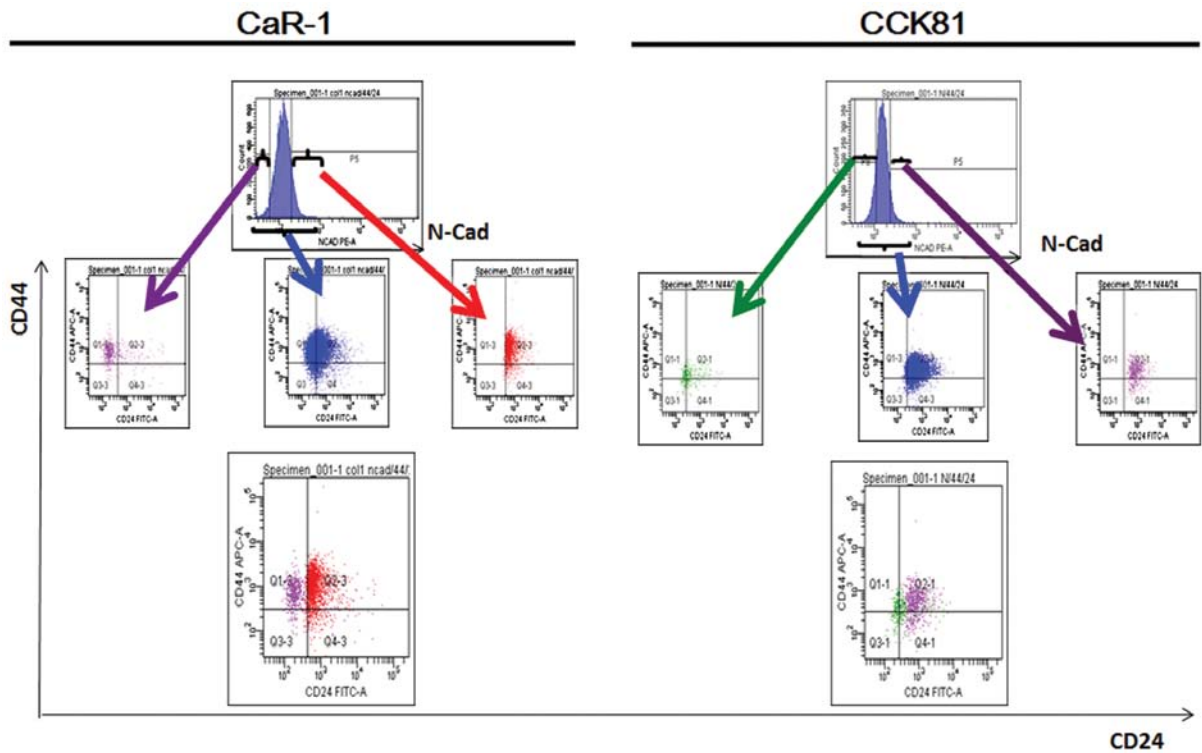


Figure 3. CD24 is enriched in the cells undergoing epithelial-mesenchymal transition (EMT). After sorting by the expression of N-cadherin during FACS, the cell lines were separated by CD24 and CD44. The data indicated that N-cadherin was more strongly associated with expression of CD24 than with expression of CD44 in CaR-1 and CCK81 cells.

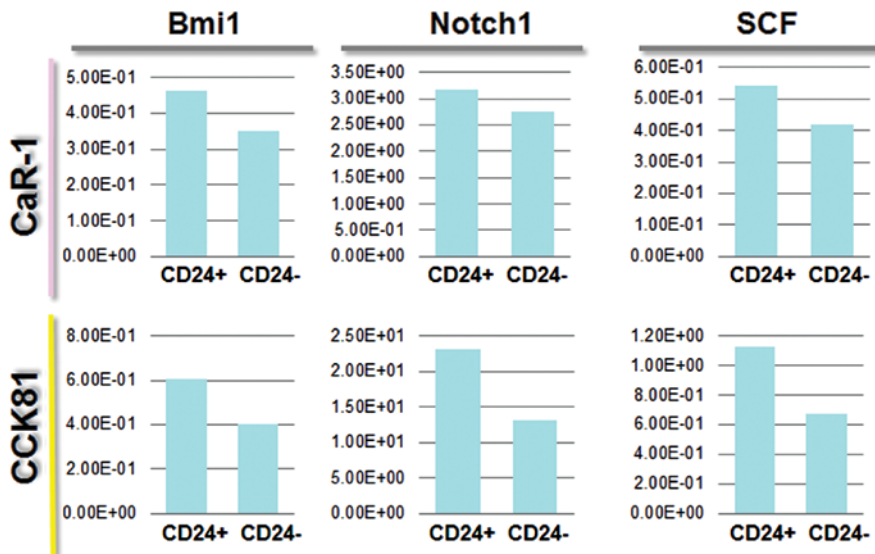


Figure 4. CD24 expression correlates with expression of stem markers. Quantitative RT-PCR was performed for the expression of several markers, including c-kit, Bmi1, SCF and Notch 1, as described in Materials and methods.

EMT, a cellular trait important for cancer metastasis. The data were consistent between the two cell lines that were studied, CaR-1 and CCK81. The cell sorting experiment indicated that CD24 is a more useful marker than CD44, for separation of EMT-prone cells, according to assessment of the expression of N-CAD, a marker of EMT. The present study indicates that CD24 is associated with EMT, and the association is more pronounced compared with other possible markers, such as

CD44. The findings are compatible with the data from other cell lines (23).

Because CD24⁺ cancer cells formed larger tumors in immunocompetent NOD/SCID mice, we determined if any stemness markers were expressed preferentially in CD24⁺ cells. The FACS experiment indicated that exposure to TGF- β in culture resulted in increased Notch 1 expression. Recent studies have indicated that Notch signaling has a critical role

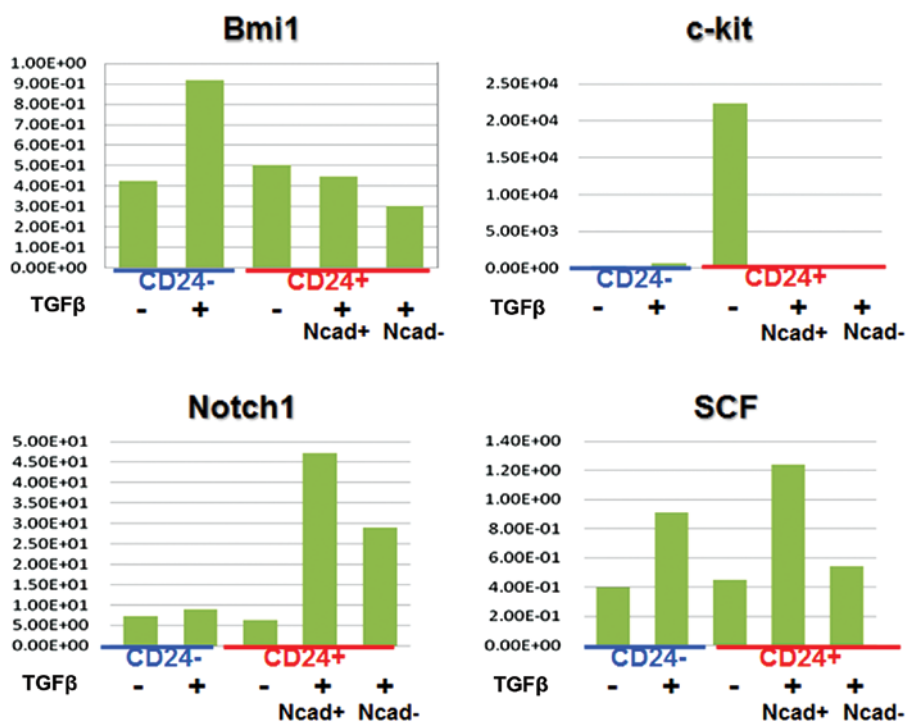


Figure 5. High sensitivity of CD24⁺ cells in response to stimulation of epithelial-mesenchymal transition (EMT). Cells were sorted by FACS with anti-CD24 and anti-N-cadherin antibodies and subjected to the culture for 72 h with or without TGF- β in the culture medium. The data on the expression of stem markers are shown.

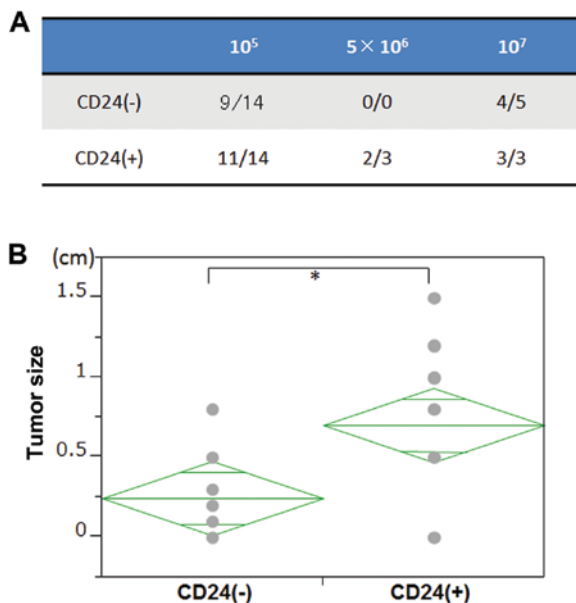


Figure 6. CD24⁺ cells show high tumorigenic activity. Cancer cells were injected into immunocompetent NOD/SCID mice subcutaneously. The data show the frequency (A) as well as tumor size (B). Asterisks show statistical significance ($p < 0.05$).

at the intersection of EMT and cancer stemness and that Notch inhibition is an attractive strategy for the treatment of several cancers, at least in part because of its ability to reverse or prevent EMT (24).

A previous study indicated that TGF- β is a possible niche signal in the bone marrow to induce hibernation of hemo-

poietic stem cells (25), a dormant phenotype of cancer cells, showing resistance to chemotherapy. The hibernation state is associated with inhibition of lipid raft clustering; this change results in inhibition of signaling of growth factors or cytokines through cell surface receptors (25). The study of *Listeria monocytogens* indicated that CD14 and CD24, which normally exhibit uniform distribution on cells undergo clustering upon treatment with the stimulation (26); the phenomenon is suggestive of lipid raft clustering and signaling through CD24. A recent study of protein clustering showed enrichment of CD24 in lipid rafts and a more random distribution of CD44 in the plasma membrane (27). Taken together, the data are indicative of the significance of CD24 as a functional marker of CSCs and suggest that this protein is a possible therapeutic target in colorectal cancer.

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