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Activation of Akt and MAPK pathways enhances the tumorigenicity of CD133+ primary colon cancer cells

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Cancer stem cells (CSCs) play an important role in carcinogenesis, resistance to treatment and may lead to cancer recurrence and metastasis. However, the molecular mechanism of CSC involved in these events needs to be further elucidated. In this study, CD133+ colon cancer cells were cultured, which showed CSC properties both *in vitro* and *in vivo* from metastatic tissue. Upstream molecules in Akt and mitogen-activated protein kinase (MAPK) pathways were preferentially expressed in these CD133+ cells, as revealed by a global gene chip. The kinase activities of Akt and extracellular signal-regulated kinase (Erk)1/2 were also significantly upregulated in CD133+ cells. In addition, the clonogenic growth of CD133+ cell was reduced greatly by inhibiting the activity of Akt and Erk1/2. The results revealed the Akt and MAPK pathways were involved in the tumorigenesis of CD133+ colon cancer cells, suggesting that molecules in these two pathways might be potential targets in the future therapy.

Background

There is increasing evidence that tumors are composed of heterogeneous cells that exhibit distinct proliferative and differentiation abilities. Only a small subset of cancer cells, which are termed 'cancer stem cells' (CSCs), showing self-renewal and differentiating abilities, can generate the original tumor (1). CSCs have been recognized as important components in carcinogenesis and are associated with recurrence, metastasis and treatment resistance. Recently, CSCs had been isolated on the basis of putative stem cell markers or side population in several solid tumors. Growing evidence has proved that the CSC model is also relevant to colon cancer. O'Brien *et al.* (2) reported that there was one colon cancer-initiating cell in 5.7×10^4 unfractionated tumor cells, counted by limiting dilution analysis. In their study, $CD133^+$ cells readily initiated a tumor upon transplantation at a low concentration into non-obese diabetic (NOD)/severe combined immunodeficient (SCID) mice, whereas $CD133^-$ and unfractionated populations did not induce tumor formation.

CD133 (prominin-1) is a five-transmembrane domain molecule with a molecular weight of 120 kDa (3). The physiological function of CD133 remains unknown. It was originally classified as a marker of primitive hematopoietic and neural stem cells (3,4). In cancer cells, it represents a marker of CSCs in various human tumors such as prostate cancer, pancreatic cancer, leukemia, brain tumor, hepatocellular carcinoma, breast cancer and colon cancer (5–11). CD133 expression was not only much higher in the colon cancer sample than normal but it could also be detected in 5 of 12 colon cancer cell lines (12). The isolated $CD133^+$ cells from colon cancer tissues or cell lines exhibited CSC characteristics both *in vitro* and *in vivo* (11). Expression of CD133 in colon cancer was correlated with unfavorable clinical outcome, and the higher level of its messenger RNA in peripheral blood mononuclear cells could predict the cancer recurrence (13,14). These data indicated the importance of CD133 as a central marker of CSC in colon cancer. Although an increasing number of reports have identified CSCs among human colon cancer cells, the genetic characteristics that distinguish CSCs from non-CSCs in the tumors remain to be elucidated. Pathways that are important for CSC survival and regeneration of the neoplasm are still not well defined.

In this study, a primary $CD133^+$ cell line from colon cancer liver metastatic tissue is used. Then, the genetic characteristics of the $CD133^+$ colon cancer cells were determined and signaling pathways that were preferentially activated in $CD133^+$ cells were identified. The importance of Akt and mitogen-activated protein kinase (MAPK) signaling pathways for the tumorigenicity of $CD133^+$ cells was found. This study may provide information for the identification of potential targets for the development of effective anticancer therapies.

Abbreviations: CSC, cancer stem cell; Erk, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; NOD, non-obese diabetic; TGF, transforming growth factor; SCID, severe combined immunodeficient; shRNA, short hairpin RNA; s.c., subcutaneous.

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Materials and methods

Tumor cells preparation

Primary colon cancer cells were originally derived from patients with metastatic colon cancer, as approved by the Research Ethics Board at Zhejiang University. The patients were fully informed and in agreement with the collection of clinical samples. After surgical resection, bulk colon cancer cells obtained from the colon cancer liver metastasis were injected subcutaneously (s.c.) into NOD/SCID mice. Tumor tissues collected from mouse xenografts were minced into ~1 mm^3 in size and washed four times in 100 mM phosphate-buffered saline with 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA). After removal of the wash solution, tissue fragments were placed in serum-free RPMI 1640 (Invitrogen, Grand Island, NY) with 1 mg/ml Collagenase Type IV and 1 mg/ml hyaluronidase (Worthington, Lakewood, NJ) and incubated for 1.5 h at 37°C to obtain enzymatic disaggregation. Every 15 min, the solution was vigorously shaken for 15 s to encourage dissociation. Cells were then sieved through a 40 μm filter and resuspended in RPMI 1640 with 10% fetal bovine serum. Cell viability was determined using the trypan blue dye exclusion assay.

Magnetic cell sorting and flow cytometry

CD133 expression was evaluated using CD133/1-phycoerythrin (Miltenyi-Biotec GmbH, Bergisch Gladbach, Germany) by flow cytometry with a FACSCalibur machine (Becton Dickinson, San Jose, CA) before separation. Then, the cells were labeled with CD133/1 microbeads and separated by passing through a mass spectrometry column that was placed in a magnetic cell sorting separator. Aliquots of $CD133^+$- and $CD133^-$-sorted cells were labeled with CD133/2-phycoerythrin (Miltenyi-Biotec GmbH, Bergisch Gladbach, Germany) and incubated for 5–10 min to evaluate the efficiency of the magnetic separation by flow cytometry. Mouse IgG1 phycoerythrin (Miltenyi-Biotec GmbH) was used as isotype control.

Cell proliferation assay

Cells were plated at a density of 1.0×10^4 cells per well in 24-well plates. Every 24 h, cells were harvested and resuspended in phosphate-buffered saline. An aliquot of cell suspension was diluted with 0.4% trypan blue (Sigma–Aldrich, St Louis, MO), pipetted onto a hemocytometer and counted under a microscope at ×200 magnification. Live cells excluded the dye, whereas dead cells admitted the dye and consequently stained intensely with trypan blue. The number of viable cells for each experimental condition was counted and represented on a linear graph. Each independent experiment was performed in triplicate.

Colony formation assay

To examine the anchorage-independent proliferation, $CD133^+$- and $CD133^-$-sorted cells were seeded 200 per well in a top layer of 0.3% low-melting

agarose (Sigma–Aldrich, St Louis, MO) in 24-well plates with a bottom layer of 0.5% agarose in RMPI 1640 medium containing 10% fetal bovine serum. After incubation at 37°C in a humidified incubator with 5% CO_2 for 3 weeks, colonies containing >20 cells were visualized under inverted microscopy and counted. In kinase inhibitor experiments, the cells were treated with 10 μM Akt inhibitor II SH-5, Akt inhibitor IV or MAPK inhibitor U0126 (Merck Chemicals, Nottingham, UK). Inhibitors or dimethyl sulfoxide (solvent control; Sigma–Aldrich) was added in the medium at a final concentration of 0.1%.

NOD/SCID mice transplantation

The sorted cells were implanted into the flanks of NOD/SCID mice to analyse their ability to initiate tumor xenografts. NOD/SCID mice were bred and maintained under defined conditions at the Laboratory Animal Research Center of Zhejiang Chinese Medicine University under conditions approved by the local animal care committee. Cells were suspended in 200 μl phosphate-buffered saline after sorting. Four-week-old NOD/SCID female mice were s.c. injected with the same number of CD133$^+$ and CD133$^-$ cells. Tumors were measured by a slide gauge every 7 days and their volume was calculated as length × width × width/2. The mice were killed by cervical dislocation 8 weeks after transplantation.

complementary DNA GeneChip scanning

Total RNA of CD133$^+$ and CD133$^-$ cell was extracted using Trizol reagent (Invitrogen) according to the manufacturer's protocol. The quantity and the quality of RNA were evaluated using an Agilent 2100 Bioanalyzer. The gene expression profile of each sample was examined using the Human Genome U133 Plus 2.0 Array (Affymetrix, Santa Clara, CA). Gene expression data were analyzed by Affymetrix GeneChip Operating Software (Version 1.4). Candidate genes whose fold change ≥2.0 were selected and validated by real-time polymerase chain reaction.

Akt and MAPK kinase assays

The kinase activities of CD133$^+$ and CD133$^-$ cells were measured by a non-radioactive kinase assay (Cell Signaling Technology, Danvers, MA). Kinase assays were performed according to the manufacturer's protocols. Briefly, immobilized Akt, Phosho-p44/p42, Phospho-p38 or Phospho-c-Jun kinase monoclonal antibodies were used to coimmunoprecipitate Akt, extracellular signal-regulated kinase (Erk)1/2, p38 and c-jun N-terminal kinase MAPKs in the cell lysates. Then, an *in vitro* kinase assay was performed by incubating with adenosine triphosphate and corresponding substrates glycogen synthase kinase-3 fusion protein (Akt), ATF-2 (p38), Elk-1 (Erk1/2) and c-jun fusion protein (c-jun N-terminal kinase) at 30°C for 30 min. Finally, the level of phosphorylated substrate was detected by western blotting with phospho-glycogen synthase kinase-3β, -ATF-2, -Elk-1 or -c-jun antibody.

shRNA lentiviral particles infection of colon cancer cells

The colon cancer cells were infected with short hairpin RNA (shRNA) lentiviral particles target to Akt1/2/3 and Erk1/2 (Sigma–Aldrich) over 48 h in the presence of polybrene (4 μg/ml) in 12-well plates. Stable colonies were selected and isolated in the presence of puromycin at the concentration of 50 μg/ml.

Statistical analysis

For continuous variables, data were expressed as mean ± SE. Results of soft agarose colony formation assays, flow cytometry analyses, cell proliferation and *in vivo* tumorigenicity assays were analyzed by analysis of variance. A difference between means was considered significant if the P value was <0.05.

Results

CD133$^+$ colon cancer cells show colony formation ability in vitro and tumor-initiating capacity in vivo

CD133 has been reported to be one of the cancer stem cell markers in colorectal cancer. Short-term cultured primary cancer cells were obtained by s.c. transplantation of human colon cancer cells from a liver metastasis into NOD/SCID mice. The frequency of CD133$^+$ was 9.7–13.2% in the metastatic colon cancer cells cultured. Single-cell suspension derived from mechanical and enzymatic dissociation of xenograft was separated into CD133$^+$ and CD133$^-$ fractions. The efficiency of separation was evaluated by flow cytometry. A considerable enrichment of CD133$^+$ cells (purity > 90%) and negative selection (purity > 95%) of CD133$^-$ cells was achieved (Figure 1A).

The proliferation, colony formation ability *in vitro* and the tumor-initiating capacity *in vivo* of CD133$^+$ cells were tested. When sorted, the proliferation rate between CD133$^+$ and CD133$^-$ cells was examined from days 1 to 7 after seeding in 24-well plates. There were no differences in growth rate between CD133$^+$ and CD133$^-$ cells in complete medium (Figure 1B). But in soft agarose, the colonies generated from CD133$^+$ cell fraction grew faster and bigger than those from CD133$^-$ (Figure 1C). CD133$^+$ colon cancer cells could form more colonies than CD133$^-$ after 3 weeks in soft agarose. It was showed that CD133$^+$ cells formed 43.6 ± 5.9 colonies per 200 cells, whereas CD133$^-$ cells formed only 11.4 ± 2.8 colonies (Figure 1D). These data revealed that CD133$^+$ colon cancer cells had greater colony formation ability than CD133$^-$ ones. To test the tumorigenicity of CD133$^+$ cells *in vivo*, NOD/SCID mice were s.c. injected with 0.2×10^4 to 1×10^6 CD133$^+$ or CD133$^-$ cells. In the case of implantation of as few as 0.2×10^4 cells, only CD133$^+$ cells were capable of tumor initiation in 2 months (Figure 1E). In contrast, CD133$^-$ formed no tumor when 1×10^4 cells were implanted (Figure 1F). A tumor could be observed till 1×10^5 CD133$^-$ cells were implanted. It was presumed that the CD133$^-$ fraction might be contaminated with a few CD133$^+$cells. Hence, CD133$^+$ cells had more tumor-forming ability *in vivo* than CD133$^-$ cells.

Global gene expression pattern of CD133$^+$ colon cancer cells

CD133$^+$ and CD133$^-$ colon cancer cells showed differences in colony formation ability *in vitro* and tumorigenicity *in vivo*. Therefore, a search was undertaken for genes that were differentially expressed between the two cell types using complementary DNA GeneChip analysis. In total, 321 genes were upregulated and 65 genes downregulated in CD133$^+$ colon cancer cells by >2-fold change (supplementary Data 1 is available at *Carcinogenesis* Online). The expression of representative genes was examined by real-time polymerase chain reaction to verify the results of the complementary DNA GeneChip analysis (Figure 2A). Pathway analysis showed that the changed genes mainly distributed within phosphatidylinositol 3-kinase/Akt, Notch, Janus kinase/signal transducers and activators of transcription, MAPK and transforming growth factor (TGF)-β pathways (Figure 2B).

Akt and MAPK signaling pathways involving in the tumorigenicity

The Akt and MAPK signaling pathways are two critical pathways, involved in cell survival, proliferation, differentiation and tumor growth (15,16). Moreover, *ABI1*, *SMG1* in Akt pathway, *TGF-β1* and *GRB2* in MAPK pathway were significantly altered in the cDNA GeneChip. The kinase activities of Akt, Erk1/2, p38, and c-jun N-terminal kinase kinase of the two cell fractions using Akt and MAPK kinase assay were tested. Different activities of Akt and Erk1/2 were found between CD133$^+$ and C133$^-$ cells. Akt was significantly activated in CD133$^+$ cell and Erk1/2 activity was upregulated by 2.38-fold (Figure 3A). These results suggested the Akt and MAPK signaling pathways could be important for the CD133$^+$ cell. Next, whether the activation of Erk1/2 and Akt was required for the colony formation of CD133$^+$ cells in soft agarose was examined. The CD133$^+$ cells were treated with 10 μM Akt inhibitor II SH-5 (IC_{50} = 13.2 μM), Akt inhibitor IV (IC_{50} = 13.1 μM) and MAPK inhibitor U0126 (IC_{50} = 16.7 μM), respectively (supplementary Figure 1 is available at *Carcinogenesis* Online). Compared with dimethyl sulfoxide-treated cells, the three inhibitors led to a 4.6-, 11.4- and 3.1-fold decrease in colony formation, respectively (Figure 3B). The importance of the Akt and MAPK pathway for CD133$^+$ cells was further confirmed by a gene-knockdown experiment. As expected, it was observed that CD133$^+$ cells transduced with Akt and Erk shRNA also showed reduction in colony formation (Figure 3C).

Discussion

The CSC hypothesis was originally based on the observation that when cancer cells of many different types were assayed for their proliferation capacities, only a minority of cells actually possessed cancer-initiating potential (17). This observation gave rise to the idea that malignant tumors comprised a small subset of cells that had great tumorigenic potential, as well as more differentiated cancer cells, which had very limited potential. These so-called CSCs were

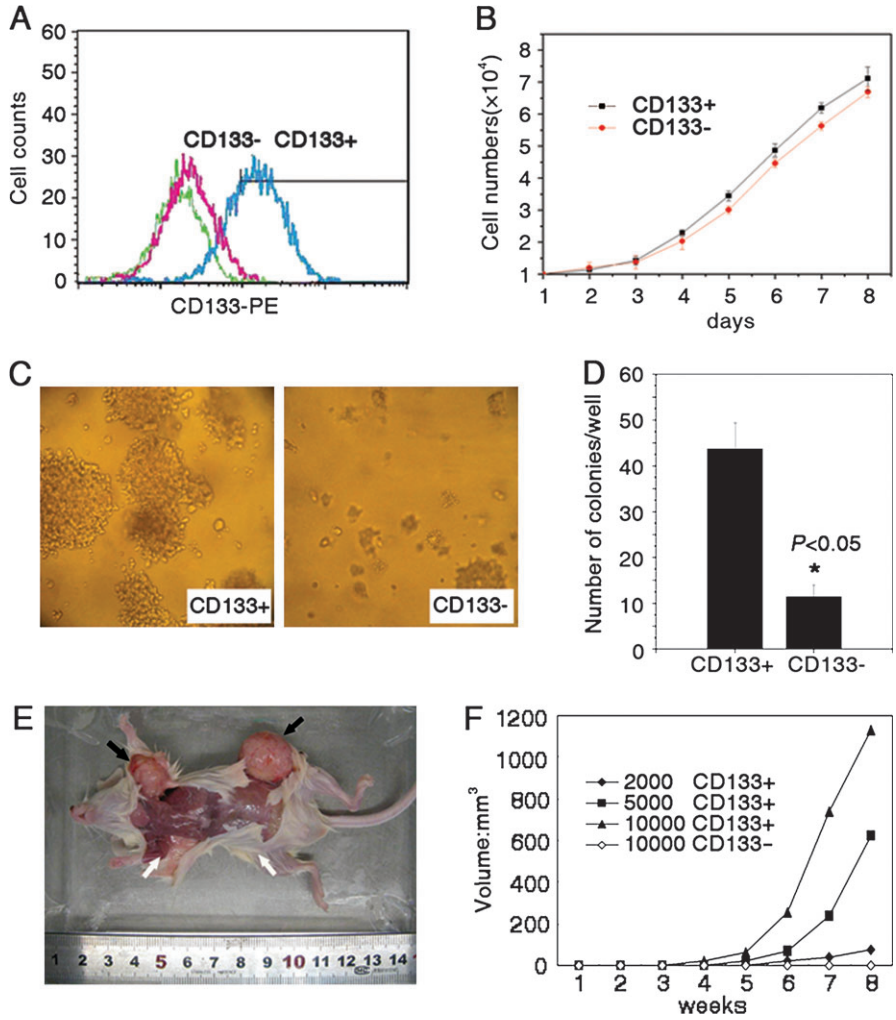
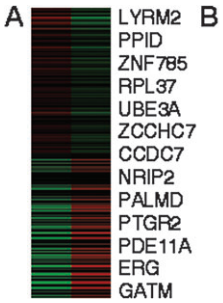


Fig. 1. CD133$^+$ colon cancer cells show tumor-initiating capacity *in vitro* and *in vivo*. (**A**) Isolation CD133$^+$ cells by magnetic cell sorting. Colon cancer cells were magnetically separated based on CD133 expression. The purities of the two separated parts were evaluated by flow cytometry. CD133$^+$ cells (Blue) and CD133$^-$ cells (Red) were separated effectively. The green curve represented the isotype control. (**B**) Cell proliferation assay. The cells were seeded 1.0×10^4 cells per well in 24-well plates. The numbers of viable cells were counted under a microscope with trypan blue staining each day. No significant difference was found in cell proliferation between CD133$^+$ and CD133$^-$ cells. (**C**) Soft agarose assay. Two hundred CD133$^+$ and CD133$^-$ cells were seeded in triplicate per well in 24-well plates with soft agarose. Colonies from CD133$^+$ (left) and CD133$^-$ (right) were visualized under inverted microscope (magnification, ×100). (**D**) Ability of colony formation of CD133$^+$ cells. After 3 weeks incubation, colonies containing >20 cells in the soft agarose were counted. CD133$^+$ cells formed more colonies (43.6 ± 5.9 per well) than CD133$^-$ cells (11.4 ± 2.8 per well) ($P < 0.05$). (**E**) Xenograft formation in NOD/SCID mice. A representative image of xenograft tumor in mice that were s.c. injected with equal numbers of the CD133$^+$ and CD133$^-$ cells. The CD133$^+$ cells could form xenograft tumors (black arrow), whereas the CD133$^-$ cells could not (white arrow) when the same number cells were implanted. (**F**) Comparison of Xenograft formation *in vivo*. Tumor volumes were measured and calculated each week. CD133$^+$ cells showed stronger ability of tumor formation.



B

Entrez Gene	Gene Symbol	Microarray	qPCR	Pathway
8842	PROM1(CD133)	2.12	2.42	
2885	GRB2	2.58	2.34	MAPK/Jak-STAT
7040	TGFB1	2.07	2.0	MAPK/TGF-beta
3572	IL6ST	2.32	2.52	Jak-STAT
3717	JAK2	-2.25	-1.8	Jak-STAT
55534	MAML3	2.6	1.9	Notch
10006	ABI1	2.35	2.8	PI3K/Akt
23049	SMG-1	4.04	5.11	PI3K/Akt
6925	TCF4	4.64	2	Wnt

Fig. 2. Global gene expression pattern of CD133$^+$ colon cells. (**A**) Global complementary DNA scanning. The gene expression profile of the two types of cells was examined using the Human Genome U133 Plus 2.0 Array. Cluster analysis was performed. Thirteen significantly changed genes were listed. (**B**) Representative genes mainly related to important pathways. Genes related to the phosphatidylinositol 3-kinase/Akt, Notch, Janus kinase/signal transducers and activators of transcription (JAK/STAT), MAPK and TGF-β pathways were changed in the CD133$^+$ cells. Representative gene expression was validated by real-time polymerase chain reaction (qPCR).

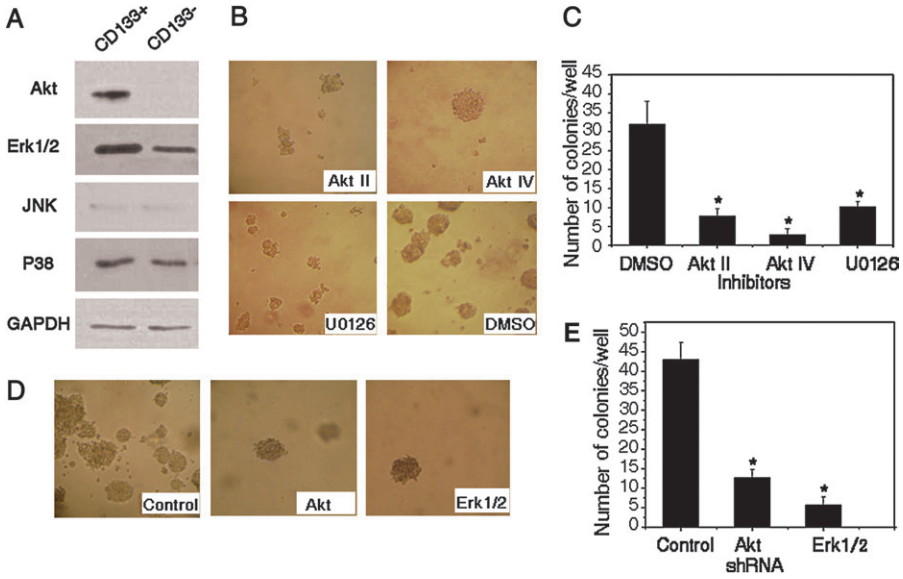


Fig. 3. Akt and MAPK pathways involved in tumorigenicity. (**A**) Akt and MAPK kinases assay. The Akt and MAPK kinases activities were tested by the non-radioactive kinase assay. Western blot analysis showed the Akt and Erk1/2 pathways were significantly activated in CD133$^+$ cell. (**B** and **C**) Inhibition of Akt and Erk1/2 activities attenuated the colony formation ability. CD133$^+$ cells were seeded in 24-well plates with soft agarose containing the Akt inhibitor II (10 μM), Akt inhibitor IV (10 μM) or MAPK inhibitor U0126 (10μM). The Akt and Erk1/2 inhibitors produced a significant reduction of colony formation, $P < 0.05$. (**D** and **E**) Downregulation of Akt and Erk1/2 decreased the colony formation. CD133$^+$ cells with stable transduction by Akt or Erk1/2 shRNA lentiviruses formed fewer colonies than control shRNA ($P < 0.05$).

proposed to possess stem-cell-like properties including the ability of self-renewal and differentiation (18). CSCs are therefore considered to be responsible for tumor development and growth (19).

CSCs can be enriched based on special cell surface markers (2), spherical colony culture methods (20) or dye elimination ability (21). Several markers are currently used for isolating colon CSCs, including CD133, CD44, CD166 and EpCAM^{high} (11,22,23). The common cell surface marker CD133 has been used to fractionate CSCs in diverse solid tumors such as brain tumor, pancreas cancer, lung cancer and colon cancer. Several studies demonstrated that CD133$^+$ colon cancer cells had tumorigenic potential *in vivo* and *in vitro*. A recent report showed that CD133 marked stem cells were susceptible to transformation into tumors retaining a fraction of mutant CD133 tumor cells (24). Thus, the CD133 was used as a marker to isolate CSCs in studies. Because CD133$^+$ cells were detectable in liver metastasis at a higher percentage when compared with primary tumors (25), primary colon cancer cells derived from liver metastasis of a colon cancer patient were cultured. Hematoxylin–eosin staining and microscopic analysis indicated that the metastatic tumor and the xenograft were consistent with the primary tumor at a histological level (supplementary Figure 2 is available at *Carcinogenesis* Online).

It was observed that the CD133$^+$ cells exhibited higher clonogenic potential in soft agarose but did not proliferate faster in a culture-containing serum than the CD133$^-$ cells. This might be due to the reason that colon cancer stem cells cultured in serum-containing medium could gradually differentiate into adherent cells with a phenotype similar to that of differentiated epithelial cells. When cultured in soft agarose, the CD133$^+$ cell proliferated slowly and formed a colony, whereas the CD133$^-$ cells died through anoikis after 5 days of culture. The CD133$^+$ cells showed higher tumorigenic potential *in vivo* than CD133$^-$ cells, consistent with other previous reports (2).

Pathways that are important for self-renewal in normal stem cells are found to be dysregulated in human malignancies. The WNT, Notch, Hedgehog and TGF-β signaling pathways had been implicated in the control of CD133$^+$ CSC function in human brain tumors (26). To understand the biological properties of CSC, one approach is to perform global gene expression profiling and to compare the CSC profile with the non-CSC in the tumor. In this study, global GeneChip scanning was performed to understand clearly the features of CD133$^+$ cells. Several genes previously reported to be associated with stem cells and cancer invasion were included in the list of changed genes. Data analyses of signaling pathway genes identified four pathways including Notch, Janus kinase/signal transducers and activators of transcription, MAPK and TGF-β, which were greatly altered in CD133$^+$ cells.

The Akt pathway is reported to play an important role in regulating tumor growth and is a well-characterized downstream target of the phosphatidylinositol 3-kinase–Akt–mTOR pathway. Hyperactive Akt signaling promotes tumorigenic cell behaviors by increasing cell survival, proliferation, invasion and angiogenesis (27). The MAPK cascade is also one of the most ubiquitous signal transduction systems and is rapidly activated by various stimuli, such as cellular stress and death. Activation of MAPKs is commonly detected in colorectal cancer (28). The activation of the Erk–MAPK pathway is proved to induce the synthesis of cyclin D1, promoting cell division. A long duration of Erk activation preferentially stimulated differentiation (29). Several lines of evidences indicated that activation of Erk/MAPK played an important part in progression of cancer. It was found the activation levels of Akt and Erk1/2 were significantly higher in CD133$^+$ cells than in CD133$^-$ cells, which suggested that the CSCs might escape from anoikis through activating both Akt and Erk.

Elucidation of the signaling pathways that govern the maintenance and survival of CSCs is essential for devising an optimal targeted therapy. Considering that the Akt and MAPK pathways may be involved in the proliferation and differentiation of CD133$^+$ cells, the clonogenic potential of the two populations in the effects of Akt and MAPK inhibitors was compared. The concentrations of the inhibitors were referenced to the IC_{50} of each inhibitor and the other studies (30,31). It was found the CD133$^+$ cells were sensitive to the inhibitors and their colony formations decreased significantly both in size and in number in soft agarose. Inactivation of Akt and Erk pathway by inhibitors abolished the preferential survival of CD133$^+$ colon cancer cells and decreased the tumorigenicity. Downregulation of Akt and Erk by shRNA transfection also attenuated the colony formation ability of CD133$^+$ cells. These results indicated that the Akt and MAPK pathways were vital for CD133$^+$ cells tumorigenicity. The inhibition of Akt and Erk1/2 more potently regulated the growth of

$CD133^+$ cells than that of $CD133^-$ and could be a useful biochemical strategy for the development of therapeutic agents for colon cancer.

Conclusions

The activations of Akt and MAPK pathways were proved to be vital for the colon CSC tumorigenicity. Inhibitors of Akt and MAPK reduced the colony formation abilities of the colon CSCs, which might provide a target for inhibiting CSC in the treatment of cancer. Further work is necessary to investigate the mechanism of Akt and Erk1/2 activation in the biological events of CSCs. Combination therapies that apply CSCs-targeting agents together with those that kill non-CSC populations need to be developed.

Supplementary material

Supplementary Data can be found at http://carcin.oxfordjournals.org/

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Conflict of Interest Statement: None declared.

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