Identification and Metastatic Potential of Tumor-Initiating Cells in Malignant Rhabdoid Tumor of the Kidney

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Abstract

Purpose: Malignant rhabdoid tumor of the kidney (MRTK) is a rare and highly aggressive malignancy of infancy. In an effort to delineate MRTK progression, we investigated the metastatic fate of some MRTK cells using xenotransplantation animal models and the tumor-initiating potential of CD133+ MRTK cells.

Experimental Design: We established two MRTK cell lines (JMU-RTK-1 and JMU-RTK-2) from patients with MRTK. We generated five luciferase-expressing MRTK cells for in vivo luminescent imaging and evaluated the metastatic fate in an orthotopic xenotransplantation model. Capacities of MRTK-initiating cells were examined in nonobese diabetic/severe combined immunodeficient mice after antibody-mediated magnetic bead sorting. Use of chemokine receptor CXCR4 expression as a metastatic marker was evaluated by flow cytometry and Western blotting.

Results: MRTK cell lines showed distant organ metastasis. JMU-RTK-1, JMU-RTK-2, and G401 cells showed considerable aggressiveness compared with SWT-1 and SWT-2 cells (P < 0.05). Moreover, as few as 1,000 CD133+ MRTK cells initiated tumor development in nonobese diabetic/severe combined immunodeficient mice by 21 days (60-100%) in all examined cell lines, although the same number of CD133- MRTK cells could not form tumors (0%). Interestingly, the metastatic potential of the CD133+ population remained unaffected compared with a nonenriched population. The potential metastatic marker CXCR4 was expressed in CD133+ and CD133- MRTK cells, and CD133+ cells seemed to play a cooperative role in terms of tumorigenicity and metastasis.

Conclusions: These results suggest that CD133+ cells may determine the metastatic fate of MRTK cells and that CD133+ cells may play an auxiliary role in tumor progression and metastasis.

There seem to be characteristics common to both tumor cells and normal stem cells in terms of what might be referred to as “stemness.” The hallmark traits of stem cells—self-renewal and differentiation capacity—are reflected by the high proliferative capacity and phenotypic plasticity of tumor cells (1). Furthermore, malignant tumor cells often lack the terminal differentiation events present in normal cells. These parallels have given rise to the hypothesis that tumors often arise from undifferentiated stem or progenitor cells: Cancer cells can undergo progressive dedifferentiation during their development (1–3). Additionally, it has been proposed that cancer stem cells—a subpopulation of cancer cells possessing tumor-initiating capability—are derived from normal stem cells (1, 4). In fact, since the identification of leukemia-initiating cells, several initiating cells in solid tumors have also been identified for breast (5), brain (6), colon (7, 8), pancreas (9), and prostate cancer (10).

The malignant rhabdoid tumor (MRT) is a rare and highly aggressive malignancy of infancy, which commonly develops in the kidney and central nervous system (11, 12). Significant progress in genetic studies has revealed that the majority of MRTs harbor biallelic inactivation of the chromatin-remodeling gene hSNF5/INI1 located in chromosome 22q11.2 (13, 14). However, MRT is resistant to most therapeutic regimens, and the overall survival rate of patients with MRT of the kidney does not exceed 25%. For example, only 8.8% of infants that were diagnosed before the age of 6 months were living 4 years after diagnosis (15). Recent clinical evidence has suggested that a high frequency of tumor-initiating cells in brain tumors (e.g., high-grade medulloblastoma) is significantly correlated with aggressiveness (6).
**Translational Relevance**

There is a great deal of clinical evidence supporting the aggressiveness of malignant rhabdoid tumor of the kidney (MRTK). However, an appropriate animal model to characterize the aggressive nature of transplantable MRTK cell lines has not been reported due to the rare malignancy. In this work, we showed the metastatic fate of some MRTK cells using luminescent imaging technology. Moreover, in an effort to understand the aggressiveness of MRTK, we were also able to identify MRTK-initiating (stem) cells from established cell lines. In light of possible distant metastasis in MRTK, we evaluated the relationship between CD133-positive MRTK cells and chemokine receptor CXCR4 expression. These results have yielded important implications concerning MRTK biology, and our transplantable cell source coupled with luminescent imaging provides a tool for new preclinical therapeutic strategies against MRTK.

Moreover, an interesting feature of MRT is the occasional occurrence of separate central nervous system primary tumors (16). Thus, the similarity between high-grade medulloblastoma and MRT in terms of aggressiveness allows us to determine whether MRT cells frequently contain initiating cells.

Herein, we established two MRT cell lines from patients with MRT of the kidney (MRTK) and show that tumor-initiating cells of MRTK are frequently present within the CD133⁺ population. Furthermore, we show the characteristic metastatic potential of the MRTK cells in an orthotopic xenotransplantation model of severe combined immunodeficient (SCID) mice. Identification and investigation of the characteristics of tumor-initiating cells in MRT can contribute significantly toward the design of aggressive MRT therapies.

**Materials and Methods**

**Cells, animals, and reagents.** JMU-RTK-1 and JMU-RTK-2 cell lines were established from two independent patients after confirming the histologic examination using the Japanese Wilms Tumor Study. Histopathologic analysis of the two cases showed that the cells were round to polygonal in shape with vesicular nuclei, prominent nucleoli, and eosinophilic cytoplasm with rare but typical cytoplasmic inclusions. Briefly, the clinical courses of the two patients are described. (a) JMU-RTK-1 cells were established from a surgical specimen derived from a 4-mo-old boy who presented with macrohematuria and an abdominal mass (4 cm × 4 cm left kidney mass as determined by abdominal computed tomography). No metastatic lesion was observed at this time. Although the patient received chemotherapy following the surgical treatment, local recurrence and pulmonary metastasis developed and the patient died 5 mo after the recurrence. (b) JMU-RTK-2 cells were established from the spinal fluid of 4-mo-old female who primarily presented an abdominal mass (a 6-cm-diameter tumor of the right kidney), although central nervous system metastasis (meningial dissemination) and local recurrence developed following right nephrectomy. The patient died 12 mo after resection of the primary tumor. The lichi Medical University ethical committee approved of the experiments described in this article. JMU-RTK-1 and JMU-RTK-2 cells were maintained in DMEM (Sigma-Aldrich) with 10% heat-inactivated FCS and supplements (17). G401 cells (18) were obtained from the Health Science Research Resources Bank (Osaka, Japan) and maintained in McCoy's 5A medium (Life Technologies) with 10% FCS and supplements. SWT-1 and SWT-2 cells (19) were donated by Dr. Masao Hirose (Naruto University of Education, Tokushima, Japan), and FRTK-1 cells (20) were provided by Dr. Michiyuki Hakozaki (Fukuoka Medical University, Fukuoka, Japan). The well-characterized SWT-1, SWT-2, and FRTK-1 cell lines were used as representative MRTK cells and maintained in RPMI 1640 (Life Technologies) with 10% FCS and supplements. The cultures were kept in a 5% CO₂ and 95% air humidified atmosphere at 37°C.

BALB/c Acl-nu/nu (BALB/c nude, 6–8 wk old) and C.B-17/Scid/scid/cfI (C.B-17 SCID) mice (8–10 wk old) were purchased from Charles River Japan, Inc., and nonobese diabetic (NOD) C.B-17-Prkdcscid/J (NOD/SCID) mice (8-10 wk old) were purchased from Charles River Japan. All experiments in this study were approved by the animal ethics review board of the Ichi Medical University and done in accordance with the Ichi Medical University Guide for Laboratory Animals and following the principles of laboratory animal care formulated by the National Society for Medical Research.

Phycoerythrin-conjugated anti-human CD133 (clone AC133; Miltenyi Biotec) and phycoerythrin-conjugated anti-human CXCR4 (clone 12G5; ebioscience) antibodies were used for the flow cytometric analysis. Isotype-matched IgG controls were purchased from BD Pharmingen. For the magnetic separation, anti-phycoerythrin MicroBeads (Miltenyi Biotec) were used for the CD133 cell enrichment.

**Establishment of luciferase-expressing MRT cells.** Firefly (Photinus pyralis) luciferase cDNA from pGL3 basic (Promega) was inserted into the pMSCVpuro retroviral vector (Clontech), generating pMSCV-luciferase (21). GP2-293 packaging cells (Clontech), a plasmid encoding the viral envelope glycoprotein (VSV-G) of vesicular stomatitis virus, using Lipofectamine 2000 (Invitrogen), Supernatants from transfected GP2-293 were cotransfected with pMSCV-luciferase and pVSV-G (Clontech), a plasmid encoding the viral envelope glycoprotein (VSV-G) of vesicular stomatitis virus, using Lipofectamine 2000 (Invitrogen). Supernatants from transfected GP2-293 were cotransfected with ~50% confluent MRTK cells in the presence of Polybrene (8 mg/mL final concentration; Sigma-Aldrich). Transduced cells were propagated in a medium containing puromycin (Sigma-Aldrich) at 15 mg/mL (luc-JMU-RTK-1, luc-JMU-RTK-2, luc-G401, luc-SWT1, and luc-SWT2).

**PCR and reverse transcription-PCR.** For reverse transcription-PCR, total RNA was extracted from cells using Isogen (Nippon Gene). Two micrograms of total RNA were used for first-strand synthesis using SuperScript III reverse transcriptase (Invitrogen). The following primers were used for hSNF5/INI1 expression (13): exon 1 sense, 5'-ATGATGTATGATGCTGGAGAAT-3'; exon 4 sense, 5'-AACGCAATCAAGGGAACGAGGACGCGA-3'; exon 4 antisense, 5'-TCCGGTTCCGTGATGATTGT-3'; exon 9 antisense, 5'-ATGCAATGCTGATGCCGGAGG-3'; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) sense, 5'-GTATGGGAAGGACTCTAGT-3'; GAPDH antisense, 5'-AGTGGCTGTCCGCTGTGAA-3'. PCR conditions for each of the primers included initial treatment at 95°C for 2 min, followed by 30 cycles comprising denaturation at 95°C for 15 s, annealing at 57°C for 30 s, and then extension at 72°C for 2 min. PCR products were analyzed by electrophoresis through a 1% agarose gel.

**Flow cytometry and magnetic bead selection.** Cells (1 × 10⁶) were washed with PBS and incubated with monoclonal antibody (mAb) for 30 min at 4°C. Following washing with 0.1% FCS-PBS, cells were analyzed using FACS-Calibur (Becton Dickinson) and FlowJo analysis software (Tree Star). At least 10,000 events were acquired for each sample. For magnetic bead selection, cells (5 × 10⁶) were treated with phycoerythrin-conjugated anti-human CD133 mAb (Miltenyi Biotec), followed by anti-phycoerythrin MicroBeads (Miltenyi Biotec), washed, and then loaded onto a MACS MS column (Miltenyi Biotec) for positive magnet-based selection. The positive and negative fractions were then analyzed by flow cytometry.

Xenograft tumor transplantation model. Cells in exponential growth phase were harvested by trypsinization and washed twice in PBS before injection. For the s.c. injections, cells (1 × 10² - 1 × 10⁶) were
injected into the s.c. space of NOD/SCID mice. To determine the minimal amount of cells capable of engraftment, limiting dilution experiments were done for CD133-positive and CD133-negative cells. Tumor appearance was evaluated using a caliper, and tumor growth at the skin was monitored by calculating the tumor volume (\(= \text{[length in mm]} \times \text{[width in mm]}^2/2\)).

For the orthotopic tumor model of the kidney, C.B-17 SCID mice were treated by injection of anti-asialo GM1 antibodies (100 mg/body, Wako) into the peritoneal cavity 1 d before the operation. The left kidney of anesthetized mice was exposed through a left flank incision and partial exteriorization. Cells (5 \times 10^6) were suspended in 0.1 mL Matrigel (BD Biosciences) and inoculated into the renal subcapsular space. Tumor growth was monitored by in vivo luminescent imaging.

**Histologic examination.** Removed specimens were fixed with 10% paraformaldehyde and embedded in paraffin. Sections were then stained with H&E.

**In vivo and ex vivo bioluminescence imaging.** In vivo tumor progression was examined using the noninvasive bioimaging system IVIS (Xenogen). Tumor-implanted mice were anesthetized with isoflurane (Abbott Laboratories), and D-luciferin (potassium salt; Biosynth) was injected into the peritoneal cavity at 3 mg/body, which was immediately followed by the measurement of luciferase activity. The imaging system consisted of a cooled, back-thinned charge-coupled device camera to capture both a visible light photograph of the animal taken with light-emitting diodes and the luminescent image. After acquiring photographic images of each mouse, luminescent images were acquired with a 1-min exposure time (21, 22). Images were obtained with a 25-cm field of view, a binning (resolution) factor of 8, 1/f stop, and an open filter. The resulting gray-scale photographic and pseudocolor luminescent images were automatically superimposed using software to facilitate identification of any optical signal and location on the mouse. Optical images were displayed and analyzed using Igor (WaveMetrics) and IVIS Living Image (Xenogen) software packages. The signal from tumors was quantified as photons flux in units of photons/s/cm^2/steradian.

For the inspection of metastasized organs, various organs of mice were resected to examine tumor-derived photons for micrometastases in the presence of D-luciferin. Direct invasion was evaluated for the following representative organs: the gut and omentum, peritoneum, retroperitoneum, diaphragm, spleen, and bladder. For metastasized organs, the lung, liver, brain, and para-aortic lymph nodes of mice were inspected by luminescent imaging (and histologic examination).

**Western blot analysis.** Cells were lysed using radioimmunoprecipitation assay buffer [50 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 1% NP-40, 0.25% sodium deoxycholate, 1 mmol/L EDTA, 0.1% SDS, 1 mmol/L NaVO_4, and 1 mmol/L NaF] containing protease inhibitor cocktail (Roche Diagnostics). Western blot analysis was conducted using standard procedures. SDS-PAGE was done using 1x sample buffer containing 5% β-mercaptoethanol. Following the transfer of proteins to nitrocellulose membranes, the membranes were incubated for 1 h with rabbit anti-human CXXCR4 (ProSci) and anti-GAPDH (Santa Cruz Biotechnology) primary antibodies. The membranes were then incubated for 1 h with secondary antibodies. Chemiluminescent detection was done using an ECL Plus Chemiluminescence Detection Kit (GE Healthcare UK Ltd.) and the photo-intensity was quantified by densitometric analysis (NIH image).

**Statistical analysis.** \(P\) values based on log-rank, Tukey-Kramer, or Fisher’s tests were obtained using Instat (GraphPad) or StatView (Abacus Concepts, Inc.). Differences between groups were considered significant if \(P < 0.05\).

**Results**

**Characteristics of established MRTK cell lines.** Two cell lines (JMU-RTK-1 and JMU-RTK-2) were established from two patients with MRTK (see Materials and Methods). Both cell lines showed heterogeneous morphology of adherent and spindle cell types on plastic culture dishes. The approximate doubling time in JMU-RTK-1 and JMU-RTK-2 was 9 and 18 h, respectively. Both cell lines were still viable after 200 passages over a 1-year period.

JMU-RTK-1 and JMU-RTK-2 cells were injected s.c. into the flanks of NOD/SCID mice (Fig. 1A). By 21 days, a visible tumor had formed in all mice that underwent the transplantation.

![Fig. 1](https://example.com/fig1.png) CharacteristicsofestablishedMRTKcelllines. A. JMU-RTK-1and JMU-RTK-2 cells (either 1 \times 10^6 or 1 \times 10^5) were transplanted into the subcutaneous space of NOD/SCID mice and tumor growth was measured at the indicated time points. B. Morphology of established MRTK cell lines following xenograft transplantation in nude mice (at 30 d following tumor implantation; H&E; original magnification, \(\times 200\)). C. Analysis of hSNF5/INI1 mRNA expression in established cell lines using reverse transcription-PCR. Top, exon1-exon9; top middle, exon1-exon4; bottom middle, exon4-exon9; bottom, GAPDH as an internal control.
Moreover, tumors transplanted into immunodeficient mice showed a similar morphology in comparison with the primary tumor of MRTK (Fig. 1B), and cells in the transplanted tumor were round to polygonal in shape, with vesicular nuclei, prominent nucleoli, and eosinophilic cytoplasm with rare but typical cytoplasmic inclusions.

It is known that loss of function in the hSNF5/INI1 gene leads to MRT development. In an effort to determine whether the established cell lines were MRT cells, hSNF5/INI1 expression was examined by reverse transcription-PCR. As shown in Fig. 1C, hSNF5/INI1 mRNA transcripts were not detected in the established cell lines. Thus, these results suggest that JMU-RTK-1 and JMU-RTK-2 cells possess a loss of hSNF5/INI1 gene function.

Metastatic frequency of MRTK cells. Recent advances in luminescent imaging technologies have facilitated the quantitative analysis of cellular processes in vivo. JMU-RTK-1 and JMU-RTK-2 cells were transduced with firefly luciferase in an

![Fig. 2. Metastatic progression of MRTK cell lines in a xenogenic orthotopic transplantation model. A, representative luciferase images of luc-JMU-RTK-1 kidney tumor at day 30 following tumor implantation (left). Right top, ex vivo inspection of tumor-derived photons; right bottom, microscopic inspection of metastasis in the lungs (left; H&E; original magnification, ×100) and the lymph nodes (LN; right; H&E; original magnification, ×50). B, a representative metastatic frequency of luciferase-expressing MRTK cells. Luciferase-expressing MRTK cells were transplanted into the left kidney of SCID mice (3-5 mice per cell line). Tumor-derived photons were examined ex vivo 30 d following tumor implantation. One of two independent experiments with similar results. C, the survival rate in orthotopically tumor-implanted mice. *, P < 0.05 (JMU-RTK-1, JMU-RTK-1, and G401 versus SWT-1 and SWT-2), log-rank test.](image-url)
effort to visualize the fate of tumor progression in the living animals. The advantages associated with the use of luciferase as a marker includes its sensitivity (as few as 100 luciferase-transduced MRTK cells can be detected over the background in vitro) and its linear dose-dependent output of light in the presence of D-luciferin (data not shown). Cells (5 × 10^5 in 0.1 mL Matrigel) were orthotopically implanted into the left renal subcapsular space of C.B-17 SCID mice. Implanted cells rapidly grew at the left kidney, and strong photons were observed around day 7 posttumor implantation. Although the rapid growth in the primary site masked weak photo-signals from potential metastasized sites, metastatic sites at 30 days posttumor implantation were visualized by e x v i v o inspection in the presence of D-luciferin (Fig. 2A). Invasion to nearby organs was very high in JMU-RTK-1, JMU-RTK-2, and G401 cells. JMU-RTK-1 cells metastasized at the lung and lymph nodes, and JMU-RTK-2 and G401 cells preferentially metastasized in the lung, liver, and lymph nodes. The metastatic frequency and the direct invasion rate to nearby organs were low in SWT-1 and SWT-2 cells (Fig. 2B). These results reflected animal survival following orthotopic tumor injection (Fig. 2C). Thus, these results suggest that JMU-RTK-1, JMU-RTK-2, and G401 cells represent potentially aggressive types in MRTK.

**CD133 expression in MRTK cell lines and tumor-initiating capacity in NOD/SCID mice.** Recent evidence obtained following the investigation of brain tumors suggests that the frequency of tumor-initiating cells may be significantly correlated with aggressiveness (7). Although the origin in MRT remains unclear, the tumor appears as a result of unique neural differentiation and is distinct from neuroblastoma (23). Thus, the similarity between brain tumors and MRT allows us to determine whether the established cell lines frequently contain MRTK-initiating cells. In an effort to enrich MRTK-initiating cells, the cell surface antigen CD133 was analyzed in MRTK cell lines using a flow cytometer. As shown in Fig. 3, the relative abundance of CD133^+ cells was ~4% to 6% in aggressive MRTK cell lines. The CD133^+ cells were enriched to 13% to 25% using antibody-mediated magnetic bead sorting. This enrichment of the CD133^+
population did not always appear to be conserved in the examined cells.

The standard used to evaluate tumor-initiating activity maintains that the candidate cell populations should be able to initiate serially transplantable tumor development. Therefore, we determined and compared the tumor-initiating capacities of CD133+ MRTK cells in NOD/SCID mice (Table 1). As few as 1,000 CD133+ MRTK cells initiated tumor development by 21 days (60-100%), although the same number of CD133- MRTK cells could not form tumors (0%). Representative serial tumor development in JMU-RTK-1 and JMU-RTK-2 cells is shown in Fig. 4A. Tumor formation was microscopically confirmed in JMU-RTK-1 cells (Fig. 4B). Thus, these results show that MRTK-initiating cells are defined by CD133 expression and that a limited population of CD133+ MRTK-initiating cells can be maintained in culture conditions on plastic dishes. When injected at 1,000, and 10,000 cells, JMU-RTK-1 and JMU-RTK-2 cells formed tumors rapidly (Fig. 4C and D), suggesting that the tumor-initiating potential in JMU-RTK-1 and JMU-RTK-2 cells is higher than that in G401, SWT-1, and SWT-2 cells.

**CXCR4 expression in CD133+ MRTK-initiating cells.** Our data (see Fig. 2B) suggest that some MRTKs possess marked tendency for distant organ metastasis. Moreover, recent emerging evidence suggests a potential role of the chemokine receptor CXCR4 in tumor-initiating cells and tumor metastasis (9). Therefore, in an effort to determine the correlation between CD133+ MRTK-initiating cells and CXCR4 expression, Western blot analysis using anti-human CXCR4 was done before and after CD133 enrichment in the MRTK cell lines (Fig. 5A). Although unsorted cells expressed CXCR4, some of the cell lines showed increased CXCR4 expression following CD133+ enrichment. Furthermore, fluorescence-activated cell sorting analysis showed cell surface expression of CXCR4 (Fig. 5B), whereas CD133- cells showed moderate expression of CXCR4.

To determine the metastatic potential in CD133-enriched MRTK cells, orthotopic injection into the left kidney (1,000 cells) was done using CD133+ luciferase-expressing MRTK cells. Although substantial tumor-derived photons were observed at the injection site (data not shown), the resulting metastatic frequency was less correlated with CXCR4 expression levels (Fig. 5C). Thus, some CD133+ MRTK-initiating cells maintained their metastatic potential and the CD133+/CXCR4+ population appeared to play an auxiliary role in tumorigenicity and metastasis.

### Discussion

We showed that tumorigenic MRTK cells are included in a rare population that expresses CD133. The remarkable features presented in this study include the following: (a) the characteristic metastatic fate of MRTK cells in an orthotopic xenotransplantation model of SCID mice; (b) CD133+ MRTK cells may play an auxiliary role in tumorigenicity and metastasis.

MRTK cells represent a most progressive rare malignancy of infantile cancers with uncertain histogenesis (11, 12, 15). However, the precise mechanism by which MRTK cells progress is a major interest in cancer biology. Using recent in vivo luminescent technology (24, 25), luciferase-labeled MRTK cell lines were used to elucidate the metastatic fate in an orthotopic xenotransplantation model of NOD/SCID mice. Tumor-burdened mice also showed progressiveness in MRTK with distant organ metastasis observed, such as occurred in the liver and lung, in addition to direct invasion of some nearby organs. Thus, these genetically modified transplantable cell lines that were established should provide a useful animal model for the future therapeutic treatment of refractory MRTK.

CD133 was initially described as a surface antigen (a transmembrane pentaspan protein) specific to hematopoietic stem cells (26, 27). Although the biological function of CD133 remains unknown, CD133 is recognized as a stem cell marker for normal and cancerous tissues. In a number of recent studies, monoclonal antibodies against CD133 have been used for the identification and isolation of a putative cancer stem cell population from malignant tumors of brain (6), colon (7, 8), pancreas (9), prostate (10), liver (28, 29), and lung (30). However, our data from the investigation of MRTK cells showed that CD133 expression remains transient and rare even following enrichment using CD133 mAbs. As shown for normal hematopoietic and endothelial progenitors, CD133 expression is limited in early progenitors and usually not detected upon differentiation (26, 27). The present data are consistent with the findings from normal and cancer stem cells as CD133+ subpopulations are exclusively tumorigenic, possess self-renewal capacity, and can differentiate into CD133- transit-amplifying tumor cells.

Shmelkov et al. (31) also reported that CD133 might not be a suitable marker for colon cancer stem cells. This was elegantly shown using transgenic mice expressing LacZ under control of the CD133 promoter. At least in MRTK, CD133+...
cells bore definite tumorigenicity and our data suggest that CD133\(^{-}\) cells may cooperate with CD133\(^{+}\) MRTK cells in terms of tumorigenicity. In human cancers, much evidence has accumulated to provide the consensus that CD133 is a reliable stem cell marker in human cancer specimens. With regard to CD133 expression, it has been shown that seven CD133 mRNA isoforms are controlled by five alternative promoters in a tissue/organ–dependent manner (32). This rather complicated CD133 gene expression appears to require short-term CD133 expression to retain the organ-specific stem cells. Therefore, further analysis of CD133 transcriptional regulation may provide clues as to how cancer stem cells are regulated between self-renewal and differentiation.

Recent data suggest that the interaction between chemokines and their receptors are also critical components in the regulation of tumor progression and metastasis in many cancer types (33, 34) and that the CXCR4/SDF-1 pathway is involved in the metastatic process of melanoma (17, 34), glioblastoma (35), and colon (36) and pancreatic (37) carcinomas. In fact, clinical studies investigating poor patient prognosis and CXCR4 expression in tumor cells showed a significant correlation for some of the aforementioned malignancies (38–40). Moreover, in the case of pancreatic carcinomas, Hermman et al. (9) showed an important correlation between CXCR4 and CD133 in cancer stem cells, in that a subpopulation of migrating CXCR4\(^{+}\)CD133\(^{-}\) cells is essential for tumor metastasis. Our data (Fig. 5) showed that both CD133\(^{+}\) and CD133\(^{-}\) populations showed CXCR4 expression, thus not entirely consistent with the above report. Our data suggest rather that CXCR4\(^{+}\)CD133\(^{-}\) cells may also contribute to metastatic tumor growth. In this regard, Dalerba and Clarke (41) recently proposed possible models for metastasis-promoting cancer cells, in which CXCR4\(^{+}\) cancer cells act in an auxiliary manner (not unlike the role of macrophages in several tumor model systems; refs. 42, 43). Our data appear to support this proposal. Furthermore, Burns et al. (44) recently reported that an alternate receptor, CXCR7 (RDC1), is expressed in some tumor cells and binds with high affinity to SDF-1. Unlike many other chemokine receptors, ligand activation of CXCR7 does not sufficiently induce migration in tumor cell lines (44) but provides cells with a growth and survival advantage (44–46). Therefore, we speculate that other chemokine receptors may also play a role in the growth and survival advantage of MRTK cells.

Given that MRTK is a progressive malignancy with a poor prognosis and which resists many chemotherapeutic regimens, a new treatment modality to control local disease and prevent systemic progression is required. Recently, it was shown that some MRTK cells express HER-2 and are sensitive to anti-HER-2 humanized mAb upon antibody-dependent cell-mediated cytotoxicity (47). Moreover, it has been shown that tumor formation from human melanoma-initiating cells is inhibited by anti-ABC5 mAb through antibody-dependent cell-mediated cytotoxicity (48).

**Fig. 4.** Tumor growth of CD133\(^{+}\) MRTK enriched cells in NOD/SCID mice. 
A, representative images of JMU-RTK-1 and JMU-RTK-2 tumors at 9 wk following CD133-enriched cell implantation in NOD/SCID mice. CD133-enriched cells (green dashed, \(1 \times 10^{4}\), and green dotted, \(1 \times 10^{3}\)) were implanted s.c. into the left back of the mice. Notably, CD133-negative cells did not form tumors even with \(1 \times 10^{5}\) cells on the right back. B, microscopic inspection of CD133-mediated cell proliferation (\(1 \times 10^{5}\) JMU-RTK-1 cells). A similar cell morphology is shown in Fig. 1B. C, JMU-RTK-1 and JMU-RTK-2 cells (\(1 \times 10^{6}\)) following CD133-mediated enrichment were transplanted into the s.c. space of NOD/SCID mice and tumor growth was measured at the indicated time points. D, various MRTK cells (\(1 \times 10^{6}\)) following CD133-mediated enrichment in the s.c. space of NOD/SCID mice and tumor growth was measured at the indicated time points. \(* P<0.05\) (JMU-RTK-1 and JMU-RTK-2, vs G401, SWT-1, and SWT-2), Tukey-Kramer test.
Although present therapeutic strategies provide only limited effectiveness against refractory malignancy, the aforementioned evidence suggests that the use of humanized specific antibodies against cancer-initiating cells should provide effective targeting. Although the number of MRTK patients in the present study was very small, our results regarding the identification and characteristics of MRTK-initiating cells provide important implications for the future design of aggressive MRTK therapies.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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