The cancer stem cell hypothesis posits that tumor growth is driven by a rare subpopulation of cells, designated cancer stem cells (CSC). Studies supporting this theory are based in large part on xenotransplantation experiments wherein human cancer cells are grown in immunocompromised mice and only CSC, often constituting less than 1% of the malignancy, generate tumors. Herein, we show that all colonies derived from randomly chosen single cells in mouse lung and breast cancer cell lines form tumors following allografting histocompatible mice. Our study suggests that the majority of malignant cells rather than CSC can sustain tumors and that the cancer stem cell theory must be reevaluated.

Keywords: breast cancer cells, cancer stem cells, lung cancer cells, single cell originated cells, tumor growth

INTRODUCTION
The fact that solid tumors and leukemias have been known for many years to be composed of a heterogeneous population of neoplastic cells has raised questions about whether all cell types comprising the malignancy sustain the cancer or only a select subpopulation. The possibility that cancers may be organized by a hierarchy of arranged classes of malignant cells wherein tumor growth is driven by a rare population of select cells, designated cancer stem cells (CSC), was suggested many years ago (see Dick, 2003 and references therein). Indeed, evidence has accumulated in recent years that CSC, which often represent a minor portion (frequently 1% or less) of the tumor, support its growth. Studies that initially documented the CSC theory were carried out with acute myelogeneous leukemia (Bonnet and Dick, 1997). In addition to hematological studies (Bonnet et al., 1997; Hope et al., 2004), findings with solid tumors, e.g., breast (Al-Hajj et al., 2003; Polyak, 2007; Stingl and Caldas, 2007), brain (Singh et al., 2004a; 2004b), colon (O’Brien et al., 2007), prostate (Wang et al., 2006) and lung cancers (Jordan, 2004), and possibly melanoma (Grichnik, 2006) (see also reviews by Clarke et al., 2006, Jordan, 2004; Massard et al., 2006; Stingl and Caldas, 2007; Yang, 2007), have all been found to be sustained by a minority of malignant cells with a capacity for self-renewal. These studies, and in particular those involving solid tumors, do not exist without controversy, however, as they are based in large part on xenotransplantation assays in which human tumor cells were grown in immunocompromised mice. The possibility exists that CSC preferentially or even exclusively adapt and proliferate when grown in a foreign species. Whether tumors are driven by a small subset or the majority of tumor cells was recently examined by allografting titrated amounts of a few to several 1000 murine lymphoid and myeloid tumor cells into histocompatible mice (Kelly et al., 2007). In this study, a relatively large fraction of recipients of the malignant cells developed tumors and the phenotypes of transplanted cancers were similar to the corresponding primary ones. Yet, this study was itself challenged with an argument that the frequency of the cells sustaining the tumor (e.g., CSC) is not important (Kennedy et al., 2007). Thus, further studies are required to resolve the CSC hypothesis. Herein, we have examined the validity of the CSC theory by isolating single cells from two different malignant cell lines (mouse lung and breast cancer cells) and
shown that they produce tumors following allografting histocompatible mice. In light of our findings and those of Kelly et al. (2007), we suggest that the CSC theory should be reexamined.

MATERIALS AND METHODS

Cell cultures and isolation of single cells
Mouse liver normal cell (BNL CL.2), lung cancer cell (LLC1) and breast cancer cell (EMT6) lines were purchased from American Type Culture Collection. BNL CL.2 and LLC1 were grown in DMEM supplemented with 10% fetal bovine serum and antibiotic-antimycotic solution (from Invitrogen Life Technologies) and EMT6 in Waymouth's MB 752/1 Medium with 2mM L-glutamine supplemented with 15% fetal bovine serum and antibiotic-antimycotic solution. Each cell line was cultured at 37°C in a humidified incubator and 5% CO₂. To establish single cell populations, cells were harvested, counted using the trypan blue exclusion method and cell suspensions prepared by serial dilution yielding suspensions of 10 cells/200 μl, 5 cells/200 μl, 1 cell/200 μl and 0.5 cell/200 μl. Each cell suspension was seeded in a 96 cell plate and incubated overnight. Only the wells containing one cell, as visualized with an inverted phase contrast microscope, were retained and inspected daily for 10 d to be sure that all single cells survived and expanded their populations. Every single, originally selected cell amplified the cell numbers and they were designated BNLCLSP-1, -2, etc, LLCSP-1, -2, etc, or EMTSP-1, -2, etc as shown in Table 1.

Tumor formation assay
LLC1 form tumors when injected into C57BL/6 mice and EMT6 form tumors when injected into BALB/c mice (Bertram and Janik, 1980; Rockwell et al., 1972). To monitor tumor formation capability of each single cell originated population, 2 × 10⁵ cells growing in the log phase were subcutaneously injected into the flanks of 5-week-old female mice and the mice were examined every 2 days for tumor formation. LLCSP cell lines and parental cells were injected into C57BL/6 mice and EMTSP and BNLCLSP cell lines and
the corresponding parental cells into BALB/c mice. After 3 weeks following injection, mice were euthanized, tumors removed and analyzed as described (Yoo et al., 2006). Animal care was in accordance with the National Institutes of Health institutional guidelines under the expert direction of Dr. Kyle Stump (NCI, NIH, Bethesda, MD).

RESULTS

We tested the CSC hypothesis by allografting non-immunocompromised mice with a colony of cells each derived from a single cell randomly taken from two different mouse cell lines, lung cancer (LLC1) and breast cancer (EMT6) cells, as described in material and methods and in the legend to Table 1. Wells containing one cell, as visualized in an inverted phase contrast microscope, were selected and examined daily over a 10 day period to ensure that all single cells survived and expanded their populations. Indeed, all single cell isolates amplified their cell numbers and the resulting populations were designated BNLCLSP-1, -2, etc, LLCSP-1, -2, etc, or EMTSP-1, -2, etc. We injected $2 \times 10^5$ cells growing in log phase into the flank of mice and the mice were monitored for tumor formation over the ensuing three weeks (Table 1). BNL CL.2 cells, which are non-malignant mouse liver cells, were used as the control cell line. None of the isolates derived from normal cells produced tumors. All isolates derived from the two malignant cell lines, however, gave rise to tumors after three weeks. Only one isolate from each cell line, LLCSP10 and EMTSP10, failed to generate tumors from every injection, i.e., LLCSP10 produced a tumor in one of four injections and EMTSP10 produced four tumors from five injections. Tumor sizes varied considerably from different isolates as well as within multiple injections from single isolates, although variations appeared to be less pronounced in tumors produced from the EMT6 cell line. The important point to emphasize, however, is that all isolates from both malignant cell lines produced tumors.

Information on cell surface marker phenotypes in mouse malignant cell lines is largely lacking compared to those in human malignant cell lines. As Sca-1 has been identified as a marker in mouse hematopoietic stem cells (e.g., see Kelly et al., 2007), we examined the expression of this cell surface marker in the parent and single cell isolates of
EMT6 (Supplemental Fig. 1) and LLC1 (data not shown) cell lines. The Sca-1 surface cell marker was expressed in both parental cells and in each of the populations generated from single cell isolates. Thus, single cell isolates produce similar populations as parent cells.

DISCUSSION AND CONCLUSIONS

The data in Table 1 argue against the idea that tumors are maintained by rare cancer stem cells. Although it can be argued that all malignant cells making up a tumor have the capacity to “dedifferentiate” to cancer stem cells which in turn drive the tumor (and the generation of the small percentage of Sca-1+ cells arising within the populations derived from single cell isolates (see Supplemental Fig. 1) support this argument), the capability of virtually every malignant cell to develop into hematopoietic stem-like cells is contrary to the cancer stem cell theory. Two distinct advantages of our study and that of Kelly et al. (2007) are that the mice were not immunocompromised and the results were derived by allografting rather than xenotransplantation of immunocompromised mice. An additional unique feature in our study is the use of random single-cell derived cultures. The fact that all independent single cell isolates from two different malignant cell lines produced tumors (Table 1) strongly suggests that most cells within a tumor cell line retain or regain the capacity to escape immune surveillance and sustain the malignancy. The low incidence of cancer stem cells in such malignancies as human acute myeloid leukemia (Bonnet and Dick, 1997) and colon cancer (Massard et al., 2006; O’Brien et al., 2007), yet the capability of only these rare cells to reproduce the respective malignancy following xenotransplantation into immunocompromised mice, suggest that such xenografting experiments must be reevaluated.

It should be noted that the tumor cells used in our study were cultured in vitro and the properties governing malignancy may be very different in cancer cells grown in vitro than those governing cancer cells within the animal. Tumor cells cultured in vitro are known to have different phenotypic and genotypic characteristics, but whether these differences have any role in sustaining malignancy is not known (Lee et al., 2006). It is possible, however, that malignant cells cultured in vitro quickly dedifferentiate and a minor CSC
population rapidly develops that is responsible for generating and driving the resulting tumors, whereas the majority of malignant cells constituting a cancer in vivo may not dedifferentiate and the cancer is sustained by a minor CSC population. Unfortunately, this possibility cannot be readily tested in our tumor model without developing more sophisticated techniques than are presently available. Importantly, the fact that Kelly et al. (2007) used mouse lymphoma and leukemia cells that had not been cultured in vitro to induce the corresponding tumors in allografted histocompatible mice suggests that the properties governing malignancy in cultured and non-cultured tumor cells may not be a factor in our and their studies. The approaches and results provided in the present and Kelly et al. (2007) studies have raised sufficient doubt in the CSC theory to challenge its validity. Furthermore, we maintain that this theory must be reevaluated, not only because it may not be correct, but also because tumor recurrence and metastasis are major concerns in cancer therapy and many of the cancer therapies currently being developed (e.g., see Huff et al., 2006; Massard et al., 2006) exclude alternative approaches. The development of therapies that target certain minor classes of cancer cells is highly significant, however, as the specific loss of such cell classes can help us better evaluate their role(s) in sustaining malignancy. In addition, therapies that eradicate solely the minor population of purported CSC should resolve any questions regarding the validity of the CSC hypothesis.

ACKNOWLEDGMENTS

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REFERENCES


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Table 1. Tumor formation derived from single cell populations.\(^1\)

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<th>LLCSP1</th>
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<th>LLCSP4</th>
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<td>0.419</td>
<td>0.706</td>
<td>0.727</td>
<td>0.725</td>
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<td>0.149</td>
<td>0.171</td>
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\(^1\)Each cell population was generated from a single cell, the resulting cells injected into the flanks of mice and the number of mice developing tumors per number of mice injected are shown in the second horizontal column from the top and the weights of tumors in g formed in mice (see Yoo et al., 2006 and “Materials and Methods” for details) are shown in the lower portion of the table.