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Communication

Molecules and Cells

Every Single Cell Clones from Cancer Cell Lines Growing Tumors *In Vivo* May Not Invalidate the Cancer Stem Cell Concept

Fengzhi Li*

We present the result of our research on the tumorigenic ability of single cell clones isolated from an aggressive murine breast cancer cell line in a matched allografting mouse model. Tumor formation is basically dependent on the cell numbers injected per location. We argue that *in vivo* tumor formation from single cell clones, isolated *in vitro* from cancer cell lines, may not provide conclusive evidence to disprove the cancer stem cell (CSC) theory without additional data.

INTRODUCTION

Many studies suggest that a distinct subpopulation of cells designated "cancer stem cells (CSC)" maintain diverse solid tumors, and direct evidence supporting the CSC theory has recently emerged from mouse models of epithelial tumorigenesis (Visvader and Lindeman, 2008). However, we have read with great interest a recent publication entitled "The Cancer Stem Cell Theory: Is It Correct?" from the journal of "Mol. Cells" (Yoo and Hatfield, 2008). In this paper, the authors demonstrated that all single cancer cell clones randomly selected grew a tumor mass *in vivo*. These authors therefore concluded that this result seriously challenges the current CSC theory and the theory must be reevaluated.

The authors stated in their paper that the CSC theory is largely based on studies from xenografting human cancer cells in immune-compromised mice, but that this may not be an appropriate experimental system for testing the CSC theory. Instead, cancer cells allografting on histocompatible mice may be a better model system to test the CSC theory. Based on their observation, 10 cancer cell colonies derived from single cells of murine lung or murine breast cancer cell lines formed tumors in their allografting mouse model. While their observation is very interesting, in our opinion this result cannot challenge or disprove the current CSC theory without additional data. Our studies indicate that the percentage of cell clones obtained from cancer cell lines at the one-cell-per-well condition varies strikingly. It is far less than 100% among cancer cell lines. Moreover, it is positively associated with the *in vivo* tumor formation aggressiveness of the cell line used. Our allografting mouse model results further revealed that tumor formation from single cell clones is basically dependent on the number of cancer cells injected.

MATERIALS AND METHODS

In vitro colony formation from individual cells

The following cancer cell lines were used in testing single cell colony formation: MCF-7 and MDA-MB-231 breast cancer cell lines, A2008 ovarian cancer cell line, PC-3 prostate cancer cell line, H1650 lung cancer cell line, and E0771 murine breast cancer cell line. Cells were seeded in 96 well plates at a density of 1 cell/well, 0.5 cell/well and 0.25 cell/well in complete growth medium with 10% FBS. Twenty-four hours later, wells with a single cell were marked under a microscope. Cell colony formation in the marked wells was observed daily under the microscope for proliferation.

In vivo tumor formation from single cell-generated colonies

An allografting C57BL/6 mouse model was used to test tumor formation using cell clones generated from individual cells of the E0771 murine breast cancer cell line with a C57BL/6 background (Ewens et al., 2006). Cells generated from a single E0771 cell in a DMEM cell cultural medium at 10^5/0.2 ml and 10^6/0.2 ml were subcutaneously injected into the left and right flanks of mice, respectively. Tumor formation was monitored every seven days for six weeks.

RESULTS AND DISCUSSION

Single cell clone formation is far less than 100%

In the recent study entitled "The Cancer Stem Cell Theory: Is It Correct?" (Yoo and Hatfield, 2008), its authors claimed that "all single cell isolates amplified their cell numbers and resulting populations …" However, the experiments performed in our laboratory indicate that clone formation from a single cancer cell in each well isolated from a cancer cell line varies strikingly among different cancer cell types and cell lines, and is far less

Departments of Pharmacology and Therapeutics, Roswell Park Cancer Institute, Buffalo, New York 14263, USA
*Correspondence: fengzhi.li@roswellpark.org

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Table 1. *In vivo* tumor formation from single cancer cell clones on allografting model

E0771 cell clone number	Cell numbers injected	Clone number shows tumor growth	Clone number shows no tumor growth
13 single cell clones	10^5	0	13
	10^6	11	2

than 100%. The percentage of cells that could form a cell clone from a single cell per well, in our experiments, is positively associated with the aggressiveness of tumor formation *in vivo* for the corresponding cancer cell line. It could be that CSCs and their close progenies may have advantages to form single cell clones *in vitro* at the condition of one cell per well, and this could be involved in a process of dedifferentiation of cells from cancer cell lines in such an *in vitro* condition.

In vivo tumor formation from single cell clones is dependent on cell numbers injected

In order to apply the favorable allografting mouse model recommended in the recent report (Yoo and Hatfield, 2008), we used a very aggressive murine breast cancer cell line (E0771) with a C57BL/6 background (Ewens et al., 2006) and isolated 13 single cell clones from the one-cell-per-well condition. The result obtained from allografting the 13 single cell clones on C57BL/6 mice is interesting: None of the 13 cell clones grew tumors when injected subcutaneously with 10^5 cells per site under the flanks of mice. In contrast, 11 out of 13 cell clones grew tumors when 10^6 cells were subcutaneously injected into the flanks of mice. One clone never grew a tumor and the other one transiently grew a tumor that disappeared at week 4 during the 6-week experimental period (Table 1).

Based on these observations, as well as what is suggested by the literature (Danovi, 2009; Quintana et al., 2008), it is likely that most cancer cell lines that are capable of forming tumors *in vivo* may have a substantially higher percentage of cells that possess tumorigenic properties. In part, the reason may be that established cancer cell lines are vulnerable to dedifferentiation *in vitro* in certain situations such as in a one-cell-per-well condition particularly. However, the formation of tumor from a single cell clone *in vivo* is highly dependent on the number of cells injected for most cancer cell lines. There may be exceptional examples. It was reported that most (if not all) cells from the C6 glioma cell line are cancer stem cells (Zheng et al., 2007). Thus, cell type differences may account for different observations, and the notion that cancer stem cells only represent a very small portion of cells in the entire cell population may not be a universal phenomenon for all types of cancers, at least for cancer cell lines. Additionally, it has been shown that different immunocompromised mouse models could significantly affect human CSC tumor formation (Quintana et al., 2008). Therefore, selection of a highly immunocompromised mouse model for studies of human CSCs should be considered. The profile of tumor formation from the 13 single cell clones from E0771 cells at the condition of 10^6 cells per injection on the allograft model is presented in Fig. 1. Given that tumor formation is heavily dependent on the number of cells injected in our experiments, it is thought that the *in vivo* tumor formation from single cell clones isolated *in vitro* from a cancer cell line should not be a conclusive evidence to disprove the CSC theory. Additionally, we think that a cancer cell's metastatic ability to initiate tumor growth at a new microenvironmental site might be considered as another important characteristic for these types of cells.

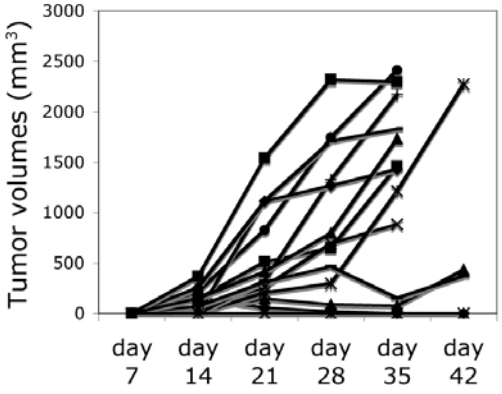

Fig. 1. Tumor formation from individual E0771 cell clones on an allografting C57BL/6 mouse model (see the "Materials and Methods" section for detail).

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