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Commentary

The concept of the so-called cancer stem cell (CSC) holds that only a minority of cells within a tumor have the ability to generate a new tumor. Over the last decade, a large body of literature has implicated the protein CD133 as a marker of organ-specific adult stem cells and in some cancers as a bona fide CSC marker. In this issue of the *JCI*, Shmelkov et al. challenge the view that CD133 is a marker of CSCs in colon cancer (see the related article beginning on page 2111). CD133 was thought previously to have a very restricted distribution within tissues; the authors have used genetic knock-in models to demonstrate that CD133 in fact is expressed on a wide range of differentiated epithelial cells in adult mouse tissues and on spontaneous primary colon tumors in mice. In primary human colon tumors, all of the epithelial cells also expressed CD133, whereas metastatic colon cancers isolated from liver had distinct $CD133^+$ and $CD133^-$ epithelial populations. Intriguingly, the authors demonstrate that the $CD133^+$ and $CD133^-$ populations were equally capable of tumor initiation in xenografts. In light of these new findings, the popular notion that CD133 is a marker of colon CSCs may need to be revised.

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Is CD133 a marker of metastatic colon cancer stem cells?

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The concept of the so-called cancer stem cell (CSC) holds that only a minority of cells within a tumor have the ability to generate a new tumor. Over the last decade, a large body of literature has implicated the protein CD133 as a marker of organ-specific adult stem cells and in some cancers as a bona fide CSC marker. In this issue of the *JCI*, Shmelkov et al. challenge the view that CD133 is a marker of CSCs in colon cancer (see the related article beginning on page 2111). CD133 was thought previously to have a very restricted distribution within tissues; the authors have used genetic knock-in models to demonstrate that CD133 in fact is expressed on a wide range of differentiated epithelial cells in adult mouse tissues and on spontaneous primary colon tumors in mice. In primary human colon tumors, all of the epithelial cells also expressed CD133, whereas metastatic colon cancers isolated from liver had distinct $CD133^+$ and $CD133^-$ epithelial populations. Intriguingly, the authors demonstrate that the $CD133^+$ and $CD133^-$ populations were equally capable of tumor initiation in xenografts. In light of these new findings, the popular notion that CD133 is a marker of colon CSCs may need to be revised.

Until now, there has been little controversy over whether the protein CD133 is a marker of cancer stem cells (CSCs). Originally described as a marker of normal hematopoietic stem cells (1, 2), it has gained more prominence as a marker of CSCs in solid primary tumors such as medulloblastomas and glioblastomas (3, 4) and subsequently of CSCs in a growing number of cancers of epithelial tissues. In their study in this issue of the *JCI*, Shmelkov et al. challenge this increasingly influential dogma; first, on the basis of CD133's wide distribution in many epithelial tissues, and second, because CD133 expression does not necessarily correlate with the ability of colon tumors to metastasize (Figure 1) (5).

Nonstandard abbreviations used: CSC, cancer stem cell; EpCAM, epithelial cell adhesion molecule.

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Normal distribution of CD133 in vivo

CD133 (also known as prominin-1) is a surface protein with five transmembrane domains. Still mysterious in its function, this pentaspan has drawn a lot of attention since its discovery in 1997 on normal human hematopoietic stem cells (1, 2). The simultaneous discovery of the mouse homolog and its implications in neurogenesis opened up new directions for studying human neural stem cells (6, 7); CD133 has been linked in several reports to multiple organ-specific stem cells and referred to as "the molecule of the moment" (8).

Prior to the current report by Shmelkov et al. in this issue of the *JCI* (5), studies of CD133 as a normal stem cell marker or a CSC marker have used primarily one monoclonal antibody against CD133, the clone known as AC133, which marks an epitope of CD133 at the cell surface (1, 2). Using a single monoclonal antibody to define a stem cell marker is usually not sufficient, and it is not clear to us why such a practice was so widely accepted. The current study by Shmelkov et al. is, to our knowledge, the first that uses a knock-in reporter mouse to track expression of CD133, both temporally and spatially, in normal tissues and during tumorigenesis in vivo. In their previous studies, Shmelkov et al. dissected the regulatory region of the human *CD133* gene, demonstrating that CD133 expression was regulated in a tissue-specific manner by multiple alternative promoters (9). Importantly, they identified similarities among the mouse and human regulatory regions of *CD133*, which paved the way for the design of their CD133 reporter

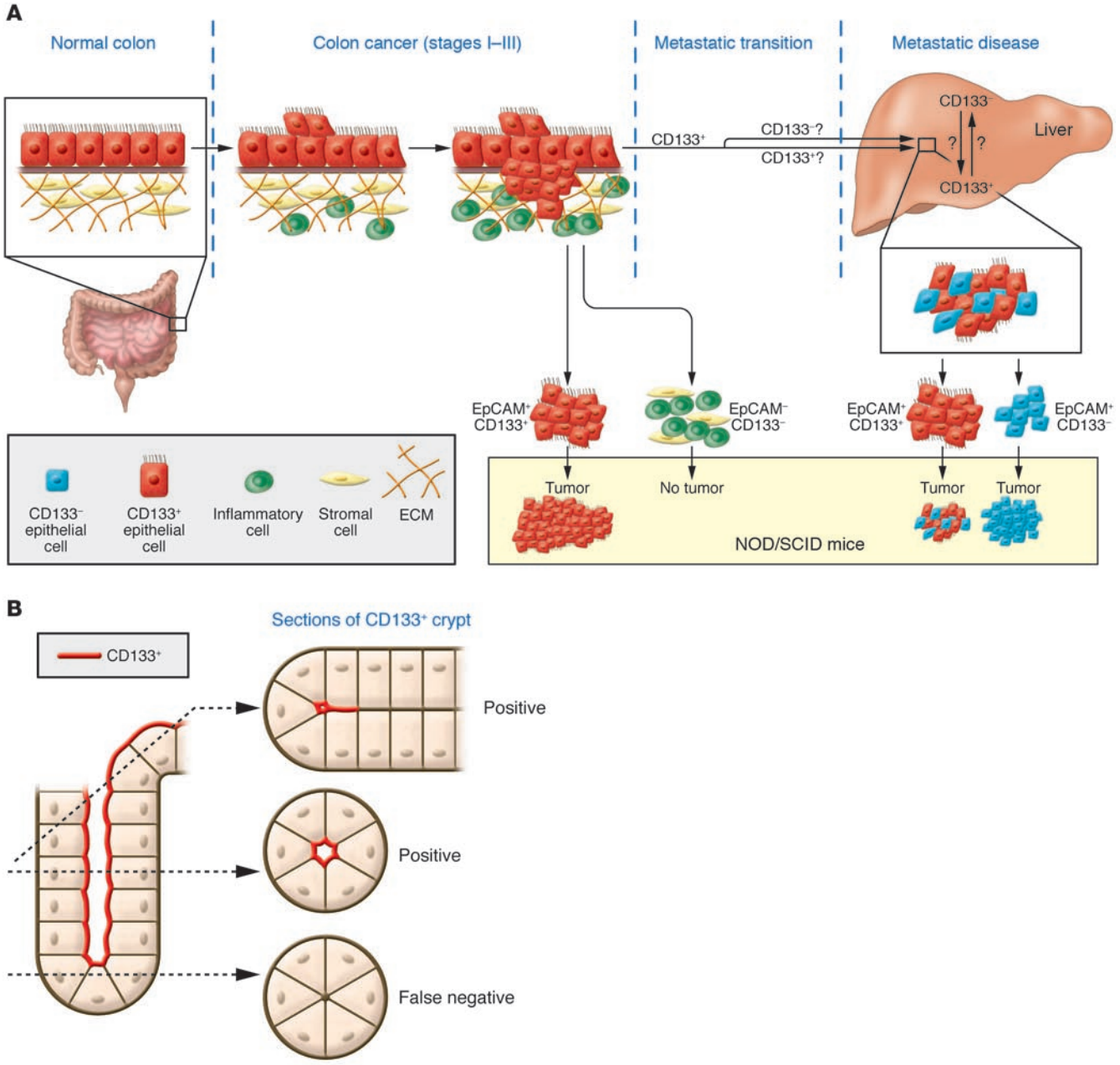

Figure 1

A new view of CD133 expression as it relates to normal colon epithelium and colon cancer stem cells. (**A**) In normal colon, all epithelial cells, identifiable by their expression of EpCAM, also express CD133. In primary tumors, malignant epithelium is EpCAM$^+$CD133$^+$, whereas stromal and inflammatory cells are the only EpCAM$^-$CD133$^-$ cells present, in addition to the extracellular matrix. Thus, the CD133$^-$ cell fraction does not contain cancerous epithelial cells, and therefore, not surprisingly, CD133$^-$ cells do not form tumors in NOD/SCID mice. Only once the disease has progressed to metastasis does a fraction of the EpCAM$^+$CD133$^-$ cells appear. During this progression, the precise point during which this subpopulation of EpCAM$^+$CD133$^-$ cells emerges is unknown. However, in their study in this issue of the *JCI*, Shmelkov et al. (5) demonstrate in liver metastases that both CD133$^+$ and CD133$^-$ populations of EpCAM$^+$ colon cancer epithelial cells are capable of forming tumors in NOD/SCID mice. Interestingly, the CD133$^-$ tumors grow more aggressively than the CD133$^+$ tumors. (**B**) Shmelkov et al. (5) suggest that the angle and location of colon tissue sections may confound the analysis of CD133 expression in vivo. CD133 protein is localized to the apical surface of the epithelial cells. Depending on the orientation of crypts in the section, CD133 expression could be underestimated if the luminal surface of cells is not exposed, resulting in false-negative staining for CD133.

mouse. In their latest study, they created a mouse with a knock-in *lacZ* reporter gene (which expresses β-galactosidase) controlled by all endogenous CD133 promoters and thus provides a far more accurate representation of CD133 tissue-specific gene expression compared with AC133 monoclonal antibody staining alone (5).

Using this genetic mouse model, Shmelkov et al. show in their current study that CD133 expression is widely distributed in the luminal layer of a number of epithelial

tissues throughout the body, a surprising finding (5). But could the authors claim that *lacZ* expression precisely reflects the expression pattern of the endogenous CD133 protein? This approach also has some potential caveats: (a) the lacZ reporter reflects the transcriptional activity of the *CD133* gene and not the expression of the CD133 protein; and (b) considering the high proliferative activity of epithelial tissues, it is possible that, due to its half-life, β-galactosidase activity could still be found in cells after the expression of CD133 was turned off. The authors, however, foresaw these caveats and addressed them with confirmatory CD133 antibody staining in their mice to substantiate the data obtained with the *lacZ* reporter. Furthermore, in adult human colon, Shmelkov et al. used the AC133 antibody to demonstrate a pattern of CD133 expression that paralleled that observed in their mouse studies. Other recent reports also have shown that CD133 is expressed on mature epithelium of pancreatic ducts in humans (10), in the proximal tubules of the kidney, and the lactiferous ducts of the mammary gland (11). Based on these findings, one could conclude that CD133 expression in humans likely reflects that in mice, and it is suggested that CD133 demarcates differentiated epithelium in these organs.

Previous reports observed that only a few cells in the colon are $CD133^+$ (12, 13), whereas Shmelkov et al. (5) demonstrate that all luminal epithelial cells in the colon express CD133 in mice and humans. They contend that the cellular localization of CD133 is on the protrusions of cell membranes (6). Thus, on the epithelial lining of many organs, CD133 could be positioned on the apical surface, at the border between the plasma membrane and the lumen, perhaps complicating the interpretation of results, as it could be mistakenly deemed to be an artifact of rim staining. Additionally, it seems that careful handling of tissues was necessary to preserve the intact brush borders, and it may be essential to examine the different areas of the tissue to make certain that the luminal surface is included in the analysis (Figure 1). However, a couple of questions remain unanswered. Different antibody clones often recognize different epitopes on the same molecule. Epitopes can arise following posttranslational modifications, such as glycosylation of residues, which can occur only under certain conditions or in certain cell types. Is the expression of the glycosylation-dependent AC133 epitope limited to rare stem cells? Are there any $CD133^+$ cells that lack the AC133 epitope?

Is CD133 a marker of CSCs in colon?

The wide distribution of CD133 among epithelial cells, as shown by Shmelkov et al. (5) using the knock-in reporter mouse strain, raised the question of whether CD133 should be used as a marker of colon CSCs. This is a question of extreme importance, as a major goal that follows identification of CSCs is elucidating unique markers that will facilitate their therapeutic targeting. Therefore, a thorough characterization of putative CSC populations relative to their normal surrounding tissue will be vital for the success of CSC-directed therapy. Our conclusion, based on the data presented in the current study by Shmelkov et al. and in two other recent reports in *Nature* (12, 13), is: (a) should a difference in CD133 expression exist between a colon CSC and a non-CSC, it would not be the presence or absence of CD133 but its relative abundance that is important; and (b) the distribution of CD133 can change profoundly among cells of primary colon tumors versus metastatic colon tumors; therefore, its functional significance may also change in different contexts.

The first report that linked CD133 expression to CSCs was published less than five years ago (3, 4). The $CD133^+$, but not $CD133^-$, cells in glioma were shown to be tumorigenic in NOD/SCID mice. Since then, many more studies proposed CD133 as a marker of tumor-initiating cells in organs such as prostate (14), liver (15), pancreas (16), and lung (17). The two studies reporting that CD133 was a marker of colon CSCs used fluorescence-activated cell sorting (FACS) to analyze CD133 expression among cells in primary tumors, which were sorted and then functionally tested for their tumor-forming ability in xenografts (12, 13). Conversely, using confocal microscopy, Shmelkov et al. report in their current study (5) that in primary colon cancer samples from humans and mice, the expression of CD133 was detected on all epithelial cells in the malignant tissue (using epithelial cell adhesion molecule [EpCAM] as a marker of epithelial cells) and that CD133 expression was excluded from the non–epithelial cell components of the tumor. The authors assume that the tumorigenic capability of the tumor cannot be contained within the stromal, inflammatory, or vascular cells. Thus, they propose that the inability of $CD133^-$ cells to initiate tumors could be simply explained by the finding that there are no $CD133^-$ epithelial cells detected in primary colon tumors.

Shmelkov et al. (5) extrapolate their findings in primary human tumors to the expression of CD133 in a mouse model of spontaneous colon cancer. They bred CD133 reporter mice with IL-10–knockout mice, the offspring of which are predisposed to colon tumorigenesis as a result of chronic inflammation. The authors demonstrated that in the murine, as in the human, primary colon tumors, the malignant epithelial cells were $CD133^+$, and the $CD133^-$ subset was represented by hematopoietic, endothelial, and stromal cells. Thus, their observations in human tumors importantly mirror those observed in spontaneous mouse colon tumors.

Perhaps the most novel and compelling finding presented by Shmelkov et al. (5) is the discovery that both the $CD133^+$ and the $CD133^-$ malignant epithelial cells ($EpCAM^+$) from metastases could form tumors in mice. The authors also analyzed patient samples and observed a significant population of $CD133^-EpCAM^+$ cells, in addition to a $CD133^+EpCAM^+$ cell population, in colon tumors that had metastasized to the liver. Following separation into $CD133^+$ and $CD133^-$ populations and serial transplantation studies in NOD/SCID mice, the authors demonstrated that both $CD133^+$ and $CD133^-$ subsets were capable of tumor initiation (Figure 1A). The implantable $CD133^-$ metastatic tumor cell subpopulation behaved more aggressively and had a faster growth rate than the $CD133^+$ subset. Intriguingly, only the $CD133^+$ metastatic cells generated subsequent tumors, which contained a $CD133^+$ population in addition to $CD133^-$ cells, whereas the $CD133^-$ metastatic cells generated only $CD133^-$ tumors. The authors hypothesize that $CD133^-$ cells derive from $CD133^+$ cells in the process of tumor progression and suggest that the emergence of $CD133^-$ cells results from downregulation of the molecules specific for the mature, differentiated epithelium, consistent with early signs of epithelial-mesenchymal transition. Accordingly, the lack of differentiation markers makes these cells less mature and possibly more aggressive than the $CD133^+$ cells.

On the surface, there appears to be a discrepancy between the current findings reported by Shmelkov et al. (5) and those of O'Brien et al. (12) and Ricci-Vitiani et al.

(13) reported in *Nature* in 2007. It is possible, however, that a distinction may exist between CSCs of primary tumors and those that form metastases. Unfortunately, the current data do not allow a determination of whether such a distinction exists, because the techniques employed in the published studies are quite diverse. Because Shmelkov et al. examined the primary tumors microscopically, and did not functionally assess tumor-initiating activity as had been done previously (12, 13), one could argue that their conclusion that CD133 expression does not segregate with the CSCs in primary colon tumors is not yet fully proven. Nevertheless, upon retrospective assessment of the FACS plots in the two previous colon cancer studies (12, 13), it appears that the entire population of normal and tumor epithelial cells indeed express at least some CD133; perhaps a more appropriate description of the colon CSC would be $CD133^{hi}$ (not $CD133^{+}$). Thus, the important distinction would not be the mere presence of CD133, but the relative abundance of the protein at the cell surface. Even so, more rigorous study will be required to establish that $CD133^{hi}$ status can be utilized is a marker of a colon CSC.

With respect to metastatic colon tumors, the data presented by Shmelkov et al. (5) are quite clear: CD133 can no longer be considered a marker of CSCs in a metastatic context. Indeed, it seems as though two distinct populations, $CD133^{+}EpCAM^{+}$ and $CD133^{-}EpCAM^{+}$ cells, have developed, and they are equally capable of self-renewal based on their ability to regenerate identical tumors over three serial passages each. To clarify these issues further, future functional studies of colon tumor–initiating activity, from primary or metastatic tumors, will need to use multiple markers in addition to CD133, possibly EpCAM and CXCR4 (16) and possibly others. Furthermore, it would be desirable if future studies used the same implantation site in order to avoid the possibility of different outcomes resulting from the targeting of immune-privileged sites (such as the kidney capsule, as in ref. 12) versus nonprivileged sites (such as the subcutaneous space utilized in ref. 13 and in the current study by Shmelkov et al.).

The report from Shmelkov et al. (5) questions some dogmas that are developing in the field of CSC research and thus inspires a number of questions: What is the stage in colon cancer progression at which $CD133^{-}$ cells emerge? Are $CD133^{-}$ cells descended from $CD133^{+}$ cells, and if so, is CD133 a marker of the metastatic stem cell? Does the prevalence of $CD133^{-}$ cells in metastasis correlate with poor prognosis? Similar to metastatic cells from the pancreas (16), do $CD133^{+}$ cells that express CXCR4 migrate to distant sites, at which locations they generate $CD133^{-}$ cells that are independently capable of self-renewal? Do $CD133^{-}$, but not $CD133^{+}$, cells migrate to their metastatic niches? If this is so, what then is the source of $CD133^{+}$ cells in metastases? Do they regain the expression of CD133 in the process of mesenchymal-epithelial transition? Ultimately, the study by Shmelkov et al. demonstrates the importance of a comprehensive validation of functional molecular markers for the isolation of stem cell populations, which may be utilized in the future for therapeutic purposes. Indeed, these new data suggest that therapeutic targeting of $CD133^{+}$ cells in primary colon tumors could be quite toxic; in addition, it would be futile to treat metastatic colon cancers with a therapy designed to target only $CD133^{+}$ cells.

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