

# Cancer stem cells don't waste their time cleaning—low proteasome activity, a marker for cancer stem cell function

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*Comment on:* Munakata K, Uemura M, Tanaka S, *et al.* Cancer Stem-like Properties in Colorectal Cancer Cells with Low Proteasome Activity. *Clin Cancer Res* 2016;22:5277-5286.

**Abstract:** A population of stem-like cells in tumors, the so-called cancer stem cells (CSCs), are being held responsible for therapy resistance and tumor recurrence. In analogy with normal stem cells, CSCs possess the capacity of long term self-renewal and multilineage differentiation. CSCs are believed to be more resistant to various therapies compared to their differentiated offspring and therefore the cause of tumor relapse. Markers for CSCs have been identified using xenograft transplantation assays and lineage tracing in mouse models, however the specificity and validity of many of these markers is under debate. Recently, low proteasome activity has been postulated as a novel CSC marker. In several solid malignancies a small subset of low proteasomal activity cells with CSC characteristics were identified, suggesting that proteasomal activity might be a functional marker for CSCs. In this perspective, we will discuss a recent study by Munakata *et al.*, describing a population of colorectal cancer cells with CSC properties, characterized by low proteasome activity and treatment resistance. We will put this finding in a broader view by discussing the challenges and issues inherent with CSC identification, as well as some emerging insights in the CSC concept.

**Keywords:** Cancer stem cells (CSCs); proteasome; CSC markers; therapy resistance

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## Introduction

Resistance to therapy, together with recurrence and metastasis, remain the biggest challenges for successful cancer treatment and account for the majority of cancer mortalities. Although major advances in the treatment of cancer patients have been made in the recent years, including personalized or targeted treatment, our knowledge of these processes is still limited. Essential for understanding the mechanisms behind therapy evasion is first to understand the mode of growth and organization of the tumor. Whereas for long the stochastic model for tumor growth has been considered as the working model,

the hierarchical or cancer stem cell (CSC) model is now a widely supported alternative. In the CSC model, only a small subset of the cancer cells has the capacity of long term self-renewal and to differentiate into specialized cells. These stem cell-like cells are held responsible for tumor expansion and progression (1-3), but also reported to be more resistant to several treatments (4-8) and to be involved in recurrence and metastasis (9-11). In this perspective, we will discuss a recent study by Munakata *et al.* (12), identifying low proteasomal activity as a marker for CSC properties in colorectal cancer cells. To put this in a broader perspective, we will also discuss some recent insights and complicating factors in the still controversial CSC concept.

## Identification of CSCs

Originally, CSCs were defined as self-renewing cells, able to reconstitute a complete hierarchically organized tumor from one cell, therefore also indicated as tumor or cancer initiating cells (6,13-16). Xenotransplantation studies in which cell populations were selected based upon (surface) marker expression, revealed LGR5+, CD133 (AC133 epitope), CD44 or CD166 as CSC markers (4,13-19). These findings were supported with lineage tracing experiments in genetically defined mouse models (8,20-22). However, these studies do not functionally test stem cells in established human cancers. Indeed, there are subpopulations present in a tumor that express the aforementioned CSC markers, but it is still unclear if these are also the cells responsible for tumor expansion. The ability of a cell to initiate a tumor in transplantation assays might be more a reflection of the ability to survive in a hostile environment than that it reflects the growth promoting capacity in established tumor tissue (23).

Other problems with the current CSC markers are their often limited specificity, different specificity in different assays and the lack of a direct relation with stemness function (24). Some of the early CSC markers, such as CD133 (4,13), have later been reported not to be distinguishing between stem or non-stem cells (25). Heterogeneity between cancer types, tumors or within a tumor can cause differences in the amount and identification of CSCs (26-28), therefore up to now, it seems impossible to appoint a universal single marker for CSC identity.

## Functional CSC markers

Given these difficulties to find markers for CSC identity, it is probably more useful to identify markers that are directly involved in stem cell functionality. Whereas a cell expressing CSC markers might have stem cell potential, it does not necessary have to be an active or functional stem cell (1). If a potential CSC becomes an active stem cell might depend on factors from the micro-environment, including stromal secretions or even chemotherapeutics (29,30). This activation of the stem cell function, usually accompanied by dedifferentiation should be marked by the activation of pathways that are specific for functional stem cells. It is expected that abrogation of such an indicator pathway would therefore result in loss of stemness. Examples of markers of stem cell functionality are Wnt-pathway activity (30), Hedgehog signaling (31), Nodal

and Activin (32) or ALDH1 activity (33). For instance, the TOP-GFP Wnt activity reporter system has been used to visually identify stem-like cells in colorectal cancer (30). However, the specificity of most of these functional markers will still depend on tumor type or even subtype, so validation remains necessary. Indeed, also Wnt signal activity has been reported not to be a universal marker of CSCs in colon cancer (34,35).

## Therapy resistance of CSCs

CSCs have been reported to be more resistant to various therapies, from conventional treatments using chemotherapy to radiotherapy. In both *in vitro* as in *in vivo* experiments, CSC populations, according to the expression of CSC markers, were enriched after treatment (36). Several mechanisms are associated with this increased resistance, in analogy with normal stem cells, CSCs exhibit enhanced DNA repair, drug efflux pumps, Reactive oxygen species (ROS) scavenging and a higher apoptotic threshold (36,37). Another mechanism might be the existence of quiescent CSCs. Since most of the therapies will act upon proliferating cells, these quiescent cells might be able to survive, become activated and repopulate the tumor (38,39).

Although there seems to be a correlation between the CSC phenotype and increased resistance, the markers used in the different experiments vary greatly (40), making it hard to conclude if they mark different CSC subpopulations or are a shared feature for all CSCs. A complicating factor is the often observed heterogeneity between (inter-tumoral) and within tumors (intra-tumoral), which might account for certain resistant populations, or might result in different CSC populations in a tumor (41). This heterogeneity can be caused by intrinsic (clonal) or extrinsic (micro-environmental) factors. Next to this, expression of CSC markers might be regulated or influenced by treatments (42-45). Nevertheless, finding new markers that reliably identify cells with stem cell functionality is essential to break through one of the major barriers for CSC research.

## Proteasome activity as a CSC marker

In 2009, the group of Pajonk was the first to show a correlation between low proteasome activity and the CSC phenotype (46). Most cancer cells are characterized by high proteasome activity (47,48), which plays a critical role in the degradation of proteins involved in cell cycle, apoptosis, DNA repair or survival pathways. Inhibition of the

proteasome will interfere with these processes and lead to accumulation of damaged or misfolded proteins, eventually resulting in apoptosis, but also results in enhanced sensitivity to chemo- and radiation therapy (49-51). Interestingly, several conventional therapies were found to inhibit the proteasome in cancer cells, which might explain, at least in part, the mechanism behind the anticancer activity of these therapies (52-54). Next to this, in contrast to normal cells, many cancer cells are highly sensitive for proteasome inhibitors such as bortezomib (49,55,56). Bortezomib, also known as PS341, an reversible 26S proteasome inhibitor, was the first proteasome inhibitor that was brought to the clinic and is now used for treatment of multiple myeloma and mantle cell lymphoma (55).

Although bortezomib has been shown to act on a plethora of targets, the actual anticancer mechanism is still not completely elucidated and might vary across different tumors (55). One of the suggested modes of action of bortezomib is to disturb the NF- $\kappa$ B mediated balance between pro- and anti-apoptotic proteins, such as the pro-apoptotic Noxa and Bcl2, an anti-apoptotic target gene, shifting it towards a more pro-apoptotic cell fate (57,58).

However, in contrast to its effectiveness in the treatment of multiple myeloma and mantle cell lymphoma, this inhibitor had an unfortunate lack of efficacy in several types of solid tumors in clinical trials (59-63). Interestingly, (cancer) stem cells have been reported to be associated with elevated expression of anti-apoptotic proteins such as Bcl2, which makes them more permissive for oncogenic transformation (64,65). High levels of Bcl2 or other anti-apoptotic proteins have been shown to reduce the efficacy of proteasome inhibitors or conventional chemotherapeutics (66,67) and therefore it is thought that CSCs have a higher apoptotic threshold due to elevated levels of anti-apoptotic proteins (37).

The lack of effect of proteasome inhibition on solid tumors (59-63) was reason to investigate if this was due to the presence of resistant CSCs in these tumors. Therefore, Vlashi *et al.*, studied the relation between expression and activity of the 26S proteasome in solid tumors and CSC activity (46). By modifying cancer cells with a green-fluorescent protein fused to a degron, a target sequence for 26S proteasomal degradation, cells with low proteasomal activity could be identified. Indeed, a very small subset of both glioma and breast cancer cells was marked by low proteasome activity. In concordance with a CSC phenotype, these cells were characterized by expression of stem cell markers, absence of differentiation markers, high sphere-

forming capacity *in vitro* and high tumorigenicity *in vivo* (46). Later, similar findings have been reported for other cancers, including pancreas, prostate, head and neck, cervical and lung cancer (68-72). Interestingly, specific targeting of these low proteasome activity cells (LPACs) resulted in tumor regression (46,73). On the other hand, LPACs were more resistant to various types of treatment, such as radiotherapy (46) or chemotherapy (68-70). In a subsequent study, the group of Pajonk found that proteasome levels were downregulated in cells overexpressing Musashi-1, via inhibition of NF-YA, the most important transcription factor for the proteasomal subunits (74). Originally, Musashi-1 was described as a positive regulator of Notch signaling (75), an established factor for self-renewal in CSCs. In line with this, Musashi-1 expression was found to be heavily upregulated in tumorigenic low-proteasome activity cells, thereby linking proteasome activity with a CSC phenotype (74).

In a recently published study, Munakata *et al.* report on the relationship between treatment resistance, proteasome activity and CSC function in colorectal cancer (12). Similar to the above described studies, they made use of cell lines modified with a reporter for low proteasome activity. Also consistent with previous findings, CRC LPACs were more resistant to radio- and chemotherapy *in vitro*, exhibited enhanced sphere formation, expression of CSC markers and tumorigenicity, compared to non-LPACs.

One step further, the authors tried to identify factors underlying the differences in cancer stemness between LPACs and non-LPACs using microarray. This resulted in the candidate factor EP300 Interacting Inhibitor of Differentiation 3 (EID3), which was significantly upregulated in cells with low proteasome activity. EID3 is member of the EID family, which has been reported to function as inhibitors of differentiation in different tissue types (76,77). Interestingly, hypermethylation of the EID3 gene has been associated with colorectal neoplasia in African Americans, suggesting a tumor suppressing function for EID3 (78). *In vitro* modulation of this factor showed a positive correlation with proliferation and resistance to radio- and chemotherapy in colorectal cancer cells, but had no effect on proteasome activity (12). In addition, expression of EID3 mRNA could serve as a negative prognostic factor in CRC patients that underwent curative resection, suggesting that cells with high levels of EID3 are capable of reconstituting the original tumor and therefore could indeed function as cancer stem-like cells.

Other genes of interest that were upregulated in LPACs

were matrix metalloproteinase 1 (MMP1) and ATP-binding Cassette Sub-family B member 1 (ABCB1, also known as multidrug resistance gene 1 MDR1). These genes involved in respectively metastasis/invasion and drug resistance in cancer (79,80), characteristics usually attributed to cancer stem-like cells.

It would be very useful to elucidate the mechanism behind the upregulation of these CSC related genes in LPACs. Is this a direct result of decreased proteasome activity or independently regulated by other factors?

### Low proteasome activity, marking all CSCs or a sub-population?

It will be important to establish if reduced proteasome activity and upregulation of the reported genes are true CSC characteristics. Since CSCs still share many characteristics with normal stem cells, the proteasomal activity in normal stem cells is of interest as well. Up to now, only a few studies have examined this, in human embryonic stem cells (hESCs), elevated proteasome activity has been observed compared to differentiated offspring (81). In neural progenitor cells, proteasomal activity is required for self-renewal (82), whereas reduced proteasomal activity is associated with senescence and aging in hESCs. The high activity of the proteasome is believed to play a role in the maintenance of the proteome integrity in these continuously dividing cells (81). This might suggest that LPACs could be a dormant stem cell population, as was supported by a high fraction of G0/G1 cells in the LPAC population (12) and a low proportion of Ki67+ LPACs in *in vivo* tumors (46). Indeed, it is believed for long that quiescence or dormancy are characteristic for adult stem cells(2), however, intestinal stem cell research of recent years reveals the presence of a specialized niche containing active cycling stem cells and a pool of more quiescent 'reserve' stem cells at the borders of the niche (1,83). Up to now, no direct proof of the existence of quiescent or dormant CSCs in tumors has been shown (2). Although this would be a good explanation for therapy resistance, it is not so obvious that these cells would also fuel tumor proliferation. It could well be that also in tumors there are specific niches that will activate a potential CSC, whereas CSCs outside this niche might convert to a quiescent phenotype.

In future studies, proteasomal reporter systems in normal hierarchically organized tissue could provide some answers about proteasome activity in normal stem cells. Lineage tracing in tumors based on proteasomal activity might

clarify the CSC functionality of LPACs.

Another possibility might be that there are several clonal sub-populations of CSCs within a tumor, each with different clonal traits. Low proteasome activity and increased therapy resistance might be a characteristic of a specific clone, which does not exclude the presence of other CSC populations. Moreover, even in genetic clones within a single tumor, functional heterogeneity has been observed, including a dormant phenotype, driven by non-genetic processes (29).

In the *in vivo* experiments by Munakata *et al.*, non-LPACs were able to initiate subcutaneous tumors (7/8) when as low as 100 cells were injected, whereas LPACs could even form tumors when only 20 cells (5/8) were injected. This suggests that also in the non-LPAC population, CSCs are present. Tumors that were derived from non-LPACs appeared to grow slightly slower. Considering the very low fraction of LPACs that were found in the cancer cell lines (<1%), this suggests that LPACs might represent a subgroup of CSC-like cells, but clearly do not mark the whole CSC population. In line with this, in 2 of the 4 colon cancer cell lines used in this study, the LPAC population did not significantly demonstrate an enhanced sphere forming capacity (12). Previous studies on low proteasome CSCs also indicate that there is probably another CSC population that is not marked by low proteasome activity (69,73). Specific targeting of low proteasome breast cancer cells at time of tumor initiation could not prevent the eventual formation of tumors, although the tumor formation process was much slower (73), again indicating the presence of another tumor initiating population.

Although the authors do not report *in vitro* transition of non-LPACs to LPACs, this might occur in these experiments. Analysis of spheroids or tumors derived from non-LPACs for the presence of LPACs could provide evidence for the plasticity of the low proteasome CSC population or for the existence of another CSC population. As is becoming more and more clear, extrinsic factors such as the microenvironment are crucial for stem cell function (30,84). Bidirectional interconversion between non-CSC's and CSC's has been demonstrated in several types of cancer (85-87). This tumor cell plasticity might be an important reason for therapy failure. When a treatment effectively targets large part of the tumor cells, also the micro-environment is altered dramatically. Cells that were originally positioned in a non-proliferative environment might suddenly receive proliferative signals and wake up from a dormant state (88).

## Conclusions

Low proteasome activity seems to be correlated with a CSC phenotype and enhanced therapy resistance in several malignancies. However, if low proteasome activity is a functional marker for CSC activity or only a marker for dormant or quiescent cells with CSC potential, is not fully resolved at this stage. Upregulation of gene signatures associated with stemness and resistance might be the result of low proteasome activity, although, on the other hand, an elevated stem cell signature might be able to downregulate the proteasome (74). In this respect it will be interesting to investigate the relation of proteasome activity to other pathways that are associated with CSC activity. Processes such as heterogeneity within the tumor and stem cell plasticity complicate CSC research as well as the treatment strategies for these tumors.

Overall, accumulating studies show evidence for the existence of a CSC population in solid tumors that is characterized by low proteasome activity and therapy resistance. Whereas most cancer cells will be effectively targeted by a combination of conventional therapies and proteasome inhibition, this specific population of CSCs is highly resistant to this treatment and most likely responsible for tumor recurrence. Addition of targeted therapies that interfere with these traits might drastically improve the treatment and survival of patients with solid tumors.

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## Footnote

*Conflicts of Interest:* The authors have no conflicts of interest to declare.

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