RESEARCH HIGHLIGHT

The High mobility group box 1/Toll-like Receptor 2 axis plays a non-inflammatory role in the self-renewal of mammary cancer stem cells

Laura Conti*, Stefania Lanzardo*, Federica Cavallo

University of Turin, Molecular Biotechnology Center, via Nizza 52, 10126 Turin, Italy

*These authors contributed equally to this work.
Correspondence: Federica Cavallo
E-mail: federica.cavallo@unito.it
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The lack of effective treatment in several metastatic cancers raises the question of whether current therapies target the right cells. These treatments may be missing cancer stem cells (CSC), a sub-population of stem cell-like cells that are able to play a critical role in cancer progression. A profound understanding of the mechanisms that regulate CSC self-renewal is therefore essential for the identification of new CSC antigens that may turn out to be the ideal target for more effective anticancer strategies. In the light of these considerations, we have performed the transcription profiling of the murine ErbB2$^+$ breast tumor cell line TUBO versus its derived CSC-enriched mammospheres. Of the all antigens that have been identified, we have focused our attention on toll-like receptor (TLR)2 which has been identified as being overexpressed in CSC. Moreover, TLR2 plays a central role in CSC biology; it is a key molecule for CSC self-renewal as its signaling inhibition impairs *in vitro* mammosphere generation and blocks tumorigenesis and lung metastases development *in vivo*. Our in-depth analysis of the downstream signal driven by TLR2 has demonstrated that it is activated by endogenous high mobility-group box (HMGB)1 with an autocrine loop, which induces IκBα phosphorylation, IL-6 and TGFβ secretion and consequently STAT3 and Smad3 activation.

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The efficacy of anticancer treatment, including immunotherapy, has constantly improved over the last decade. However, therapeutic resistance and the lack of curative treatment for metastatic disease suggest that anticancer therapies do not affect the right cell populations. In fact, tumors are no longer considered a homogeneous population of proliferating cells, but a heterogeneous mass made up of cells with different characteristics and at different grades of differentiation. These cells are organized into a hierarchy, with a small population of CSC that can generate the more differentiated cells that constitute the tumor bulk at the top. CSC display stem cell properties, such as the capacity to self-renew and to reestablish tumor heterogeneity, and are

responsible for long-term tumor progression.

A great deal of evidence has demonstrated that CSC are resistant to chemo- and radiation-therapy[1], and it has been shown that anticancer treatment can select CSC and promote tumor relapse, in some cases[2]. In view of this, CSC should be considered the ultimate target for successful anticancer strategies. This aim may be achieved both by striking antigens expressed on CSC and by better understanding the mechanisms that regulate CSC biology. Over the last few years, we have been proposing an innovative strategy for the identification of new tumor associated antigens that display a causal role in cancer progression (oncoantigens) against which preventive vaccines or other anticancer therapies can be addressed. This strategy, based on the integration of microarray data derived from progressive stages of mouse mammary carcinoma [3], has allowed us to identify several fresh tumor bulk expressed oncoantigens that are currently targeted by DNA vaccines in experimental models of tumor prevention and therapy [4]. We have now adapted this pipeline for the identification of CSC antigens.

In our recent paper *"The non-inflammatory role of Toll-like Receptor 2/ High mobility group box 1 axis in the self-renewal of mammary cancer stem cells"* [5], we enriched the CSC population by growing TUBO breast cancer cells in anchorage independent conditions. In this situation, only tumor cells with CSC characteristics can survive and proliferate, forming floating spherical colonies termed mammospheres that can be propagated for serial *in vitro* passages; they down-regulate cell-cell junctions and begin to show mesenchymal behavior, much like what happens *in vivo*. Since the surface marker phenotypes of different CSC are still the subject of some debate and, even in the same cancer type, there are significant differences in marker expression from one specimen or cell line to another [6], we decided to enrich, and not to isolate, CSC using this growth technique [7]. The TUBO cell line was derived from a carcinoma that arose in a BALB-neuT mouse [8], an ErbB-2-driven mammary carcinogenesis mouse model that recapitulates the features of human breast tumors.

The transcription profile of TUBO cells was compared with that of their derived mammospheres in order to single out CSC markers. We later performed a profound meta-analysis that brought forward the genes with a human ortholog. This analysis led to the identification of a gene signature that is associated to human mammary CSC. Of the genes identified, we focused our attention on TLR2 because its involvement in normal stem cell physiology and tumorigenesis is already known [9-11].

We demonstrated that TLR2 expression increases from TUBO through the various mammosphere passages and associates with a CSC phenotype. Furthermore, it can be said that TLR2 regulates mammosphere generation *in vitro* as a significant reduction or enhancement in the number of mammospheres generated was found when it was properly silenced or stimulated, respectively. In addition, the freshly discovered role of TLR2 in CSC self-renewal was also observed in one of the most aggressive types of human breast cancer, triple negative breast cancer (TNBC). This is particularly important since there is no standard follow-up treatment for TNBC to prevent recurrence and immunotherapy is still in its infancy.

The analysis of the expression of several endogenous TLR2 ligands showed that high-mobility-group box1 (HMGB1) [12] was naturally secreted by TUBO cells and even more by mammospheres; this autocrine loop of TLR2 stimulation, through HMGB1 production, sustains CSC-renewal and sequentially triggers NF-kB, STAT3 and Smad3 activation.

What are the consequences of TLR2 silencing on *in vivo* tumorigenesis? A striking reduction in the number of tumors generated was observed in syngeneic mice that had been subcutaneously or intravenously injected with TUBO-derived spheres in which TLR2 was silenced. This indicates that TLR2 routes the tumor initiating ability and metastatic potential of mammospheres.

These results are an important step towards insight into the mechanisms that regulate breast tumor development and metastases and are in line with recent evidence which shows that functional TLR2 is also expressed in a wide variety of tumors [13-15] and correlates with clinical grade [16]. This role is played independently of inflammation [11], as already observed in normal epithelial cells, where TLR2 stimulates proliferation, migration and angiogenesis [17-18]. It is worth noting that our paper has demonstrated the role of TLR2 in mammary CSC self-renewal for the first time, much like what has been previously reported for normal adult renal stem cells [9] and for ovarian CSC [19]. Most notably, our research has shed new light on the ambiguous role of TLR2 in tumorigenesis, since TLR2 expression on antigen presenting cells can stimulate an immune response against the tumors, while its expression on CSC may increase metastatization and tumor recurrence. Our study supports the further development of TLR2-blocking antibodies as anticancer therapies, and challenges the use of TLR2 agonists in this therapeutic setting.

We are currently confirming the role of TLR2 in ErbB2-

driven mammary carcinogenesis by evaluating tumor onset in mice that are transgenic for ErB2 and knock-out for TLR2. Further data on the role of the other genes identified as CSC antigens will be provided in the near future.

Conflicting interests

The authors have declared that no competing interests exist.

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