

Targeting highly expressed extracellular HSP90 in breast cancer stem cells, inhibits tumor growth *in vitro* and *in vivo*

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Abstract

Breast cancer stem cells (BCSC) have the ability to self-renew and resist to radiation and chemotherapy-induced cell death, allowing them to survive and to cause tumor recurrence. Here we report that extracellular heat shock protein 90 (eHSP90) is over-expressed in BCSC and related to their stemness. Moreover we show that mAb 4C5, an antibody targeting exclusively eHSP90, inhibits primary growth as well as regression of BCSC derived tumors, thus offering considerable promise as a candidate for breast cancer therapy.

Introduction

Breast CSCs (BCSC) represent a small subpopulation (0.1% to 1%) of breast cancer cells in primary tumors¹. The first report of CSC in breast cancer was by Al-Hajj et al., in 2003, who isolated these cells and designated them as CD44⁺/CD24^{-/low}². CD44⁺/CD24^{-/low} cells have evidenced stem cell features and many studies have confirmed the poor prognosis of tumors with the CD44⁺/CD24^{-/low} phenotype³. On the other hand, Dontu and colleagues developed an *in vitro* culture system that allows for propagation of human mammary epithelial cells in non-adherent non-differentiating culture conditions⁴. Cells capable of surviving and proliferating in such conditions, formed discrete clusters, enriched in progenitor cells, termed "mammospheres". Additionally, Ponti and colleagues found that 95% - 96% of cells in mammospheres cultured from cell lines and primary breast tumors were CD44⁺/CD24^{-/low}⁵.

Previous studies have shown that extracellular HSP90 (eHSP90) participates in the invasion and metastatic processes of various cancers including breast cancer⁶⁻⁸. Here, we show for the first time that eHSP90 is over-expressed in mammosphere cultures derived from the MDA-MB-231 cancer cell line and highly enriched in cells with the CD44⁺/CD24^{-/low} BCSC phenotype, thus indicating that eHSP90 could potentially be a novel BCSC marker. Moreover we show that monoclonal antibody mAb 4C5, by targeting exclusively eHSP90, has the capacity to inhibit stem cell activity both *in vitro* and *in vivo*.

Results and Discussion

eHSP90 is localized and over-expressed on BCSC.

Double immunofluorescence experiments using polyclonal anti-HSP90 and anti-CD44 antibodies performed on live mammospheres, revealed that eHSP90 and BCSC marker CD44 were co-localized on the cell surface of most cells (Fig. 1a). So as to further investigate if eHSP90 is over-expressed on BCSC as compared to the parental cell line, immunostainings were performed on both MDA-MB-231 cells and mammospheres using either anti-HSP90 or mAb4C5. Interestingly, anti-HSP90 and mAb4C5 labeling revealed a 65.6% and 66.8% increase on mammospheres than on MDA-MB-231 cells, respectively. (Figs. 1b and c).

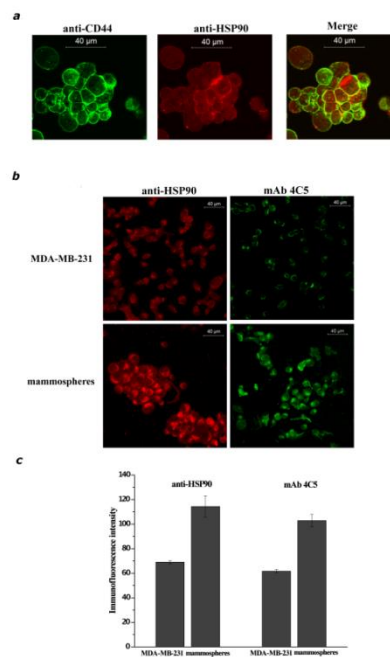


Figure 1. eHSP90 is localized and over-expressed on BCSCs.

MAb4C5 inhibits colony formation of mammospheres.

In order to examine the effect of mAb 4C5 on tumor growth in vitro we performed anchorage independent clonogenic assays. Assays were performed in the absence or presence of mAb4C5, using MDA-MB-231 cells and the corresponding mammospheres (Figs 2a and b). Our results showed that mAb 4C5-treated mammospheres showed a 73.3% reduction of their clonogenic potential as compared to control cultures (Fig. 2c). These results further support the importance of eHSP90 in BCSC function.

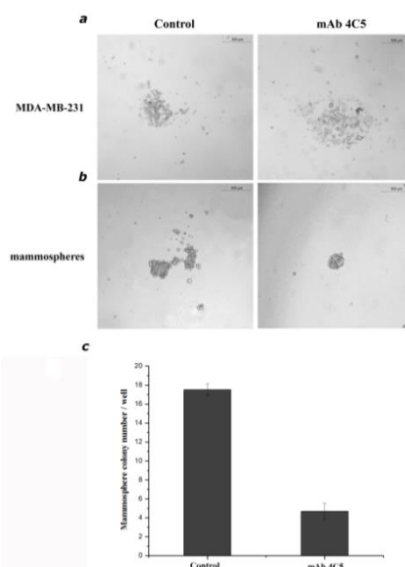


Figure 2. MAb4C5 inhibits colony formation of BCSCs.

MAb4C5 has a prophylactic effect on the development of primary tumors derived from MDA-MB-231 cells and mammospheres

To explore the effect of mAb4C5 on primary tumor growth in vivo, first we inoculated into both inguinal mammary fat-pads of each mouse, MDAMB231 cells and mammospheres, left and right respectively. As expected, the mammosphere derived tumors were significantly larger when compared to the ones derived from the parental cells. The results of our prophylactic protocol showed that 8 weeks after inoculation both the MDA-MB-231 and the mammosphere derived tumors were significantly smaller in the mAb4C5 treated mice as compared to the controls (Figs. 3a and b). Furthermore, an important reduction of proliferation marker Ki-67 positive cells was observed in mAb4C5 treated animals with respect to controls both in the MDA-MB-231 and the mammosphere derived tumors (Figs. 3c and d).

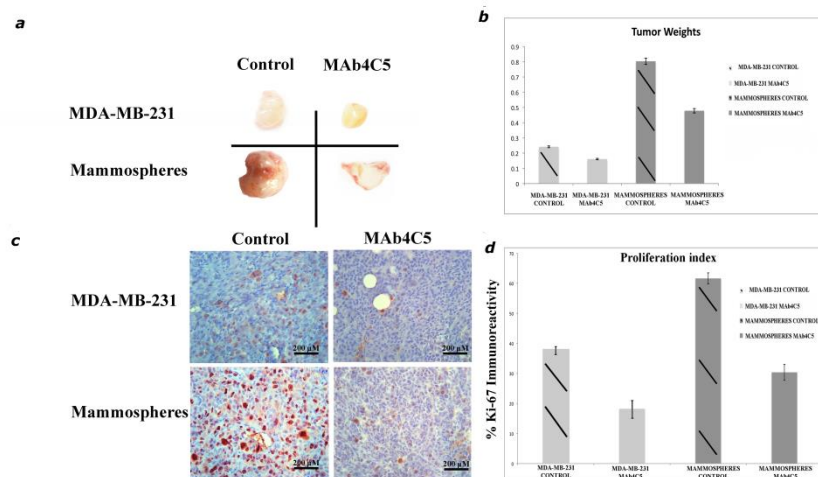


Figure 3. MAb4C5 inhibits primary tumor growth in an in vivo prophylactic model.

MAb4C5 alone and combined with paclitaxel has a therapeutic effect on the progression of primary tumors derived from mammospheres.

In our therapeutic protocol, our results showed that treatment of mice having already palpable tumors with mAb4C5, antitumor agent paclitaxel or a combination of the two, resulted in significant tumor regression in all cases as compared to controls (Fig. 4a). More precisely, in the mAb4C5, paclitaxel and mAb4C5/paclitaxel groups a 44.2%, 88.4% and 97.3% reduction of tumor weight was obtained respectively in comparison to the control group (Fig. 4c). Ki-67 immunohistochemical analysis and quantification of these tumors revealed a significantly reduced immunolabeling when compared to controls, in the mAb 4C5 treated mice (67.3%), which became even less in the paclitaxel group (90.1%), and was almost absent when the animals received a combination of the two agents (96.7%) (Figs. 4b and c).

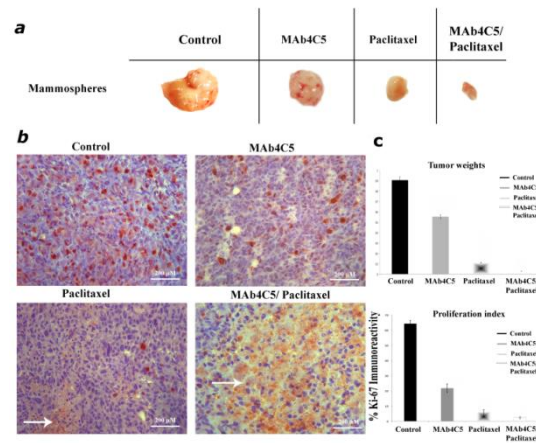


Figure 4. MAB4C5 alone and combined with paclitaxel, causes tumor regression in an in vivo therapeutic model

Concluding remarks

In conclusion, the present work shows that eHSP90 is over-expressed in mammospheres derived from the MDA-MB-231 cell line, that are enriched in cells with the CD44+/CD24-/low BCSC phenotype. Moreover it demonstrates that by targeting eHSP90 with mAb4C5, stem cell activity is inhibited in vitro, as judged by the significantly reduced capacity of mammospheres to form colonies. Finally it reveals that eHSP90 participates in BCSC derived primary tumor growth. More specifically, data is presented, demonstrating the in vivo anti-cancer activity of mAb 4C5 on MDA-MB-231 primary tumor growth and more importantly its effect either alone or in combination with paclitaxel on the regression of tumors derived from BCSC.

References

1. Lu H, Clauser KR, Tam WL, Frose J, Ye X, Eaton EN, Reinhardt F, Donnemberg VS, Bhargava R, Carr SA, Weinberg RA. A breast cancer stem cell niche supported by juxtacrine signalling from monocytes and macrophages. *Nature cell biology* 2014;16:1105-17.
2. Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF. Prospective identification of tumorigenic breast cancer cells. *Proceedings of the National Academy of Sciences of the United States of America* 2003;100:3983-8.
3. Ahmed MA, Aleskandarany MA, Rakha EA, Moustafa RZ, Benhasouna A, Nolan C, Green AR, Ilyas M, Ellis IO. A CD44(-)/CD24(+) phenotype is a poor prognostic marker in early invasive breast cancer. *Breast Cancer Res Treat* 2012;133:979-95.
4. Dontu G, Abdallah WM, Foley JM, Jackson KW, Clarke MF, Kawamura MJ, Wicha MS. In vitro propagation and transcriptional profiling of human mammary stem/progenitor cells. *Genes & development* 2003;17:1253-70.
5. Ponti D, Costa A, Zaffaroni N, Pratesi G, Petrangolini G, Coradini D, Pilotti S, Pierotti MA, Daidone MG. Isolation and in vitro propagation of tumorigenic breast cancer cells with stem/progenitor cell properties. *Cancer research* 2005;65:5506-11.
6. Eustace BK, Jay DG. Extracellular roles for the molecular chaperone, hsp90. *Cell cycle (Georgetown, Tex.)* 2004;3:1098-100.
7. Stellas D, Karameris A, Patsavoudi E. Monoclonal antibody 4C5 immunostains human melanomas and inhibits melanoma cell invasion and metastasis. *Clinical cancer research : an official journal of the American Association for Cancer Research* 2007;13:1831-8.
8. Sidera K, Gaitanou M, Stellas D, Matsas R, Patsavoudi E. A critical role for HSP90 in cancer cell invasion involves interaction with the extracellular domain of HER-2. *The Journal of biological chemistry* 2008;283:2031-41.