Dear Editor,

Breast cancer (BC) is the most common cancer among women worldwide. The high percentage of early breast cancer (EBC) at diagnosis has raised the necessity of acquiring a better control of local relapses (Demicheli et al., 2008; Benson et al., 2009). Surgery itself and the subsequent wound healing process may represent perturbing factors for local recurrence and metastasis development (Demicheli et al., 2008; Troester et al., 2009). Both clinical and experimental evidences support this hypothesis. Multicentricity is a hallmark for many BC, yet 90% of local recurrences occur at the same quadrant of the primary cancer (Benson et al., 2009). Accordingly, wound fluids (WF) drained from BC patients after surgery stimulate proliferation and invasion of BC cells in vitro (Tagliabue et al., 2003; Belletti et al., 2008). Our previous studies implicated the 70-kDa ribosomal protein S6 kinase (hereafter p70S6K) in the response of BC cells to surgery-derived stimuli (Belletti et al., 2008). p70S6K is a serine/threonine kinase and downstream target of mTOR (Fenton and Gout, 2011). Many data suggest that p70S6K is implicated in BC onset and/or progression. The chromosomal region 17q23 containing the p70S6K gene (RPS6KB1) is amplified in ~10% of all primary BC, leading to p70S6K overexpression (Sinclair et al., 2003), correlating with poor prognosis (Maruani et al., 2012) and increased risk of loco-regional recurrence (van der Hage et al., 2004). Despite these strong correlative observations, the role of p70S6K in the process of BC relapse has never been investigated, nor has p70S6K been exploited as a therapeutic target.

To investigate whether p70S6K activation was functionally involved in the response of BC cells to post-surgical WF, we generated BC cell lines with impaired p70S6K activity, by overexpressing a kinase inactive mutant (p70KR) or silencing p70S6K expression (sh-p70) (Supplementary Figure S1A and B). As additional approaches, we used the specific p70S6K1 inhibitor PF-4708671 (hereafter PF) (Pearce et al., 2010) and the clinically approved mTOR inhibitor, the rapamycin analogue Temsirolimus (hereafter Tems) (Supplementary Figure S1C).

Then, we designed an in vivo experimental model resembling the course of human BC (Figure 1A). MD-MB-231 BC cells were bilaterally injected in nude mice mammary fat pads (MFP). When primary tumors reached 150–200 mm3, masses were surgically removed under anesthesia. After recovering, mice were followed up to detect appearance of local relapse. Eight weeks after surgery, mice were sacrificed and mammary glands, recurrences (when present), and lymphnodes were collected (Figure 1A). Since impairment of p70S6K activity in BC cells gave rise to smaller tumors (Harrington et al., 2004; Supplementary Figure S2A and B), we injected $1 \times 10^6$ control (CTR) cells (left MFP) and $2 \times 10^6$ p70KR expressing cells (right MFP) in order to obtain, at surgery time, primary tumors of similar size (Figure 1B upper panels and Supplementary Figure S2C). p70S6K activity was efficiently downmodulated in primary tumors derived from p70KR MDA-MB-231 cells (Supplementary Figure S3). When control cells were injected, local relapse typically appeared at 4–8 weeks after surgery, with a recurrence rate of 64% (Figure 1B lower panels, C, and E; Supplementary Table S1). Strikingly, in mice injected with p70KR cells, the percentage of recurrence dramatically dropped to 18% (Figure 1B lower panels, C, and E; Supplementary Table S1). Tumor spreading to loco-regional lymphnodes was detected only ipsilaterally to MFPs injected with CTR cells (Figure 1C). Similar results were obtained using MDA-MB-231 cells stably silenced for p70S6K (Figure 1E; Supplementary Table S1). PCR analyses excluded the possibility that recurrences observed in CTR cells injected-MFPs were caused by p70KR-expressing cells attracted to the surgery site via circulation (Supplementary Figure S4A). Increase of S6 phosphorylation was consistently detected in all relapses with respect to paired primary tumors, further supporting the critical role played by p70S6K in local re-growth (Supplementary Figure S4B).

To exploit the potential of therapeutically targeting p70S6K, we tested in vivo specific p70S6K1 inhibition using PF (Pearce et al., 2010) and inhibition of mTOR using Tems, in vivo. We bilaterally injected MDA-MB-231 cells and then designed a 3-day schedule of peri-operative treatment (Day $-1$, Day 0, and Day $+1$ with respect to surgery, Figure 1A), in order to restrain p70S6K activity during the surgery-induced inflammatory response. While this schedule did not affect the size of primary tumors (Figure 1D), peri-operative treatment with PF resulted in highly effective suppression of recurrences (64% in controls vs. 23% in PF $600 \mu g$ and 11% in PF $1200 \mu g$). By contrast, Tems was partially effective when given at a lower dose and, surprisingly, ineffective or even harmful to the mouse when administered at a higher dose (64% in controls vs. 29% in Tems $300 \mu g$ and 67% of Tems $600 \mu g$) (Figure 1E; Supplementary Table S1). Statistical analysis demonstrated that the treatment with higher dose of PF was significantly effective in protecting against local relapse ($P = 0.01$ in Logrank test; Hazard Ratio 7.5; 95% Confidence Interval 1.3–11.4) and significantly more effective than...
Figure 1 Inhibition of p70S6K signaling prevents formation of BC local recurrence. (A) Schematic representation of the experimental design. (B) Upper panels show representative images of mice bearing primary tumors of similar size in both MFPs before surgery. Lower panels show representative images of the same mice bearing recurrence only in the left MFP injected with control cells, but not in the right MFP injected with p70KR cells. (C) Hematoxylin and eosin staining of recurrences and residual mammary glands excised from mice described in B. On the right, a lymphnodal
the treatment with higher dose of Tems ($P = 0.03$ in Logrank test; Hazard Ratio 6.7; 95% Confidence Interval 1.1–56.1).

Results obtained so far suggested that a robust p70S6K signaling was necessary for residual isolated cells to survive in the breast microenvironment after surgery and then recur. To dissect the mechanism that might also explain the inefficacy of Tems observed at a higher dose, we challenged BC cells in anchorage independent growth and evaluated their survival at different time points. A significant increase in the apoptotic rate was detected only when p70S6K1 activity was specifically hampered by either overexpressing the kinase inactive mutant p70KR or treating with PF (Figure 1F and Supplementary Figure S5). Prolonged treatment with mTOR inhibitor did not result in significantly increased apoptosis in anchorage independent growth (Figure 1F) and led to hyper-activation of AKT (Figure 1G), as previously reported by others (Harrington et al., 2004). We then confirmed these in vitro results in the mouse model of BC. Tumors excised from mice treated with higher dose of Tems displayed significantly higher levels of activated AKT (Figure 1H), suggesting that, under prolonged Tems treatment, tumor cells could activate a survival response, eventually leading to local relapse.

Given the relevance of our findings in the mouse model, we were keen to assess whether these data could have a clinical validation. To this aim, we scrutinized p70S6K activity in 26 paired BC specimens from patients who underwent lumpectomy at first and then surgical widening to clear margins at 1–2 weeks later. We observed that nearly 50% of patients displayed an increase of p70S6K activity in the second specimen with respect to the first and only 8% showed the reverse trend, strongly supporting the hypothesis that p70S6K activation was induced by surgery in human tissues (Figure 1I and Supplementary Table S2).

Many literature data suggested that p70S6K plays a pivotal role during BC progression (Sinclair et al., 2003; van der Hage et al., 2004; Belletti et al., 2008; Maruani et al., 2012). Here, using a mouse model of BC, we show that hampering p70S6K activity has a strong impact on BC cell behavior and significantly prevents the formation of local recurrence. Importantly, a 3-day schedule of peri-operative treatment with a specific p70S6K1 inhibitor was sufficient to greatly diminish the appearance of relapses. Survival, more than proliferation, of residual isolated BC cells required the activity of p70S6K1 (Figure 1F). Inhibition of mTOR should, in principle, elicit the same response, but our experiments clearly demonstrate that this is not the case. While doubling the dose of specific p70S6K1 inhibitor PF resulted in halving the frequency of recurrences (23% and 11%, respectively for PF 600 μg and PF 1200 μg), Tems treatment at two doses led to incoherent outcomes (29% and 67%, respectively for Tems 300 μg and Tems 600 μg), suggesting that complete block of mTOR signaling by a higher dose of Tems treatment may trigger the compensatory escape mechanism. Accordingly, prolonged Tems treatment was not able to efficiently induce apoptosis in vitro but led to the compensatory activation of AKT, which, in turn, fostered cell survival (Figure 1F–H).

Taken together, our findings provide a biological rationale for the use of novel peri-operative treatment targeting p70S6K pathway, which is directed to compensating the harmful consequences of surgery and wound healing process and restraining local relapse in EBC patients. Our data also suggest that for targeted therapies to be really effective not only finding the right drug for the right patient but also understanding the most effective time of administration is necessary. [Supplementary material is available at Journal of Molecular Cell Biology online. We are grateful to the patients who participated in this study, to Sara Benevol (Division of Experimental Oncology 2, CRO, National Cancer Institute of Aviano) for expert technical assistance and to all the members of the S.C.I.C.C. lab for helpful scientific discussion. This work was supported by grants from the Italian Association for Cancer Research (AIRC IG 10459 to B.B.), from Regione Friuli Venezia Giulia (to G.B.), from CRO Intramural Grant (to G.B.), from the ‘Friuli Exchange Program’ (to S.B.) and from Outgoing AIRC/Marie Curie International Fellowship in Cancer Research (to S.B.).]
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References


