Author’s response to reviews

Title: A pooled shRNA screen to identify regulators of primary mammary stem and progenitor cell function

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Author’s response to reviews: see over
Referee 1

Reviewer’s report

In this manuscript by Sheridan and colleagues, the authors present their findings of a pooled shRNA library screen conducted in mouse MaSCs targeting transcription factors in attempts to identify novel regulators of MaSC/basal cell biology. They identify Prox1 and Asap1 as factors, which promote stem cell proliferation/survival based on secondary clonogic and transplantation assays. Based on these results, they claim they have provided a proof-of-principle screening approach to interrogate the role of genes in MaSC biology. Overall, while this is an interesting, well defined and novel study, but there are concerns about the data and whether this screen does in fact constitute a proof-of-principle as the hits identified and validated from it modestly (if at all) affect MaSC/basal cell biology. Because of the modest findings, the results seem to be over interpreted in the absence of controls and the limitation of the screen is not clearly stated. Therefore, additional controls and experiments should be conducted in order to be acceptable for publication.

Based on three independent biological screens, we identified potential modulators of stem/progenitor cell behaviour with more than half of those targeting novel genes that have not been previously implicated in mammary gland development. Although the changes were modest, they were highly reproducible. In terms of limitations, consideration of these has been included in the Discussion with solutions to perceived limitations including the use of alternative knock-down technologies such as CRISPR alluded to.

Major Concerns:

1) Since this study is aimed to provide a proof-of-principle to the scientific community, and there are some important hits that are not discussed in the text but are listed in their table, the supplemental table of the hits identified from the screen should be moved to the main text.

We have moved this to the main text and discussed other hits such as those in the Wnt pathway (p6).

2) Since inhibition of PROX1 had no effect on in vivo transplantation, this actually weakens their conclusion about the robustness of the screen.

This may reflect insufficient knock-down (mentioned in the Discussion). Alternatively, PROX1 may be required for progenitor and not stem cell activity, a role that may not necessarily be evident in the transplantation assay, which primarily measures repopulating potential.

We do not believe this weakens the conclusion since the mammosphere assay is principally a progenitor assay that is also permissive for stem cell fate decisions. We are exploring regulators of stem and progenitor cells using the functional assay (stated more clearly now in the Results). In our screen, we are reading out the effect of shRNAs on progenitor and/or stem cell activity, and may be modifying the proliferative potential,
survival or lineage potential of any of these cell types. Indeed, there are much larger numbers of progenitors present than stem cells in the mammosphere assays, reflected in the transplantation frequency of stem cells (approx. 1 in 300) versus those with colony-forming potential (approx. 1 in 20). We have indicated this in the Discussion.

3) As controls, the authors should provide include the effects of established regulators of MaSCs identified in their screen so as (eg. SOX9 and/or SNAI2) to be able to show how ASAP1 and PROX1 compare to these established genes.

The reviewer raises a fair point. However, as the strategy identified a number of known regulators of stem and progenitor cells, as well as genes implicated in mammary gland development, we chose to validate novel genes identified in the screen. We have performed a comparison with Snai2 hairpins, and similar to that found by Guo et al Cell 2012, knock-down in this system led to reduced colony-forming potential, thus validating the use of our assays (now shown as Supp. Figure S3). Notably, a correlation between in vitro clonogenic assays and phenotypes in mouse models does not always apply. In fact, Snai2 deficiency is associated with a very mild phenotype in the virgin animal (Nassour et al. PLOS ONE, 2012), arguing that it alone is not pivotal to stem cell function and morphogenesis.

4) The side branching phenotype reported by the authors following transplantation of shASAP1 cells is not at all convincing. Both the wholemounts and histology appear identical to the controls.

We apologise for the discrepancy here; we agree that there is no branching phenotype here as stated on p8 in the Results.

Referee 2

Reviewer’s report

The manuscript tries to be both a methodological paper by claiming a proof-of-principle for the use of particular cells for shRNA analysis and a paper about the biology of mouse mammary stem cells. Unfortunately, it is neither of the two. For a methodological paper, there is too little news in terms of methodology. The basic processes used were all well established and described before. The modifications required to adapt them to a particular cell type and the selection process applied are something normal to this kind of study. Protocols have been published for analyses with 55,000 constructs at a time, even going directly into an animal system for selection. Thus, an in vitro analysis with some 1250 constructs is not that impressive technically. Concerning biology, the results are of too preliminary a state to warrant publication. As they are, the results represent a mere transcript profiling experiment, in which some seventy genes exhibited significant variation. However, already the validation is only partly done. In order to make sure that not any off-target effects are responsible for the changes identified, much more validation is needed, such as over-expression of the relevant genes, which needs to be performed to document a reversal of the knockdown effect or at least an effect opposite to that of the knockdown. In addition, more about the actual functional consequences
needs to be presented to document any biological relevance. What was done, was simply picking two genes for reasons unknown and doing a few preliminary validation experiments on them. This is insufficient.

We validated two novel hits identified in the original functional screen (performed three times) in colony forming assays, competitive colony forming assays and also performed in vivo transplantation experiments. It is not usual to carry out overexpression studies for in vitro functional screens, although we agree that these are warranted for libraries going directly into mice.