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LETTERS TO THE EDITOR

Multipotent PI-MECs are the true targets of MMTV-neu tumorigenesis

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Lo *et al.*[1] purport to identify the mammary tumor-inducing cells in mouse mammary tumor virus (MMTV)-neu transgenic mice to be derived from 'luminal progenitors'. Despite some new experimental data that are mainly confirmatory, the authors do not discuss the fact that multiple research groups had previously reported the originating cells of MMTV-neu tumors. It is our collective opinion that none of the advances reported by the authors are unique to their experimental approach. Using genetically tagged cells in WAP-Cre/Rosa26-lox-STOP-lox-lacZ triple transgenic mice, Henry *et al.*[2] reported that lacZ-positive parity-identified mammary epithelial cells (PI-MECs) were the targets for MMTV-neu tumorigenesis. In addition, PI-MECs were subsequently shown to be virtually 100% sorted into the CD49f-positive mammary epithelial fraction.[3] Boulanger *et al.*[4] showed that PI-MECs were multipotent and sensitive to tumor growth factor beta (TGFβ) expression, and Booth *et al.*[5] showed that MMTV-neu induced mammary cancer cells (marked by lacZ expression) were suppressed by the normal mammary microenvironment but still maintained some of their multipotency. None of these reports were referenced or discussed by Lo *et al.*,[1] although all of them are directly related to the experimental subject of this manuscript. These omissions are egregious and suggest at a minimum that the authors had not previewed the earlier literature relating to their studies. More importantly, the previous reports indicate that the targets for MMTV-neu are not 'luminal progenitors' but alveolar progenitors that are multipotent, express CD49f and are inhibited from expansion by both TGFβ expression and the absence of cyclin D1.[6] These previously published works seriously diminish the scientific impact of the paper published by Lo *et al.*[1] Further, in the light of these previous reports, the alleged novelty of the findings described in the article published by Lo *et al.* is misleading and a note of correction from *Oncogene* or these authors is appropriate.

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Cancer stem cells and cells of origin in MMTV-Her2/neu-induced mammary tumorigenesis

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The main purpose of our study was to find new biomarkers to identify and enrich the tumor-initiating cells (TIC) or cancer stem cells (CSCs) in MMTV-Her2/neu-induced mammary tumors. The cancer stem cell hypothesis is an evolving concept of oncogenesis that the definition of a cancer stem cell does not necessarily imply its origin from a stem, progenitor or differentiated cell.[1] Two previous studies attempted to address the same issue using the MMTV-Her2/neu transgenic mice model.[2,3] In order to be consistent with these previous studies,[2] we used the term 'tumor-initiating cell' instead of 'cancer stem cell' to represent the subpopulation of tumor cells that can self-renew, propagate the tumor and differentiate into many types of cells found in a tumor. Liu *et al.*[2] first reported that TICs can be functionally isolated from MMTV-Her2/neu tumors but no specific markers have been identified to enrich them. It was also found that the majority of tumor cells shown in their study were $CD24^+$. When the $CD24^+$ cells were stratified into two subpopulations, $Sca1^+/CD24^+$ and $Scal1^-/CD24^+$, using the Sca1 marker for analysis, both fractions contained similar levels of TICs and exhibited equal tumorigenicity.[2] In another study, CD61 has been reported as a biomarker for identification of the TIC subset arising in MMTV-wnt-1 tumors and 50% of $p53^{-/-}$-derived tumors, but not in MMTV-Her2/neu tumors.[3] They concluded that MMTV-Her2/

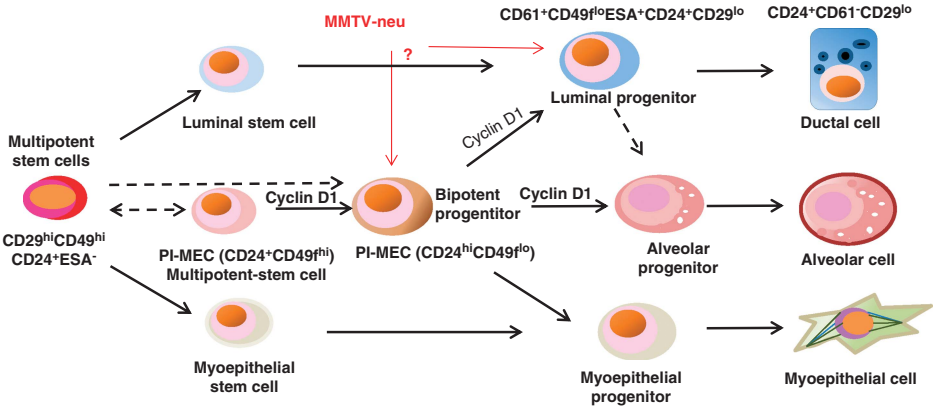

Figure 1. Model of the epithelial differentiation hierarchy in mouse mammary glands (Modified from Visvader and Lindeman[16] and Jeselsohn *et al.*[11]). Mammary multipotent stem cells which are able to regenerate entire mammary glands in mice at the single cell level are enriched in the $CD29^{hi}CD49^{hi}$ $CD24^{+}ESA^{-}$ subset.[8,23] Mouse mammary glands may consist of a hierarchy of multipotent stem, bipotent, unipotent progenitor and differentiated cells, which are delineated by different combinations of cell surface markers.[8,11,23,24] The PI-MECs reside in the $CD24^{+}CD49f^{+}$ population, which may be comprised of multipotent stem cells, bipotent progenitor and other committed progenitor cells.[11,14,15] The Cyclin D1 kinase activity is required for differentiation of common progenitor cells into both luminal and alveolar but not myoepithelial progenitors.[11]

neu tumors are composed of a homogeneous cell population and no distinct CSC population can be identified. In our study, we screened many potential biomarkers and found that a combination of two mammary stem/progenitor markers ($CD49f^{hi}CD61^{hi}$) can be utilized to identify and sort out TICs from primary tumors and their derivative cell lines. The sorted $CD49f^{hi}CD61^{hi}$ subpopulation displayed typical characteristics of TICs, such as the increased tumorsphere formation ability and enhanced tumorigenicity both *in vitro* and *in vivo*. Furthermore, we found that the integrin-β3-TGFβ pathway is critical for maintenance of this population within a tumor. To our knowledge, we for the first time found that a combination of CD49f and CD61 can be utilized to successfully isolate and enrich TICs from MMTV-Her2/neu mammary tumors.[4]

During identification of TIC markers for HER2 mammary tumors, we systematically analyzed normal, pre-malignant and malignant mammary epithelial cells using a panel of stem cell markers. Although identification of the origin of tumor cells is not the main focus in our paper, these studies indeed gave us some clues about the originating cells of MMTV-Her2/neu tumors.[4] In light of our finding and other published results, we posited that TICs in the MMTV-Her2/neu tumors are potentially derived from luminal progenitors. This hypothesis is supported by the following evidence. First, CD61 which we used to identify TICs is a putative luminal progenitor marker.[5–7] Recently, Weinberg and colleagues have also shown that $CD49f^{low}CD61^{+}$ cells are enriched in luminal progenitor activity.[7] Second, the $CD24^{high}CD49f^{med/low}$ Ma-CFC progenitor population was anomalously expanded in preneoplastic MMTV-Her2/neu mammary glands relative to age-matched, normal counterparts.[4,8] Third, the detected, expanded cell population also displayed ESA^{high}, a feature similar to human mammary luminal progenitor cells.[6] Last, gene expression profiles of MMTV-Her2/neu tumor cells are most concordant with the luminal progenitor gene signature.[9]

The previous studies have shown that the parity-induced mammary epithelial cells (PI-MECs) are the targets for MMTV-Her2/neu induced tumorigenesis, but the identity of PI-MEC remains largely elusive[10,11] (Figure 1). PI-MECs are present in both nulliparous and parous mammary gland.[10,12] In this sense, the designation of PI-MECs is a misnomer as suggested.[12] Pregnancy is not required for MMTV-Her2/neu induced tumorigenesis.[13] The PI-MECs were originally found within the $CD24^{+}CD49f^{+}$ cell population,[14] which are heterogeneous and may be comprised of multipotent, bipotent and other committed progenitor cells.[11,12,15,16] One fraction of PI-MEC ($CD24^{+}CD49f^{hi}$ population), which was identified to contain multipotent stem cells,[14,17] has been proved not to be the target for Her2-induced tumorigenesis.[11] The mammary glands in kinase-deficient cyclin D1 ($cyclinD1^{KE/KE}$) mice that are resistant to MMTV-Her2/neu-induced tumorigenesis contain increased $CD49f^{hi}CD24^{+}$ and decreased $CD24^{hi}CD49^{lo}$ cell populations compared with those in cyclin $D1^{+/+}$ mice.[11] As there is a decrease in $CD24^{hi}CD49f^{lo}$ cells in the mammary glands of $cyclinD1^{KE/KE}$ mice, Jeselsohn *et al.*[11] proposed that this fraction of PI-MECs (the $CD24^{hi}CD49f^{lo}$ population) is the target of MMTV-Her2/neu. In line with this, we observed the exact same population expanded in the pre-neoplastic MMTV-Her2/neu mammary glands.[4] Although the $CD24^{hi}CD49f^{lo}$ cell population displays bipotent activity (Jeselsohn *et al.*[11]), they are by no means homogeneous and may contain bipotent progenitor cells and other intermediates yet to be identified.[15,16,18] It remains unsolved which fraction of $CD24^{hi}CD49f^{lo}$ cells (the bipotent cells or the derived progenitors) are the direct target for MMTV-Her2/neu-induced tumorigenesis[18] (Figure 1).

The $CD24^{hi}CD49f^{lo}$ bipotent cells can potentially give rise to luminal as well as myoepithelial progenitor cells and during pregnancy alveolar progenitor cells might be directly derived from these bipotent cells[11] or from their derived luminal progenitor cells.[16] Despite a decrease in the number of $CD24^{hi}CD49f^{lo}$ cells in $cyclinD1^{KE/KE}$ mammary glands, this cell subpopulation still exhibits normal myoepithelial cell differentiation but shows a defect in cell differentiation into luminal progenitor cells as evidenced by the reduced luminal colony-formation ability and the decreased expression of luminal progenitor marker CD61.[11] If the bipotent progenitors in the $CD24^{hi}CD49f^{lo}$ cell population are the direct target of MMTV-Her2/neu and are selectively depleted due to the absence of cyclin D1 activity, we would expect that cell differentiation of both luminal and myoepithelial cell lineages should be blocked but not just luminal-lineage differentiation was inhibited as observed.[11] A potential explanation for these observations is that lack of cyclin D1 kinase activity results in loss of an important fraction of $CD24^{hi}CD49f^{lo}$ cells, for example, luminal or alveolar progenitors, which are the direct target of MMTV-Her2/neu (Figure 1). As discussed above, data from other groups

and ours suggest that luminal progenitors may be the direct target. As the biomarkers for alveolar progenitors have not been well characterized, the lineage relationship between luminal and alveolar progenitors remains unknown.[18] It has been suggested that they may be derived from the same upstream CD61^{+} luminal progenitors[5,16,18] (Figure 1). Furthermore, both overexpression and knockout of ErbB2/neu in mammary epithelial cells only impede ductal growth but have no effect on lobuloalveolar development;[19–21] it seems unlikely, at least not proven, that unipotent alveolar progenitor cells are the direct target for MMTV-Her2/neu-induced tumorigenesis. Consistently, mammary glands from cylcinD1$^{KE/KE}$ mice showed a relatively mild problem in lobuloalveolar development compared with the defect in luminal differentiation and pronounced effects were observed only after three rounds of pregnancy.[11,22] Overall, we support the hypothesis that luminal progenitor cells, which are either a fraction of or derived from PI-MECs, are more likely to be the direct target of MMTV-Her2/neu-induced tumorigenesis. More direct evidence such as the lineage tracing of cells that undergo transformation is needed to validate this hypothesis.

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