Title
Investigating YAP’s role in Human Mammary Epithelial Cell (HMEC) Immortalization and Cancer Stem Cell (CSC) Phenotype Acquisition during Early Breast Cancer Progression

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The malignant aspect of tumorigenesis in breast cancer is initiated when human mammary epithelial cells (HMEC) first immortalize (gain the ability to proliferate indefinitely) and subsequently undergo oncogenic activation. Our laboratory has developed a two-step HMEC immortalization process that we believe accurately models early breast cancer progression. Normal finite pre-stasis HMEC must overcome two distinct senescence barriers to achieve full immortalization: stasis (stress-associated senescence) and replicative senescence, respectively. HMEC bypass stasis, and become post-stasis, when errors in the retinoblastoma pathway are present. Replicative senescence, a hallmark of normal cells, can only occur after telomere shortening and DNA replication error, events that allow telomerase expression. This is followed by post-stasis, where the cell undergoes an irreversible process of senescence. The YAP transduced post-stasis HMEC have decreased levels of the lncRNA MORT and rapidly go through conversion when p53 function is abrogated. Our preliminary results have shown that YAP cells that acquire the error allowing telomerase expression conditionally immortal (CI); CI cells may still have low telomerase expression. Full immortalization is achieved when sufficient telomerase expression exists to maintain short stable telomeres, via a process we have called conversion. The transcriptional coactivator YAP, upregulated in several human cancers, is closely correlated with a variety of oncogenic effects including proliferation and induction of cancer stem cell (CSC) properties. Our preliminary data have shown that YAP-transduced post-stasis HMEC expressed properties of CI cells. Growth curves indicated that some cell lines achieved full immortality; however, these cell lines had an additional error – amplification of c-Myc, an oncogene known to induce telomerase in post-stasis or CI cells. The absence of uniform immortalization in the YAP-transduced HMEC is likely due to context-dependent effects. This is further supported given that YAP-transduced post-stasis HMEC have decreased levels of the lncRNA MORT and rapidly go through conversion when p53 function is abrogated, well-documented attributes of CI HMEC. Preliminary studies have also reported that YAP controls the differentiation of breast progenitor cells; as a result, it is likely that YAP plays a role in inducing CSC phenotypes. In this study, we attempt to gain a greater understanding of YAP’s role in HMEC immortalization and induction of CSC properties in early breast cancer progression. We measured gene expression levels and attempted to identify proteins indicative of a CSC phenotype in YAP-transduced post-stasis HMEC. Our initial results did not reveal an association between YAP and HMEC conditional immortality or induction of CSC attributes in the cultures examined. However, using a YAP construct that promotes nuclear localization (where YAP is most active) has led to a potentially promising YAP-derived cell line that we presume to be immortal. We go on to demonstrate that YAP may be acting via non-transcriptional oncogenic roles in our HMEC models. Thus, we recommend future studies into YAP and the HMEC immortalization process given that they are promising targets for breast cancer prevention therapies.

**Abstract**

**Background**

- Breast cancer is the most common cancer in women and the second most common cancer worldwide (Bray et al. 2018).
- Human Mammary Epithelial Cells (HMEC) achieve immortalization by overcoming two distinct senescence barriers (Figure 1).
- Inactivation of the retinoblastoma (RB) pathway (bypassing stasis) occurs by overexpressing cyclin D1 or inhibiting p16 in vitro.
- Reactivation of telomerase (bypassing replicative senescence) occurs via c-Myc transduction in vitro.

**The YAP Oncogene**

- Normal cells have a mean TRF > 5 kb; immortal/cancerous HMEC maintain short stable telomeres with a mean TRF of about 4 kb.
- The immortalization process is cancer-specific – ideal for identifying therapeutic targets for breast cancer prevention (prior to malignancy).

**Materials & Methods**

- Finite pre-stasis 184D HMEC specimens were obtained from reduction mammaplasty tissue.
- A p16 viral vector was added to bypass stasis; YAP and YAP-NLS transduced HMEC were harvested for quantitative RT-PCR.
- 184D control and YAP-transduced HMEC were fixed for immunofluorescence by staining for Keratin 14 (K14) and Keratin 19 (K19) for analysis of a CSC phenotype.
- HMEC were imaged at various passages for visual analysis.

**Results**

184D-p16sh-YAP HMEC

- Quantitative RT-PCR
  - Expression levels of MORT, p57, YAP, and CTGF & AXL (YAP downstream targets) were measured at passages 11 and 13.
  - A significant decrease in gene expression in YAP cells compared to controls was measured for all genes at passage 11, though this is likely due to variability in GAPDH.
  - No significant changes were observed at passage 13.
  - No significant increases in YAP, CTGF, or AXL expression in YAP cells suggests that YAP transduction and/or sustained expression was nonfunctional.

184D-p16sh-YAP-NLS HMEC

- YAP may not remain localized to the nucleus; this led to the development of the YAP-NLS cell line.
- Quantitative RT-PCR in the YAP-NLS also did not show significant increases in YAP, CTGF, or AXL expression.
- A phenotypic difference was observed compared to controls: YAP-NLS had rapid-growing, aggressive cells in addition to senescent cells.

**Conclusion**

- We cannot support or refute an association between YAP, HMEC CI, or CSC properties.
- Localization of YAP to the nucleus induces a phenotypic change in HMEC that we presume leads to a YAP-derived immortal cell line.
- YAP likely plays a role in HMEC immortalization via non-transcriptional oncogenic functions.
- In the future, we plan to measure continued growth and telomerase activity in YAP-NLS HMEC to analyze immortalization.

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