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14. ABSTRACT Breast cancer is attributed to genetic alterations, the majority of which are yet to be characterized. Oncogenic alterations that give rise to breast tumors need to be identified to develop targeted treatment options and consequently, improve clinical outcomes. We aim to identify kinases that drive breast oncogenesis. We hypothesize that a kinase gain-of-function screen in human mammary epithelial cells will identify novel breast cancer oncogenes and provide potential targets for drug intervention. The study is based on a transformation model that requires simultaneous activation of the PI3K/AKT and MEK/ERK pathways to transform human mammary epithelial cells. A pBabe-Puro-Myr-Flag kinase ORF library was screened in immortalized human mammary epithelial cells expressing myr-AKT. Three kinases PTK6, PAK1 and CAMK4 promoted robust anchorage-independent growth in soft agar and are further being validated to understand their mechanism of action and relevance in human cancer.					
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Table of Contents

	<u>Page</u>
Introduction.....	4
Body.....	4
Key Research Accomplishments.....	5
Reportable Outcomes.....	5
Conclusion.....	5
References.....	5
Appendices.....	5

Introduction

The field of cancer research is moving towards targeted therapy for more efficient treatment options. Leading the field are successful examples such as Imatinib and Gefitinib that target BCR-ABL in chronic myeloid leukemia and EGFR^{L858R} in lung cancer, respectively. Other candidates such as PLX4032 that targets BRAF^{V600E} in melanoma, thyroid and colorectal carcinoma also seem promising (Flaherty et al., 2009). In breast cancer, targeted therapies have been developed against HER2. The heterogeneity of cancers however, requires identification of more driving alterations that could be possible targets for drug development. We believe that an unbiased approach – a kinase gain-of-function screen in human mammary epithelial cells – can identify novel druggable targets that promote breast cancer and prove to be excellent targets for therapy.

Body

Human mammary epithelial cells have been immortalized with human catalytic subunit of telomerase, SV40 large T and small t antigens (HMLE). HMLEs additionally expressing HRAS oncoprotein form colonies in soft agar and tumors in nude mice (Elenbaas et al., 2001). Transforming ability of HRAS oncoproteins can be phenocopied by the combined expression of myristoylated *AKT1* (myr-AKT) and activated allele of *MEK1* (MEK^{DD}). A preciously conducted study by Boehm et al. (2007) identified *IKBKE* as a breast cancer oncogene that cooperates with HMLE -MEK^{DD} to replace the function of myr-AKT in transformation. Here, we conducted a human kinase screen in HMLE cells expressing myr-AKT to identify kinases that promote transformation, possibly in a MAPK-dependent manner.

Firstly, the transformation ability of immortalized human mammary epithelial cells expressing myr-AKT and MEK^{DD} (HMLE-A-M) at 1:25 dilution with HMLE-A cells was determined to be detectable and significant enough as the positive control for the pooled screen.

The 597 kinases in the pBabe-Puro-Myr-Flag ORF library were pooled at the DNA level into 22 pools of 25-27 kinases each. Retroviruses were produced from the DNA pools, positive and negative controls. HMLE-A cells were infected with the retrovirus, selected with puromycin and seeded in soft agar. Soft agar colonies for each pool were counted every week from week 2 to 6 post-seeding. Five pools scored higher than 1.5 standard deviations at two or more time points (Figure 1).

In order to determine individual kinases that contributed to soft agar colony formation, the five scoring pools were deconvoluted. We deconvoluted each pool separately and found 28 kinases that significantly promoted anchorage-independent growth. The ability of the kinase hits to promote anchorage-independent growth of HMLE-A cells was then directly compared to establish PTK6, PAK1 and CAMK4 as the three best candidates for further validation and characterization (Figure 2).

Preliminary studies of one of the hits, PAK1, assessed the effect of myristoylation and kinase activity in transformation of HMLE-A cells. Myr-Flag-PAK1 and Flag-PAK1 constructs were introduced to HMLE-A cells to discover no significant difference in soft agar colony formation, indicating that myristoylation of PAK1 is unnecessary for PAK1-dependent anchorage-independent growth (Figure 3A). On the other hand, HMLE-A cells expressing kinase dead PAK1 (K299R) did not show robust colony formation, in contrast to HMLE-A cells expressing wild-type *PAK1* (Figure 3B). Thus, the next step in

characterizing the role of PAK1 will be to study its activity in various relevant pathways and their contribution in PAK1-dependent transformation. Similar studies will be carried out to understand the mechanism of PTK6 and CAMK4 in transformation of HMLE-A cells.

Key Research Accomplishments

- Completed a pooled gain-of-function screen of human kinase ORF library in immortalized human mammary epithelial cells expressing myr-AKT.
- Identified three candidates: PTK6, PAK1 and CAMK4 for further evaluation
- Established the irrelevance of myristoylation of PAK1 in transformation.
- Determined the necessity of PAK1 kinase function in promoting anchorage-independent growth.

Reportable Outcomes

Abstracts

1. Shrestha Y, Schafer EJ, Boehm JS, Thomas S, Weir B, Hahn WC. Human kinase screen for novel breast cancer oncogenes. American Association for Cancer Research Special Conference: Frontier in Basic Cancer Research. October 8-11, 2009. Poster Presentation.
2. Shrestha Y, Boehm JS, Thomas S, Hahn, WC. Identification of novel breast cancer oncogenes. American Association for Cancer Research Special Conference: Translation of the Cancer Genome. February 7-10, 2009. Poster Presentation.

Conclusion

The human kinase ORF library of 597 kinases was successfully screened in a pooled format to assay anchorage-independent growth of HMLE-A cells. Deconvolution of five scoring pools revealed twenty-eight kinase hits, the three top hits of which are being studied further. Preliminary characterization of PAK1 showed that its kinase activity is required for soft agar growth. Hence, we plan to study the activity of PAK1 in PI3K, MAPK and other pathways in order to determine their role in PAK1-dependent transformation. Similar studies will be carried out for PTK6 and CAMK4.

References

- Boehm, J.S. et al. Transformation of human and murine fibroblasts without viral oncoproteins. *Mol. Cell. Biol.* **25(15)**, 6464-6474 (2005).
- Elenbaas, B. et al. Human breast cancer cells generated by oncogenic transformation of primary mammary epithelial cells. *Genes Dev* **15**, 50-65 (2001).
- Flaherty, K. et al. Phase I study of PLX4032: Proof of concept of V600E BRAF mutation as a therapeutic target in human cancer. *ASCO Abstract #9000* (2009).

Appendices

Abstract 1. Human Kinase Screen for Breast Cancer Oncogenes

The heterogeneity of breast cancer requires identification of novel genes that promote breast tumor development. Targeting such oncogenes therapeutically promises to improve clinical outcomes. We conducted an *in vitro* screen to identify human kinases that promote anchorage-independent growth of immortalized human mammary epithelial cells. The screen is based on a transformation model that requires cooperative activation of the PI3K/AKT and MEK/ERK pathways. The pooled screen identified three transforming kinases - PTK6, PAK1 and CAMK4. The functions of these candidates in the PI3K/AKT and/or MEK/ERK pathways are being currently studied to determine their role in breast tumorigenesis.

Abstract 2. Identification of novel breast cancer oncogenes

Breast cancer is the most common non-cutaneous malignancy among women in the United States. Oncogenic alterations that give rise to breast tumors need to be identified to develop targeted treatment options and improve clinical outcomes. This study aims to identify kinases that drive breast oncogenesis. We hypothesized that a kinase gain-of-function screen in immortalized human mammary epithelial cells will identify novel breast cancer oncogenes and provide potential targets for drug intervention. For this purpose, we used a human mammary epithelial transformation model that requires combined activation of the PI3K/AKT and MEK/ERK pathways. The ability to form tumors *in vivo* and show anchorage-independent growth *in vitro* was assayed in the screens. Kinases that scored in the primary screens and are altered in breast cancer cell lines and tumor samples are being characterized in functional studies to substantiate their role as breast cancer oncogenes.

Supporting Data

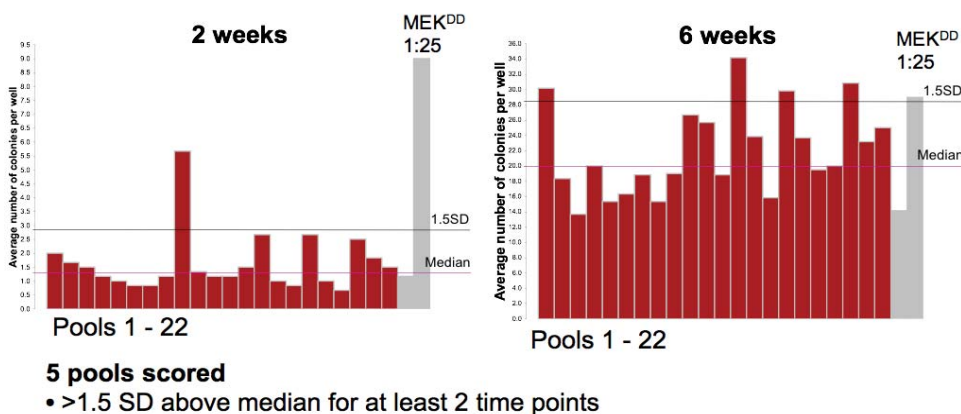


Figure 1: Pooled human kinase ORF screen in HMLE-A cells. The kinase library was divided into 22 pools. Soft agar colonies counted at 2 and 6 weeks post-plating are shown here.

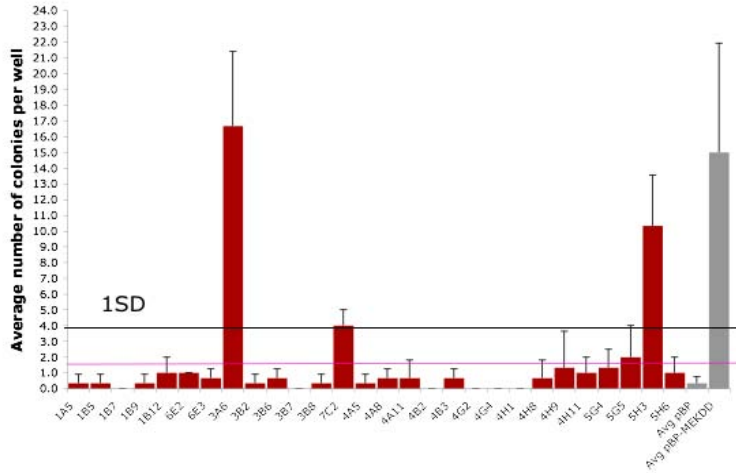


Figure 2: Three best candidates determined for further validations. Twenty-eight kinases scored as a result of deconvoluting five scoring pools. Direct comparison of their ability to promote soft agar growth determined three best candidates that are being studied further.

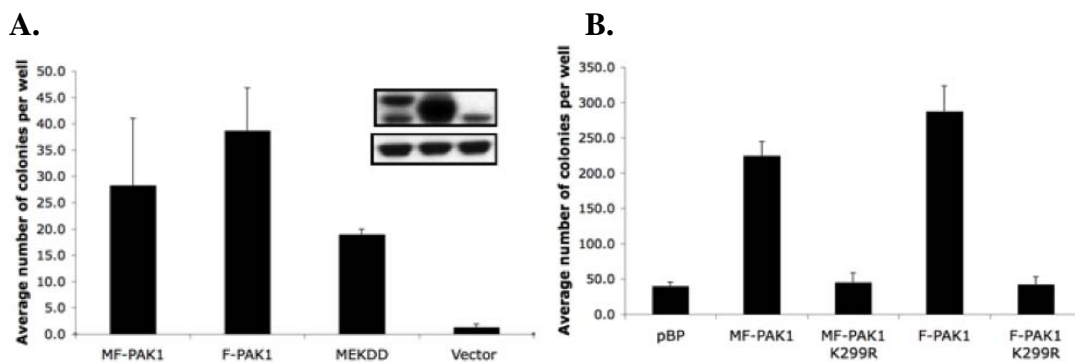


Figure 3: PAK1 characterization. A. Myr-Flag-PAK1 and Flag-PAK1 were compared with respect to their abilities to promote soft agar growth. The expression levels are shown in the inset. B. Kinase dead PAK1^{K299R} was compared with wild-type PAK1 for anchorage-independent growth of HMLE-A cells.