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TITLE: The Impact of Ethnicity-Dependent Differences in Breast Epithelial Hierarchy on Tumor Incidence and Characteristics

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Indianapolis, IN 46202

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14. ABSTRACT
The incidence of young-onset and the most aggressive triple negative breast cancer (TNBC) is significantly higher in African American than Caucasian women suggesting that the biology of normal breast epithelial cells between these two ethnic groups differ, which may contribute to altered susceptibility to tumor initiation, progression and/or metastasis. To address this hypothesis, we have generated immortalized cell lines from healthy breast tissues of African American and Caucasian women and transformed these cells with mutant p53, activated K-RAS, and PIK3CA. Unlike previous published studies, these transformed cells, particularly from Caucasian, displayed luminal progenitor phenotype. Transformed cells are being characterized for signal transduction pathway activation. Transformed cells from African American women showed aggressive phenotype with inherent plasticity and epithelial to mesenchymal transition like features. Xenograft studies are underway to determine whether these transformed cells show ethnicity-dependent differences in tumorigenecity and metastasis.

15. SUBJECT TERMS

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Introduction: As per American Cancer Society latest estimate (2006-2010) breast cancer mortality rate in women of African American ancestry (AA) is significantly higher compared with Caucasian (CA) women, despite lower incidence of breast cancer in AA. The incidence of young-onset and the most aggressive triple negative breast cancer (TNBC) is significantly higher in AA than CA suggesting that the biology of normal breast epithelial cells between these two ethnic groups differ, which may contribute to altered susceptibility to tumor initiation, progression and/or metastasis. Our goal is to elucidate whether the newly discovered unique normal breast epithelial population in AA women determines the phenotype of breast cancer in this ethnic group.

Keywords: African American ancestry (AA); Caucasian ancestry (CA); mutant $TP53^{R273C}$; mutant $KRAS^{G12D}$; mutant $PIK3CA^{H1047R}$, stem/luminal progenitor/mature cells.

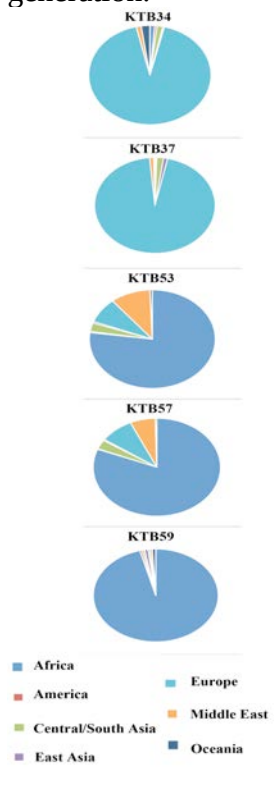
Accomplishments:

Specific Aim 1: To demonstrate that differences in normal breast epithelial hierarchy between African American and Caucasian women determine tumor behavior	**Timeline**	**Site 1**
Major Task 1 Generate transformed cells from breast epithelial cells of Caucasian (CA) and African American (AA) women	Months	
Subtask 1 Obtain IACUC and IRB approval	1-3 **Achieved**	Dr. Nakshatri
Subtask 2 Generate cells from breast biopsies of African American and Caucasian women	1-3 **Achieved**	Dr. Nakshatri
Subtask 3 Sort cells into different subpopulations using CD44/CD24, CD44/EpCAM and PROCR/EpCAM and immortalize the cells	3-6 **Achieved**	Dr. Nakshatri
Subtask 4 Perform transformation assays with SV40 T antigen/mutant p53 and activated H-Ras or FAB83B	6-12 **In Progress**	Dr. Nakshatri
Subtask 5 Subtype classification of transformed cells using assays such as PAM50	12-15 Yet to be initiated	Dr. Nakshatri
Milestone(s) Achieved: Established cell lines (at least 5 each) from breast epithelial cells of Caucasian and African American women	*Established five immortalized cell lines from African American and six from Caucasian women*	
Local IRB/IACUC Approval	3 **Achieved**	

Milestone Achieved: HRPO/ACURO Approval	6 **Achieved**	
Specific Aim 2: Establish that inter-individual heterogeneity within African Americans for unique cells determine susceptibility to breast cancer		
Major Task 3		
Subtask 1 Procure necessary tissues from IUSCC and Komen Normal Breast Tissue banks, prepare slides and standardize antibodies for IHC	1-8 **Tissues are available and latest sample availability table is provided**	Dr. Nakshatri/IHC core (Dr. Sandusky)
Subtask 2 Perform immunohistochemical characterization of healthy normal, tumor-adjacent normal and tumor (50 samples per AA and CA) total 300	9-16 **To be initiated**	Dr. Nakshatri/IHC core (Dr. Sandusky)

Rationale: Despite large body of genome-wide studies in breast cancer, molecular pathways linked to ethnicity-dependent differences in breast cancer incidence and outcome are yet to be identified. One major limitation has been the non-availability of live "normal cells" from different ethnic groups, which we have overcome. We focused on epithelial plasticity as a starting point because recent studies have demonstrated validity of this concept in vivo. It was demonstrated that cellular plasticity contributes to regenerative capacity in vivo by allowing dedifferentiation of mature epithelial cells to stem cells (Tata et al., 2013). Indeed, we recently showed elevated number of highly plastic cells in AA compared with CA women (Nakshatri et al., 2015). These inherent differences in plasticity should impact the behavior of tumor cells in CA and AA, despite the same oncogenic hit, which was examined in aim 1.

Fig. 1: Ancestry mapping of tissues used for cell line generation.



Isolation and immortalization of primary breast epithelial cells: We have generated immortalized cells lines from five AA, six CA and four from Hispanic women by overexpressing human telomerase gene (hTERT) in primary cells isolated and propagated from core breast biopsies of healthy women. Below, we describe the characteristics of few of these cell lines that have been infected with lentiviruses encoding mutant oncogenes/tumor suppressors. Two cell lines from AA (KTB59-TERT and KTB53-TERT) are of

PROCR+/EpCAM- phenotype, which represents a unique population of cells enriched in African American women and displays inherent features of epithelial to mesenchymal transition (Nakshatri et al., 2015).

Ancestry mapping to determine ethnic origin of cells used: Self-reported ethnicity does not always match with genome-driven ethnicity as determined by ancestry mapping. To ensure that we are using samples of AA and CA, all samples were subjected to highly discriminative ancestry informative 41-SNP (single nucleotide polymorphism) genomic analyses (Nievergelt et al., 2013). As shown in **Fig. 1**, all AA samples used in this study are enriched for African ancestry, whereas all CA are enriched for European ancestry.

Transformation of primary breast epithelial cells from AA and CA women: Immortalized breast epithelial cells from African American (KTB59-hTERT and KTB53-hTERT), Caucasian women (KTB37-hTERT, KTB34-hTERT and KTB6-hTERT) and a Caucasian with atypia/hyperplasia (N3-GFP-TERT) were transformed using $TP53^{R273C}$, which has lost tumor suppressor function and gained few oncogenic functions, oncogenic mutant $KRAS^{G12D}$, oncogenic mutant $PIK3CA^{H1047R}$, in dual combination of $TP53^{R273C}$ and $KRAS^{G12D}$, $TP53^{R273C}$ and $PIK3CA^{H1047R}$, $KRAS^{G12D}$ and $PIK3CA^{H1047R}$, and in triple combination of $TP53^{R273C}$, $KRAS^{G12D}$ and $PIK3CA^{H1047R}$. These cells are currently being examined for tumorigenecity, and metastasis using *in vitro* and xenograft models. Results of *in vitro* studies are described below. These mutated genes were selected for transformation based on a recent publication that showed their ability to transform normal breast epithelial cells (Nguyen et al., 2015).

Characterization of transformed cells:

A) KTB59-hTERT (AA women): KTB59-hTERT cells were infected with lentiviruses encoding mutant proteins. Infected cells were examined by fluorescence microscopy and flow cytometry for the presence of proteins using fluorescent tags (GFP, green fluorescence protein;

Fig 2: Expression pattern of overexpressed oncogenes or mutant p53 in KTB59-hTERT cells. Fluorescence microscopy was used to measure expression of proteins tagged to oncogenes or mutant p53. Green- mutant p53, Yellow- mutant PIK3CA, Red- mutant K-RAS.

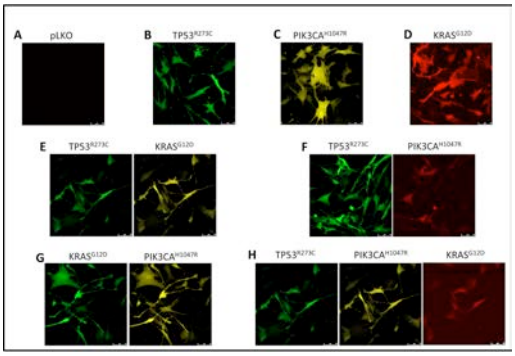
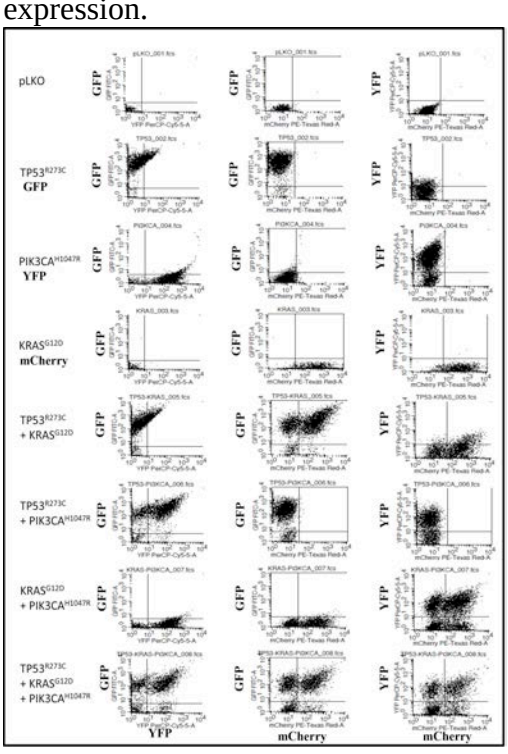
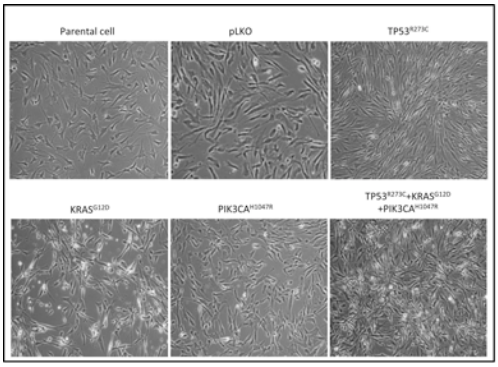


Fig. 3: Flow cytometry analyses of lentivirus-infected cells KTB59-TERT cells for oncogene and mutant p53 expression.



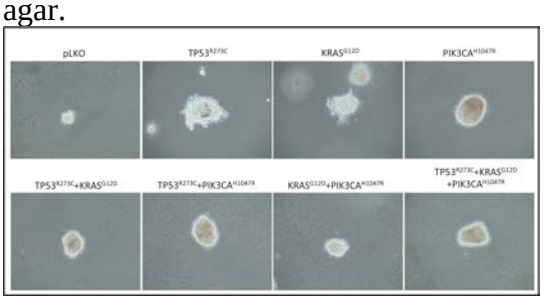
YFP, yellow fluorescence protein; or mCherry). $TP53^{R273C}$, $PIK3CA^{H1047R}$, and $KRAS^{G12D}$ are co-expressed with GFP, YFP, and mCherry, respectively, through phosphoglycerate kinase promoter within the same vector. The expression of GFP, YFP or mCherry within cells by fluorescence imaging indicated the integration of mutated genes into the genome **(Fig. 2).** GFP, YFP or mCherry populations were analyzed by flow cytometry, which further confirmed mutant gene integration **(Fig. 3).** Morphology of different cell types flow sorted for GFP, YFP and/or mCherry positivity is shown in **Fig. 4**. Cells infected with mutant $TP53^{R273C}$ were elongated, spindle shaped compared to control virus (pLKO) infected cells. Cells infected with $KRAS^{G12D}$ were elongated and less adherent. Cells infected with $PIK3CA^{H1047R}$ showed adherent characteristics and showed higher levels of cell-cell contact compared to parental cells whereas cells infected with triple combination mutants showed most aggressive phenotype with spindle shaped and less adherent morphology. Moreover, contact inhibition was lost and cells piled one above the other. Thus, the type of oncogenic insult has distinct effect on phenotype, despite common cell type origin.

Fig. 4: Morphology of KTB59-hTERT (AA) cells infected with indicated lentiviruses.



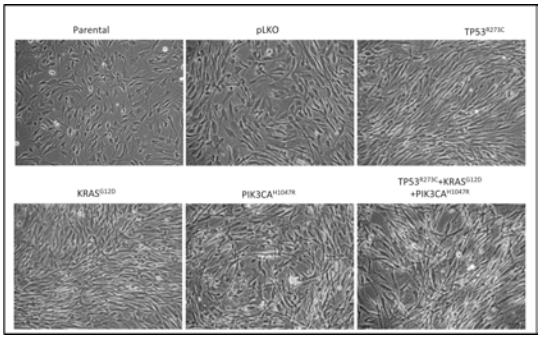
We used soft agar assay as a surrogate to further confirm transformed phenotype of oncogene or mutant tumor suppressor-expressing cells. As expected, cells expressing empty vector showed small size of colonies, while cells expressing oncogenes or mutant p53 exhibited significantly larger size colonies **(Fig. 5).** Thus, soft agar surrogate assay shows transformed phenotype of mutant p53 or oncogene-infected cells.

Fig. 5: Growth patterns of lentivirus infected KTB59-hTERT cells in soft agar.



We next established 2D cultures of transformed cells by transferring soft agar colonies to tissue culture plates. The morphology of cells prior to and after soft-agar growth was similar with triple infected cells showing aggressive phenotype **(Fig. 6).**

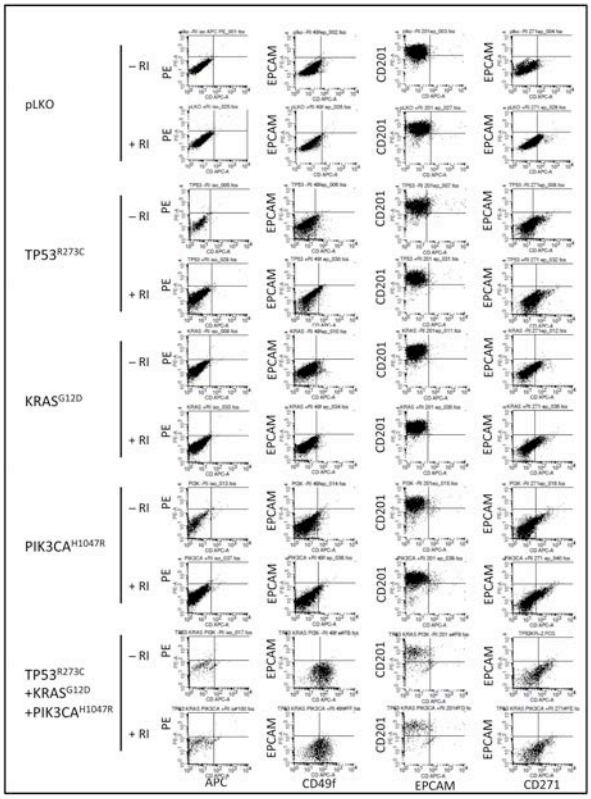
Fig. 6: Morphology of KTB59-hTERT cells overexpressing indicated oncogenes or mutant p53 replated from soft agar colonies.



We used flow cytometry to determine whether transformation affected stem/luminal progenitor and mature cell phenotypes. CD201/EpCAM combination identifies PROCR+ multipotent stem (CD201+/EpCAM-) and

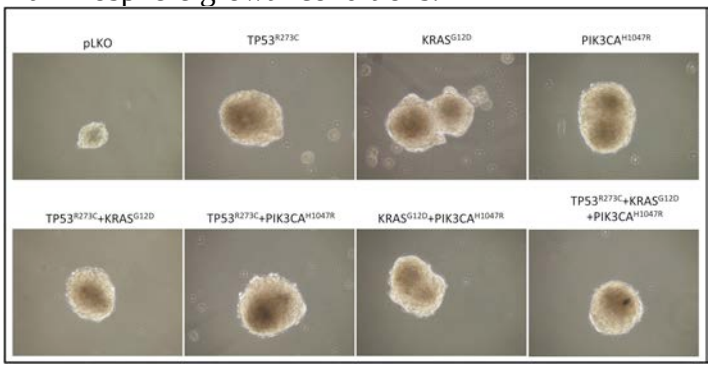
mature cells (CD201-/EpCAM+) cells. CD49f+/EpCAM-, CD49f+/EpCAM+, and CD49f-/EpCAM+ cells correspond to stem/basal, luminal progenitor, and differentiated cells, respectively. CD271+/EpCAM± and CD271-/EpCAM+ cells correspond to basal and luminal cells, respectively (Kim et al., 2012; Lim et al., 2010; Nakshatri et al., 2015; Visvader and Stingl, 2014; Wang et al., 2014). We performed these studies by growing cells in the presence or absence of ROCK inhibitor. ROCK inhibitors are believed to protect stem cells from matrix detachment-induced apoptosis (Terunuma et al., 2010). Recent studies in the lab have indicated that withdrawal of ROCK inhibitor induces differentiation of non-transformed cells. While the CD201/EpCAM and CD271/EpCAM staining patterns were similar between control and oncogene and mutant p53-infected cells, triple infected cells showed distinct CD49f/EpCAM staining pattern **(Fig. 7).** While none of the other cell types were CD49f-positive, >50 % of triple virus infected cells were CD49f-positive. Because triple-infected cells showed distinct morphology and gained CD49f expression, we believe that combination of three mutant proteins is required for efficient transformation.

Fig. 7: Stem, progenitor and differentiated cell state of KTB59-TERT cells overexpressing indicated oncogenes and/or mutant p53. Assay was done ± ROCK inhibitor (RI).



We used Mammosphere assay to characterize KTB59-hTERT transformed cells for stemness. The images were captured on 11 days after cell seeding. Empty vector transfected cells formed smaller spheres, while transformed cells exhibited significantly larger spheres **(Fig. 8).** These results suggest that transformed cells have higher self-renewal capacity.

Fig. 8: Growth pattern of oncogene and/or mutant p53 overexpressing KTB59-TERT cells under mammosphere growth conditions.



Oncogene-induced signaling pathway activation: Mutant Ras and PIK3CA are known to activate ERK and AKT pathways, respectively (Busca et al., 2016;

Yang et al., 2016). We next examined whether cells overexpressing oncogenes and/or mutant p53 show differences in the activity of these kinases. Since typical growth media for these cells contain epidermal growth factor (EGF) and insulin, which can activate these kinases, the assay was done with or without these supplements. In all cell types except triple transfected cells, phospho-ERK, particularly phospho-ERK2, was detectable only in cells grown under EGF/Insulin supplemented media **(Fig. 9).** By contrast, robust phospho-ERK (both ERK1 and ERK2) was detected in triple-infected cells even in the absence of EGF/Insulin supplemented media. Surprisingly, we did not observe AKT upregulation. Nonetheless, our results suggest that overexpression of all three mutant proteins is required for robust signaling pathway activation.

Fig. 9: pERK and pAKT levels in KTB59-hTERT cell overexpressing oncogenes and/or mutant p53.

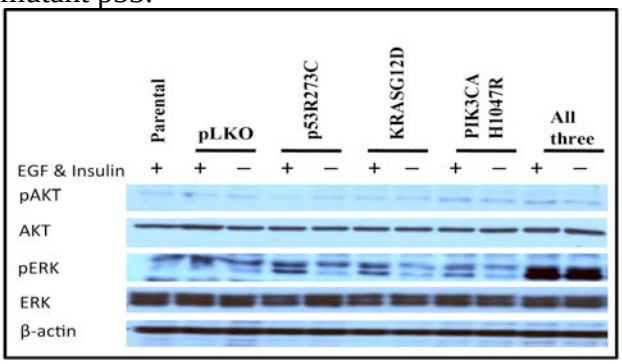
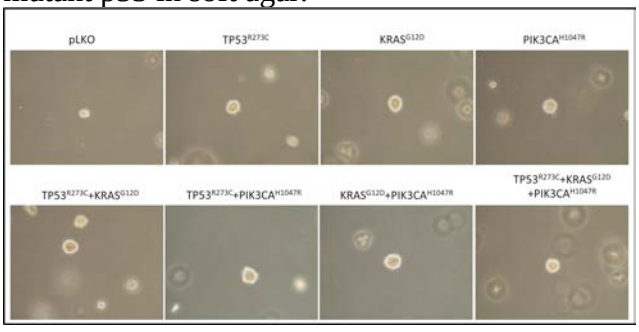


Fig. 10: Cytokeratin 14, Keratin 18 and 19 expression in three immortalized CD201+/EpCAM- cells from African American women.

Keratin expression in transformed cells: Since KTB59-TERT cells did not express EpCAM, we had to confirm that these cells are of epithelial origin. qRT-PCR demonstrated the expression of Cytokeratin 14, 18 and 19 confirming epithelial origin of these cells **(Fig. 10).** Also note that these cells contain 46 chromosomes based on karyotyping.

Ongoing studies with KTB59-TERT cells-Tumorigenecity of transformed cells: 10^6 transformed KTB59-hTERT cells (pLKO as vector control and cells obtained from three different soft-agar colonies of triple combination of $TP53^{R273C}$, $KRAS^{G12D}$ and $PIK3CA^{H1047R}$) in 100 microliter HBSS were injected into the mammary fat pad of 6-7 week old female NSG (NOD/SCID/IL2Rgnull) mice. These animals are being monitored for tumor formation.

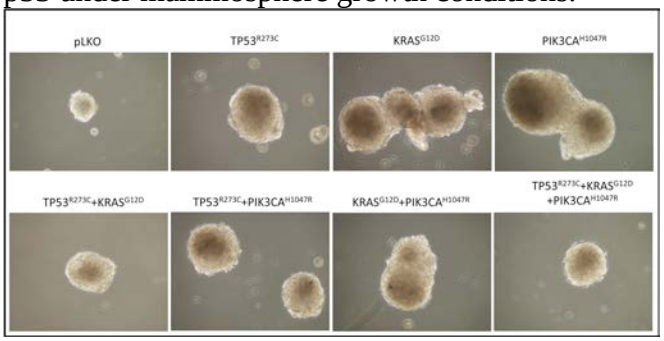
Fig. 11: Growth patterns of KTB53-hTERT cells overexpressing indicated oncogenes and/or mutant p53 in soft agar.



B) Characteristics of KTB53-hTERT (AA) cells: KTB53-hTERT cells, which are also enriched for CD201+/EpCAM- cells, were infected with GFP-$TP53^{R273C}$, YFP-$PIK3CA^{H1047R}$,

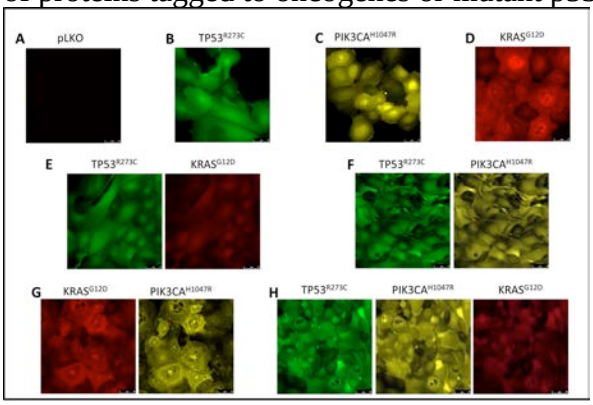
and mCherry-KRAS^{G12D} lentiviruses either individually or in combination as described above and subjected to soft-agar assay. As expected, cells expressing empty vector showed small size of colonies, while cells expressing oncogenes or mutant p53 exhibited significantly large size of colonies **(Fig. 11).** In mammosphere assay, oncogene or mutant p53-infected cells formed larger colonies suggesting enhanced self-renewal capacity of transformed cells **(Fig. 12).**

Fig. 12: Growth pattern of KTB53-hTERT cells overexpressing indicated oncogenes and/or mutant p53 under mammosphere growth conditions.



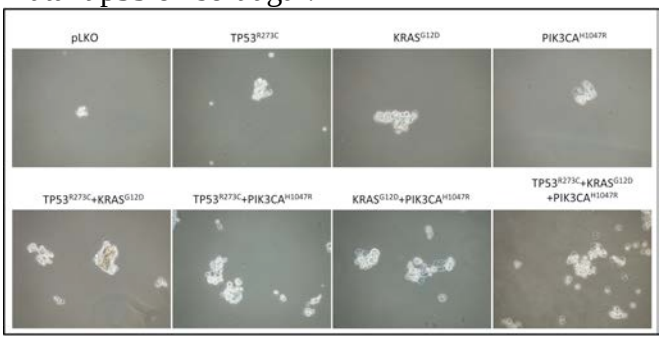
C) KTB34-hTERT (CA) cells: KTB34-hTERT cells from CA were infected with control pLKO, GFP-TP53^{R273C}, YFP-PIK3CA^{H1047R}, and mCherry-KRAS^{G12D} lentiviruses in various combinations. The expression of GFP, YFP or mCherry within cells by fluorescence imaging indicated the integration of mutant genes into cells **(Fig. 13).** Additional characterization of these cells is currently underway.

Fig. 13: Oncogene and mutant p53 overexpression in KTB34-hTERT cells (CA). Fluorescent microscopy was used to determine overexpression of proteins tagged to oncogenes or mutant p53.



D) KTB37-hTERT (CA) cells: KTB37-hTERT cells were infected with lentiviruses as above and subjected to soft-agar assay **(Fig. 14).** Oncogenes and mutant p53 significantly affected number and size of soft agar colonies. There were morphologic differences between parental and triple-infected cells. Cell-cell junctions were lost in triple-infected cells compared to parental cells **(Fig. 15).**

Fig. 14: Growth pattern of KTB37-hTERT (CA) cells overexpressing indicated oncogenes and/or mutant p53 on soft agar.



E) KTB6-hTERT (CA) cells: KTB6-TERT cells infected with either pLKO or combination of GFP-TP53^{R273C}, YFP-PIK3CA^{H1047R}, and mCherry-KRAS^{G12D} and subjected to soft-agar assay. As expected triple infected cells formed larger colonies **(Fig. 16).** Morphologically, triple-infected cells had minimum cell-cell adhesion in the presence of ROCK inhibitor, but regained cell-cell adhesion when

ROCK inhibitor was withdrawn **(Fig. 17).** This unique feature prompted us to characterize these cells for stem/progenitor cell markers. Both parental and triple-infected cells underwent some level of differentiation when grown in the absence of ROCK inhibitor, as a significant number of cells were CD49f-/EPCAM+ and CD271-/EpCAM+ **(Fig. 18).** However, in the presence of ROCK inhibitor only triple-infected cells displayed stemness or basal phenotype, as evident from the presence of CD49f+/EpCAM- and CD271+/EpCAM- cells **(Fig. 18).**

Next step: We are still in the process of transforming additional cell lines from AA women, which is proving to be difficult. Once established, these cell lines along with CA-derived cell lines described above will be subjected to xenograft assays for tumorigenecity and metastasis. These cell lines, particularly before and after triple-infection, will also be subjected transcriptome and proteome analyses to identify genes/proteins deregulated upon transformation in an ethnicity-dependent manner. Such genes/proteins will be used for the analyses described in aim 2.

Progress on aim 2: Although we have not initiated this aim, we are in constant contact with our tissue procurement facility to obtain sufficient samples for the analyses. Table below shows number of currently available samples, which are sufficient for our studies.

4. Impact: Results obtained to date suggest that there are individual differences in phenotype of cells that are transformed by defined oncogenes. We also observed that combinations of p53, K-ras and PIK3CA mutations confer aggressive phenotype to transformed cells. Since we have been successful in transforming CD201+/EpCAM- cells that are enriched in African American women compared to Caucasian and Hispanic women, we are eagerly waiting for the outcome of studies in xenograft models. We anticipate that tumors derived from these cells show resemblance to basal-

Fig. 15: Morphology of pLKO and triple infected KTB37-hTERT cells. Note differences in cell-cell junctions.

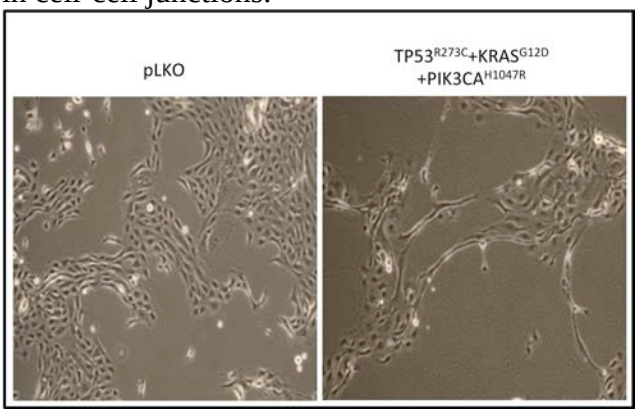


Fig. 16: Growth pattern of KTB6-hTERT (CA) cells overexpressing both oncogenes and mutant p53 on soft agar. Assay was done ± ROCK inhibitor.

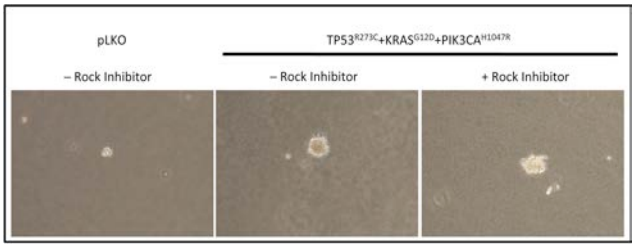
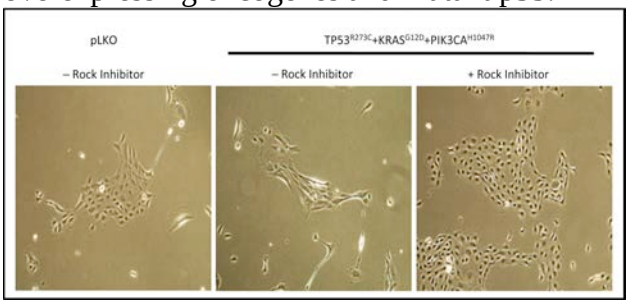


Fig. 17: Morphology of KTB6-hTERT cells overexpressing oncogenes and mutant p53.



like and mesenchymal-stem like tumors with higher intratumor heterogeneity as evidenced clinically in breast cancers of African American women (Keenan et al., 2015). Unlike previously used models of in vitro transformation of human mammary epithelial cells, which are always basal cells and do not recapitulate clinical situation, the model system employed here provides cancer cells with luminal progenitor and mature cell features, which recapitulates clinical situation. Furthermore, we have developed a reversible differentiation system by withdrawing ROCK inhibitor in the media for a specific period to determine how induction of differentiation alters behavior of transformed cells. Positive results from such efforts will provide insights into potential mediators of cancer cell differentiation, which can be exploited clinically in future.

Fig. 18: Stem/progenitor/mature cell state of parental, pLKO empty vector containing and oncogene/mutant p53 overexpressing KTB6-hTERT cells. Please see CD49f expression differences ± ROCK inhibitor (RI).

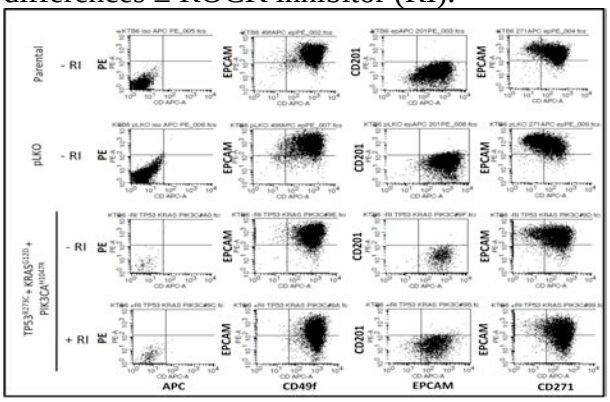


Table: List of samples available for aim2.

Ethnicity/Race	Total Samples	Tumor			Normal/Benign/Normal Adj			Matched	
		Fixed	Frozen	Total	Fixed	Frozen	Total	Fixed	Frozen
Asian	31	6	4	10	9	12	21	2	3
African American/Black	790	103	48	151	248	218	466	56	11
Caucasian	2461	357	146	503	1129	829	1958	192	47
Hispanic/Latino	26	5	3	8	7	11	18	1	1
Native Hawaiian	5	0	0	0	3	2	5	0	0
Unknown/Other	13	1	6	7	1	5	6	0	0

5. Changes/Problems: No changes

6. Products:

Publications, conference papers, and presentations: Nothing to Report
Website(s) or other Internet site(s): Nothing to Report
Technologies or techniques: Nothing to Report
Inventions, patent applications, and/or licenses: Nothing to Report
Other Products: Nothing to Report

7. Participants & Other Collaborating Organizations

(1).

Name:	Prof. Harikrishna Nakshatri
Project Role:	PI
Researcher Identifier (University ID Number):	**20200001165457**
Nearest person month worked:	11
Contribution to Project:	Prepared primary cells and supervised the entire project and

	wrote the report
Funding Support:	**Partially by W81XWH-15-1-0707**

(2).

Name:	Brijesh Kumar
Project Role:	Post-Doctoral Fellow
Researcher Identifier (University ID Number):	**20200095058465.**
Nearest person month worked:	7
Contribution to Project:	Dr. Kumar has performed the mentioned work.
Funding Support:	**W81XWH-15-1-0707**

(3)

Name:	Mayuri Prasad
Project Role:	Post-Doctoral Fellow
Researcher Identifier (University ID Number):	**20200095026778.**
Nearest person month worked:	6
Contribution to Project:	Dr. Prasad did few of the ROCK inhibitor and cells from high risk individuals
Funding Support:	**W81XWH-15-1-0707**

(3). Collaborating Organizations: Nothing to Report

8. Special Reporting Requirements

Collaborative Awards: Nothing to Report

Quad Charts: Nothing to Report

9. Appendices: No appendices

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