Differential methylation of the KRT5 gene in ER+ vs. ER- breast cancer cells

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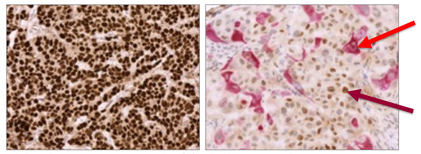
Background/Hypothesis

About 75% of newly diagnosed breast cancers are estrogen receptor α (ER) positive. While endocrine therapy (ET) is the standard of care and provides long term stabilization, intratumoral heterogeneity and ER expression is associated with resistance and recurrence. ER+ tumors often contain subpopulations of ER- cells expressing the intermediate filament cytokeratin 5 (CK5). Our group and others have demonstrated that ER-/CK5+ cells exhibit all the hallmarks of cancer stem cells (CSCs) and that CRISPR/Cas9 knockout of CK5 impairs CSC properties.
CK5+ cells can preexist or be acquired during ET. The mechanisms which determine ER and CK5 heterogeneity are unknown.



Patient derived xenografts: (L) UCD65, ubiquitously expresses ER (brown) (R) UCD15, heterogeneously expresses ER and CK5 (pink)

Epigenetic reprogramming of the ER (ESR1) promoter through CpG island methylation is known to occur during tumor progression, leading to loss of expression.

We hypothesize that the CK5 (KRT5) gene is regulated by DNA methylation in a reciprocal manner to ER in breast cancer cells.

The goal of this study was to identify KRT5 gene methylation in a series of cell lines with differential CK5 protein expression.

Methods/Approach

Table of breast cancer cell lines

Cell line	ER	CK5
UCD4	+	-
UCD65	+	-
T47D	+	-
T47D + Progestin (P)	+	+
EWD8*	-	+
MDA-MB-468	-	+

*EWD8 cells are derived from T47D cells cultured in long-term estrogen deprivation.

1. Immunocytochemistry of breast cancer cell lines for ERα and CK5 expression.
2. Process of bisulfite conversion and non-methylation specific PCR.
3. Sanger sequencing of bisulfite DNA aligned to genomic DNA sequences.
4. ChIP-qPCR for Histone tail marks H3K4me3, H3K27Ac and H3K27me3 in the proximal and distal KRT5 promoter. Single cell ATAC sequencing performed in the same cell-lines/treatments.

Fig. 1: ER/CK5 expression in breast cancer cells

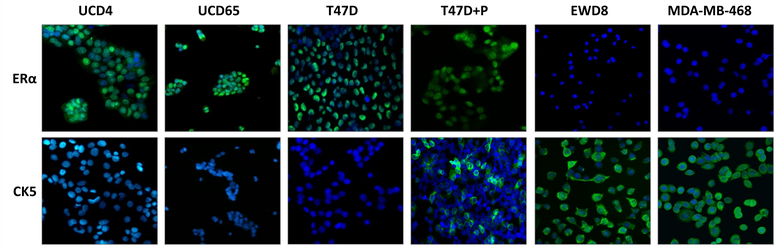


Fig 1. Relationship between ER and CK5 expression in breast cancer cells. Cells immunostained for ER and CK5 demonstrate a reciprocal relationship in expression. T47D cells treated with progestin for 24 h induce CK5 expression in a subpopulation of ER- cells.

Fig. 2: Process of Bisulfite Sequencing

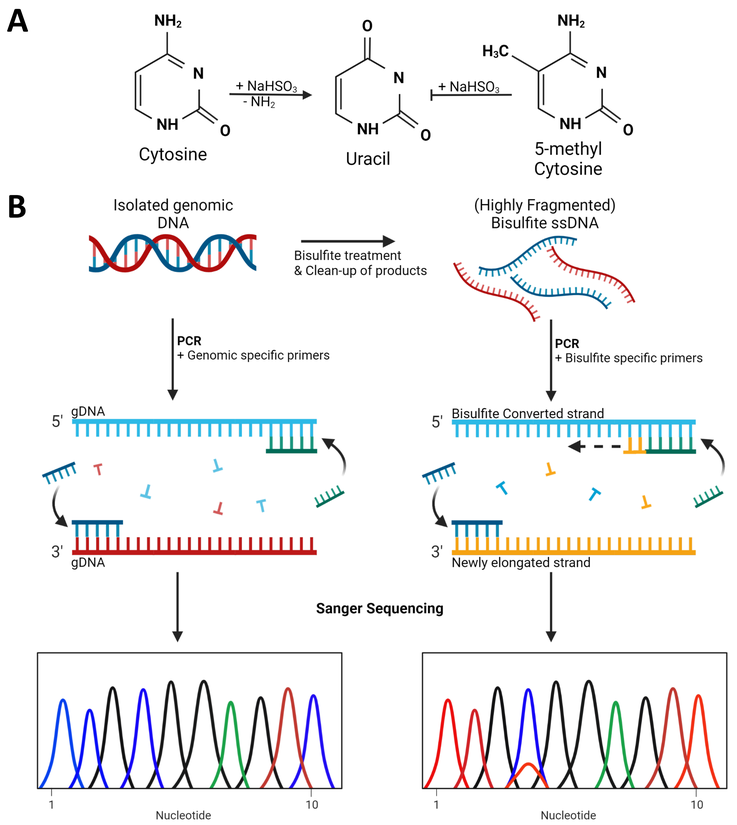


Fig 2. Bisulfite conversion and non-methylation specific PCR. (A) Treatment with sodium bisulfite selectively deaminates unmethylated cytosine residues rendering uracil. 5 methyl-cytosine (5mC) does not undergo conversion. **(B)** Purified genomic DNA is treated with sodium bisulfite (Qiagen EpiTect bisulfite). Unmethylated cytosine residues are converted to uracil with >99% efficiency. Conversion yields non-complimentary DNA. Single stranded products are then transferred to a PCR reaction containing primers designed with specificity for one strand of the uracil containing DNA, excluding regions containing CpG dinucleotides. PCR products are subsequently amplified and sequenced. Only 5mCs are preserved in the sequence, while unmethylated residues are represented as thymine. (C= blue, G= black, T= red, A= green).

Fig. 3: Methylation status of KRT5 gene correlates with protein expression

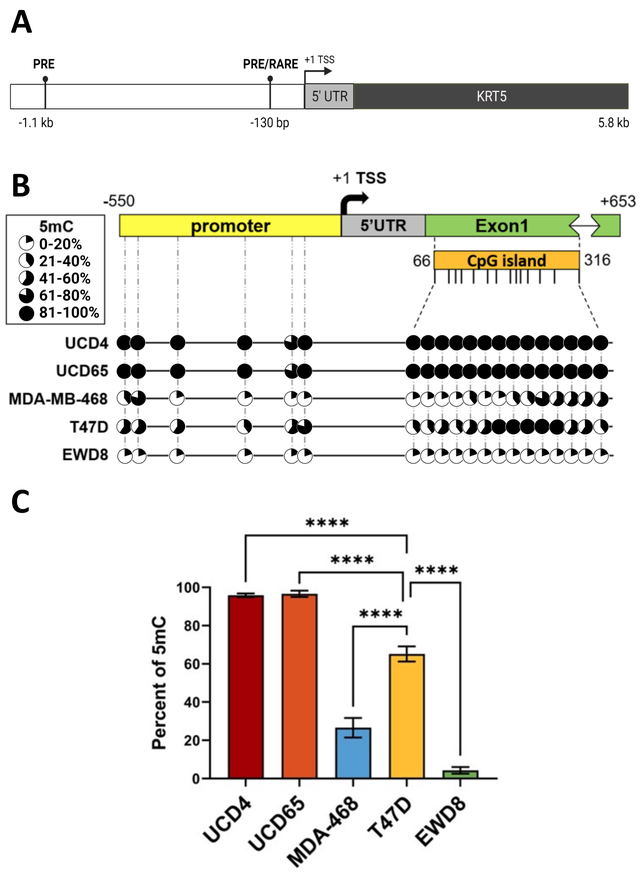


Fig 3. Differential CpG methylation at KRT5 gene in breast cancer cell lines. (A) KRT5 gene, including 1.1 kb upstream of the transcriptional start site (TSS). Designated are hormone responsive elements (HRE) for progesterone receptor (PRE) and retinoic acid receptor (RARE). **(B)** Differential CpG methylation of the KRT5 proximal promoter and exon 1 in breast cancer cells. 20 CpG dinucleotides were identified as being subject to methylation. The percent of 5mC at each loci is indicated. **(C)** Average percent of 5mC across the exon 1 CpG island in the cell lines indicated. One-way ANOVA, Tukey comparisons to T47D cells are indicated,****p<0.0001.

Fig. 4: KRT5 chromatin state suggests role of additional epigenetic programs

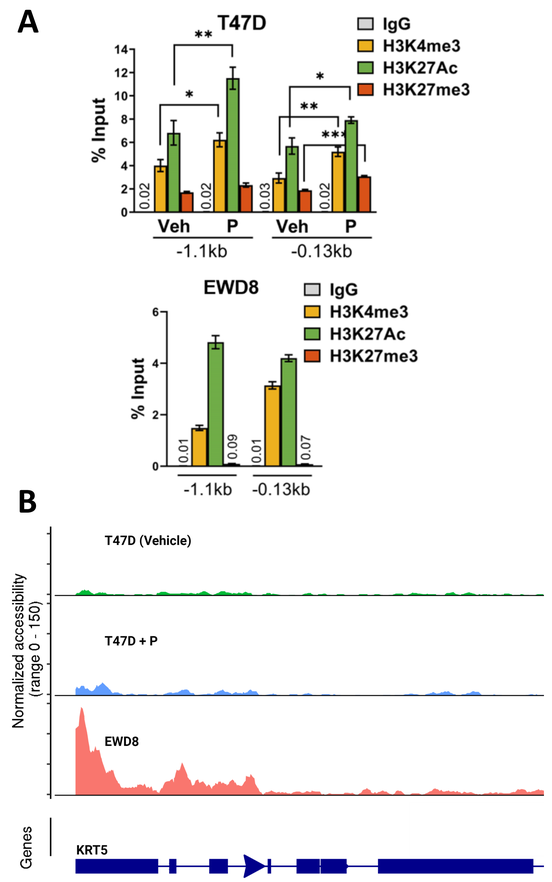


Fig 4. KRT5 chromatin accessibility. (A) ChIP-qPCR for active (H3K4me3, H3K27Ac) and repressive (H3K27me3) histone marks at the CK5 proximal (-0.13 kb) and distal (-1.1 kb) HREs in T47D cells treated with vehicle, 10 nM of the progestin R5020 (P), or in EWD8 cells. Mean +/- SEM of % input of sextuplicate samples, t-test *P*-values,*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. **(B)** Single cell ATAC sequencing tracks for KRT5 gene in the same cell lines/treatment. Peaks represent degree of accessible chromatin.

Conclusions & Future Directions

Conclusions

- KRT5 expression is subject to regulation by promoter and exon 1 methylation.
- Estrogen withdrawal is sufficient to alter KRT5 methylation status.
- Histone tail modifications suggest the role of multiple epigenetic programs in regulating CK5 expression.

Future Directions

- Determine methylation status of ESR1 and PGR in our panel of cell lines.
- Test whether inhibition of methyltransferase (DNMT1) increases CK5 expression in CK5 negative cells.
- DNMT3a-dCas9 and TET1-dCas9 system. Catalytically inactive Cas9 serving as a platform for targeted DNA methylation or demethylation of CK5 and subsequently test if this is sufficient for CSC properties.

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