CRISPR/Cas9-engineered Immortalized Breast Epithelial MCF10A Reporter Line for EMT Studies and Anti-cancer Drug Discovery

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Abstract

Metastasis is responsible for most cancer-related deaths. One mechanism of metastasis involves epithelial-to-mesenchymal transition (EMT), a process characterized by the decrease in cell adhesion and increase in cell motility. Cells undergoing EMT often display downregulation of epithelial markers (such as E-cadherin; ECAD) and upregulation of mesenchymal markers (such as vimentin; VIM). Besides metastasis, EMT has also been reported to be associated with other pathological conditions, such as acquired therapeutic drug resistance.[1] Given the roles that EMT plays in the pathological processes, it is of increasing interest as a target for anti-cancer treatment and drug discovery.[2] *In vitro* reporter models have proven to be a valuable tool for dissecting the signaling pathways that regulate the EMT process and for screening compounds targeting EMT. In previously developed EMT reporter cell lines, the reporter gene was driven by a truncated EMT marker gene promoter.[3-5] Therefore, the establishment of a more physiologically relevant reporter cell model is critical for advancing our knowledge of EMT.

ECAD, a hallmark of epithelial cells, has been implicated in the onset of metastatic dissemination.[6] Using CRISPR/Cas9 knock-in technology, we generated an ECAD-emerald green fluorescent protein (EmGFP) reporter model with immortalized breast epithelial MCF10A (ATCC® CRL-10317™) cells. In the reporter cells, the EmGFP gene was tagged at the C-terminus of ECAD, allowing for real-time monitoring of EMT progression in live cells. The targeted knock-in of the ECAD-EmGFP allele was verified at the genomic DNA, transcript (mRNA), and protein levels. Functional evaluation of the reporter cell line revealed that treatment of ECAD-EmGFP reporter cells with TGF-β led to EMT induction, as demonstrated by a reduction in ECAD-GFP expression and increase in VIM and fibronectin expression. Additional functional characterization revealed that the reporter cells possessed an enhanced migration capacity upon EMT induction with TGF-β. In summary, this MCF10A-ECAD-EmGFP reporter cell line serves as a physiologically relevant *in vitro* cell model for studying EMT cancer biology and anti-EMT drug discovery.

Results

I. Background Information

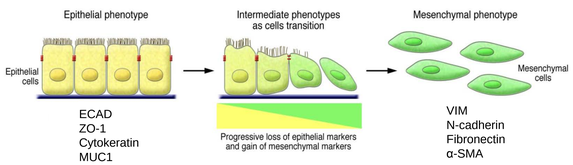

Figure 1. The dynamic and plastic model for EMT.
EMT involves a functional transition of polarized epithelial cells into mobile mesenchymal cells. During EMT, cells display progressive loss of epithelial features, and gain of mesenchymal features. A few commonly used epithelial and mesenchymal cell markers are listed: ZO-1, zona occludens 1; MUC1, mucin 1. This diagram is adapted from *J Clin Invest. 2009;119(6):1420-1428, 2009.*

II. Generation of ECAD-EmGFP Knock-in Allele

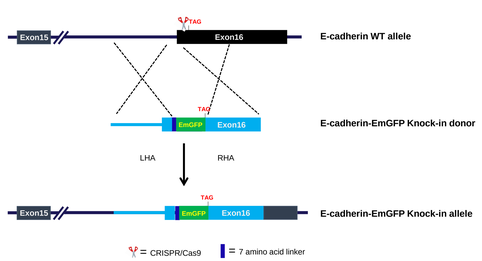

Figure 2. Gene-editing design for the generation of ECAD-EmGFP knock-in allele in MCF10A cells.
MCF10A parental cells were transfected with ECAD gRNA and knock-in donor, along with the Cas9-expressing plasmid. Transfected pooled cells were used for single cell sorting and expanded single cell clones were then subjected to genotyping to identify ECAD-EmGFP knock-in clones. In this design, the EmGFP gene is incorporated into the last exon of the ECAD gene, allowing for real-time monitoring of EMT states. LHA, left homology arm. RHA, right homology arm.

III. Identification of ECAD-EmGFP Knock-in Allele

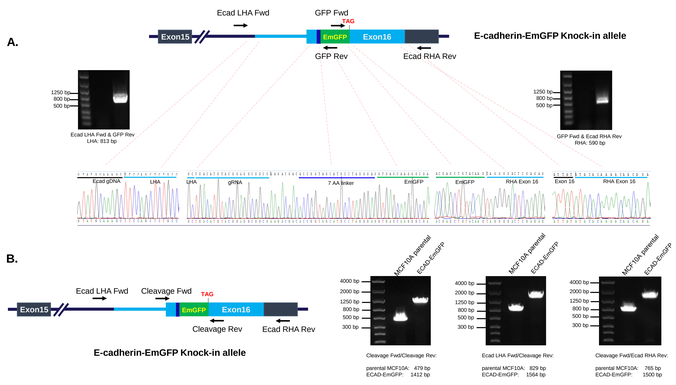

Figure 3. Identification of ECAD-EmGFP knock-in MCF10A single cell clones.
(A) Genomic DNA was extracted from expanded MCF10A single clones for left homology arm (LHA) and right homology arm (RHA) junction PCR. Junction PCR primers and their positions (Ecad LHA Fwd/GFP Rev for LHA, and GFP Fwd/Ecad RHA Rev for RHA) are displayed in the diagram. PCR amplicons were separated on agarose gels and then subjected to Sanger sequencing. Successful ECAD-EmGFP knock-in clone displays the expected sequences at both LHA and RHA junction regions. (B) To determine if ECAD-EmGFP knock-in clone is homozygous for ECAD-EmGFP knock-in allele, genomic DNA from parental and ECAD-EmGFP MCF10A cells was extracted for PCR with the primers shown in the diagram. PCR with primer pairs Cleavage Fwd/Cleavage Rev, Ecad LHA Fwd/Cleavage Rev, and Cleavage Fwd/Ecad RHA Rev detected the appropriate wild type ECAD amplicons from parental MCF10A cells, and ECAD-EmGFP knock-in amplicons from ECAD-EmGFP cells, indicating ECAD-EmGFP cells only contain ECAD-EmGFP knock-in at endogenous ECAD locus. Sanger sequencing of amplicons confirmed both wild type ECAD sequence from parental cells and ECAD-EmGFP knock-in sequence from gene edited cells.

IV. Confirmation of ECAD-EmGFP Knock-in Allele

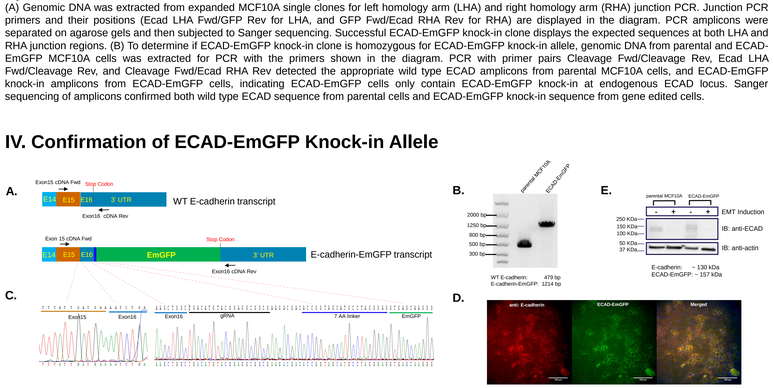

Figure 4. Confirmation of ECAD-EmGFP knock-in transcript in MCF10A single cell clone.
(A) Diagram displays the exon structure of wild-type ECAD and ECAD-EmGFP partial transcripts. (B) To confirm only ECAD-EmGFP mRNA was transcribed from endogenous ECAD allele, cDNA from parental and ECAD-EmGFP MCF10A cells was synthesized and subjected to PCR with the primers displayed in diagram (A) PCR amplicons were separated on agarose gel. A 479 bp WT ECAD transcript was detected in parental MCF10A cells anda 1214 bp ECAD-EmGFP transcript, but no wild-type ECAD transcript, was identified in ECAD-EmGFP cells, confirming the genotyping results shown in Figure 3. (C) Sanger sequencing confirmed ECAD-EmGFP knock-in junction region sequence of the transcript from ECAD-EmGFP cells. (D) Immunocytochemistry (ICC) was performed on ECAD-EmGFP cells by using an ECAD antibody (left). Cells showing ECAD positive (left, red) also express GFP (middle, green), as shown in the merged image (right, yellow), confirming the correct knock-in of ECAD-EmGFP allele. (E) To verify the expression of ECAD-EmFP fusion protein, parental and ECAD-EmGFP MCF10A cells were treated with TGF-β to induce EMT, thereby decreasing ECAD and ECAD-EmGFP expression. Non-induced and EMT induced parental and ECAD-EmGFP cells were subjected to western blot. ECAD antibody detected approximately 130 kDa. wild type ECAD protein and approximately 157 kDa. ECAD-EmGFP fusion protein in non-induced parental and ECAD-EmGFP cells, respectively. Upon EMT induction, the expression of these proteins were significantly decreased, as expected. These data confirmed that ECAD-EmGFP fusion protein was expressed in ECAD-EmGFP MCF10A cells. Actin was used as a loading control.

V. Growth Kinetics and Morphology of ECAD-EmGFP Cells

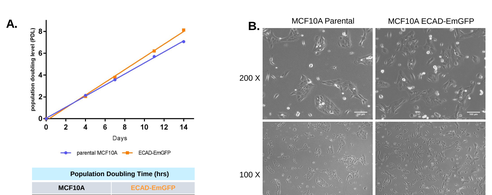

Figure 5. Growth properties and morphology of ECAD-EmGFP cells.
(A) Population doubling time of MCF10A parental (blue line) and ECAD-EmGFP (orange line) cells was measured over 14 days. The observed population doubling time is 47 hours for parental MCF10A cells and 43 hours for ECAD-EmGFP cells, this is a difference of approximately 8% and indicates the growth kinetics of ECAD-EmGFP cells are similar to that of parental MCF10A cells. (B) Parental MCF10A and ECAD-EmGFP cells were imaged at 100x and 200x magnification power. ECAD-EmGFP cells displayed a morphology similar to that of the parental MCF10A cells.

VI. ECAD-EmGFP Cells Undergo EMT Upon Induction

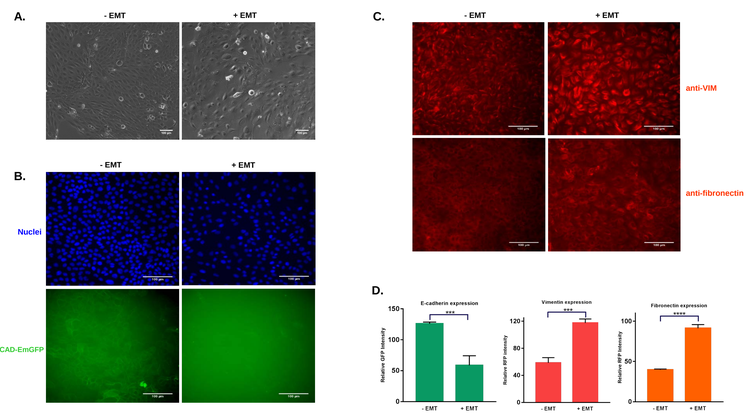

Figure 6. EMT induction in MCF10A ECAD-EmGFP cells.
MCF10A ECAD-EmGFP cells were incubated in complete growth media supplemented with either 1X StemXVivo EMT Inducing Supplement (R&D Systems; + EMT) or an equivalent volume of 1X Dulbecco's Phosphate-Buffered Saline (- EMT as a no treatment control; ATCC® 30-2200™) for 5 days. (A) The morphology of ECAD-EmGFP cells changed from a cobblestone appearance to cells of a spindle-like shape upon EMT induction. (B) Induction media incubation induced a significant decrease in ECAD-EmGFP protein expression (green; bottom left and right). The nuclei of cells were counterstained with DAPI (blue, top left and right). (C) ICC was performed on control and EMT induced ECAD-EmGFP cells. EMT induction significantly increased the expression of mesenchymal markers, VIM (red, top left and right) and fibronectin (red, bottom left and right). (D) High content imaging quantification analysis showed there is an approximately 2 fold decrease in GFP intensity (ECAD expression), and 2-3 fold increase in RFP intensity (VIM and fibronectin expression) upon EMT induction. (Student's t-test, ***p<0.001, ****p<0.0001)

VII. Increased Motility of ECAD-EmGFP Cells Upon EMT Induction

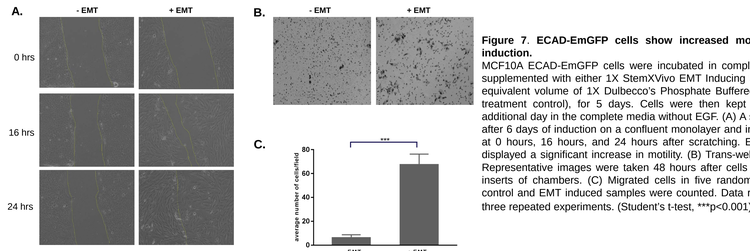

Figure 7. ECAD-EmGFP cells show increased motility upon EMT induction.
MCF10A ECAD-EmGFP cells were incubated in complete growth media supplemented with either 1X StemXVivo EMT Inducing Supplement or an equivalent volume of 1X Dulbecco's Phosphate Buffered Saline (as a no treatment control), for 5 days. Cells were then kept in culture for an additional day in the complete media without EGF. (A) A scratch was made after 6 days of induction on a confluent monolayer and images were taken at 0 hours, 16 hours, and 24 hours after scratching. EMT induced cells displayed a significant increase in motility. (B) Trans-well migration assay. Representative images were taken 48 hours after cells were seeded into inserts of chambers. (C) Migrated cells in five random fields from both control and EMT induced samples were counted. Data represent mean of three repeated experiments. (Student's t-test, ***p<0.001)

Summary

- We have successfully created an immortalized breast epithelial MCF10A ECAD-EmGFP EMT reporter cell line using CRISPR technology, in which a EmGFP gene was incorporated into the last exon of the endogenous ECAD gene.
- MCF10A ECAD-EmGFP cells undergo EMT upon induction, enabling real-time monitoring of the dynamic EMT states in live cells.
- MCF10A ECAD-EmGFP cell line is a valuable tool for studying EMT biology and for use in screening compounds targeting EMT.

References

1. Du B, Shim JS. Targeting epithelial-mesenchymal transition (EMT) to overcome drug resistance in cancer. *Molecules* 21(7): 1-15, 2016.
2. Marcucci F, *et al.* Epithelial-mesenchymal transition: a new target in anticancer drug discovery. *Nat Rev Drug Discov* 15(5): 311-325, 2016.
3. Pattabiraman DR, *et al.* Activation of PKA leads to mesenchymal-to-epithelial transition and loss of tumor-initiating ability. *Science* 351(6277): aad3680, 2016.
4. Tang HM, *et al.* An epithelial marker promoter induction screen identifies histone deacetylase inhibitors to restore epithelial differentiation and abolishes anchorage independence growth in cancers. *Cell Death Discov 2*: 16041, 2016.
5. Yun-Ju Huang R, Yo-Yan Huang T. A new dimension in drug discovery: reversing epithelial-mesenchymal transition (EMT). *Cell Death Dis* 7(10): e2417, 2016.
6. Onder TT, *et al.* Loss of ECAD promotes metastasis via multiple downstream transcriptional pathways. *Cancer Res* 68(10): 3645-3654, 2008.

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