Development of a Novel VIM-RFP Reporter Line for Colorectal Cancer EMT Study and Drug Discovery

Diana Douglas, BS, Sangeeta Kumari, MS, Luis Romero, MS, Luis G. Rodriguez, PhD, Chaozhong Zou, PhD, Robert Newman, PhD, Weiguo Shu, PhD
ATCC Cell Systems, Gaithersburg, MD 20877, USA

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Abstract

Epithelial-to-mesenchymal transition (EMT) describes a dynamic and reversible process where cells lose their epithelial characteristics and acquire mesenchymal properties. Accumulating evidence indicates that EMT displays an array of intermediate states, a phenotype referred to as "partial EMT".[1] EMT is executed in response to signaling pathway molecules and microRNAs (miRNAs) that induce the expression of specific EMT-associated transcription factors (EMT-TFs), including Zeb1/2, Snail1/2, and Twist. There is clinical evidence and an ever-growing body of research indicating that EMT plays an important role in cancer cell dissemination and distal metastasis.[2] Therefore, targeting EMT is considered a novel opportunity in anti-cancer treatment and drug development.

Vimentin (VIM), a hallmark of mesenchymal cells, is of increasing interest as a novel anti-cancer therapeutic drug target.[3] In colorectal cancer (CRC) patients, increased vimentin protein expression predicts a poor prognosis.[4] Here, we developed a novel CRC HCT-116 VIM-RFP (ATCC® CCL-247EMT™) reporter line by using CRISPR/Cas9 genome-editing technology. In this cell line, the red fluorescent protein (RFP) reporter was incorporated into the endogenous VIM gene just before the stop codon at the last exon, enabling real-time monitoring of EMT states in live cells. The VIM-RFP knock-in allele was confirmed at genomic, transcriptional, and translational levels. Functional data revealed that miRNA-200 inhibitor treatment induced the increased expression of VIM-RFP, and decreased expression of E-cadherin (CDH1), a hallmark of epithelial cells. The expression of EMT-TFs ZEB-1 and ZEB-2 was also upregulated upon induction. In addition, we showed that induced HCT-116 VIM-RFP cells displayed increased migration capacity. These data suggested that miRNA-200 inhibitor induced HCT-116 VIM-RFP cells have undergone EMT. Azacitidine, a clinically approved demethylating agent, has been extensively evaluated in a number of clinical trials as a treatment for CRC patients.[5] Studies reported that azacitidine can induce MET in a number of cancer cell lines.[2,6] We showed that azacitidine treatment of HCT-116 VIM-RFP cells can effectively induce vimentin-RFP expression, suggesting a potential application of this VIM-RFP reporter line as a platform for drug evaluation and compound screening. Taken together, the HCT-116 VIM-RFP EMT reporter line will be a valuable tool for dissecting the molecular mechanisms underlying EMT and for evaluating or screening compounds targeting EMT in CRC.

Results

I. Background Information

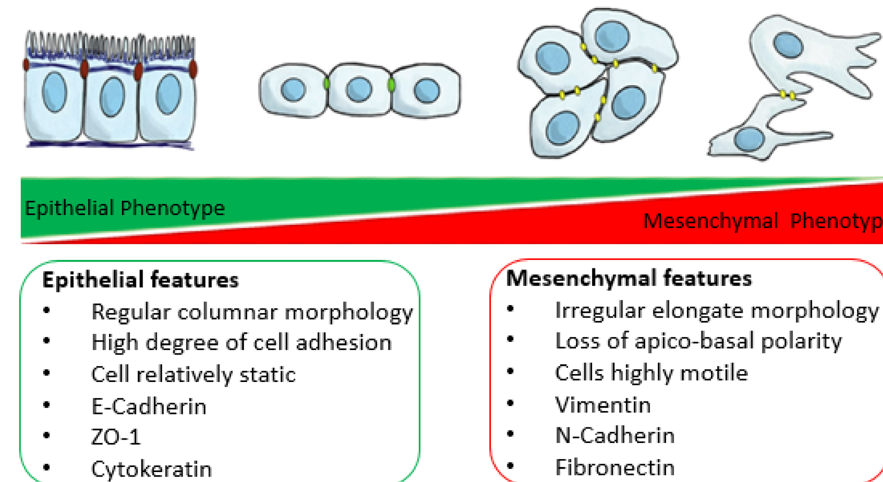

Figure 1. The dynamic and plastic model for EMT.
During EMT, cells display a progressive loss of epithelial features and gain of mesenchymal features. Rather than a binary process involving a complete conversion from the epithelial to mesenchymal state, cells undergoing EMT display an array of dynamic intermediate states, a phenotype that has been referred to as "partial EMT". This diagram is adapted from *Current Opinion in Cell Biology 55:30–35, 2018.*

II. Generation of Vimentin-RFP Knock-In Allele

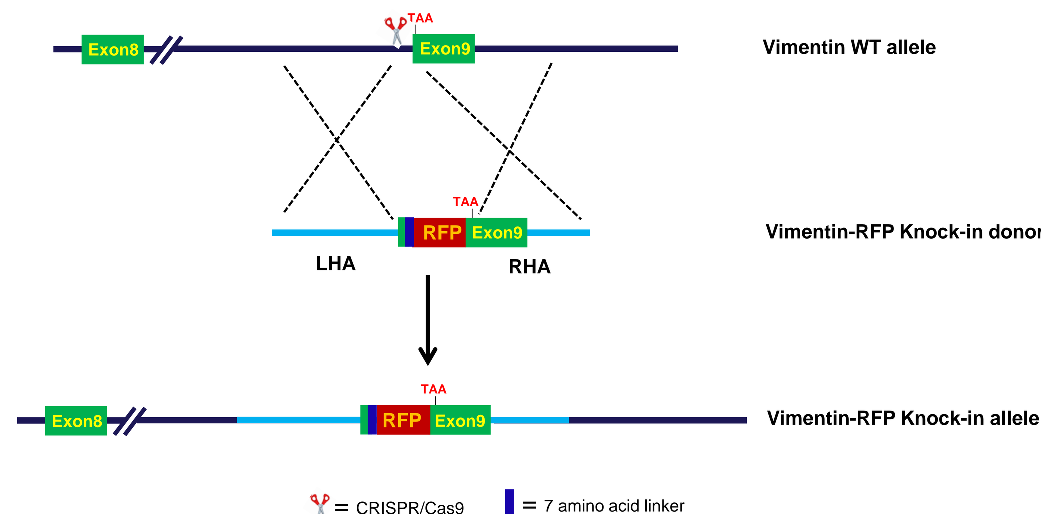

Figure 2. Gene-editing design for the generation of Vimentin-RFP knock-in allele in HCT-116 cells.
HCT-116 parental cells were transfected with Vimentin gRNA and knock-in donor, along with the Cas9-expressing plasmid. Transfected pooled cells were used for single cell sorting and expanded single cell clones were then subjected to genotyping to identify Vimentin-RFP knock-in clones. In this design, the RFP gene is incorporated into the last exon of Vimentin gene, allowing for real-time monitoring of EMT states.

III. Identification of the Vimentin-RFP Knock-in Allele

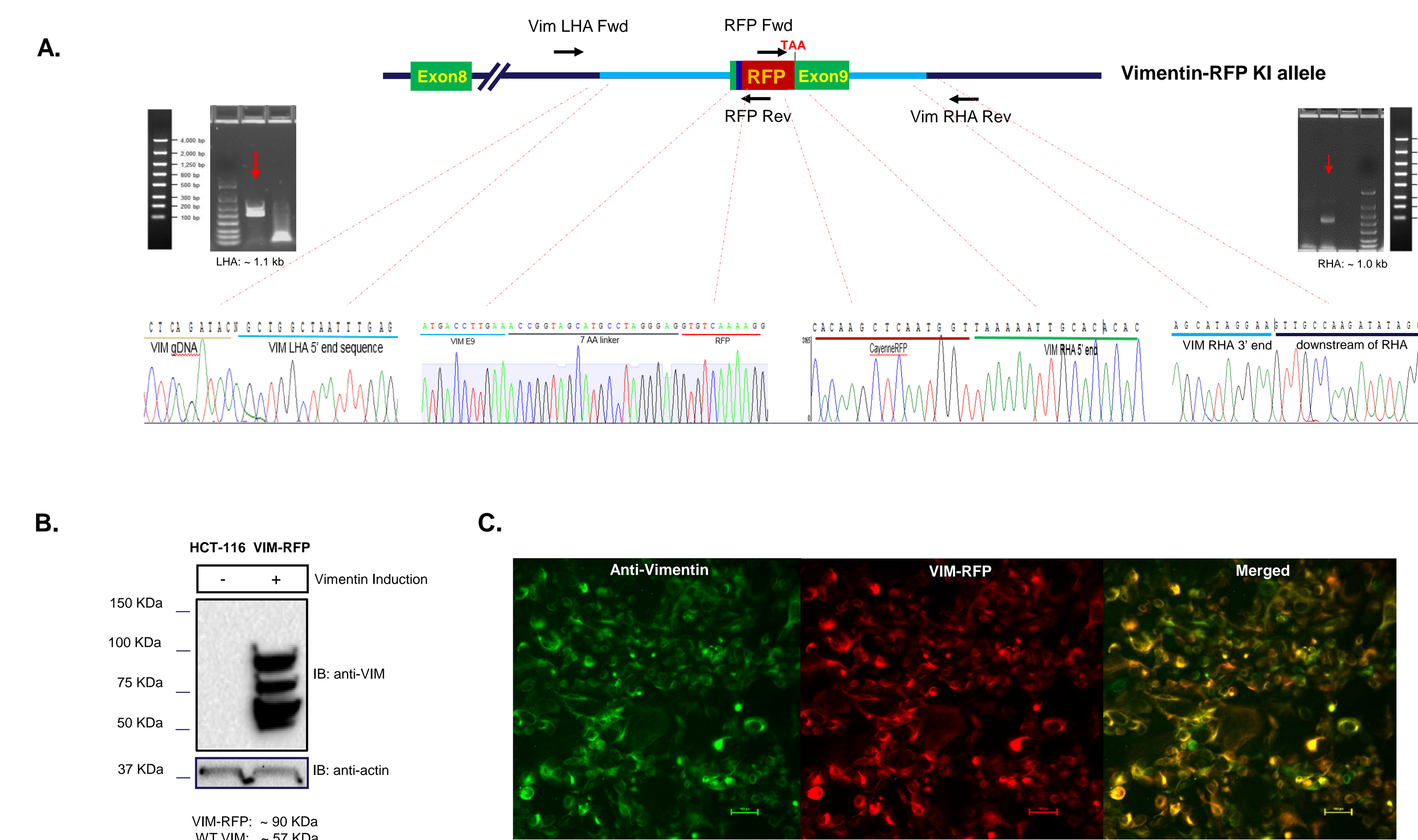

Figure 3. Identification of VIM-RFP knock-in HCT-116 single cell clones.
(A) Genomic DNA was extracted from expanded HCT-116 (ATCC® CCL-247™) single clones for left homology arm (LHA) and right homology arm (RHA) junction PCR. Junction PCR primers and their positions are displayed in the diagram. PCR amplicons were separated on agarose gels and then subjected to Sanger sequencing. Successful VIM-RFP knock-in clones display the expected sequences at both LHA and RHA junction regions. (B) Parental HCT-116 cells express low level of VIM protein. To verify the VIM-RFP knock-in allele and the expression of VIM-RFP fusion protein, VIM-RFP HCT-116 cells were treated with 5'-azacytidine to induce VIM expression. Non-treated, and 5'-azacytidine treated VIM-RFP cells were subjected to Western blot. VIM antibody detected approximately 57 kDa wild type VIM protein and approximately 90 kDa VIM-RFP fusion protein in only 5'-azacytidine induced VIM-RFP cells, but not in control VIM-RFP cells. Actin was used as a loading control. (C) Immunocytochemistry (ICC) was performed on 5'-azacytidine induced VIM-RFP cells using VIM antibody (left). Cells showing VIM positive (left, green) also express RFP (middle, red), as shown in the merged image (right, yellow), further confirming the correct knock-in of VIM-RFP allele.

IV. Growth Kinetics of VIM-RFP Knock-in Cells

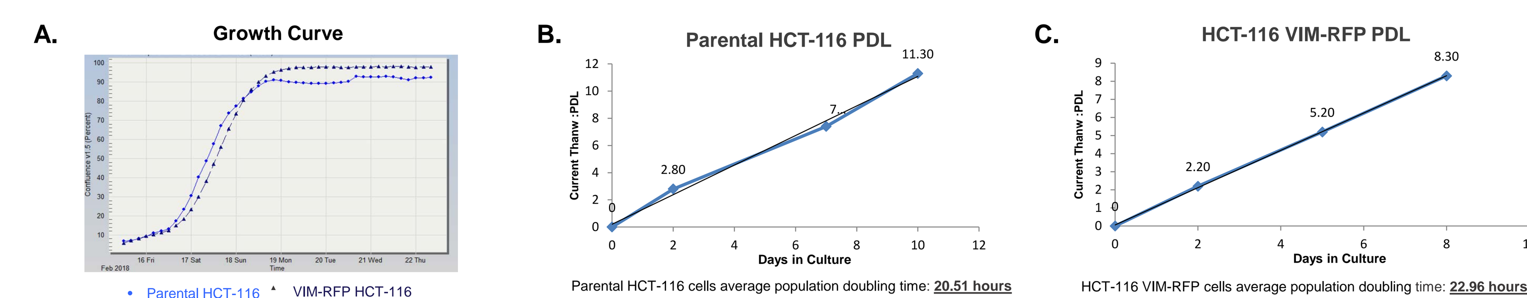

Figure 4. Growth properties of HCT-116 VIM-RFP cells.
(A) Growth curve of HCT-116 parental (blue line) and VIM-RFP (purple line) cells was measured over 7 days. (B, C) Population doubling time of HCT-116 parental and VIM-RFP cells was measured over 8-10 days. These data indicate the growth kinetics of VIM-RFP cells are similar to that of the parental HCT-116 cells.

V. Growth Kinetics of VIM-RFP Knock-in Cells

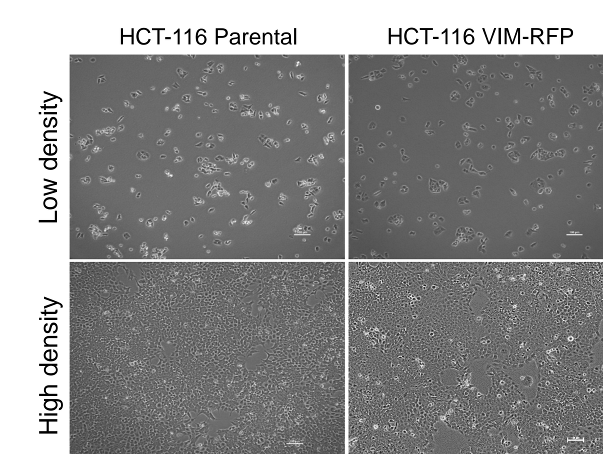

Figure 5. Morphology of HCT-116 VIM-RFP cells.
HCT-116 parental and HCT-116 VIM-RFP cells were imaged at low and high cell densities. HCT-116 VIM-RFP cells displayed a morphology similar to that of the HCT-116 parental cells.

VI. EMT Induction by Anti-miR200 Inhibitors in VIM-RFP Cells

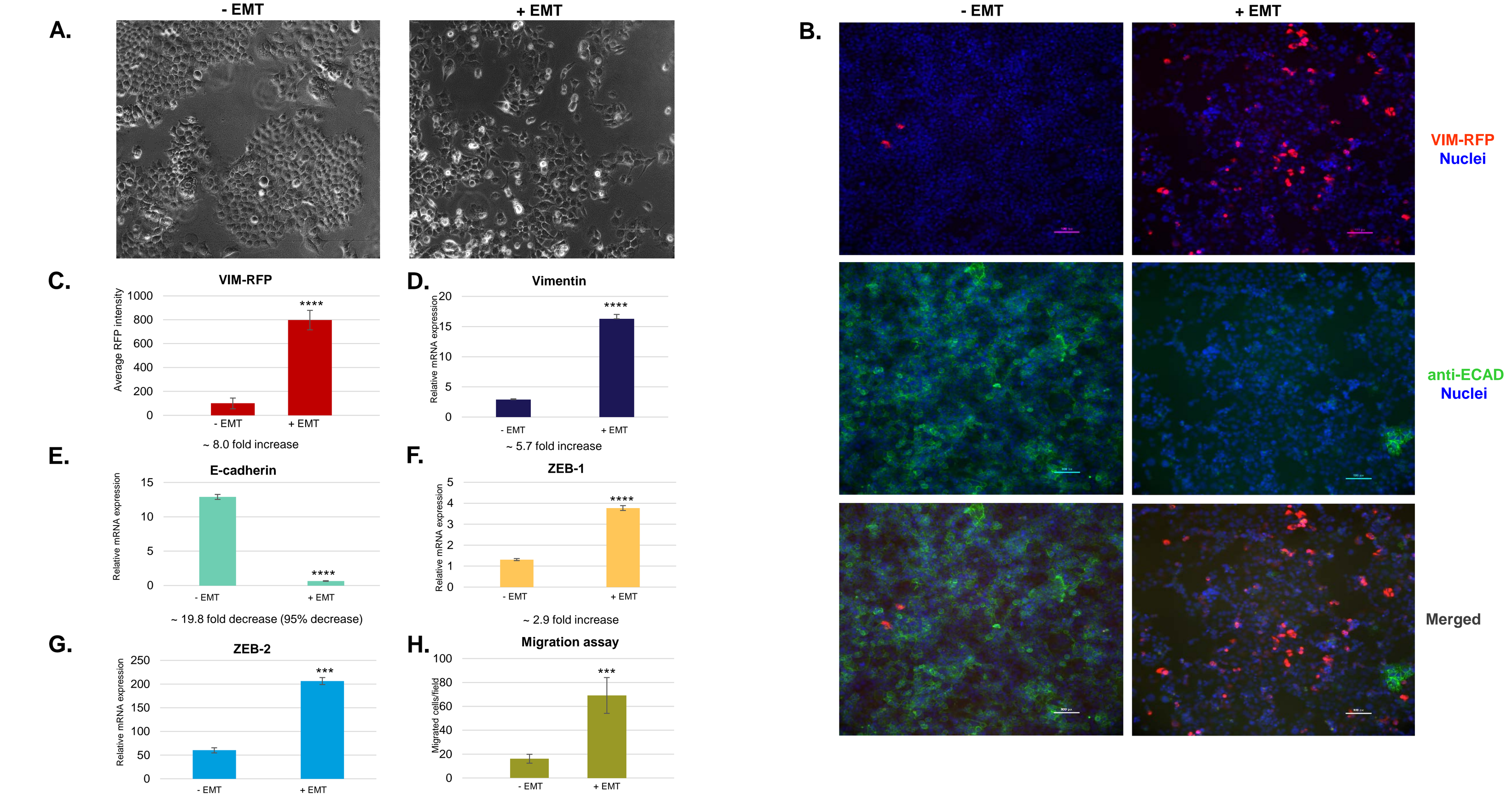

Figure 6. EMT induction in VIM-RFP cells through introduction of exogenous miR-200 family inhibitors.
HCT-116 VIM-RFP cells received six cycles of transfection with either miR-200 family inhibitors (+ EMT) or an equivalent volume of 1X Dulbecco's phosphate buffered saline (as a no treatment control, - EMT). (A) miR-200 family inhibitor treatment induced morphology changes of VIM-RFP cells. (B) miR-200 family inhibitor treatment induced a significant increase in VIM-RFP protein expression (red; top left and right), and decrease in CDH1 protein expression (green; middle left and right). The bottom panels are an overlay of the top and middle left and the top and middle right panels. The nuclei of cells were counterstained with DAPI (blue). (C) High-content imaging quantification showed there is an approximately 8-fold increase in RFP intensity upon induction. (D, E, F, G) ddPCR analysis of EMT marker gene expression revealed the increased expression of Vimentin, ZEB-1, ZEB2, and decreased expression of CDH1 upon induction. (H) Trans-well migration assay data indicated that miR-200 family inhibitor induction increased the motility of VIM-RFP cells. (Student's t-test, ***p<0.001, ****p<0.0001)

VII. Azacitidine Induction of VIM-RFP Expression

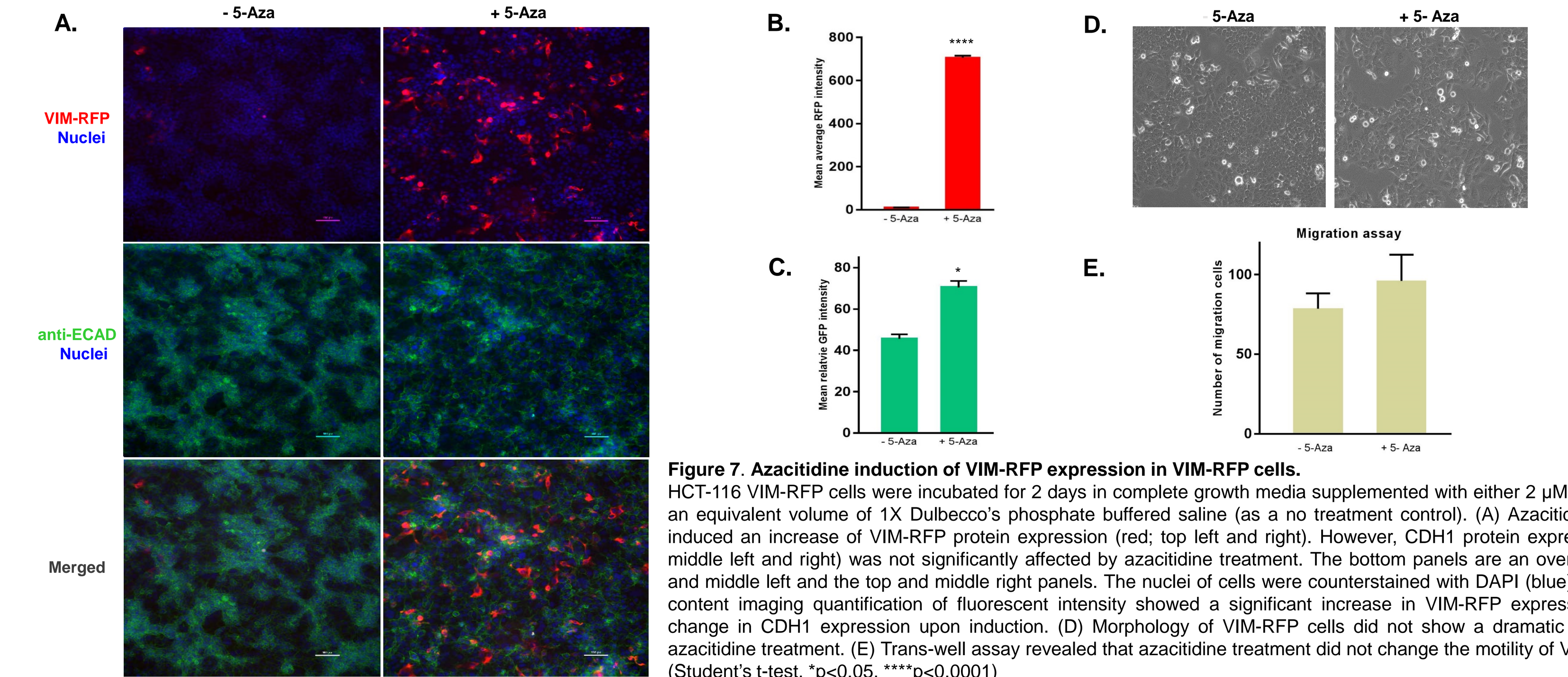

Figure 7. Azacitidine induction of VIM-RFP expression in VIM-RFP cells.
HCT-116 VIM-RFP cells were incubated for 2 days in complete growth media supplemented with either 2 μM azacitidine or an equivalent volume of 1X Dulbecco's phosphate buffered saline (as a no treatment control). (A) Azacitidine treatment induced an increase of VIM-RFP protein expression (red; top left and right). However, CDH1 protein expression (green; middle left and right) was not significantly affected by azacitidine treatment. The bottom panels are an overlay of the top and middle left and the top and middle right panels. The nuclei of cells were counterstained with DAPI (blue). (B, C) High-content imaging quantification of fluorescent intensity showed a significant increase in VIM-RFP expression, but little change in CDH1 expression upon induction. (D) Morphology of VIM-RFP cells did not show a dramatic change upon azacitidine treatment. (E) Trans-well assay revealed that azacitidine treatment did not change the motility of VIM-RFP cells. (Student's t-test, *p<0.05, ****p<0.0001)

Summary

- We created colorectal cancer HCT-116 VIM-RFP reporter cell line by using CRISPR genome-editing technology to incorporate an RFP gene into the last exon of the endogenous VIM gene.
- HCT-116 VIM-RFP cells undergo EMT upon induction, enabling real-time monitoring of the dynamic EMT states in live cells.
- The HCT-116 VIM-RFP cell line is a valuable tool for studying EMT cancer biology and for use in screening compounds that target EMT.

References

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ATCC 10801 University Boulevard, Manassas, Virginia 20110-2209 **Phone:** 800.638.6597 **Email:** SalesRep@atcc.org **Web:** www.atcc.org

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