The Generation of a NSCLC EMT Reporter Cell Line for Metastatic Lung Cancer Drug Discovery and Development

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Abstract

Cancer metastasis continues to be the leading cause of mortality in cancer patients around the world[1]. One of the prominent biological processes implicated in cancer metastasis is epithelial to mesenchymal transition (EMT). EMT has also been shown to have roles in many aspects of cancer initiation and progression, including tumorigenesis and drug resistance[2]. Despite the accumulation of a large body of data on the association of EMT with cancer, EMT has not been an active target for therapeutic development partly due to the lack of appropriate *in vitro* models. Utilizing some of the basic biology of EMT, we have created a novel advanced *in vitro* model for use in both basic research and discovery of new anti-EMT drugs.

In lung cancer, vimentin (VIM) intermediate filament proteins are associated with EMT and the metastatic spread of cancer. Vimentin expression is generally upregulated when epithelial cells transition to the mesenchymal phenotype[3]. We capitalized on this biological phenomenon using CRISPR/Cas9 gene editing to generate a VIM-red fluorescent protein (RFP) fusion reporter cell line in the A549 non-small cell lung cancer (NSCLC) cell line, which is one of the most widely used and recognized lung cancer cell lines. The A549 VIM RFP cell line harbors a C-terminal RFP tag on the vimentin gene, which enables end-point or real-time tracking of the EMT status as cells transition from epithelial to mesenchymal phenotype under defined conditions. The EMT reporter cell line was verified at the nucleic acid (genomic and mRNA) and protein levels and functional levels by cell-based assays. Bio-functional evaluation of the A549 VIM RFP cell line shows sensitivity to metastatic NSCLC drugs PP1 (SRC inhibitor) and A 83-01 (ALK5 inhibitor). These results provide the foundation for the use of this cell line in high-throughput screening (HTS) applications including the identification of new anti-EMT drugs for metastatic NSCLC. Furthermore, the A549 VIM RFP reporter cell line is also a convenient and sensitive model for basic science research on the mechanisms of metastasis.

Design of CRISPR/Cas9 Reagents to Generate VIM RFP Fusion in the Human Non-small Cell Lung Cancer Cell Line, A549 (ATCC® CCL-185™)

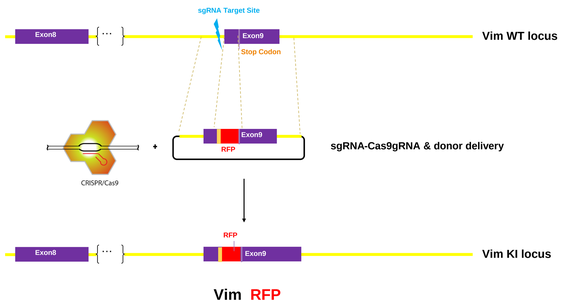

Figure 1. Identification of single guide RNA (sgRNA) target site at the VIM genomic locus. A sgRNA that was designed and built to guide Cas9 to bind and cut near the VIM stop codon, facilitated the knock-in (KI) of the VIM RFP donor template at the VIM locus upon co-transfection.

References

1. Dizon DS, *et al*. Clinical Cancer Advances 2016: Annual Report on Progress Against Cancer From the American Society of Clinical Oncology. J Clin Oncol. 34: 987-1011, 2016.
2. Lamouille S, *et al*. Molecular mechanisms of epithelial-mesenchymal transition. Nat Rev Mol Cell Biol 15: 178-196, 2014.
3. Gilles C, *et al*. Vimentin contributes to human mammary epithelial cell migration. J Cell Sci 112(Pt 24): 4615-4625, 1999.

The VIM RFP Fusion was Confirmed at the DNA and mRNA Levels

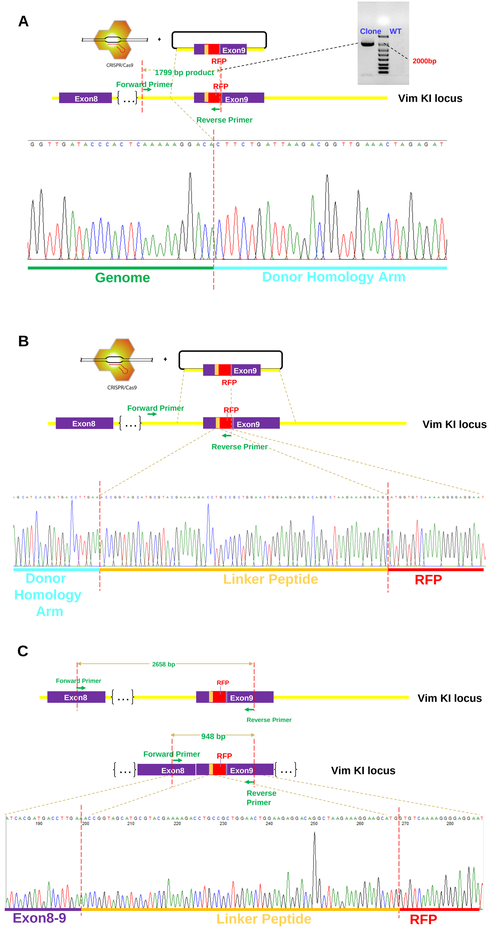

Figure 2. (A) Sanger sequencing result for the genomic DNA and donor left homology arm junction. (B) sequencing results for the donor left homology arm-linker peptide-RFP junction. (C) Sequence of VIM RFP transcript across cDNA VIM RFP junction for the isolated clone. The yellow line is the peptide sequence linking the VIM gene to the RFP sequence. The red dashed lines in the chromatogram indicate the regions where the linker peptide (yellow line) merges with the VIM exon or the RFP sequence.

Morphology and Growth Rate of the A549 VIM RFP Cell Line and Parental Cell Line are Similar

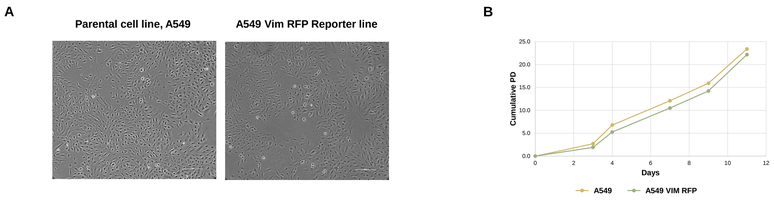

Figure 3. (**A**) Analysis of photomicrographs of the A549 cells and A549 VIM RFP cells grown under identical conditions indicates similar morphology (**B**) and similar growth rate (n=3).

A549 VIM RFP Shows Increased Mesenchymal Marker Protein Expression and Decreased Epithelial Marker Protein Expression After EMT

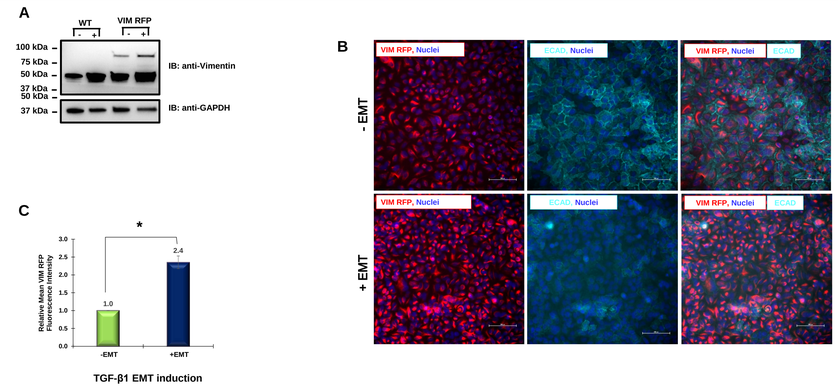

Figure 4. (A) WT A549 cells and A549 VIM RFP cells were incubated in F-12K medium containing 10% FBS supplemented with either 2.5 ng/mL TGF-β1 (+ EMT) or an equivalent volume of 1X Dulbecco's phosphate buffered saline (as a no EMT control; - EMT) for 5 days. Western blotting analysis of cell samples depict increase in WT VIM and VIM RFP expression upon TGF-β1 EMT induction. (B) Images of "- EMT" (top row) and "+ EMT" (bottom row) A549 VIM RFP cells were captured using a high content imaging system. As shown, treatment of A549 VIM RFP with TGF-β1 induced EMT and resulted in increased VIM RFP expression (red; top and bottom left). (C) The increased VIM RFP expression upon EMT induction was quantified using the system software (n=16); *p<0.05 compared with the "– EMT" control. Additionally, a decrease in E-cadherin (ECAD) expression (cyan; middle top and bottom) was observed by immunocytochemistry with an ECAD antibody-fluorescent protein conjugate. The nuclei of cells were counterstained with a nuclear stain (blue). The right panels are an overlay of the VIM RFP and ECAD expression data.

TGF-β1 EMT-Induced A549 VIM RFP Cells Have Increased Invasion Capacities

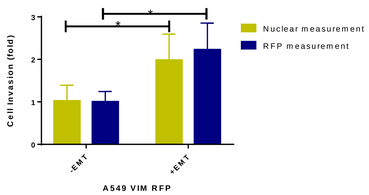

Figure 5. After a 5 day incubation with (+ EMT) or without (- EMT) TGF-β1, A549-VIM RFP cells were monitored over a 24 hr period for invasion through an 8 µm pore filter of the basement membrane of the Corning™ BioCoat™ Tumor Invasion 24 well plate. EMT-induced A549 VIM RFP cells show increased invasive capacity. The number of invading "+ EMT" nuclear and RFP cells are normalized to the "- EMT" control. The similar number of RFP positive and nuclear counterstained cells depict the utility of the VIM RFP expression to monitor invaded cells. Nuclear data represent mean±SD; n=5; RFP data represent mean±SD; n=4; *p<0.05 compared with the "– EMT" control.

Small Molecule EMT Inhibitors Block Transition in A549 VIM RFP Cells

Figure 6. Two pathways associated with EMT were targeted: TGFβ and SRC using A 83-01 and PP1, respectively. In both cases, TGFβ1-induced EMT was inhibited by the compound. The calculated Z-factor values for plates 1 & 2 depict values of 0.5 and 0.7, indicating that the assay is suitable for HTS. Error bars indicate the standard deviation over 3 wells.

Conclusion

- We have successfully generated a vimentin-RFP fusion, EMT reporter cell line via CRISPR/Cas9 gene editing
- This EMT reporter cell line (ATCC® CCL-185EMT™) has similar growth kinetics as the parental A549 cell line
- The A549 VIM RFP reporter cell line progresses from epithelial to mesenchymal phenotype upon TGF-β1 stimulation for 5 days, resulting in a strong VIM RFP signal due to upregulated vimentin expression
- The epithelial cell marker, E-cadherin, decreases in expression upon TGF-β1 stimulation, as expected for this EMT reporter
- There is increased invasion capacity and sensitivity to A 83-01 and PP1 inhibition following EMT
- The A549 VIM RFP reporter cell line can be used in HTS applications for the identification of new anti-EMT drugs for metastatic NSCLC
- A549 VIM RFP reporter cell line is a suitable and sensitive model for basic science research on the mechanisms of metastasis

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