Using CRISPR Technology to Upregulate EP4 Receptor Expression

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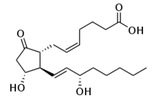
Introduction

Traditionally, in order to increase the abundance of a specific protein in cells to examine the effect on biological responses, the gene of interest is cloned into a plasmid vector under the control of a viral promoter, transfected into a cell where it more or less randomly integrates into a chromosome, and may or may not then sufficiently increase the amount of the desired protein. Typically, a 10-fold increase is considered to be high. Gene editing using CRISPR-Cas9 technology is typically used to delete or mutate segments of DNA, however a nuclease deficient Cas-9 can also be used as a delivery vehicle for transcriptional activator domains such as VP64. VP64 is composed of four tandem copies of VP16 {Herpes Simplex Viral Protein 16, amino acids 437-447*: DALDDFDLDML} connected with glycine-serine {GS} linkers. When fused to another protein domain that can bind near the promoter of gene, VP64 acts as a strong transcriptional activator. We are currently working on cloning guide RNAs for the EP4 gene promoter into the pLenti SpBsmBI sgRNA Hygro vector and then transfecting them into Ros 17/2.8 osteosarcoma cells already stably transfected with the nuclease deficient Cas9-VP64. The ultimate goal of our work is to upregulate the prostaglandin EP4 receptor in bone marrow stem cells to see if it results larger number of differentiated osteoblasts following treatment with EP4 receptor agonists.

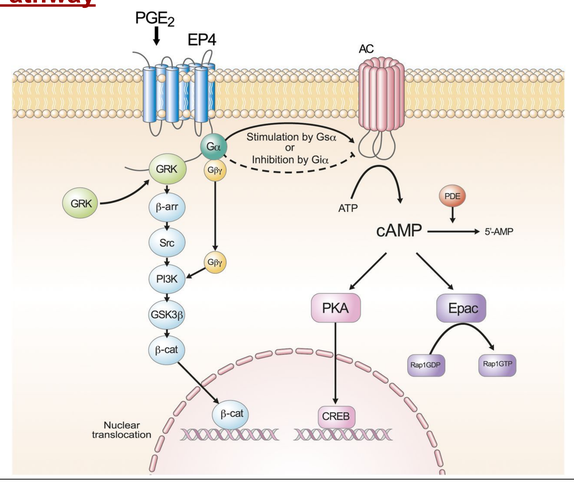
The EP4 Receptor and Importance



The EP4 receptor is one of four cell surface receptors for prostaglandin E2 (PGE2). PGE2 is a naturally occurring prostaglandin which are physiologically active lipids that function similarly to hormones. PGE2 has been implicated in regulating developmental specification of cells as well as regeneration of stem cells through the cAMP/PKA activity of the pathway. By upregulating the EP4 receptor gene PTGER4 there should be more EP4 receptors present on a cell.

Question: if significantly more EP4 receptors can be expressed on the surface of mesenchymal stem cells in bone marrow, will they, when subsequently treated with PGE2 differentiate into osteoblasts either faster or to a greater extent?

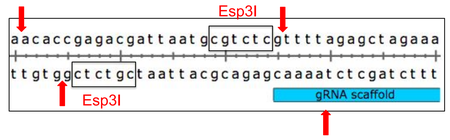
EP4 Pathway

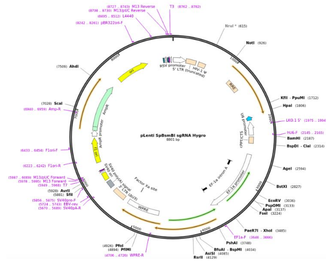


pLenti SpBsmBI sgRNA Hygro Vector

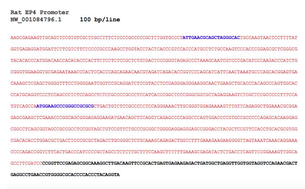
pLenti SpBsmBI sgRNA Hygro is a Lentiviral vector. Lentiviruses are retroviruses and are used as delivery vectors for gene therapy. The EP4 guide RNAs will be cloned into this vector and then will be introduced into cells along with a vector containing deadCas9 with the VP64 transcription activator domain linked to it.

Normally, the enzyme BsmBI would be used to linearize the plasmid however, it requires a 55°C incubation temperature. Instead, Esp3I (which is an isoschizmer of BsmBI) is used because it only requires an incubation temperature of 37°C. When cutting this vector with Esp3I, it leaves an overhang of 5'NNNN. The forward oligonucleotide used was P-acacC (GN19) G and P-aaaaC(N19C) G as the reverse oligonucleotide.

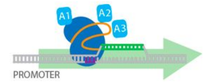




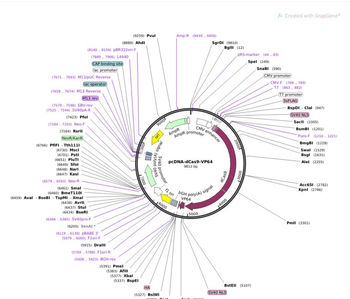
pLenti SpBsmBI sgRNA Hygro vector



rat EP4 promoter with primer sequences 58 and 607 highlighted in blue



dCas9 is fused to VP64 activator and a tagged gRNA recruits other activators



dCas9 Plasmid

Experimental Design

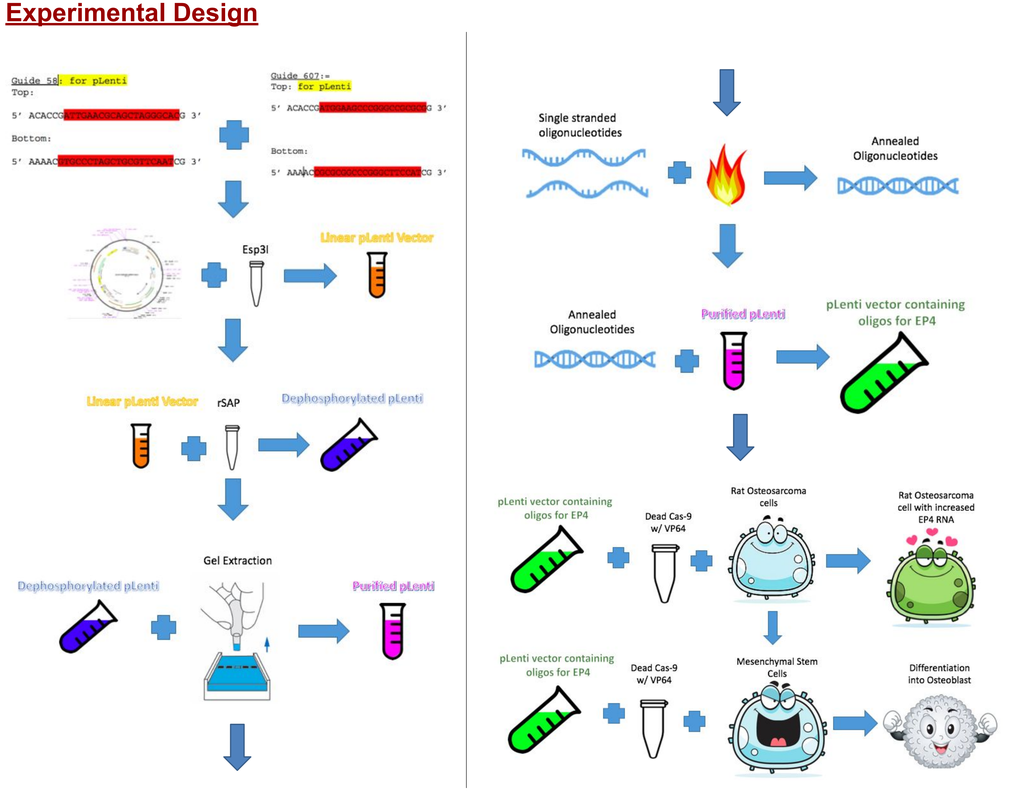


Figure 1. Flow Chart

Results So Far

1% agarose gel showing wells 1, 2, 6, and 7 having vector cut with Esp3I. Well 5 contains the uncut vector and well 4 contains size markers (NEB 2-log ladder)

Gel showing that the cut pLenti vector is the pure and correct size when compared to the ladder. The cut vector is 47.6 ng/ul vio NanoDrop

Conclusion and Futher Study

- So far, the experiment is completed up to the purified pLenti vector stage
- The next step will be to co-transfect the pLenti vector containing the EP4 guide RNAs with a plasmid containing dead Cas-9 with linked VP64 into Rat Osteosarcoma Cells (Ros 17/2.8). This will be a proof of concept to ensure that there is an increase in EP4 RNA and protein
- Another proof of concept will be to look for an increase in alkaline phosphatase enzyme activity following the EP4 agonist drug treatment (a marker of osteoblastic activity)
- The long-term goal is to use the pLenti vector to upregulate EP4 receptor expression in mesenchymal stem cells and observe any changes to cell differentiation hopefully into osteoblasts.

Acknowledgements

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