Construction of a Recombinant Exportin-7 Expression Vector

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Introduction

Erythrocytes, more commonly known as red blood cells, are a vital component of the gas exchange system, which delivers oxygen to peripheral tissues via the circulatory system. While the use of erythrocytes in oxygen transport is a common feature in all vertebrates, mammalian erythrocytes are unique in that they lack nuclei. Erythroid terminal differentiation (ETD) in mammals is the process whereby erythroblasts (precursor cells) accumulate erythroid-specific proteins such as hemoglobin, undergo extensive cellular and nuclear remodeling and ultimately shed their nuclei in a process called enucleation (Figure 1). The purpose of this project was to employ molecular genetic manipulation techniques to construct an expression vector that contains the full-length clone of the Xpo7 gene and then to express Xpo7 in Murine Erythroleukemia (MEL) cells. The Xpo7-transfected MEL cell line will then be used as a model to determine the effect of Xpo7 on their biology, to develop a western blot assay for the identification of Xpo7 protein and to identify RNA interference constructs that can cause Xpo7 gene knockdown.

Methods and Materials

I. FVA Cells

Mice were infected with the anemia-inducing strain of Friend Virus (FVA) by tail vein injection. During the course of 2-3 weeks, splenic enlargement to 10 times normal weight occurred (Right). Spleens were disrupted by forcing the spleen through a nylon mesh to form a single cell suspension. FVA-infected murine spleen erythroblasts (FVA cells) were isolated using gravity sedimentation, a technique that separated the cells by size and density. The purified FVA cells were at approximately the proerythroblast stage of development. Erythropoietin, a hormone regulator of erythropoiesis, was added to protect the FVA cells from apoptosis and induce ETD. FVA cells cultured with erythropoietin underwent terminal differentiation to reticulocytes in 48 to 72 hours.

II. Construction of the Xpo7 Recombinant Plasmid

Terminally differentiating FVA cells were homogenized and total RNA was extracted from the lyate using Pheasy9® Mini Kit (Qiagen). The Xpo7 cDNA was generated and amplified using PrimerScript™ RT-PCR Kit (Takara) and Xpo7-specific primers. The PCR product had an added adenosine (A) nucleotide on each 3’ end, which anneals to the thymidine (T) overhangs of the pGEM®-T Easy cloning vector (Promega). T4 ligase was then used to ligate and circularize the two stands and form the cloning vector (Figure 2). The cloning vector was used to transform E. coli using electroporation and the resultant transformants were selected using ampicillin-resistance and blue-white screening (Figure 3). LB broth was inoculated with a single white colony and incubated to expand the bacterial population. An aliquot of the bacterial culture was frozen in 30% glycerol stock and stored at -70°C. The recombinant plasmids were extracted from the culture using QIAprep® Spin Miniprep Kit (Qiagen). The isolated plasmids were digested with restriction enzymes SpeI, EcoRI, and BstXI (Promega); the resultant fragments were resolved using 0.8% agarose gel electrophoresis to generate a restriction map used to characterize the plasmids (Figure 4).

Results

The unexpected result may arise from an error within the GenBank® database entry for the Xpo7 DNA sequence that missed additional BstXI sites that resulted in the expected 1,522 bp band being cut into smaller fragments. Despite an anomaly with the expected BstXI pGEM™-T plasmid digest, there is a general consensus between all other fragment bands with other enzyme digest that strongly suggests that the vector contains the correct insert. The Xpo7-transfected MEL cell line will be induced to express the Xpo7 protein. The Xpo7 protein will then be purified using affinity chromatography. The purified anti-Xpo7 antibodies will be used with lysates of the transfected MEL cells to develop a western blot assay for the documentation of Xpo7 protein level and to determine the effects of Xpo7 expression on MEL cell biology. The same cell line will be used to test shRNA constructs designed to silence Xpo7 gene expression. Once the western blot assay and silencing constructs are perfected, silencing in FVA cells will be performed to elucidate the function of Xpo7 during ETD.

Future Directions

IV. Future Projects

To confirm its identity, the Xpo7 plasmid will be sequenced at Roswell Park Cancer Institute. Once the sequence is confirmed, the Xpo7 clone will be subcloned into the pGene/V5-His expression vector (Invitrogen) (Figure 5). Murine erythroleukemia (MEL) cells will then be transfected with the expression vector using electroporation and stable transfectants will be selected.