ShRNA Knockdown of ID Genes in Human Embryonic Stem Cells as a Possible Path Towards B and T Cell Development

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Abstract

Human Embryonic Stem Cells (hESCs) are pluripotent, self-renewing cells capable of becoming any cell in the human body. Previously, our lab has derived multiple cell types from hESCs, with a particular interest in hematopoietic (blood cell) development. We have been able to successfully derive natural killer (NK) cells, a type of lymphocyte with potent anti-tumor activity. However, to date we have been unable to derive other lymphocytes (T and B cells) from hESCs. Using Umbilical Cord Blood (UCB) as a comparison for early hematopoietic development, we deduced that one possible factor for this difference could be a relatively high expression of the Inhibitor of Differentiation (IDs) transcription factors in hESCs compared to UCB. ID proteins bind to and negatively regulate basic Helix-Loop-Helix (bHLH) proteins responsible for many differentiation programs within the cell. In particular, ID2 and ID3 are known to promote NK cell development and inhibit B and T cell development. My project has been to introduce shRNA constructs into hESCs to inhibit expression of ID2 and ID3 as a means to better promote T and B cell development from hESCs. Two shRNA systems have been designed. The first uses a lentiviral vector in which the shRNA construct is constitutively expressed. The second using a lentiviral vector containing a CRE-conditional shRNA expression system. We have introduced both of these vectors into Ntera2 embryonal carcinoma cells and hESCs. For the constitutive shRNAs, we demonstrate partial knockdown of *ID2* and *ID3* via qRTPCR. These constitutive knockdown ID hESCs become more difficult to culture in an undifferentiated state, and over time may have lost some of their potency. This potential problem with constitutive knockdown cells highlights the need for the inducible system, which is in progress. We anticipate that the ability to inhibit ID2/ID3 expression at specific points of hESCs differentiation into hematopoietic cells could solve this problem and facilitate development of B and T cells from hESCs.

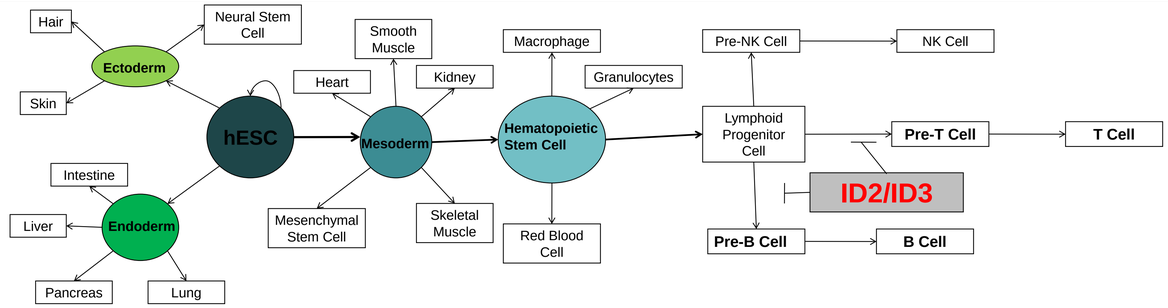
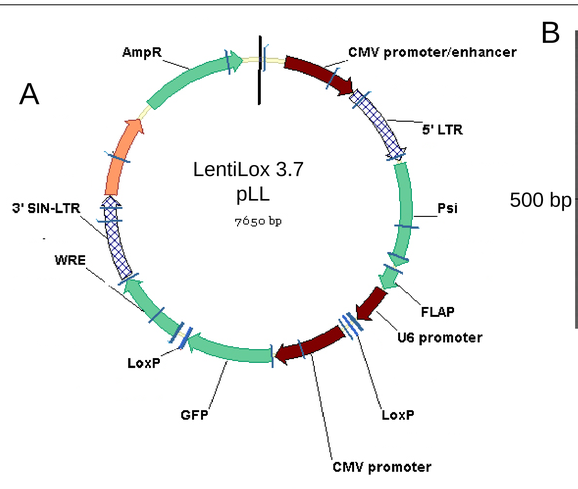
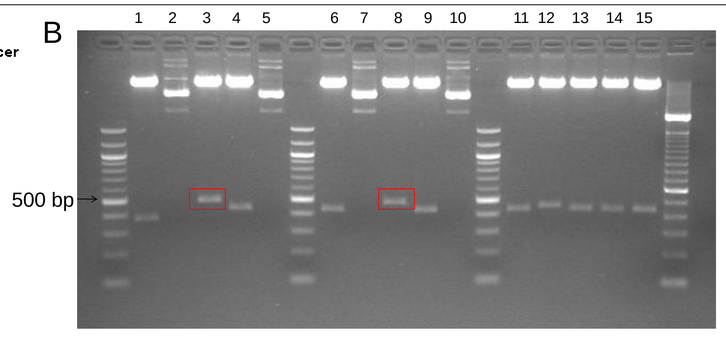


Figure 1. **Use of human embryonic stem cells (hESCs) to model human development and define role of ID2 and ID3 in T and B cell development.** hESCs have the potential to become every cell type found in the human body, as outlined in this figure illustrating the various pathways. The Kaufman lab has particular interest in blood cells, and has previously developed Natural Killer (NK) cells from hESCs, but not B or T cells. We hypothesize that inhibition of ID2 and ID3 will allow for B and T cell development.





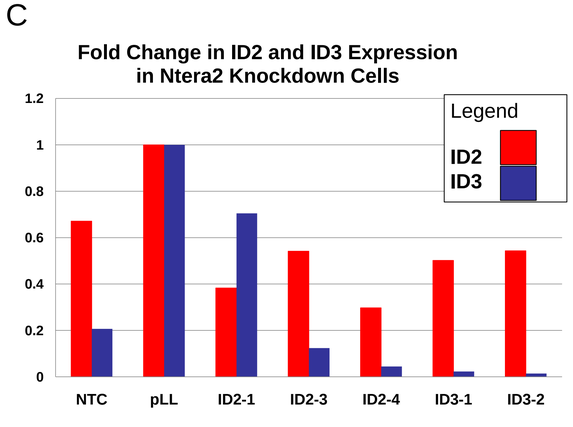


Figure 2. A. **LentiLox 3.7.** For this project we used the plasmid LentiLox vector (pLL), which was useful to us for multiple reasons. It has a viral U6 Promoter directly before the site at which a short hairpin RNA (shRNA) sequence can be inserted. shRNA binds to mRNA encoding for a specific protein (as determined by nucleotide sequence), signaling for degradation of the mRNA, preventing the protein from being created. This vector also expresses GFP, allowing for cells containing the plasmid to be detectable under a fluorescent microscope or flow cytometer. pLL also contains all of the cis-acting elements required for viral production.
B. **Plasmid Preparation.** Four shRNA sequences specific to ID2 and four shRNA sequences specific to ID3 were designed, using the guidelines provided by the McManus Lab. These sequences were inserted into a pLL vector. Plasmids were then digested to test for correct size, with a plasmid of 510 bp indicating a positive insert, and a plasmid of 460 indicating no insert. Clones positive for insert were sequenced to test for intact insert. For 6 of the 8 total shRNAs, successful clones were found. Lanes 3 and 8 had plasmids at the desired size of 510 bp, indicating that these clones were positive for the 50 bp insert.
C. **Functional Assays to Determine Best Sequence.** Ntera2 cells (a human embryonal carcinoma cell line easier than hESCs to grow and maintain) were nucleofected with each of the 6 working sequences. These cells were sorted for GFP positive cells using a flow cytometer. Relative levels of expression of ID2 and ID3 were tested against GAPDH using qRTPCR. Using this data, the ID2-1, ID2-4 and ID3-1 were selected to be used in hESCs. NTC stands for Non-Treated Control, pLL is empty (no shRNA) Lentilox plasmid, ID2-1 through ID2-4 are ID2 Knockdown Ntera2 cells with the four unique sequences, and ID3-1 and ID3-2 are the ID3 Knockdown Ntera2 cells with two unique sequences.

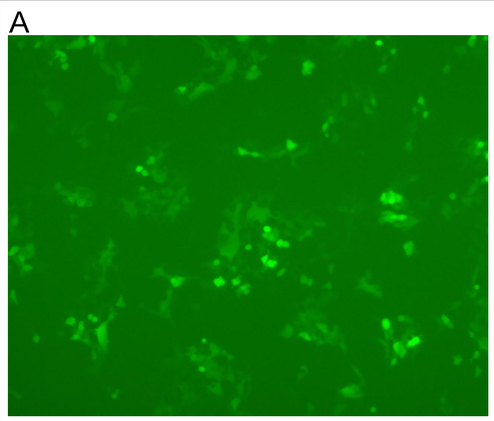


Figure 3. **Viral Production.** Lentiviral mediated gene transfer is a useful tool to introduce genes into hESCs. Four plasmids are required for transfection. These are the transfer vector (pLL containing the shRNA cassette) and 3 packaging proteins, pMDL (encoding for integrase, reverse transcriptase and structural proteins), pRev (responsible for integration of the full pLL vector) and pVSVG (which confers the viral particle the ability to transduce a wide range of cells including hESCs and Ntera2s). These four plasmids are co-transfected into 293T HEK cells, which will then begin secreting viral particles into the media, which can be collected over several days, concentrated, titered, and then be ready for infection onto hESCs.
Viral Infection. Cells are trypsinized, counted, and centrifuged. An appropriate amount of viral media to achieve a moi (multiplicity of infection, meaning number of viral particles per cell to be infected) of 2.5 or 10 is used to resuspend and replate the hESCs. The cells are now infected, and should be continued to be treated as BL2 for 2 weeks, at which point they are considered safe and should have the vector of interest (in this case pLL containing our shRNA) as part of the genome at random site or sites of integration.
A. 293T HEK cells transfected with pLL under a fluorescent microscope

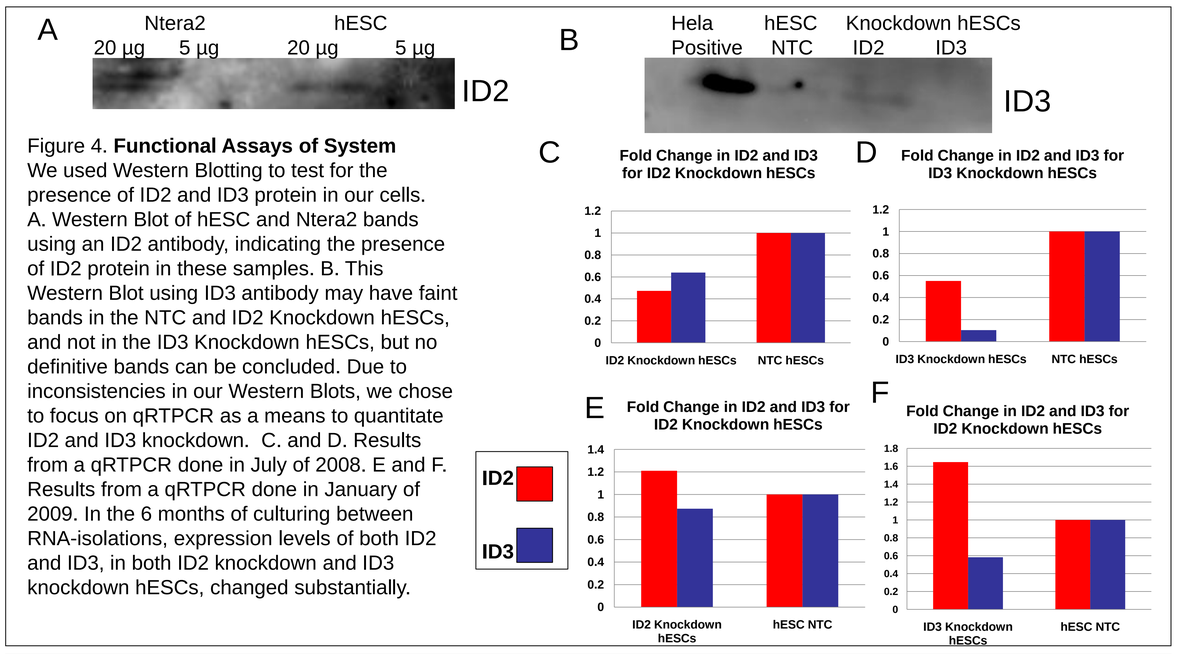
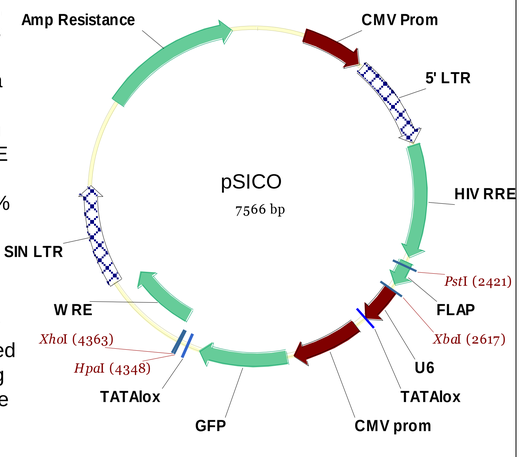


Figure 4. **Functional Assays of System**
We used Western Blotting to test for the presence of ID2 and ID3 protein in our cells. A. Western Blot of hESC and Ntera2 bands using an ID2 antibody, indicating the presence of ID2 protein in these samples. B. This Western Blot using ID3 antibody may have faint bands in the NTC and ID2 Knockdown hESCs, and not in the ID3 Knockdown hESCs, but no definitive bands can be concluded. Due to inconsistencies in our Western Blots, we chose to focus on qRTPCR as a means to quantitate ID2 and ID3 knockdown. C. and D. Results from a qRTPCR done in July of 2008. E and F. Results from a qRTPCR done in January of 2009. In the 6 months of culturing between RNA-isolations, expression levels of both ID2 and ID3, in both ID2 knockdown and ID3 knockdown hESCs, changed substantially.

Figure 5. **Future Plans.** We have moved away from the constitutive ID2 and ID3 knockdown system in favor of an inducible knockdown system, which will allow for more consistent results and easier cell culture. This new plan is already underway, with the same ID2 and ID3 shRNA sequences being used, but in a pSICO vector which can be induced via CRE administration to begin expressing the shRNA. The CRE will be administered in one of two ways; either as part of an Adenovirus, which will insert a plasmid expressing the CRE protein, or by using a TAT-CRE recombinant protein which will allow the cell to uptake CRE without necessitating a virus. These systems will allow for a population of 100% cells to have the construct in an inactive form, growing as unaltered hESCs, and then have the shRNA constructs turned on either before differentiation or at a selected, controlled time during differentiation. Currently, the inducible system has been introduced to hESCs in it's inactive form. We are currently deciding how best to acquire and apply CRE to the hESCs, and hope to have the variables of this system figured out shortly. Once that is accomplished, qRTPCRs and Western Blotting will again be used to determine the effectiveness of the construct. Once the constructs have been validated, differentiation experiments will be performed to evaluate the role of ID2 and ID3 in the development of B and T cells.



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