VISUALIZATION OF FACTOR VIII WITH FLOW-CYTOMETRY AS A TOOL FOR NOVEL GENE THERAPY APPROACH IN HEMOPHILIA A

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Graduate Students, Population, Health & Wellness

BACKGROUND

Haemophilia A is a genetic X-linked disorder, characterized by coagulation Factor VIII (FVIII) deficiency and leading to pathological bleedings. The disease occurs in a rate of 1 in 5000 males' births. The treatment is the administration of plasma-derived or recombinant Factor VIII which is expensive and leads to the development of inhibitory antibodies in around 40% of patients affected by the severe form of the disease. The disease becomes for these patients life-threatening.

New approaches to treat Haemophilia include Gene Therapy (GT). Cells corrected through genetic modifications are used to produce in Haemophilia A patients FVIII protein in a sustained manner, as long-term treatment for this disorder.

The cells of choice should be persistent and equipped with the machinery for large protein assembly and secretion. So far, target cells for Haemophilia gene correction are mostly liver cells, although they are highly immunogenic and exposed to immune-mediated destruction after GT.

Based on literature evidences, Bone Marrow Transplantation can correct Haemophilia A in mice, providing evidence that Hematopoietic Stem Cells (HSC) or their progeny are able to produce FVIII. Whereas classically FVIII protein is visualized on adherent cells through immunohistochemistry staining, Flow-Cytometry (FC) literature publications are very scarce.

FC analysis is an attractive method for analyzing hematopoietic cells, and in general, a versatile method for protein visualization. However, large proteins as FVIII are difficult to be carefully analyzed, and the method requires several steps of optimization.

AIM AND OBJECTIVES

This joint project with Dr. Muhammad Elnaggar, (postdoctoral fellow) .**aimed to** optimize a method to characterize large proteins as FVIII with a reliable FC staining protocol. To this aim:

A) Optimize and Validate a flow cytometry method to visualize FVIII in intracellular compartments and on cell membrane surfaces.

B) Evaluate the capacity of FVIII protein production in FVIII producing cells after FVIII gene transduction.

METHODOLOGY

Cell cultures and maintenance of cell lines (U937 (human pre-monocytic) cells, HECV (human vascular endothelial) cells, HELA cells) and PBMCs

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RNA extraction and quantitative PCR for FVIII Expression in RNA level in the cell lines

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Flow Cytometry detection of extracellular and intracellular Factor VIII protein through Antibodies against FVIII domains and proper IgG controls. FC analysis: FlowJo_V10

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Lentiviral vector transduction of cells at different Multiplicity Of Infection (MOIs) with the supervision of my colleague Dr. Muhammad Elnaggar

RESULTS and DISCUSSION

Optimization of The Flow-Cytometry Staining Procedure for FVIII Visualization

FVIII characterization FVIII detection in PBMCs using FVIII and IgG2a Dylight 650 NHS Ester™ labeled Antibodies

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Competition assay to assess anti-FVIII antibody by pre-incubation with recombinant FVIII protein by FC using PBMCs

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Comparison between Fc Block Vs. Mouse serum block efficiencies in reducing the intracellular non specific binding using PBMCs by FC

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Changing Antibody labelling method from NHS ester custom labelling to Zenon technology and testing on PBMCs by FC

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Gating strategy for FC analysis for FVIII (very strict/stringent gating) to insure accuracy

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Assessing effect of different cell confluence on FVIII expression

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Assessment of Lactadherin (MFGE8) blocking effect on expression of FVIII by FC

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Optimized FVIII FC staining protocol

Step		Details	Interval
1	Harvesting of cells	Wash cells once with PBS (Centrifuge). Harvest cells and count using Trypan blue & hemocytometer, and divide cells as one million cells per condition.	Centrifuge at 300xg, for 5 min at RT.
2	Live and dead staining	In 1 ml of cells in PBS, add 1 µl of live/dead dye (Incubate), according to manufacturer protocol (LIVE/DEAD™ Viability/Vitality Kit, Molecular Probes)	Incubate for 15 min in the dark at RT.
3	Staining buffer wash	Wash cells in 3 ml of staining buffer: 0.1% BSA-PBS solution (Centrifuge).	Centrifuge at 300xg, for 5 min at RT.
4	CD markers or FVIII (surface staining)	Cells are re-suspended in X µl of staining buffer for surface staining, desired labelled surface Abs are added (total volume for surface staining should be 100 µl) (Incubate).	Incubate for 30 min in the dark at RT.
5	Staining-buffer wash	Wash cells in 3 ml of staining buffer: 0.1% BSA-PBS solution (Centrifuge).	Centrifuge at 300xg, for 5 min at RT.
6	Cells Fixation	Re-suspend cell pellet in 100 µl Fixing buffer (Incubate) according to manufacturer protocol (Invitrogen™ FIX & PERM™ Cell Permeabilization Kit).	Incubate for 15 min at RT.
7	Staining-buffer wash	Wash cells in 3 ml of staining buffer: 0.1% BSA-PBS solution (Centrifuge).	Centrifuge at 300xg, for 5 min at RT.
8	Cells Permeabilization	Re-suspend the cells in 100µl of perm buffer. Add 10% final concentration of Mouse Serum (Incubate).	Incubate for 15 min at RT.
9	FVIII (Intracellular staining ǂ)	- Zenon staining: Label anti-FVIII Abs and IgG isotype control with Zenon labelling dye (according to manufacturer protocol of Zenon™ Antibody Labelling Kits Thermofisher) and add the labelled anti-FVIII Ab to the cells (Incubate). - Stably labelled anti-FVIII Abs: add to cells the labelled Abs at titrated concentration. (Incubate)	Incubate for 20 min in the dark at RT.
10	Staining-buffer wash	Wash cells in 3 ml of staining buffer which is 0.1% BSA-PBS solution (Centrifuge).	Centrifuge at 300xg, for 5 min at RT.
11	Final Cells Fixation	Re-suspend the cells in 200 µl of 2% paraformaldehyde-PBS.	–
12	Data acquisition	Acquire the results on Flow-cytometry instrument	–

ǂ During the incubation time, compensation beads are as well prepared for further FC acquisition, using UltraComp eBeads™ from Invitrogen™ with adding equivalent colors used for cellular staining.

Functional studies of FVIII production and accumulation

❖ **Lenti-viral Vector-mediated gene transfer of FVIII in U937 cells**

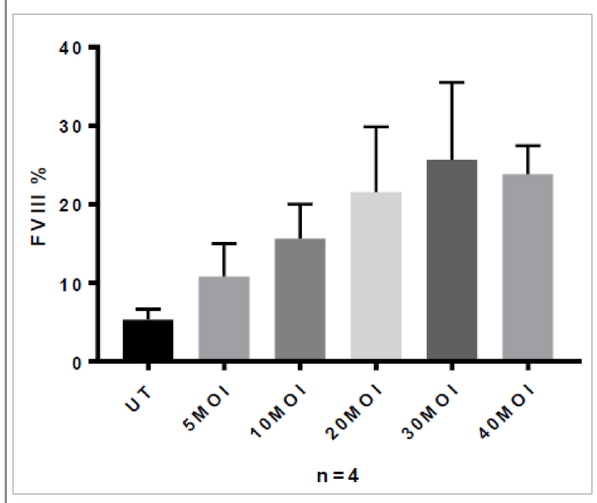
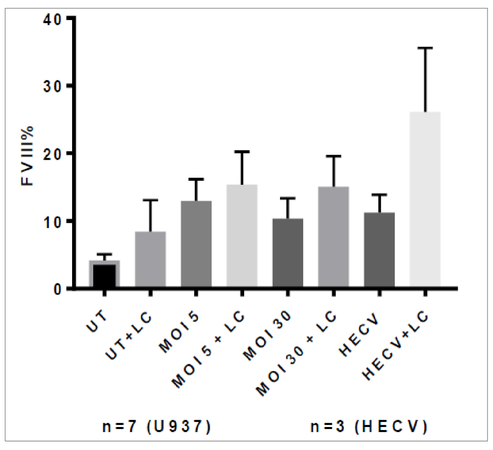


Figure 1: A 3rd generation Self Inactivating (SIN) Lentiviral vector, carrying the FVIII human gene under a Phosphoglycerate Kinase (PGK) promoter was used to transduce cell lines at different viral concentrations, measured as MOI (Multiplicity of Infection Units). MOIs of 5 to 40 (meaning 5 to 40 viral particles/cell). After a day of incubation in 37 degrees, the viruses were removed, cells washed, grown in fresh media and used for the FC experiments to be tested for FVIII expression.
As shown on the left, the genetic modification of the cells resulted in increasing FVIII expression, according to the MOI used, and plateauing at the highest MOI. This result demonstrated that FVIII transgene-derived protein can be efficiently visualized by FC. Graph shows mean+/- SEM

❖ **Evaluation of FVIII production and accumulation inside the cell using Lactacystin on U937 & HECV**

Figure 2: To study FVIII accumulation inside the cells, we blocked the proteosomal pathway using Lactacystin which is a 16S proteasome inhibition protein. Lactacystin was incubated at 50µM concentration for 4 hours prior to FC analysis with U937 cells un-transduced or transduced at 5 & 30 MOIs. Proteasome inhibition leads to accumulation of misfolded proteins in the cells.
After proteasome inhibition FVIII was higher both in un-transduced and transduced cells, as shown on the right. FVIII sharp increase after proteasomes inhibition proves that normally a good portion of FVIII is routed to proteasome for degradation. This happens both in un-transduced and transduced cells and in different cell lines.



CONCLUSION

We demonstrated that, after deep and thorough optimization, a robust FC analysis on large proteins as FVIII is possible and is of great importance. We succeeded in optimizing this protocol in PBMCs and in different cell lines, transduced and un-transduced. This suggests that FC visualization of FVIII can be broadly applied to any FVIII producing cells.

ACKNOWLEDGEMENT

I would like to give gratitude to both my supervisors (Dr. Sara Deola and Dr. Ahmed Malki). I would like to thank my colleague Dr. Muhammad Elnaggar who assisted and guided me to complete my master's thesis.