Mir-155 Downregulation by miRCURY LNA™ microRNA Inhibitor Can Increase alpha Chain Hemoglobins Expression in Erythroleukemic K562 Cell Line

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Abstract

Background: MicroRNAs (miRNA) are small non-coding RNAs that have a distinguished role in post-transcriptional gene expression. It's estimated that 10-30% of human mRNAs are regulated by miRNAs. Many miRNAs profiles change during normal erythropoiesis in which some of them are stage specific.mir-155 was downregulated 200 fold in erythropoiesis.

Materials and methods: K562cells were grown in RPMI1640 and viability tested by trypan blue. microRNA 155Inhibitor and its scramble were purchased from exiqon. K562 cells were transfected using transfection kit according to manufacture manual. After RNA extraction and cDNA synthesis, miRNA downregulation confirmed by miRNA Real time PCR, then alpha- and zeta- chain expression was investigated by RT and QRT-PCR.

Results: The viability of cells before transfection was 99% and the efficiency of mir-155 inhibitor transfection was 90%. By relative Q-PCR the zeta chain expression was increased 3.4 fold and alpha chain was increased 8.3 fold in comparison to untransfected cells.

Conclusion: This study showed that mir-155 downregulation has a distinguished role in alpha chain hemoglobin mRNA expression level. The expression of alpha chain was more than zeta chain that may be result of adult source of K562 cells. Differentiation induction by miRNA regulation without adding any growth factor can be considered as a new strategy in gene therapy and tissue engineering.

Key words: Erythroid, Hemoglobin, miRNA, mir-155

Introduction

microRNA (miRNA) are newly discovered non-coding RNA that have a distinguished role in post transcriptional gene expression. These small molecules act by binding to 3 UTR region of target mRNAs and act as fine-tune for regulation of multiple pathway.(1-4)

Each miRNA has many target genes and each gene may regulated by many miRNAs. This network of miRNA action and crosstalk of miRs with transcription factors show a large complicated network of cellular regulation in molecular level.(2)

It's estimated that 10-30% of human mRNAs are regulated by miRNAs.(3)

miRNAs may have tissue specific expression, for example miRNAs 142, 181, 223 expressed mainly in hematopoetic system. Some miRNAs such as mir-17, 24, 146, 155, 128 and 181 have role in early hematopoiesis. Mir-223 is a main miRNA to

promote granulopoiesis by blocking NFI-A and increasing of C/EBP-α transcription factors(4 -6)

About 19 miRNAs down regulated during Megakaryopoesis including miR-10a, 10b, 30c, 106, 126, 130a, 32, 143, miRNAs 223, 15a, and 16-1, exhibited a biphasic expression pattern; suggesting a stage-specific function.(7)

Many miRNAs profiles change during normal erythropoiesis that some of them are stage specific. For example mir 155, 221, 223 downregulated (200, 20, 10 fold respectively) and mir-451 upregulated 270 fold in erythrocyte differentiation.(2, 6, 8) Perhaps down-regulation of miR-221 and miR-222, unblocks KIT expression and allows erythroblast expansion.(7)

Yang used miRNA array to characterize miRNA variation of K562 cells before and after hemin treatment and showed that miR-126 exhibited up-regulation while miR-103, miR-130a, miR-210, and miR-18b exhibited down-regulation after hemin induction.(9)

Zhan et al analyzed more than 295 miRNA pofiles in erythroid differentiation by microarray. They showed that more than 100 miRNA expressed in this lineage. Also, they show similar up-regulation of miR-451, miR-24, and miR-16 and down-regulation of miR-221-222 and miR-155 during erythropoiesis.(10)

Human mir-155 is predicted based on homology to a cloned miR from mouse, Lagos-Quintana eta la described the identification of 34 novel miRNA by tissue- specific cloning in mouse. They found mir-155 (UUAAUGCUAAUUGUGAUAGGGG) in colon tissue.(11) In 2004, Kasashima et al investigated miRNAs during 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced differentiation of human leukemia cells (HL-60) into monocyte/macrophage-like cells. They found three novel and 38 known miRNAs expressed in HL-60 cells, and experimentally validated mir-155 in human HL-60 leukemia cells.(12)

Like the mouse miRNA, human mir-155 resides in the non-coding BIC transcript, located on chromosome 21. In most cases, sequences of the new mature miRNAs were perfectly conserved between human and mouse, in mir-155 case the mature form differs from that in the mouse at a single position.(13)

Eis et al. confirm that miR-155 is processed from the BIC transcript in human, and demonstrate elevated expression of miR-155 in lymphoma samples including diffuse large B cell lymphoma (DLBCL).(14)

Landgraf in collaboration with more than 20 research centers comprised miRNA expression in the hematopoietic system to all other organ systems and showed that only 5 miRNAs are highly specific for hematopoietic cells: miR-142, miR-144, miR-150, miR-155, and miR-223.

The mature sequence of mir -155 (MI0000681= CUGUUAAUGCUAAUCGUGAUAGGGGUUUU UGCCUCCAACUGACUCCUACAUAUUAGCA UUAACAG) that shown in databases represents the most commonly cloned form from Mammalian microRNA Expression Atlas Based on Small RNA Library Sequencing.(15)

While mir-155 is highly expressed in human cord blood derived hematopoetic stem cell, many reports indicate that miR-155 is down-regulated during erythropoiesis induction in primary cells or cell lines.(2, 16)

In 2009, Faraoni et al, presented mir-155 as a multifunctional microRNA. They reviewed this miRNA role in hematopoiesis, inflammation, immunity, neoplastic disease, leukemia, solid tumors, cardiovascular disease and viral infection.(17)

K562 cell line is bcr-abl positive and possesses erythroid features. Different clones of these cells express Hb Portland (ζ2γ2), Gower1 (ζ2ε2), Gower2 (α2ε2) and small quantities of Hb F (α2γ2). Although initial clones of K562 cells have shown heterogeneity in hemoglobin content. Furthermore, as a result of previous reports erythropoiesis inducer cannot induce synthesis of ß chain.(18)

So, this cell line was selected as a model for evaluation of mir-155 downregulation effect on α- and ζ- chain hemoglobin mRNA expression.

Materials and methods Cell culture: This cell line is supplied by the National Cell Bank of Iran, Pasteur Institute, Tehran, Iran. K562cells were grown in RPMI1640 medium (Gibco, USA) with 10% fetal Bovin serum (FBS) (Gibco,USA), 1X Penicillin sterptomycin antibiotic and 2mM L-glutamin at 37ºC under a humidified atmosphere consisting of 95% air and 5% CO2 for 7 days. On the day of transfection, cells were counted and tested for viability by trypan blue. Then in 6 -well format plated, 2×10^5 cells per well in 3 ml, complete growth medium.

miRCURY LNA™ microRNA Inhibitor and Scramble: miRCURY LNA™ microRNA Inhibitor purchased from exiqon(EXIQON, Denmark). This product is based on the LNA™-technology for superior specificity and biostability.

Locked nucleic acid (LNA™) nucleosides are a class of nucleic acid analogues in which the ribose ring is "locked" by a methylene bridge connecting the 2'-O atom and the 4'-C atom. LNA™ nucleosides contain the common nucleobases (T, C, G, A, U and mC) and are able to form base pairs according to standard Watson-Crick base pairing rules. However, by "locking" the molecule with the methylene bridge the LNA™ is constrained in the ideal conformation for Watson-Crick binding. K562 cells were transfected using Lipofectamine 2000 (Invitrogen, USA) according to manufacture manual, briefly one day before transfection 2×10^4 cells were plated in 2.5 cc medium with 10% FBS without antibiotic. In one tube 100 pmol mir-155 inhibitor was diluted into 250µl OPTIMEM (Gibco, USA). In another tube 5µl lipofectamin 2000 were diluted in 250µl optimum. After a 5 -minute incubation, two tubes combined and incubated for 20 minutes. Finally, this compound added to cultured plates. Transfection of scramble was done, similarly. Scramble was used for the evaluation of transfection efficiency and non-specific effects of oligonucleotides. Every 4 days, these stages were repeated. All tests were done in triplicate manner.

RNA extraction and cDNA synthesis: Total RNA was extracted from 2×10^5 cells (from 6 -well culture plate) of controlled, scrambled and anti-sense treated samples by using Biozol reagent in third and seventh days after starting of transfection. Briefly, 500µl biozol was added to counted cell and followed by phenol/cholorophorm precipitation according to the manufacture instructions. The purity examination of extracted RNA was done by spectrophotometrically at 260/280nm and gel electrophoresis.

In order to gain maximum purity, extracted RNA was treated by DNAse I (fermentas) as manufacture guidelines.

cDNA was synthesized by cDNA synthesis kit (Bioer). In sum, 20 ng of total RNA was reverse transcribed in a total volume of 20µl containing oligodt 18 primer 1 µl, AMV enzyme 0.5 µl, 5x buffer 4 µl , RNase Inhibitor 0.5 µl, dNTP mix 1 µl, RNase -free watern: up to 20 µl as recommended by manufacturer.

miRNA Real time PCR: In this study third day extracted RNA, was used for miRNA assay. The high-specificity miRNA QPCR Core Reagent Kit provides the reagents for quantitative PCR amplification of cDNA templates derived from miRNAs within a total RNA population. Because of their short length, miRNAs are difficult to detect with standard QRT-PCR protocols. As a first step, use the miRNA 1st-strand cDNA synthesis kit (Stratagene, USA) to elongate miRNAs in a polyadenylation reaction and then reverse transcribed the polyadenylated RNA into QPCR-ready cDNA. The target of interest is then amplified and detected using the high-specificity miRNA QPCR core reagent kit (Stratagene, USA). The universal reverse primer serves as the downstream primer in the QPCR reaction, the specificity of the QPCR reaction is provided by the miRNA-specific forward primer. Our forward primer for mir-155 was TTAATGCTAATCGTGATAGGGGT.

Real time PCR: Quantitative RT-PCR was carried out using SYBER GREEN master mix (Bioer), Primers α chain Forward CCGACAAGACCAACGTCAAGG α chain reverse GGTATTTGGAGGTCAGCACG,ζ chain Forward GGTGAAGAGCATCGACGACA ζ chain reverse TCTCGGTCAGGACAGAGGA, GAPDH forward GACAAGCTTCCCGTTCTCAG, GAPDH reverse GAGTCAACGGATTTGGTCGT with a thermal cycler BIOER according to manufacture protocol (94˚C 2min, 94˚C 10sec, 58˚C 15sec, 72˚C 25sec) in 40 cycles.

The alpha- and zeta- chain expression were normalized with GAPDH internal control and the relative gene expression was calculated using Ct ($2^{-\Delta\Delta ct}$) method.

Results

Cell expansion and cell transfection: K562 cell line was suspained and passaged in RPMI 1640 with 10% fetal Bovin serum (FBS), 1X Penicillin sterptomycin antibiotic and 2mM L-glutamin. On transfection day, cells were counted and tested for viability. The viability of cells before transfection was 99%.

Cells in log phase were selected for mir-155 inhibitor transfectin. Transfection was done in duplicate but before the assay, cells were mixed. After overnight incubation, medium was changed and cells were evaluated for determination of transfection efficiency. Since miRCURY LNA™ microRNA Inhibitor and its scramble were FITC-labeled, transfection efficiency was measured by flow cytometery. The mean transfection efficiency in our experiments was about 60%.

miRNA 155 downregulation confirmation: To exclude mir-155 changes after transfection short RNA U6 was selected as a normalizer for

measuring the content of mir-155. miRNA real time PCR was done on miRNA- specific cDNA synthesized from third day sample after starting transfection. Analysis of result, showed about 20 fold reductions in the expression of mir-155. The trasfection was repeated each three day for the permanent suppression of mir-155.

Reverse transcriptase PCR on alpha chain hemoglobins

RT-PCR analysis showed that transfected K562 cells with mir-155 inhibitor express both α and ζ hemoglobin chains.

Figures- 1, 2 show alpha- and zeta- chain expression on 3% agarose gel dyed with ethidum bromide indicating these transfected cells express both primitive and definitive hemoglobins.

Alpha and Zeta chain quantitative real time-PCR: By relative Q-PCR, the zeta chain expression was increased 3.4 fold and alpha chain was increased 8.3 fold in comparison to untransfected cells. The effect of scramble on alpha and zeta chains expression was not statistically important. (P>0.05), (Figure- 3)

Conclusion:

Red cells are the most abundant cell type in the human body, with more than 25 trillion in the bloodstream.

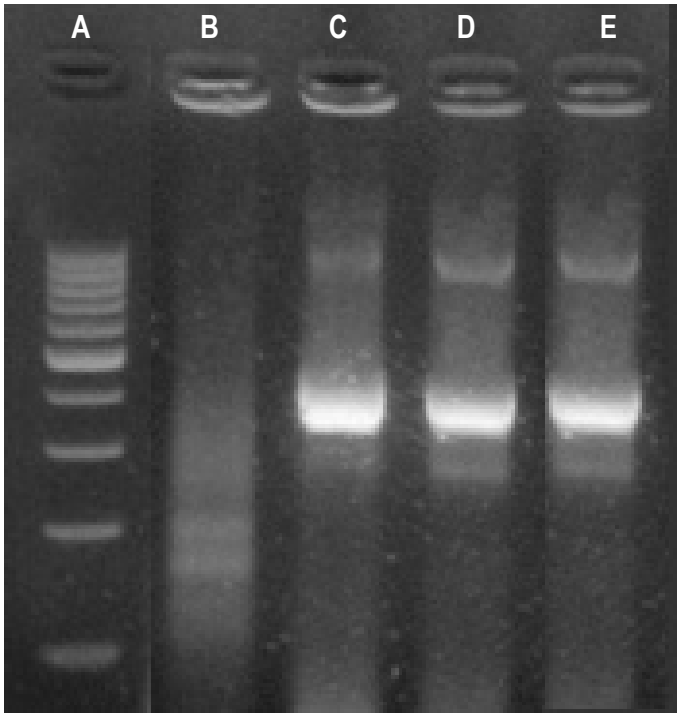

Figure 1: Alpha chain RT-PCR A: Ladder 100 bp; B: NTC (non template control); C: mir-155 inhibitor- treated cells; D: scramble- treated cells; E: untransfected control cells (product size: 407bp)

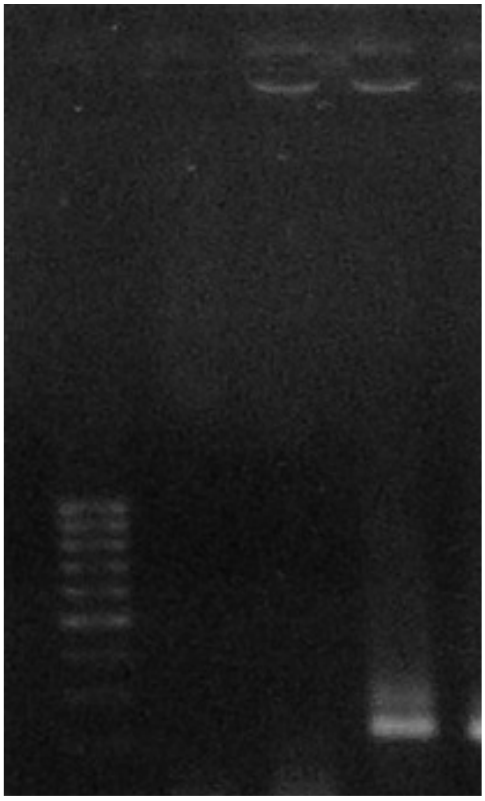

Figure 2: Zeta chain RT-PCR A: Ladder 100 bp; B: NTC (non template control); C: NPC (non-primer control); D: untransfected control cells; E: scramble- treated cells; F: mir-155 inhibitor- treated cells (product size: 208 bp)

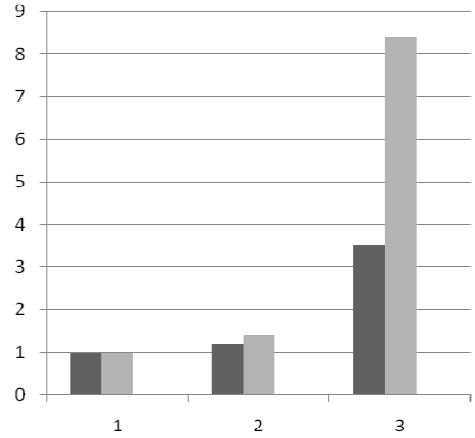

Figure 3: Effect of miR-155 dow-nregulation on erythroid differentiation of K562 cells. The anti-miR155 and scramble were transfected into untreated cells .The cells were assayed for gene expression of α and ζ chains at 7 days post- transfection. Expression of these chains was detected by qRT-PCR. Relative fold changes of gene expression were calculated by ΔΔCt method and the values are expressed as $2^{-\Delta\Delta CT.}$ Data represent the mean plus standard deviation of three independent experiments. The statistical significance between NC/Cy3 control and particular samples was calculated by Student's t-test ($p < 0.05$). 1: untreated cells; 2: scramble treated cells; 3: mir-155 inhibitor treated cells. Dark bar shows ζ gene expression and gray bar indicates α gene.

Red cells are produced in the bone marrow where they undergo progressive maturation from unilineage progenitors to morphologically defined precursors to enucleated erythrocytes.

Erythropoietin is the primary cytokine regulating erythroid cell maturation but other factors such as testosterone, estrogen, GM-CSF, IL-9, IL-3 and SCF are involved. Erythropoiesis is also regulated by transcriptional complexes containing GATA-1, SCL, EKLF, and multiple other factors.(19)

MicroRNAs have recently been found in erythroid cells and raise the possibility that gene downregulation is also important for lineage maturation.

microRNA (miRNA) are non-coding RNA that have a distinguished role in post- transcriptional gene expression. These small molecules act by binding to 3 UTR region of target mRNAs and act as fine-tune for regulation of multiple pathways such as proliferation, differentiation, apoptosis and cancer.

Recently multiple miRNAs are known to be involved in erythropoesis. miRNAs such as mir-451, mir144 and mir-210 are up -regulated in erythroid differentiation process but mir-155, mir-150, mir-221, mir-222, mir-223, mir-24 are down-regulated.

Mir-155 is a multifunctional miRNA that plays crucial role in various physiological and pathological pathways such as hematopoesis, inflammation, immunity and cancer.(17)

In this study we showed that mir-155 downregulation by antisense can increase α and ζ chains expression in K562 cells without any erythropoesis induction agent and cytokines such as hemin, DMSO, EPO and TPO.

Each miRNA has several targets and each gene has several biding sites for multiple miRNAs, so it's not far-fetched that manipulation in one or more miRNA profiles can start a cascade of events resulting in differentiation, proliferation, and apoptosis in cells.

Several studies have shown mir-155 down-regulation in the process of erythroid differentiation.(2, 3, 8, 10) Our present study is fully compliant with previous reports.

In 2007, Masakai et al, showed that the expression level of miR-155 decreased about 200-fold, and the expression of miR-451 increased about 270-fold within 12 days of cultures during normal human erythropoiesis. They introduced mir-451 and mir-155 as a key molecule in erythroid differentiation. Our manipulation in mir-155 level which resulted in an increased alpha- and zeta- chain expression is a confirmatory note on their result.(2)

Bianchi and colleagues carried out complete analysis on 194 expressed miRNA during erythropoesis and observed that their findings are completely compliant with previously reports. According to their report miR- 15b, miR-16, miR-22, miR-185, miR-181, mir-150, mir-155, mir-221, miR-222, miR-223, mir-451, miR 144, miR-188, miR-362 and miR-210 are changed during erythropoiesis. So, our finding about mir-155 doesn't have any incompatibility with their result.(3)

The alpha globin gene cluster is on chromosomes 16. This cluster has an upstream H40 regulatory region and ζ, α2 and α1 globin chain genes; there are two pseudogenes, ψ and ζ.

According to TESTA et al, report K562 clones are divided into three groups: in group I no hemoglobin can be detected but in group II Hb Portland and in group III Hb Portland and Gower I are present.(18) It should be noted that α chain synthesis was occasionally present in minute amounts of K562 clones and mir-155 suppression by antisense, increased vigorously α -chain expression in comparison with ζ chain.

Since, miRNAs are negative regulators in post-transcriptional level of gene regulation, likely our induced down-regulation of mir-155 unblock hemoglobin gene expression.

In 2007, Cavazzana-Calvo and colleagues treated an 18-year-old male patient who had HbE/β-thalassaemia. They treated the patient's HSCs with an HIV-derived lentiviral vector containing a functional β-globin gene after high dose of chemotherapy.

The HSCs containing the transferred β-globin gene gradually gave rise to healthy blood cells and levels of the normal β-globin protein increased; this resulted in the improved production and quality of red blood cells.(20)

Designing a same study with replacing globin genes responsible for miRNAs instead globin genes maybe facilitate and make faster thalassmias and hemoglobinopathies gene therapy pathways.

A better understanding of the regulation of the globin genes expressed in the embryo, fetus, and adult will ultimately lead to improved therapies for people with hemoglobinopathies, including sickle cell disease and thalassemia. Differentiation induction by miRNA regulation without adding any growth factor can be considered as a new strategy in gene therapy, regenerative medicine and tissue engineering.

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References

1. Lawrie CH. microRNA Expression in Erythropoiesis and Erythroid Disorders. Br J Haematol, 2010. 150 (2): p. 144- 51.
2. Masaki S, Ohtsuka R, Abe Y, et al. Expression Patterns of microRNAs 155 and 451 during Normal Human Erythropoiesis. Biochem Biophys Res Commun, 2007. 364 (3): p. 509- 14.
3. Bianchi N, Zuccato C, Lampronti I, et al. Expression of miR-210 during Erythroid Differentiation and Induction of gamma-Globin Gene Expression. BMB Rep, 2009. 42 (8): p. 493- 9.
4. Bhagavathi S, and Czader M. MicroRNAs in Benign and Malignant Hematopoiesis. Arch Pathol Lab Med. 134 (9): p. 1276- 81.
5. Fazi F, Rosa A, Fatica A, et al. A Minicircuitry Comprised of microRNA-223 and Transcription Factors NFI-A and C/EBPalpha Regulates Human Granulopoiesis. Cell, 2005. 123 (5): p. 819- 31.
6. Georgantas RW, Hildreth R, Morisot S, et al. CD34+ Hematopoietic Stem Progenitor Cell microRNA Expression and Function: a Circuit Diagram of Differentiation Control. Proc Natl Acad Sci U S A, 2007. 104(8): p. 2750- 5.
7. Havelange V and Garzon R, Micrornas: Emerging Key Regulators of Hematopoiesis. Am J Hematol. 2010, 85 (12): p. 935- 42.
8. Masaki S, Ohtsuka R, Abe Y, et al. Expression analysis of microRNAs in erythropoiesis. Rinsho Byori, 2008. 56 (12): p. 1086- 92.
9. Yang GH, Wang F, Yu J, et al. MicroRNAs are Involved in Erythroid Differentiation Control. J Cell Biochem, 2009. 107 (3): p. 548-56.
10. Zhan M, et al. MicroRNA Expression Dynamics during Murine and Human Erythroid Differentiation. Exp Hematol, 2007. 35 (7): p. 1015- 25.
11. Lagos-Quintana M, Rauhut R, Yalcin A, et al. Identification of Tissue-specific microRNAs from Mouse. Curr Biol, 2002. 12(9): p. 735-9.
12. Kasashima K, Nakamura Y, and Kozu T, Altered Expression Profiles of microRNAs during TPA-induced Differentiation of HL-60 Cells. Biochem Biophys Res Commun, 2004. 322(2): p. 403- 10.
13. Weber MJ. New Human and Mouse microRNA Genes Found by Homology Search. FEBS J, 2005. 272 (1): p. 59-73.
14. Eis PS, Tam W, Sun L., et al. Accumulation of miR-155 and BIC RNA in Human B- cell Lymphomas. Proc Natl Acad Sci USA, 2005. 102 (10): p. 3627- 32.
15. Landgraf P, Rusu M, Sheridan R, et al. A Mammalian microRNA Expression Atlas Based on Small RNA Library Sequencing. Cell, 2007. 129 (7): p. 1401- 14.
16. Bruchova H, Yoon D, Agarwal AM, et al. Regulated Expression of microRNAs in Normal and Polycythemia Vera Erythropoiesis. Exp Hematol, 2007. 35(11): p. 1657-67.
17. Faraoni I, Antonetti FR, Cardone J,.et al. miR-155 gene: a Typical Multifunctional microRNA. Biochim Biophys Acta, 2009. 1792(6): p. 497- 505.
18. Testa U, Vainchenker W, Beuzard Y, et al. Hemoglobin Expression in Clones of K562 Cell Line. Eur J Biochem, 1982. 121(3): p. 649-55.
19. Wang Q, Huang Z, Xue H, et al. MicroRNA miR-24 Inhibits Erythropoiesis by Targeting Activin Type I Receptor ALK4. Blood, 2008. 111(2): p. 588-95.
20. Persons DA. Gene Therapy: Targeting beta-Thalassaemia. Nature, 2010, 467(7313): p. 277-8.