Exploiting Hybrid Functional Petri Nets to Investigate Transcriptional Activity of Hemoglobin Switching

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ABSTRACT

β-thalassemia, SCD and other human β-globin gene related diseases are the major sources of mortality in the world. Bone marrow transplantation, gene therapy and supporting care with transfusion of red blood cells are possible treatments of human β-globin gene related diseases. However, none of these treatments has progressed to the level of worldwide efficient clinical therapy. Reactivation of γ-globin gene in affected adults is known to be an efficient measure to ameliorate the severity of β-thalassemia and SCD.

In this study, we propose new strategies for β-globin disorders. These approaches are centered upon induction of γ-globin gene expression. We use Cell Illustrator software tool to create HFPN model of hemoglobin switching network, validate the model with available qPCR data and perform simulations to compare the efficiency of the proposed strategies with the existing drug and RNAi-mediated therapies. Simulation results show that our drug and RNAi-mediated strategies have been postulated to lead to the potential induction of γ-globin gene expression.

Keywords: Quantitative modeling, hybrid functional Petri net, β-thalassemia, hemoglobin switching network

ÖZ

β-talasemi ve diğer β-globin geni ile ilgili anomalilerden oluşan hastalıklar dünyada mortalitenin en yüksek olduğu önemli bir halk sağlığı sorunudur. Kemik iliği nakli, gen terapisi ve kırmızı kan hücrelerinin nakli ile destekleyici bakım, β-globin geni ile ilgili anomalilerden oluşan hastalıkların olası tedavileri arasında yer almaktadır. Fakat bu tedavilerin hiçbiri dünya genelinde yeterli klinik tedavi seviyesine ulaşmış değildir. Ancak γ-globin geninin reaktivasyonu, β-talasemi hastalığının şiddetini iyileştirmek için etkin önlem olarak önerilebilir.

Bu tezde, β-globin geni ile ilgili anomalilerden oluşan bozuklukları çalışmak için γ-globin gen ekspresyonunun reaktivasyon olgusuna dayanan yeni stratejiler önerilmiştir. "Hemoglobin Switching", HFPN modeli oluşturarak mevcut qPCR verileri ile, mevcut ilaç ve RNAi metodu kullanılan tedavilerle ve önerilen stratejilerin etkinliğini karşılaştırmak koşulu ile, "Cell Illustrator" yazılımı kullanılarak in silico simülasyonlar gerçekleştirilmiştir. Simülasyon sonuçları, bizim önerdiğimiz ilaç ve RNAi aplikasyonlarının γ-globin gen ekspresyonunun yüksek indüklenmesine neden olabilecek potansiyel stratejiler olabileceğini göstermektedir.

Anahtar kelimeler: Kantitatif modelleme, hibrid fonksiyonel Petri net, β-talasemi, hemoglobin anahtarlamalı ağ

Dedicated to:

My parents and best friends, Gity and Behzad Mehraei who truly got my back during these hard years I was away from home.

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LIST OF ABBREVIATIONS

BCL11A	B-cell lymphoma/leukemia 11A
CHD4	Chromodomain Helicase DNA binding protein 4
CPN	Continuous Petri Net
dsRNA	double stranded RNA
EHPN	Extended Hybrid Petri Net
ETF	multiprotein complex of Erythroid Transcription Factors
ETFI	Erythroid Transcription Factors Inhibitor
FOG1	Friend of GATA protein 1
HbA	Adult Hemoglobin
HbF	Fetal Hemoglobin
HDAC1/2	Histone Deacetylase 1 and 2
HFPN	Hybrid Functional Petri Net
HPN	Hybrid Petri Net
KLF-1	Kruppel-Like transcription Factor 1
MBD2	Methyl-Binding Domain 2
MEL	murine erythroleukemic
miRNA	micro RNA
mRNA	messenger RNA
Myb	Myeloblastosis
NuRD	Nucleosome Remodeling Deacetylase
P/T-net	Place Transition Net
qPCR	quantitative Polymerase Chain Reaction

RISC	RNA-induced silencing complex
RNA	Ribonucleic Acid
RNAi	RNA interference
SCD	Sickle Cell Disease
shRNA	short hairpin RNA
siRNA	small interfering RNA
tBHQ	tert-Butylhyroquinone

Chapter 1

INTRODUCTION

Over the past decades, owing to the advanced technologies in biological sciences, vast amount of biological data and information have been collected from many scientific research. The accumulated data yet requires to be compiled, analyzed and interpreted. Since handling such large amount of data is not feasible manually, fields such as applied mathematics, computer science, computational science, biomathematics and bioinformatics arose as essential tools to solve this problem by designing structural biological databases, developing software tools along with models to run simulations to retrieve useful information from mass of raw biological data. Hence, the data can be collected and stored in organized biological databases, analyzed, validated through computational models, and finally the biological phenomenon can be interpreted based on the accumulated biological data.

Biological systems are indeed complex and the exact interaction among their components are yet unclear. The most common approach to represent these complex biological systems is computational modeling. Computational modeling enables us to use current known facts and available data from wet lab results not only to have better understanding of dynamic behavior of the biological systems, but also to predict the unknown results by simulations. In such computational models, biological outcomes are presented as variables, and the interaction among these variables are represented by the dynamic

behavior of complex biological systems. The initial concentration levels of entities and process rates of variable interactions are obtained and validated by the available data. Subsequently by creating the new potential scenarios and conditions, it is possible to predict the results by running simulations on the proposed models. Obtaining these simulation results could be as fast as pressing an enter button on a computer, and running such simulations and acquiring new data and information is currently the most feasible known approach. Therefore, the mentioned approach has this advantage to obtain useful information out of huge number of biological data in shorter time and lower cost compared to conducting these experiments in wet laboratory settings. These types of computational modeling are categorized in two branches. Qualitative computational modeling enables researchers to have better understanding of structure and states of the biological system. On the other hand, Quantitative computational modeling is helpful as a tool to learn about dynamic behavior of complex biological systems in details. Thus, prior to initiating such projects, choosing an appropriate computational modeling tool is essential.

β-globin gene disorders such as β-thalassemia and SCD are caused by mutations in β-globin gene. The mutations in β-globin gene leads to deficiency of β-subunits in hemoglobin of human adults. There is not yet a global treatment to cure β-globin gene related disorders. In order to reach an ideal treatment for such diseases, understanding the developmental stages of hemoglobin switching network, as complex biological systems, became crucial. During the last few decades, there have been several studies to find out which genes are involved in gene regulation in this network and what the interactions among them are [5,7,32,43].

There exist some therapies and treatments of β-globin gene disorders such as bone marrow transplantation [18,29] gene therapy [33], red blood cells transfusion [35], and chelation therapy particularly for patients who have β-thalassemia [19]. One of the current potential treatments of β-globin gene disorders is γ-globin gene induction. Although the identification of drugs, which can lead to reactivation of γ-globin gene expression is still challenging [15], it is the topic of interest in many research since increasing the level of HbF can ameliorate clinical severity and decrease the mortality rates of SCD [34] and β-thalassemia patients [47].

Since none of the mentioned approaches were ideal to cure β-globin gene disorders, it became an interesting subject for researchers to find a potential treatment by induction of γ-globin gene expression in human adult with β-globin gene disorders. However, the details related to its biological network is still unknown for scientists. Thus, quantitative computational modeling can be an alternative tool to reach better understanding of these complex biological systems.

One of those systems, which became interesting due to the necessity for designing mathematical models for studying biological systems is Petri nets [13,29,27]. Since there are variety of structured processes involved in such complex biological systems, it is more efficient to model and analyze them by using HFPN. As examples of HFPNs to describe biological systems and analyze them, it is worth mentioning HFPNs to model bio-pathways signaling [14,27], p53 transcriptional activity validation [13], role of interleukin-6 in human early hematopoiesis [46], cell fate specification during

Caenorhabditis *elegans vulval* development [29], the flower developmental network of *Arabidopsis thaliana* [22], eukaryotic cell cycle [20], and p16-mediated pathway [1].

In this study, we concentrate on finding potential treatments for β-globin gene related disorders caused by mutations in β-globin gene by constructing and analyzing the most detailed quantitative computational HFPN model based on recent biological discoveries and latest available biological data obtained by experimental observations. Our constructed HFPN model is able to quantitatively compare targeting one or many components involved in fetal-to-adult hemoglobin switching pathway by considering dynamic behaviors of the main proteins and their complexes for reactivating γ-globin gene expression via simulations. This quantitative comparison is carried out by performing a series of simulations by targeting all major components involved in silencing γ-globin gene expression in HbA to observe which strategy, whether it is based on current known drug based treatments and gene therapies or on our proposed potential strategy may lead to optimum γ-globin gene induction. By constructing HFPN model and analyzing it by carrying out simulation results, not only we shed light on how the fetal-to-adult hemoglobin switching network works in human adults, but also identified two potential strategies to treat β-globin gene related disorders by reaching optimum concentration level of γ-globin mRNA. One of these two strategies is targeting BCL11A mRNA and SOX6 mRNA by utilizing a drug called Acy-947 together with targeting ETF (GATA1, FOG1, and SOX6). The other strategy is siRNA-mediated knock down of BCL11A, HDAC1/2, FOG1, and BCL11A gene transcripts. All of the known strategies to find potential cure for such diseases in our HFPN model was validated by available qPCR

data obtained from the literature. Additionally, we proposed two potential strategies as predicted therapies [28].

The rest of the thesis is organized as follows: The basics of Petri Nets is presented in Chapter 2. The research conducted in the frame of present thesis is detailed in Chapter 3. In this chapter, we describe step-by-step construction of HFPN model of fetal-to-adult hemoglobin switching network, validate the model and identify potential optimal therapeutic strategies by performing quantitative comparative analysis of existing and newly proposed strategies. Finally, the main results are summarized in Chapter 4.

Chapter 2

PETRI NETS

2.1 Background

Petri nets are graphical and mathematical modeling tool which can be used for problem solving in various areas [30]. There are numerous examples of asynchronous, distributed, concurrent, parallel, deterministic, nondeterministic and stochastic dynamic systems which can be modeled and analyzed in terms of Petri nets. The main advantage of using Petri nets is that it enables visualization and analysis states and subsystems seperately, and demonstrates the distributed activities of the whole complex system with high accuracy and effectiveness [21]. During the last few decades, Petri nets are increasingly used in molecular biology and system biology. Petri nets represent a well-defined technique to model various complex systems and analyze them in details. Although classical Petri nets were designed to model and analyze behavior of only discrete-event systems, later the concept was expanded with such extensions as color, time, hierarchy, fuzziness, stochasticity and continuity. It is also common to use combination of the extentions for defining new type of extented Petri nets such as HFPN.

2.2 Basic Definitions and Notations

A Petri net is a directed graph consisting of places, transitions, and arcs, which are usually represented as circles, bars/boxes and arrows, respectively. A pair of place and transition can be connected by a directed arc, but there cannot be arc between two places or two transitions. An arc is directed from place to transition or vice versa. Arcs are identified by

their weights. so that k-weighted arc is labeled with the weight k, indicating the number of parallel arcs between specified pair of objects.

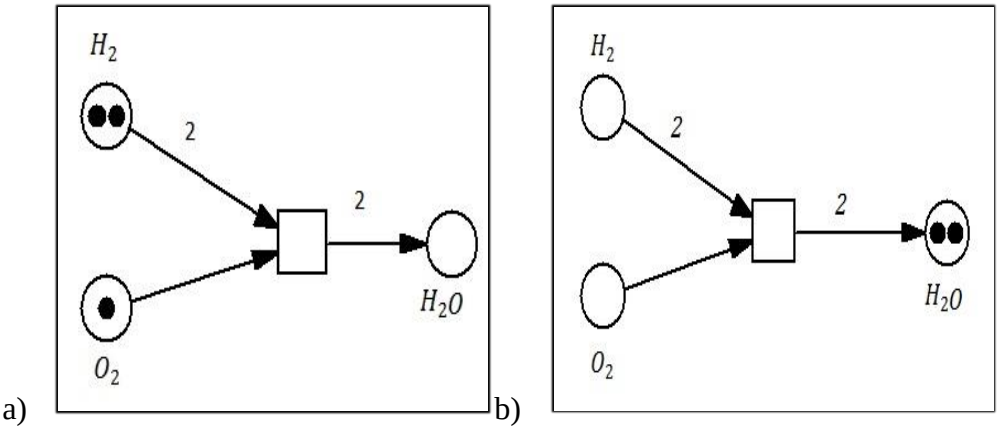
A place from which an arc runs to a transition is called the input place of the transition. Similarly, a place to which arc runs from a transition is called the output place of the transition. Input places in Petri nets are interpreted as preconditions and inputs of the model while output places are considered as postconditions and outputs. Transitions in Petri nets are usually represented as actions, events, computational steps, logic clauses, and processors which can change the states in Petri Nets.

Dynamic structure of a Petri net can be described in terms of flow of tokens. A state is recognized by distribution of the tokens among places, called a marking. The initial state of the system is represented by the initial marking M_0. The arrrangement of the tokens in a Petri net based on the places can be rearranged and create new marking states. A marking M is m-vector where m is the total number of places.

A classical Petri net or P/T-net [30] is a 5-tuple $PN = (P, T, A, W, M_0)$ where $P = \{p_1, \dots, p_m\}$ is the set of finite places, $T = \{t_1, \dots, t_n\}$ is the set of finite transitions, $A \subseteq (P \times T) \cup (T \times P)$ is the set of arcs, $W: A \to \mathbb{N}$ is the weight function, and $M_0: P \to \mathbb{N}$ is the initial marking. We assume that T and P are nonempty pairwise disjoint sets, that is, $T \cup P \neq \emptyset$ and $T \cap P = \emptyset$. A state of a Petri net can be changed to another state by firing of transitions. The weight of arc (p, t) is denoted by $w(p, t)$. A transion t is said to be enabled in M if $M(p) \geq w(p, t)$; It is disabled otherwise. Firing of enabled transition t changes $(m_1, \dots, m_n)$ to $(m'_1, \dots, m'_n)$ as follows $m'_i = m_i - w(p_i, t)$. A

self-loop is composed of a pair of arcs (p,t) and (t,p). A pure Petri net does not have any self-loop.

As an easy example to illustrate how it is possible to construct a Petri net based on a simple system, consider the reaction of hydrogen gas H_2 with oxygen gas O_2. The product of these two reactants is a water molecule H_2O. As a chemical reaction, it is expressed as $2H_2 + O_2 \rightarrow 2H_2O$ (see Figure 1).



a) b)

Figure 1: A Petri net model of the reaction of hydrogen and oxygen gasses to produce a water molecule.
Two states of Petri net: a) Before transition fires b) After transition fires.

The main drawback of analyzing heavily loaded dynamic discrete event systems with discrete or classical Petri nets is the classical problem known as state explosion which consequently leads to memory overflow [36]. Continues Petri Nets are introduced to avoid this common disadvantage of classical Petri Nets [10]. A CPN is a 5-tuple $CPN = (P,T,A,W,M_0)$. The only difference between CPN and P/T-net is that weight function and marking are positive and real valued, that is, $W: A \rightarrow \mathbb{R}^+$ and $M_0: P \rightarrow \mathbb{R}^+$.

The weakness related to CPNs is that, they can not model a systems with different types of proceesses such as both continuous and discrete ones. Biological systems consist of such different types of processes. For example, biochemical reactions in a biological systems are continuous processes, while presence or absence of biological phenomenon, or counter-like mechanisms are discrete processes. Thus, to cover both continuous and discrece processes in a model, HPNs were introduced [11,23]. HPN is a 6-tuple $HPN = (P, T, A, W, M_0, h)$ where P and T are same to those in definition of CPN with the only difference that they can both be continuous and discrete. That is, the discrete part of HPN consists of discrete places, P^D, and discrete transitions, T^D, while the continuous part consists of P^C and T^C nodes as its continuous places and transitions, respectively. An arc can be adjacent from a continous place (or transition) to a discrete transition and vice versa. Arc weights and initial marking in HPN can take their values from either positive real numbers (for continous places where $p_i \in P^C$) or natural numbers (for discrete places where $p_i \in P^D$). Weight function and the initial marking are defined as $W: A \to \mathbb{R}^+$ or $\mathbb{N}$, and $M_0: P \to \mathbb{R}^+$ or $\mathbb{N}$. $h: P \cap T \to \{D, C\}$ is called hybrid function, In such Petri nets, it is possible to use test arcs, which allow a particular component to affect the behaviour of other parts without any change in its own marking, and without the need for removing any contents from the source place after firing related transition.

Inhibitory arcs are one of the common arcs which are used in many applications. Although it is possible to subsititute inhibitory arc by equivalent net fragments, extended hybrid Petri nets are introduced to include inhibitory arcs in case of continuous places [11]. In extended HPN, weights can get infinitely small continuous values. That is, $w(p, t) \in$

$\mathbb{R}^+ \cup \{0^+\}$. This also holds for place markings, that is $M(p) \in \mathbb{R}^+ \cup \{0^+\}$ where p is a continuous place.

2.3 Hybrid Functional Petri Nets

HFPN has been developed to model and analyze biological processes [27]. In HFPN the rate of a continuous transition can be expressed as a function of concentration. Furthermore, different types of functions can be defined for the arcs connecting with continuous transitions as firing rules. Morever, each transition can be associated with delay function. The possible arc types include test input arc, discrete (input/output) arc, and continuous (input/output) arc.

Let T be a continuous transition and let $a_1, a_2, \dots, a_p$ and $b_1, b_2, \dots, b_q$ be respectively input and output arcs (continuous or test) from continuous places $P_1, P_2, \dots, P_p$ to continuous places $Q_1, Q_2, \dots, Q_q$. The contents of corresponding input and output arcs at time t are represented by $m_1(t), \dots, m_p(t)$ and $n_1(t), \dots, n_q(t)$. Three major rules about continuous transitions are:

1) The continuous transition T fires if and only if the firing condition remains true;

2) To determine consuming rate from input places P_i when a continous transition T fires, for all a_i, a funcion $f_i\left(m_1(t), \dots, m_p(t)\right) \geq 0$ is defined for T. In case a_i is a test input, f_i is considered to be equivalent to 0 which means there will not be any change in quantity of corresponding P_i;

3) To determine adding rate to output places Q_j when a Continous transition T fires, for all b_j, a funcion $g_j(m_1(t), \dots, m_p(t)) \geq 0$ is defined for the transition.

Discrete transition T consists of discrete or test type input arcs $a_1, a_2, \dots, a_p$ and output arcs $b_1, b_2, \dots, b_q$ that are directed from discrete places $P_1, P_2, \dots, P_p$ to discrete places $Q_1, Q_2, \dots, Q_q$ with contents $m_1(t), \dots, m_p(t)$ and $n_1(t), \dots, n_q(t)$ at time t, respectively. The rules applied to discrete transitions are:

(1) Same as the first rule of continuous transitions with predicate $c(m_1(t), \dots, m_p(t))$ as firing condition;

(2) Same as second and third rules of continuous transitions except that for $m_i(t)$ is defined in the set of nonnegative integers;

(3) The delay function for discrete transitions is defined by function $d\left(m_1(t), \dots, m_p(t)\right)$, where $m_i(t)$ is defined in the of nonnegative integers. Delay function lets T fire with delay $d\left(m_1(t), \dots, m_p(t)\right)$ at time t if the firing condition holds. During this delay time $d\left(m_1(t), \dots, m_p(t)\right)$, if the firing rule is changed and does not hold anymore, then T can not fire and the firing rule will be reset.

2.4 Modeling Biological Processes with Petri Nets

Petri nets as a quantitative modeling approach became applicable for modeling biological phenomon and processes since 1993 [37]. Since then, there exist many research and studies regarding this field. Petri nets have this advantage over other modeling approaches that they use simple mathematical model with intuitive graphical presentations, which enable not only qualitative analysis, but also quantitative analysis. In the following subsections, basic modeling of biological processes are demonstrated.

2.4.1 Modeling of Unimolecular and Biomolecular Reactions

A unimolecular reaction or first-order reaction is when a molecule rearranges its atoms to form other molecules [4]. When an unstable molecule A converts to a stable molecule B, it can be considered as a unimolecular reaction and denoted by $A \rightarrow B$. A Petri net illustrating unimolecular reaction is illustrated in Figure 2.

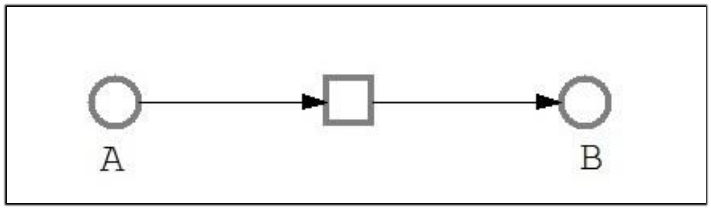


Figure 2: Petri net model of a unimolecular reaction.

Bimolecular reaction or second-order reaction represents binding of two substrates A and B to get product C as the result. Such a reaction is denoted by $A + B \rightarrow C$. A Petri net representing bimolecular reaction is illustrated in Figure 3.

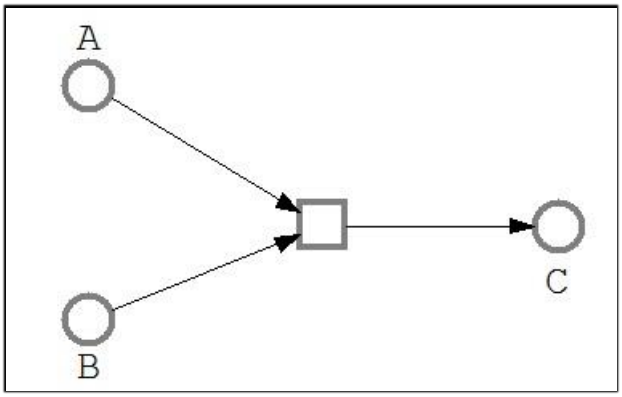


Figure 3: Petri net model of a bimolecular reaction.

2.4.2 Modeling of Biodegradation and Central Dogma of Biology

Biodegradation is the chemical dissolution of materials by bacteria or other biological means [12]. In Petri nets, it is possible to represent biodegradation by a sink transition (see Figure 4).

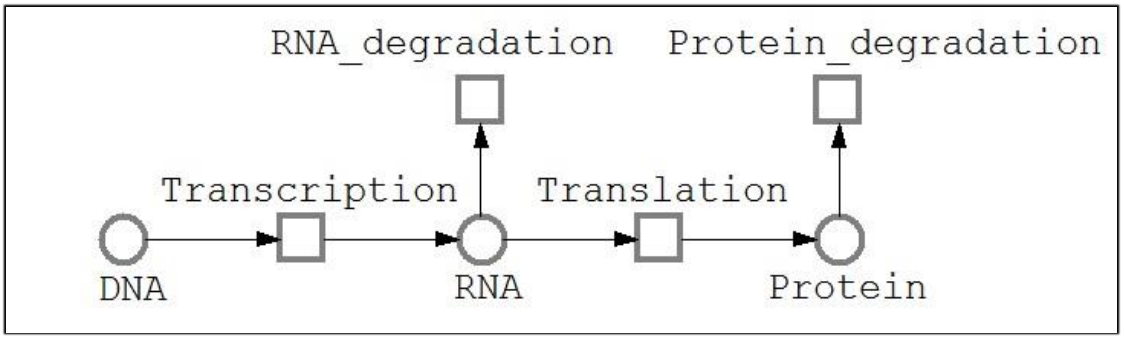


Figure 4: Petri net model of central dogma of biology.

Central dogma is related to transferring genetics sequential information such as transcription of DNA to RNA, and translation of RNA to protein. It is possible to model such descriptions in Petri nets, too (see Figure 4).

2.4.3 Modeling of Presence/Absence Type Events

As mentioned in section 2.2, HPN not only preserves the features of CPN, but it can also include discrete components. Presence or absence of a biological phenomenon can be presented by a discrete place with Boolean variable. As it is described in section 2.2 HFPN enable the model to contain inhibitory arcs. For example, when a mutation happens in a gene, which in the most extreme case stops the production of a particular protein, can be modeled in an extended HPN (see Figure 5). Another example would be the case when a drug suppresses the target gene expression by binding to its mRNA (see Figure 6).

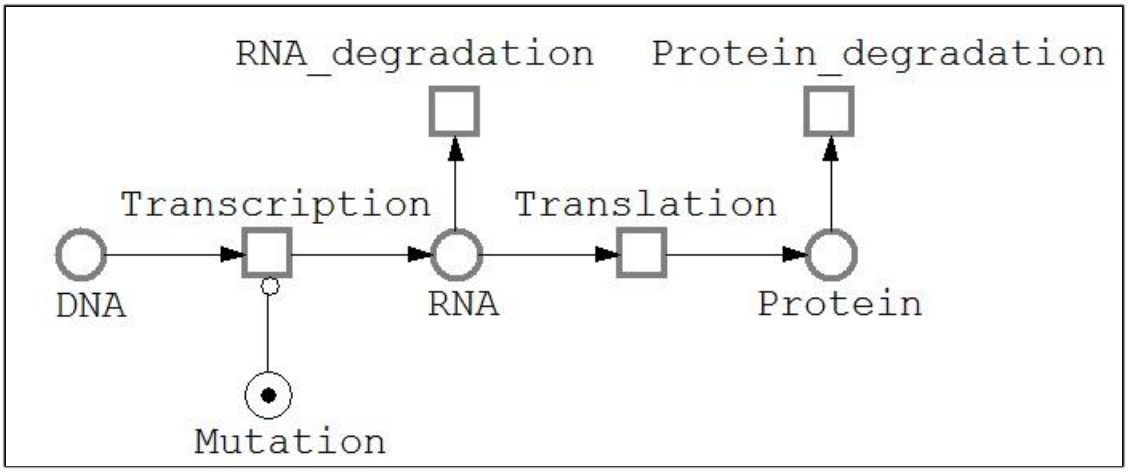


Figure 5: Hybrid Petri net model of presence of mutation by using an inhibitory arc.

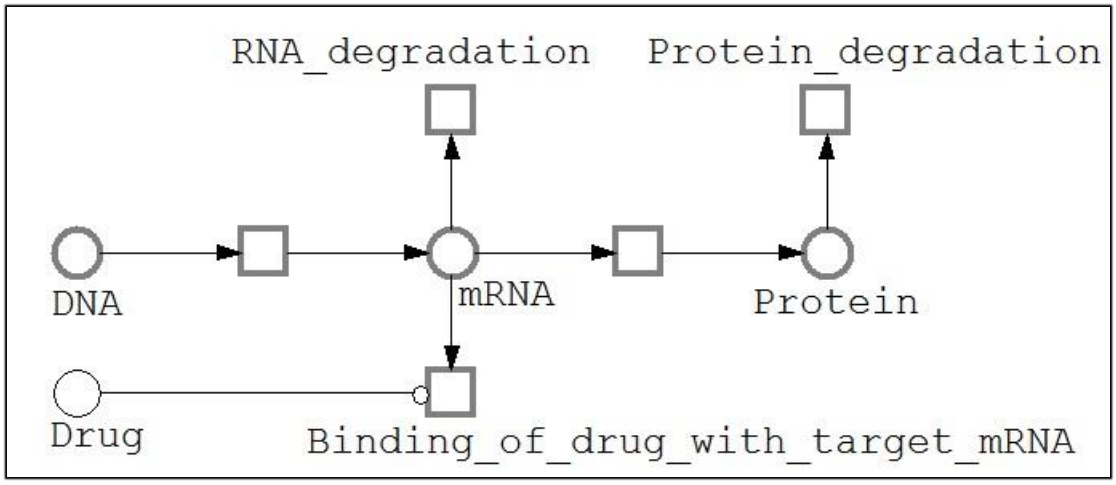


Figure 6: Extended Hybrid Petri net model of presence of drug by using an inhibitory arc.

Chapter 3

MOLECULAR TARGETS FOR β-GLOBIN DISORDERS

3.1 Biological Context

3.1.1 Introduction

Mutations in β-globin gene may lead to diseases such as β-thalassemia and SCD. β-thalassemia is a type of thalassemia disease caused by the reduction or absence of the synthesis of the β-globin chains of the hemoglobin tetramer [6]. The absence or reduction of β chains causes excessive accumulation of α-globin and precipitation, which leads to ineffective erythropoiesis [38]. Presence of mutations in β-globin gene is the main reason of causing β-thalassemia. When mutations cause absence of the synthesis of β-globin chains, it is called β^0 thalassemia. Whereas in the case where mutations result in a reduction in the synthesis of β-globin chains, this is then referred to as β^+ thalassemia [31]. In terms of the severity of the disease, we may categorize it into β thalassemia major, and β thalassemia minor [6].

3.1.2 Hemoglobin Switching Pathway

Hemoglobin switching represents the developmental stages of globin gene regulation including its two switches. First developmental switch is related to embryonic-to-fetal stage, which occurs within the first six weeks of prenatal age. At this stage γ-globin gene expression is up regulated at its maximum level while ε-globin gene expression is downregulated. Second developmental switch is related to fetal-to-adult stage, which occurs within the first six weeks of postnatal age. At this stage β-globin gene expression is

upregulated and it replaces the γ-globin gene expression significantly in a healthy adult. Hence, six months after birth, β-globin gene expression is at its maximum level while γ-globin gene expression is down regulated. Due to these developmental switches, dominant hemoglobin molecules at each stage of ontogeny are Hbε in embryonic from the moment of conception for almost three months, HbF in fetus from the third month after conception to birth, and HbA after birth respectively. These developmental stages are illustrated in Figure 7.

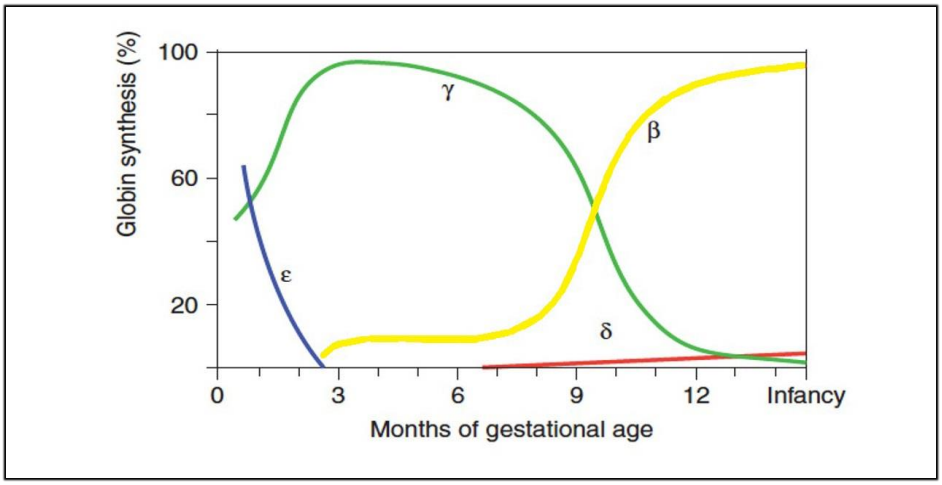


Figure 7: (Adopted from [41].) Hemoglobin Switching network: There are two developmental stages in β-globin gene family. Embryonic to fetal, which occurs within the first three months after conception, and fetal to adult hemoglobin switching, which occurs within the first three months after birth.
In adults, β-globin gene expression remains to be up regulated.

During fetal-to-adult hemoglobin switching BCL11A binds to NuRD complex and silences γ-globin gene expression [5,42]. It was observed that down regulation of BCL11A gene expression induces γ-globin gene expression [50]. Myb protein is an indirect upstream regulator of BCL11A through KLF1, which positively regulates transcription of BCL11A [39]. KLF1, GATA1, and FOG1 are transcription factors of the

β-globin gene [26,48]. Fetal-to-adult hemoglobin switching network is illustrated in Figure 8.

3.2 Developing HFPN Model of Hemoglobin Switching Pathway

We develop HFPN model of fetal-to-adult hemoglobin switching network based on the biological context taken from relevant literature [40-42,49,50]. The model consists of one generic entity, indicating the presence/absence condition for β-globin gene mutation, 27 continuous entities used to measure the level of biological components (see Table 1) and 52 continuous processes (see Table 2). The model uses 50 input connectors, 28 output connectors, 3 input inhibitors, and one input association (see Table 3). It is also assumed that levels of mRNAs and proteins are kept low by natural degradation (see Table 4).

In molecular biology research, it is quite common to observe different results for identical experiments. Therefore, it is hard task to come up with kinetic parameters such as reaction rates based on wet lab results only. In the proposed model, we set the process rates to the values specified in similar research [1,24,27], and then in line with the reverse engineering approach estimate correct values by validating the model with qPCR data existing for fetal to adult developmental stage of hemoglobin switching in case of wild type β-globin gene expression.

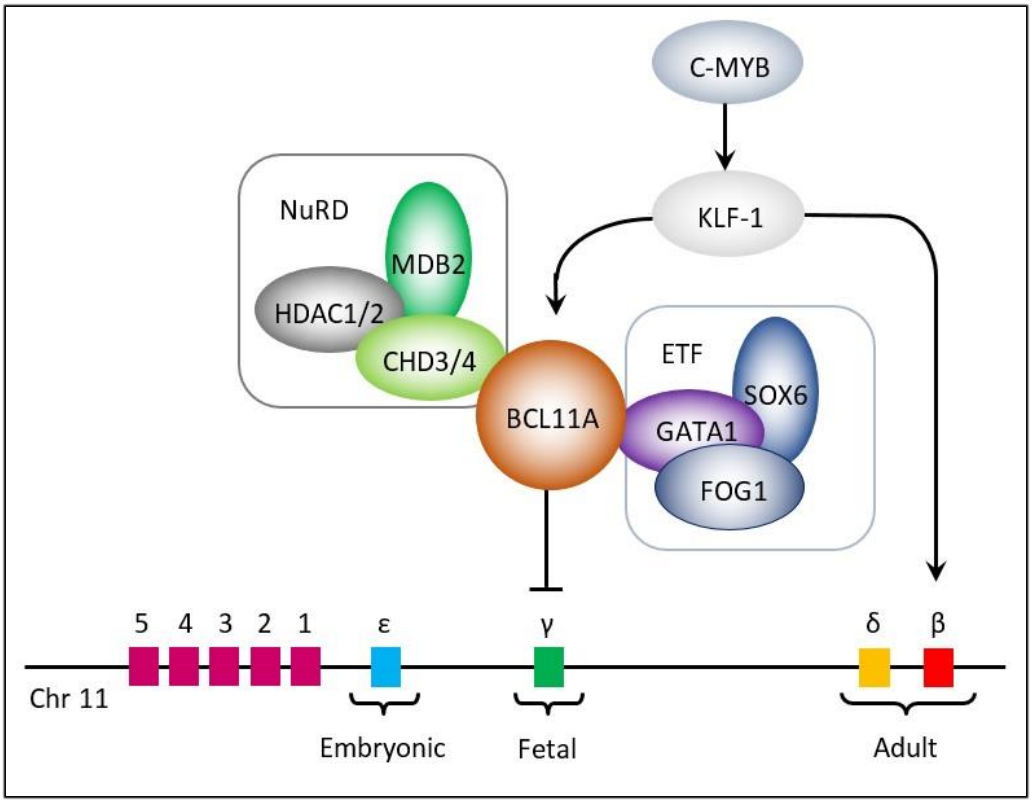


Figure 8: Fetal-to-adult hemoglobin switching network.

Table 1: HFPN entities and corresponding biological components

Entity name	Entity type	Variable	Value	Type
C-MYB	Continuous	*m1*	1	Double
KLF1 mRNA	Continuous	*m2*	0	Double
KLF1	Continuous	*m3*	0	Double
BCL11A mRNA	Continuous	*m4*	0	Double
BCL11A	Continuous	*m5*	0	Double
HDAC1/2 mRNA	Continuous	*m6*	0	Double
HDAC1/2	Continuous	*m7*	0	Double
MBD2 mRNA	Continuous	*m8*	0	Double
MBD2	Continuous	*m9*	0	Double
CHD3/4 mRNA	Continuous	*m10*	0	Double
CHD3/4	Continuous	*m11*	0	Double
NuRD	Continuous	*m12*	0	Double
BCL11A_NuRD	Continuous	*m13*	0	Double
GATA1 mRNA	Continuous	*m14*	0	Double
GATA1	Continuous	*m15*	0	Double
FOG1 mRNA	Continuous	*m16*	0	Double

FOG1	Continuous	*m17*	0	Double
SOX6 mRNA	Continuous	*m18*	0	Double
SOX6	Continuous	*m19*	0	Double
ETF	Continuous	*m20*	0	Double
BCL11A_NuRD_ETF	Continuous	*m21*	0	Double
γ-globin_BCL11A_NuRD_ETF	Continuous	*m22*	0	Double
γ-globin gene	Continuous	*m23*	0	Double
γ-globin mRNA	Continuous	*m24*	0	Double
HbF	Continuous	*m25*	0	Double
Mutation	Generic	*m26*	0/1	Boolean
β-globin mRNA	Continuous	*m27*	0	Double
HbA	Continuous	*m28*	0	Double

Table 2: HFPN processes and corresponding biological phenomenon

Phenomenon	Process	Type	Rate	Delay
Transcription of KLF1 mRNA	*T1*	Continuous	*m1*0.1*	0
Translation of KLF1	*T2*	Continuous	*m2*0.1*	0
Transcription of BCL11A mRNA	*T3*	Continuous	*m3*1*	0
Translation of BCL11A	*T4*	Continuous	*m4*0.1*	0
Transcription of HDAC1/2 mRNA	*T5*	Continuous	*1*	0
Translation of HDAC1/2	*T6*	Continuous	*m6*0.1*	0
Transcription of MBD2 mRNA	*T7*	Continuous	*1*	0
Translation of MBD2	*T8*	Continuous	*m7*0.1*	0
Transcription of CHD3/4 mRNA	*T9*	Continuous	*1*	0
Translation of CHD3/4	*T10*	Continuous	*m10*0.1*	0
Binding of HDAC1/2, MBD2 and CHD3/4	*T11*	Continuous	*m7*m9*m11*0.1*	0
Binding of BCL11A with NuRD	*T12*	Continuous	*m5*m12*0.1*	0
Transcription of GATA1 mRNA	*T13*	Continuous	*1*	0
Translation of GATA1	*T14*	Continuous	*m14*0.1*	0
Transcription of FOG1 mRNA	*T15*	Continuous	*1*	0

Translation of FOG1	*T16*	Continuous	*m16*0.1*	0
Transcription of SOX6 mRNA	*T17*	Continuous	*1*	0
Translation of SOX6	*T18*	Continuous	*m18*0.1*	0
Binding of GATA1, FOG1 and SOX6	*T19*	Continuous	*m15*m17*m19*0.1*	0
Binding of ETF with BCL11A_NuRD	*T20*	Continuous	*m13*m20*0.1*	0
Binding of BCL11A_NuRD_ETF with γ-globin gene	*T21*	Continuous	*m21*m23*0.1*	0
Activation of γ-globin gene	*T22*	Continuous	*0.01*	0
Transcription of γ-globin mRNA	*T23*	Continuous	*m23*0.1*	0
Translation of HbF	*T24*	Continuous	*m24*0.1*	0
Activation of β-globin mRNA by KLF1	*T25*	Continuous	*m3*0.002*	35
Activation of β-globin mRNA by GATA1	*T26*	Continuous	*m15*0.002*	35
Activation of β-globin mRNA by FOG1	*T27*	Continuous	*m17*0.002*	35
Translation of HbA	*T28*	Continuous	*m27*0.1*	0

Table 3: HFPN connectors and their attributes.

Connector	**Firing style**	**Firing script**	**Connector type**
c1-c50	Threshold	0	Input process
c51-c78	Threshold	0	Output process
c79-c81	Threshold	0	Input inhibitor
c82	Threshold	0	Input association

Table 4: Degradations in the HFPN model.

Phenomenon	**Process**	**Type**	**Rate**
mRNA degradation	*d1-d10*	Continuous	m_i*0.05
Protein degradation	*d11-d24*	Continuous	m_i*0.01

3.3 Numerical validation of the model

In the present research, we use Cell Illustrator software, which is licensed to Eastern Mediterranean University, to create HFPN model of human fetal to adult hemoglobin switching network, to validate the model and to perform simulations in order to identify optimal molecular targets.

We validate the model through altering calibration parameters such as initial markings (concentrations) and process rates to obtain good fit into wet lab results for the gene, mRNA and protein concentrations. In the simulation plots, x-axis stands for Petri time (pt) and y-axis presents concentration levels of genes, mRNAs, proteins, and their complexes. In these plots, each 10 pt stands for three months of gestational age. According to our assumptions fetal life starts at 20 pt and a child is born at 50 pt. We measured the γ-globin mRNA levels at 70 pt, that is, 6 months after birth.

In order to minimize our approximation regarding the wild type β-globin mRNA and γ-globin mRNA concentration levels, we have validated the model by extrapolating the simulation results for those mRNA concentration levels with respect to their relation to biological components, which are involved in fetal to adult hemoglobin switching. The simulation results regarding the fetal to adult hemoglobin switching developmental stage is illustrated in Figure 9.

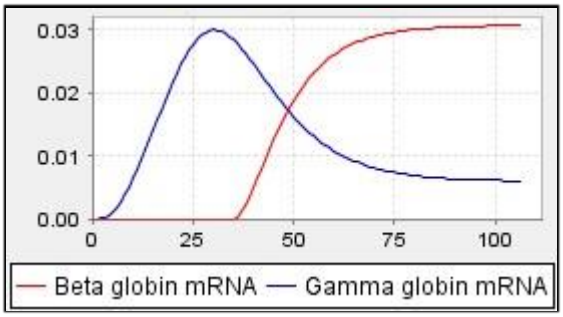


Figure 9: Simulation results for expression of β-globin and γ-globin genes illustrated in the same graph to emphasize their relation during hemoglobin switching developmental stages. Before birth, γ-globin mRNA level is greater than β-globin mRNA level. However, after birth, β-globin mRNA level is greater than γ-globin mRNA level. Thus, this is a proper fit to illustrate especially fetal to adult hemoglobin switching.

3.4 Target-based Drug Prediction for β-hemoglobin Disorders

3.4.1 Target-based Drug Therapeutic Strategies

In this thesis we explore comparative efficiency of six target-based drug therapeutic strategies. The target-based drug therapeutic strategies include: (1) The combination of Simvastatin and tBHQ to target KLF1 mRNA [25]; (2) MS-275 to target KLF1 mRNA [8,9,35]. (3) ST-20 to target KLF1 mRNA and HDAC1/2 mRNA [9]; (4-5) ACY-957 to target BCL11A mRNA and SOX6 mRNA [43,44]; (6) Identifying a potential hypothetical drug to target function of ETF.

3.4.1.1 HFPN Model and its Validation

Drug treatment in primary human erythroid cells with Simvastatin, tBHQ, and combination of these drugs revealed that Simvastatin decreased KLF1 mRNA by approximately 20%, tBHQ by approximately 25%, and the combination of Simvastatin and tBHQ by approximately 44% [25]. All three experiments resulted in HbF induction. In sickle cell and thalassemia patients, drug treatments with MS-275 in erythroid progenitors showed decrease of the KLF-1 mRNA level by 3-fold compared to untreated control [9]. Drug treatments of erythroid progenitors in sickle cell and thalassemia

patients revealed that ST-20 decreased KLF1 and HDAC1/2 mRNA levels by 2.5-fold and 6-fold respectively [9]. Drug treatment with ACY-957 in human CD34-derived erythroblasts in culture respectively decreased BCL11A mRNA and SOX6 mRNA levels by 1.4-fold and 2.3-fold, and BCL11A mRNA and SOX6 mRNA levels by 2-fold and 10-fold. Experiments with ACY-957 depended on drug dosage and time.

In this section, we extend previous HFPN model by integrating biological entities and processes related to known potential drug treatments and our proposed strategy. This extended HFPN model consists of nine generic and 27 continuous entities (see Table 5) with 60 processes, nine Boolean and 27 continuous variables (see Table 6). The generic entities in our model represent β-globin gene mutation and drugs such as MS-275. The continuous entities are considered to represent entities such as genes, mRNAs, proteins, multi-proteins, and their complexes. The processes stand for biological reactions such as gene transcription, mRNA translation, binding, mRNA and protein degradation. Boolean entities show the presence or absence of a specific drug, while continues entities represents the concentration level of biological components. There are 100 connectors in our proposed HFPN model consist of 59 input connectors, 28 output connectors, 12 input inhibitor, and one input association (see Table 7). Protein and mRNA degradation in this HFPN is the same as in Table 4.

Table 5: Relationship between biological components and HFPN entities.

Entity name	**Entity type**	**Variable**	**Value**	**Type**
C-MYB	Continuous	*m1*	1	Double
KLF1 mRNA	Continuous	*m2*	0	Double
KLF1	Continuous	*m3*	0	Double
BCL11A mRNA	Continuous	*m4*	0	Double
BCL11A	Continuous	*m5*	0	Double
HDAC1/2 mRNA	Continuous	*m6*	0	Double

HDAC1/2	Continuous	*m7*	0	Double
MBD2 mRNA	Continuous	*m8*	0	Double
MBD2	Continuous	*m9*	0	Double
CHD3/4 mRNA	Continuous	*m10*	0	Double
CHD3/4	Continuous	*m11*	0	Double
NuRD	Continuous	*m12*	0	Double
BCL11A_NuRD	Continuous	*m13*	0	Double
GATA1 mRNA	Continuous	*m14*	0	Double
GATA1	Continuous	*m15*	0	Double
FOG1 mRNA	Continuous	*m16*	0	Double
FOG1	Continuous	*m17*	0	Double
SOX6 mRNA	Continuous	*m18*	0	Double
SOX6	Continuous	*m19*	0	Double
ETF	Continuous	*m20*	0	Double
BCL11A_NuRD_ETF	Continuous	*m21*	0	Double
γ-globin_BCL11A_NuRD_ETF	Continuous	*m22*	0	Double
γ-globin gene	Continuous	*m23*	0	Double
γ-globin mRNA	Continuous	*m24*	0	Double
HbF	Continuous	*m25*	0	Double
Mutation	Generic	*m26*	0/1	Boolean
β-globin mRNA	Continuous	*m27*	0	Double
HbA	Continuous	*m28*	0	Double
Simvastatin+tBHQ as KLF1 mRNA suppressor	Generic	*m28*	1	Boolean
MS-275 as KLF1 mRNA suppressor	Generic	*m29*	1	Boolean
ST-20 as KLF1 mRNA suppressor	Generic	*m30*	1	Boolean
ST-20 as HDAC1/2 mRNA suppressor	Generic	*m31*	1	Boolean
ACY-957 as BCL11A mRNA suppressor (case I)	Generic	*m32*	1	Boolean
ACY-957 as SOX6 mRNA suppressor (case I)	Generic	*m33*	1	Boolean
ACY-957 as BCL11A mRNA suppressor (case II)	Generic	*m34*	1	Boolean
ACY-957 as SOX6 mRNA suppressor (case II)	Generic	*m35*	1	Boolean
ETFI (ETF Inhibitor)	Generic	*m36*	1	Boolean

Table 6: Relationship between biological phenomena and HFPN processes.

Phenomenon	**Process**	**Type**	**Rate**	**Delay**
Transcription of KLF1 mRNA	*T1*	Continuous	*m1*0.1*	0

Translation of KLF1	*T2*	Continuous	*m2*0.1*	0
Transcription of BCL11A mRNA	*T3*	Continuous	*m3*1*	0
Translation of BCL11A	*T4*	Continuous	*m4*0.1*	0
Transcription of HDAC1/2 mRNA	*T5*	Continuous	*1*	0
Translation of HDAC1/2	*T6*	Continuous	*m6*0.1*	0
Transcription of MBD2 mRNA	*T7*	Continuous	*1*	0
Translation of MBD2	*T8*	Continuous	*m7*0.1*	0
Transcription of CHD3/4 mRNA	*T9*	Continuous	*1*	0
Translation of CHD3/4	*T10*	Continuous	*m10*0.1*	0
Binding of HDAC1/2, MBD2 and CHD3/4	*T11*	Continuous	*m7*m9*m11*0.1*	0
Binding of BCL11A with NuRD	*T12*	Continuous	*m5*m12*0.1*	0
Transcription of GATA1 mRNA	*T13*	Continuous	*1*	0
Translation of GATA1	*T14*	Continuous	*m14*0.1*	0
Transcription of FOG1 mRNA	*T15*	Continuous	*1*	0
Translation of FOG1	*T16*	Continuous	*m16*0.1*	0
Transcription of SOX6 mRNA	*T17*	Continuous	*1*	0
Translation of SOX6	*T18*	Continuous	*m18*0.1*	0
Binding of GATA1, FOG1 and SOX6	*T19*	Continuous	*m15*m17*m19*0.1*	0
Binding of ETF with BCL11A_NuRD	*T20*	Continuous	*m13*m20*0.1*	0
Binding of BCL11A_NuRD_ETF with γ-globin gene	*T21*	Continuous	*m21*m23*0.1*	0
Activation of γ-globin gene	*T22*	Continuous	*0.01*	0
Transcription of γ-globin mRNA	*T23*	Continuous	*m23*0.1*	0
Translation of HbF	*T24*	Continuous	*m24*0.1*	0
Activation of β-globin mRNA by KLF1	*T25*	Continuous	*m3*0.002*	35
Activation of β-globin mRNA by GATA1	*T26*	Continuous	*m15*0.002*	35

Activation of β-globin mRNA by FOG1	*T27*	Continuous	*m17*0.002*	35
Translation of HbA	*T28*	Continuous	*m27*0.1*	0
Binding of Simvastatin+tBHQ to KLF1 mRNA	*T29*	Continuous	*m2*0.18*	0
Binding of MS-275 to KLF1 mRNA	*T30*	Continuous	*m2*0.4*	0
Binding of ST-20 to KLF1 mRNA	*T31*	Continuous	*m2*0.37*	0
Binding of ST-20 to HDAC1/2 mRNA	*T32*	Continuous	*m6*1*	0
Binding of ACY-957 to BCL11A mRNA (case I)	*T33*	Continuous	*m4*0.38*	0
Binding of ACY-957 to SOX6 mRNA (case I)	*T34*	Continuous	*m18*0.21*	0
Binding of ACY-957 to BCL11A mRNA (case II)	*T35*	Continuous	*m4*0.62*	0
Binding of ACY-957 to SOX6 mRNA (case II)	*T36*	Continuous	*m18*1.9*	0
Binding of ETF with its inhibitor	*T37*	Continuous	*m20*0.12*	0

Table 7: Connectors in the HFPN to identify drug based discoveries

Connector	**Firing style**	**Firing script**	**Connector type**
c1-c59	Threshold	0	Input process
c60-c87	Threshold	0	Output process
c88-c99	Threshold	0	Input inhibitor
c100	Threshold	0	Input association

In our simulation results, we have considered the case when β-globin gene had a mutation, which had led to a severe case of β-globin gene disorder. In this case, β chain production is not only insufficient, but also it is almost lost. In order to compensate for the lack of β chain by reawakening γ-globin gene expression, we have also validated our model in case of mutated β-globin gene and known drug-based therapies for the purpose of γ-globin

gene induction (combination of Simvastatin and tBHQ, MS275, ST-20 and ACY957). Finally, we propose a potential strategy by targeting ETF complex and have shown the simulation results. The snapshot of our HFPN model is illustrated in Figure 10.

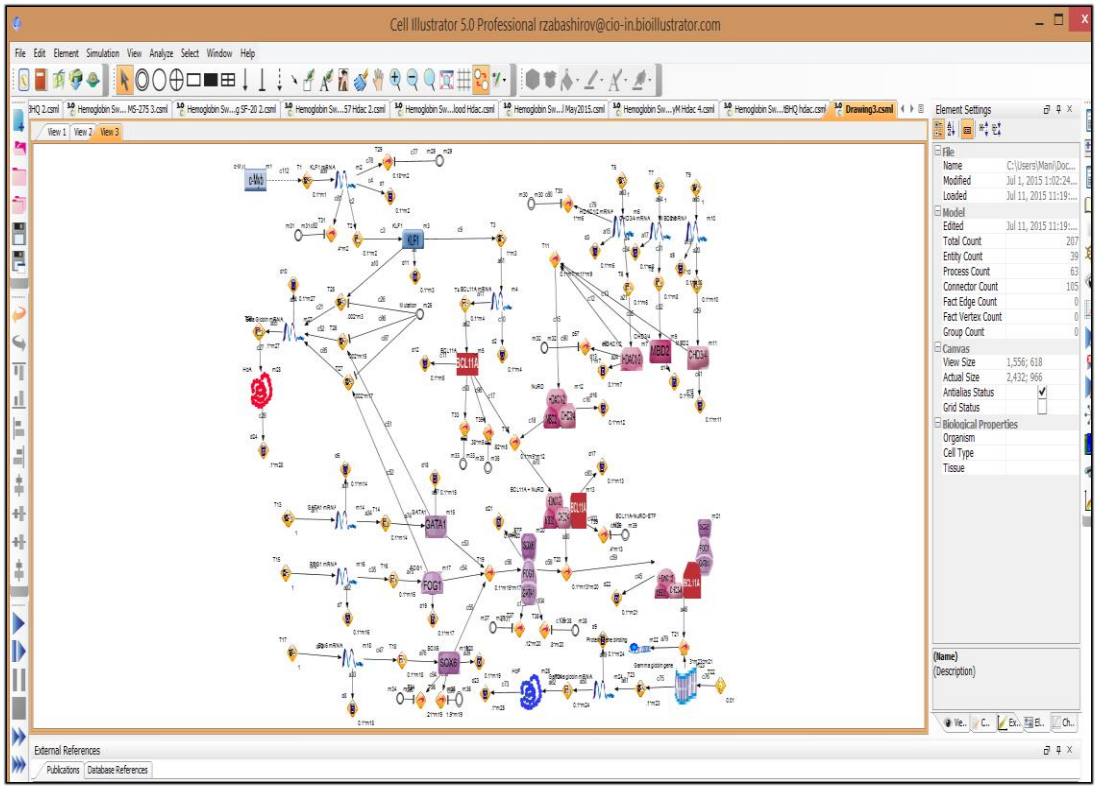


Figure 10: Snapshot of Cell Illustrator screen with HFPN model on it.

In vitro experiments carried out in human primary cells showed that a combination of Simvastatin and tBHQ decreases KLF1 mRNA level by 44% [25]. Drug treatments with MS-275 and ST-20 in erythroid progenitors cultured from patients suffering from SCD and β-thalassemia resulted in decreased KLF1 mRNA level by 3- and 2.5-fold, respectively [9]. The simulation results for the KLF1 mRNA levels are demonstrated in Figure 11. It can be easily seen that KLF1 mRNA level reaches its steady state at 25 pt and continuously remains at the same level. KLF1 mRNA level at time 70 pt in untreated cell and cells treated by a combination of Simvastatin and tBHQ, MS-275, and ST-20

measured to be 1.25, 0.70, 0.41, and 0.50, respectively, which provides a good fit for aforesaid wet lab results.

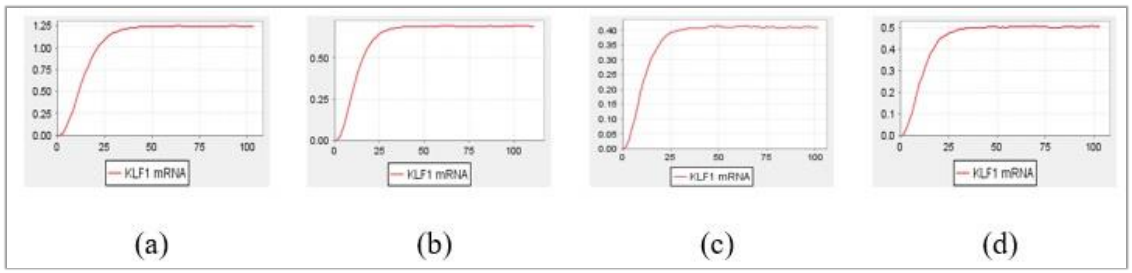


Figure 11: Simulation results for KLF1 mRNA level in (a) an untreated cell; (b) primary erythroid human cells treated by Simvastatin and tBHQ; (c) erythroid progenitors cultured from SCD and β-thalassemia patients treated by MS-275;
(d) erythroid progenitors cultured from SCD and β-thalassemia patients treated by ST-20.

Based on ST-20 drug treatment in erythroid progenitors cultured from SCD and β-thalassemia patients, HDAC1/2 mRNA level decreased by 6-fold comparing to the untreated cells [9]. Simulation results for HDAC1/2 mRNA levels for both untreated cell and ST-20 treatment are illustrated in Figure 12. HDAC1/2 mRNA level is measured at time 70 pt. Simulation results reveal that treatment with ST-20 decreased HDAC1/2 mRNA level from 1.5 to 2.5 which agrees with wet lab results.

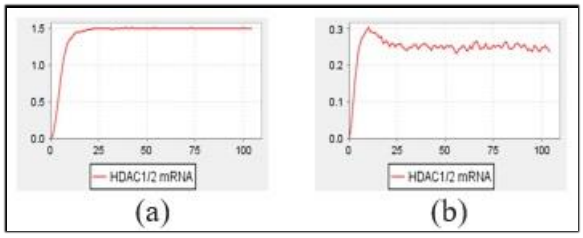


Figure 12: Simulation results for HDAC1/2 mRNA level in (a) an untreated cell;
(b) erythroid progenitors cultured from SCD and β-thalassemia patients treated by ST-20.

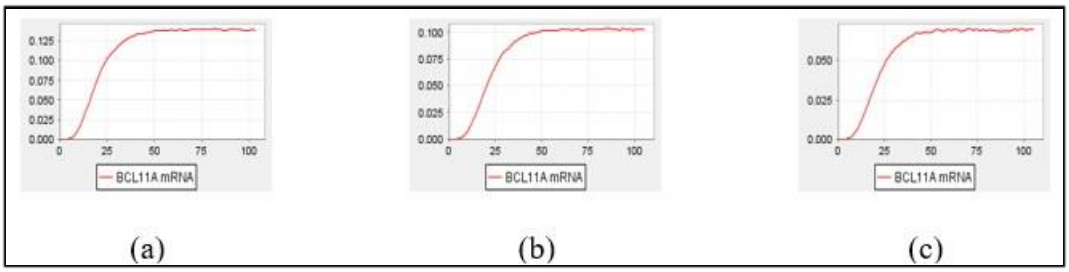


Figure 13: Simulation results for BCL11A mRNA level in (a) an untreated cell; human CD34-derived erythroblast in culture treated by (b) ACY-957 (Case I); (c) ACY-957 (Case II).

Treatment with ACY-957 having different time duration and dosage on gene chip and qPCR time course experiments with CD71(low) GlyA(neg) cells decreased BCL11A mRNA and SOX6 mRNA levels by 1.4-fold and 2.3-fold, respectively for ACY-957 case I [45]; 2-fold and 10-fold for ACY-957 case II [44]. The simulation results for ACY-957 case I and ACY-957 case II along with untreated cell simulations are illustrated in Figure 13 and Figure 14 for BCL11A mRNA levels and SOX6 mRNA levels, respectively.

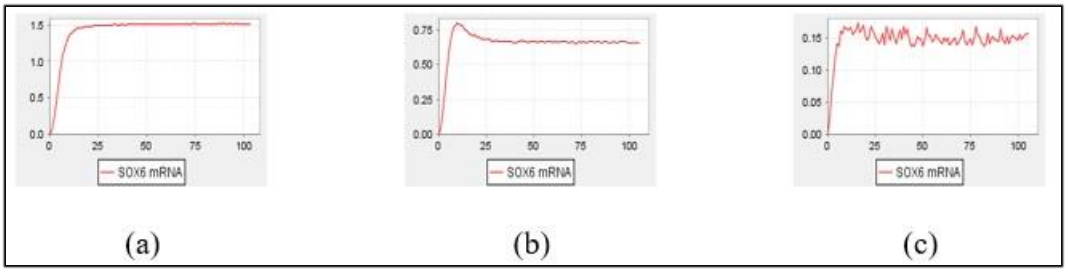


Figure 14: Simulation results for SOX6 mRNA level in (a) an untreated cell; human CD34-derived erythroblast in culture treated by (b) ACY-957 (Case I); (c) ACY-957 (Case II).

3.4.1.2 Drug Target Prediction

In our strategy, we respectively decrease BCL11A mRNA, SOX6 mRNA and ETF levels by 1.4-fold, 2.3-fold and 10-fold. The simulation results related to our strategy are illustrated in Figure 15. Use of this strategy in HFPN model results in accumulation of

maximum γ-globin mRNA levels compared to other drug strategies considered in the present thesis. Treatments with a combination of Simvastatin and tBHQ, MS-275, ST-20, ACY-957 (case I), and ACY-957 (case II) lead to increase of γ-globin mRNA levels by 3.4-, 4.1-, 3.1-, 4.4-, and 5-fold. Simulation results for our strategy shows 5.4-fold increase with respect to untreated case. Simulation results for γ-globin mRNA levels are illustrated in Figures 16 and 17.

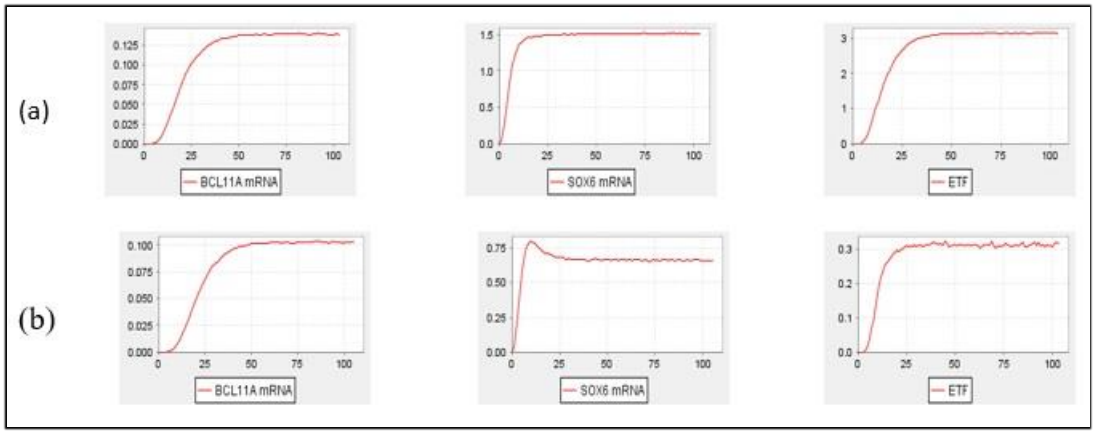


Figure 15: Simulation results for BCL11A mRNA, SOX6 mRNA, and ETF levels in (a) an untreated cell; (b) our proposed strategy.

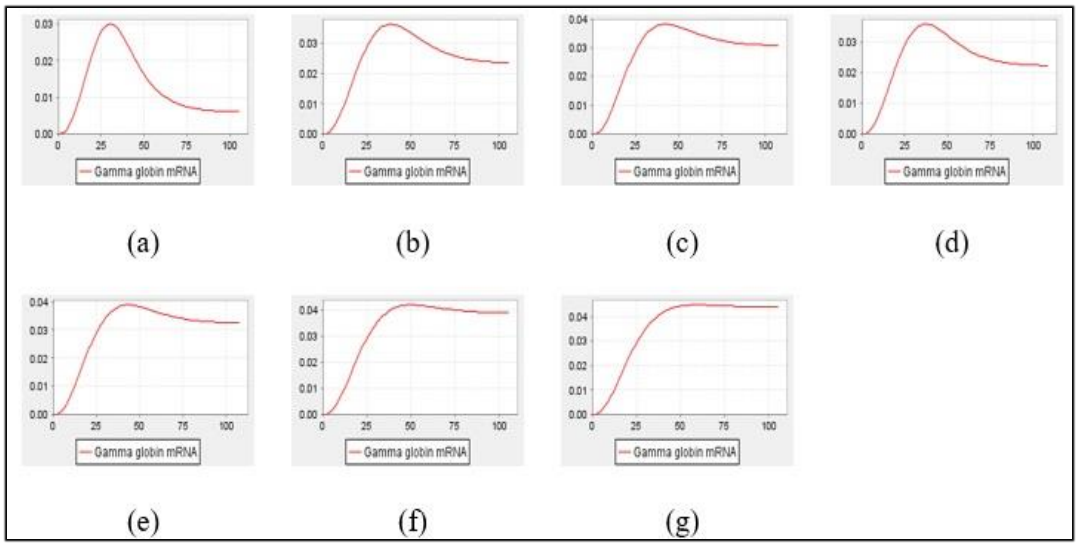


Figure 16: Simulation results for γ-globin mRNAlevels in (a) an untreated cell; (b) primary erythroid human cells treated by Simvastatin and tBHQ; (c) erythroid progenitors cultured from SCD and β-thalassemia patients treated by MS-275; (d) erythroid progenitors cultured from SCD and β-thalassemia patients treated by ST-20; human CD34-derived erythroblast in culture treated by (e) ACY-957 (Case I); (f) ACY-957 (Case II); (g) our proposed strategy.

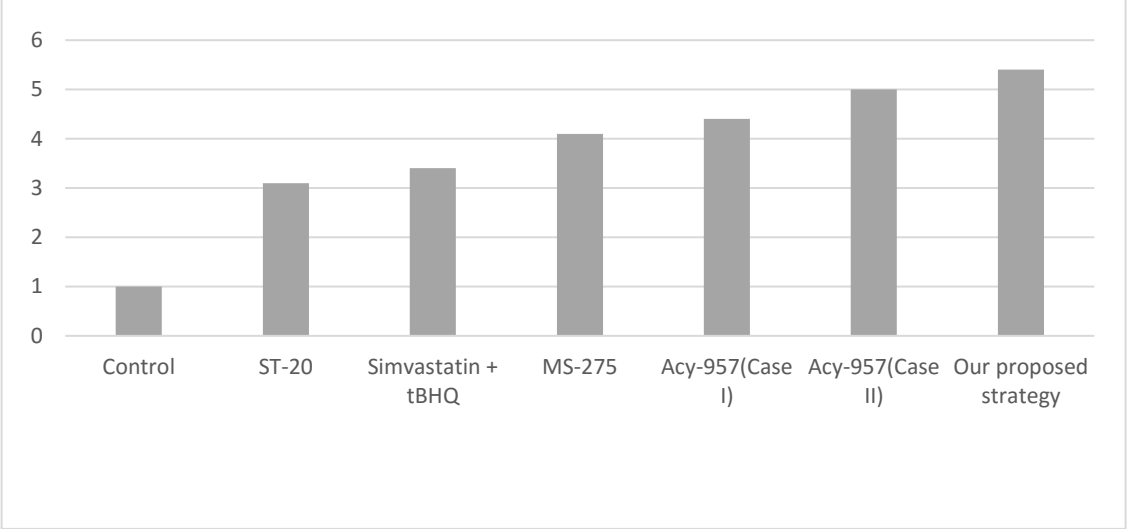


Figure 17: Comparison of an untreated cell control with the inhibitor treated samples. γ-globin mRNA level increased by 3.4-, 4.1-, 3.1-, 4.4-, 5-, and 5.4-fold in treatment with a combination of Simvastatin and tBHQ, MS-275, ST-20, ACY-957 (Case I), ACY-957 (Case II), and our strategy, respectively.

3.4.2 RNAi-mediated Approach to Treat β-globin Disorders

RNAi method was discovered in 1998 [16]. The main idea behind of its use in context of β-globin disorders is to increase γ-globin gene expression through knocking down specified genes in fetal-to-adult hemoglobin switching network.

In the thesis we explore the comparative efficacy of five RNAi-mediated gene therapeutic strategies for inducing γ-globin gene expression: (1) reducing MBD2 mRNA expression by siRNA-mediated knockdown of MBD2 [17], (2) shRNA-mediated knockdown of Myb followed by silencing of KLF-1 and BCL11A mRNAs [39], (3) shRNA-mediated knockdown of BCL11A followed by silencing of KLF-1 and BCL11A mRNAs [39], (4) siRNA-mediated knockdown of CHD4 followed by silencing of KLF-1 and BCL11A mRNAs [2], and (5) our proposed RNAi-mediated strategy of inhibiting BCL11A, FOG1 and HDAC1/2 mRNAs. Simulation results show that our strategy is the optimal one among five strategies discussed in the present work as it identifies the rational molecular targets yielding the greatest induction of γ-globin gene levels.

3.4.2.1 HFPN Model and its Validation

Our HFPN model of hemoglobin switching network for RNAi-mediated strategies consists of 8 generic and 27 continuous entities (see Table 8), 61 processes (see Table 9), and 114 arcs (see Table 10). The generic entities represent presence of siRNA and shRNA knock down approaches for some target components. We consider Myb, BCL11A, MBD2, CHD4, HDAC1/2 and FOG1 as target components. The continuous entities and processes are defined in the same way as it was done in section 3.2. The Boolean variables in this model show the presence/absence of siRNA or shRNA components. In case of presence the variable is set to 0, otherwise it is set to 1. The other Boolean variable is

related to β-globin gene mutations. The value of this variable is set to 0 in case when there is not a mutation, otherwise it is set to 1. There are 114 arcs in this extended HFPN model consisting of 68 input connectors, 15 output connectors, 15 input inhibitor, and one input association (see Table 7). The natural degradation of proteins and mRNAs are illustrated in Table 11. The details related to the initial values and the process rates are given in section 3.2. The snapshot of corresponding HFPN model taken from Cell Illustrator is demonstrated in Figure 18.

Table 8: Relationship between biological components and extended HFPN entities

Entity name	**Entity type**	**Variable**	**Value**	**Type**
MYB	Continuous	*m1*	1	Double
KLF1 mRNA	Continuous	*m2*	0	Double
KLF1	Continuous	*m3*	0	Double
BCL11A mRNA	Continuous	*m4*	0	Double
BCL11A	Continuous	*m5*	0	Double
HDAC1/2 mRNA	Continuous	*m6*	0	Double
HDAC1/2	Continuous	*m7*	0	Double
MBD2 mRNA	Continuous	*m8*	0	Double
MBD2	Continuous	*m9*	0	Double
CHD4 mRNA	Continuous	*m10*	0	Double
CHD4	Continuous	*m11*	0	Double
NuRD	Continuous	*m12*	0	Double
BCL11A_NuRD	Continuous	*m13*	0	Double
GATA1 mRNA	Continuous	*m14*	0	Double
GATA1	Continuous	*m15*	0	Double
FOG1 mRNA	Continuous	*m16*	0	Double
FOG1	Continuous	*m17*	0	Double
SOX6 mRNA	Continuous	*m18*	0	Double
SOX6	Continuous	*m19*	0	Double
ETF	Continuous	*m20*	0	Double
BCL11A_NuRD_ETF	Continuous	*m21*	0	Double
γ-globin_BCL11A_NuRD_ETF	Continuous	*m22*	0	Double
γ-globin gene	Continuous	*m23*	0	Double
γ-globin mRNA	Continuous	*m24*	0	Double
HbF	Continuous	*m25*	0	Double
Mutation	Generic	*m26*	0/1	Boolean
β-globin mRNA	Continuous	*m27*	0	Double
HbA	Continuous	*m28*	0	Double

MBD siRNA	Generic	*m'29*	1	Boolean
shMYB501	Generic	*m'30*	1	Boolean
shBCL11A	Generic	*m'31*	1	Boolean
CHD4 siRNA	Generic	*m'32*	1	Boolean
BCL11A siRNA	Generic	*m'33*	1	Boolean
FOG1 siRNA	Generic	*m'34*	1	Boolean
HDAC1/2 siRNA	Generic	*m'35*	1	Boolean

Table 9: Processes in the HFPN to identify RNAi-mediated discoveries

Phenomenon	**Process**	**Type**	**Rate**	**Delay**
Transcription of KLF1 mRNA	*T1*	Continuous	*m1*0.1*	0
Translation of KLF1	*T2*	Continuous	*m2*0.1*	0
Transcription of BCL11A mRNA	*T3*	Continuous	*m3*1*	0
Translation of BCL11A	*T4*	Continuous	*m4*0.1*	0
Transcription of HDAC1/2 mRNA	*T5*	Continuous	*1*	0
Translation of HDAC1/2	*T6*	Continuous	*m6*0.1*	0
Transcription of MBD2 mRNA	*T7*	Continuous	*1*	0
Translation of MBD2	*T8*	Continuous	*m7*0.1*	0
Transcription of CHD3/4 mRNA	*T9*	Continuous	*1*	0
Translation of CHD3/4	*T10*	Continuous	*m10*0.1*	0
Binding of HDAC1/2, MBD2 and CHD3/4	*T11*	Continuous	*m7*m9*m11*0.1*	0
Binding of BCL11A with NuRD	*T12*	Continuous	*m5*m12*0.1*	0
Transcription of GATA1 mRNA	*T13*	Continuous	*1*	0
Translation of GATA1	*T14*	Continuous	*m14*0.1*	0
Transcription of FOG1 mRNA	*T15*	Continuous	*1*	0
Translation of FOG1	*T16*	Continuous	*m16*0.1*	0
Transcription of SOX6 mRNA	*T17*	Continuous	*1*	0
Translation of SOX6	*T18*	Continuous	*m18*0.1*	0
Binding of GATA1, FOG1 and SOX6	*T19*	Continuous	*m15*m17*m19*0.1*	0
Binding of ETF with BCL11A_NuRD	*T20*	Continuous	*m13*m20*0.1*	0

Binding of BCL11A_NuRD_ETF with γ-globin gene	*T21*	Continuous	*m21*m23*0.1*	0
Activation of γ-globin gene	*T22*	Continuous	*0.01*	0
Transcription of γ-globin mRNA	*T23*	Continuous	*m23*0.1*	0
Translation of HbF	*T24*	Continuous	*m24*0.1*	0
Activation of β-globin mRNA by KLF1	*T25*	Continuous	*m3*0.002*	35
Activation of β-globin mRNA by GATA1	*T26*	Continuous	*m15*0.002*	35
Activation of β-globin mRNA by FOG1	*T27*	Continuous	*m17*0.002*	35
Translation of HbA	*T28*	Continuous	*m27*0.1*	0
Binding of MBD2 siRNA to MBD2 mRNA	*T29*	Continuous	*m8*0.76*	0
Binding of shMYB501 to KLF1 mRNA	*T30*	Continuous	*m2*0.58*	0
Binding of shBCL11A to KLF1 mRNA	*T31*	Continuous	*m2*0.02*	0
Binding of shBCL11A to BCL11A mRNA	*T32*	Continuous	*m4*1.5*	0
Binding of CHD4 siRNA to KLF1 mRNA	*T33*	Continuous	*m2*0.45*	0
Binding of CHD4 to BCL11A mRNA	*T34*	Continuous	*m4*0.21*	0
Binding of BCL11A siRNA to BCL11A mRNA	*T35*	Continuous	*m4*4*	0
Binding of FOG1 siRNA to FOG1 mRNA	*T36*	Continuous	*m16*1*	0
Binding of HDAC1/2 siRNA to HDAC1/2 mRNA	*T37*	Continuous	*m6*1*	0

Table 10: Connectors in the extended HFPN model

Connector	**Firing style**	**Firing script**	**Connector type**
c1-c68	Threshold	0	Input process
c69-c98	Threshold	0	Output process
c99-c113	Threshold	0	Input inhibitor
c114	Threshold	0	Input association

Table 11: Degradations in the extended HFPN model

Phenomenon	**Process**	**Type**	**Rate**
mRNA degradation	d1-d10	Continuous	m_i *0.1
Protein degradation	d11-d24	Continuous	m_i *0.01

It is observed that in chemical inducer dimerization (CID) dependent mouse bone marrow cells carrying β-globin yeast artificial chromosome (β-YAC) that MBD2 siRNA treatment decreased MBD2 mRNA level by 80% and induced fetal hemoglobin [17]. MBD2 mRNA simulation results in our HPFN model are illustrated in Figure 19 for both untreated cells and treated cells with siMBD2 approach. By calibrating binding rate of siMBD2 with MBD2 mRNA, we reached 5-fold decrease from 5 to 1 concentration level for MBD2 mRNA, which is a proper fit comparing with wet lab experimental results.

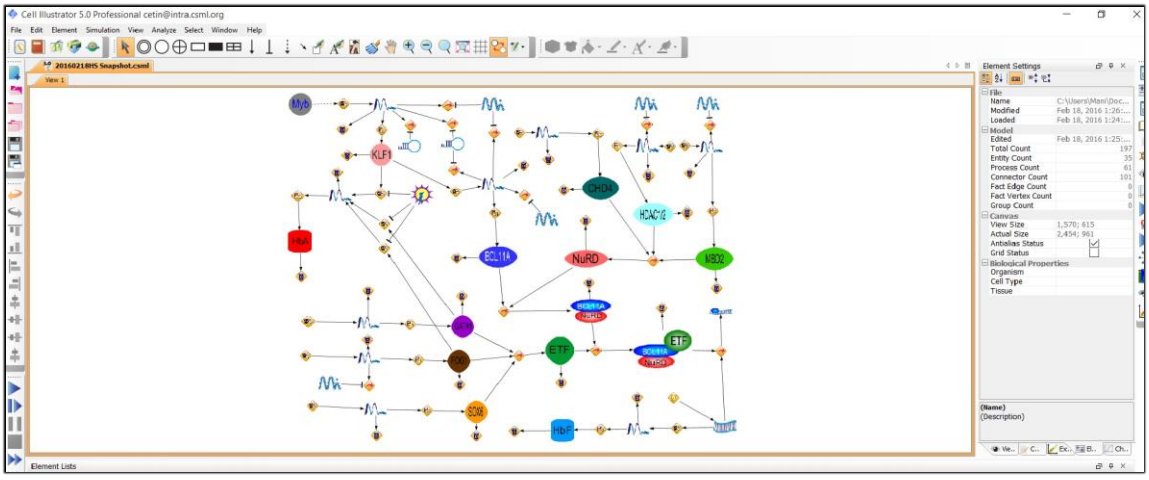


Figure 18: Snapshot of Cell Illustrator screen with extended HFPN model on it.

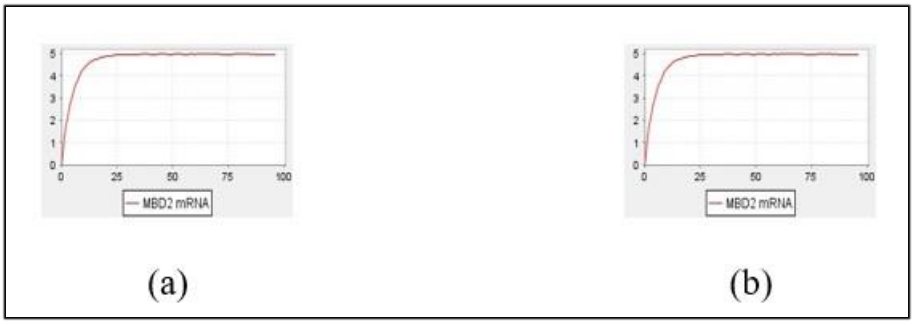


Figure 19: Simulation results for MBD2 mRNA in (a) an untreated CID cell; (b) a treated CID cell with siMBD2.

As it is recognized, Myb is a critical upstream regulator of KLF1 and BCL11A transcription factors [39]. It has been observed that shMyb knock down in MEL cells decreases KLF1 and BCL11A gene expression. Among two shMyb constructs (shMyb500 and shMyb501), which were tested on the mentioned cells, shMyb501 was more efficient in reducing KLF1 and BCL11A gene expression. shMyb501 decreases KLF1 and BCL11A mRNA concentration level by 75% and 76%, respectively [39]. Our HFPN model is validated in accordance with shMyb501 treatment, which shows 4- and 4.2-fold decrease from 1.25 to 0.31 for KLF1 mRNA and 0.14 to 0.033 for BCL11A mRNA concentration level, respectively. Simulation results are illustrated in Figure 20 and Figure 21.

In the same study on MEL cells, it is also observed that shBCL11A treatment suppresses KLF1 and BCL11A gene expression by 10% and 82%, respectively [39]. Simulation results for HFPN model demonstrates that shBCL11A treatment decreases KLF1 mRNA and BCL11A mRNA levels by 1.1- and 5.6-fold from 1.25 to 1.13 and from 0.14 to 0.0252, respectively. These simulation results are in strong agreement with experimental data taken from the aforementioned experiment. These results are presented in Figure 20 and Figure 21.

CHD4 also positively regulates KLF1 and BCL11A transcription factors by binding, and CHD4 represses gene expression of γ-globin indirectly by binding to and positively regulating BCL11A gene expression [2]. It is reported that CHD4 knockdown by siRNA approach in CID cells decrease KLF1 and BCL11A gene expression by 70% and 40%, respectively [2]. We performed simulations and observed siCHD4 approach suppresses KLF1 mRNA and BCL11A mRNA levels from 1.25 to 0.375 by 3.3-fold and from 0.14 to 0.084 by 1.7-fold, respectively (see Figure 20 and Figure 21).

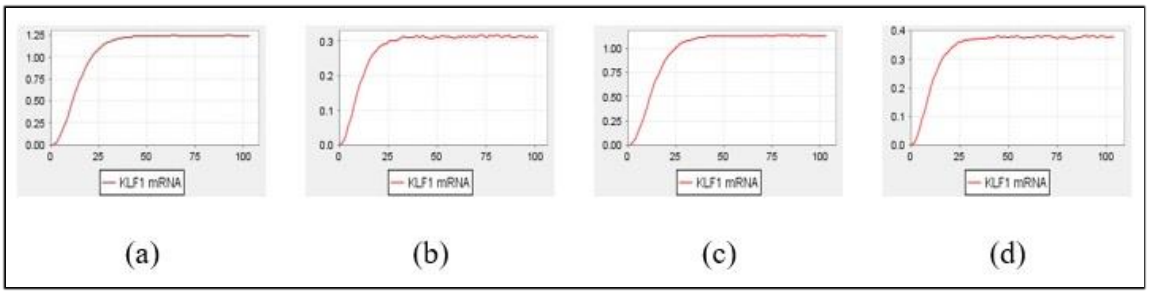


Figure 20. Simulation results for KLF1 mRNA in (a) Untreated cells;
Cells treated with (b) shMyb501; (c) shBCL11A; (d) CHD4 siRNA.

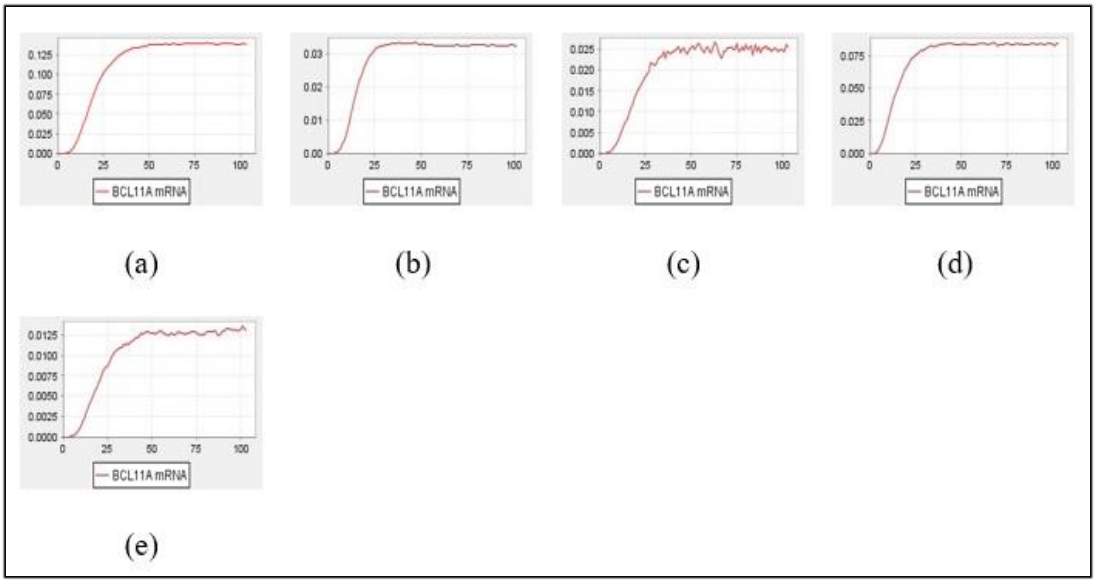


Figure 21: Simulation results for BCL11A mRNA in (a) Untreated cells; Cells treated with (b) shMyb501; (c) shBCL11A; (d) CHD4 siRNA;
(e) Our proposed strategy.

3.4.2.2 Prediction of Molecular Targets for RNAi-mediated Treatment

In this thesis we are aimed on determining the potential optimal molecular targets with RNAi-mediated approach that would lead to more γ-globin gene expression compared to existing RNAi-mediated approaches. By performing exhaustive model checking we found that BCL11A, FOG1 and HDAC1/2 are the optimal targets as knocking down of these regulators results in maximum γ-globin gene expression. As we observed decrease of BCL11A mRNA levels from 0.14 to 0.0125 by 11-fold (see Figure 22) and FOG1 and HDAC1/2 mRNA levels from 1.5 to 0.04 by 37.5-fold (see Figure 23) by hypothetical siRNA approach increases γ-globin gene expression by 6-fold over untreated control.

Figure 22: Simulation results for BCL11A gene expression in (a) an untreated cell; (b) Cells treated with our proposed RNAi-mediated strategy.

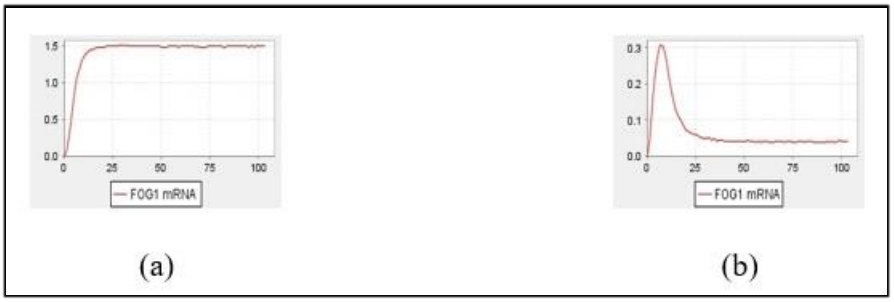


Figure 23: Simulation results for FOG1 gene expression in (a) an untreated cells; (b) Cells treated with our proposed RNAi-mediated strategy.

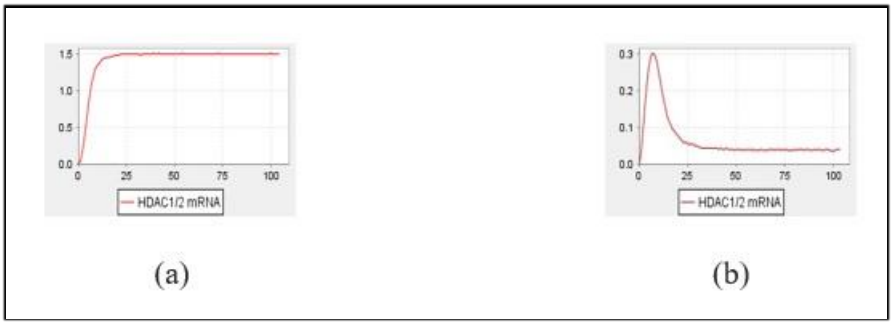


Figure 24: Simulation results for HDAC1/2 gene expression in (a) an untreated cells; (b) cells treated with our proposed RNAi-mediated strategy.

Role played by the regulators of fetal-to-adult hemoglobin switching network as potential targets for reawakening HbF by siRNA- and shRNA-mediated techniques have been investigated in numerous studies. As it is illustrated in section 3.4.2, siMBD2, shMyb501, shBCL11A, and siCHD4 were amongst the most effective down regulators so far to induce γ-globin gene expression. The question is whether there is a target-based approach producing more γ chains. To find an answer for this question, we have performed computer simulations by targeting major biological components responsible in silencing HbF during fetal-to-adult hemoglobin switching developmental stage to increase γ-globin mRNA levels. According to simulation results siMBD2, shMyb501, shBCL11A, siCHD4, and our RNAi-mediated strategy increase γ-globin mRNA levels by 1.9-, 3.4-, 4, and 5, 6-fold over the untreated control from 0.008 to 0.015, 0.027, 0.032, 0.04, and 0.048, respectively (see Figure 25). The comparison of these simulation results shows that our strategy is the optimal one so far since it leads to more γ-globin mRNA levels (see Figure 26). It must be noticed that RNAi-mediated strategy originally suggested in the present thesis even more effective than the drug-based strategies proposed still in e present thesis.

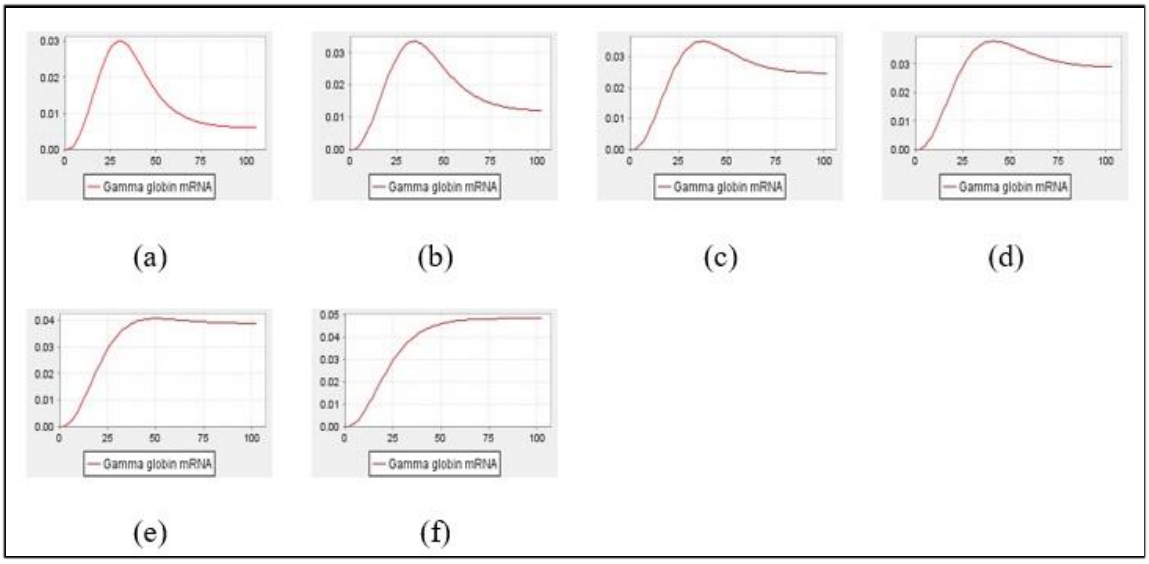


Figure 25: Simulation results for γ-globin mRNA in (a) an untreated cells vs cells treated with (b) siMBD2; (c) shMyb501; (d) shBCL11A; (e) siCHD4; and (f) our proposed RNAi-mediated strategy.

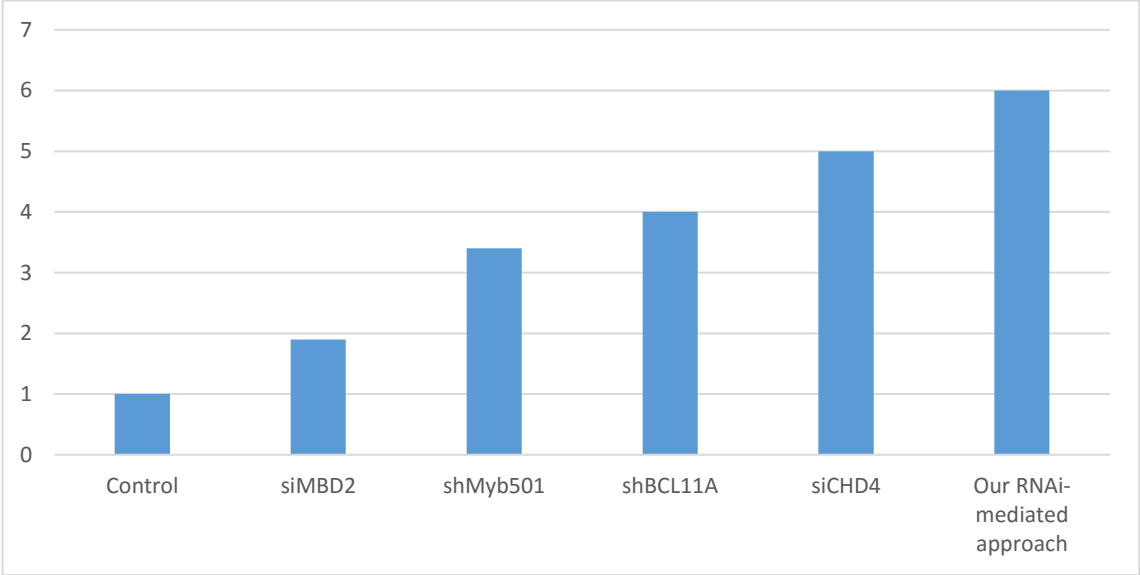


Figure 26: Comparison of untreated cells with various siRNA/shRNA treated cells.

Chapter 4

CONCLUSION

In the present work in line with quantitative modeling approach, we use HFPN and Cell Illustrator software to model fetal-to-adult hemoglobin switching and its transcriptional activities to shed light on the way it works. In addition, we use the extended HFPN models to identify potential strategies to ameliorate severity of β-globin disorders, promoting this innovation to the benefit of reverse pharmacology and HFPN-based quantitative modeling. The main results obtained in the frame of the present thesis are:

1. In accordance with the reverse pharmacology approach we pose a hypothesis regarding modulation of ETF that induce γ-globin gene expression. Comparison of simulation results for the proposed strategy with the ones obtained for already existing drug-based strategies shows that our strategy is better as it results in the highest level of γ-globin induction.

2. In line with RNAi-mediated approach we pose a hypothesis regarding modulation of BCL11A, FOG1 and HDAC1/2 that increases γ-globin gene expression. Simulation results show that our strategy is better among existing RNAi-mediated strategies as it leads to the highest level of γ-globin gene expression.

In this work, we use a deterministic HFPN with estimated crisp values for initial markings of places, and for binding rates of processes to model transcriptional activity of hemoglobin switching. However, for the future work, we are going to extend the HFPN model to a Fuzzy Stochastic HFPN model to increase its accuracy and to minimize the error.

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