Petri Net Modelling Validates RNAi-mediated Gene Silencing in Suppressing the Disease Apolipoprotein B

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Abstract. RNA interference, also known as RNAi, RNA silencing, or posttranscriptional gene silencing (PTGS), is a mechanism present in almost all eukaryotes, which functions like an intracellular immune system. The phenomenon of RNA interference takes place between the transcription of DNA and the translation to proteins. In this study, we first presented a model of the RNA silencing mechanism. The model was constructed based on four important factors: stochastic translocation of mRNA, stochastic dsRNA synthesis, formation of RISC protein, and a random supply of primed as well as unprimed dsRNA. We then applied our model to a primate-specific model for the purpose of validating and predicting the apolipoprotein B (*APOB*) mRNA suppression in a cynomolgus monkey's liver. The simulation results of our model were strongly consistent with known biological phenomena. To our knowledge, this is the first time that hybrid functional Petri net (HFPN) was used to construct a mammal-specific model which was capable of validating and predicting the suppression of *APOB* mRNA in non-human primates.

Keywords: RNA interference, RNA silencing model, systems biology, HFPN, antisense therapeutics.

1 Introduction

RNA interference, also known as RNAi, RNA silencing, or posttranscriptional gene silencing (PTGS), is a mechanism present in almost all eukaryotes, which functions like an intracellular immune system [1]. This naturally occurring phenomenon was first found in the nematode worm Caenorhabditis elegans [2]. RNA interference can be applied in a variety of ways. The most impressive use is termed antisense therapeutics. However, RNA interference is not the only way to do antisense therapeutics. Three types of antisense therapeutics can be distinguished by different inducers. The first one is single-stranded antisense oligonucleotides; the second is ribozymes; and the third, which will be discussed in this paper, is called RNA interference induced by small interfering RNA (siRNA) molecules [3]. RNA interference holds more potential since Elbashir and its colleagues showed that 21 nucleotide-long siRNA duplexes with 3'overhangs can specifically suppress gene expression in mammalian cells [4].

The single-strand RNA complement to the target mRNA is called "antisense" because the "sense" sequence of the mRNA is the one that is directly translated into a functional protein. Binding the target mRNA prevents it from carrying out its function; therefore, the single strand RNA "silences" or "interferes" with the target mRNA. The ability to interfere with a critical point in gene expression and protein synthesis is what makes the antisense molecule such an attractive therapeutic platform. Theoretically, antisense molecules could be used to cure any disease which is caused by the expression of a deleterious gene, e.g. viral infections, cancer growth, inflammatory diseases, and heredity disease [3].

In the last few years, important insights have been made in elucidating the mechanism of RNAi. A plethora of

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in vivo and in vitro experiments have divided mechanistic model for RNA interference into three steps [5,6]. The first step, referred to as the RNAi initiating step, involves the binding of the RNA nucleases (Dicer) to dsRNA and the cleavage of the dsRNA into discrete 21 to 25 nucleotide RNA fragments (siRNA). In the second step, these siRNAs join multinuclease complex (RISC) to degrade the homologous single-stranded mRNAs. In the third step, RISC is recycled and the siRNA undergoes amplification to supply additional siRNA to maintain the cleavage process. Fig. 1 illustrates this concept. Despite the fact that many studies on RNAi have used a simplistic mechanism as described, there are still many problems pending. First, most processes in biology are stochastic instead of deterministic. The RNA silencing phenomenon is without exception. For instance, dsRNA synthesis and mRNA translocation should be the stochastic phenomena. Secondly, RISC is a complex protein [7]; therefore, the formation of RISC takes time during RNA silencing. Thirdly, according to the literature [8,9], dsRNA is supplied in two different ways, the primed and unprimed mechanism. We still are not sure which way is correct. In this work, we first constructed a simple RNA silencing model to describe the basic behavior of this phenomenon and then applied this model to a mammal-specific model for the purpose of validating and predicting the suppression of apolipoprotein B (*APOB*) mRNA in primates.

2 Constructing a RNA silencing model with Petri net

In this section, we try to demonstrate the advantages of the RNA silencing model with Petri Net. We will first introduce the Petri Net theory with its characteristics and basic components along with their functions, especially those applied to RNA interference models [10,11]. Secondly, a basic mathematical model of RNA silencing will be demonstrated. Thirdly, an intuitive method of constructing the RNA silencing model will be presented. Finally, this model is applied to validate the *APOB* mRNA suppression in primates.

2.1 Hybrid functional Petri net in biological simulation

Many useful simulation systems are built on the basis of ordinary differential equations (ODEs). Unfortunately, E-Cell is rather complicated to use for modeling a simple biological pathway. Those more focused on the biological aspect may not be familiar with the complicated mathematical equations used to construct the models. Petri Net's intuitive method for construction modeling is better suited than other mathematical models for simulating biological phenomena.

Petri Net is a model employed in multiple fields, and many extensions to this simple model have been developed for various simulation purposes. The major categories of Petri Net extensions are: (a) Hierarchical Petri Nets, which allow the previously defined net to be present in a new net as an entity or process, (b) Hybrid Petri Nets, which allow the component to deal with continuous values instead of integer numbers of tokens, (c) Timed Petri Nets, which introduce the concept of deterministic time delays, (d) Stochastic Petri Nets, in which entity and process may be assigned delays which are given by a probability distribution, and (e) Colored Petri Nets, which allow more complex firing rules in the processes.

It was not until recently that Petri Net had a significant influence in biological applications. A novel extension of Petri Net called hybrid functional Petri net (HFPN) and the enhanced version, hybrid functional Petri net with extension (HFPNe), have been develop by Masao Nagasaki and his colleagues. Both methods extend and combine different kinds of Petri Nets, making it suitable for solving biological problems. Using this model, we have established an RNA silencing model with HFPNe [1,7].

HFPN contains several key components: entity (place), process (transition), connector (arc), and tokens (markings). The entity can represent a protein, gene, metabolite, or any signal factor. The processes represent reactions, binding, separation, transcription, translation and ordinary biochemical reactions. In Petri Net, entities and processes can be divided into two forms in accordance with their properties: continuous and discrete. A continuous entity contains positive real numbers and delivers tokens at every unit of time. Discrete entities contain natural numbers and can deliver tokens at every defined time interval. Connectors link each component and make a network. Tokens, or markings, indicate the value held by each entity. With those elements, one can represent components of the RNA silencing model, such as mRNA degradation, mRNA translocation, transcription, and complex formation. Different components and reactions are shown in Fig. 1.

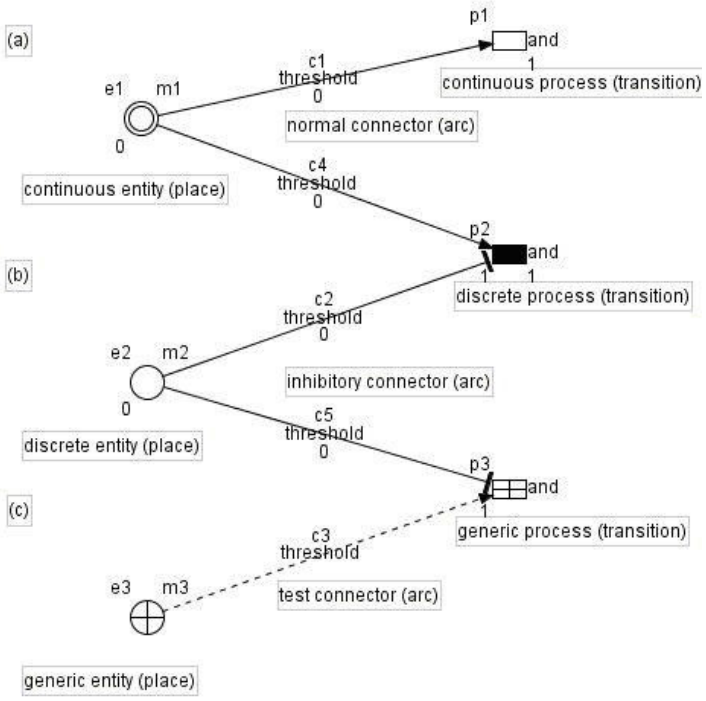


Fig 1. The different kinds of entities, processes, and connectors in HFPN. (a) continuous entity, normal connector, and continuous process. (b) discrete entity, inhibitory connector, and discrete process. (c) generic entity, test connector, generic process.

2.2 Our modified mathematical model for RNA silencing

In order to run a simulation of RNA silencing, firing rules had to be introduced into every process. In this paper, we have modified the original modes [12,13] by adding four important factors that have not been presented before. Those factors include stochastic translocation of mRNA, stochastic dsRNA synthesis, formation of RISC proteins, and a random supply of primed as well as unprimed dsRNA. We divide RNA silencing into four steps; therefore, it can be described quantitatively as a system of differential equations. The four equations are:

$$\frac{ds1}{dt} = -ans1 + gus3 + ps4 \ . \quad (1)$$

$$\frac{ds2}{dt} = ans1 - bs2s4 - gps2s4 - dss2 \ . \quad (2)$$

$$\frac{ds3}{dt} = bs2s4 - gus3 - dcs3 \ . \quad (3)$$

$$\frac{ds4}{dt} = i - bs2s4 - ps4 - dms4 \ . \quad (4)$$

The four variables, s1, s2, s3, s4 represent concentrations of dsRNA, RISC-siRNA complex, RISC-siRNA-mRNA complex, and mRNA in cytoplasm, respectively. The parameters in the above equations are listed in Table 1.

2.3 Constructing and running RNA silencing model

We have already given a brief introduction to the basic components in Petri Net. Therefore, what we are now concerned with is how to use those components to adequately represent biological reactions in the real world. In

this section, we will describe the step-by-step process of building an RNA silencing model. Those problems that have not been taken into account in previous works will be solved in this Petri Net model.

First, we need to decide the number of nodes in our Petri Net model. Considering the mechanism of RNA silencing, we will put seven elements, or entities, in this Petri Net model. These include dsRNA (s1), RISC-siRNA complex (s2), RISC-siRNA-mRNA complex (s3), mRNA in cytoplasm (s4), mRNA in nuclei (s5), monomer of RISC (s6), and ssRNA (s8). Secondly, we will include continuous processes which represent the specific reactions occurring in this pathway, such as combination, degradation, translocation, and synthesis. Finally, we establish normal arcs that connect each component and thus complete the basic model. After the main structure has been established, we apply parameters (see lists in Table 1) and processes firing rules (see lists in Table 2). This completes the whole model of RNA silencing. The complete model with all firing rules (the functions present in every process) can be seen in Fig. 3.

Table 1. Properties of each entry in the simple model we construct. (all variables are described per molecule per time unit)

Name	Type	Variable	Initial Value
dsRNA	Double	s1	0
RISC-siRNA complex	Double	s2	0
RISC-siRNA-mRNA complex	Double	s3	0
mRNA_cytoplasm	Double	s4	0
mRNA_nuclei	Double	s5	1000
RISC_monomer	Double	s6	1000
ssRNA	Double	s7	10000
Unprimed amplification rate	Double	Gu	0.4
Primed amplification rate	Double	Gp	0.002
Decay rate of RISC-siRNA-mRNA complex	Double	Dc	0.0001
Decay rate of mRNA	Double	Dm	0.1
Decay rate of siRNA	Double	Ds	0.0001
Rate of dsRNA cleavage	Double	A	20
Rate of RISC-siRNA-mRNA complex formation	Double	B	0.008
Rate of mRNA synthesis	Double	I	1000
Number of siRNAs cleaved from one dsRNA	Double	N	5
Rate of dsRNA synthesis from mRNA	Double	P	0.002

Table 2. Firing rules and properties of each process in the simple model.

Name	Type	Firing Style	Kinetic Script
synthesis_p1	continuous	and	10
Dicer_cleavage_p2	continuous	and	a*n*s1
Tetramerization_p3	continuous	and	SMass(s7*0.0010,0.2)
combination_p4	continuous	and	b*s2*s4
translocation_p5	continuous	and	100
synthesis_p6	continuous	and	p*s4
Unprimed_ amplification_p7	continuous	and	gu*s3
primed_amplification_p8	continuous	and	gp*s2*s4
degradation_p9	continuous	and	ds*s2
degradation_p10	continuous	and	dc*s3
synthesis_p11	continuous	and	I
degradation_p12	continuous	and	dm*s4

Now we discuss in detail the processes in our model:

1. The dsRNA is formed from ssRNA, which will be translocated from nuclei to cytoplasm stochastically by RNA-dependent polymerase. In our model, a process with stochastic activity (if (rand()<0.5){return true;}else {return false;}) works for this reaction.
2. The target mRNA for RNA silencing is transcribed in the nuclei and translocated into cytoplasm. We set a stochastic process to handle this phenomenon.
3. After the complex silencing works, dsRNA will be supplied through two pathways: primed and unprimed. For these two pathways to be represented in RNA silencing, we use two processes with stochastic activity.
4. RISC is a protein with four subunits. It must work after the four subunits are combined. We set a process called tetramerization to represent this reaction. The kinetic style of this process is set to stochastic mass (standard deviation=0.2, c6 stoichiometry=4, c5 stoichiometry=1, coefficient1=0.01, coefficient2=0.1).

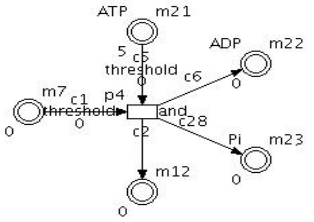
These four setting should improve this RNA silencing model so that it is closer to the real world phenomena.

2.4 Apply the RNA silencing model to validate the *APOB* mRNA suppression

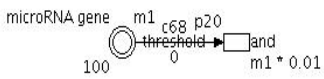
We have constructed a general RNA silencing model, and the next step is to make extensions in order to test real experimental data. Zimmermann and his colleague [14] have demonstrated that siRNA can suppress *APOB* mRNA expression in primates. In order to validate their results through modeling, the first job is to modify our Petri net model to fit RNAi-mediated gene silencing in primates using shRNA. We add primate-specific components based on known biological phenomena into our model. The components are listed as:

Table 3. Petri net presentations of some biological phenomena in the extended model.

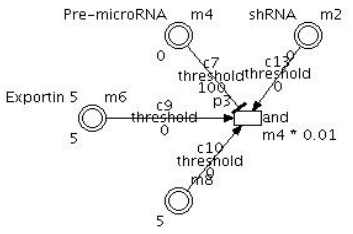
ATP Consumption



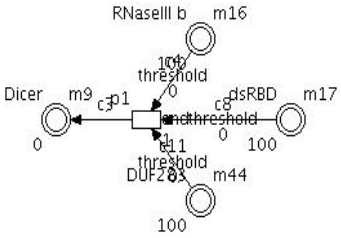
RNA Degradation



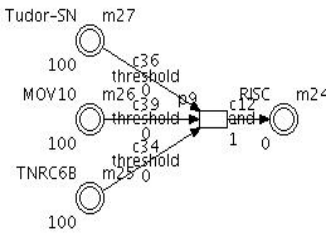
MicroRNA Transport



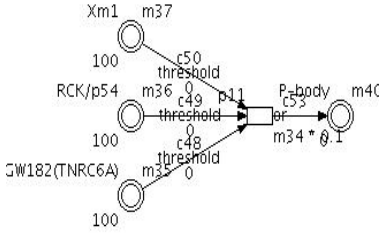
Dicer Formation



RISC Formation



Processing Body Formation



1. Endogenous microRNA is transcribed by RNA polymerase III and the product, Pri-mRNA can be cleaved by Pasha and Drosha [15].
2. shRNA competes with microRNA for exportin 5 when translocated from the nucleus to the cytoplasm [16].
3. Dicer is a complex which contains RnaseIII, dsRBD, Helicase, PAZ, ATPase, and DUF283 [15].
4. Dicer recruits Ago2 and TRBP to cooperate [17].
5. RISC is also a complex containing Tuder-SN, PRMT5, Gemin3/4, Ago1, TNRC68, and MOV10 [18].
6. P-body is composed of DCP2, DCP1a, Xml, RCK/p54, and GW182 (TNRC6A) [19].

Petri net presentations for some of above components are shown in Table 3. The parameters introduced in the extended model are listed in Table 4 and Table 5. The whole model constructed in hybrid functional Petri net is shown in Fig. 2.

Table 4. Properties of entries in the extended model. (All variables are described per molecule per time unit.)

Name	Variable	Initial Value
microRNA gene	m1	100
Pol II	m2	10
Pri-microRNA	m3	0
Pre-microRNA_nucleus	m4	0
Drosha	m5	5
Exportin 5	m6	5
Pre-microRNA_cytoplasm	m7	0
RAN-GTPase	m8	5
Dicer	m9	0
Ago2	m10	10
TRBP	m11	100
microRNA_guide_strand+DicerComplex	m12	0
microRNA_passenger_strand	m13	0
Dicer_complex	m14	0
RNaseIII a	m15	100
RNaseIII b	m16	100
dsRBD	m17	100
Helicase	m18	100
PAZ	m19	100
miRISC	m20	0
ATP	m21	5
ADP	m22	0
Pi	m23	0
RISC	m24	0
TNRC6B	m25	100
MOV10	m26	100
Tudor-SN	m27	100
PRMT5	m28	100
APOB mRNA	m30	100
Pasha	m31	5
Gemin3	m29	100
Gemin4	m32	100
Ago1	m33	100
mRNA-miRISC complex	m34	0
GW182(TNRC6A)	m35	100
RCK/p54	m36	100
Xm1	m37	100
DCP1a	m38	100
DCP2	m39	100
P-body	m40	0
mRNA_cleavage	m41	0
shRNA	m42	2.5
ATPase	m43	100
DUF283	m44	100
Mg++	m45	5
KCl	m46	10

Table 5. Firing rules and properties of each process in the extended model.

Name	Type	Kinetic Style	Kinetic Script
p1	continuous	custom	5
p2	continuous	custom	10
p3	continuous	custom	m4*0.01
p4	continuous	connectorcustom	null
p5	continuous	custom	1
p6	continuous	custom	m11*m9*0.1
p7	continuous	custom	1
p8	continuous	connectorrate	0.01+m24*m12/(0.001+m24*m12)
p9	continuous	custom	1
p10	continuous	custom	0.02556*m20*m30*m42/(8.4+m20*m30*m42)
p11	continuous	connectorrate	m34*0.1
p12	continuous	custom	m40*0.1
p14	continuous	custom	1
p15	continuous	custom	1
p16	continuous	custom	1
p17	continuous	custom	1
p18	continuous	custom	1
p13	discrete	custom	5
p19	continuous	custom	m30*0.01
p20	continuous	custom	m1*0.01
p21	continuous	custom	m3*0.01
p22	continuous	custom	m4*0.01
p23	continuous	custom	m5*0.001
p24	continuous	custom	m31*0.001
p25	continuous	custom	m6*0.001
p26	continuous	custom	m6*0.001
p27	continuous	custom	m42*0.01
p28	continuous	custom	m10*0.001
p29	continuous	custom	m11*0.001
p30	continuous	custom	m9*0.001
p31	continuous	custom	m14*0.001
p32	continuous	custom	m7*0.01
p34	continuous	custom	m12*0.01
p33	continuous	custom	m24*0.001
p35	continuous	custom	m20*0.001
p37	continuous	custom	m34*m30*0.01
p38	continuous	custom	m40*0.001

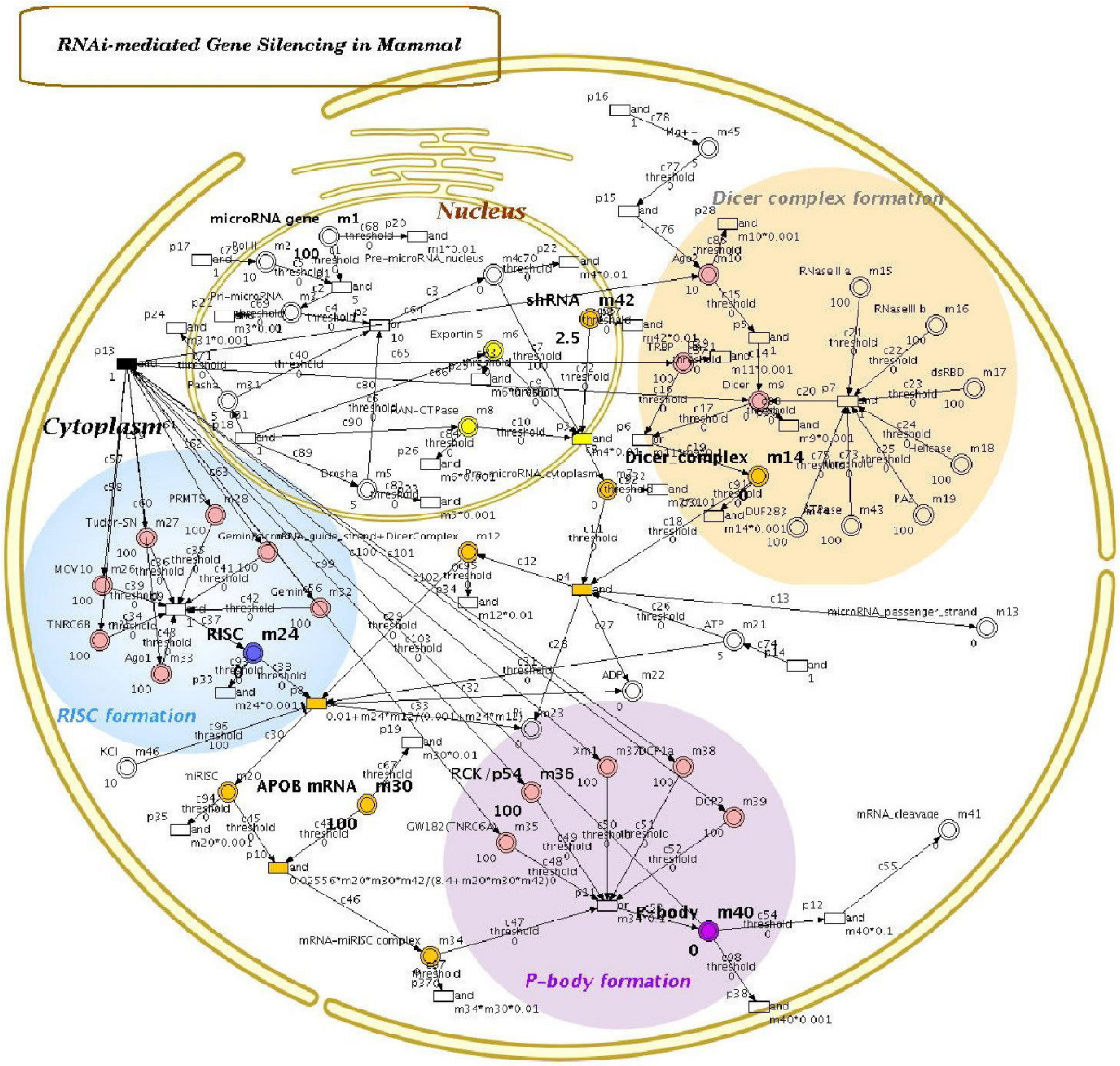


Fig 2. The primate-specific RNA silencing model.

3 Results and Discussion

The simulation results of the model were compared with some known experimental RNA silencing phenomena. In order to see the differences in outputs, we have adjusted several levels of dsRNA. Before running the simulation, we set the running time to be 50pt (pt is a time unit in GON; here we assume that a time unit is one hour). In accordance with the model we have constructed, we can get the simulation results showed in Fig. 3 to 6. After simulation, we compared the results with the known biological phenomena below:

1. During the RNA silencing process, the concentration of dsRNA will exhibit a strong initial drop.
2. Rapid generation of sequence-specific siRNA makes RISC rise in the early stages.
3. While RdRp is functioning normally, there is enough dsRNA for target mRNA, and silencing continues. mRNA will decline sharply.
4. RNA silencing is "dosage dependent", which means that the process will be influenced by the initial level of dsRNA.

All the above biological phenomena match the results presented in Fig. 3 through 6. In Fig. 3, the initial level of ssRNA=10000. We can see that dsRNA drops down and the target mRNA is silenced. In Fig. 4, we changed the level of ssRNA to 1000; however, the phenomenon of dosage dependence is not obvious. In Fig. 5, we changed the level of ssRNA again (the 1/10th of Fig. 4), and we then find that target mRNA does not drop dramatically (dosage dependence). In Fig. 6, we set the input of dsRNA; therefore, we can observe the silencing function even more efficiently.

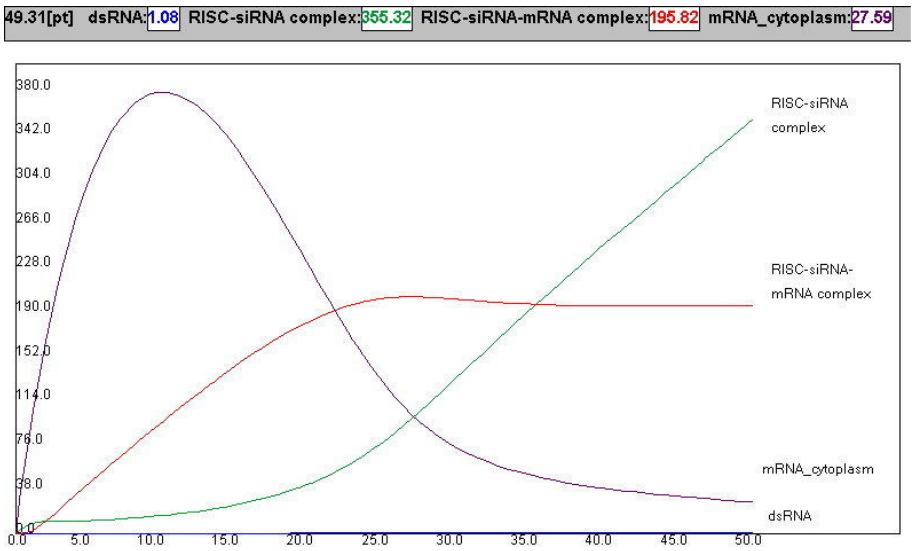


Fig 3. The simulation results of our RNA silencing model when initial ssRNA =10000.

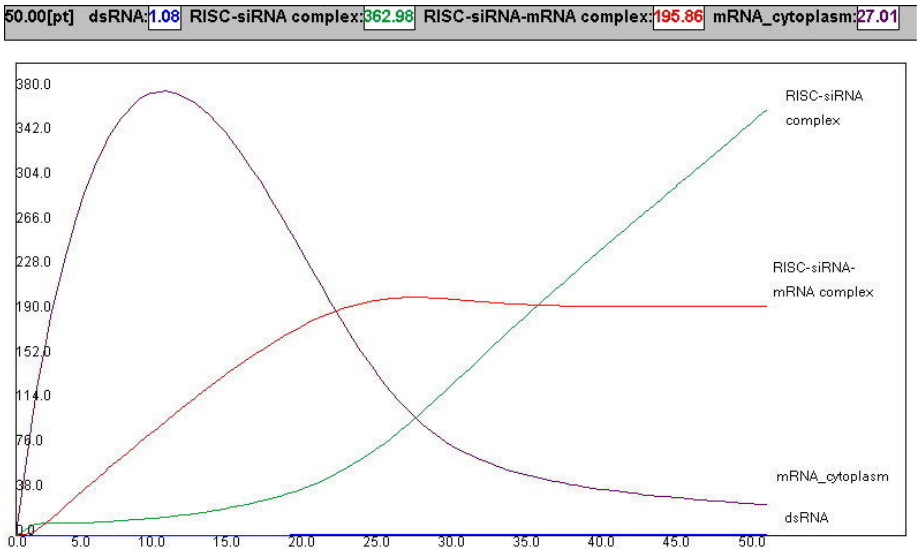


Fig 4. The simulation results of our RNA silencing model when initial ssRNA=1000.

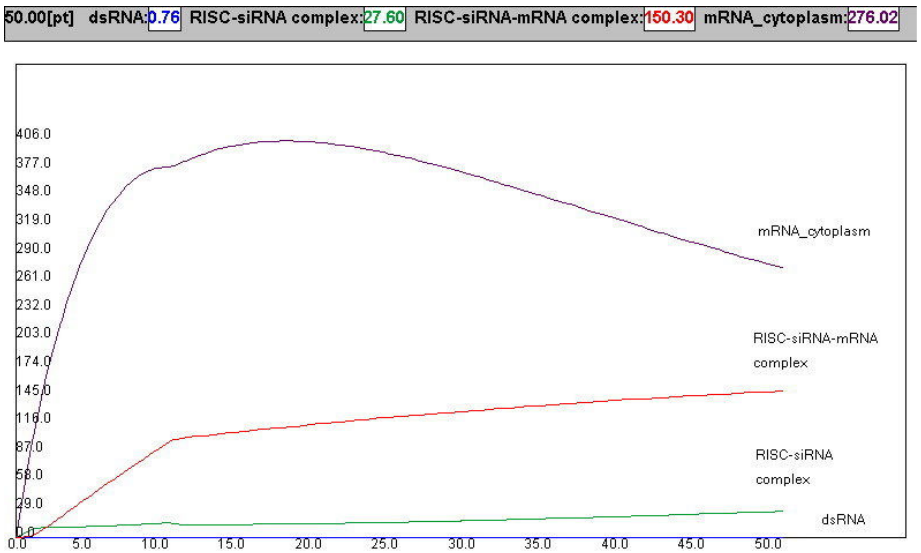


Fig 5. The simulation results of our RNA silencing model when initial ssRNA=100.

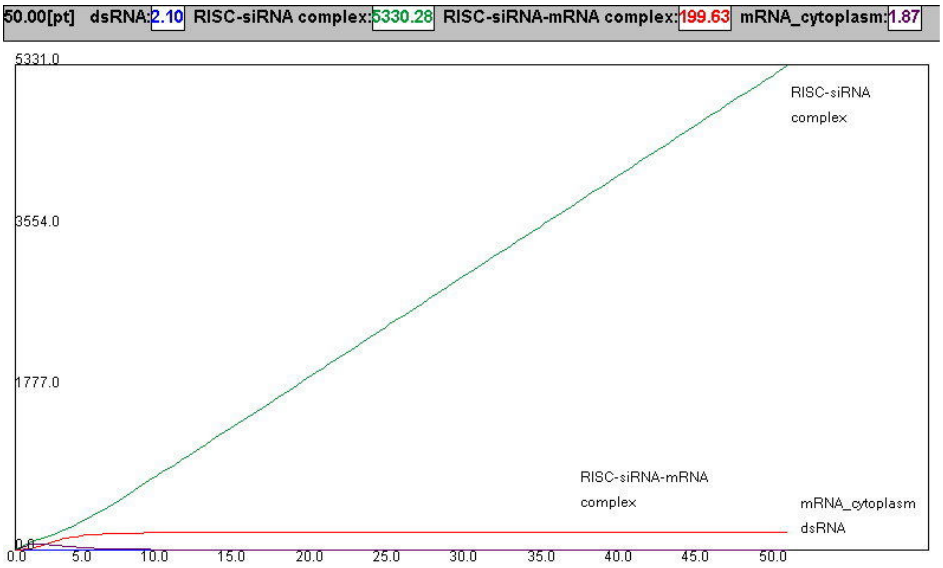


Fig 6. The simulation results of our RNA silencing model when initial ssRNA=10000 and the dsRNA input is at rate of 100/h. It may represent chemically synthesized or in vitro transcribed siRNA duplexes which can be transfected into cells

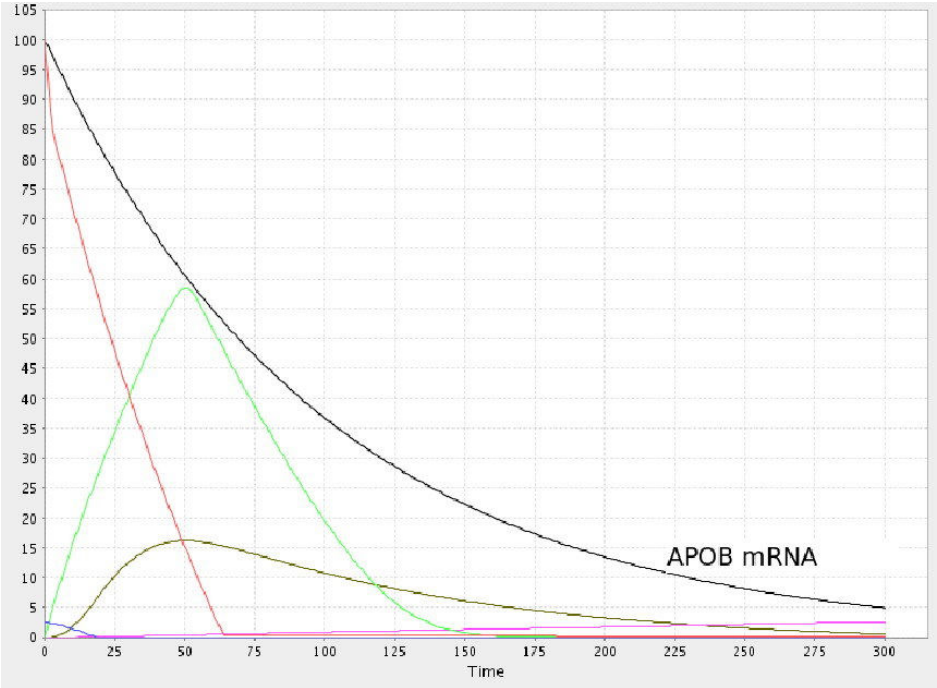


Fig 7. Suppression of *APOB* mRNA in primate by RNAi

After understanding the behavior of the RNA silencing mechanism based on the results from this simple model, it is important to extend the model and apply it to validate *APOB* mRNA expression referred to in Zimmermann's work [14]. Zimmermann and his colleague used 2.5 mg/kg siRNA to silence *APOB* mRNA in a cynomolgus monkey's liver. It was found that the silencing effect lasted for 11 days. In Fig. 7, our model showed that *APOB* mRNA was silenced by siRNA derived from shRNA (short hairpin RNA). The expression amount of the *APOB* mRNA decreased by over 90% during 11 days, a result that perfectly matches the results demonstrated in literature. `

4 Conclusion

In this study, we first presented a model of the RNA silencing mechanism. The model was constructed based on four important factors: stochastic translocation of mRNA, stochastic dsRNA synthesis, formation of RISC protein, and a random supply of primed as well as unprimed dsRNA. We then applied our model to a primate-specific model for the purpose of validating and predicting the apolipoprotein B (*APOB*) mRNA suppression in a cynomolgus monkey's liver. The simulation results of our model were strongly consistent with known biological phenomena. To our knowledge, this is the first time that hybrid functional Petri net (HFPN) was used to construct a mammal-specific model which was capable of validating and predicting the suppression of *APOB* mRNA in non-human primates.

Future studies will encompass more variables involved in this reaction and hopefully further elucidate the RNA silencing pathway and other predictions which can be applied to antisense therapeutics. KEGG [20] provides many biological pathways that may be helpful while considering the whole-cell simulation. RNA interference is still a newly discovered process, and thus the mechanism still contains many unknown variables. Furthermore, full-size simulation is a challenging endeavor and we intend to create it in our model in the near future.

5 Acknowledgment

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