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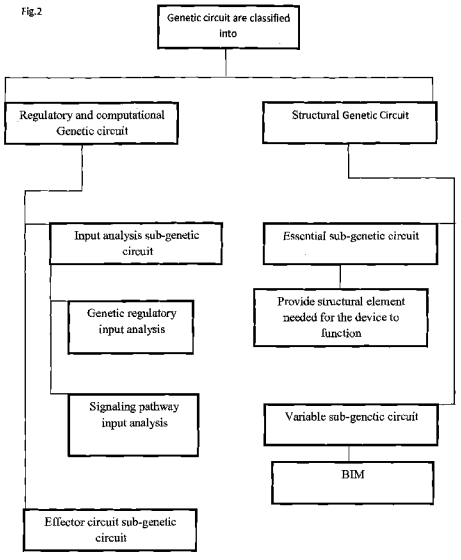
(54) Title: A NOVEL GENETIC DEVICE TO ENGINEER CELL BEHAVIOR



(57) Abstract: This invention is related to the design and production of novel genetic device to control cell behavior. This device can be used to engineer eukaryotic and prokaryotic cells, so it has a wide variety of application from biotechnological to biomedical science and biomedical engendering. Also this invention is related to the production of novel genetic circuit and recombined DNA to control cell behavior. These genetic circuits and recombined DNA can be used to engineer eukaryotic and prokaryotic cells, so it has a wide variety of application from biotechnological to biomedical science and biomedical engendering. Also this invention is contains novel methods to solve some pharmaceutical, biopharmaceutical, and medical obstacles as miRNA delivery system and develop cancer therapeutics. Also this invention contains novel methods related to the production of synthetic promoters.

A novel Genetic Device to Engineer Cell Behavior

RELATED APPLICATIONS AND INCORPORATION BY REFERENCE:

This application claims priority to PCT patent application entitled Using rna-guided foki nucleases (rfns) to increase specificity for rna-guided genome editing filed on Mar 14, 2014. Priority is also claimed to U.S. provisional patent application 09/949,470 entitled A system for antibiotic-regulated gene expression in eukaryotic cells, based on sequences from Enterobacteriaceae antibiotic resistance promoters, polypeptides that bind to the same in an antibiotic- responsive manner filed on Sep 7, 2001. Priority is also claimed to PCT patent application IB2001/001963 entitled Antibiotic-based gene regulation system filled on Oct 19, 2001. Priority is also claimed to PCT patent application CA2002/000654 entitled A system for inducible expression in eukaryotic cells filled on May 1, 2002.

Sequences list:

List of all nucleotide sequences is present in the associated sequence list.

List of all amino acid sequence is present in the associated sequence list.

Technical Field:

The invention is related to the field of adaptive T cell therapeutic specifically T cell engineering with chimeric anti-bodies receptor (CAR), also the invention is related to the field of gene delivery system specifically the miRNA delivery system, also the invention is related to the field of synthetic biology specifically synthetic gene circuit, also the invention is related to the field of molecular biology specifically gene knockdown.

Background Art:

synthetic biology is a science that is difficult to define, but one of the acceptable definitions is the use of molecular biology tools and techniques to forward-engineer cellular behavior[1]. the first genetic circuit was reported at the first month of the new millennium [1, 2], three early designs for gene circuits ware common: the first is genetic toggle, the second is the oscillator, and the third is auto-regulatory circuit[1]. These simple genetic circuits architecture had been studded for years now and a well mathematical description had been developed over years.

The use of small well characterized biological part to assemble large genetic circuits had a great effect on easing the design and the assembly of genetic circuits, and also standardizes the process of designing genetic circuits. Many databases had been developed to store and allow shear of biological part. The most common one is the Registry of Standard Biological Parts located at MIT University.

The use of Boolean logic gate function to modulate and to formalize biological parts make its easer to design circuit, and enable circuit to perform computations[3]. Increase number of genetic circuits result on the development of specialized databases to restore and allow shear of genetic circuits as Synthetic Biological Circuit database.

One of the common parts and sub-circuit element is the inducible promotors. Inducible promoter can be considered as a gene switch that enable the controllable expression of transgenes[4]. Many inducible promoters are available for mammalian and human cell for example, Tetracycline regulated gene expression[5], pristinamycin regulated gene expression[6], macrolide regulated gene expression[6], ecdysone regulated gene expression[7] and mifepristone regulated gene expression[8].

Recently the CASPIR\CAS system has been adapted to be used as TF (transcription factor) to control transcription state of gene of interest. In-order to adapt the CASPIR\CAS system to act as a transcription activator, the researchers have developed a mutant CAS9 which lack the catalytic endonuclease activity (d-cas9) then they fuse its C-terminal with activation domain (like VP16-VP64) to activate the transcription, the result molecule have the capacity to activate the

transcription of the target genes in gRNA depended manner[9, 10].gRNA is a chimeric RNA composite of cr-RNA fused with trans-RNA , it is responsible for guiding dcas9\cas9 to target specific DNA sequences[11].

The earlier reports on using dcas9\gRNA to active Transcription describes low level of expression when using one gRNA, so the design of a cluster that contain multiple gRNA for specific promoter will be a great help [12]. Indeed Nissim & et al have developed a method to drive gRNA cluster expession. There cluster were arranged as follow: 28nt -gRNA -28nt, where "28nt" represent the cys4 recognition sequence [12]. Cys4 is a RNA binding and RNA endonuclease isolated from *Moraxella catarrhalis.* It has the ability to recognize the "28nt" sequence and cut downstream of 28nt. So in this architecture the Cys 4 will bind to its sequence in the RNA and cut it to release the gRNA, which will bind to the dcas9 activating the transcription of the target promoter [12].

One limitation of such a technology was in the inability to express gRNA from type 2 RNA polymerase promoter. Indeed Nissim & et al have report some methods to solve such a problem. In-order to produce a gRNA and protein from the same promoter the authors use a varieties of methods, but here this organization (1-promotor, 2- target protein, 3- MALAT1 triplex helix, 4-(28nt -gRNA -28nt) $_{3-4}$, 5-polyadenylation signal) will be followed. The MALAT1 triplex helix is a 110 sequence isolated from MALAT1 (metastasis-associated lung adenocarcinoma transcript 1), it has the ability to form a conserved triple helix that stabilize mRNA lacking poly A tail [12, 13].

Here the dynamic of this organization will be as follow, after the transcription the produces mRNA will has the 5' cap and the 3' poly A tail. The 28nt will be recognized by the Cys 4 protein which will bind to it and cut immediately downstream of it to liberate; a-the protein coding region with 5'cap (enable its ribosomal binding) & 3' triple helix that will stabilize the mRNA as it lake Poly A tail, and b- gRNA. The2nd method is related to production of gRNA solely from RNAPII promoter. The following organization will be followed (1- promoter, (28nt -gRNA -28nt) $_{3-4}$, SV40 poly adenylation sequence).The cys4 will act on the earlier sequence to release the gRNA from the mRNA

Synthetic biology which has the ability to reprogram cell or a whole organism will have a great impact on our life. These reprogrammed cells and organism can be used to develop novel therapeutic and diagnostic tools[14]. Indeed a circuits that depend upon AND logic gate to diagnosis bladder cancer had been reported recently[15].

Many biomedical and pharmaceutical Obstacles can be solved using synthetic biology tools. Examples of these problems are development novel anti-cancer therapeutics, miRNA delivery systems, and novel diagnostic tools.

The development of new anti-cancer therapeutic is widespread interest. According to the American Cancer Society cancer represent the second most common cause of death in the US, accounting for nearly 1 of every 4 deaths. Many methods are available for cancer treatment, for example chemotherapy, radiotherapy, immunotherapy & surgery. Most of the times a combination of these methods are used to achieve a favorable therapeutic out-come. Most anti-cancer programs depend on chemotherapy alone or with combination of other Methods. Many biological process are targeted by chemotherapeutic, most of these drugs Tiger DNA synthesis process[16] , other tiger Growth regulatory & cell cycle pathways[16, 17]. A newer drug families tigers programmed cell death pathways [16, 18]. Other targets are also being investigated for example, modulation of oxidative stress[19] and targeting tumor angiogenesis [20].

Most if not all chemotherapeutics are associated with severe side effects like Anemia, pain, nerve change, hair loose, memory change, constipation and swelling. Also another problem associated with chemotherapeutics is resistant development. Chemotherapeutic resistant can be defined as a lack of response to drug-induced tumor growth inhibition [21]. For example, cisplatin which targets DNA (indeed after more than 30 year of cisplatin introduce to clinical practices, there precious mechanism is still not-fully understood[22]) has been used to treat wide variety of solid tumor like ovarian, head and neck, colorectal, bladder and lung cancers, but one common problem associated with cisplatin treatment is resistant development that lead to therapy failure[23]. Many mechanisms has been discovered to be associated with chemotherapeutic resistant, for example p-glycoprotein & P53 mutation are two common mechanism for resistant development [21, 23].

All these problems state above make the development of new anti-cancer therapeutic methods an urgent need. Indeed many methods are being developed for anti-cancer treatment like gene therapy and miRNA based therapeutic.

miRNAs (micro-interference RNA) are a class of ncRNA (non-coding RNA), they are around 22nt (there length varied from 19-25nt) long and they are potent gene expression regulator [24, 25].They regulate gene expression by binding to the 3UTR (Un-translate region) of mRNA and inhibit mRNA translation [26, 27]. miRNA represent a complex network for controlling gene expression as one miRNA may regulate many genes and one gene maybe regulated by many miRNAs[28].

The production of miRNA in cell is a multistep sequential process: firstly, the miRNA genes are transcribed into what is known as pri-miRNA (primary miRNA) [24, 25]. Secondly, pri-miRNA binds to Dorsa\DGCR8 (DiGeorge syndrome critical region 8), this protein complex which act as a micro-process cuts the pri-mRNA into 70nt hairpin containing structure known as the pre-miRNA[25, 29]. Thirdly, this pre-miRNA is recognized by the Exportin-5 which transports them to the cytosol [30]. Fourth, in the cytosol the pre-miRNA are recognized and further processed by the enzyme Dicer[31]. The Dicer enzyme produces 20-22nt long miRNA duplex, the miRNA duplex are composite of 2 strand the guided strand and the passenger strand which is also known as miRNA*[28]. Most of the reports suggest that the guided miRNA is the one that is loaded in to the RISC[28]. Finally after begin laded into RISC (RNA-induced silencing complex) the guided miRNA guide the RISC compound to repress the mRNA that show perfect pairing of 2-7nt in there 3UTR with the miRNA[32].

The discovery of microRNAs (miRNAs) as powerful regulators in a wide variety of diseases, make it is only a logical consequence to view miRNAs as therapeutic entities [33, 34]. MiRNA based therapy follows to major paths or the development of miRNA based therapeutic is separated into two main categories, the first is miRNA antagonist which inhibits the endogenous miRNA in the target cell, and the second is miRNA mimetic which acts as the endogenous miRNA in the target cell.

In the first approach, miRNA-antagonist which is also known as miRNA silencing, two main methods have been developed. The first is to target miRNA production process with ASO (antisense oligonucleotide) [35, 36] but it shows little efficiency[37]. The second is to inhibit mature miRNA using ASO, this technology is also known as AMOs (anti-miRNA oligonucleotides)[37]. AMOs technology which depend on complementary base-pair binding between miRNA and AMO represents the most efficient method to inhibit miRNA[37]. The second approach, miRNA mimetic depend upon delivery of exogenous miRNA to the target cell to restore miRNA expression level in that cell[37].

The use of miRNA therapy in cancer is very attractive as it offers many advangeous, for example it can Target many gene simultaneously which will be helpful in a heterogenic disease as cancer [38, 39]. Despite their great promising results, they provide a great challenge for the pharmaceutical industries, many problems are associated with miRNA therapy, for example they have poor tumor penetration, quickly degraded & cleared from the blood, have a potential immuno-toxicity & poor intracellular delivery with retention in the endosome compartment[38]. Developing a method to enable the selective and efficient delivery of miRNA to the cancer tissue will represent an outbreak in cancer therapeutic.

Indeed, many trials and methods have been developed to solve such problem, and one early method was the chemical modification of miRNA backbone. Peptide nucleic acids (PNA), Phosphorothioates, Locked nucleic acids, and 2′ RNAs substitutes to less reactive groups as 2′-O-methyl- (2′-O-Me), and 2′-fluoro- (2′-F) are all examples of such chemical modifications [40].

PNA is one of the most common chemical modifications associated with miRNA. They have many advangeous for example, as opposed to the ribose and deoxyribose sugar backbone, PNAs contain a polyamide backbone composed of repeating N-(2-aminoethyl)-glycine units linked by peptide bonds[37], they show nuclease & protease resistant activity[41]. A bottleneck for PNAs is their inability to cross cell membrane [37, 42]. Many modifications have been used to enhance their membrane penetrating activity. CPP (cell penetrating peptide) represent one of the most widely used methods to chemically modify PNA [42], but this method suffer from a major

drawback which is CPP mediated cellular deliver through the endocytosis pathway [43]. PLA-CPP cannot escape endosomes efficiently and hence large dose is needed to achieve therapeutic accepted level [42]. Large dose PNA produces cellular toxicity and potential off-target side effect[42].

most of chemically modified miRNAs show two major drawbacks, firstly they show off target activity and secondly a toxic metabolites that cause a sever side-effect[44]. These results make the development of a delivery system to deliver miRNA to its target selectively and efficient of invaluable important. There are many miRNA delivery systems for example, MS-2 virus like[45], Cationic polyurethanes-short branch PEI [46], PLGA (poly-lactate co-glycolate) [47],cationic lipoplexes[48], and exosomes [40, 49]. None of these delivery systems has reaches clinical trials yet.

Exosomes are a class of secreted lipid membrane vesicles, which originate from endosome compartment [50, 51]. They are released to the extracellular space when the multi-vesicular body fuses with the plasma membrane[52]. Exosomes contain lipid, proteins, mRNA and miRNA [53]. Exosomes gain much attention when their ability to deliver functional mRNA and miRNA to the target cell and mediate genetic material exchange between cells was established [54]. Using exosomes to deliver miRNA has been recently illustrated in a number of studies, which report the use of exosomes loaded with miRNA to control many pathological conditions [55].

Many methods have been developed to enable selective miRNA loading into exosomes; the common method is the transfection of exosomes producer cell. This method initially was based on transfect the donor cell with vector that drive high expression level of the miRNA of interest so enable its load into-exosomes [55]. Further analysis shows that the mechanism of miRNA selective packing in exosomes depend on a conserved motif call Exo-motif, Exo-motif which is a 4nt sequence (GGAG) is present in all exosomal miRNA it is located at 3' terminal of miRNA in 75% cases & present at random location in the other25%, this motif mediated the binding of miRNA to the hnRNPA2B1 (heterogeneous nuclear ribonucleoprotein A2/B1) which controls the selective miRNA loading into exosomes [56].

The genomic transferring technique has reshaped and enhances every aspect of biomedical science from basic research to clinical uses. It can be used to change the selectivity of T cells by transfect the cell with chimeric antigen receptors (CAR). The (CAR) combines an antigen recognition domain of a specific antibody with an intracellular domain of the CD3-zeta chain and other singling moieties [57]. This method enables the redirection of the T cell effector functions toward a specific cell that express the antigenic specific for this antibody recognition domain, in MHC independent manner [58]. This method has been used widely with cytotoxic $CD8^+$ cell to redirect its effector function toward cancer cell[59].There is a huge number of clinical trials that examine the clinical value of chimeric antigen receptors ,the results show little clinical success duo to many side-effects such as on-target but off-tumor adverse effects, Cytokine release syndrome, Off-target adverse effects and Insertion mutagenesis [60].

On-target but off tumor effect is process in which the CAR attack normal tissue express "the antigen" (i.e. the CAR specific antigen) [61]. HER2 protein is over expressed on the colorectal cancer, so a CAR-T cell specific for HER2 has been design and administrated to the patient (who had colorectal with lung and liver metastatic), 15 min after cell administration the patient suffer a cytokine-releasing syndrome symptoms and subsequently died 5 days latter from multi-organ failure, further investigation explain that the reason was the recognition of HER2 protein normally expressed by lung cells which lead to pulmonary toxicity, cytokine storm and death of the patient[60].

cytokines storm or the cytokine-release syndrome, it is a syndrome that is characterized by fevers, rigors, hypotension and hypoxia [60]. The major reason for this syndrome is the massive T cell activation upon interacting with it target antigen. The causes for these symptoms are the high level production of INF-γ, TNF-α (tumor necrosis factor)[62], IL-2, and IL-6 which leads to generalized inflammation[60]. Many methods have been developed to control them as high level cortisol dose [63], but it has been observed to cause a decrease in CAR T cells efficiency[60], Also IL-6 receptor blocker as tocilizumab (TCZ) has been developed [60] , and suicidal genes. TCZ causes many side effects but the most common one is it cause an increase in the infection rate, also it has been reported that TCZ cause some gastrointestinal disorders[64].

Conditional gene knockdown had provided invaluable tools to study biological system. The development of genetically modified animal model with specific gene knockdown enables greater and deeper understands on gene functions and on gene regulations. There are many methods that are used in conditional gene knockdown, for example Cre recombinase [65-67], and siRNA mediated gene knockdown [68-70].

Both Cre recombinase mediated gene knockdown and siRNA mediated gene knockdown depend mainly upon tissue specific promoter to their expression[71]. Although this strategy had been widely used, it limits the research ability to perform a conditional gene knock down in the highly differentiated cells.

Although a partial solution to such a problem has been achieved by the use of inducible expression system like TetR system, such systems offer the ability to control time of gene knock-down but still they do not produce a specific gene knock-down in highly differentiated cells.

DISCLOSURE OF THE INVENTION:

Section A:

This genetic device is composite of 3 genetic circuits (GC): GC1, GC2, and GC3. Each GC is further composite of cluster of Sub-genetic circuits (CSGC). Each CSGC is composite of Sub-genetic circuits (SGC). Each SGC is composite of sub-genetic circuit elements (SGCE), and finally each SGCE is composite of biological parts (BP). See Fig.1 for a flowchart description of device.

The Genetic circuit present in this device can be separated into 2 man classes:

a) Regulatory and computational circuits

b) structural circuits

GC1 and GC2 represent the first class (i.e. Regulatory and computational circuits), meanwhile the GC3 represent the second class (i.e. structural circuits). The Regulatory and computational circuit is composites of Clusters of sub-genetic circuits (CSGC). Each CSGC is composite of at least one Sub-genetic circuit (SGC).

Regulatory and computational SGC can be classified into:

a) Input analysis sub-genetic circuit (IASGC).

b) Effector sub-genetic circuit (ESGC).

In the first case (Input analysis) the IASGC performs a computational analysis (using Boolean logic gate functions) on the input signals, and generates an out-put signal. The ESGC depends on the out-put signal generated by IASGC as an input and generate the final output signal of the circuits. ESGCs mediate the effector function of the circuit, for example: siRNA, gRNA, miRNA, anti-gRNA, or protein production.

The input analysis Sub-genetic circuit (IASGC) can be classified into:

a) Genetic expression sensitive Sub-genetic circuits (GESSGC)

b) Signaling pathway sensitive Sub-genetic circuits (SPSSGC)

In the first case (i.e. GESSGC) the circuit depends on the gene expression regulation as input function, for example tissue specific gene expression as an input. In the second case the (SPSSGC) the circuit depends on cell signaling pathways as an input signal for example NF-kB activation state as an input function.

Genetic circuit 3 is a structural genetic circuit, the sub-genetic circuit (SGC) present in this circuit can be classified into :

a) essential sub-genetic circuit(ESGC)

b) variable sub-genetic circuit(VSGC)

the essential sub-genetic circuit contain the essential genes for the device to function, for example dcas9 or Cas9 will be essential if the device produce gRNA. The VSGC acts as a "gene-store". Gene store is a DNA segment that acts as an internal memory of the device, this memory contains element like miRNA, gRNA, protein, etc.; these elements will be called build in memory (BIM). The device may use the BIM to produce output signals (i.e. proteins, RNA, etc.) in response to different input signals.

See Fig.2 for a functional classification of the device elements. .

See Fig.3 for a flow chart that shows a functional classification of the device.

The device performs two levels of computations:

a) First level computations

b) second level computations

First level computations represent the first level of input signals analysis, CSGC 1 in each regulatory and computations circuits receive the input signals. These input signals are disturbed to the SGCs of the CSGC. CSGC1 analysis the input signals and generate an output signals; these output signals will be called" linker".

The linkers are of two classes:

a) Local linker (LOL) which connects the SGC of the same cluster together

b) Global linker (GL) which connects different CSGC toghters.

The linkers can be considers as the internal wiring of the device which connect functional entitles together.

The Global linker (GL) carries the result of the first level computations to the 2^{nd} level of computations. The 2^{nd} level computations are carried by the CSGC2 and CSGC3 of each circuit; also CSGC2 and CSGC3 can receive input signals before get analyzed by CSGC2 (i.e. directly from the cell). The Global link linker function is not only to carry the CSGC1 output signals but also to mediated cross linking between circuits, for example the linker generated by CSGC1 of circuit 1 may act on the CSGC2 of circuit 2 and hence mediate the interaction between different SGCs of the device. The 2^{nd} level computations analysis used LOLs to connect its SGCs together. See Fig.4 which shows the computation levels carried out by the device; it also shows the parts that carry different level of computations

The out-put signal will have different fate:

a) It might return back as input to first level computations unit present in CSGCs 1 for re-processing

b) It might return back as input to second level computations unit present in CSGC 2 & CSGC3 for re-processing

c) It might return back to act on the linkers to modulate device behavior

d) It might act on the cell to mediate device cell analysis (DCA). In DCA the device generate an out-put signal that act on the cell to generate a new input signal. This new input signal will be further processed to generate a new out-put analysis that will have a new fate.

e) It might act on the organism level to mediate Global device cell analysis (GDCA). In GDCA the device generate an out-put signal that act on the cell to generate a signal that will act on the organism level and this will lead to generate a new input signal. This new input signal will be further processed to generate a new out-put signal that will have a new fate

g)) It might act on circuit three gene stores to generate a new input signal or an output signals
h) It might act on cell as a final receiver to the signals
See Fig.5 to see the pathways that might be followed by the out-put signals

Duo to the complexity of the device I develop a coding system to enable easy identification of SGCs the coding system is composite of:
a) a first latter that referred to the type of SGC: S for essential structural SGC, V for variable structure SGC, P for SPSSGC, and G for GESSGC.
b) A number that indicate the maximum number of input signal this SGC can take
C) the latter O is referred to output signal
d) a number that indicate the maximum number of output this SGC can produce
e) the latter C is referred to the CSGC
f) a number that indicate to which this SGC belong (i.e. to which CSGC this SGC belong), incase its repeated within many CSGC a comma (,) is used and the number is write between this commas.
g) a Roman number is used to indicate to which GC this SGC belong, incase its repeated within many GC the whole code is repeated.

Examples on this nomenclature system:
a- P6O4 this code means that this SGC is a SPSSGC, can receive a maximum6 input signals, and can generate a maximum 4 output signals.

b- P6O4C2III this code means this SGC is a SPSSGC, can receive a maximum 6 input signals, can generate a maximum 4 output signals, and its located within CSGC number 2 in circuit number 3.

c- P6O4C2,3,1 III this code means this SGC is a SPSSGC, can receive a maximum 6 input signals, can generate a maximum 4 output signals, and its located within CSGC number 2 in circuit 3, CSGC number 3 in circuit 3, and within CSGC number 1 in circuit 3.

d- P6O4C2,3,1 III P6O4C2,3,1 I this code means this SGC is a SPSSGC, can receive a maximum 6 input signals, can generate a maximum 4 output signals, and its located within CSGC number 2 in circuit 3, CSGC number 3 in circuit 3, CSGC number 1 in circuit 3, CSGC number 2 in circuit 1, CSGC number 3 in circuit 1, and within CSGC number 1 in circuit 1.

The elements a, b, c, and d of the coding system are called descriptor of the SGC; meanwhile the elements e, f, and g are called locators. The descriptors are used to describe the SGC without gave any indication about its location in the device.

Herein I used Boolean logic gate function to explain the circuit behavior; the following assumptions are followed to explain biological process in the form of Boolean logic functions:

a) miRNA response element (MRE) is assumed to be an inventor gate, in which the presence of miRNA is associated with inhibition of mRNA translation and vice versa (i.e. in the absence of miRNA the mRNA will be translated). This assumption assumes that miRNA is the only determine factor in the process of mRNA translation. See Table one for Truth table of the inventor function here. In table 1 the A input represent the miRNA which has 2 states: present (1) or absent (0). B represents the translation of mRNA and it has 2 states: translation occurs (1) translation inhibited (0). So here the fact that miRNA inhibited translation has been expressed in the form of Boolean logic gate. See Fig 6 for stander symbol of inventor logic gate.

A	B
1	0
0	1

Table 1 : Truth table for inventor function

b) Promoter is assumed to be a buffer logic gate, where in the presence of input signal (presence of RNA polymerase at the TSS (transcription start site)) It produce an output signal (in the form of RNA transcript). See Table two for Truth table of the buffer function here. So here A represent the Input function

A	B
1	1
0	0

Table 2: Truth table for inventor function

and B represent the output function. See Fig7 for stander symbol of buffer logic gate.

c) The process of repressor binding to its DNA binding site as an inventor function. So in the presence of the repressor bind to its DNA response element the output signal (RNA transcript) is inhibited and Vice-versa. See Table one for Truth table of the inventor function here. See Fig.7 for stander symbol of inventor logic gate.

d) The process of the chemical inhibition of chemically regulated transcription activator or repressor as an inventor function. So in the presence of the chemical inhibitor(CI) (i.e. the chemical inhibitor of the chemically regulated transcription activator or repressor) it represses its function and vice-versa. See Table one for Truth table of the inventor function here.

e) The process of Transcription regulation by inducible promoter and chemically regulated Transcription activator can be represented as complex sequence of logic gated where: the chemical inhibitor has an inventor logic gate, the production of chemically regulated activator as a buffer function and the presence of the protein in the activate state as an AND function (see below), finally the binding to inducible promoter & activation of transcription is a buffer function. The AND function is a two input logic gate function. See Table 3 for logic gate function of AND gate. So here the production of the active state of chemical regulated activator (C) required the presence of two input function A & B. where A is the output function for protein production (the buffer gate for transcription activator production), and B is the output function for the chemical inhibitor explained earlier in assumption C (i.e. it is an inventor gate where the value of 1 shown here indicated the absence of chemical inhibitor). The state of the protein (i.e. Active or not) is a buffer function with the activation of its target promoter. See Fig.8 for symbol of AND logic gate, Fig.9 for a follow chart for the complex logic gate explained here, and Fig10 for a sketch of the logic gates explained in assumption D "wired" or connected together.

A	B	C
1	1	1
1	0	0
0	0	0
0	1	0

Table 3: Truth table for AND logic gate function

f) the process of translation as buffer function in the absence of any miRNA regulatory mechanisms. In case there is any miRNA regulatory mechanism it's a complex sequence of logic gate composite of: a buffer gate for the mRNA transcription, an inventor gate with its miRNA

existence (i.e. absences or present) and finally the translation step will be an AND logic function the receive two inputs one form inventor logic gate and the second from the buffer logic gate see Fig. 11 for a sketch of this logic gate.

g) The process of negative feedback loop (NFL) as a multistep process:
I- the transcription step: transcription process is AND gate that receive two inputs one form buffer logic gate and the other input from inventor logic gate. The input to the buffer function is the activation signal. The input to the inventor function is the repressor produced by the circuit.
II-the produced inhibitory signal is the input function for the inventor logic gate stated earlier.
See Fig12 for a sketch of this logic gate function.

h- Biological part (BP) is a DNA segment or stretch that is responsible for specific function, for example poly adenylation signal to terminate transcription or promoter to activate transcription.

j- SGCE is an element that is composite of more than one biological part, and has the ability to receive an input function and generate an out-put function.

The development of complex genetic devices like the one described here requires large variety of biological parts (BP), but there is limited variety of such BP in synthetic biology repertoires. To solve such a problem I developed the following methods:
a- gRNA depend synthetic promoters (GSP):
promoters are an essential BP in design any genetic circuits; design a genetic device with complex GCs requires a complex and variable set of promoters. The method describe here depends on the gRNA\dCas9 system. The system (i.e. GSP) is composite of a stretch of random DNA sequences (50-1000nt length), this DNA sequence will be called here as Regulator (REG), cloned upstream of eukaryotic minimal promoter.

A set of gRNAs specific for REG will be designed. The gRNA specific for REG will tiger the dcas9 to REG region, the dCas9 system will contain an activation domain fused to it, so activate the translation from GSP. This method will enable the activation of promoters in a gRNA specific manner; also it will enable easy production of synthetic promoter.

b- gRNA inhibitor:

gRNA is essential factor for dCas9 system to function, and as dCas9 system might work with a system contain many gRNA each is specific for specific DNA sequences, for example different REG, a method to inhibit specific gRNA while maintain the other functional will be highly required. As stated earlier gRNA is a chimeric RNA molecules composite of cr-RNA which guide system (i.e. dCas9) to specific DNA sequence, a PAM, and trans-RNA which is essential for the system to function. Design an RNA that bind to the gRNA via base pair will result in the formation of gRNA-anti gRNA duplex, this duplex will be recognized by nucleus RNAase that will digest this duplex. In order to allow the system to be specific to specific gRNA a 10nt sequences that has a complementary base pair with crRNA region of the gRNA molecules will be able to hybridize with the gRNA and tiger it for digestion by RNAase. The gRNA and anti-gRNA can be expressed from type II RNA promoter as described earlier.

Section B:

Herein I describe some novel SGC & SGCE that might be used independently or in the assembly of this device:

This device can be used with the currently available SGCEs and SGCs, or with the novel SGCEs and SGC described in this section. The novel SGCEs and SGC described in this section might be classified into novel designs and novel parts. In novel design part I discuss some new designs for SGCs that can perform new function or SGCs that perform currently available functions with improved design. In novel parts I discuss the use of novel biological parts in the design of novel SGCEs or with the currently available SGCE designs.

A- Novel biological parts:

I- EthR proteins and OethR operons:

EthR (SEQID:14) is a tetR\camR repressor type present in *Mycobacterium tuberculosis* it bind to its operon OethR and inhibit the transcription of the downstream gene Eth A. EthR can be used here as NRR (non-regulated repressor) to control Transgene expression. In a screen of the compound that might inhibit EthR, 2-phenylethyl-butyrate (2PEB) has been found to be an effective inhibitor [72]. Although 2PEB has been reported to be safe on mammalian cell in-vitro [72] no clinical trial with this compound has been reported. So here EthR can be used as NRR in

vivo and as RR ex-vivo.

EthR and its Operon can be modified to activate transcription by cloning at least OethR operons upstream eukaryotic minimal promoter and fuse an activation domain with EthR transcription factor. In the earlier modification when EthR bind to its Operon it will activate transcription instead of inhibiting it. So here EthR can be used as NRA (non-regulated activator) in vivo and as RA (regulated activator) ex-vivo.

The EthR system (i.e. EthR protein an O_{ethR} operon) can use of a variety of SGCEs and SGCs, for example miRNA sensor SGCs and conditional negative feedback (CNFL) SGCE:

1- miRNA sensor SGCs

the system is composite of two nucleic acid constructs:

1- The first nucleic acid constrict is composite of: an eukaryotic promoter (X), an OethR is cloned downstream of the operon, coding region of a reporter protein and finally a transcription terminator.

2- The second nucleic acid constrict is composite of: an eukaryotic promoter (Y), coding region of a EthR, a MRE (miRNA response element) and finally a transcription terminator.

In the earlier architecture the presences of miRNA specific for MRE will result in the repress of EthR and hence the production of reporter protein. This SGC is useful in studying the presence or absence of miRNA.

Some application of such a system maybe:

1- a method to study the Role played by signaling molecules in regulation of miRNA, whereas the production or repression of miRNA can be Qualified using the reporter proteins.

2- An assay to study the miRNA transportation a cross biological membranes, whereas the degree of miRNA transportation across biological membrane can be Qualified using the reporter proteins (the transported miRNA will enhance reporter protein production so the amount of miRNA transported across membrane can be correlated to the amount of reporter protein production)

3- An assay to study the anti-miRNA transportation a cross biological membranes, whereas the degree of anti-miRNA transportation across biological membrane can be Qualified using the reporter proteins (the transported anti-miRNA will inhibit reporter protein production by

inhibiting miRNA that inhibit EthR, so the amount of anti-miRNA transported across membrane can be correlated to the amount of reporter protein production)

2-conditional negative feedback (CNFL) SGCE:

the function of this genetic element is to produces a product like miRNA, gRNA or protein for a limited time and then become irresponsive to further signal for a limited time and then become responsive again and after that become irresponsive and cycle follow on as the activation signal is persistent. The time period in which the circuit response to the activation signal is described here as responsive time, meanwhile the time period where the circuit cannot response to the signal is described here as the out-time.

The system here is composite of promoter, downstream of the promoter is the repressor binding site RBS, downstream of the RBS is the coding region which contain: a) repressor coding region, b) the out-put of the circuits (another protein, miRNA, siRNA ,gRNA or anti-gRNA), and finally the transcription terminator.

The repressor used in this circuit can be of two types:

a) non-regulated repressor (NRR) for example EthR protein and GAL4 DNA binding Domain

b) regulated repressor(RR) for example MphR-KRAB protein

see Fig 13 for an interaction diagrame when this SGCE contian NRR.

see Fig 14 for an interaction diagrme when this SGCE contian RR.

see Fig 15 for a boolean logic gate diagrame when this SGCE contian NRR.

see Fig 16 for a boolean logic gate diagram when this SGCE contian RR.

The dynamic of the system will be the same incase of NRR and RR, the ony difference will be in the ability to intetfear with this SGCE self-regulated cycle using chemical inhibitor spesfifc for RR in case SGCE contians RR. the dynamic can be describe as follow: in the absence of activation signal the circuit is OFF, but once the circuit response to activation signal it start produce repressor protein and output product, so the circuit now is in responsive time. Once the concentation of repressor protein enale it to bind to RBE (Repressor binding element , can be used interchangebally with Represor binding site (RBS) the circuit will automatcally shutdown, and become iresponsive to further activation signal, so the circuit now is in out-time. Once the concentaion of the repressor start to decrease the circuit will recover again and if there is

activation signal it will eneter a 2nd cycle, but if there is no activation cycle it will be in the off state.

dynamic can be describe using boolean logic gate as follow: in order for the circuit to produce its out put two condation must be present the first is the avialblety of activation signal, and second the absences of NRR bind to the RBE. The ealier statement can be represented as AND gate (represented as U3 in Fig.16) that recive 2 inputs the first from the buffer gate (represented as U2 in Fig.18) , and 2nd is from an inventro funcation (represented as U1 in Fig.16). The producation of output signal is a buffer funcation with the activation state (represented as U6 in Fig.18). in case the crcuit use RR the only difference will be in the RR logic gate instead of simple inventor logic gate it will be an AND gate (represented as U5 in Fig.16). U5 recives two input the first form the producation buffer logic gate, and the second from the inventor gate (represented as U4 in Fig.18). of the chemicall inhibitor.

Although the earlier description has been stated in the prior art its incorporation in larger device has not been reported. Also here I suggested two novel parts to be incorporated in this device. First is EthR (SEQID 14) and second is OethR operon.

This system can be used alone for a variety of application for example conditional gene knockdown. For example it can be used to produce miRNA for specific amount of time (i.e. the system responsive time) in animal model to study the effect of temporal inhibition of certain proteins on the development of the organism; also it can produce an anti-miRNA to study the effect of temporal inhibition of certain miRNA on the development of the organism. This system can also be used to produce siRNA to cause irreversible gene knock down in vitro or in vivo.

This system can be wired with other system to produce a SGC that perform more complex function as described in the examples section below.

B- Novel desgins:

I- condiational Ossillator:

This SGC is composite of two SGCEs, the first SGCE (SGCE1) is at least composite of promoter, downstream of it is the RBE, downstream of the RBE there is a coding region contain the activation signal for second SGCE and the out-put Signal A (the out-put signal may be: a miRNA, protein, gRNA, siRNA, or anti-gRNA), downstream of the coding region is the

transcripation terminator.The second SGCE contian a promoter that depened upon the activation signal generated by the first SGCE (SGCE1), downsteam of its is RBE , downstream is the repressor and the output signal B coding region, down stream of this ORF there is miRNA response element (MRE), and finally transcripation terminator. This condational Ossillator get there name form the fact that they behave as a ossillators in the absence of spesific condation, and in the presnec of this condation they stop ossillating.

this condational osssillator can be classified into:

a) uni-codiational ossillator

b) bi-condational ossillator

in the case of uni-condational ossillator there is only one condation that must be absent for the system to ossillate, meanwhiel bi-condatinal ossillator need the absence of two condations. The difference between the uni- and bi-condational ossillator will depended upon the reressor type, uni-condational ossilltor use NRR and bi-condational ossillator use RR.

see Fig. 17 for a shows a flow chart on this circuit dynamic.

see Fig. 18 for an interacting diagram for this SGC when contian NRR .

see Fig. 19 for an interacting diagram for this SGC when contian RR.

see Fig. 20 for a boolean logic gate diagram for this SGC when contian NRR.

see Fig. 21 for a boolean logic gate diagram for this SGC when contian NRR.

the dynamic of the system here can be describe as follow, SGCE1 generate the output product A and the activation signal for SGCE2. SGCE2 generate the Repressor (whear its NRR or RR) and put-put signal B. The first conational point is the miRNA spesific for MREof SGCE2, in which if miRNA is present it repress translation and hence the repressor will not be produced. If the repressor is produced(i.e miRNA wasnot present) it will bind to RBE in both SGCEs and inhibite transcription of both the activation signal and itself. As it repress its producation it will not be further produced and after time its concentratin will fall down and then dissacioated from the its RBS causing the activation of both SGCE1 and SGCE2 and the cycle repeat it self. In case of RR the use of small chemical compounds to regulate reprssor function will add a next level of control on the behaviour of SGC.

as shown in Fig.20 the dynamic of the system in case of NRR can be explanied in the terms of boolean function as follow: the transcription activation at the SGCE1 is an AND gate (U3) that recive its inputs from two function the first is the buffer function (U2) and the second is inventor

function (U1). The buffer function recive its input from the activation signal and the inventor function receives its input from the repressor produced by SGCE2. The AND gate produce an output that fourm an input to the buffer gate of product A production (U4) and to the buffer gate for SGCE2 production (U5). The transcription activation at the SGCE2 is an AND gate (U7) that recive its inputs from two function the first is the buffer function (U5) and the second is inventor function (U6). The buffer function recive its input from the activation signal generated by SGCE1 and the inventor function receives its input from the repressor produced by SGCE2. The AND gate produce an output that fourm an input to the AND gate U9 which receive its second input from inventor gate U8 (which represent the miRNA condation). U9 produces an input to buffer function of out-put signal B production (U10) and to the inventor gates (U1&U6).

as shown in Fig.21 the dynamic of the system in case of NRR can be explanied in the terms of boolean function as follow: the transcription activation at the SGCE1 is an AND gate (U3) that recive its inputs from two function the first is the buffer function (U2) and the second is inventor function (U1). The buffer function recive its input from the activation signal and the inventor function receives its input from the repressor produced by SGCE2. The AND gate produce an output that fourm an input to the buffer gate of product A production (U4) and to the buffer gate for SGCE2 production (U5). The transcription activation at the SGCE2 is an AND gate (U7) that recive its inputs from two function the first is the buffer function (U5) and the second is inventor function (U6). The buffer function recive its input from the activation signal generated by SGCE1 and the inventor function receives its input from the repressor produced by SGCE2. The AND gate produce an output that fourm an input to the AND gate U9 which receive its second input from inventor gate U8 (which represent the miRNA condation). U9 produces an input to buffer function of out-put signal B production (U10) and to the AND gate (U11). U11 AND gate represent the ativation state of the repressor, U11 AND gate recive its input function from the U9 AND gate and from the inventor gate U12. U11 AND gate generate the input signal for the inventor gates (U1&U6).

Although the oscillating genetic circuits (OGC) have been reported earlier the architecture of the oscillator was different, most of the reported OGC has the repressilator architecture which makes the SGC complex in design and large in size. The novel design of this circuit makes it simple to construct and small in size. Also non-of the early reported circuit report the use of miRNA to

regulate the oscillating behavior of the circuit. This SGC will have a variety of application for example it can be used in the self-regulated production of transgenic proteins or the self-regulated production of miRNAs. It also might be used to control production of viral vectors.

II- SGC for complex data integration:

this CSGC is used for integration of multiple inputs (from 2 and up to 7 inputs), and generation of one final output. This CSGC has two states: ON and OFF. In ON state the CSGC generate its final output signal. In the OFF state the CSGC does not produce the output signal.

This SGC is composite of three SGCE:

a) SGCE 1which is composite of: a promoter, downstream of it is the RBS for repressor 2 , downstream of it is chemically regulated repressor1coding region, and finally the transcription terminator.

b) SGCE2 which is composite of: a promoter, downstream of it is the RBS for repressor 1, downstream of it is chemically regulated activator coding region, and finally the transcription terminator.

c) SGCE3 which is composite of: a promoter, downstream of it is the RBS for repressor 1, downstream of it is chemically regulated repressor 1 coding region and the output signal which might be (protein, miRNA, gRNA, siRNA, or anti-gRNA) and finally the transcription terminator.

The system might integrate other inputs by the following means:

a) fuse a MRE downstream of chemically regulated repressor 1 mRNA (this will enable the circuit to have a three input integration capacity

b) fuse a MRE downstream of chemically regulated activator mRNA (this will enable the circuit to have a four input integration capacity)

c) fuse a MRE downstream of chemically regulated repressor 2 mRNA (this will enable the circuit to have a five input integration capacity)

d) use a tissue specific promoter with SGCE1(this will enable the circuit to have a six input integration capacity)

e) use a tissue specific promoter with SGCE2(this will enable the circuit to have a six input integration capacity)

Fig. 22 shows an interacting diagram for an 4 input circuit. The four inputs are:

a) promoter (Pro)1 of SGCE1 is a tissue specific promoter

b) pro2 of SGCE2 is an tissue specific promoter

c) the chemical inhibitor (CI) for RR

d) the CI for chemical regulated transcription activator CRTA .

The dynamic of this example can be shown as follow: in the presence of activation signal at pro 1 it produce a RR, this regulated repressor bind to its RBE located in SGCE2 and SGCE3. At this state the circuit can be described as being in the OFF state where it does not produce any output signal. In the presence of the CI for RR and activation signal at pro 2 SGCE2 produces the CRTA. CRTA bind to its inducible promoter (ip1) and activate the production of: a) NRR that inhibits the RR production by bind to RBE located upstream of RR coding region, and b) the output of the device. At this stage the circuit can be described as being in the ON state where it does produce output. The circuit can be shutdown (i.e. transform from ON to OFF state) using two signal the first in the presence of CI for CRTA and the second is the activation signal for pro1.

Fig.23 represents the Boolean logic gate for this SGC when it can receive a seven input signals, U3 AND gate function represent the transcription state of RR where this AND gate receive two inputs the first from a buffer gate (U2) and the second from the inventor gate (U1). The U2 gate receive its input from the activation signal, meanwhile the U1 inventor gate receive its function from the output of SGCE3 activation. The translation of RR is an AND logic gate (U5) that receive its input from two functional logic gate the first is the U3 AND logic gate and the second is the inventor function U4. The U3 AND logic gate reflex the transcription, meanwhile U4 indicate the presence or absence of miRNAs. The activation state of the RR is represented with AND gate (U7) that receives its input from two functional logic gate the first is the U5 AND logic gate and the second is the inventor function U6. The U7 AND logic gate reflex the activation state of RR, meanwhile U6 indicate the presence or absence of chemical inhibitor CI. U10 AND gate function represent the transcription state of CRAT where this AND gate receive two inputs the first from a buffer gate (U9) and the second from the U8 inventor logic gate. The buffer gate (U9) receives its inputs from the activation signal, meanwhile the inventor logic gate receives its input from the U7 AND gate. The translation of CRAT is an AND logic gate (U12) that receive its input from two functional logic gate the first is the U10 AND logic gate and the second is the inventor function U11. The U10 AND logic gate reflex the transcription,

meanwhile U11 indicate the presence or absence of miRNAs. The activation state of the CRTA is represented with AND gate (U14) that receives its input from two functional logic gate the first is the U12 AND logic gate and the second is the inventor function U13. The U12 AND logic gate reflex the translation, meanwhile U13 indicate the presence or absence of chemical inhibitor CI. The activation of ip1 is an AND logic gate (U15) function that receive two inputs from two functional logic gate the first is the U14 AND logic gate and the second is the inventor function U16. U 14 AND reflex the activation state of protein, meanwhile the U16 inventor gate receive its input from U7 AND gate representing the activation state of RR. The translation of SGCE3 mRNA is an AND gate that receive its input from two functional logic gate the first is the U15 AND logic gate and the second is the inventor function U17. The U15 AND logic gate reflex the transcription, meanwhile U17ndicate the presence or absence of miRNAs. The final output is the input to buffer gate U19 represent the output signal and to the inventor gate U1.

C-Examples:

the device discussed here can be used as a whole or can be modified, for example in the following section I describe two examples: the first is based on the whole device (i.e. an example of the device composite of three GCs), and the second is based on a modified device (i.e. an example who does not follow typical design of the device) in which it contain two GCs only.
I: a device to reprogram $CD4^+$ T cell to attack cancer cell, deliver miRNA in suit to cancer cell, and engineer T cell response & cytokine release:

Firstly CAR enables the direction of T cell effector function toward a cell carry the surface antigen specific for the CAR. So the CAR enable the directions of the cell effector function to any cell in a MHC independent manner [58]. This method has been used and still develops in clinical trials with CD8+ cytotoxic T cell to direct its cytotoxic arsenal toward cancer cell [57, 73]. Here I used the same principle but with CD4+ T cells to enable the directions of its function toward cancer cells. The use of CD4+ cell modified with CAR to target tumor cell has been reported [74, 75] they show to be efficient in lysing tumor cells.

Exosomes will be used to deliver miRNA. As described earlier miRNA therapeutic agents can be loaded into exosomes using the Exo-motif concept. T cell has been shown to efficient exosomes secretory cell [76], Indeed T cell has been shown to secret or produce exosomes after TCR

activation and these exosomes was bearing the TCR\CD3\zeta complex[77] so theoretically CAR must be present in the exosomes, also T cell has been shown to enhance its exosomes release after forming IS (immunological synapse) [78]. So based on the earlier notes using T cell to deliver exosomes will enable the highly selective and efficient delivery of miRNA to the target cell, in which the selectivity is granted by the in-suit release of exosomes to the target cell and by the CAR (which is specific for the target cell) that will be load into exosomes. See Fig.24 for Flowchart on selective delivery of miRNA to target cell using $CD4^{+}$T cell reprogramed with this device. Also in this device I reported the design of two gene circuits that will enable the engineering of T cell responses as its tissue migration and cytokine release. Fig.25 shows the composition of this device.

Duo to the large size of the device, choosing of large capacity vector is a must; hence I choose the ϕ C31cloning vector to deliver the circuit to the T-cell, but it can be delivered using any other mean for example using transposes. In order to avoid the need to use three different vector (CAR- lentivector, ϕ C31 integrase expression vector , and ϕ C31 plasmid) and the need to use two antibiotic as selection marker I modify the selection protocol to be as follow: removing the anti-biotic selection marker from the CAR vector and insert it in the variable SGC of genetic circuit III, then insert the phi c31 integrase (int gene, SEQID: 36 (nucleotide sequence) & SEQID:44 (amino acid sequence)) within the CAR vector. In order for the antibiotic resistance marker to be expressed it must be successfully inserted in the genome of T cell (depending on the integrase enzyme which will not be expressed unless successful CAR-lentivector insertion occur first), which will not be achieved if the cell was not successfully transfected with both vectors.

A-Genetic circuit III function and parts:

1- VSGC (i.e. BIM):

the VSGC contain the following parts:

a- selection marker.

b- Suicidal gene.

c- The variable miRNA cluster.

d- CAR vector

Although the CAR vector is not structurally linked to the device it is functionally linked to the device. CAR vector can be cloned with the device in the same vector, so it can be structurally linked to the device.

The variable miRNA cluster contains miRNA that will be changed according to the target cell. Two general types of miRNA are present within the variable miRNA cluster the first is the therapeutic miRNA (TmiRNA) and the second is the AmiRE (see below).

There are several miRNAs that are highly represented in exosomes, for example T cell exosomes are enriched in miR-575, miR-451, miR-125a-3p, miR-198, miR-601 and miR-887 [56]. As it has been shown earlier normal exosomes contain miRNAs, and these miRNAs may have a variety of actions or effects on recipient (Target) cell, these effects depend upon the receiver cells type. These miRNAs can be classified depending on their action on recipient cell into:

1- miRNAs with therapeutically valuable effects (i.e. they produce a therapeutically wanted effect on the target cell) for example miRNA451 has been shown to exert anti-cancer effect on NPC (nasopharyngeal carcinoma) [79], also miRNA451 has been shown to exert anti-cancer effect on glioma [80] and on NSCLC (non-small cell lung cancer) [81], further more miR-125a has been shown to inhibit hepatocellular carcinoma (HCC) [82] & miRNA 198 has been shown to inhibit colorectal cancer growth & metastatic [83].

2- miRNAs with therapeutically un-favorable effects (i.e. they support tumor cell) for example miRNA9 has been identified in T cell exosomes [78], miRNA9 has been shown to support cell invasiveness & metastatic behavior of breast cancer [84]. So in this situation the use of anti-miRNA that has an Exo-motif (anti- exosomal miRNA that contain an Exo-motif (AmiRE)) will give a soluation to this problem. They will be loaded into exosomes and inhibit miRNA which has therapeutically un-favorable effect.

3- miRNAs with therapeutically neutral effects (i.e. they neither decrease the therapeutic effect nor enhance the tumor cells).

These miRNA (i.e. TmiRNA & AmiRE) will be cloned into the same miRNA hairpin cluster to drive there expression from one pri-miRNA transcript.

by choosing part b & c to be variable, this allow the circuit to be us used with wide variety of choose like, different suicidal gene can be used with no restriction to one particular type, also by making the selection marker variable, the circuit may be adapted easily to the available selection marker.

The two proteins (suicidal gene (for example HSV-TK) & selection marker) are present in one ORF connect by 2A peptide or IRES to deliver their multicistronic expiration, and they are present under the control of constitutive promoter, for example CMV promoter, to drive high level of gene expression. The miRNA are present here as miRNA hairpin cluster, under the control of the strong promoter, for example CMV promoter, to drive high level of gene expression.

The earlier genetic parts can be synthesized using the commercially DNA synthesis serves, and then cloned into a destination vector (that contain attL1& attL2). This will enable the easy transfer of the variable region from the destination vector to the expression vector (in this case will be the ϕ C31plasmid) in one step using the Gateway cloning technology.

2- ESGC:

-Parts:

1-CMV promoter (SEQID: 1), 2-coding region of (SV40NLS-cys4 (SEQID: 2), 2A peptide (SEQID: 3), dCas9-VP16 (SEQID: 4)), 3- SV40 polyadenylation signal (SEQID:5).

- Assembly:

Downstream of the CMV promote there is the Kozak box (SEQID:22) which increase the translation of RNA into proteins (i.e. increase the translation efficiency)[85], downstream of Kozak box there is the ORF, this ORF contain the SV40 NLS fused with cys4 N-terminal, and then p2A peptide has been used to connect Cys 4 to the dCas9 protein to enable its multicistronic expression. dcas9 C-terminal is fused with VP16 domain to activate transcription, finally SV40 polyadenylation signal is used as efficient transcription terminator.

B- GCIII assembly:

the circuit was assembled by fusing cHS4 (SEQID: 8) -which act as insulator element- upstream of the CMVp, and then downstream of the SV40 the attR1 (SEQID: 6) & attR2 (SEQID: 7) ware

fused. The function of attR1& attR2 are to enable the addition of the variable region to the device by Gateway cloning technology, thus enable the easy cloning of variable region (i.e. BIM). See Fig.26 for the structure arrangement of this circuit.

C-Genetic circuit I (GC I) assembly & parts:

1-SGCI:

-SGCE1:

-function: gave a controllable production of the TetR protein.

- Parts: 1- CMV promotor (SEQID: 1) 2-OethR operons (SEQID: 9)3- SV40NLS-TetR CDC (SEQID: 10) 4- SV40 polyadenylation signal (SEQID: 5).

-Assembly:

CMVp (promoter+ enhancer) are present upstream of OethR (EthR binding element) which is a (55bp that bind an EthR in an oligomer manner to cause strong inhibition for transcription) then down-stream of the OethR there is a Kozak element to increase the translation efficient, downstream of the Kozak element there is TetR CDS (coding sequence) which has been codon optimized for human translation and fused with SV40NLS to drive its Nuclear import, finally there is the SV40 polyadenylation sequence as a transcriptional terminator .

- Inputs:

1- EthR: inhibit TetR production.

-Outputs:

1-TetR protein.

-SGCE2

-function: gave a controllable production of the PIT protein

- Parts:

1-CMV promoter (SEQID: 1), 2- TetO2 (SEQID: 11), 3-PIT coding region (SEQID: 12), 4-SV40 polyadenylation signal (SEQID: 5).

-Assembly:

the CMVp is present upstream of TetO2 to regulate the transcription of the downstream gene in a tetracycline depended manner, downstream of TetO2 is the Kozak element to increase the efficacy of the downstream gene translation, downstream of Kozak sequence there is PIT coding region. Downstream of the PIT there is the SV40 Polyadenylation sequence.

-Inputs:

1-TetR: inhibit PIT production.

2- Tetracycline: activate PIT production.

-outputs:

1- PIT

c- Sub circuit element 3:

- function: gave a controllable production of the EthR and GAL4-VP16 proteins.

- Parts:

P_{PTR} promoter (SEQID: 13), TetO2 (SEQID: 11), Kozak sequence (SEQID:22),SV40 NLS-EthR coding region (SEQID: 14), 2A peptide coding region, VP16 coding region (SEQID: 15), linker amino acid (SEQID16), GAL 4 (SEQID: 17), SV40 polyadenylation signal (SEQID: 5).

-Assembly:

downstream of P_{PTR} promoter there is TetO2 operon to enable tetracycline to regulate the transcription process , downstream of tetO2 there is the Kozak sequence up-stream of the start codon of the SV40 NLS coding region, SV40 NLS is fused with EthR protein, with its Start and stop codon deleted, and its fused to the P2A peptide, that connect it to the GAL4-VP16 coding region. Finally there is the SV40 Poly Adenylation signal that act as a terminator.

-inputs:

1- TetR: inhibit the production.

2- Tetracycline: active the production.

3- PIT: essential for production.

4- Pristinamycin: inhibit the production.

-Outputs:

1-EthR protein.

2- GAL4-VP16 protein.

This SGC is a G2O1. It arrangement has been describe in section III.

2- SGC2

-SGCE1:

function: gave a controllable production of CymR and gRNA for the S1P receptor1 (S1PR1).

-parts:

1- UAS minimal promoter (SEQID: 18), 2-SV40NLS-CymR (SEQID: 19), 3MALAT1 triple helix (SEQID: 20) 4-$(28nt\text{-}gRNA\text{-}28nt)_{3\text{-}4}$ (SEQID:21), SV40 polyadenylation

signal(SEQID:5).

-Assembly:

the UAS minimal promoter which is formed form X6 UAS (6 Up stream Activation sequence) are fused to together so it act as enhancer when the GAL4-VP16 bid to it, downstream of the 6xUAS there is the D. melanogaster HSP70(heat shock protein 70) mammal promoter, the earlier arrangement enable the transcription to be depended only on the GAL4-VP16. Downstream of this promoter there is CymR coding region, downstream of it the MALT1 triple helix which is a 110nt long present immediately upstream of (28nt-gRNA-28nt) cluster, Finally the SV40 poly A signal is present.

The gRNA(s) for S1PR ware generated as follow, using the MPED (mammalian promoter enhancer database) 1KB long stretch (+1 to -1000) upstream S1PR1 TSS was retrieved and by the using of E-CRISPR [86] the following gRNA was design. The gRNA produced by this method was fused with the tracer RNA (SEQID: 22) to form the chimeric-gRNA. The cluster is shown in Fig27.

- Input:

1- GAL4-VP16: essential for production.

- Output:

1-gRNA for S1p receptor activation. 2- CymR protein.

3- SGC3:

-SGCE1:

function: gave a controllable production of the gRNA for the CCR2 receptor.

1- CMV promoter (SEQID: 1), CuO2 (SEQID: 23), (28nt-gRNA-28nt) $_{3-4}$ (SEQID:24), SV40 polyadenylation signal (SEQID:5).

-Assembly:

downstream of the strong CMV promoter there is the CuO the enable CymR repressor to bind & to repress transcription followed by the gRNA cluster and finally the SV40 polyadenylation signal. The gRNA(s) for S1PR ware generated as follow, using gene bank the CCR2 promoter was retrieved(+500nt upstream of TSS) retrieved and by the using of E- CRISPR [86] the following gRNA was design.

The gRNA produced by this method was fused with the tracer RNA tracer to form the chimeric-

gRNA the. The cluster is shown in Fig28.

-inputs:

1- CymR: inhibit production.

2- Cumate: activate the production

-outputs:

1- gRNA for CCR2.

The assembly of the circuits:

the circuit was assembled by fusing SGC1 to 2 to 3 using a 50nt spacer DNA (SEQID: 23), the cHS4 was fused up-stream of the earlier mention Assembly's to prevent any un-predicated epigenetic inference from the nearing cellular genomic data or other circuit element. A 50nt sequence was used here as a spacer to physically separate some entities' in the circuit. Fig 29 illustrates parts arrangement of this circuit.

C-GCII:

1-SGCE1

-SGCE1

function: enable the production of MphR –KRAB protein to be a function of cell activation.

- Parts:

1-IL-2 promoter (SEQID: 25), 2- MphR coding region (SEQID: 26), 3- SV40 polyadenylation signals (SEQID: 5).

-Assembly:

the IL-2 promoter is present upstream of the coding region contain SV40 NLS fused with the KRAB domain which is connect to the N-terminal of the MphR-KRAB using a flexible GS amino acid linker, downstream of this ORF there is the SV40 polyadenylation signals .

- Input:

1-TCR signaling.

-Output:

1- MphR-KRAB protein.

-SGCE 2:

-function: allow the controlled production of miRNA 146a, miRNA29a and miRNA29b.

-Parts:

1- IL-2 promoter (SEQID: 25), 2-ETR2 operator (SEQID: 27), 3-miRNAs (146a, 29a, 29b) hairpin cluster (SEQID: 28), 4- SV40 polyadenylation signal (SEQID: 5).

- Assembly:

downstream of the IL-2 promoter there is the ETR2 operator that is composite of 2 ETR binding sequences this operator enable the binding of the MphR, downstream of ETR2 there is the miRNAs cluster that is composite of miRNA (146a,29a&29b) , and finally downstream of it there is the SV40 polyadenylation signal. The miRNA cluster generated here using the following steps: 50nt upstream and downstream of each miRNA was retrieved, then fused to both the 5' and 3' end of each miRNA hairpin loops, and finally these miRNAs ware fused together.

-Inputs:

1- MphR-KRAB

2- TCR signaling

-outputs:

1- miRNA146a (SEQID: 29)

2- miRNA29a (SEQID: 30)

3- miRNA29b (SEQID: 31)

The Assembly of the circuit:

fusing cHS4 region upstream of IL-2 promoter & downstream of the SV40 poly adenylation signal of sub-circuit element 2, and then the Sv40 polyadenylation signal of sub circuit element 2 is fused with cHS4 region to generate the following arrangement(cHS4-sub-circuit element 1-cHS4-sub-circuit element 2- cHS4) the purpose of using multiple cHS4 insulator element is to prevent any un-predicated epigenetic mechanism that might be generated duo to KRAB domain

which depend on epigenetic mechanism to mediate the repressor effect, and to prevent any silencing occur at the IL-2 promoter to interfere with the other regulatory function of the device. In-summary this epigenetic insulator prevent any epigenetic reaction occur at sub-circuit 1or 2 to affect one another or to interfere with other circuit elements. Fig.30 illustrates the parts arrangement of this circuit.

d- Device assembly:

GC1, GC2, and GC3 where assembled to give the final assembly is illustrated in Fig 31. Both ends of the circuit are "sealed" with cHS4 insulator, the purpose of this "sealing" is to prevent the epigenetic interaction between the circuit & between nearby DNA sequences.

e- ϕC31 Vector Assembly:

the final device assembled in a plasmid contain the following elements:

a- pBR322 plasmid Ori (origin of replication) (SEQID: 32) which enable the propagation of plasmid in bacteria.

b- Ampicillin resistance gene (AmpR) (SEQID: 33)

c- Ampicillin resistance gene promoter (AmpR promoter) (SEQID: 34)

b & c part act as a marker for selection of bacterial transfected clone

d- attB (SEQID: 35) sequence to enable insertion of the vector the genome using the ϕC31 integrase.

See Fig (32) for a sketch of the vector ϕC31 without the device inserted & Fig (33) for the ϕC31 loaded with device.

f- Transfection protocol:

the production of this genetically modified T cell will follow the Conventional method; which consists of T cell activation, Transfection & Expansion [87]. The first step here will be the isolation of naive $CD4^+$ T cell from the patient peripheral blood mono nuclear cell (PBMC). many commercial kits are available for doing this task (i.e. $CD4^+$ naive T cell separation) for example, EasySep™ Human Naïve CD4+ T cell Isolation Kit(stem cell technologies).

One of The most common ways to activate T cell ex-vivo is by the use of anti CD3-Ab & anti CD28-Ab [87-89]. Also INF-γ, IL-12 & anti-IL-4 neutralizing Ab must be added to the culture media to drive Th1 differentiation [90-92]. In the transfection step the cell is transfected with both genetic element (i.e. CAR lentivector & ϕC31plasmid) the cell can be transfected with lentivector using the protocol described by[93-95], meanwhile the cell can be transfected with ϕC31plasmid using the protocol describe by [96, 97]. Finally the cell will be purified and re-administrated to the patient.

2- Dynamic description of the device:

I- GC1:

The migration of T cell from blood to secondary lymphoid organ in case of native t cell [98] & from blood to the site of infection[99]is essential process for effective immune response. T cell migration is orchestrated by a large network of chemokines and their receptors [98]. Chemokines can be classified into two large group the CC family & CXC family, the first family has two adjust cysteine amino acid at their N terminal while in the second the two cysteine are separated by an amino acid[98].

Although this classification has been officially and widely accepted another classification that based on the function of chemokines has been also developed, in such case the chemokines are classified into two groups: a pro-inflammatory chemokines which have an up-regulated level in case of inflammation, and a hemostatic chemokines which is constantly produced by the tissues at non-inflamed sites[98].

Indeed chemokines play a very important role in controlling tumor microenvironment, most solid tumors are composite of malignant cell and host stromal cell (i.e. non-malignant stromal cell), and chemokines are thought to control the process of stromal cell recruitment by malignant cells [100]. The infiltrated immune cells play a fundamental role in tumor microenvironment; indeed adaptive immune cells have been shown to a double-edged sword in tumor micro-environment. The role of Adaptive-immune cells in mediating anticancer effect is well established, for-example they mediate tumor-immune surveillance eliminating early stage tumor cells[101], but their roles in-supporting tumor are rising for example, Th2 has a pro-tumor effects secreting cytokines that has a M2 polarization & M2 cell promote tumor invasions, this effect

has been shown with breast cancer[101, 102]. Another factor that contribute to the tumor supporting effect on lymphocyte is the Treg ($CD4^+CD25^+$), these cells have been shown it to inhibit the function of many of the immune cells, they secret TGF-β & IL-10 which directly inhibit $CD8^+$ cytotoxic effect [101, 103].
The chemokines network in cancer stills not completely understood with many point to be elucidated but the experimental data has support that the following: chemokines (CCL2 and CXCL9) control and mediate TIL (Tumor infiltrating Lymphocyte) migration to the tumor cells [104]. CCL2 and CXCL9 mediate there action through there receptor CCR2 [105] and CXCR3A\B [106]respectively. CXCR3 A\B (different forum generate by alternative splicing) is present on the activated Th1 with high level [107], meanwhile CCR2 is expressed on T cell [108], but its expression level in the Th1 or Th2 is somewhat confusing with paper suggest it to be highly expressed and mediate Th1 immunity[109] other have a strong data to support a Th2 mediated immunity [110].

CCR2\CCL2 axe has been shown to regulated & orchestrated the monocyte\macrophage migration to tumor site[111].increase expiration of CCR2 will lead to a better tumor infiltration, indeed in the one experiment the infiltration of CD8+ cytotoxic cell was enhanced by forced over-expression of CCR2 [112]. CCR2\CCL2 Aix now is getting more importance, as it has shown that $CCR2^+$ Type 1 Cytotoxic γδ T has the ability to migrate to tumor cell and exert cytotoxic effect on them meanwhile $CCR2^-$ cannot[113].

sphingosine-1-phosphate is a lipid second messenger system that act on 5 receptors(S1PR1-5) [114] with a chemotactic activity[115], T cell mainly depend upon S1PR1& S1PR4[114]. S1PR1 (sphingosine-1-phosphate receptor1) is a G-protein coupled receptor that mediated T cell egress from lymphoid tissue [116]. sphingosine-1-phosphate level are higher in blood & lymphatic vessels and low in most tissues & it was suggested that this concentration gradient mediated the egressing mechanism [114]. Based on a series of experiment the S1P&S1PR chemotaxis system was shown to mediate its response in a "bell shape" manner, in which at high S1P level there is an internalization of the S1PR1 duo to activation render the system irresponsive to S1P chemotactic activity but at small concentration the receptor internalization is limited allow for a greater sensitivity to the S1P concentration gradient[114].

The earlier model can be explained as follow, when S1P bind to its receptor it activate the receptor, then this activation leads to receptor internalization, and as a result high concentration of S1P will bind to more S1PR1 leading to receptor internalization and hence desensitize cell to S1P. Indeed this process explain two important factors, the first is who the S1PR1-S1P mediate egress from the tissue to blood & who the T cells are migrating to tissue against S1P concentration gradient.

In the first case, T cell egress from Lymphatic to blood is related to concentration gradient of S1P [117, 118]. The second issue is how T cell migrate to tissue against the concentration gradient of S1P, Indeed earlier reports Indicate that S1P inhibit T cell chemotactic response to chemokines [117, 119], but the recent data indicate a model in which high level S1P present on the blood tiger desensitization response and this desensitization is responsible for T cell migration to tissue against concentration gradient of S1P[120].

As explained earlier S1PR1 play an important role in regulating T-cell recirculation, so it is not a surprise that S1PR1 is under tight transcription regulation. Indeed in one study memory T cells that lack S1PR1 was found to be tissue residual (resident memory T cells (Trm)) & forced expression in such a cell result in there recirculation[121]. Krüppel-like factor 2 (KLF-2) is a transcription activator that promote S1PR1 expression [122], It is highly expressed on memory & native T cell but weekly expressed on effector T cell [123].
TCR signaling has been associated with repression of KLF-2, indeed TCR signaling has been identified to initiate KLF-2 down-regulate and then this down-regulation is modulated & reinforced by cytokine receptor gamma-chain (cγ) [123].

So in-order to achieve a high therapeutic effects one must be able to control the cell localization toward the tumor tissue and to avoid its migration toward non-malignant tissues.
In-order to control the cell migration and tissue infiltration I design the GCI described above. (See Fig.34 for an interacting diagram of this Circuit).

The basic concept of this circuit design can be stated as follow, when the cell administrate to the

patient it need to be infiltrated to the tumor location this can be achieved using the highly expressed chemokines receptor CCR2 (its expression will be increased by the effect of dcas9VP16 domain), and when it cause severe side effects or when its infiltration is need to be eliminated the cell need to re-express S1PR1 receptor (the level of S1PR1 will be very low duo to the TCR signaling as explained above).

So in order to achieve such this states (i.e. the migration state characterize by high level expression of CCR2 & recirculation state characterize by high level expression of S1PR1) one must be able to control the transcription state of this proteins. The dcas9-VP16 protein as explained earlier has the ability to activate the transcription of the target gene based on gRNA molecules. So the transcription state of the two proteins can be controlled by control the transcription of the gRNA specific for them. By using chemically regulated gene-expression system the production of gRNA specific of each protein will be controlled by the administration of chemically activator or repressor for that gRNA.

Using simple architecture for this production (i.e. maintain the gRNA directly under the control of chemically regulated activator or repressor) will required multiple dose of the chemical regulator (i.e. the chemical that control system for example tetracycline in regulating TetR protein) to maintain the activation or repression state. This continuous administration of chemical compound may have serious side effect on the patient. Hence I design this circuit that has two states, the first is characterized by the production of gRNA specific for CCR2, and the second is characterized by the production of gRNA specific for S1PR1. The circuit offers the advangeous of single dose state change (i.e. using single dose of chemical regulator can change cell from state 1 which is characterized by gRNA specific for CCR2 to the 2nd state characterized by gRNA specific for S1PR1) so offer more safe and convenient control for cell localization.

The circuit has two states:

1- ON state in which the cell up regulated the transcription of sphingosine-1-phosphate receptor these changes enable the cell to recirculate to the blood duo to the high level of S1p present in blood as discussed before.

2- OFF state in which the cell have up-regulated level of chemokines receptor (CCR2) and no-expression of sphingosine-1-phosphate receptor duo-to absence of activation effect & duo to the TCR signaling as discussed before, So the cell will actively and efficiently migrate to the tumor localized tissue.
in absence of any interference the circuit is in the OFF state, In-order to activate the circuit on, intake of tetracycline will tiger the following sequence of event that turn the circuit on and maintain the cell in the on state:

a- binding of tetracycline to the TetR tiger the release of the TetR from its operon (Tet O2) present upstream of PIT CDC and (GAL4-VP16, EthR) CDC, this step allows the transcription of PIT (pristinamycin-dependent trans-activator) which is under the control CMVp (Cytomegalovirus promotor).

b- The PIT will bind to its promotor P_{PTR} (pristinamycin-responsive promoter) and activate the transcription of the GAL4-VP16 and EthR (present in one ORF (open reading frame) & are connected by P2A peptide).

c- GAL-VP16 2A peptide EthR mature mRNA will be translated into GAL-VP16 protein and EthR protein.

d- The GAL4-VP16 will bind its target promotor (UAS (upstream activation sequence) fused to eukaryotic minimal promotor (HSP70 (heat shock protein 70 from D. melanogaster))) and activate the transcription. This promoter regulates the transcription of CymR and gRNA for the activation of S1P receptor.

e- EthR will bind to its operon upstream of TetR CDC and inhibit transcription of TetR.

f- CymR will bind to its operon downstream of CMVp and inhibit the CCR2 gRNA transcription.

See Fig.35 for the circuit on the On State.

In-order to drive the shut down the circuit the administration of Pristinamycin or one of its analogues will drive the following process:

a- binding of pristinamycine to PIT release the PIT from its promotor
b- The GAL4-VP16 and EthR production will be drastically reduced
c- The decrease of EthR production will result in the activation of TetR production
d- TetR that will bind to its operons and inhibit the PIT, GAL4-VP16 and EthR production
g- The low state of GAL4-VP16 will lead to decrease in all GAL4-VP16 target genes
h- as the GAL4-VP16 is now turned off and all of its target gene are turned off
The circuit is now in the off state.
See Fig.36 for the circuit on the OFF state.
The concept of this circuit is to increase safety& efficiency of the CAR-T cell by provides a controllable switch for tissue localization and migration. The enhanced efficiency exerted by this circuit is related to its ability to increase the expression of CCR2 which has been shown in the recent reports to increase tumor infiltration & localization [112, 124, 125]. The enhanced safety of this circuit is related to its ability to recirculate activated CAR-T cell, indeed as explained earlier activate T cell decrease its circulation by down-regulate S1PR1, so here the circuit will enable the activation of S1PR1 gene independently from the activation state of the cell. This GC might be useful when there is On-target but off tumor or cytokines storm manifestation as it will enable the cell recirculation facilitating circuit II effects.
also EthR and CymR repressor have well characterized chemical inhibitors theses inhibitor are not clinically characterized, so here there use with the device will be limited to the ex-vivo stage (i.e. during the production of this genetically modified T cell ex-vivo)
II- GC2:
In-order to have a safer effective therapy CAR therapy a more efficient tools & method are needed [61]. So here I design a simple genetic circuit that is based on miRNA to control the cytokines release and cellular behavior. The circuit interaction diagram is illustrated in Fig 37. Fig 38 represents a flow chart that explains the possible outcome s of the circuit depending of cell state.
The purpose of this circuit is to gain a control over the cell proliferation & cytokine release. This

circuit forms a network with the cellular regulatory pathway, through the production of miRNAs to regulate the cell behavior (output) and also its sense the cell state (input) through the use of what can be called "representative promoter" or sensor promoter. The "representative promoter" is a promoter that has the ability to reflect the cell state. A representative promoter can be a synthetic promote that contain a DNA response element of the target transcription factor like (AP-1, NF-kB, STAT5A&B, NFAT, etc.) fused with mammal mini-promoter. Also the representative promoter can be a natural promoter like INF-γ or IL-2 promoter.

By cell using the word "cell state" I mean the activation state of the cell (there is active TCR signaling & cell is engagement with antigen or there is no TCR signaling & the cell is not activate). Here I choose the IL-2 promoter to be the "representative promoter". IL-2 gene (Gene ID: 3558) is coding for the IL-2 protein. IL-2 is a major mediator of immunological response[126] , controlling cell proliferation, differentiation [127, 128] and apoptosis\survival state[129-131].

IL-2 production represent an endpoint for TCR signaling[129]& hence its transcription state is a reflection to the TCR singling state, so here based on this concept the circuit will be designed. The circuit is based upon using the IL-2 promoter as sensor for the cell state, IL-2 promoter act as the major input to control the production of the downstream regulatory element of the circuit.

The circuit is composite of two SGCEs: the first is the MphR-KRAB producing SGCE (SGCE1) and the second part is the miRNA producing SGCE (SGCE2).

SGCE1 is composite of IL-2 promoter up-stream of the MphR-KRAB downstream of the MphR-KRAB protein there is the SV40 poly-adenylation signal that act as a transcription terminator. This arrangement couple the production of the MphR-KRAB protein to the state of cell activation as explained earlier. SGCE2 is composite of ETR (erythromycin response) downstream of the IL-2 promoter, downstream of the ERE there a miRNA cluster hairpin contain 3 miRNAs (miRNA146a, miRNA 29a, miRNA 29b) a finally there is the SV40 poly-adenylation signal that act as a transcription terminator.

As stated earlier the miRNA cluster contain the following miRNAs (miRNA146a,

miRNA29a&b).The function of miRNA 146a is diverse; firstly it has been reported to down regulate the IL-2 production which is an essential mitogen for T cells expansion also they it is able to impair the AP1 protein function and to act as a modulation of apoptosis by down regulated the FAS associated death domain (FADD) [132]. Also miRNA146a has been reported to decrease the T cell response by target the down regulation of TRAF6 and IRK1 (part of the NF-kB signaling cascade) [133]. MiRNA146a target down regulation of INF-γ production by down-regulate the STAT1 (signal transduction and active transcription protein 1), STAT1 activate the T-bet transcription factor that activate the Ifng gene (INF-γ coding gene) so by inhibiting STAT1 miRNA 146a down-regulate the INF- γ production[133]. The recent data support a fundamental role in miRNA 146a in down-regulation pro- inflammatory cytokines secretion [134].

The function of miRNA 29ab1 (miRNA29a & miRNA 29b1) subclass in Th1 has been extensively described in the last few years. There major function relies on regulate Th1 cell differentiation [133] and to down regulate INF- γ production[135]. Indeed recent research indicate that miRNA29a inhibit INF- γ production by inhibiting T-bet TF(T-bet is a TF that activate INF- γ production) [136], meanwhile miRNA29b regulate INF- γ production by inhibiting both T-bet & INF- γ [135].

Indeed using the earlier miRNA cluster to regulate cytokine storm required high level of control over their production, as over production will lead to sever inhibition of the T cell and decrease its therapeutic efficacy, meanwhile low level of production will not control or prevent cytokine storm. So the function of this circuit is to fine-tune & regulate these miRNAs production to achieve optimal efficacy & safety.

Here two factors will control the production of the miRNA: the first is the cell state represented by the IL-2 promoter, and the second is MphR-KRAB binding affinity. The MphR-KRAB binding affinity is determine by two factors: the first is its production which is a function of TCR signaling in which efficient signaling tiger high production level, and the second is the availability of erythromycin in which the presence of erythromycin decrease its affinity.

In High affinity state MphR-KRAB (HA-MphR-KRAB) binds to its response element up-stream of the miRNA cluster inhibiting the miRNA production. This high affinity state can be transformed into a low affinity state (LA-MphR-KRAB) by the intake of erythromycin which tiger MphR-KRAB release from the ETR allowing miRNAs production.

The circuit has 3 states: the first state is the ON state, meaning miRNAs are produced; this state can be achieved by the administration of erythromycin to activated T cell which causes MphR-KRAB to switch from high affinity state into low affinity state, and as there is a high activity at IL-2 promoter so the cell will be produce miRNA that will inhibit both the activation and pro-inflammatory cytokine secretion.

The second state is the steady state in which the cell produce little miRNA that modulate its behavior, this state will be achieved when weak antigenic stimulus are present to the cell. The low activation state wills tiger low production of both miRNA & MphR-KRAB. This state increase the antigenic threshold and this points can be explain as follow; when the cell interact with weak antigenic stimulus they produce week signal that drive low amount of activation to IL-2 promoter, causing the MphR-KRAB to be at low affinity state which gave a partial week inhibitory effect on miRNAs, but produce a little miRNA that inhibit and decrease the cellular response further (i.e. the low activation signal form a feedback loop that decrease the cell response further as the low IL-2 promoter activity produce few amount of MphR-KRAB that has low consternation to bind to its operon and to inhibit the miRNAs production, but this small activity make little miRNA the decrease the cell response further) the function of such state is to decrease the cellular response toward week antigen, so increase the cellular threshold toward antigenic stimulant.

The third state is the OFF state, where there is no miRNAs production duo-to engagement with strong antigen. This point can be explained as follow; in massive activation state there is massive activation at IL-2 promoter and hence MphR-KRAB level will be so high and the miRNAs production will be inhibited duo to the occupation by MphR-KRAB to it operon (ERE) i.e. when the antigenic stimulus is strong enough it form a positive feedback loop this loop further increase the activity further as miRNA production are begin inhibit after a certain point, this

point is when the MphR-KRAB are high enough to bind to its operon and inhibit the miRNAs production. This state gave the circuit the properties of rapid response to outer interference (i.e. the interference by physician or observer to decrease the high cell activation by the administration of macrolides as erythromycin). When macrolides are administrated it will drive a rapid and high level of mRNAs duo-to; disassociation of MphR-KRAB from its operon and the high level activation at IL-2 promoter which will drive high level of miRNA production that will control and inhibit the massive activation of the cell.

III- The whole device dynamic:

The whole device dynamic is explained in Fig.39 which gave also a scheme to be followed when interacting with the patient.

II: a Genetic device for conditional gene knockdown:

as dissuaded in the prior art the conditional gene knockdown is an invaluable tool to study biological system, and for the development cell lines and genetically modified animals. Herein I report a device with a typical architecture in which it contain two genetic circuits instead of three, this device will enable the specific gene knockdown in highly differentiated cells.

I- Genetic circuit1:

GC1 follow the normal architecture sated earlier in which it contain an Input analysis sub genetic circuit and an effector sub genetic circuit.

I. a- Input analysis sub genetic circuit:

this IASGC is GESSGC that depend upon tissue specific gene expression, its composite of two SGCE:

1- The first SGCE is composite of: a-tissue specific promoter(x), b- RBE downstream of the tissue specific promoter, c- coding region contain gRNA cluster specific for effector sub-genetic circuit element, and d- a transcription terminator.

2- The second SGCE is composite of: a-tissue specific promoter(y), b- regulated repressor specific for RBE of SGCE1 coding region, c- downstream of regulated repressor coding region is a miRNA response element, and d- a transcription terminator.

The dynamic f this SGC is illustrated using interaction diagram in Fig.40.
This circuit is G4O1, and as described in Fig.41 this circuit can integrate four input signals and produce a final output signals. First SGCE1 promoter activation is essential for the production of gRNA for effector Circuits (i.e. the output signal or GL1).three other input can modulate the circuit response: 1- the activation state at SGCE2 promoter activation in which the activation will tiger the production of RR mRNA, 2- the absence or the presence of miRNA specific for RR mRNA, 3- the absence or the presence of chemical inhibitor specific for RR. These four inputs can be used to deliver the precious control of the output function production.

I. b- effector sub genetic circuits:
this ESGC1 is composite of one SGCE which is composite of: a- gRNA depended promoter (i.e. the gRNA produced by IASGC), b- chemically regulated transcription activator coding region, c- downstream of chemically regulated transcription activator coding region is a miRNA response element, and d- a transcription terminator.

The earlier SGCE mediated a second level of computation by enable miRNA depend production of chemically regulated transcription activator.

II- Genetic Circuit II:

Herein Genetic circuit II is a structure genetic circuit element composite of: VSGC, and ESGC.

1- ESGC:
ESGC is composite of one SGCE which is composite of: a- a promoter, b- dcas9-VP16 and Cys4 coding region, and c- a transcription terminator.

The function of ESGC is to produce the protein essential for the device to Function: the first protein is dcas9-VP16 to drive transcription of target gene in gRNA depended manner, and the second is the cys4 which is used produce gRNA from its clusters as described earlier.

2- VSGC:

VSGC is composite of one SGCE which is composite of: a-inducible promoter that depends on chemically regulated transcription activator produce by GC1, b- SiRNA coding region or Cre-recombinase coding region, and c- a transcription terminator.

The whole device interaction diagram is shown in Fig.41.

This device can be used to regulate the production siRNA or Cre-recombinase and hence control conditional gene knock-down. The whole device can receive six inputs that modulate and control its production on siRNA or Cre-recombinase. These inputs can be classified into:

a) tissue specific promoter activation signals:
that enable the device to produce its output signal (i.e. siRNA or Cre-recombinase) in a specific subset of cells
b) miRNA Response elements:
that enable the device to produce its output signal (i.e. siRNA or Cre-recombinase) in a specific subset of cells that does not express these miRNA
c) chemical inhibitor to synthetic transcription factor:
to enable research to choose or to control the time at which the device get activated or to shut down.

The combinations of these signals enable the highly specific production of the output signals, meaning a gene knock down in highly differentiated cells at a highly specific manner.

Brief description of the drawing:

Fig.1
represents a flow chart on the functional composition of the Device

Fig.2
shows a functional classification of the device elements.

Fig.3
shows a functional classification of the device.

Fig.4

shows computation levels carried out by the device; it also shows the parts that carry different level of computations

Fig.5

shows the pathways that might be followed by the out-put signals

Fig.6

stander symbol of inventor logic gate

Fig.7

stander symbol of buffer logic gate

Fig.8

stander symbol of AND logic gate

Fig.9

a follow chart for the complex logic gate as explained in assumption D section A.

Fig.10

logic gates of the active state of chemically regulated transcription activator

Fig.11

logic gates of translation regulation by miRNA

Fig.12

logic gates negative feedback loop

Fig.13

an interacting diagram of SGCE with NRR as described in section B.A. 2 conditional negative feedback (CNFL) SGCE

Fig.14

an interacting diagram of SGCE with RR as described in section B.A. 2 conditional negative feedback (CNFL) SGCE

Fig.15

a Boolean logic gate diagram of SGCE with NRR as described in section B.A. 2 conditional negative feedback (CNFL) SGCE

Fig.16

a Boolean logic gate diagram of SGCE with RR as described in section B.A. 2 conditional negative feedback (CNFL) SGCE

Fig.17

shows a flow chart of the dynamic of SGC disscused in section B.B.I condiational Ossillator

Fig.18

an interacting diagram of SGCE with NRR as described in section B.B.I condiational Ossillator

Fig.19:

an interacting diagram of SGCE with RR as described in section B.B.I condiational Ossillator

Fig.20:

a Boolean logic gate diagram of SGCE with NRR as described in section B.B.I condiational Ossillator

Fig.21:

a Boolean logic gate diagram of SGCE with RR as described in section B.B.I condiational Ossillator

Fig.22:

an interacting diagram of SGCE as described in section B.B.II SGC for complex data integration

Fig.23:

a Boolean logic gate diagram of SGCE as described in section B.B.II SGC for complex data integration

Fig.24:

an over vew on selective and spesific miRNA delivery by cell reprogrammed with this device

Fig.25:

the composition of the device discussed on the example I

Fig: 26

Example I GCIII parts arrangement

Fig: 27

Example I S1PR1 gRNA cluster arrangement

Fig: 28

Example I CCR2 gRNA cluster arrangement

Fig: 29

Example I GC I parts assembly

Fig: 30

Example I GC II parts assembly

Fig: 31

Example I Device assemblies

Fig: 32

Example I phi (ϕ) C31 vector plasmid

Fig: 33

Example I ϕ C31 vector loaded with device

Fig: 34

Example I GC1 interaction diagram

Fig: 35

Example I GC1 interaction diagram in ON state

Fig: 36

Example I GC1 interaction diagram in OFF state

Fig: 37

Example I GCII interaction diagram

Fig: 38

represents a dynamic interaction between Circuit II parts and cell

Fig. 39

a scheme for managing the device

Fig.40

Example II GESSGC interaction diagram

Fig.41

Example II whole device interaction diagram

number	Name
1	CMVp
2	OethR
3	TetR CDC
4	SV40 Poly A signal
5	TetR
6	Tetracycline
7	TetO
8	PIT CDC
9	PIT protein
10	P_{PTR}
11	EthR&GAL4-VP16 CDC
12	EthR CDC
13	2-PEB
14	Pristinamycin
15	GAL4-VP16
16	CymR CDC & S1PR1 gRNA cluster
17	S1PR1 gRNA
18	CymR protein
19	Cumate

20	CCR2 gRNA cluster
21	CCR2 gRNA
22	CuO

NUMERALS:

Number	Name
1	IL-2 promoter
2	MphR-KRAB CDC
3	SV40 poly A signal
4	ETR
5	miRNA cluster
6	MphR-KRAB
7	miRNA 146a, miRNA 29a miRNA29b
8	Cellular TFs

Symbol	Function
A	MphR-KRAB production
b	MphR-KRAB suppress miRNA production
c	miRNA production
d	miRNA inhibit cell TF

These 2 tables represent the number and symbols described in Fig.37

DRAWINGS—REFERENCE

Symbol	interaction
a	TetR production
b	TetR repression mechanism mediated by binding to TetO2
c	PIT production
d	PIT activate Transcription
e	EthR production
f	EthR repression mechanism mediated by binding to EthR
g	Tetracycline bind to TetR removing its repression effect
h	2PEB bind to EthR removing its repression effect
K	GAL4-VP16 production
S	GAL4-VP16 bind to its promoter and activate transcription
O	Pristinamycin bind to PIT removing its activator effect
L	CymR production
M	S1PR1 gRNA production
N	Cumate bind to CymR removing its repression effect
R	CymR bind to CuO repression transcription of downstream ligand

These 2 tables represent the number and symbols described in Fig.34, Fig35, and Fig.36

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CLAIMES

WHAT IS CLAIMED IS:

1-A recombinant DNA molecule comprising :

a) an eukaryotic promoter

b) at least one EthR operator (OethR) sequence positioned 3' or 5' to the eukaryotic promoter of claim 1

c) a gene lying 3' to said operator and operably linked to said promoter.

2- A recombinant DNA molecule comprising:

a) an eukaryotic promoter

b) a coding sequence of EthR operably linked to the eukaryotic promoter of claim 2.

3- The coding sequence of EthR of claim 2, wherein the EthR comprise a coding sequence fused to nuclear localization sequence (NLS) sequence.

4. A host cell transformed with a vector comprising the DNA molecule of claim 1.

5. A host cell transformed with a vector comprising the DNA molecule of claim 2.

6- A host cell transformed with a vector comprising the DNA molecule of claim 1 and the DNA molecule of claim 2.

7- A method for suppress transcription of said gene of claim 1 in said host cell of claim 5, said method comprising:

a) transforming said mammalian cell of claim 5 with a vector comprising:

(i) an eukaryotic promoter

(ii) at least one OethR operator sequence positioned 3'or 5' to the eukaryotic promoter

(iii) the gene of claim 1 lying 3' to said OethR operator and operably linked to said promoter wherein said gene transcription will be inhibited duo to binding of said EthR to said OethR.

8- A method for studying gene expression regulation & cellular differentiation in mammalian cell in Vitro composite of:

a) a mammalian cell transfected with a vector compressing:

(i) an eukaryotic promoter

(ii) at least one OethR operator sequence positioned 3'or 5' to the eukaryotic promoter of claim 8.

(ii) a reporter gene lying 3' to said OethR operator and operably linked to said eukaryotic promoter of claim 8 wherein said reporter gene transcription will be inhibited if said EthR of claim 3 bind to said OethR

b) The mammalian cell of said claim 8 transfected with:

(i) tissue specific promoter

(ii) the coding sequence of EthR protein of claim 3 operably linked to said tissue specific promoter of claim 8

wherein the transcription of said EthR protein of claim 3 will depend upon activation state of said tissue specific promoter of claim 8, whereby inhibit the production of said reporter gene of claim 8 .

9- A method for studying miRNA function and regulation in mammalian cell in Vitro composite of:

a) a mammalian cell transfected with a vector compressing:

(i) an eukaryotic promoter

(ii) at least one OethR operator sequence positioned 3'or 5' to the eukaryotic promoter of claim 9.

(ii) a reporter gene lying 3' to said OethR operator and operably linked to said eukaryotic

promoter of claim 9 wherein said reporter gene transcription will be inhibited if said EthR of claim 3 bind to said OethR

b) The mammalian cell of claim 9 transfected with a vector composite of :

(i) an eukaryotic promoter

(ii) the coding sequence of EthR protein of claim 3 operably linked to said eukaryotic promoter of claim 9

(iii) a miRNA binding element (MRE) positioned 3' to said coding sequence of EthR protein of claim 3 wherein the translation of said EthR protein of claim 3 will be inhibited by the binding of miRNA to said miRNA binding element of claim 9

wherein the expression of miRNA in said mammalian cell of claim 9 will inhibit translation of said EthR of claim 3 whereby increase production of said reporter gene of claim 9.

10- An assay for studying miRNA and miRNA mimetic transportation across cell membrane composite of:

a) a miRNA or miRNA mimetic

b) a mammalian cell transfected with a vector compressing:

(i) an eukaryotic promoter

(ii) at least one OethR operator sequence positioned 3'or 5' to the eukaryotic promoter of claim 8.

(ii) a reporter gene lying 3' to said OethR operator and operably linked to said eukaryotic promoter of claim 10 wherein said reporter gene transcription will be inhibited if said EthR bind to said OethR.

c) The mammalian cell of claim 10 transfected with a vector composite of:

(i) an eukaryotic promoter

(ii) the coding sequence of EthR protein of claim 3 operably linked to said eukaryotic promoter of claim 10

(iii) a miRNA binding element specific for said miRNA or miRNA mimetic of claim 10 positioned 3' to said coding sequence of EthR protein of claim 3

wherein the translation of said EthR protein of claim 3 will be inhibited by binding of miRNA to said miRNA binding element of claim 10. Whereby the amount of said reporter gene of claim 10 productions can be directly correlated to the amount of said miRNA or miRNA mimetic of claim 10 transported across membrane of said mammalian cell of claim 10.

11- A method for studying gene expression regulation & cellular differentiation in vivo composite of:

a) an embryonic stem cell transfected with a vector compressing:
(i) an eukaryotic promoter
(ii) at least one OethR operator sequence positioned 3'or 5' to the eukaryotic promoter of claim 11.
(iii) a reporter gene lying 3' to said OethR operator and operably linked to said eukaryotic promoter of claim 11 wherein said reporter gene transcription will be inhibited when said EthR bind to said OethR
(iv) a tissue specific promoter
(v) the coding sequence of EthR protein of claim 3 operably linked to said tissue specific promoter of claim 11
b) incorporating said stem cell of claim 11 into a blastocyst to form a chimeric embryo
c) implanting said chimeric embryo of claim 11 into a pseudo pregnant animal
d) allowing said chimeric embryo of claim 11 to develop into a viable offspring
e) screening offspring to identify heterozygous animals expressing EthR gene and said reporter gene of claim 11
f) breeding said heterozygous animals to produce homozygous transgenic animals producing said EthR gene and said reporter gene of claim 11
wherein all animal tissues will express said reporter protein of claim 11, tissues activate said tissue specific promoter of claim 11 will not express said reporter protein of claim 11, whereby the regulation and differentiation process can be elicited.

12- A transgenic animal made by the method of claim 11.

13-A transgenic animal wherein said animal has integrated into its genome a recombinant DNA comprising:

a) an eukaryotic promoter

b) at least one OethR operator sequence positioned 3'or 5' to the eukaryotic promoter of claim 13.

c) a gene lying 3' to said OethR operator and operably linked to said eukaryotic promoter of claim 13.

14- The transgenic animal of claim 13, further comprising a gene encoding the EthR repressor protein.

15- A method to study miRNA or miRNA mimetic uptake and distribution in vivo composite of:

a) a miRNA or miRNA mimetic

b) an embryonic stem cell transfected with a vector compressing:

(i) An eukaryotic promoter (X)

(ii) at least one OethR operator sequence positioned 3'or 5' to said eukaryotic promoter of claim 15.

(iii) a reporter gene lying 3' to said OethR operator and operably linked to said eukaryotic promoter (X) of claim 11 wherein said reporter gene transcription will be inhibited when said EthR bind to said OethR

(iv) an eukaryotic promoter (Y)

(v) the coding sequence of EthR protein of claim 3 operably linked to said eukaryotic promoter of claim 15

(vi) a miRNA response element for said miRNA or miRNA mimetic of claim 15 positioned 3' to the coding sequence of EthR protein of claim 3

b) incorporating said stem cell of claim 15 into a blastocyst to form a chimeric embryo

c) implanting said chimeric embryo of claim 15 into a pseudo pregnant animal

d) allowing said chimeric embryo of claim 15 to develop into a viable offspring

e) screening offspring to identify heterozygous animals expressing said EthR gene and said reporter gene of claim 15

f) breeding said heterozygous animals to produce homozygous transgenic animals producing said EthR gene and said reporter gene of claim 15

wherein all animal tissues will express said reporter protein of claim 15, homozygous transgenic animals of claim 15 produce said EthR protein of claim 3 and hence do not produce said reporter gene of claim 15, wherein administration of said miRNA or miRNA mimetic of claim 15 repress EthR translation, whereby allow said reporter gene of claim 15 production and hence this allow detection of tissue uptake and tissue distribution of said miRNA or miRNA mimetic of claim 15.

16- A transgenic animal made by the method of claim 15.

17- A method to study miRNA function and regulation in mammalian cell in vivo composite of:

a) an embryonic stem cell transfected with a vector compressing:

(i) An eukaryotic promoter (X)

(ii) at least one OethR operator sequence positioned 3'or 5' to said eukaryotic promoter(X) of claim 17.

(iii) a reporter gene lying 3' to said OethR operator and operably linked to said eukaryotic promoter (X) of claim 17 wherein said reporter gene transcription will be inhibited when said EthR bind to said OethR

(iv) an eukaryotic promoter (Y)

(v) the coding sequence of EthR protein of claim 3 operably linked to said eukaryotic promoter(Y) of claim 17

(vi) a miRNA response element positioned 3' to the coding sequence of EthR protein of claim 3

b) incorporating said stem cell of claim 17 into a blastocyst to form a chimeric embryo

c) implanting said chimeric embryo of claim 17 into a pseudo pregnant animal

d) allowing said chimeric embryo of claim 17 to develop into a viable offspring

e) screening offspring to identify heterozygous animals expressing said genes of claim 17

f) breeding said heterozygous animals to produce homozygous transgenic animals producing said genes of claim 17

Wherein all tissues and cells of homozygous transgenic animals of claim 17 produce said EthR protein of claim 3 and hence do not produce said reporter gene of claim 15, wherein the production of endogenous miRNA specific for said miRNA response element (MRE) result in repress said EthR production, whereby allow the production of reporter gene in such a cell, whereby identify the regulation mechanisms and expression pattern of miRNA specific for said MRE of claim 17.

18- A transgenic animal made by the method of claim 17.

19- A recombinant DNA molecule comprising:

a) An eukaryotic promoter

b) at least one gRNA specific for S1PR1 operably linked to said eukaryotic promoter of claim 19

20- A host cell transformed with a vector comprising the DNA molecule of claim 19.

21- A method for regulating effector lymphocyte recirculation composite of:

a) a lymphocyte transfected with a vector compressing:

(i) a chemically regulated promoter

(ii) at least one gRNA specific for S1PR1 operably linked to said chemically regulated promoter of claim 21

b) The lymphocyte of claim 21transfected with a vector compressing:

(i) an eukaryotic promoter

(ii) a coding sequence of chemically regulated transcription factor specific for said chemically regulated promoter of claim 21

c) The lymphocyte of claim 21transfected with a vector compressing:

(i) a coding sequence of dCas9 operably linked to said eukaryotic promoter of claim 21

(ii) a coding sequence of activator domain fused to said coding sequence of dcas9 of claim 21

d) A chemical compound for regulating said chemically regulated transcription factor of claim 21

Wherein the function of said chemically regulated transcription factor of claim 21 regulated the transcription state of said gRNA of claim 21, whereby regulate gRNA specific for S1PR1, whereby regulate cell recirculation.

22- A host cell made by the method of claim 21

23- A recombinant DNA molecule comprising:

a) An eukaryotic promoter

b) at least one gRNA specific for CCR2 operably linked to said eukaryotic promoter of claim 23

24- A host cell transformed with a vector comprising the DNA molecule of claim 23.

25- A method for enhance lymphocyte cell migration to tumor tissue composite of:

a) a lymphocyte transfected with a vector compressing:

(i) an eukaryotic promoter

(ii) at least one gRNA specific for CCR2 operably linked to said eukaryotic promoter of claim 25

b) The lymphocyte of claim 25 transfected with a vector compressing:

(i) a coding sequence of dCas9 operably linked to said eukaryotic promoter of claim 25

(iii) a coding sequence of activator domain fused to said coding sequence of dcas9 of claim 25

wherein the production of said gRNA of claim 25 will active the production of CCR2 in a dCas9 depended manner whereby enhance lymphocyte cell migration to tumor tissue.

26- A host cell made by the method of claim 21.

27- A method for regulating effector lymphocyte migration to tumor tissue composite of:

a) a lymphocyte transfected with a vector compressing:

(i) a chemically regulated promoter

(ii) at least one gRNA specific for CCR2 operably linked to said chemically regulated promoter of claim 27

b) The lymphocyte of claim 27 transfected with a vector compressing:
(i) an eukaryotic promoter
(ii) a coding sequence of chemically regulated transcription factor specific for said chemically regulated promoter of claim 27

c) The lymphocyte of claim 21transfected with a vector compressing:
(ii) a coding sequence of dCas9 operably linked to said eukaryotic promoter of claim 27
(iii) a coding sequence of activator domain fused to said coding sequence of dcas9 of claim 27
d) A chemical compound for regulating said chemically regulated transcription

Wherein the function of said chemically regulated transcription factor of claim 27 regulated the transcription state of said gRNA of claim 27, whereby regulate gRNA specific for CCR2, whereby enhance lymphocyte cell migration to tumor tissue.

28- A recombinant DNA molecule comprising:
a) An eukaryotic promoter
b) at least one OethR operator positioned 3' or 5' of said eukaryotic promoter of claim 28
c) a coding sequence of chemically regulated repressor lying 3' to said OethR operably linked to said eukaryotic promoter of claim 28
29- A host cell transformed with a vector comprising the DNA molecule of claim 28.

30- A recombinant DNA molecule comprising:
a) An eukaryotic promoter
b) at least one OethR operator positioned 3' or 5' of said eukaryotic promoter of claim 28
c) a coding sequence of chemically regulated activator lying 3' to said OethR operably linked to said eukaryotic promoter of claim 30
31- A host cell transformed with a vector comprising the DNA molecule of claim 30.

32- A recombinant DNA molecule comprising:
a) An inducible promoter

b) at least one OethR operator positioned 3' or 5' of said inducible promoter of claim 28

c) a coding sequence of chemically regulated repressor lying 3' to said OethR operably linked to said inducible promoter of claim 32

33- A host cell transformed with a vector comprising the DNA molecule of claim 32.

34- A recombinant DNA molecule comprising:

a) An inducible promoter

b) at least one OethR operator positioned 3' or 5' of said inducible promoter of claim 28

c) a coding sequence of chemically regulated activator lying 3' to said OethR operably linked to said inducible promoter of claim 34.

35- A host cell transformed with a vector comprising the DNA molecule of claim 34.

36- A recombinant DNA molecule comprising:

a) An eukaryotic promoter (X)

b) at least one OethR operator positioned 3' or 5' of said eukaryotic promoter of claim35

c) a coding sequence of chemically regulated repressor (R) lying 3' to said OethR operably linked to said eukaryotic promoter (X) of claim 36

d) a poly adenylation signal positioned 3' of said chemically regulated repressor of claim 36

e) a DNA response element specific for said chemically regulated repressor (R) of claim 36 sandwiching between an eukaryotic promoter (Y) and a chemical regulated activator Coding sequence

f) The chemical regulated activator of claim 36 positioned 3' of said DNA response element specific for said chemically regulated repressor (R) of claim 36

g) a poly adenylation signal positioned 3' of said chemically regulated activator of claim 36

h) an inducible promoter specific for said chemically regulated activator of claim 36

i) the DNA response element specific for said chemically regulated repressor (R) of claim 36 sandwiching between said inducible promoter specific for said chemically regulated activator of claim 36 and a coding sequence contain EthR and a transcription activator

j) a poly adenylation signal positioned 3' of said coding sequence contain said EthR repressor and a transcription activator of claim 36

37. The DNA molecule of claim 36, wherein said eukaryotic promoters is selected from the group consisting of CMV, VIP, tk, HSP, MLP, MMTV ,Non Tissue-Specific Promoters and Tissue-Specific Promoters.

38. The DNA molecule of claim 36, wherein said chemically regulated repressor is selected from the group consisting of TetR, MphR and pip.

39. The DNA molecule of claim 36, wherein said chemically regulated activator is selected from the group consisting of PIT, MphR-VP16, MphR-VP64, MphR-NFkB-p65, RXR, mifepristone regulated gene expression and Ecdysone- regulated gene expression.

40- The DNA molecule of claim 36, wherein said poly adenylation signal is selected from the group consisting of SV40 poly A signal, bovine GH poly A signal, ADH1 transcription terminator, or any mean to terminate transcription.

41- The DNA molecule of claim 36, wherein said EthR and transcription activator are connected together using IRES or 2A peptide

42- A vector comprising the DNA molecule of claim 36.

43- A host cell transformed with a vector comprising the DNA molecule of claim 36.

44- A method for regulating recombined protein production composite of:

a) a host cell transfected with a vector comperes of:
(i) An eukaryotic promoter (X)
(ii) at least one OethR operator positioned 3' or 5' of said eukaryotic promoter (X) of claim44
(iii) a coding sequence of chemically regulated repressor (R) lying 3' to said OethR operably linked to said eukaryotic promoter (X) of claim 44
(iv) a poly adenylation signal positioned 3' of said chemically regulated repressor of claim 44

(v) a DNA response element specific for said chemically regulated repressor of claim 44 sandwiching between a eukaryotic promoter (Y) and a chemical regulated activator coding sequence

(vi) The chemical regulated activator coding sequence of claim 44 positioned 3' of said DNA response element specific for said chemically regulated repressor (R) of claim 44

(Vii) a poly adenylation signal positioned 3' of said chemically regulated activator of claim 44

(Viii) an inducible promoter specific for said chemically regulated activator of claim 44

(ix) the DNA response element specific for said chemically regulated repressor (R) of claim 44 sandwiching between said inducible promoter specific for said chemically regulated activator of claim 44 and a coding sequence contain EthR and a recombined protein to be produced by said method of claim 44.

b) Chemical compound specific for said chemically regulated repressor of claim 44

c) Chemical compound specific for said chemically regulated activator of claim 44

wherein the temporary addition of said chemical compound specific for said chemically regulated repressor of claim 44, whereby activate production of said recombined protein of said method of claim 44, and wherein the temporary addition of Chemical compound specific for said chemically regulated activator of claim 44, whereby inhibit production of said recombined protein of said method of claim 44.

45. The method of claim 44, wherein said chemically regulated repressor is selected from the group consisting of TetR, MphR and pip.

46. The method of claim 44, wherein said chemically regulated activator is selected from the group consisting of PIT, MphR-VP16, MphR-VP64, MphR-NFkB-p65, RXR, mifepristone regulated gene expression and Ecdysone- regulated gene expression.

47- The method of claim 44, wherein said poly adenylation signal is selected from the group consisting of SV40 poly A signal, bovine GH poly A signal, ADH1 transcription terminator or any mean to terminate transcription.

48- The method of claim 44, wherein said EthR and said recombined protein are connected together using IRES or 2A peptide.

49- A host cell made with method of claim 44.

50- A recombined protein produced with method of claim 44.

51-A recombined DNA compress of:

a) An eukaryotic promoter(X)

b) at least one OethR operator positioned 3' or 5' of said eukaryotic promoter of claim51

c) a coding sequence of chemically regulated repressor (R) lying 3' to said OethR operably linked to said eukaryotic promoter(X) of claim 51

d) a miRNA response element (MRE) positioned 3' to said coding sequence of chemically regulated repressor (R) of claim 51

e) a poly adenylation signal positioned 3' of said miRNA response element of claim 51

f) a DNA response element specific for said chemically regulated repressor of claim 51 sandwiching between a eukaryotic promoter (Y) and a chemically regulated activator coding sequence

g) The chemical regulated activator of claim 50 positioned 3' of said DNA response element specific for said chemically regulated repressor (R) of claim 51

h) a miRNA response element (MRE) positioned 3' to said chemical regulated activator of claim 51

j) a poly adenylation signal positioned 3' of said miRNA response element (MRE) of claim 51

k) an inducible promoter specific for said chemically regulated activator of claim 51

l) the DNA response element specific for said chemically regulated repressor of claim 51 sandwiching between said inducible promoter specific for said chemically regulated activator of claim 51 and a coding sequence contain a reporter gene

m) poly adenylation signal positioned 3' to said reporter gene

n) a tissue specific promoter

o) the coding sequence of EthR operably linked to said tissue specific promoter of claim 51

p) a miRNA response element (MRE) positioned 3' to said EthR coding sequence

q) poly adenylation signal positioned 3' to said EthR coding sequence

52. The DNA molecule of claim 36, wherein said eukaryotic promoters is selected from the group consisting of CMV, VIP, tk, HSP, MLP, MMTV ,Non Tissue-Specific Promoters and Tissue-Specific Promoters.

53. The DNA molecule of claim 51, wherein said chemically regulated repressor (R) is selected from the group consisting of TetR, MphR and pip.

54. The DNA molecule of claim 51, wherein said chemically regulated activator is selected from the group consisting of PIT, MphR-VP16, MphR-VP64, MphR-NFkB-p65, RXR, mifepristone regulated gene expression and Ecdysone- regulated gene expression.

55. The DNA molecule of claim 51, wherein said MRE positioned 3' to said coding sequence of chemically regulated of claim 51 are different from wherein said MRE positioned 3' to said coding sequence of chemically regulated activator of claim 51.

56- a vector contains recombined DNA of claim 51

57- A host cell transformed with a vector comprising the DNA molecule of claim 51.

58- a method for studying miRNA production and regulation in vitro composite of:

a) a host cell transfected with a vector comperes of:

(i) An eukaryotic promoter (X)

(ii) at least one OethR operator positioned 3' or 5' of said eukaryotic promoter of claim58

(iii) a coding sequence of chemically regulated repressor lying 3' to said OethR operably linked to said inducible promoter of claim 58
(iv) a miRNA response element (MRE) positioned 3' to said coding sequence of chemically regulated of claim 58
(v) a poly adenylation signal positioned 3' of said miRNA response element of claim 58
(vi) a DNA response element specific for said chemically regulated repressor of claim 58 sandwiching between an eukaryotic promoter (Y) and a chemically regulated activator
(vii) The chemical regulated activator of claim 58 positioned 3' of said DNA response element specific for said chemically regulated repressor of claim 58
(viii) a miRNA response element (MRE) positioned 3' to said chemical regulated activator of claim 58
(Vii) a poly adenylation signal positioned 3' of said miRNA response element (MRE) of claim 58
(Viii) an inducible promoter specific for said chemically regulated activator of claim 58
(ix) the DNA response element specific for said chemically regulated repressor of claim 58 sandwiching between said inducible promoter specific for said chemically regulated activator of claim 58 and a coding sequence contain a reporter gene
(x) poly adenylation signal positioned 3' to said reporter gene
(xi) a tissue specific promoter
(xii) the coding sequence of EthR operably linked to said tissue specific promoter of claim 58
(xiii) a miRNA response element (MRE) positioned 3' to said EthR coding sequence
(xiv) poly adenylation signal positioned 3' to said EthR coding sequence

b) Chemical compound specific for said chemically regulated repressor of claim 58
c) Chemical compound specific for said chemically regulated activator of claim 58

Wherein the production of said reporter gene of claim 58 depended upon the absences or the presence of specific set of miRNA specific for said MRE of said activator or repressor, whereby enable the production of reporter gene in specific sit of cells based on their expression of miRNAs, Wherein said Chemical compound specific for said chemically regulated repressor of claim 58 and Chemical compound specific for said

chemically regulated activator of claim 58 enable further the control of time of reporter gene expression.

59. The method of claim 58, wherein said chemically regulated repressor is selected from the group consisting of TetR, MphR and pip.

60. The method of claim 58, wherein said chemically regulated activator is selected from the group consisting of PIT, MphR-VP16, MphR-VP64, MphR-NFkB-p65, RXR, mifepristone regulated gene expression and Ecdysone- regulated gene expression.

61- The method of claim 58, wherein said poly adenylation signal is selected from the group consisting of SV40 poly A signal, bovine GH poly A signal, ADH1 transcription terminator, or any other mean to terminate transcription.

62- A host cell made with method of claim 58.

63- A method for studying miRNA production and tissue differentiation composite of:

a) An embryonic stem cell transfected with a vector compressing:
(i) A tissue specific promoter (X)
(ii) at least one OethR operator positioned 3' or 5' of said tissue specific promoter (X) of claim 63
(iii) a coding sequence of chemically regulated repressor lying 3' to said OethR operably linked to said tissue specific promoter of claim 63
(iv) a miRNA response element (MRE) positioned 3' to said coding sequence of chemically regulated of claim 63
(v) a poly adenylation signal positioned 3' of said miRNA response element of claim 63
(vi) a DNA response element specific for said chemically regulated repressor of claim 63 sandwiching between A tissue specific promoter (Y) and a chemically regulated activator coding sequence
(vii) The chemical regulated activator coding sequence of claim 63 positioned 3' of said DNA response element specific for said chemically regulated repressor of claim 63
(viii) a miRNA response element (MRE) positioned 3' to said chemical regulated

activator of claim 63

(Viii) a poly adenylation signal positioned 3' of said miRNA response element (MRE) of claim 63

(ix) an inducible promoter specific for said chemically regulated activator of claim 63

(x) the DNA response element specific for said chemically regulated repressor of claim 63 sandwiching between said inducible promoter specific for said chemically regulated activator of claim 50 and a coding sequence contain a reporter gene

(xi) a tissue specific promoter (Z)

(xii) the coding sequence of EthR operably linked to said tissue specific promoter of claim 63

(xiii) a miRNA response element (MRE) positioned 3' to said EthR coding sequence

(xiv) poly adenylation signal positioned 3' to said EthR coding sequence

b) incorporating said stem cell of claim 63 into a blastocyst to form a chimeric embryo

c) implanting said chimeric embryo of claim 63 into a pseudo pregnant animal

d) allowing said chimeric embryo of claim 63 to develop into a viable offspring

e) screening offspring to identify heterozygous animals expressing said genes of claim 63

f) breeding said heterozygous animals to produce homozygous transgenic animals producing said genes of claim 63

Wherein:

(i) The production of said reporter gene of claim 63 depended upon the absences or the presence of specific set of miRNA specific for said MRE of said activator or repressor.

(ii) The production of said reporter gene of claim 63 depended upon the differential activation of said tissue specific promoter (X) and said tissue specific promoter (Y).

(iii) The production of said reporter gene of claim 63 depended upon said Chemical compound specific for said chemically regulated repressor of claim 63 and Chemical compound specific for said chemically regulated activator of claim 63.

Whereby:

(i) Enable the production of reporter gene in specific set of cells based on their expression of miRNAs.

(ii) Enable the production of reporter gene in specific set of cells based on their differential activation of said tissue specific promoter (X) and said tissue specific promoter (Y).

(iii) Enable the production of reporter gene in specific set of cells in specific time based on the arability of said Chemical compound specific for said chemically regulated repressor of claim 63 and Chemical compound specific for said chemically regulated activator of claim 63

64- A transgenic animal made by the method of claim 63

65- A method for conditional gene knockdown in-vitro composite of:

a) a mammalian cell transfected with a vector composite of:

(i) A tissue specific promoter (X)

(ii) at least one OethR operator positioned 3' or 5' of said tissue specific promoter (X) of claim 65

(iii) a coding sequence of chemically regulated repressor lying 3' to said OethR operably linked to said tissue specific promoter of claim 65

(iv) a miRNA response element (MRE) positioned 3' to said coding sequence of chemically regulated of claim 65

(v) a poly adenylation signal positioned 3' of said miRNA response element of claim 65

(vi) a DNA response element specific for said chemically regulated repressor of claim 65 sandwiching between A tissue specific promoter (Y) and a chemically regulated activator coding sequence

(vii) The chemical regulated activator coding sequence of claim 65 positioned 3' of said DNA response element specific for said chemically regulated repressor of claim 65

(viii) a miRNA response element (MRE) positioned 3' to said chemical regulated activator of claim 65

(Viii) a poly adenylation signal positioned 3' of said miRNA response element (MRE) of claim 65

(ix) an inducible promoter specific for said chemically regulated activator of claim 65

(x) the DNA response element specific for said chemically regulated repressor of claim

65sandwiching between said inducible promoter specific for said chemically regulated activator of claim 50 and a coding sequence contain siRNA to knock down gene.

(xi) a tissue specific promoter (Z)

(xii) the coding sequence of EthR operably linked to said tissue specific promoter of claim 65

(xiii) a miRNA response element (MRE) positioned 3' to said EthR coding sequence

(xiv) poly adenylation signal positioned 3' to said EthR coding sequence

b) incorporating said stem cell of claim 65 into a blastocyst to form a chimeric embryo

c) implanting said chimeric embryo of claim 65 into a pseudo pregnant animal

d) allowing said chimeric embryo of claim 65 to develop into a viable offspring

e) screening offspring to identify heterozygous animals expressing said gene of claim 65

f)breeding said heterozygous animals to produce homozygous transgenic animals producing said genes of claim 65

66- A transgenic animal made by the method of claim 65.

67- A method for conditional multiple gene knockdown in-vitro composite of:

a) mammalian cell transfected with a vector composite of:

(i) A tissue specific promoter (X)

(ii) at least one OethR operator positioned 3' or 5' of said tissue specific promoter (X) of claim 67

(iii) a coding sequence of chemically regulated repressor lying 3' to said OethR operably linked to said tissue specific promoter of claim 67

(iv) a miRNA response element (MRE) positioned 3' to said coding sequence of chemically regulated of claim 67

(v) a poly adenylation signal positioned 3' of said miRNA response element of claim 67

(vi) a DNA response element specific for said chemically regulated repressor of claim 67 sandwiching between A tissue specific promoter (Y) and a chemically regulated activator coding sequence

(vii) The chemical regulated activator coding sequence of claim 67 positioned 3' of said DNA response element specific for said chemically regulated repressor of claim 67

(viii) a miRNA response element (MRE) positioned 3' to said chemical regulated activator of claim 67

(Viii) a poly adenylation signal positioned 3' of said miRNA response element (MRE) of claim 67

(ix) an inducible promoter specific for said chemically regulated activator of claim 67

(x) the DNA response element specific for said chemically regulated repressor of claim 67 sandwiching between said inducible promoter specific for said chemically regulated activator of claim 67 and a coding sequence contain cluster of siRNAs to knock down genes.

(xi) a tissue specific promoter (Z)

(xii) the coding sequence of EthR operably linked to said tissue specific promoter of claim 67

(xiii) a miRNA response element (MRE) positioned 3' to said EthR coding sequence

(xiii) a miRNA response element (MRE) positioned 3' to said EthR coding sequence

(xiv) poly adenylation signal positioned 3' to said EthR coding sequence

b) incorporating said stem cell of claim 67 into a blastocyst to form a chimeric embryo

c) implanting said chimeric embryo of claim 67 into a pseudo pregnant animal

d) allowing said chimeric embryo of claim 67 to develop into a viable offspring

e) screening offspring to identify heterozygous animals expressing said genes of claim 67

f) breeding said heterozygous animals to produce homozygous transgenic animals producing said genes of claim 67

68- A transgenic animal made by the method of claim 67.

69- A method for selective gene expression in tumor cells composite of:

a) tumor cell transfected with a vector composite of:

(i) A tissue specific promoter (X)

(ii) at least one OethR operator positioned 3' or 5' of said tissue specific promoter (X) of claim 69

(iii) a coding sequence of chemically regulated repressor lying 3' to said OethR operably linked to said tissue specific promoter of claim 69

(iv) a miRNA response element (MRE) positioned 3' to said coding sequence of chemically regulated of claim 69

(v) a poly adenylation signal positioned 3' of said miRNA response element of claim 69

(vi) a DNA response element specific for said chemically regulated repressor of claim 69sandwiching between A tumor specific promoter (Y) and a chemically regulated

activator coding sequence

(vii) The chemical regulated activator coding sequence of claim 69 positioned 3' of said DNA response element specific for said chemically regulated repressor of claim 69

(viii) a miRNA response element (MRE) positioned 3' to said chemical regulated activator of claim 69

(Viii) a poly adenylation signal positioned 3' of said miRNA response element (MRE) of claim 69

(ix) an inducible promoter specific for said chemically regulated activator of claim 69

(x) the DNA response element specific for said chemically regulated repressor of claim 69sandwiching between said inducible promoter specific for said chemically regulated activator of claim 69 and a coding sequence contain gene tended to be expressed.

(xi) a tissue specific promoter (Z)

(xii) the coding sequence of EthR operably linked to said tissue specific promoter of claim 69

(xiii) a miRNA response element (MRE) positioned 3' to said EthR coding sequence

(xiii) a miRNA response element (MRE) positioned 3' to said EthR coding sequence

(xiv) poly adenylation signal positioned 3' to said EthR coding sequence

70- A tumor cell transformed with method of claim 69

71- A recombinant DNA molecule comprising :

a) an eukaryotic promoter(X)

b) a DNA response element specific for a chemically regulated repressor (S) lying 3' or 5' to said eukaryotic promoter (X) of claim 71.

c) a coding sequence of chemically regulated repressor(R) lying 3' to said DNA response element specific for said chemically regulated repressor (S) operably linked to said eukaryotic promoter (X) of claim 71.

d) a DNA response element specific for said chemically regulated repressor (R) of claim 71 sandwiching between an eukaryotic tumor specific promoter (Y) and a chemically regulated activator coding sequence

e) The chemical regulated activator coding sequence of claim 71 positioned 3' of said DNA response element specific for said chemically regulated repressor (R) of claim 71

f) an inducible promoter specific for said chemically regulated activator of claim 71

g) the DNA response element specific for said chemically regulated repressor (R) of claim 71sandwiching between said inducible promoter of claim 71 specific for said chemically regulated activator of claim 71 and a coding sequence contain chemically regulated repressor (S).

72- A host cell transformed with a vector comprising the DNA molecule of claim 71.

73- A recombinant DNA molecule comprising:

a) an eukaryotic promoter(X)

b) a DNA response element specific for a repressor (S) lying 3' or 5' to said eukaryotic promoter (X) of claim 73.

c) a coding sequence of repressor(R) lying 3' to said DNA response element specific for said repressor (S) operably linked to said eukaryotic promoter (X) of claim 73.

d) a DNA response element specific for said repressor (R) of claim 73 sandwiching between an eukaryotic tumor specific promoter (Y) and a chemically regulated activator coding sequence

e) The chemical regulated activator coding sequence of claim 71 positioned 3' of said DNA response element specific for said repressor (R) of claim 73

f) an inducible promoter specific for said chemically regulated activator of claim 73

g) the DNA response element specific for said repressor (R) of claim 73 sandwiching between said inducible promoter of claim 71 specific for said chemically regulated activator of claim 73 and a coding sequence contain chemically regulated repressor (S).

74- A host cell transformed with a vector comprising the DNA molecule of claim 73.

75- A recombinant DNA molecule comprising:

a) an eukaryotic promoter(X)

b) a DNA response element specific for a repressor (S)

c) a coding sequence of repressor(R)

d) a DNA response element specific for said repressor (R)

e) A chemically regulated activator coding sequence

f) an inducible promoter specific for said chemically regulated activator of claim 73

g) a coding sequence coding sequence of repressor (S)

76- A host cell transformed with a vector comprising the DNA molecule of claim 75.

77- A recombinant DNA molecule comprising:

a) human IL-2 gene promoter

b) a human miRNA 164a steam loop sequence

c) a human miRNA 29a steam loop sequence

d) a human miRNA 29b steam loop sequence

78- A host cell transformed with a vector comprising the DNA molecule of claim 77.

79- A recombinant DNA molecule comprising:

a) a mammalian IL-2 gene promoter

b) a mammalian miRNA 164a steam loop sequence

c) a mammalian miRNA 29a steam loop sequence

d) a mammalian miRNA 29b steam loop sequence

80- A host cell transformed with a vector comprising the DNA molecule of claim 79

81- A recombinant DNA molecule comprising:

a) an eukaryotic promoter

b) a mammalian miRNA 164a steam loop sequence

c) a mammalian miRNA 29a steam loop sequence

d) a mammalian miRNA 29b steam loop sequence

82- A host cell transformed with a vector comprising the DNA molecule of claim 81

83- A recombinant DNA molecule comprising:

a) an eukaryotic promoter

b) a mammalian miRNA 164a steam loop sequence

c) a mammalian miRNA 29a steam loop sequence

84- A host cell transformed with a vector comprising the DNA molecule of claim 83

85- A recombinant DNA molecule comprising:

a) an eukaryotic promoter

b) a mammalian miRNA 164a steam loop sequence

86- A host cell transformed with a vector comprising the DNA molecule of claim 85

87- A recombinant DNA molecule comprising:

a) an eukaryotic promoter

b) a mammalian miRNA 29a steam loop sequence

88- A host cell transformed with a vector comprising the DNA molecule of claim 87

89- A recombinant DNA molecule comprising:

a) an eukaryotic promoter

b) a mammalian miRNA 29b steam loop sequence

90- A host cell transformed with a vector comprising the DNA molecule of claim 89

91- A recombinant DNA molecule comprising:

a) an eukaryotic promoter

b) a mammalian miRNA 29a steam loop sequence

c) a mammalian miRNA 29b steam loop sequence

92- A host cell transformed with a vector comprising the DNA molecule of claim 91

93- A recombinant DNA molecule comprising:

a) an eukaryotic promoter (X)

b) a repressor coding sequence operably linked to said eukaryotic promoter (X) of claim 93

c) an eukaryotic promoter (Y)

d) a repressor binding site specific for said repressor of claim 93 located downstream of said eukaryotic promoter (Y) of claim 93

e) a mammalian miRNA 29a steam loop sequence operably linked to said eukaryotic promoter (Y) of claim 93

f) a mammalian miRNA 29b steam loop sequence operably linked to said eukaryotic promoter (Y) of claim 93

94- A host cell transformed with a vector comprising the DNA molecule of claim 93

95- A recombinant DNA molecule comprising:

a) an eukaryotic promoter (X)

b) repressor coding sequence operably linked to said eukaryotic promoter (X) of claim 95

c) an eukaryotic promoter (Y)

d) a repressor binding site specific for said repressor of claim 95 located downstream of said eukaryotic promoter (Y) of claim 95

e) a mammalian miRNA 29a steam loop sequence operably linked to said eukaryotic promoter (Y) of claim 95

f) a mammalian miRNA 146a steam loop sequence operably linked to said eukaryotic promoter (Y) of claim 95

96- A host cell transformed with a vector comprising the DNA molecule of claim 95

97- A recombinant DNA molecule comprising:

a) an eukaryotic promoter (X)

b) repressor coding sequence operably linked to said eukaryotic promoter (X) of claim 97

c) an eukaryotic promoter (Y)

d) a repressor binding site specific for said repressor of claim 97 located downstream of said eukaryotic promoter (Y) of claim 97

e) a mammalian miRNA 29b steam loop sequence operably linked to said eukaryotic promoter (Y) of claim 97

f) a mammalian miRNA 146a steam loop sequence operably linked to said eukaryotic promoter (Y) of claim 97

98-A host cell transformed with a vector comprising the DNA molecule of claim 97

99- A recombinant DNA molecule comprising:

a) an eukaryotic promoter (X)

b) a repressor coding sequence operably linked to said eukaryotic promoter (X) of claim 99

c) a DNA response element specific for said repressor of claim 99 located 5' or 3' to an eukaryotic promoter (Y)

d) a mammalian miRNA 29b steam loop sequence operably linked to said eukaryotic promoter (Y) of claim 99

e) a mammalian miRNA 29b steam loop sequence operably linked to said eukaryotic promoter (Y) of claim 9

f) a mammalian miRNA 29a steam loop sequence operably linked to said eukaryotic promoter (Y) of claim 99

100- A host cell transformed with a vector comprising the DNA molecule of claim 99

101- A method to control T cytokine release compressing:

a) transfect T cell with a vector composite of :

(i) an eukaryotic promoter

(ii) a regulated repressor coding sequence operably linked to said eukaryotic promoter (X) of claim 101

(iii) a mammalian miRNA 29b steam loop sequence operably linked to said eukaryotic promoter of claim 101

(iv) a mammalian miRNA 29b steam loop sequence operably linked to said eukaryotic promoter of claim 101

(v) a mammalian miRNA 146a steam loop sequence operably linked to said eukaryotic promoter of claim 101

102- The method of claim 101, wherein said regulated repressor is selected from the group consisting of TetR, MphR and pip.

103- The method of claim 101, wherein said eukaryotic promoter is selected from the group consisting of IL-2 promoter, inf-g promoter or any other promoter that get activated by TCR signaling.

104- A recombinant DNA molecules comprising:

a) a promoter

b) a repressor binding site for a repressor (R) lying 3' or 5'' to said promoter of claim 104

c) a coding region contain a transcription activator and an out-put signal (A) lying 3' to said repressor binding site of claim 104 and operably linked to said promoter of claim 104

d) an inducible promoter whose activity is controlled by said transcription activator of claim 104

e) the repressor binding site for a repressor (R) of claim 104 lying 3' or 5' to said inducible promoter of claim 104

f) a coding region contain said repressor (R) of claim 104 and an out-put signal B

105-The genetic circuit of claim 104, wherein said output signal (A) can be miRNA, gRNA, siRNA, anti-gRNA, transcription activator, transcription repression or a recombined protein.

106- The genetic circuit of claim 104, wherein said output signal (B) can be miRNA, gRNA, siRNA, transcription activator , anti-gRNA, transcription repression or a recombined protein.

107- A host cell transformed with a vector comprising the DNA molecule of claim 104.

108- A genetic circuit comprising:

a) a first nucleic acid construct comprising:

(i) a promoter

(ii) a repressor binding site for a repressor (R) lying 3' or 5'' to said promoter of claim 108

(iii) a coding region contain a transcription activator lying 3' to said repressor binding site of claim 108 and operably linked to said promoter of claim 108

b) a second nucleic acid construct comprising:

(i) an inducible promoter whose activity is controlled by said transcription activator of claim 108

(ii) the repressor binding site for a repressor (R) of claim 108 lying 3' or 5' to said inducible promoter of claim 108

(iii) a coding region contain said repressor (R) of claim 108

109- A host cell transformed with a vector comprising the genetic circuit of claim 108.

110- A recombinant conditional genetic oscillator comprising:

a) a first nucleic acid construct comprising a First promoter operably associated with a first gene encoding a first protein

b) a second nucleic acid construct comprising a promoter operably associated with a second gene encoding a second protein

wherein

(i) the first protein, when produced, is capable of activating transcription from the promoter of part (b)

(ii) the second protein, when produced, is capable of repressing transcription from the promoter of part (b) and from the promoter of part (a)

111- the conditional genetic oscillator of claim 110 wherein said second protein is chemically regulated repressor.

112- A host cell transformed with a vector comprising the conditional genetic oscillator of claim 110.

113- A method to transform activation state of signaling pathway into measurable signal composite of:

a) a sensor promoter

b) signal out-put coding sequence operably linked to said sensor promoter of claim 113

c) an out-put signal receiver

wherein said sensor promoter of claim 113 is a promoter who can sense the activity of the signaling pathway and produce an out-put product in response to change in the pathway activation state

114- the method of claim 113, wherein said output signal can be miRNA, anti-miRNA, gRNA, siRNA, transcription activator, transcription repression or reporter protein.

115- the method of claim 113, wherein said output signal receiver can be promoter, Fluorometer or reporter protein.

116- A method to sense T cell activation state composite of:

a) a T cell transfected with a vector comprising:

(i) an eukaryotic promoter

(ii) coding sequence of a reporter protein operably linked to said eukaryotic promoter of claim 116

(iii) a miRNA response element lying 3' to said reporter protein coding sequence

b) Transforming said T cell of claim 116 with a vector comprising:

(i) a mammalian IL-2 promoter

(ii) a miRNA coding sequence specific for said miRNA response element operably linked to said mammalian IL-2 promoter

Wherein;

(i) the expression of said reporter protein of claim 116 is constitutive

(ii) the activation of T cell increase production of said miRNA of claim 116, whereby inhibiting the production of said reporter protein of claim 116, whereby enable the activation of T cell to be state in Quantitative manner

117- A method to sense T cell activation state composite of:

a) a T cell transfected with a vector comprising:

(i) an eukaryotic promoter

(ii) coding sequence of a reporter protein operably linked to said eukaryotic promoter of claim 117

(iii) a miRNA response element lying 3' to said reporter protein coding sequence

b) Transforming said T cell of claim 117 with a vector comprising:

(i) a sensor promoter

(ii) a miRNA coding sequence specific for said miRNA response element operably linked to said sensor promoter

Wherein;

(i) the expression of said reporter protein of claim 117 is constitutive

(ii) the activation of T cell increase production of said miRNA of claim 117, whereby inhibiting the production of said reporter protein of claim 117, whereby enable the activation of T cell to be state in Quantitative manner

118- the Method of claim 117, wherein said sensor promoter is composite of an eukaryotic minimal promoter, and a transcription factor binding site

119- the sensor promoter of claim118, wherein said transcription factor binding sites is selected from a group composite at least of NF-kB binding site, STAT5 binding site, NFAT binding site, AP1 binding site or c-fos binding site.

120- a device to engineer cell behavior composite of :

a) genetic circuit (GC) I

b) genetic circuit (GC) II

c) genetic circuit (GC) III

121- The device of claim 120, wherein said genetic circuit III is composite of variable sub- genetic circuit and essential sub-genetic circuit.

122- The device of claim 120, wherein said genetic circuit I and genetic circuits II are regulatory and computational gene circuit.

123- The regulatory and computational gene circuit of claim 122 is composite of: input analysis sub-genetic circuit (IASGC), and effector sub-genetic circuit (ESGC).

124- The Input analysis sub-genetic circuit (IASGC) of claim 123 is of two types: Gene expression sensitive Sub-genetic circuits (GESSGC), and Signaling pathway sensitive Sub-genetic circuits (SPSSGC).

125- The Gene expression sensitive Sub-genetic circuits (GESSGC) of claim 124 depend upon gene expression as input.

126- The Signaling pathway sensitive Sub-genetic circuits (SPSSGC) of claim 124 depend upon signaling pathway as input.

١٢٧- The effector sub-genetic circuits (ESGC) of claim 123 depend upon tissue specific transcription factors and upon the input analysis sub-genetic circuit (IASGC) of claim 123 outputs as input.

128- The Input analysis sub-genetic circuit (IASGC) of claim 124 internal parts communicate using a local linker (LOL)

129- The Gene expression sensitive Sub-genetic circuits (GESSGC) of claim 124 internal

part communicate using the local linker (LOL) of claim 127

130- The effector sub-genetic circuits (ESGC) of claim 123 internal parts communicate using the local linker (LOL) of claim 127

130- The local linker (LOL) of claim 127 can be miRNA, anti-miRNA, gRNA, siRNA, transcription activator or transcription repression

131- The device of claim 120, wherein said genetic circuit I and genetic circuits II are communicate using global linker (GL)

132- The device of claim 120, wherein said genetic circuit I internal parts are communicate using the global linker (GL) of claim ١٣٠

133- The device of claim 120, wherein said genetic circuit II internal parts are communicate using the global linker (GL) of claim ١٣٠

134- The global linker (GL) of claim 130 can be miRNA, anti-miRNA, gRNA, siRNA, transcription activator or transcription repression

135- a method of delivery miRNA to cancer cells compressing:

a) transfecting a T cell with vector composite of:

(i) an eukaryotic promoter

(ii) a miRNAs contain an Exo-motif coding region operably linked to said eukaryotic promoter (X) of claim 135

b) transfecting said T cell of claim 135 with a vector composite of:

(i) a CAR (chimeric antibody receptor) coding region operably linked to said eukaryotic promoter of claim 135

Wherein

(i) said CAR of claim 135 is specific for a cancer cell type, whereby redirecting said T cell of claim 136 effector function to said cancer cell type,

(ii) said Exo-motif containing miRNA of claim 135 whereby load said Exo-motif containing miRNA into exosomes of said T cells of claim 135

(iii) said T cells of claim 135 will release said exosomes of claim 135 contain said miRNA of claim 135 to said cancer cell of claim 135, whereby delivering miRNA in a selective and efficient manner.

136- A host cell made with the method of claim 135

137- A recombined DNA molecules composite of:

a) an eukaryotic promoter (X)

b) a CAR (chimeric antibody receptor) coding region operably linked to said eukaryotic promoter of claim 137

c) a miRNAs contain an Exo-motif coding region operably linked to said eukaryotic promoter of claim 137

138- A host cell transformed with a vector comprising the DNA molecule of claim 137.

139- A genetic device to reprogram T cell to deliver miRNA to cancer cell and to regulate its tissue migration and cytokine release composite of:

a) genetic circuit (GC) I

b) genetic circuit (GC) II

c) genetic circuit (GC) III

140- The device of claim 139, wherein said genetic circuit III is composite of variable sub- genetic circuit and essential sub-genetic circuit.

141- The variable sub- genetic circuit of claim 140 is composite of:

a) an eukaryotic promoter

b) a miRNA coding region operably linked to said eukaryotic promoter of claim 141

142- The miRNA coding region of claim 141 is composite of:

a) a therapeutic miRNA

b) a (anti- exosomal miRNA that contain an Exo-motif) AmiRE

143- The therapeutic miRNA of claim 142 wherein said therapeutic miRNA is composite of:

a) Steam loop of miRNA coding region

b) an Exo-motif

144- The therapeutic miRNA of claim 142 wherein said therapeutic miRNA is a miRNA mimetic or miRNA antagonist

145- the AmiRE of claim 142 wherein said AmiRE is composite of:

a) Steam loop of AmiRE coding region

b) the Exo-motif of claim 143

146-the essential sub- genetic circuit claim 140 is composite of:

a) an eukaryotic promoter

b) cys4 coding region operably linked to said eukaryotic promoter of claim 146

c) dcas9 coding region operably linked to said eukaryotic promoter of claim 146

147- The coding sequence of cys4 of claim 146, wherein the cys4 comprise a coding sequence fused to nuclear localization sequence (NLS) sequence.

148- The coding sequence of dcas9 of claim 146, wherein the cys4 comprise a coding sequence fused to nuclear localization sequence (NLS) sequence.

149- The device of claim 139, wherein said genetic circuit I is composite of input analysis sub-genetic circuit (IASGC) and effector sub-genetic circuit

150- The input analysis sub-genetic circuit (IASGC) of claim 149 is a Gene expression sensitive Sub-genetic circuits (GESSGC)

151- The Gene expression sensitive Sub-genetic circuits (GESSGC) of claim 150 is composite of:

a) a first nucleic acid construct composite of:

(i) a constitutive promoter (x)

(ii) an OethR operator

(iii) a TetR coding region

b) a second nucleic acid construct composite of:

(i) a constitutive promoter (Y)

(ii) a TetO operator

(iii) a PIT coding region

c) a third nucleic acid construct composite of:

(i) P_{PTR} promoter

(ii) the TetO of claim151

(iii) GAL4-VP16 coding region

(iv) the EthR coding region of claim 3

152- The coding sequence of TetR of claim 151, wherein the TetR comprise a coding sequence fused to nuclear localization sequence (NLS) sequence.

153- The coding sequence of PIT of claim 151, wherein the PIT comprise a coding sequence fused to nuclear localization sequence (NLS) sequence.

154- The effector sub-genetic circuits of claim 149 is composite of:

a) an effector sub-genetic circuits I which is composite of:

a) a nucleic acid construct composite of:

(i) a GAL4-VP16 depended promoters

(ii) a CymR coding region

(iii) a gRNA for S1PR1

b) an effector sub-genetic circuits I which is composite of:

b) a nucleic acid construct composite of:

(i) a constitutive promoter (X)

(ii) a CuO operon

(iii) a gRNA for CCR2

155- The coding sequence of CymR of claim 154, wherein the CymR comprise a coding sequence fused to nuclear localization sequence (NLS) sequence

156- The device of claim 139, wherein said genetic circuit II is composite of input analysis sub-genetic circuit (IASGC) and effector sub-genetic circuit

157- The input analysis sub-genetic circuit (IASGC) of claim 156 is a Signaling pathway sensitive Sub-genetic circuits (SPSSGC).

158- The Signaling pathway sensitive Sub-genetic circuits (SPSSGC) of claim 157 is composite of:

a) a mammalian IL-2promoter

b) a MphR-KRAB coding region

159- The coding sequence of MphR-KRAB of claim 154, wherein the MphR-KRAB comprise a coding sequence fused to nuclear localization sequence (NLS) sequence

160- The effector sub-genetic circuit of claim 156 is composite of:

a) a mammalian IL-2promoter

b) a ERT Operon

c) regulatory miRNA coding region

161- the regulatory miRNA of claim 160 is composite of:

a) miRNA 146a steam loop coding region

b) miRNA 29a steam loop coding region

c) miRNA 29b steam loop coding region

162- A method to control device of claim 139 composite of:

a) administrate Pristinamycin or one of its analogues to the patient in case of low anti-tumor activity

b) Administration of macrolides like erythromycin in case of cytokine storm

c) Keep Administration of streptomycin, and add tetracycline in case of macrolides non-responsive cytokine storm.

163- a genetic device for conditional gene knock down composite of:

a) Genetic circuit I

b) Genetic circuit II

164- The device of claim 163, wherein said genetic circuit II is composite of variable sub- genetic circuit and essential sub-genetic circuit

165- The variable sub- genetic circuit of claim 164 is composite of:

a) an chemically inducible promoter

b) a siRNA coding region or Cre-recombinase coding region operably linked to said chemically inducible promoter of claim 165

166- The essential sub- genetic circuit of claim 164 is composite of:

a) an eukaryotic promoter

b) a dcas9 and Cys4 coding region operably linked to said eukaryotic promoter of claim 166

167- The device of claim 163, wherein said genetic circuit I is composite of input analysis sub-genetic circuit (IASGC) and effector sub-genetic circuit

168- The input analysis sub-genetic circuit (IASGC) of claim 167 is a gene expression sensitive sub-genetic circuit.

169- The gene expression sensitive sub-genetic circuit (SPSSGC) of claim 168 is composite of:

a) SGCE1

b) SGCE2

170- The SGCE1 of claim 169 is composite of:

a) a tissue specific promoter

b) a repressor binding site

c) a gRNA operably linked to said tissue specific promoter of claim 170

171- The SGCE2 of claim 169 is composite of:

a) a tissue specific promoter

b) a repressor coding region specific for said repressor binding site of claim 170

c) a miRNA response element

172- the effector sub-genetic circuit of claim 167 is composite of:

a) a gRNA promoter whose activity depend upon the gRNA produced by claim 170

b) a chemically regulated transcription activator specific for said chemically inducible promoter of claim 165 coding region

c) a miRNA response element

173- a gRNA depended promoter composite of:

a- a DNA stretch

b- an eukaryotic minimal promoter located downstream of said DNA stretch of claim 173

wherein a gRNA specific for said DNA stretch of claim 173 will recruit dcas9 fused with activation domain to said DNA stretch, whereby enable gRNA depended activation of the transcription.

174- a method to design synthetic promoter composite of:

a- cloning a DNA stretch upstream of an eukaryotic minimal promoter

b- design a gRNA specific for said DNA stretch of claim 174

wherein a gRNA specific for said DNA stretch of claim 174 will recruit dcas9 fused with activation domain to said DNA stretch, whereby enable gRNA depended activation of the transcription.

175- a synthetic RNA to control gRNA degradation composite of:

a- a RNA sequence of 5-20nt , named anti-gRNA

wherein said RNA of claim 175 has a complementary base pair sequences with gRNA of claim 175, whereby forum a duplex structure with said gRNA of claim 175, whereby enable RNAase mediated digestion of said duplex, whereby the gRNA of claim 175 will be degraded.

176- a method for design a RNA sequences to control gRNA degradation composite of:

a- design an anti-gRNA to said gRNA of claim 176 in which said anti-gRNA of claim 176 has a partial or complete base pair binding with said gRNA of claim 176.

177- a method to control gRNA\dCas9 system composite of:

a- design an anti-gRNA to said gRNA of claim 177 in which said anti-gRNA of claim 176 has a partial or complete base pair binding with said gRNA of claim 177.

Wherein said RNA of claim 177 has a complementary base pair sequences with gRNA of claim 177, whereby forum a duplex structure with said gRNA of claim 177, whereby enable RNAase mediated digestion of said duplex, whereby the gRNA of claim 177 will be degraded.

178- a method to control gRNA\Cas9 system composite of:

a- design an anti-gRNA to said gRNA of claim 178 in which said anti-gRNA of claim 176 has a partial or complete base pair binding with said gRNA of claim 178.

Wherein said RNA of claim 178 has a complementary base pair sequences with gRNA of claim 178, whereby forum a duplex structure with said gRNA of claim 178, whereby enable RNAase mediated digestion of said duplex, whereby the gRNA of claim 178 will be degraded.

179- a recombined DNA molecule composite of:

a- a promoter

b- a coding sequences for an anti-gRNA

180- a host cell transfected with a vector contain recombined DNA molecule of claim 179.

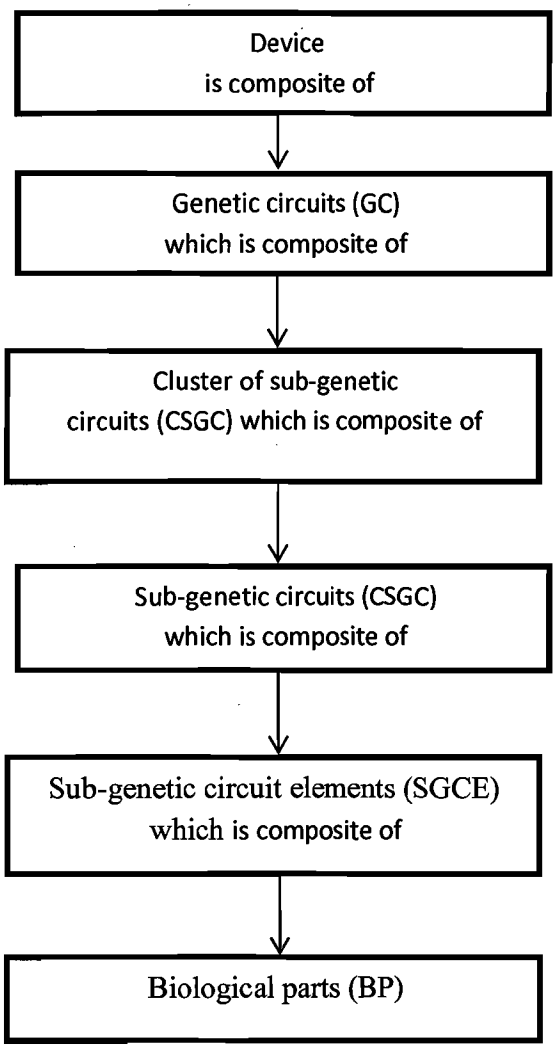

Device
is composite of
Genetic circuits (GC)
which is composite of
Cluster of sub-genetic
circuits (CSGC) which is composite of
Sub-genetic circuits (CSGC)
which is composite of
Sub-genetic circuit elements (SGCE)
which is composite of
Biological parts (BP)

Fig.1

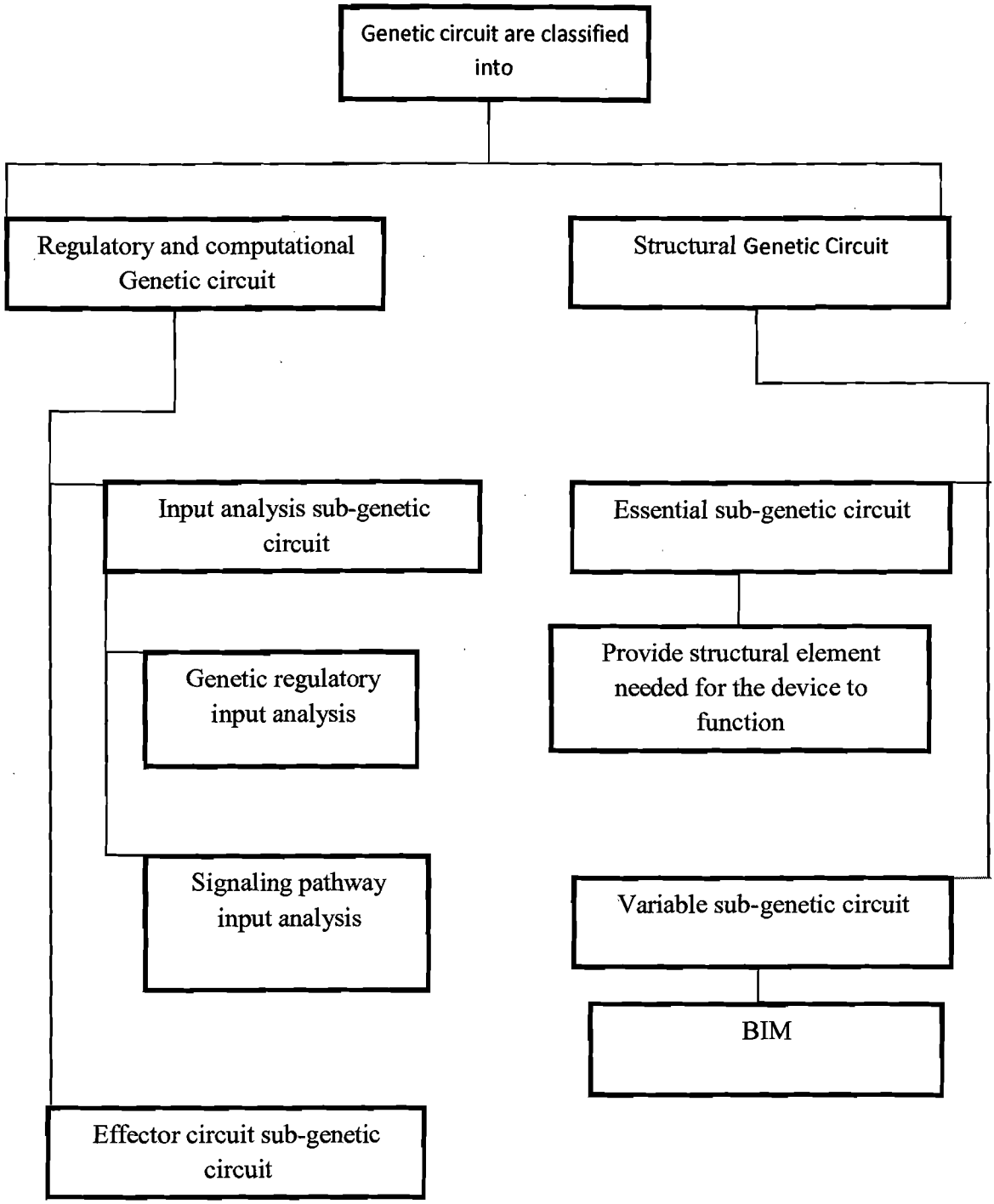

Genetic circuit are classified into
Regulatory and computational Genetic circuit
Structural Genetic Circuit
Input analysis sub-genetic circuit
Essential sub-genetic circuit
Genetic regulatory input analysis
Provide structural element needed for the device to function
Signaling pathway input analysis
Variable sub-genetic circuit
BIM
Effector circuit sub-genetic circuit

Fig.2

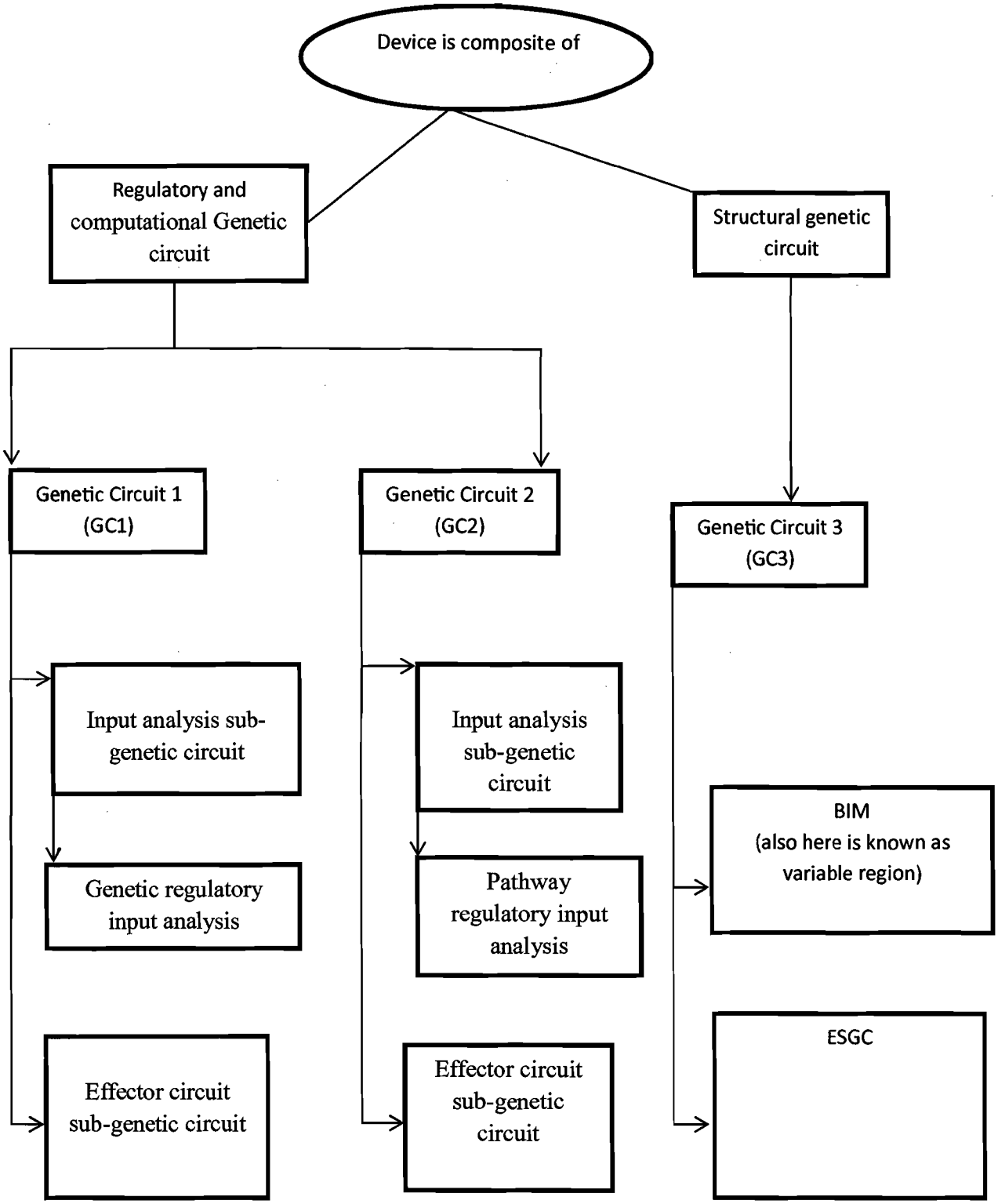

Device is composite of
Regulatory and computational Genetic circuit
Structural genetic circuit
Genetic Circuit 1 (GC1)
Genetic Circuit 2 (GC2)
Genetic Circuit 3 (GC3)
Input analysis sub-genetic circuit
Genetic regulatory input analysis
Effector circuit sub-genetic circuit
Input analysis sub-genetic circuit
Pathway regulatory input analysis
Effector circuit sub-genetic circuit
BIM (also here is known as variable region)
ESGC

Fig.3

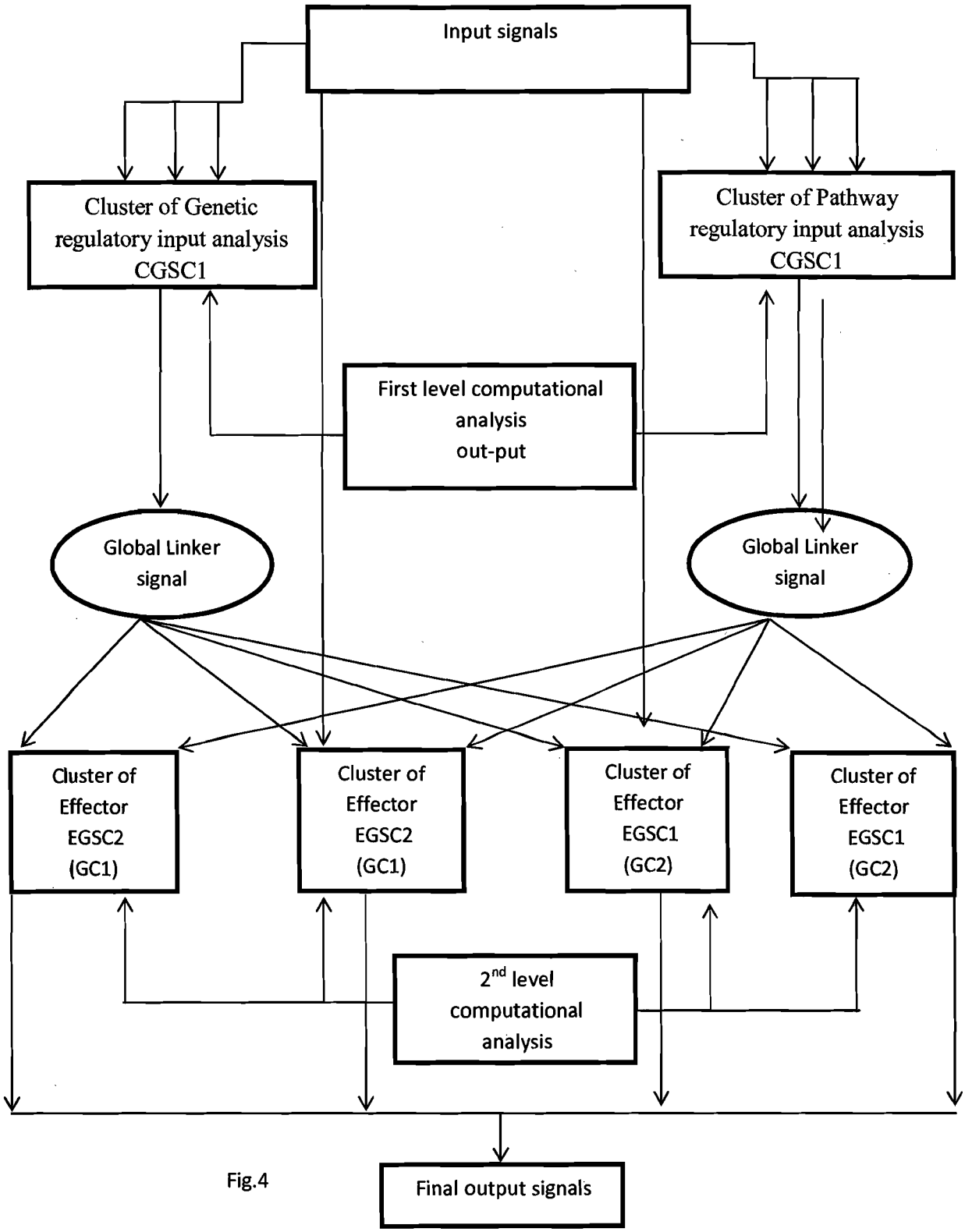

Input signals
Cluster of Genetic regulatory input analysis CGSC1
Cluster of Pathway regulatory input analysis CGSC1
First level computational analysis out-put
Global Linker signal
Global Linker signal
Cluster of Effector EGSC2 (GC1)
Cluster of Effector EGSC2 (GC1)
Cluster of Effector EGSC1 (GC2)
Cluster of Effector EGSC1 (GC2)
2nd level computational analysis
Final output signals

Fig.4

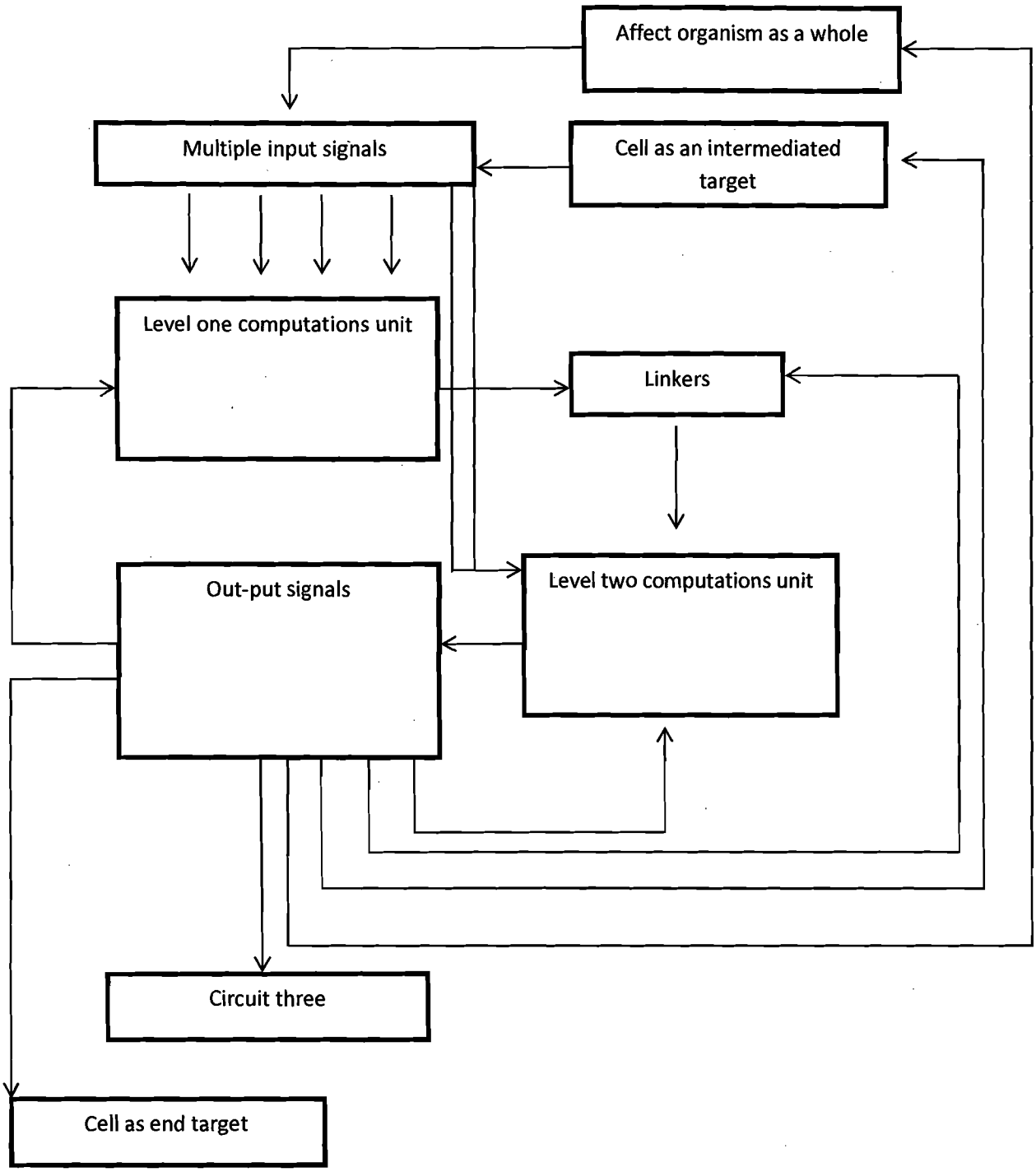

Affect organism as a whole
Multiple input signals
Cell as an intermediated target
Level one computations unit
Linkers
Out-put signals
Level two computations unit
Circuit three
Cell as end target

Fig.5

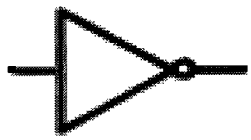


Fig.6

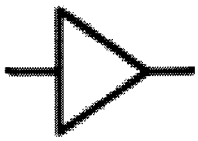


Fig.7

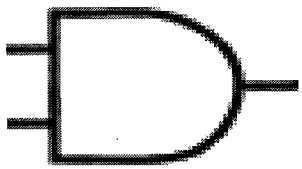


Fig.8

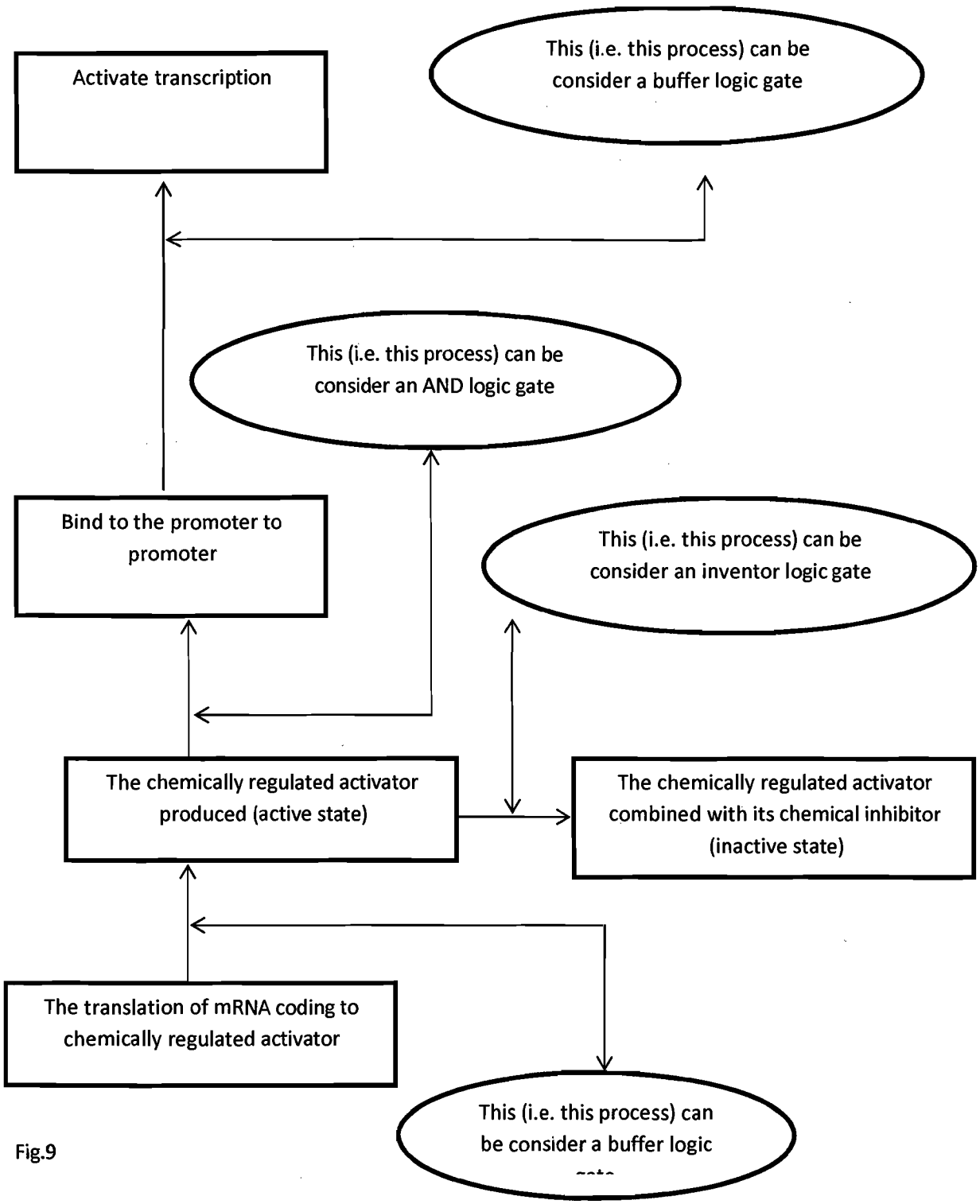

Activate transcription
This (i.e. this process) can be consider a buffer logic gate
This (i.e. this process) can be consider an AND logic gate
Bind to the promoter to promoter
This (i.e. this process) can be consider an inventor logic gate
The chemically regulated activator produced (active state)
The chemically regulated activator combined with its chemical inhibitor (inactive state)
The translation of mRNA coding to chemically regulated activator
This (i.e. this process) can be consider a buffer logic

Fig.9

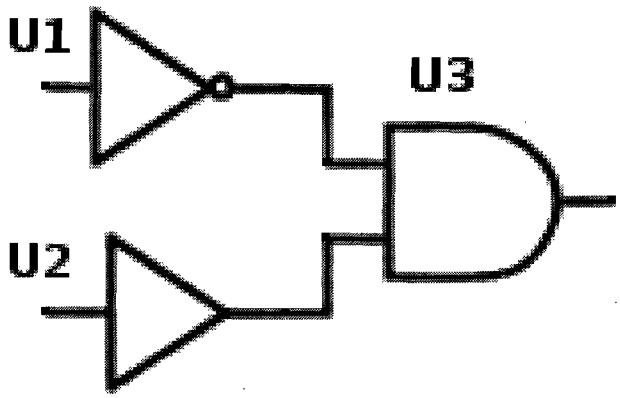


Fig.10

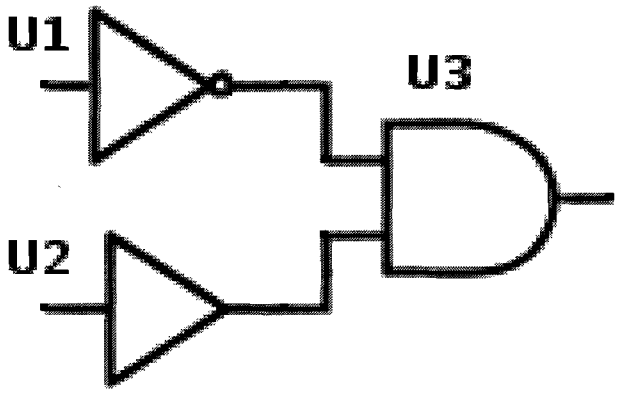


Fig.11

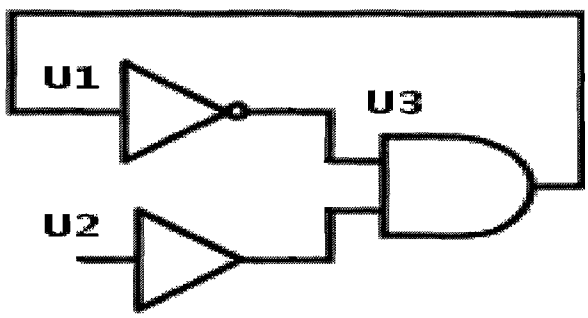


Fig.12

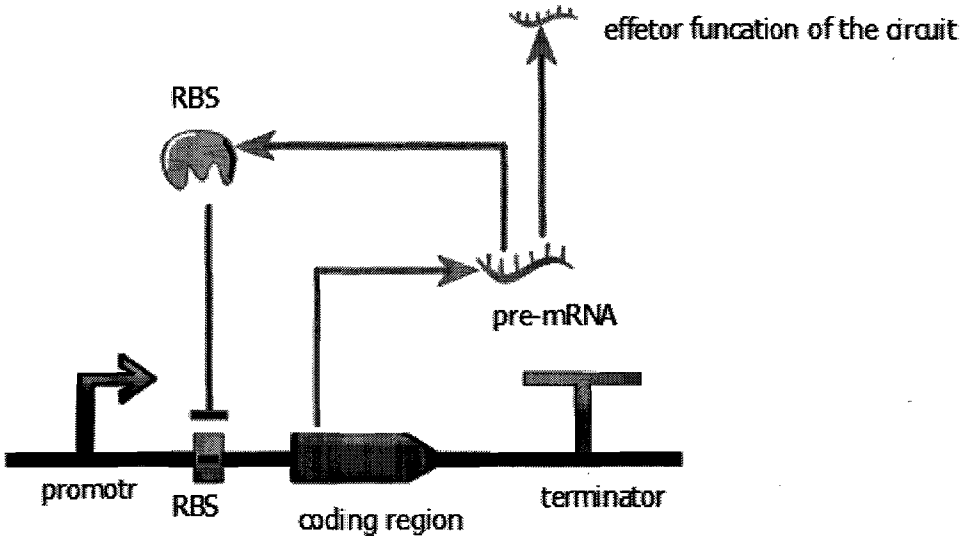

effetor funcation of the circuit
RBS
pre-mRNA
promotr
RBS
coding region
terminator

Fig.13

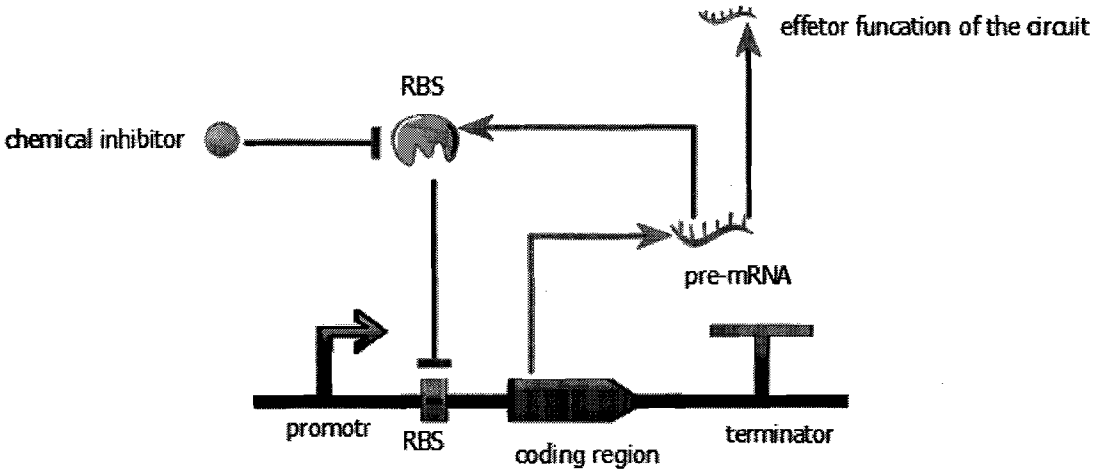

effetor funcation of the circuit
RBS
chemical inhibitor
pre-mRNA
promotr
RBS
coding region
terminator

Fig.14

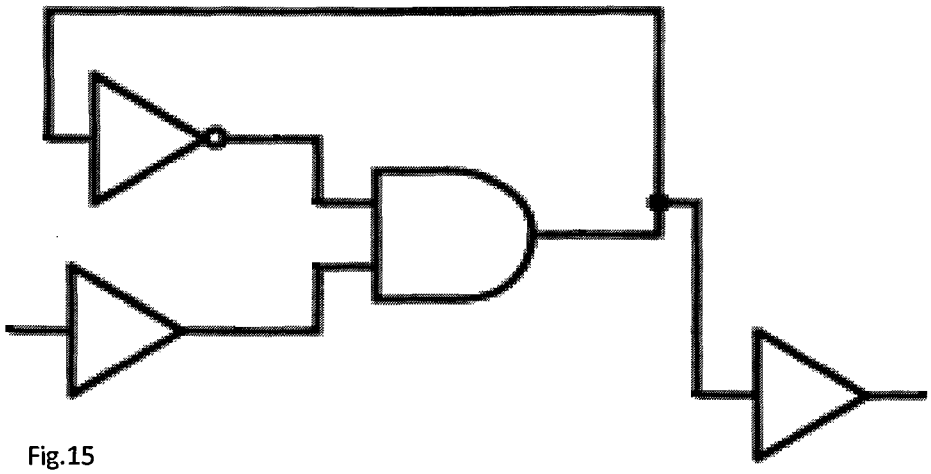


Fig.15

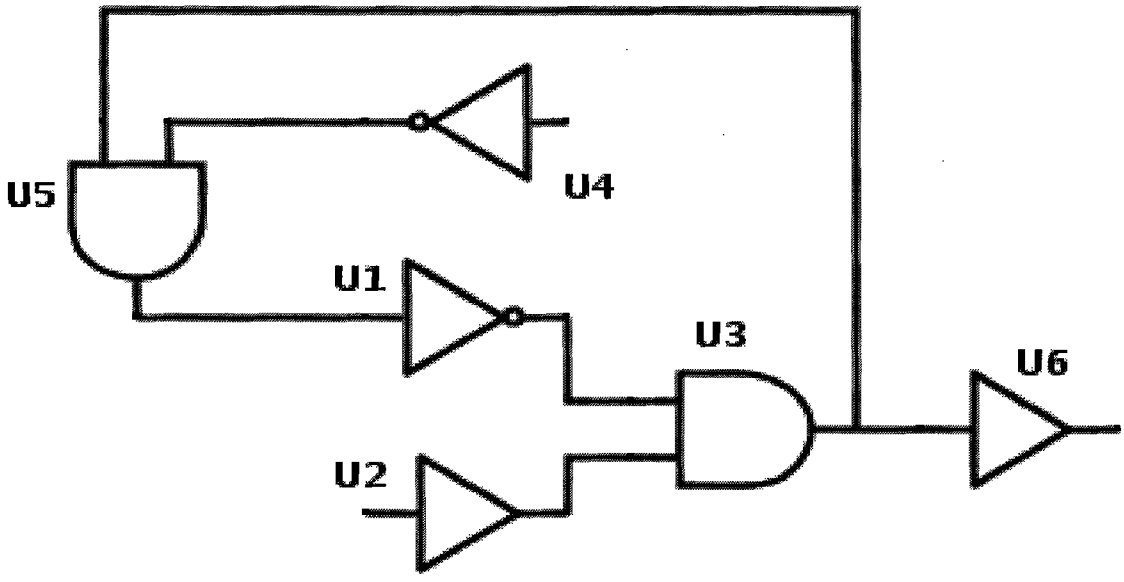


Fig.16

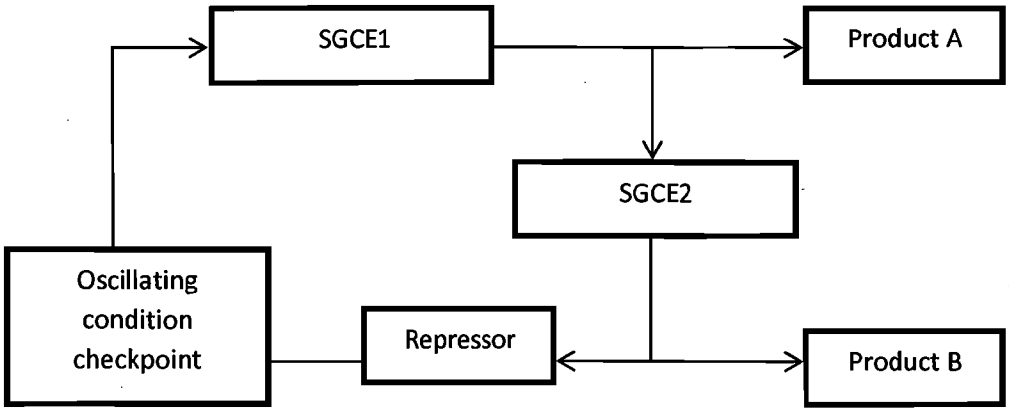

SGCE1
Product A
SGCE2
Oscillating condition checkpoint
Repressor
Product B

Fig.17

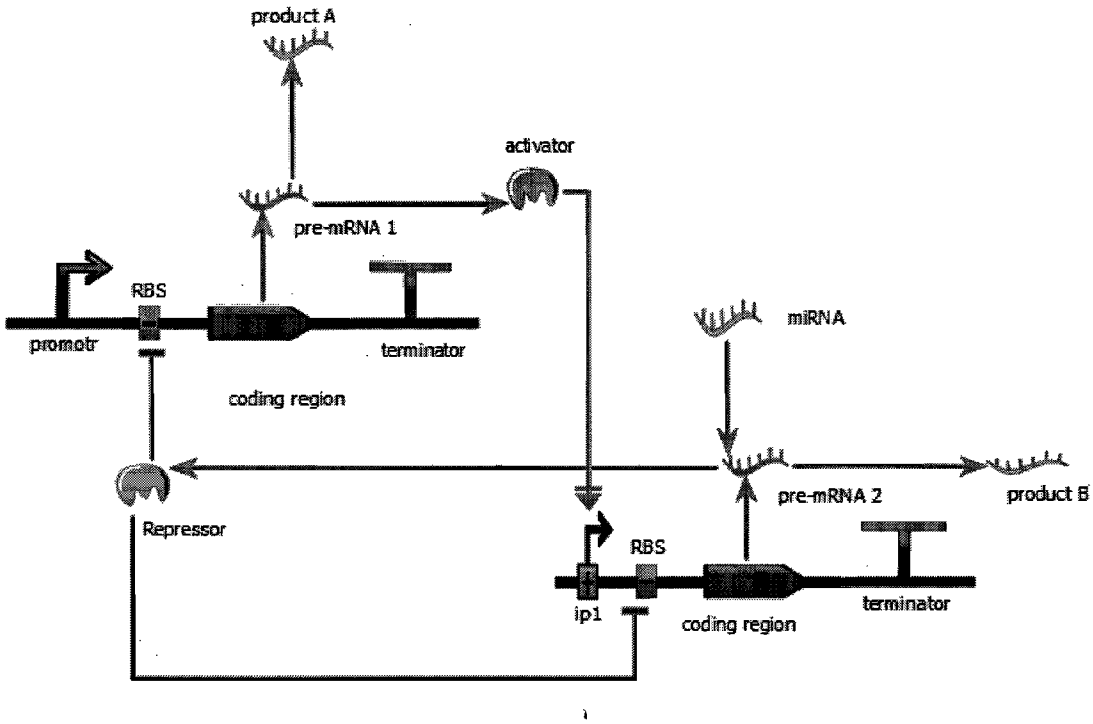

product A
activator
pre-mRNA 1
RBS
promotr
terminator
coding region
miRNA
Repressor
pre-mRNA 2
product B
RBS
ip1
coding region
terminator

Fig.18

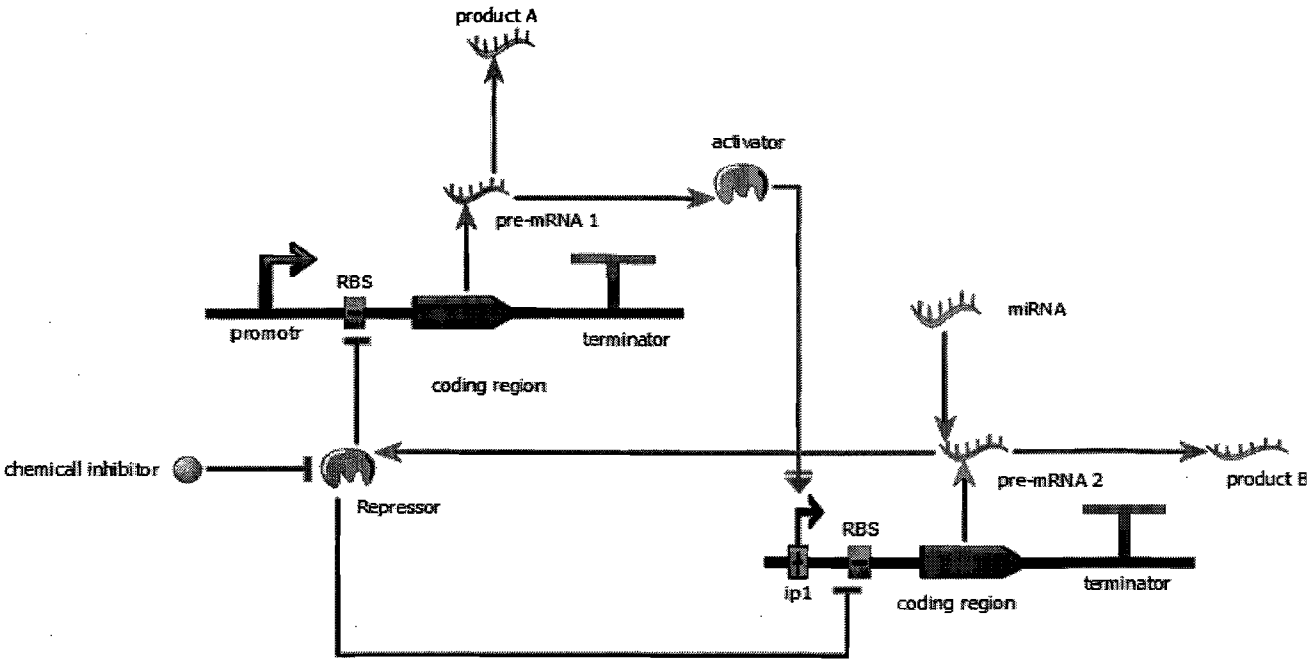

product A
activator
pre-mRNA 1
RBS
promotr
terminator
coding region
miRNA
chemicall inhibitor
Repressor
pre-mRNA 2
product B
RBS
ip1
coding region
terminator

Fig.19

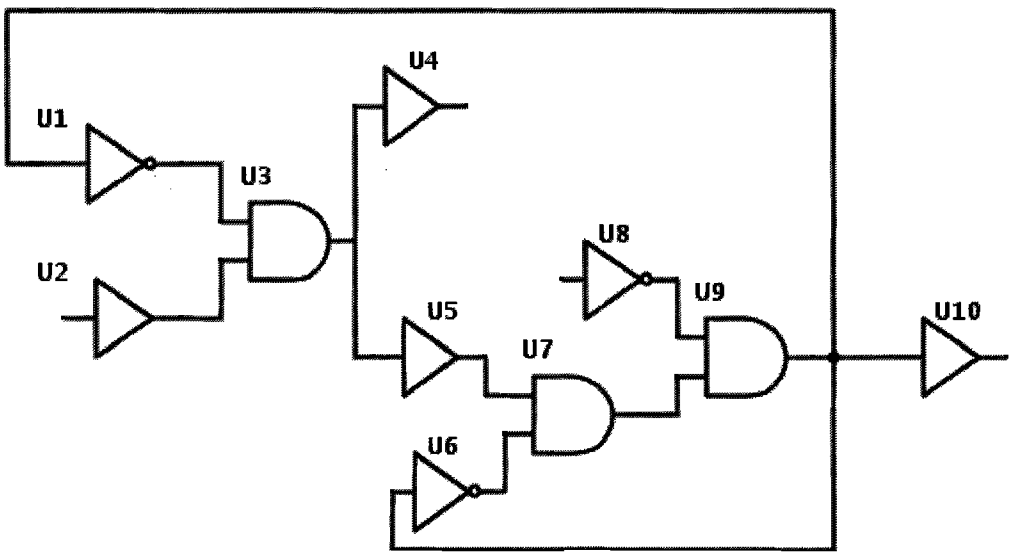

U1
U2
U3
U4
U5
U6
U7
U8
U9
U10

Fig.20

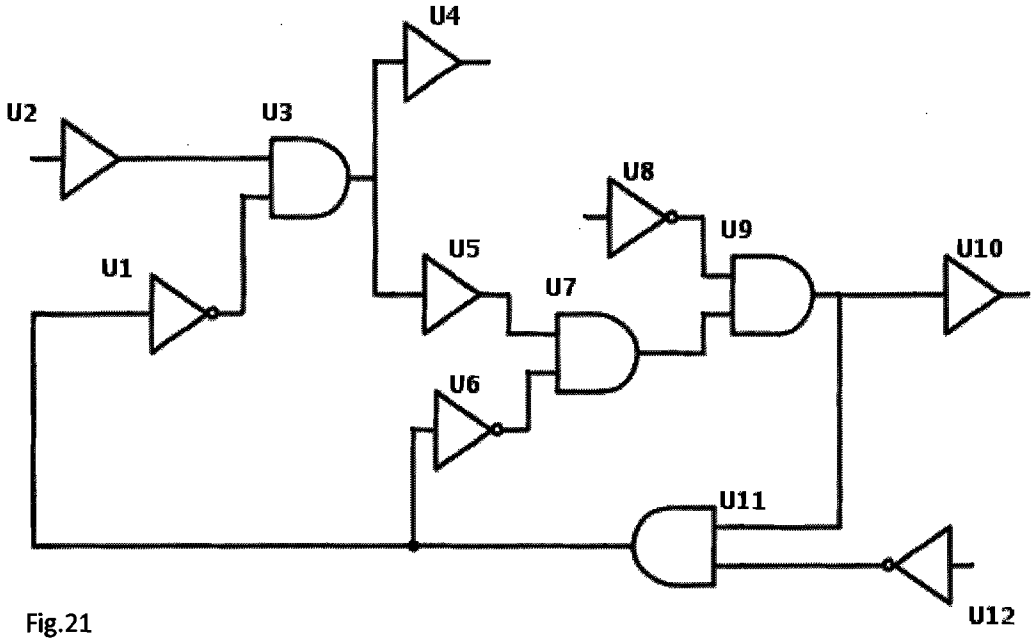

U4
U2
U3
U8
U9
U10
U1
U5
U7
U6
U11
U12

Fig.21

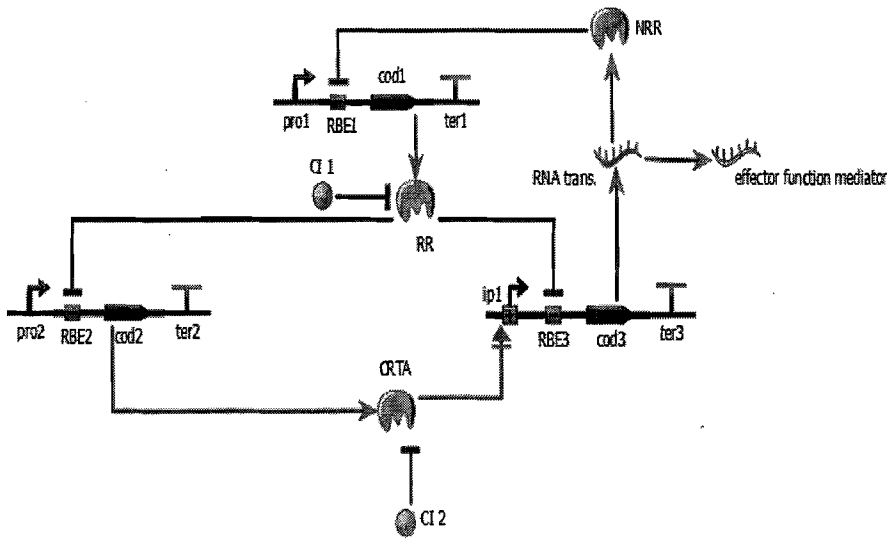

NRR
cod1
pro1
RBE1
ter1
CI 1
RNA trans.
effector function mediator
RR
pro2
RBE2
cod2
ter2
ip1
RBE3
cod3
ter3
CRTA
CI 2

Fig.22

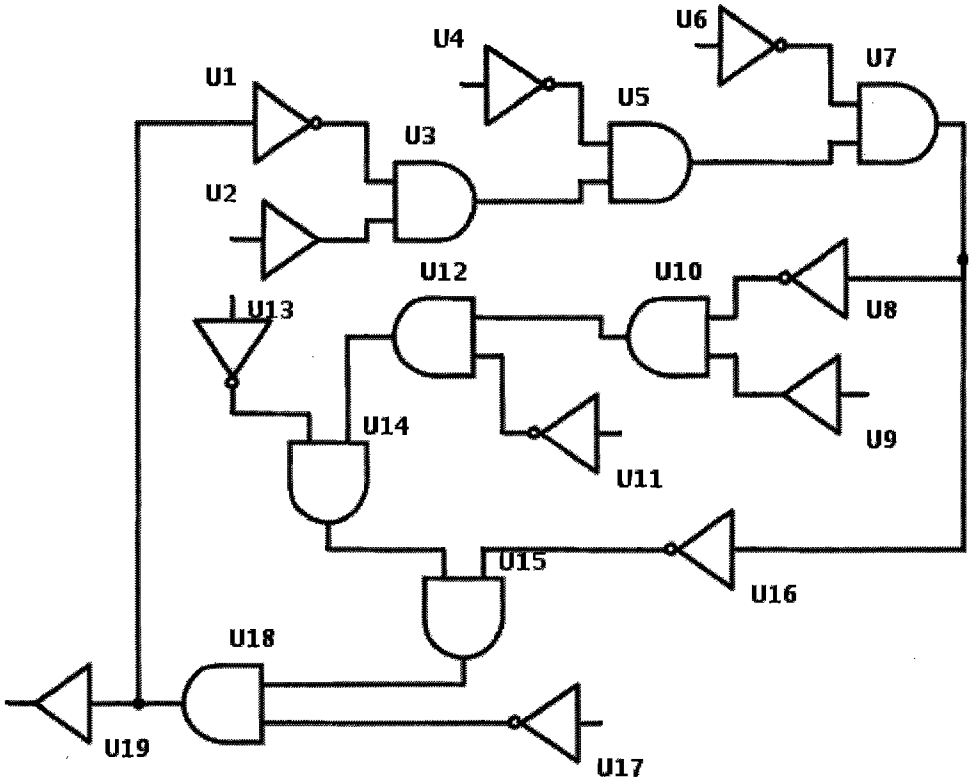


Fig.23

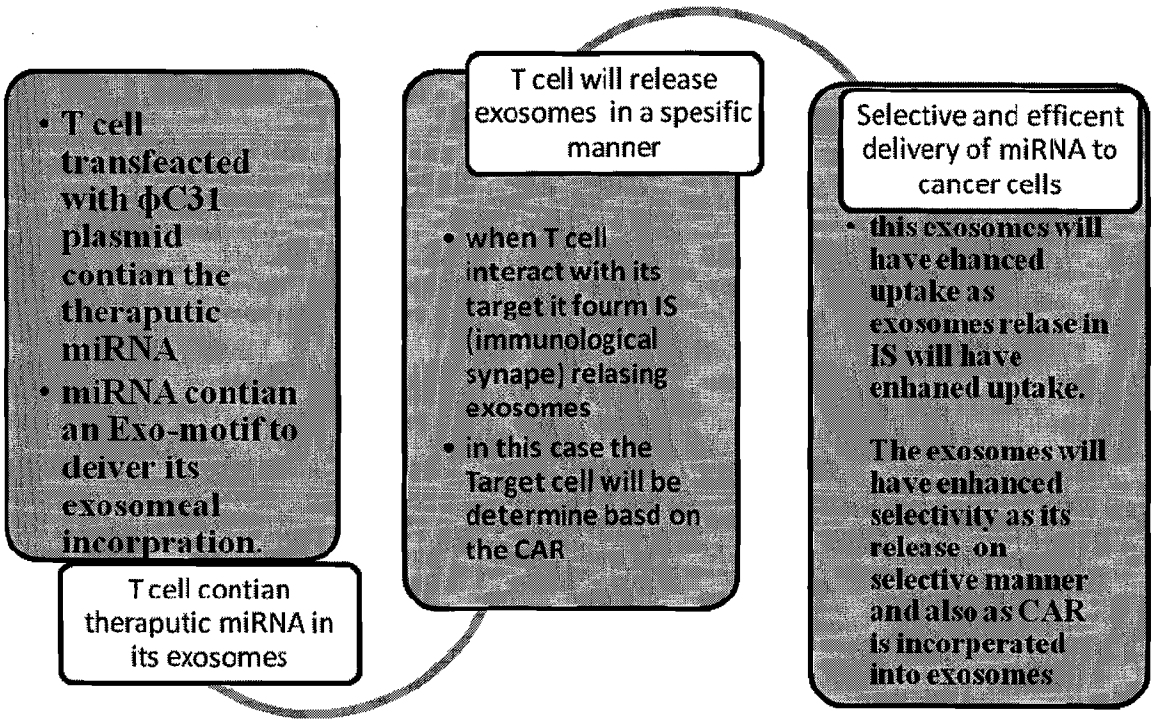

• T cell transfeacted with ϕC31 plasmid contian the theraputic miRNA
• miRNA contian an Exo-motif to deiver its exosomeal incorpration.
T cell contian theraputic miRNA in its exosomes
T cell will release exosomes in a spesific manner
• when T cell interact with its target it fourm IS (immunological synape) relasing exosomes
• in this case the Target cell will be determine basd on the CAR
Selective and efficent delivery of miRNA to cancer cells
• this exosomes will have ehanced uptake as exosomes relase in IS will have enhaned uptake.
The exosomes will have enhanced selectivity as its release on selective manner and also as CAR is incorperated into exosomes

Fig.24

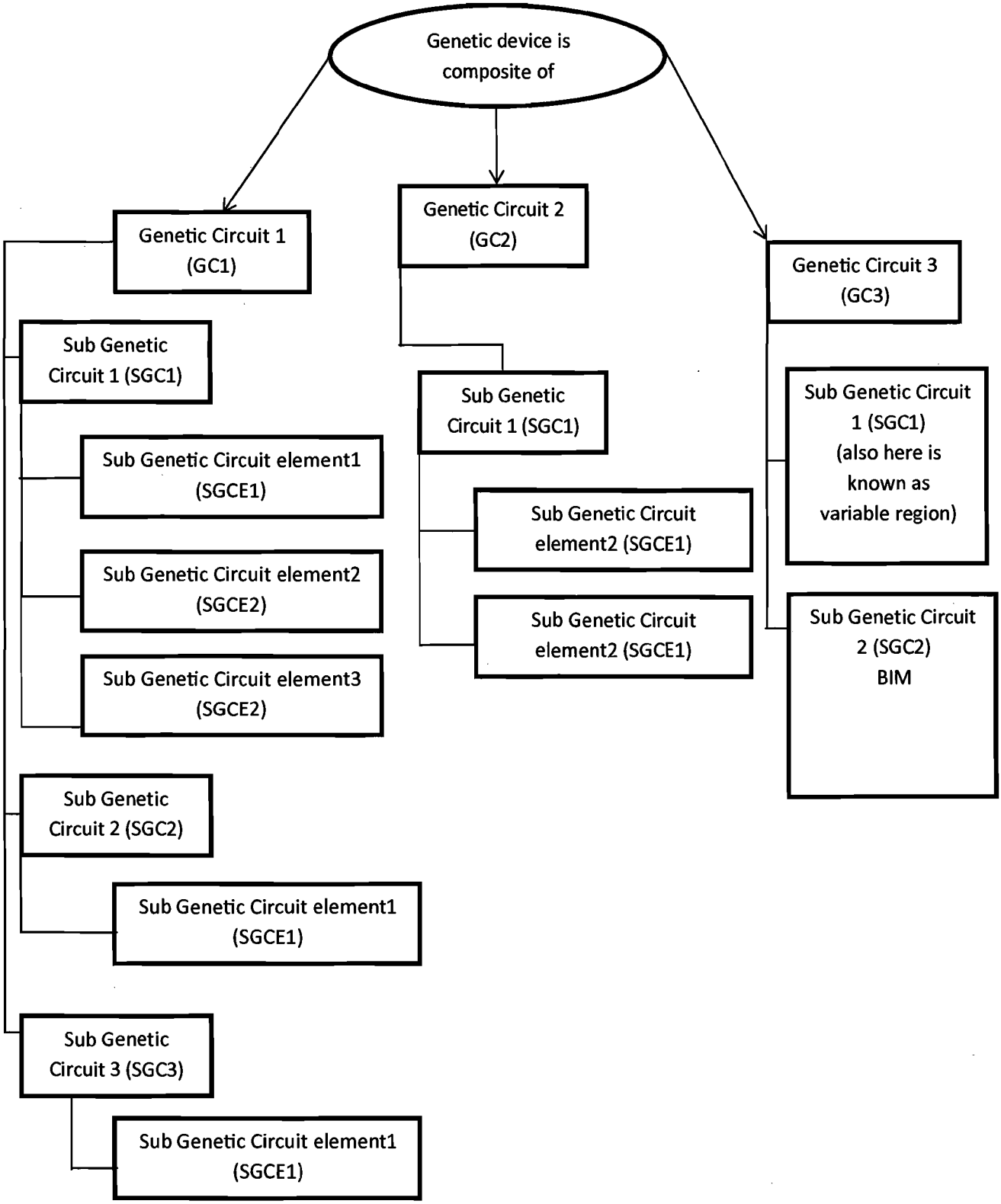

Genetic device is composite of
Genetic Circuit 1 (GC1)
Genetic Circuit 2 (GC2)
Genetic Circuit 3 (GC3)
Sub Genetic Circuit 1 (SGC1)
Sub Genetic Circuit element1 (SGCE1)
Sub Genetic Circuit element2 (SGCE2)
Sub Genetic Circuit element3 (SGCE2)
Sub Genetic Circuit 2 (SGC2)
Sub Genetic Circuit element1 (SGCE1)
Sub Genetic Circuit 3 (SGC3)
Sub Genetic Circuit element1 (SGCE1)
Sub Genetic Circuit 1 (SGC1)
Sub Genetic Circuit element2 (SGCE1)
Sub Genetic Circuit element2 (SGCE1)
Sub Genetic Circuit 1 (SGC1) (also here is known as variable region)
Sub Genetic Circuit 2 (SGC2) BIM

Fig.25

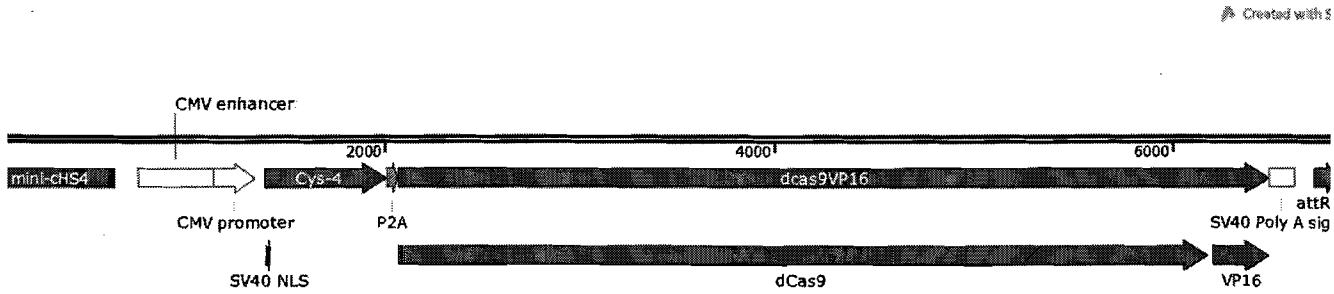

CMV enhancer
2000
4000
6000
mini-cHS4
Cys-4
dcas9VP16
attR
CMV promoter
P2A
SV40 Poly A sig
SV40 NLS
dCas9
VP16

Fig: 26

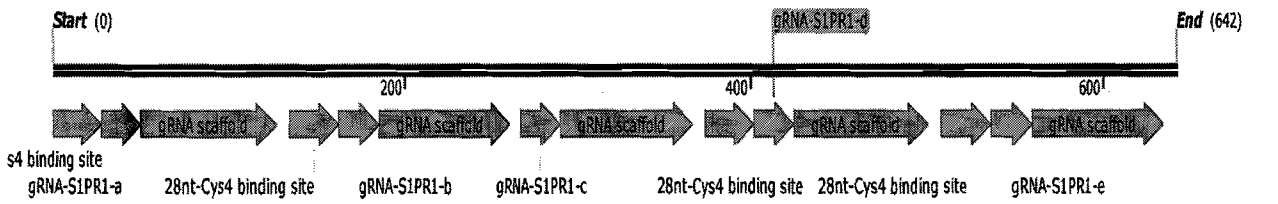

Start (0)
gRNA-S1PR1-d
End (642)
200
400
600
gRNA scaffold
gRNA scaffold
gRNA scaffold
gRNA scaffold
gRNA scaffold
s4 binding site
gRNA-S1PR1-a
28nt-Cys4 binding site
gRNA-S1PR1-b
gRNA-S1PR1-c
28nt-Cys4 binding site
28nt-Cys4 binding site
gRNA-S1PR1-e

Fig: 27

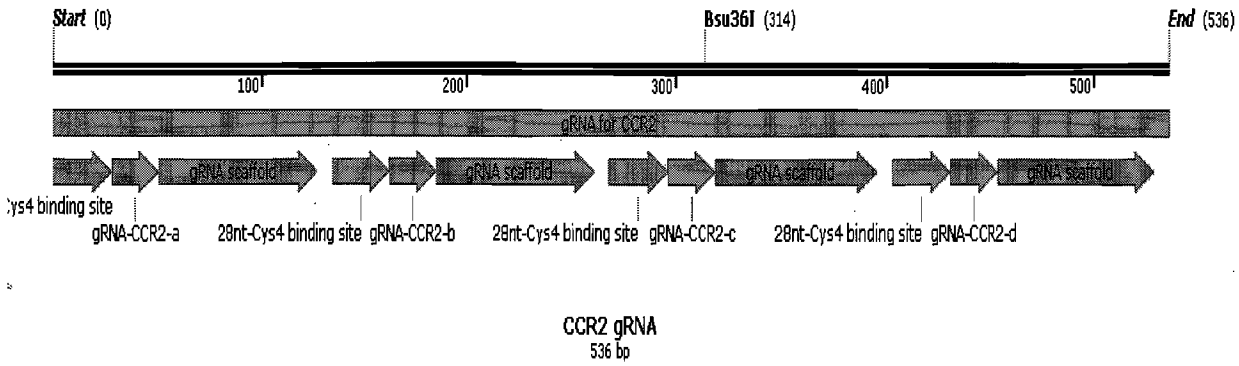

Start (0)
Bsu36I (314)
End (536)
100
200
300
400
500
gRNA for CCR2
gRNA scaffold
gRNA scaffold
gRNA scaffold
gRNA scaffold
Cys4 binding site
gRNA-CCR2-a
28nt-Cys4 binding site
gRNA-CCR2-b
28nt-Cys4 binding site
gRNA-CCR2-c
28nt-Cys4 binding site
gRNA-CCR2-d
CCR2 gRNA
536 bp

Fig: 28

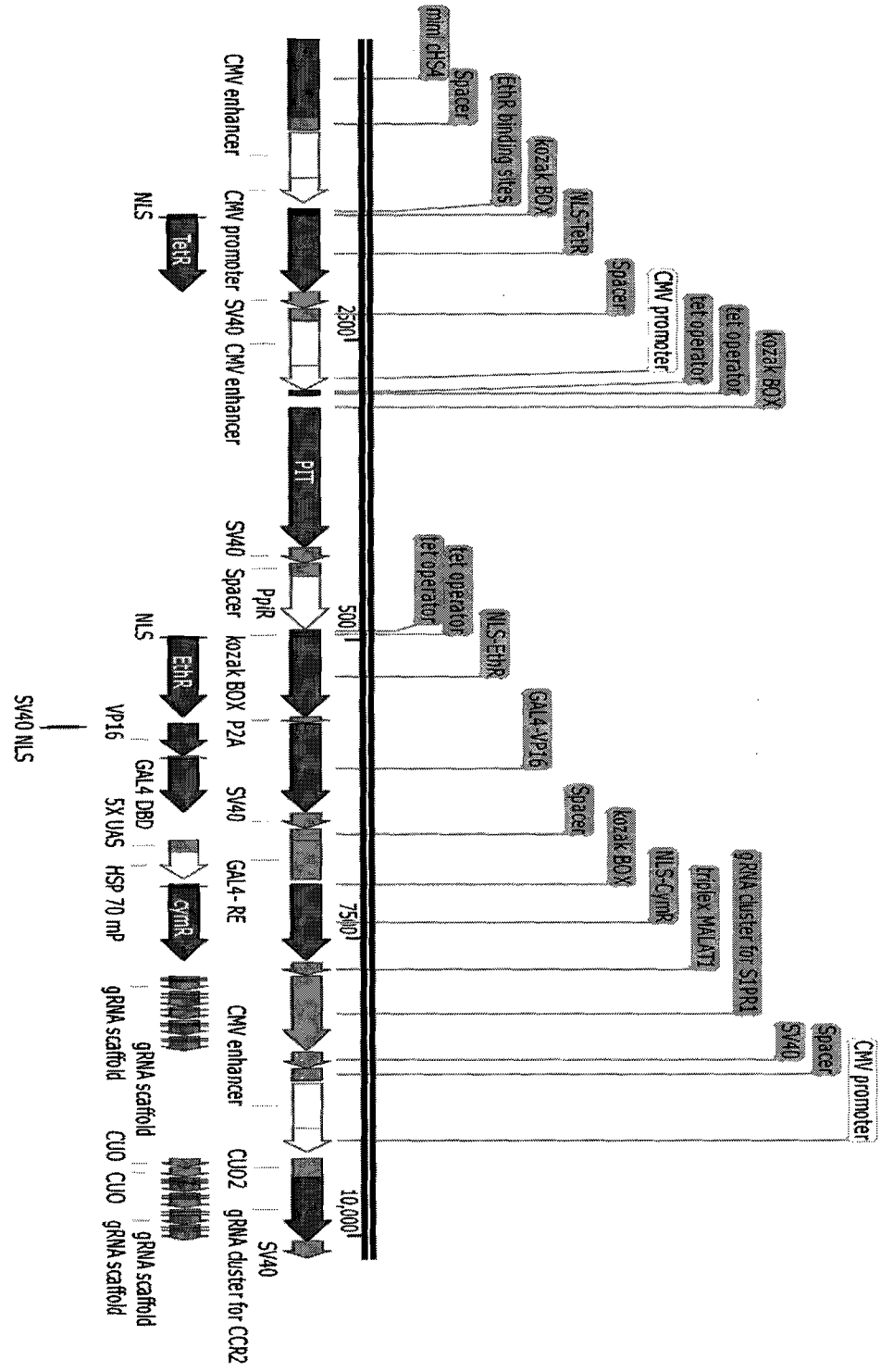

CMV enhancer
CMV promoter
NLS
TetR
SV40 CMV enhancer
PIT
SV40 Spacer
PpiR
NLS
EthR
VP16
GAL4 DBD
5X UAS
HSP 70 mP
gRNA scaffold
CUO
CUO
gRNA scaffold
SV40 NLS
2500
500
7500
10,000
mini cHS4
Spacer
EthR binding sites
kozak BOX
NLS-TetR
Spacer
CMV promoter
tet operator
tet operator
kozak BOX
tet operator
tet operator
NLS-EthR
GAL4-VP16
Spacer
kozak BOX
NLS-CymR
mutex MALAT1
gRNA cluster for S1PR1
SV40
Spacer
CMV promoter
kozak BOX P2A
SV40
GAL4- RE
CymR
CMV enhancer
CUO2
gRNA cluster for CCR2
SV40
gRNA scaffold

Fig: 29

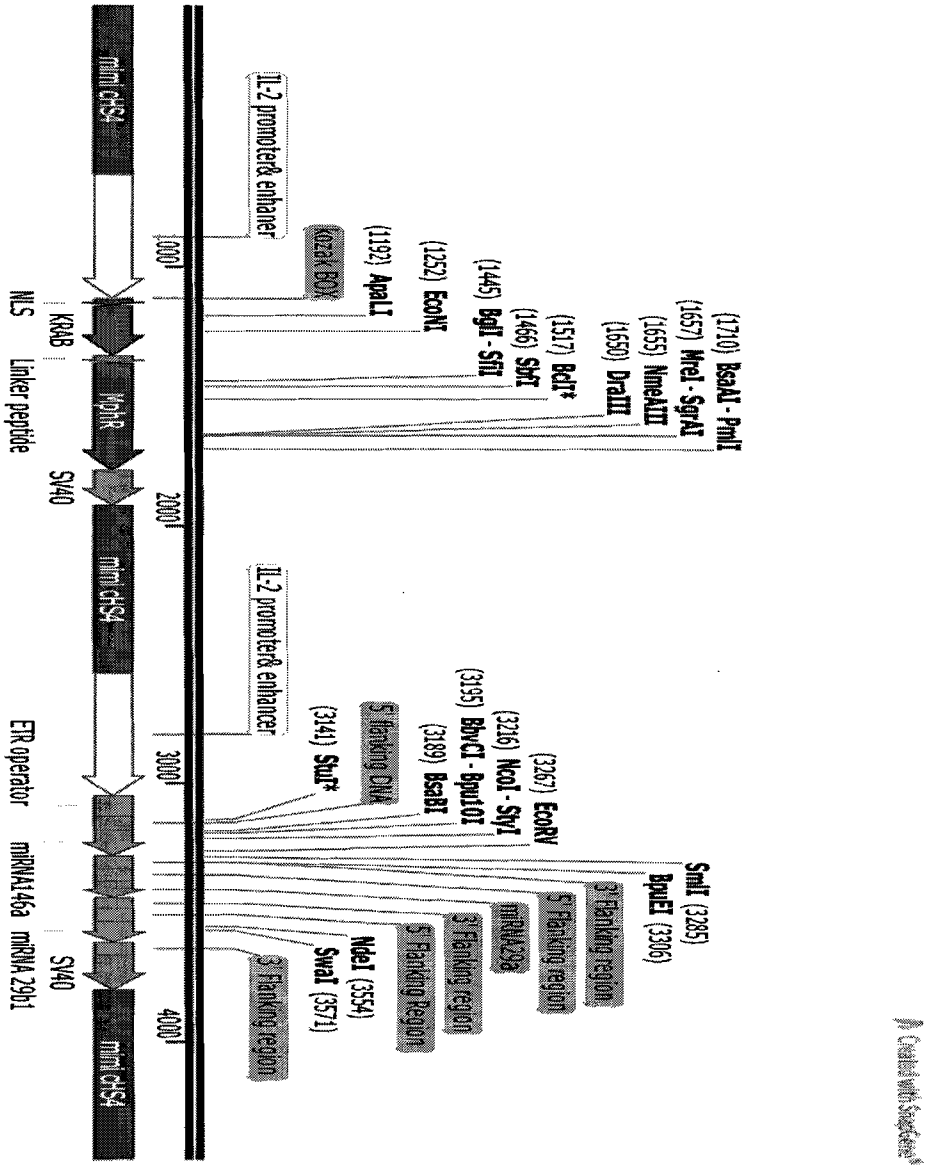

mini cHS4
IL-2 promoter& enhancer
Kozak BOX
(1192) ApaLI
(1252) EcoNI
(1445) BglI - SfiI
(1466) SfiI
(1517) BclI*
(1650) DraIII
(1655) NmeAIII
(1657) MreI - SgrAI
(1710) BsaAI - PmlI
1000
NLS
KRAB
Linker peptide
KpnR
SV40
2000
mini cHS4
IL-2 promoter& enhancer
(3141) SbuI*
5' flanking DNA
(3189) BsaBI
(3195) BbvCI - BpuEI
(3216) NcoI - SfyI
(3267) EcoRV
3000
ETR operator
miRNA146a
miRNA 29b1
SV40
SmaI (3285)
BpuEI (3306)
3' flanking region
5' flanking region
miRNA23a
3' flanking region
5' flanking Region
NdeI (3554)
SwaI (3571)
3' flanking region
4000
mini cHS4

Fig: 30

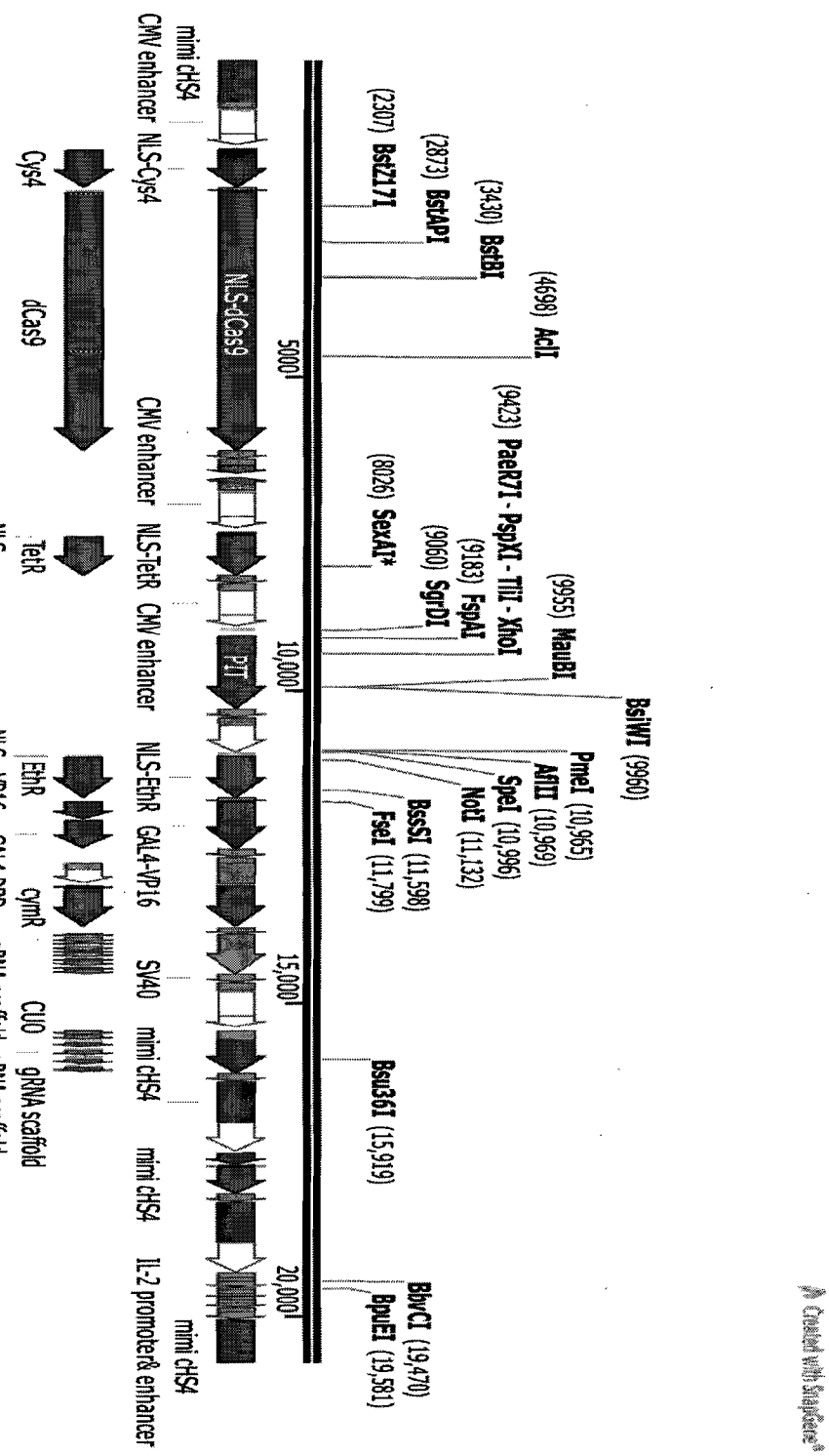

(2307) BstZ17I
(2873) BstAPI
(3430) BstBI
(4698) AclI
(8026) SexAI*
(9060) SgrDI
(9183) FspAI
(9423) PaeR7I - PspXI - TliI - XhoI
(9955) MauBI
BsiWI (9960)
PmeI (10,965)
AflII (10,969)
SpeI (10,996)
NotI (11,132)
BssSI (11,598)
FseI (11,799)
Bsu36I (15,919)
BbvCI (19,470)
BpuEI (19,581)
5000
10,000
15,000
20,000
mimi cHS4
CMV enhancer
NLS-Cys4
Cys4
dCas9
NLS-dCas9
CMV enhancer
NLS-TetR
TetR
NLS
CMV enhancer
PUT
NLS-EthR
EthR
NLS VP16
GAL4-VP16
GAL4 DBD
SV40
cymR
gRNA scaffold
mimi cHS4
CUO
gRNA scaffold
mimi cHS4
IL-2 promoter& enhancer
mimi cHS4
SV40 NLS
Created with SnapGene®

Fig: 31

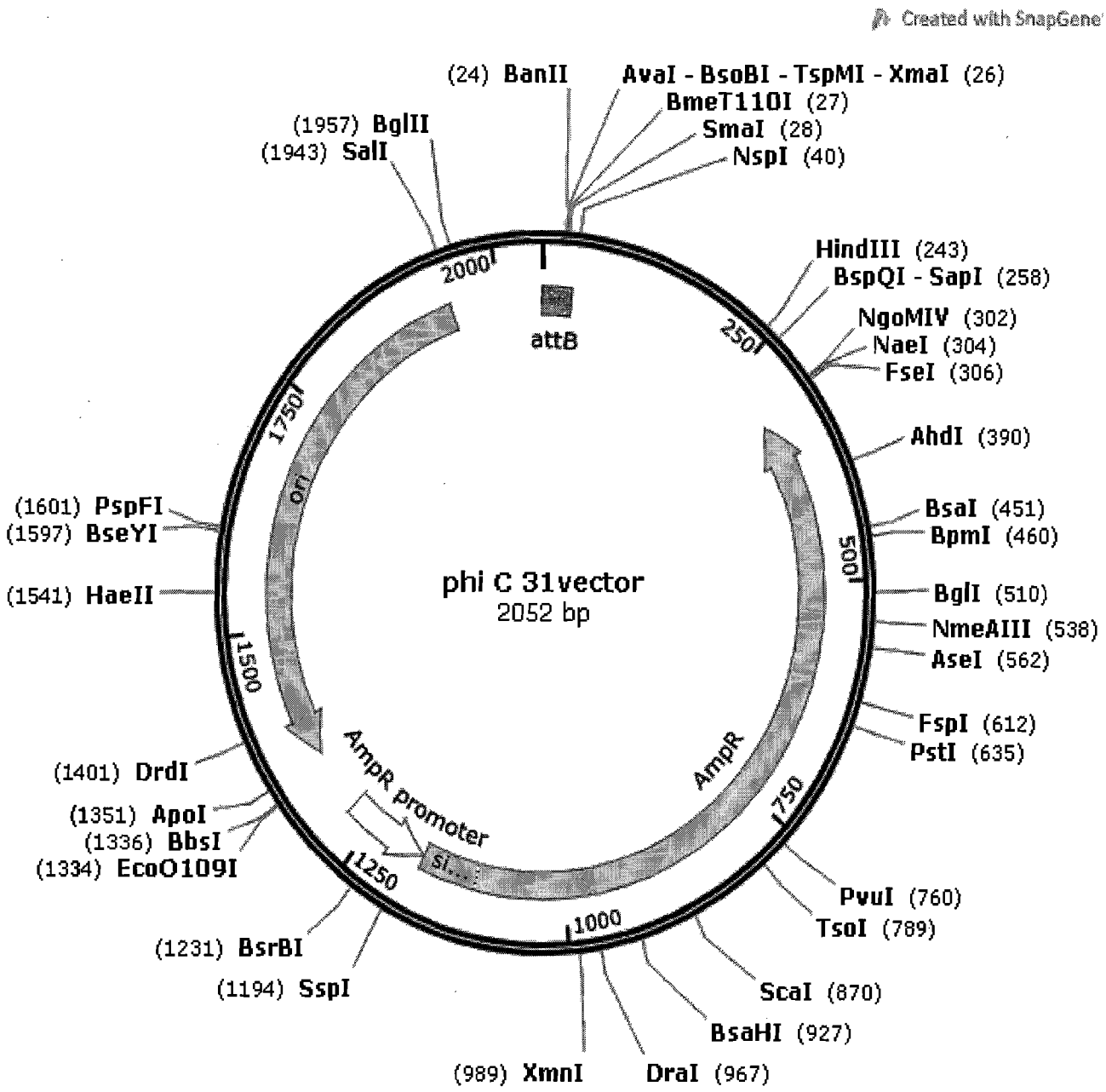

Created with SnapGene
(24) BanII
AvaI - BsoBI - TspMI - XmaI (26)
BmeT110I (27)
SmaI (28)
NspI (40)
(1957) BglII
(1943) SalI
HindIII (243)
BspQI - SapI (258)
NgoMIV (302)
NaeI (304)
FseI (306)
AhdI (390)
BsaI (451)
BpmI (460)
BglI (510)
NmeAIII (538)
AseI (562)
FspI (612)
PstI (635)
PvuI (760)
TsoI (789)
ScaI (870)
BsaHI (927)
DraI (967)
(989) XmnI
(1194) SspI
(1231) BsrBI
(1334) EcoO109I
(1336) BbsI
(1351) ApoI
(1401) DrdI
(1541) HaeII
(1597) BseYI
(1601) PspFI
attB
ori
AmpR
AmpR promoter
phi C 31vector
2052 bp
250
500
750
1000
1250
1500
1750
2000

Fig: 32

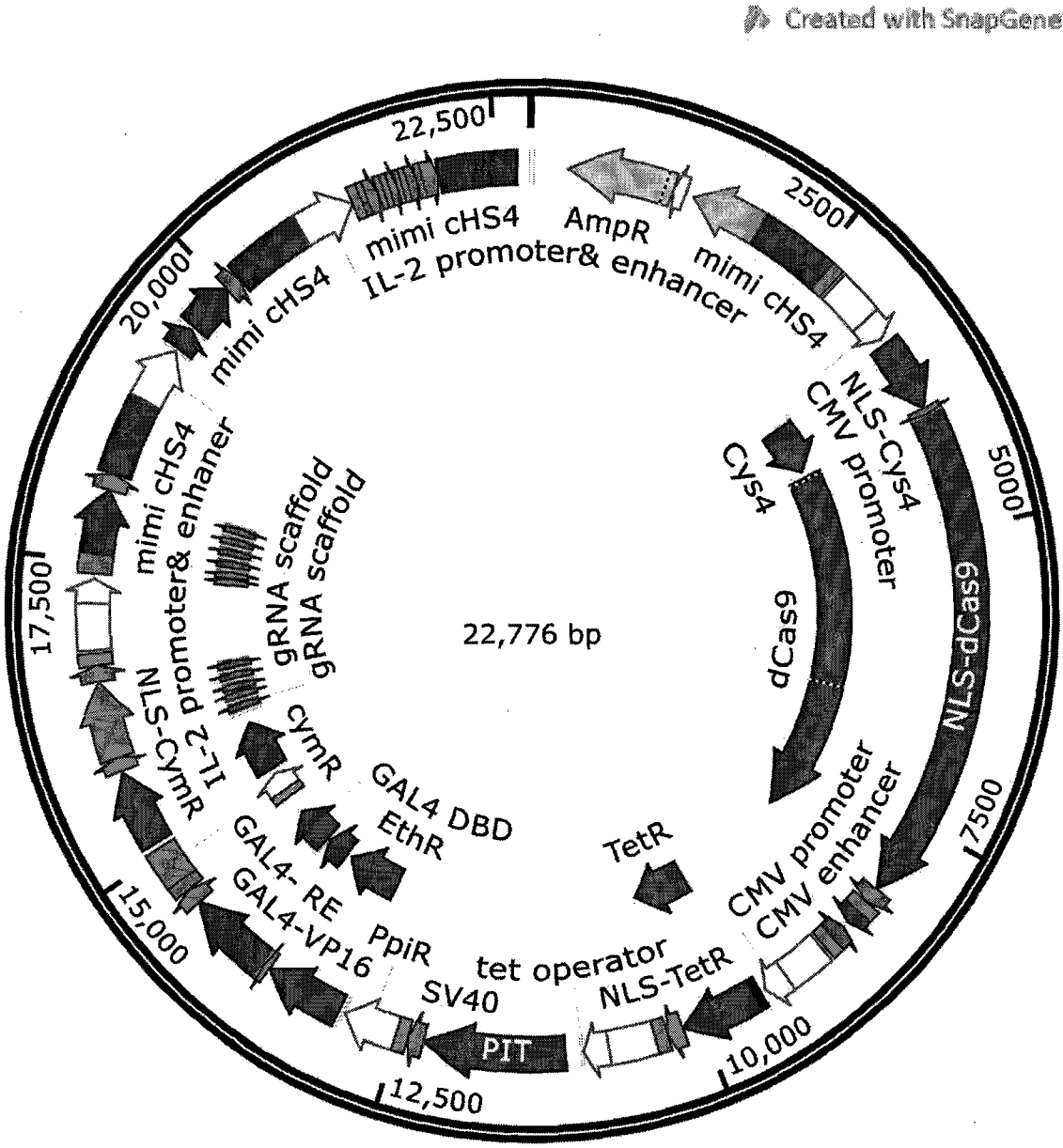


Fig: 33

ϕ C31 vector loaded with device

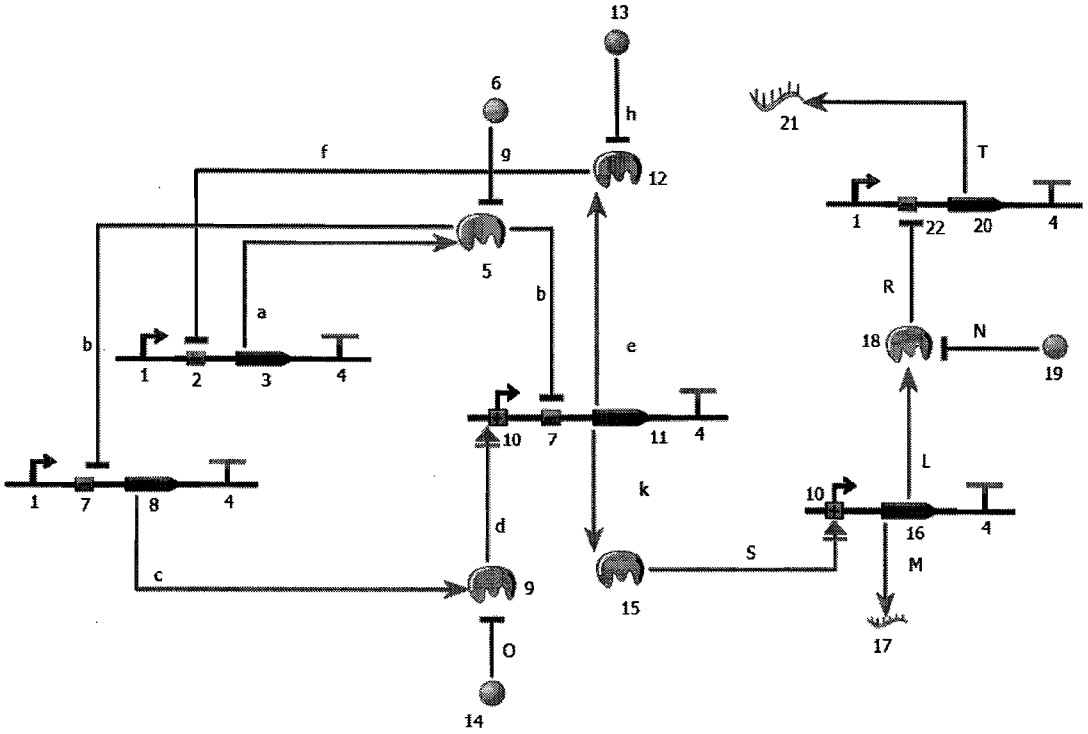

13
6
h
21
f
g
12
T
1
22
20
4
5
b
a
R
b
1
2
3
4
e
18
N
19
10
7
11
4
L
1
7
8
4
k
10
16
4
d
S
c
9
15
M
O
17
14

Fig: 34

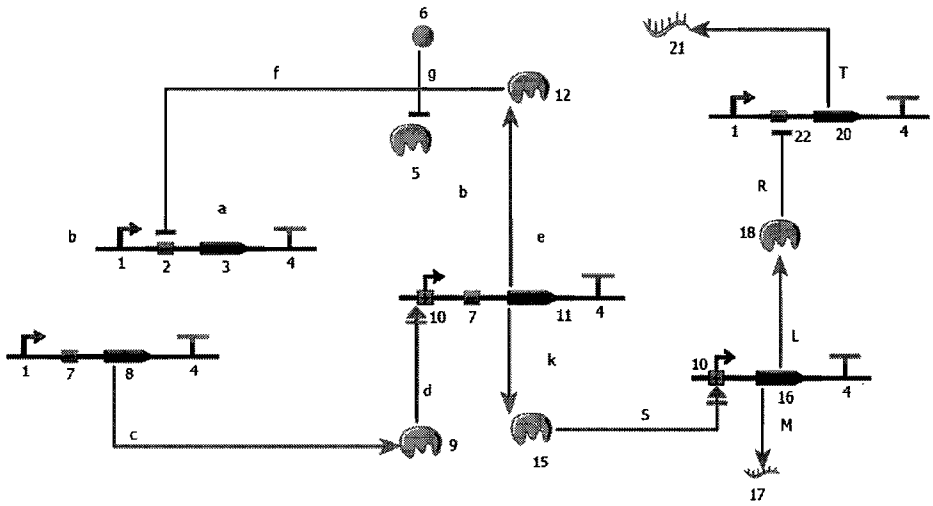

6
21
f
g
12
T
1
22
20
4
5
b
a
R
b
1
2
3
4
e
18
10
7
11
4
L
k
1
7
8
4
10
16
4
d
S
c
9
15
M
17

Fig: 35

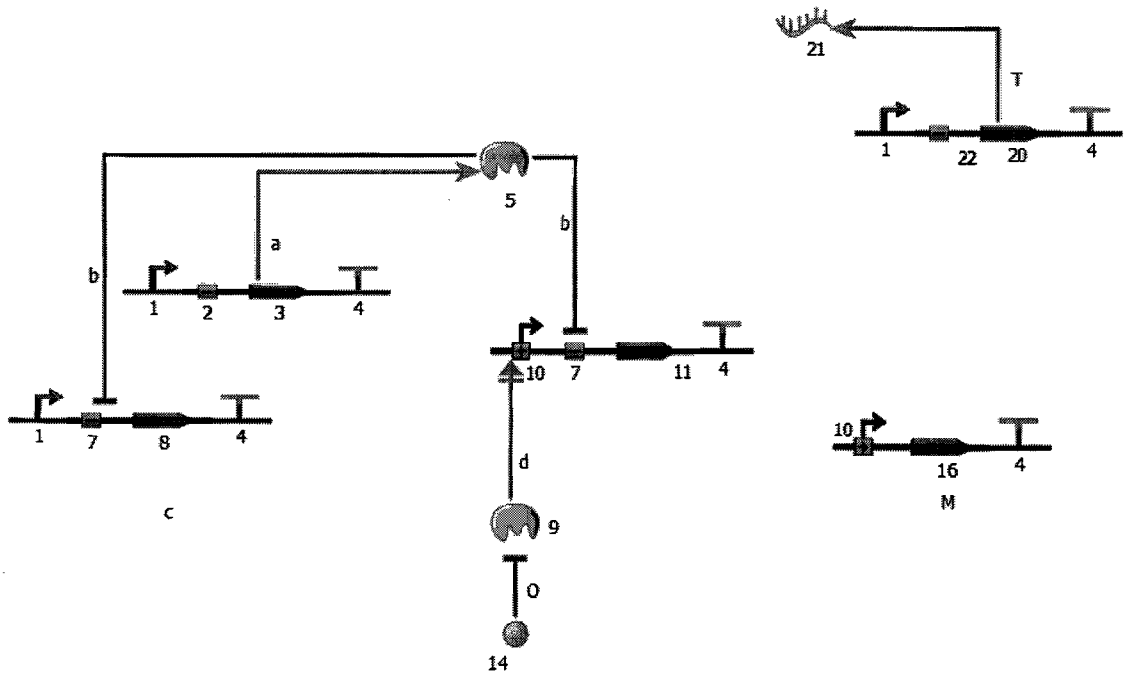

21
T
1
22
20
4
5
b
a
b
1
2
3
4
10
7
11
4
1
7
8
4
c
d
9
0
14
10
16
4
M

Fig: 36

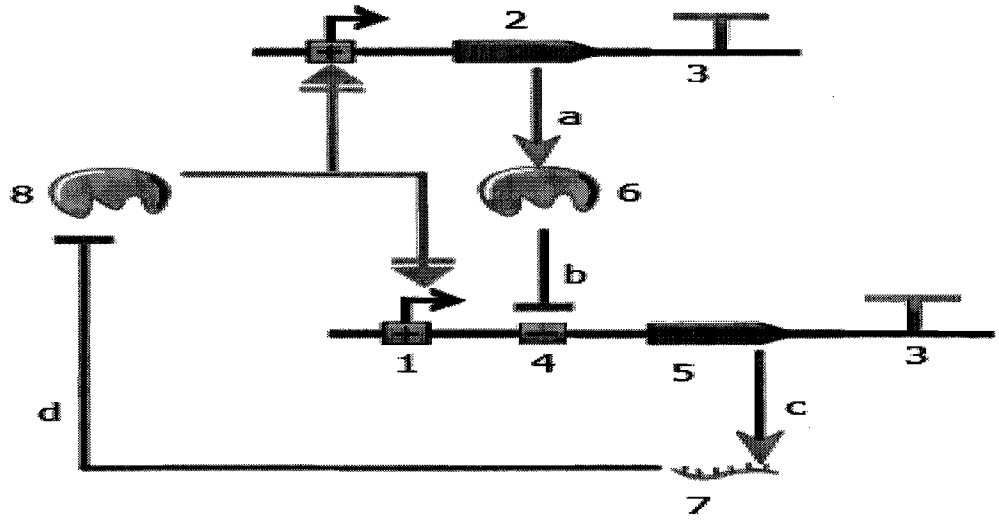

2
3
a
8
6
b
1
4
5
3
d
c
7

Fig: 37

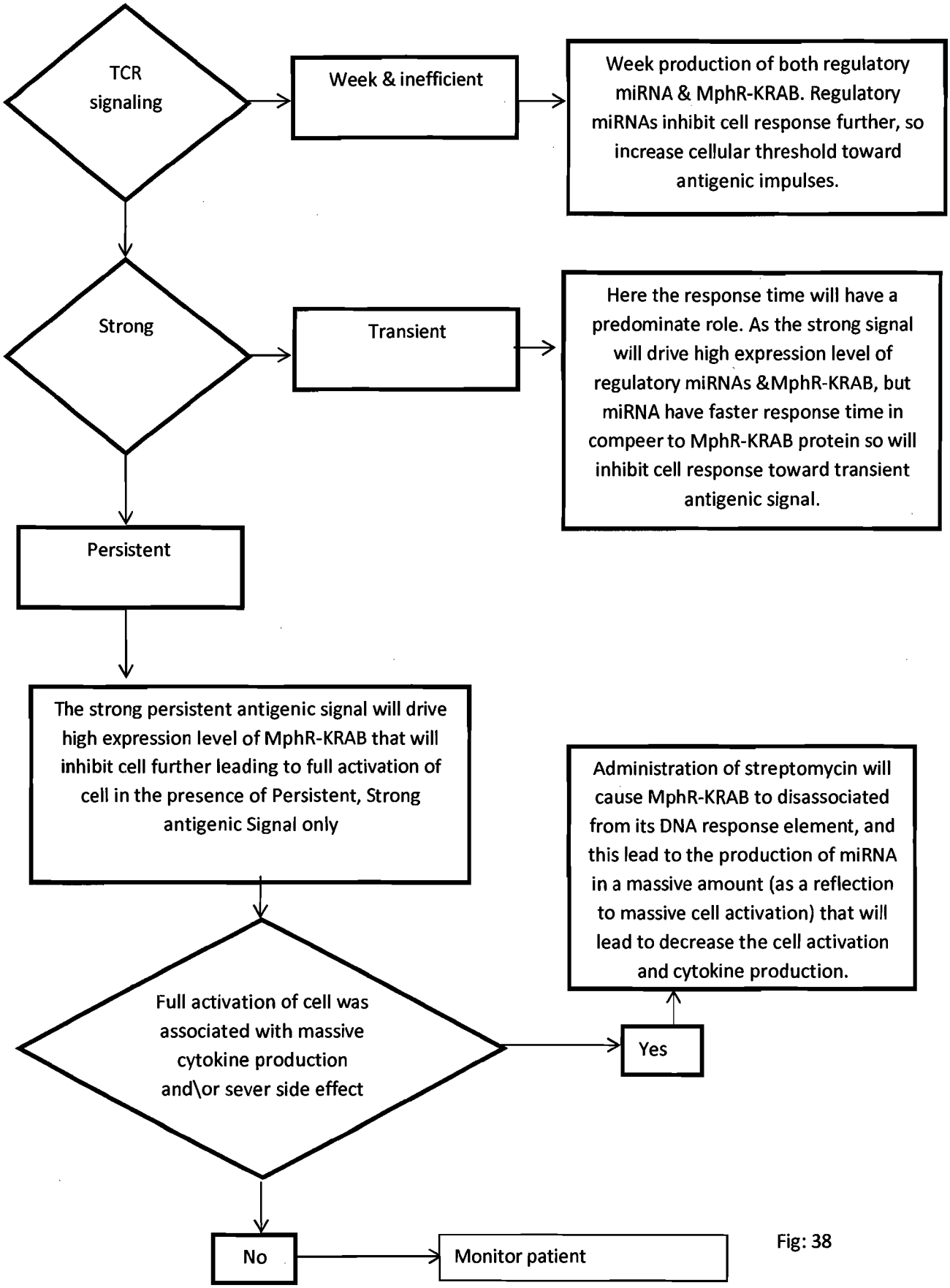
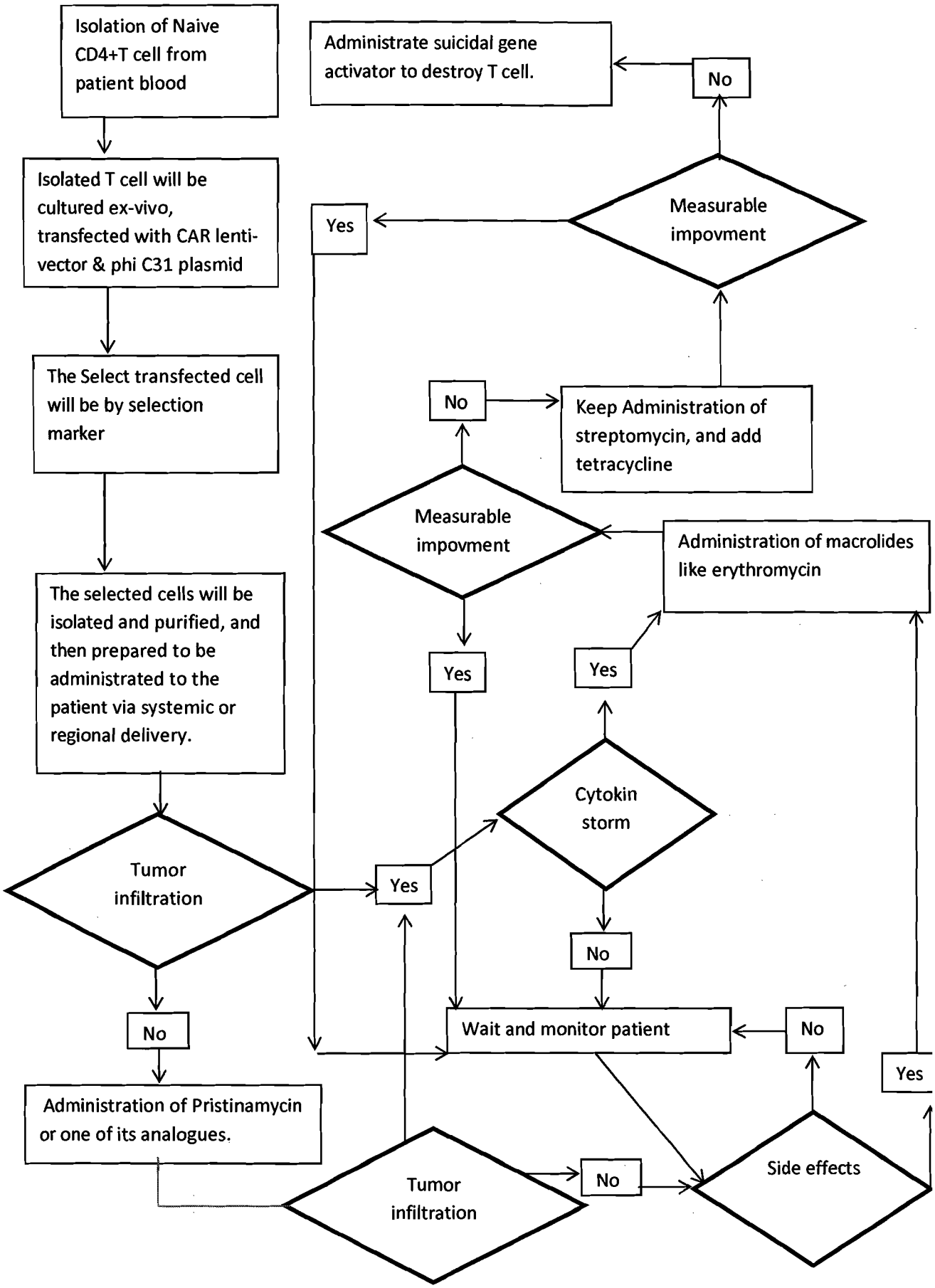

TCR signaling
Week & inefficient
Week production of both regulatory miRNA & MphR-KRAB. Regulatory miRNAs inhibit cell response further, so increase cellular threshold toward antigenic impulses.
Strong
Transient
Here the response time will have a predominate role. As the strong signal will drive high expression level of regulatory miRNAs &MphR-KRAB, but miRNA have faster response time in compeer to MphR-KRAB protein so will inhibit cell response toward transient antigenic signal.
Persistent
The strong persistent antigenic signal will drive high expression level of MphR-KRAB that will inhibit cell further leading to full activation of cell in the presence of Persistent, Strong antigenic Signal only
Administration of streptomycin will cause MphR-KRAB to disassociated from its DNA response element, and this lead to the production of miRNA in a massive amount (as a reflection to massive cell activation) that will lead to decrease the cell activation and cytokine production.
Full activation of cell was associated with massive cytokine production and\or sever side effect
Yes
No
Monitor patient

Fig: 38


Isolation of Naive CD4+T cell from patient blood
Isolated T cell will be cultured ex-vivo, transfected with CAR lenti-vector & phi C31 plasmid
The Select transfected cell will be by selection marker
The selected cells will be isolated and purified, and then prepared to be administrated to the patient via systemic or regional delivery.
Tumor infiltration
No
Administration of Pristinamycin or one of its analogues.
Yes
Cytokin storm
Yes
No
Measurable impovment
No
Keep Administration of streptomycin, and add tetracycline
Measurable impovment
Yes
Administrate suicidal gene activator to destroy T cell.
No
Yes
Administration of macrolides like erythromycin
Yes
Wait and monitor patient
No
Yes
Side effects
No
Tumor infiltration

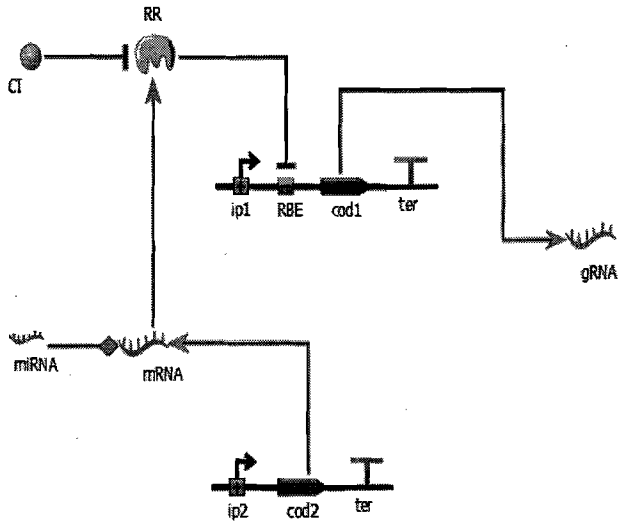

RR
CI
ip1
RBE
cod1
ter
gRNA
miRNA
mRNA
ip2
cod2
ter

Fig: 40

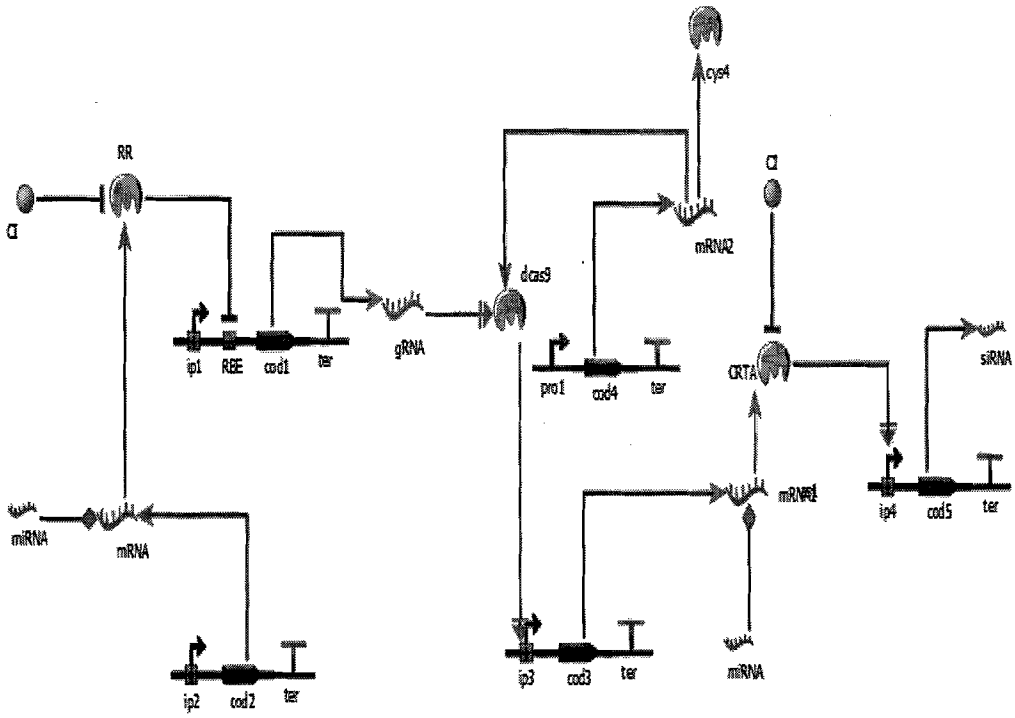

cys4
RR
CI
mRNA2
CI
dcas9
ip1
RBE
cod1
ter
gRNA
pro1
cod4
ter
CRTA
siRNA
mRNA2
ip4
cod5
ter
miRNA
mRNA
ip2
cod2
ter
ip3
cod3
ter
miRNA

Fig: 41