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(54) **Title:** TISSUE-SPECIFIC REGULATION OF TRANSGENE EXPRESSION

(57) **Abstract:** The present invention relates to nucleic acid constructs and vector comprising thereof for use in gene transfer and therapy applications, and uses thereof for obtaining a tissue- specific expression of a transgene of interest.



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TISSUE-SPECIFIC REGULATION OF TRANSGENE EXPRESSION

FIELD OF THE INVENTION:

5 The present invention relates to nucleic acid constructs and vector comprising thereof for use in gene transfer and therapy applications, and uses thereof for obtaining a tissue-specific expression of a transgene of interest.

BACKGROUND OF THE INVENTION:

10 Achieving tight regulation of transgene expression in the space and during the time has been the goal of many studies in the field of gene therapy.

 The first line of transcriptional control is based on the use of tissue-specific promoters. For instance, many natural or artificial promoters which activity is restricted to hepatocytes
15 have been used with a large panel of gene transfer vectors. However, it has been shown that these promoters are usually weak, as compared to ubiquitous promoters and can be leaky in many other cell types. Therefore, there is still a clear need to improved regulatory systems.

 It results that inducible gene expression systems have been developed and have been
20 proven valuable tools to control expression of a transgene during the time and many of them have been tested in gene therapy models. In mammalian cells a number of inducible system that respond to inducing agents such as hormones, or heat shock proteins have been devised in the past years. An inducible system may thus turn “on” or turn “off” expression of a transgene of interest according to the desired effect. The switch is usually obtained by administration of
25 a drug that will bind to the chimeric regulator and will modify its DNA binding activity.

 The tetracycline regulatable system was first described by Gossen and Bujard in 1992 (Gossen and Bujard, 1992). It is based on the fusion of the Tet repressor protein (TetR) with the VP16 transactivator of the Herpes simplex virus. The TetR from E. Coli Tn10 operon is
30 able to bind to the Tet operator DNA sequence (TetO) in a specific manner. In the presence of tetracyclin (tet) the TerR conformation changes and it is no longer able to bind to TetO sequence. When fused to VP16, the TetR-VP16 chimeric protein is able to activate DNA transcription, provided that the TetO sequence is present in the target DNA. The TetR-VP16 is still sensitive to conformational changes and in the presence of tet it is no longer able to

bind DNA and hence to activate transcription. This system was coined the Tet-Off regulatable system. Mutagenesis in TerR allowed to isolate mutants which DNA binding activity was opposite to the wild type protein. Rather to attach to DNA in the absence of tet, the reverse TetR (rTetR) can only bind DNA in the presence of the drug. This mutant system was called the Tet-On regulatable system. Using an opposite strategy, some investigators sought to develop a tet regulatable system based on the fusion of the same TetR with a transcriptional repressor domain, (Deuschle *et al.* 1995). This tetracycline-controlled transrepressor chimeric protein was engineered by fusing the KRAB (Kruppel associated box) domain of human KOX1 to the Tet repressor derived from Tn10 of Escherichia Coli. The Krab domain is a 75 aa domain found in about one third of the several hundreds of human zinc finger proteins and found exclusively in the amino terminal end of proteins that contain Krüppel-class zinc fingers in their carboxy termini. The KRAB domain acts by triggering formation of heterochromatin in the vicinity of its binding site and thus is able to shut off polymerase I, II and III promoters. To achieve heterochromatin formation, KRAB recruits KAP1 (KRAB-associated protein 1 also known as TRIM28 or KRIP1) which acts as a scaffold for various proteins such as heterochromatin protein 1, histone methyl transferase and histone deacetylase. KRAB induced transcriptional silencing can act over several kilobases. It has been demonstrated that KRAB can mediate transcriptional repression through tens of kilobases and that this system is mainly involved in the control of endogenous retroviruses in embryonic stem cells. In presence of tetracycline, the binding of tTR-Krab to DNA is prevented with concomitant restoration of the initial promoter activity. Therefore the tTR-Krab system works in a tet-“on” fashion.

It was initially shown that a large increase in transgene expression could be achieved in an *in vitro* system with a range of repression of more than 3 kb apart from the tetO target sequence (Szulc *et al.* 2006). The repressor activity of the tTR-Krab system extends to pol III promoters and lentiviral vectors in which shRNA driven by pol III promoters have been placed under control of tetO dependent tTR-Krab repression have described recently (Szulc *et al.* 2006). These vectors allow the highly efficient doxycycline-controlled expression of shRNA and hence control of knockdown of cellular genes targeted by the shRNA. It was initially thought that repression could only be achieved in integrated genome. But, several studies demonstrated efficient regulation of transgene expression in AAV and integration-defective lentiviral vectors (Barde *et al.* 2009).

However, in the context of gene therapy with desirable expression of a transgene of interest, inducible systems such as the tTR-Krab system, suffer from a number of drawbacks:

(1) The inducer drug should be orally available and penetrate the target tissue;

(2) A prolonged exposure to the drug is required to achieve long-term expression of the transgene, involving therefore that the inducer drug should have a metabolism compatible with prolonged therapeutic use;

(3) An immune response to the chimeric protein can be expected when expressed at high level for prolonged periods of time.

Recently, a new regulation system of transgene expression has been described (Brown *et al.* 2006). This system is based on RNA interference. RNA interference was first described in the nematode *Caenorhabditis Elegans*. Soon after, it was shown to occur also in mammalian cells. Naturally miRs are transcribed by RNA polymerase II from cellular sequences as primary miRs (pri-miRs). Within the nucleus, pri-miRs are cleaved by Drosha to form shorter precursor miR (pre-miR) hairpins of 60-80 nt in length. Pre-miRs are then exported to the cytoplasm, where the hairpin pre-miRs are processed by Dicer to form a RNA duplexes of 19-23 bp that have 2 nt 3' overhangs. This duplex is handed on to the RNA induced silencing complex (RISC) where one of the strands, the passenger strand, is cleaved within RISC and then released from the complex. The remaining intact single stranded guide RNA activates RISC to direct target-specific silencing through hybridization of the guide to complementary sequences. The guide strands of natural miRs typically hybridize to the 3' untranslated regions (3'UTRs) of a gene to cause translational suppression. When guide and target sequences are perfectly complementary, RISC mediates mRNA slicing through effects of its Ago2 'slicer' component. Theoretically, the RNAi pathway can be harnessed to silence any gene that is transcribed and where the pathway is functional. Because the patterns of expression of miRNAs are different among cell types, some miRNA are specifically expressed in specific cell types or in specific cell lineages. Therefore it is theoretically possible to silence any transgene in a specific cell type or lineage by adding to the transgene transcript sequences that are targets to the corresponding miRNAs (Brown *et al.* 2007).

This was demonstrated by a seminal study of Brown *et al.* 2006. In this study, the immune response to GFP was shut-off by using a lentiviral construct containing 4 tandem repeats of the miR142-3, which is specifically expressed in cells from the hematopoietic lineage. miR142-3 efficiently mediated degradation of the GFP mRNA in antigen presenting

cells and suppressed cytotoxic elimination of transduced cells including hepatocytes. International patent application N° WO 2007/000668 describes thus a gene transfer vector suitable for genetic engineering approaches, such as gene therapy, gene transfer and/or regulation of expression of a transgene comprising a miRNA target sequence. Such vectors
5 can be thus designed to be regulated by endogenous miRNAs for controlling transgene expression to achieve specific expression profiles of the vector and more particularly to obtain a miRNA-mediated approach for restricting gene expression.

SUMMARY OF THE INVENTION:

10 The present invention arises from the combination by the inventors of this miRNA-mediated approach for restricting gene expression with a repressor in order to obtain a tissue-specific expression of a transgene only in cells in which a particular miRNA is expressed. Thus, to detarget tTR-Krab repression in specific cell types the TeR-KRAB coding sequence was linked to 4 copies of miR target sequences with perfect homology to tissue specific
15 miRNA. This resulted in reduced expression of the tTR-Krab repressor in specific cell types.

Advantageously, this improved strategy provides an important, and hitherto unavailable, means of obtaining a satisfying tissue-specific expression of a transgene of interest without the drawbacks of the systems previously described in the state of the art.

20 Thus, the invention relates to a nucleic acid construct comprising:

a) a first sequence encoding for a repressor operably linked to a first promoter and to at least one tissue-specific miRNA target sequence, and

b) a second sequence encoding for a transgene of interest operably linked to a
25 second promoter and to an operator.

In a particular embodiment, the second promoter is a tissue-specific promoter.

In a preferred embodiment, the tissue-specific miRNA and the tissue-specific promoter are specific for the same organ/tissue.

30 Indeed, such particular nucleic acid construct strengthens the specificity of the tissue-specific promoter in order to obtain an optimal tissue-specific expression of the transgene.

The invention also relates to a vector comprising said nucleic acid construct as defined above.

The invention further relates to a cell infected or transduced with the vector as defined above.

5 The invention also relates to a vector or a cell as defined above for use as drug.

The invention further relates to an *in vitro* or *ex vivo* method for obtaining a tissue-specific expression of a transgene of interest comprising the following steps:

- 10 a) providing a vector of the invention as defined above, and
b) transferring said vector in a cell or a tissue of interest expressing a corresponding tissue-specific miRNA.

DETAILED DESCRIPTION OF THE INVENTION:

15 The present invention relates to a nucleic acid construct and uses thereof, carried out by the inventors by an approach combining a miRNA-mediated approach for restricting gene expression to a repressor in order to obtain a tissue-specific expression of a transgene only in cells in which a particular miRNA is expressed.

20 Therefore, when the cell expresses the tissue-specific miRNA liable to bind to the corresponding tissue-specific miRNA target sequence, the repressor is not translated since its mRNA is destroyed caused by the binding of the miRNA and therefore the transgene may be expressed in said particular tissue.

On the contrary, when the cell does not express the tissue-specific miRNA liable to
25 bind to the corresponding tissue-specific miRNA target sequence, the repressor is translated and expressed and may therefore bind the operator and prevent the transcription of the transgene.

Definitions:

30 Throughout the specification, several terms are employed and are defined in the following paragraphs.

As used herein, the terms “miRNA” or “microRNA” refer to a short and non-coding ribonucleic acid (RNA) molecule (on average 21 nucleotides long) found in all eukaryotic

cells. miRNAs are post-transcriptional regulators that bind to complementary sequence on target messenger RNA transcripts (mRNAs), usually resulting in transcriptional repression and gene silencing. Gene silencing may occur either via mRNA degradation or preventing mRNA being translated.

5

As used herein, the term “tissue-specific miRNA” refers to a miRNA which is specifically expressed in a particular organ and therefore in kind of cells found in said particular organ. For example, miR122 is a liver-specific miRNA and is specifically expressed in hepatocytes. This suggests that tissue-specific miRNAs may play a conserved
10 role in the establishment and/or maintenance of a cell or tissue type of that particular organ.

As used herein, the terms “miRNA target sequence” or “target sequence of a miRNA” refer to the complementary sequence on target mRNAs which is bound by a miRNA.

15

As used herein, the terms “tissue-specific miRNA target sequence” or “target sequence of a tissue-specific miRNA” refer to a complementary sequence recognized and bound by endogenous miRNAs cell type specific, thus regulating transgene expression in a desired subset of cells.

20

As used herein, the term “repressor” refers to a DNA-binding protein that regulates the expression of one or more genes by binding to the operator and blocking the attachment of RNA polymerase to the promoter, thus preventing transcription of genes. This blocking of expression is called repression. Repressor proteins attach to a DNA segment known as the operator.

25

As used herein, the term “operator” refers to a DNA segment to which a repressor binds.

30

As used herein, the term “operably linked” means that the components described are in a relationship permitting them to function in their intended manner. Thus, a nucleic acid sequence is “operably linked” when placed into a functional relationship with another sequence nucleic sequence. For instance, a promoter is “operably linked” to a coding sequence if the promoter causes the transcription of the coding sequence. Generally, operably linked means the linked nucleic acid sequences are contiguous.

Nucleic acid constructs of the invention:

The present invention provides for nucleic acid constructs useful for obtaining a
5 tissue-specific expression of a transgene of interest without using an inducer drug.

In a first aspect, the invention thus relates to a nucleic acid construct comprising:

- 10 a) a first sequence encoding for a repressor operably linked to a first promoter
and to at least one tissue-specific miRNA target sequence, and
- b) a second sequence encoding for a transgene of interest operably linked to a
second promoter and to an operator.

15 In one embodiment, the repressor is a chimeric repressor fusion protein.

Such chimeric repressor fusion protein comprises a DNA binding domain and a
transcriptional repressor domain.

20 The DNA binding domain can be derived from a large array of bacterial operons in
which a transcriptional regulator binds to a specific target sequence. Examples are the lacZ
operon, the CynR operon. The use of mammalian DNA binding domain may also be
considered such as the GAL4, Lex1 or Ecdysone receptor binding domain. Specific DNA
binding domain may also be engineered by designing a zinc finger domain capable to bind
DNA of known sequence.

25 The panel of transcriptional repressor domains includes the KRAB repressor, or the
ERD and SID transcriptional repressor domains.

In a particular embodiment, the chimeric repressor fusion protein is the tTR-KRAB
protein. Said protein is formed by the fusion of the DNA binding domain of the tetracycline
repressor (tTR) to the KRAB repression domain of human (tTR-KRAB), which can bind the
30 tetO sequence (called operator in the context of the present invention).

In one embodiment, the tissue-specific miRNA is selected from the group consisting of liver-specific miRNA, brain-specific miRNA, heart-specific miRNA, lung-specific miRNA, spleen-specific miRNA, kidney-specific miRNA, hematopoietic tissue-specific miRNA and muscle-specific miRNA.

5

In a particular embodiment, the tissue-specific miRNA is a liver-specific miRNA. Accordingly, the liver-specific miRNA is miR-122.

In another particular embodiment, the tissue-specific miRNA is a hematopoietic
10 tissue-specific miRNA. Accordingly, hematopoietic tissue-specific miRNA is miR142.

In one embodiment, the first sequence comprises at least one tissue-specific miRNA target sequence. More than one copy of a miRNA target sequence may increase the effectiveness of the system.

15 Accordingly, the first sequence may comprise from one to ten, preferably four tissue-specific miRNA target sequence.

In a particular embodiment, the multiple miRNA target sequences may be in tandem.

It should be further underlined that combinations of miRNA target sequences to obtain highly specific cell expression patterns of the transgene of interest.

20

In this context, some tissue-specific expression patterns of mammalian miRNAs have already been disclosed: for example, international patent application WO 2007/000668 describes N° WO 2007/000668, which is incorporated herein by reference, gives tissue-specific expression patterns of mammalian miRNAs (Table 1).

25 It should be further noted that tissue specific miRNA can be assayed by profiling using microarrays as previously described (Liu *et al*, 2008).

Alternatively, one can use the miRbase database available on the web at:

<http://www.mirbase.org/> to determine the most appropriate target sequences to be used for expression in a specific tissue or cell type.

30

For the purpose of the invention, the term “transgene of interest” is intended to mean in particular any gene encoding a protein product having a therapeutic product. The protein product may be homologous with respect to the target cell (i.e. a product which is normally expressed in the target cell when said cell exhibits no pathology). In this case, the expression

of a protein makes it possible, for example, to compensate for an insufficient expression in the cell or the expression of a protein that is inactive or weakly active due to a mutation. The therapeutic gene can also encode a mutant of a cellular protein, having increased stability, modified activity, etc. The protein product may also be heterologous with respect to the target cell. In this case, an expressed protein may, for example, add to or introduce a deficient activity in or into the cell, allowing it to treat a pathology or stimulate an immune response.

In one embodiment, the transgene of interest is selected in the group consisting of enzymes, blood derivatives, hormones, chimiokines, interleukins, growth factors, neurotransmitters or their precursors, trophic factors, tumor suppressor genes, factors involved in clotting, proteins involved in DNA repair, protein transporters, receptors, etc.

In a particular embodiment, the transgene of interest is a transgene useful for treating a liver disease. Accordingly, the transgene of interest is the hepatic enzyme bilirubin glucuronosyl transferase useful for treating the Crigler-Najjar syndrome.

As used herein, the term "promoter" refers to a DNA sequence that determines the site of transcription initiation for an RNA polymerase. A promoter useful in the present invention may comprise a RNA polymerase III promoter that can provide high levels of constitutive expression across a variety of cell types and will be sufficient to direct the transcription of a distally located sequence, which is a sequence linked to the 3' end of the promoter sequence in a cell. Suitable promoters include, for example, constitutive, regulated, tissue-specific or ubiquitous promoters, which may be of cellular, viral or synthetic origin, such as CMV, RSV, PGK, EF1[alpha], NSE, synapsin, [beta]-actin, GFAP, and the like.

In one embodiment, the first promoter and the second promoter are ubiquitous strong promoters. Accordingly, the first promoter may be the human cytomegalovirus (CMV) promoter and the second promoter may be PGK promoter. Alternatively, the liver specific mouse transthyretin (mTTR) promoter can also be used.

In a particular embodiment, the second promoter (which is operably linked to the transgene of interest) is a tissue-specific promoter. The term "tissue -specific promoter" refers to a promoter that initiates transcription of a polynucleotide selectively in a tissue of interest.

It should be further noted, that tissue-specific promoters are well-known from the skilled man in the art. For instance, some tissue-specific promoters are described and available on the website of Invivogen: <http://www.invivogen.com/tissue-specific-promoter>

5 According to this particular embodiment, the nucleic acid construct comprises:

a) a first sequence encoding a repressor operably linked to a first promoter and to at least one tissue-specific miRNA target sequence, and

10 b) a second sequence encoding a transgene of interest operably linked to a second promoter and to an operator, wherein said second promoter is a tissue-specific promoter.

In one embodiment the tissue-specific promoter is selected from the group consisting of liver-specific promoter, brain-specific promoter, heart-specific promoter, lung-specific promoter, spleen-specific promoter, kidney-specific promoter, hematopoietic tissue-specific promoter and muscle-specific promoter.

The term "liver-specific promoter" refers to a promoter that initiates transcription of a polynucleotide selectively in the liver. Thus, the liver-specific promoter useful for the present invention may be any liver-specific promoter that is capable of enhancing tissue-specific expression of an associated coding DNA sequence in the liver.

Examples of liver specific promoters include, but are not limited to, the mouse thyretin promoter (mTTR), the human factor VIII promoter (F8), the human alpha-1-antitrypsin promoter (hAAT), the human albumin minimal promoter and the mouse albumin promoter.

The term "muscle-specific promoter" refers to a promoter that initiates transcription of a polynucleotide selectively in the muscle. Thus, the muscle-specific promoter useful for the present invention may be any muscle-specific promoter that is capable of enhancing tissue-specific expression of an associated coding DNA sequence in the muscle.

Examples of muscle-specific promoters include, but are not limited to, the desmin promoter and the creatine kinase promoter.

In another embodiment, expression of the transgene is under the control of a tissue specific promoter and an enhancer. Indeed, to increase gene expression levels in a tissue of interest a tissue-specific promoter may be bound to one or more specific enhancers. As used herein, the term “enhancer” refers to a DNA sequence that increases the rate of transcription.

5

To increase gene expression levels in hepatic tissue liver-specific promoters may be bound to specific enhancers. Thus, some enhancer/promoter combinations may also be used for achieving long term expression in the liver (e.g. one liver-specific promoter combined to one or more liver-specific enhancer elements). For instance, a liver-specific enhancer

10 comprises one or more copies of the α -1-microglobulin enhancer.

In a preferred embodiment, the tissue-specific miRNA and the tissue-specific promoter are specific for the same organ/tissue.

15 In a still preferred embodiment, the tissue-specific miRNA and the tissue-specific promoter are liver-specific.

For instance, the tissue-specific miRNA is miR122 and the tissue-specific promoter is the mouse transthyretin (mTTR) promoter.

20 It should be further noted that cells, more particularly hematopoietic cells, may be isolated by methods known from the skilled man in the art before being transduced with a nucleic acid construct of the invention or a vector comprising thereof as described below.

In relation to the present invention, the repressor is thus operatively linked

25 downstream of a promoter and upstream of at least one tissue-specific miRNA target sequence to form a sequence that can be used to prevent translation of the transgene in the presence of a corresponding tissue-specific miRNA expressed in a desired cell.

In one embodiment, the operator is a tet operator sequence.

30 Said tet operator sequence comprises from two to ten, preferably from five to nine, even more preferably seven tetO binding sequences.

Preferably, said tetO binding sequences are in tandem. Each adjacent tetO binding sequences may be spaced from each other the same distance in the same nucleic acid

sequence. The distance between the two or more tetO adjacent binding sequences may also vary and/or may be modified to achieve a desired degree of regulation efficiency.

In relation to the present invention, the transgene of interest is thus operatively linked downstream of a promoter and downstream of a tet operator sequence to form a sequence that can be used to control expression of the transgene in the presence of a repressor.

Alternatively the tet operator may be placed in within the lentiviral LTR as described in the WO 2007/000668. In this case, after reverse transcription, the tet operator is present at both sides of the proviral genome integrated in infected cells.

In another embodiment, the tet operator sequence is operatively linked to a promoter to form a sequence, including operatively linked tet-O sequences, a promoter and a transgene, that can be used to control expression of said transgene in the presence of a repressor.

It should be further noted that in another embodiment the both sequences previously described may also be carried out in two distinct nucleic acid constructs. Such embodiment may be useful for *in vitro* and *ex vivo* experiments.

Vector comprising the nucleic acid constructs of the invention:

The nucleic acid constructs of the present invention may be used to deliver in the transgene of interest to a site or cell of interest. For this purpose, a vector may be used.

A vector is thus a tool that allows or facilitates the transfer of an entity from one environment to another. By way of example, some vectors used in recombinant DNA techniques allow entities, such as a segment of DNA (such as a heterologous DNA segment, such as a heterologous cDNA segment), to be transferred into a target cell. Optionally, once within the target cell, the vector may then serve to maintain the heterologous DNA within the cell or may act as a unit of DNA replication. Examples of vectors used in recombinant DNA techniques include plasmids, chromosomes, artificial chromosomes or viruses.

It should be further be noted that a vector according to the present invention include a viral or non-viral vector.

Non-viral delivery systems include but are not limited to DNA transfection methods. Transfection includes a process using a non-viral vector to deliver a gene to a target cell. Typical transfection methods include electroporation, DNA biolistics, lipid-mediated transfection, compacted DNA-mediated transfection, liposomes, lipofectin, cationic agent-mediated, cationic facial amphiphiles (CFAs) and combinations thereof.

Viral delivery systems include but are not limited to an adenovirus vector, an adeno-associated viral (AAV) vector, a herpes viral vector, a retroviral vector, a lentiviral vector, a baculoviral vector as well as a virus-like particle (VLP).

In one particular embodiment, the vector is a lentiviral vector such as a lentiviral vector selected from the group consisting of Human Immunodeficiency Virus (HIV), e.g. HIV-1 or HIV-2, and Simian Immunodeficiency Virus (SIV).

Viral delivery systems and to produce them are known by those skilled in the art. In this context, such systems and methods have already been disclosed: for example, international patent application N° WO 2007/000668, which is incorporated herein by reference, describes the major examples of viral delivery systems useful in the context of the invention as well as the methods to produce them.

In another particular embodiment, the vector is a virus-like particle (VLP).

Where virus-like particle are being used, they may be prepared according to techniques known in the art and for example as described in the international patent application published under n° WO 02/34893, which is incorporated therein by reference.

Therapeutic uses of the vectors of the invention:

As is apparent from the foregoing, the above-defined vectors can be used for the *in vivo* or *ex vivo* transfer of a transgene of interest to cells.

Accordingly, the vector is particularly indicated for treating diseases either by direct administration of the vector particle to the patient afflicted by such a disease, or by administering cells, in particular cells originating from the individual afflicted by such a disease, which have been contacted *ex vivo* with the vector.

Another aspect of the invention relates to a vector of the invention for use as drug.

As previously mentioned, the transgene of interest is selected in the group consisting of enzymes, blood derivatives, hormones, chimiokines, interleukins, growth factors, neurotransmitters or their precursors, trophic factors, tumor suppressor genes, factors
5 involved in clotting, proteins involved in DNA repair, protein transporters, receptors, etc.

Therefore, a vector of the invention is useful for treating any pathology caused by an insufficient expression of a particular protein or a mutation resulting in a protein that is inactive or weakly active. This kind of pathology usually results from recessive genetic mutations of a single gene.

10 The term "patient" as used herein denotes a mammal. Preferably, a patient according to the invention is a human.

In the context of the invention, the term "treating" or "treatment", as used herein,
15 means reversing, alleviating, inhibiting the progress of, the pathology to which such term applies, or one or more symptoms of such pathology.

Another aspect of the invention relates to an *in vitro* or *ex vivo* method for obtaining a tissue-specific expression of a transgene of interest comprising the following steps:

20 a) providing a vector of the invention as previously defined, and

b) transferring said vector in a cell or a tissue of interest expressing a corresponding tissue-specific miRNA.

Therefore, another aspect of the invention relates to a cell infected or transduced with a vector according to the invention as such.

25 The invention also relates to a said cell infected or transduced with a vector according to the invention for use as drug.

Pharmaceutical compositions

30 Therefore, the present invention also provides a pharmaceutical composition for treating a patient by gene therapy, wherein the pharmaceutical composition comprises a

therapeutically effective amount of a vector of the present invention or a cell comprising thereof. The pharmaceutical composition may be for human or animal usage. Typically, a physician will determine the actual dosage will be most suitable for the patient and it will vary with the age, weight and response of said patient.

5

FIGURES:

Figure 1: Operating principle of the invention: miRNA-based regulation of TetR-KRAB control of transgene expression. **A.** Schematic representation of the miRNA-TetR-KRAB regulatory and reporter cassettes. **B.** In the absence of tissue-specific miR, the repressor is translated and is free to bind the tetO operator, thus preventing transgene expression. However in the presence of a tetracyclin analog (Doxycycline) the repressor does not bind and transcription is activated. **C.** In targeted cells tissue-specific miR binds to its complementary target and results in degradation of the TetR-KRAB mRNA. As a result, transgene expression occurs.

15

Figure 2: Schematic diagram of the recombinant lentivirus vectors used to accomplish liver-specific transgene expression. Lentiviral vector encoding four target sequences of miR-122, TetR-KRAB and GFP under the control of a constitutively active strong CMV promoter (PKTM3), a weaker constitutively active PGK promoter (PKTM13) or a mTTR liver specific promoter (PKTM7 and PKTM9); Lentiviral vector without target sequences of miR-122 and expressing GFP from liver-specific mTTR promoter (PKTM10); Lentiviral vector encoding four target sequences of miR-142, TetR-KRAB and GFP under the control of a mTTR liver specific promoter (PKTM12); Inducible TetR-KRAB lentiviral vector system that includes a GFP gene under control of a CAG promoter as part of a bicistronic unit comprising the KRAB based repressor (PLVCT). cPPT : Central polypurine tract, IRES : internal ribosomal entry site, WPRE : woodchuck hepatitis virus post-transcriptional, RRE : rev protein Responsive element, pA : polyadenylation site, CMV : cytomegalovirus, CAG : CMV immediate enhancer/ β -actin, PGK : phosphoglycerate kinase, mTTR : liver-specific transthyretin promoter.

30

Figure 3: GFP expression in cells transduced (293T (A), HUH7 (B), or NR8383 (C)) with the different lentiviral constructs in the presence or absence of doxycycline. After 10 days of culture, with or without doxycycline, transduced cells were analyzed for GFP expression by FACS. Results are expressed as a percentage of mean fluorescence intensity

(MFI). In tetR-Krab system, the expression of GFP is optimal in the presence of doxycycline: this corresponds to 100% of the MFI.

Figure 4: Lentiviral vectors used to target transgene expression to skeletal muscle-derived cells. **A.** Schematic diagram of the lentivirus vectors used to accomplish skeletal muscle-specific expression. **B.** miR-133 expression levels in Huh7 and NR8383 cells detected by RT-qPCR. **C.** Differentiated or undifferentiated C2C12 cells (C2C12d and C2C12ud respectively) were transduced with lentiviral vectors carrying the TetR-KRAB sequence followed by four copies of miR target of miR-122 or miR-133. After 10 days of culture with or without doxycycline, transduced cells were analyzed for GFP expression by FACS. Results are expressed as a percent of MFI. Data shown are mean and error bars indicate the SD. Statistically significant differences (*, $P < 0.05$) were determined using the Student's t-test.

Figure 5: Assessment of efficacy of miRNA-regulated TetR-KRAB control of transgene expression in vivo. **A.** Schematic diagram of the AAV vectors used for in vivo studies. **B.** GFP fluorescence was measured in live animals at 5 weeks after injection with recombinant AAVs. Mice received AAVs encoding the TetR-KRAB sequence with four copies of miR target of miR-122 located downstream. Animals did (Dox+, right panel) or did not (Dox-, left panel) receive doxycycline in their drinking water. The color scale next to the images indicates the signal intensity. **C & D.** Results at 5 weeks (**C**) or 8 and 12 weeks post injection (**D**) are expressed as a percentage or in counts/mm²/second. In TetR-KRAB system, the expression of GFP is optimal in the presence of doxycycline and corresponds to 100% of the counts/mm²/second. Data shown are mean and error bars indicate the SD. Statistically significant differences (*, $P < 0.05$) were determined using the Student's t-test.

EXAMPLES

Rationale: As shown in Figure 1A, our system contains two different expression cassettes. The reporting cassette contains a marker or a therapeutic gene under control of a ubiquitously active or a liver specific promoter that has tetO binding sites. The second cassette is regulatory and encodes a TetR-KRAB-fusion protein with tissue-specific miR targets placed in the 3' untranslated region of the transcript. In cells which do not express the tissue-specific miR (Figure 1B) and in the absence of doxycycline, the transgene is not

expressed. However the transgene can be expressed in the presence of doxycycline when binding of TetR-KRAB to the tetO sequences is prevented. In contrast, in cells expressing the tissue-specific miRs with cognates in the regulatory transcript, synthesis of the chimeric repressor is inhibited (Figure 1C) and the transgene is then expressed. Therefore transgene expression is restricted to a specific target tissue, except in the presence of doxycycline when the binding of TetR-KRAB to the tetO sequences is prevented in non-target cells.

EXAMPLE1: *In vitro* experiments in hepatic and hematopoietic cells

In a first set of experiments, we evaluated the efficiency of the system in liver-derived cells and in other tissues that may be important in the context of gene therapy.

Material & Methods

Constructions of lentiviral vectors: As shown in Figure 2, lentiviral constructs contain two different expression cassettes. The first cassette contains a ubiquitous promoter driving the tetR-Krab sequence and the 4 target sequences of miR 122 or 142.3. This cassette has been placed in reverse orientation with an unidirectional polyadenylation site. The tetO heptamer sequence is placed between the two cassettes, or in the proviral long terminal repeats (LTRs) sequences. The second cassette contains the GFP gene under control of a ubiquitous or specific promoter followed by a woodchuck hepatitis virus post-transcriptional regulatory element (WPRE). This construction was synthesized as a modular system including unique restriction sites on both sides of each component. From this plasmid, different promoter of GFP and TetR-Krab were inserted. All other constructs were generated from the same backbone (PTKM 3, 7, 9, 10, 12, 13, 15). Our construction has been compared with a conventional inducible tetR-Krab system that includes GFP gene under control of CAG promoter (CMV immediate enhancer/ β -actin) as part of bicistronic unit comprising the Krab-based repressor (PLVCT) previously published. In this construct the TetO was inserted into the U3 region of the 3' LTR.

Production of lentiviral vectors: Lentiviral vector stocks were generated by calcium phosphate-mediated transient transfection of 293T cells of the vector transfer plasmid, the packaging plasmid psPAX2, and the vesicular stomatitis virus G protein (VSVG) envelope protein-coding plasmid pMD2G. Culture medium was collected at 24 and 48 h, aliquoted, and stored at -80°C until used.

Cell culture and infection: 293T human epithelial kidney cells, HUH7 hepatoma cells, and NR8383 normal rat alveolar macrophage cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum, 50 IU/ml penicillin, and 50 µg/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO₂. $2.5 \cdot 10^4$ cells per well were seeded into 24-well plates and were infected with 100ul of lentiviral supernatant during 24 hours. Doxycyclin was used at a final concentration of 100ng/ml.

MicroRNA Quantification: miRs were extracted using mirVana miR isolation kit (Ambion, Applied Biosystems, Austin, TX) according to the manufacturer's instructions. Mature miRs were measured by reverse transcriptase qPCR (RT-qPCR) using TaqMan miR assays (Applied Biosystems). In brief, 10 ng of total RNA was reverse transcribed with stem-loop RT primers specific for human miR-133a, miR-142.3p, or miR-122 (Applied Biosystems) using the TaqMan miR reverse transcription kit according to the manufacturer's instructions. qPCR was performed on the resulting complementary DNA using miR-133a, miR-142 or miR-122 specific TaqMan primers and TaqMan universal PCR master mix in a 7900 real-time PCR instrument. The reactions were incubated in a 96-well optical plate at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Expression of the U6 gene was used as an endogenous control for data normalization. Stem-loop RT primer and TaqMan primer specific for human U6 were obtained from Applied Biosystems (Austin, TX).

Total RNA Quantification: Total RNA was processed with Trizol as lysis buffer then extracted with the RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. For RT-qPCR, total RNA was initially reverse transcribed using the SuperScript III Platinum Two-Step kit (Invitrogen). Analysis of gene expression was carried out using the MESA GREEN qPCR MasterMix Plus for SYBR Assay (Eurogentec) and the TaqMan 7900 instrument. Total cDNA was added to a solution containing the primers and the Sybr Green

PCR Master Mix, then loaded into a 96-well plate. Temperature cycling conditions were: 10 min at 95 °C for activation followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The values were normalized to those obtained for the 18S gene. The results of the RT-qPCR are expressed as the Ct value for each sample. The sequences of the forward and reverse primers were the following.

FACS Analysis: Ten days after infection, transduced cells were analysed for EGFP expression by FACS. Analysis was performed on an LSRII (Becton Dickinson). Data were analysed with FlowJo software.

Statistical analysis: All results were compared statistically using the Student's t-test with GraphPad Prism software. A p value of <0.05 was considered significant.

Results

Cell lines from hepatic or non-hepatic origin were transduced with the various vectors described previously (Figure 2). We used HuH7 cells from human hepatic origin that are known to strongly express miR122. We also used 293T cells derived human embryonic kidney as well as cells from rat alveolar macrophage (NR8383 cells). NR8383 cells are part of the immune system and express miR142. In all cell lines, the expression of GFP was quantified by FACS analysis and we compared GFP expression in the presence or absence of doxycycline in each cell line and between the various cell lines (Figure 3).

We compared our system combining a miRNA-mediated approach for restricting gene expression to a repressor tetR- Krab, with a conventional inducible tetR-Krab system. In this construction, named PLVCT, The tetR-Krab cDNA was expressed from the ubiquitously active CAG-promoter as part of a bicistronic transcript also producing the GFP gene. After transduction with PLVCT, we observed very tight regulation of GFP expression. Indeed, as other studies have previously shown, in 293T cells and in the absence of doxycycline, the decrease in GFP MFI is about 98%. However the efficiency varies according to cell types. In the hepatic cell line, the decrease is 90% but only about 60% in the macrophage of rats.

We tested in different cells line our tetR-Krab regulation system in which the tetR-Krab mRNA is coupled to target sequences of the miR122 or miR142.

In 293T cells (miR122^{negative} and miR142^{negative}), we observed in absence of doxycycline a decrease in mean fluorescence with all construction (Figure 3 A). This indicates that tetR-Krab protein is produced and will bind to the tetO in the absence of doxycycline and repress the expression of the GFP. Repression of transcription of GFP gene is more efficient when the GFP is under the control of a liver-specific transthyretin promoter (mTTR) rather than under a ubiquitous promoter. Indeed, regarding the PTKM 3 and PTKM 13 constructions that contain ubiquitous promoters driving GFP, there is a decrease in GFP MFI of about 50 % in 293 cells. However, with the mTTR promoter the decrease ranged from 82% to 94% (Figure 3A). It is worth noting that the mTTR promoter which activity should be restricted to hepatocytes is leaky in other cell types.

With lentiviral constructs PTKM 9, 7, 10, 12, and 15 there was a decrease of GFP MFI of at least of 74 percent. PTKM 9 was the most efficient (GFP MFI decreased of 94%). This may be due to the fact that PTKM 9 contains a strong promoter (CMV) driving TetR KRAB expression unlike PTKM 7, 10 and 12 containing a weaker PGK promoter. The difference between PTKM 9 and 15 is the position of tetO (Figure 2). In PTKM 9, the tetO sequence is placed upstream of the promoters, between the two diverging cassettes. In PTKM 15, TetO was inserted into the U3 region of the 3' LTR. During reverse transcription, RNA 3' U3 region serves as the template for the synthesis of its 5' DNA homologue and the tetO sequence is duplicated in the integrated provirus. However, our results showed that tPTKM 15 is not more efficient than PTKM 9 suggesting that multiplying tetO sequences does not seem to impact effectiveness of repression.

In HUH7 cells, (miR122^{positive} and miR142^{negative}) we observed that in the absence of doxycycline there was no variation of GFP in all versions that contain the miR122 target sequences (Figure 3B). These results showed that, as expected, the miR122 will bind to its target sequence and will destroy the mRNA coding for the chimeric repressor. Therefore, with these constructions, transgene expression can be restricted to hepatic cells.

Lentiviral constructs PTKM 10 and 12, do not contain the miR 122 target sequences. Consequently, in HuH7 tetR-Krab protein is produced and therefore we observed in the absence of doxycycline a decrease in MFI with both constructs. This clearly shows that the absence of tetR-Krab inhibition with the miR122 target containing vectors is actually due to the presence of the miR122 targets.

In NR8383 cells (miR142^{positive} and miR122^{negative}) in the absence of doxycycline there was a decrease of GFP MFI of approximately 70% when cells were transduced with constructs that did not contain the target sequences of the miR 142 (Figure 3C). In NR8383 cells infected with PTKM 12 that contains the miR142 target sequence, transgene expression did not change in the absence of doxycycline. This indicates that the miR142 target sequence is functional in cells from the hematopoietic lineage.

Taken together, these results indicate that our new expression system provides miRNA-based specific expression and high transgene expression in cells from different tissues.

EXAMPLE 2: *In vitro* experiments in muscle cells

Material & Methods

Cell culture and infection: C2C12 myoblasts were maintained at subconfluent densities in DMEM supplemented with 10% fetal bovine serum, 50 IU/ml penicillin, and 50 µg/ml streptomycin. Near-confluent cells were induced to differentiate with DMEM containing 2% horse serum for 5 days. All cells were cultured in a humidified atmosphere containing 5% CO₂. For infection, 10⁴ cells were seeded in each well of a 24-well plate before infection with lentiviral vectors at a multiplicity of infection (MOI) of 10. Doxycycline was used at a final concentration of 1 µg/ml.

MicroRNA Quantification: miRs were extracted using mirVana miR isolation kit (Ambion, Applied Biosystems, Austin, TX) according to the manufacturer's instructions. Mature miRs were measured by reverse transcriptase qPCR (RT-qPCR) using TaqMan miR assays (Applied Biosystems). In brief, 10 ng of total RNA was reverse transcribed with stem-loop RT primers specific for human miR-133a or miR-122 (Applied Biosystems) using the TaqMan miR reverse transcription kit according to the manufacturer's instructions. qPCR was performed on the resulting complementary DNA using miR-133a or miR-122 specific TaqMan primers and TaqMan universal PCR master mix in a 7900 real-time PCR instrument. The reactions were incubated in a 96-well optical plate at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Expression of the U6 gene was used as an endogenous control for data normalization. Stem-loop RT primer and TaqMan primer specific for human U6 were obtained from Applied Biosystems (Austin, TX).

Results

We evaluated the efficiency of the system for regulating gene expression in muscle. To this end we designed lentiviral vectors containing four copies of perfectly complementary miR-133 targets downstream of the Tet-KRAB-encoding sequence. This miR is specifically expressed in adult cardiac and skeletal muscle tissues (Figure 6A). We used C2C12 myoblasts because these cells are able to mimic skeletal muscle differentiation in vitro. We confirmed that expression of miR-133 increased when C2C12 cells underwent myoblast differentiation (Figure 4B). Differentiated or undifferentiated C2C12 cells were transduced at a MOI of 10 with miR-133- TetR-KRAB regulated or miR-122- TetR-KRAB regulated lentiviral vectors. The expression of GFP was quantified by FACS analysis and we compared expression of the reporter in the presence or absence of doxycycline (Figure 4B & C). GFP expression did not change during differentiation in cells transduced with the lentiviral vector carrying miR-122 target sequences (Figure 4C). Moreover, doxycycline withdrawal caused a similar decrease in GFP expression in differentiated and undifferentiated cells. In contrast, in cells infected with the lentiviral vector carrying miR-133 target sequences we observed a significant reduction of GFP expression in undifferentiated cells after doxycycline removal, indicating that the TetR-KRAB regulation functioned according to the intended design. However, after differentiation, TetR-KRAB inhibition of GFP expression was severely impaired, indicating that the miR-133 expression silenced TetR-KRAB production.

EXAMPLE 3: *In vivo* experiments

Material & Methods

In vivo analysis: Balb/C mice (8-10 weeks of age) were injected with a total dose of 1×10^{11} viral genomes (vg) into each of the two tibialis anterior muscles. Doxycycline was administered in drinking water at a concentration of 2 g/liter supplemented with 4% sucrose. For whole-body in vivo analysis, anesthetized mice were imaged using NightOWL II LB 983 NC320 (Betthold technologies) imaging equipment. The fluorescence was quantified using the WINLIGHT software. All surgical procedures were conducted according to the guidelines of the French Ministère de l'Agriculture. Mice were anesthetized by isoflurane inhalation (3% v/v) and maintained under a 12h light/dark illumination cycle with food and water ad libitum.

Results

Efficient miRNA-mediated TetR-KRAB regulation in vivo: To test the effectiveness of our system in vivo, the cassettes were inserted into recombinant AAV vectors. We designed three DNA cassettes containing the TetR-KRAB coding sequence with four copies of miR-133 or miR-122 target sequences located in the downstream untranslated region. The control vector was devoid of miR target sequences (Figure 5A). The GFP- and TetR-KRAB-coding sequences were under the control of a CMV promoter. These vectors were injected into the two tibialis anterior muscles of subject mice (n=6 for each vector). At 2 weeks after AAV injection, three animals from each group received doxycycline in their drinking water. Mice were analyzed for GFP expression using fluorescence imaging 5 weeks after injection (Figure 5B). GFP expression in muscle was scored and we compared GFP expression in the muscle in the presence or absence of doxycycline with each vector. In mice injected with AAV constructs with or without miR-122 target sequences, we observed a strong fluorescence signal in muscle when animals received doxycycline. In contrast, in the absence of doxycycline, there was a sharp (70 to 98%) decrease in fluorescence (Figure 5C). However, in mice injected with AAV carrying miR-133 target sequences, there was no variation of GFP expression in the absence or presence of doxycycline. The same animals were re-analyzed at 8 and 12 weeks after injection. The intended regulation by doxycycline persisted in muscles injected with AAV that did not contain the miR target sequences although no significant effect of doxycycline was recorded in mice injected with the miR-133-regulated vector (Figure 5D).

DISCUSSION

Many natural or artificial promoters, with activity that is restricted to specific tissues, have been used in gene transfer vectors to achieve targeted expression of a transgene. However, it has been shown that these promoters are usually weak when compared to their constitutively active counterparts. Moreover these promoters are often leaky, which compromises their tissue specificity. Consequently an additional layer of control is required to achieve robust transgene transcription and limit expression to intended target cells. To address this, we have used tissue-specific expression of miRs to silence a transcriptional repressor. We have designed an original new system which combines use of the tetR-KRAB artificial transgene-repressor with endogenous miR silencing machinery. Specific and high level expression in targeted-tissue could be achieved, and this was independent of the promoter that was used. Expression of the TetR-KRAB component is under control of miR

target sequences that were selected according to the miR expression profile of specific cell types. In our design, translation of the TetR-KRAB repressor is shut off in permissive cells that express the miRs, which in turn results in de-repression of the transgene. This system is versatile, and we obtained targeted expression in several cells types by including selected miR targets downstream of the TetR-KRAB-encoding sequences. Muscle-specific miR-133, liver specific miR-122, or hematopoietic specific miR-142 target sequences were shown to work synergistically with the TetR-KRAB cassette and enable tissue-specific expression.

An additional advantage of the regulatory system we describe is that the size of the paired cassettes is small enough to be inserted into most vectors that are currently used for gene transfer. In the study reported here, we successfully used lentiviral and AAV vectors to demonstrate utility of the system. We are currently investigating the use of alternate vectors, such as adenoviral and integration-defective lentiviral vectors, to effect TetR-KRAB silencing.

In cells that do not express the tissue-specific miR, we observed a decrease in reporter transgene expression (between 50 and 94%), which was demonstrated in vivo and in vitro. However this effect was influenced by the promoters and is cell type-dependent. The decrease was measured as the ratio of transgene expression in the presence and absence of doxycycline. Complete shut-off of the transgene was however not achieved and could be a result of transcriptional interference between the two promoters. Indeed, viral vectors containing two transcription units in tandem have frequently been reported to be compromised by mutual promoter suppression. However, when two promoters are fused back to back, interference between them has been reported to be minimal. A critical parameter that may influence the efficiency of the system we developed is the location of the tetO sequence. In addition to the configuration described here, we designed a lentiviral vector in which the tetO sequence was inserted into the U3 region of the 3' LTR. During reverse transcription, the 3' U3 RNA region serves as the template for synthesis of its 5' DNA homologue and the tetO sequence is duplicated in the integrated provirus. We found that this arrangement was less efficient than the configuration described here, suggesting that duplicating tetO sequences does not improve effectiveness of transgene repression. We also compared our system to a previously described inducible TetR-KRAB system (PLVCT) (Szulc et al., 2006). As with the results reported by Szulc and colleagues, we also observed a decrease in GFP expression in 293T cells when incubated in the absence of doxycycline. However we found that the efficiency varied in different cell types. After doxycycline withdrawal, there was a more pronounced decrease in

GFP expression compared to cells of haematopoietic origin. Therefore our results indicate that in cells which do not express the tissue-specific miR, the decrease of transgene expression may vary. In addition, the promoter type may influence the doxycycline responses. Nevertheless, our system is still efficient and allows for improvement in the tissue specificity by significantly reducing background transgene expression.

Preventing cytotoxic immune responses to transgene products is important for gene therapy, and the system described here provides the means for achieving this. We and others have previously documented that an immune response to a transgene product can impair the desired long term expression following hepatic transgene delivery when using oncoretroviral, AAV or lentiviral vectors. Frequently, epitopes of the transgene product are presented by antigen presenting cells, resulting in an undesirable immune response and premature removal of the transgene. One of the approaches to overcome initiation of a vigorous immune response is to prevent transgene expression in antigen presenting cells, and using lineage-specific miRs to inhibit transgene expression allows for this. Brown and colleagues used target sequences of hematopoietic haemotoietic lineage-specific miR-142.3 to eliminate off-target expression in hematopoietic cells. Although this strategy accomplished sustained gene transfer in haemophilia B mice for more than 280 days (Brown et al., 2007), the method was insufficient to prevent an anti-FVIII immune response in treated haemophilia A mice. We believe that our approach improves gene transfer efficiency by allowing high expression in targeted cells, but at the same time limiting expression in non-targeted cells, especially those of hematopoietic origin.

In addition to transgene-directed immunity, development of an immune response against the exogenous trans-activators or trans-repressors has been reported in nonhuman primates. We believe that this may not compromise efficiency of transgene tissue targeting of the system we described here. If an immune response to the TetR-KRAB chimeric protein develops, it should be directed to non-targeted cells that express the TetR-KRAB protein, while targeted cells that do not express the TetR-KRAB should be spared.

In conclusion, we believe that the system we have developed and described here is original and versatile. It can be exploited to achieve specific and robust transgene expression that is critical in successfully developing gene therapy.

REFERENCES:

Throughout this application, various references describe the state of the art to which this invention pertains. The disclosures of these references are hereby incorporated by
5 reference into the present disclosure.

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CLAIMS:

1. A nucleic acid construct comprising :
 - 5 a) a first sequence encoding a repressor operably linked to a first promoter and to at least one tissue-specific miRNA target sequence, and
 - b) a second sequence encoding a transgene of interest operably linked to a second promoter and to an operator, wherein said second promoter is a tissue-specific
10 promoter.
2. The nucleic acid construct according to claim 1, wherein the tissue-specific miRNA is a liver-specific miRNA.
- 15 3. The nucleic acid construct according to claim 2, wherein the liver-specific miRNA is miR122.
4. The nucleic acid construct according to any one claims 1 to 3, wherein the tissue-specific promoter is a liver-specific promoter.
20
5. The nucleic acid construct according to according to claim 4, wherein the liver-specific promoter is the mouse transthyretin (mTTR) promoter.
6. The nucleic acid construct according to any one claims 1 to 5, wherein the
25 repressor is a chimeric repressor fusion protein.
7. The nucleic acid construct according to claim 6, wherein the chimeric repressor fusion protein is the tTR-KRAB protein.
- 30 8. The nucleic acid construct according to any one claims 1 to 7, wherein the operator is a tet operator sequence.
9. The nucleic acid construct according to claim 8, wherein the tet operator sequence comprises seven tetO binding sequences.

10. A vector comprising a nucleic acid construct as defined in any one claims 1 to 9.

11. The vector according to claim 10, wherein the vector is a viral vector.

12. A cell infected or transduced with the vector as defined in claim 10 or 11.

13. A vector as defined in claim 10 or 11 or a cell as defined in claim 12 for use as drug.

14. A vector as defined in claim 10 or 11 or a cell as defined in claim 12 for use in the treatment of a liver disease.

15. An *in vitro* or *ex vivo* method for obtaining a tissue-specific expression of a transgene of interest comprising the following steps:

a) providing a vector of the invention as defined above, and

b) transferring said vector in a cell or a tissue of interest expressing a corresponding tissue-specific miRNA.

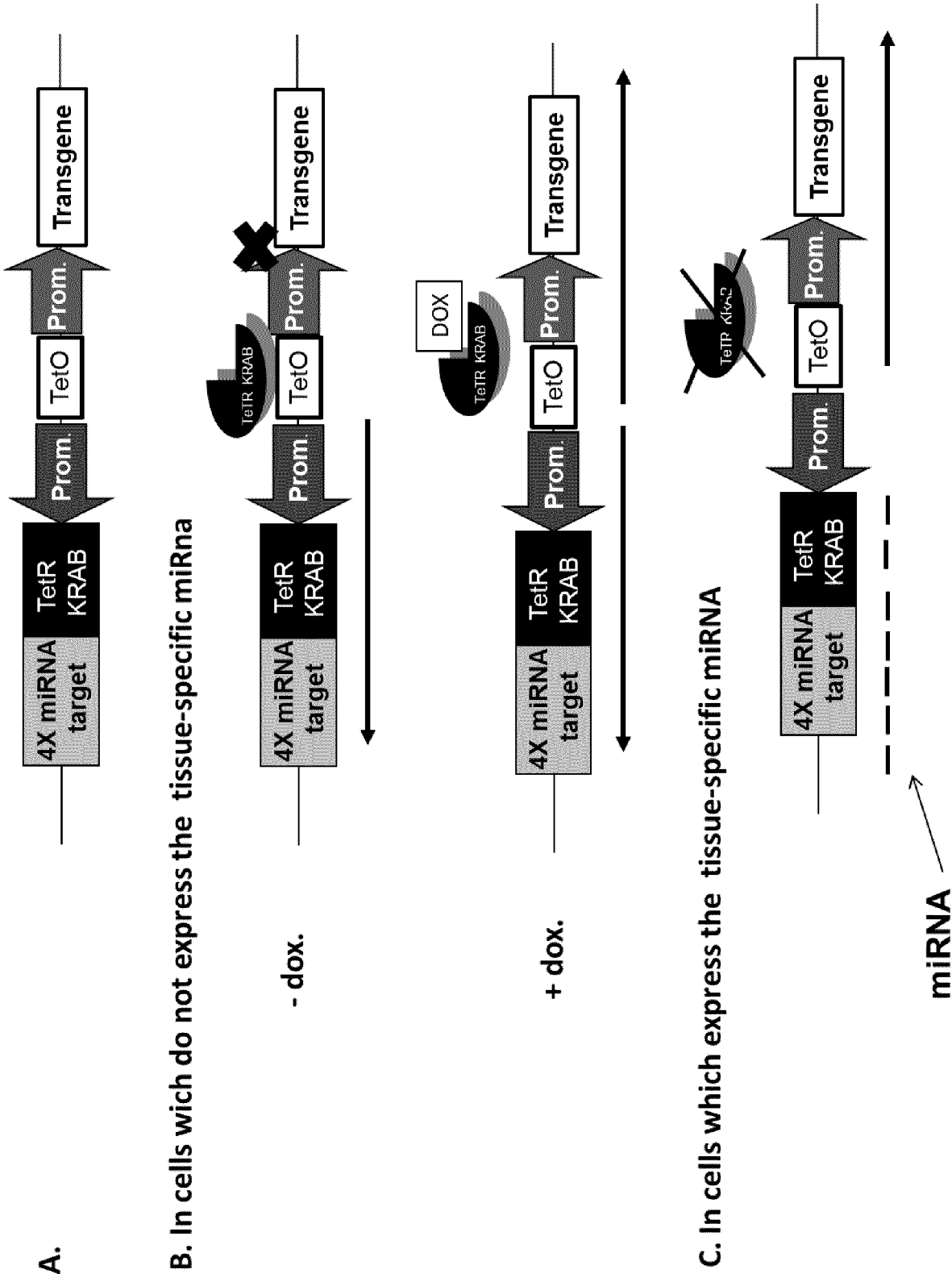


Figure 1

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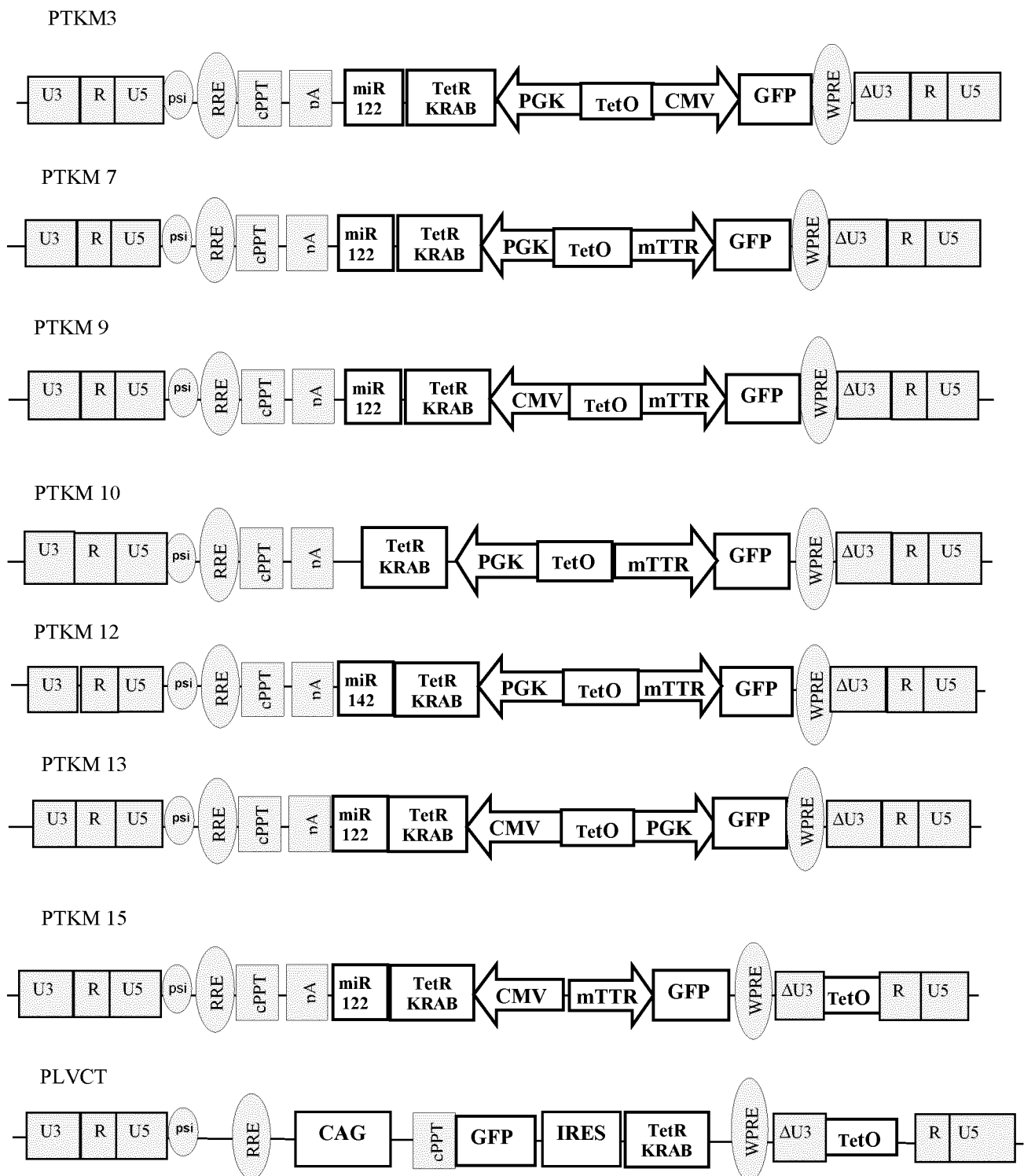
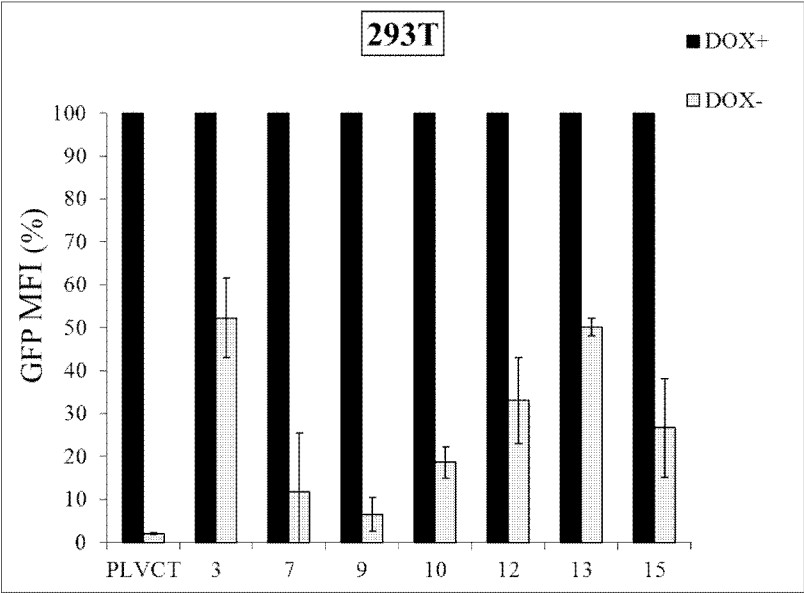


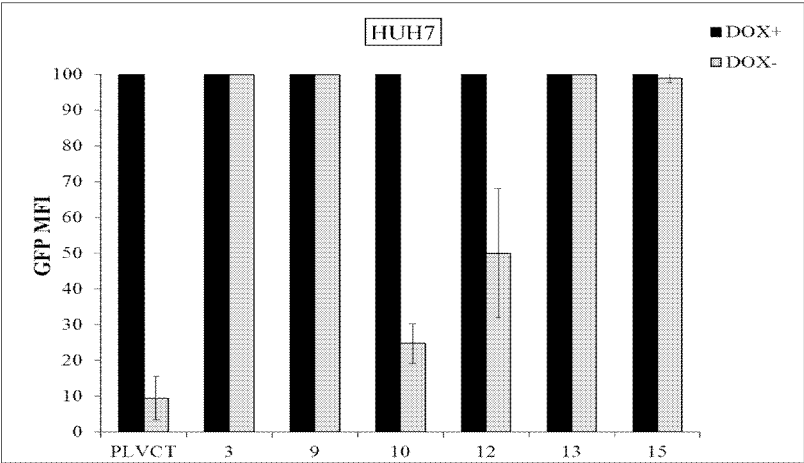
Figure 2

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A



B



C

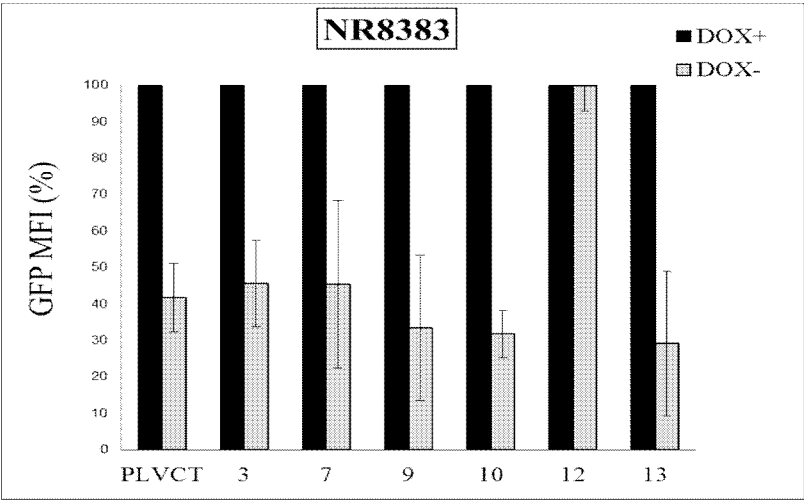


Figure 3

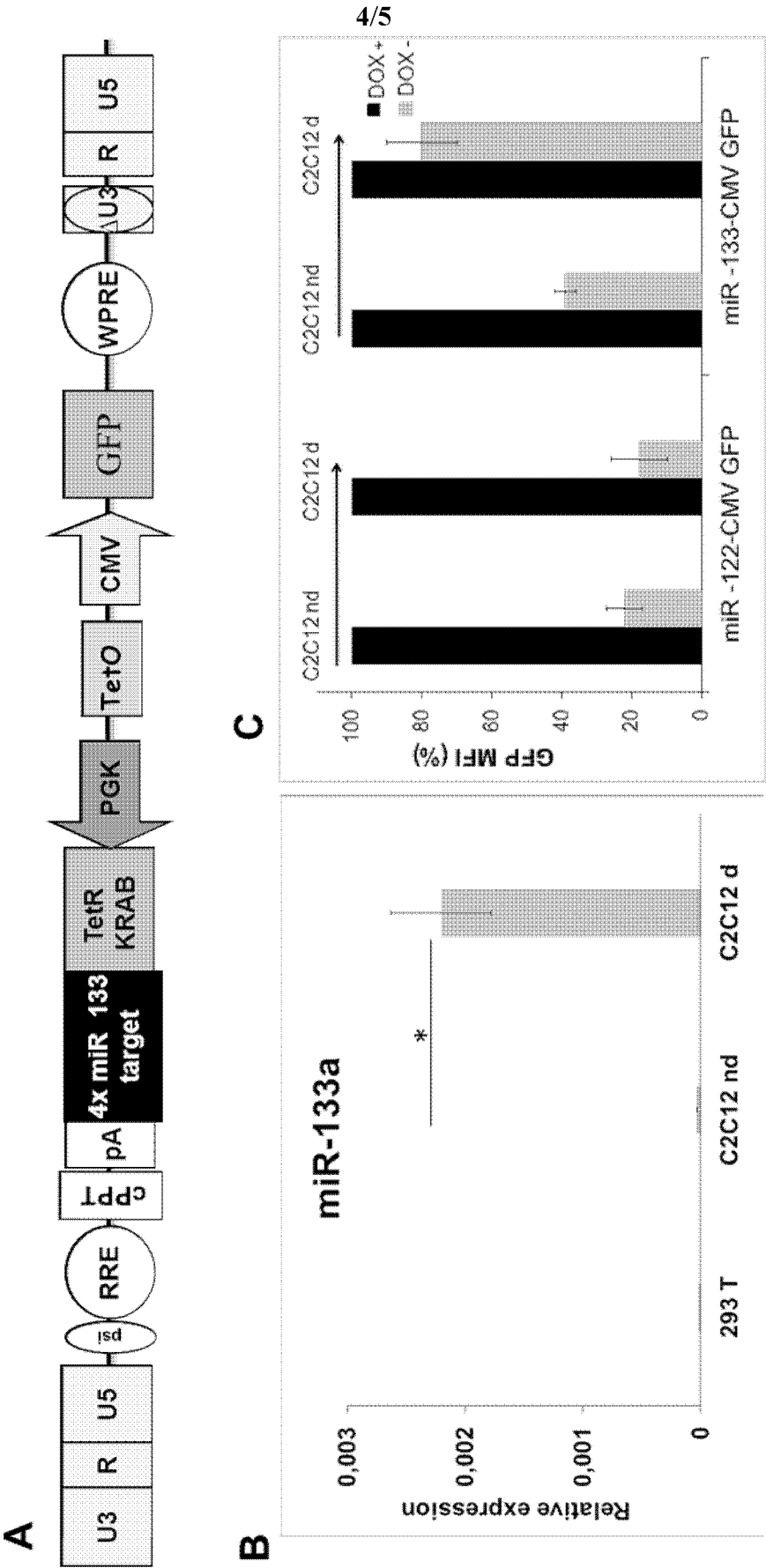


Figure 4

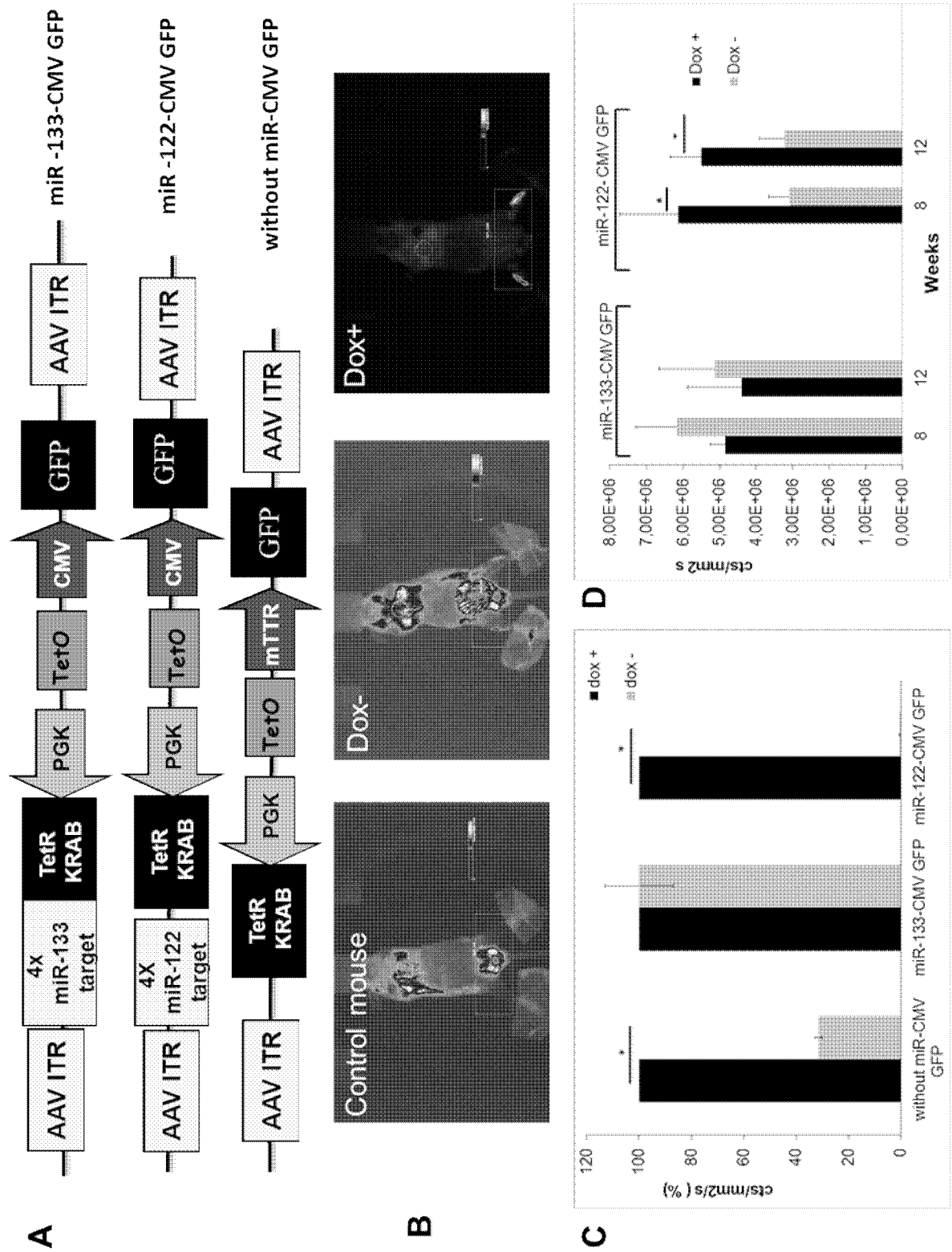


Figure 5

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2012/057090

A. CLASSIFICATION OF SUBJECT MATTER

INV. C12N15/867 C12N15/86
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2009/286242 A1 (HANNON GREGORY J [US] ET AL) 19 November 2009 (2009-11-19) abstract paragraph [0053] paragraph [0193] paragraph [0210] paragraph [0247] paragraph [0262] - paragraph [0265] paragraph [0295]	1-15
X	US 2007/054872 A1 (REPPEN THOMAS W [US] ET AL) 8 March 2007 (2007-03-08) paragraph [0010] - paragraph [0012] paragraph [0015] paragraph [0032] - paragraph [0033] paragraph [0040] ----- -/-	1-15



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

27 June 2012

Date of mailing of the international search report

05/07/2012

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2
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Authorized officer

Brero, Alessandro

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2012/057090

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2009/137421 A1 (MO YIN-YUAN [US] ET AL) 28 May 2009 (2009-05-28) figures 3, 6 -----	1-15
A	SZULC J ET AL: "A versatile tool for conditional gene expression and knockdown", NATURE METHODS, NATURE PUBLISHING GROUP, GB, vol. 3, no. 2, 1 February 2006 (2006-02-01), pages 109-116, XP003018664, ISSN: 1548-7091, DOI: DOI:10.1038/NMETH846 cited in the application the whole document -----	1-15
A	BROWN BRIAN D ET AL: "Endogenous microRNA can be broadly exploited to regulate transgene expression according to tissue, lineage and differentiation state", NATURE BIOTECHNOLOGY, NATURE PUBLISHING GROUP, NEW YORK, NY, US, vol. 25, no. 12, 16 November 2007 (2007-11-16), pages 1457-1467, XP002471752, ISSN: 1087-0156, DOI: 10.1038/NBT1372 cited in the application the whole document -----	1-15
A	BROWN B D ET AL: "Endogenous microRNA regulation suppresses transgene expression in hematopoietic lineages and enables stable gene transfer", NATURE MEDICINE, NATURE PUBLISHING GROUP, NEW YORK, NY, US, vol. 12, no. 5, 23 April 2006 (2006-04-23) , pages 585-591, XP002394629, ISSN: 1078-8956, DOI: DOI:10.1038/NM1398 cited in the application the whole document -----	1-15
A	WO 02/092134 A1 (CELL GENESYS INC [US]) 21 November 2002 (2002-11-21) page 3, line 3 - line 4 -----	4,5

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2012/057090

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2009286242	A1	19-11-2009	NONE
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