Assessment of gene transfer efficiency

Regulation of gene expression in gene therapy

17th October 2014
Questions

How to assess that our cells are transfected?

How to assess the transfection efficiency?

How to normalize the results after transfection?
Reporter genes

Genes used for determination of the transfection efficiency. Expression of a reporter has to be easily detectable. It has to be a gene which is not naturally present in transfected cells.
Reporter genes are used to study

- when,
- where, and
- how much a gene is expressed
Reporter genes

- chloramphenicol acetyltransferase (CAT)
- β-galactosidase
- secreted alkaline phosphatase (SEAP)
- luciferase – various forms are used
- green fluorescent protein (GFP)
- β-glucuronidase
Detection of the expression of reporter genes

- autoradiographic tests
- colorimetric tests
- fluorescence emission
- chemiluminescence emission
- protein detection: ELISA tests
<table>
<thead>
<tr>
<th>Reporter gene</th>
<th>species</th>
<th>product</th>
<th>use</th>
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<tbody>
<tr>
<td>lacZ</td>
<td>E.coli</td>
<td>β-galactosidase</td>
<td>Widely used reporter system, The enzyme hydrolyzes the colorless substrate X-gal to a blue precipitate</td>
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<tr>
<td>luc</td>
<td>Photinus pyralis (firefly)</td>
<td>luciferase</td>
<td>Highly sensitive reporter enzyme that oxidizes luciferin and generates a bioluminescent products (photons)</td>
</tr>
<tr>
<td>CAT</td>
<td>E.coli</td>
<td>Chloramaphenicol acetyltransferase</td>
<td>A useful reporter for in vitro assays but proteins gives poor resolution in situ CAT transfers nonradioactive acetyl groups to radioactive chloramphenicol</td>
</tr>
<tr>
<td>GUS</td>
<td>E.coli</td>
<td>β-glucuronidase</td>
<td>Generally used reporter in plant systems, hydrolyzes colorless glucuronides (e.g. X-gluc) to yield colored products for localization of gene expression</td>
</tr>
<tr>
<td>GFP</td>
<td>Aequorea victoria (jellyfish)</td>
<td>Green fluorescent protein</td>
<td>A very widely used system, reporter that emits fluorescence after irradiation with UV,</td>
</tr>
</tbody>
</table>
Chloramphenicol acetyltransferase (CAT)

• 1st reporter gene used to monitor transcriptional activity in cells

• Bacterial enzyme that transfers acetyl groups from acetyl-CoA to chloramphenicol, detoxifying it

Ways of Detection:

• Reaction quantified using radiolabeled substrates (\(^{14}\text{C}-\text{chloramphenicol}\))
  - qualitative rather than quantitative

• ELISA (non-radioactive) is recently available
Chloramphenicol acetyltransferase (CAT)

*Advantages*: low background in eukaryotic cells, high stability - protein is very stable (half-life is 50 hours)

*Disadvantages*: use of radioisotopes

It becomes historical...
CAT assay: TLC

TLC: Thin Layer Chromatography
CAT – detection of activity

Acetylated forms

Chloramphenicol

origin

1  2  3  4
non-transfected  transfected
CAT assay: ELISA

- Anti-CAT – antibody to CAT
- Anti-CAT-DIG - digoxigenin-labeled antibody to CAT
- Anti-DIG-POD Fab fragment - antibody to digoxigenin conjugated to peroxidase (anti-DIG-POD) and binding to digoxigenin
- ABTS - peroxidase substrate

Diagram:

1. Anti-CAT
2. Anti-CAT-DIG
3. ABTS substrate

Green color
β-galactosidase
**β-gal (β-galactosidase)**

- *E. coli* enzyme (encoded by *lacZ*) that hydrolyzes galactosidase sugars such as lactose
- Widely used to monitor transfection efficiency
- Many assay formats: colorimetric, fluorescent, chemiluminescent

Transfected cells

Non-transfected cells
β-galactosidase

In bacteria

Lactose ⇒ Galactose + Glucose

5-bromo-4-chloro-3-indolyl-B-D-galactoside

After transfection

5-bromo-4-chloro-3-indolyl-B-D-galactoside (colorless) via β-gal (β-Galactosidase) to galactose (colorless) + 4-Cl-3-Br-indigo (deep blue)
\textbf{Advantages:}

simple colorimetric assays, non-radioactive

\textbf{Disadvantages:}

background activity in mammalian cells (reduced at higher pH values),
**β-galactosidase**

**Staining of the cells:**
β-galactosidase performs hydrolysis of β-galactosidates, usually X-gal (5-bromo-4-chloro-3-indolyl-B-D-galactoside)

**Various quantitative tests:**

- **colorimetric:** ONPG - o-nitrophenyl-β-galactoside – β-gal hydrolyzes ONPG into galactose nad ortho-nitrophenol, the latter compound has a yellow color which can be measured spectrophotometrically

- **fluorimetric:** MUG – 4-methylumbelliferyl-D-galactopyranoside

- **chemiluminescent:** 1,2-dioeksyetan
Expression of β-galactosidase in various cells after lipotransfection

Cells from monkey kidney

Vascular smooth muscle cells (rat)
Histochemical staining for β-galactosidase expression in rat hepatocellular carcinomas

Heller et al, 2000
SEAP (secreted alkaline phosphatase)

- Mutated form of human placental alkaline phosphatase
- Secreted outside the cell (you can measure sample repeatedly and non-destructively by sampling culture medium)
- Heat-stable (eliminate endogenous AP activity by heating samples in 65°C)
- Colorimetric or chemiluminescent assay, read on plate reader
Luciferases

- firefly *Photinus pyralis* & sea anemone *Renilla reniformis*
Luciferases

- short lasting emission
- short half-life of luciferase
- high sensitivity
1. Luciferin is activated by luciferase in an ATP-dependent step
2. Luciferin-adenylyl intermediate is formed
3. When oxygen is present this intermediate is rapidly converted to a peroxy luciferin product
4. Peroxy luciferin decays to oxyluciferin with the emission of photons.
Renilla reniformis luciferase

Sea Pansy
**Luciferases – reactions**

**A**
- Luciferin + ATP + O₂ → Oxyluciferin + PR₃ + AMP + CO₂
- Luciferase

**B**
- Coelenterazine + O₂ → Coelenteramide + CO₂
- Renilla luciferase

*Renilla luciferase – coelenterazine is a substrate*
Luciferase measurement in vitro

Remove medium. Rinse with PBS.
Add 1X CCLR.
Scrape cells, transfer to microcentrifuge tube.
Briefly centrifuge, then transfer supernatant to new tube.
Mix 20μl of cell lysate and 100μl of Luciferase Assay Reagent in the tube.

VEGF promoter luciferase

VEGF promoter activity [% of control]

0 2 4 6 8
control DMOG

#
**Dual luciferase system**

- fast method, measurement in one tube - both the level of reporter which is coupled to regulated promoter and the level of the second reporter gene which is coupled to a constitutive promoter that is stable in the various experimental conditions.

1. The firefly luciferase activity (the "experimental" reporter) is measured

2. The firefly luminescence is rapidly quenched, with simultaneous activation of the Renilla luciferase luminescent reaction (the "control" reporter activity).
In vivo bioluminescence imaging
In vivo bioluminescence images of a mouse inoculated with leukemia-model cells which express firefly luciferase gene stably. The bioluminescence images visualize whole-body expression of luciferase gene quantitatively. Proliferation of the tumor cells can be assessed repeatedly in an individual mice.
At our Faculty…

IVIS (Image Visualisation and Infrared Spectroscopy)

Molecular Biotechnology for Health

www.wbbib.uj.edu.pl/bmz
HO-1 overexpression increases proliferation of myoblasts transplanted intramuscularly into immunodeficient mice
Green fluorescent protein

- from *Aequorea victoria* (jellyfish): *two different proteins*: equorin and GFP; equorin emits blue light after Ca$^{2+}$ binding, then the light is absorbed by GFP, which emits green light.

- Expression of GFP in other organisms causes green fluorescence after stimulation with blue light or UV.
The Nobel Prize in Chemistry 2008

Osamu Shimomura

discovered GFP during the study of the bioluminescent protein aequorin, the mechanism by which certain jellyfish glow (1961)

Martin Chalfie

took the cDNA of GFP and first expressed it in bacteria and worms; he demonstrated that GFP could be used as a molecular tag

Roger Y. Tsien

reported the S65T point mutation that greatly improved GFP fluorescent characteristics. His lab also evolved GFP into many other color variants
Color variants of GFP

Cold Spring Harb. Protoc.; 2009; doi:10.1101/pdb.top63
Fluorescent proteins

- **Yellow fluorescent proteins** – e.g. EYFP, one of the brightest and most popular FPs, Citrine variant is brighter and more resistant to photobleaching, acidic pH, and other environmental effects

- **Orange fluorescent proteins** - currently, the most useful orange FPs are mKusabira Orange (mKO) and its faster folding derivative mKO2, TagRFP, mOrange2, and tdTomato (a tandem dimer)

- **Red fluorescent proteins** - several members of mFruit series, like mApple, mCherry, and mPlum – they differ e.g. in their brightness: mApple is one of the brightest-red FPs, whereas mCherry is only half as bright as mApple

- **Cyan fluorescent proteins** - replacing Tyr66 in the chromophore of GFP with tryptophan produces a cyan-emitting variant CFP, a commercially available derivative AmCyan1; Clontech

- **Blue fluorescent proteins** - blue variants of GFP result from the substitution of histidine for tyrosine at position 66 in the chromophore
A San Diego beach scene drawn with an eight color palette of bacterial colonies expressing fluorescent proteins derived from GFP and the red-fluorescent coral protein dsRed. The colors include BFP, mTFP1, Emerald, Citrine, mOrange, mApple, mCherry and mGrape. Artwork by Nathan Shaner, photography by Paul Steinbach, created in the lab of Roger Tsien in 2006.
The color of mice: in the light of GFP-variant reporters

Bright-field photograph of four agouti coat-colored mouse pups.

Dark-field photograph taken under epifluorescence optics revealing the transgenic color of the mice.

EGFP - green FP variant
ECFP - cyan FP variant giving a bluecyan fluorescence
EYFP - yellow FP variant
Green fluorescent protein
Green fluorescent pigs...

When lit up in the dark, the pigs glow green

In daylight, their eyes and skin are green-tinged

http://news.bbc.co.uk/2/hi/asia-pacific/4605202.stm
Fluorescent zebrafish

daylight

UV light
Regulation of gene expression in gene therapy
Successful gene therapy in the clinical setting

- should provide very tight regulation of gene expression

- the regulation should to be achievable using a compound that is nontoxic

- is able to penetrate into the desired target tissue or organ

- it has a half-life of a few hours (as opposed to minutes or days) so that when withdrawn or added (depending on the regulatable system used) gene expression can be turned “on” or “off” quickly and effectively

- the genetic switches should be nonimmunogenic in the host
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Mammalian expression plasmid

pCMV/GFP

- For expression in cells
- CMV Promoter
- cDNA
- SV40 Promoter
- Neomycin resistance
- Ampicillin resistance
- Bacterial origin of replication

GFP

To determine transfection efficiency

Polyadenylation site

To generate stable cell line

pUC

For amplification of the plasmid in bacteria

Bacterial origin of replication
Promoters

Constitutive

Viral
- SV40
- CMV

Eucaryotic
- \( \beta\)-actin
- Cell-type specific
  - tumor-specific – promoters of genes which are expressed mostly in tumors but not in the healthy tissues

Inducible

SV40 - Simian virus 40
CMV – human cytomegalovirus
Inducible promoters

Natural Physiologically regulatable

Regulated by antibiotics
- Doxycycline-regulated
- Tetracycline-regulated
- Rapamycin-induced

Regulated by hormones
- progesteron
- ecdyson

synthetic Complex (chimeric)
Hypoxia inducible factor – a crucial mediator of hypoxia-induced gene expression

- **Erythropoiesis & iron metabolism**
  - Erythropoietin
  - Transferrin
  - Transferrin receptor
  - Ceruloplasmin
  - Heme oxygenase-1

- **Vasomotor control**
  - NOS II

- **Cell proliferation & viability**
  - IGF-1, IGFBP-1&3
  - TGFβ3, NOS II

- **Angiogenesis**
  - VEGF
  - VEGFR-1

- **Energy metabolism**
  - GLUT1,2 & 3
  - PEPCK
  - LDH A
  - PGK3
  - Aldolase A & C
  - PFK L & C
  - Pyruvate kinase
  - Enolase

**O**

**HIF**
Activation and degradation of HIF-1α

Mole et al., IUBM, 2001
HIF-1α

HYPOXIA

O2 down

PROLYL HYDROXYLASES

2-OXOGLUTARATE

CO2

SUCCINATE

NORMOXIA

HIF-1α

stabilization

e.g. VEGF

proteasomal degradation

OH OH

pVHL
HIF-1 binds to hypoxia response element present in regulating regions of many genes

HBS - consensus HIF-binding site
HAS – HIF ancillary sequence
HRE – a hypoxia regulated sequence

phosphoglycerate kinase 1

OBHRE - optimized hypoxia response promoter

OBHRE promoter *in vitro*

Hypoxia

Normoxia

OBHRE promoter *in vivo*

Hypoxic Tumour

Normoxic Liver
Tumor Specific Promoters are active specifically in tumor cells, such as hepatocellular carcinoma, pancreatic cancer, prostate cancer, melanocytes... They consist of a single fragment from the 5’ region of a given gene. Each of them comprises a core promoter and its natural 5’UTR.

COX-2 is an inducible isoform of cyclooxygenase. Expression of COX-2 is undetectable under physiological conditions but up-regulated in many malignant tumors.

Survivin, a member of the IAP (inhibitor of apoptosis) gene family, is expressed during embryonic and fetal development but is undetectable in normal adult tissues. Survivin plays an important role in the growth and progression of a variety of cancers and was shown to be overexpressed in most common human cancers.

The alpha-fetoprotein (AFP) gene is normally expressed in fetal but not adult livers. However, about 70% of hepatocellular carcinoma (HCC) are known to overexpress AFP.

Prostate specific antigen (PSA) is a serine protease primarily synthesized by both normal prostate epithelium and the vast majority of prostate cancers.
How to regulate gene expression e.g. in the heart
HIF-regulated expression

VEGF – vascular endothelial growth factor
FGF – fibroblast growth factor

DMOG – acts as a hypoxia, activates HIF
Hypoxia-dependent promoters

Three Models of Hypoxia Switch

A

| pGL-HRE/SV40 | HRE | SV40 | Luciferase |
| pGL-HRE/MLC | HRE | MLC | Luciferase |

B

| pCEP4/HIF-1alpha | CMV | HIF-1alpha |
| pGL-HRE/MLC | HRE | MLC | Luciferase |

C

MLC-ODD double plasmid system

Transactivator plasmid
pGS-MLC-ODD

MLC
GAL4 DBD
ODD
p65 AD

Reporter plasmid
p65 AD
ODD
GAL4 DBD
pGene-Luc

6 X GAL4 UAS

Y. Tang et al. / Methods 28 (2002) 259–266
Cell-type specific gene regulation/
Regulation of gene under special conditions
Promoters

- *natural*
  - Viral
    - constitutive (broad specificity)
  - Eucaryotic
    - inducible
- *synthetic complex (chimeric)*
  - cell/tissue specific promoter
Cell specific promoters

Tissue Specific Promoters are active in a specific type of cells or tissues such as B cells, monocytes, leukocytes, macrophages, muscle, pancreatic acinar cells, endothelial cells, astrocytes, lung...

Keratin 14 – keratinocytes
Flk-1 (VEGFR-2) – endothelial cells
Tie-2 – endothelial cells
Endoglin – endothelial cells
Desmin - muscles

*Cell-specific expression can be additionally increased by using the viral serotypes targeting specific cell types*
Inducible promoters

natural

Regulated by antibiotics

- tetracycline-regulated
- rapamycin-induced

Regulated by hormones

- progesteron
- ecdysone
1. TRANSACTIVATOR – fusion protein, consists of domain which binds to promoter and domain responsible for transcription activation

2. INDUCIBLE PROMOTER – to which transactivator binds

3. INDUCER – a molecule (drug, hormone) which binds to transactivator and it changes the affinity of the transactivator to promoter
Regulatable systems for gene expression

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Tetracycline regulatable system

- tet-off
- tet-on

Tetracycline is used to switch off or switch on the expression of a gene
Several different Tet-regulatable systems

A. basic composition = two expression cassettes

B. TetOff (tTA = TetR-VP16)

C. TetOn (rtTA = rTetR-VP16)

D. KRAB TetOn (tTS = TetR-RKAB)

E. combined TetOn (rtTA + tTS)

F. autoregulatory loop TetOn (rtTA)
Regulatable systems for gene expression

1. TRANSACTIVATOR – tTA = tet repressor + VP16
   - fusion of the coding sequence of the *E. coli* Tet repressor with the carboxy terminal domain of the Herpes simplex VP16 protein, which activates a number of mammalian gene promoters, apparently by binding directly to key transcription factors involved in the formation of the RNA polymerase II preinitiation complex

2. INDUCIBLE PROMOTER

3. INDUCER – TETRACYCLINE
Tetracycline

- has been used as an antibiotic for decades and it has been well characterized in a clinical settings (doxycycline, lymecycline and minocycline)

- is nontoxic at doses required for gene activation

- is rapidly metabolized and cleared from the body, making it an ideal drug for the rapid increase in gene expression as well as rapid decrease in expression of the desired transgene

However,

because the protein was derived from bacteria, it may be immunogenic
Regulatable systems for gene expression

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2. INDUCIBLE PROMOTER

3. INDUCER – TETRACYCLINE
- based on the *E.coli* Tn10-encoded **tetracycline resistant operon**

- **Tetracycline resistance operon** consists of two genes:

a) the resistance gene **TetA** – codes for a membrane protein that exports invaded tetracycline out of the bacterial cell

b) the regulator gene **TetR (repressor)** – codes for a dimeric DNA-binding protein
Tetracycline-dependent regulatory system

Bacteria:

In the absence of tetracycline, TetR protein inhibits its own expression as well as the expression of TetA by binding to operator sequence (tetO) of the tet operon. Tetracycline or other antibiotics (doxycycline) prevent this cooperation by binding to the TetR and inducing its allosteric change.

Gene therapy:

TetR was converted to a transcriptional transactivator, called tTA, by fusing the VP16 transactivation domain of Herpes simplex virus to the C-terminus of TetR.
Tet-off system

- tet-responsive transcriptional activator (tTA) is expressed from the strong CMV promoter.

- tTA is a fusion of amino acids 1–207 of the tet repressor (TetR) and the negatively charged C-terminal activation domain (130 amino acids) of the VP16 protein of herpes simplex virus.
Tet-off system

- PTS – tissue or cell type specific promoter
- tetO - operator sequence of the tet operon
Figure 6. Change in luciferase activity is detectable within 1 hr of induction or suppression.
Mutations that convert TetR to rTetR (and tTA to rtTA)
Tet-on system

Diagram showing the Tet-on system with Pts, rtetR, VP16, rtTA, Dox, tetO, Pcmv, Target Gene, No/Little Target protein, and Target Protein.
NIH3T3 fibroblasts have been transfected with Tet-On plasmid, selected in medium with G418 and then again transfected with plasmid pBI-X which encodes reporter gene \text{LacZ}.
Modifications of Tet-dependent systems

- TetR or rTetR cDNA is regulated by CMV promoter
- TetR or rTetR cDNA is regulated by cell-specific promoter

<table>
<thead>
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<th>Table 1</th>
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<td><strong>New tissue-specific tTA (or rtTA) transgenic mice.</strong></td>
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<tr>
<th>Tissue</th>
<th>Promoter</th>
<th>References</th>
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<tr>
<td>Brain</td>
<td>Neuron-specific enolase</td>
<td>[22]</td>
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<td></td>
<td>Calmodulin-dependent protein kinase II</td>
<td>[23]</td>
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<tr>
<td>Lung</td>
<td>Clara cell 10 kDa</td>
<td>[15]</td>
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<tr>
<td>Class II positive cells</td>
<td>MHC class II</td>
<td>[25]</td>
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<td>Mammary gland</td>
<td>Mouse mammary tumor virus</td>
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<tr>
<td>Pancreas</td>
<td>Proinsulin gene II</td>
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<td>Epithelium</td>
<td>Cytokeratin</td>
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<tr>
<td>Skin</td>
<td>Keratin 14</td>
<td>[29]</td>
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<tr>
<td>Liver</td>
<td>LAP (C/EBPβ)</td>
<td>[30]</td>
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</table>

MHC, major histocompatibility complex; LAP (C/EBPβ), liver enriched activating protein (CCAAT/enhancer binding protein β).
New versions of tet-dependent gene expression

<table>
<thead>
<tr>
<th>tTA</th>
<th>Tet-off</th>
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<td>(a fusion between TetR and VP-16)</td>
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<table>
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<th>rtTA</th>
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<table>
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<tr>
<th>tTS</th>
<th>Tet-on</th>
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<tr>
<td>+ tTS</td>
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</table>

**tTS** consists of TetR fused to the KRAB repressor domain
KRAB – Kruppel-Associated Box – transrepressing domain of human Kid-1 protein
Combined use of tetracycline-controlled transcriptional silencer (tTS) and rtTA system
Features of tet-induced expression system

**Tet-on systems:**

- Older versions – a significant basal activity; fully active only at high Dox doses
- Novel versions: display a considerably lower basal activity in the OFF state

  However, tightness of the control may be partially lost at higher vector doses

**rtTA/tTS system** – significantly reduced the basal expression in vivo, provided that vector architecture was optimized

Tetracycline is rather **non-immunogenic** in mice

Dox, an analogue of Tet, is a well-documented antibiotic drug
  (used in the clinics for more than 40 years)

Dox is usually well-tolerated; can be taken orally. It can be also applied intravenously.

The bioavailability of Dox is similar, not dependent on the way of administration
The tissue penetration is excellent and includes the brain. Concentrations are the highest in liver, kidney and digestive tract, as it is eliminated primarily via urine.

**Potential problems with Dox:**

a) accumulates in bones; its slow release from bones may slow-down the silencing of a Tet-On system,

b) risk of raising resistance to antibiotics

c) can stain developing teeth (even when taken by the mother during pregnancy)
Inducible promoters

Natural

Artificial Complex (chimeric)

Regulated by antibiotics
- tetracycline-regulated
- Rapamycin-induced

Regulated by hormones
- progesteron
- ecdysone
Dimerizer-induced gene expression

Drug-binding domains

Activation domain

Dimerizer

mRNA

DNA-binding domain

Minimal promoter

Target gene

Target gene

Current Opinion in Biotechnology
Rapamycin® (sirolimus) is an immunosuppressive agent

Sirolimus is a macrocyclic lactone produced by *Streptomyces hygroscopicus*. The chemical name of sirolimus (also known as rapamycin) is $(3S,6R,7E,9R,10R,12R,14S,15E,17E,19E,21S,23S,26R,27R,34aS)$-$9,10,12,13,14,21,22,23,24,25,26,27,32,33,34,34a-hexadecahydro-9,27-dihydroxy-3-[(1R)-2-[(1S,3R,4R)-4-hydroxy-3-methoxycyclohexyl]-1-methylethyl]-10,21-dimethoxy-6,8,12,14,20,26-hexamethyl-23,27-epoxy-3H-pyrido[2,1-c][1,4] oxazacyclohentriacontine-1,5,11,28,29 (4H,6H,31H)-pentone. Its molecular formula is **C$_{51}$H$_{79}$NO$_{13}$** and its molecular weight is 914.2.

Rapamycin = sirolimus (SRL)
Sirolimus (SRL) binds to FK506-binding protein (FKBP) and then such complex binds to the mammalian target of rapamycin (mTOR)

The SRL–FKBP–mTOR complex inhibits biochemical pathways that are required for cell progression and it blocks cytokine signal transduction
two chimeric human peptides composed of ZFHD1 (the DNA binding unit) and FKBP12, and truncated cellular protein FRAP and truncated NF-kB p65 protein. In the presence of rapamycin, the ZFHD1-FKBP protein binds to the FRAP-p65 chimera to form a complex that activates 12 ZFHD1 sites in the ZFHD1-dependent promoter.
Inducible promoters

- Natural
  - Regulated by antibiotics
    - tetracycline-regulated
    - Rapamycin-induced
  - synthetic Complex (chimeric)
    - Regulated by hormones
      - progesteron
      - ecdysone
The system is activated by binding of the synthetic steroid mifepristone (RU486) to a truncated human progesterone receptor, which is fused to VP16 activation domain, an eukaryotic transactivator derived from HSV-1 and a yeast Gal4 DNA-binding domain.

**RU 486 activates the transactivator by promoting the binding of the GAL4-DNA-binding domain to its consensus elements**

doses of mifepristone used for the system activation are below the threshold required to induce abortions in women
1. **TRANSACTIVATOR – VP16-GAL4-PR-LBD**

A fusion protein consisting of the coding sequence of the yeast GAL4 transactivator protein, which is linked at its carboxy terminal end to the coding sequence of a truncated ligand-binding domain of the human progesterone receptor.

2. **INDUCIBLE PROMOTER**

3. **INDUCER – RU486**
The optimal gene regulatory system should exhibit

(a) good regulation and induction kinetics,

(b) quick response to the administration or removal of the inducer,

(c) strong transgene regulation

(d) negligible cytotoxic or inflammatory responses associated with the regulatory elements within the switch system.