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ORIGINAL PAPER

Current status of transcriptional regulation systems

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Abstract Many attempts have been undertaken to control transgene activity in mammalian cells. This is of importance for both applied biotechnology and basic research activities. State of the art regulatory systems use elements for transgene regulation which are unrelated to host regulatory networks and thus do not interfere with endogenous activities. Most of these regulation systems consist of transregulators and transregulator responding promoter elements that are derived from non mammalian origin. Apart from the tetracycline (Tet) regulated system which is most widely used for conditional gene expression at the moment, a number of new systems were created. These systems have been significantly refined and their performance makes them suitable for regulating transgenes not only in cellular systems but also in transgenic animals and for human therapeutic use.

Keywords Viral transduction · Autoregulatory expression · Tet-system · Synthetic promotor · Gene regulation

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Abbreviations

eGFP	Enhanced green fluorescent protein
EPO	Recombinant human erythropoietin
GOI	Gene of interest
IRES	Internal ribosomal entry site
lmw	Low molecular weight
pA	Polyadenylation site
P_{TA}	Transactivator dependent promoter
SEAP	Secreted form of human placental alkaline phosphatase
TAg	Simian virus 40 large T antigen

Principles of artificial transgene regulation circuits

Methods for adjusting heterologous gene expression in mammalian cells, tissues and animals provide a powerful tool for basic research (e.g. gene function analysis) and biotechnology. Biotechnological applications range from in vitro processes like drug discovery, production of biopharmaceutical proteins till *in vivo* applications as adjustable interventions in gene therapies, tissue engineering as well as transgenic animals.

Diverse transgene control modalities responsive to different types of low molecular weight (lmw) inducers have been constructed (Fussenegger 2001). These gene regulation systems are either in OFF-type (gene expression in absence of

inducer) or in ON-type (gene expression in presence of inducer) configurations. The OFF-type systems consist of a transactivator and a transactivator dependent promoter. In most cases the transactivator is a fusion protein between a lmw responsive DNA-binding moiety and a mammalian transactivation domain, often the Herpex simplex virus derived VP 16 protein. The transactivator dependent promoter consists of operator modules (sites that mediate the binding of the transactivator) adjacent to a minimal eukaryotic promoter. In the absence of regulating lmw compounds the transactivator binds to the operator sequences and the transcriptional activation domain exerts its effects on the minimal promoter. In the presence of the lmw ligands the interaction of promoter and transactivator is abolished due to conformational changes (Fig. 1A). From the ON-type systems two variants exist. One system harbours the DNA-binding moiety of a prokaryotic response regulator that binds to operator sequences and acts as a transrepressor (e.g. the T-Rex system. Invitrogen). The operator sequences are placed within a eukaryotic promoter so that in case of transregulator binding transcription is blocked. Unlike the OFF-systems the gene induction of these ON-systems is therefore due to dissociation of the transregulator from the DNA. To achieve more efficient repression and derepression this configuration was modified in a way that the transregulator was fused to a eukaryotic repressor domain, e.g. the KRAB domain of the human kox-1 gene (Deuschle et al. 1995). In the absence of regulating lmw compounds binding of the transrepressor to the operator silences the promoter driven transgene expression. The addition of lmw ligands leads to the dissociation of the transregulator and thereby

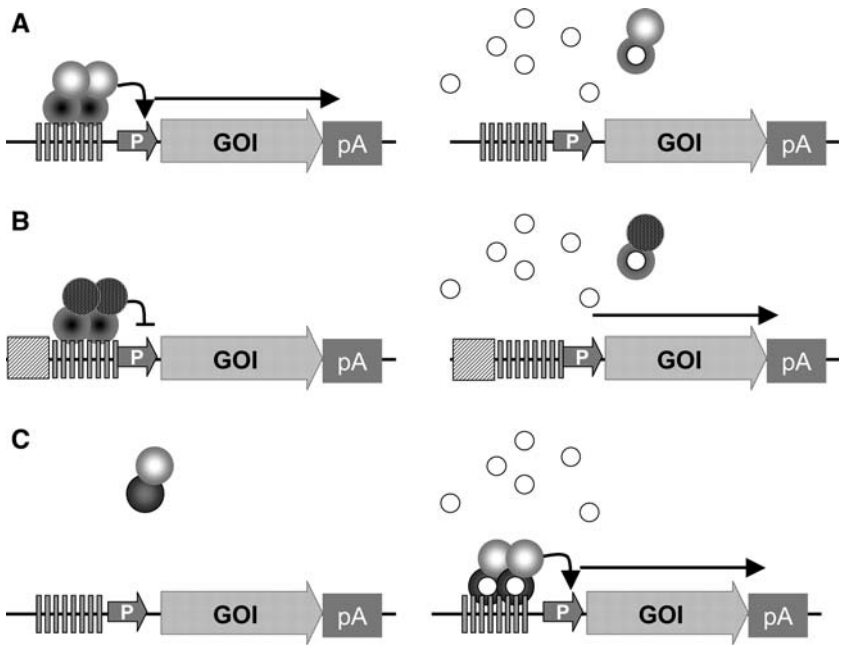

Fig. 1 Principles of on- and off-regulated transcription. The artificial expression units depicted are regulated through their promoters. The elements represent as follows: prokaryotic operator repeats are depicted by eight vertical rectangles; minimal eukaryotic promoters are symbolized by grey arrows with a P insert; eukaryotic enhancing elements are symbolized by a dotted box; the gene of interest (GOI) is shown as a grey arrow; the transcription termination elements are symbolized by pA; the hybrid transregulators are symbolized by fused balls wherein the dark-grey ball functions as the DNA-binding moiety and the light-grey ball as the activator (**A**, **C**) or repressor (**B**) domain. Low molecular weight (lmw) ligands (open circles) bind to the transregulator and modulate DNA-binding or dimerization and thereby regulate transcription of the target promoters. (**A**) OFF-system, based on a ligand-mediated dissociation of a transactivator from the operator. (**B**) ON-system, the dissociation of a transrepressor through the ligand facilitates promoter-activation by allowing the enhancer to stimulate the promoter. (**C**) ON-system, based on the ligand-dependent

induces promoter specific transcription (Fig. 1B). The second variant of the ON-systems has been developed for the Tet system. In this case, the Tet-dependent transactivator (tTA) was converted to a reverse transactivator (rtTA) which binds to the Tet operator only in the presence of tetracycline. The rtTA was created by mutation and screening in *E. coli* and *Saccharomyces cerevisiae* (Gossen et al. 1995). The fusion of this transregulator to a mammalian transactivation domain formed the basis to built the Tet-on system (Fig. 1C). More recently, an improved reverse transactivator has been introduced which shows higher affinity towards the inducer doxycycline and has a lower background activity in the uninduced state (Urlinger et al. 2000a).

The development of artificial regulation systems started 1987 with the publication of Hu and Davidson by adoption of the lac operator system from *E. coli* to mammalian cells. A non-exhaustive list of currently used systems is presented in Table 1.

The extensive use and numerous improvements have lead the Tet system to the highest refinement including a series of more stable inducers (e.g. doxycycline). Thus, in the following mainly examples obtained with the Tet system will be described. It should be noted, however, that a great potential exists if other systems are brought to the same level of performance. This will allow constructing more complex artificial networks with a precise function in mammalian cells and whole organisms.

Binary systems

The most obvious design of systems based on Tet-dependent expression is binary with two independent expression units for the gene to be

Table 1 Transcription based heterologous regulation systems

System	Operator target of regulator	Regulatory element of the transregulator	Regulating inducer	Reference
Lac I–lac0	Lac O	LacI	IPTG	Hu and Davidson (1987)
Tet-on	Tet O	Rev.tetR	Tetracycline analogues	Gossen and Bujard (1992)
Tet-off	Tet O	TetR	Tetracycline analogues	Gossen et al. (1995)
Gal4-based	Gal 4	Steroid receptor	Steroid homologues	Wang et al. (1994)
Ecdysone-based	Ecdysone responsive elements	Ecdysone receptor variants	Ecdysone analogues	No et al. (1996)
Dimerization-based	e.g. Gal4	Ligand dependent dimerization proteins (e.g. FKBP and Cyclophilin	FK506 and Rapamycin FK 506 Rapamycin	Spencer et al. (1993)
Cold-inducible expression	Alphaviral PSG promoter	Alphavirus replicase	Cold	Boorsma et al. (2000)
Temperature-inducible expression	rheO	Rhea	heat (37°C)	Weber et al. (2003)
Streptogramin-based	Plr (pristinamycin-responsive promoter)	PIP (Pristinamycin-induced protein)	Streptogramins (Pristinamycin)	Fussenegger et al. (2000)
Macrolid-based (E.Rex)	ETR	MphR	Macrolid antibiotics (Erythromycin)	Weber et al. (2002)
Gas-inducible AIR	Actylaldehyde inducible element	Alc repressor	Acetylaldehyde	Weber et al. (2004)
Nicotine-inducible NICE	6HNic-Operator	Hdno repressor	6-Hydroxy-nicotine	Malphettes et al. (2005)

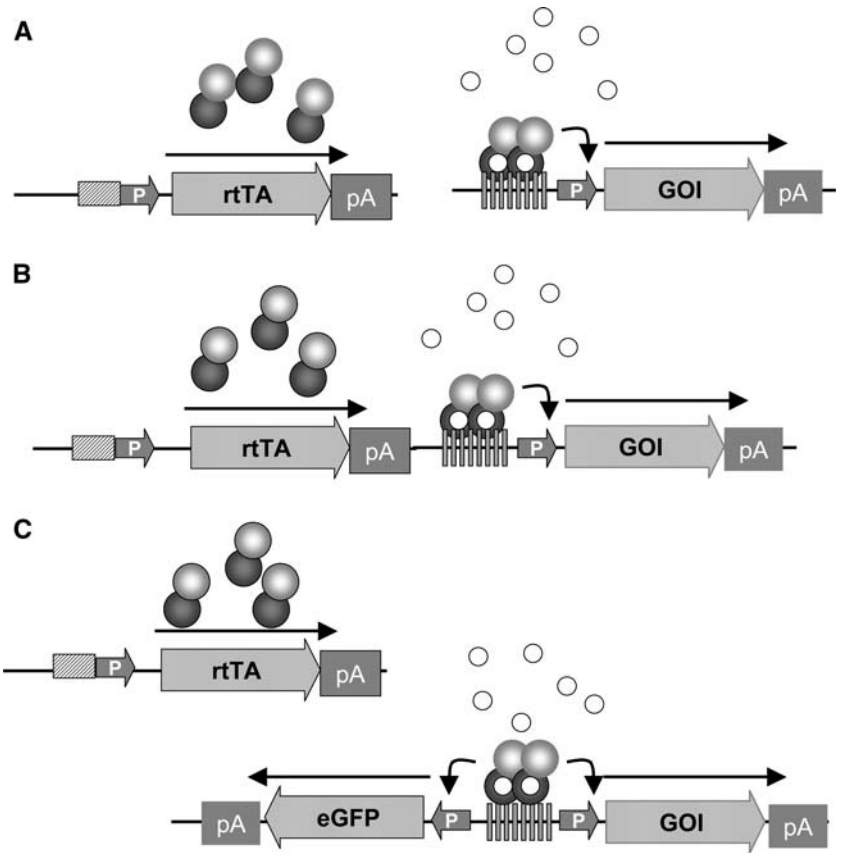

Fig. 2 Binary systems. (**A**) Cells carry independent cassettes for constitutive expression of the transactivator and for the transactivator dependent expression of the GOI. (**B**) If both expression units are encoded on a single vector, close proximity of both expression units is provoked. (**C**) Co-regulation of the GOI with a reporter gene (e.g. eGFP) via a bidirectional transactivator responsive promoter simplifies screening

regulated and for the regulatory gene (Fig. 2A). In the binary configuration one expression module harbours the constitutively expressed transactivator (e.g. tTA) while the gene of interest (GOI) is controlled by the transactivator dependent promoter (P_{TA}) which is on a separate plasmid. In order to create a strictly controlled gene regulation system some requirements have to be fulfilled. This concerns both expression units:

Transactivator

The transactivator is essential for the induction of transgene expression. It has to be expressed at a balanced level since high levels of transactivator are often not well tolerated by cells. One possible explanation for this phenomenon is the so-called squelching effect of the transactivating domain, in particular this has been shown for the Herpes Simplex Virus derived VP16 protein. High levels of VP16 results in depletion of cellular transcription factors (Gill and Ptashne 1988). Efforts have been made to reduce the toxicity of the transactivator, either by modulating the VP16 transactivating domains (Baron et al. 1997) or by replacing the VP16 domain to transactivating domains of non-viral transactivators like p65 and E2F4 (Urlinger et al. 2000b; Akagi et al. 2001).

Transactivator dependent promoter

To obtain a high inducibility and a low basal activity the P_{TA}-GOI (transactivator-dependent promoter driving a GOI) cassette has to be integrated into a 'neutral' chromosomal site. Otherwise, nearby cellular enhancers affect the basal expression level of the P_{TA} cassette. Since most gene transfer protocols rely on random integration, screenings have to be performed to isolate cells with good regulation properties. Gossen and Bujard reported in 1992 that upon screening of HeLa cells regulation factors of 10^5 can be achieved. However, such high factors might be

restricted to certain cell lines (Howe et al. 1995; Freundlieb et al. 1999). This could be due to cell specific variations in expression of endogenous transcription factors like the GATA factors (Gould and Chernajovsky 2004).

From a practical point of view the above listed considerations require the sequential transductions of the transactivator and the GOI cassette to create a binary regulated expression module. A number of cell lines of different species expressing either the tTA and the rtTA are commercially available (http://www.clontech.com) which can help to speed up the establishment of well regulated cells. Further, direct coupling of a reporter gene (e.g. eGFP) to the GOI has been shown to fasten the screening process for regulated expression. This can be achieved—either via bicistronic arrangements (not depicted) or using bidirectional regulatable promoters (Fig. 2C).

Autoregulated expression cassettes

An attractive alternative is the expression of both the GOI and the transactivator under control of the transactivator dependent promoter.

In these so-called autoregulated systems a minimal basal expression of the transactivator in the repressed state is required. Upon induction, the transactivator activates its promoter in a positive feedback loop, thereby increasing both the transcription of the transactivator itself and the GOI. The first autoregulated system is based on the Tet-system and consists of two vectors (Shockett et al. 1995) (Fig. 3A). Transcriptionally

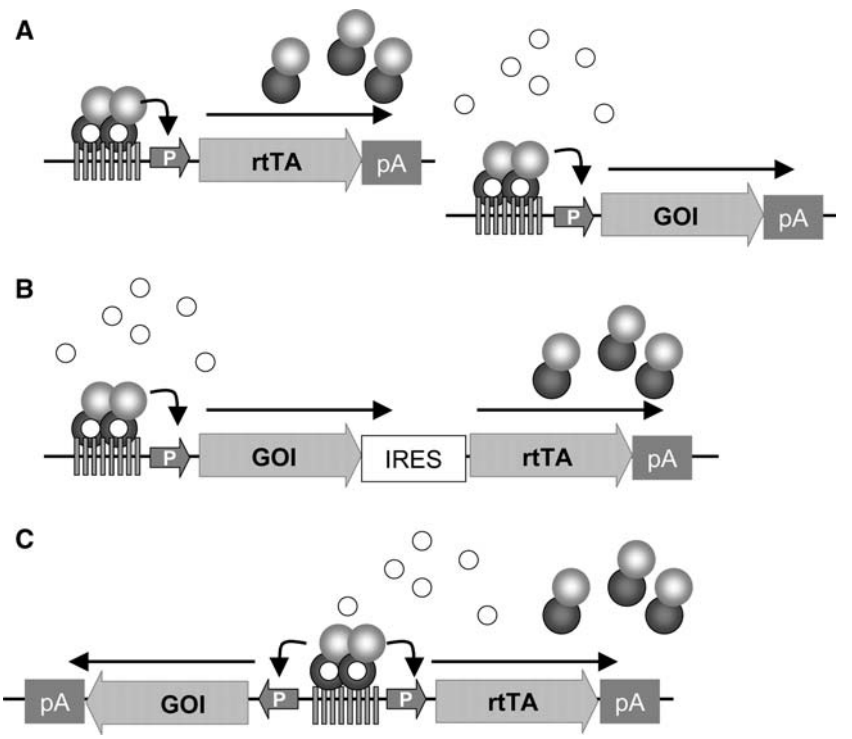

Fig. 3 Different configurations of autoregulated systems. In the uninduced state autoregulatory expression modules display a low, basal expression of both the transactivator and the GOI. Upon induction the transactivator binds to the P_{TA} and induces the expression of the GOI as well as its own expression thereby creating a positive feedback loop. (**A**) Binary configuration. This approach utilizes two vectors, one encoding the transactivator the other encoding the GOI. Both are under control of the P_{TA}. (**B**) Bicistronic configuration. The P_{TA} drives the expression of a mRNA that encodes the GOI and the transactivator. An IRES element is used to couple expression of both genes. (C) Bidirectional configuration. A bidirectional transactivator dependent promoter facilitates the expression of two opposite genes (transactivator and GOI). The autoregulated principle is exemplified with the Tet-on system

autoregulated systems were also developed for other regulation systems like the streptogramin (Fussenegger et al. 2000), the macrolide (Weber et al. 2002; Fux et al. 2003) or the acetaldehyde (Weber et al. 2004) regulation system. Autoregulated systems gained interest as they allow to generate cells with a regulatable transgene upon a single transduction step. This is a major advantage as this strategy dramatically shortens the time to establish such cell lines. Further, in clinical settings like gene therapy the compactness of the autoregulatory concept is favourable. Due to these advantages in the following the focus will be laid on the single vector approach.

To generate such positive feedback loops two set-ups have been applied.

Bi- or multicistronic cassettes

In this set-up the ligand-dependent promoter drives the expression of a mRNA that encodes the transactivator and the GOI(s) (Fig. 3B). The transactivator is usally placed in the second cistron because often the second cistron is expressed at a lower rate. However, there is no strict requirement for this set-up as strict regulation was also achieved when the transactivator was positioned in the first cistron (Fussenegger et al. 1997). The same report also demonstrated that this configuration is not restricted to two cistrons but allows the expression of up to four different genes. The expression from genes of downstream cistrons strongly depends on the strength of the used IRES elements. Unfortunately, this property is not predictable as not only the IRES as such but also the composition of the cistrons may influence the strength of IRES-mediated translation (Hennecke et al. 2001).

Bidirectional cassette

This configuration for positive autoregulation became feasible with the creation of a transactivator-dependent bidirectional promoter (Baron et al. 1995). This promoter can be used to drive the expression of two mRNAs with opposing direction, one encoding the transactivator, the other encoding the GOI (Fig. 3C). In 1999 it was demonstrated that this configuration leads to strict transgene regulation (Strathdee et al. 1999). In the meantime this concept has proven useful for a variety of applications. It was demonstrated that the autoregulated expression of Hepatitis B surface antigen and core antigen can confer immunity against Hepatitis B Virus upon DNA vaccination (Kwissa et al. 2000). Another approach made use of this autoregulatory setting to investigate the infection process of retroviruses, thereby modulating the level of the envelope gene expression (Spitzer et al. 2003). Even tumour growth can be tightly controlled in models where the expression is regulated in a Tet-dependent manner (Felsher and Bishop 1999; Jain et al. 2002; Kröger et al. 2003).

The transcriptional autoregulation proved also useful for the creation of a conditional immortalization system. Here the transactivator and the immortalizing gene—the SV40 large T antigen (TAg)—are both transcribed from the bidirectional Tet-dependent promoter. Despite the low expression level in the repressed state this conditional immortalization system allows strict control of cell proliferation (May et al. 2004a). Furthermore, the activation of this immortalizing construct had no side effects on the cell which is in contrast to the temperature-driven regulation step for the most commonly used conditional immortalization system, the temperature-sensitive TAg (May et al. 2005a). Gene expression profiling of the Tet-dependently immortalized cells revealed that about 10% of all genes were affected by immortalization. These changes were completely reversible (May et al. 2004a, 2005b). Using this system it was possible to couple stable SEAP and EPO production with growth arrest, a feature that might lead to the generation of improved producer cell lines (May et al. 2004b).

The reports on conditional immortalization are exemplary for many publications clearly demonstrating that the basal expression level of autoregulated systems is not necessarily followed by a biologic effect. Obviously, in most cases transgenes have to be expressed above a certain threshold level to exert their effects. If this threshold level is below the basal expression level of the autoregulated module this system is silent in the uninduced state.

Viral transduction systems

While the sequential transduction of the transactivator and the P_{TA}-GOI cassette is feasible in cell culture and for animals, implementation of the two step systems in primary cells and for therapeutic purposes is unfavourable. Two independent vectors to transduce the two components have been used for co-infection of primary cells (Koponen et al. 2003; Rendahl et al. 1998). Since co-infection of two vectors is frequently not efficient in many primary systems single step transfer vectors have been established. In analogy to the above mentioned design of expression cassettes two different strategies were followed: transduction of both a constitutively expressed transactivator and the P_{TA}-GOI cassette on a single vector (Fig. 2B) or transfer of the components in an autoregulatory arrangement (Fig. 3B, C).

A particular problem of single vector transfer is the close proximity of the transactivator and the P_{TA} cassette. This frequently leads to an elevated basal expression of the GOI. In this respect, autoregulatory systems were successfully established in a variety of different viruses types like γ-retroviruses, adenoviruses, adeno-associated viruses and lentiviruses. This has not been restricted to the Tet-system as other regulations systems were employed like the streptogramin (Mitta et al. 2004), the macrolide (Weber et al. 2002; Fux et al. 2003) or the acetaldehyde (Hartenbach and Fussenegger 2005) regulation system. A non-exhaustive list of the different regulation systems established in viral vectors is given in Table 2.

Regulated expression in transgenic animals

Basically, the same considerations as discussed above are also valid for the establishment of animals with regulated gene expression. Regulated systems have been established predominantly in mice but can also be expanded to larger animals like pigs (Kues et al. 2006, in press). In comparison to cells even more effort has to be made to screen animals for appropriate regulation since a certain tissue specificity is induced by the nature of the integration site. This site not only influences the level of basal expression but also the tissue specificity of expression. Ubiquitous expression was described from an autoregulated design (Shockett et al. 1995). Depending on the purpose of the transgenic animal regulated expression of the GOI might be restricted to certain tissues or developmental stages. This is required to regulate the GOI exclusively in cells of interest. Mice have been established in which the transactivator is expressed from tissue specific promoters (e.g. Mayford et al. 1996; Saam and Gordon 1999). A list of mice covering both indicator mice (P_{TA}-reporter) and mice with tissue-specific expression of the transactivator can be obtained elsewhere (http://www.tet-systems.com.).

Graded versus digital induction characteristics

An important aspect of transcriptionally regulated systems is the ability to adjust the transgene expression to a defined level. The binary set-up (transactivator constitutively expressed, transgene

Table 2 Single step viral vectors

Virus vector type	Constitutitve	Autoregulated
Adenoviral	Mizuguchi and Hayakawa (2002), Mizuguchi et al. (2003), Xu et al. (2003), Tietge et al. (2003)	Mizuguchi et al. (2003), Unsinger et al. (2004), Gonzalez-Nicolini and Fussenegger (2005)
Adeno-associated	Jiang et al. (2004)	Chtarto et al. (2003), Agha-Mohammadi et al. (2004), Fitzsimons et al. (2001)
γ-retroviral	Hwang et al. (1996), Paulus et al. (1996), Iida et al. (1996)	Hofmann et al. (1996), Unsinger et al. (2001), Kuhnel et al. (2004)
Lentiviral	Kafri et al. (2000)	Reiser et al. (2000), Vigna et al. (2002), Mitta et al. (2004), Hartenbach and Fussenegger (2005), Markusic et al. (2005)

regulated; see above) of regulated systems differs from autoregulated systems (transactivator and transgene regulated; see above) in this respect. In systems in which the transactivator is constitutively expressed a gradual increase of the inducer concentration leads to a gradual increase in transgene expression (Kringstein et al. 1998). In contrast, autoregulated cassettes behave different. It was demonstrated that autoregulatory systems do not exhibit such a behaviour. Rather, submaximal inducer concentrations lead to two distinct cell populations—an expressing and a non-expressing one. With the stepwise increase of the inducer concentration the ratio between expressing and non-expressing cells is changed without affecting the expression level (Becskei et al. 2001; May et al., unpublished results). This transcriptional response can therefore be refered to as a digital expression pattern. Importantly, this phenomenon is only detected upon single cell analysis (e.g. using eGFP or histology), because gene expression analysis in lysates will even out such differences.

This regulation pattern of the autoregulatory cassettes is independent of the transactivator, the set-up or the transduction method used (May et al., unpublished results) and is conserved from prokaryotes to lower eukaryotes up to mammals. As feedback loops are common concepts of natural networks efforts have been made to describe these modules mathematically. These studies unravelled that positive autoregulatory loops by itself create this transcriptional response (for review see Rao et al. 2002; Kaern et al. 2005). The molecular basis for this digital expression pattern are stochastic events in gene activation. Although stochasticity in gene activation has been previously considered to be unfavourable it has been shown recently that it is advantageous in certain settings. Molecular processes that take place during development or cell differentiation are often based on stochastic effects. E.g. during the differentiation of the hematopoietic system heterogenic cell populations are created from a homogenous isogenic population. This is likely to occur through stochastic activation of defined transcription factors (Hoang 2004). In addition, stochastic effects have also been implicated in the development of diseases like the formation of cancer (Magee et al. 2003).

Investigation of effects of stochastically expressed genes is challenging as the underlying molecular basis/network for digital or graded expression are complex and often not well understood. Cells are generally equipped to generate both digital and graded expression. For example the dissection of the network influencing the Gal1 promoter in yeast revealed that different transcriptional activators and repressors generate different transcriptional responses from the Gal1 promoter (Biggar and Crabtree 2001). An artificial system consisting of a Tet-dependent transactivator and a Tet-dependent repressor yielded a digital expression response through competition of both components for the same DNA motif. In contrast, both components alone produced a graded transcription response (Rossi et al. 2000). In addition the combination of both the repressor and the transactivator resulted in a regulation system which showed higher induction rates than systems which relied only on one of the components (Rossi et al. 1998). On the other hand studies using p53 elucidated that this transcription factor can elicit different transcription responses on different target promoters (Lahav et al. 2004; Joers et al. 2004).

Autoregulatory systems seem to be an ideal tool to elucidate the effects of stochastic gene expression. They reduce the complexity of natural occuring networks and allow the generation of synthetic stochastic expression units. These units could also serve as a basis for the creation of larger synthetic networks which could be used for tissue engineering approaches. A first attempt to create a more complex network employed two sets of transactivators with different binding specifities towards different operator sequences. This property was used to control two transgenes through adjusting the inducer concentration (Baron et al. 1999). Another study proved that also the combination of different regulation systems is feasible. This was used to create an expression circuit which stringently controlled a suicide gene (Imhof et al. 2000). Such a strategy with different regulation systems was also employed for the creation of a mammalian toggle switch (Kramer et al. 2004) and a hysteretic switch (Kramer and Fussenegger 2005). Especially the use of viruses for transduction of

autoregulatory expression cassettes should expand the range of cells for the analysis of stochastic expression and for the creation of stable synthetic gene circuits in mammalian cells.

Conclusion

In the future, more therapeutic interventions will make use of regulated systems. Thus, these systems will most likely play a significant role in the further development of tissue engineering. Examples in animals as disease models have shown the excellent performance of regulated transgene expression. Further, synthetic biology will rely on such artificial regulatory networks.

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