universität wien

DISSERTATION

Titel der Dissertation

„Regulated gene expression in encapsulated cells“

Verfasserin

Mag.rer.nat. Viktoria Ortner

angestrebter akademischer Grad

Doktorin der Naturwissenschaften (Dr.rer.nat.)

Wien, 2012

Studienkennzahl lt. Studienblatt:	A 091 490
Dissertationsgebiet lt. Studienblatt:	Molekulare Biologie
Betreuerin / Betreuer:	Dr. Erwin Heberle-Bors

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1. ABSTRACT

The heat shock response is the most prominent reaction of cells to different stressful conditions. As a result, the cells strongly induce the promoters of heat shock proteins to cope with the stress. This property is the basis for an application of such promoters in therapeutic gene expression. The aim of this work was to establish a system for heat inducible expression in encapsulated cells.

A newly designed artificial heat shock promoter (HSE-promoter) was characterised for its application in regulated gene expression in detail. First its inducibility was tested in different cell lines and compared to other heat responsible promoters. In order to analyse the promoter in more detail a stable cell line was generated. Different established heat shock inducers were tested for their ability to activate the artificial HSE-promoter and compared to induction of endogenous heat shock promoters. In addition, detailed expression kinetics of both the artificial and the endogenous promoter were performed. By this means it was demonstrated that the artificial HSE-promoter leads to higher reporter gene expression with lower background levels in different cell lines. Furthermore, the artificial promoter responded almost exclusively to heat and not to other natural triggers like hypoxia. Cell survival after heat induction was analysed and resulted in a non-significant decrease in viability. These superior properties make this promoter an ideal tool in gene or cell therapy applications.

Cell therapy provides a strategy to administer genetically modified cells to patients. When incorporated into cellulose sulphate microcapsules, these cells are protected from the host immune system and remain localised to the area of implantation. Thus even heterologous cells survive in the patient and can produce therapeutic substances over several months in the target tissue. In order to externally regulate the expression of the therapeutic gene, the heat inducing properties of magnetic nanoparticles were applied. Therefore the cells were exposed together with magnetic nanoparticles to an alternating magnetic field. The experiments revealed a tight regulation of the HSE-promoter over several orders of magnitude by variation of nanoparticle concentration, induction time and magnetic field strength. After encapsulation of the cells together with magnetic nanoparticles a first proof-of-principle *in vitro* was performed. The magnetic field induced the cells to express high levels of the marker gene luciferase. Taken together it was possible to develop an inducible expression system for encapsulated cells tightly controlled from the outside.

2. ZUSAMMENFASSUNG

Der Hitzeschock Signalweg ist der wichtigste Abwehrmechanismus von Zellen gegen verschiedene Stressfaktoren. Dabei werden hauptsächlich Hitzeschockproteine produziert, die ein wichtiger Faktor beim Überleben sind. Die Produktion dieser Proteine wird über hoch-induzierbare Bereiche in ihren Promotoren gesteuert, den sogenannten Hitzeschock Elementen (HSE). Diese Eigenschaft macht den Promoter zu einem hervorragenden induzierbaren Expressionssystem für therapeutische Proteine. Ziel dieser Arbeit war es eine Methode für Hitze regulierbare Expression in verkapselten Zellen zu etablieren.

Dafür wurde ein künstlicher Hitzeschock Promotor (HSE-Promotor) verwendet und genau charakterisiert. Der Promotor wurde in verschiedenen Zelllinien getestet und auch mit anderen Hitze-induzierbaren Systemen verglichen. Zur genaueren Charakterisierung wurde eine stabile Zelllinie mit integriertem HSE Promotor hergestellt. Mit dieser Zelllinie wurde getestet, ob der Promotor auf verschiedene Stress-Faktoren reagiert und wie diese Reaktion mit dem natürlichen Signalweg vergleichbar ist. Es konnte gezeigt werden, dass der HSE Promoter sehr hohe Produktionsraten erreichen kann, wobei ohne Aktivierung kaum Expression zu sehen war. Zusätzlich reagiert der HSE Promoter zwar stark auf Hitze, nicht jedoch auf viele andere natürliche Stressfaktoren, was ihn zu einem idealen Kandidaten für eine Anwendung in der Gen- und Zelltherapie macht. Auch das Überleben der Zellen nach Stressbehandlung zeigte kaum eine Beeinträchtigung.

Bei der Zelltherapie können genetisch modifizierte Zellen an Patienten angewendet werden. Verkapselung schützt diese Zellen vor dem Immunsystem, daher können die heterologen Zellen im Patienten überleben und Wirkstoffe produzieren. Allerdings fehlen bis jetzt effiziente Methoden, um diese Wirkstoffproduktion zu regulieren. Wir haben ein induzierbares Expressionssystem auf Basis eines artifiziellen Hitzeschock Promotors entwickelt. Durch Verkapselung der Zellen zusammen mit magnetischen Nanopartikeln kann die notwendige Erwärmung in den Kapseln durch ein alternierendes Magnetfeld erreicht werden. Um das System zu etablieren wurde die HSE Zelllinie mit magnetischen Nanopartikeln gemischt und durch ein magnetisches Wechselfeld aktiviert. Dabei konnten optimale Bedingungen für die Proteinexpression gefunden werden und zusätzlich eine Regulation dieser Produktion durch verschiedene Magnetpartikel-Mengen, Induktionszeiten und Magnetfeldstärken erreicht werden. Um einen Proof-of-Principle für das System zu erbringen, wurden die Zellen gemeinsam mit den Magnetpartikeln verkapselt und im Magnetfeld induziert. Dieser Versuch zeigte eine hohe Proteinproduktion durch die Induktion im Magnetfeld. Somit konnte der Erfolg dieser neuartigen Methode gezeigt werden.

3. INTRODUCTION

3.1. GENE AND CELL THERAPY

In the last decades, gene and cell therapy manifested as milestone in the treatment of both, inherited and acquired disease. With this approach the problems of a wide range of absent, misexpressed or deregulated gene products can be compensated by introducing genetic material with a therapeutic effect. In principle there are two different ways to manipulate the dysfunctional pathways. First, one could deliver genetic material encoding for the absent protein directly to the target tissue. This process is called gene therapy as genes or partial DNA sequences are used. Cell therapy represents another approach where cells are manipulated to produce the therapeutic substance and the cells are then transferred to the site of action. In both cases the natural pathways are bypassed by synthetically generated proteins or peptides.

3.1.1. GENE THERAPY

In principle, gene therapy is the delivery of genetic material to cells of the patient. In the simplest case, this information codes for a missing or mutated protein like in most of genetic diseases. Apart from this, the genetic information can also contain information for artificial proteins, peptides or RNA. This kind of therapeutic technique is mainly used in cancer treatment, in order to interfere with tumour cell pathways. All current gene therapy approaches target somatic cells and do not interfere with inheritance. Gene therapy can be classified by the kind of transport to the tissue. DNA can be introduced as plasmids or by modified viral vectors (Emery 2004).

3.1.1.1. Nonviral gene delivery

Naked DNA has to be somehow protected from degradation and guided into the cells. This way to the transcription site in the nucleus is paved with barriers such as the cell or nucleus membrane. DNA has to overcome certain defence mechanisms like nucleases or immune responses and finally has to be capable of expression without silencing by methylation or simple dilution in dividing cells. Because of these problems, the efficiency of naked DNA transfer is reduced.

In the last years, several different techniques arose like packaging of the DNA into so called liposomes, integration into matrices or carrier particles like nanoparticles (reviewed in (Al-Dosari et al. 2009). One way to protect and guide DNA is the enclosure with cationic lipids (Felgner et al. 1987; Wasan et al. 2000) or polymers like polyethylenimine (PEI) (Boussif et al. 1995). The negatively charged DNA is able to form colloidal complexes with the positively charged lipids, the so called lipoplexes, or to form polyplexes, when polymers are used. Thus, the cellular uptake and the intracellular delivery are enhanced by electrostatic interaction with the glycoproteins and proteoglycans in the cell membrane or by endocytosis. In addition, the genetic information is protected from enzymatic digests. The transfection efficiency of cationic lipids is strongly dependent on the geometric structure of the positively charged lipid, the number of charged groups per molecule, the nature of the lipid anchor and the linker bondages. Of equal importance is the DNA-lipid ratio (Wasungu et al. 2006) and the characteristics of the so-called helper- or co-lipids such as cholesterol or

dioleoylphosphatidylethanolamine (DOPE). Employment of the co-lipids results in less toxicity but reduced delivery rate. Lipoplexes comprise also the problem of lipid particle accumulation in the important routes of transport like the blood stream (Liu et al. 1997) and the rapid clearance from circulation. Introduction of neutral polymers such as polyethylene glycol (PEG) can slow down clearance (Harvie et al. 2000) but, like DOPE, reduces the transfection efficiency. Similar to lipid-based vesicles also polymers are used for gene delivery of which PEI is most commonly employed. This polymer shows low to medium toxicity at lower molecular weight (<25 000 Da) and in linear form, in opposite to high molecular weight or branched appearance (Fischer et al. 1999; Gosselin et al. 2001). PEI was first used for DNA delivery in 1995 (Boussif et al. 1995) and leads to release of the genetic material into the target cell via endosome disruption by the so-called proton sponge effect (Akinc et al. 2005). Nowadays, also other polymers like aminoesters or oligoamines which are polymerised by disulfide linkers or polyamino acid derivates are established, which present higher biocompatibility (Park et al. 2006).

Another nonviral delivery approach is the coating of inorganic nanoparticles with DNA. These particles range from 10-100 nm and are most of the times compared of metals like iron, gold or silver, inorganic salts or ceramics (Sokolova et al. 2008). According to their small size, these particles are able to enter the cell via specific membrane receptors and show low toxicity and no immune response (Davis et al. 2007; Cai et al. 2008).

Beside chemically-based vectors also mechanical forces are used to introduce the genetic material leading to spatially controlled gene delivery. The first approach is to simply inject the DNA close to the cells (Wolff et al. 1990) or to use jet injection (Wendell et al. 2006). This transfer mechanism is very simple and lacks problems with tissue and cell barriers or targeting as the genetic material is introduced into the cell by physical damage with needle injection or generation of pores in the target cells caused by the high-speed ultra thin liquid DNA stream of the jet injection. Another transfer method first established in plants is the gene gun (Klein et al. 1992), where DNA-coated heavy-metal particles are accelerated by highly pressured gas to high velocity and the delivery is based on the impact of the particles in the tissue. Because of the direct interaction with the cells, these methods are predominantly used for targeting skin as well as for immunizations (Song et al. 2000; Goudy et al. 2008). However they also comprise the potential of localized tissue damage like edema, pain or bleeding.

Another mechanism to introduce DNA into cells is the temporal disruption of the cell membrane with an electric field, the so called electroporation or the application of ultrasound resulting in heating and temporary increase in membrane fluidity. Transport of DNA into the cells is guided by electrophoresis in the established electric field, but this technique is limited by the accessibility of the target tissue to the electrodes used. Sonoporation could be improved by contrast agents (Bekeredjian et al. 2005b) or the use of microbubbles (Endoh et al. 2002) which are air-filled bubbles in a range of 1-6 µm with a vesicle membrane of polymers or phospholipids. These bubbles enhance the ultrasound action by release of local shock waves after ultrasound treatment and a resulting disruption of cell membranes and an increase of DNA delivery rate. Ultrasound-mediated delivery in general is safe, non-invasive and reaches internal organs without any surgery. Targeted delivery could also be optimised by including site-specific ligands (Bekeredjian et al. 2005a).

Based on the different nonviral techniques used for gene delivery it is possible to target the delivery by including tissue or cell specific recognition factors. Constructing nonviral delivery systems is less complicated than viral vector design, enables the transfer of large DNA

molecules and implies the possibility of repeated administrations. Nevertheless, there are certain disadvantages using plasmid DNA for gene therapy. First of all, the efficiency of naked DNA transfer is lower than using viral packaging strategies. Second, the gene expression is only temporary, as the genetic information is not integrated into the host genome.

3.1.1.2. Viral gene delivery

As viruses are the natural way to introduce foreign genetic information into a cell, this system was adopted for gene therapy. The infection of a cell with a virus most of the time leads to a massive production of new virus particles and subsequent activation of the immune system or to disease. Therefore, the first step was to reduce the potential danger of virus infection by creating recombinant versions which harbour the potential to infect cells without replication properties and to transport the artificial therapeutic genetic information into the cells. Today, four different virus families are used for this approach, the recombinant retrovirus vectors, recombinant adenovirus vectors, lentiviral vectors or adenovirus-associated viral (AAV) vectors. They can be divided into two major groups, the integrating vectors (retrovirus, lentivirus and AAV) and the none integrating (adenovirus, herpesvirus), depending on their potential to stably integrate genetic information into the host genome.

Recombinant retrovirus based vectors are one of the most commonly used delivery systems for gene therapy. The natural retroviruses, like the oncoretroviruses (Moloney Murine Leukemia virus) or the spumaviruses (human foamy virus) are single stranded RNA viruses with a genome of 7-10 kb coding for three essential domains, the structural proteins (*gag*), the coding regions for reverse transcriptase and integrase (*pol*) and the region coding for envelop glycoproteins (*env*). For their use as gene therapy vectors, the region coding for the structural capsid proteins (*env*) is separated from the regions for replication (*pol*) and structural proteins (*gag*) and both parts are introduced into cell culture cells to generate so-called packaging-cell lines (Nolta et al. 1990). In the vector, gag, pol, and env are replaced by the therapeutic gene but the packaging signal Ψ and remaining sequences like the long terminal repeats (LTR) are still present. By introducing the vector into the packaging cell line, the primary transcript of the therapeutic gene, is expressed. As this transcript contains the packaging signal, it is guided to the cell membrane and new virus particles are produced. These recombinant particles consist of the therapeutic transcript and proteins for replication, integration or structural proteins provided by the packaging cell line. The recombinant retrovirus now has the capacity to infect cells and transfer genetic information into the host genome, but without the possibility to replicate and infect other cells. By manipulating the envelop proteins, these vectors can be targeted to specific cells or tissues. Retroviral delivery systems integrate normally as one unit into the host genome and therefore stable transduction is provided also in highly replicating cells without chromosomal rearrangements. In addition, gene transfer is efficient and retroviral vectors are considered as safe for clinical trials (Kohn et al. 2003). On the other hand, retroviral vectors show differences in gene expression levels and sometimes they are also silenced, which might be due to the chromosomal region of integration, by the so-called chromosomal position effect (Emery et al. 1999; Emery et al. 2000). As for all integrating viruses the problem of insertional mutations due to random integration of the genetic information is a main disadvantage. In addition, the recombinant vector envelope is built of glycoproteins and is therefore relatively fragile compared to other viruses with capsids. Another major disadvantage results from the restriction of retrovirus infection to dividing cells like hematopoietic cells, as they need a breakdown of the nuclear membrane caused by cell division to deliver the genetic

information to the site of transcription and therefore non-dividing cells are excluded from this type of gene delivery system.

Another integrating virus of the retrovirus family, the lentiviruses, are the basis of an upcoming generation of gene delivery vectors. In addition to the common retroviral proteins gag, pol and env, lentiviruses like the human immunodeficiency virus (HIV) contain in their single stranded (ss) RNA genome an additional set of proteins, tat, rev, nef, vif, cpu and vpx (Frankel et al. 1998). These proteins enable the virus to form a so-called preintegration complex in the cytoplasm of infected cells, followed by active transport into the nucleus. Because of this special feature, lentiviruses can also integrate genetic information in non-dividing cells (Lewis et al. 1992; Bukrinsky et al. 1993). In lentiviral vectors, most of the viral genes are removed by the therapeutic gene but sequences responsible for preintegration complex formation and nuclear import are still present (Kafri et al. 1997; Zufferey et al. 1997). To increase safety and efficiency of the lentivirus based vectors, several improvements like insertion of a central polypurine tract (Follenzi et al. 2000; Zennou et al. 2000), the woodchuck post-transcriptional regulatory element (WPRE) (Zufferey et al. 1999) or the use of SIN (self-inactivating) vectors (Miyoshi et al. 1998; Zufferey et al. 1998) have been established. Similar to the other retrovirus based vectors also lentiviral based gene delivery systems harbour the problem of non-specific integration into the host genome

Beside the RNA virus based gene delivery vectors, also DNA based viral vectors are used. Among them, the adenovirus based vectors are the most extensively and first used systems for gene therapy approaches. Adenoviruses are double-stranded (ds) DNA viruses with a large linear genome of ~36 kb coding for several regulatory and structural proteins, including the glycoproteins for the viral capsid. The genetic information can be transduced to dividing and non-dividing cells by entering via receptor-mediated endocytosis (Bergelson et al. 1997; Tomko et al. 1997). However, the DNA is not integrated into the genome but is present as an episome, which can be lost, when cells have a high division rate. Recombinant adenoviral vectors use only specific viral regulatory elements like the packaging signal or the inverted terminal repeats and combine them with the therapeutic gene (Chamberlain et al. 2003). In addition, there is an advanced vector available, where all viral genes are deleted, the so-called gutless vector. Like in retroviral vector production, the recombinant adenoviral vector was transfected together with a helper plasmid in a packaging cell line. The helper plasmid harvests most of the adenoviral genome information except the information for replication (E1, E3). In contrast, the packaging cell line provides the information for the viral protein E1 and so a replication deficient adenoviral virus particle containing only the therapeutic information can be produced. The recombinant adenoviral vectors possess a wide tropism, high expression levels and the possibility to transfer large therapeutic genes to the target cells. In contrast to the retroviral vectors, adenoviral vectors do not integrate into the host genome and therefore lack the problem of insertional mutagenesis (reviewed in (St George 2003)). The expression of the transgene delivered by recombinant adenoviral vectors is observed for a relatively short period (5-20 days) (Dai et al. 1995) and might be linked to the strong immune response of the host system to the capsid proteins. Even recombinant adenoviral vectors need the viral capsid proteins and therefore host cells react with cytotoxic T-cells (Kafri et al. 1998). This major immune response raises problems for a repeated administration of adenoviral vectors for gene delivery, which is necessary as the transgene is not integrated but it is located on an episome.

To overcome the problem of the major immune response, other DNA virus vectors were established. One of them is the adeno-associated virus (AAV), which is an ssDNA virus of the parovirus family. This capsidated virus has a genome of ~ 5 kb coding for two proteins,

rep for replication and integration and cap for the structural proteins flanked by two inverted terminal repeats. This virus has a wide cell tropism and showed weak immunogenic capacity. AAV enters the cell via clathrin coated pits and endosomal disruption via acidification (reviewed in (Ziello et al. 2010)) but is not able to replicate and exit the cell, so it needs the help of other viruses like adenovirus (Berns et al. 1996). When AAV is used as a gene therapy vector, the two genes rep and cap are replaced by the therapeutic gene and this vector is then transferred into a packaging cell line where rep and cap and additionally also some important adenoviral genes like E1and E2 are expressed (Xiao et al. 1998). In the target cells the help of the adenoviral proteins is missing and therefore the virus can no longer replicate or exit the cells. Natural AAV has the possibility to integrate on specific sites at chromosome 19 through the rep protein, but lacks this site specific integration in recombinant vectors lacking the rep gene (Kotin et al. 1990). Beside the site specific integration, AAV has also the tendency for homologous recombination (Miller et al. 2002). AAV does not always integrate into the host genome as it also exists as an episome, dependent on different factors like the cell type. Due to the small size of the virus genome, only small therapeutic genes up to 4.5 kb can be delivered. In addition, the rep protein and also some of the adenoviral helper proteins are cytotoxic and cytostatic and so the production of recombinant AAV viral vector in packaging cell lines is limited. Recombinant AAV based delivery systems sometimes bare the risk of insertional mutations and are also associated with small chromosomal rearrangements (Miller et al. 2002) which is a disadvantage for therapy approaches.

Recombinant viral gene delivery vectors with a wide range of cell tropism are the first choice for gene therapy, but there is also the idea of using viral vectors targeting specific tissues. The most prominent virus for tissue or cell type restricted gene delivery is herpes simplex virus (HSV). This ds DNA virus has a ~ 150 kb linear genome with approximately 90 genes, flanked by inverted repeat sequences. Among the genes there are two subgroups, the essential genes for virus growth and the nonessential genes, which are for example required for virus-host interactions (Roizman et al. 2007). The HSV genome is surrounded by an icosahedral capsid, the tegument, which contains structural and regulatory proteins, and the outer envelope, built of 13 glycoproteins. HSV infects cells by binding of the glycoproteins, enters the cells via endocytosis (Nicola et al. 2004) or by fusion of the virion at the plasma membrane (Satoh et al. 2008). The de-enveloped virus is then transported to the nucleus and persists as an episome in the latent phase or can undergo a lytic replication. HSV is a neurotrophic virus and can be transported anterograde and retrograde along axons and pass synapses by components of the capsid and tegument (Diefenbach et al. 2008). For the use as a recombinant gene delivery vector, the essential genes for replication are deleted from the viral genome and introduced in a packaging cell line (Todo 2002; Hu et al. 2003). HSV based vectors are highly infectious, can easily be produced, show long term transgene expression when kept in the latent phase and are specific for neuronal tissue. Therefore, this kind of recombinant vector is an ideal tool for gene therapy approaches based on neuronal diseases.

In general viruses are an excellent tool to transfer genetic information into host tissue as they have high efficiency in gene delivery, can overcome tissue specific and cellular barriers and transfer the therapeutic gene into the nucleus. On the other hand several disadvantages are known like inefficient long term expression in adenovirus based systems or insertional mutations for retroviruses.

3.1.1.3. Clinical gene therapy approaches

The possibility of generating feasible transport systems for gene delivery around 1990 and the growing understanding of genetic diseases resulted in the first clinical gene therapy trial by Blaese and Anderson (Blaese et al. 1995) in 1990. Within this approach, a retroviral delivery system was used to transfer the gene for adenosine deaminase (ADA) into T-lymphocytes of two patients with severe combined immunodeficiency (SCID). This first clinical trial showed only a temporary response in one of the two patients and a quite weak response in the other patient. Nevertheless, this trial could proof the general safety of gene transfer to humans.

In 1997, a clinical trial on the treatment of ornithine transcarbamylase (OTC) deficiency was initiated at the University of Pennsylvania. OTC deficiency is an X-linked recessive metabolic defect in the urea cycle. A deletion or complex mutation in the OTC gene leads to high levels of ammonia in the blood which can result in cerebral edema, coma and death in severe cases. The therapeutic approach was to introduce a wild type OTC gene with a recombinant adenoviral vector into the liver of the patients by injecting the vector into the hepatic artery (Batshaw et al. 1999). One patient, Jessie Gelsinger, who received a high dose of the vector, began to experience severe complications within hours after administration and died a few days later from multiple organ failure caused by a massive inflammatory response to the vector (Raper et al. 2003). Due to this unexpected tragedy, the gene therapy program was stopped at the University of Pennsylvania and also other trials at this time were halted to review the safety risks.

One year later, the group of Fisher and Cavazzana-Calvo reported the first success in a gene therapy approach by treating X-SCID, an X linked severe combined immune deficiency in 10 infants (Cavazzana-Calvo et al. 2000). X-SCID is caused by a deletion in the gamma-chain of a receptor on maturating immune cells, resulting in severe defects in differentiation of T-cells, B- cells and natural killer (NK) cells. The group of Fisher used a recombinant retroviral vector containing the interleukin-2 receptor gamma chain and transduced the patient´s bone marrow cells. In nine out of the ten treated infants, a reconstitution of 100 % was observed and patients developed a relatively normal T-cell repertoire and good NK- and B-cell function (Cavazzana-Calvo et al. 2000). Nevertheless, two patients developed leukaemia which was based on an integration of the viral vector in the promoter region of the proto-oncogene LMO-2, resulting in overexpression of LMO-2 and other oncogenes. They were subsequently treated with a standard therapy for leukaemia and respond well (Hacein-Bey-Abina et al. 2003). Although the initial trial showed an amazing success in treatment of X-SCID, the random integration of the vector with fatal consequences again showed that gene therapy approaches lack the overall safety, which is necessary for treatment of patients.

In the following years, gene therapy research was curbed by these two distressing events and since then, many improvements on vector safety and trial design were made. Today, over 2000 gene therapy approaches are in clinical trials. Most of them are based on viral drug delivery using either adenoviral vectors or retroviral vectors (Edelstein et al. 2007). In the last years, the primary targets of gene therapy shifted from monogenetic diseases to the treatment of more complex disorders like cancer. The first gene therapy is provided by SiBiono GeneTech, which achieved approval in China in 2004 for treatment of head and neck cancer with an adenoviral vector encoding the tumour suppressor p53, but little informations about the therapy are available (Guo et al. 2006). In Europe, Amsterdam Molecular Therapeutics filed for approval with the EMA (European Medical Agency) in the

beginning of 2010. They use an AAV vector harbouring the human lipoprotein lipase gene to cure lipoprotein lipase deficiency (reviewed in (Sheridan 2011)). In June 2012, this approach was approved as first gene therapy in Europe or the US by the EMA (and will respond to the whole gene therapy community.

3.1.2. CELL THERAPY

In contrast to gene therapy, where genetic information is introduced into host cells by different viral or non-viral delivery systems, cell therapy uses autologous or heterologous cells which can be genetically engineered ex vivo, selected for their specifications or just expanded and then transferred into the patient. For genetically engineered cells, this method has the advantage of an extensive selection for stable cell clones producing the optimal amount of the therapeutic substance or the right specifications. Beside this selection another advantage compared to gene therapy is the fact that the patient is not exposed to viral or non-viral drug delivery systems which might cause severe problems when transferred directly into the host.

3.1.2.1. Autologous cell approach

When naive cells are used for cell therapy, they are placed at the site of action to enhance regeneration or correct the function of the tissue. The most prominent therapy using naive cells are chondrocytes for cartilage repair (Peterson et al. 2000; Robinson et al. 2000), keratinocytes and fibroblasts for burn and wound repair (Navsaria et al. 1995; Carsin et al. 2000) or Schwann cells for restoring myelin in CNS lesions (Baron-Van Evercooren et al. 1997).

All these approaches have the limitation of cell growth, as differentiated adult cells have a reduced potential to expand, even if cultivated under enhanced conditions *ex vivo.* Because of this, new approaches working with precursor cells or adult stem cell populations have been established. However, embryonic stem cell (ESC) research is limited by ethical issues and the possibility of ESC to form teratomas.

Depending on the initial problem, different precursor or stem cells are used and triggered to differentiate into the desire cell type. This differentiation either happens *in vivo* by the help of the surrounding tissue of ex vivo using several differentiation factors. Some of the new approaches using progenitor or stem cells try to enhance the generation of the suitable cell type by genetically engineering the precursors to express the triggering factors. One of the most promising attempts is the transfection of bone morphogenic protein 2 (BMP2) into human bone marrow mesenchymal stem cells (BM-MSC) to favour the *in vivo* differentiation into bone tissue. BMP2, which is already clinically approved as a protein therapy, has been shown to enhance bone formation by inducing stem cells or progenitor cells (Smoljanovic et al. 2009) in a dose dependent manner. Too high concentrations of BMP-2 lead to massive bone formation and too low amounts do not show an effect. Therefore genetically engineered MSC using transient transfection vectors like adenovirus or inducible expression systems like the tetracycline repressor (TetR) system turned out to be more potent than the standard treatments in clinical trials.

A further possible therapeutic use for genetically engineered stem cells or progenitor cells is the regeneration in cardiovascular diseases in order to treat or compensate the loss of cardiomyocytes (Haider et al. 2008). Mainly BM-MSC or myoblastic progenitor cells

expressing myogenic genes like Nox4 (Xiao et al. 2009), angiogenic genes like vascular endothelial growth factor (VEGF) (Yang et al. 2010) or factors for engraftment or cell homing (Elmadbouh et al. 2007) are used.

In addition to proliferative or differentiating factors, genetically engineered cells can also express artificial gene products or factors normally not expressed in this cell type. For example autologous hematopoietic stem cell (HSC) therapy is tested as an effective therapy for monogenetic diseases of the hematopoietic system. In clinical trials, HSC´s were engineered to express the functional genes for the treatment of storage disorders like the gene for interleukin-2 receptor (IL-2R) γ chain for the treatment of SCID (reviewed in (Fischer et al. 2000)) or the expression of the ATP-binding cassette transporter ABCD1 to cure adrenoleukodystrophy (ADA) (Cartier et al. 2009).

Another cell type used for autologous cell therapy approaches to treat several kinds of cancer is the genetically engineered T-cell approach. As these cells are part of the immune system, they naturally can act on tumour cells but are normally suppressed because of the "self "origin of tumour antigens or the low amount of antigen presented on the tumour cell. When artificial T-cell receptors (TCR) recognising specific tumour-antigens are generated and expressed in cytotoxic T lymphocytes, this leads to a specific killing of tumour-cells (Dotti et al. 2009; Schmitt et al. 2009). One major problem of tumour specific T-cells is the expression of tumour antigen in other cells or tissues of the organism which might cause a severe self immune reaction (Brentjens et al. 2010; Morgan et al. 2010a), so toxicity and safety of this approach still have to be determined. The implementation of virus specific cytotoxic T-cells or other cell types of the immune system into the clinic as a tool to treat viral infections is already in progress as here the antigen is virus specific and does not naturally appear in the host tissue (Hegde et al. 2009).

Recently it was discovered that also differentiated human adult cells can be reprogrammed by expression of four different stem cell transcription factors, Oct4, Sox-2, c-myc and Klf4 into so-called induced pluripotent stem cells (iPSC) (Takahashi et al. 2006; Yamanaka 2007; Yu et al. 2007) . The overexpression of this "stem-cell factors" is either initiated by viral gene delivery or non-viral cargo variants into the differentiated cells. The pluripotency of these induced stem cells was demonstrated in the last years by differentiation into multiple cell types like hematopoetic cells (Schenke-Layland et al. 2008), human hepatocytes (Schenke-Layland et al. 2008), neurons (Schenke-Layland et al. 2008) or cardiomyocytes (Gai et al. 2009). Therefore development of patient specific iPSC might overcome the problems of limited stem cell isolation efficiency and will probably turn out to be an excellent tool to generate genetically modified differentiated cells to treat several kinds of disorders.

Although precursor or stem cells have a high potential to work as a pool for generation of differentiated cells, they always bare the risk of residual pluripotency and the resulting risk of tumour formation. As well, the differentiation into specific cell types is still not completely understood and often results in only partial differentiation. Beside this also the survival of differentiated cells originating from precursor or stem cells is reduced *in vivo*.

Using naive or engineered autologous cells for cell therapy is ideal as these cells would not initiate an immune response. The major disadvantage of these "self-repair" strategies is the limitation of the modified cells to one patient. Treatment of any genetic disorder or disease would need an exclusive cell line for each patient, which results in enormous costs and is very time consuming. Therefore it is more efficient to use one cell type for different patients

and to reduce the immune response to these heterologous cells with well established immune suppressors.

3.1.2.2. Heterologous cell approach

The use of heterologous cells for cell therapy or transplantation has the benefit of a broader donor pool but always has to deal with the immune response to the allogenic or xenogenic cells. Nevertheless, this approach is routinely used in the clinic due to the optimisation of immunosuppressive drugs like rapamycin, cyclosporine or FK506. Most prominent heterologous cell therapy approaches are the allogenic bone marrow transplantation or transplantation of different organs like heart, kidney or lung, but none of these approaches is used for tissue engineering. In addition, also allogenic iPSC are a promising tool to generate different genetically modified cell types for many patients.

The use of most heterologous cells is in a pre-clinical state. Here engineered cell lines are used like human cell lines for pancreas regeneration expressing proinsulin with a furin-cleavable site, whereas expression of furin is regulated by a glucose sensitive promoter (Tatake et al. 2007). F3 cells (immortalised human NSC cells) expressing VEGF to repair spinal cord injury (Kalamvoki et al. 2007). Some attempts for the use of allogenic cells or cell lines to regenerate tissue are already in the clinical phase. The use of allogenic cultured retinal pigment epithelial cells from cadaveric sources to treat Parkinson´s diseases (Subramanian et al. 2002; Watts et al. 2003) or the use of the human NT2 cell line for the treatment of stroke (Kondziolka et al. 2000; Nelson et al. 2002) have shown promising results.

Cell therapy approaches based on cell lines are advantageous because these cell lines can be easily expanded, transfected and selected for their specifications ex vivo and have the potential to proliferate *in vivo*. Hence there is the risk of a growth benefit compared to the naive cells resulting in excessive amount of modified cells. In general the application and modification of heterologous cells shows the clear advantage of availability for different patients compared to the limited treatment with autologous cells and therefore reduced costs and developing time. However, the immune reaction to the heterologous cells limits their application, even if many immunosuppressive drugs are used in the clinic today. Another critical issue of cell therapy is the mobility of modified cells within the host organism which might lead to interaction with other cell types or tissue than the expected and selected once.

Taken together, different approaches using autologous or heterologous cells to regenerate tissue or to treat disorders, show high potential in pre-clinical experiments and also in clinical trials. However, still a better understanding of the underlying mechanisms and a safe and detailed manufacturing protocol is needed before they can be transferred into the clinics.

3.1.2.3. Encapsulation

The use of heterologous cells for cell therapy approaches has the clear benefit of a large pool of well characterised cell clones compared to the use of the limited supply of autologous cells. When storage diseases or inherited disorders have to be treated, un-modified autologous cells are not suitable for therapy. Nevertheless, heterologous approaches always have to deal with immune response against the foreign cells. The commonly performed down regulation of immune response by immunosuppressive drugs is the only way to overcome this problem, although even in these settings resistance or tolerance of the

immunosuppressivors can lead to rejection of the implanted cells or tissue. One way to protect allogenic or even xenogenic cells and tissues from the immune system is microencapsulation. In this technique, cells are separated from the surrounding tissue by an artificially generated semipermeable membrane. This membrane prevents large molecules, antibodies or immune cells from the contact with the encapsulated cells, but at the same time allows diffusion of low molecular weight components like nutrition, substrates or oxygen into the capsule. Due to the semipermeability also possible therapeutic products and metabolic products of the enclosed cells can be exported to the surrounding tissue. The microencapsulation of cells or tissues therefore is an excellent method to introduce therapeutic heterologous cells into an organism without activation of the immune response.

In principle microcapsules are produced by generating stable control-sized droplets of cells and polymer, which then interact with a polylinker or compelxation agent to obtain a solid microcapsule membrane around the droplet. Today different encapsulation techniques are established, the matrix-core/shell microencapsulation, the liquid-core/shell microencapsulation and the cell-core/shell microencapsulation (reviewed in (Rabanel et al. 2009)). Using matrix-core/shell encapsulation, cells are mixed with a hydrogel component as alginate and cross linked by divalent cations like calcium (Kierstan et al. 1977) or barium (Zekorn et al. 1992). The most commonly produced capsule type here are the Ca^{2+}- or Ba^{2+}-alginate microcapsules or the coated agarose capsules produced by thermal gelation (Iwata et al. 1989; Iwata et al. 1992; Yang et al. 1994). Another way to form matrix-core/shell capsules is the complexing of a polyanionic polymer (e.g. alginate) with a polycation (e.g. poly-L-lysine (PLL)) (Lim et al. 1980). In this mainly applied approach the core of the capsule is gel – like and the cells can arrange in this inner matrix, but growth and cell survival are limited by the reduced diffusion rate of nutrition and therapeutic products. In addition matrix – core/shell microcapsules are less mechanically stable than capsules of other approaches and show limited long term stability (Hoffman 2002).

To improve cell survival often liquid or semi-liquid inner cores of microcapsules are preferred. These types of capsules are generated by extrusion of a cell polymer mixture to produce small droplets which interacts with the second polymerisation agent provided in a reaction bath. The core of the capsules stays liquid or semi-liquid, while the membrane is formed at the interface of the two polymers. The membrane formation occurs either by interfacial precipitation or ionic or complex coacervation. The first method uses an aqueous cell-polymer solution co-extruded with a water insoluble polyacrylate polymer in an organic solvent. After solvent removal the polyacrylate precipitates at the interface of the droplet and forms a membrane (Sefton et al. 1987; Sugamori et al. 1989). Depending on the polyacrylate and the solvent used in this technique, possible limitations are the response of the encapsulated cells to the remaining, often toxic solvent and the reduced biocompatibility with the membrane. Nevertheless, capsules obtained with hydroxyethyl methaacrylate-metaacrylic acid (HEMA-MAA) demonstrate high mechanical stability and sufficient biocompatibility (Crooks et al. 1990; Lahooti et al. 1999). Microencapsulation by ionic or complex coacervation uses the extrusion of a cell – polyelectrolyte suspension in an ionic polymer receiving bath. At the interface ionotropic gelation or insoluble polyelectrolyte-complex formation of the two ionic polymers leads to membrane formation. Examples of this approach are the hollow alginate capsules produced by extrusion of $CaCl_2$ and carboxymethylcellulose in a sodium alginate bath (Blandino et al. 1999; Chai et al. 2004) or capsules built up of cellulose sulphate and poly-diallyl-dimethyl-ammonium chloride (pDADMAC) (Merten et al. 1991). The latter capsules display larger pores than other capsules but show excellent mechanical stability and long term *in vivo* survival (Pelegrin et

al. 1998; Dautzenberg et al. 1999; Schaffellner et al. 2005). In addition hollow core capsules can also be produced by liquefying the hydrogel core of for example Ca^{2+} - alginate beads using a chelator or the exchange by divalent or monovalent cations (Weber et al. 2004; Breguet et al. 2007).

In the last years an additional technique to shield the cells was established. Cells are directly coated with polymers resulting in thinner membranes (6 – 50 nm) than those of the traditional approaches. Thin membranes can be produced by the different layers of positively and negatively charged polymers like pDADMAC and poly styrene sulfonate (PSS) (Germain et al. 2006). Other methods for direct coating are the interaction of gaseous siliceous alkaoxide precursors with the surface of the cells or tissue, which results in a thin porous silica layer (Muraca et al. 2000; Boninsegna et al. 2003) or the interfacial polymerisation coating using different polyethylene glycol diacrylate (Cruise et al. 1999; Xie et al. 2005).

To protect heterologous cells from the environment, the properties of the semipermeable membrane are of enormous importance. This barrier has to be biologically inert, biocompatible, mechanically stable and permeable for smaller molecules, but excluding high molecular components. All these characteristics are dependent on the concentration, reaction time, temperature and type of polymers used for encapsulation. Most microencapsulations are performed using alginate, a natural polymer existing in brown seaweeds and the bacterium *Pseudomonas* (Govan et al. 1981). Alginates are a family of unbranched copolymers of α-L-glucuronic acid (G) and β-D-mannuronic acid (M) differing in mixture and arrangement of M and G depending on their original source (Smidsrod et al. 1990). Due to the composition of the polymer the biocompatibility differs. It has been reported that a high M content in the polymer is associated with increased interleukin-6 (IL-6) and tumour necrosis factor (TNF) mediated immune response (Otterlei et al. 1991). In some cases also overgrowth of the capsules containing either high amounts of G (Clayton et al. 1991) or M (Soon-Shiong et al. 1991) was observed. It is not clear if a high content of one of the two uronic acids is the basic problem for biocompatibility as contradictory results have been published over the years. Another possible reason for evoking an immune response might be the tendency of this natural polymer to be contaminated with endotoxine, certain proteins and polyphenols (Orive et al. 2002).

In contrast to alginate, cellulose sulphate is a chemically well defined material with a highly reproducible production. Capsules generated of pDADMAC and sodium cellulose sulphate (SCS) show high mechanical stability, high long term stability and are well tolerated by the donor (Dautzenberg et al. 1999). In addition, cellulose sulphate allows a better pore size control and a high survival rate and metabolic activity of encapsulated cells (Karle et al. 1998; Lohr et al. 1998; Pelegrin et al. 1998).

The kind of polymer and microencapsulation technique also determines other important factors for successful cell survival of the encapsulated cells. One major aspect is the size of the generated microcapsule, which should be in the range of 100 – 700 µm, optimally around 300 – 400 µm (Sakai et al. 2006). Above this size the diffusion of oxygen and nutrition to cells located at the centre of the capsule is too low for proper cell survival (Sugiura et al. 2005). Additionally, larger capsules might activate the immune response easier than small capsules and also the rough surface of the capsules will lead to increased immune response and overgrowth (Zhang et al. 2008c). The spherical shape of the generated capsules has the advantage of an optimal surface-to-volume ratio for protein and nutrition diffusion, which is linked to a higher cell survival compared to cell immobilisation on different synthetic scaffolds, an alternative approach for cell therapy.

Another important parameter for cell viability and biocompatibility is the pore size of the semipermeable membrane which determines the mass transport properties. The influx of molecules necessary for cell viability and metabolism critically depends on the pore size (Yuet et al. 1995), but also on the cell type used. On the other hand also the size of the largest molecule able to pass the membrane, the molecular weight cut-off (MWCO), has to be regulated to block interaction of immune system components with the cells, but enable therapeutic and metabolic product diffusion. This parameter is application dependent and differs also between allogenic and xenogenic cell encapsulation (Uludag et al. 2000), with reduced MWCO for the latter one.

The idea of protecting foreign cells from the immune system by an immunoprotective membrane was introduced around 1930 when tumour cells were covered with a polymer membrane and transplanted into an animal (Bisceglie 1933). Later Chang et. al. introduced the term “artificial cells” for microencapsulation of cells. Chang used endocrine cells which survived after implantation and were able to produce hormone (Chang 1966; Chang et al. 1966). One of the first approaches of encapsulating naive cells or tissues was the protection of allogenic or even xenogenic Langerhans islets for the treatment of diabetes, which showed promising results in animal studies (Lim et al. 1980; Altman et al. 1984; Thanos et al. 2009) and in clinical trials (Calafiore et al. 2006; Elliott et al. 2007). Later also other tissues or cells were encapsulated for treating different diseases. Cells of the choroid plexus (CP) which express some important neurotrophic factors (Emerich et al. 2005) were used in animal models for treatment of neurological disorders as Huntington disease (Borlongan et al. 2004) or Parkinson´s disease (Emerich et al. 1992; Ekser et al. 2008; Laguna Goya et al. 2008). In the 1990th the first human trial using xenogenic bovine chromaffin cells to reduce pain was performed (Buchser et al. 1996).

Despite the use of naive heterologous cells or progenitor cells, in the last decade the encapsulation of genetically engineered cells became more popular, as with genetically modified cells a defined and even artificial therapeutic protein can be produced independent of the cell type. To treat neurological disorders protective neurotrophic factors are expressed like cilliary neurotrophic factor (CNTF) in baby hamster kidney cells (BHK) for Huntington´s disease (Bachoud-Levi et al. 2000) or amyotrophic lateral sclerosis (ALS) (Aebischer et al. 1996) treatment in clinical trials. Another attempt is the use of fibroblasts expressing brain-derived neurotrophic factor (BDNF) for spinal cord injuries or C2C12 cells expressing glia-cell line derived neurotrophic factor (GDNF) in Parkinson´s disease treatment (Kishima et al. 2004; Lindvall et al. 2008). In addition enclosed mesenchymal stem cells (MSC) modified to produce glucagon-like peptide 1 were used in clinical trials to treat traumatic brain injuries like stroke (Heile et al. 2009).

Another approach is the reconstitution of dysfunctional or damaged tissues. Bone and cartilage defects represent a promising field for encapsulation techniques. Studies have shown that encapsulated MSC expressing BMP-2 *in vitro* lead to cartilage and bone formation (Ding et al. 2007) and fibroblasts expressing human transforming growth factor ß1 (TGF-ß1) result in cartilage regeneration in mice (Paek et al. 2006). The use of immunoprotected MSC expressing Sox-9 has also been shown to improve chondrogenesis *in vitro* (Babister et al. 2008). Beside bone formation also regeneration of heart failure was under investigation using encapsulated Chinese hamster ovarian cells (CHO) expressing vascular endothelial growth factor in a rat model (Springer et al. 2000; Zhang et al. 2008a).

Microencapsulation offers an optimal tool for expression of therapeutic proteins leading to the reconstitution of deregulated or dysfunctional proteins in metabolic and genetic disorders.

This approach includes encapsulated epithelial cells expressing ß-glucuronidase I tested for mucopolysaccharidosis Type VII treatment (Nakama et al. 2006), myoblastic cells producing erythropoietin for ß-thalassemia treatment (Murua et al. 2007; Murua et al. 2009), Factor VIII or factor XI expressing mouse C2C12 myoblasts for haemophilia A and B treatment (Hortelano et al. 1996; Hortelano et al. 1999; Garcia-Martin et al. 2002), growth hormone production in encapsulated myoblasts to treat dwarfism (al-Hendy et al. 1995) or the oral administration of encapsulated *Escherichia coli* transfected with an urease gene to restore normal urea levels in renal failure models (Prakash et al. 1996).

In recent years also the treatment of cancer was in focus of encapsulation approaches. Primary tumours seem to be the ideal target for therapeutic proteins produced by capsules as tumours are locally delimited and dependent on nutrition and blood supply from the surrounding tissue. Hence, inhibition of angiogenesis is one promising approach to stop tumour growth. Several attempts to express anti-angiogenic factors by encapsulated cells have been published like BHK cells expressing endostatin, a natural inhibitor of angiogenesis in mice (Joki et al. 2001) or expressed by human embryonic kidney (HEK) cells in rat models (Read et al. 2001). Angiostatin expressed by encapsulated C2C12 myoblasts also showed a reduced tumour growth in mice (Cirone et al. 2003). In addition, macrophage stimulated killing of tumour cells was initiated by encapsulated human cell lines expressing inducible nitric oxygen synthase in a dose dependent manner using inducible expression systems like the TetR system in mice (Xu et al. 2002). Stimulating the immune response against cancer cells was also the basis of *in vivo* experiments using encapsulated cells expressing interleukin 2 (IL-2) (Cirone et al. 2002). Beside the use of naturally occurring proteins to inhibit cancer growth, also artificially introduced therapeutic substances were converted by expressed proteins into active anti-cancer drugs (Salmons et al. 2010). Löhr et. al. showed that encapsulated HEK 293 cells overexpressing the 2B1 isoform of cytochrome P 450 (CYP2B1) could significantly reduce pancreatic tumours in mice when the pro-drug ifosfamide was converted to its active form (Lohr et al. 1998). The results of this *in vivo* study led to clinical trials where the tumour size was successfully reduced and the median survival of the patients could be increased (Lohr et al. 1999; Lohr et al. 2001). This approach was also shown to be effective for the treatment of mammary cancer in mice (Kammertoens et al. 2000) and dogs (Winiarczyk et al. 2002). In addition to the promising results of pro-drug conversion by CYP2B1 also other enzyme/pro-drug combinations were published (Portsmouth et al. 2007) like the delivery of encapsulated cells expressing cytosine deaminase (CD) followed by administration of 5-fluorocytosine in mouse models or a combination of two different suicide genes (Kammertoens et al. 2000). If these strategies are combined with traditional therapy forms like radiation, the beneficial effect could be increased (Ryschich et al. 2005).

When compared to other administration approaches for the treatment of metabolic or genetic disorders, cell encapsulation has the advantage of a controlled *de novo* synthesis of therapeutic proteins in a well defined cell system without the problem of a fast release of high or even toxic concentrations of the protein as for example in the case of broken protein capsules. In contrast to other cell based techniques, microencapsulation also protects the naive or modified cells independently of their origin from the immune system and prevents migration of the engineered cells. When combined with regulated gene expression systems, encapsulated cells raise a promising field of dose-dependent treatment of several disorders. Although many clinical trials based on encapsulation approaches are in progress, the scaling up of the encapsulation process is difficult and cells and encapsulation material have to be

further investigated for their biocompatibility and safety in patients (Orive et al. 2010; Salmons et al. 2010)

3.2. INDUCIBLE SYSTEMS

Stable and sufficient expression of proteins is one key aspect of most gene expression systems. In this case a robust promoter which is resistant to cell dependent up- and down-regulations is used. Such promoters are often derived from a housekeeping gene like the human phosphoglycerate kinase 1-gene (PGK) or the human telomerase reverse transcriptase (hTRT) or viral promoter sequences as the cytomegalovirus (CMV) intermediate early transcript or Rous sarcoma virus long terminal repeats (RSV). They result in constitutive expression of the encoded protein independent of the environment and are predominantly used for the production of proteins in many systems like gene therapy approaches or cell therapeutical trials. In the last years it turned out, that expression levels of therapeutic proteins sometimes need to be tightly regulated as overproduction might result in severe complications whereas too low levels of proteins would not show an effect. Therefore, regulated gene expression systems were employed to induce the production of a protein in a temporal or even spatial manner. For the application of an inducible gene expression system in the clinics, it has to accomplish some important issues. First, the system must show high inducibility in case of activation, but low basal expression. Second, the system should not interfere with endogenous pathways and third, it should show adjustability to intermediate levels over a wide range of inducer signals. In addition, the system should not evoke an immune response to components used for regulation. Today several inducible gene expression systems are known (see Table 1), fulfilling the above mentioned criteria at least in part. Some of these systems are already employed in clinical trials, but still there is substantial need for improvements.

induction system	**components**	**inducer**	**side effects of inducer**	**kinetics *in vivo***	**reference**
Tet-repressor (TetR)	Tet-repressor fusions (TA/rtTA)	Doxycycline	intermediate	slow	(Gossen et al. 1992; Gossen et al. 1995; Urlinger et al. 2000; Lamartina et al. 2002)
Progesterone receptor (GeneSwitch®)	Truncated progesterone receptor-GAL4 or p65	Mifepristone (RU486)	strong	slow	(Nordstrom 2003; Taylor et al. 2010)
ecdysone receptor-human retinoid X receptor (RheoSwitch®)	EcR/RXR linked to GAL4/VP16	Muristone A or RSL1	weak	slow	(No et al. 1996; Palli et al. 2003; Karzenowski et al. 2005)
Rapamycin dimerisation	FKBP linked to ZFHD-1 + FRAP linked to p65	Rapamycin derivatives	intermediate	slow	(Rivera et al. 1996; Amara et al. 1997; Liberles et al. 1997; Pollock et al. 2002b)
hypoxia induction	Hypoxia inducible elements	hypoxia	intermediate	fast	(Phillips et al. 2002; Su et al. 2004)
heat shock induction	Hsp72-, Hsp70B- or artificial promoters	heat	weak (for localized heat)	fast	(Brade et al. 2000; Braiden et al. 2000; Huang et al. 2000; Vekris et al. 2000; Brade et al. 2003; Guilhon et al. 2003b)

TABLE 3-1 DIFFERENT INDUCTION SYSTEMS

3.2.1. TWO COMPONENT SYSTEMS

Most of these inducible systems act via a small molecule, which in turn induces the expression of a transactivator followed by expression of the protein of interest. As this system needs to be activated in two steps (first the transactivator, then the actual protein) they are termed two-step or two component inducible systems. In contrast, the one step or one component systems act via direct activation of an inducible promoter. In addition to the above mentioned common criteria for the use of inducible gene expression in the clinics, the inducer substances should be orally bioavailable, be able to reach the target tissue and have a drug metabolism profile compatible with prolonged therapeutic use. The most prominent ligand-dependent regulatory gene expression systems are the tetracycline (Tet)-dependent induction system, the mammalian steroid receptor based-, the rapamycin based- and the insect steroid receptor based systems.

3.2.1.1. TetR System

The tetracycline (Tet) induced gene expression system originates from the *Escherichia coli* (E. coli) Tn10 tetracycline resistance operon, where the expression of the Tet resistance gene is repressed in the absence of Tet. The inhibition of gene transcription is due to the binding of the Tet repressor (TetR) to promoter regions upstream of the Tet operon, the so called operator sequences or Tet response elements (TetO or TRE). In the presence of Tet, the TetR binding to the TRE is blocked and the expression of the Tet resistance gene is started. In 1992 Bujard and Gossen established an inducible expression system based on the E.coli TetR system (Gossen et al. 1992) by fusing the TetR with the herpes simplex virion protein 16 (VP16) transcriptional transactivator domains, generating an artificial tetracycline dependent transcriptional activator (tTA). In a second expression cassette, multimerised TREs are fused upstream of a CMV minimal promoter to drive expression of the gene of interest. When Tet is absent from the system the TetR can bind to the TRE and drive gene expression. Due to the repression of gene expression in the presence of Tet the whole system is called Tet-Off (Gossen et al. 1992). Although the Tet-Off system allows precise control of target gene expression it is limited to approaches where expression should be maintained active for long times as continuous exposure to Tet is required to silence the system. In addition, the induction kinetics is dependent on the clearance of Tet from the system.

An opposite Tet-system which activates gene expression in the presence of Tet was established by Gossen and colleagues a few years later, called Tet-On system. In this system the TetR was mutated at four sites, leading to a change in four amino acids. This mutated form is termed reverse Tet repressor protein (rtTetR) and leads to a reverse behaviour of the protein. When fused to VP16, the repressor (rtTA) binds to the TRE only in the presence of Tet and thus activates the transcription of target genes. Compared to the Tet-Off system, the activation of the transgene is faster, but higher concentrations of Tet are needed to activate expression. To overwhelm this problem, other mutations of the original tTA were generated and two new rtTA mutants, the $rtTA^S$-S2 and the $rtTA^S$-M2 mutant, which showed improved sensitivity to Tet and reduced background activity compared to the original TetOn system (Urlinger et al. 2000; Lamartina et al. 2002) were established.

An attempt to reduce the potential immune reaction against the system is the combination of the TetR with a mammalian transcriptional repressor like Krüppel-associated box (KRAB) of the Zink finger protein Kox1 (Deuschle et al. 1995) or the rodent Kid-1(Witzgall et al. 1994a;

Witzgall et al. 1994b). This TetR-KRAB (rTS) system is free of the viral VP16 and therefore thought to be less immunogenic. In general zinc finger proteins inhibit RNA polymerases via their KRAB domain. A combination of the KRAB domain with the TetR gets attached to the TRE combined with a normal CMV promoter in the absence of Tet and inhibits gene expression. When Tet is present, rTS detaches from the TRE and gene expression starts.

One problem of the Tet systems discussed above is the background activation in the absence of Tet. To minimize basal activation two different approaches were established. On the one hand the rtTA and tTS system can be combined to reduce background activation. The tTS is cloned into a bicistronic promoter downstream of the transactivator and tTS and rtTA are separated by an internal ribosomal entry site (IRES) (Freundlieb et al. 1999). In the presence of Tet, the rtTA will bind to the TRE and the rTS is detached and gene expression will start. Without Tet the two proteins reverse their activity and the TRE-attached rTS reduces the basal expression in the absence of the inducer. This system allows precise control of the on/off state, but has the problem that three different foreign proteins have to be expressed. This might influence the immune response and also the incorporation into vector systems due to size restriction. On the other hand a single cassette Tet system was established using a Tet-inducible promoter P_{CMV-1} driving the expression of both proteins, the rtTA and the gene of interest (Haberman et al. 1998). Due to the fact that rtTA is not expressed in the absence of Tet it cannot induce background gene expression compared to the situation in the Tet-On system, where the expression of rtTA is under control of a constitutive promoter. Nevertheless, the expression of the gene of interest is dependent on the induction of the Tet-inducible promoter and the following expression of the rtTA. Therefore this inducible system shows much slower expression kinetics compared to the other systems.

To optimise induction of Tet response systems and avoid the immunogenic properties of tetracycline, also derivatives or analogues of the original antibiotic were tested. One of them, doxycycline (Dox), is used in the clinics for more than 30 years and could increase the response of the different TetR systems up to hundred fold compared to the original antibiotic (Gossen et al. 1995). In summary, the TetR systems provide a feasible tool to regulate gene expression *in vivo* through their inducer substance Tet or Dox. These systems have already been used for gene and cell therapy approaches as Tet/Dox are used in the clinics for several years and their pharmacokinetics and potential toxicity are well investigated. Nevertheless, the TetR system has some disadvantages for regulating therapeutic gene expression due to the fact that the inducer substance has to be administered orally, has to diffuse to the place of action and can interact with the system as long as it is available in the tissue. Hence the regulation of gene expression is in the range of days (Sommer et al. 2002) but some approaches might need tighter and faster control of expression levels. In addition, Tet and Dox are antibiotics and exhibit this function in the patient including undesired side effects.

3.2.1.2. Steroid hormone receptor systems

Another attempt to regulate gene expression via small inducer substances is the use of steroid hormone receptors, which represent one of the largest groups of mammalian transcription factors. Wang et. al. introduced in 1994 a system using the human progesterone receptor in a C-terminally truncated version (Wang et al. 1994) also known as GeneSwitch® (Valentis, Inc) system. This ligand binding site mutated receptor has lost its ability to bind progesterone, but still can bind to the agonist mifepristone (MPF or RU486). When fused with the yeast GAL4 DNA binding domain and a transactivator domain like

VP16, the specificity and inducibility of the system is improved. The chimeric transcription factor is under the control of a constitutive promoter, and expressed as an inactive monomer which is in a complex with heat shock proteins and other chaperones (Pratt et al. 1997). The gene of interest is under the control of an inducible promoter, built of six binding sites for the GAL4 DNA-binding domain linked to a TATA box element (Abruzzese et al. 1999). In the presence of MPF a conformational change releases the inactive chimeric transcription factor from the complex with chaperones and forms a stable homodimer which in turn can bind to the GAL4 response elements in the promoter and drive the expression of the therapeutic protein. When the inducer is removed, the chimeric transcription factor reverts in its inactive monomeric form. To reduce the background activation of the system and to enhance inducibility, the VP16 activation domain was exchanged and the p65 activation domain of the human NF-κB was introduced into the inducible gene expression system (Abruzzese et al. 1999; Burcin et al. 1999; Abruzzese et al. 2000). The use of p65 also reduces the potential immunogenicity of the system in humans, as it now consists of 86 % human sequences. MPF, which was originally used in medical termination of pregnancy, has antiprogestin and antiglucocorticoid activity. In humans most of the substance is bound to α-1-glycoprotein hence only a small fraction is available for inducing gene expression (Heikinheimo et al. 1989; Sarkar 2002). For these reasons MPF has to be administered in higher concentrations and due to its progesterone agonistic activity might also be involved in endogenous pathways.

To overwhelm the problem of interaction with endogenous pathways, non-mammalian or even artificial systems can be employed. In 1992 the *Drosophila melanogaster* steroid receptor system, regulating metamorphosis by responding to the hormone 20-OH ecdysone, was established as an inducible gene expression system (Christopherson et al. 1992). In the presence of ecdysone, the ecdysone receptor (EcR) dimerises with the Ultraspiracle gene product, a Drosophila homologue of the human retinoid X receptor (RXR). This active heterodimer than can bind to ecdysone response elements in the promoter region to activate gene expression (Horodyski et al. 1993). To optimise the system, the transactivation domain of EcR was replaced by a transactivation domain of VP16 and co-expression of the RXR instead of the Ultraspiracle gene. In addition, the EcR was modified in the P box of the DNA binding domain, which allows the specific binding to artificial response elements, a hybrid of the naturally occurring glucocorticoid response elements and the response elements of RXR or EcR (No et al. 1996). When Muristone A, a synthetic analog of ecdysone, is orally administered, the two constitutively expressed receptors form active heterodimers and activate the expression of the gene of interest by binding to the artificial hybrid binding elements. In the absence of Muristone A, the dimer is no longer stable, dissociates and gene expression is stopped. A further improvement of the system was commercialised by New England Biolabs, called RheoSwitch® Mammalian Inducible Expression system. This system is based on the improvements made by Palli et al. (Palli et al. 2003) and Karzenowski et al. (Karzenowski et al. 2005), using a fusion protein of a highly modified EcR ligand-binding domain with the DNA binding domain of GAL4 and an activator fusion protein of RXR ligand binding and the VP16- activator. In the presence of a synthetic ligand, the RSL1, which showed no side activity in humans, the active dimer induces gene expression at high levels and without the ligand almost no background activation (Palli et al. 2003; Karzenowski et al. 2005). One advantage of these systems is that the active dimer has no natural binding sites present in the host and therefore the basal expression is lower than in other systems like the Tet system. In addition, substances as the hydrophobic Muristone A or small molecule analogs (Saez et al. 2000) can easily diffuse through the tissue and even cross the blood-brain barrier, making this system an ideal tool for gene expression in the brain. Although this

inducible gene expression system shows high inducibility and low background activation it has to overexpress two transgenic receptors simultaneously, which might lead to problems in the efficiency of the system. Like in the Progesterone receptor system, also this induction model harbours a receptor potentially involved in metabolic pathways, the RXR (Subbarayan et al. 2000) and therefore might result in complications when used for longer periods.

3.2.1.3. Dimerisation systems

A different way to regulate gene expression by small inducer molecules is the dimerisation approach (reviewed in (Pollock et al. 2002a)). Here the capability of rapamycin to heterodimerise two proteins is used to bring two parts of a transcriptional activator in close proximity and therefore induce gene expression through special binding elements of the activator. The original heterodimer consists of FK506-binding protein (FKBP) and FKBP-rapamycin-associated protein (FRAP), also known as mammalian target of rapamycin (mTor) and dimerisation is established via the FKBP rapamycin binding (FRB) domain of FRAP. For the use as an inducible expression system, an activation domain (AD) can be attached to FRB domains and a DNA binding domain (DBD) can be linked to FKBP domains to induce expression of certain proteins in the presence of rapamycin (Ho et al. 1996; Pollock et al. 1999). One of the most prominent combinations for clinical settings is the use of zinc finger homeodomain 1 (ZFHD-1) as a DBD linked to three copies of the human FKBP. NF-κB subunit p65 is used as an activation domain and fused to a single FRB domain. Both chimeric proteins are expressed from one cassette by a bicistronic promoter and separated by IRES. In the absence of the dimeriser, both proteins show no binding affinity to the inducible promoter, consisting of 12 modified ZFHD-1 binding sites followed by a TATA box. In the presence of rapamycin, the two chimeric proteins dimerise and can bind tightly to the response elements to induce expression of the gene of interest (Rivera et al. 1996). As rapamycin was introduced into the clinics as an immunosuppressive agent, other analogues, known as Rapalogs were established, which showed no or less immunosuppressive activity in combination with mutations in the FRAP domain of the AD fusion protein (Amara et al. 1997; Liberles et al. 1997; Pollock et al. 2002b). When compared to the TetR or ecdysone regulation system, the dimerisation system showed no background activation but less maximal inducibility (Senner et al. 2001; Go et al. 2002). Although this system provides robust expression over long periods, it lacks the possibility of fine tuning the expression of therapeutic proteins like most other inducible gene expression systems.

In general, two component expression systems show high target gene expression in response to an orally administered inducer. This inducer is typically well characterised for clinical applications and is used in moderate doses. Nevertheless, these systems might evoke an immune response or the inducer leads to some kind of resistance in clinical use. In addition, for all of these systems an activator or transcription factor has to be produced in the host constitutively, which might also interfere with endogenous pathways or evoke an immune response. Another general disadvantage is the fact that the inducer has to be taken up orally and has to be transported or to diffuse to the site of action, which limits the fine tuning possibilities of all the two component induction system approaches.

3.2.2. ONE COMPONENT SYSTEMS

In contrast to the two component inducible gene expression systems described above, also inducible promoters can be used, which are activated by external stimuli and therefore are referred to as one component systems. In principle these one component systems have the advantage that no activator or artificial transcription factor has to be expressed in the system and that the inducer is not a small molecule, which has to reach the expression system, but an external environmental signal or patho-physiological signal that stimulates the expression of the therapeutic protein by employing the endogenous machinery. The most prominent systems use hypoxia or hyperthermia for induction.

3.2.2.1. Hypoxia inducible promoters

If cells are exposed to hypoxic conditions, they activate a signaling pathway, mediated by hypoxia inducible factor-1 (HIF-1), which then activates gene expression of certain protective proteins by binding to special response elements (reviewed in (Rocha 2007)), the so-called hypoxia response elements (HRE) (Binley et al. 1999). If these HRE in combination with a TATA box or minimal promoter are placed in front of a therapeutic gene, the expression of the protein will be induced if hypoxia is established in the tissue or cells harbouring the artificial system. As in tumours hypoxia can result from poor vascularisation, the employment of this inducible system may be a proper way to induce gene expression linked to tumours. With this approach, mainly suicide genes are expressed in tumours, sometimes also combined with tissue- or tumour specific promoters like the integration of HRE in an α-fetoprotein promoter, driving the suicide gene for the HSV-1 thymidine kinase (HSV-tk) to treat hepatocarcinoma (Ido et al. 2001). Another application of the hypoxia inducible expression system is myocardial ischemia, as here severe hypoxic events can cause myocardial fibrosis and lead to death. Using a combination of heart specific promoters enhanced with several HREs, protective and angiogenic factors can be expressed specifically in the ischemic tissue to improve cardiac function (Phillips et al. 2002; Su et al. 2004). In general, this inducible system turns out to be an ideal tool for gene and cell therapy of hypoxic tissues, but is limited to this special patho-physiological condition. Regulation of the hypoxia inducible system is only possible by reversing the hypoxic conditions and therefore is very slow and ineffective. Taken together, this system can only be used for long term expression in special situations.

3.2.2.2. Heat shock promoters

Another system triggered by external signals is the heat shock pathway (see also 3.5). This ancient stress response mediates the induction of target genes like chaperones in response to several kinds of stress. This is achieved through heat shock factor 1 (HSF1) binding to special recognition sites in the promoters, the heat shock elements (HSE) (reviewed in (Shamovsky et al. 2008). The most prominent proteins driven by these promoters are the heat shock proteins (HSPs) and within this group mainly the HSP70 proteins, HSP70-1, Hsp70-2, and in humans also HSP70B and HSP70B´. Promoters of these genes show high inducibility in response to heat, but also to other stressors like, heavy metals, irradiation, nutrition depletion or infections. As they are involved in normal cellular processes like the correct folding of proteins, they show moderate basal levels which are tightly regulated and also integrated in other cellular signaling pathways. Nevertheless, their inducibility through externally applied heat and the fast down regulation when the external heat is removed makes them an interesting tool for induced gene expression. Early attempts used natural

heat shock promoters, but due to their high basal activity and complex regulation, most of them showed proper inducibility, but high background (Dreano et al. 1986; Brade et al. 2000; O'Connell-Rodwell et al. 2004). The most employed natural promoter for therapeutic approaches is the highly heat-inducible HSP70B promoter, driving expression of mainly suicide genes in tumours in response to heat (Braiden et al. 2000; Huang et al. 2000; Brade et al. 2003; Guilhon et al. 2003b). Several attempts to improve the natural promoters were performed like reducing the natural promoter to minimal sequences consisting of the HSEs and a TATA box, called minimal HSP70 promoter (Vekris et al. 2000; Smith et al. 2002) or the introduction of additional HSE into a HSP70 promoter (Brade et al. 2000). All these improvements lead to high inducibility of the expression systems in response to heat and low background activation. In addition, these systems show a better defined reaction to different heat durations and temperatures, making regulation of the system more precise. One big advantage of heat induced gene expression is the accurate spatial control of heat generation, established in recent years by focused ultrasound (FU), or magnetic nanoparticle mediated heat generation by an alternating magnetic field (AMF) (for details see 3.4) (reviewed in (Rome et al. 2005)). These heat generation techniques are already in clinical use for hyperthermia treatment of solid tumours (Thiesen et al. 2008) and can efficiently be combined with heat-responsive gene expression systems. As natural heat shock promoters respond to different kinds of stress, the employment of heat shock promoters for regulated gene expression might be activated under certain cellular conditions even without heat treatment. This broad responsiveness is reduced in the artificially generated heat shock promoter variants almost exclusively to heat induction. Nevertheless, a possible activation by oxidative stress, cytokines or lack of nutrition, which is most prominent in tumours, might cause severe problems. Applying heat to a specific tissue might also result in problems as additionally to the inducible gene expression system also the natural heat shock response will be activated. Therefore, an exact spatial control of heat generation with well defined temperatures has to be used for this system.

3.3. NANOPARTICLES

Nanoparticles have a size of a few nanometres up to tens of nanometres and have a broad range of application, from electro-technics up to biomedicine. Although they consist of different materials like polymers or silica, mostly magnetic nanoparticles are employed in bioscience (reviewed in (Pankhurst 2003)). For most applications, iron oxide particles in a range of 5-60 nm were used, consisting of either magnetite (Fe3O4), maghemite (γ-Fe2O3) or hematite (Fe2O3). Beyond these iron oxides also cobalt, nickel, manganese or zinc ferrites as well as gold or platinum were applied in biomedicine. Magnets can be produced either by sintering or casting, but for magnetic nanoparticles typically the wet chemical routes are performed due to efficiency and the possibility to control size, composition and even shape of the resulting particles, which have a high influence on their magnetic properties. For example, magnetic iron oxide particles are synthesised by co-precipitation of an aqueous solution of Fe^{2+} and Fe^{3+} salts (e.g. chloride) in the presence of a base. Depending on the molecular ratio and the established pH a complete precipitation of the salts like Fe_3O_4 at a pH between 9-14 and a molecular ratio of Fe^{2+} to Fe^{3+} of 1:2 will occur in a non-oxidising oxygen free environment (reviewed in (Babincova et al. 2009)). Beside the efficient and cheap generation of magnetic nanoparticles, they can also be coated with organic or inorganic materials. On the one hand, this coating prevents agglomeration of nanoparticles and on the other hand, certain different macromolecules can be fixed to the nanoparticle surface. This

shielding with biological molecules improves the application of the particles in biomedicine. Another synthesis route is the decomposition of organic precursors in the presence of a surfactant, as this approach results in small and narrow size distributed nanoparticles.

Magnetic nanoparticles are defined as particles which can be manipulated by a magnetic field based on Coulomb´s law. Magnetic fields can penetrate human tissue and therefore magnetic nanoparticles can be influenced inside the tissue by an externally applied magnetic field. This potential of manipulation from the outside makes magnetic particles a promising tool for biomedical problems. Their application cover a broad range from separation, drug targeting, contrast agents for magnetic resonance imaging (MRI) or transfection reagents to the use as “nanoheaters” in hyperthermia approaches.

3.3.1. MAGNETIC SEPARATION

Magnetic particles are attracted by an established magnetic field, which is the basis for magnetic separation. In this application, magnetic nanoparticles are coated with polymers like dextran, phospholipids or polyvinylalcohol (PVA) (reviewed in (Molday et al. 1982; Chanana et al. 2009)). For separation the targets have to be tagged or labelled with the magnetic particles, mostly performed by antibodies which recognise special epitopes on the surface of the targets. When a magnetic field is established, the nanoparticles start to agglomerate at a high magnetic field strength and therefore, the target, which could be a virus, bacterium or protein, is separated from other components. Applications for magnetic separation range from the detection of malaria parasites in blood samples (Paul et al. 1981), enrichment of rare tumour cells like breast cancer cells (Schindlbeck et al. 2008) or the detoxification of blood from certain poisons (Chen et al. 2007).

3.3.2. CARRIER PARTICLES

Similar to the approach of separating macromolecules by coupling to magnetic nanoparticles these particles can be used as carriers for DNA or drugs. By applying a magnetic field, they will be transported to a selected area within a cell or a tissue. This concentration at the site of action bears a great benefit for different applications including cancer treatment with cytotoxic agents. Magnetic carriers therefore reduce the systemic distribution of a potentially harmful therapeutic substance and the necessary total amount. To act as carriers, the particles have to be covered or coated with a biocompatible material like dextran, biotin, avidin, carbodiimine, polyethylenimine or inorganic materials like silica (Plank et al. 1998; Arias et al. 2001; Gomez-Lopera et al. 2001; Santra et al. 2001; Reimer et al. 2003; Mykhaylyk 2007; Mykhaylyk et al. 2010). When the coated nanoparticles reach their target tissue, the attached substance is released from the carrier. Different approaches were established to release the therapeutical substance like the cleavage from a pH-sensitive linker through pH changes, enzymatic cleavage or temperature dependent release. In contrast, nanoparticles can also remain covalently linked to their therapeutic substance, as for example to radioactive substances (Hafeli et al. 1995; Hafeli et al. 2003). They can be applied in hydrogels or liposomes to trace the embedded drug to a designated area. The release from the hydrogel or liposome can either be triggered by rupture of the carrier vesicle or by thermal regulation (reviewed in (Brazel 2009)). The use of magnetic nanoparticles in drug release and targeted drug delivery increased dramatically during the last years (Dobson 2006; Yang et al. 2006). Most of the research focused on the treatment of cancer using

cytotoxic drugs linked to nanoparticles, for which an efficient drug delivery and tumour remission could be demonstrated in different animals (Pulfer et al. 1998; Alexiou et al. 2000; Goodwin et al. 2001). In recent years also some clinical trials using magnetic nanoparticle mediated drug targeting dealing with cancer treatment were performed (Lubbe et al. 1996b; Alexiou et al. 2000; Lanza et al. 2002). Most of the time, the transported drug is a cytotoxic drug for cancer treatment, but also siRNA, viral vectors or DNA are used (reviewed in (Plank et al. 2011)). Nevertheless, the use of nanoparticles always bears the risk of accumulation in smaller blood vessels which is one typical application route and might result in embolisation (Lubbe et al. 1996a; Lee et al. 2007; Hafeli et al. 2009). In addition, nanoparticles loaded with drugs have the problem of a small payload of each particle resulting in low doses of therapeutic substance at the point of application. Although some problems have to be solved, magnetic nanoparticle mediated drug delivery is a promising tool for the treatment of several diseases in the near future.

3.3.3. MAGNETIC RESONANCE IMAGING

Another application of magnetic nanoparticles is diagnostic imaging using magnetic resonance imaging (MRI). This non-invasive technique visualises soft tissue and metabolic processes by application of a strong magnetic field and radiofrequency (RF) radiation. Due to the magnetic field, the nuclear magnetisation of hydrogen atoms is aligned resulting in a detectable signal which differs between the tissues. Therefore, a spatial determination of the signal is possible. The resulting image strongly depends on the contrast between background and tissue specific factors. Contrast agents are used to enhance the signal difference of a specific tissue to the background signals. The most popular contrast agents of the last years are based on gadolinium chelates and iron oxide nanoparticles. Gadolinium has a short half-life of around 12 min. Another problem with gadolinium based agents is the fact that they do not cross the blood brain barrier (BBB) and can lead to nephrogenic disorders (Marckmann et al. 2006). Iron oxide nanoparticles can enhance the MRI by distorting the local magnetic properties of a region because of their large magnetic moment leading to a stronger signal change. In addition, iron oxide particles are biocompatible and due to coating can increase long term circulation of the contrast agent. Magnetic nanoparticles smaller than 40 nm are able to cross the blood brain barrier to some extent, offering for the first time a contrast agent suitable for brain specific imaging without direct injection into this area. Although normal magnetic nanoparticles have several advantages in MRI their great potential is their ability to target specific areas depending on their biofunctional coating. Most prominent in this field is the coupling of antibodies to specifically target tumour cells expressing special proteins on their surface, like the detection of rectal cancer (Toma et al. 2005) or breast cancer (Funovics et al. 2004). Beside the use of antibodies also small peptides or other small ligands can be linked to the particles (Sun et al. 2008, Weitman et al. 1992; Ross et al. 1994) Today there are some magnetic nanoparticles already in clinical use for imaging of abdominal viscera, like Feridex I.V. or Combidex (Advanced Magnetics, Inc., Cambridge MA) but till now none of the targeted particles have been approved

3.3.4. HEAT GENERATION

The ability of heat generation in an alternating magnetic field is another important feature of magnetic nanoparticles. Depending on the kind of magnetic nanoparticle, the magnetic field

strength and the frequency, the nanoparticles absorb the applied energy and convert it into heat. Heat generation is due to the established magnetic moment of the nanoparticle in a magnetic field. If this field changes its direction at a certain frequency, the established magnetic moments of the individual particles also change cyclically. At high frequencies, the net movement of the magnetic dipole is zero and the applied energy is directly turned into heat by Brownian and Néel relaxation processes (reviewed in (Pankhurst 2003; Fortin et al. 2008; Jordan et al. 2009)). Larger particles of more than 100 nm convert the applied energy of the alternating magnetic field (AMF) into heat by eddy current heating, particles of an average size between 100 nm and 40 nm generate heat due to hysteresis heating (reviewed in (Pankhurst 2003; Jordan et al. 2009)). Beside their size also the composition and physical properties of the magnetic nanoparticles is important for heating characteristics. One important characteristic is the Curie point (T_c), which is defined as the temperature where the particles switch to a paramagnetic state and stop absorbing the energy from the AMF and therefore stop heating. Below the Curie point, the particles return to magnetic behaviour and convert the applied energy again into heat. The T_c of a magnetic nanoparticle depends on its chemical composition as for example Fe_2O_3 has a T_c of around 600°C and Mn-Zn-ferrite nanoparticles have a T_c of around 44°C (Meijer et al. 1995; Kotte et al. 1998). Therefore, the choice of the material and the corresponding T_c is important for the different applications as potential overheating of a tissue or a material can be avoided by the use of a low Curie point material (Gazeau et al. 2008). In addition, also the settings for the AMF are important for heat generation. Generally the frequencies used for the AMF generation are in the range of 50 kHz up to 10 MHz and magnetic field strength up to 55 kA/m are tolerated by organisms (Ivkov et al. 2005) although they normally have been in the range of 10-20 kA/m in clinical trials (Thiesen et al. 2008).

The main application of the nanoheaters is in hyperthermia treatment (see also 3.4.) of cancer but in the last years also thermally induced release of therapeutic substances from nanoparticles or thermo-responsive carrier materials as well as heat induced gene expression were established.

In hyperthermia treatment, where a tissue or normally a tumour is heated to temperatures above 42°C to induce specific cancer cell killing, mostly iron oxide nanoparticles were used. These particles are either injected directly into the tissue or applied within a suspension (magnetic fluid) or embedded in a carrier (Wust et al. 2002; Lao et al. 2004; Thiesen et al. 2008; Latorre et al. 2009). Like for other therapeutic applications the high biocompatibility of the coated nanoparticles is a big advantage and also the targeting to specific areas represents a further benefit for the treatment. Using magnetic nanoparticles to induce heat is important for hyperthermia treatment at a local area compared to more widespread heating techniques used before. Localised thermotherapy reduces the potential harmful side effects of heating up a tissue from the outside and is therefore the preferred treatment.

Similar to their use as nanoheaters for hyperthermia treatment, the heat generated by application of an AMF can be used to disrupt or open carrier systems in drug delivery. The development of thermoresponsible polymers as vehicles for therapeutic substances leads to the involvement of nanoparticles within this area. First of all the magnetic nanoparticles can be used to guide the carriers or to enable real time imaging of the transport and in a second step they can induce the drug release. For the use as thermoresponsive drug carriers, nanoparticles with a Curie temperature slightly above the physiological temperature are preferred and polymers have to change their properties or shape in response to small temperature changes. In general, the drug is embedded in the polymer hydrogel and is somehow released through temperature increase. Drug release could either be triggered by

squeezing, where the hydrated gel starts to shrink at a higher temperature and so the drug is exuded (Bikram et al. 2007) or the drug is released from a thermosensitive polymer in the hydrogel. The latter type of drug release is still under basic investigation, but both systems still have to be improved for the use in clinics.

Another approach combines heat responsive gene expression with magnetic nanoparticles producing heat in response to an AMF. On the one hand, a heat inducible promoter driving the expression of a protein can be linked to magnetic nanoparticles and expression starts when the AMF is applied to the particles. Using this system and Mn-Zn-ferrite magnetic nanoparticles coupled to an Hsp70 promoter driven expression construct, a highly regulated and efficient gene expression system could be established in mice (Tang et al. 2008). Another attempt is to include the magnetic nanoparticles in so called magnetic cationic liposomes (MCL) and transfect target tissue with a heat inducible expression construct. In one approach, TNF-α was expressed in response to heat generation after AMF treatment in mice (Ito et al. 2001) in another one MCL were used for heating of glioma tumour tissue in rats (Yanase et al. 1998). Although both approaches show promising results in animal studies, further research has to be done to transfer this application into clinical trials.

3.4. HYPERTHERMIA

If cells or tissues are exposed to temperatures above 40°C they induce several defence mechanisms to survive. In normal tissue, increased perfusion helps to deal with the higher temperatures and stress dependent protein denaturation is reversed by activating the heat shock response. At temperatures above 44°C, the resulting damage is too severe and the cells start to die. In contrast to normal tissue, regions affected by cancer react more sensitive to increased temperatures. On the one hand the mutated cells need more energy and oxygen supply because of their deregulated growth. Therefore, tumours often stimulate angiogenesis resulting in new blood vessel formation. Most of these blood vessels derive from already tumourigenic cells or regions near the tumour and are therefore not regularly shaped, have chaotic structures and do not sufficiently supply the tumour with nutrition and oxygen resulting in hypoxic regions. In addition to the low oxygen levels, also waste products are not sufficiently removed from areas with deregulated vascularisation leading to an acidic environment (Vaupel et al. 1989; Vaupel 2004). In response to higher temperatures, cells in this area induce apoptosis through intra-nucleosomal DNA-cleavage (Sellins et al. 1991). On the other hand the primary defence against stress, the heat shock response is also deregulated in most tumours. The key player of this response, heat shock factor 1 (HSF1), was already identified to promote tumour growth (Dai et al. 2007) and members of the heat shock protein 90 (Hsp90) family localise to the mitochondrion of cancer cells to inhibit stress initiated membrane rupture and therefore prevent tumour cell apoptosis (Kang et al. 2007). By initiating the heat shock response with increased temperatures, cancer cells start to express Hsp on their surface, which is noticed by the natural killer cells (Multhoff et al. 1995a; Multhoff et al. 1995b). Despite the activation of the innate immune response also the adaptive immune response is stimulated by the increased release of tumour-associated antigens or self-antigens (Zhang et al. 2008b).

This tumour-specific reaction to heat is used in clinical applications to specifically kill cancerous regions in the body. Dependent on the temperature two different types of treatment are distinguished. Above 46°C, cells are directly killed by temperature dependent protein denaturation. This treatment is called thermoablation. On the other hand,

hyperthermia is performed at lower temperatures (40-44°C) leading to a tumour-specific destruction of the cells, while normal tissue is unaffected. To reach these higher temperatures, energy has to be introduced, described as power-density specific adsorption rate (SAR), measured in W/kg. To establish hyperthermia conditions above 42°C in a target region, at least 20-40 W/kg have to be applied (Tilly et al. 2001). Depending on the method and material used, different SAR is reached within the tumour or even within the whole patient.

3.4.1. TYPES OF HYPERTHERMIA

Hyperthermia treatment can be classified into three different types, depending on the affected area. If the temperature is increased only in the tumour region, this method is called local hyperthermia. Treatment of larger regions like organs or limbs by heated fluids is called regional hyperthermia and heating the whole patient is called whole body hyperthermia (reviewed in (van der Zee 2002)).

3.4.1.1 Local hyperthermia

For heating of tumour tissue in local hyperthermia, several different methods are used today like radio wave (RFA), laser (Vogl et al. 2001), microwave (Fan et al. 1996) or ultrasound wave based systems. RFA uses a needle-like probe, directly injected into the tumour which releases high frequent current to heat the tissue (Goldberg 2001) or an applicator consisting of four dipole antenna arranged in a ring (Seebass et al. 2001). Another attempt to increase the temperature locally is the use of a transducer generating high-frequency ultrasound waves which are focused to a single point within the tumour (ter Haar 2001). Compared to other heating techniques, this high-intensity focused ultrasound (HIFU) can be adjusted better and reach deeper regions within the body. Although HIFU and also infrared are able to precisely heat a certain area, their therapeutic depth is limited to a few centimetres and therefore, these treatments are limited to tumours near the body surface. The use of HIFU to treat prostate cancer was tested in a different clinical trials (reviewed in (Rewcastle 2006)) resulting in clinical application. Regional hyperthermia using radio waves to treat several kinds of cancer entered clinical trials up to phase III, either combined with chemotherapy (Issels et al. 2010), radiation (van der Zee et al. 2000) or without additional treatment (Wust et al. 1998). To treat deep-seated tumours different heating sources have to be positioned next to the area of interest. Examples for such heating sources are microwave antennas, ultrasound transducers or radiofrequency arrays. As these antennas or arrays have to be positioned near the tumour to ensure a therapeutic temperature increase, these methods are highly invasive. In addition, the positioning of the heating elements in case of microwave or radiofrequency antennas is important in respect to interference and focus.

Another type of intestinal heating is the application of nanoparticles in combination with an electromagnetic field. In this situation, the nanoparticles are injected into the tumour and the energy is applied from the outside. The first therapeutical concepts for the use of magnetic nanoparticles in combination with an alternating magnetic field to treat cancer was introduced in the 1950s (Gilchrist et al. 1957) and was further improved by several groups in the last years (reviewed in (Thiesen et al. 2008)). For this application mainly ferromagnetic nanoparticles are used because of their excellent biocompatibility, their known metabolic pathways and the heating potential (see also 3.3.4.) but also other metal nanoparticles consisting of manganese (Mn) or zinc (Zn) ferrites are in focus. Ferromagnetic nanoparticles are normally coated to prevent agglomeration and injected directly into the tumour (Jordan et

al. 1997), or into blood vessels supplying the tumour (Archer et al. 1989; Archer et al. 1990). Treatment of malignant diseases using magnetic nanoparticles either dispersed (magnetic fluid hyperthermia) or as integrated magnetic material have entered clinical phase and showed promising results treating prostate carcinoma (Johannsen et al. 2005), bone metastases (Matsumine et al. 2011) or glioblastoma (Jordan et al. 2001). In 2010 the German company MagForce Nanotechnologies received the first clinical approval for the treatment of brain tumours using magnetic nanoparticles (Gneveckow et al. 2004), which will promote the development of other nanoparticle based therapies for clinical use.

Beside the use of a magnetic field combined with ferromagnetic nanoparticles also other nanoparticles are used to locally produce heat within the tumour. Gold (Au) nanoparticles increase temperature in the tissue when this area is exposed to radio waves, which are low-frequency electromagnetic waves with good tissue penetration and low tissue specific adsorption rates. The nanoparticles, either tagged with antibodies or alone, are injected into or near to the tumour and exposed to a radiofrequency field produced by an external field generator (Kanzius RF generator) (Curley et al. 2000; Glazer et al. 2010). Beside radio waves also near-infrared can be used to heat Au nanoparticles for hyperthermia applications (Gobin et al. 2010). In comparison to the direct implantation of arrays or antenna into the tumour the entire nanoparticle based approaches are less invasive and can be regulated from the outside of the patient. As the nanoparticles can be modified on their surface, also tumour targeting strategies like antibody coupling to the particles directed against tumour surface markers (Gazeau et al. 2008; Cherukuri et al. 2010) can be utilised. In addition, the applied electromagnetic energy has a high tissue penetration with low energy-dependent specific adsorption rate (SAR). This kind of treatment is more convenient and less invasive, but the heat production is always dependent on the power of the applied field and the amount of nanoparticles in the tumour. Nanoparticle distribution in the tissue is not entirely regulated and therefore, the local heat production cannot perfectly be controlled. To avoid overheating and the resulting damage of healthy tissue, the temperature in the hyperthermia region has to be controlled properly, but standard methods like MRI cannot be used as they will interfere with the magnetic field. Therefore new and precise temperature sensoring systems have to be developed for magnetic field induced hyperthermia, like fibre optic-probes injected into the tumour (Gneveckow et al. 2004) or addition of NMR active substances (Klingeler et al. 2008). Another limitation is the generation of the magnetic field for heat induction with ferromagnetic nanoparticles. The electromagnetic field strength has to be at least 5-18 kA/m, depending on the particles used, and therefore efficient magnetic field generators forming strong magnetic fields have to be employed, which is challenging for the constructors. Nevertheless, local hyperthermia is now in clinical trials or even approved for clinics and demonstrates the great power of this new cancer treatment.

3.4.1.2. Regional hyperthermia

When not only a distinct tumour area but a whole organ or region is heated, this is called regional hyperthermia. The classic example is the temperature increase within the limb by heated fluids (Coit 1992; Ceelen et al. 2000) and is based on bypassing a large supplying artery and a limb-draining vein. This treatment is well tolerated by the patient up to 43°C (Eggermont et al. 1996) and can be combined with other treatments like chemotherapy. Another example is the positioning of antenna into hollow organs combined with counter electrodes positioned on the body surface. Temperature increase in hollow organs like urethra, cervix or oesophagus can also be generated by hot water tubes but here the risk of overheating is very high and the technique has to be further improved for clinical trials.

3.4.1.3. Whole body hyperthermia

The application of local or regional hyperthermia is designed for the destruction of primary tumours or a defined area, but not for the treatment of metastasis. To affect areas all over the patient whole body hyperthermia is applied. For all the different methods the whole body temperature has to be increased up to 41.8-42°C for at least 1 hour. Consequently, energy has to be applied to the body and at the same time, the natural energy loss through the body surface has to be minimised. With perfect thermal isolation the body would heat up to 42°C within 3 hours without any additional energy. This perfect isolation cannot be established and the duration of the heating would be too long. The first attempts to heat up the patient were extracorporeal heating or contact heating but these methods showed several side effects like burn or systemic reactions and low efficiency. Hence, other heating techniques with acceptable adverse effects were developed like radiant systems. This heating technique applied water-steam and long-wavelength infrared in an isolated chamber to increase the total body temperature to 42°C within 60-90 min (Robins et al. 1985; Wehner et al. 2001). Although the new developed methods showed less severe side effects, all whole body hyperthermia treatments can cause overheating of the skin resulting in thermal lesions and together with the applied deep anaesthesia or sedation also systemic toxicity can arise (Bull et al. 1982; Wust et al. 2000).

3.4.2. INTERACTION WITH OTHER TREATMENTS

For the treatment of cancer it is state of the art to not only use a single strategy but a combination of different treatments like radiotherapy and chemotherapy. When hyperthermia is combined with other treatments this enhances the therapeutic effect. Combined with radiotherapy, hyperthermia is one of the most potent radiosensitisers known. Radiotherapy destroys the tumour cells by the formation of oxygen radicals due to ionising radiation, but most of the cancerous tissues have a reduced perfusion rate and are therefore more resistant to this therapy. Hyperthermia increases the perfusion rate in the tumour resulting in a higher supply with oxygen radicals resulting in a higher radiosensitivity (Song et al. 1997). These radicals can now induce DNA damage which is further potentiated by the fact that hyperthermia in tumour cells interferes with cellular DNA-repair mechanism which is linked to the deregulated heat shock response (Kampinga et al. 2001). In addition, cells that are in the S-phase are highly resistant to radiotherapy, but are sensitive to hyperthermia. The high complementary action of hyperthermia and radiotherapy enhances the therapeutic outcome up to a factor of 5 and at the same time the radiation dose can be reduced to minimise radiation induced toxicity. Several clinical trials combining hyperthermia with radiation have been performed in the last years focusing on cervical cancer (reviewed in (Lutgens et al. 2010)), breast cancer (reviewed in (Zagar et al. 2010)) or soft tissue sarcoma (Prosnitz et al. 1999).

The combination of hyperthermia with chemotherapy also showed therapy synergistic effect. In general the temperature dependent enhancement of perfusion and the change in cytoskeleton architecture, which leads to enlarged pores, promotes the uptake of chemotherapeutic drugs by the tumour (Dahl 1995). Like for thermoradiotherapy, also the combination of chemotherapy with hyperthermia allows reduction of drug concentrations and is was shown that mild heat treatment can potentiate the therapeutic outcome (Urano et al. 1999). Different drugs show diverse effects when combined with higher temperatures. The cell-specific toxicity of platinum based drugs and alkylating agents like ifosfamide was shown to enhance linearly depending on the temperature increase (Istomin et al. 2008). For other

drugs, like anthracyclines only weak or even no additional effect was detected, but in some cases, like for doxorubicin, a counteraction was observed when hyperthermia treatment was performed. This reduction is due to the thermal instability of some chemotherapeutics (Ng et al. 1996). The combination of heat with chemotherapeutic agents was tested for different drugs in clinical trials, like in soft tissue sarcoma, where whole body hyperthermia was combined with ifosfamide, carboplatin and etoposide (ICE chemotherapy) resulting in response rates between 24 and 33 % (Westermann et al. 2003).

In general, hyperthermia has emerged to be an efficient method for cancer treatment although the different heating techniques still have to be improved. The approach for deep-seated tumours using nanoparticles is a novel non-invasive strategy for tumours mostly resistant to the commercial chemo- and radiotherapies and will boost the development of other nanoparticle based treatments. Beside the efficient tumour killing ability, hyperthermia is also the most potent enhancer of radiotherapy and most chemotherapeutic agents.

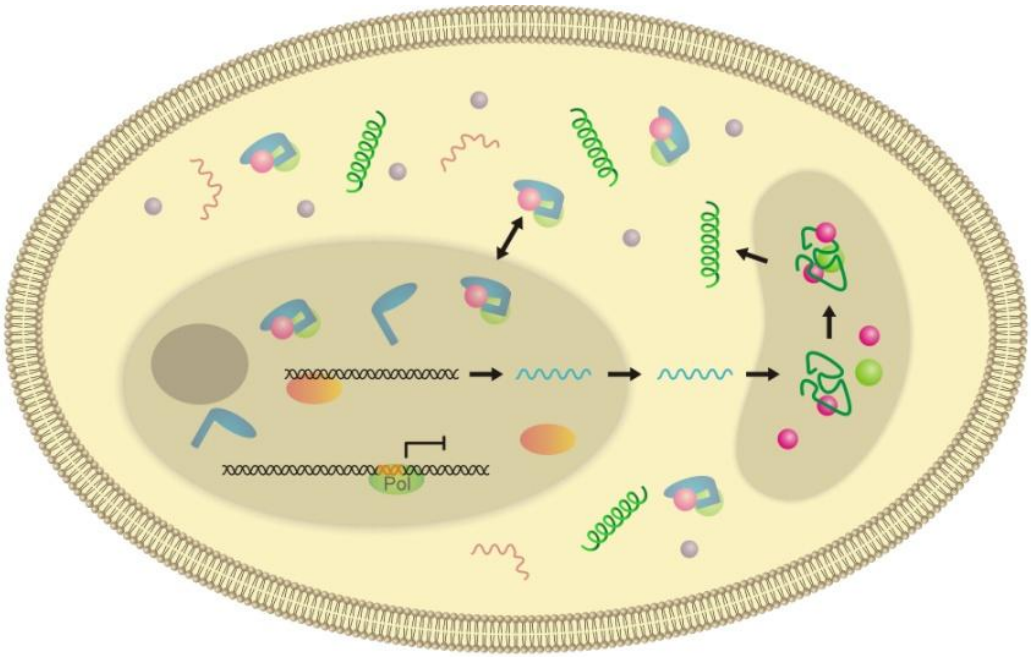
3.5. HEAT SHOCK RESPONSE

The heat shock response is a highly conserved stress response of all cells from bacteria to humans but with differences in the involved proteins or the regulation. Therefore, in this work mainly the human situation will be discussed. The heat shock response was first discovered by Ritossa in 1962 (Ritossa 1962) after the treatment of *Drosophila busckii* and *Drosophila melanogaster* larvae with higher temperatures. In response to this treatment, chromosome puffs in the polytene chromosomes of salivary glands were observed in regions encoding stress response proteins, the so called heat shock proteins. From this time on, a lot of effort was put into the analysis of this fast and robust reaction to heat. Although the heat shock pathway was initially discovered as a reaction to higher temperatures, soon afterwards it was shown that cells used this response when exposed to several kinds of stress, environmental and internal. The reaction to such conditions is an ancient mechanism of cells to survive stress like heat, radiation, increased or decreased pH and oxygen levels, exposure to heavy metals, but also bacterial or viral infections. In addition, the heat shock response is also initiated by inflammation, certain protein disorders or aging. When cells are exposed to severe stress, like heat, several problems arise in the cells. Firstly, the naive proteins in the cells start to partially unfold and also the cytoskeleton is affected. At mild stress actin is converted into stress fibres and in severe situations the whole cytoskeleton collapses including actin, tubulin and intermediary structures. Secondly, the different cell organelles lose their correct position within the cell, start to degrade and also the intracellular transport is disrupted (Welch et al. 1985). Thirdly, the whole transcriptional machinery is hit by stress resulting in inhibited RNA splicing (Vogel et al. 1995) and downregulation of protein synthesis (Storti et al. 1980; Dinh et al. 2001). Beside the intracellular effects, stress also acts on the cell membrane initialising hyperfluidisation, changes in lipid composition and rearrangement of microdomains (Nagy et al. 2007; Vigh et al. 2007). To prevent cells from this undesired effects the heat shock pathway is induced.

In principle, the pathway is turned on by an external or internal trigger, leading to the activation of key mediators, which then turn on expression of stress responsive genes. Although several stimuli are known today, they all result in protein denaturation (Figure 3-1 B 1) followed by activation of the heat shock factor (HSF), the major player in the heat shock response. HSF is activated by trimerisation (Figure 3-1 B 2) and modifications like phosphorylation (Figure 3-1 B 3) leading to an enhanced DNA binding capacity at special

recognition sites, the heat shock elements (HSE) (Figure 3-1 B 4). Due to induction via stress, active HSF trimers turn on the expression of several “survival proteins”, most of them preventing cell death and enhancing survival like the heat shock proteins (HSPs). They act as molecular chaperones preventing incorrect protein folding or refolding of partially unfolded proteins. Beside the role in survival, the heat shock proteins are also involved in *de novo* protein synthesis (Figure 3-1 A), several other cellular response mechanisms and certain diseases. Therefore, this pathway is relevant in most cellular reactions but still, its regulation and activation are not completely understood.

A



B

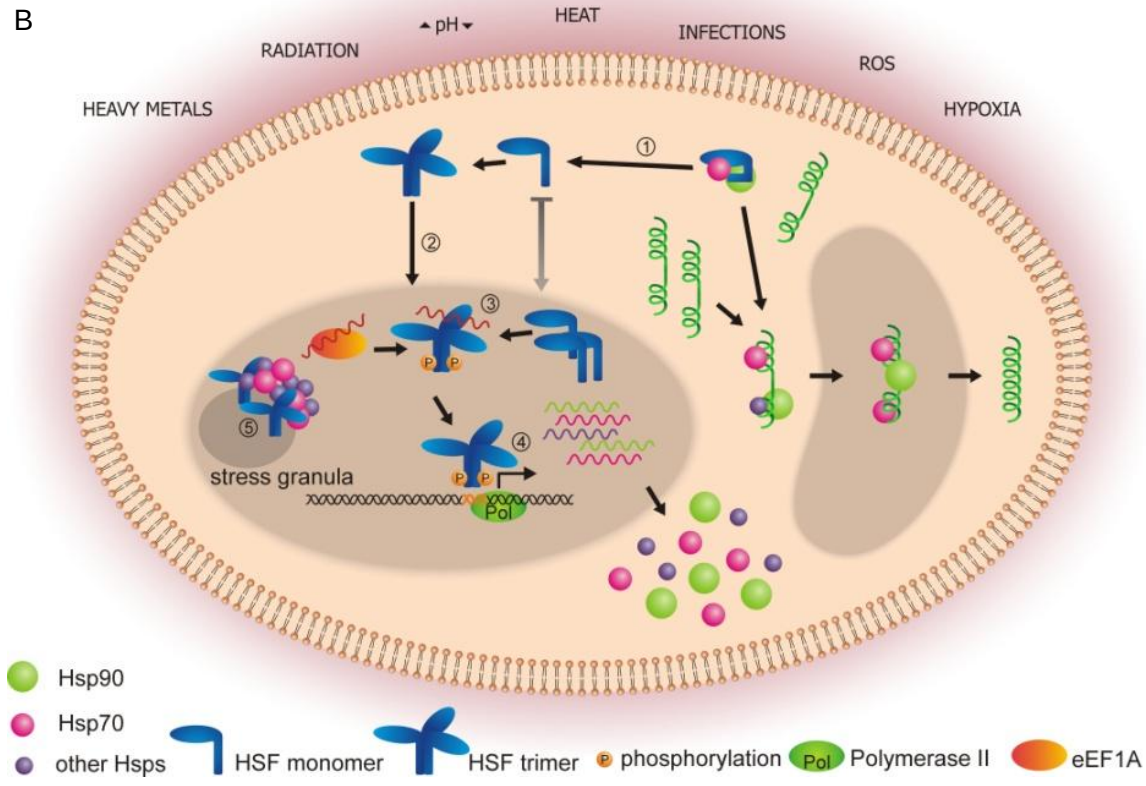


FIGURE 3-1 THE HUMAN HEAT SHOCK RESPONSE. In unstressed cells HSF1, the key mediator of the heat shock response, is engaged in an inactive complex with Hsp90 and Hsp70/40. HSF is localised in the nucleus or the cytoplasm and the RNA polymerase is locked at the promoter of Hsp genes. In response to several different stress factors, like heat, radiation, heavy metal exposure or hypoxia, proteins in the cell start to denature and the Hsp´s of the inactivation complex are recruited to the misfolded proteins. As a result, HSF monomer is released, starts to trimerise, is modified at several positions and binds to the HSE in the promoter of target genes (mainly Hsp) and also to the stress granula. In addition, the membrane gets hyperfluid which also triggers HSF activation. As an additional activator, a heat responsible RNA, heat shock RNA 1 (HSR1) together with the elongation factor 1a (eEF1A) promote HSF1 activation. After binding of the HSF1 trimer to the HSE the RNA polymerase II is unlocked and transcription starts**.**

3.5.1. HEAT SHOCK PROTEINS

One of the most dominant groups of proteins regulated during the heat shock response are the so called heat shock proteins. These proteins primarily assist the refolding of cellular proteins after stress dependent denaturation and therefore belong to the family of chaperones. As the Hsps constitute 1-2 % of total protein in unstressed cells, it is clear that they also have important functions in normal cells. In principle, heat shock proteins all act as cytoprotective agents, both in normal and stressed cells. Some of the family members are involved in *de novo* protein synthesis, others are important for cytoskeleton maintenance (Hsp27) or act as enzymes (Hsp32). Heat shock proteins are divided into five classes according to their molecular weight, the small Hsps (15-30 kDa), Hsp60s, Hsp70s, Hsp90s and the sub-group of Hsp110s.

3.5.1.1. Small heat shock proteins

The small heat shock proteins are a group of conserved ATP independent chaperones with a molecular mass ranging from 15-30 kDa. They all share a conserved C-terminal domain of eight beta strands forming an intermolecular beta sheet, the so called alpha-crystallin domain (Hayes et al. 2009) and some subunit interaction or stabilisation domains. The members of this family display their role in mainly preventing protein aggregation by the formation of large hetero- or homo-oligomers out of monomeric or dimeric modules (Benesch et al. 2008). In humans 10 different small heat shock proteins (HSPB 1-10) are known, some of them are ubiquitously expressed like Hsp27 (HSPB1), Hsp20 (HSPB6) or Hsp22 (HSPB8) while others are only expressed in special tissues like heart and muscle (HspL27 (HSPB3), cvHsp (HSPB7) or Mkbp (HSPB2)), testis (CT51 (HSPB9) and ODF1 (HSPB10)) or eye lens (alpha-crystallin A chain (HSPB4)) (Kampinga et al. 2009). Beside their role in protein aggregation prevention, some small Hsps also interact with components of the cytoskeleton. Hsp27 for example is bound to the barbed ends of F-actin (Lavoie et al. 1993), Hsp20 is important for actin cross linking (Tessier et al. 2003) and α-crystallin interacts with intermediate filaments as vimentin or desmin (Nicholl et al. 1994; Perng et al. 1999). The overall potential of the small Hsps to form large oligomers up to 100 kDa is dependent on different post-translational modifications, mainly phosphorylation of serin residues. The phosphorylation status of the proteins determines their accumulation to larger aggregates as de-phosphorylation results in larger oligomers whereas higher levels of this post-translational modification lead to smaller oligomers (Parcellier et al. 2005). In addition to the oligomerisation status also the cellular localisation is influenced by the phosphorylation status of the proteins. Hsp27 for example is bound to F-actin as a dimer or tetramer and after stress treatment, Hsp27 is phosphorylated and translocates to the nucleus where it forms larger multimers and actin polymerisation in response to stress is initiated (Guay et al. 1997; Garrido et al. 1998; Brunet Simioni et al. 2009). In response to several kinds of stress, the small Hsps are rapidly modified and as a result change their oligomerisation form and their binding properties (Lee et al. 1997). They

can work together with Hsp70 in protein re-folding, the degradation machinery of the proteasome (Lanneau et al. 2007) or interact with key apoptotic proteins to prevent stress induced cell death (Bruey et al. 2000; Charette et al. 2001). Mutations in the small Hsps lead to neuro-myopathies (Irobi et al. 2004) or cataract (Koteiche et al. 2006).

3.5.1.2. Heat shock protein 60

Heat shock proteins 60 (Hsp60) belong to the family of chaperonins, which are oligomeric ring-shaped structures and together with a second protein, Hsp10 perform correct folding of proteins in an ATP dependent manner. The structure of this chaperonin was extensively studied in the *Escherichia coli* (*E.coli*) analog of Hsp60, the GroEL chaperonin (Ranson et al. 2001). In principle, the protein is built of three domains, the apical domain which is responsible for substrate binding and interaction with the co-chaperone, the equatorial ATP-binding domain and the intermediate domain, which acts as a linker between the other two domains. The Hsp60 chaperonin machinery consists of two heptameric rings, connected via the intermediate domain of each Hsp60. These ring structures are flanked by the heptameric ring-shaped co-chaperone Hsp10/GroES (*E.coli*) at the top and the bottom of the cavity. The chaperonin oligomer can engage unfolded proteins up to 60 kDa and convert it to the correct folded proteins in the presence of ATP. Two transitional states are defined for the Hsp60 cavity, the peptide-accepting state, where the non-native peptide is captured by a flexible hydrophobic part in the so-called “open” state of the chaperonin (Weissman et al. 1995; Sigler et al. 1998). After internalisation of the naive peptide, the lid and bottom heptamers of Hsp10 close the cavity and ATP-dependent protein folding is performed, termed peptide-folding state (Farr et al. 2000). The Hsp60, as other Hsps, is highly conserved from bacteria to humans and shares ~95 % sequence and structural homology in different bacteria and up to 70 % homology in certain protein domains when compared to humans. Nevertheless, the human Hsp60 exhibits special oligomeric states and can be present as a mono-, hepta- and tetradecamer which is in contrast to the oligomeric state of other chaperonins (Levy-Rimler et al. 2002). Human Hsp60 (HSPD1) is translated into a preprotein and targeted to mitochondria via an N-terminal mitochondrial targeting sequence (MTS). During the uptake into the mitochondria the preprotein is cleaved and assembles to its functional oligomeric chaperonin structure (Singh et al. 1990; Venner et al. 1990). Hence, the primary location for Hsp60 is the mitochondrium, although recently it was also shown to be localised in the cytoplasm or the cell membrane in stressful conditions (Pfister et al. 2005) or during diseases like cancer (Ghosh et al. 2008) or atherosclerosis (Wick 2000). As Hsp60 displays a high homology with bacterial Hsps the innate and adaptive immunity of humans somehow can cross-react with the human Hsp60 when expressed on the cell surface (Young et al. 1989; Derbinski et al. 2010). Although Hsp60 is constitutively expressed it is up-regulated to some extent in response to heat (Naylor et al. 1996; Vargas-Parada et al. 2001) and plays also a bifunctional role in apoptosis. On the one hand, Hsp60 located in the cytoplasm was shown to release active caspase-3 in an ATP-dependent manner (Samali et al. 1999; Xanthoudakis et al. 1999) and therefore exhibits a pro-apoptotic role. On the other hand, cytosolic Hsp60 also prevents apoptosis by forming complexes with Bax and Bak (Kirchhoff et al. 2002) and is associated with the survival of tumour cells (Ghosh et al. 2008).

3.5.1.3. Heat shock protein 70/100 family

The proteins of the Hsp70 (HSPA) family are a highly conserved group of ATP-dependent chaperones ranging from 66 to 78 kDa. All members of the family share two conserved domains, the N-terminal adenine nucleotide-binding domain of ~ 44 kDa (ATPase domain or

NBD) and the ~ 27 kDa C-terminally located peptide binding domain (PBD) or substrate binding domain (SBD). The SBD consists of two motifs, one substrate binding ß-sandwich motif built of two ß-sheets and an α-helical domain responsible for substrate binding affinity (Mayer et al. 2001). The linker domain between NBD and SBD is important for the conformational changes in chaperone function and is therefore highly conserved. As Hsp70 is a ATP-dependent foldase, the substrate is bound to the SBD in the presence of ATP and a co-chaperon (Hsp40) with low affinity, after hydrolysis to ADP the substrate binds with high affinity and ADP is released using nucleotide exchange factors (NEF) like heat shock protein binding protein 1 (HspBP1) (Shomura et al. 2005) or Bag (Sondermann et al. 2001). This switch between the two conformations is repeated till the protein has its correct folding. In normal cellular environment the Hsp70s are involved in *de novo* protein synthesis, protein transport across membranes and assist the generation of protein complexes using different co-chaperones as Hsp40, Bag, Hsp-organising protein (HOP) (Chen et al. 1998), HSC70-interacting protein (HIP) (Hohfeld et al. 1995) or carboxyl terminus of Hsp70-interacting protein (CHIP) (Ballinger et al. 1999). These co-chaperones modulate the chaperone activity of members of the Hsp70 family by binding to the two conserved domains. When cells are exposed to several kinds of stress, some members of the Hsp70 family (Hsp72, Hsp70B´) are transcriptionally up-regulated and prevent protein aggregation or unfolding and refold partially denatured proteins. The group of Hsp70s is highly conserved except in some hyperthermophilic archaea (Gribaldo et al. 1999) and shares about 60 % sequence homology even with the major bacterial Hsp70, the DnaK. In humans today 13 members are known in the family of the HSPA (former known as Hsp70s) (Kampinga et al. 2009) with different localisation in the cell and different expression levels or inducibility in response to stress. Some HSPA genes do not contain introns (HSPA1A/B, HSPA1L, HSPA2, HSPA6 and HSPA7) while all other members have different numbers of introns.

Three of this proteins are encoded in the major histocompatibility (MHC) locus on chromosome 6, the HSPA1A (Hsp72), the HSPA1B (Hsp70-2) and the HSPA1L (Hsp70-Hom or Hsp70t). HSPA1A and HSPA1B are two highly homologous proteins differing in only two amino acids and seem to be interchangeable. Both HSPA1A and HSPA1B are intronless genes differing in their 3´untranslated region (3´ÚTR) (Walter et al. 1994) and both proteins are stress inducible, the HSPA1A at higher levels upon prolonged activation in severe stress and HSPA1B as a stronger inducible Hsp activated even at mild stress (Akcetin et al. 1999). HSPA1A is the best studied of the inducible chaperones and is long known to be sensitive to several kinds of stress like heat (Wu et al. 1985), hyperoxia (Chambellan et al. 2006) or hypoxia (Benjamin et al. 1990), depletion of nutrition (Nissim et al. 1992; Bergeron et al. 1996), heavy metals (Murata et al. 1999) or radiation (Nogami et al. 1993; Trautinger et al. 1999) and works as a cytoprotective agent to prevent and repair protein denaturation (reviewed in (Morimoto 1991; Mathew et al. 1998; Morimoto 1998; Mathew et al. 2000; Soti et al. 2005; Voellmy et al. 2007; Shamovsky et al. 2008; Akerfelt et al. 2010; Morimoto 2012). HSPA1A and HSPA1B are located in the cytoplasm in unstressed cells and translocate to the nucleus in stressful conditions. In addition to their prominent role in the heat shock response by preventing protein degradation, HSPA1A plays also an important role in apoptosis, as the protein inhibits the activation of caspase (Mosser et al. 2000), the apoptosis signal-regulating kinase 1 (Ask-1) (Park et al. 2002) or the activation of c-Jun N-terminal kinase (JNK) (Park et al. 2001a) after stress. It also stabilises the protein kinase B (PKB)/Akt and primes the protein kinase C (PKC) for phosphorylation (Gao et al. 2002), blocks NFκ-B activation by inhibition of I-κBα kinase activation (Shanley et al. 2000) or the release of cytochrome c by preventing Bax translocation (Stankiewicz et al. 2005). Another important feature of the inducible HSPA1A is the suppression of the immune response via

inhibiting the transcription (Cahill et al. 1996; Cahill et al. 1997; Housby et al. 1999) or the negative regulation of inflammatory cytokines (Ianaro et al. 2001). On the other hand, HSPA1A can also be found extracellularly or associated with the membrane in response to stress, which in fact stimulates the immune response. The release of HSPA1A from the cell is either the result of necrotic cell death (Basu et al. 2000) but also intact cells like B-cells (Clayton et al. 2005), glia cells (Guzhova et al. 2001) or peripheral blood mononuclear cells (PBMC) transport HSPA1A to the extracellular space using exosomes (Lancaster et al. 2005). When expressed at the cell surface in response to stress or diseases, the heat shock protein can interact with cytotoxic T-cells and natural killer cells (Multhoff 2002; Lehner et al. 2004) without the involvement of MHC. Extracellular HSPA1A is also associated to the stress response and can stimulate inflammatory cytokine production (Multhoff et al. 1999), inducible nitrogen oxide synthase (iNOS) (Panjwani et al. 2002), tumour necrosis factor α (TNF-α), interleukin production (Asea et al. 2000) or activation of the complement cascade (Prohaszka et al. 2002). Beside its role in stress response, HSPA1A is also involved in normal cellular functions as cell-cycle control, development and differentiation or cell growth. HSPA1A is regulated mainly by HSF in the heat shock response, but is also affected by other cellular signaling pathways like, transforming growth factor beta (TGFβ) signaling (Takenaka et al. 1992), JAK-STAT signaling (Stephanou et al. 1999) and the mitogen-activated protein kinase/ extracellular signal-regulated kinase (MAPK/ERK) pathway (Song et al. 2001). The third protein encoded in the MHC locus, HSPA1L is localised in the cytoplasm (Milner et al. 1990) and the gene is induced after heat stress to some extent without tremendous increase in protein levels (Hageman et al. 2011), but high constitutive expression levels in testis (Ito et al. 1998).

HSPA2 (Hsp70.2) another member of the family is also highly expressed in testis but also to some extent in other tissues. This protein was shown to play an important role in spermatogenesis (Dix et al. 1996; Govin et al. 2006), but is as well expressed in other tissues (Scieglinska et al. 2011). Beside other members of the family the HSPA2 gene contains HSE which are not functional and the gene is not expressed in response to stress (Wisniewski et al. 1990). It is located in the cytoplasm and is predicted to play a role in tumour cell growth (Rohde et al. 2005).

Most of the HSPA family members are localised in the cytoplasm, but there are also some chaperones which are specific for certain organelles. The constitutively expressed HSPA5 (GRP75 or BiP) is localised in the lumen of the endoplasmic reticulum (ER) and responsible for the import and folding of secretory proteins and other ER specific proteins (Munro et al. 1986). HSPA5, like HSPA1A, is involved in the apoptotic pathway by interaction with p53 (Wadhwa et al. 2002). The mitochondrial chaperone HSPA9 (mortalin, mtHsp75 or GRP75) is also constitutively expressed and like HSPA5 is not up-regulated in response to stress (Bhattacharyya et al. 1995). This chaperone is necessary for the import of proteins into the lumen of mitochondria where the proteins are correctly assembled by Hsp60. Another compartment specific HSPA is HSPA13 (Stch), which is associated with microsomes (Otterson et al. 1994). Like the other more distantly related family members HSPA12A and HSPA12B (Han et al. 2003), this gene is not induced in response to heat stress. Another distant member, the HSPA14 (Wan et al. 2004) shows some inducibility in response to stress and seems to play a role in immune response.

In normal cells, the most abundant HSPA is the housekeeping chaperone HSPA8 (Hsc70 or Hsp73). This constitutively expressed protein maintains polypeptides in an unfolded stage to transport them across membranes. HSPA8 also targets denatured proteins to degradation via lysosomes.

In large mammalians but not in mice, there is another inducible member of the Hsp70 family expressed, the HSPA6 (Hsp70B´) (Leung et al. 1990; Noonan et al. 2008) and the highly homologous HSPA7 (Hsp70B), which is also highly inducible, but does not encode a functional protein (Parsian et al. 2000). HSPA6 shows ~ 80 % overall sequence homology and even 100 % homology in the peptide binding domain when compared to HSPA1A, but is exclusively expressed under severe stress (Noonan et al. 2007a). In addition, the inducibility even increases, when cells are kept at low cell density (Noonan et al. 2007b). HSPA6 unlike all other HSPA family members was not shown to be able to refold proteins, maybe because this chaperone has an abnormal N-terminal ATPase domain. However, it is able to rescue p53 activity even without the normally necessary co-chaperone DNAJ (Hsp40) (Hageman et al. 2011). The HSPA6 promoter contains 4 HSE (Schiller et al. 1988; Noonan et al. 2007b) and is strongly induced in response to severe proteotoxic stress, but regulated more tightly compared to HSPA1A as it persists just for some hours, whereas HSPA1A expression can continue for several days (Noonan et al. 2007a).

Closely related to the HSPA family are the four known HSPH proteins, HSPH1 (Hsp105), HSPH2 (Hsp70RY, HSPA4, APG-2, Hsp110), HSPH3 (HSPA4L, APG-1) and HSPH4 (Grp170, ORP150, HSP12A). These proteins were previously classified as member of Hsp70s as they consist of the same ABD and SBD but an extended linker domain and a longer C-terminal end (Liu et al. 2007; Shaner et al. 2007). Due to these structural differences these proteins were recently classified into a new sub-family of heat shock proteins (Lee-Yoon et al. 1995; Kampinga et al. 2009). Except HSPH4 (Grp170), which is an ER-specific protein (Chen et al. 1996), all other HSPHs are located in the cytoplasm. The proteins of this family act as NEF for HSPA (Dragovic et al. 2006) and in addition have chaperone activity on their own. Nevertheless, they are not able to fold proteins, but bind immediately to aromatic stretches in unfolded peptides, compared to the aliphatic residues preferred by the HSPA chaperones (Xu et al. 2012a). Associated to HSPA proteins the HSPHs are involved in different cellular processes like *de novo* folding of proteins (Yam et al. 2005), translocation of proteins into the ER (Shaner et al. 2005), protein degradation or refolding under stress conditions (Albanese et al. 2006). Two members of the family were shown to be induced in response to stress, HSPH1 (Hsp105) (Subjeck et al. 1983) and HSPH3 (APG-1) (Kojima et al. 1996; Kaneko et al. 1997) while HSPH2 (Hsp70RY) was shown not to be induced by heat stress (Fathallah et al. 1993; Santos et al. 1998).

In conclusion the proteins of the HSPA and HSPH family are located in several compartments in the cell and are involved in the protein life cycle from *de novo* synthesis up to the refolding of stress denatured proteins and assist transport across membranes and degradation. Therefore, these groups of chaperones play an important role in normal cell processes, but some of the members are also key players in stress response.

3.5.1.4. Heat shock protein 90 family

In normal cellular environment, the group of Hsp90 proteins (HSPC family) are among the most strongly expressed cytoplasmic components (Borkovich et al. 1989). They regulate many processes in cellular compartments and their expression can be further induced in response to stress (Jakob et al. 1995; Yonehara et al. 1996). In bacteria, only a single Hsp90, called high-temperature protein G (HtpG), is known whereas the group of archaea lacks this family of heat shock proteins (Stechmann et al. 2004). In humans, five different HSPC proteins are known today, HSPC1 (Hsp90, Hsp87, Hsp90AA1), HSPC2 (Hsp90AA2),

HSPC3 (Hsp90AB1, Hsp90β), HSPC4 (GRP94, endoplasmin, HSP90B1) and HSPC5 (Hsp75, Hsp90L, TRAP1) (Chen et al. 2005; Kampinga et al. 2009).

Hsp90 proteins share three highly conserved regions, a 25 kDa N-terminal ATP-binding domain (ABD), a middle domain and a 50 kDa C-terminal dimerisation domain. In general, Hsp90 acts as a homodimer, in which the dimerisation is mediated via the C-terminal region. This region consists of α- helices and β-sheets and two of these α-helices per monomer form the tetra-helical dimer structure (Minami et al. 1994; Harris et al. 2004). In addition to dimerisation, the C terminus of cytosolic HSPC members also harbours a motif to recognise the tetratricopeptide repeat (TPR) domain, the Met-Glu-Glu-Val-Asp or MEEVD motif (Young et al. 1998) which is similar to the Hsp70 TPR binding motif. The C-terminal domain is linked to the N-terminus by a middle domain, containing two αβα motifs linked to α helices. This middle domain contains many recognition sites important for client protein interactions (Meyer et al. 2003). The ABD at the N-terminus is highly conserved and is build of a α and β-sandwich motif (Prodromou et al. 1997a; Prodromou et al. 1997b) which is also the binding site for the major Hsp90 inhibitors like geldanamycin (Stebbins et al. 1997). Other conserved amino acids form a lid like structure, which closes the ATP-bound pocket and together with loops of the middle domains are required for efficient ATP hydrolysis (Meyer et al. 2003). In the ATP bound state, the N-terminal domain also stabilises the Hsp90 dimer and enhanced activity (Cunningham et al. 2008). For the compartment specific HSPC members tumour necrosis factor receptor-associated protein 1 (TRAP1, HSPC5) which is located in the mitochondria and the ER specific Grp94 (HSPC4, HSP90B1) a leader sequence is also located at the N-terminus. In addition, the Grp94 has a highly conserved KDEL sequence in the C-terminal region which locates this chaperone to the ER (Chang et al. 1989). Cytosolic HSPC members like HSPC1 (HSP90AA1), HSPC2 (HSP90AA2) or HSPC3 (HSP90AB1) harbour an additional flexible and charged linker sequence, which is located between the N-terminus and the middle domain. This linker is essential for ATP hydrolysis dependent conformational changes in the cytosolic Hsp90 (Hainzl et al. 2009).

In contrast to other chaperones, the conformational states of the Hsp90 chaperone cycle are less conserved. Hsp90 itself has weak ATPase activity which is enhanced by interaction with co-chaperones (Nadeau et al. 1993). Without ATP, the Hsp90 dimer is attached to each other just at the C-terminal part also called the opened state of the cycle. After binding of ATP to the N-terminal binding site, the lid closes and due to conformational changes, an additional dimerisation site at the N-terminus is formed. This conformation is termed closed state. The hydrolysis of ATP triggers the conversion to an intermediate form which is not understood till now. The release of ADP then converts the Hsp90 back into the opened state. Within this cycle, the conformational changes in the chaperone are rate limiting steps (Graf et al. 2009; Hessling et al. 2009) and some co-chaperones are involved in the Hsp90 client maturation like p23 (Freeman et al. 2000; Young et al. 2000).

Proteins of the HSPC family play an important role in several cellular processes but they have only limited influence on *de novo* protein synthesis (Nathan et al. 1997). The chaperones bind together with co-chaperones to partially folded intermediate conformations of their target proteins, the Hsp90 clients, and stabilise these conformations or enhance maturation (Pearl et al. 2006). Recognition of Hsp90 clients does not involve a special motif, as shown for other chaperones, but seems to be linked to the different co-chaperones and client binding found for all three conserved regions of Hsp90. These attached proteins, around 20 for cytosolic eukaryotic Hsp90, influence Hsp90 in different ways. A subgroup of co-chaperones enhances (AHA1, Cpr6) (McLaughlin et al. 2002; Panaretou et al. 2002) or

inhibits Hsp90 ATPase activity (like HOP, Cdc37 or p23) (Prodromou et al. 1999; Siligardi et al. 2002). Others recruit special clients as for example Cdc37, which recruits protein kinases or Cpr6, FKBP51 and FKBP52 which recruit progesterone and glucocorticoid receptors (Riggs et al. 2004; Caplan et al. 2007). In addition, co-chaperones also influence the Hsp90 chaperone cycle.

The cytosolic Hsp90s (HSPC1-3) are responsible for most processes associated with this family and are, except for one co-chaperone known for Grp94 (Liu et al. 2010), the only ones that need these associated proteins. In combination with the different co-chaperones, Hsp90 proteins are involved in the regulation of many different clients like steroid hormone receptors (Bresnick et al. 1989; Nathan et al. 1995), protein kinase (Xu et al. 2001), but also eNOS (Garcia-Cardena et al. 1998), myosin (Rayment et al. 1993; Liu et al. 2008) and transcription factors like STAT3 (Sato et al. 2003). HSPC family members are also involved in antigen processing (Li et al. 2002; Kunisawa et al. 2006), protein trafficking and secretion (Chen et al. 2006; Lotz et al. 2008), RNA processing (Boulon et al. 2008; Zhao et al. 2008), telomere stability (Holt et al. 1999) but also protein degradation (McClellan et al. 2005). In addition, Hsp90 proteins combined with the TPR domain containing co-chaperones such as HOP interact with chaperones of the HSPA family (Hsp70) and their co-chaperones Hsp40 to facilitate client maturation. These clients include progesterone receptor (Cintron et al. 2006) or HSF1, thus priming its activation (Nadeau et al. 1993).

Although Hsp90 is highly expressed in normal cells, the expression can be further increased by environmental stress via HSF1 (Sorger et al. 1987) or immune response via STAT1 or STAT3 (Stephanou et al. 1997; Ripley et al. 1999; Ammirante et al. 2008) leading to a kind of self-regulation of the chaperone. Hsp90 and many of its co-chaperones were also shown to be up-regulated in certain diseases including cancer (McDowell et al. 2009; Whitesell et al. 2012) or neurodegenerative disorders (Salminen et al. 2011) reflecting their important role in cellular processes.

The proteins of the HSPC family are in general essential for many cellular processes in unstressed environment and in addition play an important role in stress response. They are located in several compartments and, except for the cytosolic proteins, act on the stabilisation of immature clients without additional help. In case of cytosolic Hsp90, the co-chaperones trigger the protein specific activity and enhance the action of the chaperone.

3.5.1.5. Other heat shock proteins

The above mentioned groups of heat shock proteins play several key functions in cells, either in regulating normal cellular processes or in enhancing survival after the exposure to different kinds of stress. In addition to these chaperone families, there exist also other heat shock proteins involved in cellular functions. One of these protein groups are the Hsp40 (DnaJ) proteins, which act as major co-chaperones for Hsp70 proteins. They are characterised by a highly conserved J-domain and are further subdivided in humans into DnaJA and B depending on the position of the J-domain and the presence of an additional cysteine-rich region (Cheetham et al. 1998). DnaJ proteins are localised in the cytoplasm or the nucleus (Hageman et al. 2009) and a subset of these proteins is induced in response to stress (Hageman et al. 2011).

Most heat shock proteins are involved in the folding or stabilising of protein structures. Beside these members, also special heat shock proteins exist, which do not affect protein synthesis or maintenance, but have other roles. Hsp32, for example, is not a chaperone but

shows enzymatic activity as haemoxygenase-1 (HO-1) catalysing the conversion of pro-oxidant haem into biliverdin (reviewed in (Chan et al. 2011)). Hsp32 is increased in response to stress (Stocker 1990) and thereby activates nitric oxide synthase (Weiss et al. 1994). Another example for a specialised heat shock protein is Hsp47, a collagen specific protein. Hsp47 is responsible for the appropriate three-dimensional arrangement of procollagen chains and prevents accumulation of these chains. The protein is localised in the ER and is further induced in response to stress (Nagata 1998).

In general, heat shock proteins of all different families mediate important cellular functions, but are even more important for cells exposed to any kind of harmful conditions. Heat shock proteins are involved in protein homeostasis, signaling transduction cascades, cellular architecture and survival.

3.5.2. HEAT SHOCK FACTORS

Heat shock factors (HSF) are key mediators of the cellular stress response. These proteins act as transcription factors which are constitutively expressed (Fiorenza et al. 1995) but exist in a repressed state in normal cells. Upon activation via stress the repressed HSF monomers are released from the inactivating chaperone complex and trimerise to generate a high affinity DNA binding domain recognising special elements, the so called heat shock elements, in the promoter of target genes (reviewed in (Anckar et al. 2011)). HSF are highly conserved throughout all organisms, but they differ in the number of family members. In invertebrates like yeast, only one HSF is known whereas in mammals four different HSFs are found, (HSF1-4 in mouse, HSF1, HSF2 and HSF4 in humans) and plants contain even more family members (Nover et al. 2001; Akerfelt et al. 2010).

The structure of the HSF members is highly conserved and contains an N-terminal DNA binding domain (DBD), a region responsible for trimerisation, a central regulatory domain (RD) and a C-terminally located transactivation domain (TAD) (Figure 3-2).

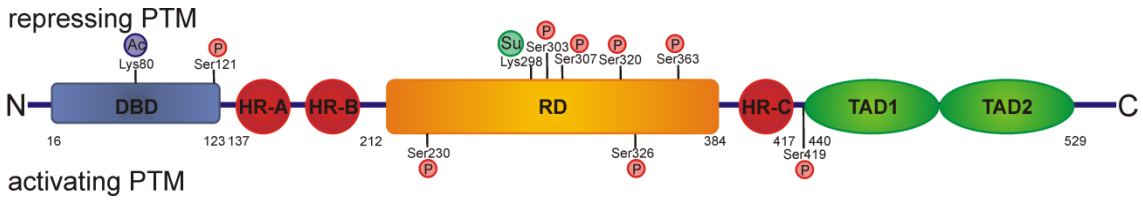


FIGURE 3-2 STRUCTURE OF HUMAN HSF1. The human HSF1 is composed of an N-terminally located DNA binding domain (DBD) which recognises the heat shock elements (HSE) in the promoter of target genes. Next to it there is a trimerisation domain built of two α-helices with hydrophobic hepta repeats (HR-A and HR-B). In the middle, a regulatory domain (RD) is located which is responsible for most post-translational modifications either inhibiting or enhancing HSF1 function. Between the RD and the two transactivation domains (TAD1 and TAD2) there is another hepta repeat located (HR-C) which interacts with HR-A and HR-B in the inactive monomeric form of the HSF. The TAD is located at the C-terminus and facilitates transcriptional activation of target genes. Important repressing and activating post-translational modifications are shown at the distinct positions. Ac: acetylation (blue), p: phosphorylation (red), Su: sumoylation (green); PMT: post-translational modification.

The DBD is the highest conserved domain in the HSF and is built of a winged helix-turn-helix motif, where the wing stabilises DNA binding by protein-protein interaction (Littlefield et al. 1999). In its trimerised form, the HSF DBD recognizes the HSE in the promoter of target genes (Figure 3-3). HSE are inverted repeats of at least three pentamers with a consensus sequence of nGAAn (Amin et al. 1988) and are located at the major groove of the DNA helix

(Wu 1995). In addition to DNA binding, the DBD also mediates interaction with other proteins to influence transactivation of the HSF (Bulman et al. 2001). Next to the DBD a domain responsible for trimerisation is located (Figure 3-2). This region is composed of two sub-domains, which each contain an amphiphilic helix with an array of hydrophobic heptad repeats (HR-A and HR-B) like in leucine zipper motifs (Sorger et al. 1989; Peteranderl et al. 1992). In the trimeric form of the HSF, the two leucine zipper motifs HR-A and HR-B form a triple-stranded coiled-coil structure (Peteranderl et al. 1999), which is further stabilised by intermolecular disulfide bonding (Ahn et al. 2003). Trimerisation is inhibited in the inactive state of HSF1 by the interaction of HR-A and HR-B with an additional HR located between the regulatory domain and the transactivation domain, the HR-C. This domain is thought to interact with the other two HR domains in the inactive monomer by conformational proximity (Figure 3-3) (Rabindran et al. 1993). In between the HR-A/B and HR-C domains, a central regulatory domain (RD) is located (Figure 3-2) (Green et al. 1995). Within this area most post-translational modifications (PTMs) (reviewed in (Xu et al. 2012b)) are found and this region inhibits the transactivation domain. The RD also harbours the intrinsic function to assay heat stress even without other parts of the HSF (Newton et al. 1996). At the C-terminus HSF1 has a transactivation domain (TAD), divided into two sub-domains, the TAD1 and the TAD2. Both domains are rich in hydrophobic and acidic residues and seem to be largely unfolded (Pattaramanon et al. 2007) although TAD1 seems to harbour a α-helical short hydrophobic linear motif, which interacts with the basal transcription factor TATA box binding protein (TBP)-associated factor,TAF-9 (Choi et al. 2000). In contrast, TAD2 is rich in proline residues and therefore seems to be nonhelical (Newton et al. 1996). The acidic parts of the TADs are necessary for initiation of target gene transcription, whereas the hydrophobic residues are responsible for elongation by interaction with chromatin remodelling complexes (Brown et al. 1998; Sullivan et al. 2001). In addition, HSF also contain a bipartite nuclear localisation signal (NLS) next to the HR-A and HR-B (Sheldon et al. 1993; Vujanac et al. 2005) which is responsible for location of the transcription factor in the nucleus (Mercier et al. 1999).

HSF is activated by a multi-step process converting the inactive monomer into a transcriptionally active trimeric version. In the monomeric state, HSF is attached to a chaperone complex which prevents formation of trimers. Hsp90 is bound to the RD but also the DBD and inhibits the binding of HSF to the target DNA (Ali et al. 1998; Bharadwaj et al. 1999). On the other hand trimerisation of the monomers is inhibited at least in hHSF1 and 2 by the interaction of HR-C with the oligomerisation domain of HR-A and HR-B (Rabindran et al. 1993). Human HSF4 lacks the additional C-terminal HR-C, and therefore was shown to be in a constitutive trimeric state, highlighting the importance of HR-C as a trimerisation inhibitor region (Chen et al. 1993; Nakai et al. 1997). In addition to the block of DNA binding and trimerisation, also the transactivational capacity of HSF is inhibited by binding of the chaperone Hsp70 together with its co-chaperone Hsp40 to the TAD (Abravaya et al. 1992; Shi et al. 1998) (Figure 3-3). In response to stress stimuli, Hsp90 and Hsp70/40 are released from the complex with HSF monomer (reviewed in (Morimoto 2002)) and the HSF monomers trimerise either as homotrimers or as heterotrimers containing HSF1 and HSF2 (Ostling et al. 2007; Sandqvist et al. 2009). Within this trimerisation process, HSF becomes localised in the nucleus by shuttling the trimers into the nucleus and blocking nuclear export of the nuclear transcription factor (Mercier et al. 1999; Vujanac et al. 2005). Another step of regulation contains several posttranslational modifications like phosphorylation, sumoylation and acetylation. These post-translational modifications either inhibit or promote transcriptional activation of HSF (Figure 3-2 and Figure 3-3). In the inactive monomeric form, HSF is phosphorylated at least at 5 sites, in the DBD at serine residue 121 by MAPK- activating

protein kinase 2 (MK2) (Wang et al. 2006), three times in the RD at serine (Ser) 303, 307 by glycogen synthase kinase 3beta or(GSK3β) (Chu et al. 1998) and MAPK (Chu et al. 1996; Kline et al. 1997) and 320 by protein kinase A (Murshid et al. 2010) and once at residue 363 between HR-C and the TAD1 by c-Jun NH2-terminal kinase (JNK) (Dai et al. 2000), but only inhibition of phosphorylation at Ser303 and Ser307 resulted in activation of target gene transcription (Batista-Nascimento et al. 2011). In addition to phosphorylation, also sumoylation at lysine298 using SUMO E2 conjugating enzyme (Ubc9) (Anckar et al. 2006) was shown to repress transactivational activity of HSF. This sumoylation was shown to be dependent on the previous phosphorylation of Ser303, which together with Lys298 form the so called phosphorylation dependent sumoylation motif (PDSM) (Hong et al. 2001; Hietakangas et al. 2003). Another level of HSF repression is the acetylation of numerous lysine residues after stress-dependent activation to reduce HSF DNA recognition property, trimerisation and sub cellular localisation (Westerheide et al. 2009). Acetylation of lysine 80 mediated by histone acetyltransferase p300 and CREB for example, leads to reduced binding affinity of HSF as this residue was shown to directly interact with the DNA backbone (Littlefield et al. 1999). The inhibitory effect of acetylation can be diminished in stressful conditions by the deacetylase sirtuin 1 (SIRT1) resulting in a prolonged binding of HSF to the DNA (Westerheide et al. 2009). In contrast to the numerous PTM repressing HSF activity only three phosphorylation events have been linked to activation, the phosphorylation of serine residue 230 by calcium/calmodulin-dependent kinase CaMKII (Holmberg et al. 2001), the Polo-like kinase 1 (PLK1) (Kim et al. 2005) mediated modification at serine 419 and the phosphorylation of serine residue 326 by an unknown kinase, which triggers interaction of HSF with the co-activator Daxx1 (Boellmann et al. 2004).

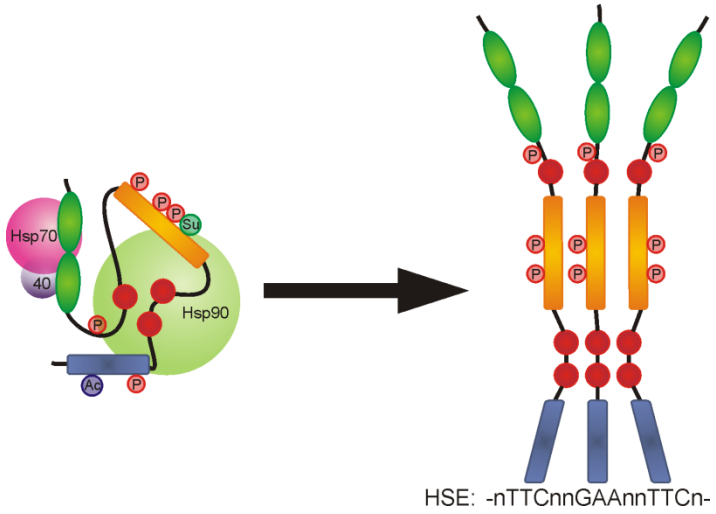


FIGURE 3-3 HSF1 TRIMERISATION. Human HSF1 is initially present as a monomer in unstressed cells. This monomer is kept in an inactive state by post-translational modifications (phosphorylation, acetylation and sumoylation) at several sites. The TAD is inhibited by binding to a complex of Hsp70 and its co-chaperone Hsp40. Hsp90 is bound to the RD, the HRs and the DBD. In response to stress, the inhibitory chaperones are released from the complex. HSF trimerises and is phosphorylated at two sites in the RD and one residue in between HR-C and TAD1. The HSF1 trimer now binds to the HSE in the promoter of target genes and activates transcription.

In mammals four different HSFs are known, of which HSF1 is the key mediator of stress responsive target gene activation. In addition, HSF1 also regulates expression of several genes in normal cellular environment (Murray et al. 2004; Trinklein et al. 2004) without increase in response to stress. These genes are involved in the regulation of cell cycle, lipid metabolism or cell proliferation (Page et al. 2006). In parallel to the translocation to the promoters of stress inducible genes, HSF1 in human cells also localises to distinct sub-nuclear structures, the so-called nuclear stress bodies (NSB) in response to stress (Biamonti 2004). NSBs are only found in human cells and are transiently formed within minutes after stress in the nucleus where they are located close to the nucleoli (Cotto et al. 1997; Chiodi et al. 2000). The HSF within the NSB binds to a region at chromosome 9q12 and regulates the transcription of a subclass of satellite III repeats. This results in the generation of large non-

coding RNA transcripts with unknown properties (Jolly et al. 2004; Rizzi et al. 2004). The knockout of the Hsf1 gene in mice results in diminished Hsp induction, a reduced cellular integrity in response to stress and abolished thermotolerance (McMillan et al. 1998) but also several developmental defects like female infertility, placenta abnormalities, as well as growth retardation and deregulated cytokine production (Xiao et al. 1999; Inouye et al. 2004; Takii et al. 2010).

HSF2 has the same structural organisation as HSF1 but is active in non-stressed cells and binds to HSE without stress trigger. As HSF2 is constitutively active on the promoter of target genes, it seems to regulate their expression in normal environment and especially during development (Mezger et al. 1994). HSF2 also binds to the Hsp promoter in response to stress, but only in combination with HSF1 (Ostling et al. 2007). In addition, HSF2 also localises to the nSB in heterotrimers together with HSF1 in response to stress (Alastalo et al. 2003). Heterotrimers of HSF1 and HSF2 seem to modulate the stress-inducible transcription of target genes as they are formed during the acute phase of the heat shock response. However, during the elongation phase HSF2 is no longer present and HSF1 homotrimers regulate transcription (Sandqvist et al. 2009). Mice lacking the Hsf2 gene showed developmental defects like altered brain morphology, reduced ovulation and defective meiosis (Kallio et al. 2002) as well as increased apoptosis in testis and reduced sperm quality (Wang et al. 2003). Cells lacking Hsf2 do not show a complete inhibition of stress triggered Hsp expression like in Hsf1 knockout cells rather than a repression of some Hsps (Hsp40, Hsp25). Therefore it seems that HSF2 has a modulatory effect on some but not all Hsp promoters (Ostling et al. 2007).

HSF4 is the only member of the family which is not involved in stress response. Due to the lack of HR-C, HSF4 is constitutively trimerised. The transcription factor is expressed in human lens cells where it regulates the transcription of non-stress inducible target genes (Fujimoto et al. 2004). In addition, HSF4 is involved in the regulation of DNA damage repair (Cui et al. 2012). Although all HSF bind to HSE, the consensus sequence for HSF4 differs significantly from that of HSF1 as here only the G nucleotide is conserved leading to a nGnnn pentameric sequence (Hayashida et al. 2011). When HSF4 is absent in mice, they start do develop cataracts in early postnatal periods (Bu et al. 2002) caused by increased activation of transcription factors and a decrease of chaperones in lens cells (Fujimoto et al. 2004).

HSF3, the last member of the mammalian HSFs, is not found in humans and was originally thought to be an avian specific HSF where it is activated in response to severe stress (Tanabe et al. 1997). In the last years, HSF3 was also identified in mice, but in this case HSF3 was shown to activate non-classical heat shock target genes like PDZK3 in response to stress (Fujimoto et al. 2010).

3.5.3. HEAT SHOCK REGULATION

The heat shock response is known for more than 50 years but still the regulation and the initial events triggering this universal defence mechanism are only partially understood. Early in the research of the heat shock response it was shown, that not only heat triggers the activation. Up to now, several different stress factors have been shown to induce the heat shock response like radiation, hypoxia, hyperoxia, low or high pH, depletion of nutrition, exposure to heavy metals, reactive oxygen species (ROS), bacterial or viral infections, DNA

damage and many others (reviewed in (Voellmy 1994; Morimoto et al. 1996; Morimoto 1998; Shamovsky et al. 2008)).

Today at least four different mechanisms for the conversion of an external stimulus to the activation of target gene expression are proposed which might all together be part of a complex regulatory network. The best analysed mechanism of heat shock response activation is based on stress dependent protein denaturation. Exposure of cells to different kinds of stress most of the time results in an increased number of denatured or partially unfolded proteins in the cell leading to increased need of molecular chaperones, which stabilise folding intermediates, refold proteins or trigger degradation (see also 3.5.1.). Among these chaperones, Hsp90 and Hsp70 are prominent chaperones necessary for proteostasis, which are recruited to the stress induced non-native proteins. As Hsp90 and Hsp70 are also the major components of the HSF inactivation complex, chaperones are released from the monomer and HSF is able to trimerise and is further modified and starts transcription of target genes (Bharadwaj et al. 1999; Morimoto 2002). Among the activated target genes, the heat shock proteins represent a large sub-group. Therefore, the necessary chaperones are up regulated and prevent protein unfolding. In response to the accumulation of Hsps in the cell, their expression is reduced, suggesting a self-regulatory mechanism in heat shock protein expression. In agreement with this model, the introduction of non-native protein into a cell results in up-regulation of Hsp expression (Ananthan et al. 1986). In addition, blocking of Hsp90 binding to the HSF by pharmacological inhibitors like geldanamycin also results in HSF trimerisation and binding to the HSE (Zou et al. 1998; Kim et al. 1999; Guo et al. 2001). Hsp90 is also bound to the RD of trimeric HSF in a complex with FKBP52 and p23 resulting in attenuation of HSF DNA-binding (Ali et al. 1998; Bharadwaj et al. 1999). In contrast, Hsp70 and its co-chaperone Hsp40 do not influence DNA binding of HSF trimer, but inhibit the transactivation capacity of HSF in dose dependent manner (Abravaya et al. 1992; Baler et al. 1996; Shi et al. 1998). The reduced activity of HSF seems to involve the Hsp70-interacting transcriptional co-repressor CoREST (Gomez et al. 2008). Another evidence for the role of Hsp70 in heat shock regulation is based on experiments where overexpression of Hsp70 was shown to inhibit heat activation of HSF (Baler et al. 1996) and that Hsp70 is able to bind its own mRNA and reduce its expression (Balakrishnan et al. 2006). Although protein denaturation is definitely a key event in heat shock response activation, additional sensoring mechanisms must be involved because the recruitment of HSF to the promoter and transcription of target genes was shown to take place within seconds up to a few minutes after heat shock initiation (Boehm et al. 2003; Zobeck et al. 2010), which is too fast for the protein denaturation based activation model.

Another sensory mechanism for heat shock response is directly linked to temperature. It was shown that a ribonucleoprotein complex is able to facilitate HSF1 activation. The complex contains the translational elongation factor eEF1A and a non-coding, constitutively expressed RNA, the heat shock RNA-1 (HSR-1) (Shamovsky et al. 2006). Mechanistically, this RNA might be related to the thermosensoric bacterial $\sigma 32$ RNA (Kugel et al. 2006), which is involved in the transcriptional activation of heat shock genes by temperature-dependent conformational changes (Morita et al. 1999). Another thermosensor mechanism is linked to the RD of HSF which has been determined as heat responsive (Newton et al. 1996) and additional cysteine disulfide bounds in the DBD which act as sensors (Ahn et al. 2003; Lu et al. 2009). The activation of the heat shock response via heat induced conformational changes is a fast response. As it was previously shown that the threshold temperature for heat shock response can be down regulated when cells were cultivated at lower temperatures (Abravaya et al. 1991), it is not likely that the thermosensors are the initial

activation event. In addition, expression of human HSF1 in *Drosophila* results in a decrease of heat shock temperature of around 10°C down to the normal heat shock temperature of fruit flies (Clos et al. 1993).

In cells exposed to stress not only intracellular proteins are affected but also membrane composition and fluidity differs from unstressed cells (Carratu et al. 1996; Horvath et al. 1998). The cell membrane starts to get hyperfluid and to rearrange sub-domains in response to mild stress (Curran et al. 2000; Shigapova et al. 2005), resulting in activation of membrane associated enzymes like phospholipase A_2, which in turn activates protein kinase C and therefore promotes phosphorylation of HSF (Jurivich et al. 1994; Holmberg et al. 1997). In addition, the rearrangement of microdomains also results in the activation of membrane associated signalling cascades like the Ras/Rac1 pathway (Han et al. 2001) or growth factor receptors (Park et al. 2005), but also increase in intracellular Ca^{2+} concentration (Kultz 2005) and cholesterol glycoside (Kunimoto et al. 2002), which all result in increased Hsp expression (reviewed in (Vigh et al. 2007)). Experiments using membrane fluidiser resulted in a decrease of the heat shock threshold temperature (Balogh et al. 2005) indicating the important role of cellular membranes as sensors for mild temperature increase, but not the initial trigger for heat shock response.

The sensor mechanisms described above all act at the cellular level but the *in vivo* situation seems to be even more complex. Experiments in *Caenorhabditis elegans (C. elegans)* could show that the heat shock response was not controlled by single cells but some kind of tissue spanning response could be observed. The initiation of the overall heat shock response could further be narrowed down to specific neuronal structures, thermosensory neurones and their post-synaptic cells (Prahlad et al. 2008). Although the research on whole organism heat shock regulation is still at the beginning, the results will be quite important to understand heat shock regulation and the associated diseases.

To convert an external stimulus into a signal able to activate heat shock response is one level of regulation. Another level of regulation is focused on HSF1 activation, as this is the key event for target gene expression. This activation involves not only the heat shock pathway but is also induced by other cellular signaling cascades. Regulating the HSF1 activation targets either post-translational modification, HSF1 localisation or trimerisation (reviewed in (Akerfelt et al. 2010; Anckar et al. 2011), see also 3.5.3.). The next level of heat shock regulation concentrates on transcriptional activation of target genes. At this level, the active HSF1 trimer binds to the HSE in the promoter of target genes as for example Hsp72, the major inducible heat shock protein. The promoter of Hsp72 contains at least 3 HSE ((Wu et al. 1986; Tsutsumi-Ishii et al. 1995) and is free of nucleosomes, leading to a fast binding of HSF1 upon stress activation. In the promoter of unstressed cells, RNA polymerase II is engaged, but kept in a paused state (Rougvie et al. 1988; Core et al. 2008) in *Drosophila* but also mammals (Brown et al. 1996; Meininghaus et al. 2000) by binding to negative elongation factor (Narita et al. 2003; Wu et al. 2003). In addition, nucleosomal packaging of the gene enhances RNA polymerase pausing (Brown et al. 1996; Lis 1998).

Upon activation, HSF1 trimer binds to the HSE in the promoter of target genes and RNA polymerase II is released from its paused form. This release is due to the binding of positive transcription elongation factor b (P-TEFb) (Bres et al. 2008) and the interaction of HSF1 with BRG1, the ATPase subunit of SWIF/SNF chromatin remodelling complex (Sullivan et al. 2001). Nucleosome displacement occurs across the whole gene and is triggered by poly(ADP)-ribose polymerase 1 (PARP-1) (Tulin et al. 2003; Ouararhni et al. 2006). The recruitment of HSF to the promoter and the start of RNA polymerase II driven transcription in

response to nucleosome disruption all occur within seconds after heat shock activation (Zobeck et al. 2010). Beside the chromatin remodelling interaction, HSF1 also interacts with TATA-binding protein (TBP) or basal transcription factors (TFIIB), which are part of the preinitiation complex (Mason et al. 1997; Yuan et al. 2000) and recruits and co-activates the so-called mediator complex to the promoter. This complex transmits the activation signal to the basal transcription machinery (Park et al. 2001b). In addition, Stress-responsive activator of p300 (Strap) is necessary at the promoter bound to HSF1 for acetylation of histones via the HAT domain of the co-recruited p300 (Xu et al. 2008). After expression, when target genes like heat shock proteins reach a certain level, the heat shock response starts to attenuate. This is caused by binding of Hsp70 to the HSF1 trimer in a self-regulatory manner (Abravaya et al. 1991; Abravaya et al. 1992; Baler et al. 1992) and the additional binding of heat shock factor binding protein 1 (HSBP1) resulting in a decreased expression of target genes (Satyal et al. 1998).

3.5.4. HEAT SHOCK RESPONSE IN DISEASES

The heat shock response represents a highly conserved mechanism of cells to handle different harmful conditions. In case of a deregulation of this pathway, several disorders like cancer or protein degradation associated diseases arise. HSF1 has been shown to be involved in cancer development as Hsf1 -/- mice are highly resistant to tumourigenesis (Dai et al. 2007) and HSF1 levels are higher in tumourigenic cells. In addition, levels of HSP are also up regulated in tumour cells (Jaattela 1999; Tang et al. 2005). The up-regulation of HSP results in anti-apoptotic signaling activation and the deregulation of important transcription factors and signaling molecules like NFκ-B or Raf1, which therefore promotes survival of cancer cells (Mosser et al. 2004). Due to the important role of the heat shock response in tumour formation the modulation of the response was established as a new therapeutical approach to treat several types of cancer (reviewed in (Westerheide et al. 2005; Murshid et al. 2011)).

The heat shock response and the involved heat shock proteins primarily are responsible for the correct folding of proteins and the prevention of protein aggregation. Consequently, problems in the heat shock pathway are associated with diseases depending on misfolded proteins. Neurodegenerative diseases like Huntington disease, Parkinson disease or Alzheimer disease were all shown to be linked to the heat shock response (Kakizuka 1998; Neef et al. 2011). Hsp70 was shown to interact with huntingtin aggregates in cell culture experiments (Kim et al. 2002). Tau proteins, which are the cause of Alzheimer´s disease, when they are deposited into neurofibrillary tangles, are client proteins of Hsp90 (Salminen et al. 2011) and the mitochondrial Hsp70 member mortalin is associated with Parkinson disease (Jin et al. 2006). A common feature of all of these neurodegenerative diseases is their increased incidence with age. This increase might be linked to a lower activation of the heat shock response in aged cells (Soti et al. 2003; Shamovsky et al. 2004) due to reduced DNA binding capacity of HSF (Jurivich et al. 1997; Gutsmann-Conrad et al. 1998). Supporting this theory, HSF1 was shown to be required for long life in *C. elegans* (Morley et al. 2004), where it is involved in the highly conserved insulin/insulin-like signaling pathway, which is important for life span determination (Kenyon et al. 1993; Cohen et al. 2008).

In summary, the heat shock response is a highly regulated cellular mechanism of cells dealing with harmful environmental conditions. Although it was discovered as a stress activated response, the components of the heat shock pathway also play important roles in

normal cellular situations and deregulation of the response results in severe neurodegenerative diseases as well as in cancer.

3.6. AIM OF THE PROJECT

The aim of this thesis was to establish a new approach for regulated gene expression in encapsulated cells and to perform first proof-of-principle experiments. For this, different already established methods like cell therapy, encapsulation technology, hyperthermia and heat inducible gene expression had to be combined.

4. MATERIALS AND METHODS

4.1. DNA/RNA METHODS

4.1.1. CLONING

4.1.1.1. General procedure

For the generation of new plasmids the backbone vector (=vector) and the plasmid containing the insert sequence (=insert) were digested over night with restriction enzymes (Thermo) generating corresponding types of overhang or blunt ends (see Tab.4-1). Afterwards the backbone vector was purified using a DNA purification kit MSB® Spin PCRapace Kit (INVITEK) according to the manufacturer´s protocol and optionally treated with alkaline phosphatase (FastAP, Thermo) according to the manufacturer´s. The digested plasmid containing the insert was loaded on a 1 % agarose gel, the DNA fragment of the expected size was cut out of the gel and purified with Invisorb® Spin DNA Extraction Kit (INVITEK) as described by the manufacturer. After purification both, the vector and insert were controlled for their amount and proper length using agarose gel electrophoresis. For ligation a T4 DNA ligase (Thermo) was used according to the manufacturer´s protocol and a ratio of insert : vector of 3 :1 was used. Next day the ligation mix was transformed into E.coli Top 10F´ and incubated at 37°C for 24 hrs on LB-Agar plates (LB-medium + 1.5 % agar). For the selection of the colony producing the correct plasmid, 10-20 colonies were picked, inoculated over night at 37°C in LB- medium (1 % bacto trypton, 0.5 % yeast extract, 0.5 % NaCl, 1 ml 5M NaOH) + 100 µg/ml Ampicillin (LB-Amp) and DNA was isolated by a rapid alkaline extraction method (see also Miniprep). Isolated DNA was digested with specific restriction enzymes to verify the correct sequence of the produced plasmid. One colony containing the expected plasmid was inoculated in 75 ml LB- Amp and incubated over night in the shaker at 37°C. Next day DNA was isolated (see also Midiprep) and again controlled by restriction enzyme digest. Purified DNA was now used for transfection of cells.

TABLE 4-1 RESTRICTION ENDONUCLEASES

Restriction enzyme	Restriction site
Cla I	5'...A T↓C G A T...3' 3'...T A G C↑T A...5'
Bgl II	5'...A↓G A T C T...3' 3'...T C T A G↑A...5'
Nco I	5'...C↓C A T G G...3' 3'...G G T A C↑C...5'
I-SceI	5'...T A G G G A T A A↓C A G G G T A A T...3' 3'...A T C C C↑T A T T G T C C C A T T A...5'

4.1.1.2. Agarose gel electrophoresis

To control the correct size of the digested plasmids up to 100 ng were analysed by agarose gel electrophoresis. Fragments between 200-3000 bp were separated on 1 % agarose gels, for smaller fragments a higher concentration of 2 % agarose (BIOZYM) in SB-buffer (2 M NaOH, 7.3 M Boric acid) was used. To determine fragment size a mixed DNA ladder (100 bp ladder + λ BstEII (Thermo)) was employed. Electrophoresis standard settings were 120 V for 20 min. DNA was visualised by intercalating etidiumbromide (10 µg/ml) by UV light using an Alphamager$^{(R)}$ Mini (Cell Biosciences).

4.1.1.3. Miniprep

Selected colonies were inoculated in 2 ml LB-Amp medium over night at 37°C on a shaker. Next day bacteria were harvested by centrifugation resuspended in cell suspension buffer P1 (50 mM Tris, 10 mM EDTA, 100 µg/ml RNase A,), cells were lysed using buffer P2 (0.2 M NaOH, 1 % SDS), and reaction was stopped by the addition of neutralisation buffer P3 (3 M KAc, 11.5 % Glacial acetic acid). Cellular components and denaturated proteins as well as chromosomal DNA was removed by centrifugation (20 min 14 000 rpm) and extracted DNA was precipitated with PEG (12 %), washed with 70 % ethanol and DNA pellet was resolved in 20 µl dH_2O. 1 µl of DNA was used for restriction analysis.

4.1.1.4. Midiprep

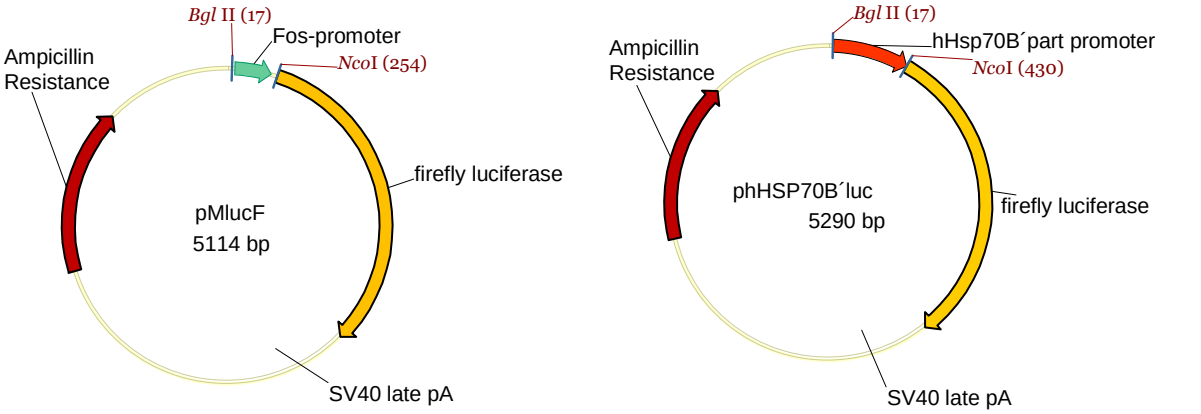
One colony selected after control restriction analysis was inoculated in 75 ml LB-Amp medium over night on a shaker at 37°C. Next day cells were harvested by centrifugation and DNA was extracted using JETSTAR 2.0 Midi Kit (Genomed) according to the manufactures' protocol with one modification. For cell lysis and washing steps, only half of the recommended volume was used. DNA yield was determined by OD_{260} measurement and the isolated plasmid was verified by restriction analysis.

4.1.2. PLASMIDS:

4.1.2.1. Constructed plasmids

<u>hHsp70B´ part promoter driven firefly luciferase expression construct:</u>

A 400 bp fragment of the human Hsp70B´promoter containing 300 bp of the promoter harbouring the HSE an additional 100 bp leader sequence was amplified by PCR from genomic DNA and the resulting PCR fragment was ligated into pGemT Easy (Promega), transformed into *E.coli*, cell clones were selected (Miniprep) and plasmid DNA was isolated (Midiprep). The pGemT vector containing the Hsp70B´part promoter was digested using *Bgl II* and *Nco I* and the promoter was inserted into pMlucF (Figure 4-1 A) (*Bgl II, Nco I*). The resulting expression construct harbours the human Hsp70B´part promoter driving the expression of firefly luciferase in response to the heat shock pathway activation (Figure 4-1 B).

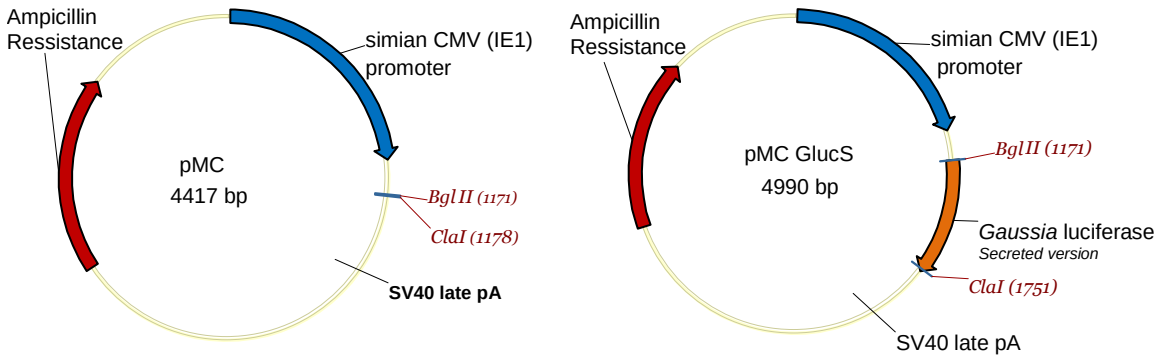


A B

FIGURE 4-1 PLASMIDS FOR GENERATION OF phHSP70Bluc. A) Backbone vector pMlucF. B) Newly generated plasmid phHsp70B´luc expressing firefly luciferase in response to Hsp70B´ partial promoter activation. For selection within the cloning procedure the plasmid contains an ampicillin resistance gene.

Constitutive *Gaussia* luciferase expression construct:

The coding sequence of the secreted form of *Gaussia* luciferase was amplified by PCR from a *Gaussia* luciferase encoding plasmid (pGLuc Basisvector) and the resulting PCR fragment was ligated with pGemT Easy (Promega), transformed into *E.coli*, cell clones were selected (Miniprep) and plasmid DNA was isolated (Midiprep). The pGemT vector containing the *Gaussia* luciferase was digested using *Bgl II* and *ClaI* and the promoter was inserted into the pMC backbone (Figure 4-2 A) (*Bgl II, ClaI*). The resulting expression construct harbours a constitutive active CMV promoter driving the expression of the secreted form of *Gaussia* luciferase (Figure 4-2 B).



A B

FIGURE 4-2 PLASMIDS FOR GENERATION OF pMCGluc S. A) Backbone vector pMC. B) newly generated plasmid pMC GlucS constitutively expressing the secreted version of *Gaussia* luciferase. For selection within the cloning procedure the plasmid contains an ampicillin resistance gene.

4.1.2.1. Other plasmids

Artificial HSE promoter luciferase construct:

An HSE promoter construct containing a core of eight idealised HSE flanked by two CMV minimal promoters was used (Bajoghli, 2004) driving the bidirectional expression of firefly luciferase and GFP. For the generation of a stable cell line, a puromycin resistance was introduced into the HSE promoter (Figure 4-3).

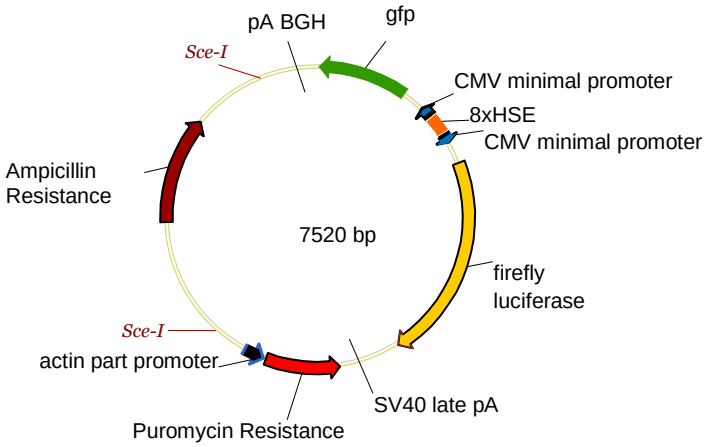


FIGURE 4-3 pSGH2luc puro. The artificial HSE promoter contains a core of 8 idealised HSE flanked by two minimal CMV promoters driving the expression of the two reporter genes gfp and firefly luciferase in a bidirectional manner. For selection of stable cell clones a puromycin resistance gene was introduced. Two Sce-I sites are located in the plasmid to allow linearization. For selection within the cloning procedure the plasmid contains an ampicillin resistance gene.

<u>Constitutive firefly luciferase expression construct:</u>

This plasmid contains a CMV promoter driving the expression of firefly luciferase in a constitutive manner (Figure 4-4).

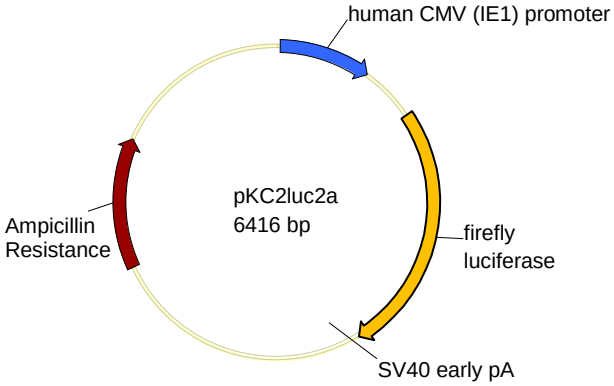


FIGURE 4-4 pKC2luc2a. This expression construct encodes the sequence of firefly luciferase and a constitutively active CMV promoter. For selection within the cloning procedure the plasmid contains an ampicillin resistance gene.

<u>Zebrafish Hsp70 promoter luciferase construct:</u>

The expression construct harbours a truncated zebrafish (zf) Hsp70 promoter (Grabher et al. 2004) driving the expression of firefly luciferase in response to heat shock pathway activation.

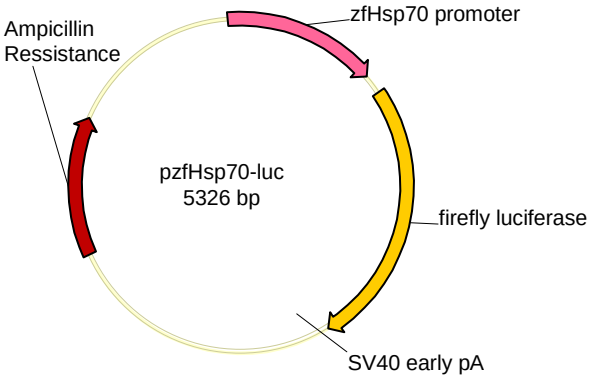


FIGURE 4-5 pzfHSP70-luc. The plasmid encodes the truncated version of zf Hsp70 promoter driving the expression of firefly luciferase. For selection within the cloning procedure the plasmid contains an ampicillin resistance gene.

4.1.3. ISOLATION OF GENOMIC DNA

Cells were seeded on 10 cm cell culture dishes (PAA) and grown to ~80 % confluence. Afterwards medium was removed, cells were washed with PBS and harvested in 7.5 ml PBS. Cells were centrifuged and pellet was resuspended in Bmod buffer (400 mM Tris, 5 mM EDTA, 150 mM NaCl, 0.1% SDS, pH 8.0) + Proteinase K (10 mg/ml) and incubated for 1 hr at 60°C on a shaker. To remove cell debris the suspension was centrifuged for 10 min at maximum speed and DNA was isolated using a DNA purification kit MSB® Spin PCRapace Kit (INVITEK) according to the manufacturer´s protocol with slight modifications (the elution was performed using 2x 25 µl dH_2O).

4.1.4. RNA ISOLATION

Cells were incubated for different durations at 43°C or incubated with different inducers and lysed directly or up to 48 h after recovery at 37°C. Total RNA was isolated according to the manufactures protocol using Invisorb® Spin Tissue RNA Mini Kit (INVITEK) and RNA was eluted using 20 µl nuclease free water (from Kit).

4.1.5. cDNA SYNTHESIS

RNA lysate were used for cDNA. Residual DNA was removed with DNAse I (Thermo) according to the manufacturer's instructions and RNA was transcribed into cDNA (total volume 20 µl) using random hexamer primers (100 µM, Thermo) using RevertAid™ H Minus M-MuLV Reverse Transcriptase (Thermo)according to the manufacturer's protocol for RevertAid™ H Minus First strand cDNA synthesis kit (Thermo).

4.1.6. PCR

For the amplification of promoter regions or coding sequence of *Gaussia* luciferase a PCR using *Phusion* polymerase (Thermo) was performed.

Per reaction:
10 ng plasmid DNA /100 ng genomic DNA
25 pmol primer forward
25 pmol primer reverse
1 µl 10mM dNTPs (Thermo)
1 U Phusion polymerase (Thermo)
10 ml 5x HF-buffer (Thermo)
H_2O to 50 µl

Temperature protocol:
98°C 30 sec
98°C 10 sec
55°C 30 sec } 40 cycles
72°C 15 sec
72°C 5 min

After PCR amplification product was controlled with electrophoresis and purified using DNA purification kit MSB® Spin PCRapace Kit (INVITEK) according to the manufacturer´s protocol

and A-tailing was performed for 20 min at 72°C afterwards and 3 µl A-tailing product was ligated with 1 µl pGemT Easy (Promega) according to the manufacturer´s protocol..

A-tailing – per reaction:
6 µl PCR product
1 µl 10x PCR-buffer (Thermo)
0.5 µl 25 mM $MgSO_4$
0.5 µl *Taq* Polymerase (AGROBIOGEN)
2 µl 0.1 µM dATP

TABLE 4-2 PRIMER FOR CLONING PCR

Primer	Sequence
Gaussia luciferase forward	5´-AGA TCT GCT AGC ACC ATG GGA GTC AAA GTT-3´
Gaussia luciferase reverse	5´-TCT AGA TTA ATC GAT TCC ACC TCC GTC ACC ACC GGC CCC CT-3´
hHsp70B promoter forward	5´-GAG ATC TCC AGC CCG GAG GAG CTA GAA-3´
hHsp70B promoter reverse	5´- GCC ATG GCT GAA GCT TCT TGT CGG-3´

4.1.7. qPCR

For qPCR, Taqman hydrolysis probes were designed using Primer Express V2 and cDNA was analysed in an Mx3000P (Strategene) qPCR cycler. As an endogen control hGAPDH was used.

Per reaction:
1.5 µl cDNA
2 µM primer forward
2 µM primer reverse
2 µM Taqman hydrolysis probe
1x buffer B (80 mM Tris, 20 mM $(NH_4)_2SO_4$, 0.02% Tween 20)
or 1x buffer ABI (10 mM Tris, 50 mM KCl)
3-3.5 mM $MgCl_2$
0.2 mM dNTP mix (Thermo)
0.025U *Taq* polymerase (AGROBIOGEN)
water to 25 µl

Temperature protocol:
5 min 95 °C
30 sec 95°C
60 sec 60°C
} 40 cycles

All cDNA tested in qPCR were performed in triplicates and to determine concentration a standard curve (based on 1:4 dilution series of standards Table 4-3) was added on each plate. cDNA were normalised to the hGAPDH levels. Standard dilution series were performed in duplicates.

TABLE 4-3 qPCR SETTINGS

Gene	buffer	$MgCl_2$	mean eff.%	Standard
hGAPDH (NM_001256799.1)	buffer B	3.5 mM	97-99%	GAPDH PCR product
Luc	buffer B	3.5 mM	96-98%	Firefly luciferase plasmid
mHsp72 (HSPA1A, NM_005346.4)	buffer ABI	3 mM	92-95%	mHsp72 plasmid
hHsp70RY (HSPA4 NM 002154)	buffer B	3 mM	93-95%	cDNA C5 cells 1hr 43°C+ 2 hrs 37°C
eGFP	buffer B	3 mM	97-99%	eGFP plasmid

TABLE 4-4 PRIMERS FOR qPCR:

Gene	sequence	T_m
hGAPDH forward	5´-GGA AGG TGA AGG TCG GAG TCA A-3´	64°C
hGAPDH reverse	5´- ACC AGA GTT AAA AGC AGC CCT G-3´	62°C
Firefly luciferase forward	5´- TGG ATT ACG TCG CCA GTC AAG-3´	61°C
Firefly luciferase reverse	5´-TTC GGT ACT TCG TCC ACA AAC A-3´	60°C
hHsp72 (HSPA1A) forward	5´-AAC CAG GTG GCG CTG AAC-3´,	58°C
hHsp72 (HSPA1A) reverse	5´-TGG AAA GGC CAG TGC TTC AT-3´	58°C
hHsp70RY (HSPA4) forward	5´-GTG GGC ATA GAC CTG GGC TTC CA-3´	62°C
hHsp70RY (HSPA4) reverse	5´-TCC AAT TGA ACG ATT CTT AGG ACC A-3´	63°C
GFP forward (Paar et al. 2007)	5´-GCA GTG CTT CAG CCG CTA C-3´	62°C
GFP reverse (Paar et al. 2007)	5´- AAG AAG ATG GTG CGC TCC TG-3´	60°C

TABLE 4-5 PROBES FOR QPCR

Gene	Sequence	T_m
hGAPDH	5´-HEX-ATT TGG TCG TAT TGG GCG CCT GGT C-BHQ1-3´	69°C
Firefly luciferase	5´-FAM-CGC GAA AAG TTG CGC GGA GG-BHQ1-3´	65°C
hHsp72 (HSPA1A)	5´-FAM-AAC ACC GTG TTT GAC GCG AAG CG-BHQ1-3´	66°C
hHsp70RY (HSPA4)	5´-FAM-CTA CGT CGC TGT GGC CCG CG-BHQ1-3´	69°C
GFP modified (Paar et al. 2007)	5´-FAM-CCG ACC ACA TGA AGC AGC ACG ACT T-BHQ1-3´	69°C

4.2. CELL CULTURE

4.2.1. CELL LINES

HEK 293 (ATCC: **CRL-1573)**

Human embryonic kidney cells (fetus). Cells were grown at 37°C in a humidified environment of 5 % CO_2 in DMEM high glucose (PAA), supplemented with 10 % FCS, 100 U/ml penicillin, and 100 U/ml streptomycin sulphate (1x Penicillin/Streptomycin, PAA). Propagation was performed using 1xtrypsin (PAA) and a sub-culturing rate of 1:10-1:15 was used.

U2Os (ATCC: HTB-96)

Human osteosarcoma epithelial cells (female origin). Cells were grown at 37°C in a humidified environment of 5 % CO_2 in DMEM high glucose (PAA), supplemented with 10 % FCS, 100 U/ml penicillin, and 100 U/ml streptomycin sulphate (1x Penicillin/Streptomycin, PAA). Propagation of the cells was performed using 1xtrypsin EDTA (Gibco, 0.25 %trypsin, 0.03 % EDTA) and a sub-culturing rate of 1:10 was used.

HeLa (ATCC: **CCL-2**)

Human epithelial cervix Adenocarcinoma cell line (female). Cells were grown at 37°C in a humidified environment of 5 % CO_2 in DMEM high glucose (PAA), supplemented with 10 % FCS, 100 U/ml penicillin, and 100 U/ml streptomycin sulphate (1x Penicillin/Streptomycin, PAA). Propagation was performed using 1xtrypsin (PAA) and a sub-culturing rate of 1:10-1:15 was used.

MCF-7 (ATCC: **HTB-22**)

Human epithelial mammary gland Adenocarcinoma cell line (female). Cells were grown at 37°C in a humidified environment of 5 % CO_2 in DMEM high glucose (PAA), supplemented with 10 % FCS, 100 U/ml penicillin, and 100 U/ml streptomycin sulphate (1x Penicillin/Streptomycin, PAA). Propagation of the cells was performed using 1xtrypsin EDTA (Gibco, 0.25 %trypsin, 0.03 % EDTA) and a sub-culturing rate of 1:10 was used.

PANC-1 (ATCC: **CRL-1469**)

Human pancreas epitheloid carcinoma cell line (male). Cells were grown at 37°C in a humidified environment of 5 % CO_2 in DMEM high glucose (PAA), supplemented with 10 % FCS, 100 U/ml penicillin, and 100 U/ml streptomycin sulphate (1x Penicillin/Streptomycin, PAA). Propagation of the cells was performed using 1xtrypsin EDTA (Gibco, 0.25 %trypsin, 0.03 % EDTA) and a sub-culturing rate of 1:10 was used.

SW480 (ATCC: **CCI-228**)

Human epithelial colorectal adenocarcinoma cell line (male, Tumour stage Dukes´type B). Cells were grown at 37°C in a humidified environment of 5 % CO_2 in DMEM high glucose (PAA), supplemented with 10 % FCS, 100 U/ml penicillin, and 100 U/ml streptomycin sulphate (1x Penicillin/Streptomycin, PAA). Propagation of the cells was performed using 1xtrypsin EDTA (Gibco, 0.25 %trypsin, 0.03 % EDTA) and a sub-culturing rate of 1-5 up to 1:10 was used.

Stable HSE promoter cell line:

HEK 293 cells were transfected with the HSE promoter construct (pSGH2luc puro) using PEI as a transfection reagent. Cells were cultivated for 2-3 days and then stably transfected cells were selected with puromycin (1 µg/ml) as a selection marker. After one to two weeks, single clone colonies were picked using single cloning discs (Invitrogen) and cultivated with puromycin. First tests were performed using 1h HS 43°C with ensuing GFP detection and luciferase reporter gene assay.

Stable cell line C5:
The stable cell line C5 showed robust GFP expression and highest luciferase inducibility and therefore was used for further experiments.

Stable cell cline D4:
The stable cell line D4 showed no GFP expression but high luciferase inducibility and therefore was used for the comparison of promoter activation in different HSE promoter cell lines. In contrast to the missing GFP expression, sequencing of the promoter showed full length integration of the promoter region.

Stable cell line A6:
The stable cell line A6 showed robust GFP expression but constitutively high luciferase expression and therefore was used for the establishment of the luciferase assay in encapsulated cells. In contrast to the constitutive activation, sequencing of the promoter showed full length integration of the promoter region.

All stable cell lines were grown at 37°C in a humidified environment of 5 % CO_2 in DMEM, supplemented with 10 % FCS, 100 U/ml penicillin, and 100 U/ml streptomycin sulphate.

4.2.2. TRANSFECTION

For transient transfection experiments 0.3x 10^5 cell/ well of a 24 well plate (PAA) were seeded and incubated for 24 hrs at 37°C. In total 400 ng DNA (tested plasmids + backbone vector pBluescript to fill up to 400 ng total DNA amount) in 100 µl DMEM without serum was mixed with 100 µl serum-free DMEM containing the transfection reagent (depending on the used cell line, see Table 4-6) and incubated at room temperature for 30 min. Afterwards, medium was removed from the cells and 200 µl transfection mix was added and incubated for 2-4 hrs at 37°C (depending on the transfection reagent). Transfection was stopped by the addition of 1 ml DMEM containing serum and cells were incubated additional 48 hrs before lysis.

TABLE 4-6 TRANSFECTION CONDITIONS

Transfection reagent per well	Cell line	Incubation time
0.8 µlTurbofect (Thermo)	HeLa, SW480, U2Os	2 hrs
1.6 µl PEI (0.0435% PEI 25000)	HEK 293, PANC-1, MCF-7	4 hrs

4.2.3. STABLE CELL LINE GENERATION

To generate a stable HSE promoter cell line, 2x10^6 HEK 293 cells per 10 cm dish were seeded and transfected with 1 µg of the HSE promoter construct pSGH2luc puro (Figure 4-3), which was previously linearised with *I-SceI* + 9 µg herring sperm DNA (GIBCO) to reach a total DNA amount of 10 µg and 8 µl PEI. Two days after transfection puromycin (100 µg/ml) was added for selection of stable construct integration and medium was changed frequently the next two weeks. Single cell clones were picked using single cloning discs (SIGMA) and the cell clones were tested for their inducibility after heat treatment.

4.2.4. STRESS INDUCTION

Heat treatment:

A defined number of cells were seeded into cell culture dishes (0.3x10^5 cells for 24 well plates, 2x10^5 cells /well for 6 well plates) and cultivated for 3 days at 37°C. For heat treatment, cells were placed on an iron plate in a cell culture incubator at a temperature of 43°C for a distinct time. To recover from the heat stress, cells were returned to 37°C or directly used for the experiments.

Induction with chemical inducers:

For the induction with heavy metals or pharmacological substances, cells were seeded as described above and after 3 days at 37°C medium was removed. Different concentrations of inducer in DMEM containing 10 % FBS were added to the cells for 1 hr and afterwards medium was changed. To recover, cells were returned to 37°C or directly used for the experiments.

4.2.5. VIABILITY ASSAY

Trypan blue assay:

To analyse the survival after heat treatment, medium was removed, cells were trypsinised, washed with PBS and all washing fractions were combined with the removed medium and the detached cells. Cells were collected by centrifugation for 5 min at 900 rpm, resuspended in 1 ml PBS and an aliquot of 50 µl was mixed with the same amount of a 0.5 % Trypan blue solution and incubated for 2 min. The number of dead cells was determined in a bright field microscope by counting the blue cells and the total cells in a Neubauer chamber.

4.2.6. HYPOXIA TREATMENT

Cells were prepared as for induction with heat treatment and after 3 days transferred to a hypoxic chamber (STEMCELL Technologies Inc.) floated with a mixture of 10 % CO_2 and 90 % N_2. Cells were incubated in the chamber for 6 – 20 hrs at 37°C and afterward recovered under normal oxygen levels at 37°C.

To mimic hypoxic conditions cells were incubated with 1 – 100 mM $CoCl_2$ like described for induction with chemical inducers.

4.2.7. DEGRADATION ASSAY

HeLa cells were transfected with plasmids encoding constitutively expressed firefly luciferase (10 ng pKC2luc2a Figure 4-4) or *Gaussia* luciferase (1 ng pMC GlucS Figure 4-2) and pBluescript backbone vector (389 ng) to reach the optimal amount of 400 ng total DNA for transfection with 0.8 µl Turbofect. 48 hrs after transfection cells were incubated for 1 hr with different inducers and lysed directly afterwards in 50 µl luciferase lysis buffer. Firefly luciferase as well as *Gaussia* luciferase activity were determined using the dual luciferase assay.

4.3. PROTEIN METHODS

4.3.1. LUCIFERASE ASSAY

Cells or capsules were lysed in luciferase lysis buffer (0.1 M Tris pH 7.5, 1 % Triton X and 1mM DTT) (50 µl for cells, 70 µl for capsules), capsules were mechanically destroyed by a pestle, and incubated for 15 minutes at room temperature. Capsules were centrifuged for 5 min at 1000 rpm and supernatant of capsule or cell lysate was used for luciferase activity measurement in a LUMAT LB 9705 luminometer.

Firefly luciferase assay:
For firefly luciferase activity determination 50 µl cell lysate was provided, 100 µl D-luciferin solution (0.2 mM D-luciferin, 20 mM Tris pH 7.5) and ATP solution (5 mM ATP, 25 mM Tris pH 7.5, 15 mM $MgCl_2$) were injected by the luminometer and relative light units (RLU) were determined for 10 sec.

Dual luciferase assay:

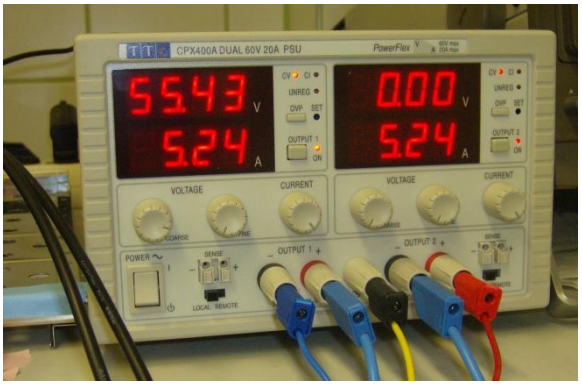
For detecting firefly and *Gaussia* luciferase, 40 µl of the cell lysate was used for firefly luciferase assay and 10 µl of the cell lysate was used for Gaussia luciferase assay. Within this assay the luminometer injected 100 µl substrate solution (2.5 mM EDTA, 6.25 mM Tris pH 7.5, 3 µM Coelentrazine) and determined RLU production for 10 sec.

Top assay the background levels of both measurements RLU of the lysis buffer was detected and subtracted from the sample values.

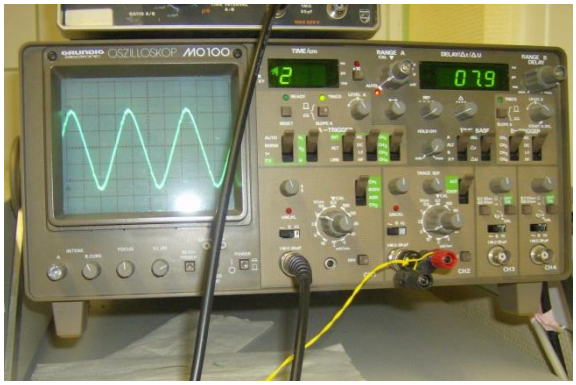
4.4. OTHER METHODS

4.4.1. MAGNETIC FIELD GENERATOR

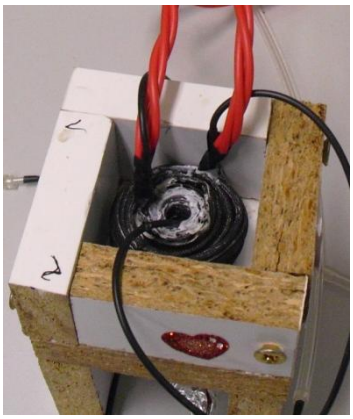
The magnetic field generator was designed and built by Christian Halter, Group of Johann Walzer, University of Applied Sciences, FH Campus Wien, Department for Engineering using a frequency generator, an oscilloscope and a induction coil (Figure 4-6 A-D) to establish an alternating magnetic field of ~ 30 kA/m using a frequency of 60 kHz and 27 A input current. The induction coil was manufactured from highly conductive brass tubes (Figure 4-6 C). During operation, the induction coil itself heats because of the high current and was therefore permanently cooled using deionised water (20°C) (Figure 4-6 E). For the experiments without capsules an additional cooled water jacket was used to ensure a temperature of 37°C for the cells.

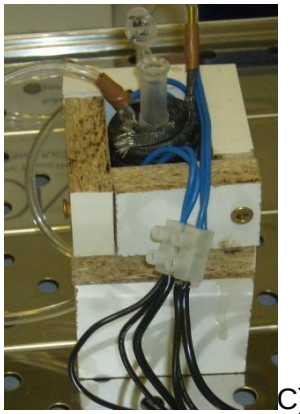


A) power supply



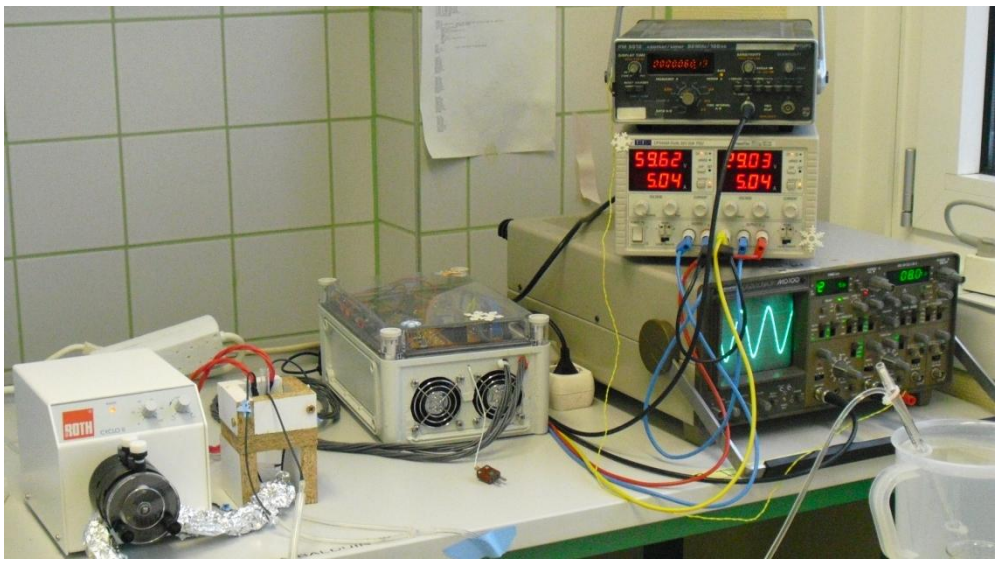
B) oscilloscope





C) Induction coil

D) frequency generator and power amplifier



E) The magnetic field generator including the water cooling system

FIGURE 4-6 THE MAGNETIC FIELD GENERATOR. The homemade magnetic field generator contains a power supply, an oscilloscope, a frequence generator and a water cooled induction coil.

4.4.2. INDUCTION IN THE MAGNETIC FIELD

For the induction experiments in the alternating magnetic field (AMF), 10^5 cells o 140 capsules per 200 µl culture medium were transferred to a reaction tube, pre-incubated at 37°C and put into the coil for defined durations of 30 minutes. Directly after the induction, the temperature of the suspension was determined using a digital fine tune thermometer and cells or capsules were supplied with 2 ml medium and transferred to 37°C for recovery. Luciferase activity was determined 6 h after induction.

For the determination of nanoparticle mediated heat production, the particles were suspended in 200 µl culture medium, transferred to a reaction tube, pre-incubated at 37°C and put into the coil for defined durations of 5 - 30 minutes. Directly after the induction, the temperature of the suspension was determined using a digital fine tune thermometer.

4.4.3 ENCAPSULATION

All cell encapsulations were performed by Cornelius Kaspar at the Department for Pathobiology, Institute of Virology, University of Veterinary Medicine, as described in Ortner et al. Materials and Methods.

5. RESULTS

5.1. CONCEPT

Gene and cell therapy approaches have shown the tremendous potential of the *in vivo* production of therapeutic proteins in the treatment of diseases (see also 3.1.1-3.1.2). Nevertheless, some problems still limit the clinical use of these therapies. Firstly, the production of therapeutic substances needs to be regulated to ensure optimal pharmacodynamics, but in most cases the production is constitutive and may lead to side effects due to drug overdose. Secondly, when cells are used for treatment they are either affected by the immune system if they are of heterologous origin or they can only be used in individual patients when autologous cells are used. In addition, modified cells always comprise the risk of unwanted reactions within the tissue, even when autologous cells are used (see 3.1.1 and 3.1.2.). Thirdly, also when inducible expression systems are used, the inducer has to be introduced into the whole system first and has to reach the side of action, which drastically increases the time until the system can be turned on (see 3.2.). In recent years, many different strategies were invented to solve one or more of the discussed problems. Within this thesis, an additional strategy for regulated *in vivo* production of therapeutical proteins was established and elaborated. This new approach uses an artificial heat inducible promoter (Bajoghli et al. 2004) stably integrated into a human cell line. The cells are encapsulated with cellulose sulphate to generate a barrier against the host immune system, but the membrane still allows the transport of macromolecules to the surrounding tissue. To induce the expression system by heat, magnetic iron oxide nanoparticles are coencapsulated. When an alternating magnetic field is applied to the capsules, the nanoparticles start to produce heat and in turn activate the artificial heat shock expression system. This activation strategy can also be applied to capsules within a patient, as the magnetic field is able to activate the nanoparticles even at a distance of several centimetres (Figure 5-1).

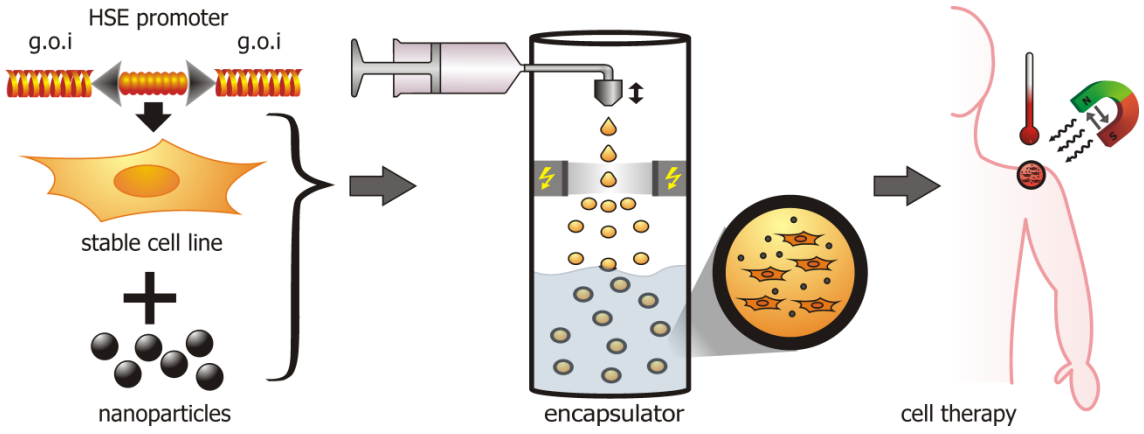


FIGURE 5-1 CONCEPT OF HEAT INDUCED GENE EXPRESSION IN ENCAPSULATED CELLS. A stable cell line harbouring an artificial heat shock promoter is encapsulated together with magnetic nanoparticles using cellulose sulphate. These capsules can then be implanted into tissues and modified cells are protected from the host immune system by the capsule membrane. When an alternating magnetic field is applied from the outside, the magnetic nanoparticles start to produce heat which in turn activates the artificial heat shock promoter leading to the expression of therapeutic proteins.

This approach combines previously well established clinical methods like hyperthermia treatment (see 3.4) with promising pre-clinical strategies like heat shock activated gene expression (see 3.2.2) and encapsulation (3.1.2.3.). Taken together a promising new method to activate gene expression in encapsulated cells with an external stimulus was established: In addition, problems with the host response were avoided by encapsulation and this method might also reduce the amount of necessary drug by a local and regulated production.

5.2. STABLE CELL LINE

The first step of the project was to generate a stable cell line, harbouring an artificial heat inducible promoter. This stable cell line has the benefit of constant levels of promoter construct integrated and therefore consistent activation behaviour, whereas transient transfections would always vary because of different transfection efficiencies.

5.2.1. PROMOTER

The artificial heat inducible promoter was previously established as an inducible expression system for medaka (Bajoghli et al. 2004). The promoter is built of eight idealised heat shock elements with a sequence of *A GAA CG TTC TA GAA C* compared to the general consensus sequence *n GAA nn TTC nn GAA n*. The eight idealised HSEs are flanked on both sites by a CMV minimal promoter driving the expression of two different genes in a bidirectional manner. For the application in mammalian cells and for the selection of stable cell clones, the promoter was integrated into an expression vector harbouring a puromycin resistance gene under the control of a constitutive actin promoter. To analyse the induction of the promoter and the expression of the genes of interest, on one side a firefly luciferase reporter gene and on the other side of the bidirectional promoter a gene encoding green fluorescent protein (GFP) was placed. The ampicillin resistance gene in the expression vector is used for selection during the cloning procedure and BGH or SV40 late poly adenylation signals (pA) are located at the end of the two reporter genes (Figure 5-2).

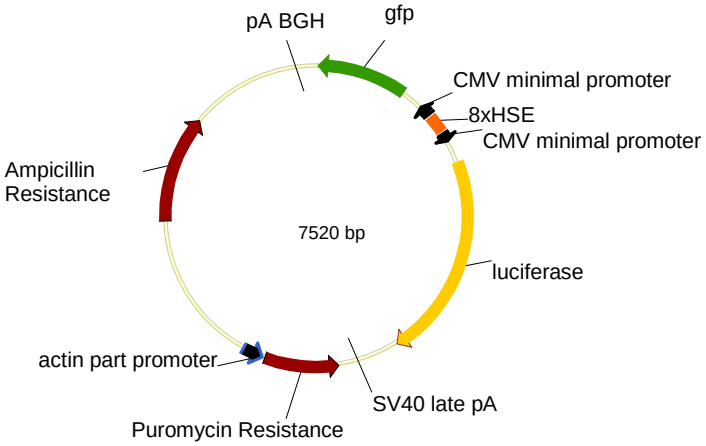


FIGURE 5-2 THE ARTIFICIAL HEAT SHOCK PROMOTER. Vector map of pSGH2 luc puro. The promoter is build of a core of eight idealised HSEs flanked by two minimal CMV promoters. These CMV promoters drive the expression of the two reporter genes firefly luciferase and GFP in a bidirectional manner. An ampicillin resistance gene is integrated into the plasmid for selection during the cloning procedure and a puromycin resistance gene under the control of an actin promoter is used for selection of stable cell clones.

5.2.2. COMPARISON OF DIFFERENT HS PROMOTERS

Heat shock promoters have previously been used for the generation of inducible expression systems but they often had problems with high background activation and low induction levels (see 3.2.2.2.). To verify the high inducibility of the artificial HSE promoter and to compare our system to established heat shock promoters, the different promoter constructs driving the expression of firefly luciferase were analysed after heat treatment. HEK 293 cells were transfected with the different promoter-luciferase constructs and incubated at 43°C for 30 min or 1 hr. Luciferase activity was determined 6 hrs after heat treatment using a luciferase assay.

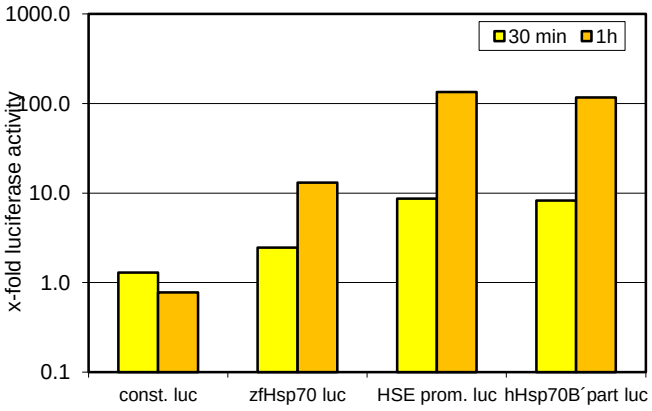


FIGURE 5-3 LUCIFERASE EXPRESSION CAPACITY OF DIFFERENT HEAT SHOCK PROMOTERS. HEK 293 cells were transfected with different heat shock promoters driving the expression of firefly luciferase, incubated for 24 hrs at 37°C and heat shock treatment was performed for 30 min or 1 hr at 43°C. Luciferase activity was measured 6 hrs later. The luciferase activities were normalised to the expression levels at 37°C. This diagram shows one representative experiment.

const. Luc: human CMV promoter, *zfHsp70*: zebrafish homolog of Hsp72 promoter, *HSE prom.*: artificial heat shock promoter, *hHsp70B´ part*: 400 bp promoter of human HSPA6 (Hsp70B´).

The artificial HSE promoter showed high inducibility of more than 100 fold in response to heat treatment for 1 hr similar to the hHsp70B´part promoter (Figure 5-3), which is known to be the highest inducible heat shock promoter for expression systems. In comparison, the zebrafish Hsp70 promoter could induce luciferase expression at 1 hr heat treatment around 10 fold which is similar to the levels of a 30 min heat treatment of the HSE promoter. This experiment clearly showed the potential of the artificial HSE promoter as a tool for inducible expression systems.

5.2.3. EXPRESSION IN DIFFERENT CELL LINES

In addition to high background activation of heat shock promoters or designed heat inducible promoters, most of them also showed tissue or cell type specific activation differences (3.2.2.2.). To analyse the potential cell type specific behaviour of the artificial HSE promoter, several different human cell lines were transfected with the HSE promoter construct and luciferase activity was determined after standard heat shock and at 37°C.

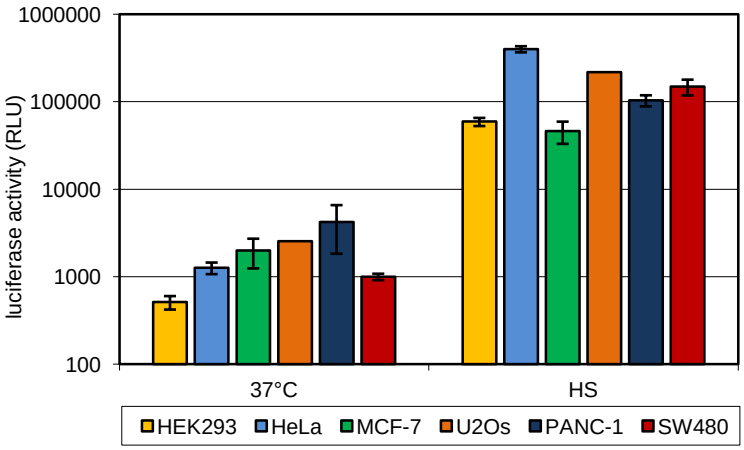


FIGURE 5-4 PROMOTER ACTIVITY IN DIFFERENT CELL LINES. HEK 293, HeLa, MCF-7, U2OS, PANC-1 or SW480 cells were transfected with the HSE promoter construct (pSGH2luc puro) in a 24 well scale ($0.3x10^5$cells/well, 10 ng pSGH2 luc puro) and incubated at 37°C for 1 day. After standard heat treatment (HS) (1hr, 43°C) cells were incubated for 4 hrs at 37°C and afterwards luciferase activity was determined in heat treated cells and transiently transfected cells without heat treatment as a negative control. This diagram shows one representative experiment. *RLU*: relative light units *HS*: heat shock

Tumour cell lines of different origin like cervix (HeLa) (Figure 5-4light blue), mammary gland (MCF-7) (Figure 5-4green) or colorectum (SW480) (Figure 5-4 red), the osteosarcoma cell line U2OS (Figure 5-4 orange) and a pancreas epitheloid carcinoma cell line (PANC-1) (Figure 5-4 dark blue) were transfected with the artificial HSE promoter driving the expression of firefly (10 ng pSGH2luc puro). The inducibility of the promoter in the different cell lines was compared to the induction in the non-cancer cell line HEK 293 (Figure 5-4 yellow). In general most tumours cell lines showed higher luciferase activity 6 hrs after heat treatment (1 hr, 43°C) (above 100 000 relative light units (RLU)) compared to a 2-5 fold lower luciferase activity in the adenovirus transduced HEK 293 (around 60 000 RLU). The transfected mammary gland carcinoma cell line MCF-7 showed similar luciferase activity after heat treatment like HEK293, but for all tumour cell lines the basal activation of the HSE promoter driven luciferase expression was 2-8 fold higher (500 RLU for HEK 293, 1000-4000 RLU for SW480 – PANC-1) than in HEK 293. Although basal luciferase expression and the maximum luciferase activity after heat treatment differ in the tested cell lines, all of them showed at least a 100 fold induction of luciferase expression after heat treatment, which clearly shows no cell type specific limitations of our HSE promoter. Nevertheless, for the application in cell therapy a cell line with extreme low background activity is crucial to avoid leaky expression of therapeutic substances. In addition, the cell line should be well characterised. As HEK 293 cells showed the lowest basal activation of the HSE promoter and the cells are not of tumourigenic origin, these cells were used to generate a stable cell line harbouring the artificial promoter.

5.2.4. GENERATION OF A STABLE CELL LINE

After testing different cell lines, the adenovirus transduced human embryonic kidney cell line (HEK 293) was chosen as suitable for our inducible expression system because of the low basal activity of the promoter and the application of the cell line in approved medical treatments (Hacker et al. 2009).

For the generation of the stable cell line, the HSE promoter construct pSGH2 luc puro (Figure 5-2), which includes the two reporter genes firefly luciferase and GFP, was introduced into the HEK 293 cells by transfection with polyethylenimine (PEI). Cells were cultivated for 2-3 days and then stably transfected cells were selected with puromycin (1 µg/ml). After one to two weeks, colonies were picked. The selected cell clones were cultivated with puromycin and first tests were performed using 1 h heat shock (HS) at 43°C with ensuing GFP detection and luciferase reporter gene assay. Cell lines with GFP

expression and/or high luciferase inducibility were selected and used for further experiments (Figure 5-5 A+B).

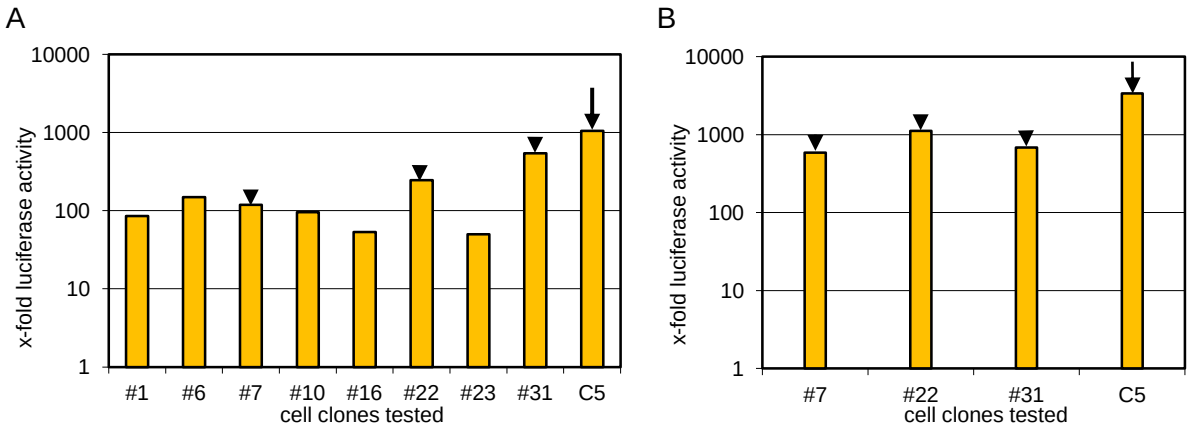


FIGURE 5-5 STABLE CELL LINES: HEK 293 stably transfected with pSGH2 luc puro were incubated at 43°c for 1 hr (HS) and luciferase induction was measured 4 h after HS. Therefore $2x10^5$ cells were seeded in 3.5 cm dish and 3 days later cells were incubated at 43°C in an incubator (+CO_2) for 1 h. Cells were lysed 4 h after heat shock and a luciferase reporter gene assay was performed. All luciferase expression levels were normalised to cells incubated at 37°C. A) shows a representative experiment of the screening procedure, the highest inducible cell clone is marked with an arrow, other highly inducible clones are marked with an arrowhead. B) A representative experiment of the best four cell clones (A: arrow + arrowheads) that were tested again to verify the results.

In total, two different batches of single cell clones were generated within the project. The first batch resulted in the selection of one highly inducible cell clone, C5. In the second batch, different cell clones (48 clones in total) were analysed in respect to GFP and luciferase expression after heat treatment and compared to the highest inducible cell line, C5, derived from the first round of stable transfection (Figure 5-5 A). The tests resulted in the selection of three additional cell clones, #7, #22 and #31 (Figure 5-5 arrowhead). All of them were used in further heat induction experiments to compare their inducibility to those of the C5 cell line (Figure 5-5 B). In the end, the stable cell line C5 showed the highest induction levels (Figure 5-5 arrow) and therefore was used for all further experiments.

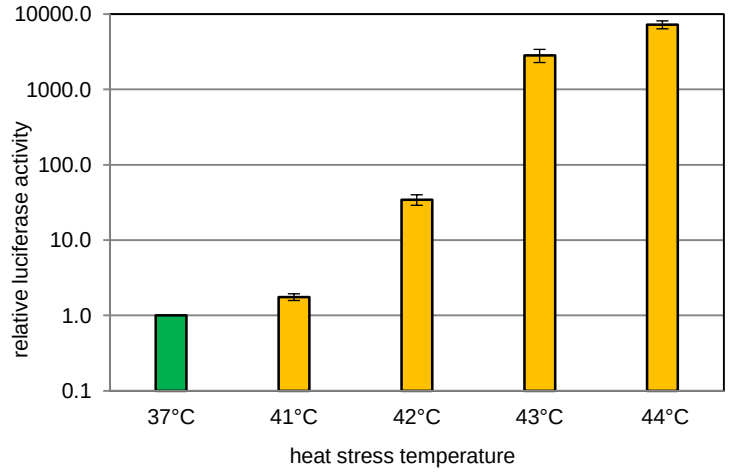
5.3. PROMOTER ANALYSIS

As a first step of the project, a stable cell line harbouring the artificial heat shock promoter was generated. With this stable cell line, the behaviour of the artificial HSE promoter now could be analysed in detail. Natural heat shock promoters do not exclusively react to one kind of stress, but they induce the stress response through a variety of different triggers like, heat, hypoxia, heavy metals or some pharmacological components (3.2.2.2.). Hence, the application of heat shock promoters as inducible expression systems always have to deal with this co-activation by different triggers. An optimal promoter would respond only to one or few stimuli, like heat, whereas not responding to other stress triggers, like hypoxia. The restriction to one or at least a limited number of externally applied induction signals increases the safety of the induction system for clinical application as the promoter would not be activated by background stress signals like hypoxia, low/higher pH or depletion of nutrition, all naturally occurring in the patient.

5.3.1. HEAT INDUCIBILITY

The first kind of stress that was analysed was heat, as this was also the trigger planned for induced expression within the capsules. In the literature, different temperatures ranging from 41°C to 45°C were used to induce the different heat shock promoters. For our artificial HSE promoter a temperature range of 41-44°C was tested (Figure 5-6).

A



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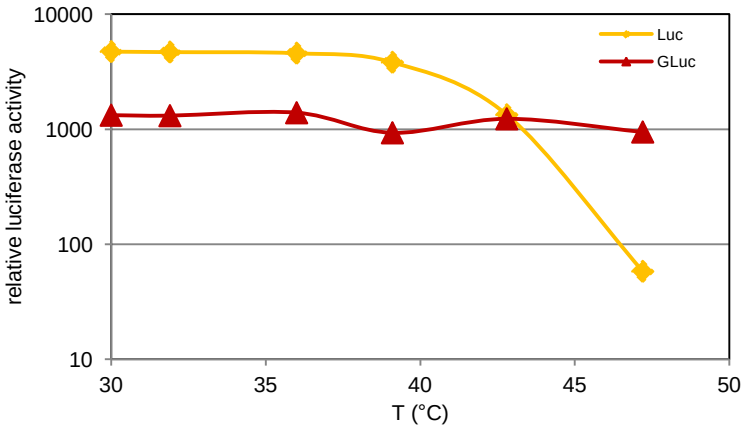

FIGURE 5-6 TEMPERATURE DEPENDENT PROMOTER ACTIVATION AND PROTEIN DENATURATION. A) This figure was recently published in Ortner et al. 2012 (see appendix Fig.2a) and adopted here. $2x10^5$ C5 cells per well of a 6-well plate were incubated at 41-44°C for 1 hr and recovered for 6 hrs at 37°C. The expression of luciferase was determined using a luciferase assay. Luciferase activity was normalised to the expression levels at 37°C. The experiment was performed in sextuplicates. Error bars: +/- SEM n=6. **B) Protein denaturation assay**. HeLa cells were transiently transfected with constitutively expressing firefly luciferase (CMV Luc) and *Gaussia* luciferase (CMV GLuc) constructs and incubated for 2 days at 37°C. Cells were trypsinised and resuspended in 200 µl medium in a PCR tube. Cells and medium were incubated for 15 min at different temperatures in a PCR gradient cycler (30, 31.9, 36, 39.1, 42.8 and47.2°C) and lysed afterwards to analyse firefly luciferase and *Gaussia* luciferase activity.

The induction of the stable cell line C5 at different temperatures showed highest activation of the promoter at 44°C (~7000 fold). At 43°C the stable cell line showed a ~ 3000 fold induction which decreased to ~30 fold when the temperature was reduced by 1°C. A further reduction to 41°C resulted in ~2 fold induction of the promoter which is close to the basal levels (Figure 5-6 A). Although 44°C showed the highest expression levels of luciferase, 43°C were chosen as standard heat treatment temperature. The exclusion of 44°C is based on the severe stress at this temperature which results in increased cell death (see also 5.5.1.). In addition, this high temperature stress might also influence the cell survival when repeated heat treatments are performed. The increase in temperature resulted in an exponential activation of the artificial HSE promoter when 41°C were compared to 42 or 43°C. In contrast the increase to 44°C resulted only in a 2.5 fold higher expression of the reporter gene. Nevertheless, with this experiment the heat-dependent induction of the promoter could be shown, as the promoter activity increased with rising temperatures. In addition, an assay to determine the degradation of proteins was established. Therefore, HeLa cells were transiently transfected with two different constitutively expressing luciferase constructs. One construct harbours firefly luciferase, which is known to be sensitive to

temperature changes and the other luciferase, derived from *Gaussia*, has previously been shown to be highly stable (Wiles et al. 2005). The cells were incubated for 15 min at temperatures between 30 and 47°C and luciferase activity was determined directly afterwards. Firefly luciferase showed decreased activity at temperatures above 39°C down to around 1.2 % of the initial activity at 47°C (Figure 5-6B, yellow line). In contrast, the activity of *Gaussia* luciferase was not influenced by increasing temperatures (Figure 5-6 B, red line). A similar approach to test stress-dependent denaturation of proteins by determining the activity of firefly luciferase has previously been used (Nguyen et al. 1994; Torok et al. 2003). Here highly stable *Gaussia* luciferase was added as a reference.

5.3.2. INDUCTION WITH HYPOXIA

In addition to heat, also other stress triggers are able to induce natural heat shock promoters. The stress factors are divided into external signals, activator components and environmental conditions. External signals are all kinds of radiation but also temperature. Additionally, in the tissue there are different situations initiating the stress response like depletion of nutrition, changes in pH or too low levels of oxygen. As our stable cell line harbouring the artificial heat shock promoter should be used for encapsulation and these capsules have a diameter of 700 µm up to 1 mm, the problem of oxygen diffusion has to be kept in mind. Therefore it was analysed, if hypoxic conditions within the capsules might induce the artificial promoter consisting of heat shock elements (HSE), although hypoxia is thought to be induced through different recognition sites in the natural heat shock promoters. To test the induction of the artificial promoter in response to hypoxic conditions, cells were incubated in a hypoxic chamber for several hours and luciferase expression was measured at several time points after hypoxia treatment.

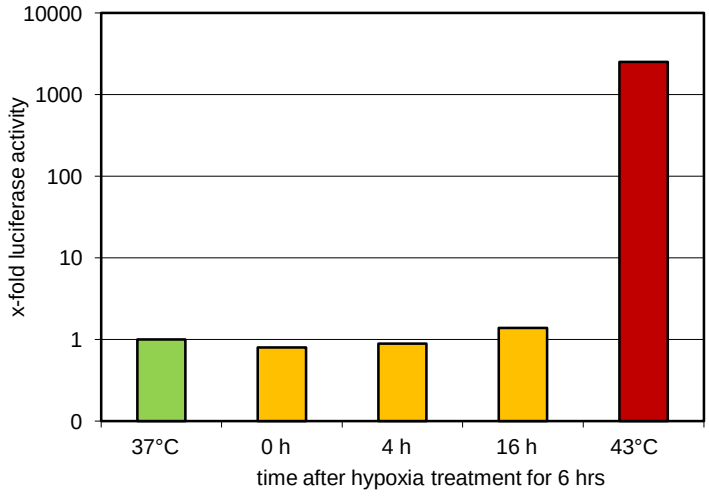

FIGURE 5-7 6 H OF HYPOXIA TREATMENT. Cells of the cell line C5 were incubated at hypoxic conditions and luciferase expression was measured at different time points after hypoxia. Therefore, $2x10^5$ cells were seeded and 3 days later cells were incubated for 6 h under hypoxic conditions (10 %CO_2, 90 % N_2) at 37°C in an incubator (+CO_2). Cells were lysed directly, 4 h and 16 h after hypoxia treatment and a luciferase reporter gene assay was performed. As a positive control, cells were incubated for 1 h at 43°C and lysed 6 h later. The luciferase expression levels were normalised to cells incubated at 37°C (negative control). This figure shows one representative experiment.

This experiment using hypoxic conditions for 6 h showed no induction of luciferase expression after 0-16 h (Fig.10, yellow bars). When cells were incubated at for 1 hr at 43°C for and 6 h at 37°C, luciferase expression increased around 2000-fold (Figure 5-7 red bar) compared to cells incubated at 37°C ((Figure 5-7, green bar) as a control. As in the literature different periods of hypoxia treatment from 6 h - 20 h were reported the experiment was repeated using 20 h of hypoxia treatment. In addition, also hypoxia treated cells were incubated at 43°C for 1 h directly after hypoxia treatment and lysed 6 h later to verify the inducibility of the promoter even after hypoxia.

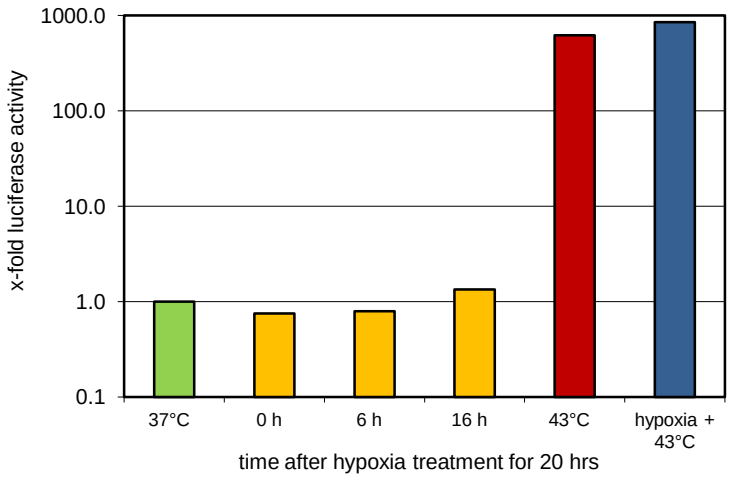

FIGURE 5-8 20 h OF HYPOXIA TREATMENT. Cells of the cell line C5 were incubated at hypoxic conditions and luciferase expression was measured at different time points after hypoxia. Therefore, $2x10^5$ cells were seeded and 3 days later cells were incubated for 20 h under hypoxic conditions (10 %CO_2, 90 % N_2) at 37°C in an incubator (+CO_2). Cells were lysed directly, 6 h and 16 h after hypoxia treatment and a luciferase reporter gene assay was performed. As positive controls, cells were incubated for 1 h at 43°C and lysed 6 h later and cells incubated for 20 h under hypoxic conditions were incubated for 1 h at 43°C and lysed after 6 h. The luciferase expression levels were normalised to cells incubated at 37°C (negative control). This figure shows one representative experiment.

Like in the previous experiment with 6 h of hypoxia treatment ((Figure 5-7), an induction of the artificial promoter and the resulting luciferase expression in cells, which were treated for 20 h under hypoxic conditions, could not be detected (Figure 5-8, yellow bars). To ensure, that the stable cell line was still inducible after hypoxia treatment, cells were incubated for 1 h at 43°C in addition to the incubation under hypoxic conditions which resulted in ~ 600 fold luciferase expression (Figure 5-8, blue bar) compared to ~850-fold induction when cells were incubated for 1 h at 43°C without previous hypoxia treatment (Figure 5-8, red bar). As a result we could show that the stable cell line is inducible by heat treatment, with or without previous hypoxia treatment. However, 20 h of hypoxia treatment alone were not sufficient to induce the artificial promoter.

The different durations of hypoxia treatment did not result in an induction of the artificial promoter, but in all control cells incubated at 43°C the expected increase of luciferase levels could be shown indicating that the cells were alive and functional. In order to exclude problems with the experimental settings we introduced a positive control for hypoxia, which in our case was a natural Hsp70-promoter of *Danio rerio* fused to luciferase as a marker gene.

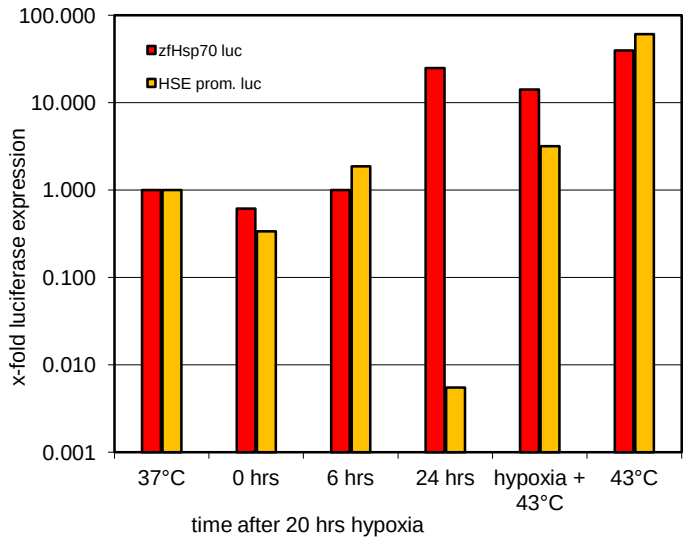

FIGURE 5-9 20 h OF HYPOXIA TREATMENT WITH TRANSIENTLY TRANSFECTED HELA CELLS. The HeLa cells were transiently transfected with a natural heat shock promoter-luciferase construct or the artificial heat shock promoter construct and induced by hypoxia treatment. Luciferase expression was measured at different time points after hypoxia. One day in advance, $1,5x10^5$ cells were seeded, transfected with 50 ng natural zebrafish Hsp70 promoter construct (zfHsp70luc) or the artificial heat shock promoter construct (HSE prom. luc) and 1 day later cells were incubated for 20 h under hypoxic conditions (10 %CO_2, 90 % N_2) at 37°C. Cells were lysed directly, 6 h or 24 h after hypoxia treatment and a luciferase reporter gene assay was performed. As positive controls, cells were incubated for 1 h at 43°C and lysed 6 h later and cells incubated for 20 h under hypoxic conditions were incubated for 1 h at 43°C and lysed after 6 h. The luciferase expression levels were normalised to cells incubated at 37°C (negative control). This figure shows one representative experiment.

When compared to the literature, the highest expression of reporter protein in HeLa cells was observed 6-10 h after 20 h hypoxia treatment so the late induction in this experiment might include other stress factors. Like shown for the HSE promoter cell line, also in HeLa cells the artificial heat shock promoter did not respond to hypoxia at all time points (Figure 5-9, yellow bars) verifying the previous observations.

Although the natural heat shock promoter of zebrafish was induced to some extent after hypoxia treatment, the induction levels were relatively low, which might be due to the transient transfection, but might also result from problems with the experimental settings of the hypoxic chamber. To exclude the possibility, that the artificial promoter could be induced to some extent by hypoxia not detectable with the transient transfection and to exclude problems with the experimental settings of the hypoxia an additional test of hypoxia induced activation was performed. In the literature, cobalt chloride was shown to mimic hypoxic conditions (An et al. 1998). Hence, the next step was to incubate the stable cell line harbouring the artificial HSE promoter with different concentrations of cobalt chloride. Within this experiment, the induction of the stably integrated artificial promoter could be directly compared to the expression of an endogenous heat shock protein (Hsp72) without the fluctuations and lower expression rate resulting from transient expression.

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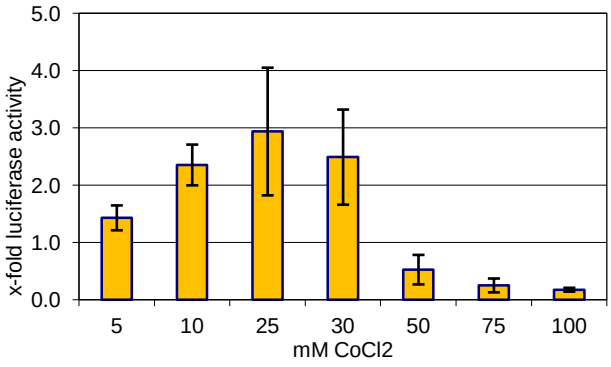

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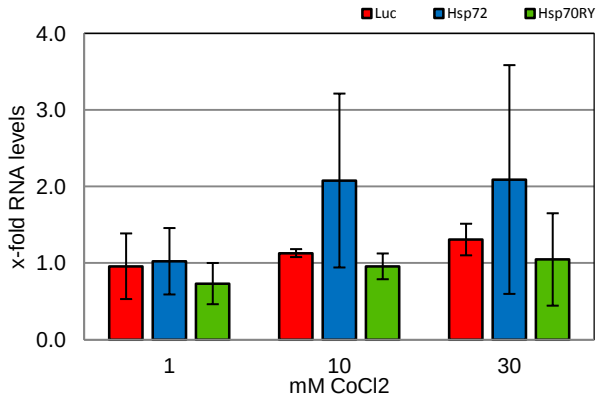


FIGURE 5-10 HYPOXIA MIMICKED BY COBALT CHLORIDE. The stable cell line C5 was incubated with different concentrations of cobalt chloride in medium for 1 hr at 37°C. Afterwards the medium was removed and the cells were incubated at 37°C with fresh medium. A) Luciferase assay. $0.3x10^5$ C5 cell per well of a 24-well plate were seeded 3 days before incubation with different concentrations of CoCl for 1 hr. Afterwards the cells were incubated for 6 hrs at 37°C. The expression of luciferase was determined using a luciferase assay. Luciferase activity was normalised to the expression levels at 37°C. B) $2x10^5$ C5 cells were seeded in 6 well plates, incubated 3 days at 37°C, treated for 1 hr with different concentrations of CoCl (1 mM, 10 mM, 30 mM), medium was changed and after 2 hrs at 37°C total RNA was isolated, transcribed into cDNA and qPCR was performed for luciferase, Hsp72 and Hsp70RY. All RNA levels were normalised to the internal reference GAPDH and to the RNA levels at 37°C. The measurements were performed in triplicates and for all experiments a summary of three separate experiments is shown. Error bars: +/- SEM n=3.

Mimicking hypoxic condition by the incubation with cobalt chloride showed weak induction of the artificial promoter up to 3 fold at 25 mM $CoCl_2$ (Figure 5-10 A), which is negligible in comparison to the more than thousand fold induction in response to heat treatment (Figure 5-6). Incubation of the stable cell line at higher concentrations of $CoCl_2$ resulted in a decrease of luciferase activity below basal levels which is due to increased cell death (50 – 100 mM, Figure 5-10 A). In contrast to luciferase driven by the artificial promoter (Figure 5-10 B, red bars) and the Hsp70RY (Figure 5-10 B, green bars), the endogenous Hsp72 was

shown to be up-regulated in response to cobalt induction in a concentration dependent manner at least two fold (Figure 5-10 B, blue bars).

These experiments showed that the artificial promoter cannot be properly induced by either hypoxia (Figure 5-7-Figure 5-9) or by mimicking hypoxic conditions with cobalt chloride (Figure 5-10), whereas the endogenous Hsp72 promoter is activated Figure 5-10 B) as well as a zebrafish Hsp70 promoter driven luciferase expression construct (Figure 5-9, red bars). In some of the experiments, a slight activation of the artificial promoter could be observed but rather seemed to be a result of fluctuations of the basal levels than a real induction of the promoter.

5.3.3. INDUCTION WITH HEAVY METALS

Beside this natural stress conditions, a variety of chemical substances are known to induce the heat shock response. Most prominent are the heavy metals which mainly activate the promoter via HSF1 but also pharmacological inducers modulating the pathway are known. To test the ability of our artificial promoter to be activated after heavy metal induction two well established heavy metal inducers, cadmium and zinc were analysed. Promoter activation was determined by measurement of luciferase activity (Figure 5-11 A-B) but also of mRNA levels (Figure 5-11 E-F) and compared to the activation on the natural highly inducible Hsp72 (HSPA1A) promoter. As a control for a non-inducible heat shock protein, Hsp70RY was used and all mRNA levels were normalised to the internal reference GAPDH. Both assays analyse the promoter activity but not the initiating pathway. As the key event of the heat shock response is the denaturation of proteins, it was also analysed if the heavy metals are able to generate protein denaturation. Therefore, two different luciferases were constitutively expressed in cells and incubated with the heavy metals (Figure 5-11 C-D). Firefly luciferase was previously shown to be sensitive for denaturation whereas *Gaussia* luciferase is highly stable (Figure 5-6 B). This feature is used in the degradation assay to determine on the one hand the denaturation capacity of the heavy metals by firefly luciferase and *Gaussia* luciferase was used as an internal reference for expression and cell number. The induction of the stable cell line C5 with heavy metals for 1 hr showed a concentration dependent expression of luciferase from 3 fold at 100 µM $CdSO_4$ up to 200 fold luciferase activity at 1600 µM $CdSO_4$. At 3200 µM the luciferase activity dropped down to levels below the basal activation, which is most probably due to the loss of cells and cell death at this high concentration (Figure 5-11 A). The high inducibility of the artificial promoter could also be verified by analysing mRNA expression levels after Cd^{2+} induction. Within this assay, a peak of 15 fold induction of luciferase mRNA could be assayed at 1600 µM $CdSO_4$ (Figure 5-11 E red). When compared to the expression of the highly inducible endogenous Hsp72, Cd^{2+} was shown to increase mRNA levels up to 16 fold at 400 µM $CdSO_4$, which is a shift to lower concentrations compared to the artificial luciferase. At higher concentrations the mRNA levels started to decrease again (Figure 5-11 E, blue). The expression of Hsp70RY was not influenced by the incubation with cadmium (Figure 5-11 E, green). In case of protein denaturation the incubation of cells with cadmium resulted in a 25 % decreased firefly luciferase activity at high concentrations of $CdSO_4$ (1600 µM) but still high expression of *Gaussia* luciferase (2 fold at 1600 µM compared to 2.3 fold at 100 µM) suggesting a protein denaturation effect of cadmium on the firefly luciferase without significant changes in *Gaussia* luciferase activity.

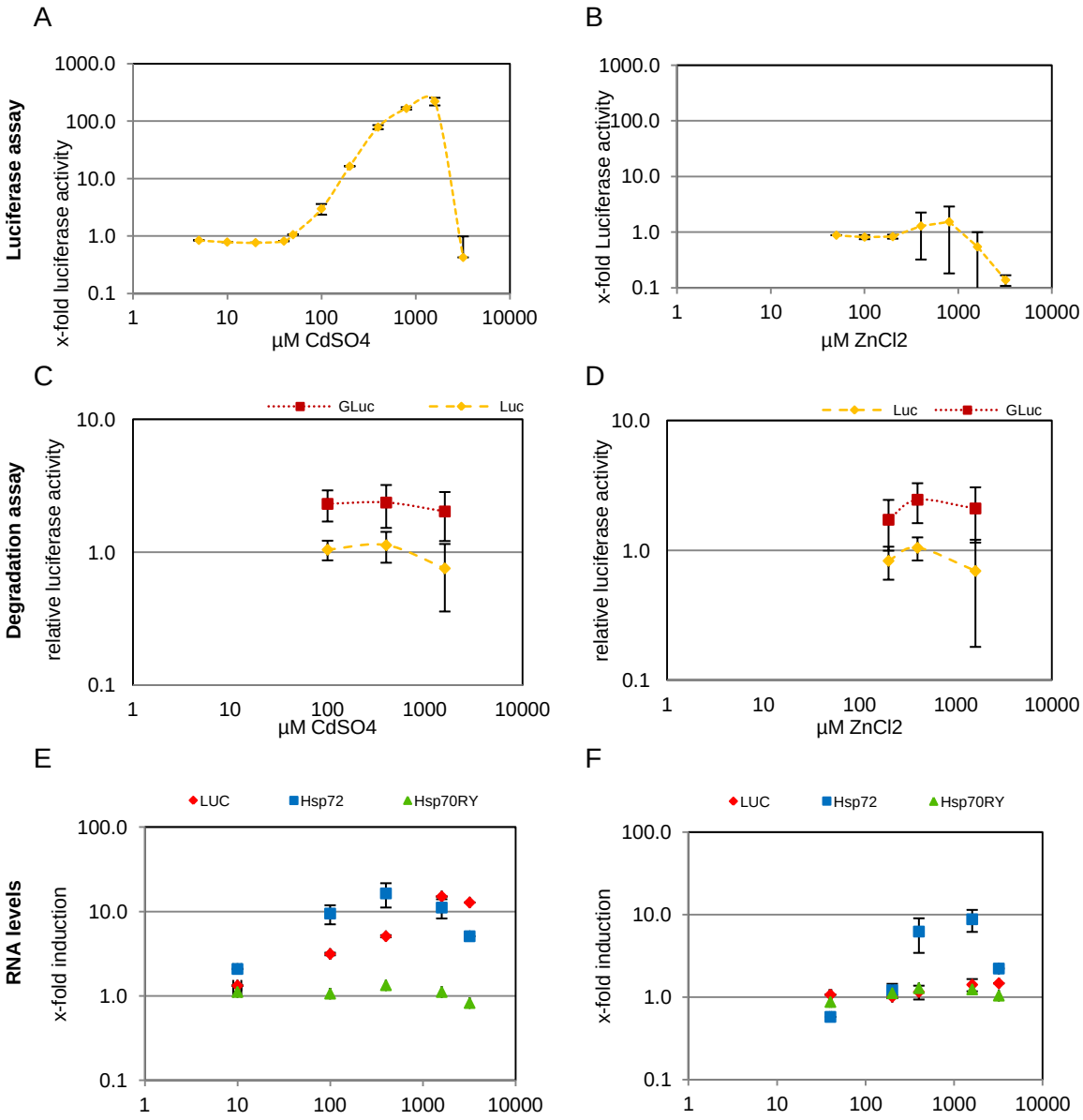


FIGURE 5-11 INDUCTION OF THE STABLE CELL LINE WITH HEAVY METALS. The stable cell line C5 was incubated with different concentrations of cadmium sulphate (A, C, E) or zinc chloride (B, D, F) in medium for 1 hr at 37°C. Afterwards medium was removed and cells were incubated at 37°C with fresh medium. A+B).Luciferase assay. $0.3x10^5$ C5 cells per well of a 24-well plate were seeded 3 days before incubation with different concentrations of $CdSO_4$ or $ZnCl_2$ for 1 hr. Afterwards the cells were incubated for 6 hrs at 37°C. The expression of luciferase was determined using a luciferase assay. Luciferase activity was normalised to the expression levels at 37°C. B+C) Degradation assay: $0.2x10^5$ HeLa cells per well of a 24-well plate were incubated for 1 day at 37°C, transiently transfected with plasmids encoding constitutively expressed firefly luciferase and *Gaussia* luciferase. After 2 days cells were incubated for 1 hr with different concentrations of $CdSO_4$ (100, 400, 1600 µM) or $ZnCl_2$ (200, 400, 1600 µM) and the activity of firefly as well as *Gaussia* luciferase were determined directly afterwards. E+F) $2x10^5$ C5 cells were seeded in 6 well plates, incubated 3 days at 37°C, treated for 1 hr with different concentrations of $CdSO_4$ (10, 40, 100, 400, 1600 and 3200 µM) or $ZnCl_2$ (40, 200, 400, 1600 and 3200 µM), medium was changed and after 2 hrs at 37°C total RNA was isolated, transcribed into cDNA and qPCR was performed for luciferase, Hsp72 and Hsp70RY. All RNA levels were normalised to the internal reference GAPDH and to the RNA levels at 37°C. The experiments A-D were performed in triplicates and a summary of three separate experiments is shown. Error bars: +/- SEM n=3.

For the induction of the stable cell line C5 with zinc, no activation of the artificial promoter could be observed neither on protein levels (Figure 5-11 B) nor at mRNA levels (Figure 5-11 F, red). In contrast, the mRNA levels of the endogenous Hsp72 increased up to 9 fold at 1600 µM $ZnCl_2$ in a concentration dependent manner (Figure 5-11 E, blue). At a higher concentration of 3200 µM the Hsp72 mRNA levels started to decrease again like shown for the induction with $CdSO_4$. The levels of Hsp70RY were not affected by zinc incubation

(Figure 5-11 F, green), similar to Cd^{2+}. Analysing the protein denaturating capacity of zinc, a 30 % reduction of firefly luciferase activity was observed without changes in *Gaussia* luciferase activity (Figure 5-11 D). Therefore, zinc seems to act on protein stability at high concentrations of 1600 µM without activation of the artificial HSE promoter.

To conclude, the artificial HSE promoter was shown to be robustly activated by incubation with cadmium but not zinc although both heavy metals were able to induce the expression of the endogenous Hsp72 (Figure 5-11 E-F, blue).

5.3.4. INDUCTION WITH PHARMACEUTICAL COMPONENTS

In the last decades, heat shock proteins and the heat shock response in general became a promising research area to treat several diseases like neurodegenerative diseases but also cancer (see also 3.5.4.). Therefore, a lot of effort was put into the development and analysis of substances, which are able to modulate the heat shock response or the expression of heat shock proteins. The artificial promoter was designed for the use in cell therapy applications. Hence, the interference of some of this stress response modulating components with our artificial promoter was analysed. For this propose the activation of the promoter was tested on protein levels, mRNA levels and compared to the endogenous activation of Hsp72. To analyse also the indirect activation of the promoter via protein denaturation, a degradation assay was performed. In total three heat shock promoter inducing components, the Hsp90 inhibiting antibiotic geldanamycin, the anti-inflammatory drug carbenoxolone and the protease inhibitor tosyl phenylalanyl chloromethyl ketone (TPCK) and one HSF inhibiting substance, the flavonoid quercetin, were tested. Testing the stable cell line harbouring the artificial HSE promoter for the induction with different pharmacological inducers showed only partial initiation of protein expression (Figure 5-12 A-D). In case of TPCK a concentration dependent activation of luciferase expression could be observed with a maximum of 50 fold at 80 µM. At higher concentrations the luciferase activity dropped down to levels below basal activity suggesting increased cell death (Figure 5-12 B). Nevertheless, the activation is not linked to protein denaturation, as TPCK showed no effect in the denaturation assay, but seemed to increase cell death at higher concentration of TPCK (Figure 5-12 F). In contrast carbenoxolone only showed induction at the highest concentration of 1500 µM (Figure 5-12 C) which is linked to the high degradation capacity of the substance (Figure 5-12 G). The activator geldanamycin (Figure 5-12 A) as well as the inhibitor quercetin (Figure 5-12 D) were not able to induce the expression of luciferase and did not show any effects on protein denaturation (Figure 5-12 E and H). At mRNA levels, all three inducers showed increased levels of Hsp72 mRNA. Geldanamycin showed a robust Hsp72 expression of ~80 fold at 9 µM (Figure 5-12 I, blue) but like the experiments at protein levels no increase in luciferase mRNA (Figure 5-12 I, red). For the induction with TPCK the mRNA levels of the endogenous Hsp72 increased up to 60 fold at 75 µM (Figure 5-12 J, blue) but in contrast to the protein activity assay (Figure 5-12 B) no increase of luciferase mRNA levels could be observed (Figure 5-12 J, red). Incubation of the C5 cells with carbenoxolone resulted in an increase of both, Hsp72 (up to 50 fold, Figure 5-12 K, blue) and luciferase (~ 9 fold) mRNA levels (Figure 5-12 K, red) corresponding well to the strong protein denaturation (Figure 5-12 G). Interestingly, also the HSF inhibitor quercetin was able to induce the endogenous Hsp72 gene to some extent (up to 4.5 fold, Figure 5-12 L, blue), but no increase in luciferase mRNA levels were observed (Figure 5-12 L, red).

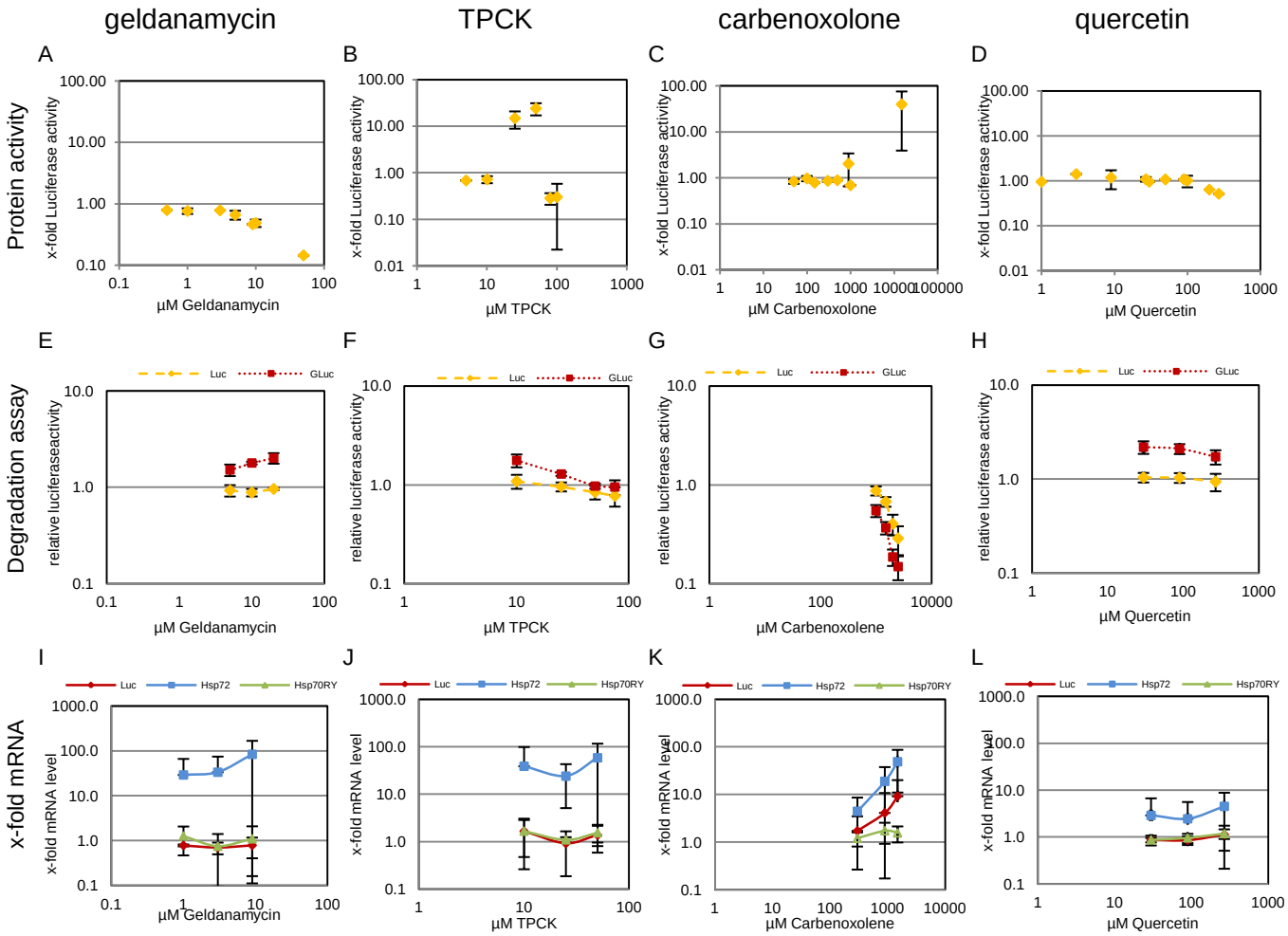


FIGURE 5-12 INDUCTION WITH DIFFERENT PHARMACOLOGICAL HEAT SHOCK MODULATORS. The stable cell line C5 was incu concentrations of geldanamycin (A, E, I), TPCK (B, F, J), carbenoxolone (C, G, K) or quercetin (D, H, L) in medium for 1 hr at 37°C. Afte removed and the cells were incubated at 37°C with fresh medium. A-D) Luciferase assay. 0.3x10^5 C5 cells per well of a 24-well plate v before incubation with different concentrations of the different pharmacological components A) geldanamycin (0.5, 1, 3, 5, 9, 10 and 5(10, 25, 50, 80 and 100 µM), C) carbenoxolone (50, 100, 150, 300, 500, 900, 1000 and 1500 µM) or D) quercetin (1, 3, 9, 25, 30, 50, 9(µM) for 1 hr. Afterwards cells were incubated for 6 hrs at 37°C. The expression of luciferase was determined using a luciferase assay was normalised to the expression levels at 37°C. E-H) Degradation assay: 0.2x10^5 HeLa cells per well of a 24-well plate were incubate transiently transfected with plasmids encoding constitutive expressed firefly luciferase and *Gaussia* luciferase. After 2 days cells were inc different concentrations of E) geldanamycin (5, 10 and 20 µM), F) TPCK (10, 25, 50 and 75 µM), G) carbenoxolone (1000, 1500, 2000 quercetin (30, 90 and 270 µM) and the activity of firefly as well as *Gaussia* luciferase were determined directly afterwards. I-L) 2x10^5 C5 well plates, incubated 3 days at 37°C, treated for 1 hr with different concentrations of I) geldanamycin (1, 3 and 9 µM), J) TPCK (10, carbenoxolone (300, 900 and 1500 µM) or L) quercetin (30, 90 and 270 µM), medium was changed and after 2 hrs at 37°C total transcribed into cDNA and qPCR was performed for luciferase, Hsp72 and Hsp70RY. All RNA levels were normalised to the internal ref to the RNA levels at 37°C. All measurements were performed in triplicates and for all graphs a summary of three separate experiments i +/- SEM n=3.

For all tested components, the NEF Hsp70RY mRNA levels were constant, independent of concentration or substance type (Figure 5-12 I-L, green). In conclusion, only one tested pharmacological inducer, the serine proteinase inhibitor TPCK, was able to induce the artificial promoter, at least at protein levels, whereas geldanamycin and carbenoxolone could not induce luciferase expression except at highly denaturating concentrations of carbenoxolone. As shown in the literature, the tested components could induce the endogenous Hsp72 gene and had no effect of the constitutively expressed Hsp70RY.

The artificial HSE promoter was designed for applications in regulated gene expression in cell therapy. Therefore, it was important to analyse, if additional triggers can activate the promoter beside heat. In this section, it could be shown, that the artificial HSE promoter does not respond to naturally occurring additional stress triggers such as hypoxia as well as two

important pharmacological components, the anti-cancer drug geldanamycin and the anti-inflammatory drug carbenoxolone. TPCK, a serine protease inhibitor was the only component tested that was able to induce luciferase expression. These results verify the application of the artificial HSE promoter in cell therapy as it is strictly regulated by heat, but hardly any other stress factors or treatments. In our experiments only TPCK could induce the promoter.

5.4. CHARACTERISATION OF PROMOTER KINETICS

Regulated gene expression systems have been previously used in medical approaches (see also 3.2.). Although these inducible systems showed high expression after activation, at least for the two component systems the expression kinetics is very static and a short time regulation is not possible (see 3.2.1.). For other one component systems the induction worked faster but the resulting amounts of product most of the time are very low (see 3.2.2.). The artificial promoter was shown to be highly inducible producing high amounts of protein without the problem of high basal activity (5.2.) known for other inducible systems. In addition, the promoter was shown to be strictly heat dependent without activation by most other stress conditions tested. The next step was to analyse the kinetics of the artificial HSE promoter after induction to prove the postulated use as a tightly regulated expression system in gene and cell therapy approaches.

5.4.1. PROTEIN KINETICS

The first step was to determine the kinetics of protein expression after heat treatment. Hence, the stable cell line C5 was incubated for 1 hr at 43°C and luciferase activity was determined at different time points afterwards. To ensure, that the expression kinetic is due to the activation of the artificial HSE promoter and not based on specific integration events in the stable cell line C5 a second HEK 292 based cell line harbouring the artificial HSE promoter was analysed the same way.

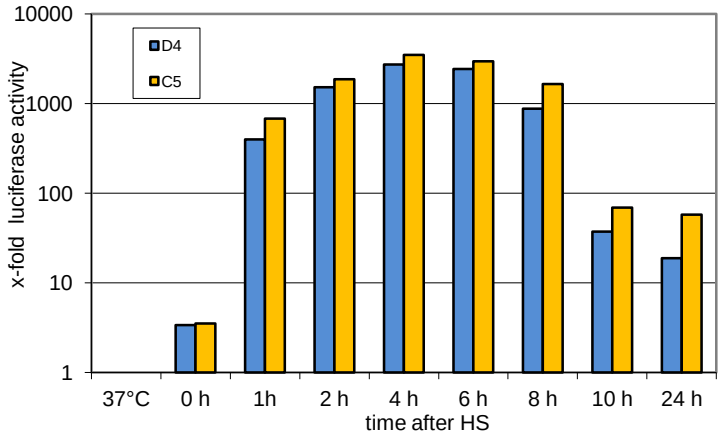


FIGURE 5-13 COMPARISON OF EXPRESSION KINETICS IN DIFFERENT SINGLE CELL CLONES. Two different HEK 293 based stable cell lines (C5 and D4) harbouring the artificial HSE promoter driving the expression of luciferase (pSGH2 luc) were seeded in 6 well plates ($2x10^5$cells/well) and after 3 days at 37°C incubated at 43°C for 1 h. Cells were lysed directly, 1, 2, 4, 6, 8, 10 and 24 hrs after heat treatment and a luciferase assay was performed. Luciferase activity was compared to cells incubated at 37°c. This figure shows one representative experiment.

Treating the two different stable cell lines for 1 hr at 43°C resulted in increasing luciferase activity up to 4-6 hrs after heat treatment (~3000 fold) and a decrease down to almost basal levels (~20-50 fold) 24 hrs later (Figure 5-13). Both cell lines showed the same kinetics although in general the stable cell line C5 (Figure 5-13, yellow bars) showed higher luciferase expression than stable cell line D4 (Figure 5-13, blue bars). This experiment therefore verifies that the observed kinetics of protein expression is not an artefact of the

stable cell line C5 but represents the kinetics of the HSE promoter as also a second independent cell clone showed the same expression kinetics.

5.4.2. mRNA KINETICS

The artificial heat shock promoter consists of a core structure of 8 idealised heat shock elements flanked by two minimal CMV promoters. If the promoter is activated, two reporter genes, GFP and luciferase are expressed by bidirectional promoter activation. To assay promoter activation kinetics in detail a series of different heat shock experiments was performed by activating the promoter through different incubation times at 43°C and assaying the induction at different time points afterwards. So far the measurement of luciferase protein levels was used to get a first idea about the promoter kinetics. However, to get more detailed data independent of the protein stability the respective mRNA was quantified and compared to the endogenous situation by detecting the mRNA levels of the major inducible heat shock protein Hsp72. In addition, GFP mRNA levels were analysed to determine the bidirectional expression behaviour of the artificial HSE promoter.

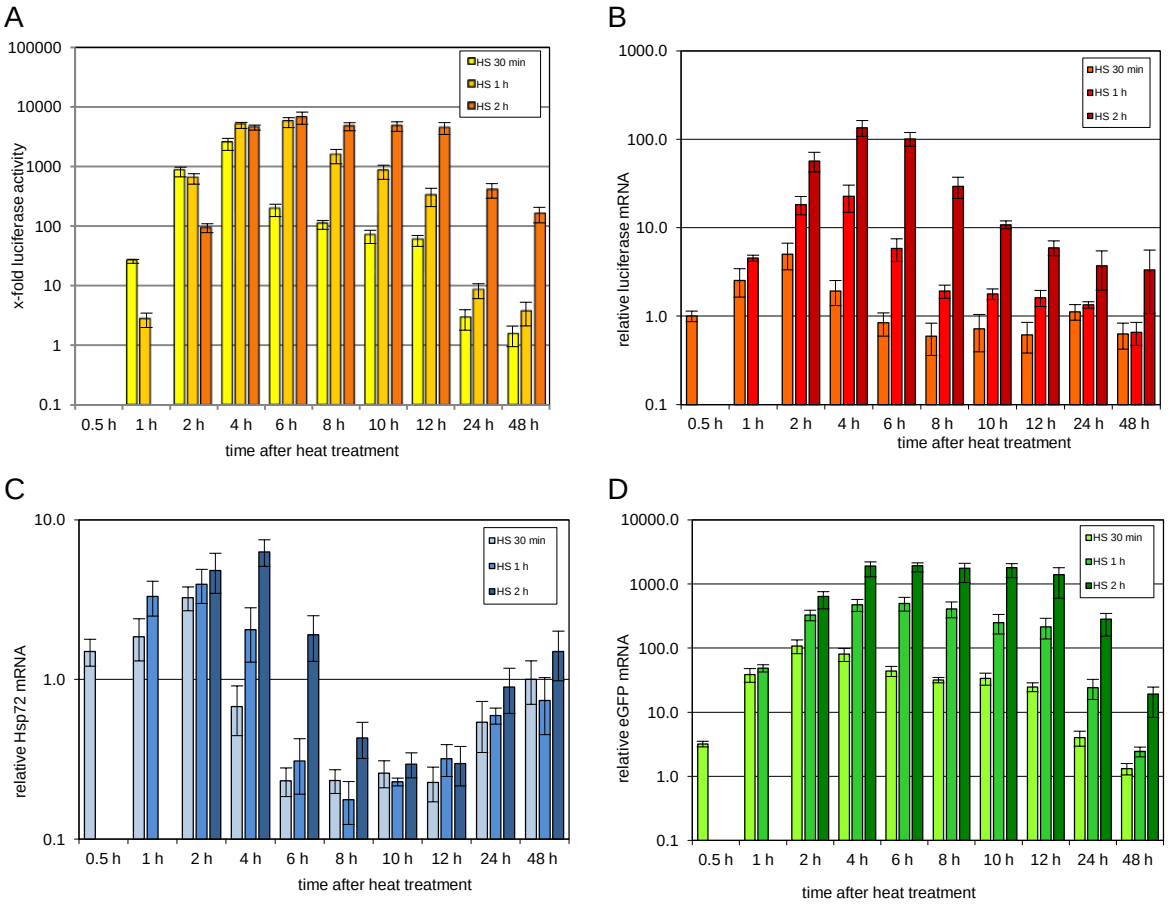


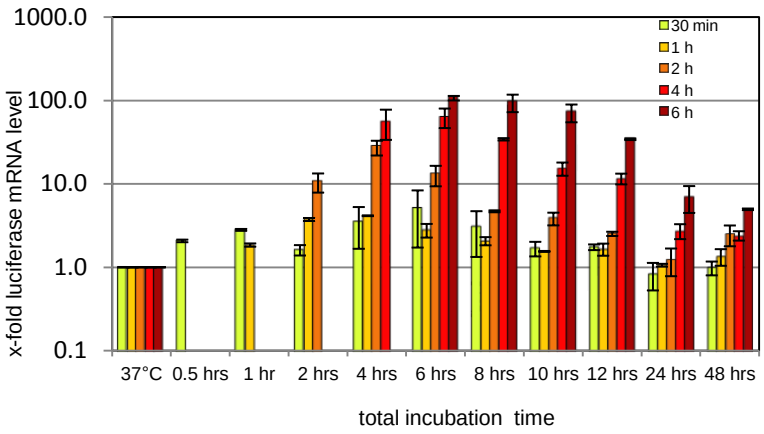
FIGURE 5-14 PROTEIN AND MRNA EXPRESSION KINETICS AFTER HEAT TREATMENT. This figure was recently published in (Ortner et al. 2012) as Fig.3 and adopted here (see also appendix). The stable cell line C5 was seeded in 6 well plates (2x10^{5}cell/well), after 3 days at 37°C cells were incubated for 30 min to 2 hrs at 43°C and lysed 0.5, 1, 2, 4, 6, 8, 10, 12, 24 or 48 hrs afterwards. A) Luciferase assay after different durations of heat treatment for 30 min (light yellow), 1 hr (dark yellow) or 2 hrs (orange). Luciferase activity was determined at 0.5 – 48 hrs afterwards. B-D) Stable cell line C5 was incubated for 30 min (light), 1 hrs (normal) or 2 hrs (dark) at 43°C and total RNA was isolated 0.5 – 48 hrs afterwards, transcribed into cDNA and qPCR was performed for luciferase (B), Hsp72 (C) and Hsp70RY (D). All RNA levels were normalised to the internal reference GAPDH and to the RNA levels at 37°C. The experiments A-D were performed in triplicates and for all graphs a summary of three separate experiments is shown. Error bars: +/- SEM n=3

The stable cell line C5 was incubated for 30 min up to 2 hrs at 43°C and either luciferase protein expression (Figure 5-14 A) or the corresponding mRNA levels (Figure 5-14 B) were analysed up to 48 hrs afterwards. Similar to the previous experiment, the luciferase protein showed peak activity of ~6000 fold at 6 hrs after 1hr incubation at 43°C (Figure 5-14 A, yellow bars) and a reduction to basal levels 24-48 hrs afterwards, whereas the reduction of heat treatment to 30 min resulted in a shift in peak activity to 4 hrs and ~ 2000 fold expression levels (Figure 5-14 A, light yellow bars). Extension of heat treatment led again to a shifted and also wider peak of protein activity but at later time points (6-10 hrs, ~5000 fold) (Figure 5-14 A, orange bars). On mRNA levels, the same kinetics was observed with peaks at 2 hrs for 30 min (Figure 5-14 B, orange bars), 4 hrs for 1 hr incubation (Figure 5-14 B, red bars) and 4 - 6 hrs peak levels when cells were incubated for 2 hrs at 43°C (Figure 5-14 B, dark red bars). In general, the mRNA levels showed the similar kinetics but the maxima were reached 2 hrs earlier compared to the protein levels. For the expression of the endogenous Hsp72, the kinetics was again similar to that of luciferase but the maxima of mRNA levels were reached at 2 hrs for 30 min (Figure 5-14 C, light blue bars) and 1 hr heat treatment (Figure 5-14 C, blue bars) and at 4hrs for the 2 hrs incubation at 43°C (Figure 5-14 B, dark blue bars). The levels of Hsp72 started to increase again after 12 - 24 hrs, which might be due to prolonged incubation of the cells without refreshing medium. This resulted in a kind of nutrition depletion which is also known to induce the natural heat shock promoters.

Analysing the expression of GFP confirmed the bidirectional design of the promoter, as GFP mRNA levels showed the same kinetics, but a slower decrease independent of heat treatment duration (Figure 5-14 D). The maximum levels of mRNA were reached at 2 hrs after heat treatment for the 30 min incubation (Figure 5-14 D, light green bars), at 4 hrs for the 1 hr incubation (Figure 5-14 D, green bars) or at 4 - 6 hrs after 2 hrs incubation at 43°C (Figure 5-14 D, dark green bars). In principle, the mRNA levels of GFP were around 10 fold higher than the corresponding luciferase mRNA levels which again were around 10 fold higher than the endogenous mRNA levels. For all tested mRNAs, as for the protein a shift of maximum expression was observed when the duration of heat treatment was extended, but in general the expression was high around 2 - 6 hrs after heat treatment and dropped down to basal levels at 24 - 48 hrs. In addition, the bidirectional expression potential of the promoter was verified and similar kinetics of the natural and artificial promoter were observed.

The previous experiment showed that the artificial promoter is highly inducible with distinct kinetics. Different durations of heat treatment resulted in a time variation of the maximum expression levels. For a detailed analysis of this shift in peak levels to later time points, the stable cell line was induced for 30 min to 6 hrs at 43°C and mRNA levels of luciferase and Hsp72 were analysed.

A



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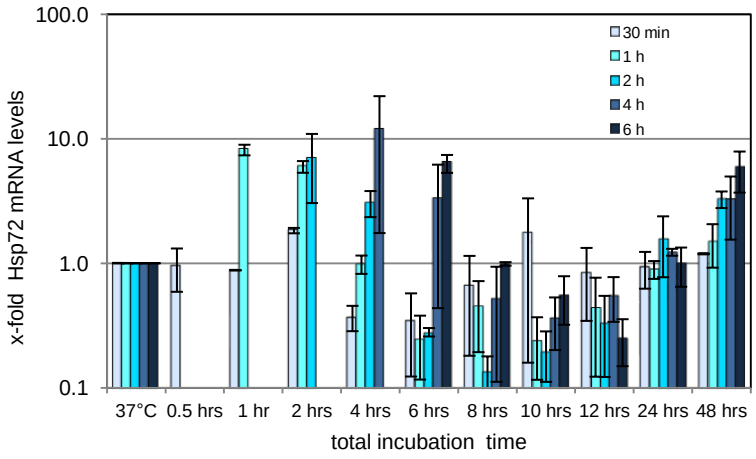

FIGURE 5-15 PROMOTOR mRNA KINETICS FOR DIFFERENT INDUCTION TIMES. The stable cell line C5 was seeded in 6 well plates ($2x10^5$cells/well) and kept at 37°C for 3 days. Afterwards the cells were incubated for 30 min to 6 hrs at 43°C and then again at 37°C, and total RNA was isolated 0.5 – 48 hrs after start (time of total incubation: heat treatment + recovery at 37°C), transcribed into cDNA and qPCR was performed for luciferase (A) or Hsp72 (B) All mRNA levels were normalised to the internal reference GAPDH and to the mRNA levels at 37°C. The measurements were performed in triplicates and for all graphs a summary of three independent experiments is shown. Error bars: +/- SEM n=3

Like in the previous experiment, incubation for 30 min at 43°C resulted in peak mRNA levels at 2 hrs for both the activation of the artificial HSE promoter (Figure 5-15 A, light yellow bars) and the endogenous promoter (Figure 5-15 B, light blue bars). Extending heat shock duration again resulted in peak luciferase mRNA levels at later time points (2 hrs for 1 hr heat treatment, 4 hrs for 2 hrs heat treatment and 6 hrs for 4 hr incubation at 43°C) to maximum levels at 8 hrs for 6 hr incubation at 43°C (Figure 5-15 A, dark red bars). The levels of endogenous Hsp72 again had their peak levels 2 hrs earlier than luciferase, but they decreased to basal levels after 8 hrs independent on the heat shock duration. As observed before, the levels increased again 24 - 48 hrs later (Figure 5-15 B).

To conclude, the artificial HSE promoter shows the same expression kinetics but higher total levels as the natural Hsp72 promoter, except the late induction observed for the natural Hsp72 after 24 - 48 hrs. In addition, it could be shown, that the artificial promoter reaches its maximum activity 2-6 hrs after heat treatment and returns to basal levels after 24 hrs, which is important for a tight regulation compared to several days of promoter activity, when for example the tetracycline based induction system is used. Another important observation was the shift in maximum expression levels when prolonged heat shock durations were performed. This heat shock duration dependent expression adds an additional level of regulation as the amount of expressed protein can be regulated by the duration of heat treatment.

5.5. SURVIVAL

Exposing cells to stress factors like high temperatures, results in the activation of an emergency program to survive. The activation of the heat shock response therefore is a defence against stress induced cell death. Therefore, the application of heat induced gene expression always bears the risk of increased cell death.

5.5.1. SURVIVAL AFTER HEAT TREATMENT AT DIFFERENT TEMPERATURES

In the beginning of the project, the stable cell line C5 was tested for its expression capacity at different heat shock temperatures. The result showed highest induction levels at 44°C and slightly lower induction at 43°C. Nevertheless 43°C was chosen as the heat shock standard temperature to avoid to high stress. Now the survival of the cells after treatment at different temperatures was analysed to substantiate the selected heat shock conditions.

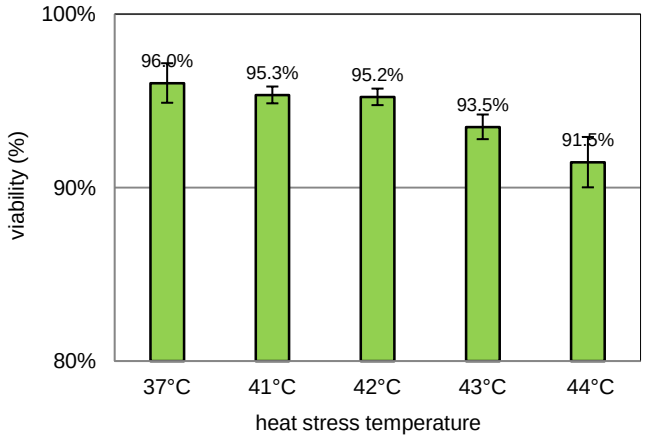

FIGURE 5-16 SURIVAL AFTER DIFFERENT HEAT SHOCK TEMPERATURES. This figure was recently published in (Ortner et al. 2012) as Fig.2b and adopted here (see also appendix). $2x10^5$ C5 cell per well of a 6-well plate were incubated at 41 - 44°C for 1 hr and recovered for 24 hrs at 37°C. The viability of the cells was determined by Trypan blue assay. The experiment was performed in sextuplicates. Error bars: +/- SEM n=6.

Analysing the survival of the cells after heat shock at different temperatures showed no decreased viability when cells were incubated at 41°C or 42°C, but a decrease of almost 3 % when incubated at 43°C. At the highest temperature tested, the viability dropped down to ~90 % which is still high (Figure 5-16). Nevertheless, if each heat treatment would result in ~10 % death rate, this would lead to a significantly reduced amount of cells over the time of application. Therefore, the selected condition of 43°C are more suitable for cell therapy application as the viability is still comparable to untreated cells combined with a more than thousand fold induction of protein expression.

5.5.2. REPEATED HEAT TREATMENT

A critical point of the whole project is the survival of cells upon repeated heat shock as for later applications in cell therapy the cells in the capsules would have to tolerate multiple heat shock treatments over several weeks. Therefore it is necessary to assay the survival of the cells first in a cell culture system. In order to achieve a time window of 2 weeks (10 heat shock treatments) for the experiments, the cells were incubated at reduced FCS concentrations (0.5 % after 4 days at 10 % FCS standard condition) to reduce proliferation to a minimum level. In parallel to the viability also luciferase expression was analysed to demonstrate the inducibility of the artificial HSE promoter at repeated heat shock treatments.

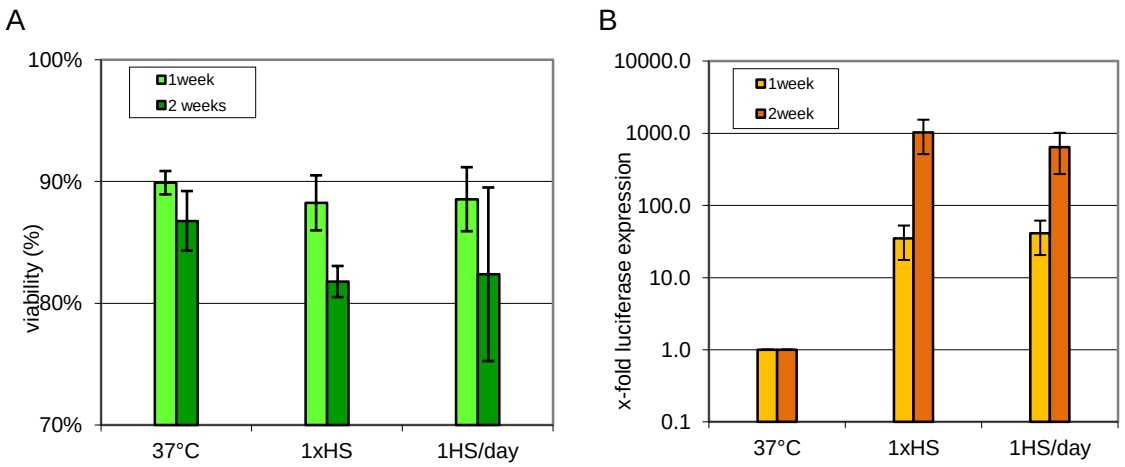

FIGURE 5-17 SURVIVAL AND PROTEIN EXPRESSION AFTER REPEATED HEAT TREATMENT. The stable cell line C5 was incubated every day (1HS/day) or once (1xHS) at 43°C for 1h or incubated at 37°C with reduced concentration of FCS (0.5 %). After one and two weeks the cells were washed twice with PBS, trypsinised, cells were collected and resuspended in medium. One half of the cells were analysed for their viability (**A**) the other half was subjected to a luciferase activity assay (**B**). **A)** Viability of the cells incubated for one week (light green) or two weeks (dark green) was determined using the Trypan blue assay**. B)** The remaining cells were lysed and a luciferase reporter gene assay was performed. The luciferase activity was normalised to basal activity in cells incubated at 37°C for one (yellow) of two (orange) weeks. The experiment was performed in triplicates. SEM, n=3

Compared to the viability of the cells at standard conditions (37°C, 10 % serum), the reduction of serum to 0.5 % and the prolonged incubation alone resulted in a decreased survival of ~90 % for 1 week at 37°C (Figure 5-17 A, light green bars) or ~87 % for a 2 weeks incubation at 37°C (Figure 5-17 A, dark green bars). This reduced viability is most probably due to the highly artificial incubation conditions at low serum. The incubation of these cells once at 43°C resulted in a decreased survival of 88 % after 1 week of culture and 82 % for the 2 weeks of culture. Interestingly, with the repeated heat treatment of cells the protein expression in response to heat treatment could still be strongly induced either by a single heat treatment or repeated heat shock (Figure 5-17 B). The induction levels of cells incubated for 1 week with reduced levels of serum reached ~ 50 fold (Figure 5-17 B, yellow bars) independent of the number of heat treatments, whereas the cells incubated for 2 weeks at reduced serum showed even higher induction rates of ~ 1000 fold for one heat treatment and 600 fold for repeated incubation at 43°C (Figure 5-17 B, orange bars). This increasing induction might be due to a general upregulation of the stress response pathway at these highly artificial conditions and a resulting amplification of heat shock pathway activation. Although the cultivation conditions within this experiment were highly artificial, it could be demonstrated that cells easily survive repeated heat treatment and that the artificial HSE promoter can still be induced repeatedly to high levels.

5.6. THE MAGNETIC FIELD GENERATOR

Beside the stable cell line harbouring the heat inducible expression system, the magnetic field generator for generation of magnetic nanoparticle induced heat had to be established. The magnetic field generator for production of the oscillating magnetic field was built by the Department of Applied Electronics and Information Technology, University of Applied Sciences, FH-Campus Wien. The construction includes a wave generator, power amplifier and a coil. Wave generator and power amplifier are commercially available pre-made units and the coil was constructed according to the required dimensions and field strength of the

magnetic field. In a first series of experiments the optimal frequency could be narrowed down between 40 and 80 kHz, as there was an optimum in temperature increase around 40 kHz. Nevertheless, only short time activation (up to 1 min) could be tested as the coil heated up fast. Another output of the first experiments was the switch from a sinus curve to a rectangle of the current input for the coil, as even better results could be obtained with this version (the rectangle input allows dramatically cheaper versions for the power amplifier). For the next version of the magnetic field generator the power was strongly increased, the generator and the amplifier were combined into one element and the coil was replaced by a new version. To avoid previous observed heating of the coil a water cooling system was introduced by using a brass tube instead of a normal wire to run cooling water directly through the coil. With the improvement of the water cooling system it was for the first time possible to activate the magnetic nanoparticles at higher power (up to 80 kHz and 20 A) and for durations up to 30 min. To avoid strong temperature increase of the amplifier cooling block, the amplifier and the frequency generator were combined and an air cooling system was introduced. With these improvements we could minimise heating of the amplifier-frequence generator. All data shown below were obtained with this last version of the magnetic field generator in order to establish conditions for the activation of the encapsulated cells with magnetic nanoparticles.

5.6.1. DETERMINATION OF CONDITIONS FOR HEAT ACTIVATION

To activate encapsulated cells with magnetic nanoparticles in the magnetic field a temperature of at least 43°C in the capsules has to be reached. For this purpose, first a suspension of 1 % magnetic nanoparticles in standard cell culture medium was used to test different settings with varying frequencies or current to find conditions for induction of the artificial HSE promoter. A suspension of 1 % magnetic nanoparticles (SIGMA) in medium was sonicated for 1 min at 50 % power with a sonificator. 100 µl of the suspension were transferred to a 2 ml reaction tube and put into the middle of the coil. Activation was performed by using different settings of current and the following frequencies: 40 kHz (light blue), 50 kHz (green), 60 kHz (petrol), 70 kHz (blue) and 80 kHz (dark blue). Temperature was measured before starting the magnetic field generator and immediately after 5min (Figure 5-18 A) or 30 min (Figure 5-18 B) activation.

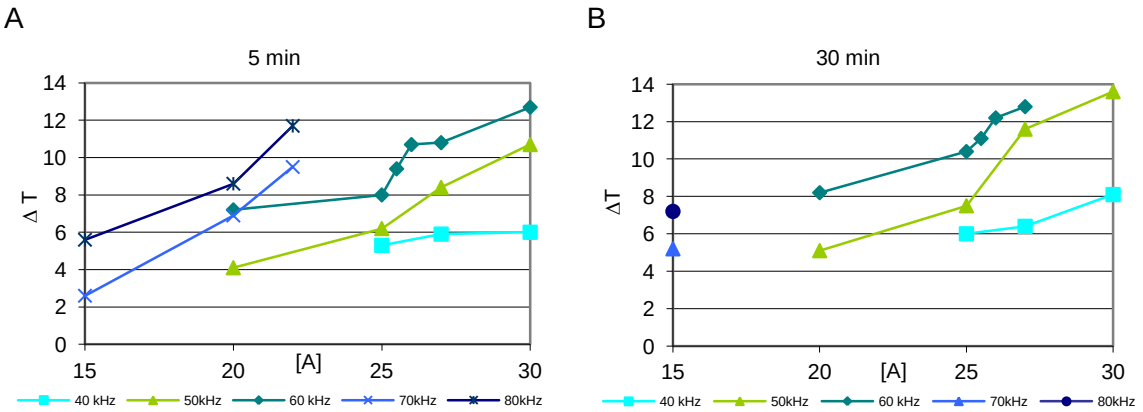

FIGURE 5-18 HEAT GENERATION OF 1 % NANOPARTICLES IN AN ALTERNATING MAGNETIC FIELD. A 1 % suspension of magnetic nanoparticles (Sigma) in cell culture medium without serum (DMEM) was sonicated at 50 % power and incubated for 5 min (A) or 30 min (B) in the alternating magnetic field. The magnetic field was established using different frequencies of 40 kHz (light blue), 50 kHz (green), 60 kHz (petrol), 70 kHz (blue) or 80 kHz (dark blue). In addition to the different frequencies also increasing current was used (15, 20, 23, 25, 26, 27 and 30 A) for the incubation of 5 min (A) or 15, 20, 25, 26, 27 and 30A for the 30 min incubation (B). Temperature increase was determined by measuring the starting temperature and the temperature directly after the end of incubation time using a temperature sensor.

The incubation of the 1 % magnetic nanoparticles for 5 min showed a frequency dependent increase in temperature with highest heat generation (12°C) at 80 kHz and 23 A (Figure 5-18 A, dark blue) but due to technical limitations of the system, neither the current could be increased nor the incubation time could be extended. Similar to the situation at 80 kHz also incubation at 70 kHz showed a temperature increase of ~10°C but again was limited in duration and applied current (Figure 5-18 A, blue). For the alternating magnetic field generated at 60 kHz, the current could be increased up to 30 A and this led to a robust heat generation of the magnetic nanoparticles up to 12.7°C at 60 kHz and 30 A (Figure 5-18 A, petrol). Induction using 50 kHz (Figure 5-18 A, green) or 40 kHz (Figure 5-18 A, light blue) resulted in lower heat generation of 10.6°C for 50 kHz and 6°C for 40 kHz. The induction for 5 min at different frequencies and increasing current clearly showed that higher frequencies and high current up to 30 A resulted in best heat generation. As the incubation time of 5 min is clearly too short for the induction of the artificial HSE promoter, the incubation time was extended to 30 min. At this experimental design, best conditions for heat shock activation were shown to be at 60 kHz and 25-27 A (Figure 5-18 B, petrol). With these adjustments, a temperature increase of 12°C could be reached in the supernatant of the magnetic nanoparticle suspension and also the required duration of 30 min was possible. There were also other conditions with a high increase of temperature (50 kHz and 30 A, Figure 5-18 B, green), but with these settings the duration of activation was limited due to heating of the coil although a cooling system was used. Magnetic fields using frequencies above 60 kHz could not be tested at currents above 20 A as here the same technical limitation as for 50 kHz and 30 A were seen. Testing of different current and frequence combinations for the generation on an alternating magnetic field showed highest temperature increase at 60 kHz and 27A in a 30 min incubation of 1 % magnetic nanoparticles. So these conditions were used as a standard for the induction of the artificial HSE promoter in all following experiments.

5.7. INDUCTION OF CELLS AND NANOPARTICLES IN THE MAGNETIC FIELD

The aim of the project was the induction of gene expression in encapsulated cells harbouring the artificial HSE promoter. In the previously described experiments, the magnetic field generator settings were determined to produce the necessary amount of heat. The next step now was to analyse, if these settings would generate appropriate heat to induce the expression of luciferase in the stable cell line. Therefore C5 cells were incubated in the magnetic field together with magnetic nanoparticles but without encapsulation. In the previous experiments, a concentration of 1 % magnetic nanoparticles was used and resulted in a temperature increase of 12.7°C. As the amount of magnetic nanoparticles is directly linked to their heat generation (see Ortner, Kaspar et al. 2012, Supplementary Figure S1, appendix) first different nanoparticle concentrations were tested for their ability to induce the artificial promoter when incubated together with the stable cell line in the magnetic field generator for 30 min at 60 kHz and 27A. In addition, different currents and induction times were tested to find optimal conditions for the activation of protein expression in encapsulated cells.

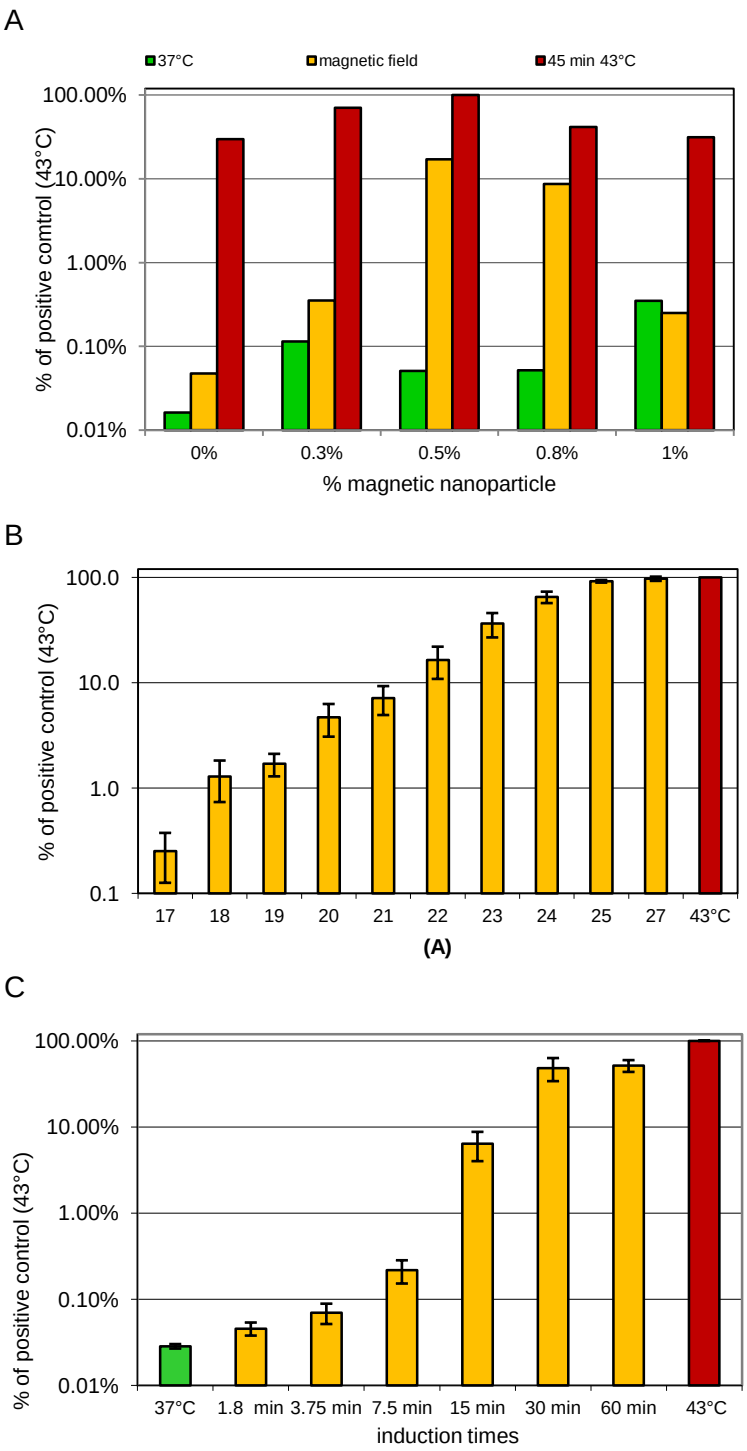

FIGURE 5-19 ACTIVATION OF THE ARTIFICIAL HSE PROMOTER USING DIFFERENT SETTINGS. This figure was recently published in (Ortner et al. 2012) as Fig.4 and adopted here (see also appendix). A) 1x10^{5}cells were incubated without or with 0.3 %, 0.5 %, 0.8 % or 1 % magnetic nanoparticles at 37°C (green bars), for 45 min at 43°C (red bars) or for 30 min in the magnetic field generator at 60 kHz and 27A (yellow bars). After treatment cells were incubated for 6 hrs at 37°C and luciferase expression was determined in a luciferase assay. The luciferase expression levels of cells + 0.5 % nanoparticles incubated at 43°C for 45 min were set as 100 % and all other luciferase activities were normalised to these levels. The figure shows one representative experiment. B) 1x10^{5}cells were incubated with 0.5 % magnetic nanoparticles for 45 min at 43°C (red bar) or for 30 min in the magnetic field generator at 60 kHz and 17-27A (yellow bars). After treatment cells were incubated for 6 hrs at 37°C and luciferase expression was determined in a luciferase assay. The luciferase activity of cells incubated for 45 min at 43°C was set to 100 % (positive control) and all other luciferase levels were normalised to this levels. This figure shows a summary of five independent experiments. SEM n=5. C) 1x10^{5}cells were incubated with 0.5 % magnetic nanoparticles at 37°C (green bars), for 45 min at 43°C (red bars) or for 1.8 -60 min in the magnetic field generator at 60 kHz and 23 A (yellow bars). After treatment cells were incubated for 6 hrs at 37°C and luciferase expression was determined in a luciferase assay. The luciferase activity of cells incubated for 45 min at 43°C was set to 100 % (positive control) and all other luciferase levels were normalised to this levels. This figure shows a summary of five independent experiments. SEM n=5.

When cells were incubated in the magnetic field generator at 60 kHz and 27 A without nanoparticles they showed no increase in luciferase expression (Figure 5-19 A, green bars) and also the inducibility of the heat shock promoter was verified with heat treatment at 43 °C for all tested nanoparticles (Figure 5-19 A, red bars). For heat treatment an incubation time of 45 min at 43°C was used as temperature kinetics of the incubator showed that it takes ~15 min to establish 43°C within the reaction tube. Nanoparticle concentrations of 0.3 – 1 % were used for induction of luciferase expression in the magnetic field at 60 kHz and 27 A. When cells were induced with different amounts of nanoparticles luciferase expression showed peak levels at 0.5 % nanoparticles and decreased with higher nanoparticle concentrations of 0.8 % and 1 % but also with lower nanoparticle concentrations of 0.3 % (Figure 5-19 A, yellow bars). Higher amounts of nanoparticles seemed to generate temperatures above 43 -

44°C, which resulted in increasing cell death and therefore reduced luciferase expression. Lower amounts were not able to generate enough heat for induction of the promoter. As 0.5 % magnetic nanoparticles showed best induction levels at 60 kHz and 27 A, these settings were used for the following experiment, inducing the stable cell line C5 together with magnetic nanoparticles in the alternating magnetic field. Additionally, cells were incubated in the magnetic field generator with increasing current to generate an alternating magnetic field with increasing strength. When cells were induced with 0.5 % magnetic nanoparticles at 60 kHz and increasing current (17-27 A) expression levels of luciferase also increased (Figure 5-19 B, yellow bars) up to levels similar to the positive control cells (45 min 43°C) (Figure 5-19 B, red bar). As a third parameter, the incubation time was varied when cells together with 0.5 % magnetic nanoparticles were incubated in the magnetic field generator at 60 kHz and 23 A. The lower current was used to avoid extended heat production when the incubation time was increased up to 60 min. Nevertheless, the experiment showed an incubation time dependent increase in luciferase expression (Figure 5-19 C, yellow bars) up to levels of the heat treatment control (Figure 5-19 C, red bar).

In summary, the experiments testing different parameters of alternating magnetic field induction resulted in a new possibility to regulate the expression of proteins either by varying the nanoparticle concentration, the magnetic field strength or the induction time. This regulation was shown to act over several orders of magnitude and is therefore an important tool for the accurate dosage of therapeutical substances in future medical approaches of the system.

5.8. KINETICS AFTER MAGNETIC FIELD TREATMENT

After determining the optimal conditions for the induction of the artificial HSE promoter of 0.5 % magnetic nanoparticles induced in the alternating magnetic field at 60 kHz and 27 A the kinetics of protein expression was analysed. This kinetics will give important hints how the future therapeutical substance will be expressed in the encapsulated cells. Therefore, cells were induced together with 0.5 % magnetic nanoparticles (SIGMA) for 30 min in the alternating magnetic field at 60 kHz and 27 A, aliquoted afterwards to $1x10^5$ cells in 1 ml medium and incubated for different durations at 37°C. Luciferase protein expression was determined at different time points after induction using a luciferase activity assay. As a positive control for the inducibility of the artificial promoter, the cells with or without magnetic nanoparticles were incubated for 45 min at 43°C and luciferase activity was determined 6 hrs later. To analyse, if there is any effect of the magnetic field treatment on the promoter not involving the particles, also cells without magnetic nanoparticles were induced at 60 kHz and 27A in the alternating magnetic field.

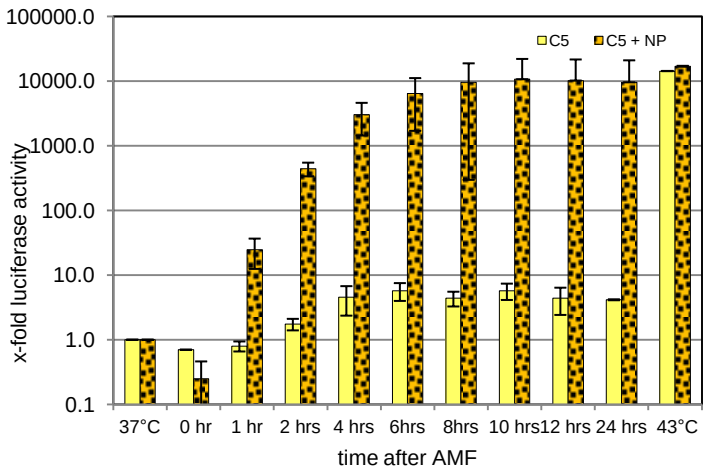

FIGURE 5-20 PROTEIN EXPRESSION KINETICS AFTER INDUCTION IN THE ALTERNATING MAGNETIC FIELD. $1x10^6$ cells of the stable cell line C5 in 200 µl medium were incubated without (light yellow bars) or with 0.5 % magnetic nanoparticles (dotted yellow bars) for 30 min at 60 kHz and 27A. Cells were resuspended with 800 µl medium and aliquoted to 100 µl. The aliquots containing $1x10^5$cell were incubated with 1 ml of medium for 0-24 hrs at 37°C and luciferase activity was determined directly after AMF treatment, 1, 2, 4, 6, 8, 10, 12 or 24 hrs afterwards. Luciferase expression was normalised to expression levels of cells incubated at 37°C either without magnetic nanoparticles or with 0.5 % nanoparticles. As a positive control for promoter induction, cells were treated for 45 min at 43°C and luciferase expression was determined 6 hrs later. This figure shows a summary of 2 independent experiments. SEM n=2, AMF: alternating magnetic field.

Analysing the expression kinetics of luciferase after treatment in the magnetic field showed slight background activation of the promoter of ~4 fold 4-12 hrs after induction, when just cells without magnetic nanoparticles were exposed to the alternating magnetic field (Figure 5-20, light yellow bars). In contrast, heat treatment resulted in a more than 1000 fold induction. When cells were induced together with 0.5 % magnetic nanoparticles in the alternating magnetic field, the expression of luciferase increased in the first 8 hrs to maximum levels of 10000 fold and high activity was determined up to 24 hrs later (Figure 5-20, yellow dotted bars). The increase up to a maximum of 8 hrs corresponds to the expression kinetics of cells incubated for 2 hrs at 43°C (Figure 5-15A) whereas the prolonged high activity was not shown for the 2 hrs induction at 43°C. Therefore it seems that induction in the magnetic field resulted in similar activation kinetics of protein expression but after reaching maximum levels, the high protein expression is somehow prolonged up to 24 hrs. To analyse, if the prolonged maximum activation of luciferase expression, is due to extended promoter activation or an effect of protein stability, the experiment was repeated and the corresponding mRNA levels of luciferase were analysed and compared to the expression of the natural Hsp72. Similar to protein levels, the mRNA kinetics is comparable to an induction of cells for 2 hrs at 43°C (Figure 5-15 B-C) for both, the artificial HSE promoter (Figure 5-21 A, dark red dotted bars) as well as the natural Hsp72 promoter (Fig. 24 B, dark blue dotted bars). When mRNA levels were analysed 6-24 hrs after induction of the cells with magnetic nanoparticles in the alternating magnetic field, the previously observed plateau of high luciferase activity could not be confirmed at mRNA levels as here the levels start to decrease again after 4 hrs back to basal levels at 24 hrs (Figure 5-21 A, dark red dotted bars). The same kinetics was observed for Hsp72 mRNA levels (Figure 5-21 B, dark blue dotted bars) although for Hsp72 the kinetics showed slower decrease of mRNA levels down to basal values compared to a fast reduction and levels below that at 37°C for the 2 hrs incubation at 43°C (Figure 5-15 C). The magnetic field treatment of cells without magnetic nanoparticles showed no increase of luciferase levels except at 8 hrs where an increase of 1.8 fold was observed (Figure 5-21 A, light red bars) and a slight increase of Hsp72 levels up to 4 fold 1-12 hrs after magnetic field induction (Figure 5-21 B, light blue bars). The analysis of mRNA kinetics after alternating magnetic field induction at 60 kHz and 27 A resulted in only background activation of the artificial promoter but slight activation of the natural promoter when cells were incubated without magnetic nanoparticles.

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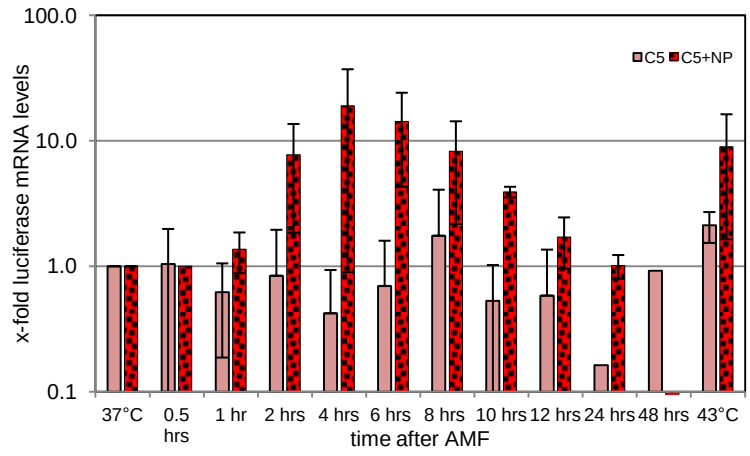

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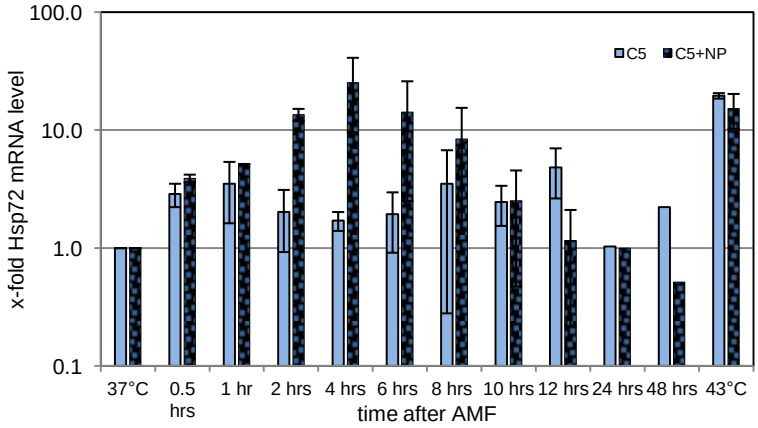

FIGURE 5-21 mRNA KINETICS AFTER INDUCTION IN THE ALTERNATING MAGNETIC FIELD. $1x10^6$ cells of the stable cell line C5 in 200 µl medium were incubated without (light bars) or with 0.5 % magnetic nanoparticles (dotted dark bars) for 30 min at 60 kHz and 27A. Cells were resuspended with 800 µl medium and aliquoted to 100 µl. The aliquots containing $1x10^5$cell were incubated with 1 ml of medium for 0-24 hrs at 37°C and RNA was isolated directly after AMF treatment, 1, 2, 4, 6, 8, 10, 12 or 24 hrs afterwards, transcribed into cDNA and A) luciferase specific or B) Hsp72 specific quantitative PCR was performed using GAPDH as an internal reference. All expression levels were normalised to GAPDH levels and to the expression levels at 37°C. As a positive control for promoter induction, cells were treated for 45 min at 43°C and luciferase expression was determined 6 hrs later. This figure shows a summary of 2 independent experiments. SEM n=2, AMF: alternating magnetic field.

In contrast to the prolonged high activity of luciferase protein after induction of the cells with magnetic nanoparticles in the alternating magnetic field, the mRNA levels of luciferase as well as Hsp72 showed a peak at 4 hrs after treatment and decreased then down to basal levels at 24 hrs afterwards. Therefore, the extended maximum protein activity is not the result of higher mRNA levels. One possible explanation would be that the protein is somehow stabilised after magnetic field treatment and as a consequence shows high expression up to 24 hrs after the incubation.

5.9. ESTABLISHMENT OF LUCIFERASE MEASUREMENT IN CAPSULES

The concept of a heat inducible gene expression system using encapsulated cells and activation through heat generation by magnetic nanoparticles exposed to an alternating magnetic field should be established within this work. First, a stable cell line harbouring the artificial HSE promoter was generated and characterised (5.2-5.5). Next, the stable cell line together with magnetic nanoparticles was exposed to an alternating magnetic field to optimise the induction conditions (5.6-5.8) for the following proof-of-principle experiments. Another major factor of the concept was the encapsulation of cells using sodium cellulose sulphate as a polymer and pDADMAC as a polylinker. All encapsulations as well as the establishment of con-encapsulation of cells with magnetic nanoparticles were performed by Cornelius Kaspar at the Department of Pathology, Institute of Virology, University of Veterinary Medicine, Vienna. The generated capsules had a size of approximately 700 µM

and contained initially 10 000 cells. The membrane of the capsules is semipermeable and allows diffusion of nutrients and O_2 into the capsules, but prevents the interaction of the encapsulated cells with cells of the immune system. In addition, the membrane to some extent mechanically shields the cells. As the transport into the capsule as well as the export of substances is based on diffusion, a standard luciferase assay using the addition of the substrate D-luciferin and the co-factors ATP and Mg^{2+} as a starting point for the detection of luciferase driven light production by the conversion of the substrate, this slow diffusion of the co-factors into the capsule might not be suitable for the detection of luciferase activity of encapsulated cells. Hence, a new protocol to determine luciferase activity of encapsulated cells had to be established. First, the assay kinetics was determined in intact capsules. For the establishment a stable cell line constitutively expressing luciferase was used to reduce possible fluctuations of luciferase expression due to heat induction. The stable cell line A6 like the stable cell line C5 harbours the artificial heat shock promoter, but accidently is not inducible by heat treatment and expresses luciferase in a constitutive manner. Sequencing of the promoter region of A6 cells showed that the promoter sequence is identical to the initially transfected construct so the constitutive expression behaviour of the cell line might be due to an integration of the promoter near a constitutive endogenous promoter. Nevertheless, this stable cell line emerged as a perfect tool to establish a luciferase assay in encapsulated cells. The first step was to analyse the effect of diffusion of D-luciferin and ATP into the capsule and the resulting delay in light production in the luciferase activity assay. Therefore, intact capsules were incubated in luciferase lysis buffer as in a standard luciferase assay and transferred to the luminometer. After starting the measurement, the substrate and the co-factors were injected automatically to the capsules in lysis buffer and after 10 seconds the detector started to measure the relative light production per second. When plotted against the total measure time, the kinetics of light production could be observed (Figure 5-22 A). 30 seconds after starting the measurement, the light production reached a plateau of maximum light production for more than 200 seconds. In the standard luciferase assay the detection time is limited to 10 seconds. Therefore, the extended production of light over long periods would result in false results as only the first 10 sec were used for the calculation of luciferase activity. In addition, the kinetics showed almost no difference in RLU/sec when 3 capsules were compared to 6 capsules (Figure 5-22 A). The measurement of 9 capsules in contrast showed higher RLU/sec. Nevertheless, the slow kinetics due to diffusion of the substrate into the capsules showed, that capsules have to be opened to determine luciferase activity properly. To compare, if luciferase activity of the released cells from the capsules would correspond to the levels of free cells, one capsule containing approximately 10000 cells was lysed according to the protocol in Ortner et al. 2012 and luciferase assay kinetics was compared to different amounts of cells of the stable cell line A6 without encapsulation. The experiment shown in Figure 5-22 B could proof that cells released from the capsule have the same assay kinetics as free cells and the RLU levels for all tested cells dropped down to almost basal levels 20 seconds after starting the measurement. In addition, the approximately 10000 encapsulated cells (Figure 5-22 B, blue) showed similar levels of RLU compared to their corresponding standard of 10000 cells (Figure 5-22 B, dark red). Reducing the amount of cells stepwise from 10000 cells down to 625 cells also showed that the detected RLU are directly proportional to the amount of luciferase producing cells. As the released cells from the capsules showed the same assay kinetics as the free cells, a standard luciferase assay could be performed for capsules were the membrane was either dissolved by special reagents (see Material and Methods of Ortner et al. 2012) or somehow disrupted to release the encapsulated cells. Lysis of the capsule membrane needs incubation of 1 hr. Therefore, a faster way to release the cells was established by disruption of the capsule membrane mechanically with a pistil.

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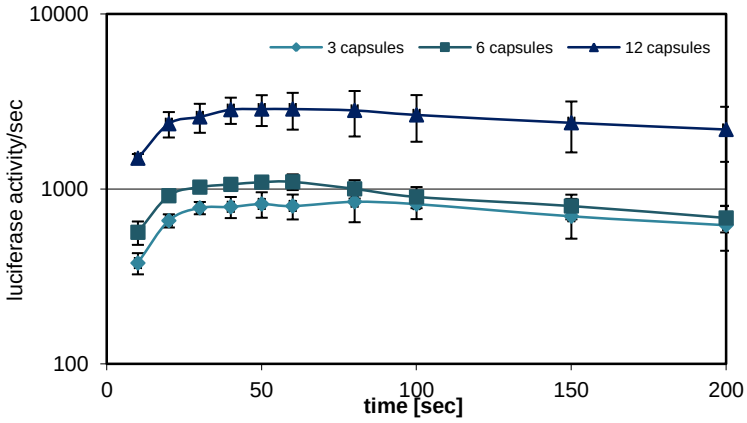

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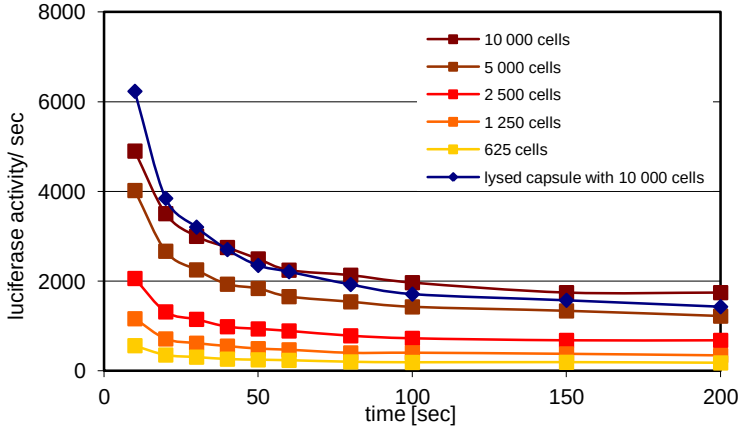

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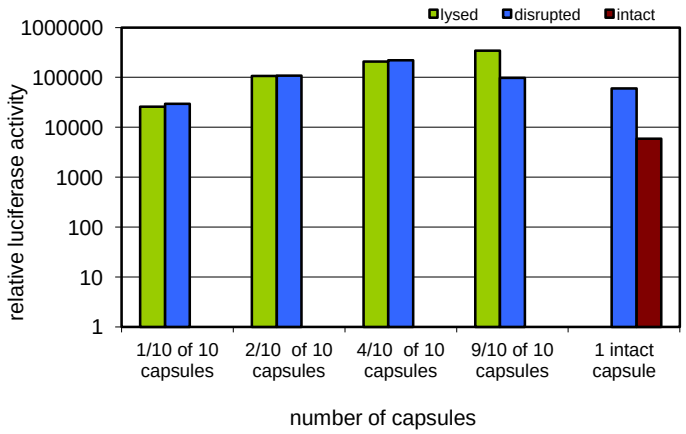

FIGURE 5-22 LUCIFERASE KINETICS IN CAPSULES. Approximately 10000 cells of a stable cell line expressing luciferase in a constitutive manner (stable cell line A6) per capsule were encapsulated by Cornelius Kaspar. **A)** 3 capsules (light blue), 6 capsules (blue) or 9 capsules (dark blue) were washed with PBS and incubated for 15 min in luciferase lyse buffer. Afterwards, the capsules were transferred to the luminometer and relative luciferase activity was determined in a time course by measuring the relative light units (RLU) for 10-200 seconds after the addition of the substrate D-luciferin and the required co-factors ATP and Mg^{2+}. Luciferase activity per second was plotted against detection time to show the kinetics of the luciferase measurement. **B)** One capsule of the stable cell line A6 was lysed (blue) (see Materials and Methods (Ortner et al. 2012)) and luciferase measurement kinetics was compared to that of 10000 cells(dark red), 5000 cells (brown), 2500 cells (red), 1250 cells (orange) or 625 cells (yellow) of the stable cell line A6. Relative luciferase activity was determined in a time course by measuring the relative light units (RLU) for 10-200 seconds after the addition of the substrate co-factors. Luciferase activity per second was plotted against detection time to show the kinetics of the luciferase measurement. **C)** 10 capsules or 1 capsule containing approximately 10000 cells of the stable cell line A6 were either lysed (see Material and Methods (Ortner, Kaspar et. al. 2012) for detailed information) (green bars) or mechanically disrupted in luciferase lysis buffer using a pistil (blue bars). Aliquots corresponding to the cell amount of 1 capsule (1/10), 2 capsules (2/10), 4 capsules (4/10) of 9 capsules (9/10) were compared to 1 disrupted capsule in a standard luciferase assay. As a control one intact capsule was measures (red bar). All figures show representative experiments.

Comparison of luciferase activity of A6 cells released by lysis (Figure 5-22 C, green bars) or after disruption of the membrane (Figure 5-22 C, blue bars) by a pistil showed no difference in luciferase activity using the standard luciferase assay except one sample, where an aliquot of 9 capsules (out of 10 capsules lysed or disrupted) showed lower luciferase activity when capsules were disrupted compared to capsule lysis. This decreased value might be due to experimental problems as all other aliquots showed the same levels of luciferase expression. When the luciferase activity of two capsules was compared to the activity of four capsules a 2 fold increase was observed compared to a 3 fold increase when one capsule or 2 capsules

were used (Figure 5-22 C). Testing one intact capsule resulted in a 10 fold decreased luciferase activity using the standard luciferase assay (Figure 5-22 C, red bar). In summary, a new protocol of luciferase activity determination using the standard luciferase assay could be established. To detect the total activity of luciferase within the capsules the membrane has to be opened to release the cells. The release is achieved in the new established protocol by mechanical disruption of the capsule membrane with a pistil, which in turn is much faster than the established lysis of the capsule membrane. The new protocol for determination of luciferase of encapsulated cells was shown to be equally efficient as the lysis of capsules and the measurement was shown to be directly dependent on the amount of luciferase producing cells.

5.10. PROOF-OF-PRINCIPLE

The experiments so far resulted in suitable conditions for heat shock activation, where the necessary temperature increase could be reached within 30 min and robust protein expression could be observed and a protocol for luciferase activity measurement was established for the capsules. Therefore, first tests with the stable cell line C5 co-encapsulated with magnetic nanoparticles could be performed. In contrast to the results of the stable cell line without encapsulation, in this experiment 1 % magnetic nanoparticles were used to ensure a proper heating even if the cells and particles are possibly more loosely arranged in the capsule and the temperature distribution within the capsule is not known. The experiments using encapsulated cells were performed together with Cornelius Kaspar.

80 capsules of the stable cell line C5 encapsulated together with 1 % magnetic nanoparticles (SIGMA) (capsules ~50 % confluent) in 200 µl DMEM were transferred to a 2 ml reaction tube and put into the middle of the coil. The temperature of the supernatant was measured with a temperature sensor and the capsules were activated via the magnetic field generator at 60 kHz 25 A, 25.5 A, 26 A and 27 A for 30 min. Immediately after activation the temperature of the supernatant was measured again and then the capsules were transferred to a 6-well plate and cultivated for 24 h in a cell culture incubator at 37°C. After 24 h the capsules were assayed for GFP expression with the UV microscope to determine if an activation of the heat shock response could be obtained with the magnetic field generator. As it was observed that some but not all capsules showed GFP expression these GFP positive capsules were collected to analyse the expression of luciferase. The capsules which showed no GFP expression were also analysed for expression of luciferase to see if in these capsules a week heat shock response could be detected. As different settings for the induction of heat shock with the magnetic field generator were used, for each setting 20 capsules of the batch of capsules which showed or did not show GFP expression were analysed with a luciferase reporter gene assay.

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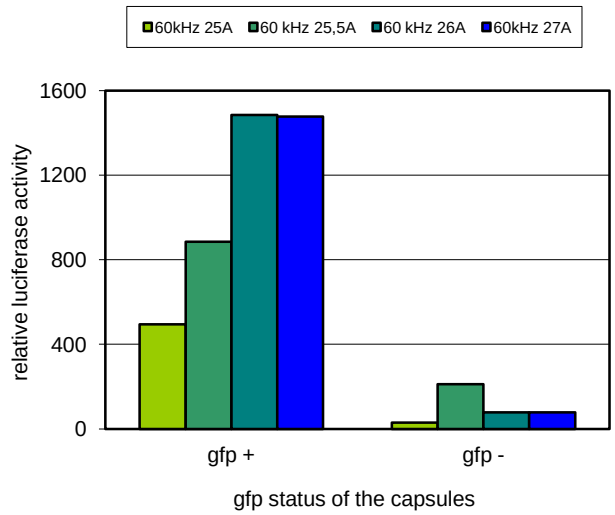

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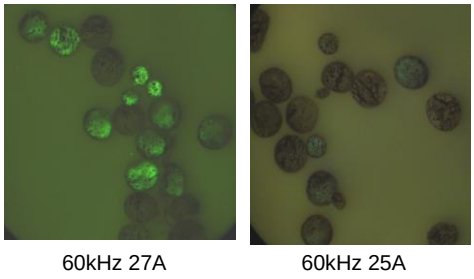

C

conditions		total caps	GFP+	% GFP +
60 kHz	27 A	143	86	60,1 %
60 kHz	26 A	85	55	64,7 %
60 kHz	25.5 A	103	46	44,7 %
60 kHz	25 A	122	12	9,8 %

FIGURE 5-23 PROOF-OF-PRINCIPLE: The stable cell line C5 was encapsulated by Cornelius Kaspar together with 1 % magnetic nanoparticles (SIGMA). Cells were grown in the capsules to 50 % confluence. 80 capsules per 200µl DMEM were treated in the magnetic field generator for 30 min at the following conditions: Frequency: 60 kHz, Current 25 A, 26 A, 25.5 A and 27 A. Capsules were afterwards cultivated again at 37°C for 24 h. GFP expression was assayed via UV microscopy and capsules showing GFP expression were separated. 20 capsules of the batch of the GFP expressing capsules and 20 capsules of the batch of capsules showing no GFP expression were crushed in 50 µl Lysis buffer with a pestle and a luciferase reporter gene assay was performed. **A)** Results of the luciferase reporter gene assay. X-fold luciferase expression was calculated relative to the expression in capsules incubated at 37°C (negative control). **B)** Fluorescence microscopy of capsules treated with magnetic field in different conditions. magnification = 10x **C)** Table of number of capsules expressing GFP within the different conditions of activation via a magnetic field.

With all four settings used for the magnetic field generator, expression of luciferase in response to heat shock activation could be observed, ranging from 500-fold for 25 A (Figure 5-23 A, light green bars) up to 1500-fold for 26-27 A (Figure 5-23 A, petrol and blue bars) and also ~ 60 % of the capsules activated at 26-27 A showed GFP expression and even with 25 A, 10 % of the capsules showed GFP expression (Figure 5-23 C). These results showed that induction of the heat shock response within the capsules could be performed with the magnetic field generator and that the resulting luciferase and GFP expression was proportional to the current input into the magnetic field generator. Interestingly also in the capsules which showed no GFP signal luciferase expression (Figure 5-23 B) up to 200-fold could be observed (Figure 5-23 A). This expression of luciferase in capsules without GFP could be the result of a basal activation of the heat shock promoter and therefore basal luciferase expression. Another explanation could be that the capsules were just slightly activated in the magnetic field, due to unknown reasons, and because of this slight activation the expression of GFP could not be detected using fluorescence microscopy. Nevertheless luciferase expression could be detected under these settings and this activation seems to be specific for the nanoparticles as the temperature of the surrounding medium of the capsules never exceeded 41°C which is not enough to induce the heat shock response. This experiment showed the first proof-of-principle for our concept of regulated gene expression in encapsulated cells by incubation in an alternating magnetic field.

6. DISCUSSION

6.1. CONCEPT

The concept of regulated gene expression in encapsulated cells provides a new approach for the treatment of many diseases. It combines different established strategies like heterologous cell therapy, heat induced gene expression, hyperthermia and encapsulation technology. In contrast to gene therapy, where genetic information is introduced directly into the host system, in cell therapy genetically engineered cells are introduced into the patient. This method avoids the problem of transfer and integration of the genetic material into host cells often resulting in non-successful production of therapeutic proteins (Hacein-Bey-Abina et al. 2003). In cell therapy the cells are pre-selected for correct integration and production of therapeutic substances. In addition, potential side effects caused by non-viral or viral delivery pathways (Raper et al. 2003) are eliminated due to the preselection. Nevertheless, cell therapy has to deal with certain problems like evoking an immune response, survival of the transplanted cells or delivery to the site of action (Nelson et al. 2002; Subramanian et al. 2002; Tatake et al. 2007; Morgan et al. 2010b). To protect heterologous cells from the immune response, encapsulation can be applied. This technology is well established for certain cell therapies (Bachoud-Levi et al. 2000; Lindvall et al. 2008), as it shields the cells by surrounding them with a semipermeable membrane. This barrier allows the transport of nutrition and proteins into and out of the capsule, but excludes the interaction with the host immune system (Chang 1964; Hauser et al. 2004; Orive et al. 2010).

A major advantage of encapsulation is the localisation of the heterologous cells in a capsule directly in, or next to the target tissue. In our approach this concentration of the cells in the capsule bears the additional advantage, that all cells can be induced by magnetic nanoparticle mediated heat generation in an equal manner. For hyperthermia treatment of tumours nanoparticles are directly injected into the tissue, which is then heated by application of an external magnetic field. Depending on their properties, like specific coating the distribution of the nanoparticles is a significant problem. This is not the case when they are encapsulated. In addition, this strategy allows the removal of the nanoparticles from the patient after the therapy. Furthermore, when magnetic nanoparticles are encapsulated together with cells, a lower amount of particles can be used as a sufficient concentration is reached within the small region of the capsules.

The application of heat generation by magnetic nanoparticles together with a heat inducible expression system can ideally be combined with encapsulation technology. In general, inducible expression systems were shown to be more efficient for therapeutic applications than a constant production of pharmacologic substances. In the treatment of most diseases the defined spatial and temporal production of the encoded proteins is essential. Examples are the production of BMP-2 to induce bone formation (Smoljanovic et al. 2009) or the expression of cytotoxic enzymes in cancer treatment (Brade et al. 2003). Without a distinct regulation these treatments would result in malformation of tissues or too high concentrations of a toxic agent. In addition, a regulated expression can be turned on at the most beneficial time point within the therapy and therefore the overall dose of the therapeutic substance can be reduced, without loss of the required effect. Heat inducible expression systems have been already established for the activation of suicide genes together with hyperthermia for cancer treatment. In this case, gene therapy and hyperthermia are used to express suicide genes

like HSVtk (herpes simplex virus thymidine kinase), which together with ganciclovir lead to enhanced killing of tumour cells (Freeman et al. 1996). The advantage of a heat inducible system is the induction from the outside by different heating techniques like focused ultrasound or magnetic nanoparticle mediated heat generation by an alternating magnetic field. In contrast, other inducible expression systems need the help of an inducer substance to start expression, like for example doxycycline in the TetR system. This inducer first has to reach the site of action in the body, resulting in a delayed activation of the system (Gossen et al. 1992). In addition, the inducers as well as the expression system itself often are of non-human origin (Gossen et al. 1995; Abruzzese et al. 2000). This might lead to stimulation of an immune response or even a toxic reaction in the patient (Wang et al. 1994; Witzgall et al. 1994a; Deuschle et al. 1995). By using the highly conserved heat shock response as basis for an expression system the above mentioned problems are avoided. Using natural heat shock promoters the induction system shows high expression levels due to elevated temperatures, but is also activated by other stress factors like depletion of nutrition or oxygen or toxic components. Therefore, primarily artificial heat shock promoters lacking most regulatory elements are used for application in gene and cell therapy (Rome et al. 2005). These modified promoters reduce the response to other stress factors like hypoxia and were shown to be highly inducible (Vekris et al. 2000; Borrelli et al. 2001; Rohmer et al. 2008). In one approach, magnetic nanoparticles were coated with plasmid DNA harbouring a heat inducible expression system based on a minimal Hsp70B´ promoter, injected into mouse tissue and an alternating magnetic field was applied. Induction in the magnetic field resulted in high reporter activity (Tang et al. 2008). Truncated heat shock promoters were also used in an adeno-virus based gene therapy approach, where high intensity ultrasound was applied to induce the promoter resulting in ~100 fold induction of marker gene expression (Liu et al. 2006). Another attempt combines a truncated Hsp promoter with a TetR induction system leading to highly enhanced induction but also a to slower expression kinetics (Yamaguchi et al. 2012). In this experimental procedure the cells are transiently transfected with the construct, which leads to a transient but efficient production of protein, although the respective cells first have to be reached within the tissue. With the introduction of the stable cell line in our system the heat inducible promoter is already integrated within the cells. Together with the co-encapsulation of cells and magnetic nanoparticles our approach combines all advantages of the individual methods. In addition problems concerning the immune response, distribution of cells and nanoparticles, as well as fluctuations in transfection efficiency are avoided. Within this thesis the novel concept of regulated gene expression in encapsulated cells could be established and verified by a first proof-of-principle experiment.

6.2. THE HEAT INDUCIBLE CELL LINE

One of the key components of our concept is the heat inducible expression system. For the experimental design of such a system natural promoters of the highly inducible heat shock protein family Hsp70 are used. In previous attempts either natural Hsp72 (Dreano et al. 1986; O'Connell-Rodwell et al. 2004) or Hsp70B promoters were used (Brade et al. 2000; Huang et al. 2000; Guilhon et al. 2003a). These promoters showed high inducibility with weak basal expression for the Hsp70B promoter. In case of the Hsp72 promoter, the basal expression was higher due to the involvement of the chaperone Hsp72 in normal cellular homeostasis. In addition, both natural promoters did not exclusively respond to heat but also to other stressful conditions like hypoxia, depletion of nutritions or exposure to toxic

components. This basal expression might therefore result in problems of such systems in gene or cell therapy when they are enhanced by stressful conditions within the patient as for example observed in tumour tissue. To minimise the background activation a reduction of natural promoter sequences down to a minimal version containing only 400 bp of the promoter including the HSEs (Vekris et al. 2000; Borrelli et al. 2001; Smith et al. 2002) or the insertion of additional HSEs (Brade et al. 2000) were established. These modified promoters resulted in even higher induction rates with lower basal activation. Nevertheless, these promoters still contain additional regulatory elements beside the HSEs and therefore at least partially respond to other inducers (Voellmy et al. 1985; Schiller et al. 1988; Borrelli et al. 2001). In contrast the promoter used in this project is not based on a natural promoter, but is a completely artificial sequence of eight idealised HSEs (Bajoghli et al. 2004). The uncoupling of the HSEs from natural promoters resulted in high expression levels in response to heat treatment similar to the minimal Hsp70B promoter. Both promoters showed around 10 fold higher induction levels than the natural Hsp70 promoter **(**Figure 5-3) in transient transfection experiments. Compared to untreated cells the basal expression was higher when the natural promoter was used than with the Hsp70B minimal promoter or the newly established artificial HSE promoter. When the artificial HSE promoter is stably integrated into HEK 293 cells, the basal expression levels were close to the detection limit of luciferase, but expression levels after heat treatment increased up to 3000 fold (Figure 5-5 and Figure 5-6). Natural heat shock promoters often are regulated in a tissue specific manner and all natural promoters showed cell type dependent differences in induction levels (Rohmer et al. 2008). To analyse this for the artificial HSE promoter, different cell lines were tested for their ability to express the reporter protein in response to heat treatment when transiently transfected. After induction a robust expression of luciferase was observed in all five cell lines tested. The basal expression levels increased when carcinoma cell lines were used instead of the HEK 293 cell line (Figure 5-4). For the application in encapsulated cells, HEK 293 cells were selected as here the basal levels without heat induction were lowest and a high inducibility of the promoter could be observed. But other cell lines also gave reasonable results which underlines the broad applicability of the artificial promoter. In addition, this promoter was also successfully applied in other species under different conditions (Bajoghli et al. 2004), further demonstrating the potential of this artificial expression system.

6.3. PROMOTER KINETICS

For the application in cell or gene therapy, the kinetics of the expression system has to be defined. In case of the most prominent version, the TetR system, the regulation of target gene expression within mouse tissue is in the range of days (Sommer et al. 2002). This slow response to induction is due to the fact that the inducer first has to diffuse to the site of action. Also other established inducer dependent systems show similar kinetics. Therefore, a system, which can be activated by an external trigger, has important advantages for medical application. Previous research of heat responsive gene expression systems showed high expression levels up to 24 hrs after heat induction and a reduction to basal levels after 48-72 hrs depending on the initial heat shock temperature (Braiden et al. 2000; Vekris et al. 2000; Smith et al. 2002; Brade et al. 2003). The here described artificial HSE promoter exhibits even tighter kinetics. The highest induction levels were observed 4-6 hrs after heat treatment and basal levels were reached within 24-48 hrs (Figure 5-14). In addition it could be demonstrated, that expression levels and maximum induction could be regulated by the

duration of heat treatment. The resulting shift of maximum expression to later time points by increased heat shock duration allows an additional level of regulation for the expression system. When mRNA kinetics of the artificial promoter construct was compared to the expression of the endogenous Hsp72 gene, maximum reporter gene mRNA levels were reached 2 hrs earlier than the endogenous Hsp72 mRNA levels. This shift of mRNA peak levels to earlier time points is most likely the result of the isolated HSEs in the artificial promoter. Within this experiment a decrease in Hsp72 levels below basal levels was observed 8-12 hrs after treatment, returning to basal levels at later time points, which was not observed for the artificial promoter. This temporal decrease of Hsp72 might be the result of a feedback regulation system (Baler et al. 1996; Balakrishnan et al. 2006; Gomez et al. 2008). The analysis of promoter kinetics was also used to examine the bidirectional design of the artificial promoter. Indeed, it could be demonstrated that both reporter genes, luciferase as well as GFP, showed the same mRNA kinetics in response to heat treatment. This bidirectional design might be used to express different components of therapeutic substances in later medical applications.

6.4. INADVERTENT ACTIVATION BY OTHER STRESS FACTORS

Although heat responsive gene expression is a fast and easy way to produce all kinds of peptides or proteins in a regulated manner, this system is underrepresented in cell and gene therapy approaches. One reason for this is the possible background activation by other stressful conditions including hypoxia, depletion of nutrition or interference with pharmacological substances. It has previously been described that natural heat shock promoter systems respond to most of these triggers (Pirkkala et al. 2001; Siddiqui et al. 2008). When exposing the artificial HSE promoter to different stress factors we could demonstrate that only two out of seven known factors (cadmium sulphate and a serine protease inhibitor) were able to induce expression, whereas all other tested stress conditions resulted in no increase of luciferase activity (but activated the natural promoter). For hypoxic conditions even extended exposure did not show any expression fromthe artificial HSE promoter (Figure 5-7 and Figure 5-8), although it was still inducible by heat afterwards. Mimicking hypoxia by cobalt chloride resulted in 2-3 fold higher expression levels but only at the highest tolerated cobalt chloride concentrations **(**Figure 5-10). In contrast the natural Hsp72 promoter showed induction levels up to 25 fold when exposed to hypoxic conditions (Figure 5-9). The discrepancy between the two promoters may be explained by the additional regulatory elements located in the natural promoters. Hypoxia was previously shown to activate specialised elements, the so-called hypoxia responsive elements (HREs) (Arany et al. 1996; Gray et al. 2005), via the key regulator hypoxia-inducible factor 1 (HIF-1). These HREs are also located in heat shock promoters (Huang et al. 2009). However it was shown that HSF-1 can be activated in response to hypoxia and acts via HSE (Benjamin et al. 1990). Additionally, HIF-1 leads to transcriptional upregulation of HSF-1 by direct binding to HRE in the Hsf1 promoter (Baird et al. 2006). Due to this cross talk between the two pathways it was not clear whether the HSE promoter would react to hypoxia.

Similar to the exposure to hypoxic conditions also the incubation of the stable cell line with zinc chloride was not able to induce the artificial promoter. Nevertheless, $Zinc^{2+}$ showed robust expression of endogenous Hsp72 (Figure 5-11), suggesting an activation pathway independent of HSE. Zn^{2+} was previously described as one of the major inducers for the metal response pathway via MRE-binding transcription factor 1 (MTF-1) (Heuchel et al. 1994; Palmiter 1994). The Hsp72 promoter as well as other heat shock promoters contain also

metal response elements (MRE) (Wu et al. 1986) beside the HSE and the metal response pathway is highly sensitive to Zn^{2+} (Murata et al. 1999). Therefore the observed Hsp72 activation can be explained by MRE induction, whereas HSEs do not seem to be activated by Zn^{2+}. In contrast to induction with zinc chloride, Cd^{2+} was able to induce both, the natural Hsp72 promoter and the artificial HSE promoter. On the protein level as well as on the mRNA level a robust concentration dependent activation of the reporter gene was observed. Similar to the situation in heat treatment the endogenous Hsp72 promoter showed peak levels of induction at lower concentrations compared to the artificial promoter (Figure 5-11). This activation of heat shock promoters was also previously observed in different mammalian cells (Wagner et al. 1999; Lee et al. 2002; Valbonesi et al. 2008).

The exposure of the heat inducible expression system to heavy metals is mainly of interest for the analysis of heat shock pathway regulation. For the proposed application in cell therapy, this response to heavy metals is less important, as here other stress factors might be more important. Among them, also pharmacological substances used for the treatment of diseases have previously been shown to interfere with the heat shock pathway (Morimoto 2008; Akerfelt et al. 2010). The anti-cancer drug geldanamycin, an established activator of the heat shock response was shown to be a specific inhibitor of Hsp90 (Whitesell et al. 1994) by blocking the interaction with client proteins. This process leads to the activation of HSF1. Exposure of cells to geldanamycin further results in phosphorylation of HSF1 and enhanced binding to the HSE (Kim et al. 1999) resulting in increased expression of heat shock proteins. Similar to these results the stable cell line showed high levels of Hsp72 mRNA (up to 100 fold induction), when exposed to increasing concentrations of geldanamycin. Interestingly, the drug failed to induce the artificial HSE promoter (Figure 5-12 A and I). To ensure that the concentrations used are sufficient for induction also higher concentrations of geldanamycin were used. However, high amounts of the drug did not induce the artificial promoter, but resulted in increased cell death (Figure 5-12 A).

In contrast, the serine proteinase inhibitor TPCK was able to induce luciferase protein expression (Figure 5-12 B). In line with the literature (Rossi et al. 1998), also endogenous Hsp72 expression was induced by TPCK (Figure 5-12 J). Interestingly, the mRNA levels of the reporter gene luciferase did not increase upon exposure to the serine protease inhibitor (Figure 5-12 J), whereas protein expression increased in a concentration dependent manner (Figure 5-12 B). Protein expression was determined 6 hrs after induction (60 fold increase), whereas mRNA levels were analysed 2 hrs after induction. These time points were chosen as standard settings after a detailed analysis of the promoter kinetics in response to heat treatment (Figure 5-14). Induction of the heat shock response by TPCK might however result in a delayed expression starting after 2 hours.

The anti-inflammatory drug carbenoxolone has also previously been shown to induce the heat shock response and the expression of heat shock proteins (Nagayama et al. 2001; Kawashima et al. 2009). This could be verified by an increase in Hsp72 mRNA levels (Figure 5-12 K). Induction of the artificial HSE promoter could be observed only at higher concentrations (1000-15000 µM) at the mRNA level (Figure 5-12 K) and also at the protein level (Figure 5-12 C). Nevertheless, this activation of the promoter did not seem to be a specific effect of the anti-inflammatory drug rather than an effect of high amounts of denaturated proteins. When carbenoxolone was analysed for its capacity to denature proteins using the degradation assay, high amounts of inactive luciferase could be observed together with an increase in cell death indicated by the decrease in both proteins, the denaturation sensitive firefly luciferase as well as the highly stable *Gaussia* luciferase (Figure 5-12 G). In addition to heat shock inducers, also one inhibitor was tested for its influence on

the artificial promoter. Quercetin, a flavonoid, was previously shown to decrease HSF1 expression levels (Nagai et al. 1995) leading to a reduced expression of heat shock proteins (Hosokawa et al. 1992). When the stable cell line was exposed to increasing concentrations of quercetin, slightly increased Hsp72 mRNA levels could be observed (2-3 fold Figure 5-12 L), which was independent of the concentration. However, this increase seems to be an unspecific effect. In addition, no activation of the artificial promoter could be observed on the mRNA level (Figure 5-12 K), as well as on the protein level (Figure 5-12 D), but also no signal reduction could be observed for exposure to quercetin.

6.5. REPEATED ACTIVATION OF THE INDUCIBLE CELLS

The general idea of this project was to establish a new approach for heat induced gene expression of therapeutical substances in encapsulated cells. The capsules containing a producer cell line would be transplanted into a patient and gene expression would then be regulated from the outside. Beside the parameters for a single release cycle, it is however equally important how the system would behave upon repeated delivery of the therapeutic substances. Since repeated release rounds also require repeated application of stress to the cells, the viability of the cells under these conditions might play a critical role. The induction of gene expression by heat has some advantages as discussed above, but this activation route also bears the potential to be harmful for the cells. The heat shock response is one of the major cellular defence mechanisms to resist environmental stress, but strong or extended stress will nevertheless kill the cells. When exposed to temperatures above 41°C the cells activate the heat shock response, but in parallel also the viability starts to decrease. Temperatures in the range of peak heat shock activation (43-44°C) at the same time resulted in a reduction of cell survival down to 94 % (43°C) or 90 % (for 44°C) (Figure 5-16). Although this decreased cell viability is still in a tolerable range it has to be considered that repeated heat treatments at 44°C might continuously reduce the cell population. To reduce the problem of cell death after heat induction, a temperature of 43°C was chosen although this does not result in the highest induction levels. Nevertheless the cell viability is high at 43°C (Figure 5-16) and the expression levels are induced more than 1000 fold (Figure 5-6 A). When the viability of the cells after repeated heat treatment was analysed, no difference in cell survival could be detected, whether the cells were exposed once or every day to heat during a period of two weeks (Figure 5-17). The survival of the cells incubated for two weeks was generally reduced by ~ 6-7 % compared to cells incubated for one week, but this was independent of the number of heat exposures. This reduction is most likely due to the artificial culture conditions used in this experiment as reduced serum concentrations of 0.5 % were necessary to cultivate cells over a period of two weeks without propagation. Nevertheless, even with these highly artificial cultivation conditions it could be shown that repeated heat treatment does not result in increased cell death and the system is still inducible to the same levels as for a single heat treatment (Figure 5-17 B). Furthermore these experiments were performed under the assumption that each release cycle in the patient would be performed under maximum expression levels. The main advantage of the presented system is however a fine tuned adoption of the expression to the needs of the patient. This in turn means reduced stress levels during induction. Activation of the system by heat is therefore suitable for repeated application.

The artificial HSE promoter and the corresponding stable cell line have been shown to fulfil all criteria necessary for a cell therapy application. The promoter is highly inducible by heat treatment with a well defined expression maximum in the range of 4-8 hrs. In contrast to

previously published attempts, the artificial HSE promoter does not respond to environmental stress conditions like hypoxia or most tested pharmacological inducers. The system only showed activation when exposed to Cd^{2+} or the serine protease inhibitor TPCK. In addition it was shown that repeated induction did not influence cell viability making this system perfectly suitable for therapeutic applications.

6.6. HEAT GENERATION BY MAGNETIC NANOPARTICLES

To induce the heat responsive gene expression system in the encapsulated cells, in this approach magnetic nanoparticles were employed. These particles are in the size range of 10-100 nm and composed of magnetic material. When magnetic nanoparticles are exposed to an alternating magnetic field they absorb energy and convert it to heat depending on the magnetic field strength, frequency and kind of particles. Particles of a size between 40-100 nm convert the applied energy directly to heat by the mechanism of hysteresis heating, when high frequencies are used (Pankhurst 2003). Similar to the size of the particles also their chemical composition regulates the amount of heat production. This regulation is due to the Curie point (T_C), a material dependent property of the nanoparticles. The T_C represents the maximal heat production which can be performed by the particle independent of the applied energy. Iron oxide nanoparticles, as used in the here described approach have a T_C of ~600°C whereas other magnetic materials like Mn-Zn-ferrite have a T_C slightly above heat shock temperature (44°C) (Meijer et al. 1995; Kotte et al. 1998). A lower Curie point has the advantage that potential overheating of the tissue or cells can be avoided. In the concept of regulated gene expression in encapsulated cells iron oxide nanoparticles were used, but heat generation could be properly regulated by different concentrations of nanoparticles and by different frequencies (Figure 5-18). However, for applications in medicine particles with a lower Curie point would increase the level of safety for the method. Another aspect for clinical application is the applied magnetic field and the frequency, as too high magnetic field strengths and frequencies would cause tissue damage in the patient. 60 kHz, applied in this project perfectly fit into the range of 50 kHz – 10 MHz used for medical applications, although the magnetic field strength (~ 36 kA/m) is relatively high. In previously published clinical trials a magnetic field strength of 10-20 kA/m showed no harmful effects on patients, but fields up to 55 kA/m are still tolerated (Ivkov et al. 2005). For the here described approach it could be shown that the application of the high magnetic field strength, together with the iron oxide nanoparticles did not cause any cytotoxic effects in the encapsulated cells (Ortner et al. 2012). The applied magnetic field strength is regulated by the settings of the magnetic field generator. Here a homemade generator consisting of commercially available pre-units was employed. The induction coil was built form brass-tubes forming an inner diameter of ~ 1.5 cm, which is sufficient for small reaction tubes. Therefore, experiments with larger vessels or even animals could not be performed. Depending on the type of coil, the power supply and the windings, the power of the magnetic field is determined. For the applied magnetic field generator there was a technical limitation to 45-48 kA/m due to the power supply and the generation of heat within the induction coil. To reduce the self-heating of the coil, the brass tubes were permanently cooled with water. In addition, a water jacket was inserted between the coil and the reaction tube to balance the temperature of the induction coil. Despite these limitations the magnetic field system was sufficient to generate the field strength needed for the proof-of-principle experiments. The clinical application of hyperthermia (see below) however demonstrated that sufficiently powerful magnetic field

generators can be built even for patient applications and that the problems of heat production in the coil can be solved even in large scale.

Another way to improve the method would be the coating of magnetic nanoparticles. As magnetic nanoparticles are co-encapsulated with cells a possible harmful effect on cell survival has to be kept in mind. In general iron oxide particles are mostly coated to decrease their aggregation tendency and to improve their biocompatibility (Soenen et al. 2010; Mahmoudi et al. 2011). In the first proof-of-principle experiments presented within this thesis, commercially available uncoated iron oxide particles were used. No severe effects of the particles on cell viability could be observed. Nevertheless, for further encapsulation experiments particles with polyethylenimine coating were used to improve biocompatibility (Ortner et al. 2012).

6.7. INDUCTION WITH MAGNETIC NANOPARTICLES

The data discussed so far showed that the established expression system is highly inducible after heat exposure with well defined kinetics. The time for activation is in the range of hours, compared to expression kinetics in the range of days for the well established TetR induction system in animal experiments (Gossen et al. 1992; Sommer et al. 2002). These differences in activation time result from the different inducers used. In the TetR system a small molecule which has to first reach the site of action activates the expression. On the other hand heat inducible systems directly respond to elevated temperatures, a condition that can be reached in the tissue within minutes. This technology is also applied in hyperthermia treatment, where tissue temperature is increased to treat diseases, but primarily cancer (Johannsen et al. 2005; Rewcastle 2006). For the necessary heat generation different strategies can be applied like radio waves (Issels et al. 2010), laser (Vogl et al. 2001), high focused ultrasound (Rewcastle 2006) or magnetic nanoparticles combined with an alternating magnetic field (Jordan et al. 2001; Johannsen et al. 2005). In 2010, the German company MagForce received the first clinical approval for their nanoparticle based hyperthermia treatment of brain tumours (Gneveckow et al. 2004).

For the system presented here magnetic nanoparticles were used to generate the required heat. Testing different frequencies and magnetic field strengths, suitable conditions for HSE promoter activation by magnetic nanoparticles could be defined for our system at 60 kHz and 36 kA/m (27 A current). As nanoparticle concentration influences the heat generation (Ortner et. al, Supplementary data S1, see also appendix), cells without encapsulation were first tested for the amount of magnetic nanoparticles needed to activate gene expression. It could be demonstrated that increasing concentrations of particles resulted in increased expression of marker proteins up to a maximum for 0.5 % nanoparticles (at the settings used for this experiment). When higher particle concentrations were used, the expression levels decreased (Figure 5-19 A) most probably due to too high temperatures. This decrease can be explained by a higher cell death rate at these temperatures, as even the increase of heat shock temperature form 43°C to 44°C resulted in reduced viability (Figure 5-16). Below its peak levels the artificial HSE promoter showed increased inducibility, when either the temperature was raised or heat treatment duration was prolonged (Figure 5-6 and Figure 5-14). Hence, regulation of expression levels was analysed by applying different current and therefore different magnetic field strength or by variation of the induction time (Figure 5-19 B and C). These experiments demonstrated the possibility to regulate the expression levels over several orders of magnitude by variation of nanoparticle concentration, magnetic field

strength or induction time. The possibility to vary the amount of produced protein is of high importance for the application in medicine as for most diseases a dose dependent therapy is needed. Most treatments used today are based on constitutive expression and even if an inducible system is used, a fine-regulation of the system cannot be facilitated. This fact highlights the potential of the system for application in cell therapy approaches.

The expression kinetics after alternating magnetic field treatment showed a similar behaviour as observed for heat treatment up to 8 hrs after induction (Figure 5-14 A). At later time points the protein levels were still at a maximum when the cells were induced in the magnetic field, whereas they dropped down to basal levels when exposed to standard heat treatment (Figure 5-20). This prolonged expression seems to be the result of a stabilisation effect of the protein as the mRNA levels did not show this behaviour. Kinetics of both, the artificial HSE promoter (Figure 5-21 A) as well as the endogenous Hsp72 promoter (Figure 5-21 B) showed a decrease in mRNA levels after 4-6 hrs, which was again similar to the kinetics observed for heat treatment (Figure 5-14 B and C). It is not clear if the effect of prolonged protein activation after magnetic field treatment is limited to the expression of the marker protein luciferase, or represents a general phenomenon. However, this has to be kept in mind for the production of therapeutic substances.

6.8. PROOF-OF-PRINCIPLE

All induction experiments discussed so far were performed with a combination of cells and magnetic nanoparticles but without encapsulation. They were however critical to define settings for the optimised induction conditions and kinetics. As these experiments resulted in conditions where robust expression was induced within the cells, the final proof-of-principle experiment could be performed with encapsulated cells. For this experiment cells were co-encapsulated with 1 % nanoparticles, which is higher that the previously used 0.5 %. This increase of particle concentration was used to ensure that enough heat is generated to induce the expression system, as in the capsule cells and nanoparticles might not be equally distributed. To avoid too high temperatures different magnetic field strengths were applied to the capsules. A first observation of the treated capsules revealed the presence of GFP expression. However, not all capsules within one setting were GFP positive, indicating variations among the capsules. The amount of capsules expressing GFP increased with higher magnetic field strength. This observation might be due to the lower heat generation capacity of the nanoparticles at reduced field strength, which was not sufficient to induce all capsules within the tested batch. Therefore only capsules expressing GFP were used for the luciferase activity assay to analyse the induction levels. With the applied settings, induction rates up to 1500 fold could be measured. As a control the activation of the promoter in capsules without a detectable GFP expression level was analysed and even here a 200 fold induction of the promoter could be determined. The performed proof-of-principle experiment therefore clearly showed that the artificial HSE promoter based expression system could be induced in cells co-encapsulated with magnetic nanoparticles when an alternating magnetic field is applied. Although around 40 % of the induced capsules showed no expression of the marker protein GFP, even in these capsules the heat inducible expression system was activated to some extent. For future therapeutical applications it would be possible to preselect the capsules depending on their expression rates and thus avoid variability among the transplanted capsules. Coexpression of GFP with the bidirectional promoter would present one simple way to achieve such a preselection. In summary the proof-of-principle for the here described new approach could be demonstrated.

6.9. OUTLOOK

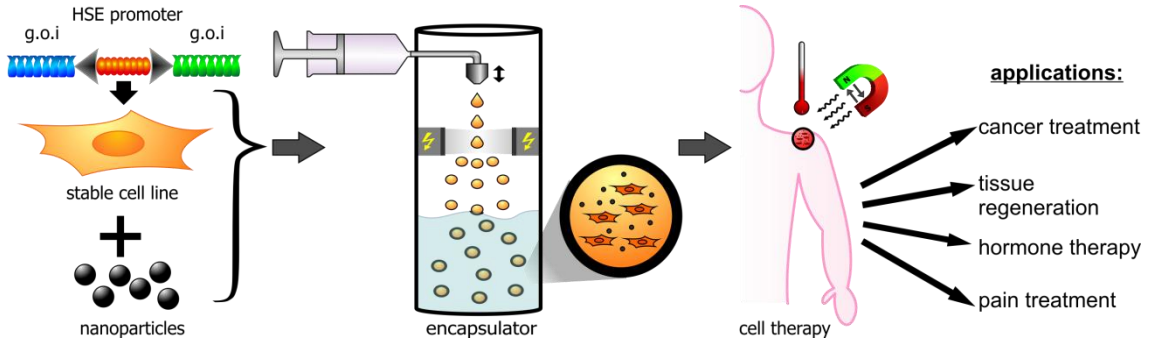

FIGURE 6-1 OUTLOOK. The externally induced expression of biologicals in encapsulated cells provides several advantages for application in medicine. A heat inducible expression system is stably introduced into a cell line which is then carefully characterised. The cells are then encapsulated together with magnetic nanoparticles to protect them from the immune response. Afterwards the capsules can be injected into the patient near to the affected tissue. When an external alternating magnetic field is applied, the nanoparticles within the capsule will produce heat which in turn activates expression of the encoded therapeutic protein. This regulation can be applied for the treatment of diseases where a dose dependent release of a therapeutic substance is critical.

The newly established method to induce the production of therapeutic substances in encapsulated cells regulated by an external trigger can be applied for the treatment of many diseases. The system is perfectly suitable for a dose regulated therapy by expressing therapeutic proteins, but also viral delivery systems. One potential application area is the field of cancer treatment. Here the main propose is to kill tumour cells by applying cytotoxic agents. In the last years several different substances were employed to act on cancer and most of them were applied via viral delivery systems. Among them are different cytokines such as TNF-α (Gossen et al. 1992) or IL-12 (Huang et al. 2000), suicide genes like HSV-tk (Barzon et al. 2009) or enzymes like iNOS (Bian et al. 2012). All these substances result in unspecific killing of the tumour cells and are therefore also harmful for normal cells. Regulating the expression of these therapeutic substances from the outside allows the adoption to the patients needs. In addition, the temporal regulation results in a more efficient cell killing as the most beneficial time point for action of the different proteins can be chosen. The treatment can be stopped once the required toxicity is reached or repeated multiple times if necessary. Inducible expression systems are already employed in cancer treatment (Gossen et al. 1992; Huang et al. 2000). By applying encapsulated cells, the previously described substances, but also viral delivery systems can be placed near to the affected tissue leading to reduced impact on the surrounding tissue.

Another area of application is regenerative medicine. Here tissues or cells are stimulated by different growth factors or morphogenic proteins to recover after severe damage. The success of these therapies is often linked to a controlled release of the stimulating proteins as overexpression might result in severe problems. One example is the regeneration of bones where BMP2 is released to initiate bone formation *in vivo*. BMP2 is already established as a protein therapy, but was shown to result in massive bone formation if the dose of the protein is too high (Smoljanovic et al. 2009). Another application for inducible expression in encapsulated cells would be the treatment of cardiovascular diseases, where VEGF is used for regeneration of vascularising tissue (Yang et al. 2010) or the regeneration after stroke by a regulated expression of EFG (Cooke et al. 2011) or glucagon-like peptide 1 (Heile et al. 2009).

The application of regulated protein production by encapsulated cells might also be beneficial when hormonal dysfunctions are treated. Here a regulation of expression in the range of hours is beneficial for the therapy and accumulation after constitutive or slow expression kinetics would result in too high amounts of the hormone. The treatment of dwarfism by expression of growth hormone in encapsulated cells has already been tried (al-Hendy et al. 1995) and the addition of a regulated expression system would substantially improve this approach. In addition, hormones are also used for the treatment of neural damage (Devesa et al. 2012). Similar to the hormone therapies also for the treatment of pain a fast responding expression system is of major importance. Within this area, a fast and fine-tuned regulation of expression is necessary to adopt the production to the needs of the patient (Milligan et al. 2012).

In the last years several approaches for the treatment of diseases based on cell or gene therapy have been established. In addition, the pharmacokinetics of therapeutic substances becomes more and more important. The here established approach of a heat inducible expression system combined with external induction and encapsulation is therefore of high relevance for the improvement of already existing therapies. With this method, high levels of proteins can be expressed by the encapsulated cell line within short time without any basal activation or problems with the host immune response. In addition the expression system can be regulated in a dose-dependent manner from the outside via magnetic field strength or the induction time. To summarise, this approach is a new way to express therapeutic substances in a defined, well regulated manner and can be controlled from the outside of the patient.

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8. APPENDIX

8.1. LIST OF FIGURES

8.2. LIST OF TABLES

8.3. ABBREVIATIONS

AAV - adenovirus-associated virus
ABD - ATP-binding domain
AD - activation domain
ADA – adenosine deaminase
ADA – adrenoleukodystrophy
ALS – amyotrophic lateral sclerosis
AMF - alternating magnetic field
Ask-1 - apoptosis signal-regulating kinase 1
BBB - blood-brain-barrier
BDNF – brain-derived neurotrophic factor
BHK – baby hamster kidney cells
BM-MSC – bone marrow mesenchymal stem cells
BMP2 – bone morphogenic protein 2
CaMKII - calcium/calmodulin-dependent kinase
CD – cytosine deaminase
CHIP - carboxyl terminus of Hsp70-interacting protein
CHO – Chinese hamster ovarian cells
CMV - Cytomegalovirus
CNTF – cilliary neurotrophic factor
CP – choroid plexus
CYP2B1 – 2B1 isoform of cytochrome P 450
DBD - DNA binding domain
DNA – deoxyribonucleic acid
DOPE - dioleoylphosphatidylethanolamine
Dox - doxycycline
ds – double-stranded
E.coli - Escherichia coli
EcR - ecdysone receptor
eEF1A - elongation factor 1a
EMA – European Medical Agency
ER - endoplasmic reticulum
ESC - embryonic stem cell
FKBP - FK506-binding protein
FRAP - FKBP-rapamycin-associated protein
FRB - FKBP rapamycin binding
FU- focused ultrasound
GAPDH - Glyceraldehyde 3-phosphate dehydrogenase
GDNF – glia-cell line derived neurotrophic factor
GFP - green fluorescent protein
HEK – human embryonic kidney
HEMA-MAA – hydroxyethyl methaacrylate-metaacrylic acid
HIF-1 - hypoxia inducible factor-1
HIP - HSC70-interacting protein
HIV – human immunodeficiency virus
HO-1 - haemoxygenase-1
HOP - Hsp-organising protein
HR - hepta repeats
HRE - hypoxia response elements
HSBP1 - heat shock factor binding protein 1
HSC – hematopoietic stem cell
HSE- heat shock element
HSF1 - heat shock factor 1
HspBP1 - heat shock protein binding protein 1
HSPs - heat shock proteins
HSR1 - heat shock RNA1
HSV – herpes simplex virus
HSV-tk - HSV-1 thymidine kinase
HSVtk- herpes simplex virus thymidine kinase
HtpG - high-temperature protein G
hTRT - human telomerase reverse transcriptase
IL-2 – interleukin 2
IL-2R – interleukin-2 receptor
IL-6 – interleukin 6
iNOS - inducible nitrogen oxide synthase
iPSC – induced pluripotent stem cells
IRES - internal ribosomal entry site
JNK - c-Jun NH2-terminal kinase
JNK - c-Jun N-terminal kinase
KRAB - Krüppel-associated box
LTR – long terminal repeats
MAP/ERK - mitogen-activated protein kinase/ extracellular signal-regulated kinase
MCL - magnetic cationic liposomes
MCS – mesenchymal stem cells
MHC - major histocompatibility
MK2 - MAPK- activating protein kinase 2
MPF/RU486 - mifepristone
MRI - magnetic resonance imaging
mTor - mammalian target of rapamycin
MTS - mitochondrial targeting sequence
MWCO – molecular weight cut-off
NBD - nucleotide binding domain
NEF - nucleotide exchange factor
NK – natural killer cells
NLS - nuclear localisation signal
NSB - nuclear stress bodies
OTC – ornithine transcarbamylase
PARP-1 - poly(ADP)-ribose polymerase 1
PBMC - peripheral blood mononuclear cells
PBS - peptide binding domain
pDADMAC – poly-diallyl-dimethyl-ammonium chloride
PDSM - phosphorylation dependent sumoylation motif
PEG - polyethylene glycol
PEI – polyethylenimine
PGK - phosphoglycerate kinase 1-gene
PKB - protein kinase B
PKC - protein kinase C
PLK1 - Polo-like kinase 1
PLL – poly-L-lysine
PMT - post-translational modification
PSS – poly styrene sulfonate
P-TEFb- positive transcription elongation factor b
PVA- polyvinylalcohol
RD - regulatory domain
RF - radiofrequency
RGD - Arg-Gly-Asp
RLU – relative light units
RNA – ribonucleic acid

ROS - reactive oxygen species
RSV - Rous sarcoma virus long terminal repeats
rTS - TetR-KRAB system
rtTA - reverse tetracycline dependent transcriptional activator
rtTetR - reverse Tet repressor
RXR - retinoid X receptor
SAR - specific adsorption rate
SBD - substrate binding domain
SCID – severe combined immunodeficiency
SCS – sodium cellulose sulphate
Ser - serine
SIN - self-inactivating
SIRT1 - sirtuin 1
ss – single stranded
Strap - Stress-responsive activator of p300
TAD - transactivation domain
TBP - TATA box binding protein
Tc- Curie point
TCR – T-cell receptors
Tet - tetracycline
TetR – tetracycline repressor
TGF –ß1 –transforming growth factor ß1
TNF – tumor necrosis factor
TPCK - tosyl phenylalanyl chloromethyl ketone
TPR - tetratricopeptide repeat
TRAP1 - tumor necrosis factor receptor-associated protein 1
TRE - Tet response element
tTA - tetracycline dependent transcriptional activator
UTR - untranslated region
VEGF – vascular endothelial growth factor
VP16 - herpes simplex virion protein 16
WPRE – woodchuck post-transcriptional regulatory element
ZFHD-1 - zinc finger homeodomain 1

8.4. CURRICULUM VITAE

Personal information	Name:	Mag. rer. nat. Viktoria Ortner
	Date of Birth:	29. Juni 1982
	Place of Birth:	Villach
	Citizenship:	Austria
Education	2007 -2012	PhD thesis at the University of Applied Sciences, Department of Applied Life Sciences, FH Campus Wien Title: Regulated gene expression in encapsulated cells
	2005 - 2007	Diploma thesis at the Institute of Medical Biochemistry, Medical University Vienna Title: Intracellular interaction partners of LRP6 and influence of lactoferrin on reelin signaling
	2001 - 2007	Master study Molecular Biology, University of Vienna
	2001	Matura (qualification for university entrance), Federal Training and Research Institute for Industrial Chemistry Focus: Biochemistry, Biotechnology and Genetic Engineering, Rosensteingasse 1170 Wien
Professional experience	2004 - 2005	research fellow Dr. Endler, Molecular Biology, General Hospital Vienna (AKH Wien)
	2006 - 2007	employee, Ecolab GmbH, Vienna

Publications:

Ortner, V., Caspar, C., Toellner, L., Halter, C., Walzer. J., Guenzburg, W., Dangerfield, J., Hohenadl, C. and Czerny, T.; Induced gene expression of encapsulated cells by magnetic field; Journal of Controlled Release, 158 (2012) 424-432

Ortner, V., Kaspar, C. and Czerny, T. (2012) Inducible systems for cell therapy and encapsulation approaches, e-book, *submitted*

Ortner, V., Ludwig, A. and Czerny, T. (2012). Efficient heat shock pathway activation during short time incubation at high temperatures; *manuscript in preparation*

Ortner, V., Eder, N., Riegel, E. and Czerny, T. (2012). The role of HSF1 in heat shock protein activation by heavy metals; *manuscript in preparation*

Conference contributions:

Kaspar, C., Ortner, V., Czerny, T., Günzburg, W.H., Kluger, C., Dangerfield, J.A., Hohenadl, C.; Thermoregulation of gene expression in encapsulated cells by magnetic field-directed, nanoparticle-mediated heat induction. **Joint Meeting 2008 of the ÖGBM, ÖGGGT, ÖGBT and ANGT, Graz, Austria, 21th – 24th September 2008**

Ortner, V., Kaspar, C., Platzer, T., Halter, C., Walzer, J., Günzburg, W.H., Dangerfield, J.A., Hohenadl, C. and Czerny, T.; Magnetic nanoparticle mediated gene expression in cell therapy. **Neujahrsempfang Vet. Med.; Vienna, Austria, 23rd January 2009**

Ortner, V., Kaspar, C., Platzer, T., Halter, C., Walzer, J., Günzburg, W.H., Dangerfield, J.A., Hohenadl, C. and Czerny, T.; Magnetic nanoparticle mediated gene expression in cell therapy. **BioNanoMed 2009 International Congress; Krems, Austria, 26 th – 27 th January 2009**

Czerny, T., Ortner, V., Kaspar, C., Halter, C., Walzer, J., Günzburg, W.H., Dangerfield, J.A., Hohenadl, C.; Regulierte Genexpression in der Zelltherapie durch Anwendung magnetischer Nanopartikel. **3. Forschungsforum der österreichischen Fachhochschulen, Villach, Österreich, 15th – 16th April 2009**

Kaspar, C., Ortner, V., Tschulenk, W., Mykhaylyk, O., Halter, C., Walzer, J., Günzburg, W.H., Dangerfield, J.A., Czerny, T. and Hohenadl C.; Magnetic nanoparticle induced gene expression in encapsulated cells. **1st Annual Meeting of the Austrian Association of Molecular Live sciences and Biotechnology (ÖGMBT); Innsbruck, Austria, 21st-23rd October 2009**

Ortner, V., Kaspar, C., Ludwig, A., Günzburg, W.H., Dangerfield, J.A., Hohenadl, C. and Czerny T.; Characterisation of an artificial heat shock promoter for cell therapy applications. **1st Annual Meeting of the Austrian Association of Molecular Live sciences and Biotechnology (ÖGMBT); Innsbruck, Austria, 21st-23rd October 2009**

Ludwig, A., Ortner, V., Platzer, T., Jung, G. and Czerny T.; Short time induction of heat shock response at high temperatures. **2nd ÖGMBT Annual Meeting 2010; Wien, Austria, 27th-29th October 2010**

Ortner, V.; Eder, N.; Czerny, T.; Role of heat shock factor in heavy metal induced stress response**. 4th ÖGMBT Annual Meeting, Graz, Austria, 17th-19th September 2012**

8.5. DANKSAGUNG

Ich möchte mich bei meinem Betreuer und Doktor-Vater Thomas bedanken. Dafür, dass er mich immer ermutigt hat, für all die Diskussionen und Projekt-Ideen und dafür, dass er immer für mich da war. Auch für die letzten Monate möchte ich mich bedanken, in denen er meine Dissertation sicherlich auswendig gelernt hat und das Verständnis für mich. Sein Ideenreichtum und sein Wissen, wie auch die menschliche Größe werden mir immer ein Vorbild sein.

An Lisi und Gerlinde: Ohne euch wären die vergangenen Jahre niemals so wunderbar und voller schöner Momente gewesen. Vielen Dank für alle wissenschaftlichen und auch nicht-wissenschaftlichen Gespräche und für all die unvergesslichen Events. Lisi, durch dich ist nicht nur Weihnachten noch schöner geworden. Gerlinde, ich werde unser gemeinsames Leben und die Kaffee-Pausen sehr vermissen. Danke euch zwei für all die Unterstützung und für eure Freundschaft.

Ich möchte mich auch bei all den anderen Personen bedanken, die unser Labor zu einer großen Familie machen. Stefan, der nicht nur die ausgefallensten Halloween-Kostüme hat sonder für mich auch eine Art kleiner Bruder geworden ist. Noreen, die eine begeisterte Bachelor-Studentin war und mit der ich gerne mein Projekt geteilt habe. Adrian und Christian, meine ersten Diplomanden, die mir ewig in Erinnerung bleiben werden. Sebastian, Carmen, Thomas, Markus und alle anderen Diplomanden und Bachelor Studenten und auch Marianne und Christoph für die nette Atmosphäre und den Zusammenhalt im Labor.

Besonderer Dank gilt auch meinen Freunden Sarah und Rene, für ihre Aufmunterungen, die vielen gemütlichen Abende und für ihre Geduld und Hilfe bei CorelDRAW und allen anderen Problemen. Ich bin sehr glücklich euch in meinem Leben zu haben!

An Adriane, die für mich das beste Geschenk was, dass mir meine Arbeit machen konnte. Danke für alle die schönen Stunden, die Unterstützung bei all meinen Vorhaben und deine Freundschaft.

Ich möchte mich auch bei meiner Außenstelle Jana in Eisenach bedanken, dass unser Kontakt auch nach mehreren Jahren getrennter Wege nie abgerissen ist und sie mich wie kein anderer per e-mail aufbauen und ermuntern kann.

Danke an meiner Familie: Monika, die für mich wie eine zweite Mutter und Freundin geworden ist und ihrer Familie, die mich bedingungslos aufgenommen hat. Agapilais, meine Schwester, die mich einfach kennt und versteht und mir immer zur Seite steht. Den Saberi´s in Regensburg, die immer für mich da sind. Danke auch an Hermann, Siegfried, Rudi und Judith und ihren Familien für die Unterstützung während der ganzen Zeit.

Mein größter Dank jedoch gilt meiner Mama. Sie hat mir durch ihren Mut, ihre Entschlossenheit und ihr großes Herz gezeigt, wie man sein Leben meistern soll. Nur durch ihren unerschütterlichen Glauben an mich, ihre Liebe und ihre Unterstützung hab ich es bis hierher geschafft.

8.6. PUBLICATION

Magnetic field-controlled gene expression in encapsulated cells; Ortner, V and Kaspar, C et al., Journal of Controlled Release 158 (2012) 424–432