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”Induction of heat shock promoters by Laser and Microwave”

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1 Introduction

1.1 Medaka fish

1.1.1 Biology and ecology of medaka

Oryzias latipes (also known as medaka) is a small egg-laying teleost which can be found all over southeast Asia. Medaka can live in fresh as well as in brackish water. Medaka is often found in rice paddies (Naruse, 2004). Its resistance to varying conditions established medaka as a model organism for development. Medaka shows a sexual dimorphism, which makes it easy to differentiate between males and females. It gives the advantage of keeping males and females in one tank, in the case that a strain is not used for a certain time. The medaka genome is known completely (Roest Crollius, 2005). Under regular daily photoperiod with more than 13 h of artificial lighting ovulation occurs about 1 h before the onset of the light period. Under normal conditions (26 °C) the fish hatch after 10 days (Iwamatsu, 2004). The development of medaka is divided into 39 stages (Iwamatsu, 2004), which are based on diagnostic features of the developing embryo. Medaka is a good model organism for the study of development because of its clear eggs.

1.1.2 Medaka as a model organism in developmental biology

Medaka fish show a lot of advantages, which made it a common model organism in developmental biology. Medaka fish are very tolerant against pH value and can tolerate high ranges in temperature. Native medaka, which live in the Tokyo area are able to survive at 0 °C in winter and 40 °C in summer (Shima, 2004). Medaka fish are very fertile and can produce progeny every day in high amounts. In contrast to zebrafish, medaka eggs stay attached to the females belly and can easily be scraped off using an inoculation loop. To mate medaka, the males and females have to be set together in one tank. Two different systems are common to produce eggs for a daily use. In the first

version, males and females are together in one tank (at an optimal ratio of one male and 4 female fish). 1h after the light cycle starts, the eggs can be collected. This system produces a huge progeny every day, but has the disadvantage, that for embryonic development the eggs have to be collected always at the same time. The other method is to separate males and females and set them together to mate. After 1h the eggs can be collected. This method has the advantage that it is rather flexible and the embryos can be collected, when they are needed. The clear eggs of medaka fish make it easy to observe organogenesis and embryo development. A high number of mutant stocks are available, which enables to identify gene functions. To generate transgenic medaka strains is easier than in most other vertebrate model organisms because of high fertility rates and its possibility to be microinjected.

1.2 The heat shock response

The heat shock response (HSR) is highly conserved and can be found in animal and plant species as well as in bacteria and fungi. Its first discovery in 1962 was made by Ritossa, who discovered chromosome puffs on polytene chromosomes in Drosophila melanogaster upon heat or chemical treatment (Ritossa, 1962). Over decades it has been shown, that the heat shock system is a highly conserved mechanism, which is found in plants, animals and fungi leading to the suggestion, that it has an ancient role as a repair mechanism. Generally the cellular heat shock response leads to a strong induction of numerous heat shock proteins (HSPs), most of them acting as chaperons that help in protein folding (Wu, 1995; Trinklein, 2004). The heat shock response is characterized by activation of heat shock factors (HSFs), which bind to heat shock elements (HSEs) that are characterized by an array of inverted repeats of the general motif nGAAn (Trinklein, 2004). HSE copies are found in the promoters of genes encoding several known heat-inducible proteins, including the *Drosophila* and human hsp70 genes (Sarge, 1993).

1.2.1 Heat shock factors

The vertebrate HSF1 and HSF2 are closer related to each other than to HSF of other eukaryotic species as fly or yeast (Wu, 1995). In human three different HSFs are known named HSF1, HSF2 and HSF4. HSF3 was exclusively found in avian cells so far (Nakai, 1993). Analysis of HSF1 knockout mice indicates the involvement of HSF1 in extraembryonic development, carcinogenesis, and circadian control (Xiao, 1999; Dai, 2007; Reinke, 2008). While HSF1 is the most important factor regarding heat shock response, HSF2 plays a role in neuronal specification and spermatogenesis (Kallio, 2002; Chang, 2006; Akerfelt, 2008). Expression of HSF4 is restricted to the brain and lung, and is required for ocular lens development and fiber cell differentiation (Nakai, 1997; Bu, 2002; Fujimoto, 2004; Min, 2004, Yamamoto, 2009). As HSF1 shows all general characteristics of the known heat shock factors (Wu, 1995; Pirkkala 2001), it is important to understand its structure and its functional domains. Heat shock response involving HSF1 is more dependent on the activation of HSF1 than on its synthesis or stability (Wu, 1995). HSF1 is present in cells in a latent state and becomes activated upon heat stress (Wu, 1995; Sarge, 1993). The activation of HSF1 is a two step process (Wu, 1995). First the HSF1 monomers oligomerize to form trimers. The second step of HSF1 activation is its binding to heat shock promoters (Wu, 1995).

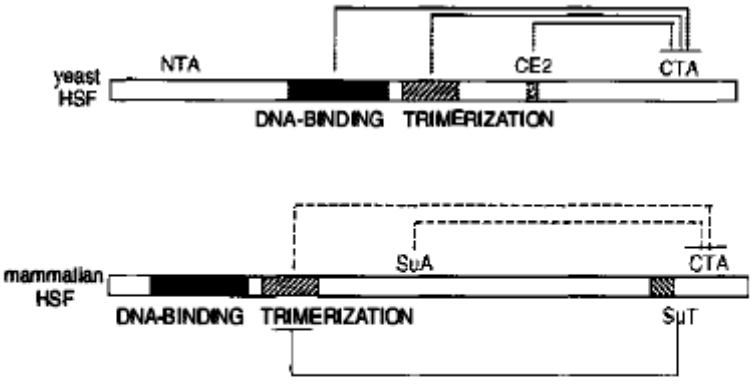


Figure 1. **Structure of yeast and mammalian HSFs.** The DNA-binding domain is marked as a solid black block in the HSF. The cross-hatched block is the trimerization domain. The N-terminal activator (NTA) and the C-terminal activator (CTA) are found at the N-terminal and the C-terminal end, respectively. (dotted) CE2 (short conserved element) is only found in yeast HSF and acts as a suppressor of the transactivation. SuT, suppressor of trimerization domain; SuA, suppressor of activation domain (Wu, 1995)

All HSFs show common structures, which are important for the activation and function. The most important structures are the following:

DNA binding domain

trimerization domain

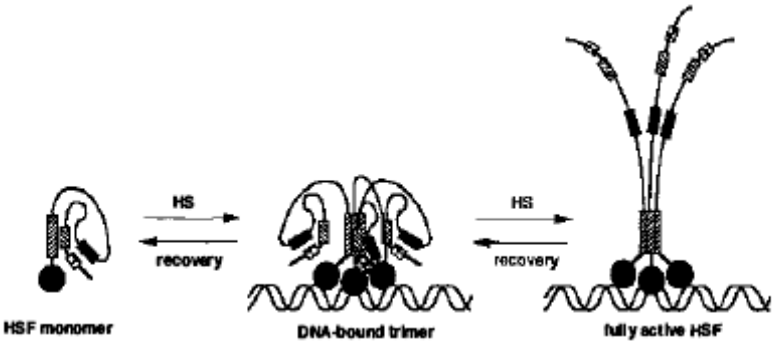
The DNA binding domain of HSFs consists of a Helix-Turn-Helix motif, which is found at the amino-terminal end of the protein (Wu, 1995). A crystal structure and two solution structures of the DNA-binding domain of HSF are known. The domain consists of 3 α-helices, which are packed against a four-stranded, anti-parallel β-sheet (Wu, 1995). This characteristics place the DNA binding domain (DBD) of HSF in the α+β class of helix-turn-helix proteins (Wu, 1995; Weber, 1987). This class of proteins was exemplified by Weber (Weber, 1987) in the bacterial catabolite activator protein (CAP). The difference between the DBD and CAP is that the HSF DBD has an α-helical bulge and a proline-induced kink that significantly distorts the second helix and by an extended turn of 5-7 residues separating the second and third helices (Wu, 1995). The third helix is known to be necessary to recognize the 5-basepair repeat found in the HSEs (Wu, 1995). Consistent with this fact, it is also known, that this helix shows the most conserved region in the whole HSR system (Wu, 1995). The trimerization domain of HSF1 is a conserved region in all HSFs (Wu, 1995). It is marked by three arrays of hydrophobic heptad repeats (HR-A/B) characteristic for helical coiled-coil structures, i.e., leucine zippers (Pirkkala, 2001; Sorger, 1989; Cohen, 1990, 1994; Landschulz, 1988, Wu, 1995). The trimerization of proteins showing a leucine zipper motif is rather unusual, as they normally form homo- or heterodimers (Pirkkala, 2001). Isoforms of HSFs can be found when alternative splicing appears. These isoforms provide another regulatory level to control gene expression (Pirkkala, 2001).

1.2.2 Monomer to trimer transition increases DNA-binding affinity

Trimerization of HSF is central to the acquisition of high-affinity binding to the HSE for higher eukaryotic HSFs (Wu, 1995). Drosophila, human, and mouse HSFs are maintained in a latent, monomeric state until the onset of heat stress,

when monomers are converted quantitatively into trimers (Wu, 1995; Baler, 1993, Sarge, 1993, Westwood, 1993; Westwood, 1991, Sistonen, 1994). HSF1 is found in unshocked cells in a latent state as monomers. These monomers bind poorly to DNA (Wu, 1995). Experiments in *Drosophila melanogaster* revealed that upon heat shock HSF1 forms trimers, which have a much higher affinity for binding of DNA (Wu, 1987; Taylor, 1991). Possible models for HSF regulation are shown in Figure 2.

A



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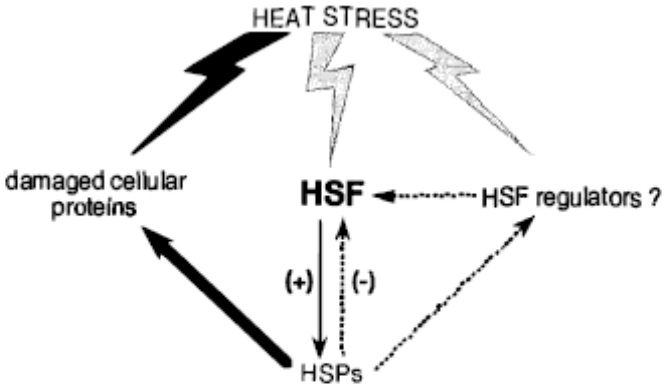

Figure 2. **Possible models for HSF regulation upon heat stress.** (A) Intra- and intermolecular coiled coil interactions of HSFs prevent trimerization under non-stress conditions. HSF monomers trimerize upon heat stress. Full activation is achieved by longer durations of heat stress. (B) The regulation of HSF due to heat stress. Darker arrows are defined heat shock pathways. So far undefined pathways to HSF and HSF regulators are marked lighter. Filled lines are defined, dotted lines undefined interactions. (Wu, 1995)

1.2.3 Suppression of trimerization

Under conditions, at which no stress is applied to the cell, the trimerization of HSFs is suppressed by a region near the carboxy terminal end of the HSF (Wu, 1995). This region contains hydrophobic heptad repeats followed by a cluster of hydrophobic residues and is called HR-C (Rabindran, 1993; Wu, 1995). The suppressor region is more conserved in animals than in plants (Pirkkala, 2001). This may be a reason for the fact, that HSF are in a constitutive trimer formation in plants and *S. cerevisiae* (Jakobsen, 1988; Jakobsen, 1991). Experiments leading to mutations in these hydrophobic arrays in *Drosophila* led to a trimerization and DNA binding of HSFs (Wu, 1995). Otherwise mutations in the less conserved heptad repeats have no functional consequences in the suppression of trimerization in yeast (Chen, 1993; Wu, 1995)).

1.2.4 Phosphorylation as a step in the heat shock response

Early findings have suggested, that phosphorylation of HSFs might be a part in the activation of HSFs (Sorger, 1987; Sorger 1988, Sarge 1993). The electrophoretic mobility of HSFs was changed due to a stress response, which could be reversed by phosphatase treatment (Sorger, 1987; Sorger 1988; Larson 1988). Later results explained at which positions amino acids of HSF1 are phosphorylated after exposure to heat stress. Further work on the subject revealed a number of serine residues, which are phosphorylated during heat stress. HSF1 activated by heat treatment was phosphorylated on Ser^{121}, Ser^{230}, Ser^{292}, Ser^{303}, Ser^{307}, Ser^{314}, Ser^{319}, Ser^{326}, Ser^{344}, Ser^{363}, Ser^{419}, and Ser^{444}. Especially the phosphorylation of Ser^{326} is interesting, as it is the only serine residue, which contributes significantly to the activation of HSF1. Phosphorylation on Ser^{326} increased rapidly during heat stress as shown by experiments using a $pSer^{326}$ phosphopeptide antibody (Guettouche, 2005). The experiments performed by Guettouche also revealed that none of the Thr or Tyr residues were found to be phosphorylated in heat shock activated HSF1. The

phosphorylation of Ser^{326} plays a critical role in the activation of HSF1. It was shown, that the phosphorylation of Ser^{326} and the trimerization of HSF1 are lying in the same time frame. The results implied that Ser^{326} is the most critical target for phosphorylation, the other residues to be found phosphorylated in heat shocked HSF1 showed a different time frame for phosphorylation compared to the trimerization of HSF1. Luciferase reporter assays indicated that phosphorylation of Ser^{326} causes the heat induced activity of HSF1 to increase two-fold. The transactivation assays in which endogenous HSP70 was used as the endpoint revealed that this enhancement of HSF1 activity translates into a fivefold increase in accumulation of HSP70 (Guettouche, 2005). It remains not completely understood, which functions the phosphorylations of the other Ser residues have. Possible explanations are that phosphorylations of these Ser residues are needed for different types of stress (chemicals as $CdCl_2$) or that they play a role in the deactivation of HSF1, when a heat shock already has been resolved (Wu, 1995; Guettouche 2005). The C terminal regulatory domain of yeast HSF1 has been shown to be important in phosphorylation of HSF upon heat shock (Hashikawa, 2004).

1.2.5 Suppression of HSF1 by Heat Shock Factor Binding protein 1

Beside the trimerization suppressor domain of HSF1 a second possibility exists to negatively affect the activation of HSF1. In 1998 a novel 76-amino-acid long protein was found, which proved to be important in the inactivation of HSF1. The protein was called Heat shock factor-binding protein 1 (HSBP1). At least five independent isolates of HSBP1 were obtained in a screen using a Madin–Darby canine kidney (MDCK) cDNA library (Satyal, 1998). The full-length canine HSBP1 cDNA was used to isolate human HSBP1 by a nucleic acid hybridization screen of a human cDNA library (Satyal, 1998). HSBP1 contains 2 hydrophobic repeats, which are important for the interaction with HSF1 (Satyal, 1998). Sequence analysis showed a highly helical protein with two tandem arrays of heptad repeat devoid of other readily recognizable structural motif(s) (Tai, 2001). HSBP1 binds to the hydrophobic heptad repeats of HSF1, when it

is in its (active) trimeric form (Satyal, 1998), but not to monomeric HFS1. HSBP1 also binds to Hsp70 and is therefore thought to be a regulator during heat stress (Satyal, 1998). In vivo experiments in *C. elegans* revealed, that an overexpression of HSBP1 decreases the ability of organisms to cope with heat stress (Satyal, 1998). Later findings revealed that HSBP1 is highly conserved in plants and animals and all homologues are relatively short proteins, ranging in length from 74 to 99 residues (Tai, 2001). The complete role of HSBP1 in the regulation of the heat shock response has not been completely understood so far. It may prevent HSF1 from accidental oligomerization (Liu, 2009).

1.2.6 Repression of HSF activation by Hsp complexes

Beside the repression through HSBP1, it was found, that Hsps can negatively regulate the activity of HSFs. At first, it was discovered by protein-protein interaction experiments, that a complex of Hsp70/Hsp40 would be a repressor of HSF1 activation (Abravaya, 1992; Baler 1992; Schlesinger, 1993). Initially, monomeric HSF was thought to be stabilized under normal conditions by interaction with HSP70/HSP40 (Shamovsky, 2008). The complex was thought to interact with denatured protein during HS and to release HSFs, which trimerizes spontaneously and acquired DNA-binding ability, leading to the production of increased amounts of HSP70 and HSP40 (Shamovsky, 2008). After sufficient amounts of these chaperones were synthesized to bind all available non-native proteins, excess HSP70 would bind to HSF trimers causing them to dissociate and revert to the inactive, monomeric state (Shamovsky, 2008; Morimoto, 1992; Shi, 1998). Hsp70 has been shown to be insufficient to repress HSF under normal conditions (Shamovsky, 2008; Rabindran, 1994). In vivo experiments revealed, that geldanamycin, a repressor of Hsp90, significantly increases the activity of HSF1 (Pirkkala, 2001; Zou, 1998). The conclusion, that Hsp90 is a member of a complex, which inhibits HSF1 activation, was later confirmed by findings in *Xenopus laevis*, where a complex of Hsp90 and HSF1 plays a key role in modulating different steps of the HSF1 activation-deactivation pathway (Pirkkala, 2001; Ali, 1998; Bharadwaj, 1999).

1.2.7 Regulation of HSF by eEF1A and HSR1

According to Shamovsky (Shamovsky, 2006) HSF may also be regulated by translongation elongation factor 1 (eEF1A) and HSR1 (heat shock RNA1), a non coding RNA, which is thought to be from bacterial origin and became a part of the eukaryotic genome by horizontal gene transfer or bacterial infections of the cell (Kim, 2010). eEF1A normally regulates the actin cytoskeleton architecture in cells (Gross, 2005; Shamovsky, 2006). It is a highly conserved protein, which plays a role in a variety of cellular processes (Negrutskii, 1998; Shamovsky, 2006). HSR1 is a non coding RNA, which appears to be highly conserved (Shamovsky, 2006). Computer models of HSR1 reveal a secondary structure that could change due to a change in temperature (Shamovsky, 2006). Together purified eEFA1 and HSR1 are able to activate HSF1 in vitro at physiological concentrations (Shamovsky, 2008). As this activation can be induced by a physiologically relevant temperature increase, it is possible, that HSR1 is the cellular thermometer, which is necessary to activate the heat shock (Shamovsky, 2008). A high amount of eEF1A can be released during HS, because of a general shutdown of protein synthesis (Panniers, 1994) and a collapse of the cytoskeleton (Welch, 1985), both mechanisms, in which eEFA1A plays a role during normal conditions. As the HS begins these mechanisms are not longer maintained and eEF1A is available for HSF1 activation. As eEF1A is now available in high amounts it can trigger the HS response via interaction with HSR1 to activate HSF. A model of this regulation is shown in Figure 3 (Shamovsky, 2006).

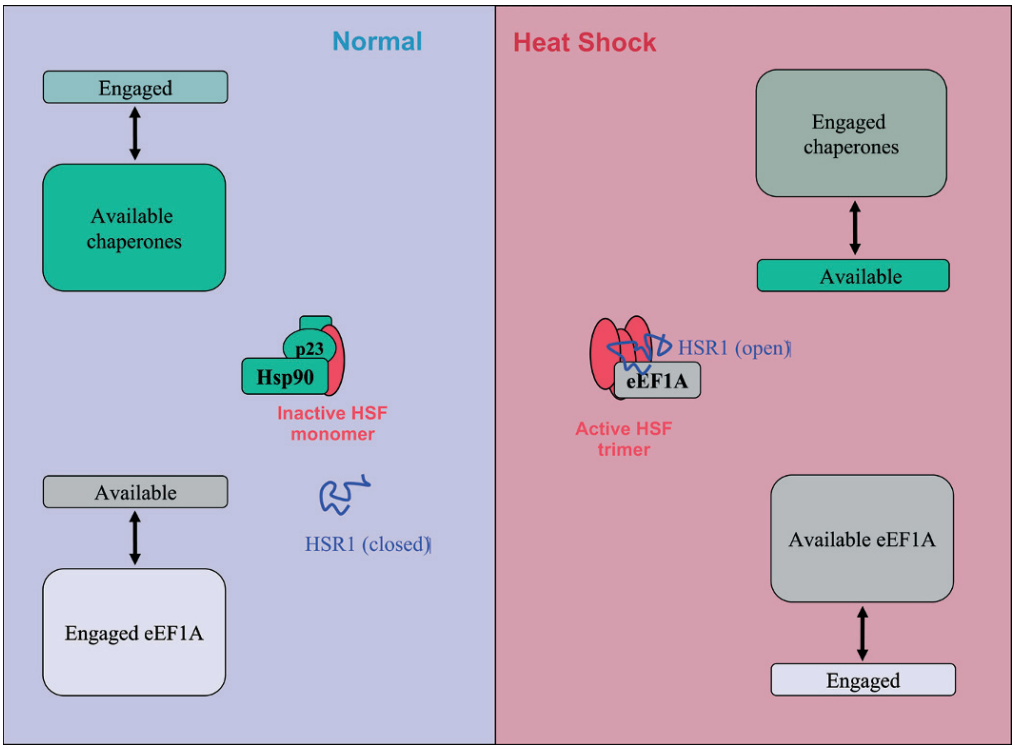

Figure 3. **Model for the activation of HSF1 under control of eEFA1 and HSR1.** Under normal (non-stressed conditions) eEF1A cannot play a role in the activation of HSF1, as it is used in translation and cytoskeleton maintenance. HSF1 is in its inactive monomer form bound by Hsp90 and co-chaperones. Also inactive is HSR1 due to the normal temperature. When a heat shock occurs HSR1 changes its conformation to the “open” form. This conformation activates HSF1, which trimerizes. As the cytoskeleton collapses during heat shock and also the translation is stopped a high amount of eEF1A is available, which now activates HSF1. As a part of a heat shock response, chaperones are necessary to repair misfolded proteins and are not longer able to stabilize the complex, which inhibits HSF1 activation. See text for details (Shamovsky, 2006)

1.2.8 Heat shock elements

The DNA binding of HSFs to DNA was discovered in 1986 by Bienz and Pelham (Bienz, 1986). HSFs bind to short sequences called heat shock elements (HSE), which are found cis-acting in target genes for HSFs (Sakurai, 2007). The HSE consists of repeats of the pentanucleotide sequence 5`-nGAAn-3`. This sequence was originally found in the promoter region of the human Hsp70. HSE are highly conserved among eukaryotes, which indicates, that the binding of HSF to HSE is highly sequence depending (Cunniff, 1991). Extensive experiments on the sequence of HSE revealed that the 3 nucleotides at the positions 2-4 are most important for binding of HSF to HSE, while the

nucleotides at the positions 1 and 5 are not as important as the core, but also can have an influence in the binding activity of HSF (Cunniff, 1993). Variations of the sequence of artificial sequences and the Hsp70 sequence revealed a 57-fold higher affinity for HSF1 binding to HSE (Cunniff, 1993). According to Cunniff and Morgan the ideal 5-basepair motif to stimulate binding of HSFs to the HSE is AGAAC (Cunniff, 1993). The number of repeats can vary, but a minimum of 3 is required for an efficient heat inducible expression (Fernandes, 1994). HSF recognizes at least three inverted repeats of the 5-basepair-motif (Pirkkala, 2001). 3 different types of HSE are known: a continuous perfect-type HSE, consisting of consecutive inverted repeats of the nGAAn unit (nTTCnnGAAnnTTCn); and a discontinuous gap-type or step-type HSE, which contains one insertion [nTTCnnGAAn(5bp)nGAAn] or two insertions [nTTCn(5bp)nTTCn(5 bp)nTTCn], respectively, between the nGAAn units (Hashikawa, 2007; Sakurai 2007). Human HSFs have been tested for their ability to bind to the different types of HSE constructs. All of the three human HSFs are able to bind to HSE with continuous inverted repeats of nGAAn (Yamamoto, 2009). In addition, human HSF4 exhibits a relatively higher affinity for discontinuous HSEs containing gaps between nGAAn units (Sakurai, 2007). These results show that the configuration of nGAAn units in the promoter is important in determining which human HSF members are involved in the regulation of the gene (Yamamoto, 2009).

1.3 Artificial promoter design

To generate an ideal artificial heat shock promoter multimerized HSEs with the idealized sequence AGAACGTTCTAGAAC (Cunniff and Morgan, 1993), alternatingly separated by 3 and 6 bp, were generated by oligonucleotide ligation (Bajoghli, 2004). A total number of eight HSEs was inserted upstream of a CMV minimal promoter. This promoter was used for a construct with two marker genes. One is the luciferase gene, which is flanked by 5V and 3V globin UTRs and the SV40 polyadenylation (pA) signal (Bajoghli, 2004). On the other

side of the construct the GFP gene was inserted with the same minimal promoter, same UTRs and pA signal. I-Sce meganuclease sites were used to ensure a high performance of insertion into the genome (Thermes, 2002). The complete construct was named gfp:HSE:luc (Bajoghli, 2004).

Figure 4. **A schematic structure of the artificial heat shock promoter.** The promoter contains 8 repeats of multimerized heat shock elements. The black arrowheads left and right to the HSEs are minimal promoters. GFP, used as a reporter gene and the gene of interest are expressed from the promoter. The arrows indicate I-SceI meganuclease sites. Abbreviations pA, SV40 polyadenylation signal; HSE, heat shock element; g.o.i., gene of interest (Bajoghli, 2004)

1.3.1 Heat shock treatment of the artificial promoter

Experiments testing the heat shock response in Medaka using the artificial promoter construct (via luciferase measurement) to different durations and temperatures performed by Bajoghli revealed an ideal heat shock temperature of 39 °C (see Figure 5A). The embryos tested in this series of experiments were heat shocked and the luciferase amount was measured 5 hours or 24 hours (for transient embryos) after the heat shock treatment. A heat shock at 39 °C showed a 630-fold-induction compared to the control group of embryos, which were kept at 28 °C. Temperatures higher than 41 °C led to a high number of dead embryos. A second series of experiments was performed to find the optimal duration for a high induction upon heat shock. The results showed that the optimal duration was 2 hours to receive a strong heat shock response (see Figure 5B).

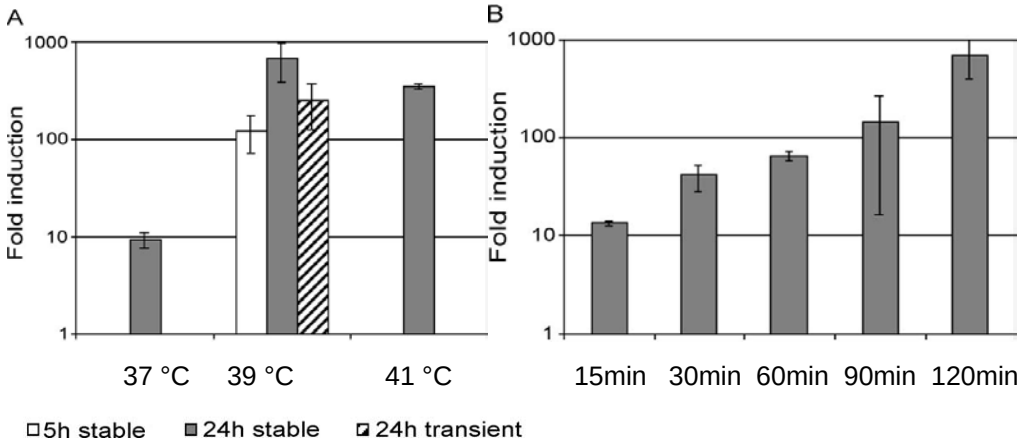

Figure 5. **Quantification of heat shock induction of the HSE promoter in medaka.** Individual embryos were heat shocked at stage 19 (Iwamatsu, 2004) and a luciferase measurement was performed. The induction was calculated by dividing induced values by basal activity values of uninduced individuals. (A) Different temperatures for a 2 hour heat treatment were tested. The luciferase activity was measured 5 (5h stable) or 24 hours (24h stable) after the heat shock. To compare the results a transient infection group was tested 24 hours after the heat shock (24 h stable).(B) Testing the optimal duration for a 39 °C heat shock, luciferase activity was measured 24 h after the heat treatment. Between two and seven individual embryos were tested (five for the uninduced control used as reference) and 17 for the transient medaka experiment (20 for the uninduced control). (Bajoghli, 2004)

Tests to compare the abilities of the artificial promoter against the natural HSP70 zebrafish promoter were performed in cell culture and led to a result that showed that the artificial promoter shows a higher luciferase activation after a heat treatment of the cells than the HSP70 promoter (Bajoghli, 2004).

1.4 Laser

Laser (acronym for **L**ight **A**mplification by **S**timulated **E**mission of **R**adiation) is a physical effect, which emits electromagnetic radiation. The nomenclature was denoted to the predecessor of the laser, the maser (Microwave Amplification by Stimulated Emission of Radiation), which was developed earlier than the first laser. The name laser was first used by Gordon Gould in 1957 (Gould, 1959). The main characteristics of laser light are coherent, low divergence and monochromatic light. The main difference between laser light and other light sources is its coherence, which means, that every photon is in the same phase as the other emitted photons by the laser. Other light sources emit incoherent light of random phase (Lipson, 1997). Laser light has a high amount of energy

(Tipler, 1994) due to its high coherence, which allows for example to weld metal with a laser. The first laser, a ruby crystal laser, was built by Theodore H. Maiman in 1960 (Maiman, 1960). Ruby is an Al_2O_3 modification with a Cr^{3+} mount of about 0.05 % (Cr^{3+} is responsible for the red colour of the crystal (Tipler, 1999). The next types of lasers, which were developed, were gas lasers, using Helium and Neon as gain medium. Generally lasers consist of the following parts: an energy source (pump), the gain medium, a material, which has properties that allow the amplification of light and an optical cavity. The optical cavity, in which the gain medium is stored, consists in the simplest construction of 2 mirrors. When the gain medium is pumped with energy (using light, discharges, electric currents or another laser [called pump laser]) (Walther, 2004), an atom or molecule of the gain medium reaches a higher energy status. By spontaneous emission, the electron goes back into its lower energy level and the energy, which is won by this phenomenon, is released by the emission of a photon. The time, at which the photon is emitted and its direction are random. If an electron in the elevated energy status is hit by a photon, the emission of a second photon is caused. The photon, which is now emitted, has the same direction, polarity, coherence and wavelength. When enough energy is pumped into the gain medium more photons are emitted, than absorbed. To reach this objective, the gain medium has to be a material, in which the electrons stay for a rather long time in the elevated energy status before they go back into the lower energy level. The light, which consists of photons that have the same direction, wavelength, coherence and polarization, is reflected between the two mirrors of the optical cavity. One of the mirrors reflects all the light, while the other only reflects about 99 %, thus leading to a high number of photons, which are reflected inside the optical cavity. Each time the photons hit an atom in a meta state, a new photon is released, leading to a further amplification of light. A part of the light exits from the optical cavity and creates the laser beam. This beam consists of photons with identical wavelength, direction, polarization and coherence. To achieve the amplification a material is needed, in which more atoms are in a high quantum status, than in a lower energy status. This status is called inversion. This is a rare effect, which

can only be found in some materials. This materials, which are used as gain medium, should also have a meta stable status, from which the atoms do not go back into the ground level too fast, to prevent loss of energy, which would make it impossible to achieve an amplification of light. Lasers can use liquid, solid or gaseous materials as gain media. Typical gas gain media are He/Ne, N_2 and CO_2. Dye lasers use an organic dye as active medium. Solid state lasers use glass, crystals or metal oxides as gain media. Each gain medium has a different wavelength of the emitted light. Lasers have been used in many different ways in technical and scientific uses, as for example cutting and welding of metal, spectroscopy, laser ablation, laser interferometry, fluorescence microscopy, bloodless surgery, kidney stone treatment, eye treatment and dentistry.

1.5 Localized and single cell heat shock promoter induction

The localized induction of heat shock promoters is a useful tool in developmental biology for a number of reasons. Not only can the overexpression of a gene be tested, it also allows creating phenotypes, in which a gene only expressed in a specific tissue. This helps to understand the working of a gene in a specific tissue. Furthermore the expression of genes in single cells allows following cell movement and the function of specific cells. Localized heat shock induction has already functionally been used in 1988 in *Drosophila* melanogaster using a hot needle to induce a heat shock response (Monsma, 1988). In contrast the use of laser technology is a much more exact way to execute local heat shock induction. Laser has been used to induce heat shock responses in zebrafish (*Danio rerio*), medaka fish (*Oryzias latipes*), *Caenorhabditis elegans*, *Arabidopsis thaliana* and in the butterfly *Bicyclus anyana*. The induction of heat shock responses by laser has been performed in *C. elegans* (Stringham, 1993), *Danio rerio* (Halloran, 2000), *Drosophila melanogaster* (Halfon et al, 2007). However the induction of single cells is a goal, which is harder to achieve, but has been proven so far in *C. elegans* (Stringham, 1993), *Danio rerio* (Halloran, 2000; Sato Maeda, 2006; Deguchi,

2009) and *Drosophila melanogaster* (Halfon, 2007). Single cell induction of heat shock promoters is limited to a number of organisms, which show big cells that can easily be targeted under a microscope.

2 Methods

2.1 Medaka farming

Medaka fish were kept in fish tanks at 27 °C in a 14 hour light cycle. The light was turned on from 8:30 to 22:30. The fish were fed three times a day. In the morning (between 8:30 and 9:00) and in the evening (between 16:00 and 18:00) the fish were fed with live 1-day-old artemia larvae. At noon (between 12:00 and 14:00) fish were fed with flakes.

2.1.1 Mating and collecting of medaka embryos

After the morning feeding the fish were set together to mate. One male fish was set in a tank containing 3 to 4 female fish. 30 minutes after mating the embryos were ready to be collected. The female fish was caught with a net and the embryos around the belly were gently scraped off with a sterile plastic inoculation loop and put into a Petri dish containing ERM Ringer solution. The embryos were gently rolled on a sheet of filter paper to separate them. Under the microscope the embryos were cleaned and dead embryos were separated and discarded. The ERM Ringer was changed and the embryos were incubated at 27 °C until used for the experiments.

2.2 Microinjection

2.2.1 Preparation of the Petri dish

Agarose (1.5%) was heated and dissolved in 1x Yamamoto’s buffer in the microwave oven. The liquid agarose was poured into a 10 cm Petri dish to a level of about 2 mm. A stamp with 6 lanes for the embryos was gently laid into

the liquid agarose. The dish with the liquid agarose and the stamp was transferred into the refrigerator and kept until the agarose had hardened. Afterwards the stamp was removed leaving 6 lanes inside, where the embryos were put. The dish was stored at 4 °C until it was used.

2.2.2 Preparation of injection needles

1mm glass capillaries (Clark Electromedical Instr., GC100-10 borosilicate glass capillaries, standard wall without filament, 1.0 mm O.D. x 0.58 mm I.D) were pulled on a Sutter P97 needle puller. The following adjustments were used: pressure 800, heat 560, pull 50, velocity 80, time 200.

2.2.3 Preparation of the injection solution and injection

Collected, cleaned and separated medaka eggs were transferred to the Petri dish containing the agarose with the 6 lanes. At 4 °C the embryos were gently pushed into the lanes to prevent them from slipping away. The solution, that had to be injected was mixed with 1x Yamamoto's buffer and pipetted into the needle using an Eppendorf Microloader tip. The needle was adjusted to the microinjector (Eppendorf Femtojet) and gently driven to the direction of the embryos. The tip of the needle was opened by gently touching it with a pair of forceps. The needle was then pushed through the chorion of the embryo into the cell of the embryo. The adjustments for the Eppendorf microinjector were about 80-100 hPa holding pressure and 300-400 hPa injection pressure. After injection, the Petri dish was filled with 1xERM Ringer and incubated at 27 °C.

2.3 Liquid crystal injection into embryos

For the purpose of inducing heat shocks in single cells, I tried to inject liquid crystals into medaka embryos to have a control mechanism for the temperature.

The suspensions were made by weighing in the crystals and pipetting the water necessary to reach the wanted concentration into a 1.5 ml reaction tube. Then the suspensions were vortexed for 1 minute and immediately used for the experiments. As it showed the suspensions often contained bubbles. To prevent these bubbles, the suspensions were kept at underpressure. Dispersions of liquid crystals were produced in the following concentrations: 20%, 10 %, 5 %, 1 %. All of these concentrations were tested for a temperature reaction in a water bath at the temperatures, where the liquid crystals should show their change of color. The microneedles were pulled with a Sutter P97 micropuller. The glass capillaries were attached to the puller and the program was started. The following parameters were chosen for the needles: Heat=560 Pull=50 Velocity=60 Time=200. 1 µl of the suspensions were pipetted into the glass needles using an Eppendorf microloader tip. The needle was adjusted to the Eppendorf Femtojet microinjector and the plate containing the embryos put under the dissecting microscope. The needle was gently driven to the embryos and opened inside the liquid in the plate using a pair of forceps. The adjustments at the microinjector were 80 hPa holding pressure and 500 hPa injection pressure. The needle was driven into the cell of the embryo and the injection started.

2.4 Transport of embryos

Since all of the laser experiments were performed at the Institute of Biophysics at the University of Linz, Austria the embryos used for the experiments had to be prepared for the transport. Medaka embryos were collected in the morning before the experiments were performed, separated and cleaned by rolling them on a piece of Whatman paper. A 10 cm Petri dish was filled about 5 mm high with liquid agarose (5%) and a stamp for the lines for the embryos was put into the liquid agarose. After hardening of the agarose the stamp was removed, leaving 6 lanes, in which the embryos were put using a pair of forceps. The dish was filled up with ERM Ringer and sealed with 3 layers of parafilm to prevent

the ERM Ringer of leaking. Until the next day the dish was put into the incubator at 17 °C. On the next morning the dish was put into a Styrofoam box and transported to Linz, where the experiments were performed. After the laser experiments were done, the single embryos were put into 3.5 cm Petri dishes, filled with ERM Ringer, which were sealed using 3 layers of parafilm. The dishes were put into a Styrofoam box and brought back to Vienna to screen for positively induced embryos.

2.5 Transgenic lines

2.5.1 HS- Wnt1

For the experiments, where a qualitative heat shock response was tested a medaka strain bearing a HS-Wnt1 construct was used as described by Bajoghli (Bajoghli, 2007).

2.5.2 pSGH2 luc puro

For establishing of a transgenic line a construct containing gfp:HSE:luc was injected into medaka embryos in the one-cell stage at 10 ng/µl together with I-sceI meganuclease (0.5 U/µl). For the screening process 14-day-old larvae were heat treated at 39 °C for 15 minutes. 24 hours after the heat treatment the embryos were screened for GFP expression under UV light. Of the F0 generation 1 embryo showed GFP expression. The embryo was raised till maturity and crossed with a WT medaka fish. The F1 generation was also raised to maturity and crossed with WT fish. The F2 embryos were used for the experiments on the luciferase expression.

2.6 Laser techniques

2.6.1 Agarose mounting heat shock

First it had to be tested, if the mounting of embryos would induce a heat shock, which would later possibly be identified as a heat shock due to the laser treatment. HS-Wnt1 embryos were used for the tests. The embryos were collected, cleaned and separated. Embryos were incubated at 27 °C in the incubator until the embryos reached stage 14 (Iwamatsu, 2004). 3% agarose was heated in 1.5 ml reaction tubes in a heating block at 37 °C until the agarose was liquid. Using a plastic pipette with a broad tip, a drop of agarose was taken up and immediately used to take up one of the embryos. The drop of agarose containing the embryo was afterwards poured into a 3 cm Petri dish. To cool down the agarose, ERM Ringer with a temperature of 27 °C was poured over the drop to harden the agarose. The immobilized embryo was incubated for 24 hours at 27 °C in the incubator. After incubation the embryos were screened for GFP expression.

2.6.2 Approach with dechorionized embryos

For the laser treatment HS-Wnt1 embryos were collected and used for the experiments. 3% agarose was heated in a microwave oven until it was liquid and then poured into a 10 cm Petri dish until the dish was half full. Afterwards a plastic stamp was inserted into the hot agarose. After cooling and hardening of the agarose the stamp was removed, leaving 6 lanes, in which the medaka embryos were placed using a pair of forceps. The dish was filled with hatching solution and closed with parafilm for the transport. For the first kind of approach using the laser the embryos had to be dechorionized. The chorion of the Medaka embryo consists of 2 layers. The inner layer is hard and inflexible, while the outer layer is soft. The hard inner layer can be dissolved using hatching

enzyme. 30 to 40 embryos were put into a well of a specially prepared 6-well-plate.

2.6.2.1 Preparation of the dechorionization plate

A specially prepared 6-well-plate was used for the dechorionization of embryos. The walls of the wells were covered with 3% agarose dissolved in ERM Ringer. After the covering had hardened, the well was filled about half with liquid agarose. The cap of a 1.5 ml reaction tube was inserted into the liquid agarose until the agarose had hardened completely. The cap was removed and the well was filled with ERM Ringer to prevent the agarose from drying out.

2.6.2.2 Dechorionization

30 embryos were put into a well of the above mentioned specially prepared 6-well-plate. The liquid covering the embryos was sucked off with a pipette and the embryos were arranged so that no embryo was lying on any other. Afterwards 100 µl of freshly thawed hatching enzyme was put into the well. The embryos all had to be covered completely with the hatching enzyme solution. A small piece of filter paper was put into the well to prevent the evaporation of the enzyme. The well was incubated at 27 °C. After 30 minutes the embryos were observed under a dissecting microscope to screen, if the enzyme had already begun to work. The start of the reaction was seen, when the inner layer of the chorion was slightly digested. This was seen, when lunar like craters were found in the chorion. Every 15 minutes after the first observation the embryos were controlled for the digestion of the chorion. When the whole inner layer of the chorion was dissolved, the reaction was stopped by diluting the enzyme. About 5 ml of ERM Ringer were gently pipetted into the well. The soft layer, which was still covering the embryos, was removed with a pair of sharp forceps. The dechorionized embryos were very sensible and were only touched by a plastic pipette. The embryos had to be immobilized for the laser treatment using

agarose. Agarose was heated until melting in a microwave oven and aliquoted into 1 ml aliquots in 1.5 ml plastic reaction tubes. The tubes were put into a preheated heating block at 37 °C. A single embryo was sucked into a plastic pipette with as little liquid as possible and then released into the liquid agarose. Immediately the embryo was sucked into the pipette again surrounded by a small amount of agarose. The drop of agarose containing the embryo was released onto a glass objective slide lying in a 3.5 cm Petri dish. Directly afterwards the dish was filled with ERM Ringer to cool the embryo and prevent a heat shock response due to the liquid agarose. The agarose hardened because of this treatment immediately. The result was an immobilized embryo in a drop of agarose, which was ready for the laser treatment of the embryo. The embryo on the slide was put out of the Petri dish and put into the bracket of the laser objective, covered with ERM Ringer. The experiments were performed with a Spectra-physics Millenia V laser with a wavelength of 532 nm. As microscope a Zeiss Axiovert 200 model was used. After focusing of the embryo the laser was turned on to heat the embryo and activate a heat shock in the embryo. After each experiment, the glass slide with the immobilized embryo was put in a new 3.5 cm Petri dish. The Petri dish was filled up with fresh ERM Ringer and closed with parafilm. 24 hours after the experiment the embryos were screened for GFP expression using an UV microscope.

2.6.3 Approach with non dechorionized embryos

Additionally to dechorionized embryos an approach was tested, in which the embryos did not have to be dechorionized. HS-Wnt1 embryos were immobilized with agarose to prevent them from moving out of the focus of the laser beam. Agarose was heated in a microwave oven until it melted. The liquid agarose was aliquoted into aliquots of 1 ml into 1.5 ml plastic reaction tubes. The reaction tubes were put into a prewarmed heating block with a temperature of 37 °C. An embryo was taken up using a plastic pipette with as little liquid as necessary. The embryo was released into the liquid agarose and immediately taken up again in a drop of liquid agarose. The drop containing the embryo was

dropped onto a glass slide for the laser. The agarose was cooled down by pouring ERM medium on the slide with the embryo. The embryo was then used for the laser treatment. After each experiment, the glass slide with the immobilized embryo was put in a new 3.5 cm Petri dish. The Petri dish was filled up with fresh ERM Ringer and closed with parafilm. 24 hours after the experiment the embryos were screened for GFP expression using an UV microscope.

2.7 Microwave experiments

To test if a microwave oven can be used to induce a heat shock in medaka embryos a standard microwave oven was connected to a laptop computer to enable to control the microwave oven in a more exact way than by the standard timer. A program for the computer was created which allowed turning on the microwave for exact time intervals (milliseconds). The program was used to turn on the microwave for a certain time interval. The time, in which the microwave was turned on and off, was determined before the experiment. The total time was called a burst. A row of the bursts was called pulse. The number of pulses during the experiment determined the total time of the experiment.

Burst: time on (t_{on})=x time off (t_{off})=y time burst (t_b)=z

Pulse: time on (t_{on})=z time off (t_{off})= a number of pulses= n

Total time of the experiment: T=n*(a+z)

HS-Wnt1 medaka embryos were collected and raised to stage 16 (Iwamatsu, 2004). Exactly 3 ml ERM Ringer were pipetted into a 3.5 Petri dish and up to 6 embryos were put into the dish. In the first approach the dish containing the embryos was put onto the center of the rotary disc of the microwave and the program chosen for the heat treatment was started. As the results showed, this

approach was not very exact leading to different results and death rates of the embryos. For the second approach a 2l beaker filled with water and ice was put into the center of the rotary disc of the microwave oven to keep the temperature in the oven constant. It was ensured, that the rotary disc function of the microwave was turned off. The Petri dish was put onto one of the 6 positions of the rotary disc, which were tested. After each experiment, the temperature in the dish was measured using an electronic thermometer (Testo). The dishes were incubated at 27 °C in an incubator. The embryos were screened for GFP expression 24 hours after the experiment using an UV microscope. The expression of GFP was distinguished into strong, medium and weak expression. A strong expression pattern showed GFP over the whole embryo. Expression was considered as medium, when a) only a part of the embryo showed GFP expression or b) the expression was clearly weaker than in a strong-expression-embryo. The embryos were considered as weak positives, when the expression of GFP was seen only in certain cells or was weak at all. To test the performance of the heat treatment, embryos which showed no GFP expression were heat treated using the standard method by heat treating them at 39 °C for 2h using a heating block. To test the survival rates upon the experiments wild type embryos were collected and raised until they reached stage 14 (Iwamatsu, 2004)

2.8 PCR cycler techniques

Normally heat treatments in *Oryzias latipes* are performed using a heat block. Because the time intervals for the experiments have to be very exact and also contained breaks between the heat treatments a PCR cycler was used to perform the heat treatment experiments. First the exact temperatures of the heating block and also the PCR cyclers were controlled using an electronic thermometer (Testo). As a second test a row of similar survival rate experiments was performed in a heating block and a PCR cycler. The results

showed that the survival rates are equal and it is eligible to compare the experiments and use the PCR cycler for the further experiments.

To test if other time durations and temperature can induce heat shocks in medaka embryos HS-Wnt1 embryos were used for the experiments. HS-Wnt1 embryos were collected and incubated until they reached stage 14 (Iwamatsu, 2004). PCR tubes were filled with 100 µl ERM Ringer, a single embryo was placed into the tube using a pair of forceps and the tube containing the ERM Ringer and the embryo was closed with a lid. The PCR tubes were then put into the PCR cycler and the PCR program was started. When the program was finished the tubes were put out of the cycler and the embryos were taken out of the tube and put into 3.5 cm Petri dishes with fresh ERM medium. Under the microscope dead embryos were removed from the batch and discarded. The embryos were then incubated at 27 °C. 24 hours after the heat treatment the embryos were screened for GFP expression using an UV microscope. The expression of GFP was distinguished into strong, medium and weak expression. A strong expression pattern showed GFP over the whole embryo. Expression was considered as medium, when a) only a part of the embryo showed GFP expression or b) the expression was clearly weaker than in a strong-expression-embryo. The embryos were considered as weak positives, when the expression of GFP was seen only in certain cells or was weak at all.

2.8.1 Survival rate experiments

To test the survival rates upon different heat treatment versions wild type embryos were collected and incubated at 27 °C until the embryos had reached stage 14 (Iwamatsu 2004). PCR tubes were filled with 100 µl ERM Ringer, a single embryo was placed into the tube using a pair of forceps and the tube containing the ERM Ringer and the embryo was closed with a lid. Each heat treatment variant was tested with the same number of embryos. A control batch of embryos was incubated at 27 °C in the incubator. The PCR tubes were then put into the PCR cycler and the PCR program was started. 3% agarose was heated in a microwave oven until it was liquid and filled into a 10 cm Petri dish.

A stamp was put into the liquid agarose and the dish was cooled at 4 °C in the refrigerator to harden the agarose. After hardening the stamp was removed leaving 6 lanes in the agarose. When the program was finished the tubes were put out of the cycler and the embryos were taken out of the tube and put into the lanes of the prepared cm Petri dish. Dead embryos were removed and discarded. 24 hours after the heat treatment the dead and live embryos of each heat treatment variant were counted and the survival rate was calculated and compared to each other.

2.8.2 Luciferase measurement

To compare the different heat treatment variations a more exact way to test the heat treatments had to be involved. A medaka strain bearing the luciferase gene under the control of the heat shock promoter was used to test the new heat treatment variations. pSGH2 luc puro medaka embryos were collected and incubated until they reached stage 12 (Iwamatsu, 2004). PCR tubes were filled with 100 µl ERM Ringer, a single embryo was placed into the tube using a pair of forceps and the tube containing the ERM Ringer and the embryo was closed with a lid. The PCR tubes were then put into the PCR cycler and the PCR program was started. When the program was finished the tubes were put out of the cycler and the embryos were taken out of the tube and put into 3.5 cm Petri dishes with fresh ERM medium. Under the microscope dead embryos were removed from the batch and discarded. The embryos were then incubated at 27 °C. 6 hours after the heat treatment the embryos had to be tested for the amount of luciferase expression. A single embryo was put into a 1.5 ml reaction tube containing 50 µl of Lysis buffer. The embryo was homogenized with a pestle and vortexed. 20 minutes after the lysis the luciferase amount was measured. The following parameters were used for the measurement:
Injection solution I: 100 µl; injection solution II: 100 µl; sequence of injections: I→II; delay between injections: 0.7 seconds; background measurement: no; delay last injection/measurement: 0.0 seconds; measurement time: 30 seconds

2.8.3 Step heat treatments

As the results of the survival rate heat treatments showed, it seemed to be a problem, that a fast increase in the temperature in a rather little time interval highly increases the death rate of the embryos. To avoid this problem a number of different heat treatments was tested, in which the temperature was increased in small steps (2 °C/min) and also with a constant rate per time. As the rate was limited by the software of the PCR cycler, the slowest rate was chosen. The heat treatments were performed as the other survival rate heat treatments. Dead embryos were discarded directly after the heat treatment. The embryos were put in 3 ml Petri dishes filled with ERM Ringer and incubated at 27 °C. 24 hours after the heat treatment the survival rate was determined.

2.9 Reagents

Liquid crystals

A liquid crystal suspension (product code R36C10W/S40100G) was ordered from Hallcrest (Hallcrest, Unit 20, Downing Road, West Meadows Industrial Estate DE21 6HA Derby , Derbyshire, UK) with the following color spectrum:

35.5 °C red
37.2 °C green
44 °C blue

Immersion oil

Zeiss Immersol 518 F

2.10 Solutions

Hatching solution

NaCl	17 mM
KCl	0.4 mM
CaCl2	027 mM
MgSO4	0.65 mM
Methylene blue	0.0001 %

PBS buffer

NaCl	137 mM
KCl	2.7 mM
Na_2HPO4	4.3 mM
KH_2PO4	1.4 mM

1.5 % Agarose

4.5 g of agarose were suspended in 300 ml distilled H_2O and boiled in a microwave oven until all of the agarose was dissolved.

Yamamoto + 1 % PEG

49.5 ml of Yamamoto were mixed with 0.5 ml PEG

ERM Ringer

NaCl	17 mM
KCl	0.4 mM
$CaCl_2 \times H_20$	1.0 mM
$MgSO_4 \times 7\ H_2O$	0.65 mM

Hatching enzyme

Wild type medaka embryos were collected and raised in hatching medium at 27 °C until the hatching glands of the embryos were clearly visible. The embryos were transferred into a 1.5 ml reaction tube, the liquid was sucked off, 0.75 µl PBS per embryo was added and the embryos were homogenized on ice with a pestle. The reaction tube was stored overnight at 4 °C. On the next day the enzyme was centrifuged for 10 min at 4 °C, 14000 rpm. The supernatant was aliquoted in 100 µl aliquots in 1.5 ml reaction tubes and kept at -20 °C until use.

Luciferase measurement

Lysis buffer

3 µl Dithiothreitol
Filled up with lysis buffer to 3 ml

Injection solution I

60 µl luciferin
60 µl Tris pH 7.5
Filled up with distilled water to 3ml

Injection solution II

75 µl ATP 1M
75 µl Tris ph7.5
45 µl $MgCl_2$ 1M

The solution was filled up with distilled water to 3ml

3 Results

3.1 Liquid crystals

In order to reliably induce a heat shock response in medaka embryos by radiation, a marker was needed to record the temperature in the embryo. For this reason a liquid crystal suspension was ordered from Hallcrest (Hallcrest, Unit 20, Downing Road, West Meadows Industrial Estate DE21 6HA Derby , Derbyshire, UK). The liquid crystals change their color in a range of temperatures which are critical for induction of the heat shock response. The optimal heat shock temperature of the used heat shock promoter is at 39 °C (Bajoghli, 2004). In case this temperature was reached a change in the color of the crystals would show it. In addition they would also indicate too high temperatures by a different color. The liquid crystals had the following color scheme:

temperature [°C]	color
35.5	red
37.2	green
44	blue

Table 1. **Color scheme of Hallcrest liquid crystals (product code R36C10W/S40100G).**

The crystals, which were delivered as a white slurry suspension, were kept in a refrigerator upon use. To test, if the color change was the same as expected, a test series was performed. As the crystals would later be injected into the embryos, small volumes were used to see the color change. Therefore glass capillaries were used filled with the crystals. As the suspension was very viscous the decision was made to dilute the suspension. Suspensions of the following concentrations were produced: 1%, 5%, 10%, and 20%. The liquid crystals for the suspension were weighed in into a 1.5 ml reaction tube and the water necessary for the different concentrations was pipetted onto the crystals.

The reaction tube was closed and the suspension was vortexed for 1 minute and immediately used for the experiments. 1 µl of the suspension was pipetted into a 1 mm glass capillary (Clark Electromedical Instr., GC100-10 borosilicate glass capillaries, standard wall without filament, 1.0 mm O.D. x 0.58 mm I.D) and the capillary was closed using a small strip of adhesive tape to prevent the suspension from leaking out of the capillary. A water bath was set to the required temperature and the capillaries were heated up in the water bath. After 5 minutes the capillaries were taken out of the water bath and put into a 10 cm Petri dish. The capillaries were put under a dissecting microscope (Nikon SMZ645) and the colour of the liquid crystals was observed. As it turned out it was not possible to see a change in the colour of the crystals, because the light, which came from the bottom was too bright. Reducing the power of the light had no effect, so another variant had to be tested. A black cardboard was used as a background and a separate light source from the top was used to see if the crystals showed the expected colours at the different temperatures. The liquid crystal suspension indeed showed the expected color differences at the different temperatures in glass capillaries. However the colors of the crystals were not clear and showed their colors only for a short time, because of the fast cooling after removal from the water bath. To have a longer time frame to score for the color changes, the next experiments included that the Petri dish was filled with water from the heated water bath and the capillaries were put into the water filled dish. This version of the test made it easier to see the different colors at different temperatures. As a side effect, when looking at the capillaries it turned out, that the crystals had a strong tendency to separate from the water and falling down onto the bottom of the capillary. When the capillaries were heated to a higher temperature (37.2 or 44 °C), the change of the color over the time due to the cooling of the water in the dish could be seen through the dissecting microscope. The colors of the liquid crystals are shown in Figures 6 and 7.

Figure 6. **Liquid crystals in a glass capillary at 35.5 °C.** A 5% liquid crystal suspension was heated to 35.5 °C in a glass capillary and observed under a dissecting microscope against a black background. The crystals show the expected red color. (20x magnification)

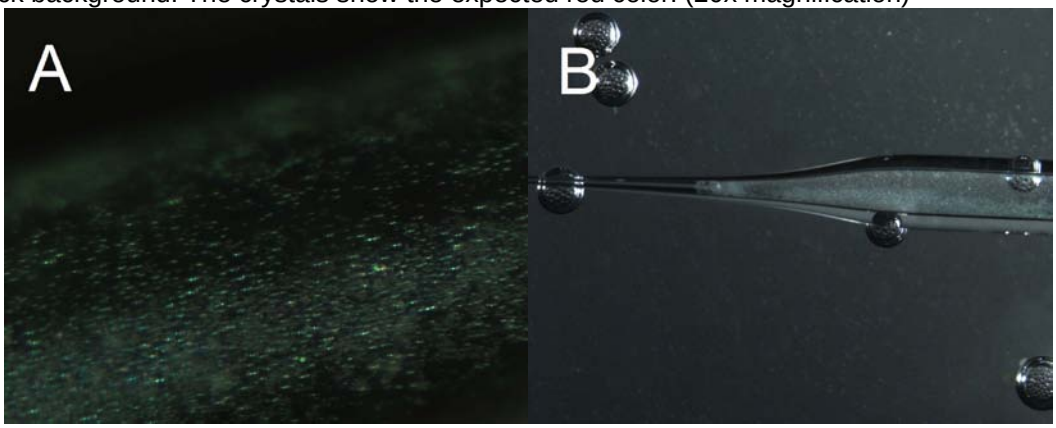

Figure 7.**Liquid crystals in glass capillaries at 37.2 and 44 °C**. A 5% liquid crystal suspension was heated to 37,2 °C (A) and 44 °C (B) in a glass capillary and observed under a dissecting microscope against a black background (magnifications: left 100x; right 10x)

3.2 Injection of liquid crystals into embryos

Despite of the results from the temperature tests, which showed, that the liquid crystals had a strong tendency to precipitate in a suspension it was tested if it was possible to inject the liquid crystals into medaka embryos. The main reason to test the injection of liquid crystals into the embryos was to find out, which temperature the embryo had reached upon laser treatment. Because not much was known, how much energy a single cell could tolerate, it would be very useful to control, if a temperature, high enough to induce a heat shock had been reached. Using the liquid crystals it might be possible to determine the temperature in different parts of the embryo.

The embryos were collected as described in Chapter 2.1.1 and transferred to the pre-cooled 4 °C prepared Petri dish immediately after collection and separation, to arrest their development. An agarose plate was made as described in Chapter 2.2.1 and suspensions of liquid crystals were produced in the following concentrations: 1%, 5%, 10%, and 20% as mentioned in Chapter 2.3. Needles with the following parameters were pulled in the needle puller: Heat=560 Pull=50 Velocity=60 Time=200.

Injection into the single cell of a one-cell embryo failed first because of blockage of the tip of the needle. Concentrations above 1% blocked the tip of the needle in all cases (n=20). Next the crystals were injected into the yolk of the embryo. This was tried because in that case a needle with a larger opening could be used, which was necessary to prevent the blocking of the needle tip. The needle was produced in the needle puller with the following adjustments: Heat=574 Pull=64 Velocity=40 Time=200.

The results showed that the yolk was too unstable for injection of the crystals. Not only was a high pressure necessary to inject a liquid crystal suspension into the yolk, which made it hard to control the injection process, the bigger needle also caused a big hole in the yolk, which often poured out and collapsed afterwards. All of the embryos (n=30) died after the injection of liquid crystals into the yolk. After these experiments it was decided to start the laser experiments without the use of liquid crystals.

3.3 Laser experiments

3.3.1 Single cell induction experiments

Inside the chorion the embryos can freely rotate, which would make it difficult to target individual cells with the laser beam. To avoid this problem the embryos had to be dechorionized and mounted in agarose. It had to be confirmed, that the mounting in the heated agarose does not induce a heat shock. The experiments were performed with HS-Wnt1 embryos, as these embryos had

GFP as a marker gene, which was expressed due to heat stress. The embryos were collected, cleaned and separated. Embryos were incubated at 27 °C in the incubator until the embryos reached stage 14 (Iwamatsu, 2004).

A specially prepared 6-well-plate was used for the dechorionization of embryos. The walls of the wells were covered with 3% agarose dissolved in ERM Ringer. After the covering had hardened, the well was filled about half with liquid agarose. The cap of a 1.5 ml reaction tube was inserted into the liquid agarose until the agarose had hardened completely. The cap was removed and the well was filled with ERM Ringer to prevent the agarose from drying out.

30 embryos were put into a well of the mentioned 6-well-plate. The liquid covering the embryos was sucked off with a pipette and the embryos were arranged so that no embryo was lying on any other. Afterwards 100 µl of freshly thawed hatching enzyme was put into the well. The embryos all had to be covered completely with the hatching enzyme solution. A small piece of filter paper was put into the well to prevent the evaporation of the buffer. The well was incubated at 27 °C. After 30 minutes the embryos were observed under a dissecting microscope to see, if the enzyme had already digested the chorion. First signs of this digestion appeared, when the inner layer of the chorion was digested and lunar like craters could be seen in the chorion under the dissecting microscope. Every 15 minutes after the first observation the embryos were controlled for the complete digestion of the chorion. When the whole inner layer of the chorion was dissolved, the reaction was stopped by diluting the enzyme. About 5 ml of ERM Ringer were gently pipetted into the well. The soft layer, which was still covering the embryos, was removed with a pair of sharp forceps. The dechorionized embryos were very sensible and were only touched by a plastic pipette. Agarose was heated until melting in a microwave oven and aliquoted into 1 ml aliquots in 1.5 ml plastic reaction tubes. The tubes were put into a preheated heating block at 37 °C. A single embryo was sucked into a plastic pipette with as little liquid as possible and then released into the liquid agarose. Immediately the embryo was then sucked again into the pipette surrounded by a small amount of agarose. The drop of agarose containing the embryo was released into a 3.5 cm Petri dish. Directly afterwards the dish was

filled with ERM Ringer to cool the embryo and prevent a heat shock due to the liquid agarose. The agarose hardened because of this treatment immediately.
The immobilized embryo was incubated for 24 hours at 27 °C in the incubator. After incubation the embryos were screened for GFP expression using a Nikon Diaphot 300 microscope under UV light. None of the embryos showed expression of GFP (total number n=98). For each test series a control group of HS-Wnt1 embryos was heat treated for 2h at 39 °C using a heating block. The embryos of the control group showed expression of GFP, indicating, that the agarose treatment did not induce a heat shock response. An embryo mounted in agarose is shown in Figure 8. Therefore the agarose mounting method could be used to immobilize the embryos for the laser treatment.

	n	GFP expression	Rate of heat shock induction
Agarose mounting	98	0	0 %
Control group	90	90	100 %

Table 2. **Summarized results for agarose mounting.** HS-Wnt1 embryos were dechorionized and mounted in agarose. A control group was heat treated for 2 hours at 39 °C. Expression of GFP was controlled 24 hours after treatment.

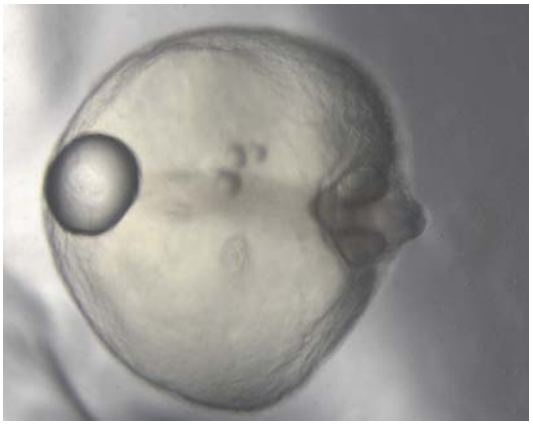

Figure 8. **HS-Wnt1 embryo mounted in agarose.** A HS-Wnt1 embryo was dechorionized and immobilized in 3% agarose. (Magnification 50x)

All of the laser experiments were performed at the Institute for by Biophysics of the University of Linz, Austria. In the first laser experiments, dechorionized transgenic embryos were used to induce a heat shock response in single cells. Dechorionized embryos were immobilized using liquid agarose on a glass slide, which was attached to the bracket of the objective of the Zeiss Axiovert 200

microscope. A Millenia V laser by Spectra-Physics with a wavelength of 532 nm was used for the experiments. Using the monitor a single cell of the embryo was chosen for the heat treatment. After aiming at a single cell, the laser was turned on for a certain duration to heat the cell and induce a heat shock. Because of the little knowledge on the subject, how much energy would be necessary to induce a heat shock, several different laser power adjustments and durations were tested. All of them failed to induce a heat shock in a single cell. The different adjustments, which were tested, are shown in Table 3.

n	power [mw]	duration	lens use	positives
1	1000	40 min	n	0
1	1000	45 min	n	0
1	1000	35 min	n	0
2	220	50 min	n	0
1	220	60 min	n	0
1	330	30 min	n	0
1	330	10 min	n	0
1	250	60 min	n	0
2	400	30 min	n	0
2	200	30 min	n	0
1	330	45 min	y	0
1	400	15 min	y	0

Table 3. **Laser adjustments and results for heat treatment of dechorionized embryos.** Transgenic embryos were dechorionized, immobilized in 3 % agarose and treated with a 532nm laser beam. Expression of GFP was inspected 24 hours after the experiment. (Lens use: a 10x magnification lens was used to aim at the embryo)

Despite of the agarose mounting of the embryos, during the experiments the embryos were often able to move inside the agarose, which prevented the laser ray to focus on the same cell during the whole experiment. But also completely immobilized embryos, which had previously shown, not to be able to rotate inside the agarose, which had kept the same position inside the agarose before and after the experiment failed to be induced by the laser. After the experiment the glass slide containing the mounted embryo was transferred into a 3.5 cm Petri dish, which was filled with ERM Ringer. 24 hours after the experiment, the

embryo was screened for GFP expression. None of the dechorionized, agarose mounted embryos showed expression after 24 hours.

Also a number of non-dechorionized embryos were laser treated in this way, just to see if a heat shock could be induced in a part of the embryo. These experiments were performed in parallel to the experiments aiming for a heat shock in single cells. The embryos were not dechorionized and also not mounted in agarose, because this mounting cannot prevent an embryo from moving inside the chorion. A single embryo was transferred into the sample holder of the microscope, filled with ERM Ringer. The embryos treated this way were arranged, so that the laser beam would hit the embryo. In some of these experiments a 100x objective was used. A drop of Zeiss Immersol 518F immersion oil was placed onto the outer lens of the objective, which was then driven to the glass slide, on which the embryo was mounted. The setting used for the experiments is shown in Figure 9.

Figure 9. **Laser device and microscope used for the treatment of dechorionized embryos**

After focusing a single cell the laser treatment was started. The laser conditions, which were tested for this purpose, are shown in Table 4.

n	power [mw]	duration	objective use	positives
1	55 mW	1 min	y	0
1	55 mW	2 min	y	0
2	55 mW	30 sec	y	0
2	27,5 mW	1 min	y	0
1	700 mW	15 min	n	0
1	330 mW	50 min	n	1

Table 4. **Laser adjustments and results for non-dechorionized embryos.** Transgenic embryos were treated with a 532nm laser beam. Expression of GFP was inspected 24 hours after the experiment. (Lens use: a 10x magnification lens was used to aim at the embryo; objective use: the 100x magnification objective was installed upstream of the embryo and the laser beam went through the objective to focus the embryo)

In these experiments one embryo showed expression of GFP, which implies that a heat shock had happened. The positive embryo was found after looking for induction of a single cell in the embryo. The positive embryo was obtained after a 50 minute laser treatment with a power of 330 mW. The GFP expression revealed, that the heat shock response was not induced in all cells of the embryo, indicating, that inside the embryo a temperature difference existed. The embryo showed a strong GFP expression in the head region, but nearly no GFP was found in the tail region (see Figure 10 A-D). The embryo showed also severe defects leading to an overall degeneration of the embryo. The same phenotype can be found in embryos, which are exposed to high temperatures. The embryo died 48 hours after the laser treatment.

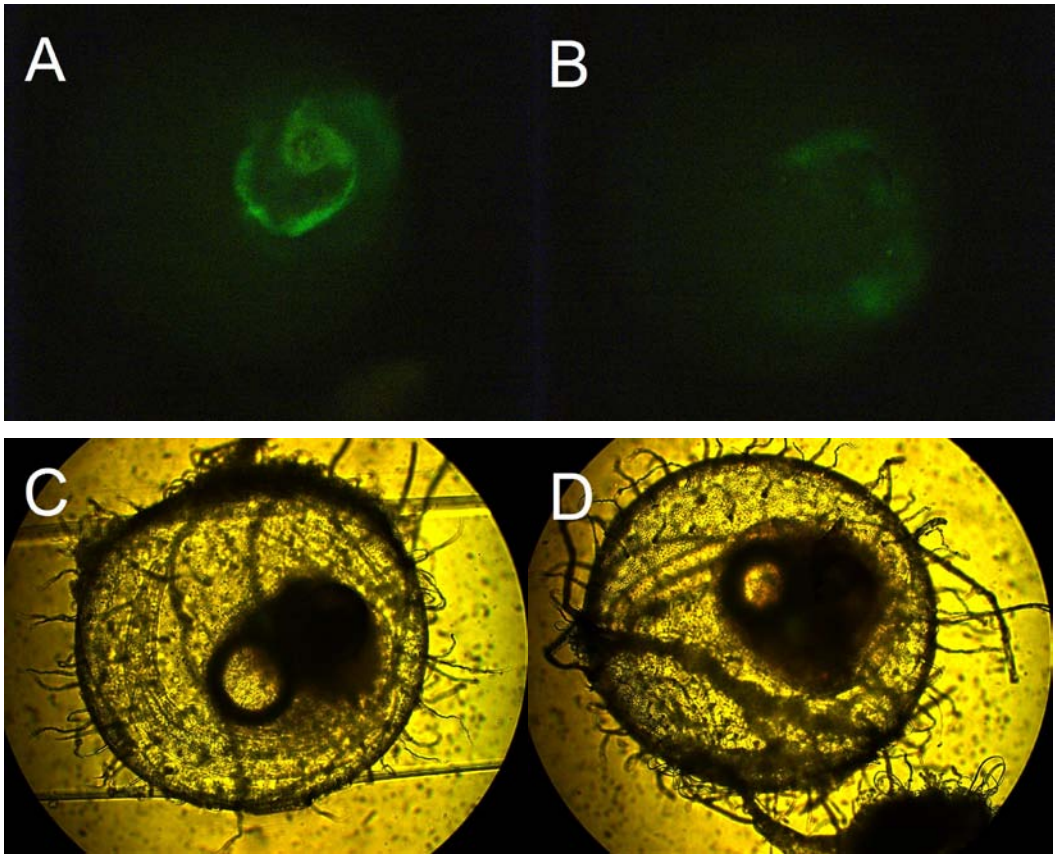

Figure 10. **GFP expression of a laser induced heat shocked embryo.** The embryo was treated for 50 minutes with a laser beam with a power of 330 mW. (A) Dorsal view of embryo (UV); strong GFP expression. (B) Lateral view of embryo1 (UV); strong GFP expression. (C) Lateral view of the embryo under visible light. (D) Dorsal view of the embryo1 under visible light. The embryo shows denatured protein (brown dot) in the head region; structures of the head and brain are not longer distinguishable (A-D: 100x magnification)

3.3.2 Whole embryo laser treatment

After the disappointing results of the experiments to induce a heat shock by laser in a single cell, the decision was made, to test laser treatments of whole medaka embryos. Because the experiments were performed in Linz, where the facility could only be used once a week, it was decided to continue the experiments with another objective. The embryos were treated in large numbers in order to test many different laser adjustments. These experiments were performed by directly exposing the embryo to the laser beam. The setting was changed in a way that a 3.5 cm Petri dish could be placed on a ring, through which the beam of the laser went. The dish with the embryo in ERM Ringer was placed onto the holding ring and the laser was turned on shortly to adjust the

dish, so the laser beam would directly hit the embryo. Additionally a 10x magnification lens was inserted upstream into the system in order to hit the embryo with a more focused laser beam and therefore higher energy in a smaller area. The setting, which was used, is shown in Figure 11.

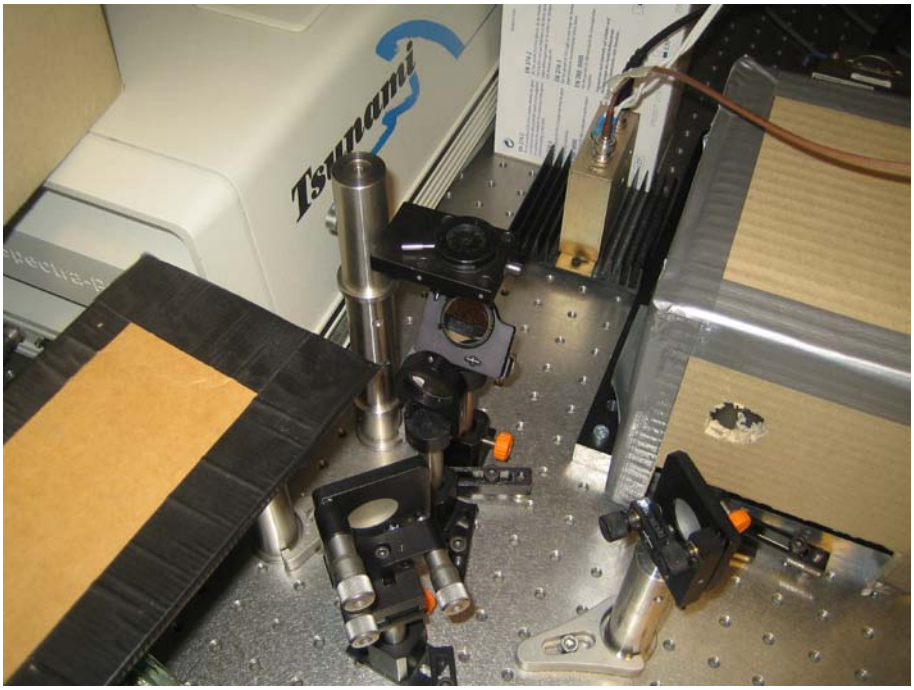

Figure 11. **Setting for laser treatment of non-dechorionized embryos.** Embryos were transferred to a 3.5 cm Petri dish, which was placed onto the holding ring. The laser beam was aimed at the embryo and treated with the laser beam.

The conditions which were tested at this arrangement are shown in Table 5.

N	power [mw]	duration	pulse	lens	positives
1	400 mW	52 min	-	y	0
1	400 mW	15 min	-	y	0
1	600 mW	10 min	-	y	0
2	350 mW	15 min	-	y	0
17	400 mW	10 min	-	y	1
2	400 mW	20 min	-	y	1
1	400 mW	20 min	-	n	0
1	700 mW	5 min	-	n	0
1	300 mW	30 min	-	n	0
1	300 mW	10 min		n	0
1	400 mW	10 min		n	0
9	350 mW	20 min	30 sec on/3 min off	y	0
5	500 mW	20 min	-	y	0
3	500 mW	20 min	-	n	0
5	600 mW	20 min	-	n	0
3	200 mW	10 min	-	y	0
3	300 mW	10 min	-	y	0

N	power [mw]	duration	pulse	lens	positives
3	400 mW	10 min	150 ms on/100ms off	y	0
2	500 mW	10 min	150 ms on/50 ms off	y	0
2	300 mW	10 min	150 ms on/50 ms off	y	0
3	300 mW	10 min	200 ms on/200 ms off	y	0
2	300 mW	10 min	150 ms on/100ms off	y	0
3	300 mW	10 min	150 ms on/50ms off	y	0
3	400 mW	10 min	150 ms on/50ms off	y	0
4	400 mW	10 min	200 ms on/200 ms off	y	0
2	400 mW	10 min	200 ms on/100 ms off	y	0
3	400 mW	10 min	200 ms on/150 ms off	y	0
2	400 mW	10 min	200 ms on/50 ms off	y	0
5	400 mW	10 min	100 ms on /50 ms off	y	0
2	400 mW	8 min	3min 400 mW/1min 800mW (repeated 2 times)	y	0
1	500 mW	13 min	-	y	0
1	400 mW	10 min	3m 400 mW/1m800mW4m400mW 1m800mW3m400mW	y	0
3	400 mW	10 min	3m 300 mW/1m700mW3m300mW 1m700mW2m300mW	y	0
1	400 mW	10 min	3m 200 mW/3m600mW 3m200mW1m600mW	y	0
1	400 mW	8 min	3m 200 mW/1m600mW (repeated 2 times)	y	0
1	400 mW	10 min	5m 400 mW/1m1000mW 3m400mW1m1000mW	y	0
1	400 mW	10 min	5m 400 mW/1m900mW 2m400mW1m900mW	y	0
1	400 mW	10 min	3m 400 mW/1m900mW 5m400mW1m900mW	y	0
1	400 mW	10 min	4m 400 mW/1m800mW (repeated 2 times)	y	0
1	40 mW	6 min	1m 800 mW/3m400mW 1m800mW1m400mW		
1	400 mW	10 min	1m 400 mW/1m900mW (repeated 5 times)	y	0
1	400 mW	9 min	3m 400 mW/1m900mW 2m400mW1m900mW2m400mW	y	0
1	400 mW	10 min	1m 900 mW/3m400mW1m900mW 3m400mW1m900mW1m400mW	y	0
1	400 mW	7 min	1m 900 mW/5m400mW/1m900mW	y	0
3	400 mW	10 min	-	n	0
3	800 mW	10 min	-	n	0
3	600 mW	10 min	-	n	0
2	1000 mW	10 min	-	n	0
7	1000 mW	10 min	-	y	1
3	1200 mW	10 min	-	y	0
4	1500 mW	10 min	-	y	0
4	200 mW	10 min	-	y	0
4	400 mW	10 min	-	y	0
4	600 mW	10 min	-	y	0
5	800 mW	10 min	-	y	1
7	1000 mW	10 min	-	y	0

Table 5**. Laser conditions for non dechorionized embryos directly exposed to laser beam without use of the microscope**. Abbrevations: m=minute; mW=milli Watt; ms=millisecond; sec=second (Lens use: 10x magnification lens)

When the dish containing the embryo was adjusted correctly, that the laser beam would aim at the embryo, the laser was turned on with the adjustments to be tested. After each single test, the embryo was incubated at 27 °C for 24 hours and screened for GFP expression. A total number of 4 positive embryos were obtained from these experiments. The conditions, at which the positive results occurred, are shown in Table 6.

power [mw]	duration	pulse	lens use	positives
1000	10 Min	-	y	1
800	10 Min	-	y	1
400	20 min	-	y	1
400	10 Min	-	y	1

Table 6. **Conditions at which positive heat shock results were obtained.**

The first positive embryo obtained in one of these experiments showed a strong GFP signal in the head, but failed to show expression in the whole embryo (see Figure 12A). The embryo developed normally and hatched after 10 days. The next positive embryo had an identical phenotype, but the GFP expression had only happened in the dorsal part of the embryo (see Figure 12B and C). Both embryos were not damaged as a result of the laser treatment and hatched after 10 days. The fourth (see Figure 12D) and fifth embryo (data not shown) showed weak expression of GFP, which was only seen in the dorsal region of the embryo. These embryos also developed normally and hatched after 9 days of incubation at 27 °C.

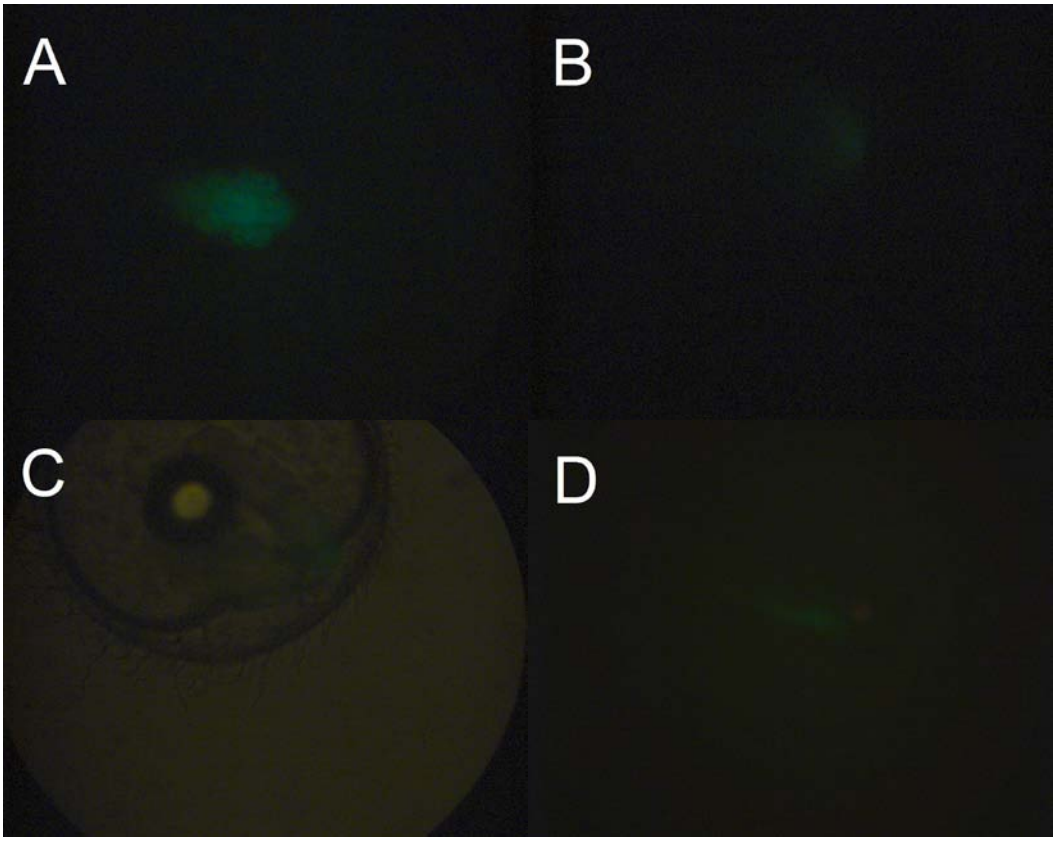

Figure 12. **Positive embryos after whole-embryo laser treatment.** (A) Dorsal view of an embryo (UV), which shows a strong GFP signal in the head region. The embryo was obtained after a 10 minute laser treatment with a power of 1000 mW. Another embryo, showing the same phenomenon is seen in (B). The picture shows a dorsal view of the embryo, which shows weak GFP expression in the dorsal region. The embryo was laser treated for 10 minutes at a power of 800 mW. (C) The same embryo as in (B) under visible light. (D) Another embryo showing a weak GFP expression in the dorsal region. The embryo was obtained after a 20 minute 400 mW laser treatment.

The laser experiments showed that a heat shock response can be induced due a laser treatment of a medaka embryo. The experiments yielded in 5 positive embryos. The conditions under which the positive embryos were obtained were tested again but failed. So the problem arose that the application of a laser to activate a heat shock response in medaka embryos did not work reproducibly. Therefore different conditions leading to reproducible results had to be found. The experiments showed that laser treatment of a medaka embryo can be used to induce a heat shock response in transgenic medaka embryos. The adjustments used for the first positive embryo were repeated with a number of embryos, but the treatments failed to reproduce further positive results. Single cell induction could not be achieved due to a number of problems. The first problem of the experiment was to immobilize the embryos in a proper way.

Dechorionization of the embryos in combination with mounting of the embryos in agarose was not in every case a solution, because some of the embryos were still able to turn around in the agarose, which made it impossible to focus on a single cell. Due to the movement of the embryos, the cells, which were focused, were only treated for a few seconds, which is too short to induce a heat shock response.

3.4 Microwave experiments

Analyzing the results from the laser experiments it became clear, that short exposures at elevated temperatures could be used to induce a heat shock in transgenic medaka. To further investigate short heat shock induction we selected microwaves as the heat source. As a regular microwave has normally a timer, which cannot be controlled as precisely as needed for this experiment, it was decided to control the microwave oven using a computer to shut the oven on and off. In collaboration with Christian Halter and Johann Walzer of the Technics Department of the FH Campus Wien an external control was build for a microwave oven controlled by a PC. A program was written, which made it possible to turn off the microwave for certain short time intervals (milliseconds) with high precision. As the power of the microwave is high, the microwave could not be turned on the whole time of the experiment. The calculation for the duration, in which the microwave is turned on in an experiment, is described in Chapter 2.7. The ultimate purpose of these experiments was to develop a heat treatment method, which induces a strong expression of the gene of interest and a high survival rate of the embryos at the same time. In the first tests up to 6 embryos were transferred into a 3.5 cm Petri dish filled with 3 ml of ERM medium. The dish was placed onto the center of the rotary disc of the microwave oven. The settings, which had to be tested, were entered in the computer connected to the microwave oven. The door of the microwave oven was closed, it was made sure that the rotating wheel function of the microwave was turned off and the program was started. After the experiment, the embryos

were transferred into a 10 cm Petri dish and incubated at 27 °C. Dead embryos were separated and discarded immediately. To test the survival rates of a certain program wild type embryos were incubated for 2 hours and the survival rate was determined by dividing the embryos, which were still alive by the total number of embryos used for the experiment. To test the efficiency of the heat treatment, transgenic embryos were used, which express GFP upon a heat treatment. These embryos were incubated for 24 hours at 27 °C and afterwards screened for GFP expression under UV light. At the time, as the microwave experiments were performed no transgenic line was available, which could be used to measure quantitatively the efficient induction of the heat shock response (e.g. a strain containing an inducible luciferase gene). We therefore compared the results by the quality of the GFP signal of positive embryos. The expression of GFP was distinguished into strong, medium and weak expression. A strong expression pattern showed GFP in the whole embryo. Expression was considered as medium, when a) only a part of the embryo showed GFP expression or b) the expression was clearly weaker than in a strong-expression-embryo. The embryos were considered as weak positives, when the expression of GFP was seen only in certain cells or was weak at all. To test if any transgenic embryos had been used, which did not show expression after the microwave treatment negative embryos were heat treated using the standard method at 39 °C for 2h using a heating block. None of the embryos, which were negative after the microwave treatment showed expression of GFP after the 2 hour 39 °C heat treatment, implying, that all of the transgenic embryos showed a heat shock response, when they were still alive. This confirms that the heat treatment by microwaves is powerful enough to induce heat shock responses in transgenic medaka embryos. The first approach, in which the dish containing the embryos was placed at the center of the turning disc showed, that the results were not reproducible (see Table 7).

n	type	stage	tb on	tb off	d pulse	t pulse off	t p on	n pulse	T on	DR	GFP
10	Wnt	11	200	2800	420	1	420	1	6,7	100%	2
10	Wnt	12	200	2300	420	1	420	1	8,0	100%	0
10	Wnt	12	200	1800	420	1	420	1	10,0	100%	0
10	Wnt	15	200	1800	420	1	420	1	10,0	100%	0
9	Wnt	14	200	2800	180	1	180	1	6,7	0%	2
9	Wnt	15	200	2300	180	1	180	1	8,0	0%	0
11	Wnt	14	200	1800	180	1	180	1	10,0	0%	0
12	Wnt	12/14/16	200	1800	180	1	180	1	10,0	25%	1
13	Wnt	12/14/16	200	1500	180	1	180	1	11,8	0%	3
10	Wnt	3/4	200	1200	180	1	180	1	14,3	100%	0
17	Wnt	12/14/16	200	1500	180	1	180	1	11,8	29%	0
14	Wnt	12/14/16	200	1300	180	1	180	1	13,3	57%	1
5	Wnt	12	200	1800	180	0,2	180	1	10,0	100%	0
6	Wnt	12	200	1500	180	0,2	180	1	11,8	100%	0
5	WT	12	200	2700	298,7	1	600	2	6,9	20%	0
5	WT	13	200	2300	297,5	1	600	2	8,0	100%	0
5	Wnt	13	200	2700	298,7	1	600	2	6,9	100%	0
5	Wnt	13	200	2700	298,7	1	600	2	6,9	100%	0
5	Wnt	13	200	2700	298,7	1	600	2	6,9	100%	0

Table 7. **Conditions and results for microwave heat treatments.** Embryos were heat treated in a microwave oven in a 3.5 cm Petri dish in ERM Ringer. Type:type of embryo; WT:wild type; Wnt:HS-Wnt1; tb on:time burst on [ms]; tb off:time burst off [ms]; d pulse:duration of pulse [sec]; t p off:time pulse off[sec]; t p on:time power on[sec]; n pulse:number of pulses; T on:time on[%];DR:death rate[%];GFP:number of positive embryos

Under the same conditions different results regarding the survival rate and success of the heat treatment were obtained. It was therefore hard to define, which conditions could be used for a strong heat shock response. To avoid this problem we tried to make the system work more accurate and create reproducible conditions. In the next experiments a 2 l beaker filled with water and ice was placed at the center of the turning disc to cool the air inside the microwave oven. The Petri dishes containing the embryos were placed on 6 different spots on the disc each in a distance of 2 cm of the inner border of the disc. The best results were obtained, when the Petri dish containing the embryos was placed close to the microwave source (at a distance of 2 cm of the inner border of the disc). In addition also the temperature of the dish containing the embryos was measured, when the experiment was finished. In the later experiments the temperature of the medium was also measured before and after the experiment. The results of the experiments are shown in Table 8.

n	type	stage	tb on	tb off	d pulse	T p off	t p on	n pulse	T on	DR	GFP	Ts	Te
5	WT	16	200	3800	600	1	600	1	5,0	0%			38,2
5	WT	16	200	3400	601,2	1	600	1	5,6	0%			41,9
5	WT	22	200	2300	600	1	600	1	8,0	40%			49,3
5	WT	23	200	2700	600	1	600	1	6,9	100%			47,1
5	Wnt	30+	200	2700	600,3	1	600	1	6,9	0%	0		49,8
2	Wnt	16	200	3800	600	1	600	1	5,0	0%	0		
2	Wnt	22	200	3800	120	0,2	600	5	5,0	0%	2	24,4	47,4
3	Wnt	19	200	4000	121,8	0,2	612	5	4,8	0%	2	26,3	39,1
3	Wnt	19	200	3800	120	0,2	600	5	5,0	100%	0	24,9	38,1
3	Wnt	16	200	4000	120	0,2	612	5,1	4,8	100%	0	27,9	46,6
4	Wnt	16	200	4000	120	0,2	612	5,1	4,8	0%	1	28,6	42,9
4	Wnt	23	200	4000	600,6	0,2	600	1	4,8	0%	1	25,4	42,4
4	Wnt	19	200	4000	600,6	0,2	600	1	4,8	50%	1	25,9	46,9
4	Wnt	19	200	4000	600,6	0,2	600	1	4,8	50%	1	24,3	44,9
4	Wnt	19	200	4000	600,6	0,2	600	1	4,8	50%	0	25,8	51,1
4	Wnt	16	200	4000	600,6	0,2	600	1	4,8	50%	1	24,4	42,1
4	Wnt	16	200	4000	600,6	0,2	600	1	4,8	75%	0	25,2	42,7
4	Wnt	16	200	4000	600,6	0,2	600	1	4,8	75%	0	25,1	42,7
4	Wnt	16	200	4000	600,6	0,2	600	1	4,8	75%	1	25,3	42,8
4	Wnt	16	200	4000	600,6	0,2	600	1	4,8	75%	1	26,8	46
3	Wnt	16	200	4000	600,6	0,2	600	1	4,8	100%	0	26,2	46,2

Table 8. **Conditions and results for microwave heat treatments.** Embryos were heat treated in a microwave oven in a 3.5 cm Petri dish in ERM Ringer positioned 2 cm from the inner border of the rotary disc next to the electric cage of the microwave oven. type:type of embryo; WT:wild type; Wnt:pSGH Wnt1; tb on:time burst on [ms]; tb off:time burst off [ms]; d pulse:duration of pulse [sec]; t pulse off:time pulse off[sec]; t p on:time power on[sec]; n pulse:number of pulses; T on:time on[%]; DR:death rate[%];GFP:number of positive embryos; Ts:T start of experiment[°C]; Te:T end [°C]

The results showed that it is possible to induce the heat shock pathway using a microwave oven. Detailed results are shown in Tables 7 and 8. A general result of the microwave experiments was a low reproducibility, when the experiments were performed with embryos from the same batch. A small number of embryos showed GFP expression after the treatment. Embryos which were alive and did not show expression of GFP were afterwards heat treated using the standard procedure of 2h at 39 °C in a heating block. This allowed identifying negative transgenic embryos. During the second approach the conditions were better controlled, however still the results were not fully reproducible. The experiments however demonstrated that microwave treatment with short durations is able to activate the heat shock response leading to a strong expression of GFP.

3.5 PCR cycler experiments

The laser experiments showed that treatments with short durations can induce heat shock responses. The results from the microwave experiments furthermore demonstrated strong heat shock responses. The idea therefore came up to develop a new heat treatment, which should generate a stronger heat shock response compared to the standard version used for heat treatment of medaka embryos (2 hours at 39 °C in a heating block). Also a goal of these experiments was to generate a variant with a higher survival rate of the embryos. The computer controlled microwave oven can not be controlled precisely enough to obtain reproducible results; therefore the next experiments were performed in a PCR cycler. It was not clear, which temperatures and durations of heat treatment would induce a strong response. Therefore a number of different conditions were tested. First it would be necessary to analyze the heat resistance of wild type medaka embryos.

3.5.1 Heat treatment of gastrulating medaka embryos

3.5.1.1 Comparison heating block vs. PCR cycler

First the heat treatment in a PCR cycler was compared to that of a heating block. An equal number of wild type medaka embryos from the same batch were incubated in a heating block as well as in a PCR cycler. The embryos were collected, dead embryos were separated and discarded and incubated at 27 °C until they reached stage 14 (Iwamatsu, 2004). For the heating block experiment, each 10 embryos were transferred to a 1.5 ml reaction tube filled with 0.5 ml of ERM Ringer. The heating block was heated up to 39 °C and the tube containing the embryos was incubated for 2 hours. After the experiment, dead embryos were discarded and the remaining embryos were incubated at 27 °C for 24 hours. In the cycler, single embryos were transferred to PCR reaction

tubes filled with 100 µl of ERM Ringer. The tubes were put into the PCR cycler and a program was started, that heated the tubes to 39 °C for 2h. After the heat treatment had been performed, the embryos were transferred to a 10 cm Petri dish containing ERM Ringer and incubated at 27 °C. Dead embryos were discarded. After 24 hours of incubation for each of the groups the death rate was calculated by dividing the number of living embryos by the total number of embryos of each group. The results (Table 9) showed the same survival rate of WT embryos at the same conditions (temperature, duration).

	temperature	duration	n	dead	alive	Survival rate
Heating block	39 °C	2h	40	1	39	97,5 %
PCR cycler	39 °C	2h	40	1	39	97,5 %

Table 9. **Comparison of death rate for heating block and PCR cycler.** Wild type embryos were collected rose to stage 14 and heat treated either at a heating block or using a PCR cycler for 2 hours at 39 °C. The results showed that the efficiency of the 2 devices is equal.

3.5.1.2 Survival rates

To compare heat treatment variants and the resulting luciferase activities, first the survival rates at the different temperatures and durations had to be determined. This was done to ensure, that for the subsequent luciferase experiments no embryos were wasted. The following heat treatment variants were selected: 1 minute HS, 10 minute HS, 2 hour heat treatment, 2x 1 minute HS at an interval of 5 minutes, and a heat treatment, at which every 5 minutes a 1 minute HS was applied for a total duration of 2 hours. Before and after each heat treatment, the temperature was set to 27 °C. Wild type medaka embryos were collected (see Chapter 2.1.1) and incubated in ERM Ringer until they reached stage 13 (Iwamatsu 2000). Heat treatments were applied using a PCR cycler. Dead embryos were collected and discarded directly after the heat treatment. The embryos were allowed to recover in ERM Ringer for 24 hours. Then the survival rate was determined. The experiments began at the lower temperatures and were continued for each heat treatment variant until the

survival rate dropped to 0% (at 2 temperatures within 1 °C). The results of this experiment are shown in Table 10

2 h					
temperature [°C]	39	40	41	42	43
survival rate [%]	100	95	85	0	0

10 min									
temperature [°C]	39	40	41	42	43	44	45	46	47
survival rate [%]	100	9	100	95	100	80	0	0	0

2 h every 5 Min HS for 1 minute								
temperature [°C]	39	40	41	42	43	44	45	46
survival rate [%]	95	100	95	75	60	35	0	0

2x1Min HS, 5 min interval											
temperature [°C]	39	40	41	42	43	44	45	46	47	48	49
survival rate [%]	100	100	100	100	100	100	95	98	70	0	0

1 min											
temperature [°C]	39	40	41	42	43	44	45	46	47	48	49
survival rate [%]	100	100	100	100	100	100	96	95	90	0	0

Table 10. **Survival rate of wild type embryos during gastrulation for different heat treatments at different temperatures.** For each experiment a number of n=60 embryos was used.

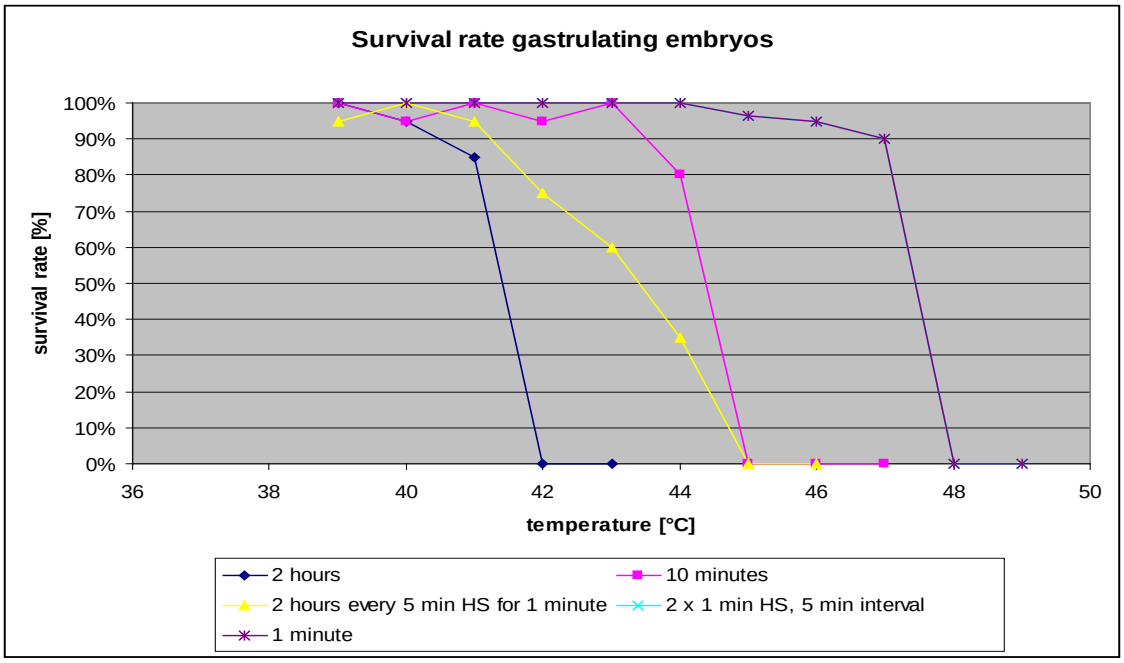

Figure 13. **Survival rate of wild type embryos during gastrulation for different heat treatments at different temperatures.** Embryos were collected, incubated until they had reached stage 13 (Iwamatsu, 2004) and heat treated at different temperatures and different durations. The survival rate was determined by counting the living embryos (24 hours after the heat treatment) and dividing the number by the total number of embryos used for the experiment.

Each of the chosen heat treatments followed a similar graph, shifted to a higher temperature for shorter heat treatments. Embryos showed resistance until a certain temperature was reached, where the embryos died at a high percentage.

3.5.1.3 Step and ramp heat treatments

The results of the survival rate showed, that a fast increase in the temperature in a rather small time interval highly increases the death rate of the embryos. To avoid this problem a number of different heat treatment conditions was tested, in which the temperature was increased in small steps and also, using a PCR cycler, with a constant rate per time. The results of the step and ramp heat treatments were compared to standard heat shock treatment with a direct temperature increase from 27 °C to the 43.5 °C.

3.5.1.4 Luciferase experiments

pSGH2 luc puro medaka embryos were collected, incubated until the embryos had reached stage 14 (Iwamatsu, 2004) and the heat treatments were performed (see Chapter 2. for the exact protocol). Afterwards the embryos were incubated at 27 °C in ERM Ringer and allowed to generate the luciferase induced by the heat shock. The embryos were homogenized and the luciferase measurement was performed. The results showed that from the different heat treatment versions the 10 min heat treatment showed the highest amount of luciferase production (diagram). As the goal was to develop a strong heat shock response, the variants with the lowest activities were not further investigated. First each of the heat treatment durations was tested at temperatures, based on the survival rate experiments. The temperatures were first tested in intervals of 1 °C and after positive results (high luciferase production) in 0.5 °C steps. The heat treatment condition, which showed the highest amount of luciferase, was found at 10 minutes and 43 °C (mean value). In fact some values of the 43.5

°C/10 minute condition showed higher luciferase activities, but a smaller mean value in average. The standard variant for heat shock treatment (2h 39 °C) showed a rather low luciferase activity, which was 33-fold lower than that of the 10 min 43 °C heat shock treatment.

	Temperature [°C]	Luciferase activity (mean value)	SD	n
2 hours	39	342704	251051	15
	40	2940774	3500971	9
	41	2146132	1663274	10
10 minutes	41	141106	35369	3
	42	449621	84366	2
	43	11458192	5628294	14
	43.5	10816831	3114715	29
	44	9822609	4155907	11
	44.5	7623775	1287367	4
1 minute	45	**345813**	79496	3
	46	1192640	475132	7
	47	2753678	820775	4

Table 11. **Luciferase activities of heat treatment variants.** Luciferase activities measured 6 hours after different heat treatment variants. SD=Standard deviation

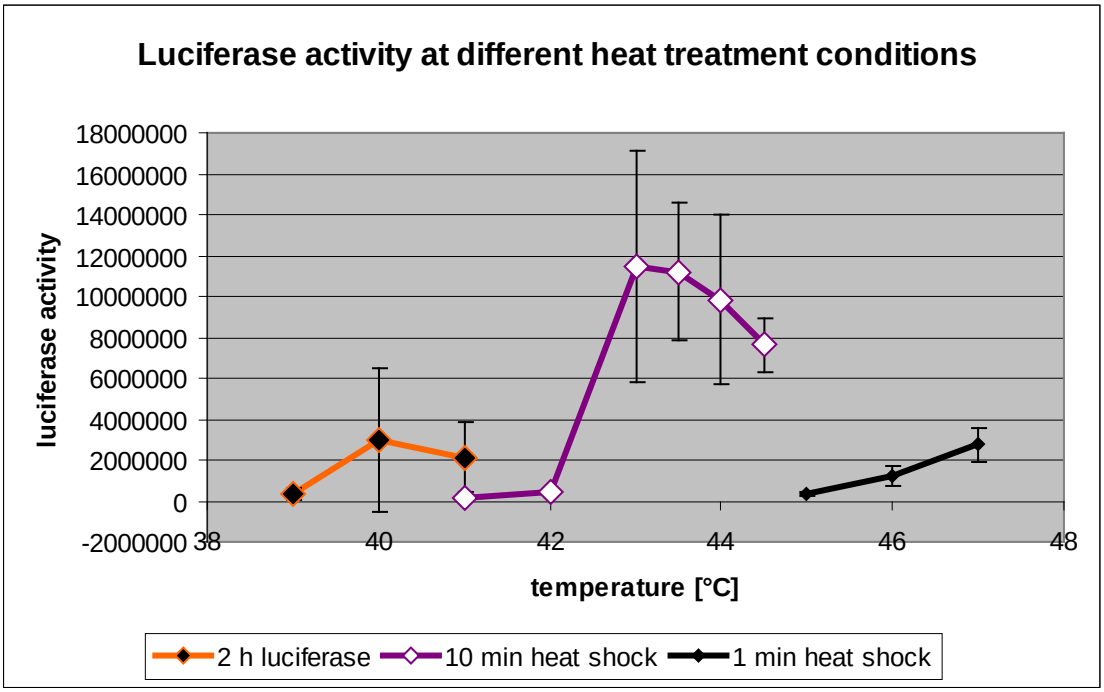

Figure 14. **Luciferase activity as a result of different heat treatment durations at different temperatures.** Mean values are shown. See Table 11 for details. Error bars represent the SD.

3.5.1.5 Comparison of survival rate and luciferase production

The results from the heat treatment experiments were summed up in Figure 15 by comparing the survival rate of the embryos and the luciferase production graphically. The goal of the work was to find heat treatment conditions which resulted in a high survival rate and also a high amount of luciferase. Therefore the heat treatment variants with the lowest induction of luciferase were not further investigated. The graph shows that a heat shock treatment of 10 minutes at a temperature of 43 °C proved to be most effective.

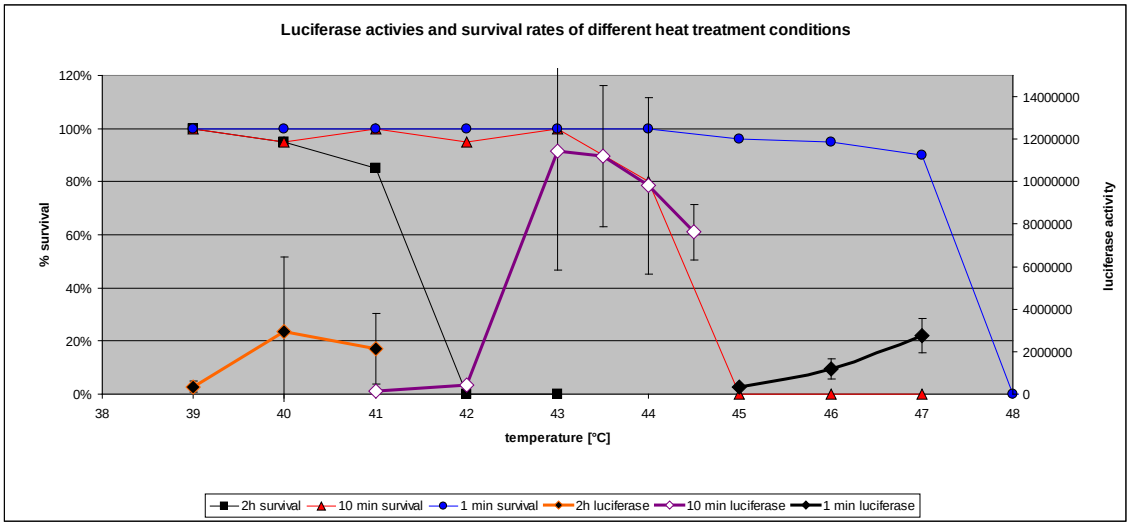

Figure 15. **Luciferase activities and survival rates of different heat treatment conditions.** For the survival rate, wildtype medaka embryos were incubated until stage 13, heat treated, incubated at 27 °C for 24 hours and the survival rate was determined. For the luciferase activity, pSGH2 luc puro medaka embryos were incubated until they had reached stage 14, heat treated and incubated at 27 °C. After the incubation the luciferase activity was measured. Mean values are shown. Error bars represent the SD

3.5.2 Old-embryo-experiments

In addition to the heat treatment experiments performed with embryos at stage 14 (Iwamatsu, 2004) a similar experimental approach was performed using 7-day-old embryos. This approach was necessary to show, that the new heat treatment versions can also be used to induce a heat shock response in embryos at older stages. Such heat treatment conditions can be applied, when the overexpression of a gene has to be tested, that has early lethal effects.

3.5.2.1 Survival rate

Survival rate experiments were performed with wild type medaka embryos which were incubated in ERM Ringer for 7 days at 27 °C. The ERM Ringer was changed every second day and dead embryos were discarded every day. In this case, compared to the stage-12-embryos, only 3 different heat treatment variants were tested. In the survival rate and luciferase experiments with the young embryos, conditions with 2x1min and 2h every 5 min were not effective (data not shown), which means, that these heat treatment variants did not show a high luciferase amount or a high survival rate. After the heat treatment experiments the embryos were incubated in ERM Ringer. Dead embryos were discarded after the heat treatment and after 6h. The death rate was determined 24h after the heat treatment. The results are shown in the following graph:

2 h								
temperature [°C]	38	39	40	41	42	43		
survival rate [%]	100	100	95	73	0	0		
10 min								
temperature [°C]	41	42	43	44	45			
survival rate [%]	100	100	79	49	0			
1 min								
temperature [°C]	44	45	46	47	48	49	50	51
survival rate [%]	100	95	97	62	0	0	0	0

Table 12. **Survival rate of wild type embryos during gastrulation for different heat shock treatments at different temperatures.** For each experiment a number of n=60 embryos was used.

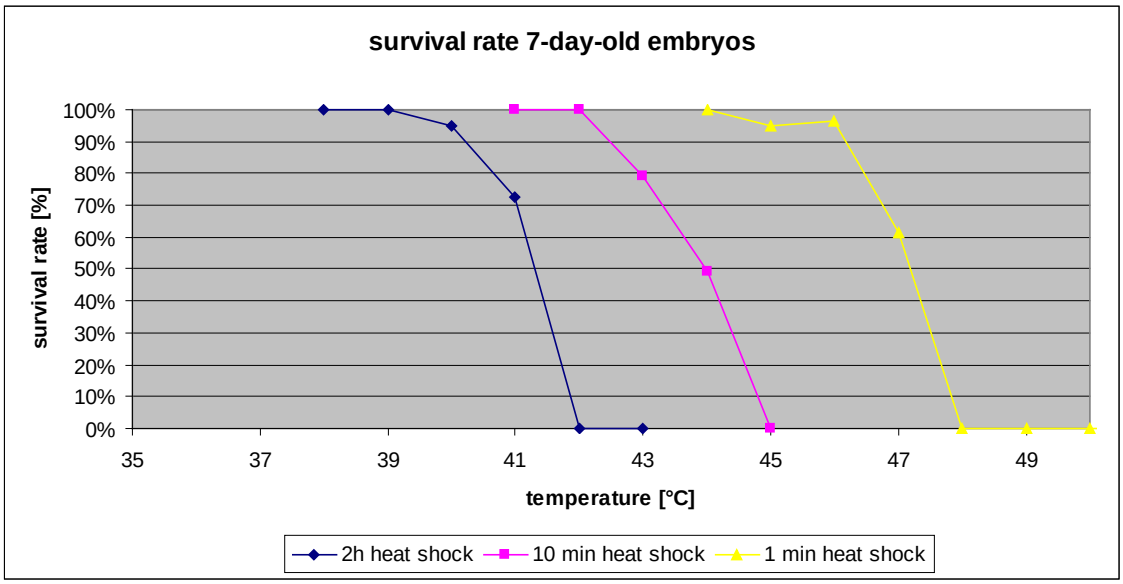

Figure 16. **Survival rates of 7-day-old embryos for 3 different heat shock treatments at different temperatures.** Wild type embryos were incubated for 7 days at 27 °C and heat treated at different temperatures for different durations in a PCR cycler. The survival rate was determined 24 hours after the heat shock treatment. See Table 12 for details.

The results show that the 7-day-old embryos react similarly to the heat treatments as the embryos during gastrulation (stage 14; Iwamatsu 2004). However, the graphs are shifted to the left, meaning, that the older embryos cannot resist the same temperatures as the embryos during gastrulation. This result is likely due to the more complex metabolism of the further developed embryo. For the following luciferase experiments it was decided to test the heat treatment variant, which had the most promising results in the young embryos. Only a 10 minute 43.5 °C heat treatment variant was tested because of a limited number of embryos.

3.5.2.2 Luciferase experiments

The survival rate results for the old embryos indicated, that the luciferase experiments could be performed at a temperature of 41 °C for a 2 hour heat treatment, because the survival rate was high. The luciferase experiments were performed with pSGH2 luc puro medaka embryos, which were 7 days old. After collecting and cleaning of the embryos they were incubated in fresh ERM

Ringer for 7 days at 27 °C. Each day dead embryos were separated and the ERM Ringer was changed every second day. The experiments were performed with 2 strains of pSGH2 luc puro medaka, which had been developed. As the 2 strains had different levels of luciferase activities the strains were only compared to itself. After the heat shock treatment the embryos were incubated in fresh ERM Ringer for 6 hours at 27 °C to allow luciferase production. Then the luciferase production of the embryos was measured and used for the next graph.

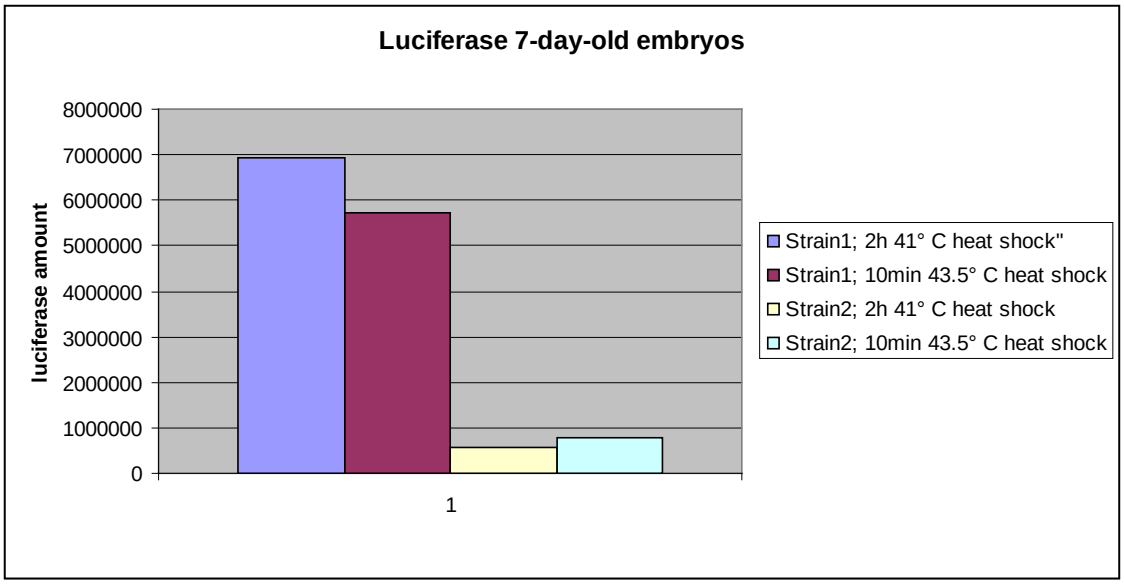

Figure 17. **Comparison of 2h 41 °C to 10min 43.5 °C heat shock treatments for 2 different strains of pSGH2 luc puro.** 7-day-old embryos were used for the experiments, mean values are shown

The results showed that the shorter heat treatment variant resulted in a comparable luciferase amount compared to the longer (2h 41 °C) heat treatment. Interestingly in one strain, the longer heat treatment showed a higher amount of luciferase than the shorter heat treatment variant. This effect could be the result of more cells of the embryo surviving the heat treatment. In the other strain used for the experiment the shorter heat treatment was more effective than the long heat treatment variant. In both cases the amounts of luciferase are rather similar and do not show strong differences in the amount of luciferase as it is the case for embryos during gastrulation.

3.5.3 Step and ramp heat treatments

In parallel experiments, embryos which were heat treated at 43.5 °C had a decreased survival rate if, they were incubated before at 17 °C (data not shown). An incubation at 17 °C is often used for overnight incubations to delay the development of the embryos. Based on these results the decision was taken to test if the problem of high temperature heat treatments is the temperature of the actual heat treatment or the high temperature step, needed to reach the heat shock temperature. For this purpose embryos were heat treated at 43.5 °C, but steps and ramps were used to reach the temperature. For the step version of the heat treatment the program used in the PCR cycler started with an incubation of 10 minutes at 27 °C followed by an increase in the temperature of 2 °C per minute. When the obtained temperature was reached, the embryo was kept for 10 minutes at this temperature and then the temperature was decreased again with steps of 2 °C per minute until it reached 27 °C again.
The ramp heat treatment was performed by starting with a 10 minute 27 °C incubation followed by a temperature gradient, which increased the temperature 0.1 °C per minute. This gradient was limited by the software of the PCR cycler. When the heat treatment temperature was reached (43.5 °C) the embryo remained at it for 10 minutes. Afterwards the temperature was decreased again with a gradient of -0.1 °C per minute. At the end of the program the embryo was incubated at 27 °C.

3.5.3.1 Survival rate

Wild type embryos were collected and incubated to stage 12 (Iwamatsu, 2004) and heat treated in the PCR cycler. The complete procedure is explained in Chapter 2.8. After the heat treatment the embryos were transferred into 10 cm Petri dishes and incubated in ERM Ringer at 27 °C. Dead embryos were separated and discarded. 24 hours after the heat treatment the survival rate was determined by dividing the living embryos by the total number of embryos

used for the experiment. As the results showed a high variability between different days under otherwise identical conditions, we decided to compare the survival rate of the ramp and step heat treatments to the 10 minute heat treatment at 43.5 °C. For each experimental series (all performed on one day with embryos from the same batch) the survival rate of the 10 minute 43.5 °C heat treatment was taken as reference. The survival rate of this heat treatment was taken, because it had at this time the most promising results. The results showed that the step and ramp heat treatments had a higher survival rate of embryos than the 10 minute 43.5 °C heat treatments. The survival rate of the step heat treatments was 1.35 to 1.78 times higher, while the ramp heat treatments were 1.63 to 1.68 times higher (data not shown). Therefore the step and ramp heat treatments always had a higher survival rate than the 10 minute 43.5 °C heat treatments. According to these results no clear decision could be made, if the step or the ramp heat treatments showed higher survival rates, because of the variations in the results for each series of experiments.

3.5.3.2 Luciferase activity of step and ramp heat treatments

As the results from the survival rate were promising, the experiments were performed with pSGH2 luc puro embryos at stage 12 (Iwamatsu, 2004). At this time the wildtype mating partners of the transgenic fish had been changed, which resulted in a limited number of embryos. Therefore the luciferase activities of the step and ramp heat treatments were compared at an effective heat treatment temperature of 43.5 °C with the activities of the 10 minute heat treatment.

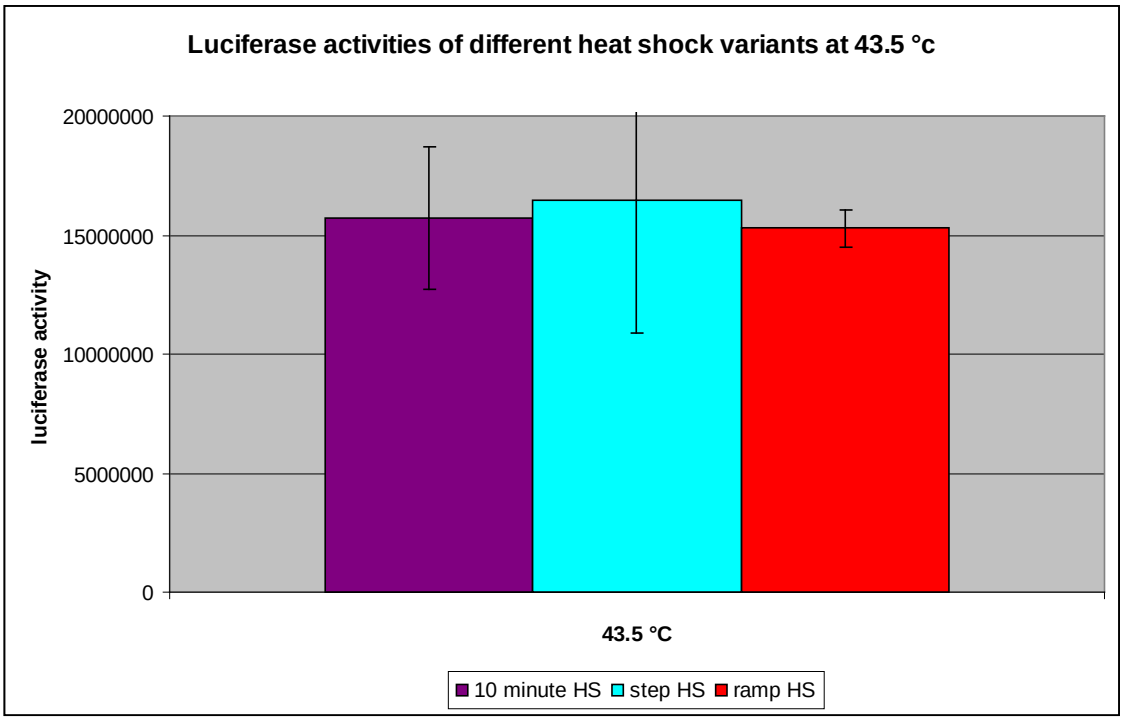

Figure 18. **Comparison of heat treatment at 43.5 °C.** pSGH2 luc puro embryos were heat treated at stage 12 and the luciferase activities measured. Mean values are shown. See text for details of the heat treatment variants. Error bars are shown in ± SD.

The results from the luciferase measurement (see Figure 18) showed, that all of the heat treatment variants can be compared, as the activities are all in the same range. When compared to the variant, which is normally used to induce a heat shock response medaka embryos (2 hours at 39 °C) all of the heat treatments showed a much higher level of luciferase activity. Especially the step heat treatment with its high survival rate and high induction of luciferase is an effective heat treatment method for medaka embryos.

4 Discussion

4.1 Laser experiments

4.1.1 Single cell induction

Localized heat shock induction is a useful tool in developmental biology. It has first been performed in *Drosophila melanogaster* (Monsma, 1988) using a hot needle. Laser has been used successfully in C. elegans (Stringham, 1993), *Danio rerio* (Halloran, 2000) and *Drosophila melanogaster* (Halfon et al, 2007) to induce heat shock responses. However single cell induction by laser treatment has so far only been performed in *C. elegans* (Stringham, 1993), *Danio rerio* (Halloran, 2000; Sato Maeda, 2006; Deguchi, 2009) and *Drosophila melanogaster* (Halfon, 2007). This method is useful for the study of cell fate and for the observation of genes, which would be only induced in a single cell or a small number of cells of an embryo. As the initial goal of the experimental series was to achieve an induction in a single cell the first aim was to record the temperature in the cells. For this purpose the use of liquid crystals was thought to be an ideal instrument. The experiments showed, that the liquid crystals, which were used are in the expected color range. However it was not possible to inject the crystals into medaka embryos. In most experiments, the crystals blocked the tip of the needle immediately. When needles with a bigger tip were used, a proper injection into the embryo was not possible. The majority of the embryos died immediately. The injection of liquid crystals into the yolk of the embryos also failed. As all experiments to inject liquid crystals into medaka embryos failed, the experiments were continued without the use of a temperature control.

For the laser treatment, the embryos were dechorionized and mounted in liquid agarose because of their ability to move inside of the chorion. This phenomenon is well known for medaka embryos (Iwamatsu, 2004). The

embryos were targeted under the microscope and a cell was chosen to be hit by the laser beam. In some cases, despite of the mounting, the embryos were able to rotate in the agarose. A possible explanation for this observation is that between the embryo and the agarose a thin layer of liquid formed, which enabled the embryos to move. It could also be possible, that the movement of the embryo was due to a structural instability, as the embryo has a structure like a liquid filled balloon, which is able to bend and stretch in different directions. However, in those experiments where the embryos kept their position, no GFP expression could be detected. Therefore the next step was to first screen for optimal laser adjustments and concentrate on single cell induction later.

Thus, a number of non-dechorionized embryos was exposed to a laser-beam and one of the embryos indeed showed expression of GFP 24 hours after the treatment. The embryo showed a strong signal in the head region, but not in the tail region. It also had severe defects in the head region, which was most probably due to the heat. The same effects can be found in embryos, which are exposed to high temperatures. The result of the experiment could not be confirmed in subsequent experiments with the same adjustments. It is possible, that the embryos in general are too transparent to absorb enough laser light and that this absorption is different in embryos which have already malformations. As the results for a highly focused laser beam were negative, next whole embryos were exposed to the laser beam, without focusing on a single cell.

4.1.2 Whole embryo treatment

The treatment of medaka embryos exposed to a non-focused laser beam yielded 4 positive embryos. However the experimental settings which were used were quite different and again were not reproducible. Positively heat shocked embryos were obtained at durations, which are known to be long enough to induce a heat shock response by laser treatment (Halloran, 2000; Sato Maeda, 2006; Deguchi, 2009). At these durations the used heat shock promoter had also been shown to be induced by heat treatment of the embryos on a heating block (Bajoghli, 2004). Most of the positively induced embryos showed the

expression of GFP only in part of the embryo and never in the whole embryo. The most interesting fact of the results of this experimental series was that the exposure of the positive embryos in all cases was relatively short. None of the positive embryos was exposed to the laser beam for longer than 20 minutes. 3 of the embryos showed GFP expression after a laser treatment with a duration of 10 minutes. As all of the embryos were manually positioned into the laser beam, it is a possible explanation for the poor results, that the laser beam did not exactly hit the embryo but the yolk instead. It is also possible, that the embryos turned during the treatment as it also has happened in the first approach (see Chapter 4.1.1). It is commonly known, that medaka embryos rotate inside the chorion to escape from exposure to light. This phenomenon can be seen, when embryos are exposed to UV light under the microscope. However, after analyzing the results the idea came up, that short durations of a heat shock treatment might be sufficient for a strong heat shock response.

4.2 Microwave experiments

As the laser experiments had shown, that short durations are efficient to induce a heat shock response in transgenic medaka embryos we tried to use a modified microwave oven to induce a heat shock response. The results of the microwave experiments showed that short exposure to heat below 10 minutes are sufficient to induce a strong signal. In a first series of experiments poor reproducibility of the results was observed. Despite of the exact control of the microwave in millisecond intervals the results of the experiments could not be reproduced exactly enough. To enable a more sensitive system the experimental hardware was modified. The temperature sensor of the thermometer was directly installed in the microwave oven for the purpose of measuring the temperature of the medium during the experiment. As a second innovation a 2L beaker filled with ice water was placed onto the center of the rotary disc of the microwave oven to keep the temperature of the air inside the microwave oven constant. Using these new conditions the experiments could

be controlled better, but again the reproducibility was not good enough. The temperatures in the samples at the end of the experiments fluctuated. The experiments with the microwave oven were therefore not continued, instead a PCR cycler was used for better control of the temperature during the experiments. However the most promising part of the microwave experiments was to see, which temperatures are necessary to induce a strong heat shock response and at which temperature the embryos die. This frame of temperatures was used to start the following PCR cycler experiments. The temperature frame is consistent with the findings of Bajoghli et al (Bajoghli, 2004) for the used heat shock promoter (see also Chapter 1.3.1).

4.3 PCR experiments

PCR cycling machines can be controlled very accurately making them an ideal tool to test heat shock conditions. As the major problem of the microwave experiments was the poor reproducibility of the results, the expectations were high to receive reproducible results with a PCR cycler. Having the knowledge from the laser experiments which showed how long the durations of heat exposure have to be and the temperature range from the microwave experiments, the experiments were started. First the exact temperature was determined, at which a high survival rate of the embryos occurred. The experiments revealed that the survival rate stays rather constant up to a point at which it decreases dramatically. This phenomenon was seen for all tested durations. It seems that upon a specific temperature too many cells die, which leads to the death of the embryo. We decided that a survival rate of 80 % or higher would be sufficient for the standard procedure of heat treating medaka embryos (2 hours, 39 °C on a heating block) often weak heat shock responses are seen (data not shown). We therefore hoped to obtain a better heat shock response for these new conditions. With these results for the survival rate of the embryos the first experiments with luciferase measurements were started. As the heat shock promoter showed the ideal 15-basepair unit

AGAACGTTCTAGACC (Cunniff, 1993), which was multimerized 8 times (Bajoghli, 2004) a high luciferase production was obtained. The ideal heat shock temperature was found at 43.5 °C. This result is in good agreement with the findings of Bajoghli et al (Bajoghli, 2004), using the same promoter construct. In these experiments the ideal heat shock temperature was found at 43 °C. Surprisingly the results revealed that the heat treatment with a duration of only 10 minutes at a temperature of 43.5 °C showed a 33-fold higher luciferase amount than the standard variant of 2 hours at 39 °C while the survival rate was nearly the same. This fact could be very helpful in cases where a strong response is needed. In addition to the short duration it is possible to determine the time of induction exactly.

The experiments, in which older embryos were used to measure the luciferase production upon heat shock treatment showed similar results as for early embryos. The survival rate graphs (see Chapter 3.5.2.1) had similar curves, but all were shifted to lower temperatures, indicating that the older embryos cannot easily resist high temperatures. This is most likely due to the fact that the older embryos are further developed and show a complex organism compared to the young ones. In particular they already have a complex metabolism. If defects due to heat treatment appear and too much cells are killed, it is no longer possible for the embryo to regenerate. The luciferase measurements revealed that also in the case of 7-day-old embryos the 10 minute 43.5 °C heat treatment showed a higher production of luciferase than the standard heat treatment for 2 hours at 39 °C. As the survival rate had demonstrated that the 7-day-old embryos can resist a 2 hour heat treatment at an elevated temperature of 41 °C it was tested, if it shows comparable results to the 10 minute 43.5 °C heat treatment. 2 strains were tested (see Chapter 3.5.2.2) one of them had a higher luciferase amount at the short heat treatment, while the other showed higher luciferase amounts, when heat treated for 2 hours at 39 °C. The most probable explanation for this result is that in the strain, which showed the higher luciferase amount more cells survived and therefore more luciferase was produced.

Step and ramp heat treatments showed a higher survival rate than standard heat treatment variants, in which the step from the incubation temperature to the heat treatment temperature was high (data not shown). After this discovery the step and ramp heat treatments were tested. Surprisingly the luciferase amount of the step heat treatment was higher than the variant, in which the increase in temperature was done in one step. The ramp heat treatment showed also a high luciferase signal, just slightly less than the 10 minute heat treatment without any steps or ramps. However the number of samples was low and the experiments have to be repeated with a higher sample number. Despite of this fact, it seems that the embryos can resist an elevated temperature much better, when the increase in the temperature is done in small intervals, as it is done in the step and ramp heat treatment versions. The heat shock response is at least as strongly activated by the step heat treatment as by the 10 minute version without steps. For an exact measurement, the experiment should be performed by measuring luciferase amounts at different intervals for the steps and ramps to find the optimal step and ramp time and temperature intervals. Such experiments were restricted in our case by the technical specifications of the used PCR cyclers. It should be possible to investigate the mechanism further by using different cycling machines, which allow smaller temperature steps and lower slopes.

Taken together it was possible to develop a new method for heat shock treatment of medaka embryos, which results in considerably stronger activation of the heat shock response which is an important tool for the induction of gene expression in transgenic embryos.

5 References

Abravaya, K., Myers,M. P., Murphy, S. P. and Morimoto, R. I. (1992). The human heat shock protein hsp70 interacts withHSF, the transcription factor that regulates heat shock gene expression. Genes Dev. 6, 1153–1164.

Akerfelt, M., Henriksson, E., Laiho, A., Vihervaara, A., Rautoma, K., Kotaja, N., Sistonen, L. (2008) Promoter ChIP-chip analysis in mouse testis reveals Y chromosome occupancy by HSF2. Proc Natl Acad Sci USA 105, 11224–11229.

Ali, A., Bharadwaj, S., O'Carroll, R., Ovsenek, N. (1998) HSP90 interacts with and regulates the activity of heat shock factor 1 in Xenopus oocytes. Mol. Cell. Biol. 18, 4949–4960

Bajoghli, B., Aghaallaei, N., Heimbucher, T., Czerny, T. (2004). An artificial promoter construct for heat inducible misexpression during fish embryogenesis. Developmental Biology 15;271(2):416-30.

Bajoghli, B; Aghaallaei, N; Soroldoni, D; Czerny, T. (2007). The roles of Groucho/Tle in left-right asymmetry and Kupffer's vesicle organogenesis. Dev Biol. 303(1):347-61.

Baler, R., Dahl, G., Voellmy, R. (1993). Activation of human heat shock genes is accompanied by oligomerization, modification, and rapid translocation of heat shock transcription factor HSF1. Mol Cell Biol. 13(4):2486-96.

Baler,R., Welch, W J, Voellmy, R. (1992) Heat shock gene regulation by nascent polypeptides and denatured proteins: hsp70 as a potential autoregulatory factor. J. Cell Biol. 117, 1151–1159.

Bharadwaj, S., Ali, A., Ovsenek, N. (1999) Multiple components of the HSP90 chaperone complex function in regulation of heat shock factor 1 in vivo. Mol. Cell. Biol. 19, 8033–8041

Bienz, M., (1986). A CCAAT box confers cell-type-specific regulation on the Xenopus hsp70 gene in oocytes. Cell 46, 1037–1042.

Bu, L., Jin, Y., Shi, Y., Chu, R., Ban, A., Eiberg, H., Andres, L., Jiang, H., Zheng, G., Qian, M., et al. (2002) Mutant DNA-binding domain of HSF4 is associated with autosomal dominant lamellar and Marner cataract. Nat Genet 31, 276–278.

Chang, Y., Ostling, P., Akerfelt, M., Trouillet, D., Rallu, M., Gitton, Y., El Fatimy, R., Fardeau, V., Le Crom, S., Morange, M. et al. (2006) Role of heat-shock factor 2 in cerebral cortex formation and as a regulator of p35 expression. Genes Dev 20, 836–847.

Chen, Y., Barlev, NA., Westergaard, O., Jakobsen BK. (1993). Identification of the C-terminal activator domain in yeast heat shock factor: independent control of transient and sustained transcriptional activity. EMBO J. 12: 5007-18

Cohen, C., Parry, DA. (1990) Alpha-helical coiled coils and bundles: how to design an alpha-helical protein. Proteins 7:1-15.

Cohen, C., Parry, DA. (1994) Alpha-helical coiled coils: more facts and better predictions. Science. 263(5146):488-9.

Cotto, JJ., Kline, M., Morimoto, RI. (1996) Activation of heat shock factor 1 DNA binding precedes stress-induced serine phosphorylation. J. Biol. Chem. 271, 3355–3358

Cunniff, NF., Morgan, WD., (1993). Analysis of heat shock element recognition by saturation mutagenesis of the human HSP70.1 gene promoter. J Biol Chem. 15;268(11): 8317-24.

Cunniff, NF., Wagner, J., Morgan, WD. (1991) Modular recognition of 5-base-pair DNA sequence motifs by human heat shock transcription factor. Mol Cell Biol. 11(7): 3504-14.

Dai, C., Whitesell, L., Rogers, AB., Lindquist, S. (2007) Heat shock factor 1 is a powerful multifaceted modifier of carcinogenesis. Cell 130, 1005–1018.

Deguchi T., Itoh M., Urawa H., Matsumoto T., Nakayama S., Kawasaki T., Kitano T., Oda S., Mitani H., Takahashi T., Todo T., Sato J., Okada K., Hatta K., Yuba S., Kamei Y. (2009). Infrared laser-mediated local gene induction in medaka, zebrafish and Arabidopsis thaliana. Dev Growth Differ. 51(9): 769-75.

Fernandes, M., Xiao, H., Lis, J.T. (1994). Fine structure analyses of the Drosophila and Saccharomyces heat shock factor–heat shock element interactions. Nucleic Acids Res. 22, 167–173.

Fujimoto, M., Izu, H., Seki, K., Fukuda, K., Nishida, T., Yamada, S., Kato, K., Yonemura, S., Inouye, S., Nakai, A. (2004) HSF4 is required for normal cell growth and differentiation during mouse lens development. EMBO J 23, 4297–4306.

Furutani-Seiki, M., Wittbrodt, J., (2004). Medaka and zebrafish, an evolutionary twin study. Mech Dev. 121**,** 629-37.

Gould, G., (1959): The LASER, light amplification by stimulated emission of radiation. In: The Ann Arbor Conference on Optical Pumping. 1959.

Guettouche, T., Boellmann, F., Lane, WS., Voellmy, R. (2005). Analysis of phosphorylation of human heat shock factor 1 in cells experiencing a stress. BMC Biochem. 11;6:4.
Gross, SR., Kinzy, TG. (2005) Translation elongation factor 1A is essential for regulation of the actin cytoskeleton and cell morphology. Nat. Struct. Mol. Biol. 12, 772–778.

Halfon, MS., Kose, H., Chiba, A., Keshishian, H. (2007). Targeted gene expression without a tissue-specific promoter: Creating mosaic embryos using laser-induced single-cell heat shock. PNAS 1997;94;6255-6260

Halloran MC., Sato-Maeda M., Warren JT., Su F., Lele Z., Krone PH., Kuwada JY., Shoji W. (2000). Laser-induced gene expression in specific cells of transgenic zebrafish. Development 127, 1953-1960

Hashikawa, N., Mizukami, Y., Imazu, H., Sakurai, H. (2006). Mutated yeast heat shock transcription factor activates transcription independently of hyperphosphorylation. J Biol Chem. 17;281(7): 3936-42.

Hashikawa, N., Sakurai, H. (2004). Phosphorylation of the yeast heat shock transcription factor is implicated in gene-specific activation dependent on the architecture of the heat shock element. Mol Cell Biol. 24(9): 3648-59.

Hashikawa, N., Yamamoto, N. Sakurai, H. (2007) Different mechanisms are involved in the transcriptional activation by yeast heat shock transcription factor through two different types of heat shock elements. J Biol Chem 282, 10333–10340.

Hietakangas, V., Ahlskog, JK., Jakobsson, AM., Hellesuo, M., Sahlberg, NM., Holmberg, CI., Mikhailov, A., Palvimo, JJ., Pirkkala, L., Sistonen, L. (2003). Phosphorylation of serine 303 is a prerequisite for the stress-inducible SUMO modification of heat shock factor 1. Mol Cell Biol. 23(8): 2953-68.

Iwamatsu, T. (2004). Stages of normal development in the medaka *Oryzias latipes*. Mechanisms of Development 121, 605-618

Jakobsen, BK., Pelham, HR. (1988) Constitutive binding of yeast heat shock factor to DNA in vivo. Mol. Cell. Biol. 8, 5040–5042

Jakobsen, BK., Pelham, HR. (1991) A conserved heptapeptide restrains the activity of the yeast heat shock transcription factor. EMBO J. 10, 369–375

Kallio, M., Chang, Y., Manuel, M., Alastalo, TP., Rallu, M., Gitton, Y., Pirkkala, L., Loones, MT., Paslaru, L., Larney, S. et al. (2002) Brain abnormalities, defective meiotic chromosome synapsis and female subfertility in HSF2 null mice. EMBO J 21, 2591–2601.

Kim, DS., Lee, Y., Hahn, Y. (2010). Evidence for bacterial origin of heat shock RNA1. RNA 16(2), 274-9.

Landschulz, WH., Johnson, PF., McKnight, SL. (1988) The leucine zipper: a hypothetical structure common to a new class of DNA binding proteins. Science 240(4860):1759-64.

Larson, JS., Schuetz, TJ., Kingston, RE. (1988). Activation in vitro of sequence-specific DNA binding by a human regulatory factor. Nature. 22;335(6188): 372-5.

Lipson, SG., Lipson, HS., Tannhauser, DS. (1997) Laser. In: Lipson, SG., Lipson, HS., Tannhauser, DS. Optik. Springer Verlag, Heidelberg, 1997, pp 403-404

Liu, X., Xu, L., Liu, Y., Tong, X., Zhu, G., Zhang, XC., Li, X., Rao, Z. (2009). Crystal structure of the hexamer of human heat shock factor binding protein 1. Proteins. 75(1): 1-11.

Maiman, T.H. (1960). Stimulated optical radiation in ruby. Nature 187 (4736): 493–494.

Min, JN., Zhang, Y., Moskophidis, D., Mivechi, NF. (2004) Unique contribution of heat shock transcription factor 4 in ocular lens development and fiber cell differentiation. Genesis 40, 205–217.

Monsma SA., Ard R., Lis JT., Wolfner MF. (1988). Localized Heat-Shock in Drosophila melanogaster. The Journal of Experimental zoology 247:279-284

Morimoto, R.I. (1993). Cells in stress: transcriptional activation of heat shock genes. Science 259, 1409– 1410.

Morimoto, RI., Sarge, KD., Abravaya, K. (1992) Transcriptional regulation of heat shock genes. A paradigm for inducible genomic responses. J. Biol. Chem. 267, 21987–21990.

Nakai, A., Morimoto, RI. (1993) Characterization of a novel chicken heat shock transcription factor, heat shock factor 3, suggests a new regulatory pathway. Mol. Cell. Biol. 13, 1983–1997

Nakai, A., Tanabe, M., Kawazoe, Y., Inazawa, J., Morimoto, RI., Nagata, K. (1997) HSF4, a new member of the human heat shock factor family which lacks propertiesof a transcriptional activator. Mol Cell Biol 17, 469–481.

Naruse, K., Hori, H., Shimizu, N., Kohara, Y., Takeda, H. (2004). Medaka genomics: a bridge between mutant phenotype and gene function. Mech Dev. 121, 619-28.

Negrutskii, BS., El`skaya, AV. (1998) Eukaryotic translation elongation factor 1 alpha: Structure, expression, functions, and possible role in aminoacyl-tRNA channeling. Prog. Nucleic Acid Res. Mol. Biol. 60, 47 –78.

Panniers, R. (1994) Translational control during heat shock. Biochimie 76, 737–747.

Pirkkala, L., Nykänen, P., Sistonen, L. (2001). Roles of the heat shock transcription factors in regulation of the heat shock response and beyond. FASEB J. 15(7):1118-1131.

Rabindran, SK., Haroun, RI., Clos, J., Wisniewski, J., Wu, C. (1993) Regulation of heat shock factor trimer formation: role of a conserved leucine zipper. Science 259, 230–234

Rabindran, SK., Wisniewski, J., Li, L., Li, GC., Wu, C. (1994) Interaction between heat shock factor and hsp70 is insufficient to suppress induction of DNA-binding activity in vivo. Mol. Cell. Biol. 14, 6552–6560.

Ramos DM., Kamal F., Wimmer EA., Cartwright AN., Monteiro A. (2006). Temporal and spatial control of transgene expression using laser induction of the hsp70 promoter. BMC Dev Biol. 20;6:55.

Reinke, H., Saini, C., Fleury-Olela, F., Dibner, C., Benjamin, IJ., Schibler, U. (2008) Differential display of DNA binding proteins reveals heat-shock factor 1 as a circadian transcription factor. Genes Dev 22, 331–345.

Ritossa, F. (1962). A new puffing pattern induced by temperature shock and DNP in Drosophila. Experientia 18, 571–573.

Roest Crollius, H., Weissenbach, J., (2005). Fish genomics and biology. Genome Res. 15, 1675-82.

Sakurai, H., Takemori, Y. (2007). Interaction between heat shock transcription factors (HSFs) and divergent binding sequences: binding specificities of yeast HSFs and human HSF1. J Biol Chem. 282(18):13334-41.

Sano, K., Inohaya, K., Kawaguchi, M., Yoshizaki, N., Iuchi, I., Yasumasu, S. (2008). Purification and characterization of zebrafish hatching enzyme – an evolutionary aspect of the mechanism of egg envelope digestion. FEBS J. 275(23): 5934-46

Sarge KD., Murphy SP., Morimoto RI. (1993). Activation of Heat Shock Gene Transcription by Heat Shock Factor 1 Involves Oligomerization, Acquisition of DNA-Binding Activity, and Nuclear Localization and Can Occur in the Absence of Stress. Mol. Cell. Biol. 13, 1392–1407.

Sato-Maeda, M., Tawarayama, H., Obinata, M., Kuwada, J. Y. & Shoji, W. (2006). Sema3a1 guides spinal motor axons in a cell- and stage-specific manner in zebrafish. Development 133, 937–947

Satyal, SH., Chen, D., Fox, SG., Kramer, JM., Morimoto, RI. (1998) Negative regulation of the heat shock transcriptional response by HSBP1. Genes Dev. 12(13): 1962-74.

Schlesinger, MJ., Ryan, C. (1993) An ATP- and hsc70- dependent oligomerization of nascent heat-shock factor (HSF) polypeptide suggests that HSFitself could be a ”sensor” for the cellular stress response. Protein Sci. 2, 1356–1360.

Shamovsky, I., Ivannikov, M., Kandel, ES., Gershon, D., Nudler, E. (2006) RNA-mediated response to heat shock in mammalian cells. Nature 440, 556–560.

Shamovsky, I., Nudler, E. (2008). New insights into the mechanism of heat shock response activation. Cell Mol Life Sci. 65(6): 855-61.

Shi, Y., Mosser, DD., Morimoto, RI. (1998). Molecular chaperones as HSF1-specific transcriptional repressors. Genes Dev. 12, 654–666.

Shima A, Mitani H. (2004). Medaka as a research organism: past, present and future. Mech Dev. 121(7-8):747-52.

Sistonen, L., Sarge, KD., Morimoto, RI. (1994). Human heat shock factors 1 and 2 are differentially activated and can synergistically induce hsp70 gene transcription. Mol Cell Biol. 14(3):2087-99.

Sorger, PK., Lewis, MJ., Pelham, HR. (1987). Heat shock factor is regulated differently in yeast and HeLa cells. Nature 329: 81-144

Sorger, PK., Nelson, HC. (1989) Trimerization of a yeast transcriptional activator via a coiled-coil motif. Cell 59, 807–813

Sorger, PK., Pelham, HR. (1988). Yeast heat shock factor is an essential DNA-binding protein that exhibits temperature-dependent phosphorylation. Cell 54: 855-64

Stringham EG., Candido EP. (1993). Targeted single-cell induction of gene products in Caenorhabditis elegans: a new tool for developmental studies. The Journal of Experimental zoology 266(3): 227-33

Tai, LJ., McFall, SM., Huang, K., Demeler, B., Fox, SG., Brubaker, K., Radhakrishnan, I., Morimoto, RI. (2002) Structure-function analysis of the heat shock factor-binding protein reveals a protein composed solely of a highly conserved and dynamic coiled-coil trimerization domain. J Biol Chem. 277(1):735-45.

Taylor, ICA., Workman, JL., Schuetz, TJ., Kingston, RE. (1991). Facilitated binding of GAL4 and heat shock factor to nucleosomal templates: differential function of DNA-binding domains. Genes Dev. 5: 1285-98

Thermes, V., Grabher, C., Ristoratore, F., Bourrat, F., Choulika, A., Wittbrodt, J. and Joly, J. (2002). I-SceI meganuclease mediates highly efficient transgenesis in fish. Mechanisms of Development 118, 91-98

Tipler, PA. (1994). Physik. Spektrum Akadem. Verlag GmbH, Heidelberg, München pp. 1292-1298

Trinklein ND., Murray JI., Hartman SJ., Botstein D., Myers RM. (2004). The role of heat shock transcription factor 1 in the genome-wide regulation of the mammalian heat shock response. Mol Biol Cell. 15(3): 1254-61.

Walther, T., Walther, H. (2004). Was ist Licht? Verlag C.H. Beck oHG, München, pp. 54-68

Weber, IT., Steitz TA. (1987). Structure of a complex of catabolite gene activator protein and cyclic AMP refined at 2.5 A resolution. J. Mol. Biol. 198(2): 311-26

Welch, WJ., Suhan, JP. (1985) Morphological study of the mammalian stress response: Characterization of changes in cytoplasmic organelles, cytoskeleton, and nucleoli, and appearance of intranuclear actin filaments in rat fibroblasts after heat-shock treatment. J. Cell Biol. 101, 1198–1211.

Westwood, JT., Wu,C. (1993). Activation of Drosophila heat shock factor: conformational change associated with a monomer-to-trimer transition. Mol Cell Biol. 13(6):3481-6.

Westwood, JT., Clos, J., Wu, C. (1991). Stress-induced oligomerization and chromosomal relocalization of heat-shock factor. Nature. 31;353(6347):822-7.

Wittbrodt, J., Shima, A., Schartl, M. (2002). Medaka-A model organism from the far east. Nature Vol 3, 53-64

Wu, C. (1995). Heat shock transcription factors: structure and regulation. Annu. Rev. Cell Dev. Biol. 11, 441– 469.

Wu, C., Wilson, S., Walker, B., Dawid, I., Paisley, T., et al. (1987). Purification and properties of Drosophila heat shock activator protein. Science 238: 1 247-53

Xiao, X., Zuo, X., Davis, AA., McMillan, DR., Curry, BB., Richardson, JA., Benjamin, IJ. (1999) HSF1 is required for extra-embryonic development, postnatal growth and protection during inflammatory responses in mice. EMBO J 18, 5943–5952.

Yamamoto, M. Hatching Gland and Hatching Enzyme. In: T. Yamamoto, (Ed.), MEDAKA (killifish): Biology and Strains. Keigaku Pub. Co., Tokyo, 1975, pp. 73-79.

Yamamoto, N., Takemori, Y., Sakurai, M., Sugiyama, K., Sakurai, H. (2009). Differential recognition of heat shock elements by members of the heat shock transcription factor family. FEBS J. 276(7):1962-74.

Zou, J., Guo, Y., Guettouche, T., Smith, D. F., and Voellmy, R. (1998) Repression of heat shock transcription factor HSF1 activation by HSP90 (HSP90 complex) that forms a stresssensitive complex with HSF1. Cell 94, 471–480

6 Anhang

Zusammenfassung

Der Heat Shock Response Mechanismus ist ein hochkonserviertes Reparatursystem und spielt eine wichtige Rolle, wenn ein Organismus zu hohen Temperaturen ausgesetzt ist. Bei einem Temperaturanstieg trimerisieren Heat Shock Faktoren (HSFs) und binden an spezielle Heat Shock Promotoren in der DNA. Dies führt zu einer Produktion von Heat Shock Proteinen, von denen die meisten als Chaperone fungieren, die Proteine, die aufgrund erhöhter Temperatur beschädigt wurden, reparieren und wieder in ihre ursprüngliche Konformation bringen. Heat Shock Promotoren werden häufig verwendet, um die Überexpression oder die zeitlich versetzte Expression eines Entwicklungsgenes zu untersuchen. Mittels Klonieren wurden Heat Shock Promotoren als Promotoren für Gene verwendet, deren Funktion untersucht werden sollte. Ein künstliches Promotor Konstrukt wurde in die Embryos von *Oryzias latipes* injiziert. Medaka Fische (*Oryzias latipes*) ist ein kleiner Fisch aus der Familie der Reisfische der oft in der Entwicklungsbiologie als Modellorganismus verwendet wird. Seine gut erforschte Embryogenese und seine durchsichtigen Eier machen ihn zu einem idealen Organismus für die untersuchten Fragestellungen. Im ersten Teil der Arbeit wurde versucht mittels eines Laserstrahles in einer einzelnen Zelle einen Heat Shock hervorzurufen, um ein Verfahren zu entwickeln, um später die Expression von Entwicklungsgenen in bestimmten Zellen oder Geweben zu untersuchen. Zwar konnte gezeigt werden, dass es mit einem Laser möglich ist einen Heat Shock Promotor zu induzieren, doch gelang dies nicht in einzelnen Zellen aufgrund der technischen Spezifikationen des Lasers. Im zweiten Teil der Arbeit wurde versucht Heat Shock Promotoren mit Hilfe eines adaptierten Mikrowellengerätes, bei welchem die Zeit in Intervallen von Millisekunden gesteuert werden konnte zu induzieren. Es zeigte sich, dass dies möglich ist und es konnten Daten gewonnen werden, mit denen weitergearbeitet wurde. Mit Hilfe der Daten aus den Laser- und Mikrowellenexperimenten wurde eine

Heat Shock Variante entwickelt, bei der Medaka Embryos im PCR Cycler behandelt wurden, um einen Heat Shock zu induzieren. Es konnte eine Methode entwickelt werden, deren Induktionsrate 33-fach höher ist, als jene, mit der Medaka Embryos üblicherweise behandelt werden, um eine Heat Shock Induktion zu erreichen.

Abstract

The heat shock response system is a highly conserved repair mechanism and plays an important role, when an organism is confronted with high temperatures. Upon rising of the temperature heat shock factors (HSFs) trimerize and bind to heat shock promoters in the DNA. This leads to an expression of heat shock proteins (HSPs), which act mostly as chaperones that repair proteins, which are damaged because of too high temperatures and bring them back to their original conformation. Heat shock promoters are often used to analyze the overexpression or the expression of a developmental gene at a different point in time than normal. Via cloning heat shock promoters have been used as promoters for genes whose function had to be analyzed. An artificial promoter construct was injected into the embryos of Oryzias latipes. Medaka fish (Oryzias latipes) is a small fish, which is often used in developmental biology as a model organism. Its embryogenesis is well known and its clear made it an ideal organism for the experiments. In the first part of the diploma thesis it was tried to use a laser to induce a heat shock in medaka embryos in a single cell to develop a method to analyze the functions of developmental genes in certain cells or tissues. Indeed it could be shown that it is possible with the help of a laser to induce a heat shock, but it could not be done in single cells because of the technical specifications of the laser. In the second part of the thesis it was tried to induce heat shocks using an adapted microwave oven, in which the durations could be controlled in intervals of milliseconds. It showed, that it is possible and data were obtained, which were used for further work. With the help of the data from the laser and the microwave experiments a heat treatment variant was developed, in which the embryos were heat treated in a PCR cycler to induce a heat shock. It was possible to develop a method for a heat treatment of medaka embryos in which the induction rate is 33-times higher than in the standard heat shock treatment method for medaka embryos.

List of abbrevations

CAP	catabolite activator protein
CE2	conserved element 2
CMV	cytomegalovirus
CTA	C-terminal activator
DBD	DNA binding domain
eEF1A	translongation elongation factor1
ERM	embryo rearing medium
GFP	green fluorescent protein
g.o.i.	gene of interest
HR	heptad repeats
HSBP1	heat shock factor binding protein
HSE	heat shock element
HSF	heat shock factor
HSP	heat shock protein
HSR	heat shock response
HSR1	heat shock RNA1
NTA	N-terminal activator
pA	polyadenylation signal
Sce	*Saccharomyces cervisiae*
SuA	suppressor of activation
SuT	suppressor of trimerization
MDCK	Madin–Darby canine kidney
p23	prostaglandin E synthase 3
Ser	serin
Thr	threonin
Tyr	tyrosin
UTR	untranslated region
SV40	simian virus40
WT	wildtype

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