Harnessing the Heat Shock Response to Raise Refined Therapeutic Outcomes

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HARNESSING THE HEAT SHOCK RESPONSE TO RAISE Refined THERAPEUTIC OUTCOMES

By
Alexis K. Hall

A DISSERTATION
Submitted to the Faculty of the University of Miami in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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HARNESSING THE HEAT SHOCK RESPONSE TO RAISE REFINED
THERAPEUTIC OUTCOMES

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Activated Heat Shock Transcription Factor 1 (HSF1) has received attention in recent literature as a therapeutic effector in diseases of protein misfolding, as an immune modulating adjuvant in tumor regression, and as a trigger for gene therapy transcription. In its normal function, activated HSF1 enhances heat shock protein (Hsp) expression when additional molecular chaperoning is required (i.e., in situations of proteotoxic stress, including thermal stress) in a process known as the heat shock (HS) response. Thus, HSF1 acts as an environmental sensor, and a harness based on the biology of this capability enables transcription of genes for engineered purposes. The hypothesis of this thesis is that a harness of the heat shock response, when paired with a therapeutic mechanism, will refine novel therapies. Extensions to the concept of deliberately activating HSF1’s normal functions for therapeutic purposes are examined through in vitro trials and in vivo preliminary studies that feature the use of HSF1 as a regulator of therapy.

Successful in vitro work translated to pioneering preclinical studies, launched at the University of Florida’s Center for Environmental and Human Toxicology. Collaboration supported the development of an innovative project to treat solid tumors using a recombinant virus system. The system was designed
to facilitate intratumoral delivery of a previously characterized molecular switch, which was newly engineered to control cytotoxic gene transcription that produced dramatic consequences in cells of human origin. Central to the targeting of the \textit{in vivo} therapy, is a transient, initial trigger: a thermal dose, delivered to solid tumors, which localizes HSF1 activation (a constitutively active mouse HSF1 construct was also produced to aid clarification of physiological consequences associated with deliberately upregulating HSF1 activity \textit{in vivo}). Gene transcription was expected to ensue to both cause and sustain tumor regression through other regulatory elements of the molecular switch. Results demonstrated practical potential to achieve a therapeutic outcome of solid tumor regression and define contemporary challenges that continuing research directions (e.g.: production of additional viral vectors, an improved animal model, and a refined heat system) now confront in order to target and safely regulate even more potent, novel therapeutic agents.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>LIST OF FIGURES</strong></td>
<td>vi</td>
</tr>
<tr>
<td><strong>Chapter</strong></td>
<td></td>
</tr>
<tr>
<td><strong>1 INTRODUCTION TO THE HEAT SHOCK RESPONSE</strong></td>
<td>1</td>
</tr>
<tr>
<td>Heat Shock Transcription Factor 1 and the Heat Shock Response</td>
<td>1</td>
</tr>
<tr>
<td>Normal function as related to chaperones</td>
<td>2</td>
</tr>
<tr>
<td>Activity-related studies</td>
<td>2</td>
</tr>
<tr>
<td>Therapeutic outcomes are possible with a harnessed Heat Shock Response</td>
<td>9</td>
</tr>
<tr>
<td>Extensions to the concept of harnessing the heat shock response in terms of enhanced molecular chaperoning</td>
<td>9</td>
</tr>
<tr>
<td>Extensions to the concept of harnessing the heat shock response in terms of activating gene transcription</td>
<td>11</td>
</tr>
<tr>
<td>The SafeSwitch conceptual answer to practical considerations relevant to gene therapy</td>
<td>19</td>
</tr>
<tr>
<td>Specific aims</td>
<td>25</td>
</tr>
<tr>
<td><strong>2 HARNESSING THE HEAT SHOCK RESPONSE</strong></td>
<td>26</td>
</tr>
<tr>
<td>Pairing the SafeSwitch harness of the Heat Shock Response with a potent gene target: GALV(+)</td>
<td>26</td>
</tr>
<tr>
<td>Rationale for the approach of pairing SafeSwitch regulation with the GALV(+) gene target</td>
<td>27</td>
</tr>
<tr>
<td>Initial trial of SafeSwitch regulated expression of the GALV(+) gene target</td>
<td>28</td>
</tr>
<tr>
<td>Creation of the GALV(+) SafeSwitch adenovirus shuttle vector</td>
<td>32</td>
</tr>
<tr>
<td>Creation of the GALV(+) SafeSwitch stable cell line construct</td>
<td>34</td>
</tr>
<tr>
<td>Screening cancer cell lines for good expression of the GALV(+) target gene</td>
<td>35</td>
</tr>
<tr>
<td>Species-specific limitations to GALV(+)‑mediated cell to cell fusion</td>
<td>37</td>
</tr>
<tr>
<td>Screening rat lines for good expression of a GALV(+) SafeSwitch</td>
<td>40</td>
</tr>
<tr>
<td>Delivering the GALV(+) SafeSwitch to rat cells in vitro via transient transfection</td>
<td>44</td>
</tr>
<tr>
<td>GALV SafeSwitch cell lines</td>
<td>45</td>
</tr>
<tr>
<td>Addition of a conditionally replicative, oncolytic adenovirus lowers the activation threshold for SafeSwitch regulated cell death in vitro</td>
<td>51</td>
</tr>
<tr>
<td>The Oncolytic SafeSwitch system</td>
<td>52</td>
</tr>
<tr>
<td>Combining the GALV(+) SafeSwitch and the Oncolytic SafeSwitch in vitro</td>
<td>55</td>
</tr>
<tr>
<td>In vitro section summary</td>
<td>58</td>
</tr>
</tbody>
</table>
2.9 Side by side comparison of GALV(+) SafeSwitch-mediated cell to cell fusion as a function of heat treatment temperature in the rat F98 #6 line and the human HT-1080 #4 line......... 47

2.10 The rat F98 #6 line displays less profound GALV(+) mediated fusion phenotype in response to full switch activation than the human HT-1080 #4 line........................................ 48

2.11 GALV(+) SafeSwitch cell line construct transiently and stably transfected in three human cell lines............................... 49

2.12 The HT-1080 derived #4 cell line shows dramatic GALV(+) SafeSwitch-mediated cell death at 48 hours post ligand application and heat treatment........................................... 50

2.13 A demonstration of the efficacy of the Oncolytic SafeSwitch in recombinant Ad-infected HeLa cells................................. 53

2.14 Cell death in the HT-1080 #4 line as a function of: heat treatment temperature, mifepristone, rAd1 infection, and HSF1(+) transfection................................................................. 56

3.1 The rat unilateral circulating water bath treatment heats a single leg of up to four sedated rats at one time......................... 63

3.2 Average tumor volume related to percent increase of growth as indicators of treatment outcomes in nude rats bearing tumors formed from HT-1080 #4 cells................................. 66
3.3 Effects in nude rats bearing tumors formed from WT HT-1080 cells, following early electroporation of HSF1(+) and GALV(+) SafeSwitch DNA……………………………………………………………. 68

3.4 Repeated intratumoral electroporation with the GALV(+) SafeSwitch and HSF1(+) constructs inhibits WT F98 tumor growth in nude rats…………………………………………………………… 70

3.5 The rat bilateral circulating water bath heat treatment was designed to heat both legs and the lower back of up to five sedated rats at one time…………………………………………………………… 72

3.6 Intramuscular in vivo electroporation of fLuc SafeSwitch DNA in rats…………………………………………………………………………………………………… 74

3.7 Intramuscular in vivo electroporation examines Hsp70 promoter activity and transcriptional activity of SafeSwitch TA constructs as a function of mifepristone modes of administration in Fischer 344 rats and in nude mice……………….. 76-77

3.8 An immunocompetent rat model, which examines tumor location and influences on tumor outgrowth that are associated with expression of human mutant HSF1 constructs and a fluorescent protein……………………………………… 81

3.9 Activating the GALV(+) SafeSwitch in vivo in an immunocompetent tumor outgrowth model that also examined co-expression of human HSF1(+) and HSF1(-)/AVST……………………………………………………………………………… 84
3.10 The mouse bilateral circulating water bath heat treatment
was designed to heat both legs and the lower back of up to
eight sedated mice at one time................................. 88

3.11 Comparative in vivo and in vitro luciferase reporter results of
SafeSwitch-mediated cell fusion occurring with the use of
stably transfected HT-1080 #4 cells............................. 90-91

3.12 Early and repeated heat treatments in an outgrowth
experiment utilizing nude mice bearing tumors formed from
HT-1080 #4 cells...................................................... 92

3.13 Outgrowth study in nude mice bearing control tumors or
tumors formed from HSF1(+)- transfected HT-1080 #4 cells… 94

3.14 A combination of the Oncolytic SafeSwitch and the GALV(+)
SafeSwitch in tumors formed from HT-1080 #4 cells in nude
mice offers potential to cause tumor regression................. 97

3.15 The Combination Approach to SafeSwitch Tumor Therapy,
entirely delivered to tumors formed from WT HT-1080 cells,
caused regression in a nude mouse............................... 100

3.16 The Combination Approach to SafeSwitch Tumor Therapy
utilizing tumors formed from HT-1080 #4 cells in nude mice
also yields regulated regression outcomes...................... 102

3.17 Laser heating method to create a focused thermal dose…… 105
3.18 Therapeutic effect of the Combination Approach to SafeSwitch Tumor Therapy delivered genes to tumors in nude mice, which were formed from WT HT-1080 cells, is activated by a focused, laser-delivered thermal dose………………………………………………………………… 107

3.19 Laser thermal dose compared to HSF1(+) as a means to activate the SafeSwitch to cause tumor regression in nude mice bearing tumors formed from HT-1080 #4 cells………… 109

4.1 Harnessing the constitutive activity of mouse HSF1 with a new construct: mHSF1(+)……………………………………………………………… 129

4.2 Rosa 26 ImHSF1(+) regulates activity of the constitutively active mouse HSF1…………………………………………………………… 130-131

4.3 Regulated activity of mHSF1(+) and consequential Hsp70 production in mouse and human cell lines that are stably transfected with Rosa 26 ImHSF1(+)…………………………………… 133-134
Chapter 1 – Introduction to the Heat Shock Response

Heat Shock Transcription Factor 1 and the Heat Shock Response

The heat shock (HS) response is a term that denotes a collective set of responses within cells, which occurs when there is a shift in cellular balance in response to unfavorable events. Events, such as an applied thermal dose of approximately 44°C for thirty minutes in mammalian cells, immediately cause denaturation of a substantial fraction of intracellular proteins. Several physiological conditions, including oxidative damage that may result in proteotoxic stress, are capable of producing a fraction of stress-unfolded proteins of a magnitude that is sufficient to trigger the notable transcriptional activity observed with the HS response (e.g., Zou et al., 1998). The characteristic end result of a HS response is an increased level of heat shock protein (Hsp) chaperones. The most characterized functions of these molecular chaperones encompass their ability to accelerate refolding of proteins to functional conformations and their ability to facilitate protein degradation via the ubiquitin-proteasome pathway. Biochemistry studies (e.g., Zuo et al., 1994), have revealed that activation of Heat Shock Transcription Factor 1 (HSF1) is the driving force behind the transcriptional enhancement, which results in the noted Hsp production changes. The factor accomplishes this function in a manner that is akin to an environmental sensor.

HSF1-mediated transcriptional enhancement, as well as the often cytoprotective effects of the cohort of molecular chaperones that are upregulated
with its characteristic activity, has stirred attention to the potential for therapeutic applications. Recent published investigations (reviewed in Voellmy, 2005b), which utilize HSF1, have examined aspects of its potential to ameliorate diseases that feature aberrantly folded protein. Therapeutic potential may extend to clinical tumor regression outcomes, as well. In the latter situation, HSF1 activity may be harnessed to result in cancer cell death by toggling immunity and by promoting gene therapy transcription.

*Normal function as related to chaperones*

The normal function of activated HSF1 is to enhance Hsp expression when additional molecular chaperoning is required to mitigate proteotoxic stress. There are several modifications that occur to HSF1 (see Voellmy, 2006, for a comprehensive review) in order to produce the active factor that causes the HS response. The HSF1 monomer must be rendered available to trimerize, it must translocate to the nucleus, and once within the nucleus, it must bind to DNA as well as acquire transcriptional competence. This section presents a brief overview of HSF1 regulation, as many reviews have recently been published (e.g., Voellmy, 2007, 2006, 2004a and 2004b).

*Activity-related studies*

The contemporary view regarding the state of HSF1, prior to activation, is that it is maintained in a monomeric form in the cytoplasm of unstressed cells through a malleable heterocomplex that involves Hsp90 (e.g., Nair et al., 1996,
Figure 1.1 Schematic of the dynamic processes that regulate HSF1 activity and the heat shock response. The “I” indicates immunophilin, which is present, along with Hsp90 and p23, in a multichaperone complex that binds the inactive, monomeric form of HSF1. The numbers “70” and “40” refer to those specific Hsp species, and “52” refers to FKBP52. The lightning bolt symbolizes HS or another activator of the stress response, which affects the formation of subsets of HSF1 complexes, as well as the various players (Daxx, CamKII kinase, and ERK1/GSK3) that act on those subsets. Starburst symbols flag phosphorylation events that are associated with factor activation, whereas the open circles denote phosphorylation events that are known to be associated with factor deactivation. The shapes associated with HSF1 after activating phosphorylation has occurred indicate proteins which may be part of the complex formed by that subset of trimerized HSF. The crescent moon symbolizes Symplekin, and the hexagon shapes symbolize the Symplekin-interacting polyadenylation factors: CstF-64 and CPSF. These proteins are included in the diagram based on a report by Xing et al (2004), which suggests that Symplekin functions as a scaffold for associated proteins to add themselves to the complex, specifically in heat-stressed cells. Dynamic processes thus occur. These processes may play roles in the regulation of the transcriptional competence attained by active HSF1, which is able to bind heat shock element (HSE) sequence upstream of inducible heat shock protein (Hsp) genes. In the HS response, only the subset of active HSF1 that eludes repressive chaperone complex formation is able to cause transcription of the indicated genes: Hsp90, Hsp70, and Hsp40 (among others). mRNA transcripts are respectively translated to produce the characteristic HS proteins.
and Guo et al., 2001), as well as p23 (Bharadwaj et al., 1999) and immunophilin (Duina et al., 1998). Fig. 1.1 was inspired by an illustration from Voellmy (2006), in which the available information of HSF1 regulated activation was integrated with findings from Pratt and Toft (1997) as well as Marchler and Wu (2001). Competitive binding of Hsps and co-chaperones, created by an equilibrium shift following the formation of aberrantly folded proteins, releases monomeric HSF1 and facilitates homotrimerization. A subset of the trimerized HSF eludes the formation of chaperone complexes, which notably contain FKB52 and Hsp90 (Guo et al., 2001) in addition to several other chaperones. That subset is able to successfully translocate to the nucleus of the stressed cells, and it increases in fractional size in a manner that is proportional to the intensity of stress over time.

Within the nucleus, trimeric HSF1 binds DNA at highly conserved heat shock element (HSE) sequences that are upstream of stress response genes. HSEs comprise a pattern of three or more pentameric-nucleotide repeats (nGAAn) that alternate in orientation (Williams and Morimoto, 1990). With additional activation changes, which include phosphorylation and sumoylation, trimeric HSF1 acquires the competence necessary to amplify transcription rate and processivity. Rapidly, this activity on HS promoters is reflected in the production of increased levels of HS proteins.

Fig. 1.2 presents the basic anatomy of the human HSF1 monomer and was also inspired by a figure published by Voellmy (2006). The HSE DNA-binding domain is composed of a Helix-Turn-Helix motif and is located at the N-terminus. The C-terminus is noted for a pattern of repeat sequence and the
Figure 1.2 Functional properties as they relate to sequence of human HSF1 and two deletions that form mutants commonly utilized in activity studies. The sequence present for each of the two transcription factor mutants, HSF1(-) (a.k.a. HSF1(-)/AVST) and HSF1(+), are indicated with dotted, horizontal lines. The dotted lines relate to the wild type (WT) sequence represented by the hatched bar (across the top of the figure). The regions that lead to HSF1 transcriptional competence are indicated with solid lines or points and are grouped in order of event occurrence: trimerization (the domain that enables factor self-association is further flagged by the symbol for trimeric HSF1: three crossed bars), nuclear localization, DNA-binding, and transcriptional activation / regulation. The Transcriptional Activation Domains, which are in the vicinity of the absent sequence in the HSF1(-) construct, and the Regulatory Domain, which extends just beyond the gap in the HSF1(+) construct, are noted in order to relate each indicated region to the functional property acquired by the respective deletion mutant. The horizontally oriented, italicized numbers, located just below the sequence bar, demarcate the relative amino acid positions. CTR stands for C-terminal repeat sequence. LZ1, LZ2 and LZ3 indicate the hydrophobic repeat regions known as leucine zippers. Respective amino acid sequence numbers (above the sequence bar) note twelve residues that are known to be modified by phosphorylation. Starburst symbols flag phosphorylation sites that have been linked to factor activation, whereas the open circles denote sites that are known to be associated with factor deactivation. Additional sequence information that pertains to WT HSF1 and the two mutants is presented in Appendices 2 and 4.
location of the Transcription Activation Domain. The Regulatory Domain is important to the transcriptional activity of the factor and spans the middle of the sequence. That domain is noted to be the region deleted in the constitutively active mutant, HSF1(+) (Xia et al., 1999, as well as Zuo et al., 1994 and 1995).

Two proximal hydrophobic repeat sequences, which are known as leucine zippers (LZ1 and LZ2) are located just 5' to it. A third leucine zipper (LZ3) is 3' to it, though it is located more distally. The areas known to permit trimerization as well as those that enable its repression, along with the mapped nuclear localization signals and the sequence that is DNA binding, are noted in the figure. Two domains responsible for transcription activation are located on the C-terminus of the sequence and flag the relevant region that is missing in the dominant negative mutant, HSF1(-)/AVST (Xia et al., 1999, and Zuo et al., 1995).

Hyperphosphorylation is another characteristic of activated HSF1. Studies into the consequences of thermal stress in cells of human origin have revealed that a series of phosphorylation events results in changes to twelve, and perhaps more, residues in HSF1 that are highlighted in Fig. 1.2 (and are also noted in Appendix 4). Only some of the consequences of these events are clear (see Fig. 1.1). Phosphorylation of human HSF1 at Ser\textsuperscript{230} (by CamKII kinase) and Ser\textsuperscript{326} is associated with both factor activation and transcriptional enhancement (Holmberg et al., 2001; Guettouche et al., 2005). Following thermal stress, Daxx, a co-activator for HSF1, is disassociated from promyelocytic leukemia oncogenic domains (PODs) and binds to and enhances transcriptional activity of HSF1 (Nefkens et al., 2003 and Boellmann et al., 2004). Conversely, phosphorylation
of human HSF1 at Ser$_{303}$, Ser$_{307}$, and Ser$_{363}$ by GSK-3, ERK, and JNK, while a consequence of HS (He, B. et al., 1998; Dai et al., 2000; Xavier et al., 2000), are rather associated with factor deactivation and transcriptional repression (Knauf et al., 1996; Chu et al., 1996; Kline and Morimoto 1997; Xia et al., 1998; Hietakangas et al., 2003 and Guettouche et al., 2005 for Ser$_{303}$ and Ser$_{307}$, as well as Chu et al., 1998 and Dai et al., 2000 for Ser$_{363}$). Dephosphorylation of the respective residues occurs during stress recovery (Hietakangas et al., 2003).

Products of DNA mutagenesis have facilitated basic studies that pertain to cellular consequences of enhanced or abrogated HSF1 function. Two human HSF1 mutants have been particularly instrumental (reviewed in Voellmy, 2005a). The respective deleted sequence is indicated in Fig. 1.2. One lacks amino acid residues 203-315 (see Appendix 4 for DNA sequence information), which comprises approximately two-thirds of the HSF1 Regulatory Domain. It is known as the constitutively active factor, HSF1(+) (Xia et al., 1999), though it was originally coined the “BH-S mutant” and described by Zuo et al. (1994 and 1995). When it is expressed at high cellular concentrations, it is found to be constitutively trimeric, DNA binding and capable of transactivating an Hsp70 promoter-driven reporter gene in the absence of stress. Conversely, the HSF1(-) / AVST mutant (a.k.a. HSF1(-) in Xia, 1999, and AV-ST in Zuo, 1995) yields a dominant negative phenotype. The latter mutant lacks a large portion of the second activation domain located on the C-terminus of WT HSF1 (see sequence information in Appendix 4). The theory regarding the associated phenotype, which is observed when the mutant is overexpressed, is that this deletion renders
it incapable of HS promoter transactivation. Since this mutant maintains DNA binding capability, it may be able to negatively influence the competitive binding of endogenous (WT) HSF1 to the same promoters. In this manner, WT HSF1 would no longer be capable, even in an active state, of causing transcription of the downstream genes. Through overexpression studies, the above mutants have enabled the direct study of cellular effects that are influenced by the transcriptional state of HSF1, since those mutants circumvent regulation that normally accompanies the HSF1 oligomeric states.

Activity regulation that acts on oligomeric and monomeric forms of HSF1 has been examined, and the schematic presented in Fig. 1.1 highlights key aspects that occur prior to and subsequent to cellular stress. As mentioned previously, HS shapes the plastic complexes that are comprised of chaperones, co-chaperones and HSF1 forms. The magnitude of the HS response proportionately follows an equilibrium shift, which produces an expanded number of a particular subset of HSF1 oligomeric forms. A fraction of that subset is able to translocate to the nucleus, where it increases transcriptional activity. Conversely, stress recovery drives a return to the previous equilibrium state of the factor and chaperone association, which then restores basal repression of HSF1 activity. Thus, the biology of HSF1’s function as an environmental sensor is, at least, partially understood.
Therapeutic outcomes are possible with a harnessed Heat Shock Response

Since endogenous HSF1 normally acts within cells as an environmental sensor that triggers gene transcription to restore homeostasis following HS, a natural extension of this concept is that there might be a way to engineer a therapy that harnesses this aspect of its functional biology. There are two applications for such a therapy. The first would enhance chaperoning in a preconditioning or supplementary manner. The second would capitalize upon its regulated, transcriptional enhancement of genes downstream of HS promoters. These applications are discussed in this section.

Extensions to the concept of harnessing the heat shock response in terms of enhanced molecular chaperoning

Proactive or reactive, deliberate enhancement of molecular chaperoning may offer a means to impact a variety of human diseases that share a commonality concerning problems with protein folding, aggregation, and trafficking (see Chaudhuri and Paul, 2006 for a table that lists several of these diseases). Of particular interest is the broad range of neurodegenerative disorders that fall into this category and include: Parkinson’s disease, Alzheimer’s disease, amyotrophic lateral sclerosis (ALS), and polyglutamine diseases (Taylor et al., 2002). Evidence has supported the hypothesis that enhanced Hsp production might offer protection against the associated toxicity and ameliorate disease states (Auluck et al., 2002; Cummings et al., 2001; Kazemi-Esfarjani and Benzer, 2002; as well as Slavotinek and Biesecker 2001).
Additionally, some evidence also points to the fact that a coordinated chaperone response, which an HSF1-orchestrated increase in production of Hsp70, Hsp40, and Hsp27/Hsp25 would comprise, may offer greater therapeutic benefit than that afforded by upregulation of a single chaperone (e.g., Chan et al., 2000; Saura et al., 2003; Carver et al., 2003; Dabir et al., 2004; Zourlidou et al., 2004; Auluck et al., 2005; and Cohen et al., 2006). Pharmacological chaperones (Arakawa et al., 2006) or specific, pharmacologic means to safely activate endogenous, intracellular HSF1, may eventually become available as advances in drug discovery continue (e.g., Westerheide and Morimoto, 2005).

In absence of such a pharmacologic means to safely activate HSF1, a suitable model is required to elucidate the cytoprotective function and physiological consequences of a coordinated chaperone response. A model would enable important determinations, including: quantification of the maximum amplified levels of Hsps that would be achievable by active HSF1, measurement of the length of time that elevated levels could be sustained, assessment of the physiological tolerance of such levels, and evaluation of the merit of an HSF1-based strategy to mitigate or prevent respective disease progression. Should a pharmacologic means to safely activate HSF1 be identified, therapeutic efficacy tests may be performed in animal models that are available and that recapitulate aspects of neurodegenerative and other diseases (Shen et al., 2005, and Fujimoto et al., 2005). Studies, including models of neurodegenerative disease (e.g. chemical lesion animal models) and ischemic stroke-reperfusion injury, suggest that protective effects may also be achieved by increasing molecular
chaperoning to combat consequences of endoplasmic reticulum stress (e.g., Christians, et. al, 2002; Lindholm et al., 2006; Szegezdi et al., 2006; Azfer et al., 2006; Martindale et al., 2006; and a commentary by Benjamin, 2006).

Furthermore, use of chemical chaperones [e.g., bile acids (Ramalho et al., 2006) and sodium 4-phenybutyrate (Qi et al., 2004; Singh et al., 2006; Kubota et al., 2006)] mitigated abnormalities in cell and animal models of dysfunction, each of which recapitulated a condition related to diabetes, obesity, aging or disorders associated with polyglutamine expression, tauopathies, and Alzheimer's Disease (Loo and Clark, 2007; Paul, 2007; Tveten et al., 2007; Ansar et al., 2007; Hansen et al., 2007; and Ozcan et al., 2006). Thus, treatment approaches that enhance molecular chaperoning offer great promise and warrant further exploration.

*Extensions to the concept of harnessing the heat shock response in terms of activating gene transcription*

Capitalizing on HSF1's ability to regulate the transcriptional enhancement of genes downstream of HS promoters may ideally answer the current bottleneck present in cancer gene therapy studies. Cancer gene therapy studies are hindered by safety concerns surrounding *in vivo* gene delivery systems that lack tumor-specific targeting and efficiency in gene delivery (as commented by Vile et al., 2000). The biological function of endogenous HSF1, which triggers gene transcription subsequent to an applied HS, offers a means to engineer targeting of cytotoxic gene transcription to tumors in a manner that may synergistically work with standard-care cancer treatments. Targeting of potent gene products is
especially important, since efficient gene delivery vectors could otherwise produce gene transcripts in undesired locations that could harm an organism.

Pertinent to *in vivo* gene delivery, adenovirus (Ad) has an extensive history as the subject of basic science studies and as a means to apply therapy in trials (Wilson, 1996; Dobbelstein, 2004, Lichtenstein and Wold, 2004; Young et al., 2006; Verma and Weitzman, 2005; Davis and Fang, 2005; as well as Davison et al., 2003). Ads are recognized for their capability to infect a broad spectrum of both dividing and non-dividing cells in tissues (Wilson, 1996). The viral vector backbone for gene delivery is correlated with a low incidence of clinically significant effects in immunocompetent individuals. Though there are six species of Ads that comprise the plethora of serotypes, most Ad vectors are derived from serotype 5 (Ad5).

Ad5, like all Ads, has a linear, double-stranded genome. More than 50 native proteins are transcribed by host cell RNA polymerase II from the genome, which is approximately 36 kilobase pairs (kbp) in size. Ad genes are classified according to their expression during the viral replication cycle: early, delayed or late. The early genes are synthesized into RNA before viral DNA replication ensues and include: E1A, E1B, E2, E3 and E4. Thus, they are of particular interest to recombinant Ad gene therapy.

When Ad does replicate, it does so as an episomal element, since adenoviral DNA usually does not integrate within host DNA. E1A is a key viral protein that launches a cascade of viral gene transcription. Consequently, E1A is essential for the assembly of infectious virus particles and also causes the host
cell to enter into the stationary phase of the cell cycle. Recombinant Ad was first prepared by deleting this one gene. The relevant functions of other early genes include viral DNA replication and transcription modulation by E2; evasion and modulation of host immune responses as well as cell lysis by E3; and RNA transport, DNA replication, and transition to late gene expression and apoptosis by E4. Since the products of E3 genes are not essential for viral replication, cloning capacity was expanded to 7.5 kbp with the further deletion of that sequence.

Lack of E1-function may be complemented in infected human embryonic kidney (HEK) 293 cells, which permit recombinant Ad progeny to be produced and packaged into non-enveloped, icosahedral capsids (Graham et al., 1977). Other cell lines are now available that are more well defined for the Ad sequence that they complement in trans (Fallaux et al., 1996 and Imler et al., 1996). Cesium chloride and column chromatography are routine methods utilized to purify replication-deficient Ad for gene therapy purposes, which often yield high titers that are in the range of $10^{12}$ pfu/ml. Thus, adenovirus is a logical vehicle for cancer gene therapy delivery.

As mentioned earlier, the immediate and inherent problem to gene therapy delivery by adenovirus is targeting. Despite the fact that therapeutic effects of intratumorally injected adenovirus primarily remain within the vicinity of the introduced needle-track (Ram et al., 1997), adenovirus is capable of traveling through the bloodstream to many tissues that support its infection. The need for targeting was appreciated by Bramson et al. (1997) and demonstrated by them
through an experiment that illustrated systemic dissemination of an intratumorally administered Ad vector.

HSF1’s ability to respond to environmental perturbations offers an attractive solution to tumor targeting concerns. Since transcription of inducible Hsp genes is increased or activated in essentially any type of cell upon transient exposure to an elevated temperature, the promoters of those Hsp genes may be utilized in recombinant adenovirus to drive transcription of introduced genes. In the case of anti-cancer gene therapy, where the product is cytocidal, there may be additional benefits to using a thermal dose to activate HSF1. Burd and colleagues (1998) described some studies into the mechanisms that yielded antitumor activity after mild, prolonged hyperthermia was applied. Their report establishes potential for hyperthermia to be an effective, nontoxic adjuvant to other synergistic cancer therapies. In the case of transient activation of HSF1, which briefly increases Hsp gene transcription, the chaperone proteins that are produced typically persist for days. There are many synergistic functions of Hsps that have been documented: they facilitate adenovirus replication and expand the number of viral progeny (Haviv et al., 2001); their expression has been associated with a reduction in the immune system-mediated response to the adenovirus vectors themselves (reviewed in Calderwood, 2005); and they dampen the expression of TNFα and other cytokines (Hsp70 inhibits NFKB and HSF1 inhibits NF-IL6). Fortuitously, the molecular chaperone function of Hsps may also act to raise anti-tumor immunity (Djeha et al., 2005; Melcher, 1998; and reviewed in Sreedhar and Csermely, 2004) through: 1) enhancing presentation...
of antigens from lysed cancer cells to antigen-presenting cells (Asea et al., 2000; Vabulas et al., 2002; Suzue et al., 1997; Todryk et al., 1999; Singh-Jasuja et al., 2000; and Castellino et al., 2000); and 2) quenching macrophage-mediated phagocytosis, which elevates secondary necrosis in apoptotic cells, in turn enabling increased cancer-cell antigen presentation to dendritic cells (Gough et al., 2004).

Gough et al. (2004) observed and documented tumor regression outcomes as an indicator of the practical consequences of HSF1 activation in an in vivo examination. The group utilized the constitutively active HSF1(+) mutant in their studies to examine whether sequellent gene expression inhibited secondary tumor growth. Two genetically similar, murine-derived (CMT93neo), stably transfected cancer cell lines were utilized, which differed only in the respective inclusion or exclusion of a strongly-promoted HSF1(+) gene. In an experiment conducted in immunocompetent C57BL/6 mice, tumors were formed with those lines. The tumors, which resulted from each line, grew at comparable rates. Tumors were surgically excised once the diameter reached 1 cm in size. Three weeks later, the mice were challenged again with injections of wild type CMT93 cells in the opposite flank. Results, presented in the group's publication (Gough et al., 2004), demonstrated a dramatic increase in tumor immunity, which was specific to mice that bore primary tumors that expressed HSF1(+). It is possible that the simple expression of a human gene in the mouse host led to production of an immune response. Exposure of the human factor and tumor antigens to the immune system could occur upon natural cell death. However, it
is likely that the overexpression of mouse Hsps in the cells of the line that expressed HSF1(+) stimulated immunity to the WT CMT93 cancer cells. To further the results of their first experiment, the group conducted a second experiment that utilized an active means of inducing cell death in the primary tumors. Gough et al. (2004) created new stably transfected cell lines that expressed HSV TK, which produced apoptosis in those cells when gancyclovir was systemically introduced, and which either did or did not also express HSF1(+). In this second experiment, cells were injected into the intraperitoneal space of immunocompetent C57BL/6 mice. The mice were then treated five days later with an appropriate, systemic dose and course of gancyclovir that was designed to cause cancer cell death. As in the group’s first experiment, mice were given a second challenge with WT cancer cells. Previously unchallenged mice served as an additional control. The findings, also presented in the paper by Gough et al. (2004), again demonstrated dramatic inhibition of secondary tumor growth that was correlated with HSF1(+) expression. Thus, active HSF1 furthered the outcomes of a gene therapy that was designed to cause cancer cell death in vivo.

The above scientific evidence indicates that Hsps, applied heat, and activated HSF1 may benefit adenoviral delivery of gene therapies to cause tumor regression. Thus, a promoter responsive to active HSF1 would be a convenient means to activate therapeutic gene transcription. However, two central concerns warrant discussion when considering the exclusive use of such promoters to drive toxic gene expression. These concerns include the risk for auto-activation
and the inherent limitations of Hsp promoters to sustain promoter activation.

Auto-activation is of concern for an unsupervised patient. Such a situation might occur if adenovirus was received that happened to persist long after the desired therapy duration. Gene expression could then inadvertently be triggered and could increase the potential for adverse consequences. In fact, a temperature that is only a few degrees above normal may increase gene transcription from HS promoters (Parsell and Lindquist, 1993; Welch, 1993; Cotto and Morimoto, 1999; and reviewed in Voellmy, 2004a). Many sources of significant protein unfolding exist that are capable of triggering a HS response, and include: fever, drug reactions, ischemia and strenuous exercise (Mehta et al., 1998; Benjamin et al., 1990; Locke et al., 1995; Venkataseshan and Marquet, 1996; Salminen et al., 1997; Moseley, 1998; Bajramovic et al., 2000; and Shastry et al., 2002), as well as heavy metals or other sources that ultimately cause cellular toxicity.

Generally, the degree to which Hsp expression is enhanced is a function of the intensity of exposure. There is much diversity, however, in the expression profile of Hsps. Some Hsp genes have promoters that are only marginally induced with heat, while others have high basal transcription levels. In actuality, most HS promoters have properties that would hinder practical applications.

One HS promoter is noted, though, to have an expression profile that is well-suited to target gene therapy. The human Hsp70b promoter is highly induced after a transient heat treatment. In fact, in vitro levels have been measured as high as several thousand-fold over background expression, which is essentially negligible (Voellmy et al., 1985; Schiller et al., 1988; Dreano et al.,
In vivo, tremendous control of target gene transcription has also been achieved (Huang et al., 2000; Vekris et al., 2000; Smith et al., 2002; Guilhon et al., 2003; and Xu et al., 2004). Of relevant interest, Ad5, among other viruses, induces Hsp70 protein in infected cells (Nevins, 1982; Kennedy et al., 1985; and LaThangue et al., 1984). This increased production of Hsp70, however, has been attributed to Hsp70a gene transcription as a consequence of E1A products (Simon et al., 1987), which act independent of HSF1 activation (Nevins, 1982; Phillips et al., 1991). Thus, the Hsp70b promoter is acceptable and capable of targeting a gene product in the context of a recombinant Ad5 delivery method.

Yet, the Hsp70b promoter is subject to auto-activation as described above and lacks innate means to maintain persistent Hsp promoter activation. Coupled to the rapid activation of Hsp gene transcription from the Hsp70b promoter, is the inherent cascade, described earlier, that contributes to the down-regulation of the promoter's activity within hours after the initial HS response, even if continuous heat is present (Abravaya, 1991). The limited response time associated with this Hsp promoter does not permit much opportunity to amplify a therapeutic effect, though repeated heat treatments could theoretically reactivate gene expression. To overcome these limitations, molecular switches that utilize the Hsp70b promoter were designed and tested by Vilaboa and colleagues (2005). Other switches, utilized in the context of viral delivery for gene therapy purposes, have recently been reviewed by Goverdhana et al. (2005). The concept of layered gene transcription offers general appeal to broaden therapeutic potential and to confer greater safety. The system presented by Vilaboa and colleagues (2005)
specifically limits the potential disruption of cell physiology, while harnessing heat-assisted benefits. It neither depends upon long-term Hsp promoter activity nor upon frequent delivery of thermal doses, intended to reactivate endogenous HSF1. Furthermore, it is designed to essentially exclude the possibility of auto-activation. Their system appears to ideally answer central concerns surrounding the use of Hsp promoters in the design of tumor treatments.

The SafeSwitch conceptual answer to practical considerations relevant to gene therapy

SafeSwitch is a unique, layered, transcriptional activation concept, which has been published and reviewed (Vilaboa et al., 2005; Vilaboa and Voellmy, 2006, respectively). The concept enables the use of the Hsp70b promoter to trigger and spatially define sustained gene expression. Potentially disastrous, unsupervised, systemic expression in patients subsequent to the therapeutic intervention period is prevented by a requirement for an appropriate small-molecule ligand in order for therapy to occur; ligand withdrawal causes toxic gene transcription to cease. Thus, inadvertent activation of the target gene cannot occur prior to nor subsequent to the designated period. The design, coined “SafeSwitch” by Dr. Richard Voellmy, is composed of two parts (see Fig 1.3 for a schematic of the SafeSwitch concept tested by Vilaboa et al., 2005). One part comprises an engineered transactivator (TA) gene that is transcribed from a dual-control promoter cassette, which is responsive to: a) the small molecule-activated, dimerized protein product of the transactivator gene, itself; or
to b) transient heat through the use of the Hsp70b promoter. The other SafeSwitch part is a transactivator-responsive promoter, identical to the similarly responsive promoter in the promoter cassette, which in this part exclusively promotes transcription of the linked target gene of interest. The SafeSwitch concept was designed to harness the transient application of a thermal dose through activation of endogenous, cellular HSF1. Factor-mediated transcription from the Hsp70b promoter in the promoter cassette produces an inactive, monomeric transactivator protein as a consequence. When the appropriate small-molecule ligand is also present, that transactivator then takes on an active conformation, which in turn produces transcription for additional transactivator protein as well as target gene product. Thus, target gene expression may be sustained in a manner that is dependent on the persistent presence of a small-molecule ligand, as well as on the continued production of ligand-responsive transactivator, but which is later independent of Hsp70b promoter activity.

Several gene switches that feature the SafeSwitch concept were tested extensively by Vilaboa and colleagues in trials (Vilaboa et al., 2005). An example of a thoroughly tested SafeSwitch described in both Vilaboa et al. (2005) and Vilaboa and Voellmy (2006) is sketched here in Fig. 1.3. The concept utilizes a clinically approved, small-molecule ligand, known as mifepristone (Wang, 1997a and 1997b; as well as Sarkar, 2002) to activate a progesterone receptor-based transactivator, called GLP65. The engineered GLP65 transactivator is a protein consisting of a yeast Gal4 DNA-binding domain (residues 2-93) fused to a human progesterone receptor ligand-binding domain (residues 640-914) and a NFκB
subunit p65 transactivation domain (residues 283-551) (Nordstrom, 2002).

Enhanced homodimerization, which produces DNA-binding competence, is enabled by the presence of the synthetic anti-progestin, mifepristone (RU486),

\[
\begin{align*}
\text{+ mifepristone} & \quad \text{TATA} \quad \text{Gal4 UAS} \\
\text{GLP65} & \quad \text{pHsp70b HSE} \\
\text{Firefly Luciferase} \\
\end{align*}
\]

Figure 1.3 The fLuc SafeSwitch schematic. The SafeSwitch comprises two components. Component (A) produces a transactivator (TA) as a consequence of a harnessed HS response. Component (B) consists of a target gene that is linked to a promoter, which responds with activity when the TA that is produced by component (A) acquires transcriptional competence. Dual regulation of the molecular switch is achieved by activation of endogenous HSF1 within cells via a transient heat treatment as well as by mifepristone ligand addition. A representation of the dynamic process associated with HSF1 activation, which results in Hsp70b promoter activation, is shown in the lower half of the figure using the same symbols that were used in Fig. 1.1. The GLP65 gene is then transcribed and translated into a monomeric protein product (illustrated with the half-moon shape). That product does not act as a transactivator, unless the mifepristone ligand is also present. Mifepristone ligand addition (shown with the starbursts in the top half of the figure) permits the GLP65 TA to readily dimerize, which results in transcriptional competence. Feed-forward transcription then occurs as a consequence of the TA’s activity on the Gal4 upstream activating sequence (Gal4 UAS). This facilitates transcription from the minimal TATA promoter and causes production of both more TA, as well as the firefly luciferase target gene product that reports the associated promoter’s activity.
but not by normal progestins (Wang, 1997a and 1997b; Sarkar 2002; as well as Nordstrom, 2002). The promoter cassette that causes production of the GLP65 transactivator combines the Hsp70b promoter with a minimal, Gal4-responsive promoter. Continued presence of the mifepristone ligand enables GLP65 production to be sustained beyond the time of Hsp70 promoter activation. When mifepristone ligand is withdrawn, self-association of the GLP65 protein is lost. Consequently, its transcriptional competence drops and protein levels of both GLP65 and the target gene, which here produces an unstable, firefly luciferase, decline at a rate that reflects each protein’s respective cellular half-life.

The SafeSwitch provides an answer to the concern regarding inadvertent auto-activation *in vivo* with the layered requirements: a systemically-introduced mifepristone ligand furthers the initial trigger of a thermal dose to cell or tissue targets; and sustained transcription limitations of the Hsp70b promoter abrogate expression that is not concurrent to mifepristone presence. On the other hand, the Gal4 promoters permit continued transgene product production when mifepristone ligand presence is not limited. Vilaboa et al. (2005) published *in vitro* and *in vivo* results that relate to their testing of the fLuc SafeSwitch system, which is illustrated by the sketch in Fig. 1.3. Their findings depicted stringent regulation of the firefly gene target gene.

Vilaboa et al. (2005) produced notable *in vitro* results with a HeLa-derived cell line, which is stably transfected with the genes (outlined in Fig. 1.3) that comprise the fLuc SafeSwitch system. Their results convincingly indicate that the SafeSwitch robustly and closely controlled luciferase activity. The amount of
luciferase produced when mifepristone was also present was proportional to the intensity of the applied thermal dose and reached a maximum level that was nearly three orders of magnitude more than the basal level. Background expression of luciferase in untreated cells or cells that received either the thermal dose or the mifepristone ligand, but not both, was nearly negligible. They further characterized the fLuc SafeSwitch in the HeLa cell line as a function of time and mifepristone presence, following a single, transient application of a thermal dose. A high level of target gene expression appeared exclusively dependent on both an initial thermal dose as well as on the continuation of mifepristone ligand over the six day period that was measured. Within the group that received ligand but that did not also receive an initial thermal dose, the mifepristone ligand did not create target gene activity at any point during that time course. Vilaboa and colleagues further demonstrated reversibility in vitro with the fLuc SafeSwitch HeLa cell line. Parallel cultures either had the mifepristone ligand removed, one day post thermal dose application, or mifepristone ligand was continued after the heat treatment. The published, comparative results (Vilaboa et al., 2005) indicated a 60-70% decline in the reporter gene activity just one day after ligand removal, which continued to drop to imperceptible levels within two more days.

Vilaboa and colleagues tested the fLuc SafeSwitch concept in vivo, as well. The fLuc SafeSwitch DNA components, outlined in fig 1.3, were injected and then electroporated into the gastrocnemius muscles of adult C57BL/6 mice. Results (Vilaboa et al., 2005) demonstrated that minimal background target gene expression was present one day later. Impressively, a hundred-fold increase in
luciferase activity was achieved over the highest basal level by the additions of a systemic dose of mifepristone (0.5 mg/kg suspended in corn oil and administered via intraperitoneal injection) and a thermal dose (achieved by immersion of the electroporated region in a 43.2°C water bath for thirty minutes). Additionally, Vilaboa et al. (2005) examined *in vivo* gene expression of the fLuc SafeSwitch as a function of time and mifepristone ligand continuation. All animals received the injections of mifepristone ligand the first day, then either did or did not also receive the above thermal dose. Subsequent to that first day, all animals continued to receive daily injections of mifepristone ligand (0.25 mg/kg), except for one group that did receive the initial thermal dose but then had the ligand withdrawn after that first day. Mice were sacrificed either on day 1 or day 6 post initiation of treatment to complete the respective luciferase assay. The findings were comparable to the fLuc SafeSwitch HeLa cell line *in vitro* study outcomes (Vilaboa et al., 2005). Coupling a thermal dose to sustained, systemic administration of mifepristone ligand resulted in a significantly high level of target gene expression on both day 1 and day 6, which was not observed in the groups that did not receive an activating heat treatment. Furthermore, results achieved in the group that had the ligand withdrawn demonstrated that the high level of target gene expression was reversible within a five day span of time. Thus, the SafeSwitch concept was applied *in vivo*, and the stringent regulation that it afforded yielded the desired high levels of target gene expression only when both permissive conditions (localized heat and systemic mifepristone ligand) were met. With the SafeSwitch concept, it may be possible to harness the HS
response in order to safely and effectively target transcriptional enhancement of a delivered gene therapy. This would ideally answer the current bottleneck present in cancer gene therapy studies.

Specific aims

The hypothesis of this thesis is that a harness of the HS response, when paired with a therapeutic mechanism, will produce outcomes relevant to the refinement of novel therapies. Extensions to the concept of deliberately activating HSF1’s normal functions for therapeutic purposes are examined in this work through in vitro trials and further in vivo preliminary studies that feature the use of HSF1 as a regulator of therapy. The specific aims are stated hereafter:

1. To pair a previously characterized, molecular switch that features a harnessed HS response with a gene target that mediates a potent mechanism of cell death, so that the combination’s therapeutic merit can be assessed in vitro and can translate to an in vivo proof of principle tumor regression model.

2. To advance the combination to an in vivo therapeutic situation.
   a. To establish conditions to activate the gene switch in vivo.
   b. To evaluate the molecular switch system for its therapeutic potential.

3. To create a constitutively active mouse HSF1 that potentially could be utilized to measure the consequences of deliberate HSF1 activation in vivo.
Chapter 2 – Harnessing the Heat Shock Response

Extensions to the concept of deliberately activating HSF1’s normal functions to achieve therapeutic purposes are explored in this chapter through in vitro studies that form the basis of the in vivo work presented in chapter three. As mentioned in the preceding chapter, the current bottleneck in cancer gene therapy studies is the development of in vivo gene delivery systems that are specifically targeted to tumor cells, that are efficient in gene delivery, and that are safe (concerns commented on by Vile et al., 2000). This chapter addresses the development of an applied strategy to unlock the bottleneck surrounding targeting and safety concerns that otherwise limit potent, novel gene therapies. Here, a previously characterized, molecular switch that employs the SafeSwitch concept (presented in chapter one), which features a harnessed HS response, is paired with a gene target to mediate a mechanism of cell death. The combination’s therapeutic merit is assessed through the completion of in vitro studies that quantitatively examine syncytia formation and that qualitatively examine cancer cell death.

Pairing the SafeSwitch harness of the Heat Shock Response with a potent gene target: GALV(+)

Gene therapy has demonstrated potential for the treatment of solid tumors. In fact, 66.5% of all gene therapy clinical trials in a database, which was compiled by Edelstein et al. (2007) and that is representative of 1,309 trials
conducted in 28 countries (as of July 30th, 2007), addressed cancer. A broad spectrum of diverse genetic means, which inhibit tumors, has been explored. Methods have ranged from inhibition of angiogenesis within tumors, attempts to decrease chaperone-mediated autophagy, attempts to restore normal function of regulatory genes (e.g., p53), as well as the creation of cytotoxic potent outcomes. A typical observation with Ad5 in vivo gene delivery is that only cells proximal to the introduced needle track are directly infected and thus able to express the target gene. One approach to overcome this gene delivery limitation capitalizes on the creation of a bystander effect as it contributes to intended cell death.

Rationale for the approach of pairing SafeSwitch regulation with the GALV(+) gene target

Fusogenic glycoproteins display bystander effects that offer benefit to cancer gene therapy purposes. There are several in the published literature that have received attention and include: mutant Newcastle disease virus fusion protein [NDV/F(L289A)], used in studies conducted by Ebert et al., 2004 and Shinozaki et al., 2005a and 2005b; vesicular stomatitis virus G glycoprotein (VSV-G), used by Errington et al., 2006; measles virus hemagglutinin protein (H) and virus fusion (F) glycoprotein (MV-H/F), used by Hoffmann and Wildner (2006); and respiratory syncytial virus (RSV-F), used by Hoffmann et al. (2007). The modified, retrovirus glycoprotein, GALV(+), has a particularly broad capacity to cause cytotoxicity in cells of human origin. Its cytotoxicity stems from its ability to fuse cells in a manner that creates unsustainable syncytia. This characteristic
has been shown to be effective in cancer gene therapies, including several that utilize heat activation (Bobkoba et al., 2002; Emiliusen et al., 2001; Galanis et al., 2001; Diaz et al., 2000; Higuchi et al., 2000; Bateman et al., 2000; Fielding et al., 2000; Braiden et al., 2000; and Brade et al., 2003). GALV(+) is discussed in greater depth later on in this chapter.

Efficacy, targeting, and safety are the primary concerns for gene therapies, particularly those with bystander effects. The SafeSwitch concept, discussed in chapter one, offered the potential to impart the required means to safely target GALV(+) anti-tumor efficacy, which could then be delivered by adenovirus in vivo. A novel approach is presented here, which pairs these two previously tested concepts to constitute a new, theoretically effective therapy for the treatment of solid tumors.

Initial trial of SafeSwitch regulated expression of the GALV(+) gene target

Two separate constructs were first paired: an Hsp70/Gal4-GLP65 transactivator construct, which was previously characterized by Vilaboa et al. (2005) (see schematic A in Fig. 2.1), and a Gal4-GALV target construct (shown below in schematic B of Fig. 2.1). The Gal4-GALV construct was produced by subcloning the GALV(+) gene, which was obtained from Dr. Richard G. Vile (Molecular Medicine Program, Mayo Clinic, 200 First Street SW, Rochester, Minnesota 55905, USA), from the original CMV-promoted plasmid vector to a Gal4-promoted expression vector (the Invitrogen pGene vector). The resultant construct transcribed the GALV(+) gene, in response to activity from the
upstream Gal4 promoter. This activity was examined in the experiment presented in Fig. 2.2.

The experiment that followed the preparation of the initial GALV(+) construct sought to answer whether SafeSwitch regulation could stringently control the potent, cytotoxic bystander effects of the GALV(+) gene. The experiment was designed to serve as a launching point for studies that would follow, so many parameters were addressed. Some of these parameters included the effective ratios of switch components, the potential for basal expression in unintended states, and the establishment of a baseline phenotype with permissive conditions.

**Figure 2.1 GALV(+) as a target gene component for a SafeSwitch.** (A) Transcription of the Hsp70/Gal4-GLP65 transactivator component is initially triggered with transient heat (illustrated by the symbol for active HSF1 bound to heat shock element sequence (HSE) upstream of an Hsp70b promoter). Transcription is then sustained by the consequential production of GLP65 transactivator (TA) protein when mifepristone ligand is both present and not limiting. Transcription occurs from the minimal TATA promoter when Gal4 upstream activating sequence (Gal4 UAS) is bound by the dimerized TA; (B) The target gene component, Gal4-GALV(+), is contained on a separate construct and is transcribed when bound by the dimerized TA. The symbols utilized are explained in Fig. 1.3.
The Gal4-GALV(+) and Hsp70/Gal4-GLP65 constructs initially were transiently transfected in HeLa cells (see Fig. 2.2) in a variety of combinations. Each DNA combination was examined in four possible scenarios of component

![Figure 2.2](image)

**Figure 2.2 Transient transfection results of SafeSwitch regulated GALV(+) expression in HeLa cells.** The full-length, unattenuated transactivator was tested for its ability to regulate the gene target: GALV(+). Only cells subjected to both heat shock (HS) and mifepristone (mife) that were also transfected with both components of the SafeSwitch, show the syncytia phenotype at 24 hours post HS (the two top-left squares). The rest of the cells appear approximately similar. The effect appears slightly dose dependent, with less developed syncytia apparent when half the amount of Hsp70/Gal4-GLP65 was co-transfected with Gal4-GALV(+) and then subsequently subjected to an activating HS and treatment with mifepristone. The notations across the top indicate that the following amounts of construct DNA were transfected: (1/2+) Hsp70/Gal4-GLP65 = 3 ng; (+) Hsp70/Gal4-GLP65 = 6 ng; (+) Gal4-GALV(+) = 0.1 μg; (-) = 0 ng. The notations to the left of the composite of pictures indicate that the following treatments were applied: HS (+) = 44°C /30 min heat treatment; mife (+) = exposure to 10 nM mifepristone; (-) = not subjected to the indicated treatment.
addition that could potentially lead to switch induction. The combinations were
examined for the ability to lead to a phenotypic change: either with or without HS
and / or either with or without the mifepristone ligand. The DNA combinations
included either construct alone, neither construct, or one of two ratios of the
construct combination: a 33.3:1 (designated “1/2+”) and a 16.7:1 (designated “+”)
ratio of Gal4-GALV(+) to Hsp70/Gal4-GLP65. These ratios were extrapolated
from Vilaboa et al. (2005) characterizations of the Hsp70/Gal4-GLP65 switch and
reflect the respective gram weights of DNAs that were transiently transfected into
triplicate wells of HeLa cells for similar treatment conditions. Cells were visually
inspected for appropriate gene regulation and representative fields were
photographed. Qualitative results of both ratios appeared quite similar under all
conditions except those depicted in the left, top two panels of Figure 2.2. No
gene expression was apparent under any condition, except when both
components of the switch were transfected and then subsequently subjected to
the transient HS treatment and to the sustained addition of mifepristone.
However, fewer syncytia developed when less Hsp70/Gal4-GLP65 was co-
transfected with amount of Gal4-GALV(+) held constant. Thus, the figure has
been simplified to present pictures that relate only to the additional control
conditions for the more sensitive transfection ratio.

The success of this transient transfection experiment to stringently
regulate GALV(+)-mediated syncytia formation and consequential cell death
prompted the consideration of viral vectors to deliver the genes to cancer cells in
vivo. Such a delivery means would benefit from a simplified system, one that
utilized only one viral vector to deliver all components. A single construct, GALV(+) SafeSwitch (schematic is presented in Fig. 2.3), which was able to conform to adenovirus packaging constraints, was deemed to be beneficial toward a therapeutic end and was designed accordingly. An attenuated version of the transactivator (TA) component in the Hsp70/Gal4-GLP65 SafeSwitch construct, Hsp70/Gal4-RKC GLP65 TA, had also been characterized (Vilaboa et al., 2005) and was employed in the production of GALV(+) SafeSwitch. Experiments by Vilaboa et al. (2005) had shown that a 1:1 ratio of the attenuated transactivator to Gal4-promoted target gene was well-regulated. This single construct, GALV(+) SafeSwitch, also simplified the production of stably transfected cell lines, after the relevant genes were subcloned to a new vector that coded for a selective antibiotic marker (see Fig. 2.4). The stably derived lines were designed to add clarity to results and to eliminate variability in gene delivery.

Creation of the GALV(+) SafeSwitch adenovirus shuttle vector

Cost-benefit analysis of the various means and materials to produce purified, high-concentration stocks of viral vectors in sufficient quantities to do later in vivo experimentation identified that commercial production through the vendor, ViraQuest, Inc. (North Liberty, Iowa), was the most expedient and cost-effective option. An efficient means of achieving adenovirus recombination first incorporates the desired new gene sequence into an intermediate shuttle vector (Anderson et al, 2000). Three constructs were prepared by subcloning the
appropriate components to the vendor-provided shuttle vector (referred to here as VQ-shuttle). These included the therapeutic construct, GALV(+) SafeSwitch, which featured the components sketched in Fig. 2.3 (relevant sequence information is listed in Appendix 1), and two control constructs. The control constructs either lacked the GALV(+) gene or included an Hsp70b promoter directly upstream of the GALV(+) gene to deregulate its transcriptional control.

The commercially produced viral vectors were tested in several cell lines. Disappointingly, they were found incapable of adequately producing GALV(+) expression in induced states and likely did not produce sufficient levels of transactivator to cause the Gal4(+)promoted target gene expression (data not shown). However, the VQ-shuttle therapeutic construct, GALV(+) SafeSwitch, of Fig. 2.3 that was utilized to produce the vectors was successfully tested \textit{in vitro} through transient transfection in several cell lines and \textit{in vivo} through DNA electroporation. \textit{In vivo} DNA electroporation has recently been reviewed by Prud’homme et al. (2006) and was determined to offer potential as a viable means to directly deliver therapeutic DNA to tumors. Many groups have utilized the method to deliver DNA to tumors in animals (Cemazar et al., 2004; Goto et al., 2004 and 2000; Heller et al., 2000; and Wells et al., 2000). Additionally, Soden et al. (2004) has presented data on flexible electrodes, and the technology may be developed to deliver DNA via electroporation in a clinical setting. Thus, DNA electroporation was able to serve as a means to test the proof of principle of the GALV(+) SafeSwitch concept in absence of a viral means to deliver the genes \textit{in vivo} (described in greater detail in chapter three).
Creation of the GALV(+) SafeSwitch stable cell line construct

As mentioned above, it was viewed as beneficial to create stably derived, cancer cell lines that incorporate the GALV(+) SafeSwitch genes in order to aid interpretation of in vitro findings. Stably derived cell lines were also viewed as instrumental to the production of xenograft and homograft tumors that, in theory, could uniformly express the therapeutic genes when the system was activated. Thus, confounding aspects of gene delivery would be circumvented while empirical determinations of appropriate activation situations were ascertained.

Figure 2.3  The GALV(+) SafeSwitch genes inserted into the VQ-shuttle vector for adenovirus production. RKC GLP65 indicates the attenuated version of the GLP65 TA gene, which is transcribed from the Hsp70/Gal4 promoter cassette in a manner that is opposite in orientation to the adjacent target gene component, Gal4-GALV(+). SafeSwitch components were inserted into the VQ-shuttle vector in a step-wise process. However, the single switch unit shown above may be excised from the final construct (GALV(+) SafeSwitch) with restriction endonucleases: Ssp I and BbvC I. Ssp I, BbvC I, and Not I are underlined to indicate that they are useful sites, though none are single cutters in the plasmid as a whole. Other sites noted in the schematic that are not underlined are unique. Those that yield blunt ends are flagged (*). The sequence that stretches between the Not I sites is listed in Appendix 1. Since a Pac I site exits toward the 3’ end of the GALV(+) sequence, viral recombination procedures utilize the alternative vector site: Nhe I.
The GALV(+) SafeSwitch cell line was created by subcloning a single large fragment (see Appendix 1 for that sequence), which encompassed the genes outlined in Fig. 2.3, from the ViraQuest shuttle vector into the Invitrogen vector containing the mammalian Blasticidin antibiotic-resistance gene. The insertion direction of the genes subcloned to the Invitrogen pEF-Bsd vector was specifically chosen so that there would be no read-through expression of the GALV(+) component from the elongation factor 1 alpha (EF1α) promoter, the promoter linked to the production of antibiotic resistance for clonal selection. This construct was first successfully tested via transient transfection of HeLa cells, before a cell line was established and considerations of the cell line for in vivo tumor modeling were explored (see pictures of HeLa cells in Fig. 2.4 that transiently expressed the construct).

Screening cancer cell lines for good expression of the GALV(+) target gene

A review of the literature revealed how the constitutively active form of the fusogenic membrane glycoprotein, GALV(+), functioned (Weiss and Tailor, 1995 as well as Bobkova et al., 2002) (see Fig. 2.4 for an illustration). The trimerized GALV(+) protein lacks the repressive R-peptide, which is found on the C-terminus of the cytoplasmic tail of the natural form of the protein. The R-peptide of the natural form of the gibbon ape leukemia virus envelope glycoprotein is typically cleaved by a viral protease during maturation of that virus (Bobkova et al., 2002). Trimeric GALV(+), which is then able to bind to the PiT-1/GLVR1 receptor on cells (Tailor et al., 1993 and Johann et al., 1993), causes the
Figure 2.4 Schematic of the gene expression cascade in the GALV(+) SafeSwitch construct for cell line production. The HeLa cells in the picture on the bottom left of the figure, though transiently transfected with the GALV(+) SafeSwitch construct, illustrate confluent cells that ultimately manifest a clear outcome of the gene expression cascade outlined in the figure. Cells such as these are triggered to activate endogenous HSF1 (eHSF1, which is depicted with the symbol utilized in Figs. 1.1 and 1.3) by a transient heat treatment (HS = 44°C / 30 min), as is indicated with the lightning bolt. Transcription of the Hsp70/Gal4-RKC GLP65 TA (half-moon shape) component is then initially triggered from the Hsp70 promoter when activated eHSF1 binds the heat shock element sequence (HSE). Production of the TA is sustained only when the mifepristone ligand presence (represented with the starburst shapes) permits the TA to dimerize, which leads to transcriptional competence. The RKC GLP65 TA then promotes its own production from the Gal4 promoter in the associated promoter cassette. It also transactivates the target gene, GALV(+), from the Gal4 promoter on the adjacent but oppositely oriented sequence. GALV(+) then homotrimerizes and binds to the indicated transmembrane domain of the Pit-1 receptor on cell membranes. In turn, this outcome yields the syncytia phenotype that is visible in the HeLa cells in the picture on the bottom right of the figure. 3‘ of the GALV(+) gene is a strong, eukaryotic promoter: elongation factor 1 alpha (EF1α). That promoter enables production of resistance to blasticidin antibiotic, which permits stably transfected cell lines to be clonally-derived. Restriction endonuclease sites, which improve genomic recombination events by linearization and that also excise extraneous vector sequence, are noted at the perimeters of the sketch.
observable cell to cell fusion that may encompass as many as one hundred nuclei in the formation of syncytia. The literature (Johann et al., 1993; Tailor et al., 1993 and 2000; as well as Grabarczyk et al. 2002), however, also hints at a limitation of the ability of the PiT-1/GLVR1 receptor to bind trimeric GALV(+), which has important implications regarding the selection of an in vivo model that might utilize its fusogenic properties to cause cell death.

Species-specific limitations to GALV(+)-mediated cell to cell fusion

A murine, homograft tumor model would be a convenient approach to illustrate tumor regression that utilized regulated, GALV(+)-mediated cell fusion. However, selective pressure appears to have acted on rodents that were reservoirs for the Gibbon Ape Leukemia Virus. The consequence is that rodents with receptors that were permissive to entry of this virus were eradicated, and only species with mutated receptors remained. Most mice and rats that persist today have significant alterations in Region A (amino acids 550-558) of the receptor, a region correlated with Gibbon Ape Leukemia Virus susceptibility as well as with GALV(+) binding and syncytia expression. However, rats as well as one obscure mouse line (Mus molossinus), which is not commercially available in the United States, were noted to still be permissive for Gibbon Ape Leukemia Virus infection in a table published in the work of Weiss and Taylor (1995). Their table illustrates the Region A sequence for the receptor in humans, cats, dogs, mice and rats. Like humans, the companion animals (dogs and cats) appeared to have an unmodified receptor for GALV and were correspondingly noted as
permissive for infection. In sum, species-specific differences in the PiT-1/GLVR1 receptor sequence are seen among animals (Bateman et al., 2000; Fielding et al., 2000; Galanis et al., 2001; Higuchi et al., 2001; Fernandes et al., 1999; as well as Weiss and Taylor, 1995).

**Figure 2.5 Schematic of the luciferase overlay method to quantify cell fusion.** Two sets of cells contain complimentary genes (A). Set one (top oval in the diagram) expresses the GALV(+) SafeSwitch. Set two (bottom oval) is converted to reporter cells via transient transfection with a mixture of reporter constructs. Approximately 3% (range of 1-5% may be used) of the reporter DNA mixture produces renilla luciferase (rLuc) and acts as an internal control, since it is constitutively transcribed from a cytomegalovirus virus (CMV) promoter. The remaining reporter in the mixture conditionally expresses a firefly luciferase (fLuc) gene from the Gal4 response elements (Gal4 RE), which comprise four, 17-bp Gal4 binding sites (17x4) and an associated minimal TATA promoter. Thus, the construct is known as 17x4-TATA fLuc. After the cells containing the GALV(+) SafeSwitch are subjected to an appropriate heat treatment (indicated with the lightning bolt) that produces the RKC GLP65 TA, a small fraction of reporter cells are added and allowed to settle as an overlay. In Panel (B) the addition of mifepristone hormone enables the produced TA to acquire transcriptional competence, which leads to the production of more TA and allows GALV(+) mediated fusion to occur among the heated cells and any adjacent reporter cells. In Panel (C) cellular contents commingle within the produced syncytia. This allows the TA that is produced by cells treated with both heat and mifepristone ligand to cause transcription of the fLuc gene. Cells are then harvested and assayed for their production of both firefly and renilla luciferases. Outcomes indirectly report the level of available, transcriptionally competent, dimerized TA. Panel (D) illustrates the concept of Relative Luciferase Activity (RLA), which offers significance to the fLuc measurements obtained. Without RLA calculations, results may be confounded with variables (e.g., transfection variability). The RLA is calculated by dividing the results observed for the conditionally-expressed firefly luciferase, with the respective results obtained in those same cells from the constitutively expressed renilla luciferase. Note: RLA is also routinely calculated in experiments that quantify the activity of an Hsp70b promoter linked to a firefly luciferase (Hsp70 fLuc) gene in order to report the activation state of HSF1. Fold Induction is further calculated by dividing the RLAs achieved in treated conditions with the RLAs obtained from the comparable, basal condition.
Alternative methods with potential to yield an *in vivo* SafeSwitch fusogenic model were also identified. Overexpression of the PiT-1 receptor, to enable sufficient GALV(+) binding in order to observe syncytia phenotype, or use of a different fusogenic membrane glycoprotein, comprise the primary alternatives.
Overexpressing the PiT-1 receptor to create a syngeneic model that overproduced the natural receptor or that expressed the human version is technically challenging. A transgenic mouse would need to be produced and evaluated for a sufficient increase in copy number and receptor expression in tissues relevant to safety and function testing. An additional challenge confronting that approach is that transgenics typically do not uniformly express the introduced genes in all tissues. Yet another consideration with that approach is that a corresponding, stably derived, novel cancer cell line would also need to be produced. Arguably, the new cancer line that would then be used to form tumors might not accurately reflect all properties of naturally occurring, unengineered tumors. Other fusogenic glycoproteins have been utilized for similar purposes [e.g., the Newcastle disease virus (NDV) F protein mutant (L289A)]; however, the tropism for cytotoxicity also appears to have limitations, as was identified in Ebert et al. (2004).

*Screening rat lines for good expression of a GALV(+) SafeSwitch*

The conclusion was that the simplest syngeneic model might evolve from the identification of a stably derived rat cell line that could respond to GALV(+)mediated fusion. The first tested line, MatBIII, did not produce syncytia after it was transiently transfected and subsequently subjected to an applied heat treatment and to ligand introduction. An experiment to ascertain whether GALV(+)mediated fusion could be identified in a different rat line was next performed (Fig. 2.6). Cell fusion was quantified with the luciferase overlay
method described in Fig. 2.5. Briefly, this method quantifies cell fusion in terms of light units that report transcription events, which are only possible to produce if protein contents mix within syncytia formed from cells of different populations.

In Fig. 2.6, human HeLa cells served as a standard measure of high-level cell-to-cell fusion potential and results are compared among parallel cultures of three rat cell lines (a model of adenocarcinoma: MatBIII, as well as two models of undifferentiated malignant glioma: RG2 and F98), which are syngeneic to

![Figure 2.6](image)

**Figure 2.6 GALV(+)‐mediated fusion in a parallel comparison of rat cell lines and the human HeLa cell line standard.** Relative 17x4 TATA luciferase fold induction is graphed for each of the four rat cell lines (MatBIII, XC, RG2, F98) and the human (HeLa) cell standard in Panels: (A) the graph of the luciferase overlay results (see Fig. 2.5 for a description of this method); and in (B) an illustration of fusion-independent, maximum fold induction. In (B), one population of cells was co‐transfected with the necessary components to cause reporter transcription: the GALV(+) SafeSwitch as well as the reporter constructs. Thus, the upper limit of transcription potential was obtained in each line examined. In brief, duplicate wells of cells were transfected with identical mixtures of DNA. Then parallel cultures were either treated or not treated with 10 nM mifepristone ligand the next day, prior to the 44°C/30 min heat treatment. In the case of overlay studies presented in Panel (A), reporter cells were suspended and added to the cultures within eight hours of the HS treatment. Cell lysate was harvested for luciferase quantification the following day.
purchasable rat lines, and one outbreed rat line (a model for Rous sarcoma, called XC, which was originally isolated from a White-Weston rat). The luciferase reporter results presented in Panel B, which were not dependent upon the occurrence of cell-to-cell fusion, indicate that either rat cell line, RG2 or F98, was able to produce the transactivator component of the switch in sufficient quantity to act on a Gal4 promoter. However, Panel A of Fig. 2.6 reveals that significantly less fusion was found to occur among the rat cells in comparison to the human HeLa cell line. The RG2 cell line displayed approximately one-tenth the fusion measured in the HeLa cells, and the F98 cell line displayed fusion measurements that were almost one-third that observed in the human line.

![Graph](image)

**Figure 2.7** Comparing the HS response in the RG2 and F98 rat lines with the human HeLa cell line standard. Relative Hsp70 luciferase was calculated as described in Fig. 2.5 (D) for each cell line transfected in duplicate wells with the mixture of firefly and constitutive renilla reporter constructs. Respective plates of cells containing all three lines were subjected to the indicated temperature treatment 24 hours after transfection. Cells were harvested for luciferase readings the following day.
Another practical consideration involved the technical aspects of applied heat. To simplify initial tumor analysis, it was planned to form subcutaneous tumors in vivo with the use of the cell lines. Heat would then be applied to the skin lying superficial to the tumors in the hope that it would be conducted to the adjacent cancer cells in a manner that would effectively trigger the activity of endogenous HSF1 in those cells. Thus, the HS responses of the two candidate rat cell lines, RG2 and F98 (reviewed in Barth, 1998), were compared to the well-characterized response in the human HeLa cell line. The experiment utilized the Hsp70 Luciferase reporter construct to quantify results, which is outlined in Fig. 2.5 (Panel D) and its known sequence information is listed in Appendix 5.

The quantified results presented in Fig. 2.7 illustrate greater production of an Hsp70 reporter in the rat lines and suggest a stronger HS response than that in the human HeLa cell line. The F98 line demonstrated the highest Hsp70 promoter activity at the lowest temperature. The lower activating temperature for the HS response is of particular interest and was a full degree less than that required in the human cell line. A possible reason for this could be species-specific differences relevant to regulating the human Hsp70b promoter utilized in this assay in the context of the rat cell. However, it is also known that there are cell type-mediated differences in the HS responses within cells of different origin with corresponding differences in the consequences of applied heat. The F98 rat line demonstrated the greatest sensitivity to an applied heat treatment in the experiment presented in Fig. 2.7. Thus, the F98 rat line was selected for further experimentation to determine whether a syngeneic model could be developed.
**Delivering the GALV(+) SafeSwitch to rat cells in vitro via transient transfection**

Transient transfection methods were optimized in the F98 cell line as well as in a different rat cell line (LCRT, see Toth et al., 2005 and 2007 for a description), which was selected to support another SafeSwitch model developed by Vilaboa that is introduced in Fig. 2.13. Representative results of the optimized

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**Figure 2.8** **Transient transfection expression of GALV(+) SafeSwitch-mediated cell fusion in select rat lines.** Syncytia phenotype is examined here in cells transiently transfected with the GALV(+) SafeSwitch and then subjected to the indicated conditions in (A) and (B) the following day (+HS = 44°C /30 min heat treatment; +mife = exposure to 10 nM mifepristone ligand treatment; (-) = not subjected to the indicated treatment). Panels (C) and (D) are enlarged pictures of the boxed cells (top left picture in each composite) in Panels (A) and (B). At 48 hours post applied treatment(s), cells were fixed with methanol and then stained with Wright-Giemsa solution prior to being photographed.
transient transfection are presented in Fig. 2.8. Quantified cell fusion potential with the F98 rat cell line appeared encouraging in terms of the results obtained from the transient transfection (Fig. 2.6 and 2.7) of the GALV(+) SafeSwitch construct combined with the reporter constructs in the context of the sensitive luciferase assay. It is possible to see some syncytia (particularly in Panels C and D of Fig. 2.8), which formed in a manner that was dependent on both heat and mifepristone treatments. However, the syncytia phenotype appears to be much more limited than that previously observed in the human (HeLa) cell line (see Fig. 2.2). A similar phenotype was identified in the LCRT rat cell. Results indicate a low level of induced syncytia formation in these rat cell lines.

It was postulated that a stably derived rat cell line might be optimized to display the desired level of GALV(+) SafeSwitch expression in the induced conditions. If such a line were able to be developed into a model, it would facilitate important studies that would benefit from the information gathered in the context of a competent immune system. Alternatively, a stably transfected human cell line would support a xenograft model for initial regression studies, albeit extrapolation of conclusions relevant to an actual human therapeutic situation would be limited without a measure of immune-mediated effects.

**GALV SafeSwitch cell lines**

To answer the question of whether a stably transfected, F98-derived cell line would improve the induced gene expression phenotype, clonal selection and expansion was carried out in the presence of Blasticidin antibiotic following
transfection of a linearized fragment of the cell line construct (described in the schematic of Fig. 2.4). A similar procedure was performed for three human cell lines: HeLa (cervical carcinoma), HT-1080 (human fibrosarcoma) and U87-MG (human glioblastoma; astrocytoma). The latter two commercially available human cell lines were used in xenograft studies that expressed GALV(+) to achieve therapeutic outcomes (published by Brade et al., 2003 and Allen et al., 2004, respectively).

The resultant F98-derived rat line (clone F98 #6), which displayed desirable induction characteristics, was directly compared to the HT-1080-derived human line (clone HT-1080 #4). The first experimental data, presented here in Fig. 2.9, quantified syncytia formation in each line across a range of temperatures with the use of the luciferase overlay method. Like the transiently transfected original F98 line (see Fig. 2.7), greater relative light readings were achieved with the F98 #6 rat line at a temperature that was measured to be two degrees less than that required to produce maximal induction in the parallel human cell line culture. Results suggested the possibility that the GALV(+) SafeSwitch fusion effect (in the presence of mifepristone) might be more pronounced, at a lower temperature, in the stably transfected rat F98 #6 line, than what was required to generate a strong phenotype in the human cells (see Fig. 2.2). However, an alternative explanation is that syncytia formed within rat cells are more easily sustained, permitting continued reporter protein production, than syncytia in human cells that cause rapid cell death. The findings presented in Fig. 2.10 distinguish the difference.
Pictures of representative fields were taken of the derived cell lines at a range of temperatures, in either the presence or the absence of the mifepristone hormone, at twenty-four hours post heat treatment. Panels A and B of Fig. 2.10 display the fields corresponding to the optimized temperature (extrapolated from the data presented in Fig. 2.9). Panel C of the same figure presents a closer inspection of the limited cell fusion noted with the F98 #6 cells, whereas Panel D displays the characteristic, encompassing cell fusion visible in the cultures of HT-1080 #4 cells after both permissive treatment conditions were applied. Fusion was essentially absent in all control conditions. Thus, the qualitative comparison

**Figure 2.9 Side by side comparison of GALV(+) SafeSwitch-mediated cell to cell fusion as a function of heat treatment temperature in the rat F98 #6 line and the human HT-1080 #4 line.** Each derived line was seeded into duplicate wells for each temperature examined. The cells were then either treated or not treated with mifepristone ligand prior to being subjected to the indicated temperature in degrees Celsius of applied heat for 30-minutes. The luciferase overlay method (Fig. 2.5), which utilized a parallel suspension of unheated cells that were transfected with reporter construct DNA and that settled on top of the treated cells, was utilized to quantify fusion events. Cell lysate was harvested the following day for luciferase readings and the fold induction of the RLA was calculated (procedure for the above is explained in Fig. 2.5).
of the two lines indicates that the human HT-1080 #4 line best displays the desired characteristics regarding controlled cell killing. The conclusion was to concentrate on the use of human lines in a xenograft model in order to accurately reflect the level of cancer cell killing that could be expected to be achieved by GALV(+) SafeSwitch activation in people.

Figure 2.10 The rat F98 #6 line displays less profound GALV(+) -mediated fusion phenotype in response to full switch activation than the human HT-1080 #4 line. Parallel cultures of each line were either treated with 10 nM mifepristone (+ mife) or not (-mife) and then were subjected to the indicated heat treatment [identified to the left of the pictures in Panels (A) and (B)]. Note: the optimal HS treatment temperature, which was utilized here, was determined for each line from the experiment presented in Fig. 2.9. Panels (C) and (D) are enlarged pictures of the boxed cells in Panels (A) and (B), respectively. At 24 hours post applied treatment(s), cells were fixed with methanol and then stained with Wright-Giemsa solution prior to being photographed.
The three human cell lines (HeLa, HT-1080, and U87-MG) were stably transfected (see the above section: GALV SafeSwitch cell lines) to isolate the respective derived lines. These three human lines were also transiently transfected to ascertain each line’s response to the newly introduced genes. All

**Figure 2.11 GALV(+) SafeSwitch cell line construct transiently and stably transfected in three human cell lines.** Three human cell lines (HeLa, HT-1080, and U87-MG) were transiently [Panels (A), (B), and (C)] and stably [Panels (D), (E), and (F)] transfected with the GALV(+) SafeSwitch construct. The following notations indicate that the respective conditions were applied: +HS = 44°/30 min heat treatment; -HS = cells were maintained at the standard incubation temperature of 37°; +mife = exposure to 10 nM mifepristone hormone treatment; -mife = no hormone was added. Boxed pictures (top left picture of each composite) indicate cultures in which syncytia were expected to be visible. Cells were photographed with the use of a phase-contrast microscope 48 hours post applied treatment(s).
lines were screened and compared to evaluate the qualitative display of the desired phenotype under different combinations of the applied mifepristone ligand and transient heat treatments (see Fig. 2.11).

The HeLa cell line displayed desired characteristics in the transient transfection situation (Fig. 2.11, Panel A). However, a derived line was not able to be isolated, which exclusively displayed induction that was dependent on an

![Figure 2.12](image)

**Figure 2.12** The HT-1080 derived #4 cell line shows dramatic GALV(+) SafeSwitch-mediated cell death at 48 hours post ligand application and heat treatment. Parallel cultures of cells were maintained at 37°C (-HS) or were exposed to a 44°C/30 min. heat treatment (+HS), either in the presence (+mife) or in the absence (-mife) of 10 nM mifepristone, as is indicated in the figure. Cells were photographed with the use of a phase-contrast microscope 48 hours post treatment application(s).
applied heat treatment and the presence of the mifepristone ligand. Fig. 2.11 (Panel D) reveals the unregulated phenotype of the derived line (HeLa #H), which depended solely on an activating heat treatment. Both stably transfected (U87-MG #23) and transiently transfected (Fig. 2.11, Panels C and F, respectively) U87-MG cell lines did display some aspects of the desired phenotype. However, the HT-1080 cell lines (Panels B and E of Fig. 2.11) were superior in the display of the desired characteristics in either scenario.

The HT-1080 cell line was examined further in order to qualitatively assess the potential for background cell killing after prolonged induction. The representative results presented in Fig. 2.12 illustrate that maximal induction and minimal deregulated gene expression were obtained within a forty-eight hour time frame. Specifically, minimal cell death is detectable in the +HS / -mife condition, and negligible cell death is present in the -HS / +mife condition when compared to the -HS/ -mife condition. This result represents a near ideal illustration of GALV(+) SafeSwitch-controlled cell death. The line was selected for an additional in vitro study that examined the effect of its combination with another SafeSwitch system.

**Addition of a conditionally replicative, oncolytic adenovirus lowers the activation threshold for SafeSwitch regulated cell death in vitro**

Synergistic combination cancer therapy has recently emerged in the literature as a promising approach to augment effects achieved by a single therapeutic approach. One effective combination that has been tested in
preclinical trials utilizes a single adenovirus or an adenovirus pair, if necessary to accommodate gene packaging constraints, to deliver an oncolytic virus and a suicide gene (Hernandez-Alcoceba et al., 2000). The principle of an oncolytic system is that an initial inoculum of virus that is introduced to a cancer cell population will cause an amplification of effects under the ideal conditions: virus will replicate in the infected cancer cells, which are then killed by the cytolysis that simultaneously releases viral progeny to infect neighboring cells.

*The Oncolytic SafeSwitch system*

Vilaboa and colleagues developed the following Oncolytic SafeSwitch system. Their system utilizes the delivery of two recombinant viruses. Together, they conditionally cause viral replication in coinfected cells, when also subjected to both an activating thermal dose and mifepristone ligand presence. The science that underlies that system is that one virus, rAd2, contains the SafeSwitch with the unattenuated version of the transactivator and the other virus, rAd1, contains the SafeSwitch gene targets. The gene targets in this system are two regular, early viral replications genes: E1A and E4. In rAd1 (an E3-deleted recombinant adenovirus), the natural promoters for E1A and E4 were replaced with Gal4 promoters, which respond to the GLP65 transactivator that is conditionally produced by rAd2. rAd2 was rendered unable to produce either E1 or E4 protein products and contained the Hsp70b Gal4 promoter sequence to cause production of the GLP65 transactivator protein. In this manner, the Gal4 promoters in rAd1 could be effectively activated subsequent to a heat treatment.
Figure 2.13  A demonstration of the efficacy of the Oncolytic SafeSwitch in recombinant Ad-infected HeLa cells. Columns (A) and (B) demonstrate the replication and disbursement of the co-infected combination of rAd1 and rAd2 at 48 hours. Subconfluent cultures of HeLa cells were infected with a 25:1 ratio of rAd1 to rAd2. In column (A), there were 100x more cells than paired infectious virus particles (MOI of 0.01). In column (B), an order of magnitude more of each virus was utilized to generate the higher MOI of 0.1. A (+) or a (-) sign in the same row as the pictures indicates whether the respective cultures did or did not received the treatment indicated at the top of the column (HS for columns (A) and (B) = a two hour heat treatment at 42°C; HS in column (C) = a 1.5 hour treatment at 42°C; mife = 10 nM mifepristone ligand for all columns). The treatments were administered four hours post infection. Eight days after the treatment(s) were applied, cells were fixed and immunostained using a hexon protein antibody prior to photodocumentation. Column (C) illustrates survival of HeLa cells co-infected with the Oncolytic SafeSwitch under a variety of treatment combinations (mife and HS indicated as above). A 1:1 ratio of rAd1 to rAd2 infectious virus particles was used with a MOI of 25 to co-infect HeLa cells. Cells were stained with crystal violet 5 days post treatment application and were photographed. These are unpublished results by Vilaboa and Voellmy and are printed here with their permission.
of co-infected cells that were also exposed to mifepristone ligand in order to express all viral genes, with the exception of E3. The consequent adenovirus replication naturally causes the infected cells to lyse upon viral maturation. Oncolytic cell lysis promotes the release of newly packaged rAd1 and rAd2 viral particles, which may then co-infect adjacent cells. In theory, the Oncolytic SafeSwitch process may then be repeated with additional thermal doses. The limitations to the repetition include continued presence of the mifepristone ligand and co-localization of the rAd1 and rAd2 particles to the same cells, in which endogenous HSF1 activity can again be triggered. A demonstration of the effectiveness that Vilaboa et al. (unpublished results, printed here with their permission) achieved with the Oncolytic SafeSwitch viral pairing in HeLa cells is presented in Fig. 2.13.

Vilaboa and Voellmy provided evidence of regulated replication of the rAd1/rAd2 pair in a co-infection experiment in subconfluent cultures of human HeLa cells (unpublished results, presented here with their permission in Fig. 2.13 columns A and B). Subsequent to infection, the cells were subjected to the indicated conditions: either the permissive conditions that comprise a transient thermal dose and mifepristone ligand presence or a condition that lacked one or both of these treatments. Immunohistochemical analysis, performed eight days later with a hexon antibody, revealed infected cells and demonstrated viral spread in cultures that were subjected to both permissive conditions. Their results provide evidence for controlled replication and distribution of virus in infected cultures. Cultures that were exclusively subjected to only one treatment
or to neither treatment did not reveal a similar replication phenotype. Thus, the Oncolytic SafeSwitch system displayed the desired phenotype under all conditions that were examined.

Column C of Fig. 2.13 shows pictures from an experiment that utilized a 1:1 ratio of Oncolytic SafeSwitch viral components to again coinfect subconfluent cultures of human HeLa cells, this time at an MOI of 25. The effective oncolytic ability of the rAd1/rAd2 pair, despite the higher MOI that was used for this experiment, also was shown to be well-regulated. Almost complete cell death was revealed with crystal violet staining of rAd1/rAd2 coinfected cells when permissive conditions were applied, and almost no cell death was detected in the control conditions that lacked one or both treatment requirements. Additionally, cell density was compared to parallel cultures of cells, which included infected but otherwise untreated cells as well as uninfected cells (data not shown), and a similar cell number was identified among all cultures. Hence, the Oncolytic SafeSwitch stringently regulated replication of the rAd1/rAd2 pair.

**Combining the GALV(+) SafeSwitch and the Oncolytic SafeSwitch in vitro**

The next question to answer was whether a combination of the GALV(+) SafeSwitch and the Oncolytic SafeSwitch would yield an enhanced therapeutic effect. Such a combination utilized in tumors in vivo could, theoretically, improve the bystander effect achieved with each round of applied heat treatment when mifepristone ligand is also present. In theory, the production of syncytia would improve the likelihood of coinfection, which is necessary to cause viral
replication. In turn, the lysis of infected cells, a byproduct of viral maturation, would ensure that the syncytia resulted in cell death. The outcome of both mechanisms could increase the potential dispersion of progeny to neighboring

![Figure 2.14](image)

**Figure 2.14** Cell death in the HT-1080 #4 line as a function of: heat treatment temperature, mifepristone, rAd1 infection, and HSF1(+) transfection. Parallel cultures of cells were either transfected with CMV-HSF1(+) or not (component addition to the cells in the pictures is indicated by the (+) notation to the left of each row of photos, which relates to the respective heading across the top; a (-) sign indicates that the component was not added). The next day, cultures indicated by a (+) sign under the rAd1 heading were infected and 10 nM mifepristone was applied as indicated (pictures of cells that received mifepristone are further flagged by the dark border box). The indicated heat treatment was administered roughly four hours later. Cells were fixed with methanol and stained with Wright-Giemsa 48 hours post heat treatment.
cells [e.g., as reported by Li et al. (2001)]. While a first round of heating would be expected to produce more potent outcomes than either method would yield on its own, a second round of heating could then also be expected to yield controlled, yet profound, cell killing. Since the GALV(+) SafeSwitch System already incorporated components similar to that of rAd2, it was conceivable that only two vectors (rAd1 and a GALV(+) SafeSwitch vector) would need to be delivered to tumors for therapy to occur. To further simplify gene delivery in preliminary studies, the human HT-1080 #4 cell line was employed. The line, which contains the stably transfected GALV(+) SafeSwitch, was utilized in an experiment in combination with rAd1, which was obtained from Dr. Nuria Vilaboa (Laboratorio de Metabolismo Oseo, Hospital Universitario La Paz, Paseo de la Castellana 261, 28046 Madrid, Spain). A demonstration of in vitro results that illustrate the regulation of the combination is presented in Fig. 2.14. An additional component to the experiment utilized the constitutively active human HSF1(+) construct to deregulate SafeSwitch transcriptional control.

The combination of the stably transfected GALV(+) SafeSwitch and rAd1 in the human HT-1080 #4 cell line was more efficient at causing cell death after a single round of applied treatment than what was achievable with the GALV(+) SafeSwitch alone. This was evidenced by the apparent difference in post-treatment cell number (indicative of associated cell death) among otherwise comparable treatment conditions (compare the middle two rows of pictures to the top row of pictures). The SafeSwitch combination treatment also appeared to be dependent on an applied heat treatment as well as the addition of mifepristone.
Some cell death, however, was visible at the highest tested temperature in the absence of the mifepristone ligand and the mifepristone requirement was deregulated when HSF1(+) is expressed at high levels from the CMV promoter (bottom two rows of pictures). The dramatic results obtained when active HSF1 was incorporated underline the potential for cytotoxic effects, highlight the importance of a localized heat treatment, and may indicate an avenue to explore to improve switch activation (i.e.: to regulate HSF1(+) production from a Gal4 promoter within the context of Safe-Switch control, in order to overcome technical limitations that may be associated with delivering an activating thermal dose).

**In vitro section summary**

The aim of the work presented in this chapter was to pair a previously characterized molecular switch that features a harnessed HS response with a gene target that mediates a potent mechanism of cell death, so that the combination’s therapeutic merit can be assessed in vitro and can translate to an in vivo proof of principle tumor regression model. To achieve this aim, the SafeSwitch system described in chapter one was first paired exclusively with a gene target that produces a glycoprotein, GALV(+). The fusogenic consequence of GALV(+) expression both imparts a substantial bystander effect and causes cell death as unsustainable syncytia are formed.

The related in vitro results of transfected and stably derived cell lines were presented in this chapter. They illustrate a generalized outcome of tightly regulated, controlled syncytia formation. That outcome of phenotype regulation
was imparted by triggering the activation of HSF1 in cells through transient, applied heat that initiated gene transcription when the mifepristone ligand was also present. That regulated process was especially effective in causing death in cells of human origin.

The commercially available cell lines, which were utilized in these studies, were characterized in terms of their expression of the molecular switch and the target gene components through studies that employed transient transfection techniques. Select human and rat lines were further characterized for such gene expression through the production of stably transfected lines created in this work. The selected lines also formed the basis for the xenograft and homograft in vivo models for solid tumor regression, which the next chapter describes.

The final in vitro experiments, presented in this chapter, indicate that greater cell death was achieved when the GALV(+) SafeSwitch system was paired with a complementary SafeSwitch system: a conditionally replicating oncolytic adenovirus that was developed by Vilaboa and colleagues. Both SafeSwitch systems independently demonstrate cytotoxic gene transcription in vitro. Both were also designed with future gene delivery aspirations in mind to achieve tumor regression in vivo. Overall, Safe-Switch regulated therapeutic gene transcription caused controlled cytotoxic effects in vitro, which offers promise for safe and successful in vivo tumor regression results.
Chapter 3 – Advancing the Model

Extensions to the concept of deliberately activating HSF1’s normal functions to achieve therapeutic purposes are explored in this chapter through *in vivo* studies that extend the *in vitro* work, which features the use of HSF1 as a regulator of therapy and is presented in chapter two. This chapter addresses the development of *in vivo* models that test the pairing of the SafeSwitch design with gene targets, in order to mediate potent mechanisms of cell death in the context of a therapeutic situation. To support the design of future SafeSwitch therapies (discussed in greater detail in chapter four), the practical considerations that surround the creation of a SafeSwitch tumor regression model are detailed.

Challenging, complex, preclinical research was launched at the University of Florida’s Center for Environmental and Human Toxicology. The aim of the innovative, collaborative project was to treat various cancers using a recombinant virus system. The primary goals were: 1) to establish conditions required to activate SafeSwitch-regulated transcription *in vivo*; and 2) to evaluate the SafeSwitch system’s therapeutic potential to produce tumor regression via gene transcription that yielded controlled, cytotoxic consequences *in vitro*. The *in vivo* therapy was designed to be specifically targeted by the initial delivery of transient heat to solid tumors, which theoretically would trigger localized HSF1 activity. Gene transcription was expected to ensue to both cause and sustain tumor regression through other regulatory elements of the molecular switch.
Combining cells and animals to form the SafeSwitch model for solid tumor regression trials

The first item to address was the combination of an animal model and an \textit{in vitro} tested cell line that would best pair to produce a suitable example of Safe-Switch regulated tumor regression. The available models included nude mice and immunodeficient rats (reviewed in Rolstad, 2001), which would support xenograft studies, and syngeneic rats to support homograft studies. It was believed that a rat model would offer convincing evidence for the controlled function of the system. Rats are equipped with a mutated (but somewhat functional) PiT-1 receptor, which may enable any regulated gene transcription in host tissues to be manifest. It was also believed that the size of the rats might allow the specific application of heat, exclusively to a tumor-bearing limb. Mice do not have a receptor that is permissive for GALV(+) binding, and a system to successfully localize heat to a single limb or a tumor on a limb was lacking in availability. Focused application of heat was viewed as a more realistic therapeutic situation. Thus, rats were chosen as the first model system to work with.

\textit{Rat model screening}

Two avenues of rat model development appeared attractive: an immunodeficient xenograft rat model and an immunocompetent rat model. The latter model would utilize tumors formed from the F98 rat line, which is syngeneic with Fischer 344 rats. An immunodeficient xenograft rat model (rnu/rnu nude rats
as host), however, would utilize human cells that were especially susceptible to the effects of the GALV(+) SafeSwitch therapy. The immunocompromised model with human cells was initially viewed as most likely to demonstrate quantifiable tumor regression.

To compare in vivo growth characteristics, all the in vitro tested cell lines (WT lines and derived lines of F98 rat origin as well as of human origin: HT-1080, U87-MG, and HeLa), were used in preliminary experiments. Small numbers of nude (rnu/rnu) rats were utilized to compare subcutaneous tumor growth kinetics at a site that was superficial to the gastrocnemius muscle. Despite literature indications that they might grow in immunodeficient rats (Liang et al., 1997 and Ota et al., 1993), the HeLa cells did not successfully form tumors in this model. The cells of U87-MG origin formed tumors very slowly, and the stably transfected line, derived of that origin, did not appear to form tumors as well as the original U87-MG line. However, the HT-1080 and the F98 lines, as well as the related, derived lines, all grew readily in the model. Thus, the HT-1080 lines and the F98 lines were selected for further experimentation that attempted to activate the molecular switch in vivo in order to cause tumor regression.

To eliminate the possibility that gene delivery to tumor cells could contribute to complexities that might confound tumor regression results, the stably transfected lines that were derived from HT-1080 cells (HT-1080 #4) and F98 cells (F98 #6) were utilized. The tumors that they formed in vivo were next evaluated subsequent to applied treatments consisting of mifepristone and / or heat treatment (described in Fig. 3.1) or no treatment. The objective was to
determine whether the *in vivo* response could approximate the *in vitro*
observations with those lines. Heat treatment was initially delivered to tumors
formed in the hind limbs of the rats via a unilateral heating method. This system
was developed by John Munson, a collaborator at the University of Florida’s
Center for Environmental and Human Toxicology (Gainesville, FL), and the basic
principles are illustrated in the picture presented in Fig. 3.1.

Results of early studies with heat and mifepristone ligand treatments (data
not shown) surprisingly did not reveal dramatic tumor regression that would have

![Figure 3.1 The rat unilateral circulating water bath treatment heats a single
leg of up to four sedated rats at one time.](image)

Rats were pharmacologically sedated and secured to a board base (top of picture) that had strategically placed holes, which connected to 50 mL tubes below the board (middle of the picture). Each tube, near its base, was in turn connected to tubing (middle of the picture), which allowed water influx to be driven by a pump. A hole for water outflow, located just below the board and close to the top of each 50 mL tube, enabled a constant circulation of water around an immersed rat limb. The limb was inserted into the tube through the hole in the board. The pump (top right of the picture, shown extending into the basin filled with water) was also equipped with a thermostat-controlled heating system and completed the design. Water temperature was further monitored to maintain the designated temperature between 43 and 44°C by both mercury and digital (bottom front of the picture) thermometers.
paralleled the results achieved in vitro (see Figs 2.10-2.12). This raised concern centering on the effectiveness of the applied heat and the delivery of a suitable level of mifepristone ligand. Regarding mifepristone, pharmacokinetics and dose requirements for GLP65-based gene switches had been documented in mice (Babij et al., 2003 and Pierson et al., 2000) and in rats (Nordstrom, 2003). In early experiments related to this thesis work, mifepristone ligand treatment consisted of a solution of 1 mg mifepristone dissolved per mL of corn oil that was administered at 1µL/ g/ day delivered via intraperitoneal (IP) injection with a tuberculin syringe and a 25 gauge needle; on the first day of treatment, a double-dose was administered to ensure the possibility of switch activation with concomitant applied heat. The mifepristone ligand mode of delivery was later switched to sustained-release mifepristone pellets (purchased from Innovative Research: 15g/ 60-day release), which were implanted subcutaneously at the scruff of the neck prior to commencement of the applied therapy. There appeared to be a confounding difference in the cytotoxicity observed in vitro and in vivo. Studies into potentially contributing components that might underlie the difference were launched.

*Electroporation studies to answer important questions*

The concerns regarding the applied heat treatment initially concentrated on the heating parameters. Questions that were systematically addressed included: 1) the timing and frequency of the applied heat treatment, i.e.: whether a heat treatment delivered soon after implantation would yield desirable
outcomes in tumor outgrowth measurements; and 2) whether a small heat effect could be amplified with repeated heat treatments to achieve a therapeutic outcome. Intratumoral delivery of the constitutively active human HSF1(+), via DNA electroporation, was also used to trouble-shoot heating limitations.

Practical constraints associated with utilizing small numbers of animals in each group exacerbated difficulty in drawing conclusions about the possible occurrence of subtle effects, since tumor growth characteristics, observed over time, often varied as much within groups as between groups. It was not clear whether the applied heat alone could inhibit or alternatively potentiate tumor growth through upregulating protective aspects of the molecular chaperoning system. Direct delivery of the constitutively active HSF1(+) construct via intratumoral DNA electroporation offered potential as an alternative means to the water bath heat treatment, in order to address switch activation (see Figs. 3.2 – 3.4). To comparatively track tumor changes over time, data is presented in terms of Average Tumor Volume (calculated by multiplying \( \pi/6 \times \text{the greatest tumor height} \times \text{the widest tumor diameter} \times \text{the narrowest tumor diameter} \)) and / or the Average of the Percent Increases in the Tumor Volume (an average is calculated, along with standard deviation of the percent differences in each group, of the new tumor volumes; the initial tumor measurement, taken at the start of the treatment, is utilized as reference). Experiment results, presented in Figs. 3.2 and 3.3, illustrate the power and the advantage of presenting tumor measurement data as an average of the percent increase in tumor volume per group, instead of the simple average of tumor volume measurements.
Figure 3.2 Average tumor volume related to percent increase of growth as indicators of treatment outcomes in nude rats bearing tumors formed from HT-1080 #4 cells. Bilateral tumors were started in eight nu/nu (nude) rats with a subcutaneous injection of approximately one million cells suspended in 50 µL of PBS from the HT-1080 #4 line in the region that lies just superficial to the gastrocnemius muscle. When tumors reached a size that was approximately 400 mm³, half the nude rats received sustained-release mifepristone pellets that were implanted subcutaneously, close to the scruff of their neck. They formed the groups that were labeled as the (+) mife condition. Rats in the (-) mife groups did not receive pellets. The left limb of each rat was heated for 30 minutes with the unilateral circulating water bath apparatus outlined in Fig. 3.1 set to 43.8°C. The associated measurements were grouped as (+) HS treatment readings for rats that either did or did not also have mifepristone pellets. The measurements associated with the unheated right limb formed the basis of the (-) HS groups for those same rats. Direct DNA injection of 15 µg of CMV-HSF1(+) was followed by electroporation. Conducting calipers flanked the tumor region in rats in the (+) HS groups. The electroporation of HSF1 (+) served as an attempt to reactivate the switch eight days post application of the heat treatment. Panel (A) presents the data as average tumor volume measurements from each group. Panel (B) graphs the same data from Panel (A) as a percentage of growth on the noted day, relative to the tumor size measured on the day of heat treatment.
Fig. 3.2 presents data from an experiment that utilized the HT-1080 #4 cell line to form tumors in nude rats. Four groups were formed that examined combinations of mifepristone and heat treatment. HSF1 (+) was delivered via DNA electroporation eight days later, in an effort to further trigger switch activation. In Panel A of the figure, it first appears that the group that received both mifepristone ligand and heat / later HSF1(+) electroporation had tumors that grew larger than any other group. This is the direct opposite of the anticipated effect. Upon closer inspection of the data presented in Panel B (Fig. 3.2), which considers tumor growth rate as a function of starting tumor size, the results indicate relatively similar tumor growth in all groups. A conclusion, which could be drawn from the results, was that the constitutively active HSF1(+) construct, delivered at that time point, was not effective as a means to facilitate inhibitory effects on tumor growth.

The experiment presented in Fig. 3.3 sought to answer whether an earlier delivery of HSF1(+), concurrent to the initial delivery of GALV(+) SafeSwitch genes to WT HT-1080 tumors, would yield results that would then differ from the control groups. Electroporation was applied to small tumors, formed from WT HT-1080 cells, in order to deliver the GALV(+) SafeSwitch construct and the HSF1(+) DNA. The results (Fig. 3.3, Panel A) were confounding: the (+) mife/ (+) HS (HSF1(+)) electroporation group), which was expected to demonstrate regression, grew the largest tumors among the rats. Note, however, that the average starting tumor size in that group was slightly larger than that of the comparative groups on the day of electroporation (labeled day 0 in Fig. 3.3,
Figure 3.3  Effects in nude rats bearing tumors formed from WT HT-1080 cells, following early electroporation of HSF1(+) and GALV(+) SafeSwitch DNA. Bilateral tumors were formed in six nude rats implanted with WT HT-1080 cells, using the methodology described in Fig. 3.2. Half the rats also received mifepristone pellets (forming the (+) mife condition). When tumors reached a size that was approximately 200 mm³, they were electroporated subsequent to direct DNA injection of either 15 µg of the GALV(+) SafeSwitch construct alone (indicated as the (-) HS group) or 7.5 µg of it in combination with 7.5 µg HSF1(+) (indicated as the (+) HS group). Note: HS in this figure exclusively refers to HSF1(+) treatment, not to a form of thermal treatment. Panel (A) graphs the average tumor volume, whereas Panel (B) presents the same data as a graph of the average of the percent increase in tumor volume on the noted day, which relates observed measurements to the starting tumor size.
Panel A). Panel B (Fig. 3.3) graphs the same data as a group average of the percent increases in tumor volume observed on the noted day, with measurements standardized to the starting tumor size. The data presented in that manner illustrates the expected effect: untreated tumors appeared to have a more rapid growth rate than treated tumors. However, both groups with unheated tumors had large error bars for these measurements. These error bars reflect the spectrum of tumor size measured in those groups. In fact, some tumors were not detectable on day 2. This result highlights the reason for a greater focus, in later experiments, to standardize tumor size and means of measurement. An additional concern was that the characteristic necrotic core of the tumors generated with the HT-1080 cells might be inhibitory to gene transcription or expression.

To expand upon the approach utilized in Fig. 3.3, another experiment was performed in nude rats that utilized an early, but this time a repeated, delivery of HSF1(+), concurrent to the delivery of the GALV(+) SafeSwitch construct to tumors. Subcutaneous tumors were formed in the experiment presented in Fig 3.4, this time with WT F98 rat cells. Great attention was given to standardize the starting tumor size. Initial treatment commenced when the tumors had grown slightly larger (an average increase in size of 25%) than those on day 0 in the experiment presented in Fig. 3.3, so as to represent differences that might be observed between treatment groups as clearly as possible. Animals were either treated or not treated with mifepristone. All groups received the GALV(+) SafeSwitch construct and half of the groups also received the HSF1(+) construct.
on the indicated days (0, 8, and 13). Results (Fig. 3.4) indicate that tumor growth appeared to be the most in the group that received neither HSF1(+), nor mifepristone. Error bars in that group grew large after the second administration.

Figure 3.4 Repeated intratumoral electroporation with the GALV(+) SafeSwitch and HSF1(+) constructs inhibits WT F98 tumor growth in nude rats. Bilateral tumors were formed in six nude rats implanted with WT F98 cells (approximately 1.15 million cells, suspended in 50 µL of PBS, injected subcutaneously above the gastrocnemius muscle). Half the rats also received 15 mg/60-day sustained release mifepristone pellets (forming the (+) mife condition). When tumors reached an average size of approximately 250 mm³, the tumor on the right leg was electroporated with 15 µg of the GALV(+) SafeSwitch; and the tumor on the left leg was electroporated with that construct combined with 5 µg of the CMV-HSF1(+) construct (as is indicated by the (+) sign across from each component in the column under each group). DNA injection and electroporation was repeated both eight and thirteen days later.
of treatment (day 8). Though the group that received both HSF1(+) and mifepristone had the smallest tumors, differences between that group and the groups that received either HSF1(+) or mifepristone (but not both) were small. An explanation for the activity of the GALV(+)

SafeSwitch in the absence of mifepristone (observed in the second set of columns) is that the HSF1(+) gene was transcribed from a strong, constitutive promoter, which appeared to cause deregulated activation of the GALV(+) SafeSwitch in the absence of mifepristone in vitro (see Fig. 2.14). Another consideration is that full expression of the switch in the rats, which received HSF1(+) and mifepristone (first set of columns), may have stimulated a rudimentary immune system response that affected growth of the control tumor located on the contralateral leg (third set of columns).

The last consideration highlighted a reason to limit treatments to one per animal, instead of one per leg of the same rat (i.e.: +/- HS). Locally activating HSF1 would yield systemic consequences, if an immune system response was successfully triggered. Since more animals would need to be included in each study to represent the same number of treatments, a new approach to heating rats was developed. The so-called rat bilateral system enabled 25% more rats to be heated simultaneously than the previous, unilateral heating system. It was developed by John Munson (University of Florida, Gainesville, FL) and is depicted in Fig. 3.5.

The rat bilateral heating system (Fig. 3.5), delivers heat to the entire lower body of rats, which also allowed tumors that were formed subcutaneously, proximal to the base of the tail, to be heated. It was postulated that this new
tumor location would allow more accurate tumor measurements to be taken, without the confounding consideration of the gastrocnemius muscle size. Additionally, it was noted during necropsy that tumors often tended to invaginate the space between the sheaths associated with the gastrocnemius muscle at the

Figure 3.5 The rat bilateral circulating water bath heat treatment was designed to heat both legs and the lower back of up to five sedated rats at one time. This heating method was designed for immunocompetent rats, however nude rats are shown in this picture. Some of the same components, which were utilized in the unilateral heating method (pictured in Fig. 3.1), are employed here: The basin filled with water, the water pump equipped with a thermostat-controlled heating system, and thermometers that further monitored temperature (visible in the bottom right picture of the composite). Rats were pharmacologically sedated and wrapped in a rectangular porous suit (top left picture), which was designed with holes for the arms and legs and Velcro to secure the fit (top right picture). A wire connected the top of the suits to a rod that stretched above the basin, which then enabled the rats to be securely suspended at an appropriate height to immerse the tumor in the circulating water bath (bottom left picture).
previous location. A consideration was that the effect of this growth into the 
muscle sheath might shield effective heat penetration to the cancer cells. A 
dorsal, lower back tumor location was believed to be more accessible for both 
heating and caliper measurement purposes.

To address whether SafeSwitch transcription actually occurred in the 
heated rats, experiments were designed that utilized DNA electroporation of the 
same reporter constructs that were first presented in Fig. 2.5. In Fig. 3.6, 
luciferase production is presented as a consequence of gastrocnemius muscle 
injection and co-electroporation in two rats treated with reporter constructs and 
an unattenuated GLP65 SafeSwitch TA (containing the Hsp70/Gal4 promoter 
cassette). To keep the quick experiment simple and small, the unilateral water 
bath heat treatment was utilized to heat a limb of one rat that received 
mifepristone via IP injection. The other rat was designated for control condition 
outcomes: it received the unilateral water bath treatment but was not given the 
mifepristone ligand. Transcription was measured in terms of luciferase 
production.

The data graphed in Fig. 3.6 appeared promising, even with the 
rudimentary unilateral water bath heat treatment. The calculated results indicate 
switch activation was greater than one hundred times the background level 
achieved in the opposite limb of the rat that was given mifepristone, and 
activation was more than 1000 times the level determined in either limb of the 
other rat, which was not given mifepristone. Thus, SafeSwitch components 
caused regulated gene transcription in rats, and light units were measured that
were orders of magnitude higher than background levels. This pilot study concluded that *in vivo* activation of an electroporated switch is, indeed, possible in a rat model. Note that this SafeSwitch combined the full-length, unattenuated

![Figure 3.6 Intramuscular *in vivo* electroporation of fLuc SafeSwitch DNA in rats.](image)

The 17x4 TATA firefly luciferase target was co-electroporated with the Hsp70/ Gal4-GLP65 TA (unattenuated SafeSwitch) construct at a 20:1 ratio along with the CMV-renilla luciferase construct, which was included at 5% of the concentration of the firefly construct. Gene introduction began with a pretreatment procedure: 500 units (U) of hyaluranidase were injected into the gastrocnemius muscles of two Fischer 344 rats. Subsequently, 50 μg of total DNA was injected intramuscularly. Electroporation then followed with calipers flanking the entire muscle. Six days later, both rats had one limb treated with the unilateral heating method of Fig. 3.1 (42.3°C / 30 min) to generate the respective (+) HS condition in the figure. On the day of heat treatment and for the five days that followed, one rat was administered a daily intraperitoneal (IP) injection of mifepristone suspended in corn oil, a final mifepristone dose of 0.25 mg/kg per day, which yielded the groups with the (+) mife notation. (-) HS/ (-) mife groups did not receive the respective treatments. On the sixth day, the gastrocnemius muscle was harvested and homogenized in PBS containing PMSF, and luciferase measurements were read with a luminometer.
transactivator (Hsp70/Gal4-GLP 65) with the Gal4-firefly luciferase reporter construct, whereas the GALV(+) SafeSwitch construct and stably-transfected cell lines utilized the attenuated form of the transactivator. The questions raised by the successful experiment outcomes included: whether the full-length version of transactivator was superior to the attenuated form in vivo and whether similar results could be achieved with intratumoral DNA electroporation.

The experiment presented in Figure 3.7 was designed to address: 1) whether mifepristone modes of administration made a substantial difference on measured reporter activity; 2) whether a substantial difference in the relative luciferase activity (RLA) could be measured in vivo between the attenuated RKC GLP65 TA and the unattenuated GLP65 TA; and 3) whether substantial differences existed in the HS responses (measured with an Hsp70 luciferase reporter) in mice and in rats that were subjected to respective bilateral water bath heat treatments (see Fig. 3.5 for the rat system; Fig. 3.10 introduces a similar system that was used to treat mice). Mice were included in this study, since they are well-characterized with regard to the effective mifepristone levels that produce GLP65 TA activation in vivo and since they offered potential as an alternative model system to explore.

The bars of the graphs in panels A and B of Fig. 3.7 illustrate Hsp70 results (n=1; obtained in one leg of each animal), which differ considerably between subjects and which should not have been dependent on mifepristone presence. These findings must be considered when examining the 17x4 TATA luciferase results (n=1; obtained in the contralateral leg of each animal of panel A
to produce the unattenuated GLP65 condition and in three additional rats that produced the RKC GLP65 condition) presented in panels C and D. Those results indicate that the unattenuated GLP65 TA system may have been more

**Figure 3.7 Intramuscular in vivo electroporation examines Hsp70 promoter activity and transcriptional activity of SafeSwitch TA constructs as a function of mifepristone modes of administration in Fischer 344 rats and in nude mice.** Panels (A) and (B) graph Hsp70 RLA results that were obtained with one limb each of three mice and three rats. Panel (B) differs from Panel (A) in that it presents the information on a log-scale, which enables the appreciation of basal levels of background RLA. For the same reason as that stated for Panel (B), Panel (D) is a log-scale graph of the data that is presented on a linear scale in Panel (C). The data represented in the graphs of panels (C) and (D) were obtained with 17x4 TATA Luc reporter, which was utilized in an additional three rats as well as in the opposite limb of the same three mice and three rats noted above. The additional rats were employed in order to complete a side by side comparison of switch activation that was achieved with either the attenuated or the full length TA constructs. The gastrocnemius muscles of each animal were electroporated after an intramuscular injection delivered the indicated firefly luciferase reporter construct. Specifically, all three mice and half of the six (total) rats, each had the left leg injected with Hsp70 firefly luciferase and the right leg injected with the target gene (the 17x4 TATA firefly luciferase reporter) as well as the unattenuated GLP65 TA (introduced in a 20:1 ratio comparable to that used in Fig. 3.6). The right leg of the remaining three rats were injected with a 5:1 ratio of 17x4 TATA reporter construct to attenuated RKC GLP65 TA construct. The CMV-renilla luciferase construct served as an internal control for all injection mixtures; the amount included was 5% of the respective amount of firefly construct. Electroporation and tissue harvesting methodology were identical to that in the previous experiment (presented in Fig. 3.6) and the schedule was similar. However, a bilateral circulating water bath treatment was utilized in this experiment to heat limbs of both rats and mice (noted with "P" or "IP" in the figure). The bilateral methods are outlined in Fig. 3.5 for rats, which received a heat treatment of 43.8 degrees for 30 minutes, and in Fig. 3.10 for mice, which received a heat treatment that was almost one degree lower (43.0 degrees/ 30 min.). Five days post DNA electroporation and two days post the time that heat treatment was administered to some animals, tissue was harvested to measure luciferase activity. Animals indicated by the following symbols: (-) - neither received the heat treatment or mifepristone; (P) - received mifepristone pellets on the day of electroporation (mice pellet = 1 mg/ 60-day sustained release and rat pellet = 15 mg/ 60-day sustained release); and (IP) – received the first mifepristone dose simultaneous to the heat treatment (three days post electroporation). The IP animals continued to receive daily intraperitoneal injections (50 mg/ kg for mice and 250 mg/ kg for rats) until tissue was harvested.
effective. Overall, both heated mice appeared to display a higher level of relative luciferase activity, obtained with either system, than rats. Since Hsp70 readings (Panel A) were also higher in the mice than the rats, this finding may suggest that a focused effort to improve delivery of an effective, uniform thermal dose may optimize switch activation when mifepristone is present. Results presented in panel C demonstrate that a substantially greater 17x4 TATA luciferase effect was achieved in the mouse that was administered mifepristone via IP injection than in the mouse that received the mifepristone pellet. The basal activity appeared to be lower in the mouse, which did not receive mifepristone or heat, than in the similarly treated rat. However, the attenuated RKC GLP65 corresponded to the
lowest basal promoter activity (a caveat is that Hsp70 luciferase data was not obtained for any of the RKC GLP65 treated rats).

To tie these muscle treatment results to earlier tumor treatment results, it was postulated that tumor cells might be limited in their capacity to express introduced genes, perhaps as a function of rapid growth that saturates ability to produce additional proteins. In the context of such a situation, precluding basal expression (under control conditions \textit{in vivo}) could be less important than potentiating tumor regression (under the desired conditions, in order to meet hypothetical, minimum expression threshold requirements for tumor regression outcomes to occur).

In absence of an immediate method to improve thermal delivery to tumors, other means to augment outcomes in the rat model were considered. An immunocompetent model offered potential to magnify any tumor regression outcomes, which might have been limited in the immunocompromised model, since an immune system response to cancer cells could improve therapeutic effects. Tumor immunity could be potentiated by therapy-induced cell death combined with the activation of HSF1 that is required to initially trigger gene transcription. Furthermore, the Fischer 344 rat immunocompetent model that was developed utilized F98 cells. \textit{In vitro}, cells of that origin demonstrated a lower threshold for the activation of the HS response than cells of human origin, though they also displayed less syncytia formation as a consequence of GALV(+) expression than the human cell line alternatives (see Figs 2.7, 2.9, and 2.10). Additionally, the theory lingered that cytotoxic gene transcription could be further
enhanced by an optimized co-delivery of HSF1(+) DNA. Hence, the immunocompetent model was utilized in the next set of experiments.

**Immune system considerations in the rat model**

Tumors, grown in three locations (back and intramuscularly in each leg), were charted in the experiment presented in Fig. 3.8, which utilized the WT F98 cell line in a syngeneic model that featured an intact immune system. Thus, this model enables the examination of immune-system related effects of applied heat and HSF1(+) expression. A consideration in utilizing this model, however, is that expression of foreign proteins (e.g., the GLP65 TA component of the SafeSwitch or human HSF1) might inhibit tumor growth over time. Normal presentation of antigens derived from foreign proteins could produce an immune response that also targets the cancer cells under control conditions. The experiment associated with Fig. 3.8 was designed to test immune influences on tumor cell growth and also examined tumor growth in new locations in an effort to accurately represent or standardize measurements.

WT F98 cells were either left untransfected or were transiently transfected with one of three CMV-promoted constructs that were anticipated to stimulate an immune response once cells expressing the constructs were transplanted to Fischer 344 rats: 1) green fluorescent protein (GFP); 2) human HSF1(+); 3) human HSF1(-) / AVST, the dominant negative mutant mentioned in chapter one and described by Xia et al. (1999) and Zuo et al. (1995). The HSF1(-) / AVST construct was included in order to contrast any downregulated HSF1-specific
effects with outcomes of upregulated activity conferred by HSF1(+). Distinct observations associated with the expression of each mutant would disentangle activity-associated consequences from a generalized immune-system mediated response, which would be expected to be similar for both mutants. The two versions of human HSF1 protein, though lacking different portions of the wild type HSF1 sequence, have an otherwise identical base sequence (see Fig. 1.2, Appendix 2 and Appendix 4) that an intact host immune system would likely view as similarly “foreign”. The GFP was included to contrast effects of HSF1 expression with effects associated with expression of any foreign protein in the model.

New locations were injected with cells: 1) a subcutaneous spot, proximal to the base of the tail (forming the back tumor in Fig. 3.8, Panel A), which was removed from the influences of the gastrocnemius muscle that might affect caliper measurements or uniform delivery of heat to the tumor; and 2) intramuscularly, in order to use the sheath of the gastrocnemius muscle to standardize caliper measurements and heating effects of the contained tumor (Fig. 3.8, Panel B). Resulting tumor outgrowth was followed for just over a month and was charted for each location. The average volume measured in each group that formed back tumors (panel A) parallels the average volume achieved in each group with leg bearing tumors (panel B). Observations regarding the back tumor location included: 1) the amount of normal tissue, which necessarily was included in the readings, was minimized; 2) the tumors grown on the back of the
Figure 3.8 An immunocompetent rat model, which examines tumor location and influences on tumor outgrowth that are associated with expression of human mutant HSF1 constructs and a fluorescent protein. Fischer 344 rats were implanted with WT F98 cells, which were either not transfected (labeled WT F98) or were transfected the previous day with the indicated construct: CMV-GFP, CMV HSF1(+); or CMV-AVST. Two tumor locations were utilized for each 50 µL injection of approximately one million cells suspended in PBS: the lower back area (just above the tail) or direct injection into the right gastrocnemius muscle. The F98 cell line is a fibrosarcoma, and it was believed that deliberate growth within the muscle sheath might yield standardization to tumor measurements. Measurements were followed over a 25 day period that began one week after cell implantation.
rats were more likely to be fully accessible to heat treatments; and 3) the back location least limited animal mobility, which might allow longer studies to be conducted. These observations offered compelling reasons for the back location to be viewed as an improved tumor growth site for use in future studies.

Tumor growth appeared equally inhibited among all groups of cells transfected with foreign proteins, when compared to tumor growth of the WT F98 cells (Fig. 3.8). This was an anticipated outcome of antigen presentation to an intact immune system, in conjunction with cancer cells. The result might lend support to the theory that enhanced tumor regression outcomes may be achieved in the rat immunocompetent model for SafeSwitch control of gene therapy transcription, even though syncytia formation was limited in the F98 line. Some considerations, however, must be taken into account: 1) transfection may have reduced the ability of the cells to grow; 2) immune system sensitivity of this level may also enact an immune response against cancer cells that only transiently produce ineffective, monomeric TA, which also would be foreign to an animal model host [i.e.: transient production of TA could occur as a consequence of transcription from the Hsp70 promoter in absence of mifepristone, caused solely by active HSF1; such a condition could also cause some antitumor effects (e.g., smaller tumors in heat-alone treated groups receiving the therapy)]; and 3) signaling cascades that result from induced molecular chaperone production by active HSF1 may further enhance an immune system response.

Rat versions of the human HSF1 mutants, if they were available, would best examine such activity-dependent signaling cascades for a contribution to
cancer cell immunity, since antigen responses to a foreign protein would be eliminated. A constitutively active mouse version of HSF1 was able to be produced for another purpose and is presented in Chapter 4 (as well as in Appendices 2 and 3). A comparison of the *Mus musculus*, (a.k.a.: house mouse, NCBI accession #P38532) and the *Rattus norvegicus* (a.k.a.: Norway rat, NCBI accession #CAA58149) sequences indicates an approximate protein homology of 98%. While not identical, this similarity is improved over the 89% homology shared between that same mouse factor and human HSF1 (accession #Q00613). However, since a parallel mouse HSF1(-) / AVST mutation was not readily available and characterized, in order to differentiate activity-specific effects from a general immune response to the somewhat foreign protein, the mouse version was not tested in this model. Additional considerations in using the human factor mutants included an ample supply of the required HSF1 DNA, which had been thoroughly characterized through several studies conducted in the Voellmy laboratory (Xia et al., 1999, as well as Zuo et al., 1994 and 1995) and by others who requested the construct from the lab. Furthermore, if successful, the ultimate thereapy would utilize the human constructs in the context of human cells.

The experiment, presented in Fig. 3.9, was designed to investigate whether tumor growth was inhibited or alternatively enhanced by a thermally activated GALV(+) SafeSwitch, which was either utilized alone or in combination with the constitutively active or dominant negative form of HSF1 (again included
Figure 3.9 Activating the GALV(+) SafeSwitch in vivo in an immunocompetent tumor outgrowth model that also examined co-expression of human HSF1(+) and HSF1(-)/AVST. Rat F98 #6 cells were either left untreated or were separately transfected in vitro with the indicated mutant HSF1 construct two days prior to their use to form tumors. Approximately one million cells (in 50 µL PBS) were injected subcutaneously, low on the back, near the base of the tail of each Fischer 344 immunocompetent rat. 15 mg/60-day sustained release mifepristone pellets (mife) were implanted in the indicated rats (+). Three days later, rats in all groups (except those that did not receive mifepristone pellets) were treated with a bilateral heat treatment (Fig. 3.5) of 43.8°C/30 min. Tumor outgrowth was then followed. Average observed tumor volume per group, along with the calculated standard deviation, was graphed in the figure.
to differentiate general immune system responses associated with expression of the human HSF1 proteins from any specific, activity related response). Instead of also delivering the GALV(+) SafeSwitch to cells, the F98 #6 line was utilized to express the genes that could produce necrotic cell death by the formation of unsustainable syncytia GALV(+). This was expected to improve the chances for exposure of cellular contents to the immune system. In this experiment, a heat treatment was administered to all groups, except the control group (which also did not receive mifepristone), just three days after cells were implanted, in an attempt activation of the GALV(+) SafeSwitch as early as possible.

At seven days post bilateral heat treatment (HS) the control group that did not receive mifepristone or heat had tumors that grew much larger than those in any other group, which appeared to be consistent with the desired phenotype. However, this difference was short-lived in the parallel group, which also was not transfected with HSF1 constructs but which was treated with heat (to produce activation of endogenous HSF1) and mifepristone ligand. That group caught up to the first group’s average tumor size by the 16th day post heat treatment. At that point, the resulting tumor burden borne by animals in those two groups necessitated euthanasia. An approach, such as that attempted in experiment 3.4 (early and repeated treatment to activate the GALV(+) SafeSwitch), likely would not maintain differences in measurements between those two groups, unless it caused a successful immune-mediated effect to be mounted.

The remaining two groups, which were only transiently transfected with HSF constructs, remained inhibited in tumor growth for the entire duration of the
experiment. No differences that might be ascribed to activity differences of the expressed mutant HSF1 were able to be noted during the 22-days that growth was measured, except that only four of the six injections (noted n=4/6 in the figure) of cells that were transfected with the HSF1(-)/ AVST mutant successfully formed (albeit small) tumors during the course of the experiment. All injections with other cells in this experiment produced tumors. It is conceivable that this observation could be attributed to an abrogation of HSF1-specific activity (e.g., consequential inhibition of chaperone-supported angiogenesis or chaperone-mediated autophagy that otherwise supports tumor growth). Presumably, the dominant inhibition effect observed in the HSF1-transfected cells was mediated by the common, general, immune-system response to human HSF1. An alternative explanation, that transfection of the cells inhibited their \textit{in vivo} growth characteristics (specifically due to HSF1-mediated effects or generically as a consequence of expressing additional proteins), could not be ruled-out.

Another explanation for the lack of sustained tumor regression, associated with expression of the GALV SafeSwitch \textit{in vivo}, is that a tumor is a heterogeneous population of cells. In fact, it has been reported that over half of the mass of cells that form a tumor are normal host supporting cells, which primarily consist of fibroblasts, tissue macrophages, and endothelial cells (Balkwill et al., 2005). Inflammation and tissue remodeling cause normal cells from the host to be recruited to that environment and enable their continued proliferation (Balkwill et al., 2005). Molecular chaperones that are upregulated by the activation of HSF1 likely play an important role in that process. The
infiltration of normal cells that support tumor growth increases over time and may even be facilitated by the inflammation that applied heat or DNA electroporation may cause. The result may create a scaffold of normal cells, which could effectively shield the cancer cells from the bystander effects observed in vitro. A possible means to circumvent this limitation would be to transplant an entire tumor mass from a human host, adapting a skin pedicle protocol similar to that described by Hahn et al. (1993). Such a model would be challenging to develop and would require additional regulatory approval. It was decided to instead investigate a simple, readily available, xenograft mouse model. That model capitalized upon some desired characteristics, which offered hope that any inhibitory effects due to normal cell infiltration might be overcome: 1) the human HT-1080 line was tremendously sensitive to GALV(+) -mediated cell death (see Figs.: 2.10, 2.11, and 2.12); and 2) reporter activity in the mouse model was superior to that in the rat model (observed in Fig. 3.7).

**Explorations with xenografted nude mice further SafeSwitch model development to measure tumor regression outcomes**

It was possible that minor effects associated with SafeSwitch activation in vitro in the #6 cell line were further diluted in vivo (e.g., Fig 2.8 and 2.10) by the infiltration of untransformed, normal host cells. Thus, the xenograft mouse model was reexamined. Nude mice have been documented to support the growth of many human cell lines (reviewed in Van Dyke and Jacks, 2002). The focus for the use of this model was to optimize advantages that it afforded, in order to
produce measurable tumor regression outcomes, which appeared to be limited in the previous models.

Efforts to amplify switch activation effects in a nude mouse model

Preliminary studies sought to quantify the potential for human cell fusion to occur in the nude mouse model through an adaptation of the *in vitro* luciferase

![Figure 3.10](image)

The mouse bilateral circulating water bath heat treatment was designed to heat both legs and the lower back of up to eight sedated mice at one time. This 30-minute heat treatment method was almost identical to that utilized in Fig. 3.5 with rats. However, the water temperature that was utilized was lower (42.5-43°C) than that employed with the rats (43-44°C), since the nude mice had difficulties with thermal regulation at a higher temperature. Additionally, it was possible to simultaneously treat up to eight mice at one time, and the suits that suspended the mice were tailored to their size. Each mouse was pharmacologically sedated and then wrapped in the rectangular, porous suit, which was designed with holes for the arms and legs (right picture). The suit was clipped together in the back and at also at the top, where a string connected the top clip to the rod that stretched above the basin. A ballast lead weight attached at the bottom stabilized the mice that were immersed in the water bath up to their abdomen.
overlay method to the *in vivo* conditions. Untransfected HT-1080 #4 cells were mixed with WT HT-1080 cells, which were transiently transfected with reporter constructs, and the mixture was injected into the gastrocnemius muscle of three groups of three mice each: 1) a control group, which received no further treatment; 2) a heat treated group, which received mifepristone in a subcutaneous pellet (P) form (1 mg / 60-day sustained release); and 3) a heat treated group, which received mifepristone via intraperitoneal (IP) injection. In parallel, the same cells that were injected into the animals were also plated as two separate cultures *in vitro*. One culture received 10 nM mifepristone. Both groups of animals were treated with the indicated mifepristone regime. Then, appropriate animals and the *in vitro* cultures were subjected to similar, parallel heat treatments. The *in vivo* heat treatment utilized the mouse bilateral water system (see Fig. 3.10 for the description of this heating method, which Fig. 3.7 also referenced and employed). Two days later, the *in vitro* cultures and the gastrocnemius muscles were harvested and extract was prepared for luminometer readings.

Firefly luciferase results (Fig. 3.11) generally favored the *in vitro* conditions (Panel B). An order of magnitude difference was observed for the overall RLA between the *in vitro* and the (IP) *in vivo* condition (Panel A). However, that difference corresponded to only a 25-fold (approximate) calculated difference in the associated fold inductions. Furthermore, only a two to three-fold difference was observed for the overall RLA between the *in vitro* and the (P) *in vivo* condition (Panel A). In fact, the associated fold inductions were nearly identical.
This result indicated some potential, in that in vivo model, for sufficiently strong transcription to produce measurable, SafeSwitch-mediated tumor regression outcomes.

**Figure 3.11** Comparative in vivo and in vitro luciferase reporter results of SafeSwitch-mediated cell fusion occurring with the use of stably transfected HT-1080 #4 cells. Panel (A) presents in vivo GALV(+) SafeSwitch activity and Panel (B) presents parallel in vitro GALV(+) SafeSwitch activity. In order to adapt the luciferase overlay method (Fig. 2.5) to the experiment conditions, parallel cultures of cells were grown: HT-1080 #4 cells and WT HT-1080 cells. The WT HT-1080 cells were transfected with the 17x4 TATA firefly and CMV-renilla luciferase reporter mixture (100:3 ratio of the respective reporter constructs). The next day, cell populations were mixed in a 5:1 ratio of untransfected cells to transfected cells. The mixture was divided to form two in vitro cultures that were plated [Panel (B)], and was also utilized to intramuscularly inject the gastrocnemius muscles of mice (0.5 million cells in 20 µL PBS per injection). Three groups of three mice each were examined. One group (P= administered pellet containing mifepristone) received mifepristone pellets (1 mg/60-day sustained release) that were implanted subcutaneously, dorsal to their shoulder blades, four days earlier. The next day, that group and the group noted (IP), which was administered intraperitoneal mifepristone (0.25g/kg), were heated according to the 30-minute, bilateral heat treatment method presented in Fig 3.10. The (IP) group also received the first injection of mifepristone ligand. Thus, those groups were noted as: (+) HS/ (+) mife. That same day, 10 nM mifepristone ligand was added to one culture of cells to yield the (+) mife condition, and both cultures were heated with the use of a circulating water bath [(+) HS]. Water baths for both the in vitro and the in vivo treatments were set to 42.8°C. The following day, the mice in the (IP) group received a second mifepristone injection. Two days post-heat application, cells were harvested from the tissue culture dishes, and the gastrocnemius muscle was harvested from the animals. Extract from the muscle was formed via homogenization in the same buffer (Passive Lysis Buffer, Promega) that was utilized to lyse the cells analyzed in vitro. Luminometer readings were taken and the average RLA and standard deviation were graphed for the in vivo study. Fold induction is indicated with hatched bars for the (P) and the (IP) groups relative to the (-) group, which did not receive mifepristone or heat treatment [(+) HS/(-) mife]. The in vitro RLA and fold induction (n=1) was also graphed for the (+) HS/ (+) mife reading relative to the (+) HS/(-) mife reading. Note that the hatched bars in each graph relate to the right Y-axis (fold induction).
Given the importance in keeping the balance tipped toward cell fusion with a negative influence likely caused by normal cell infiltration over time, an outgrowth experiment was designed, in which implanted cells were either left untreated or were subjected to mifepristone and heat treatment as early as possible. The area where cells were injected was heated just three days post implantation and then heated again one week later. The columns presented in Fig. 3.12 (Panel A), illustrate the average tumor volumes on the days indicated for the (+)HS / (+)mife group, which changed little over time, and for the (-)HS / (-)mife group, which tripled in average size over the course of the experiment. Fig. 3.12 (Panel B) presents the data in terms of the cumulative average percent increase in tumor volume (from day 7 to the end of the study) for each group (left set of columns) and the average tumor weight, which was assessed post-mortem for each group (right set of columns). The average of each group’s percent increase in the tumor size parallels the observed average final tumor weights.
The outcome appeared to paint a trend of slightly smaller tumors for the treated group that received an early and a repeated heat treatment, though large error bars were particularly evident in the presentation of the data in Fig. 3.12, Panel B.

![Graph](image)

**Figure 3.12 Early and repeated heat treatments in an outgrowth experiment utilizing nude mice bearing tumors formed from HT-1080 #4 cells.** Tumors were started close to the base of the tail of ten mice that were subcutaneously injected with one million HT-1080 #4 cells suspended in 100 µL PBS. Half the mice were implanted with mifepristone pellets (1 mg/ 60-day sustained release), forming the (+) mife condition. That same group of mice received the first heat treatment [(+] HS = 43°C/ 30 min.), which was administered three days after cells were implanted. A second heat treatment (HS = 43°C/ 30 min.) was applied one week later (ten days after cells were first implanted). One mouse was lost due to thermal regulation problems post each heat treatment. Only mice that completed the study were included in the graphs above. Thus, the (+) HS/ (+) mife group has an n=3. Panel (A) indicates the average tumor volume and the associated standard deviation of measurements taken on the indicated days throughout the study. The first set of columns in Panel (B) presents the calculated average of the percent increases in tumor volume of each group during the interval between the second heat treatment and the end of the study (nine days later). The tumor volumes measured on the day of the second heat treatment served as the reference points. The second set of columns in Panel (B) presents the average final tumor weights and the associated standard deviation for tumors that were harvested at the conclusion of the study.
The next question to address in this immunodeficient model was the effect of CMV-HSF1(+) on tumor outgrowth. Here no immune system inhibition of tumors expressing foreign proteins were expected, which is why the model was able to easily support the growth of the human HT-1080 cells. In fact, the results presented in Fig. 3.13 were found to be quite opposite of results achieved with the immunocompetent rat model (see Figs. 3.8 and 3.9), which demonstrated limited tumor growth when human HSF1 mutants were expressed.

The experiment design for Fig. 3.13 employed immunodeficient mice bearing tumors, which were formed either from cells that were untransfected or from cells that were transfected with HSF1(+). Mice bearing tumors formed from untransfected cells and half of the mice injected with transfected cells, were otherwise untreated. The remaining half of the mice that received transfected cells were treated with both HS and mifepristone. The results, graphed in Fig. 3.13, display the percent increase in tumor size from day ten to day 25 post an early, single heat treatment administered to that last group. Tumors, formed in mice that received cells transfected with the CMV-HSF1(+) construct, appeared to grow to a similar size, independent of HS or mifepristone treatment. However, there was a difference in tumor size associated with whether or not the tumors were formed from transfected cells. Transfected cells appeared to produce larger tumors in the nude mice, though error bars were again substantial in size and the timing of the cell preparation for injection (untransfected cells were harvested first) possibly could account for the observed growth differences.
To maximize the potential for differences among treatment groups and to eliminate guesswork in trying to decipher significance, a different factor was next tested in combination with the GALV(+) Safe Switch Therapy. The combination had already been tested \textit{in vitro} (see Fig. 2.14) and consisted of the GALV(+) SafeSwitch (in the human HT-1080 #4 cell line) and the addition of the SafeSwitch rAd1 Oncolytic virus (introduced in chapter two). That combination

![Graph](image)

**Figure 3.13 Outgrowth study in nude mice bearing control tumors or tumors formed from HSF1(+) transfected HT-1080 #4 cells.** Parallel cultures of HT-1080 #4 cells were grown. One set was transfected with CMV-HSF1(+). The next day, one million cells (suspended in 50 µL PBS) were implanted subcutaneously at the base of the tail of mice and mice in the (+) mife group were administered mifepristone pellets (1 mg/60-day sustained release). Three days post implantation, the dorsal region of mice in the group indicated as (+) HS was subjected to the bilateral water bath heat treatment (= 42.7°C/30 min., Fig. 3.10). The average of the percent increase in tumor volume growth over the 25 days was calculated and graphed. Not all mice formed tumors, so the number forming tumors (the group size) is indicated as a fraction of the total number of mice originally implanted with cells.
was found to synergistically enhance in vitro cell death. Thus, it was next proposed to investigate the in vivo addition of rAd1 to the GALV(+) SafeSwitch, utilizing the nude mouse model.

*The nude mouse model for SafeSwitch controlled therapy is enhanced with an oncolytic adenovirus*

SafeSwitch control of the Oncolytic SafeSwitch component, rAd1 (used in Figs. 2.13 and 2.14), theoretically, would permit the same specificity of the viral agent to achieve tumor cell killing in vivo when combined with the GALV(+) SafeSwitch, while potentially intensifying effects that might produce regression outcomes. Syncytia formation, even if limited, would add a bystander effect to the first round of cell infection, which results in lysis. While it is likely that both SafeSwitch components will initially be delivered to the same cells, it is possible that one component will end up in one cell and the other component in a neighboring cell. Activation of the GALV(+) SafeSwitch alone could produce syncytia that would unite the TA produced within that cell with rAd1 genes in the neighboring cell. This union would then enable the transcription that is required to produce the oncolytic effect and ensures that desired cell death results. Should the GALV SafeSwitch construct ultimately be able to be packaged into an adenoviral construct that could co-replicate with complementary rAd1 expression, there would be additional, similar benefits afforded with new, subsequent applications of thermal dose and mifepristone, which could extend the bystander
effects to cells, newly infected with viral progeny. If therapeutic effects were as potent as the *in vitro* results of Fig 2.14 would indicate them to be, the synergistic combination of Oncolytic and GALV(+) SafeSwitch therapeutic approaches would benefit from the added safety conferred by the additional requirement for a repeated heat treatment to reactivate viral replication and the associated oncolytic effect. An experiment was conducted to determine effects achieved *in vivo* with the Combination Approach to SafeSwitch Tumor Therapy.

In order to ascertain whether improved results could be obtained by the Combination Approach to SafeSwitch Tumor Therapy, which combines the GALV(+) SafeSwitch expression with the rAd1 component of the Oncolytic SafeSwitch that is delivered to cells, preliminary experiments were planned with small numbers of animals. Nude mice were implanted with nearly two million HT-1080 derived line #4 human cancer cells, injected subcutaneously at the base of their tail. Once resulting tumors were at least 3 mm in diameter, the mice received intratumoral injections of the SafeSwitch Oncolytic virus. Three concentrations of virus were utilized: 17.5 x10^7 PFU; 1.75 x10^7 PFU; and 0.175 x10^7 PFU. 1 mg mifepristone/60-day sustained release pellets were employed to deliver the mifepristone ligand. The applied heat treatment was administered via the mouse bilateral water bath (Fig. 3.10), which was controlled to maintain a temperature of 43 degrees for 30 minutes. Tumor outgrowth was followed in order to ascertain if the desired outcome of a ten percent difference was achieved between treatment groups.
Figure 3.14  A combination of the Oncolytic SafeSwitch and the GALV(+) SafeSwitch in tumors formed from HT-1080 #4 cells in nude mice offers potential to cause tumor regression. Panel (A) shows regression results of the tumor that originally was similar in size to those shown in Panels (B) and (C). Panel (D) is an untreated tumor, which illustrates tumor growth over time. Two million HT-1080 #4 cells suspended in 100 µL were used to form tumors proximal to the base of the tail of each nude mouse shown above. Tumors that resulted in Panels A-C were originally of uniform size, however the tumor that resulted in the mouse shown in Panel D was significantly larger. The mouse in Panel D was left untreated and the mice with uniform tumors were implanted with mifepristone (mife) pellets (1mg/60-day sustained release). The next day, the mice that received mifepristone were also intratumorally injected with a 25 µL solution of PBS containing the amount of virus indicated (rAd1 PFU quantity listed across the top). The following day (two days after the mice were treated with mifepristone) those same mice also received a heat treatment (1st HS = 43.1°C/30 min.) with the bilateral water bath (Fig. 3.10). Tumors were photographed (top row of pictures) five days later. Remarkable results were obtained in the mouse in Panel A that received the highest concentration of virus. The mice in Panels B and C were subjected to a second heat treatment (2nd HS = 43.0°C/30 min.) six days after the first heat treatment. All mice were photographed at 19 days after the first heat treatment (bottom row of pictures).

<table>
<thead>
<tr>
<th>rAd1:</th>
<th>17.5 x10^7 PFU/25uL PBS</th>
<th>1.75 x10^7 PFU/25uL PBS</th>
<th>0.175 x10^7 PFU/25uL PBS</th>
<th>0.0 PFU/25uL PBS</th>
</tr>
</thead>
<tbody>
<tr>
<td>mife:</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
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<tr>
<td>1st HS:</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>2nd HS:</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

5 days post 1st HS:

19 days post 1st HS (8 days post 2nd HS):

A.  B.  C.  D.
The results of the initial pilot study exceeded the desired outcome expectations and are presented in Fig. 3.14. The mouse in Panel A, which received the highest virus titer, yielded what appeared to be complete tumor regression. However, lower titers did not appear sufficient to cause tumor regression. A likely explanation was that not enough TA was produced in infected cells to cause viral transcription to occur. The ratio of TA to target gene could be important to the production of the desired response. Normal, mouse cell infiltrate may have been infected along with the responsive human cells, resulting in an effective titer that was less than a theoretical “threshold” for tumor regression to result. Furthermore, the response could be inhibited by insufficient heat delivery to activate eHSF1, which initially produces the TA in the HT-1080 #4 cell line. In any of these scenarios, a second round of heat treatment, administered in the continued presence of mifepristone, would not add to results without co-administration of more rAd1. In fact, a second round of heat treatment did not add to the results observed in panels B and C of Fig. 3.14. The exciting result observed in panel A of that figure prompted the design of a second pilot study that utilized delivery of the therapy to WT cells.

The second in vivo pilot study, utilizing the Combination Approach to SafeSwitch Tumor Therapy, was designed to offer more physiological relevance in that it did not utilize the advantage of stably transfected SafeSwitch genes in situ. The entire gene therapy was delivered to WT tumors, which were formed in four nude mice that collectively had tumors of two sizes (two small and two large
tumors). DNA-electroporation delivered the heat-triggered, mifepristone-activated, GALV(+) SafeSwitch construct DNA to cells, and the otherwise undiluted rAd1 component of the Oncolytic SafeSwitch was coinjected with DNA prior to electroporation. Animals were divided into two groups with one small and one larger tumor each. One group received mifepristone ligand pellets (15 mg mifepristone with a 60-day sustained release). The group that was implanted with the mifepristone pellets also received the bilateral heat treatment. One of the remaining untreated mice had a larger tumor that mandated euthanasia to comply with the animal protocol that supported this research, since it showed signs of ulceration on the day of the first data point. Thus, the data from that animal is not included in the figure.

The results presented in Fig. 3.15 were encouraging. While the untreated tumor (last graph and bottom row of pictures) that was documented in parallel did not respond to the rAd1/GALV(+) electroporated SafeSwitch therapies, the mouse with the larger tumor that received the treatment (first graph and top row of pictures) responded with dramatic regression. That regression was sustained and visualized over the two week period that followed the single heat treatment. The smaller [(+) HS/ (+) mife] tumor (middle graph and pictures) was expected to respond similar to the other heat and mifepristone treated tumor. It was hypothesized that there may have been a problem with heat delivery to that tumor or that electroporation may not have effectively delivered the genes to the cells. Additionally, the tumor growth characteristics appeared different in that smaller tumor and those characteristics could have altered the potential for a
Figure 3.15 The Combination Approach to SafeSwitch Tumor Therapy, entirely delivered to tumors formed from WT HT-1080 cells, caused regression in a nude mouse. Approximately one million WT HT-1080 cells suspended in 50 µL were injected to form subcutaneous tumors in nude mice proximal to the base of their tail. Two small tumors of equivalent size and one large tumor resulted. The one mouse with a larger tumor and one of the mice with a smaller tumor were selected for the (+) mife condition and were implanted with 1 mg/60-day sustained release pellets. Tumors in all mice were injected with a 20 µL mixture of 7.5 µg GALV(+) SafeSwitch DNA and 7x10^7 PFU rAd1 Oncolytic SafeSwitch virus. The tumors were then electroporated. The next day, the mice that received mifepristone pellets were subjected to the bilateral heat treatment [(+) HS = 43°C/30 min.]. Panel (A) indicates the growth of each tumor over time as a graph of the increase in tumor volume as a percentage of the starting tumor size. Panel (B) shows the corresponding pictures of the respective tumors. From left to right in Panel A, the tumor graphed of the first mouse [(+) HS/ (+) mife] corresponds to the top row of pictures in Panel B; the tumor graphed in the middle [(+) HS/ (+) mife] of Panel A corresponds to the middle row of pictures in Panel B; and the last tumor graphed [(-) HS/ (-) mife] in Panel A corresponds to the bottom row of pictures in Panel B.
response to occur. The tumor tissue appeared to be primarily composed of a
different type of cells (e.g., scar tissue), since it lacked the typical phenotype
often observed with HT-1080 cells grown in vivo, which features a large,
amorphous necrotic core. The role that the necrotic core might play in altering
gene delivery and the effect that it might have on heating properties were
unknown.

A larger study followed, in order to investigate the regulation and
reproducibility of the outcomes observed in Figs 3.14 and Figs. 3.15. One was
designed and carried out with thirteen nude mice, bearing tumors formed from
HT-1080 #4 cells. Use of the stably-transfected cell line eliminated the need to
electroporate the GALV(+) SafeSwitch genes. The presence of the TA gene in
every cancer cell that was introduced to the mice ensured that the genetic
potential existed in those cells for the transcriptionally active form of TA to be
produced, which is expected to occur only when the additional requirements for
heat and mifepristone are met in the regulated system. Thus, any unregulated
expression of the delivered rAd1, in the context of the GALV(+) SafeSwitch and
when either heat and/or mifepristone are absent, might be reflected in measured
tumor size differences. The mice were divided into four groups that either
received heat treatment and subcutaneous sustained-release mifepristone
pellets (four mice originally composed this group) or that did not receive one or
both of those activating components (three mice formed a group to represent
each condition). All tumors were injected with rAd1 Oncolytic SafeSwitch and
mice in the group scheduled to receive heat applications were treated the
Figure 3.16 The Combination Approach to SafeSwitch Tumor Therapy utilizing tumors formed from HT-1080 #4 cells in nude mice also yields regulated regression outcomes. Nearly one million HT-1080 #4 cells (suspended in 100 µL PBS in an effort to generate even tumor sizes) were injected subcutaneously proximal to the base of the tail in 13 nude mice. Seven of the mice also received subcutaneous implants of mifepristone pellets (1 mg/60-day release) dorsal to their shoulder blades, which formed the (+) mife groups. When tumors reached approximately 100 mm in size, all tumors were injected with 1.4x10⁷ PFU (20 µL injection volume) rAd1 Oncolytic SafeSwitch. The next day, half of the mice that did not receive mifepristone pellets and four of the mice that did were administered a 43°C / 30 minute bilateral heat treatment, creating the (+) HS condition. Mortality of two of the original four mice that received both heat treatment and mifepristone pellets did not permit their numbers to be represented. Panel (A) graphs the percent increase in tumor size for all groups, and Panel (B) utilizes pictures of tumors borne by the two mice, which formed the (+) HS/ (+) mife group, to illustrate the regression phenotype.
following day. Unfortunately, two of the mice in the group that was expected to demonstrate tumor regression experienced problems with thermal regulation and died, a frequent complication associated with the rudimentary heat treatment. Thus, their tumor regression was not able to be followed. However, the mice that were able to be followed in the (+) HS/ (+) mife group were documented pictorially to illustrate the positive regression phenotype (Fig. 3.16, Panel B). Tumor volume measurements for all groups (Fig. 3.16, Panel A) indicate that: 1) growth inhibition was apparent in the (+) HS / (-) mife groups, but regression outcomes were not able to be observed; and 2) growth inhibition was not apparent in the unheated groups.

Based on the result observed in the larger study with the HT-1080 #4 cells as well as the positive result from the WT HT-1080 pilot electroporation experiment, another large experiment was planned. That experiment utilized the WT HT-1080 cells and was designed to address several control situations. It included the following groups of mice: those that received intratumoral injections of rAd1 and GALV(+) SafeSwitch (and then were electroporated), which were also given heat treatment and implanted with placebo pellets; those that received intratumoral injections of rAd1 and GALV(+) SafeSwitch (and then were electroporated), which were not given heat treatment but were implanted either with mifepristone pellets or with placebo pellets; those that neither received intratumoral injections of rAd1 nor GALV(+) SafeSwitch (nor were electroporated), which were implanted with mifepristone pellets or placebo pellets but either did or did not receive a heat treatment; those that received intratumoral
injections of rAd1 without the GALV(+) SafeSwitch (these were not electroporated) but were implanted with mifepristone pellets and either were given or did not receive heat; and those that received intratumoral GALV(+) SafeSwitch (were electroporated) without rAd1 also present, which received both a heat treatment and a mifepristone pellet. A total of 45 mice were used with a minimum of five mice in each of the eight groups. A new lot of 1 mg mifepristone pellets (60-day sustained release) was utilized in this experiment.

Surprisingly, no significant difference among groups was observed in measurements taken over the following month (data not shown). An attempt to repeat the treatment also failed (data not shown). It was postulated that a higher dose of mifepristone pellets may be required to compensate for lot to lot differences in mifepristone pellet fabrication. In fact, later trials (data not shown), which utilized pellets from the same lot as those in the above experiment, also did not produce regression. Forward-looking measures to optimize chances for success sought to improve the heating method that was utilized, in addition to switching from mifepristone pellets formulated from 1 mg mifepristone to pellets formulated from 5 mg of mifepristone (to be released over a 60-day period).

The nude mouse model is further enhanced with a method of focused heat

A series of pilot experiments, utilizing just a few mice and the laser apparatus depicted in Fig. 3.17, was carried out (data not shown) by John Munson (University of Florida, Gainesville, FL). His observations of temperature readings achieved in normal, cadaver mouse flank that was heated with the laser
indicated potential for strong, reproducible intratumoral switch activation. His initial results were further supported by tumor regression outcomes that were produced in a couple of live, nude mice bearing tumors formed from HT-1080 #4 cells (data not shown). Those results occurred when the rAd1 vector was intratumorally injected, mifepristone ligand was present, and the laser heat was applied. His success prompted the design of a larger laser experiment. Particular concerns were whether the laser heat alone inhibited tumor growth and whether the surface of the skin was burned by the laser heat.

Figure 3.17  Laser heating method to create a focused thermal dose. The laser apparatus and set up is illustrated in the top picture in the composite. A closer view of the 810 nm laser set at 8.5 Amps is visible in the bottom left picture. Tumors were blackened with ink to enhance energy absorption and to make the targeted tissue color more uniform (see the black spot on the nude mouse in the middle bottom picture). Mice were heated for five minutes by holding the blackened area of the mouse against the hole in the shield, which keeps the focal length from the laser fixed. This method did not require sedation to deliver the thermal dose.
The results of the larger experiment, presented in Fig. 3.18, demonstrate that tumor regression was able to be achieved, even with this formative system. Tumors exposed to laser heat alone comparably paced growth of tumors not treated with the laser. Thus, the concern that the laser heat alone could inhibit tumor growth was not founded in this experiment. Additionally, tumors (formed in mice that were given both adenovirus and GALV(+) therapies, but that neither received a heat treatment nor the mifepristone ligand) did not demonstrate any growth inhibition. In fact, they grew at a rate that was comparable to that of the control groups, which neither received adenovirus nor GALV(+) SafeSwitch components. Tumors that were subjected to the heat treatment, mifepristone ligand, and rAd1, but that did not include the GALV(+) SafeSwitch that would contribute the TA component, grew like the above described control groups. This finding illustrates that adenoviral infection effects alone did not contribute to tumor regression. On the other hand, with laser heat methodology to deliver the thermal dose, the GALV(+) SafeSwitch treatment, which was designed to be a stand-alone treatment when mifepristone ligand and an adequate transient thermal dose were administered, did appear to demonstrate some inhibition of tumor growth under desired conditions, even in absence of rAd1. The tumor growth inhibition, however, appeared to be similar in magnitude to outcomes of control conditions that were observed when both GALV(+) and rAd1 were combined along with the presence of either heat treatment or mifepristone ligand (but not both). The inhibition of tumor growth under control conditions indicates some deregulation, apparent only with the potent combination of cytotoxic
Figure 3.18  Therapeutic effect of the Combination Approach to SafeSwitch Tumor Therapy delivered genes to tumors in nude mice, which were formed from WT HT-1080 cells, is activated by a focused, laser-delivered thermal dose. 1.1 million WT HT-1080 cells were suspended in a 100 µL volume of PBS and were used to form subcutaneous dorsal tumors in nude mice proximal to the base of their tail. Eleven days later, 5 mg/60-day sustained-release mifepristone pellets were implanted in the mice indicated with (+) mife above. Fifteen days after tumor growth was started, tumors were approximately 225 mm³. At that time, 2x10⁷ PFU virus and/or 7.5 µg GALV(+) SafeSwitch construct DNA was intratumorally injected and electroporated as indicated above by a (+) or a (-). The next day, tumors in the animals received a focused thermal dose with the developed laser heating method (Fig. 3.17). Tumor volume was measured over time and graphed as an average of the increases in tumor volume that were expressed as a percent of the initial volume. The “-1” or “-2” flagged by a “^” just below the chart columns indicates attrition in the number of mice (n) by that amount from that point on in the study. Mice were euthanized and excluded from the study once their tumors ulcerated or exceeded size constraints for inclusion criteria in the study.
effects. Full tumor regression was achieved in the sixteen days following heat
treatment in the group that received all therapeutic components: laser heat
treatment, mifepristone ligand, as well as rAd1 and GALV(+) SafeSwitch
therapies.

Since the laser heat methodology appeared to improve treatment
outcomes with the GALV(+) SafeSwitch therapy alone, the next experiment
sought to compare results among different treatment groups, utilizing laser heat
and mifepristone to form the treatment groups and the stably transfected HT-
1080 #4 cells to form the tumors. Use of this line eliminated the need for the
electroporation process. This enabled an examination of the regression
outcomes desegregated from any influence that the electroporation technique
might have on adenovirus delivery / expression. The addition of the constitutively
active HSF1 mutant, when laser heat treatment was not administered, was also
examined in the context of the GALV(+) SafeSwitch therapy. Many control
groups were utilized to try to obtain a complete picture.

Results presented on the right side of the graph (last two groups) in Fig.
3.19 illustrate that electroporation did not affect the outcome when rAd1 was
delivered to the *in situ* GALV(+) Safe Switch in this model of the Combination
Approach to SafeSwitch Tumor Therapy. However, the adjacent group that
received mifepristone, but which received the constitutively active form of HSF1
instead of laser heat to complete switch activation, actually appeared to have
tumors that increased in size at a rate that was similar to the group that received
neither Adenovirus nor mifepristone (left-most group), but which did also receive
Figure 3.19 Laser thermal dose compared to HSF1(+) as a means to activate the SafeSwitch to cause tumor regression in nude mice bearing tumors formed from HT-1080 #4 cells. 1.3 million HT-1080 #4 cells were suspended in PBS in a 50 µL injection volume to form bilateral, subcutaneous tumors on either side of the flank proximal to the base of the tail in nude mice. Ten days later, 5 mg/60-day sustained-release mife pellets were implanted and a single intraperitoneal dose of mifepristone (0.5 mg/kg) was administered to the mice in the groups indicated by: (+) mife. That same day, a constant 20 µL volume was used to intratumorally inject the following: 1) rAd1: (+) / HSF1(+): (-), which was formulated with 1x10⁷ PFU rAd1 and 2.36 µg endotoxin-free filler empty vector DNA; 2) rAd1: (+)/ HSF1(+): (+), which was formulated with 1x10⁷ PFU rAd1 with 11.15 µg CMV-HSF1(+); 3) rAd1: (-)/ HSF1(+): (+), which was formulated with 15 µg CMV-HSF1(+). Electroporation was then performed on tumors in groups that are indicated by the notation, “electroporated: (+)”. The next day, tumors of mice in groups marked as “Laser HS: (+)” received the focused thermal dose from the laser heating method described in Fig. 3.17. The purpose of the experiment was to determine the potential of HSF1(+) electroporation to activate the switch versus the laser heat switch activation effects under the new conditions. Tumor volume measurements for all groups were graphed as a percent of increase in tumor volume; measurements obtained the day after the heat treatment was administered served as the points of reference. As in the last experiment, the “-1” flagged by a “^” just below the chart columns indicates attrition in the number of mice (n) by that amount from that point on in the study. Mice were euthanized and excluded from the study once their tumors grew too large to meet study inclusion criteria or ulcerated.
HSF1(+). The latter group had tumors that grew at the fastest rate, though a
caveat is that the associated error bars are particularly large. In contrast, the
second group from the left, which received mifepristone but otherwise was similar
to the left-most group, demonstrated some tumor growth inhibition. This
outcome is unlike the results in Figure 3.13, in which cancer cells were
transiently transfected ex vivo with HSF1(+). In that experiment, cells were
implanted and heat was applied early in order to follow tumor outgrowth over
time. The different outcomes could be ascribed to methodology-specific effects
(e.g., angiogenesis). Though differences in results between groups treated with
just laser heat and mifepristone were not compelling, there did appear to be a
trend of inhibition on tumor growth when laser heat was applied. However, this
effect did not appear to be dependent on mifepristone addition. Thus, there may
be some deregulation of the switch occurring with concentrated laser heat
delivery to the #4 cell line. A means to further standardize heating, perhaps with
the addition of real-time feedback (which may report the internal tumor
temperature that is achieved) may best support feasibility, therapeutic benefit,
and safety studies that are required to transfer the SafeSwitch tumor regression
application to clinical trials.

In vivo section summary

The aim of the work presented in this chapter was to advance the use of
the GALV(+) SafeSwitch to an in vivo therapeutic situation. The GALV(+)
SafeSwitch was designed to be specifically targeted by the initial delivery of
transient heat to solid tumors, which theoretically would trigger localized HSF1 activity. The specific targeting of the therapy was designed to be achieved by activating endogenous HSF1 through a localized delivery of transient heat to solid tumors. Gene transcription was expected to ensue to both cause and sustain tumor regression through other regulatory elements of the molecular switch. The purpose of the experiments was to establish the conditions that caused transcription of the SafeSwitch gene target in vivo, in order to assess requirements that might lead to tumor regression outcomes.

To achieve this aim, the development of a model was approached from several directions. The GALV(+) SafeSwitch system was tested in a variety of animal backgrounds including: immunodeficient rat xenograft studies, immunocompetent rat homograft studies, and nude mouse xenograft studies. Two heating methods were tried with rat models, and two additional heating methods were utilized with the mouse model. Additionally, DNA electroporation of the constitutively active human HSF1 construct was explored.

Many challenges were encountered during the quest to achieve reproducible tumor regression outcomes in vivo, which were not factors evident in the in vitro tests that illustrated controlled, cancer cell death. These challenges were identified, explored, and subsequently defined. The main technical difficulties centered on an effective means to appropriately heat tumors, which would consistently activate transcription of the SafeSwitch TA in vivo. Other difficulties were ascribed to the non-permissive murine receptor for the GALV(+) component, since the published literature substantiated this possibility (Johann et
al., 1993; Tailor et al., 1993 and 2000; as well as Grabarczyk et al. 2002). Low
GALV(+) -mediated syncytia production in murine cells was best visualized in the
vitro studies with the F98 line (e.g., Figs 2.8 and 2.10), and likely limited in vivo
outcomes, as well (e.g., 3.4 and 3.9). The above limitations might have been
further exacerbated in the in vivo situation, since a heterogeneous population of
cells comprise a tumor mass. A focused, directed heat dose did improve the
regression results that were achieved in the nude mouse model bearing tumors
formed from human HT-1080 cells.

Laser-assisted thermal dose delivery along with the virulent combination
of the GALV(+) SafeSwitch system and the recombinant, conditionally-replicative,
Oncolytic SafeSwitch adenovirus, rAd1, reproducibly appeared to yield effective
tumor regression results when mifepristone was present (Figure 3.18). Animals
that simultaneously received both SafeSwitch therapies, which also received
ligand and a heat treatment, had smaller tumors than animals treated with the
combination of SafeSwitch therapies in absence of either mifepristone or a
thermal dose. The Combination Approach to SafeSwitch Tumor Therapy yielded
the most promise for potential to achieve controlled regression outcomes. The
final in vivo experiments in this chapter highlight the importance of the future
directions for the treatment of solid tumors, which Chapter 4 describes.
Chapter 4 – Future Directions

Extensions to the concept of deliberately activating HSF1’s normal functions for therapeutic purposes were examined in the previous chapters through the design and the completion of successful \textit{in vitro} trials and \textit{in vivo} preliminary studies that feature the use of HSF1 as a regulator of therapy. The outcomes measured have defined challenges for the \textit{in vivo} SafeSwitch concept to successfully improve tumor regression outcomes. The future directions that extend from these challenges will impact the feasibility, therapeutic benefit, and safety studies that are required to advance the studies to the clinical trial realm. Future directions include the following: 1) a GALV(+) SafeSwitch adenoviral vector that is complementary to rAd1 must be produced; such a vector would effectively illustrate the advantage of SafeSwitch regulation to control virulent cytotoxicity, which is expected to result from the combination virotherapy; 2) further groundwork needs to be completed for an immunocompetent animal model, which would ascertain immune system contributions to therapeutic outcomes as well as offer greater physiological relevance for people; and 3) technical challenges concerning the method of applied heat treatment need to be overcome.

The outcomes measured also identified some interesting notes concerning the \textit{in vivo} effects of the constitutively active mutant HSF1. The third aim of this thesis work offers an avenue for future work to explore, which may describe additional consequences of deliberate HSF1 activation \textit{in vivo}. That aim was to
create a constitutively active mouse HSF1, which can be utilized as a tool to examine effects that result from its harness of the HS response. Though this third aim leads to a distinct direction of future studies, those studies might have important implications with regard to the basic science that underlies the processes affected by constitutive upregulation of HSF1 activity in vivo and might also offer relevance to concerns regarding pharmacologic activation of the factor. This chapter presents work that contributes to future directions, which seek to refine therapies that feature a harnessed HS response.

**Current challenges for SafeSwitch therapy progress toward clinical studies**

Three aspects are currently important in order to effectively ascertain the SafeSwitch concept’s preclinical potential to improve tumor regression outcomes. These include vector considerations, model considerations, and the technical considerations of the applied heat treatment. Those areas of future work are discussed in this section to facilitate the design of trials that will determine the feasibility, therapeutic benefit, and safety of the SafeSwitch clinical concept that this dissertation presents.

*Refinements to the vectors for gene delivery*

It is expected that a virus, which delivers the GALV(+) SafeSwitch in combination with the existing rAd1 Oncolytic SafeSwitch, would provide even greater effectiveness than that observed in the experiments presented at the conclusion of chapter three. Expression of the rAd1 Oncolytic SafeSwitch genes
would also enable co-replication and spread of GALV(+) SafeSwitch viral progeny. This would enhance desired virulence with additional heat treatments, even as it underscores the need for stringent confinement of gene expression to the targeted area by the SafeSwitch system. Ads are known to replicate in dividing as well as non-dividing cells. A new GALV(+) SafeSwitch vector, in the context of an animal model that is permissive for both adenovirus replication and GALV(+) mediated fusion, would best illustrate the advantage of regulation conferred by the SafeSwitch therapeutic concept. Such a demonstration might be particularly powerful in the context of a competent immune system that mimics the human clinical condition.

Improvements to vector design and gene arrangement should be considered as this future work is undertaken. The simplest design objectives need to be considered as a new GALV(+) SafeSwitch adenoviral shuttle construct is created to produce the new vector. Some considerations that need to be carefully thought out to ensure a successful virus product include the following: 1) substitution of the full-length, unattenuated GLP65 transactivator for the attenuated form in order to attempt to improve in vivo therapeutic outcomes; and 2) means to strengthen gene expression from the viral vector (e.g., gene rearrangement). It is possible that the proximity of the Gal4 promoters in the present design and the reverse orientation of the transactivator component within the vector, both limited the potential to produce transcripts. Another related, effective approach may be to include insulator sequence (reviewed by Bell and Felsenfeld, 1999). This might shield the promoters from cancer cell silencing (Li
et al., 2006) and would shield them from position-dependent effects in the vector. Two such options include the murine H19 insulator sequence (Kaffer et al., 2000) and the 5’ region of the chicken beta-globin gene (Chung et al., 1993). Also, more complex changes such as base substitution in the promoter region to hinder CpG methylation modification and/or downstream codon optimization could also yield beneficial results. (Valencik and McDonald (2001) report an example of improved gene expression in a transgenic application as a consequence of such optimization.) An important third consideration in producing a virus is the adenovirus packaging constraint, which must be accommodated as modifications (such as the above) are made. A new shuttle vector that allows recombination to remove the E4 region of the adenovirus vector would likely yield the required room to make the essential changes (e.g., to utilize the full-length, unattenuated version of the transactivator). With deletion of E1, E3 and E4 sequences, cloning capacities of around 10 kbp are possible (He, T.-C. et al., 1998) and the vectors may be packaged in existing cell lines for such purposes (e.g., Armentano et al., 1995; Wang et al., 1995; Gao et al., 1996; Yeh et al., 1996).

It is important to also note, however, that clinical cancer trials with currently available adenoviral vectors have not been powerful mediators of therapy for reasons that extend beyond a lack of a regulated, bystander effect to distribute virus within the tumor. Problems include a lack of cell-specific infectivity, a lack of a permissive animal model, and the concerns related to viral immunogenicity. The clinical results have prompted continued basic science
investigations into the development of better adenoviral vectors for gene therapy purposes.

Receptors for adenovirus were examined by researchers in the hope that viral capsid modifications would improve results. The icosahedral capsid of adenovirus contains twelve pentons in addition to 240 hexons. For each penton, a fiber projects from a knob on the penton base, which mediates host-cell entry. Some cell types were found to be deficient in the primary virus fiber receptor, the Coxsackie and adenovirus receptor (CAR), and mutations in the CAR-binding region of the adenoviral knob have been found to inhibit infection of many cell types (Roelvink et al., 1999 and Jakubczak et al., 2001). The secondary means of adenovirus entry into tumor cells utilizes an RGD (Arg-Lys-Asp) motif in the penton base protein, which binds the integrin $\alpha_v\beta_3$ receptor that is abundantly expressed on many solid tumor cells (Basak et al., 2004; Dmitriev et al., 1998; Kirby et al., 1999 and Nemerow, 2000).

Means to circumvent the viral receptor bottleneck have been explored through virus fiber modifications relevant to the CAR-independent pathways (Ranki et al., 2007; Zhu et al., 2006; Takayama et al., 2003; and Wu et al., 2002). However, Zhu et al. (2006) noted that capsid modifications appeared to harm viral replication, even while they enhanced viral infectivity, which might attenuate effects of capsid-modified oncolytic Ads. A parallel concern is that many of the recombinant adenovirus in vivo trials have utilized virus that lacked E3 sequence as well as E1A and E1B. This compromises potential therapeutic efficacy in terms of ability to lyse cells and to evade host immune responses that are
directed at viral clearance (Hermiston et al., 1993; Lichtenstein et al., 2002; Gooding, 1994; Shisler et al., 1996; Wang et al., 2003). Thus, it is possible that their efficacy was abrogated.

Basic science efforts are also currently focused on making adenovirus vectors safer as a bioactive compound. Generally, adenovirus-delivered therapies have been tolerated well in both vaccine and gene therapy clinical trials. However, a lack of compelling potency in terms of achieving therapeutic outcomes has led to a general consensus that it might be preferable to concentrate resources on the design of more adequate adenoviral vectors, instead of on premature clinical trials (Breyer et al., 2001).

Adenovirus adverse effects have primarily been observed when high vector doses were necessitated. The associated mild symptoms include transient low-grade fever, which is a first indicator of toxicity, and pneumonia. However, the potential does exist for serious side effects, as is best illustrated by the first major adverse event: the fatality of 18-year-old Jesse Gelsinger. In that case, death was associated with an inflammatory reaction, which followed the systemic administration of a high-dose of a rAd-delivered gene therapy treatment that was designed to treat ornithine transcarbamylase deficiency (Raper et al., 2003).

A recent publication (Cheng et al., 2007) has described the mechanism that Ad5 employs to signal dendritic cells and some monocytes to stimulate adaptive immune responses in a CAR and integrin RGD receptor-independent manner, which did not permit infection of epithelial nor fibroblast cells. Cheng et
al. (2007) reported that infection in these cells and the consequential
immunogenic and pyrogenic response were found to be mediated by the shaft of
the Ad5 fiber in a heparin-sensitive manner. This finding putatively suggests a
distinct Ad receptor and may offer immediate benefit to vaccine studies in terms
of designing appropriately targeted vectors. However, further basic science
information is needed to engineer an improved adenovirus vector design to
deliver gene therapy to tumors in a manner that would enable repetitive use and
address vector safety concerns. Thus, an overall demand exists for greater
potency and safety with Ad vectors to optimize therapeutic outcomes.

Viral vectors, other than adenovirus, could be designed that incorporate
the SafeSwitch concept. The advantage of the SafeSwitch concept is that the
Hsp70 promoter, which is designed to specifically target initial activity to the
treatment region, is ubiquitous. However, activating the Hsp70 promoter with an
effective thermal dose is technically challenging in animal models.

A concept that is competitive to the use of the Hsp70 promoter, which
would circumvent the technical aspects of triggering the therapy with an applied
heat treatment, would utilize tumor specific promoters. Such promoters would
preferentially, or even specifically, transcribe genes only in the context of cancer
cells. Survivin, is one such promoter that has recently emerged in the literature
(Zhu et al., 2006) as an attractive candidate for the treatment of mesothelioma.
The survivin promoter displays low (but not entirely absent) toxicity in human liver
tissue and low gene transcription in normal human cell lines. Examples of
promoters in this category include prostate-specific antigen (for use with prostate
cancers) and the alpha-fetoprotein promoter (for use with hepatocarcinomas) (Lee et al., 2000 and Huber et al., 1991) among many others. The limitation to this approach, with the notable exception of the survivin promoter (Zhu et al., 2004), is that the range of neoplastic tumors that potentially could be specifically treated tends to be narrow or not synergistic to other chemotherapy treatments. Additionally, as improvements to adenoviruses and the virulence of the genes that they deliver continues to progress, the safety concern regarding even low levels of expression of therapeutic genes in certain normal cells may gain importance. Since the Hsp70b promoter can be activated in essentially all cell types, even deep within the body when an appropriate method to deliver the triggering thermal dose is utilized, the specific targeting is preserved in the context of a SafeSwitch system. Thus, the SafeSwitch concept, in principle, remains an excellent candidate for future development.

Refinements to the animal model

A troublesome complication encountered in ascertaining the effectiveness of the SafeSwitch therapy in vivo, involved animal model tumor regression considerations. This complication is not unique to the SafeSwitch approach and has surfaced as a concern with regard to poor clinical trial outcomes. Outcomes of little resultant clinical activity were found when oncolytic adenovirus was utilized to treat cancer in people, despite compelling in vitro and in vivo findings. This has brought into question the relevance of the animal models utilized in the preclinical studies. Regarding adenoviral replication, transplantable homograft
mouse tumors are generally non-permissive for therapeutic effects, though screens of murine epithelial cells have revealed some cell lines that produce infectious virus products, albeit 25 to 100-fold less than that produced in the comparative human cell control lines (Ganly et al., 2000 and Hallden et al., 2003). Xenograft models are also not ideal in that they necessitate a suppression of the immune response, which limits the extrapolation of results that are pertinent to human use.

One small animal model, the cotton rat (Sigmodon hispidus), has been examined in the literature and found to be susceptible to both adenovirus infection as well as replication in the cornea, lungs, and respiratory passages. Additionally, the inflammatory consequences were found to mimic the human condition. One syngeneic cell line utilized to form tumors in this model is the LCRT line, which was employed in the experiment that generated Fig. 2.8. Two additional lines, CCRT and VCRT were examined in a recent study (Steel et al., 2007) that evaluated adenovirus infection and replication capability \textit{in vitro} and \textit{in vivo}. All three cotton rat cell lines were found to support viral replication \textit{in vitro}, however only the LCRT and the VCRT lines displayed tumor growth inhibition \textit{in vivo} after infection with oncolytic adenovirus. This variability underscores both the complexity of the \textit{in vivo} situation to achieve tumor regression and the importance in developing an appropriate model.

Jogler et al. (2006) published some impressive side-by side comparison studies of adenovirus replication, which compared results achieved in human lung (A549) cells to kidney and lung studies in cells of cotton rat origin as well as
cells related to mice, rabbits, hamsters, guinea pigs, and woodchucks. Their findings indicated that cotton rats, while permissive for viral replication, may not attain the same effective level of replication as is attainable in either the human lung cells or in swine primary kidney cells (as well as in the established PK15 kidney cell line). Their results extended similar findings of Torres et al. (1996), which indicated efficient Ad5 replication and infection in porcine cells. Thus, swine may be a better intermediate model to bridge the gap that spans nude mouse studies and clinical trial tumor studies that would utilize Oncolytic SafeSwitch adenovirus replication.

A swine SafeSwitch model has other advantages that recapitulate important aspects of the human condition and that offer relevance to the therapeutic outcomes potentially achievable in people. The literature indicates that the porcine model *Sus scrofa* (pig) has an appropriate PiT-1 receptor for GALV(+)‐mediated cell‐to‐cell fusion (Lavillette and Kabat, 2004), so ideally, the full virulence of the combination of GALV(+) and Oncolytic SafeSwitch therapies may be assayed. Swine have been utilized as a biomedical model for decades (Swanson et al., 2004), because of their comparative physiology and anatomical size (especially convenient in the available miniature pig lines) as well as similarity in organ development and in disease progression (Lunney, 2007). A high degree of sequence homology between humans and pigs (Swanson et al., 2004) extends to a proteomic similarity of the porcine pregnane X receptor protein. This receptor regulates p450 cytochrome CYP3A, which metabolizes many prescription drugs in humans with results more comparable than the
analogous receptor in mice (Xie and Evans, 2002). Levels of adenovirus uptake
and pharmacodynamics in porcine liver have been characterized (Cho et al.,
2007) with a notably improved toxicity profile over comparable mouse studies
that are confounded by Kupfer cell clearance.

Pigs are economical models with established animal husbandry
procedures. They produce large litters, are capable of deliberate breeding within
a year, and have a short gestation period. Inbred, cloned and transgenic lines
are available (e.g., Lee et al., 2005) along with numerous defined and
established cell lines. Additionally, transplantable cell lines that are capable of
forming hematological tumors (Cho et al., 2007) and subcutaneous tumors
(Adam et al., 2007) have recently been developed that expand the use of swine
cancer models from melanoma (e.g., Vincent-Naulleau, 2004) to other types of
cancer.

The method of genetically engineering primary porcine fibroblasts (Adam,
et al., 2007) so that transformed cell lines are produced, which can then be used
to form subcutaneous and deep tumors comparable in size to human tumors, is
an attractive approach for preclinical applications. The method utilized by Adam
et al. (2007) to reportedly create a robust, rapid and reproducible means to
genetically induce tumors, capitalizes on cell similarities between swine and
humans. One disadvantage is that the RT-PCR vectors that Adam et al. (2007)
employed introduce a combination of human and murine genes, thus the animals
must be partially immunosuppressed to support the growth of the transformed,
previously isogenic cells for tumor formation in vivo. However, as genomic
studies continue to be completed, cDNA sequence that is specific to a cloned, inbred line of miniature pigs might become available. The relevant swine sequence might then be packaged into a retroviral vector akin to those utilized by Adam et al. (2007). The desired outcome would be a similar methodology that then yields an immunocompetent cancer model, which features genetically defined tumors that may be grown to the desired size, in the desired location, and in the desired number of animals for ideal studies to be completed in vivo.

The ideal immunocompetent model would permit: adenovirus replication, comparable to that observed in people, as well as GALV(+) SafeSwitch-mediated cell to cell fusion of normal host cells and of the introduced cancer cells. Successful SafeSwitch study results achieved in such a model would offer compelling evidence to make the logical leap to clinical studies. Even the pig model with partial immunosuppression (Adam et al., 2007) offers significantly more similarity to human studies than murine models.

The GALV(+) therapy could first be tested via transient transfection of normal pig cells in parallel to the resultant, genetically transformed porcine cancer line. Adenovirus replication could be confirmed in vitro in that line as well. The required SafeSwitch studies could then be completed in nude mice as well as in the pigs. The preliminary nude mice studies would benefit from a refined method to apply heat treatment, considering results obtained by Locke et al. (2005) in which ultrasound heating was directly compared to water bath hyperthermia treatments. A focused, refined heat treatment method could then, theoretically, be scaled up to treat the tumors on miniature pigs and / or
equipment that would be utilized to treat people may be easily adapted to apply heat to the pigs (note: Cohen et al. (2007) utilized magnetic resonance imaging (MRI)-guided focused ultrasound on brain targets in pigs; also Wust et al. (2002) provides a review of hyperthermia methodologies utilized in the treatment of cancer).

**Refinements to the applied heat treatment**

Improvements to the methodology utilized to deliver the thermal dose in the tumor regression model ought to both improve the therapy’s effectiveness as well as the clinical relevance of outcomes achieved. Small animal hyperthermia systems (e.g., the SAHUS designed by Singh, 2004 and Novak, 2005), which would apply the required, focused heat treatment exclusively to a targeted tumor in a consistent fashion, are technically challenging to develop. The thermal dose achieved by the system is dependent on time as well as on the sustained temperature reached in the dynamic *in vivo* environment, which features tumor-specific vasculature effects, tumor color effects, as well as thermal regulation effects. Thus, it is important to establish a feedback loop to control the energy that is directed to the tumor.

The design criteria for small animal hyperthermia systems vary with animal species, tumor model, thermal objectives and other experimental parameters, e.g., an imaging system. The simultaneous heating of multiple animals necessitates additional controls, cost, and complexity. Fortunately, others have developed elaborate electromagnetic (approximately 8-3000 MHz)
means to heat tissue that incorporates MRI mapping of the temperatures achieved (reviewed in both Moonen, 2007 and Rome et al., 2007). MRI mapping has also been utilized to spatially define reporter gene expression, which sensitively measures transcription from an Hsp70b promoter (Guilhon et al., 2003; Xu et al., 2004; Rome et al., 2005). Furthermore, a simpler, ultrasonic means (approximately 0.5 to 10 MHz) has been utilized to do basic studies in a rodent model (Smith et al., 2002; Singh et al., 2004; and Novak et al., 2005). Ultrasound is particularly attractive, since its short wavelength, which is typically about 1 mm, permits the energy to be concentrated in a very small area.

Smith et al. (2002) published data that support the simple method of ultrasound heat in a rodent model application, which employed an adenoviral vector to deliver a luciferase gene that was controlled by an Hsp70b promoter. The vector was injected intradermally and then a thermal dose was applied to the specific region with the use of the ultrasound transducer. Their results (Smith et al., 2002) revealed that ultrasound heating was: 1) capable of producing high levels of luciferase; 2) levels could be tuned according to the exposure dose, as is characteristic of a HS response based on Hsp70b promoter activation; and 3) that negligible reporter gene expression was detected in untreated animals.

A means to introduce feedback control in the context of ultrasound heating has been developed by Singh et al. (2004) and further examined by Novak et al. (2005) to heat a variety of tumors. The design of their SAHUS features a feedback element. It was found to consistently deliver localized heat to tumors in mice, with minimum systemic effects and in such a way that a narrow
temperature range (41-41.5 degrees) was sustained for up to 60 minutes (Novak et al., 2005). Additionally, their system enabled simultaneous animal heating, reusability, personnel access, and microPET imaging.

Personal communication with Dr. Eduardo G. Moros (Professor & Director, Division of Radiation Physics & Informatics in the College of Medicine at the University of Arkansas in Little Rock) has indicated that such an apparatus may be designed and made available for future SafeSwitch preclinical studies. If such a system were able to be employed to deliver a consistent, comparable thermal dose to a variety of tumors, the data derived from such studies would add significant clarity to results, improving upon results achieved with any of the heating methods that were utilized in this dissertation work. It is expected that consistent, focused heat treatments to tumors will enable quantification of reproducible regression outcomes. Confounding variability in thermal dose, which Roemer (1999) addresses, would be circumvented.

A constitutively active mouse HSF1 to further studies in murine models

This thesis work has illustrated the following clear outcomes regarding the physiological effects of HSF1: they are powerful, possible to harness, and all is not known regarding the consequences of deliberate HSF1 activation. A tool, which would at will produce an active factor in a murine model or would otherwise enable a more complete examination of the physiological effects of constitutively active HSF1, may allow ramifications of sustained HSF1 activation to be explored. The third aim of this thesis work was to create a constitutively
active mouse HSF1, which could then be utilized to measure the physiological consequences of deliberately upregulating HSF1 activity in vivo. Thus, the product of this aim might yield information of significance to studies that currently seek a yet undiscovered pharmacologic means to safely activate HSF1. Additionally, a constitutively active mutant HSF1 of mouse origin has interested other investigators who have requested its use for their applications.

**Creation of the constitutively active mouse HSF1**

A constitutively active mouse HSF1 construct was designed by first integrating known sequence information from the previously characterized human HSF1 in the context of conserved mouse and human amino acid sequence (see Appendix 2 for an alignment that highlights the relevant sequence). The cDNA for mouse HSF1 was amplified and placed in an intermediate vector for the Stratagene quick-change PCR procedure. The mutagenesis utilized primers designed and fabricated to delete the region coding for the mouse amino acids, which correspond to amino acids 203-315 in the human sequence (relevant sequence is highlighted in Appendix 2). This active mouse factor, mHSF1(+), was sequenced and subsequently subcloned to the Invitrogen pcDNA3.1(+) expression vector, which was also utilized in studies that characterized human HSF1(+) as well as wild type (WT) HSF1.

In an experiment, which was designed to qualitatively compare the relative activity of each WT and HSF1(+) factor expressed in parallel, the strong, constitutive cytomegalovirus promoter (CMV) of the Invitrogen pcDNA3.1(+)
expression vector enabled overexpression in mammalian cells. Each construct was transiently transfected into mouse embryonic fibroblasts (NIH 3T3 cells) and assayed for an ability to cause transcription of a co-transfected Hsp70 Luciferase reporter (see Fig. 2.5 and known sequence information in Appendix 5). Unlike the WT versions of HSF1, the mutated mouse factor was found to constitutively

![Figure 4.1 Harnessing the constitutive activity of mouse HSF1 with a new construct: mHSF1(+).](image)

A transient transfection, overexpression experiment in NIH 3T3 cells illustrates the constitutive transcriptional activity of both mouse and human forms of HSF1(+), as is measured by the ability to activate transcription of an Hsp70 Luciferase reporter. Cells were separately transfected with one of the following expression constructs: CMV-hHSF1, CMV-mHSF1, CMV-hHSF1(+), or CMV-mHSF1(+), each of which was combined in a 10:1 ratio with Hsp70 Luciferase reporter and a 500:1 ratio with CMV-renilla luciferase. Cell lysate was harvested the following day for luminometer readings of luciferase activity. RLA (see Fig. 2.5) was calculated and graphed.
activate the HS response in terms of measured, relative Hsp70-luciferase transcription, even in absence of stress (see Fig. 4.1). The qualitative level of transcription that was able to be achieved was comparable to the analogous human mutation.

Further in vitro characterization

The mutated mouse factor was further characterized for activity in a collaboration that led to its use and incorporation in a figure published by

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**Figure 4.2 Rosa 26 ImHSF1(+) regulates activity of the constitutively active mouse HSF1.** Panel (A) illustrates the Rosa 26 ImHSF1(+) schematic to achieve mifepristone-dependent regulated expression for the constitutive activity of mHSF1(+), which was developed to form the single-construct design for ubiquitous, yet controlled, expression of mouse HSF1(+). It is similar to the harnessed HS response molecular switch (SafeSwitch), except that an ubiquitously active Rosa 26 promoter replaces the Hsp70/Gal4 promoter cassette, so there is no feed-forward aspect nor thermal dose requirement for gene expression in this system. Additional differences include the orientation of the target gene relative to the promoter for the GLP65 gene (all promoters are oriented in the same direction in this construct) and the antibiotic resistance for clonal cell selection here is Hygromycin. Panel (B) presents transient transfection results that reveal the tight regulation of Hsp70 promoter activity achieved by the Rosa 26 ImHSF1(+) design. Quadruplicate parallel cultures of NIH 3T3 cells were transiently transfected with one of each of the following three constructs: CMV-β-Gal, CMV-mHSF1(+), or Rosa 26 ImHSF1(+) (abbreviated ImHSF1(+) in the figure), each combined with reporter construct mix (a 10:1 ratio of the construct to Hsp70 firefly luciferase reporter DNA and a 500:1 ratio of it to CMV-renilla luciferase reporter DNA). The next day, half of the cells in each group were treated with a 10 nM concentration of mifepristone ligand. Two days post transfection, cell lysate was harvested for luminometer readings of luciferase activity, and results for comparable conditions were averaged. Results were further calculated to generate RLA and the Fold Induction that are graphed in the figure on a logarithmic scale. The scale allows the silenced expression of Hsp70 luciferase, achieved with the Rosa 26 ImHSF1(+) construct in absence of mifepristone ligand, to be appreciated in comparison to the basal levels, achieved by the transfection of a CMV-β-Gal control construct that does not depend on mifepristone ligand for expression.
Petrucelli et al. (2004). Their data presented the result that overexpression of Hsp70 protein was achieved as a consequence of overexpression of the mouse HSF1(+) constitutively active factor. Furthermore, such expression of the mouse HSF1(+) construct led to an inverse influence on tau expression in their model. Enhanced tau turnover was ascribed to the ubiquitin ligase protein, CHIP.

Harnessing the activity of the mutant factor

In order to harness the constitutive activity of mouse HSF1(+), the mutated gene was subcloned to an Invitrogen pGene vector downstream of a Gal4
promoter. That vector is commercially designed to be paired with the complimentary Invitrogen pSwitch system, which produces the active form of GLP65 in the presence of mifepristone ligand. In the context of that system, it successfully demonstrated the expected expression profile (data not shown). Further subcloning was then performed to generate a novel, inducible single construct: Rosa 1mHSF1(+), which is illustrated in Fig. 4.2 (Panel A).

The inducible single construct featured the ubiquitously active promoter, Rosa 26 (Kisseberth et al., 1999 and Soriano, 1999; obtained from Eric P. Sandgren of the University of Wisconsin School of Veterinary Medicine). This promoter was selected to facilitate production of mouse HSF1(+) in both stably transfected cell lines and in transgenic mice. The effectiveness of the inducible single construct was first proven in transiently transfected mouse cells (NIH 3T3, see Fig. 4.2, Panel B) in comparison to promoter activity afforded by CMV-mHSF1(+), which is not dependent on mifepristone ligand presence. Promoter activity results (Fig. 4.2, Panel B) of the latter are within an order of magnitude of the mifepristone-induced Rosa 26 ImHSF1(+) expression. Overall, the fold induction of reporter activity observed with the CMV-promoted constructs is nearly negligible, yet the fold-induction observed with the Rosa 26 ImHSF1(+) construct is nearly a 100-fold. The effectiveness of the inducible single construct was further proven in stably transfected mouse (derived from NIH 3T3 cells) and human (derived from HEK 293 cells) cell lines, which were produced. The appropriate transcription activity and protein production that was identified in these lines is presented in Fig. 4.3.
Figure 4.3: Regulated activity of mHSF1(+) and consequential Hsp70 production in mouse and human cell lines that are stably transfected with Rosa 26 ImHSF1(+). Panels (A) and (B) show comparative tight regulation of an Hsp70 Luciferase reporter and Hsp70 protein, respectively, observed in a mouse 3T3 cell line and a human 293 cell line. Each line is stably transfected with the Rosa 26 ImHSF1(+) construct. Panel (A) presents western blot results for Hsp70 levels in the Rosa 26 ImHSF1(+) 3T3 cell line and in a comparable unresponsive cell line when each line was treated with or without 10 nM mifepristone ligand. Panel (B) reveals a similar tight regulation of the Hsp70 promoter activity through the sensitive luciferase reporter activity. The Rosa 26 ImHSF1(+) line and the indicated 3T3 comparative control line were plated in parallel for both experiments. The cells used to generate results presented in Panel B were transiently transfected with reporter constructs (a 10:1 ratio of each construct to Hsp70 firefly luciferase reporter DNA and a 500:1 ratio of each construct to CMV-renilla luciferase reporter DNA) in duplicate fashion for each condition examined. The next day, a 10 nM treatment of mifepristone ligand was applied. Cell lysate was harvested 24 hours later, and reporter activity was measured on a luminometer to calculate RLA and fold induction (see Fig. 2.5). To generate the results presented in Panel (A), one of two duplicate plates of cells for each line (here the original NIH 3T3 parent line or the Rosa 26 ImHSF1(+) 3T3 derived line) were treated with 10 nM mifepristone. Lysate for each condition was harvested 48 hours later by boiling cells obtained from the respective condition in the presence of SDS Sample buffer. Protein concentrations were equilibrated via a Bradford assay and Western analysis was conducted utilizing StressGen SPA-810 antibody. Like Panel (A), Panel (C) characterizes protein expression obtained from a line stably transfected with the Rosa 26 ImHSF1(+) construct [a.k.a.: R26 ImHSF1(+)]. The western blot results examine mifepristone-dependent expression of several proteins: Hsp70 (detected with StressGen SPA-810 antibody); Tubulin (beta Tubulin was detected with Abcam ab6046 antibody as a control); TA = GeneSwitch GLP65 Transactivator, which illustrates expression of the integrated genes, and is detected with Lab Vision 1638 antibody that also detects endogenous NF-kBp65 protein; and endogenous human HSF1 as well as the mutant mouse HSF1(+) (both detected with StressGen SPA-901 antibody). Cell lines examined in parallel include the WT parent HEK 293 line and two derived lines: 1) a 293 line stably transfected with the Rosa 26 (R26) ImHSF1(+) construct; and 2) a control line otherwise identical to (1), except that the construct used to produce it deliberately lacked the mHSF1(+) gene (referred to in the figure as the R26 no ImHSF1(+) 293 line). Cell treatments and Western analysis was identical to that utilized for Panel (A)’s experiment. Hsp70 protein was enhanced only in the R26 ImHSF1(+) 293 line and not in the control lines; TA is present in all cell lines except wild type HEK 293 cells; HSF1(+) is visible and highly upregulated in the R26 ImHSF1(+) cell line, but not in the control lines. Panel (D) reports mifepristone-dependent Hsp promoter activity in the two 293-derived lines. Transient transfection methodology was similar to that utilized to generate the results presented in Panel (B).
Despite successful, well-regulated production of the desired proteins in the cells lines that were isolated, a different scenario was encountered in an attempt to produce those proteins in transgenic mice. The transgenic mice were created by microinjection of the relevant (prokaryotic sequence was excised), linearized construct gene sequence. Mice that were positive for transgene presence (as was determined by both PCR and southern blot methods) were induced with an appropriate dose of mifepristone ligand that ought to have produced the anticipated gene expression. However, production of the inducible Hsps (Hsp70, Hsp40, Hsp25), and the phosphorylated form of HSF1 were not detectable by western blot methods that sensitively detected these proteins in extract from *in vitro* treated, NIH 3T3 mouse cells (data not shown). Further analysis of progeny
from the founder animals revealed that the transactivator, even in an induced state, often was not detectable in their tissues (data not shown). Founder animals were identified, however, to positively transmit the introduced gene sequence to their offspring (data not shown).

It was hypothesized that integration position-dependent silencing of the Gal4 promoter and/or the Rosa 26 promoter may have occurred \textit{in vivo}. This possibility argues for the inclusion of insulator sequence in future gene switches designed for ubiquitous \textit{in vivo} expression. A new subcloning plan for an improved construct was assembled, which integrated insulator sequence. The plan may be utilized to produce a transgenic animal in a collaborator’s lab.

If an animal model for the deliberate activation of Hsp gene transcription via induced production of mouse HSF1(+) is able to be produced, it might be possible to ascertain the merit of a strategy to pharmacologically increase the cohort of Hsps to mitigate or prevent the following diseases: neurodegenerative disease, chemotoxicity, sepsis, ischemia / reperfusion injury, as well as age-related oxidative damage. As mentioned in chapter 1, new drug applications of chemical chaperones to address diseases of chaperoning have already demonstrated preclinical promise.

\textbf{Summary of thesis conclusions}

HSF1 is known to act within the intracellular environment as a sensor. Advances in biomedical technology have enabled the fabrication of a harness of this factor, which capitalizes upon its ability to sense environmental changes in
order to subsequently transcribe genes for engineered purposes. The hypothesis of this thesis is that the SafeSwitch harness of the HS response, when paired with a therapeutic mechanism, will produce outcomes relevant to the refinement of novel therapies. Extensions to the concept of deliberately activating HSF1’s normal functions for therapeutic purposes were examined in this work through successful \textit{in vitro} trials and \textit{in vivo} preliminary studies that featured the use of HSF1 as a regulator of therapy.

After successful completion of the \textit{in vitro} work that comprised the first aim, the preclinical work of the second aim was conducted at the University of Florida’s Center for Environmental and Human Toxicology. The aim of the continuing, innovative, collaborative project is to treat various cancers using a recombinant virus system that employs SafeSwitch control of therapeutic gene expression. Under SafeSwitch control, the activity of the gene therapy was designed to be dependent on mifepristone ligand presence and to be specifically targeted by the delivery of transient heat to solid tumors.

As the second aim was addressed, limitations within the GALV(+) SafeSwitch \textit{in vivo} model were identified. It became evident that in the models that were developed, the GALV(+) SafeSwitch was not powerful enough to cause tumor regression on its own. However, it was effective in a new therapeutic approach, which combined the GALV(+) SafeSwitch with the complimentary Oncolytic SafeSwitch. The latter SafeSwitch-controlled therapy conditionally causes the replication of adenovirus. Tumor regression outcomes were achieved
in a xenograft model and were further improved with developments centering on the applied heat treatment.

Future directions focus on three main areas: production of additional SafeSwitch viral vectors, an improved animal model, and a refined heat system to standardize observed regression outcomes. Preclinical studies that encompass elements produced from outcomes of these future directions would enable the clinical potential of the SafeSwitch strategy for tumor regression to be extrapolated from findings. Should the results yield convincing data in terms of feasibility, therapeutic merit, and safety, they could be presented for Institutional Review Board approval to launch related clinical studies.

To explore the contribution of the immune system to such a clinical concept, which deliberately activates HSF1, as well as to produce a tool to answer questions that were raised regarding physiological consequences of HSF1 activity, a constitutively active mouse HSF1 was produced through the completion of the third aim. The novel construct was tested extensively in vitro. However, an in vivo model in the form of a transgenic animal that featured the construct was not able to immediately be produced.

In summary, three specific aims were accomplished and novel therapeutic approaches were refined in the course of this thesis work. The first aim paired the previously characterized SafeSwitch with the gene target, GALV(+), to examine the feasibility of controlled syncytia production in vitro. The SafeSwitch effectively regulated the potent expression of the gene target, which resulted in deliberate cell death as a consequence of high levels of cytotoxic gene
transcription. This finding was particularly evident in results achieved with cells of human origin that were either stably or transiently transfected as a means to deliver the genes. The second aim then translated the *in vitro* work to an *in vivo* proof of principle tumor regression model, which demonstrated the practical potential to achieve a therapeutic outcome of solid tumor regression. The third aim produced a constitutively active mouse HSF1 construct, which may be utilized by others to clarify the physiological consequences of deliberately upregulating HSF1 activity *in vivo*. The outcomes measured *in vivo* have defined the challenges that a SafeSwitch therapy for tumor regression now confronts, and the concept may offer a means to safely target and effectively control even more potent therapies.
Chapter 5 – Experimental Methods

In vitro methods

Cell culture techniques

Several mammalian cell lines were utilized to complete this work. Each line was maintained at 37°C in a 5% CO₂ incubator. The primary cell lines were purchased from ATCC, with the exception of two cell lines that were obtained directly from the indicated labs [1) the LCRT line, which was obtained from William S. M. Wold, Department of Molecular Microbiology and Immunology, Saint Louis University School of Medicine, St. Louis, Missouri, and was described by Toth et al., 2005 and 2007; and 2) the HEK 293 line, which was utilized in chapter four, was obtained from Dr. Anthony H. V. Shapira, Institute of Neurology, University College London, London NW3 2PF, UK, and was described by Tabrizi et al., 2000]. All lines were cultured consistent with the supplier’s recommended media formulations and passaging procedures. The ATCC lines utilized within this work included: 1) MatBIII (a.k.a.: 13762 MAT B III, ATCC: CRL-1666), a Fischer 344 rat mammary gland adenocarcinoma; 2) F98 (ATCC: CRL-2397), a CD Fischer 344 rat undifferentiated malignant glioma; 3) RG2 (ATCC: CRL-2433), a CD Fischer 344 rat undifferentiated malignant glioma; 4) XC (ATCC: CCL-165), a White-Weston rat line Rous sarcoma; 5) NIH 3T3 (ATCC: CRL-1666), originally isolated from Swiss mouse embryo cultures; 6) HeLa (ATCC: CCL-2), human adenocarcinoma; 7) HT-1080 (ATCC: CCL-121), human fibrosarcoma; and 8) U87-MG (a.k.a. U-87 MG, ATCC: HTB-14), human
glioblastoma/ astrocytoma. Transfection techniques for each line utilized the respective recommended Invitrogen DNA transfection system, e.g., Lipofectamine, Lipofectin, PLUS reagent, or Lipofectamine 2000 and followed the technical tips and guidelines provided by the manufacturer relevant to each line.

Stable cell lines were typically produced with the transfection of a single construct that contained a selective marker, which was linearized with restriction endonucleases that also removed as much prokaryotic sequence as possible. The specifics for the cell lines produced are described in the related sections. To broadly describe the procedure, a QIAEX II gel extraction kit (Qiagen) was utilized to purify the endonuclease-digested fragment electrophoresed through an agarose gel. An antibiotic kill-curve on untransfected cells was routinely performed to empirically determine the effective antibiotic strength. The purified DNA fragment was utilized to transfect one well of a 24-well dish of the target cells, following Invitrogen transfection protocol recommendations for the respective line. The day after transfection, the cells in the transfected well, as well as the untransfected antibiotic control cells, were each treated with 200 μL of trypsin-EDTA to detach them from the tissue culture dish surface. The trypsin-treated cells were then resuspended in 11 mL of appropriate growth media containing serum. A repeat-pipetter was next utilized to dispense 100 μL into each well of a 96-well dish. 10 mL of additional growth media was then added to the remainder of the cells to again dispense 100 μL into each well of a 96-well dish, thereby creating a log dilution. This procedure was typically repeated for a third serial dilution.
The day after cells were seeded in the 96-well dish (48 hrs after transfection), 100 μL of 2x selection media (growth media containing twice the concentration of selective antibiotic empirically determined to be effective at killing untransfected cells in approximately one week’s time) was added to each well. Thus, the final concentration of the selective antibiotic in each well was 1x. Daily observations were performed to monitor single colony formation and to note any wells that formed multiple colonies. Wells with cells that appeared to be resistant to the antibiotic were compared to the respective control plate, which was initially plated with a similar cell concentration. Fresh media was added every two days (maintaining the 1x antibiotic concentration), until the wells became full. At that point, the old media on cells was aspirated and entirely replaced with fresh media. This method ensured that secreted growth factors would not be overly depleted while cell number was low. Plates that had no cells left or that were overgrown with cells in every well were discarded. Single-cell colonies often began to appear within a week or two, but it generally took at least two to three weeks before the next step was possible.

The adherent cells were detached and the entire cell suspension was transferred to a 24-well dish containing media without selective antibiotic. The day after transfer, cells were evaluated for 10-20% confluency in the new dish. Media containing selective antibiotic was added if that criteria was met, in order to reselect and expand that line. If cells fell outside of that confluency range, they were reseeded to a new well or allowed to expand in number prior to addition of selective media, as appropriate. Once cells approached confluency in
the new well, they were split into wells of new dishes for phenotype testing (e.g., a microscopy screen or a luciferase-based screen, such as the luciferase overlay method described in Fig. 2.5). A stock of cells was maintained in reserve for expansion, if a clone with desired characteristics was identified. The derived line was usually able to be maintained in media that contained half the antibiotic concentration required for its initial selection.

Cell treatments included heat and mifepristone ligand addition. Heat treatments were carried out in tissue culture dishes that were sealed with parafilm, which was wrapped around the edges. The dishes were then floated on a heated, circulating water bath for the treatment duration. A typical heat treatment was 44°C for thirty minutes. Mifepristone ligand was added to cells to create a final concentration of 10 nM from a working solution of 1x10^{-5} M, which was purchased from Invitrogen directly or was diluted from a stock solution of 1mg/ mL mifepristone (Sigma) in ethanol.

Dual-Luciferase Assays were utilized throughout the thesis work. The Dual-Luciferase Reporter Assay System (Promega) was exclusively employed and samples were typically measured on a plate reader luminometer equipped with dual-injectors. An assay developed within this work to quantify the incidence of cell to cell fusion was described in Figure 2.5 and was coined the luciferase overlay method. In that assay, cells to be analyzed for gene cell fusion, which expressed a GLP65 TA and that contained the Gal4-GALV(+) sequence, were overlayed with a second set of cells, which were transiently transfected with luciferase reporter gene components. When the GALV(+) component was
successfully transcribed and the protein that was produced was able to cause fusion events with adjacent reporter-transfected cells, Gal4-promoted firefly luciferase was then also able to be produced. In assays that more directly measured the HS response, firefly luciferase reported transcription from an Hsp70b promoter (see Appendix 4 for related construct information). For all luciferase-based assays, approximately 3% (range of 1-5% was optimized) of the reporter DNA mixture produced renilla luciferase (pRL-CMV purchased from Promega) and acted as an internal control, since it was constitutively transcribed from a cytomegalovirus virus (CMV) promoter.

Relative Luciferase Activity (RLA) was calculated to equilibrate outcomes with respect to transfection efficiency and related sources of variability. The RLA was calculated by dividing the results observed for the conditionally expressed firefly luciferase, with the respective results obtained in those same cells from the constitutively expressed renilla luciferase. Fold Induction was next able to be calculated by dividing the RLAs achieved in treated conditions with the RLAs obtained from the comparable, basal condition.

Cell phenotype was documented either by phase contrast Nikon microscope photography or standard inverted scope pictures of cells fixed with methanol and stained with Wright-Giemsa solution. The method utilized is noted in the respective figure legend. Staining with the Sigma Wright-Giemsa kit was a simple procedure in which media was aspirated and a minimal amount of methanol was added to each well for five minutes. Wright-Giemsa solution was then added to wells in an amount that quadrupled that of the minimal amount of
methanol. The stained cells were then stored at 4°C overnight or until ready to be imaged. The solution was then aspirated and the cells were rinsed several times to remove unbound stain before the addition of a final minimal amount of PBS to aid photography.

Virus for infection studies was purchased from ViraQuest, Inc. (North Liberty, Iowa) or was obtained from Dr. Nuria Vilaboa (Laboratorio de Metabolismo Oseo, Hospital Universitario La Paz, Paseo de la Castellana 261, 28046 Madrid, Spain), who supplied the rAd1 adenovirus. Infection was carried out with a concentrated cell suspension, which was mixed with an appropriate amount of virus to create the MOI noted in the experiment. A minimal amount of the mixture was added to wells of a tissue culture dish (e.g., 500 µL per well of a 6-well dish) and incubated on a mutating platform to facilitate interactions between cells and virus particles. Two hours later, four times the volume of growth media was added and cells were returned to standard incubation conditions to adhere overnight. The next day, media was changed and cells were prepared for phenotype assays (e.g., mifepristone was added).

**DNA methods**

Plasmid DNA was isolated utilizing standard molecular biology techniques and often employed the GenElute Plasmid Miniprep Kit (Sigma) or an appropriate larger preparation kit from Qiagen (e.g., the Endo-free plasmid Maxi or Giga Kits). Electrophoresed DNA was extracted from agarose with the use of a QIAEX II Gel Extraction Kit (Qiagen). DNA was routinely eluted with HPLC grade
water or buffer supplied with the respective kit. PCR for cloning purposes typically utilized \textit{Pfu} Turbo or \textit{Pfu} Ultra (Stratagene) and guidelines provided by the manufacturer were followed. Products were often initially cloned into an intermediate vector prior to additional cloning steps with the use of the ZeroBlunt Topo PCR Cloning Kit (Invitrogen). DyeDeoxy Terminator (Applied Biosystems Inc.) cycle sequencing reactions verified double-stranded DNA products created through cloning procedures. Sequencing was performed by the University of Miami Miller School of Medicine’s DNA Sequencing Core Facility, with the use of their ABI model 373A DNA sequencer (Applied Biosystems Inc.). The resultant nucleotide sequences were analyzed with IntelliGenetics computer software (IntelliGenetics). XL10-Gold Ultracompetent cells (Stratagene) were preferentially employed for subsequent transformations, particularly post ligations involving a large gel-purified product that was expected to ligate with low efficiency.

The Gal4-GALV construct was produced by subcloning the GALV(+) gene, which was obtained from Dr. Richard G. Vile (Molecular Medicine Program, Mayo Clinic, 200 First Street SW, Rochester, Minnesota 55905, USA), from the original CMV-promoted plasmid vector (the PCR3-GALV construct described by Bateman et al., 2000) to a Gal4-promoted expression vector (the Invitrogen pGene vector). Sequence was removed from the pCR3.1 vector (Invitrogen) by EcoR I digestion and inserted into pGeneA-V5 His (Invitrogen) by ligation to the similarly digested, alkaline phosphatase-treated vector).
The GALV(+) SafeSwitch construct was formed in a step-wise manner in the backbone of the VQpacAd5K-NpA vector (noted within this work as the VQ-shuttle vector). This vector was provided by ViraQuest, Inc. (North Liberty, Iowa), since it was their preferred shuttle vector for their adenovirus production method. Figure 2.3 presents a sketch of the final construct and Appendix 1 lists relevant sequence information.

The GLP65 gene is a synthetic fusion protein comprised of a GAL4 DNA-binding domain (amino acids 1-93 of the native protein in yeast, which allows the factor to bind the GAL4 promoter); a truncated ligand binding domain (amino acids 640-914 of the native human progesterone receptor protein, which enables mifepristone-specific binding that activates the factor); and an activation domain (amino acids 283-551 of the native human p65 subunit of NF-kB, which permits activation of gene transcription). It is transcribed from an Hsp70b promoter linked to six GAL4 response elements and a TATA box in a promoter cassette (described in Vilaboa et al., 2005; as well as Vilaboa and Voellmy, 2006). The Gal4-GALV gene uses the identical GAL4 response elements, without the Hsp70b promoter component.

In brief, the attenuated version of the GLP65 TA gene, RKC GLP65 (described in Vilaboa et al. (2005); modifications to GLP65 TA sequence included: deletion of essentially all untranslated sequence located on the C-terminus and mutagenesis to the associated Kozak sequence, which affected nucleotides at the -3 and +4 positions so that translation initiation was no longer favored), was inserted in reverse orientation into the VQ-shuttle vector between
Kpn I and Pme I restriction sites (the Pme I site was destroyed by non-similar blunt ligation). The target gene component, Gal4-GALV(+), was inserted in normal orientation (opposite the orientation of the adjacent TA component) between Hind III and Not I, making use of the vector's downstream polyA sequence.

Though SafeSwitch components were inserted into the VQ-shuttle vector in a step-wise process, the entire sequence was able to be excised from the final construct (referred to as the GALV(+)/SafeSwitch) with the restriction endonucleases: Ssp I and BbvC I. The BbvC I end was blunted with the use of DNA Polymerase I, Large (Klenow) Fragment (New England BioLabs), for insertion into the EcoR V-digested pEF-Bsd vector (Invitrogen). Subcloning to the latter vector was important for stable cell line production, since it contained the mammalian Blasticidin antibiotic-resistance gene for clonal selection. The insertion direction of the genes integrated within the Invitrogen pEF-Bsd vector was identified through restriction mapping that utilized Dra III restriction endonuclease sites. The clone was selected that demonstrated that the GLP65 gene was inserted in an orientation that was opposite that of other genes in the vector. In this way, the GALV(+) sequence was 5' to the vector's elongation factor 1 alpha (EF1α) promoter for antibiotic resistance. This arrangement (see Fig. 2.4) precluded undesired read-through expression from the related promoters.

Other plasmids were utilized in experiments with the above constructs. This included CMV-GFP (Clontech). Additionally, CMV-β-Gal, Wild type (WT)
CMV-HSF1, CMV HSF1(+) and CMV-AVST, were all obtained from the Voellmy lab (University of Miami Miller School of Medicine, Miami, FL). The human HSF1 constructs are described in Appendix 4.

A constitutively active mouse HSF1 construct was prepared by first amplifying sequence from a mouse 17-day embryo Quick-clone cDNA library (Clontech), which was created from a pool of 200 normal Swiss-Webster/NIH embryos. The PCR product was initially cloned into the pCR-Blunt II-TOPO vector (Invitrogen) of the ZeroBlunt Topo PCR Cloning Kit. This construct was utilized for QuikChange mutagenesis (Stratagene). Primers were designed following manufacturer’s suggestions and corresponded to mouse sequence (see Appendix 2 and Appendix 3) homologous to the area that flanked the deleted region, the area that coded for amino acids corresponding to amino acids 203-315 of the human CMV-HSF1(+) sequence (see Appendix 4). This active mouse factor, mHSF1(+), was sequenced and subsequently subcloned from that vector to the Invitrogen pcDNA3.1(+) expression vector through the use of Hind III and EcoR I restriction sites that were common to both vectors. Wild type (WT) CMV-mHSF1 was also subcloned to that same vector and is described in Appendix 3, as well.

The Rosa 26 ImHSF1(+) [a.k.a.: R26 ImHSF1(+)] and the control construct [R26 no ImHSF1(+)] were created in step-wise fashion. QuikChange (Stratagene) site-directed mutagenesis was performed to first introduce a mutation in the pSwitch plasmid (Invitrogen), which contained the GLP65 TA. The QuikChange primers utilized were the following (5’ to 3’):
CGGGAGATCATTCGAGCTTAAGAGCACTTTGTACAGGTCGAAGCGGAGTACT
G, as well as the reverse complimentary sequence. This mutagenesis
introduced both a BsrG I and an Afl II restriction site. The Rosa26 promoter was
obtained from Eric P. Sandgren, University of Wisconsin School of Veterinary
Medicine, Madison, WI and was amplified utilizing the following primer pairs: 1)
R26-F-BsrGI: ATATGTACAGTCGACTAGATGAAGGAGAGCCTTTC; and 2)
R26-R-PacI: ATATTAATTAACCGCAAACGCACCAAGC. These primer pairs
included the indicated restriction site (BsrG I and Pac I) at their 5’ ends. The
PCR product was then subcloned to the pCR-Blunt II-TOPO vector (Invitrogen).
The introduced BsrG I and the previously present Pac I sites of the new pSwitch
construct were then utilized to replace the existing promoter with the similarly
digested Rosa26 promoter, in order to create the Rosa26-pSwitch construct.
Lastly, the WT and mHSF1(+) constructs were subcloned from the pcDNA 3.1(+)
(Invitrogen) vector to the pGene (Invitrogen) vector, utilizing the common Hind III
and EcoR I restriction sites. The new constructs were amplified with primers: 1)
pG-F-Afl II: AGCGCTCTTTAGTGCCACCTGACGTCGACGG; and 2) pG-R-
BsrGI: TATTGTACAGAAGCCATAGAGCCCACC. The PCR products were
maintained in pCR-Blunt II-TOPO vector (Invitrogen) via a subcloning step to that
vector. The BsrG I and Afl II digested products were then separately ligated to
the similarly digested Rosa26-pSwitch plasmid to make the final constructs.

Western blots of cell lysate were prepared utilizing standard molecular
biology techniques. In brief, cells were directly transferred from wells to tubes
containing SDS sample buffer. Lysate was boiled three minutes and then
equilibrated for overall protein content utilizing a Bradford assay prior to being loaded on an SDS-PAGE gel. After electrophoresis, gels were transferred to PVDF membranes with a semi-dry transfer apparatus. Membranes were blocked with blocking buffer obtained from the ECL Plus kit (Amersham) and incubated with primary antibodies (e.g., Hsp70: StressGen SPA-810 antibody; Tubulin: Abcam ab6046; TA: Lab Vision 1638; human and mouse HSF1 and HSF1(+): StressGen SPA-901 antibody). The protocol for the ECL Plus kit was followed for washings and secondary antibody incubation as well as for development and exposure to film.

**In vivo methods**

Two rat lines and two mouse lines were utilized in this work and were obtained from Harlan. Male Fischer rats served as the immunocompetent model, and rnu/rnu male athymic rats and athymic mice served as the immunocompromised models. Immunocompetent C57BL/6xSJL/J mice were utilized in the transgenic mouse studies conducted and the University of Miami Miller School of Medicine (Miami, FL).

**Tumor studies**

Generally, six-week old animals were utilized to form tumors. Approximately 1-2 million cells in a volume of 50 or 100 μL PBS were injected either subcutaneously or intramuscularly in the desired animal model. Tumor cells were grown *in vitro* until the time of *in vivo* passaging. At that point, they
were detached from their dishes utilizing trypsin-EDTA (0.25% and 0.05%, respectively), which was inactivated by the addition of growth media containing serum. Cells were washed once with Dulbecco's phosphate-buffer saline (PBS) without calcium or magnesium if passaged into nude mice, but were washed twice when passaged into either rat model. Lastly, cells were resuspended in the injection solution of PBS.

Resulting tumors were generally grown to at least 100 mm$^3$ prior to the commencement of therapy, unless an outgrowth experiment was performed. Tumor size was periodically measured with calipers and tumor volume was calculated using the formula: $\pi/6 \times$ the greatest tumor height $\times$ the widest tumor diameter $\times$ the narrowest tumor diameter (a total of three diameter measurements). A value of “1” was assigned when no tumor was detectable. An average of the percentage of increase in tumor volumes was calculated when possible, in an attempt to equilibrate tumor growth relative to starting tumor size. To perform this calculation, difference between the new size and the original tumor size was expressed as a percent of the original tumor size.

Animals were either implanted with subcutaneous pellets (purchased from Innovative Research, Inc.) containing mifepristone (1 mg or 5 mg/60-day sustained-release pellets for mice and 15 mg/60-day sustained release for rats). Alternatively, mifepristone ligand treatment consisted of intraperitoneal injections (50 mg/ kg for mice and 250 mg/ kg for rats) of mifepristone suspended in corn oil. Generally, 1 mg mifepristone was dissolved per mL of corn oil, which was administered at a dose of 1µL/ g/ day delivered via intraperitoneal (IP) injection.
with a tuberculin syringe and a 25 gauge needle. The first day, a double-dose was administered to ensure the possibility of SafeSwitch activation if the HS response requirement was met as well.

When procedures necessitated anesthesia, pentobarbital (50 mg/kg for rats and (40mg/kg for mice, each administered intraperitoneally) was utilized. Anesthesia was required both for electroporation procedures and for water bath heating protocols. Electroporation was performed subsequent to injection of material containing nucleic acids (e.g., a mixture of rAd1 (7 x 10^7 pfu) and GALV(+) SafeSwitch (7.5 μg) in a volume of 20 μl). Electrodes (1 cm²) were placed non-invasively on the skin flanking the desired region (e.g., the gastrocnemius muscle or the tumor). Conducting gel was added and the field strength of 1.8 kV/ cm, per 50 ms pulse, was applied for a maximum of 8 pulses and at an interval of one second.

Several procedures were utilized to apply heat to the animals. Two heating methods were tried with the rat model, and two additional heating methods were utilized with the mouse model. The water bath treatments included the rat unilateral circulating water bath heat treatment (pictured and described in Fig. 3.1); the rat bilateral circulating water bath heat treatment (Fig. 3.5); and the mouse bilateral circulating water bath heat treatment (Fig. 3.10). Common components for these methods included a basin filled with water, a water pump equipped with a thermostat-controlled heating system, and thermometers that further monitored temperature. The differences in the methods included a means to treat a single leg (with the rat apparatus) or the
means utilized to suspend the pharmacologically sedated animals in the bath so that their lower half was treated. Animals were generally heated for 30 minutes in water that was approximately 43.5°C for rats and approximately 43°C for mice. Since it was apparent that a focused, localized heat treatment would improve the therapy, a fourth means to deliver a thermal dose was developed by John Munson (University of Florida, Center for Environmental and Human Toxicology, Gainesville, FL). It is pictured and described in Fig. 3.17 and was called: the laser heating method. An 810 nm diode laser set to 8.5 Amps delivered 1.5W of power with a three second pulse duration, which resulted in a fluence of 145 Joules/cm² energy to a 1 cm diameter section of skin (over a tumor) that was blackened with ink. Mice were physically restrained for only five minutes at a fixed distance from the laser in order to receive the activating thermal dose. Since the method was relatively brief, pilot studies did not utilize pharmacologic means of physical restraint.

Procedures utilized to screen transgenic mice

The UM Transgene Facility performed direct embryonic microinjection of the purified R26 ImHSF1(+) and the matching control construct, R26 no ImHSF1(+). Founder mice were identified among progeny obtained from the UM Transgene Facility by PCR and southern blot screens. Tail snip PCR was performed with the use of an XNAT2, Extract-N-Amp Tissue PCR Kit (Sigma), which rapidly isolated DNA from the cut end of the tail and enabled quick assembly of appropriate PCR reactions. The following primers (written 5’ to 3’):
differentiated endogenous mouse HSF1 sequence (2,110 bp), which contained introns, from the introduced mouse HSF1(+) cDNA (424 bp), which consisted of the same sequence sans introns: 1) GTAGTCCACATTGAGCAGGG; and 2) AGGATGGAGTCAATGAAGGC. A 55°C annealing temperature, along with a 2 min. 40 sec. extension time, was utilized. Products were electrophoresed through agarose, and a 2.1 kbp band resolved (when stained with ethidium bromide) for all samples that were successfully amplified. Positive samples had an additional 424 base pair band, which indicated the presence of the desired cDNA sequence.

The matching, negative control line was screened with the following primer pair (also written 5’ to 3’), which required a 62°C annealing temperature along with a two minute extension time to generate a single 878 bp PCR product in positive samples: 1) AATTAGATCT/GCTCTTACGCGGGTCGAAGC; and 2) TAT/TGTACAGAAGCCATAGAGCCCACC. These primers were originally created for a different purpose. That purpose required the addition of sequence to the 5’ end, and those bases are noted by italics and are written 5’ to the “/” symbol. Hence, only sequence that is 3’ to the “/” symbol is necessary to successfully screen samples of this type. PCR products were analyzed via agarose gel electrophoresis. The bands that resolved, which were of the determined size, indicated mice that contained DNA sequence corresponding to the 5’ end of the DNA construct that was microinjected (i.e.: sequence encompassing the Gal4 promoter and the 5’ end of the Rosa 26 promoter).
When positive results were obtained by either PCR reaction, results were confirmed by southern blot. New genomic DNA preps were prepared from the frozen tail that was retained after the XNAT2 procedure was completed. Standard proteinase K / phenol extraction procedures were employed (e.g., the procedure outlined on The Jackson Laboratory’s website: [http://www.jax.org/imr/tail_phenol.html](http://www.jax.org/imr/tail_phenol.html)). 10 μL of the resulting genomic DNA was digested with Bgl II restriction endonuclease, a single-cutter within the sequence and a frequent cutter within the mouse genome, so that sequence could be electrophoresed on an agarose gel and so that differences in the integration position could resolve as bands of slightly different size. Samples were electrophoresed beside a DNA ladder and a positive control. The positive control consisted of the linearized construct DNA that was used in the microinjection procedure (approximately 4.76 kbp in size), which was diluted in Bgl II - digested genomic DNA (isolated from a mouse of the same origin that was known not to be transgenic), in order to achieve representation equivalent to one copy of the gene in the genomic DNA extract. The DNA was transferred to a nylon membrane and was probed with a 32p-labelled 693 base pair sequence (Afl III and Bgl II digest product), which corresponded to a fragment of the TA that is present in both the R26 ImHSF1(+) and the matching control construct.

Five of twenty-four progeny received from the Facility contained the R26 ImHSF1(+) transgene and four potential founders from another twenty-four progeny were found to contain the control construct. Mice were intraperitoneally administered 200 μg mifepristone/ per mouse dissolved in sesame oil vehicle.
Animals were sacrificed after a series of injections ranging from two to five days. Brain, heart, and liver were harvested and homogenized with a dounce homogenizer using Passive Lysis Buffer (Promega) to suspend the protein extract. Western blots were then performed in a manner similar to technique outlined for the cell studies.

Statistical methods applied

Standard deviation in the graphs was plotted for data that represents a sample of the population. It was calculated using the unbiased, n-1 method. The following formula was utilized: the square root of (the sum of the squares of the difference of the means (x), divided by the sample size (n) minus one). See below:

\[ \sqrt{\frac{\sum (x - \bar{x})^2}{n-1}} \]
References


Shinozaki, K., Ebert, O., and Woo, S.L. (2005b) Eradication of advanced hepatocellular carcinoma in rats via repeated hepatic arterial infusions of recombinant VSV. *Hepatology* 41: 196-203.


APPENDIX 1 – GALV(+) SafeSwitch Construct

GALV(+) SafeSwitch components, which were inserted into the commercially available (ViraQuest, Inc.: www.viraquest.com) VQpacAd5K-NpA vector in a stepwise-fashion, may be excised from the GALV(+) SafeSwitch construct (11,711 bp total) for additional subcloning procedures (see related schematic of genes and restriction sites in Fig. 2.3).

A Not I restriction endonuclease digestion cuts out both:

1) The attenuated transactivator (presented here in 3’-5’ orientation) and its promoter cassette (3,114 bp in size).

2) The Gal4-GALV(+) sequence (2,942 bp in size, without the vector’s polyA; one must be added 3’ to GALV(+) in the new recipient vector)

*The three Not I sequences are indicated: GCGGCCGC
(The location of some additional restriction sites are noted with bold letters).

In order from top to bottom of the 6,064 bp of DNA sequence that follows:

*The top, black, underlined sequence (1,965 bp) represents the attenuated GLP65 sequence, presented here in 3’-5’ orientation.

*The gray, non-italicized, underlined sequence (344 bp) represents the Gal4 promoter, presented here in 3’-5’ orientation.

*The gray, italicized, underlined sequence (461 bp) represents the Hsp70b sequence of the promoter cassette, presented here in 3’-5’ orientation.

*The gray, non-italicized, underlined sequence (344 bp) represents the Gal4 promoter, this time in 5’-3’ orientation. The regions that are bolded and not underlined indicate the six Gal4 binding sequences. The minimal TATA box promoter is both bolded and underlined.

*The italicized, underlined sequence (2,004 bp) at the bottom is the region that codes for GALV(+). Note that it requires a polyA to follow for proper expression.

GALV(+) SafeSwitch Sequence:

```
GCGGCCGC CCGGAGGATCCTTAGGAGCTGATCTGACTCAGCAGGGCTGAG
AAGTCTCATGTCGCCGCAATGGAGGAGAAGTCTTCATCTCCTGAAAGGAGGCC
ATTGGGGAGGCCCGGCGGGCCCCAGTGGAGCAGGAGCTGGGTCGGGGGGCC
TCTGGGCCCCTGTCACTAGGCGAGTTATAGCCTCAGGGTACTCCATCAGC
ATGGGCTCAGTTGTGGGCGCCACAGTAGATGCCCTGGTTCAGCAAGCTG
CTGAAACTCGGAGTTGTCGACGGATGCCAGGTCTGTGAACACAGCTGGGT
CTGTGCTGTTGCCAAGCAAGGCCCGGCCCCAGTGGAGCAGGAGCTGGGTCGG
GGCAAGTGGGCGGCAAGGCTGAGGAGGCTGGGGGGCCGGGAAGGCCGAAGC
CTGGCTGATCTGCCAGAAGGAAACACCATGGTGGGAAACTCATCATAGTG
TGATGGTGCTCAGGGATGACGTAAAGGGATAGGGCTGGGGTGCTGGCTTG
GGGACAGAAGCTGCGGGAAGGCACAGCAATGCGTCGAGGTGGAGG
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178
APPENDIX 2 – Mouse and Human HSF1 Alignment

Mouse: 1. MDLAVGSEAEPLKALVDPQDIALCPSGSPYFHVTDCQGFAEVLVKT 60
       MDLAVGSEAEPLKALVDPQDIALCPSGSPYFHVTDCQGFAEVLVKT

Human: 1. MDLAVGSEAEPLKALVDPQDIALCPSGSPYFHVTDCQGFAEVLVKT 60
       MDLAVGSEAEPLKALVDPQDIALCPSGSPYFHVTDCQGFAEVLVKT

Mouse: 61. FKYDINASATFLVQEPFKVKEQKGLPKEPDFTFQHPEFTQGQKELLRIKVT 120
        FKYDINASATFLVQEPFKVKEQKGLPKEPDFTFQHPEFTQGQKELLRIKVT

Human: 61. FKYDINASATFLVQEPFKVKEQKGLPKEPDFTFQHPEFTQGQKELLRIKVT 120
        FKYDINASATFLVQEPFKVKEQKGLPKEPDFTFQHPEFTQGQKELLRIKVT

Mouse: 122. SVPXKEDIRHILDSVYRLDVGSLDSGQKEDSNFLAIHLNNEHALRVAVSLRQA 180
       SVPXKEDIRHILDSVYRLDVGSLDSGQKEDSNFLAIHLNNEHALRVAVSLRQA

Human: 121. SVPXKEDIRHILDSVYRLDVGSLDSGQKEDSNFLAIHLNNEHALRVAVSLRQA 180
       SVPXKEDIRHILDSVYRLDVGSLDSGQKEDSNFLAIHLNNEHALRVAVSLRQA

Mouse: 131. CQCVNKLQLFLSGLVRIMNLPGQYVFQVXRPQ168 240
       CQCVNKLQLFLSGLVRIMNLPGQYVFQVXRPQ168

Human: 131. CQCVNKLQLFLSGLVRIMNLPGQYVFQVXRPQ168 240
       CQCVNKLQLFLSGLVRIMNLPGQYVFQVXRPQ168

Mouse: 241. SLPSFAYSSLVQLYVAVTQTVTVTLPPXPTTVSASYTVAGVAVLVE 300
       SLPSFAYSSLVQLYVAVTQTVTVTLPPXPTTVSASYTVAGVAVLVE

Human: 241. SLPSFAYSSLVQLYVAVTQTVTVTLPPXPTTVSASYTVAGVAVLVE 300
       SLPSFAYSSLVQLYVAVTQTVTVTLPPXPTTVSASYTVAGVAVLVE

*Phosphorylation of Ser-230 by CaMKII promotes the transcriptional activity of HSF1

Mouse: 351. EPSEPPEPPEVEFAPFSEMTQARYFSLKSVKPSFGDRSFATQPPPPPFGDK------D 358
       EPSEPPEPPEVEFAPFSEMTQARYFSLKSVKPSFGDRSFATQPPPPPFGDK------D

Human: 351. EPSEPPEPPEVEFAPFSEMTQARYFSLKSVKPSFGDRSFATQPPPPPFGDK------D 358
       EPSEPPEPPEVEFAPFSEMTQARYFSLKSVKPSFGDRSFATQPPPPPFGDK------D

*Phosphorylation of Ser-308, Ser-327, and Ser-336 by GSK-3, ERK, and JNK repress the transcriptional activity of HSF1

Mouse: 356. QADAPXPSTPSECVKLSVLKESNLHSLDAMHSLDNLQMTLSEFSTVTRALDLD-----412
        + P+ P STPSECVKLSVLKESNLHSLDAMHSLDNLQMTLSEFSTVTRALDLD

Human: 360. RPPSEPPEPPEPSECVKLSVLKESNLHSLDAMHSLDNLQMTLSEFSTVTRALDLDDLS 419

Mouse: 413. ---------------IGELLLRGERPPFEAEWXCGKPSQCKYV eagerly 453
        IGELLLRGERPPFEAEWXCGKPSQCKYV eagerly

Human: 420. PSALPEPLLEDLSSLSICELLPEPFPPEAEWNSXWXHMTQWFLFEL 479

Mouse: 454. AVDSDAEELVLAISAGGDSAFDADHDPDTHLLLGPTVQA 509
        +IVDSIA+LYVLK+++ IFIEED - -ELTSHIFGTE RQAKEPTE

Human: 430. AVDSDAEELVLAISAGGDSAFDADHDPDTHLLLGPTVQA 529

Sequence homology between Human and Mouse HSF1. Mouse sequence (NCBI accession # P38532) is shown in bold gray type on top; human sequence is below in standard gray type (NCBI accession # Q00613); homology is identified in black type between the two sequences. Two HSF1 deletion mutants are flagged here with black, italicized, underlined font. The HSF1(+*) mutant (middle of the sequence, corresponding to the deletion of amino acids 203-315 in human HSF1) confers constitutive activity to the resultant mutant (see related Figs.: 1.2 and 4.1) and is highly conserved between the human and mouse HSF1 sequence. The region deleted in the human HSF1(-)/AVST dominant negative mutant (bottom of the figure) is located at the 3’ end of the sequence and proximal flanking areas are also conserved. Serine residues known to be phosphorylated are in black font, bolded and italicized, along with the sumo-modified residue, Lys 298.
APPENDIX 3 – CMV - Mouse HSF1 Constructs

CMV-HSF1 sequences: Each construct’s sequence was inserted into Invitrogen’s pcDNA 3.1 (+) vector (Invitrogen Cat# V79020; map available under Technical Resources – Vector Data on the Invitrogen website: www.invitrogen.com), utilizing the Hind III (AAGCTT) and EcoR I (GAATTC) restriction sites. Sequencing of the inserted sequence can be achieved with a T7 forward primer and Invitrogen’s pcDNA3.1/BGH reverse primer (5’-3’: TAGAAGGCACAGTGCAG). The constructs are ampicillin resistant.

Requests for DNA are met with the implied agreement that the receiver agrees to use the DNA only for research purposes, will acknowledge the source, and will not pass it on to others without written permission from Dr. Richard Voellmy.

Transcribed mouse HSF1 sequence: amino acids that are underlined, bolded, and italicized were deleted to form the constitutively active mHSF1(+) sequence.

MDLAVGPGAAGPSNVPAFLTKWTLVTSDLICWSPSNGPHVFQDQQQFAKEVLPKYPYFKHNMSFVR
QLNMYGRKVKVHIQGQLVKEPERDDTFHPCFLRQEQLLENIKRKTSTLKEKIRQDSRVTLLT
DVQLMKQECMDSSLKLMKHNEALWREVASLQRKHAQQKVVNKLIQFLISLVQSNRILGVKRKIIPLML
SDSNSAHSPYKGRQYSLHEHVHGPGOPYSPAYSSWSSLYSSDASVTSSSGPIISDITELAPTSPLASPGRSI
DERPLSSSTLVKQEPSPPSHsRPLVLEASPGRSPSSMDTPLSPTAFIDSILRESERPSTPAASNTAPMDTTGA
QAPALTPSTFEKCLSVACLKNEELSDHDAMDSNLDNLSTLMHGFSVDTSALLDLFSPSVTMDSLLP
DLDDLASIQELLSPQEPFRPITEAENSNPDSEGKLVHYTAQFLPFLDPDADVTGSSLTELPSVGESSYFSEGDDYTDIDPTISLLTGTEHPHAKDPTVS@

Note: The bolded, italicized region that is not underlined indicates 66 extra base pairs (22 aa) that were identified when the clone was sequenced. WT mHSF1 was initially isolated via PCR from a mouse 17-day embryo Quick-clone cDNA library, which was created from a pool of 200 normal Swiss-Webster/NIH embryos (Clontech cat #7140-1; lot #0080213). The isolated WT mHSF1 served as the template for the production of mHSF1(+). Thus, both constructs have the same additional sequence. This additional sequence may be a splicing variation. Both clones perform similar to their human HSF1 counterparts in their ability to activate the promoters of inducible heat shock proteins.

CMV-mHSF1 (WT) mouse version 7,300 bp:
Hind III / EcoR I digest releases 2 fragments: 1,913 bp insert; 5,387 bp vector
hHSF1(+) ORF: 1,578 bp total- 1,512 bp are identical to the database.

AAGCTTCAAGCCCTCTGCTTGTCGCACGGCCGCGGCGCGCGGGGG
ACGGCGAAGCCGCGGCGGCGACACTAGCTACGCTTCGCCATCTCTCATCAA
AGGCCAACCCCGCTCTGGTGGTCCGAGATCTGTGCGTGCG
GCCGCCTGGGTAGGGCGGCGCGCGACAAGCTACGCTTGCGCCCTGCCCTA
TGACCGCCCTGTGACGGCGAACCGGAGCAAGAGGCGCTACGGTGCGGCCC
GAGTGAGGAGCACAGTCCACGTTTGACCAGGCGGCGGAGG
CMV-mHSF1(+) constitutive active mouse HSF1 6,961 bp:
Hind III / EcoR I digest releases 2 fragments: 1,574 bp insert; 5,387 bp vector
mHSF1(+) ORF: 1,239 bp total - 1,173 bp are identical to the database.
AACCGGAGCCCAAGCCCCCAGCTCCCGACCCCCCTCCACCCCTGAGAAGTG
GCCTCAGGTAGCCTGCTTAGACAAGAAAGAGCTGCTGACAGCAGCGCT
CTTCAGTGAGAACCAGCTGCCCCTGCTGAGACGCTGGGAGAGGCCCT
CCATGCCCAGATTGAGCTCGCTGACTGACGGACAGCGGCTGAGCCTG
CAGGAGCTTCTGTCAAGAGCGCTTCGACAGTACGGATGAGCTGTG
ACGGATGATCCACCATCTCTCTTCTGACAGGCACTGAACCCATAAAG
CCAAGGACCCCACTGCTCATTGAGTGGAGCCTAGGCTGCTTGCTT
GTGCTGGCCCAACCTACTCAGATGGACATGGCTGGTCTGGCAGGAGAC
AAAGCATGTTGGATGCTCAGGACCCCTAGGTCAGGACCAACACTCCGAG
TGGAGCACAGATGGAACTTGGCTGCGGCACTTGATGTCAGGAGGAA
GATCCCTGAAGGCTGACAGCTGCTGCTGGCCTTTACCCAGCCCGAGGCTACT
CTCTGTCACAGCTCCACAGCCACACTTTGGAAGTTTGGCCCTGGAATTC
**CMV-HSF1 sequences:** Each construct’s sequence, which was inserted into Invitrogen’s pcDNA 3.1(+) (Invitrogen’s map of this vector and sequence information is provided at the end of this document), can be excised with Hind III (AAGCTT) and EcoR I (GAATTC). Sequencing of the inserted sequence can be achieved with a T7 forward primer and Invitrogen’s pcDNA3.1/BGH reverse primer (5’-3’: TAGAAGGCACAGTCGAGG). Constructs are ampicillin resistant. Note the single amino acid difference from NCBI’s sequence of the factor (accession #Q00613) at position 448 (P->L).

****Requests for DNA are met with the implied agreement that the receiver agrees to use the DNA only for research purposes, will acknowledge the source, and will not pass it on to others without written permission from Dr. Richard Voellmy.

**CMV-hHSF1 (WT): 7359 bp**

Hind III / EcoR I digest releases 2 fragments: approx. 2.1 kbp insert; 5,387 bp vector

hHSF1 (WT) ORF: approx. 1590 bp:
Human AA sequence - amino acids 203-315 are bolded and italicized:

```
MDLPVGPGAAGPSNVPAFLTKLWTLVSDPDTDALICWSPSGNSFHVFDQGQFAKEVLPKYFKHNNMASFVR
QLNMYGFRKVHVIEQGGVLKPERDDTFEQHPCLRQEGQLENIKRKVTSTVLKSEDIKIRQDSVTKLTT
DVQLMKGKQECMDSKLLAKMHEINALWREVASLRQKHAQQQKVVPNKLIQFLISLVQSNRILGVKRIPLML
NDSGSAHSMPKYSRQFSLEHVHGSYPASPSPPSAYSSSLSAYPDAVASSGPISIDETELAPASMASPGGSI
```

**CMV-hHSF1(+) (constitutively active construct = aa 203-315 deleted)** 7,020 bp:

**Hind III / EcoR I digest releases 2 fragments: approx. 1.75 kbp insert; 5,387 bp vector**

**hHSF1(+) ORF**: 1,251 bp
CMV-hHSF1 HSF1(−)/AVST (dominant negative construct)

The 454-523 amino acid deletion to hHSF1 (WT) is bolded and italicized. The single amino acid changes of 448 P→L and 453 E→D are also bolded and underlined:

MDLPVGPAGSPSVPAFLTLKWLTVSDFPTDALICWSPSNSFHVFDQGQFAKEVLPKYFKHHNMASFVRLQLNMYFGRKVHVHEQGGLVKPERDDTEPQHPFCRLRQEQQLLENIKRKVTSVSTLKSEDIKIRQDSTKLLTDVQLMKGKQECMDSKLAMKHKENALWREVASLRQKHAQQQKVKNLQLQFLSLVQSNRILVQRKIPLMLNDSGAHSMPKYSRQFSLEHSVGSYAPSAPYSSSLLAPDAVASSGPIISDITELAPASPMASPGEI

DERPLSSPLVRKVKEPPSPQSPVREEASPGPSSVDTLSPTALIDSLIRESEPASVTALTDARGHDTTEGRPPPSPTSTPEKCLSVACLDKNESSPLMLDSNLDLQTMSSLHGSFVDSALLDDRFLPSVTVD

MSLPDDLSSLSSLQELLSPQEPWPRPEAEANSDPSKQVLHYTAQPLPLLDPGDSVDGSNDDLVPVLFLGEGLD SYFSEGDFGFAEDPTESLLTGSEPPKADPTVS

hHSF1 HSF1(−)/AVST deletion (aa 454-523)

Hind III / EcoR I digest releases 2 fragments: approx. 1.9 kbp insert; 5,348 bp vector

HSF1(−)/AVST ORF: 1,383 bp
*Sequence of this construct between EcoR I (GAATTC) and Xba I (TCTAGA) is from the MCS of Invitrogen’s pcDNA 1.2 and does not correspond to the MCS of pcDNA 3.1 (+) (Cat# V79020; maps available under Technical Resources – Vector Data on the Invitrogen website: www.invitrogen.com)*
APPENDIX 5 – Hsp70b-fLuc

Comments for Hsp70b-Luc construct as assembled from compiled DNA sequence (5.1 kbp of sequence of the approximate 8.5 kbp total construct sequence):

- AmpR gene is present in the vector, but is not described
- The following sites and bp numbers are listed relative to the sequence provided below.

**In accepting this material, you agree that you will only use it for research purposes, will acknowledge the source, and will not pass it on to others without written permission from Dr. Richard Voellmy.**

Inserts Human Sequence and Restriction Site Information:

Single cutters in this part of the sequence: Aat I / Stu I (1218), Msl I (1460), Bcl I (1625), Sca I (1729), Psp 1406 I (1887), Ava III / Eco T22 I / Nsi I (2033), Bgl II (2157), Dra III (2378), Ase I / Asn I / Vsp I (2641), Bal I / Msc I (2813), Afl II (2820), Ssp I (2861), Srf I (3256), Hind III (3475).

- 1218-3480 = 2262 bp of chromosome 1 genomic contig (gi 51458934 / nt004487 bp 11982500-11984780)
- 3108-3480 (end of Hind III site) = 372 bp HSE and RNA leader (Bolded)

Stu I (AGGCCT): Known to cut at bp 1218 (a Stu I / Hind III digest release three bands of approximate size: 3.2, 2.8, 2.45; a 2275 bp piece should also be seen among many bands in a Stu I/Sal I digest)

Bgl II (AGATCT): Known to cut at bp 2157. See Hind III and Sal I for double-digestion product sizes.

Pst I (ctgcag): Four bands resolve in a single-digest, each of approximate size: 4.8 kbp, 2.5 kbp, 708 bp, 477 bp; Known cut sites are: 2306, 2783, and 3491.

BamH I (GGATCC): A single-digest releases three fragments of approximate size: 6 kbp, 2 kbp, and 497 bp. Known cut sites are at 3008 and 3505. Addition of Hind III to the BamH I digest makes the last band slightly smaller (by approximately 30 bp).

Sma I (CCCGGG): Many cut sites; the last one (bp 3256) below indicates the -108 start of transcription position (An “A” that is NOT bolded). Underlined areas correspond to Direct and Inverted Repeats. Italicized letters in the bolded region signify “heat shock consensus”.

Hind III (aagctt): Linearizes at 3475 leaving an 8.5 kbp product; appears to be a single cutter in the plasmid. A Bgl II (AGATCT)/ Hind III digest yields 3 bands of approximate size: 4.2 kbp, 2.9 kbp, and 1.4 kbp.

Sal I (GTGCAC) and Bgl II (AGATCT): Three fragments resolve in a Bgl II/Sal I digest each of approximate size: 4.1 kbp, 3 kbp, and 1.4 kbp (Bgl II: 2157 - Sal I: 3493)

Xba I (TCTAGA): Cuts twice in the luciferase gene at 3499 & 3633 releasing a 134 bp fragment and an approximate 8.5 kbp vector fragment in a single-enzyme digest

EcoR I (GATATC): Releases four fragments in a single enzyme digest that resolve at the following approximate sizes: 4.2kbp, 2kbp, 1.1kbp, 655 bp; two of the known cut sites are in the luciferase gene at bp: 3518 & 4173

Vector sequence is italicized:

- 293-1213 bp corresponds to vector sequence (includes the ori)
- 3475-5025 bp corresponds to luciferase reporter sequence (italicized and underlined)
List of Publications


VITA

Alexis K. Hall was born in Saigon, Vietnam, on June 16th, 1972 as the daughter of parents, Robert D. Hall and Lois B. Hall, who were living there to conduct diplomatic business. Related travel opportunities allowed her early education to be well-rounded, including attendance at Notre Dame (1984-1985) and Marymount (1985-1988) International Schools of Rome, Italy. In June 1990, she graduated from South Lakes High School in Reston, Virginia. That August, she commenced her undergraduate work at the Georgia Institute of Technology. Co-op experience in biomechanics during her second year of the Mechanical Engineering program led her to more specifically examine biological mechanisms for their potential to achieve therapeutic outcomes. Work and academic efforts yielded an A.S. degree in pre-Chiropractic in July 1994 from Scott Community College (Eastern Iowa District) and an A.A. in Biology from Broward Community College in August 1998. Her academic research experience in the development of novel therapeutics began with projects that were completed through the College of Liberal Arts: Honors Program, at Florida Atlantic University (FAU). She received her B.S. in Biological Sciences from FAU in August 2000 and immediately began her graduate work at the University of Miami (Leonard M. Miller) School of Medicine. Mentored by Dr. Richard Voellmy, she proposed her first thesis project in June 2002. She was then afforded an exceptional level of autonomy and experienced the uncommon opportunity to develop administrative responsibility while completing her graduate studies. She managed the daily operations of the Miami lab for the year and a half prior to the preclinical stage of her research, which was conducted from April 2006 to June 2007 in Gainesville at the University of Florida’s Center for Environmental and Human Toxicology. As a liaison for the collaborative project, which continues to innovatively build safety into gene therapy designs, she aided the launch of challenging, complex studies. Interest in the regulatory aspects of research administration led her to commence her career in Biosafety (Division of Environmental Health and Safety) at the University of Florida in December 2007. Her Ph.D. degree in Biochemistry and Molecular Biology was granted in May 2008 by the University of Miami.