C h a p t e r 2

NONINVASIVE BIOLOGICAL CONTROL VIA TEMPERATURE MODULATION

2.1: Engineered Control over Biological Systems

Engineered bacterial and mammalian genetic and cell-based therapies offer unprecedented opportunities for fine-grained control of function, but they also display the potential for severe and sometimes fatal side effects. The management and suppression of these toxicities is at the forefront of current biomedical research. Challenges and opportunities for improvement exist at all stages of therapeutic implementation: during gene editing or delivery, during *in vivo* administration, and after infusion of the drug product. Advances in control over these processes have progressed in recent years, but current implementations generally lack spatial specificity or noninvasive access. As such, there remains a significant need for the implementation of a biomodulation modality with these characteristics.

Control of gene editing has been investigated in the context of the linear space of DNA sequence, and also in terms of the three-dimensional spatial coordinates within the target organism. The recognition of viral genotoxicity has prompted investigation into site-specific delivery and integration methods. Avenues of interest include non-integrating retroviruses which enable high efficiency episomal gene delivery[1] and also integration targeting via fusion of the viral integrase with sequence-specific DNA-binding proteins[2–4]. Recently, RNA-programmable recombinases generated via fusion to CRISPR components have also

been explored[5]. Non-viral methods of gene delivery are also candidates for novel control strategies. Site-directed nucleases such as Cas9, Zinc Finger Nucleases (ZFNs), and TALENs significantly reduce the risk of genotoxicity by utilizing sequence-specific interactions with DNA to guide cleavage, although the latter two methods require protein engineering development for each new target, resulting in significant investment in labor and a delivery vector with a relatively large cargo capacity[6].

Controlling dosage or potency of delivery is highly desirable for biological therapies with potentially severe side effects. Some genetic payloads are amenable to inducible expression via promoters with intrinsic sensitivity to chemical stimuli such as exogenous drug administration (such as doxycycline derivatives[7]) or by communicating with neighboring cells to threshold gene expression based on population density[8]. Strategies have also been developed to titrate cell behavior at the post-translational level. The high risk of severe cytokine release syndrome in CAR-T therapy has prompted the development of several modified chimeric antigen receptor designs which can be inducibly activated to affect intracellular signaling and subsequent T-cell activation but which otherwise remain quiescent[9,10].

Temporal and spatial control of gene delivery and activation has also been widely investigated. As with dosage modulation, temporal control is often achieved via use of chemically inducible promoters to drive the transgene of interest, with doxycycline-inducible systems in particular finding widespread use in the literature[11,12]. Temporal control can also be implemented on the protein level using ligand-inducible activation or dimerization systems, as has been implemented for chemically-triggered Cas9 gene editing[13]. Spatial

control of biological therapy is also of interest to restrict activity to specific physical regions within a patient. Spatial localization can be conferred via environmental sensing if the target site of the therapy can be sufficiently differentiated from healthy tissue via molecular markers. This strategy has been explored extensively in CAR-T therapy, wherein homing strategies have been developed using split primary and secondary (co-stimulatory) T-cell surface receptor signaling to affect AND logic[14], and dominant inhibitory receptors to enact NOT logic[15]. Novel combinatorial sensing systems can integrate multiple signals in parallel. In a recently reported tri-partite configuration, a CAR is utilized to sense the primary tumor antigen while a chimeric co-receptor against the normally immunosuppressive cytokine TGF-β provides co-stimulation and a third chimeric receptor against IL-4 provides a tertiary signal for expansion and cytokine production[16]. Platform technologies such as synthetic notch receptors, which couple arbitrary extracellular ligand binding to any desired internal transcriptional program, are also under investigation for improved CAR-T homing to target tissues[17].

A new direction in controlling the spatial targeting of next generation therapies relies on engineering biological systems to sense external stimuli which can be precisely administered by a scientist or clinician. Image-guided interventions such as radiation therapy, laser and RF ablation, and focused ultrasound are established clinical techniques that utilize geometric information about the spatial coordinates of the disease site to direct the application of the treatment[18], but which have largely been ignored as cues for the controlled induction of biological activity.

One of the most successful spatially-directed modes of biological modulation is optogenetics, the control of biological materials with light. This technology has enabled spatiotemporal control via applications such as conjugation of photocleavable inhibitory compounds to viral capsids[19], engineering photoactivatable nuclear entry of the viral payload[20], and delivery of light-activatable biological cargo[21]. Optical control of non-viral genetic editing systems such as Cas9[22] and ZFNs[23] has also been developed. A key drawback of optical approaches is the poor accessibility of deep tissues; because light scattering through tissue occurs on the length scale of millimeters, at-depth access relies on invasive surgical intervention[24]. This places a severe constraint on the applications wherein this form of spatial targeting is relevant because a multitude of factors such as patient age and frailty, as well as the proximity of disease to highly sensitive tissues such as brain regions and blood vessels, can render surgery unsuitable or impossible[25–27]. To overcome this limitation, technologies with noninvasive penetrance through human tissue are being investigated.

A variety of noninvasive techniques are already in use for disease ablation, such as X-ray irradiation, magnetic hyperthermia, and focused ultrasound. These systems are under investigation for biomodulation, albeit not as extensively as their optogenetic counterparts. One such system utilized oxidative stress and DNA damage-responsive transcription factor binding sites to construct an artificial X-ray responsive promoter, which demonstrates up to 20-fold upregulation of gene expression upon exposure to X-ray photons[28]. However, DNA damage induced by ionizing radiation can result in deleterious or potentially oncogenic mutations, suggesting that other approaches may be safer moving forward. Magnetic fields are highly tissue-penetrant, enabling noninvasive imaging (MRI)[29], and are the subject of much recent work for modulation of biological function. Organ-specific genome editing has

been achieved via delivery of CRISPR components in a baculovirus coated with magnetic nanoparticles which enhance cellular uptake under a regionally-applied alternating magnetic field, enabling payload delivery that outpaces immune inactivation of the vector[30]. Magnetic nanoparticles have also been utilized to drive gene expression from a heat-inducible promoter via RF-induced hyperthermia[31], to control neural function directly via activation of MNP-affixed ion channels[32], and for drug delivery via mechanically-induced disruption of endothelial junctions by magnetic actuation[33]. Two drawbacks limit the utility of MNP-based control strategies. First, the difficulty in focusing magnetic fields limits the achievable spatial resolution and no sub-organ targeting has yet been demonstrated using this approach[34,35]. Additionally, as abiological components, synthetic magnetic nanoparticles cannot replicate as engineered cells or biomolecules multiply, thereby limiting the duration for which they can be stimulated. Biologically encoded magnetic nanoparticles and nanostructures, which could be expressed constitutively by engineered cells, are under development. The iron chelating protein Ferritin was genetically fused to the TRPV1 calcium channel and was able to stimulate an RF-mediated channel opening[36]; however, the weak paramagnetism of Ferritin has raised some controversy regarding the mechanism of this magnetic actuation[37]. Stronger biomagnetic structures exist, such as magnetosomes from naturally magnetotactic bacteria[38] but these have yet to be expressed in a heterologous, therapeutically useful host organism. Efforts are under way to engineer biomolecules capable of generating more highly magnetized biological structures and recent advances have enabled magnetic trapping of *E. coli* expressing these structures at defined spatial coordinates[39]; however, actuation of biological functions or behaviors using engineered highly magnetic biomolecules has yet to be achieved.

Focused ultrasound (FUS) represents an alternative tissue-penetrant signaling modality with high potential for intrinsic and engineered biomodulation. Focused ultrasound is a pressure wave emitted by a single concave piezoelectric element or by a set of transducer elements in a concave array[40]. The ultrasonic waves are produced at an intensity such that they propagate through tissue with minimal biological effect but constructively interfere at the transducer's focal zone, locally increasing the amplitude of the mechanical perturbation and the resulting energy deposition. As a result, the tissue at the focal point undergoes significant mechanical stress and, if ultrasound is supplied at a sufficient intensity and duration, heating[41]. The volume of the focal zone is wavelength-dependent and can be quite small, with an achievable cross-sectional full width half max (FWHM) of less than 10 microns in aqueous media[42] or less than 100 microns in low-impedance tissues such as the eye[43] using ultra-high frequency (~>40 MHz) transducers. Attenuation, and therefore depth penetration, is also frequency-dependent; while ultra-high frequency ultrasound is useful for ocular control or imaging[44], most FUS transducers operate in the 0.5 – 3 MHz range, producing a theoretical diffraction limited resolution of approximately 0.25 – 1.5 mm according to the Abbe diffraction limit. In practice, resolution can be diminished by scattering or nonlinear propagation of the sound wave, as well as transducer geometry. Typical FWHM values for *in vivo* focused ultrasound range from 1 - 5 mm laterally and 1 – 4 cm axially, and this frequency range can accommodate focal depths on the order of 10 cm[45–47] in tissue.

Focused ultrasound has previously been investigated for biological manipulation via stimulation of endogenous cellular components. One method of ultrasound biomodulation is via non-thermal perturbation. The precise mechanism of this mode of stimulation is under active investigation, but is generally thought to be mediated by mechanosensitive cellular

components such as ion channels[48]. One of the most widely studied applications of this technique is neuromodulation of the brain, which has been demonstrated in cell cultures and tissue slices[49] as well as in living animals[50,51]. Stimulation of other CNS components such as the retina has also been achieved with high precision[43], as has modulation of the peripheral nervous system[52]. Image-guided ultrasound pulse planning using penetrative imaging modalities such as MRI enables quantitative prediction of through-skull sound propagation and consequently the application of focused ultrasound to the brain in a transcranial, fully noninvasive manner[53,54]. This in turn has enabled noninvasive neuromodulation of the brain in human subjects[55].

Biomodulation via engineered responses to mechanostimulation is gaining traction as a viable method of interfacing with cells and tissues. Local chemical stimulation can be achieved in the brain by taking advantage of the specialized tissue at the interface between the vasculature and the brain, known as the Blood-Brain Barrier (BBB), which is resistant to the diffusive trafficking of biomolecules and most small-molecule drugs[56]. Mechanically-induced aberrations in the BBB can be imparted with spatial precision using FUS, resulting in transient gaps through which drugs can transport and induce their biological activity in a localized manner[57,58]. This approach has been extended to gate the brain access of therapeutic antibodies[59] and even of recombinant viruses which were evolved to resist trafficking through the BBB without physical disruption[60], thus enabling spatial control over transgene delivery in the brain. While localized BBB disruption enables selective brain access, other tissues are more permeable to foreign molecules and require other approaches to affect ultrasound-mediated control. Mechanical control can be exerted via ectopic expression of stretch-sensitive ion channels such as TRP-4, as has been demonstrated in *C. elegans*[61]. A

similar strategy relying on the native expression of the mechanosensitive cation channel Piezo1 in T-cells has been utilized to gate transcription from the calcium/NFAT signal transduction pathway in CAR-T lymphocytes[62]. However, both of these strategies rely on perturbing ionic flux, which is typically regulated by multiple channel and pump proteins[63], and these techniques are dependent on local pressure amplification by inorganic microbubbles which will dilute out as the cells divide. As such, while mechanical control is an intriguing mechanism for noninvasive biological stimulation, there remains a vacancy in the repertoire of biological components for a fully genetic method to control cell function in response to focused ultrasound.

2.2: Ultrasound Hyperthermia as a Noninvasive Biological Stimulus

One of the primary clinical applications for focused ultrasound is hyperthermic tissue ablation. High intensity pulses sustained for sufficient duration are able to destroy tissue at the focal point by locally raising the temperature past the thermal limit of tissue viability, enabling selective and noninvasive destruction of diseased regions[64]. Such ablative treatments are currently utilized for elimination of uterine fibroids and a variety of solid tumors[47,65]. Transcranial ultrasound ablation has also been utilized to relieve symptoms of essential tremor[66] and Parkinson's disease[67]. Over 80,000 patients had undergone image-guided HIFU therapy as of 2014, demonstrating the widespread clinical adoption of this treatment modality and hardware capability[68]. Although the primary clinical application of focused ultrasound is destructive in nature, not all stimulation regimes result in tissue ablation. Using real-time monitoring via technology such as MRI thermometry alongside rapid feedback control, mild hyperthermia can be maintained at sub-ablative temperatures[69].

Such moderate thermal elevation can therefore be utilized as a signal to control biological responses.

Focused ultrasound is not unique in its ability to noninvasively elevate tissue temperature[70]. Radiofrequency electromagnetic radiation can capacitatively heat tissue; however, the absorbance of RF radiation by tissue restricts >200 MHz radiowaves to an operating depth of less than 4 cm[71]. For deeper applications, energy can be deposited by inductive heating via oscillating magnetic fields which induce eddy currents in tissue (Magnetic Induction), or which rapidly reorient paramagnetic or ferromagnetic particles (Magnetic Particle Hyperthermia). Capacitative RF heating between two electrodes also has good depth penetration, but the heating is concentrated at the electrodes themselves, rendering spatial control difficult at intermediate locations. The spatial specificity of Magnetic Particle Hyperthermia is typically mediated by the location of the particles rather than the location of the field gradients due to the poor spatial resolution of field focusing[34]. Finally, phased RF array heating uses constructive interference between electromagnetic waves to affect localized temperature elevation; however, the focal volumes tend to be large relative to the size of the patient[72] and interference along bone-tissue interfaces can result in deviations from the expected heating pattern[73]. Altogether, focused ultrasound is currently unique in its ability to noninvasively direct controlled temperature elevation at millimeter length scales.

Biomodulation via hyperthermia has previously been achieved mainly via stimulation of endogenous temperature-sensitive promoters. While most bacterial heat shock promoters demonstrate relatively low fold-change in expression upon induction[74,75], mammalian heat

shock promoters (particularly those of the HSP70 family) show robust switch-like behavior. Genetically engineered biological reporter genes such as luciferase and fluorescent proteins have been gated by HSP70 promoters and triggered via laser-induced hyperthermia *in vitro*[76] and *in vivo*[77]. Magnetic hyperthermia has also been utilized to activate stress-inducible promoters for expression of imaging[78] or therapeutic transgenes, typically to induce tumoricidal payloads[79,80]. Despite the multitude of research and clinical studies invested in magnetic nanoparticle-mediated hyperthermia, the technique suffers from fundamental limitations in spatial resolution (with spatial specificity generally conferred by localized physical administration of the nanoparticles), constraints on input power due to generation of eddy currents in off-target tissues, and the inability of synthetic magnetic nanoparticles to replicate along with the cells which they are to control.

Critically, HSP70 has also been activated *in vivo* using FUS stimulation in genetically modified murine models[81,82] in wild-type animals wherein the HSP70 and reporter were delivered as viral transgenes[83], and in *ex vivo*-engineered cells after implantation into a model organism[84]. Hinting at the potential for thermal control of semi-autonomous cell therapies, a recent study generated chimeric animals wherein hematopoietic stem cells from an HSP-reporter mouse were administered intravenously into a reporter-null recipient mouse, homed to tumor sites (and bone marrow), and underwent selective activation via MRI-guided HIFU hyperthermia[85].

While the heat shock promoter system provides a convenient endogenous control strategy for transcriptional programming, it suffers from several important limitations. First, because

the primary stimulus for this signal transduction pathway is an upregulation of unfolded proteins, the heat shock pathway has crosstalk with other inputs, such as chemical and metabolic stresses[86–89], low pH[90], oxidative stress[91], signaling molecules such as some prostaglandins[92,93], drugs such as anti-inflammatory agents[94] and proteasome inhibitors[89], energy deprivation[95], and potentially mechanical stress[96]. An important corollary of this activation mechanism is that the thermal threshold for the activation of the heat shock response is highly dependent on the thermostability of the cell proteome rather than on an intrinsic molecule of the heat shock machinery. This prediction has been confirmed by the observation that the human HSF1 transcription factor, which coordinates the overall heat shock promoter activation, demonstrates an altered setpoint when expressed in *Drosophila* cells which more closely matches the induction threshold of the host organism's HSF1[97]. This result implies that tuning the activation threshold of heat shock promoters may be challenging. An additional factor which may interfere with HSP-dependent control of cell function in the clinical setting is the activation of heat shock promoters by hypoxia[98–102]. This phenomenon suggests that care should be taken in the use of these promoters in motile cell types which could potentially reach naturally hypoxic niches in the body such as the bone marrow[103] or cartilage[104], and in patients suffering from cancer-associated anemia[105] and consequent hypoxia[106]. A further layer of complexity is added by the differential performance of heat shock promoters across various cell types[107,108] and by the impaired HSP promoter response in some cell lineages, particularly in the brain[109–112]. In bacteria, the inducibility of heat shock promoters is typically low (on the order of ten-fold)[74] and they can suffer from significant basal leakage. Additionally, due to the mechanistic complexity of the heat shock pathway[113] (discussed in more detail below), the prospect of engineering this

system to tune its induction threshold and switching sharpness is a daunting task. Finally, because the modular switch in the heat shock pathway is a transcriptional promoter, it would be difficult to utilize this system to exert rapid control over cell function on the timescale of seconds to minutes, as would be possible by fusing a thermal switch directly to a protein of interest. More rapid thermoswitching mechanisms have been explored in the context of temperature-sensitive untranslated regions in RNA, such as naturally occurring ROSE[114] and FourU[115] elements as well as synthetic sequences[116], which form thermo-labile stem-loop structures to prevent translation or polymerase procession[117]. While more rapid than transcriptional control, this strategy is still slower than direct protein regulation, often demonstrates switching only over a broad temperature range, and typically demonstrates low fold-changes in resulting gene expression[118–120]. Additionally, this gating paradigm is largely restricted to prokaryotes. As such, a tunable biological system with intrinsic thermosensitivity and a simplicity that lends itself to control of cell function on the transcriptional and post-translational levels would be highly desirable for noninvasive modulation of biological activity.

2.3: Temperature in Biological Systems

In order to utilize temperature for biomodulation, the effects of this variable on the cell must be understood and accounted for. Temperature is a globally pervasive parameter in biological systems. The thermodynamics of all reactions are dictated by the relative scaling of their energies to kT, where k is the Boltzmann constant; thus, temperature affects equilibrium reaction quotients[121]. Additionally, the rate of a reaction is dictated by its activation energy[122],

which is also scaled by kT; therefore reaction rates are also temperature-dependent. Structural transitions in macromolecules also occur with thermal dependence. These transitions can be confined to the submicroscopic scale, as is the case for free cytosolic proteins, or they can influence the macroscopic structure of the cell, as is the case for the thermal denaturation of the cytoskeletal protein spectrin above 43 °C[123]. As such, thermal perturbations can induce widespread effects on the structure and function of the cell.

2.4: The Cellular Response to Temperature

As may be expected, cells have evolved numerous strategies to cope with stress associated with fluctuations in temperature. Upon sufficient hyperthermia (or other forms of damage such as hypoxia, osmotic stress, mechanical stress, ionizing radiation, organic denaturants, and heavy metals[124,125]), cells upregulate the transcription and synthesis of a set of 50-200 (depending on the organism) proteins termed "heat shock proteins" (HSPs)[125]. These proteins, which are typically named according to their molecular weight, attempt to protect the cell by preventing or reversing damage to the cell's components. These proteins can be grouped into seven functional classes. The most abundant class represents molecular chaperones, which act to disaggregate denatured proteins and protect monomers from re-aggregation. A second class consists of proteolytic enzymes which degrade irreversibly aggregated or misfolded proteins. The third class consists of nucleic acid damage repair proteins which detect non-native covalent modifications of DNA or RNA and attempt to repair them. A fourth, understudied class of proteins alters metabolic flux, presumably to compensate for changes in reaction rates due to altered enzyme stability or thermal

equilibrium. This class of proteins is overrepresented in the stress response of unicellular organisms, while multicellular organisms appear to rely more heavily on chaperones. The fifth class of HPSs consists of transcriptional regulators which effect further changes in gene expression to upregulate downstream stress response pathways or inhibit stress-sensitive pathways. The sixth group of HSPs assists in maintaining the structural integrity of the cytoskeleton, while the final class performs miscellaneous membrane-associated functions such as regulating membrane fluidity and transporting toxins out of the cell.

The most well studied, class of heat shock response proteins are chaperones. This diverse family of proteins acts to prevent aggregation of misfolded proteins, stabilize unfolded intermediates until they spontaneously sample their native conformation, assist in the targeting of proteolytic degradation, and aid in other functions such as protein translocation between organelles[126]. Notably, chaperones are more highly inducible in multicellular organisms, whereas unicellular organisms distribute the workload of damage repair more evenly among the many classes of stress-inducible genes. In accordance with their stoichiometric mode of activity, chaperones are the most highly upregulated set of proteins in the stress response of most organisms, and are also strongly constitutively expressed[125]. Chaperones recognize conserved features of denatured proteins such as hydrophobic patches and motifs, and also specific sequences. Chaperones can be further divided into subclasses. “Holdases” such as sHSPs are typically expressed at high levels only upon detection of denaturation or stress and are ATP-independent agents that simply bind to unfolded proteins and sequester them from aggregation. In contrast, “foldases” such as Hsp70 and its constitutive paralog Hsc70 bind unfolded or misfolded protein segments and then utilize

ATP hydrolysis to undergo a conformational change to modify their internal environment, presenting the client protein with an alternate energy landscape which may induce it to refold into its native state. This group of chaperones is typically expressed from both inducible and constitutive promoters as separate, homologous genes. Another group of heat shock proteins that assist in chaperone-mediate refolding is the HSP100 family, which pulls single polypeptide chains through a central pore in an ATP-dependent manner, thereby removing it from a misfolded state or pulling it out of an aggregate and subsequently enabling spontaneous or chaperone-assisted refolding. This family of heat shock proteins is generally restricted to bacteria and lower eukaryotes, implying that higher eukaryotes have an alternate mechanism for disaggregating proteins.

The trigger for HSP induction is complex, although the foundational events appear similar between bacteria and higher organisms. The master regulator transcription factor of the heat shock response, termed σ32 in bacteria and HSF1 in mammalian cells, is sequestered by constitutively expressed members of the chaperone family itself and prevented from affecting transcription[125]. Accumulation of unfolded proteins shifts the equilibrium of chaperone binding away from the heat shock regulator and toward their unfolded clients, thereby releasing the transcription factor and initiating the expression of stress response genes. In bacteria, induction of HSPs induces a feedback inhibition loop to control σ32 function via modulation of its translation, activity, and degradation[127]. Translation of σ32 is further regulated by an intrinsic RNA thermometer in the 5' UTR of its transcript[128]. In higher organisms, HSF1 regulation is an even more complex phenomenon wherein intrinsic structural thermosensitivity of the HSF1 regulatory domain, together with post-translational

modifications such as phosphorylation, acetylation, and SUMOylation, modulate HSF1 activity[113]. Additionally, the accessory protein eEF1A1, which loads charged tRNAs into the ribosomal A site, is released during stress-associated translational inhibition and redistributes to the nucleus, where it both stabilizes the interaction of HSF1 to its cognate binding sequence in heat shock promoters and also facilitates mRNA export from the nucleus via the 3'UTR of HSP promoter transcripts[129].

2.5: Temperature in Cellular Viability

Despite the wide array of cytoprotective responses to thermal perturbation, temperature still influences the growth and survival of cells. Investigation of the molecular mechanisms of temperature-associated changes in viability has been undertaken, but no single "weak link" has been established as the critical mediator of survival. While both biochemical reaction imbalances and structural transitions of cellular components could potentially influence viability, the activation energies of most metabolic reactions are on the order of 10 kcal/mol whereas structural transitions are typically on the order of 100 kcal/mol[123]. Because the thermal energy required to impair cell viability is also on the order of 100 kcal/mol, it is assumed that these structural transitions, rather than metabolic unbalancing, are the principal cause of hyperthermia-mediated cell death[123]. These transitions are typically attributed to protein unfolding, although the identity, number, and quantity of damaged proteins that result in cell death have not been elucidated. Protein denaturation results in inactivation of function due to loss of structure and typically also in aggregation due to exposure of previously buried hydrophobic residues. Other large-scale transitions, such as changes in membrane fluidity,

have been determined unlikely to contribute significantly to the impairment of viability upon hyperthermia[130]. While large-scale transitions in DNA occur only at temperatures around 90 °C, it is possible that structural reorganization in microdomains influences viability, although this has not been investigated in detail. Covalent breaks in DNA are not thought to occur in response to mild hyperthermia (up to 47 °C), although DNA damage can occur secondary to thermal stress due to inactivation of the protein machinery responsible for DNA damage repair[131]. Likewise, the unfolding of RNA species such as tRNAs and UTR regions could also influence cell health. Cytoskeletal reorganization also occurs upon even mild hyperthermia (for durations on the order of 30 minutes), but different cytoskeletal components (e.g. microfilaments, microtubules, or intermediate filaments) are disrupted in different cell types and it is unclear if a link exists between structural reorganization and viability[132]. This reorganization can be reversible and the thermal dosage threshold for reversibility differs between cell types, and likely is dependent on the stage of the cell cycle at which the cells are exposed to hyperthermia[132]. Disruption of the cortical cytoskeletal system, such as the interface between actin and integrins, is likely to affect signal transduction but the effect of this disruption on viability is unclear.

Differential scanning calorimetry analysis of mammalian cells show that erythrocytes, which lack a nucleus, show a single structural transition at a T_m of 70 °C while V79 cells, which contain a nucleus, demonstrate a more complicated transition profile with an onset near 40 °C[130]. Thus it is tempting to conclude that the lethal structural transitions occur in the nuclear compartment, a hypothesis bolstered by evidence that heat-treated cells have altered nuclear density and demonstrate intra-nuclear protein aggregation. However, both the nuclear and

cytosolic compartments demonstrate endothermic peaks at temperatures in excess of 50 °C, but also onset of excess C_p near 40 °C, indicating that both compartments contain a small fraction of thermo-labile components which may be the determining factors for temperature tolerance[130]. It should be noted that the critical transition or transitions responsible for cell death may be within or between supramolecular structures rather than within single macromolecules.

Structural transitions such as protein unfolding occur even during physiological temperatures, and the ability of cells to survive depends on their capacity to compensate for or repair these insults[123]. Indeed, it has been suggested that a small fraction (on the order of 0.2%) of cells in culture at 37 °C is lost by virtue of "thermal noise" that cells cannot recover from[133]. In accordance with this postulate, cells constitutively express a baseline level of chaperone machinery that is responsible for repairing stress-mediated denaturation and damage[125]. The ability of cells to compensate for such damage has also been characterized *in vitro*; CHL V79 cells are able to tolerate 5% denaturation with minimal effect on viability whereas 10% protein denaturation results in nearly full lethality[123]. The ability of cells to compensate for some amount of thermal denaturation appears in multiple kingdoms of life, as differential scanning calorimetry analysis of several bacterial strains demonstrates that the optimal growth temperature is generally several degrees higher than the onset of thermal structural transitions.

The timing of heating with respect to cell cycle also influences thermotolerance. Structural aberrations have been observed in the macroscopic structure of chromatids during

hyperthermia. Correspondingly, cells are most heat-sensitive during mitosis, less so during the S-phase of the cell cycle, and minimally thermo-sensitive during G1[123]. Induction of hyperthermia during S-phase typically leads to a DNA replication blockade, resulting in entry into mitosis prior to full replication of the chromosomes and subsequent death via mitotic catastrophe[131]. Interestingly, cellular synthesis of DNA while the DNA repair machinery is inactivated appears to also strongly contribute to cell death – when DNA synthesis is chemically inhibited during and after heat shock, cells are rescued from what would be lethal DNA lesions[131]. Correspondingly, thermal damage to cell cycle checkpoint proteins can induce cell death by permitting the cell to proceed to mitosis without first completing DNA replication[131].

Most models of cellular thermal viability are phenomenological in nature. It has generally been observed that cells *in vitro* display a biphasic survival profile in response to hyperthermia (**Fig. 2-1**)[130]. The curve is typically characterized by a pre-exponential phase for the first few minutes of hyperthermia, after which point survival decays in an exponential manner. It should be noted that the decrease in survival can be partially ameliorated by pre-exposing cells to mild heat stress, as is depicted in curve C. While this increase in robustness is typically attributed to pre-expression of heat shock proteins, which aid the cell in repairing the effects of

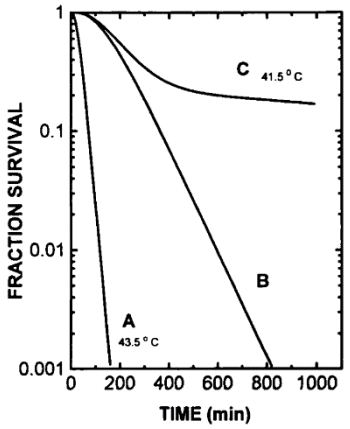

Figure 2-1: *In vitro* cell viability as a function of time at varying temperatures. Note the initial slow rate of death followed by an acceleration and, at permissive temperature, a stabilization of viability due to chaperone production. Reproduced from (Lepock 2003[123]) courtesy of Taylor & Francis Publishing.

thermal denaturation, it is unclear why some cells are able to remain viable while others succumb to heat stress.

The threshold at which cells accumulate significantly more damage than they can repair (i.e. the temperature at which viability decays sharply) is dependent on many factors. While the aforementioned V79 CHO cells demonstrated decreased viability upon even brief exposure to temperatures of 43.5 °C, other cell types such as PC3 prostate cancer cells demonstrate little change in metabolism or survival at the same temperatures and tolerate short, high temperature hyperthermia (48 °C for 10 minutes) or milder, long duration hyperthermia (44 °C for 60 minutes) with little measurable change in viability[124]. Additionally, the use of 3D cultures, which more accurately mimic the tissue environment, appears to increase post-heat shock viability relative to 2D culture controls via unidentified mechanisms, calling into question the applicability of a wealth of early data collected on monolayer cultures[124]. It should be noted that Arrhenius analysis indicates that human cells are slightly more thermotolerant than rodent cells, as indicated by their higher "thermal break point" in the slope of the Arrhenius plot and the general shallower slope shape indicating slower killing[134,135] (**Fig. 2-2**). In clinical practice, temperature has been found empirically to influence tumor viability in an integrated dose-dependent fashion[70]. A variety of mathematical

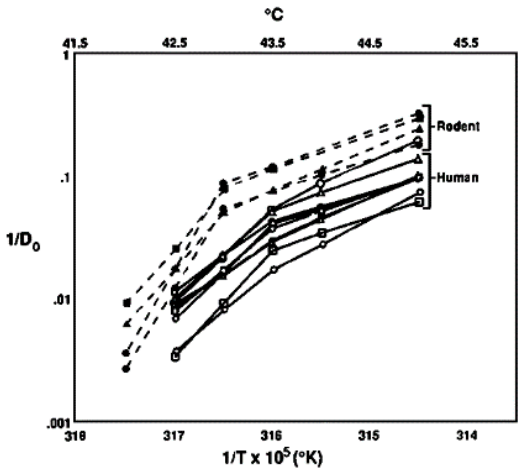

Figure 2-2: An example of the exponential dependence of *in vitro* cell viability on hyperthermic dose. The killing rate for human cells is generally lower than that of rodent cultures. Reproduced with permission from Roizin-Towle and Pirro (1991)[135].

models have been established to predict cell viability as a function of thermal dose. To date, all of these models are empirical in nature, with all constants fit to data rather than derived from fundamental thermodynamic or biological considerations[131]. The dependence of cell survival on temperature above the physiological baseline follows an exponential relationship, as demonstrated in **Fig. 2-2**. To standardize equivalent thermal exposures, the convention in the field is to use the "Cumulative Equivalent Minutes at 43 °C" (CEM 43 °C), which is defined by **Equation 1-1**

$$\boldsymbol{Equation\ 1-1}: CEM\ 43\ ^{\circ}C = tR^{(43-T)}$$

where t is the duration of treatment, T is the average temperature for the given treatment interval, and R is an empirically determined constant set to 0.5 or 0.25, depending on if T is greater than or less than the thermal "break point" of human cells (43 °C on average)[136]. A large body of literature has been assembled over the past four decades regarding tissue viability after varying CEM43 doses[134]. These data encompass many different temperatures and durations and indicate that, within the same tissue type and species, the CEM43 conversion is robust regardless of the input temperature. This metric is therefore the "gold standard" for prediction of biological effects upon hyperthermic exposure.

2.6: Thermal Regimes for Safe Biomodulation

The establishment of the CEM43 metric of thermal dosage enables the categorization of temperature/duration landscapes according to their predicted biological effects. While the

non-specific effects of hyperthermia such as protein denaturation and metabolic reaction rate imbalances are unavoidable in thermal therapy, the most important parameter with respect to temperature-switchable therapeutic agents is the thermal dosage limit below which tissue damage is negligible. In the context of cancer therapeutics, damage to malignant tissue surrounding the thermoswitchable agents can be dismissed or even be deemed beneficial. However, in a general setting the bystander tissue must be spared of the toxic effects of hyperthermia as much as possible. **Fig. 2-3** depicts a representative thermal dosage landscape which could be used to inform stimulation intensities and durations. The area below a threshold curve denotes a region of parameters which would be predicted to be safe for stimulation. Above this curve, non-specific cell death is expected to occur and the corresponding parameters would therefore be unsuitable for stimulation of therapeutic agents. The mode of cell death upon exposure to hyperthermia varies between cell types, thermal doses, and possibly the timing of exposure relative to the cell cycle[131]. Within the so-called hyperthermic range of 40 – 47 °C, most cell death occurs via mitotic catastrophe or apoptosis (or a combination thereof). In the ablation range of 48 – 60 °C, protein denaturation and aggregation is much more widespread and the cell typically coagulates. The objective of FUS hyperthermia-activated therapy would be to operate within the bounds of the safe thermal envelope to ensure that the effect on tissue health is specifically modulated by the function of the therapeutic agent.

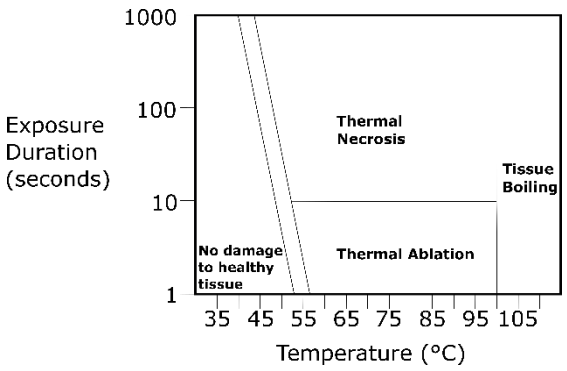

Figure 2-3: Schematic of the thermal landscape and the regimes in which different hyperthermic temperature / duration exposures can fall. Image adapted from The Focused Ultrasound Foundation.

Determining a safe thermal regime requires evaluating viability and damage in native living tissue, as cells in culture can display altered temperature sensitivity[124] while *ex-vivo* tissue can suffer from confounding effects such as poor perfusion and oxygen delivery[137]. Additionally, anatomical context is crucial for understanding and predicting thermal damage. Much as *in vitro* cell viability under hyperthermic conditions depends on the identity of the cell line, thermal damage thresholds also differ between tissues within the same species and between different species[137]. Due to the experimental and ethical difficulty of conducting experiments on thermal viability in human patients and organs, animal models have been utilized as proxies for most studies. A comprehensive review of previous literature across a variety of animal models by the MRI + EUREKA research consortium's Thermal Workshop on RF Hotspots led to the recommendation of a CEM43 limit of 9 minutes for RF-induced heating during MRI imaging[138]. It should be noted that the panel also recommended a ceiling of 39 °C exposure regardless of duration. While this thermal dosage limit was set conservatively for imaging applications and therapeutic interventions may warrant more relaxed criteria, the data analyzed by the consortium suggests that a CEM43 on the order of 10 minutes is a reasonable preliminary approximation of the thermal envelope within which focused ultrasound may be utilized for safe biomodulation. As the field evolves and more applications of FUS-stimulated therapeutics are demonstrated in pre-clincal models (particularly in the context of non-tumor tissue, such as in the intestine for management of gastrointestinal disorders), more rigorous thresholds will be established, likely in a tissue-specific manner.

2.7: Temperature in Cancer Medicine and Immunology

One of the primary potential applications of next-generation biological therapeutic agents is the treatment of cancer. Hyperthermia-mediated modulation of engineered therapeutic agents has the potential to synergize with a variety of beneficial effects of temperature elevation for tumor destruction[139]. While ablation of tumor mass by temperatures in excess of 60 °C directly reduces tumor burden, mild (sub-ablative) hyperthermia and fever-range (39 °C – 41 °C)[140] temperatures may also benefit cancer treatment by modifying the tumor microenvironment and potentiating the immune response.

One of the primary intrinsic benefits of hyperthermia is its ability to mobilize and activate the immune system. Temperature elevation induces immunostimulatory changes in the tumor and associated tissue and also directly in various immune cell compartments. Hyperthermia can alter the surface protein expression profile of tumor vasculature to facilitate extravasation of cytotoxic T-lymphocytes from the bloodstream into the tumor[141]. As a result of either this altered expression profile or of increased blood perfusion, 42 °C microwave hyperthermia enables enhanced tumor access by T-cells, Natural Killer (NK) cells, and dendritic cells while decreasing the population and activity of immunosuppressive T-reg and myeloid-derived suppressor cells[142]. Thermal shifts can also alter the surface proteome on tumor cells to dysregulate the camouflaging balance of surface receptors upon which cancer cells rely for immune evasion. In some tumors, 43 °C hyperthermia enhances MHC-I expression on the tumor surface, increasing the probabiliry of recognition by patrolling CTLs[143]. In contrast, hyperthermia can inhibit MHC presentation in other tumor

types, thereby promoting their recognition and destruction by Natural Killer (NK) cells[144]. Hyperthermia sensitizes some cancer lines to NK cell-mediated lysis via HSF1-mediated overexpression of MICA, a target of the NKG2D activating receptor in NK cells[145,146]. Hyperthermia can also induce some tumors to produce chemokines such as CCL2, CCL5, and CXCL10, which actively recruit pro-inflammatory immune cells to the site of disease[142].

The adaptive immune system also demonstrates temperature-responsive stimulation. In CTLs, hyperthermia increases the rate of contact with antigen presenting cells, possibly via increasing the membrane fluidity of the T-cells[147]. Fever-range hyperthermia induces PKC relocalization[148] and potentiates activation of cytotoxic function in a TCR pathway-dependent manner, as indicated by increased phosphoroylation of the LAT and PKCθ signaling mediators[149]. Mild hyperthermia also increases expression of the death ligand FasL on T-cells, potentially via augmentation of NF-κB and NFAT nuclear translocation or via direct HSF1-mediated transcription[150]. Exposure to 42 °C further augmented pro-inflammatory cytokine production by tumor-infiltrating T-cells[142].

In addition to cells of the lymphoid lineage, myeloid immune cells are also biased toward activation at elevated temperatures. Mild hyperthermia recruits monocytes to the tumor, which can then differentiate into macrophages and present tumor-associated antigens to the adaptive immune system[151]. The induction of monocyte trafficking may be driven largely by the overproduction of CXCL2 and other chemokines by heat-treated tumors[152]. In macrophages, fever-range hyperthermia in combination with recognition of soluble damage motifs promotes increased phagocytosis and synthesis of cytotoxic effector molecules[153–155].

These damage signals can also augment IL-12 secretion by monocyte-derived dendritic cells and bone marrow dendritic cells, resulting in increased T-cell proliferation[156,157]. Hyperthermia also promotes dendritic cell maturation[158] and secretion of the inflammatory cytokine TNF-α[142].The general immunostimulatory effect of mild hyperthermia has been corroborated by the observation of abscopal, $CD8^+$ T-cell dependent tumor retardation in a mouse model where a contralateral tumor was treated with magnetic hyperthermia[152]. When devising therapeutic stimulation protocols for engineered thermo-responsive cancer therapies, it will be worthwhile to optimize the heating parameters for both the triggering of the temperature switch and also the effector functions of the relevant immune cells, which can diminish upon prolonged exposure to super-febrile thermal regimes[154,155].

Heat shock proteins, whose conventionally studied role is as molecular chaperones to promote homeostasis, also function as signaling molecules to promote immune activity. This behavior is prominent in tumors undergoing therapeutic hyperthermia or fever. Hsp70, Hsp90, Hsc70, and gp96 are released by damaged tumor cells and bind to TLR4 on the cancer cell surface in a paracrine manner, thereby inducing chemokine production and promoting dendritic cell infiltration[142]. Hsp70, Hsp90, and Calreticulin are overexpressed and trafficked to the membrane of chemotherapeutically stressed cancer cells where they promote phagocytosis and maturation of dendritic cells[159]. These proteins also ligate the immune surface receptor CD91[160] on dendritic cells, wherein they induce cross-presentation of chaperoned antigens and upregulation of co-stimulatory surface receptors[161]. In dendritic cells, internal Hsp90 activity appears necessary for maturation, thereby intrinsically linking

this process to fever and hyperthermia[158]. Ligation of TLR4 by damage-associated Hsp70 from tumor cells also promotes maturation[160].

There is clear consensus in the literature that temperature elevation promotes the immune response against tumors. However, the mechanisms of immune action can differ depending on context, such as the identity of the tumor cells and their specific response to hyperthermia (e.g. MICA upregulation, MHC up or down-regulation, chemokine release, etc.) and also of the immune cells which are able to infiltrate into the tumor structure during the application of thermal stimulus. Additionally, most reported works suggest that temperature-stimulated immune activation is optimal at or below 41 °C, above which deleterious effects and damage begin to outpace stimulatory pathways[140]. It is important to note, however, that inducing a focal thermal elevation above fever conditions at the tumor core to stimulate engineering thermo-responsive therapeutics will result in temperature dissipation along a gradient to the periphery. Tumor-infiltrating immune cells further from the focal stimulation zone are expected to experience more optimal temperatures in the fever range and may therefore act as a secondary mechanism to promote tumor rejection.

In addition to potentiating the immune response against tumors, local hyperthermia has complementary effects with other therapeutic modalities[139]. The most significant benefits are likely to occur due to temperature-associated increase in blood perfusion[70]. This enhanced blood transport reoxygenates hypoxic tumors, which are otherwise resistant to radiotherapy[162]. It also increases the local availability of passively transported therapeutic agents such nanoparticles. Cells in S-phase of the replication cycle, while relatively resistant

to radiation therapy, are sensitized to hyperthermia[123]. Actively mitotic cells are also sensitive to hyperthermia, with experiments in CHO cells demonstrating complete destruction of the centriole upon a thermal dose of 45 °C for 15 minutes[132]. A variety of clinical trials have demonstrated that combination therapy incorporating local hyperthermia improves treatment outcomes across multiple cancer types[71] and this modality is regarded as a leading adjuvant for chemotherapy and radiation therapy[163].

2.8: Ultrasound Hyperthermia: Potential for Biological Control

Noninvasive technologies have the potential to address many of the shortcomings of next-generation therapeutic agents, such as engineered cells and viruses. Focused Ultrasound Hyperthermia is a signaling modality capable of nearly full-body access and confined spatial resolution on the order of millimeters, rendering it an ideal choice for communicating with endogenous biological agents with spatiotemporal precision. FUS hyperthermia can be measured in real time to ensure that a sub-ablative thermal regime is maintained, and mild hyperthermic exposures have now been sufficiently characterized to ensure minimal deleterious effects to the stimulated tissue. The hardware for HIFU stimulation is clinically available and appears to be gaining market penetrance. As such, the infrastructure for specific ultrasound biomodulation is available and awaiting novel engineered biological agents to take advantage of its potential.

2.9: References

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