REVIEW

Cellular Stress Response Pathway System as a Sentinel Ensemble in Toxicological Screening

Steven O. Simmons,* Chun-Yang Fan,† and Ram Ramabhadran* ‡

*Integrated Systems Toxicology Division, National Health and Environmental Effects Research Laboratory, U.S. EPA, Research Triangle Park, North Carolina 27711; and †Curriculum in Toxicology, University of North Carolina-Chapel Hill, Chapel Hill, North Carolina 27599

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High costs, long test times, and societal concerns related to animal use have required the development of in vitro assays for the rapid and cost-effective toxicological evaluation and characterization of compounds in both the pharmaceutical and environmental arenas. Although the pharmaceutical industry has developed very effective, high-throughput in vitro assays for determining the therapeutic potential of compounds, the application of this approach to toxicological screening has been limited. A primary reason for this is that while drug candidate screens are directed to a specific target/mechanism, xenobiotics can cause toxicity through any of a myriad of undefined interactions with cellular components and processes. Given that it is not practical to design assays that can interrogate each potential toxicological target, an integrative approach is required if there is to be a rapid and low-cost toxicological evaluation of chemicals. Cellular stress response pathways offer a viable solution to the creation of a set of integrative assays as there is a limited and hence manageable set (a small ensemble of 10 or less) of major cellular stress response pathways through which cells mount a homoeostatic response to toxicants and which also participate in cell fate/death decisions. Further, over the past decades, these pathways have been well characterized at a molecular level thereby enabling the development of high-throughput cell-based assays using the components of the pathways. Utilization of the set of cellular stress response pathway-based assays as indicators of toxic interactions of chemicals with basic cellular machinery will potentially permit the clustering of chemicals based on biological response profiles of common mode of action (MOA) and also the inference of the specific MOA of a toxicant. This article reviews the biochemical characteristics of the stress response pathways, their common architecture that enables rapid activation during stress, their participation in cell fate decisions, the essential nature of these pathways to the organism, and the biochemical basis of their cross-talk that permits an assay ensemble screening approach. Subsequent sections describe how the stress pathway ensemble assay approach could be applied to screening potentially toxic compounds and discuss how this approach may be used to derive toxicant MOA from the biological activity profiles that the ensemble strategy provides. The article concludes with a review of the application of the stress assay concept to noninvasive in vivo assessments of chemical toxicants.

Key Words: alternatives to animal testing; cell culture; mechanisms; transgenic models; signal transduction; stress response reporter gene assays; cellular stress.

Economic and societal considerations are requiring the development of rapid high-throughput methods for toxicity assessment—a vital need and priority in the pharmaceutical and environmental toxicology arenas. The vast array of compounds that require evaluation, and the time- and cost- and labor-intensive nature of traditional whole animal testing, combined with the societal pressure to reduce animal use, have heightened the need to search for alternative methods for toxicity testing. The methods being investigated range on one end from computational approaches through the use of in vitro cell culture models to the use of alternative species on the other. These efforts, although not intended to eliminate the use of rodent testing in the near term, have the potential of going a long way towards the 3 R’s of “reducing, refining, and replacing” the use of vertebrate animals for toxicity testing (http://www.alttox.org/trc/tox-test-overview/). A recent report by the National Research Council envisions toxicological tests for characterization of toxicants in the next twenty year time frame moving to in vitro screening using human cells, thereby greatly reducing or eliminating the use of laboratory animals (NRC, 2007). Although the attainability of totally animal-free testing of toxicants at any time frame is debatable, the efforts to reduce animal usage through the use of in vitro test methods bring toxicological screening closer to this goal. However, in vitro tests are only capable of measuring cell-autonomous or cell type-specific toxicities and are unlikely to detect many organism-level toxicities and are unlikely to detect many organism-level toxicities.

1 Present address: Syngenta Biotechnology, Inc., Research Triangle Park, NC 27709.

2 To whom correspondence should be addressed at Integrated Systems Toxicology Division, National Health and Environmental Effects Research Laboratory, US EPA, Research Triangle Park, NC 27711. Fax: 919-541-3335. E-mail: ramabhadran.ram@epa.gov.

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toxicities such as those caused by interference with cell-cell communication or those caused by metabolites of the parent test chemicals. Despite this caveat, cell-based toxicity tests, because of their ability to handle large numbers of chemicals, can provide an ideal tool for classification and prioritization for further screening and, in some cases, for obtaining mode of action (MOA) information based on key biochemical events revealed at a cellular level. The development of cell-based toxicological assays is further enabled by advances in techniques of genetic manipulation of cells in culture (Batt et al., 1995).

Efforts in the drug discovery arena have led to the development of a number of exquisite tools and in vitro methodologies for high-throughput screening that can be directly translated to toxicological applications (Houck and Kavlock, 2008). These assays can be used to measure the activation/inactivation of cellular signaling pathways using surrogate reporters that provide rapid readouts economically. These reporter technologies can also be extended to in vivo evaluations, in alternate species, and especially in vertebrate model organisms such as zebrafish whose genome has been sequenced and in which the methods of transgenesis are well-developed (Hill et al., 2005). The use of these alternative species models reduces the dependence on conventional rodent testing, thus reducing costs and increasing throughput.

Even with the availability of modern tools to measure modulation of cellular pathways, it is difficult to choose assay targets as indicators for measuring toxic events because of the large numbers of cellular pathways that have been documented as well as their involvement in both normal cell physiology and in toxic responses. The recent report by the National Research Council (NRC) of the U.S. National Academy of Sciences (NRC, 2007, page 38) envisions the delineation of “toxicity pathways” defined as “cellular response pathways that, when sufficiently perturbed in an intact animal, are expected to result in adverse health effects” as a part of the vision of the future of toxicology. However, the nature and number of such pathways are loosely defined, making the goal of pathway definition a long range effort. Therefore the NRC report fails to identify most of the potential target pathways for chemical screening in the immediate future. The focus of this review is to propose and elaborate on an approach for screening and classifying compounds using a cellular stress response pathway ensemble, which consists of a limited and manageable number of pathways that are activated in a cell-autonomous manner by toxic physical and chemical insults and that also participate in cell fate decisions such as apoptosis when the cell is overwhelmed by stress. Because the stress response pathways have been relatively well-defined at the biochemical level through extensive research in the past several decades, they could also permit preliminary inference of MOA of compounds based on the key events that are known to activate these pathways. These inferences could then be used to generate hypotheses for further testing using specific cell biological, functional genomic and pharmacological, and whole animal experiments.

Because of their ubiquitous presence in all metazoan cells, the cellular stress response pathways might be considered the guardians of the primary cellular infrastructure. This infrastructure, which could be thought of as a “basic cell,” is comprised of minimal components including the basic macromolecules such as nucleic acids, proteins, complex carbohydrate polymers and lipids as well as the superstructures made from macromolecules such as the nucleus, mitochondria, endoplasmic reticulum (ER), and lysosomes. Damage to this cellular infrastructure initiates activation of one or more stress response pathways in an effort to repair the damage to restore homeostasis, and the activation occurs at doses or exposure times lower than those required to elicit apical toxic events such as apoptosis or necrosis. However, as will be discussed later, the stress response pathways, in addition to their role in homeostasis also feed into the network whereby a cell decides (cell fate decision) to initiate apoptosis should efforts aimed at restoring homeostasis fail. Because of this involvement in cell fate decision, the stress response pathways are likely to be intimately linked to adverse effects elicited by toxicants. Extending this concept further beyond the “basic cell,” every differentiated cell can be thought of as consisting of the infrastructure that makes up the “basic cell” that is overlaid with the cell type specific proteome and cellular structures needed for the specialized functions of that specific cell type. From this perspective, the stress response pathways would also respond to cytotoxic stimuli arising from the interaction of environmental chemicals with the components unique to each type of differentiated cell (such as neurons, myocytes or T-lymphocytes) that leads apical to toxic events such as apoptosis. This reasoning would suggest that stress assay screens, as described further below, can be used in a wide range of cell types to compare and contrast the responses of various cell types to toxicants, hence permitting inference of organ specific responses. Clearly, an organism is a complex assemblage of many of the specialized cells in to organ systems, endowed with functions such as uptake of toxicant and metabolism that is not present in the in vitro systems. Hence, the in vitro screening concepts discussed in this review measures only “cell-autonomous” toxicity events. However, the extended use of stress response assays to measure key events in whole animals is discussed in the last section of this review.

In the sections below, the major cellular stress response pathways are reviewed, highlighting their common architecture that enables them to respond rapidly to environmental insults, their role in cell fate decisions, their importance to biological systems as shown by consequences of loss or gain of functions in vivo, and their applicability to screen and evaluate toxicants both in vitro and in vivo. The extensive citation of illustrative examples in the discussion that follows is also intended to convey the extent of detailed mechanistic understanding of the stress response pathways that has accrued through decades of study. Nonetheless, the major goal of this review is to discuss the germane aspects of these pathways comprehensively and yet maintain brevity. Given the vast amount of literature devoted to each of these pathways,
wherever possible other reviews that incorporate and synthesize the primary sources are cited rather than the sources themselves. Although a large number of articles and reviews have been written on the individual stress response pathway, none that provides a global overview of the entire stress system and the common architecture exists. It is hoped that this review integrating the stress system biology would encourage systems biology approaches to the study and applications of this system in toxicological evaluations. It is also of note that four of the eight pathways discussed in this review have been suggested as toxicity pathways and as possible candidates for use in high-throughput assays by the NRC Report on its vision and strategy for toxicity testing in the 21st century (NRC, 2007). By extension, based on mechanistic similarities discussed below, the other four not mentioned in the NAS report can also be considered toxicity pathways and they are equally amenable to high-throughput assays.

TABLE 1
The Major Adaptive Stress response pathways

<table>
<thead>
<tr>
<th>Stress response pathway</th>
<th>Chemical inducers</th>
<th>TF</th>
<th>Activated gene promoters</th>
<th>RE (5'→3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxidative stress</td>
<td>Quinones, hydroperoxides, heavy metals, trivalent arsenicals</td>
<td>Nrf2</td>
<td>HMOX1, NQO1, GST2A</td>
<td>TGCTGAGTCAa</td>
</tr>
<tr>
<td>Heat shock response</td>
<td>Heat, Heavy Metals</td>
<td>HSF-1</td>
<td>HSPA6</td>
<td>GAANNTTTe</td>
</tr>
<tr>
<td>DNA damage response</td>
<td>Etoposide, Methyl Methanesulfonate, N-Dimethylhydroxylamine, Cyclophosphamide, UV radiation</td>
<td>p53</td>
<td>CDKN1A, GADD45A, MDM2, BCL2, TP53</td>
<td>YYYCWGRRRF</td>
</tr>
<tr>
<td>Hypoxia</td>
<td>Hypoxia, Cobalt, Desferrioxamine, Quercetin, Dimethyloxylglycine</td>
<td>HIF-1</td>
<td>VEGF, TF, EPO</td>
<td>TACGTGCTg</td>
</tr>
<tr>
<td>ER stress</td>
<td>Tunicamycin, Thapsigargin, Caprin, Brefeldin A</td>
<td>XBP-1,</td>
<td>HSPA5, DNAJ8B</td>
<td>GATGACGTG (ATF6)h</td>
</tr>
<tr>
<td>Metal stress</td>
<td>Heavy Metals</td>
<td>MTF-1</td>
<td>MT1E, MT2A</td>
<td>GATGACGTGK (XBP-1)i</td>
</tr>
<tr>
<td>Inflammation</td>
<td>Metal, PCBs, Exhaust Particles, Smoke Particles</td>
<td>NF-kB</td>
<td>IL1A, TNFA</td>
<td>CNNTGCRCYCGGGNCb</td>
</tr>
<tr>
<td>Osmotic stress</td>
<td>High salt, polyethylene glycol, mannitol</td>
<td>NFAT5</td>
<td>Akr1B1, Slc6A12, Slc5A3</td>
<td>GGGATTCCCc</td>
</tr>
</tbody>
</table>

a Alam et al. (1999).
b Okinaga and Shibahara (1993).
c el-Deiry et al. (1992).
e Wang et al. (2000).
f Clauss et al. (1996).
g Lin and Green (1988).
h Andersen et al. (1987).
i Kunsch et al. (1992).
j Miyakawa et al. (1998).

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CELLULAR STRESS RESPONSE PATHWAYS AND COMMON PATHWAY ARCHITECTURE

Despite their high degree of structural and functional organization, multicellular organisms react to environmental changes primarily at the level of cells which are the fundamental units of biological organization. Cells respond to normal metabolic signaling via endogenous growth factors and small molecules to promote growth and maintenance of the organism. Cells also respond to exogenous stressors such as chemical toxicants, xenobiotics, heat, and ionizing radiation through a number of specific adaptive stress response pathways that attempt to mitigate damage and maintain or re-establish homeostasis. Stress response pathways are present in all cell types of metazoans and are activated at significantly lower toxicant concentrations than those causing overt cellular injury (Kultz, 2005a). Hence they can serve as a “canary in the coalmine” to measure cell-autonomous toxicity of environmental agents across all cell types. These pathways are highly conserved in most metazoans including mammals, highlighting their central and obligatory roles in all organisms in responding to environmental insults. Several key cellular stress in response pathways have been characterized individually through elegant studies in vitro and the major signaling components and molecular mechanisms have been identified. Adaptive stress response pathways are signal transduction pathways that ultimately result in the transcriptional activation of cytoprotective genes. These genes are recognized as early responders to cellular stress with specificity of response being determined
by the nature of the stress stimulus and its cellular target (Kultz, 2005a). The major stress response pathways, their classical inducers and the prototypic downstream genes that respond to their activation are listed in Table 1 and are discussed below.

Adaptive stress response pathways act as “first responders” to chemical toxicity, radiation, and other physical insults. One of the key characteristic of stress response pathways that enables their rapid response to stressors is found in the common architecture shared by the pathways, which allows cells to mount their initial response without the delay required for de novo gene transcription and translation. This common architecture is comprised of three basic components as shown in Figure 1. The transcription factor (TF) is a DNA-binding protein that interacts with the promoter regions of its target genes via its canonical DNA-binding sites, known as “response elements” (REs), to activate the expression of the target genes. The second component, referred to as the sensor, is a protein that physically interacts with the TF in the cytosol, sequestering the TF from the nucleus under normal cellular conditions. In addition to its role in cytoplasmic sequestration of the TF, the sensor may direct TF degradation, providing an additional layer of regulatory control. The result of the sensor-TF complexation is to maintain inactivity of the TF under normal cellular conditions while providing a mechanism that permits rapid activation in response to an appropriate stimulus. The third element is the transducer which can be defined as an enzymatic protein, such as a kinase, that conveys a biochemical cue (such as phosphorylation) from a signaling pathway(s) upstream of the sensor/TF complex in the event of cellular stress. The transducer may directly modify the TF, providing a critical activating signal or modify the sensor which in turn, destabilizes the sensor/TF complex. Liberated, stabilized, and activated, the TF translocates to the nucleus where it activates its target genes. However, unlike the sensor and TF that are unique to a stress response pathway, there are groups of transducers that transmit stress signal to each stress response pathway and sometimes to more than one stress response pathway, providing the basis for the activation of multiple pathways by the same stressor as discussed in the “cross-talk” section below. Figure 1 shows a general schematic in which exposure to toxicants or physical stresses activates the transducer which in turn modifies the sensor and/or TF. Table 2 lists the three common elements associated with each of the major stress response pathways whose specific components and mechanisms are discussed below illustrating the depth at which these pathways are understood at a biochemical level. It is the thesis of this review article that the parallel measurement of the transcriptional activation of stress pathway-specific genes resulting from the activation of the specific pathway TF using reporter assay technology would provide a measure of cellular stress induced by xenobiotics.

Before proceeding to a detailed description of the stress pathways, it is necessary to clarify and distinguish the stress response pathways from other cellular pathways that are transcriptionally activated by xenobiotics and are currently being used to assay toxicity using techniques similar to those proposed in this review for measurement of the stress pathway activation. Prototypic examples of this set of pathways are
members of the nuclear receptor family known as xenobiotic metabolism pathways and endogenous hormone response pathways (Beischlag et al., 2008; Moore et al., 2006; Puga et al., 2009) which are also identified as toxicity pathways in the NRC report (NRC, 2007, pp. 64–65). The xenobiotic metabolism group is composed of nuclear receptor families pregnane X receptor, constitutive androstane receptor, peroxisome proliferator-activated receptor, and aryl hydrocarbon receptor, all of which are TFs that are activated by xenobiotics. Similarly, the endogenous hormone response pathways are composed of hormone receptor TFs that respond to hormones such as estrogen, androgen, thyroid hormone and progesterone. In fact, assays based on the activation of many, if not all, of the metabolism and endogenous hormone response pathways is already extensively used in toxicity testing, notably at the National Computational Toxicology Center of the Environmental Protection Agency (EPA) (http://epa.gov/ncct/toxcast/files/ToxCast_Assays_01aug2007.pdf). However, the distinguishing feature of the xenobiotic metabolism pathways and endogenous hormone response pathways is that they all require for their activation the binding to the receptor of a specific chemical agent(s), exogenous or endogenous (e.g., xenobiotics, estrogen, thyroid hormone). A second distinguishing feature of the xenobiotic metabolism and hormone response pathways is that their presence and activity is in many cases is restricted to particular tissues such as the liver or reproductive organs. Further, they may be restricted to specific cell types within the tissues, and in some cases such as the estrogen and androgen receptors show sex-specific variations (Bookout et al., 2006). In contrast, the stress pathways are activated by a wide variety of stressors, both physical and chemical, that affect the common cellular biochemical processes. In addition, the stress pathways are ubiquitously expressed in all normal cell types (of both sexes), although the extent of their response can be variable between cell types depending on the biochemical milieu of the particular cell. These two properties of broad stressor response and ubiquitous presence make stress pathways ideal for general measurement of toxicity in contrast to the targeted, compound-specific assays that are based on xenobiotic metabolism and endogenous hormone response pathways.

In the following sections, the specifics of the 3-component design of the eight major cellular stress pathways are briefly discussed with the intent illustrating their common architecture and also conveying to the reader the depth of knowledge in the area that enables the design of exquisite assays. The existing deep knowledge of the pathways is also ideal for inferring the MOA of xenobiotics as discussed below.

**Oxidative Stress Response**

Perhaps the most central of the adaptive stress response pathways is the oxidative stress response, which responds to the diverse number of environmental toxicants and cellular reactions capable of reactive oxygen species (ROS) production. The oxidative stress response is coordinated by the TF, nuclear factor (erythroid-derived 2)-like 2 (Nrf2: Kang et al., 2005; Kensler et al., 2007). (In accordance with the accepted convention, protein names appear as standard text and the names of the corresponding encoding genes are italicized. Thus Nrf-2 represents the protein encoded by the **NFE2L2** gene or the Nrf2 gene. Human genes are presented in all capital letters, rodent genes with first letter capitalized, and genes of all other species are in lower case. This system is not strictly used with protein nomenclature.). Under normal cellular conditions, Nrf2 is sequestered in the cytoplasm by the sensor, Kelch ECH associating protein 1 (Keap1: Kang et al., 2005). Keap1-Nrf2 complexes are stabilized by interactions with the actin cytoskeleton. Keap1 interacts with two other proteins, Roc1 and Cull3; the Keap1-Roc1-Cul3 complex recruits E2 ubiquitin ligase, which in turn drives the proteosomal degradation of Nrf2 (Hong et al., 2005). Due to Keap1-directed ubiquitination, Nrf2 protein has an average half-life of about 20 minutes under normal conditions (Itoh et al., 2003). Together, these control mechanisms permit only a low basal level of Nrf2-directed gene expression. Oxidative stress however, imposes several important changes on the Keap1-Nrf2 dynamics that lead to the activation of Nrf2. First, several key oxidative stress-responsive transducers such as the mitogen-activated protein kinases (MAPKs, ERK, p38), protein kinase C (PKC), and phosphatidylinositol 3 kinase (PI3K) phosphorylate both Keap1-and Nrf2 (Huang et al., 2002; Kang et al., 2000) are activated. Phosphorylation of Keap1 induces a conformational change that lowers the affinity of Keap1 for Nrf2, whereas phosphorylation of Nrf2 provides both a stabilizing effect and an activating signal. Second, Keap1 is a cysteine-rich protein, providing a sulfhydryl-rich target for direct interaction with electrophiles, including ROS (Dinkova-Kostova et al., 2002). Electrophiles react with the cysteine residues of Keap1, inducing critical conformational changes in Keap1 which serve to liberate and stabilize Nrf2. Third, a liberated and stabilized Nrf2 translocates to the nucleus, where it dimerizes with small Maf proteins (Kataoka et al., 2001). Nrf2/Maf heterodimers interact directly with the antioxidant response elements (AREs) within target gene promoters, displacing the transcriptional repressor Bach1 (Dhakshinamoorthy et al., 2005; Reichard et al., 2007). This de-repression and activation of Nrf2 target genes leads to increased target gene expression. Nrf2-induced genes, such as those encoding Heme oxygenase-1, Glutathione S-transferase A2, and NAD(P)H quinone oxidoreductase (NQO1) serve to neutralize ROS and electrophiles, biosynthesize glutathione, direct xenobiotic efflux and remove oxidized proteins. The net result of induction of these target genes is to scavenge ROS and conjugate electrophiles which serve to limit oxidative damage and to detoxify the cell.

**Heat Shock Response**

Heat shock response is a major stress response pathway and one of the earliest to be discovered. It is a highly conserved cellular response pathway designed to protect cells from diverse
physical and chemical insults (Westerheide and Morimoto, 2005). Activation of this pathway triggers the transcriptional upregulation of a family of genes known as "heat shock" proteins. Although originally described for their role in adaptation to hyperthermia, members of the heat shock protein family were subsequently shown to respond to a number of chemical toxicants that also cause protein denaturation. The heat shock response works to rapidly activate the expression of heat shock chaperone proteins that bind to unfolded proteins in an attempt to minimize denaturation, prevent protein aggregate formation and facilitate the refolding of denatured proteins. Under normal conditions, heat shock transcription factor (HSF-1) is held in the cytoplasm in its inactive state through direct protein sequestration by heat shock protein 90 (Hsp90: Boellmann et al., 2004). Under stress conditions, the HSF-1 monomer rapidly trimerizes and localizes to the nucleus (Voellmy and Boellmann, 2007). Concurrent with multimerization, HSF-1 is further activated via phosphorylation by calcium/calcmodulin-dependent protein kinase II (CaMKII) and protein kinase CK2 (formerly referred to as casein kinase II: Holmberg et al., 2001; Soncin et al., 2003). The activated HSF-1 trimer upregulates transcription of target genes by binding the heat shock response element (HSE) present in the target gene promoters (Voellmy, 1994). HSF-1 target genes encode chaperones such as Hsp70 and Hsp27, inflammatory mediators such as interleukin 6, and cellular pumps such as Multidrug resistance 1 protein. One HSF-1 target gene, Heat shock binding protein 1 (HSBP1), binds and deactivates the HSF-1 trimer, providing a negative feedback loop (Satyal et al., 1998).

**p53-Mediated DNA Damage Response**

Perhaps the most-studied among the stress responses is the genotoxic stress response pathway that responds to agents that damage DNA. The cellular response to DNA-damaging agents is largely orchestrated by the p53 family of TFs, with p53 itself being the principal member (Harkin and Hall, 2000; Horn and Vousden, 2007; Riley et al., 2008). Under normal conditions, p53 is constitutively expressed, but is negatively regulated by the pathway sensor, Mdm2. In addition to physical sequestration of p53 in the cytoplasm, Mdm2 also directs p53 to ubiquitin-mediated proteasomal degradation, thus reducing p53 protein half-life to mere minutes. However, a variety of stress events such as DNA damage induced by genotoxic chemicals, ionizing radiation, hypoxia, mitotic spindle damage or ribonucleotide depletion trigger a series of events that stabilize p53 protein by inhibiting its degradation. This stabilization is largely driven by the transducers for this pathway, namely ataxia telangiectasia mutated (ATM), jun N-terminal kinase (JNK), Chk1, and Chk2 kinases, which phosphorylate the p53 transactivation domain, thus blocking the site for direct Mdm2 interaction (Fuchs et al., 1998; Morgan and Kastan, 1997; Shieh et al., 2000). Additional phosphorylation signals provided by the MAPKs and CK2 can modify the carboxyl terminus of p53, enhancing the tetramerization required to render p53 transcriptionally active (Prowald et al., 1997; Wu, 2004). Once activated, p53 regulates divergent groups of target genes. The products of one group of target genes such as CDKN1A (p21) and cyclin G arrest the cell cycle at the G1/S or G2/M checkpoints in the cell cycle (Gartel and Radhakrishnan, 2005). Another group of target genes includes GADD45A and XPC, whose products regulate DNA repair (Thoma and Vasquez, 2003; Zhan, 2005). A third cluster of p53 target genes, characterized by Bax and Bcl-L, mediate apoptosis (Reed, 2006). Lastly, p53 stimulates the expression of its sensor Mdm2, providing a negative feedback loop to surviving cells; however, Mdm2 is considered a late response target, which provides a temporal window for active p53 to affect cell cycle, DNA repair, and/or apoptosis (Lahav et al., 2004). Growth arrest is inevitable following p53 activation, but the exact mechanisms through which p53 determines whether to repair DNA damage or induce apoptosis is not yet understood.

**Hypoxia Response Pathway**

Certain chemical toxicants such as metals can deplete oxygen availability in cells, and causes hypoxia. The intracellular partial pressure of oxygen is constantly monitored by a family of enzymes known as prolyl hydroxylases (PHD: Lee et al., 2007). Under normoxic conditions, PHD proteins catalyze the non-reversible hydroxylation of proline residues within hypoxia-inducible factor alpha (HIF-1α), the hypoxia-inducible TF subunit that directs the transcriptional response to decreased oxygen availability (van den Beucken et al., 2006). Hydroxylation of HIF-1α fosters its direct interaction with von Hippel-Lindua tumor suppressor protein (VHL), leading to proteasomal degradation of HIF-1α. Hypoxia impairs PHD activity, stabilizing HIF-1α subunits. Other signal transducers also stabilize HIF-1α including p38 and PI3K kinases (Duyndam et al., 2003; Gao et al., 2002; Yen et al., 2005). Stabilization and accumulation of HIF-1α enables its heterodimerization with the HIF-β subunit, the aryl hydrocarbon receptor nuclear translocator. HIF heterodimers bind DNA via the hypoxia response elements (HREs) within target gene promoters and recruit transcriptional coactivators such as p300 and cAMP response element binding protein (CBP: Arany et al., 1996; Zakrzewska et al., 2005). HIF-regulated target genes enable cells to adapt to conditions of decreased oxygen tension by increasing oxygen transport (globin-2), iron transport ( transferrin), stimulating angiogenesis (vascular endothelial growth factor [VEGF]), affecting blood vessel diameter (endothelin 1), modulating glycolysis (aldolase), and regulating glucose uptake (glucose transporter 1) as discussed in Wenger et al. (2005).

**ER Stress Response**

The ER is a critical eukaryotic organelle because of its central role in lipid and protein biosynthesis as well as protein folding and maturation. Specifically, the ER lumen is the site
where nearly 30% of the newly translated cellular proteins are properly modified and folded into their mature forms for membrane incorporation and secretion (Zhang and Kaufman, 2008). Conditions that perturb ER function disrupting a cell’s ability to produce properly folded proteins are collectively referred to as ER stress or unfolded protein response (UPR). Chemical toxicants such as tunicamycin and thapsigargin cause an accumulation of unfolded protein aggregates in the ER lumen (Harding and Ron, 2002; Schroder, 2008; Zhang and Kaufman, 2008). If ER stress results in irreversible damage by the persistence of this stress, the cells undergo apoptosis, a mechanism that is believed to be involved in disease states such as type-1 diabetes (Zhang and Kaufman, 2008). The cellular response to ER stress has three major effector branches, making it one of the more multifaceted stress response pathways known (Harding and Ron, 2002). However, although seemingly more complex compared with the pathway architectures already discussed, all three components are linked by a unifying ER chaperone protein, BiP (Grp78), which is the sensor equivalent for the ER stress pathway. Under normal conditions, BiP resides in the ER lumen and binds the luminal domains of the membrane-spanning proteins involved in each of the three pathway branches to maintain their quiescence. Along the first branch, general protein biosynthesis is transiently paused by the stress-mediated phosphorylation of eukaryotic translation initiation factor 2 (eIF2) by a resident ER kinase, PRKR-like endoplasmic reticulum kinase (PERK). As unfolded proteins accumulate in the ER lumen, BiP preferentially binds the unfolded proteins over the luminal domain of PERK. The release of BiP from the PERK luminal domain triggers the homodimerization and autophosphorylation of PERK. Activated PERK then inactivates the translation initiation factor, eIF2, via direct phosphorylation. Under normal cellular conditions, eIF2 recruits the charged initiator methionyl tRNA to the ribosome and thus is critical for translational initiation, but phosphorylation by PERK prevents eIF2 from binding GTP which is necessary for initiation of translation. This general attenuation of translation, however actually enhances the translation of certain mRNAs such as those encoding the TF ATF4 (Harding et al., 2000, 2003) which in turn regulates target genes involved in amino acid biosynthesis and import, as well as mitochondrial function and apoptosis (Harding et al., 2003). The second and third branches mediate activation of genes critical for reestablishing ER function. As new protein synthesis is paused in response to ER stress, BiP dissociates from a second ER-specific protein partner termed inositol requiring enzyme 1 alpha (IRE1α). Like PERK, the release of BiP triggers the activation of IRE1α by autophosphorylation. Activated IRE1α splices an mRNA species encoding an ER stress-specific TF termed X-box binding protein 1 (XBPI: Calfon et al., 2002). Although most protein synthesis has been halted, certain mRNAs such as ATF4 and the spliced XBPI mRNA are translated (in the cytosol) during ER stress owing to a unique sequence in their 5’ untranslated regions. The resulting XBPI protein translocates to the nucleus where it directs the expression of several ER stress-responsive genes, including those encoding ER-specific chaperones p58 and ERdj4 (Lee et al., 2003). Only the XBPI protein derived from the spliced mRNA is transcriptionally active. The third major branch involves the transcription factor ATF6 which spans the ER membrane, and is constitutively expressed but maintained in an inactive form due to its association with BiP under normal conditions. Under ER stress, BiP dissociates from ATF6 triggering its translocation to the Golgi apparatus where it is cleaved by the S1P and S2P serine proteases into its transcriptionally active form (Ye et al., 2000). Activated ATF6 upregulates the expression of additional ER stress-related chaperones such as Grp78 (BiP) and CHOP (GADD153: Ma and Hendershot, 2004), although there are genes that are jointly regulated by ATF6 and XBPI. These ER stress responsive target genes work to clear the ER of unfolded proteins by refolding proteins and removing damaged proteins. With respect to the general theme of conservation of stress response pathway architecture, the ER stress response pathway is atypical in that the sensor (BiP) restrains the enzymatic transducers (PERK and IRE1α), but, in keeping with the classical scheme, suppresses the transcription factor, ATF6.

Metal Stress Response

Certain metals can activate yet another stress response pathway termed the metal-response pathway (Lichtlen and Schaffner, 2001). The metal stress response pathway is perhaps the most atypical with respect to architecture among the major adaptive stress response pathways. The metal-response pathway centers around the activity of a specific TF called metal transcription factor-1 (MTF-1) which is constitutively expressed in all cell types examined (Andrews, 2001; Lichtlen and Schaffner, 2001). MTF-1 has six amino-terminal zinc-finger domains, termed F1-F6, and these zinc fingers enable MTF-1 to be an intracellular sensor of free zinc ion levels (Laity and Andrews, 2007). Zinc fingers F1-F4 bind zinc tightly at normal intracellular zinc concentrations and form the basic structure of the DNA-binding domain, but one with a relatively low affinity for DNA. By comparison, zinc fingers F5 and F6 bind zinc at a lower affinity. However, at elevated intracellular zinc concentrations, zinc occupancy of zinc fingers F5 and F6 increases, thereby stabilizing the DNA-binding domain of MTF-1 and conferring a higher affinity for its consensus DNA-binding sequence, termed the metal-response element (MRE: Stuart et al., 1985). Activated MTF-1 then induces the expression of its target genes, such as those encoding metallothionein proteins (MTs: Saydam et al., 2002). Metallothionein family members are small, cysteine-rich proteins that serve to chelate metal ions in the cytosol (Nordberg and Nordberg, 2000). Another classic MTF-1 target gene product, zinc transporter (ZnT-1) is critically involved in zinc uptake as well as heavy metal efflux from the cell. MTF-1
Target gene products work to lower intracellular free zinc levels, destabilizing the F5 and F6 zinc fingers of MTF-1, reducing the DNA-binding ability, providing a negative feedback loop for gene regulation. Until recently, it was thought that this mechanism accounted for the metal-responsive activities of MTF-1; however, this model did not account for two incongruous observations regarding MTF-1 activation. First, MTF-1 was activated by heavy metals other than zinc that cannot function to stabilize the F5 and F6 zinc fingers (Chen et al., 2004). To account for this observation, it has been hypothesized that heavy metal such as cadmium displace zinc from other cellular proteins, thereby increasing intercellular zinc levels consistent with the proposed model (Lichten and Schaffner, 2001). More recently, a cysteine-rich domain located near the carboxyl terminus of MTF-1 was shown to be required for MTF-1 activity and was shown to have affinity for other metals like cadmium. It has been suggested that this domain may bind a putative repressive cofactor and that heavy metal binding produces a conformational change that disrupts this interaction and possibly allows the recruitment of coactivators. The second observation inconsistent with the original model for MTF-1 activation was that MTF-1 could in the absence of heavy metals trigger the expression of its target genes in response to oxidative stress (Andrews, 2000; Dalton et al., 1996). This observation led to the discovery that multiple transducers, including PKC and CK2 that phosphorylate MTF-1, and showed that these modifications are critical for MTF-1 activation. Although a metal ion-responsive sensor that negatively regulates (as in other stress response pathways) MTF-1 under normal cellular conditions has been proposed, no such sensor has been identified.

Nuclear Factor Kappa B–Mediated Inflammatory Stress Response

In addition to its roles in normal cell growth and development, nuclear factor kappa B (NF-kB) is the key transcriptional driver of immune and inflammatory responses (Guijarro and Egidio, 2001; Schoonbroodt and Piette, 2000). Active NF-kB is comprised of homo- or heterodimers assembled from members of the NF-kB and Rel protein families. By far, the best characterized among these is the p50 (NF-kB/p65 (RelA) heterodimer. In most cells, NF-kB (p50/p65) is held in a latent state in the cytoplasm by its physical interaction with its inhibitory sensor, IκB (Hayden and Ghosh, 2008). The IκB family has several members including IkBa, IκBb, IκBe, IκBg, IκBz, and Bcl-3. IκB proteins interact with NF-kB, masking NF-kB surfaces required for nuclear localization and DNA-binding. Although, there is continual nuclear-cytoplasmic shuttling of IκBa-NF-kB complexes, IκBa possesses a strong nuclear export signal that causes the localization equilibrium of the complex to be heavily biased towards the cytoplasm. By contrast, IκBb allows no nuclear shuttling of bound NF-kB. IκB-NF-kB complexes are destabilized by a diverse set of stimuli, including exposure to environmental toxicants such arsenic (Fry et al., 2007), divalent metal ions (Goebeler et al., 1995; Liao et al., 2007; Persichini et al., 2006), cigarette smoke (Anto et al., 2002), and diesel exhaust particles (Kafoury and Madden, 2005). These stimuli induce a signaling cascade that culminates in the activation of the pathway transducer termed IκB kinase (IKK: Perkins, 2007). IKK is comprised of three subunits: IKKα, IKKβ, and IKKγ. The first two subunits comprise the catalytic domain of the IKK complex, whereas IKKγ is the regulatory subunit that integrates upstream signaling events and activates the IKK complex. IKK directly phosphorylates IκB proteins which results in the disruption of the NF-kB-IκB interaction, the subsequent targeting IκB for proteasomal degradation and the liberation of NF-kB. The released NF-kB rapidly translocates to the nucleus where it triggers the expression of its target genes via NF-kB binding sites designated κB sites. NF-kB gene targets include stress response genes such as cytochrome P450s (CYP2E1, CYP2C11, CYP7B: Abdel-Razzak et al., 2004; Dulos et al., 2005; Morgan et al., 2002), superoxide dismutase (SOD1: Das et al., 1995), NAD(P)H quinone oxidoreductase (Yao and O’Dwyer, 1995), and heat shock protein 90 (Hsp90AA1: Ammirante et al., 2008), as well as genes that encode cytokines (Son et al., 2008), and regulators of apoptosis (Grimm et al., 2005). One other NF-kB target gene of note is NF-kB1A (IκBα), which shuttles into the nucleus to attenuate the NF-kB response, thus providing a critical negative feedback loop in the NF-kB signaling pathway (Sun et al., 1993).

Osmotic Stress Response

Exposure to certain toxicants can create changes in the osmolyte concentration in the extracellular environment of a cell, causing a rapid change in the movement of water across its cell membrane resulting in a condition known as osmotic stress (Kultz, 2005b; Kultz and Csonka, 1999). Of the major stress response pathways, the osmotic stress response pathway is perhaps the least understood with respect to mechanisms. This pathway is a vital component of normal physiology in the renal medulla. Perhaps it is this renal-specific relevance that has held back wider studies of the pathway, but its implementation as a stress response pathway in cells extends beyond the kidney. At the center of this pathway is the transcription element binding protein (TonEBP) also known as NFAT5. TonEBP is member of the Rel family, the same family as the NF-kB dimerization partner p65 (RelA). In contrast to other Rel family members, little is known about the mechanisms underlying TonEBP activation in response to hypertonicity. No known sensor has been identified for this pathway, although TonEBP is localized primarily in the cytoplasm under normal conditions. Two kinases clearly play a role in triggering TonEBP activation: p38 kinase in...
conjunction with the GTPase activity of Rac1 is required for TonEBP activation in human kidney cells (Dahl et al., 2001; Ko et al., 2002) and ATM kinase also activates TonEBP in response to hyperosmolarity, although this activation appears to be limited to osmotic stress-induced DNA damage (Irrazabal et al., 2004; Zhang et al., 2005). There is also some evidence to suggest protein kinase A (PKA) has a role in TonEBP activation in the kidney (Ferraris et al., 2002). The mechanisms through which hypertonicity stimulates the activities of these transducers are not yet understood. Activated TonEBP translocates to the nucleus where it collaborates with activities of these transducers (Roa et al., 2003) to regulate genes involved in solute transport (BGT1, ALDOA), organic solutes response (BGTL, ALDOA), and chaperones (Hsp70; Kultz, 2005b).

**BEYOND HOMEOSTASIS: TRANSITION TO APICAL CYTOTOXIC EVENTS**

As discussed up to this point, the central role of stress response pathways is posited to be to counteract deviations from the normal state imposed on a cell by unfavorable environmental conditions such as exposure to toxic compounds, and reestablish a homeostasis. In general, stress response pathways are induced at doses that are orders of magnitude below those required to elicit apical the event of cell death through apoptosis or necrosis. If the stress response pathways are successful in maintaining homeostasis, the cell avoids consequences of toxic exposure and enables the organism to recover. Thus, the validity of measuring stress response pathway activation as precursor events for toxicity and the relevance of these measurements as predictors of toxic consequences may be questioned. However, several cases have been described where the stress TFs activate proteins also activate apoptotic process, and therefore participate in cell fate decisions. Stress response pathways are also intimately linked to the pathways that lead to apoptosis, which are activated when the homeostatic responses are overwhelmed by stress. This would make sense from the perspective that the function of homeostasis at an organism level includes the elimination of cells that have sustained substantial damage, and hence, may undergo oncogenic transformation or necrosis with associated tissue damage. It is also logical that the stress response pathways would assess the damage to the cells through yet unclear mechanisms and play a role in triggering apoptosis when the goal of cellular homeostasis is thwarted. Thus, the key component of the stress response pathway, the activated TF, is the balance point around which a decision is made by a cell to commit to survive or to self destruct through apoptosis. The recent NRC report (NRC, 2007, pp. 36–37) recognizes homeostatic stress response pathways as toxicity pathways: “Thus, in the cellular response network dealing with oxidative stress, the antioxidant pathways activated by Diesel Exhaust Particles are normal adaptive signaling pathways that assist in maintaining homeostasis; however, they are also toxicity pathways in that they lead to adverse effects when oxidant exposure is sufficiently high.” This statement in the context of oxidative stress also applies to other stress response pathways as discussed below.

Although the details of this balancing mechanism are not clear, there are several examples where TFs of key stress response pathways are also known to activate proapoptotic genes, with the TF p53 being a prime example. In addition to its proapoptotic function of promoting cell cycle arrest to enable repair of DNA damage, as discussed in an earlier section, p53 activates a battery of proapoptotic genes from extrinsic and intrinsic apoptotic pathways that interact with proapoptotic genes to make a cell fate decision (Li et al., 2008; Yu and Zhang, 2005). Further, p53 has been shown to translocate into mitochondria augmenting the onset of apoptosis (Marchenko et al., 2000). Similarly, HIF-1, although activating protective genes and mechanisms, is also known to induce proapoptotic genes, and this process may involve p53 stabilization (Greijer and van der Wall, 2004). The ER stress-activated homeostatic responses have also been shown to stimulate apoptosis through the CHOP/ATF4 TF (Ohoka et al., 2005; Zhang and Kaufman, 2008). Manganese has been reported to induce ER stress that eventually leads to the activation of multiple caspases in striatal dopaminergic neuronal cells (Chun et al., 2001). Similarly, the NF-kB pathway has also been suggested to have a proapoptotic arm that functions through the activation of transcription of pro-death receptors (Herr and Debatin, 2001; Li et al., 2005). Although examples of proapoptotic activities of other TFs have not been reported, the cross-wiring of stress response pathways and the activation of multiple stress response pathways by chemical toxicant, observed in studies described in the following section, would suggest that stress response pathway induction is closely linked also to cell fate/death decisions.

Therefore, from a toxicological perspective the activation of a stress response pathway may be considered a harbinger of cell death if the stress is enhanced by increasing dose of toxicant or if the stress persists over extended time periods. Thus, the first sign of activation of any one of the stress response pathways can be considered a key event that delineates the “No Observable Effect Level” dose (NOEL) and/or “Least Observable Effect Level” dose (LOEL). Alternately, the dose of the stressor at which activation of subsequent stress response pathways occur may be pegged as the key event, because this transition event indicates the failure of the first activated pathway to maintain homeostasis thereby causing a spill-over activation of the second and other stress response pathways. Clearly these concepts need to be refined based on new experimental data to evaluate the potential of stress response pathway activation assays as predictors of toxicological endpoints.
As the stress response pathways are involved in cell death decisions, their appropriate regulation has been shown to be critical for the normal growth and development of multicellular organisms. A number of studies have been performed in transgenic mice which were engineered to delete genes encoding the sensor or the TF components of the various stress response pathways. As shown in Table 3, these studies have demonstrated that the phenotypic consequences of dysregulated constitutive stress response TF activity by ablating the sensor or conversely preventing TF activation by ablating the encoding gene have serious biological consequences ranging from loss of fertility to embryonic lethality.

Although deletion of either HIF1α (Rocha, 2007; Semenza and Prabhakar, 2007) or MTF1 (Lichtlen and Schaffner, 2001) results in embryonic lethal phenotypes, deletion of the genes encoding HSF-1 (Christians and Benjamin, 2005a, b), p53 (Lee and Bernstein, 1995), or Nrf2 (Kensler et al., 2007), produce viable but compromised animals. For example, HSF1 or HSF2 gene disruption leads to multiple defects, including female infertility (Christians and Benjamin, 2005a, b). TP53 (p53) knockout mice of both genders suffer from early tumor development but also show a number of other phenotypic differences from wild-type animals (Lee and Bernstein, 1995). Loss of Nrf2 results in mice that are more sensitive to xenobiotic and drug exposures as well as inflammatory stress responses at a multigorgan level (Kensler et al., 2007). As described in the previous section, the NF-κB pathway is composed of a host of TFs such as NF-κB1, NF-κB2, c-Rel, and RelA that are used in different contexts (Li and Verma, 2002; Pasparakis et al., 2006). With the exception of RelA, individual deletions of any of these TFs results in viable offspring, but with compromised immune and hematoepoietic systems (Gerondakis et al., 2006). Unlike the other stress response pathways, the ER stress response pathway is composed of the three distinct components (PERK, ATF6, and IRE1/XBP-1) discussed earlier. Homozygous deletion of EIF2AK2 (PERK), which controls the eIF2α phosphorylation results in viable animals but these mice develop pancreatic degeneration and skeletal dysplasia (Zhang et al., 2002). ATF6, the membrane-tethered TF that is activated by proteolysis upon ER stress, is encoded by two related genes ATF6α and ATF6β. Homozygous deletion of either ATF6α gene results in viable mice, but with compromised response to chronic and acute stress as well as decreased survival (Wu et al., 2007; Yamamoto et al., 2007). However, simultaneous homozygous deletion of both genes results in embryonic lethality, underscoring a critical role for ATF6 in development (Yamamoto et al., 2007). Embryonic lethality also results from homozygous deletion of ERN1 (IRE1) gene, which regulates the stress-induced splicing of XBP1 mRNA during ER stress (Urano et al., 2000). Deletion of the transcription factor XBP-1 gene also results in embryonic lethality (Reimold et al., 2000). In the metal stress response pathway, homozygous deletion of MTF-1 results in embryonic lethality (Lichtlen and Schaffner, 2001). Lastly, homozygous deletion of the osmotic stress transcription factor NFAT5/TonEBP, a member of the NF-κB family, also results in embryonic lethality. These studies underscore the importance of stress TFs to the normal development and functioning of the organism.

However, in contrast to genetic loss of stress-responsive TFs where consequences varied, genetic loss of the sensor has proved lethal in all cases. Disruption of Mdm2 (Marine et al., 2006), KEAP1 (Wakabayashi et al., 2003), NFKBIA (IκB; Gerondakis et al., 2006), VHL (Kapitsinou and Haase, 2008), which respectively regulate p53, Nrf2, NF-κB, and HIF-1α all result in embryonic or postnatal lethal phenotypes. Embryonic lethality is also observed upon homozygous deletion of Gpr78/ BiP which regulates the three arms of the ER stress response pathway (Luo et al., 2006). Similarly, deletion of the β isoform of Hsp90 which is involved in HSF sequestration also leads to embryonic lethality (Voss et al., 2000). Interestingly however, where data are available, the simultaneous genetic deletion of both the sensor and corresponding TF genes rescues the embryonic lethal phenotype that results from deleting the sensor gene alone as evidenced by the viability of double knockout mice such as p53-Mdm2 (Jones et al., 1995; Marine et al., 2006) and Nrf2-Keap1 (Motohashi and Yamamoto, 2004). These results show that while the lack of the stress TF activity generally compromises a cell or an organism, the unregulated activation of the stress TFs leads to significant biological impairment incompatible with normal development. Based upon the compromised stress response observed in these animal models and in cultured cells from mice that suffer embryonic lethality through loss of stress response pathway gene regulation, it is reasonable to extrapolate that the aberrant activation of TF induced by xenobiotics exposure at a cellular level maybe detrimental during development (Go et al., 2004; Wang et al., 2004; Yamamoto et al., 2007). Indeed, cells derived from mice where sensor or TF genes are conditionally deleted in specific cell types in post-embryonic stages, also show compromised stress response in those particular cell types upon exposure to a variety of agents (Gerondakis et al., 2006; Wang et al., 2004). These studies underscore the central role of stress response pathways in maintenance of homeostasis at both the cellular and whole organism levels.

The conclusions above derived using gene ablation studies in animals and in cells derived from them are in general agreement with the limited number of studies have also been performed with transgenic mice overexpressing either the TF or the sensor of a few stress response pathways. These studies, unlike those using homozygous knockout animals, are more difficult to interpret because the levels of expression, its timing, and its tissue specificity are variable due to factors such as promoter choice and the site of integration of the transgene (Overbeek, 2002). Nevertheless, where available, these studies
support the thesis that appropriate expression of the stress response pathway TFs and sensors is vital to normal development and maintenance of the animals, as shown by the following examples. In a large set of studies focused on the genotoxic stress pathway, overexpression of wild-type TF p53 or various mutant versions (which essentially down regulate the activity of endogenous p53 via dominant negative effects), results in animals that are cancer prone and/or have developmental abnormalities (Choi and Donehower, 1999; Godley et al., 1996; Godley et al., 1999; Jones et al., 1998; Lundgren et al., 1997). Analogous overexpression studies with nearly similar results in animals that are cancer prone and/or have developmental abnormalities (Choi and Donehower, 1999; Godley et al., 1996; Godley et al., 1999; Jones et al., 1998; Lundgren et al., 1997). Analogous overexpression studies with nearly similar conclusions have been reported for the ER stress transcription factor ATF-4 (Bagheri-Yarmand et al., 2003).

Taken together, the in vivo gene ablation and overexpression studies described above strongly suggest that chronic activation of the stress response pathway components by exposure xenobiotics can lead to adverse consequences to both developing and mature organisms.

**CROSS-TALK BETWEEN STRESS RESPONSE PATHWAYS**

Although a large amount of cell biology research in the last several decades has been dedicated to investigation of individual cell signaling pathways, it has become evident that many pathways share common elements and are therefore extensively “cross-wired” and hence subject to “cross-talk.” The mechanistic basis for the induction of multiple stress response pathways by the same stressor, as discussed in details further below, is that the activation of stress response pathways involves not only the stress response pathway-specific TFs shown in Table 2, but the cooperation of and competition for a number of additional TFs including other stress TFs as well as shared non-stress TFs. For instance, in inducing oxidative stress responsive genes, the transcription factor Nrf2, in addition to interacting with proteins of the basic transcription complex, has been shown to form heterodimers with proteins MafK, MafF, MafG, Jun, and CBP/p300 (Ryter et al., 2006). In its transcriptional activation process, NF-κB and p53 have been shown to interact with TFs and coactivators such as c-Jun and C/EBP-β, CBP/p300 (Ikeda et al., 2000; Xiao, 2004). Because of this cross-wiring the persistent and/or xenobiotic-induced dose-dependent activation of any one stress response pathway is capable of eventually leading to the activation of other stress response pathways as the cell progresses toward cytotoxicity through apoptotic or necrotic death; this is akin to a “conflagration” or an “avalanche” effect that begins at a specific point source, but engulfs the cell leading to its eventual destruction. Although strict groupings are difficult, the “avalanche” effect resulting from activation of multiple stress response pathways can be divided as originating through four broad, overlapping mechanisms as discussed below.

The first mechanism operates at the level of transcription through interactions between TFs at the stress responsive gene promoters, many of which have multiple stress TF binding sites. For instance, the promoter for the Hmox1 gene, which responds to oxidative stress, contains Nrf2 binding sites as expected as well as REs for MTF-1, HIF-1, HSF-1, and NF-κB (Ryter et al., 2006) rendering Hmox1 responsive to multiple stress stimuli. One of the heat shock-responsive genes, Hspa2, is induced under osmotic stress because it contains DNA motifs specific for the osmotic stress factor, NFAT5/TonEBP (Woo et al., 2002). It has also been shown that TFs that are primary agents in a particular stress response pathway may influence the activation of other stress response pathways. For instance MTF-1 contributes to HIF-1 activation during hypoxia (Murphy et al., 2005). A link between the transcriptional activities of NF-κB and HIF-1 has been demonstrated in vitro as well as in vivo (Wright and Duckett, 2009). Similarly, activation of p53 by oxidative stress leading to DNA damage has been shown to suppress transcription from Nrf-2 responsive promoters (Faraonio et al., 2006). p53 has been reported to play a role in metal-responsive gene expression driven by MTF-1 (Ostrakhovitch et al., 2007). Cross-talk between HSF-1 and MTF-1 has also been reported (Saydam et al., 2003; Uenishi et al., 2006).
In the second mechanism of stress response pathway cross-talk, a xenobiotic impacts intracellular conditions such that there is not only activation of the toxicant’s primary target stress response pathway, but that of additional stress response pathways as well. As reviewed earlier, cadmium and other heavy metals are thought to activate MTF-1 by displacing zinc from intracellular stores where zinc is held in complexes with MTs (Lichten and Schaffner, 2001). Zinc is also known to induce the production of ROS by translocating into mitochondria and to the moderate induction of p53 (Rudolf et al., 2005). The activation of the MTH1 promoter by heat shock is hypothesized to be due to the increase in intracellular concentration of heavy metals, mainly zinc, through a loss of in intracellular sequestration of heavy metals (Saydam et al., 2003). HSFs are activated by global ATP depletion in kidney cells (van Why et al., 1999). Heat stress has been shown to lead to the release of NF-κB from its inactive complex and its translocation into the nucleus resulting in activation of NF-κB target genes (Kretz-Remy et al., 2001). Chemicals that induce oxidative stress primarily activate the Nrf2 pathway, but prolonged exposures and/or increased toxicant concentration leads to the activation of the NF-κB pathway (Gloire et al., 2006). Oxidative stress is also known to cause DNA damage, resulting in the activation of the p53 pathway (Finkel and Holbrook, 2000). Double-strand breaks in DNA which activate p53 through the ATM pathway have also been shown to activate the NF-κB pathway (Habraken and Piette, 2006). During ER stress there is leakage of calcium from the ER where it is concentrated, and the calcium released from the ER enters the mitochondria and induces the production of ROS, thus producing oxidative stress and the attendant induction of the oxidative stress pathway (Zhang and Kaufman, 2008). ER stress, through increased demand for protein refolding, generates ROS which triggers phosphorylation and activation of Nrf2 via the PERK pathway, and the activation of IRE1 leads to the activation of NF-κB through a pathway involving IRE1-α, TRAF2, and IKK (Zhang and Kaufman, 2008). Finally, hypoxic stress has been shown to induce the expression of XBP-1 mRNA and activate its splicing to produce active XBP-1 protein (Romero-Ramirez et al., 2004). In addition, it is also possible that a chemical toxicant may itself independently activate more than one stress response pathway by its ability to interact with several distinct molecular target in the cells, albeit at different doses. This scenario is best illustrated by the side effects of pharmaceuticals that interact with their intended target receptor at low concentrations to produce therapeutic effects, but with other cellular components at higher doses to produce toxic side effects.

A third mechanism of stress response pathway interaction involves the inhibition of a central cellular process common to several of the stress pathways which can, in turn, alter the global stress responses of the cell. This mechanism is best illustrated by the effects of agents that inhibit proteasomal function. The proteasomal degradation of the sensors and the resultant release/stabilization of the TFs plays a central role in the modulation of several of the stress response pathways. In keeping with this expectation, proteasome inhibitors have been shown to cause the activation of heat shock response (Kawazoe et al., 1998; van Why et al., 1999), genotoxic response (Williams and McConkey, 2003), and Nrf2-mediated oxidative stress response (Meiners et al., 2006) and counter-intuitively, to the attenuation of hypoxia response (Kaluz et al., 2006). A similar imbalance of sensor/TF occurs during ER stress which leads to the activation of the NF-κB pathway. In this case, the attenuation of translation induced through the IRE1-α pathway leads to a decrease in the synthesis of the sensor IkB with the resultant decrease in its levels, and release and activation of NF-κB to upregulate its target gene battery (Zhang and Kaufman, 2008). It is well-established that protein phosphorylation and dephosphorylation reactions are widely used cellular regulatory mechanism that controls the activation or suppression of cellular pathways including stress response pathways. For instance, modest concentrations of heavy metals such as zinc and vanadium, which a inhibit broad range of phosphatases, have been shown to activate cellular receptors as well as TFS and kinases (transducers) that activate certain stress response pathways (Samet et al., 1998, 1999; Wu et al., 2002).

Lastly, stress response pathways share in common many of the enzymatic transducers that provide the critical biochemical signals that trigger pathway activation in response to toxicant exposure. Activation of these transducers can lead to the activation of multiple stress response pathways as shown by the following examples. Among the most common stress response pathway transducers are MAP kinases. One MAPK, p38, is involved in the activation of the oxidative stress, hypoxia, and osmotic stress responses (Emerling et al., 2005; Yeh and Yen, 2006; Zhou et al., 2008). PKC is also implicated in the activation of multiple stress response pathways including oxidative stress and metal stress responses (Kim and Kim, 2004; Yu et al., 1997). PKA plays a role in the activation of both the heat shock and osmotic stress response pathways (Ferraris et al., 2002; Kim et al., 1997). The ATM kinase, generally regarded as the p53-activating kinase has also been shown to regulate MTF-1 (Heinloth et al., 2003). Many more such examples of enzymatic transducers shared by multiple stress response pathways have been reported in the literature, but will not be discussed here in the interest of brevity.

Why do the major stress response pathways share so many transducing signals in common? It seems that much like keys on a piano, different combinations of transduction signals are “struck” upon exposure to different toxicants to form stress response pathway-specific “chords.” It would be reasonable to conclude that if one or more requisite transduction signals are active for one stress response pathway that another stress response pathway that shares that transduction signal is “primed” for activation when it’s remaining signal(s) is triggered. It should also be noted, however, that there are also pathway-specific transduction signals, such as IKK for NF-κB, that act in concert with
combinations of common transducers to activate specific stress response pathways (Hayden and Ghosh, 2008).

Thus, through these four broad activation mechanisms and their overlaps, chemical toxicants will produce patterns of dose-dependent activation of the stress response pathway ensemble to produce a pattern of activation or a “signature” that is potentially chemical specific as depicted in the simulated data in Figure 2. Conversely, an examination of the global stress response pathways activity as a group or an ensemble over a range of doses is likely to show patterns of stress gene activation that may be specific for a compound or for a group of compounds that share a common mode of toxic action. This forms the basis for the screening approach discussed below.

STRESS RESPONSE PATHWAY ENSEMBLE AS A MEASURE OF IN VITRO TOXICITY RESPONSE

Much of the work completed to date on stress response pathway activation has focused on identifying the mechanistic underpinnings of single pathways using well-characterized chemical activators. This work has led to a basic understanding of the key molecular events that occur during activation of the various stress response pathway signaling cascades. These efforts now allow for a comprehensive strategy to be implemented that exploits the key features of the common stress response pathway architecture to engineer in vitro assays that can be used for high-throughput toxicity testing. An ensemble of these assays would effectively monitor the coordinated activation of multiple pathways and thereby provide a method to assess the potential of a compound to cause toxic responses in vivo. A stress assay ensemble approach has the potential to reveal stress response pathway activation patterns, generating compound-specific “stress signatures” that could then be used to cluster chemicals with similar stress response pathway activation profiles. Stress signatures could then provide mechanistic clues of the underlying mode/mechanism of action of a toxicant.

The common elements of stress response pathway architecture provide convenient points for assay development. Whereas many of the key stress response pathways share multiple transduction signals in common, the signaling information conveyed in an activated stress response pathway converges on a pathway’s key TF. The TF therefore serves as a pathway-specific nodal indicator of stress response pathway activation. Stimulation of a stress response pathway leads to the activation of its key TF which then directs the expression of unique set of target genes. This observation avails two attractive strategies to measure stress response pathway activation, both employing reporter gene technology. One strategy would be the use of promoter elements derived from the unique target genes themselves to drive the expression of a surrogate reporter protein, such as firefly luciferase that provides a conveniently-detected and quantitative readout. Many stress-responsive target genes are expressed at low levels under normal cellular conditions, but are rapidly and robustly induced following stress response pathway activation. This characteristic of stress-induced target genes enables the use of reporter gene assays with excellent signal-to-background ratios, a critical requirement for a successful high-throughput assay. Putative genomic promoters that signal the activation of each of the major stress response pathways are listed in Table 1. The second related strategy is to direct reporter gene expression using synthetic promoters containing multimerized REs for the key TF of a given stress response pathway; the canonical RE sequences for the stress responsive TFs are also shown in Table 1. The induction of genomic promoters are tightly regulated by complex interactions of multiple TFs, many of which are not stress-induced but work cooperatively with the central stress-activated TF for each pathway as exemplified by the HMOX1 discussed earlier. The use of synthetic promoters largely isolates the activity of a single TF, in this case the stress-induced TF. Combining these two reporter gene strategies captures the specificity (synthetic promoters) and sensitivity (genomic reporters) of stress response pathway activation (Simmons and Ramabhadran, 2007). Further, because reporter genes can be delivered by highly efficient and portable vector systems such as lentiviral vectors, these reporter genes can be assessed across a broad variety of cell types including established cell lines and primary cells (Simmons and Ramabhadran, 2008) as well as more metabolically competent cell models such as primary hepatocytes.

The effort to understand the signaling mechanisms underlying stress-responsive gene promoter activation led to the development of reporter gene assays whose utility in identifying stressors and studying toxicant MOA is well-documented. A reporter gene driven by the human hsp70 promoter was used to identify and study diverse chemical toxicants such as heavy metals, diamines, and organochlorines such as chlorophenol derivatives, tetrachlorohydroquinone, and 1-chloro-2,4-dinitrobenzene (Ait-Aissa et al., 2000; Boellmann et al., 2004). An HMOX1 reporter construct has been used to measure oxidative stress response resulting from exposures to heavy metals such as cadmium and oxidizers such as tert-butylhydroquinone (t-BHQ) and phenylarsine oxide (Alam et al., 1996; Palmiter, 1994). Environmental toxicants such as copper chloride and cobalt chloride (Martin et al., 2000; Koizumi et al., 2007; Shan et al., 1999). Similarly, reporter genes for p53 targets such as GADD45A have been used to identify DNA-damaging agents such as carboplatin, etoposide, 2,4-dichlorophenol and methyl nitrosourea (Hastwell et al., 2006; Singh et al., 2007; Walmsley, 2008). Synthetic reporter gene constructs activated by HIF-1 have been used to detect hypoxia-inducing chemicals such as copper chloride and cobalt chloride (Martin et al., 2005; Mojsilovic-Petrovic et al., 2007). MTF1-responsive reporter genes detect not only toxic levels or metals such as cadmium, zinc, and copper, but also oxidative stressors such as t-BHQ (Dalton et al., 1996; Palmiter, 1994). Environmental toxicants such as manganese, lead and dioxin have been shown to stimulate a NF-kB synthetic reporter gene (Pyatt et al., 1996; Ramesh et al., 2002; Yao and O’Dwyer, 1995). To date, these
reporter genes have been employed in stand-alone assays to measure the activation of a single stress response pathway in response to toxicant exposure. An ensemble of selected stress reporter gene assays that concurrently monitor the status of the key stress response pathways globally by high-throughput screening would enable prioritization of the large inventory of untested chemical toxicants for targeted testing in more complex assays. Such an approach would move beyond the identification of particular toxicants with single stress response pathways to a paradigm wherein a more comprehensive stress signature, specifically one that captures comparative stress response pathway activation and cross-talk, can be obtained (Fig. 2).

Previous studies have demonstrated the feasibility of a singular stress reporter assay approach to identifying chemicals with potential toxic properties. There have also been efforts to package individual assays into a comprehensive battery (Dorsey et al., 2002; Fischbach and Bromley, 2001). The CAT-Tox panel of 14 stress-based reporter genes using a laborious and now outdated detection method was developed by Todd et al. (1995). The approach proposed here couples a refined list of promoters that cover the major stress response pathways with improved reporter gene assay technology ideal for high-throughput or ultra high-throughput screening. Preliminary data show that the stress assay ensemble can be used to measure the activation of several stress-activated genes in response to diverse chemical insults (Simmons and Ramabhadran, 2007, 2008), and that chemical toxicants produce compound-specific stress signatures. It is anticipated that comparison of the stress signatures produced by well-studied chemical toxicants with known MOAs with those of newly-tested chemicals (of unknown MOAs) will provide a starting point for generating hypotheses of MOAs. The reporter assay format is inexpensive and can be fully automated, making it amenable not only to screen large numbers of chemicals, but also to generate dose-response and/or exposure duration (time course) data. Additionally, cell type specific responses of the stress ensemble in a range of human cell lines derived from different tissues have been examined (Simmons and Ramabhadran, 2008). Significant commonalities were observed, but also notable differences, namely in the minimal dose, maximal extent of activation, and the relative activation points of different pathways in the stress signatures of the compounds tested across cell lines. Such testing in a range of cell lines can potentially help identify the target tissues for the toxicant.

The stress assay ensemble will be particularly useful for the study of chemical mixtures from two perspectives. First the stress response signatures can be used in parallel to concurrently measure the dose-dependent activation of stress response pathways (A). Graphical depictions of the individual pathway dose responses and their conversion to heat maps which facilitate visual comparison of stress response signatures. (B) A comparison of stress signatures of two hypothetical pro-oxidant compounds. Compound X induces the oxidative stress and metal stress response pathways (red pixels) at lower concentrations, activates additional stress response pathways at higher concentrations, and is cytotoxic at the highest concentration (green pixels). In contrast, Compound Y triggers the oxidative stress and inflammation response pathways at lower concentrations with only modest activation of additional stress response pathways and no observed cytotoxicity.

**FIG. 2.** Implementation of the stress response assay ensemble (simulation). Individual stress pathway assays can be used in parallel to concurrently measure the dose-dependent activation of stress response pathways (A). Graphical depictions of the individual pathway dose responses and their conversion to heat maps which facilitate visual comparison of stress response signatures. (B) A comparison of stress signatures of two hypothetical pro-oxidant compounds. Compound X induces the oxidative stress and metal stress response pathways (red pixels) at lower concentrations, activates additional stress response pathways at higher concentrations, and is cytotoxic at the highest concentration (green pixels). In contrast, Compound Y triggers the oxidative stress and inflammation response pathways at lower concentrations with only modest activation of additional stress response pathways and no observed cytotoxicity.
obtaining compound signatures, study of mixtures as well as for gaining insights to the MOA as discussed further below.

FIG. 3. Use of stress response profiles to identify chemicals with similar activity patterns (simulation). (A) Stress response profiles of eight hypothetical compounds. Compounds can be profiled across the stress reporter assays using a dose-response metric such as a LOEL or 50% activity concentration (AC50). (B) Clustering techniques such as hierarchical clustering (shown here) can be used to group together those chemicals with similar stress response profiles. Compounds with similar stress response profiles may likely share a common MOA. (The color scheme for the heat map for this figure has been deliberately chosen to be different from Fig. 2 to emphasize the difference between the concepts addressed in these figures).

obtaining compound signatures, study of mixtures as well as for gaining insights to the MOA as discussed further below.

INSIGHTS INTO MOA FROM STRESS RESPONSE PATHWAY ENSEMBLE ANALYSIS

MOA is defined as “a description of key events or processes by which an agent causes a disease state or other adverse effect” and it is distinguished from “mechanism of action,” which is defined as “a detailed description, often at the molecular level, of the means by which an agent causes a disease state or other adverse effect” (NRC, 2007, p. 38). From this perspective, the stress response pathways represent a set of homeostatic pathways that are named according to key toxicity events elicited in cells, such as oxidative stress, genotoxic stress, metal stress, ER stress, etc. These key events can hence be considered as a starting point for MOA definition because the stress response pathways are linked to adverse cellular effects. The preliminary MOA information gained from the screens can also be useful for more detailed mechanistic studies where desirable.

As discussed in an earlier section describing transgenic and knockout animal studies delineating the importance of stress response pathway regulation during development, persistent alterations in the stress response pathways can lead to adverse effects at cellular and whole organism level. Further, the alterations in stress response pathways have been linked to many
**TABLE 4**

<table>
<thead>
<tr>
<th>DNA construct</th>
<th>Compound</th>
<th>Induction pattern</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transgenic mice</td>
<td>Hsp70-luc</td>
<td>Cadmium</td>
<td>Lung</td>
</tr>
<tr>
<td></td>
<td>Hsp70-hGH</td>
<td>Metals</td>
<td>Blood</td>
</tr>
<tr>
<td></td>
<td>H01-luc</td>
<td>Chloroform</td>
<td>Kidney</td>
</tr>
<tr>
<td></td>
<td>NF-κB-luc</td>
<td>Silica</td>
<td>Epithelial cell</td>
</tr>
<tr>
<td></td>
<td>p53-luc</td>
<td>γ-irradiation</td>
<td>Intestine, spleen, liver, etc.</td>
</tr>
<tr>
<td>Transgenic Zebrafish larvae</td>
<td>hsp70-GFP</td>
<td>Cadmium</td>
<td>Gill, skin, liver, olfactory tract</td>
</tr>
<tr>
<td></td>
<td>hsp70-GFP</td>
<td>Copper</td>
<td>Gill, skin, retina, myotube</td>
</tr>
<tr>
<td></td>
<td>hsp70-GFP</td>
<td>Arsenic</td>
<td>Gill, skin, liver, olfactory, neuronal cell, retina, myotube</td>
</tr>
<tr>
<td></td>
<td>hsp27-GFP</td>
<td>Arsenic</td>
<td>Heart, muscle</td>
</tr>
</tbody>
</table>

Secondly, this method for clustering chemicals may allow for the detection of modes or mechanisms not directly measured by the constituent assays, thus potentially eliminating the need to design assays that measures every conceivable mode or mechanism. The inclusion of additional mechanistically-based assays such as stress response assays will undoubtedly expand the potential of this strategy to correctly group chemicals based on a larger universe of potential biological activities.

**STRESS RESPONSE PATHWAY ACTIVATION AS TOXICITY MEASURE IN VIVO**

A previous section described *in vitro* studies where the promoter elements from stress responsive genes have been used to create reporter genes that are useful in measuring stress response pathway activation in cultured cells. Many of these reporter genes were subsequently used to engineer transgenic mice that have proved useful for toxicology studies. Although stress response pathways activation in cultured cells serve as an indicator of cell-autonomous toxicity, the use of transgenic animal models expressing stress reporters harnesses the advantages of stress response pathway-based assays with the integrated systems biology of an intact organism. These models permit the real-time imaging of live animals and hence multiple-time-point monitoring of animals following exposure to toxicants thereby reducing the number of animals needed for such studies. The primary advantage of using live stress-reporter animals is the evaluation of stress response pathway activation at an organ systems level that accounts for the combined pharmacokinetic and pharmacodynamic parameters of toxicant exposure that cannot be incorporated into *in vitro* assays. The efficacy of this approach has been validated by a series of excellent examples in literature discussed below, where dose-response and exposure duration effects of toxic events were measured in transgenic mouse models, and more recently in fish models, expressing recombinant stress reporters (see Table 4).
Transgenic mice expressing a luciferase reporter under the control of the murine heat shock-responsive Hsp70-1 promoter were tested in vivo against a broad range of toxicants to evaluate their ability to induce the heat shock response in the lungs. Although well-characterized inducers such as cadmium and heat activated the reporter gene in concordance with the endogenous Hsp70 gene, other respiratory stressors such as ozone, paraquat, and parathion also triggered a marked heat shock response in these reporter mice (Wirth et al., 2002). Similarly, transgenic mice engineered to express human growth hormone (hGH) protein (as the reporter) under the control of human HSP70 promoter when exposed to sodium arsenite, cadmium chloride, copper sulphate, or methylmercurium chloride by intraperitoneal injection showed elevated plasma hGH levels that correlated well with the toxicant dose (Cannon and Tennant, 1997; Sacco et al., 1997).

Transgenic mice expressing a luciferase reporter under the control of the oxidative stress-responsive mouse heme oxygenase (HO-1) promoter were used to study the effects of inhaled particulate matter employing luminescent quantification analysis to detect luciferase activity (Roberts et al., 2007; Weir et al., 2005). This study demonstrated the utility and the efficacy of the HO-1 luciferase transgenic mouse model to detect particle-induced oxidative lung injury in vivo. However, repeated evaluations were not possible in a real-time mode because detection required the sacrifice of the treated animals. In another study using a similar transgenic mouse model, a noninvasive luminescent imaging technique using firefly luciferase was used to compare the differing effects of chloroform on males versus females and the effects of testosterone treatment on chloroform-induced toxicity (McMullen et al., 2002; Weir et al., 2005). The same approach was also used to study the effects of a diverse set of toxicants such as cadmium, doxorubicin, and thioacetamide (Malstrom et al., 2004). These studies showed that the noninvasive imaging technique allowed for extensive dose-response and time-course study of toxicants effects with repeated real-time imaging of the same animals, thus reducing animal use.

Along the same lines, the mouse synthetic NF-κB-luciferase transgenic mouse model has been used to study the effects of fibrogenic particles, namely silica and asbestos, on inflammatory responses (Hubbard et al., 2001). These studies showed that, in vivo, silica initiates inflammation and epithelial cell alterations through activation of multiple TFs. Similarly, a transgenic mouse line in which firefly luciferase gene expression was driven by the p53-responsive P2 promoter from the Mdm2 gene has been used to non-invasively characterize the genotoxic response to γ-irradiation at the whole animal level (Hamstra et al., 2006). This and similar models may prove to be particularly useful for studying DNA-damaging agents in vivo.

These examples highlight the potential utility of stress reporter transgenic mice in toxicological evaluation of chemicals. Similarly, transgenic reporter mouse lines that report hypoxic stress (Schmid and Young, 2006) and ER stress (Hiramatsu et al., 2006) have been described. Although these and other such animal stress reporter models have a great potential to advance in vivo toxicology, for both MOA studies and toxicological screening, new models using cost-effective alternative species rapidly emerging in other fields such as developmental biology and genetics are also proving useful in toxicology.

Zebrafish (Danio rerio) has emerged as an alternate vertebrate species (Amatruda and Zon, 1999; Lieschke and Currie, 2007) well-suited for in vivo toxicological screening of environmental chemicals. Compared with invertebrate model organisms such as the fly or the worm, fish are more phylogenetically related to mammals, and thus have a higher degree of genetic conservation. In addition, zebrafish offers advantages in cost, labor and screening throughput compared with rodents (Hill et al., 2005; Oxendine et al., 2006; Reimers et al., 2004). Zebrafish and a related species, medaka (Oryzias latipes; Oxendine et al., 2006) offer a number of additional advantages, especially for developmental toxicity studies, including a small body size, high fecundity, ex vivo development, large clutch sizes, short time to reproductive maturity, relatively low cost of husbandry, and lastly transparency of embryos which allows for real-time observation of development. The entire genomes of both fish species have been nearly sequenced, thereby providing the bioinformatics foundation required for genetics and transcriptional studies using techniques such as real-time PCR as well as for the generation of transgenic models. These recent advances have facilitated the generation of both transgenic zebrafish and medaka (Meng et al., 1999; Ozato et al., 1992) and have opened avenues for the application of reporter fish to the screening of environmental compounds.

A notable transgenic zebrafish line employed in toxicological screening is the zebrafish hsp70 promoter green fluorescent protein zebrafish (GFP: Blechinger et al., 2002), which has been used to measure heavy metal toxicity induced by cadmium during larval stage. This study demonstrated that GFP expression acts as an accurate and reproducible indicator of endogenous hsp70 gene induction and that GFP expression was induced in a dose-dependent manner. Another study (Seok et al., 2006) utilized zebrafish embryos transiently expressing GFP under the control of the human HSP70 promoter to detect copper sulfate contamination; these embryos were sensitive enough to detect copper sulfate at doses well below the LC50 concentration. This model system was extended to the detection of arsenite which produced a dose-dependent induction of GFP expression (Seok et al., 2007). Transgenic hsp27-GFP zebrafish, where GFP expression was controlled by zebrafish hsp27 heat shock promoter, also responded to heavy metals and arsenic in a dose-dependent manner and the GFP expression pattern was shown to correlate with endogenous hsp27 mRNA (Wu et al., 2008). These studies show that transgenic adult and embryonic zebrafish stress reporter models are useful to detect and study environmental toxicants.

Although the embryos and larvae are ideal for developmental toxicology studies due to their transparency, use of adult
transgenic reporter zebrafish are often hampered by autofluorescent pigmentation of the fish. However, use of pigmentless mutant fish strains developed recently can help overcome the background fluorescence issues. Exemplifying this point, transgenic adult zebrafish were further engineered to express luciferase or GFP reporter transgenes under the control of synthetic aromatic hydrocarbon response elements (AHREs) and were successfully used to detect dioxin and polychlorinated biphenyl (PCB) pollutants in drinking water (Carvan et al., 2000). More recently, a new “transparent” strain of zebrafish has been reported that should prove equally or more useful than the golden mutant (White et al., 2008).

These studies, although few in number, demonstrate the utility of stress-response reporter fish which respond to chemical toxicants and other environmentally relevant compounds such as engineered nanoparticles for toxicological screening. More work is needed to develop novel transgenic fish models that can be used to study additional stress response pathways and to develop transgenic reporter fish using synthetic promoters that isolate the activity of key stress-activated TFs. Undoubtedly, these models will prove invaluable to studying mechanisms of toxicity using the integrated systems of a whole animal combined with the low cost, rapid assay time and high throughput potential that fish models offer.

SUMMARY

In the foregoing sections, the need for rapid screening of compounds for toxic potential is discussed and how, because of their ubiquitous presence and shared architecture, a small set of cellular stress response pathways can be potentially used for screening toxicants. As is the case with all biological pathways, the stress response pathways are extensively cross-wired and their continued activation in vitro and in vivo can lead to cell death and developmental consequences, thus linking their activation to adverse effects. A scheme for concurrent evaluation the stress response pathways in vitro using reporter gene assays in a rapid and cost-effective manner is proposed and the potential use of transgenic animals expressing stress reporters in toxicology to capture in vivo effects is also discussed.

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REFERENCES


Ma, Y., and Hendershot, L. M. (2004). Herp is dually regulated by both the endoplasmic reticulum stress-specific branch of the unfolded protein response and a branch that is shared with other cellular stress pathways. J. Biol. Chem. 279, 13792–13799.


