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Redox stress and signaling during vertebrate embryonic development: regulation and responses

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Abstract

Vertebrate embryonic development requires specific signaling events that regulate cell proliferation and differentiation to occur at the correct place and the correct time in order to build a healthy embryo. Signaling pathways are sensitive to perturbations of the endogenous redox state, and are also susceptible to modulation by reactive species and antioxidant defenses, contributing to a spectrum of passive vs. active effects that can affect redox signaling and redox stress. Here we take a multi-level, integrative approach to discuss the importance of redox status for vertebrate developmental signaling pathways and cell fate decisions, with a focus on glutathione/glutathione disulfide, thioredoxin, and cysteine/cystine redox potentials and the implications for protein function in development. We present a tissue-specific example of the important role that reactive species play in pancreatic development and metabolic regulation. We discuss NFE2L2 (also known as NRF2) and related proteins, their roles in redox signaling, and their regulation of glutathione during development. Finally, we provide examples of xenobiotic compounds that disrupt redox signaling in the context of vertebrate embryonic development. Collectively, this review provides a systems-level perspective on the innate and inducible antioxidant defenses, as

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Footnote: When referring to gene and proteins we adhere to the Zebrafish Nomenclature Guidelines (<https://wiki.zfin.org/display/general/ZFIN+Zebrafish+Nomenclature+Guidelines#ZFINZebrafishNomenclatureGuidelines-2>). Briefly, genes and proteins from different organisms are presented as such: zebrafish *gene*/Protein, mouse *Gene*/PROTEIN, and human *GENE*/PROTEIN. In cases where no specific model system is being addressed we use the human nomenclature.

well as their roles in maintaining redox balance during chemical exposures that occur in critical windows of development.

Keywords

Nrf2; glutathione; toxicology; pancreas; redox proteomics

1. Introduction

Embryonic development is a complex and highly choreographed process, requiring exquisite temporal and spatial regulation. The challenge to the embryo is not only to control this process but also to shield it from being disrupted by internal or external stressors. Vertebrate embryos have evolved to be especially resilient to perturbation, through what has been called the “intrinsic robustness of developmental programs” in the face of a variable environment [1]. At the same time, experimental studies often show that embryonic and larval stages are among the most sensitive to adverse effects of chemicals (reviewed in [2–6]). The sensitivity of developing animals to chemical toxicants likely results from the complex nature of developmental processes combined with innate and inducible mechanisms that protect against toxic outcomes; for example, xenobiotic-metabolizing and anti-oxidant enzyme systems) are typically not yet fully functional in developing animals [2].

Redox signaling is an important component of developmental processes, and redox status is tightly regulated during development [7–10]. Consequently, disruption of redox status through generation of reactive oxygen species (ROS) or otherwise altering the control of intracellular redox potential (i.e. causing redox stress [11–15]) can interfere with these developmental processes. These disruptions can include altered cell fate decisions that can lead to structural and functional changes in developing animals [9], including in specific tissues such as the pancreas [16].

In this review, we take a multi-level, integrative approach to discuss the roles and regulation of redox signaling during development and its disruption by xenobiotic chemicals. We first summarize the importance of redox signaling and the consequences for developmental outcomes. We provide an overview of biochemical redox systems and modifications, and discuss a class of transcription factors important in the molecular regulation of redox signaling. While there are numerous redox-sensitive cell types, we examine a tissue-level example in which redox modulations affect the development and function of the pancreas. Finally, we provide specific examples of agents that disrupt redox signaling and control in developing vertebrates that result in adverse outcomes. Overall, this review provides a systems-level perspective on innate and inducible antioxidant defenses and their roles in helping to maintain redox balance in the face of chemical threats during the developmental period.

2. Redox signaling vs. stress during development

Intracellular ROS are important components of signaling cascades and regulate numerous physiological processes critical to embryonic development [17–19]. ROS are classically

defined as oxygen-containing molecules with an unpaired electron such as hydroxyl radicals ($\cdot\text{OH}$) and superoxide ($\text{O}_2^{\cdot-}$). However, this definition has been expanded to include nonradical oxygen-containing molecules such as hydrogen peroxide (H_2O_2). The realm of ROS is further expanded when one considers interactions with other molecular structures containing nitrogen or sulphur. For a detailed discussion of the classification of ROS and free radicals we refer the interested reader to the excellent review by Sies et al. [12]. We consider here the impact of reactive species, i.e. oxidants, on redox signaling and the adaptive and adverse outcomes that can result from oxidant stress.

The effects of reactive species are modulated by the presence of robust and diverse antioxidant defenses and the homeostatic redox state of these systems. These vary depending upon the source of the oxidant, the cellular compartment in which they occur, and the proximity of a favorable electron donor. Glutathione, among other antioxidant defenses, has been shown to have specific and sometimes compartmentalized characteristics. For example, GSH redox potential (E_h) in the cytoplasm is different from that in the mitochondria and that in the nucleus [20, 21]. Another critical component that determines the fate of a reactive species is whether the reactive species is a primary vs. secondary oxidant [22]. Primary oxidants are regulated by catalase, peroxidases, and superoxide dismutase; this regulation allows for specific functions within signaling pathways. Secondary oxidants are defined as those that are not well mitigated or controlled, such as the actions of hydroxyl radicals and protein or lipid-based radicals [22]. Collectively, the nature of the reactive species along with the state of antioxidant defenses determines what kind of redox stress or signaling effects will occur.

Redox signaling and redox stress [12–15] can either be active (specific) or passive (non-specific) in nature, with active effects targeting specific signaling pathways, and passive effects causing damage to macromolecules [7]. Taking this idea a step further (Fig. 1), reactive species can cause physiologic effects such as prompting cell fate decisions [23] or stimulating insulin secretion in pancreatic beta cells [24], among numerous other effects. Responses can also be adaptive in nature, such as the upregulation of enzymes involved in GSH synthesis and utilization, or through induction of other cytoprotective systems. On the other end of the spectrum, passive damage to macromolecules can cause adverse or toxic responses including cell death or loss of function. It is therefore important to distinguish between redox signaling and redox stress in these effects, particularly in the context of embryonic development.

In the context of embryonic development, it may be more appropriate to use the term “redox stress” [12–15] instead of “oxidative stress.” Often it is the change in redox status of a specific couple, not necessarily whether it is oxidative or reductive, that is important in causing stress in the embryo. For example, the GSH E_h changes dynamically at specific stages of development [25, 26], it is not a simply a directional change in the redox status that can be detrimental. Rather, prevention of a programmed oxidative change or of a rebound in cellular reductive capacity can interfere with the normal developmental program of the cell. This can result in alterations in cell proliferation, cell differentiation, cell migration, cell polarity, or programmed cell death. Therefore, a specific redox signal may signal cellular differentiation of a progenitor cell at one developmental stage, but that same redox signal

might result in apoptosis at a different developmental stage. Any deviation in cellular responses from the normal developmental program can have consequences for cell organization, growth, and ultimately the health and function of the organism. We use the term “redox stress” to capture both oxidative and reductive changes to redox states that may be involved in the perturbation of cellular homeostasis [13, 14].

3. Importance of redox status to developmental signaling pathways and cell fate

Embryogenesis is characterized by defined developmental stages. A critical developmental stage is the period of organogenesis, during which cells become more terminally differentiated in structure and biochemical function. Preservation of spatial and temporal signaling is a prerequisite for normal organogenesis. Conversely, disruption of critical signaling events can result in structural abnormalities, loss of cellular function or spontaneous abortion of the developing conceptus.

Redox status has an important role in regulating cellular differentiation during development, and early experiments have revealed the importance of redox control on important developmental events, including proliferation, differentiation, apoptosis, and left-right asymmetry [7, 8, 18, 26–31]. Below, we review some of the major couples that are involved in redox reactions and states of the cell and their relationship with developmental events.

3.1. Redox potentials during development

Intracellular redox status can be a potential indicator of cellular function. While many redox couples are relatively small molecules, their effect on the development of the proteome can be quite significant. In general, most research in this area has focused, albeit not exclusively, on those couples with cysteine residues. Cysteines can be easily oxidized, and then reduced, to facilitate detoxification of reactive species. In many cases, the oxidized and reduced forms contribute directly to the redox potential (E_h), which can be calculated using the Nernst equation. The E_h is defined as the half-cell reduction potential and the reducing capacity of a specific redox couple [27]. More negative E_h values represent a greater reducing capacity than more positive values, which exhibit a greater oxidizing capacity. As these numbers fluctuate, they exhibit a level of regulatory control of redox-sensitive elements. Interestingly, each individual couple exhibits a unique midpoint potential (E°), and thus, each couple is capable of exercising independent regulation of controllable redox sensitive systems [11, 32].

One of the basic tenets of developmental toxicology is that an embryo will exhibit greater sensitivity to exogenous toxicants during specific periods, or “critical windows,” of development [33]. Embryos are highly susceptible to toxicity during organogenesis, and redox states are also more susceptible to dysregulation during this period. Specific factors that disrupt redox status and contribute to poor developmental outcomes can fall into one of three subcategories: 1) environmental, 2) pharmaceutical/recreational or 3) physiological influences. Environmental influences include exposure to pesticides, heavy metals, industrial waste and nanoparticles [34–38]. Pharmaceutical or recreational drugs such as specific

therapeutics (phenytoin, valproic acid), chemotherapeutics (thalidomide, cyclophosphamide, hydroxyurea), environmental exposures (tobacco smoke, nanoparticles) and recreational drugs (ethanol, and cocaine) [37, 39–47] with oxidizing properties can also cause developmental disruptions. Lastly, physiological influences such as temperature and hypoxia can disturb redox states; these are usually associated with specific illness or disease, such as high glucose levels in diabetics [48–50]. While the nature of the influence may dictate the susceptibility of certain redox couples, the disruption of specific redox states can result in the loss of specific signaling pathways. Thus, expanding our understanding of the redox couples provides insight into mechanisms of control and dysfunction.

3.1.1. GSH/GSSG—Redox regulation of development has been most widely studied in the context of the glutathione (GSH) and glutathione disulfide (GSSG) redox couple. GSH is a tripeptide of glutamate, cysteine, and glycine, where glutamate and cysteine are linked through the gamma (γ) configuration in the R-group of glutamate, rendering GSH insensitive to degradation via intracellular peptidases. Glutathione synthesis occurs as a result of two enzymatic processes regulated by glutamylcysteinyl ligase (GCL) and glutathione synthetase (GSS) [51]. The GCL catalyzed reaction requires glutamate and cysteine, where cysteine is considered the rate-limiting precursor. The ratio of GSH to GSSG is an indicator of intracellular redox status but is best described as an E_h . GSSG is recycled by glutathione reductase (GSR) back to GSH. GSH is an essential regulator of embryogenesis and cell cycle progression [52–57]. In GCL knockout mouse embryos, GSH is not produced and embryos die during gastrulation [53]. Even beyond early whole-conceptus development, cell fate decisions have been shown to be closely related to GSH E_h , with more oxidized E_h associated with differentiation, and more reduced E_h with proliferation [58–60]. These results are suggestive that GSH does not merely act as an antioxidant detoxifying reactive chemical species, but rather may serve other regulatory developmental roles.

Early studies designed to better understand the role of GSH E_h in cellular function focused initially on cellular proliferation. For example, in one study where normal fibroblasts were grown at low density (20% confluence), GSH E_h was measured to be -220 mV; however, as cells became increasingly confluent, proliferation slowed due to contact inhibition, and the GSH E_h shifted to a more oxidized state of -185 mV at 100% confluence [61]. By comparison, transformed fibrosarcoma cell lines that are not influenced by contact inhibition and continually proliferate did not show any changes to their GSH E_h , which was maintained regardless of cell density [61]. Artificial reduction of the GSH E_h through supplementation of cysteine precursors (L – 2-oxothiazolidine-4-carboxylic acid [OTC] and N-acetylcysteine [NAC]) produced pro-reducing shifts of -10 mV and -8 mV, respectively. More reducing GSH E_h supported sustained proliferation [61]. Treatment of a GSH synthesis inhibitor, buthionine sulfoximine (BSO), oxidized GSH E_h by $+24$ mV, which correlated with a decrease in proliferation by over 50% [61]. Although specific redox-sensitive targets have not yet been fully characterized, changes in GSH E_h correlate with a decrease in ROS production during M phase of the cell cycle compared to quiescent and S phase cells, suggesting direct GSH E_h control of the cell cycle [62].

In general, the embryo is most susceptible to redox dysregulation during the period of greatest differentiation (gastrulation/organogenesis). Within the embryo, as cells begin to differentiate into specific germ layers, shape and function shift toward more distinct characteristics specific to a cellular phenotype. Early studies to better understand the role of GSH E_h began by measuring the redox potentials of differentiating cells in culture. In 3T3-L1 preadipocytes, undifferentiated cells contain substantially more reduced GSH than fully differentiated adipocytes; concomitantly, GSSG concentrations increased by nearly three-fold during differentiation [63, 64]. Together, these changes in GSH and GSSG concentrations produced a GSH E_h shift of nearly +22 mV [64]. Although pre-differentiation redox potentials vary, similar incremental shifts in GSH E_h have also been observed in different types of cell culture models of differentiation, including myogenic and gastroenterocytic cells [60, 65, 66]. In more recent work, promotion of osteoclast differentiation could be exacerbated through loss of nuclear factor (erythroid-derived 2)-like 2 (NRF2) signaling (*Nrf2*^{-/-} cells), increased ROS generation and oxidation of GSH E_h [67]. Interestingly, in this same differentiation model, pretreatment with the antioxidants, NAC or diphenyleneiodonium, reduced differentiation in both NRF2-sufficient and -deficient osteoclasts, suggesting a direct role of GSH E_h in osteoclast differentiation. Together, these various studies support a regulatory role of GSH E_h in differentiation of numerous phenotypes.

While the concept of GSH E_h regulation of differentiation originated from *in vitro* work in cellular models, recent studies with zebrafish embryos *in vivo* support the *in vitro* cell differentiation data. During zebrafish (vertebrate) development (0–120 hours post fertilization; hpf), changes to GSH E_h were observed during periods of highest embryonic differentiation [25] (Fig. 2). At 0 hpf, GSH E_h was approximately -230 mV, but these values became increasingly oxidized to -175 mV by 18 hpf. After 18 hpf, GSH E_h slowly became more reduced, approaching 0 hpf levels by 72 hpf. Thus, more oxidized GSH E_h correlated with important developmental periods characterized by rapid differentiation, most notably gastrulation and organogenesis, occurring around 5–10 hpf and 10–24 hpf, respectively. Between 24–48 hpf in the pharyngula period, a period of finer differentiation and morphogenesis, GSH E_h was slightly more reduced but remained oxidized compared to pre-differentiation levels. Interestingly, during the period of greatest differentiation (0–24 hpf), total GSH was relatively low compared to latter periods of development; this may suggest that after exposure to an oxidant, maintenance of specific developmental GSH E_h may be more difficult earlier in development than during embryonic stages highlighted by less differentiative events (Fig. 2).

It is important to note that the GSH E_h is directed by enzymes that synthesize, recycle, and utilize GSH in other reactions, as well as by the cofactors required for such reactions. Therefore, the interpretation of the data provided by the Nernst equation must be considered in the context of the biological system, and not by simple thermodynamic relationships [68, 69]. The GSH E_h is the product of enzymes functioning within the system, but is not necessarily in equilibrium with other redox couples or protein thiol modifications. However, the GSH E_h is still one of the most well-regarded and predictive measures that provides important information regarding redox status and the biological response to an oxidant.

3.1.2. Thioredoxin—Thioredoxins (TRX) are a family of oxidoreductases capable of reducing oxidized protein substrates. This is made possible by a thioredoxin motif (-CGPC-), which plays an important role in its biochemical function. Cysteine residues in proteins can be oxidized to sulfenic acids (PrS-OH) during periods of oxidative stress. In some cases, TRX can reduce protein substrates and restore protein redox states. Much attention has been given to TRX specifically for its potential regulatory role in the maintenance of redox-sensitive transcription factors, including nuclear factor kappa B (NF- κ B), AP-1 and NRF2 [21, 70–73]. Since many of these transcription factors are important during development, TRX-mediated control of redox-sensitive transcription factors is likely a central function of the TRX couple. However, its role as an oxidoreductase extends beyond basic regulation of redox-sensitive transcription factors. In conjunction with the peroxiredoxin family of proteins, TRXs can detoxify ROS and directly mitigate oxidative stress, thereby regulating the availability of important oxidative second messengers, such as H₂O₂ [74].

Less is known about the function of TRX during development. Knock-out of Trx1, the cytosolic and nuclear TRX, is embryo-lethal and mice die early in gestation [75]. Interestingly, deletion of Trx2, the mitochondrial TRX, does not cause embryo death until later in gestation, when massive apoptosis occurs on gestational day (GD) 10.5 [76]. This timing coincides with the maturation of the mitochondria and the increasing reliance on aerobic metabolism. Many ROS are produced through an active electron transport chain, and thus, Trx2 is likely required to mitigate ROS over-production and damage. Measuring Trx2 redox states during early to mid-organogenesis shows a distinctive shift in Trx2 E_h beginning between GD 9–10 [77], further suggesting a supportive role to reconcile a shifting metabolism from glycolysis to oxidative phosphorylation.

3.1.3. Cys/CYSS—Cysteine (Cys) and cystine (CySS) are small biothiols that typically make up 2–10% of the total intracellular small biothiol concentrations. Even though they only constitute a small fraction of the total small biothiols, they are believed to play a role independent of other thiols in redox signaling [78]. While related, Cys E_h and GSH E_h have been shown to be independently regulated during cellular differentiation. For example, in one study using human mesenchymal stem cells, cells were differentiated into either osteocytes or adipocytes. Glutathione E_h was followed over a 21 day period and showed redox trends similar to differentiation-related redox shifts seen in other cell lines, where intracellular GSH E_h became increasingly oxidized with terminal differentiation, beginning at –260 mV (day 0) and terminating at –230 mV (day 21) [79]. Interestingly, in cells undergoing adipogenesis, Cys E_h , like GSH E_h , became increasingly oxidized, from –160 mV (day 0) to –148 mV (day 21), but in cells undergoing osteogenesis, Cys E_h became increasingly reduced, from –160 mV (day 0) to –190 mV (day 17). These findings demonstrate—at least in the context of pluripotent cells—that various couples exhibit unique, independent redox potentials and thus support the redox regulation of specific cell fates through specific couples.

3.2. Implications of GSH homeostasis for protein function

As the field of redox developmental biology continues to grow, a better understanding and appreciation for different redox nodes of control will strengthen our ability to identify critical and susceptible redox-sensitive pathways and periods. For now, most information on redox regulation of cellular function is centered on the GSH E_h -mediated control of the redox proteome. Protein cysteine residues help to maintain protein structure and function, and they can be reversibly modified with GSH (*S*-glutathionylation/mixed disulfides; Pr-SSG). This key post-translational modification can control protein function [80]. However, not all Cys residues in proteins are targets of *S*-glutathionylation; generally, cysteines with a low pK_a can form a thiolate anion ($\text{Pr-SH} \rightarrow \text{Pr-S}^\bullet$) making them more prone for *S*-glutathionylation.

S-glutathionylation of susceptible target proteins can be mediated through changes to GSH E_h , following direct oxidation of critical residues or catalyzed through glutaredoxin (Grx) [81]. As GSH E_h is oxidized, GSSG accumulates and the environment becomes increasingly pro-oxidizing. This promotes oxidation of susceptible Cys residues and increases *S*-glutathionylation (Fig. 3). Alternatively, overproduction of ROS can directly oxidize Cys residues into a sulfenic acid (Pr-SOH). In the presence of reduced GSH, the protein sulfenic acid can be converted to a GSH conjugate and yield water ($\text{Pr-SOH} + \text{GSH} \rightarrow \text{Pr-SSG} + \text{H}_2\text{O}$). During periods of heavy ROS production, sulfenic acids can be further oxidized into sulfinic acids (Pr-SO₂H). While Pr-SOH are more easily reversed and reduced, Pr-SO₂H reduction is generally slower and dependent on the action of enzymes, such as sulfiredoxin (Srx) [82]. However, oxidation to a sulfonic acid (Pr-SO₃H) is believed to be irreversible, resulting in complete loss of protein function. By shunting Pr-SOH to an *S*-glutathionylated state, proteins can be largely protected from partial or complete deactivation to more oxidized species (e.g. Pr-SO₂H or Pr-SO₃H). Lastly, *S*-glutathionylation of cysteines can also occur through glutaredoxin (Grx) activity, where Grx mediates a reaction between glutathione radicals (GS[•]) and the target protein Cys residue.

Reversal of *S*-glutathionylation can be accomplished either through changes to the GSH E_h or enzymatically (Grx). *S*-glutathionylated proteins can interact with a pro-reducing GSH E_h , where GSH is highly abundant and generates a reducing environment. Under this redox environment, *S*-glutathionylated proteins are reduced back and GSSG is generated ($\text{Pr-SSG} + \text{GSH} \rightarrow \text{Pr-SH} + \text{GSSG}$), restoring original protein redox states. Glutaredoxins are a family of proteins that are similar to the TRX family, containing a similar active motif, -CPYC-, but use GSH as a co-factor in their reactions [83]. For example, Grx can mediate protein de-*S*-glutathionylation by using reduced GSH as a co-factor for protein GSH removal, yielding GSSG as a byproduct. Glutathione disulfide can then be reduced into two GSH through the enzymatic reactions of glutathione disulfide reductase (GSSG-Rd) [51].

Since GSH E_h is known to fluctuate during development, *S*-glutathionylation of protein targets during differentiation may serve as an important regulator of many developmental programs. As with many known human developmental toxicants and oxidative environmental insults, untimely shifts in GSH E_h often occur [32]. These abnormal changes to the intracellular environment may result in aberrant temporal and spatial redox-sensitive signaling that may promote dysmorphogenesis, functional loss, or embryo-lethality.

4. Tissue-specific ROS regulation and responses during development: Pancreatic beta cells

A fine line distinguishes the ability of reactive species to act as redox signals or to cause redox stress. This ability can be cell-specific, being largely dependent on the robustness of the antioxidant defense systems of the cell type. As a whole, developmental antioxidant enzyme activities vary dependent upon gestational age and may play a pivotal role in specific tissue development and cellular differentiation [84–86]. While there are many examples of embryo tissues that use reactive species for signaling during development, pancreatic beta cells are reported to have relatively low antioxidant defenses, and oxidative stress is a key mechanism underlying beta cell dysfunction in diabetes [17, 87–89]. Beta cells are organized in clusters in the Islets of Langerhans, a highly vascularized structure, facilitating delivery of exogenous compounds *in vivo*. For these reasons, beta cells may be highly susceptible to redox stress, especially during the developmental period. Here we focus on the roles of redox signaling in pancreatic beta cell development and function.

4.1. A fundamental role for redox signaling in development of pancreatic beta cells

Pancreas development is regulated by a variety of molecular signals reviewed in detail elsewhere (e.g. [90, 91]). A key redox-sensitive transcription factor essential for pancreatic development is pancreatic and duodenal homeobox 1 (PDX1), which signals differentiation of pancreatic precursor cells, distinguishing them from other endoderm cells in the developing gut. PDX1 becomes progressively restricted to beta cells where it regulates the promoter activity of *preproinsulin* [92]. PDX1 is essential to the maintenance of beta cell function and survival, with ablation of its signaling leading to pancreatic agenesis [93].

The activity of PDX1 is sensitive to redox signaling. During hyperglycemia, as blood glucose and ROS climb, the DNA binding activity of PDX1 decreases; this activity is restored with antioxidant treatments such as NAC [94, 95]. Kaneto *et al* [94] further showed that increased ROS in rat islets caused activation of the c-Jun N-terminal kinase (JNK) pathway, leading to a marked reduction of insulin production, and blocking the JNK pathway had a cytoprotective effect on beta cells experiencing oxidative stress.

Developmental exposure to pro-oxidants has also been shown to affect beta cell growth *in vivo*. Sant *et al*. [96] recently demonstrated a reduction in beta cell mass and a shortened pancreas phenotype in the zebrafish embryo after developmental exposure to *tert* butyl hydroperoxide. Hoarau *et al* showed that H₂O₂ specifically activated the ERK-1/ERK-2 pathway in the developing pancreas and initiated beta cell differentiation [17]. Additional support for the importance of H₂O₂ redox signaling can be found in a study by Rovira *et al*. [97], in which treatment of zebrafish embryos with the pharmaceuticals disulfiram and methiopropamine resulted in disrupted proliferation and premature differentiation of pancreatic beta cells, and thus, smaller islets. These pharmaceuticals inhibited the conversion of retinaldehyde to retinoic acid; retinoic acid can ablate H₂O₂ mediated cell signaling in embryonic stem cells. Another study found that undernourished rat fetuses had small islets with high levels of activated ERK-1/ERK-2, further implicating ROS in beta cell development [98]. While a shift towards oxidative conditions in the beta cell can interfere

with development and function of these cells, a reduction or loss of redox signaling due to inappropriate reductive redox conditions is similarly detrimental. For example, exposure to high concentrations of NAC during pancreatic development in rats reduced the number of pancreatic progenitor cells; this finding persisted into adulthood, as rats exposed to NAC *in utero* had a 2.5- fold decrease in the number of beta cells [17].

4.2. Redox signaling modulates beta cell function

Modulation of antioxidant and cytoprotective gene expression has also been shown to affect the function of beta cells. Mitochondrial ROS triggers glucose-stimulated insulin secretion [99]; conversely, an increase in cellular antioxidant defenses is associated with impaired insulin secretion [99, 100]. However, understanding the role of redox signaling in beta cell function is complicated, with numerous contradictory findings. Some studies report that low levels of oxidative stress are required to stimulate insulin production [101–104]. *Nrf2*-null mice have lower serum insulin and elevated blood glucose [105], while Kelch-like ECH-associated protein 1 (KEAP1) knockdown mice that constitutively activate NRF2 on the Leptin-deficient background (ob/ob) have increased antioxidants but exacerbated systemic insulin resistance [106]. Others have demonstrated that loss of NRF2 in beta cells aggravates diabetes, while repeated NRF2 activation attenuates diabetes [107, 108]. In a hypomorphic KEAP1 knockdown model, constitutive activation of NRF2 had a cytoprotective effect on pancreatic beta cells, making them more resistant to oxidative stress [108]. In addition, when KEAP1 hypomorphs were crossed with a diabetic mouse model strain (db/db), islets with activated NRF2 were highly responsive to glucose and the diabetic phenotype in the db/db mice was rescued [108]. The KEAP1-knockdown mice also were protected against induction of diabetes and obesity by a high-calorie diet [108]. Many factors may explain these contradictory studies including differences in timing, genetic background, endogenous antioxidant defenses, duration and repetition of the oxidative challenge, among others. Additional studies are required to fully understand the role of reactive species in beta cell function, and whether there are any inherent differences between developing beta cells and those found in the mature adult.

5. Regulation of redox signaling during development

During development, redox signaling plays a critical role in cell fate decisions. The cellular response to reactive species depends on existing redox homeostasis and antioxidant defenses. Following oxidative stress, up-regulation of antioxidant defenses (including GSH) can occur in a process referred to as the antioxidant response. More broadly, there are other molecular responses such as other stress-response genes, DNA repair genes, or redox-sensitive post-translational modifications; this is defined as the oxidative stress response (OSR) [109].

5.1. NRF2 and related proteins regulate the oxidative stress response

NRF2 is a CNC-bZIP transcription factor that forms a molecular link between chemical exposure, oxidative stress, and antioxidant defenses such as GSH. It is the “master regulator” of the adaptive OSR [110, 111]. The role of NRF2 in mediating the response to oxidative stress is evolutionarily conserved in metazoans [112–114]. Although not the focus

of this review, NRF2 orthologs have been studied extensively in the invertebrate model species *Drosophila melanogaster* (CncC [115–117]) and *Caenorhabditis elegans* (Skn-1 [118–122]). Although there are some mechanistic differences in how the invertebrate and vertebrate NRF2 proteins are regulated, they share functional roles including in regulation of developmental processes and antioxidant defenses [112–114].

Vertebrate NRF2 is ubiquitously expressed and constitutively bound to KEAP1, which sequesters it in the cytoplasm, targeting it for ubiquitination and degradation. When NRF2 is activated by oxidant-induced disruption of NRF2-KEAP1 interactions, it accumulates in the nucleus and associates with small MAF proteins. NRF2-MAF complexes bind to antioxidant response elements (AREs) in the promoter regions of numerous cytoprotective genes, including those involved with synthesis and maintenance of GSH homeostasis [123–127]. NRF2 is highly pleiotropic, serving functions in diverse processes such as inflammation, cancer, DNA repair, lipid metabolism, Phase II and Phase III metabolism, and autophagy [124, 128–135]. Consequently, disruption of NRF2 function during embryonic development might be predicted to have far-reaching effects.

Most of what we know about NRF2 function is from studies in adult tissues and cells, which have been the subject of extensive investigation [111, 136–138]. We know much less about the roles of NRF2 during embryonic and fetal development [2, 32, 139] but there is an emerging understanding that NRF2 as well as other NRF-related proteins have important roles in maintaining redox homeostasis during development [140]. Studies in mammals have shown that vertebrate embryos have the capacity to respond to oxidative stress with increased expression of anti-oxidant genes [9, 32, 141–144], and in a few cases have demonstrated a specific role for NRF2 through the use of *Nrf2* knockout mice [145, 146]; other studies have inferred a role for NRF2, although without such direct evidence [139, 142, 143]. Studies using partial or complete loss-of-function of zebrafish *Nrf2a* (one of two co-orthologs of *Nrf2* in this species [135]) have shown that this protein is involved in regulation of the oxidative stress response and protection against some oxidants in embryos [2, 135, 140, 147–153]. Results from NRF2 overexpression studies also demonstrate the importance of NRF2 during development. For example, genetic activation of NRF2 through knockdown or knockout of its regulator KEAP1 has dramatic, tissue-specific effects on developing mice [154–156].

Other Nrf family proteins (Nfe2, Nrf1 [Nfe211], Nrf3 [Nfe213] and the related Bach1 and Bach2 proteins) are not as well understood as Nrf2 but may also be involved in regulation of redox homeostasis during development. In contrast to Nrf2-null mice and Nrf3-null mice, which develop normally [157, 158], Nrf1-null mice die *in utero* either by GD 7.5 or GD 17.5 and exhibit a defect in definitive erythropoiesis [159, 160]. Studies in Nrf1^{-/-}/Nrf1^{+/-} chimeric mice show that Nrf1 is required for the development of the liver but not other tissues, that the effect appears hepatocyte-specific, and that Nrf1^{-/-} hepatocytes exhibit increased oxidative stress and undergo apoptosis late in pre-natal development [161]. Disruption of both Nrf2 and Nrf1 results in pronounced oxidative stress, apoptosis, and embryo-lethality at an earlier stage than seen in the Nrf1^{-/-} mice, demonstrating that Nrf1 and Nrf2 both have developmentally important, partially overlapping but distinct roles [124]. Consistent with that, knockout studies show that Nrf1 and Nrf2 regulate distinct sets of

genes [133, 162]. Thus, both Nrf1 and Nrf2 appear to be involved in regulating redox status during development, with non-redundant roles. Nrf3 is expressed in embryos [163, 164] but its role in regulating redox homeostasis during development is not at all understood. Nfe2 is best known for its roles in regulating hematopoiesis [165–167], but a few studies suggest that it may also have redox-related functions during development [168, 169]. The related Bach proteins, which generally act as repressors in opposition to Nrf proteins [170, 171], also appear to be important in developmental regulation of redox homeostasis [172, 173].

5.2. Nrf regulation of glutathione during development

Nrf proteins have an undisputed role in the maintenance of antioxidant defenses and the response to oxidative stress; broadly, activation of Nrf proteins in embryos can provide protection from subsequent oxidant challenges (e.g. [142]); conversely, knockout or knockdown renders embryos more sensitive to oxidant challenges (e.g. [148]). However, until recently, the contribution of Nrf proteins to the natural ontogeny of antioxidant defense systems such as glutathione was largely uncharted. We recently conducted a comprehensive analysis of the role of Nrf1a, Nrf1b, Nrf2a, and Nrf2b in the ontogeny of the glutathione redox system in the zebrafish embryo [140]. Knockdown of Nrf1a or Nrf1b altered glutathione redox state through 72 hpf; GSH/GSSG E_h values in Nrf1b morphants were more oxidized, but otherwise followed the temporal changes observed in controls. In contrast, GSH/GSSG E_h in Nrf1a morphants deviated from these temporal fluctuations, and had a more reduced GSH/GSSG E_h . Knockdown of Nrf2a resulted in an overall oxidized GSH/GSSG E_h , with the greatest impact after 48 hpf. Nrf2b morphant embryos were initially more reduced like Nrf1a morphants, until 24 hpf when they began to resemble control embryos. However, none of these knockdown targets completely prevented the doubling of total glutathione that occurs between 36–48 hpf, suggesting that the various Nrf1 and Nrf2 proteins may act together to regulate GSH redox status during development, or that there are other important regulators of the development of the glutathione redox system besides Nrf1 and Nrf2. Still, these findings demonstrate that deficient Nrf1 and Nrf2 signaling during embryonic development can perturb the glutathione redox system in temporally specific ways, even in the absence of an exogenous oxidant challenge [140].

6. Developmental disruptions resulting from chemical-induced redox stress

Controlled fluxes of oxidized and reduced states during embryonic development influence cell fate decisions such as proliferation, differentiation, apoptosis, and senescence [8, 144, 174]. Deviations from the normal sequence of redox states can result in altered cell fate decisions that impact the organism in ways that range from subtle to extreme, with potentially significant teratogenic or later-life health outcomes [4, 10]. Here we provide an example of a pharmaceutical (Thalidomide) and examples of environmental factors (endocrine disruptors and metals) that have implicated redox stress resulting from exposure to these xenobiotics, and the impact on embryonic development.

6.1. Thalidomide

During the late 1950's and early 1960's, the pharmaceutical Thalidomide was prescribed to pregnant women for many different symptoms, including as an anti-nausea agent for

morning sickness. Originally deemed safe for human use in several countries, Thalidomide caused over 10,000 cases of congenital abnormalities, most notably phocomelia and amelia, the abnormal shortening or absence of the limbs. Other effects were also observed, including central nervous system damage, eye malformations, and internal organ deformities, albeit to a lesser extent [175, 176]. Subsequent studies showed that Thalidomide impacted fetal development at the specific critical window of 34–50 days gestation, causing redox misregulation of the transcription factor NF- κ B. This heterodimer is composed of p50 and p65; when the Cys₆₂ residue in the DNA binding domain of p50 is oxidized, NF- κ B fails to properly regulate gene transcription [177]. Thalidomide was shown to preferentially induce oxidative stress and GSH E_h shifts in sensitive species but not resistant species, which correlated to a loss of NF- κ B signaling [178–180]. Subsequent loss of NF- κ B signaling prevents gene expression required for normal limb bud outgrowth [180].

Other research identifies cereblon (CRBN), a protein that directly binds thalidomide and inhibits its E3 ubiquitin ligase function, as an independent action of thalidomide causing ROS formation [181, 182]. Knockdown of CRBN in zebrafish embryos phenocopied thalidomide treatment of the fish embryos, resulting in truncated pectoral fins and a hypomorphic otic vesicle, while not affecting other aspects of development. Another thalidomide target is prostaglandin H-synthase, which has been shown to form a reactive intermediate that enhances ROS and DNA damage in rabbit embryo culture [183]. Today, thalidomide is carefully monitored to avoid its use in pregnant women, but it has found clinical relevance in the treatment of multiple myeloma and some types of leprosy [184, 185].

6.2. Endocrine Disrupting Chemicals

Bisphenol-A, or BPA, is a widely used polymer additive used to increase the rigidity of plastic products. Research over the last decade has generally confirmed BPA as an endocrine-disrupting chemical, and has also demonstrated that embryonic exposure can cause redox stress. For example, in zebrafish, BPA enhanced formation of hydroxyl radicals and caused lipid peroxidation, glutathione depletion, and reductions in activities of catalase, superoxide dismutase, glutathione-s-transferase, and glutathione peroxidase [186]. BPA exposure has also been shown to alter morphology and islet composition of the pancreas in fetal mice [187]. In *ex vivo* cultures of primary mouse pancreatic islets, BPA exposure decreased the mitochondrial membrane potential, which increased cellular ROS levels and led to apoptosis through activation of the NF- κ B pathway [188].

Another widely used endocrine-disrupting chemical is the pesticide atrazine. Atrazine has particular low-dose effects on developmental outcomes such as altered amphibian population sex ratios [189]. Developmental exposures to atrazine in *Drosophila melanogaster* showed increased ROS generation and altered expression of many antioxidant response genes, including *keap1* and *gss* [190]. In a rat model, atrazine also caused lipid peroxidation in addition to decreasing ATPases activity and altering Ca²⁺ homeostasis [191]. Changes in antioxidant enzyme activity and lipid peroxidation have been noted in carp [192] and zebrafish embryos [193] exposed to atrazine.

Perfluorinated compounds (PFCs) are a class of surfactant chemicals used in a variety of consumer products and fire safety equipment. Despite the phase out of some PFCs over the last 2 decades, PFCs contain carbon-fluorine bonds and are highly persistent. PFCs have been shown to alter cellular redox homeostasis in cultured hepatocytes in a dose-dependent manner [194]. In embryos, PFC exposures have been repeatedly shown to induce oxidative stress and impaired endoderm development [153, 194–196]. The molecular mechanisms that contribute to developmental impacts of PFCs include induction of PPAR and PXR/CAR signaling pathways, as well as the TNF- α /NF- κ B pathway, among others (reviewed in [197]).

6.3. Metals

Human exposure to methylmercury (MeHg) occurs primarily from consuming fish that have bio-accumulated large concentrations in their tissues. The most notorious example of human MeHg poisoning occurred in Minamata, Japan where MeHg released in industrial wastewater between the 1930's and the 1960's poisoned over 10,000 individuals. In adults, health endpoints of moderate poisoning included neurological damage, hearing and speech impairments, and limb ataxia, whereas extreme poisoning cases led to coma or death. Neurological damage and other effects from gestational exposures have also been well characterized and reviewed by Grandjean et al [198]. The mechanisms behind these effects were investigated in a genome-wide transcriptional analysis of exposed zebrafish embryos, and it was postulated that MeHg generally acts as a developmental toxicant by disrupting cellular redox homeostasis [199]. Through RNA sequencing of exposed zebrafish embryos, another study further suggests that binding of MeHg to selenocysteine blocks the availability of selenium and results in altered intracellular redox states [200]. In postnatal animal studies, juvenile mice chronically exposed for 2–6 weeks with MeHgCl had significantly altered glucose-insulin homeostasis, increased ROS in pancreatic islets, and reduced antioxidant-related defenses [201, 202]. Progressively higher levels of ROS generation in the juvenile mouse pancreas led to lipid peroxidation in plasma and islets [201].

Arsenic is a metalloid to which humans are exposed through groundwater wells. Many wells worldwide exceed the World Health Organization's (WHO) maximum recommended level of 10 parts per billion, and high exposure levels have been linked to increased incidence of bladder cancers [203]. Arsenic has been shown to activate the NRF2-mediated antioxidant response system in mouse cerebral cortex and hippocampus [204]. In zebrafish embryos, teratogenic arsenic exposures were associated with reduced GSH content, reduced activities of catalase and glutathione peroxidase, and increased lipid peroxidation [193]. In rats, pregnant dams exposed to arsenite bore pups with female-only effects of altered weight and glucose metabolism, and both lipid peroxidation and glutathione levels were significantly increased in liver tissues [205]. Other developmental toxicology studies in mice (e.g. [206, 207]), chick (e.g. [208]), and South American toad (*Rhinella arenarum*) [209] have noted similar effects.

Collectively, these examples show that developmental exposures to diverse chemicals result in biological changes that converge on shared mechanistic pathways related to redox homeostasis and antioxidant defense systems.

7. Conclusions

The developing vertebrate embryo uses a variety of finely tuned molecular signals to control and regulate organogenesis. Reactive species are one such multifaceted signal for embryonic development with repercussions for cell fate determination, tissue organization and growth. Reactive species may exert passive or active effects contributing to a spectrum of outcomes on redox signaling and redox stress. The application of redox proteomics in combination with biochemistry, molecular signaling, and tissue-specific effects holds great promise for understanding the role of redox signaling during embryonic development, and provides insight into the mechanisms by which redox signaling can be disrupted by developmental exposure to chemicals.

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Abbreviations

ARE	antioxidant response element
BSO	buthionine sulfoximine
E_h	Redox potential
GCL	glutamylcysteinyl ligase
GRX	glutaredoxin
GSH	glutathione
GSR	glutathione reductase
GSS	glutathione synthetase
GSSG	glutathione disulfide
Keap1	Kelch-like ECH-associated protein 1
NAC	N-acetyl cysteine
Nrf2	nuclear factor (erythroid-derived 2)-like 2
OSR	oxidative stress response
PDX1	pancreatic and duodenal homeobox 1
ROS	reactive oxygen species

TRX thioredoxin

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Fig 1.

Effects of reactive oxygen species are mediated by antioxidant defenses and redox state and can have either active or passive actions producing a range of functional or deleterious effects. Low or moderated levels of reactive species such as ROS may have active or specific effects, while high amounts of un-regulated reactive species can cause passive, non-specific damage to cellular targets. Active effects are more likely to control physiologic effects, and passive effects toxic or stressful events. Both redox stress and redox signaling may contribute to adaptive effects such as hyperplasia or upregulation of antioxidant defenses.

Figure 2.

Zebrafish GSH redox ontogeny. Data is compiled from Timme-Laragy et al. (2013) and is reprinted with permission from Hansen and Harris (2015). Important developmental events are highlighted during zebrafish development. Corresponding GSH E_h are measured at various times hpf. Interestingly, the periods characterized by the greatest degrees of differentiation, including gastrulation, organogenesis and pharyngula periods, exhibit more oxidized GSH E_h levels, supporting a direct role in the regulation of specific developmental processes.

Figure 3.

Overview of S-glutathionylation modification of target proteins. Low pKa cysteine residues can be modified via S-glutathionylation, i.e. addition of a GSH moiety. Reaction 1: under reducing conditions (more negative GSH E_h), S-glutathionylated proteins can be reduced through reactions with GSH to yield the reduced protein and GSSG. Additionally, reaction 1 can also be performed enzymatically through the action of glutaredoxin (Grx). Reaction 2: under oxidizing environments (more positive GSH E_h), reduced target proteins can be reversibly modified through GSSG reaction with target cysteines via S-glutathionylation. H₂O₂, an oxidant, can act directly on the protein to promote the formation of -SOH.

Reaction 3: S-glutathionylation of sulfenic acid moieties with GSH yield an S-glutathionylation derivative and water. Reaction 4: reduction of sulfenic acid containing target proteins through the oxidoreductase activities of Trx proteins. Trxs become oxidized and can be reduced enzymatically through thioredoxin reductases and NADPH (not shown). Reaction 5: reduction of sulfinic acid containing target proteins into sulfenic acids through sulfiredoxin (Srx). Conversion to sulfenic acids is a slow reaction but is ultimately reversible. Reaction 6: Hyperoxidation of target proteins to yield sulfonic acid residues. These modifications are irreversible and result in loss of protein function.

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