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Review

Glutathione during embryonic development ☆

Jason M. Hansen [a,*], Craig Harris [b]

[a] Department of Physiology and Developmental Biology, College of Life Sciences, Brigham Young University, Provo, UT 84602, United States
[b] Department of Environmental Health Sciences, School of Public Health, University of Michigan, Ann Arbor, MI 40109-2029, United States

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ABSTRACT

Background: Glutathione (GSH) is a ubiquitous, non-protein biothiol in cells. It plays a variety of roles in detoxification, redox regulation and cellular signaling. Many processes that can be regulated through GSH are critical to developing systems and include cellular proliferation, differentiation and apoptosis. Understanding how GSH functions in these aspects can provide insight into how GSH regulates development and how during periods of GSH imbalance how these processes are perturbed to cause malformation, behavioral deficits or embryonic death.
Scope of review: Here, we review the GSH system as it relates to events critical for normal embryonic development and differentiation.
Major conclusions: This review demonstrates the roles of GSH extend beyond its role as an antioxidant but rather GSH acts as a mediator of numerous processes through its ability to undergo reversible oxidation with cysteine residues in various protein targets. Shifts in GSH redox potential cause an increase in S-glutathionylation of proteins to change their activity. As such, redox potential shifts can act to modify protein function on a possible longer term basis. A broad group of targets such as kinases, phosphatases and transcription factors, all critical to developmental signaling, is discussed.
General significance: Glutathione regulation of redox-sensitive events is an overlying theme during embryonic development and cellular differentiation. Various stresses can change GSH redox states, we strive to determine developmental stages of redox sensitivity where insults may have the most impactful damaging effect. In turn, this will allow for better therapeutic interventions and preservation of normal developmental signaling. This article is part of a Special Issue entitled Redox regulation of differentiation and de-differentiation.

1. Introduction and Background

1.1. Structure, function, and roles of GSH

Glutathione (GSH) is a nearly ubiquitous small molecule made up of three amino acids: glutamate (Glu), cysteine (Cys), and glycine (Gly) which serves in a number of important roles as an antioxidant, cellular protectant, regulatory signaling molecule, and in the maintenance of intracellular redox state [1–3]. The structural features of GSH that distinguishes it from other small peptides, relates to the relatively unique γ-glutamyl bond formed between Glu and Cys. This covalent bond occurs through the side chain carboxylic acid of Glu and renders the compound inert to normal intracellular peptidase-mediated degradation. This lack of degradation and other regulatory factors allow GSH to accumulate in cells in sufficient concentrations to enable it to function as an effective redox buffer. The critical roles and activities of GSH, however, reside in the redox active thiol (Cys-SH) of the Cys amino acid. The inherent chemical and physical properties of the redox-active thiol moiety of GSH permits cycling through oxidized and reduced states under physiological conditions, serving as a free radical scavenger, a catalytic reductant, a electron donor, a physiological storage depot for Cys, and a critical determinant of the redox steady state [2,4]. Biochemical and physical factors can lower the pKa of the active thiol via deprotonation of Cys-SH to form the thiolate anion ($Cys\text{-}S^-$) and greatly enhance the reactivity of GSH toward electrophiles, radicals, and other reactive chemical species [5]. Early studies of the roles and functions of GSH in embryogenesis and development in general, focused primarily on its demonstrated roles as a protectant against toxic chemicals and other deleterious environmental insults [6–13]. While clear evidence supports a different, important function of GSH in embryo protection, more recent studies suggest a much broader and more important role of GSH and its related actions during embryogenesis [14–16]. This review of GSH during embryogenesis will present a summary of the roles and functions of the GSH from both temporal and spatial perspectives.

1.2. Anatomical structure of the conceptus

The developing embryo presents a complex and very dynamic integration of evolving form and function where anatomical structures

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* Corresponding author at: Department of Physiology and Developmental Biology, College of Life Sciences, Brigham Young University, 3047 Life Sciences Building, Provo, UT 84602, United States. Tel.: +1 801 422 2158.
E-mail address: jason_hansen@byu.edu (J.M. Hansen).

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appear and disappear, cell growth rates peak and decline, and cell differentiation establishes new shapes and functional activities of subsets of cells, with all of these events occurring within a very short time span. During preimplantation phases of development many of the factors that regulate GSH and its various roles and functions are believed to be controlled from the maternal environment through maternal nutrients and signals within the oviduct and its fluids. Once implantation has reached a point of completion the conceptal relationships with the maternal environment change dramatically, especially in mammalian species like rodents and humans where complete interstitial implantation into the myometrium isolates the conceptus from direct contact to many maternal fluids that contain oxygen, nutrients, and developmental regulatory factors. As the process of embryogenesis unfolds, newly differentiated tissues are often spatially separated from other tissues by creating fluid compartments such as the yolk sac fluid (YSF) and amniotic fluid (AF) which aid in their isolation [17]. This is important for establishing discrete signaling domains and gradients for creating unique anatomical forms and allowing the performance of various important physiological tasks. Division of duties in spatially isolated tissues include the capture, uptake, and processing of nutrients by the visceral yolk sac (VYS), which are required by the whole conceptus for support of normal growth and development [18,19]. It is important in this regard to view the VYS and embryo separately because they perform different functions and have unique needs concerning the status and requirements of GSH and related low molecular weight thiols [20,21]. The complete ontogeny of GSH precursor uptake, utilization, biosynthesis, and turnover, as well as the many enzyme pathways and activities that modulate this complex network in the developing conceptus is far from complete and remains the subject of continuing research.

1.3. Glutathione during development

1.3.1. Preimplantation: fertilization to implantation

An understanding of how GSH relates to the dynamics and regulation of development cannot be undertaken without a clear working knowledge of embryogenesis and the anatomical and functional changes that define them. Embryogenesis can be divided into three major segments, each having unique structural and functional characteristics. Following ovulation, the oocyte swept off of the surface of the ovary by the fimbriae and into the distal segment of the oviduct. Fertilization takes place in the distal portion of the oviduct and the embryo undergoes cleavage divisions as it moves down from the duct to the uterus. Prior to fertilization, the mouse oocyte was shown to have optimal GSH concentrations at around 7 mM. High GSH concentrations at this stage have been reported to be necessary for normal fertilization because ovarian inhibition of GSH biosynthesis resulted in a 90% depletion of GSH in the oocyte and preventing sperm nuclear decondensation [22]. This effect can be rescued by the addition Cys to the culture medium. Following successful fertilization, the zygote undergoes a series of cleavage divisions, where cells are dividing cytoplasm and becoming smaller with the formation of each new daughter cell. With the dividing embryo maintaining the same approximate overall volume as the unfertilized oocyte, GSH levels drop in a near linear fashion from 1.2 pmoles/oocyte to around 0.12 pmoles/embryo at the blastocyst stage (Fig. 1) [23]. During the entire time, 4–5 days, the embryo moves down from the oviduct toward the uterus GSH levels continue to decrease.

1.3.2. Implantation

Very little is known about GSH status during the critical implantation stages. Implantation of the conceptus occurs at the blastocyst stage and, in most commonly studied species, including humans, results in the complete interstitial embedding of the conceptus into the uterine endometrial wall. Animal models for implantation each has inherent drawbacks and has not been widely used to study the effects of chemical toxicity where measurements of GSH and its roles in protection could be assessed. Reports of altered implantation and reproductive failure point to interactions with immune system components and the resulting generation of excess ROS by these cells as contributors to the failure to implant. The apparent reduction of the GSH pool suggests that the conceptus may be increasingly vulnerable to chemical and environmental insults as the blastula stage is reached.

1.3.3. Postimplantation — organogenesis

Interstitial implantation in mammals results in the temporary isolation of the early embryo from uterine fluids and direct maternal circulation. The consequences of this placement are a conceptus that resides in a relatively hypoxic environment and one that has adapted several unique strategies for providing the growing conceptus with sufficient nutrients (histiotrophic nutrition pathways) [19,24]. Due to the extremely small size and the experimental inability to separate intimately connected tissues in the very early conceptus, all measurements of GSH prior to the late egg-cylinder stage (GD 7 in mouse and GD 9 in rats) have been made in whole conceptuses. In subsequent stages of development, anatomical dissection becomes a practical possibility and tissues

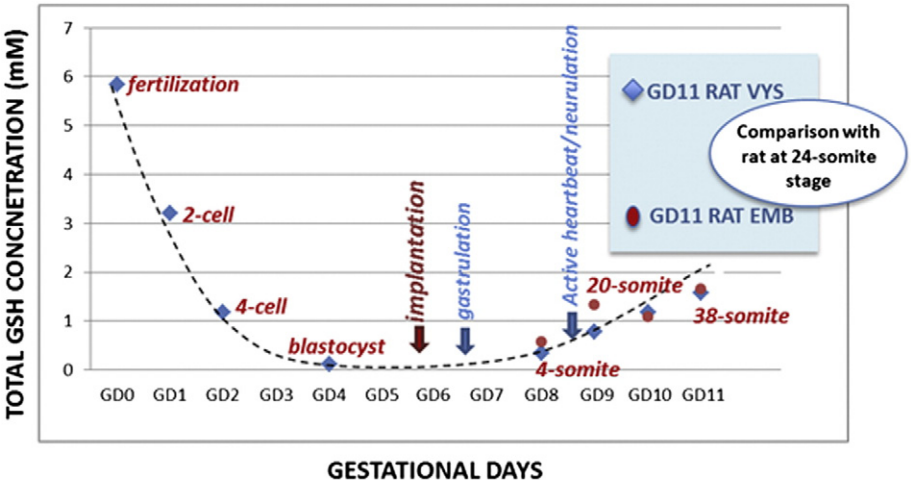

Fig. 1. GSH concentrations during mouse preimplantation and early postimplantation mouse conceptus. Prior to fertilization, GSH concentrations in the oocyte are high (~7.0 mM) but decline following fertilization and through subsequent cleavage division. GSH concentrations reach a nadir in the blastocyst at GD 5 when implantation is initiated. Once implantation is complete and organogenesis has commenced, levels begin to increase in both embryo and VYS remain nearly equal between the two tissues. Total glutathione concentrations in the rat are provided for comparisons (insert box) and show much higher concentrations at a similar stage of development and also show a greater difference between embryo and VYS. Data from Harris et al. unpublished and [23].

of interest can be removed and analyzed individually. Prior to the establishment of the three primary germ layers, endoderm, mesoderm, and ectoderm, cells of the conceptus have two identities based on their germ line of origin. Cells of the blastocyst inner-cell mass (the source of totipotent embryonic stem cells) and subsequent cells of the embryo proper have a maternal imprint while the trophoblast cells and their various derivatives have a paternal imprint [25,26]. Apart from specific anatomical locations and intended fate these two germ lines appear to have very different strategies for maintaining and utilizing GSH.

As the stage of embryonic development reaches the gastrulation period (late GD 7 and GD 9, respectively, for mouse and rat) anatomical and functional roles of the embryo and VYS begin to become distinct. The physical and functional relationships appear to be very important with regard to GSH uptake, synthesis and turnover. Rodent conceptuses differ markedly from humans with regard to the anatomical structures and associations between the embryo proper and the VYS. In rodents the VYS is inverted (brush border endothelium facing outward) and this extraembryonic membrane completely encloses the embryo proper and the bounding amniotic sac. In the human, the brush border endothelium faces inward and the VYS exists as a closed sac that extends outward from the embryo proper. Partly because of these anatomical differences, much debate has occurred about whether the well-characterized nutritional pathways described in the rodent are also relevant to the vestigial VYS in the human. Differences in nutrient uptake and supply could have a significant impact on the maintenance and regulation of GSH synthesis, steady state, and turnover because they are the same pathways that control amino acid precursor supply and flux. Most published studies of embryonic biotransformation, regulation, signaling, and nutrient/precursor supply remove and ignore the VYS. Because of its paramount importance to the developing mammalian conceptus and its central role in controlling GSH steady state during organogenesis, it will be included here wherever data is available.

The period of mammalian embryogenesis that extends from the onset of gastrulation and concludes with the complete closure of the secondary palate marks the beginning and the end of organogenesis. It is during this period that the three primary germ layers are established through gastrulation and via multiple folding events that occur in the embryo. Afterwards, the CNS begins to take shape through neural fold elevation and closure (from the center in opposite directions) to form a closed neural tube. Hematopoiesis begins toward the anti-mesometrial pole of the VYS in distinct blood islands while the conceptal vasculature is forming in the embryo and VYS. Within hours, the visible heart anlage becomes contractile and begins to beat, instantaneously perfusing and oxygenating tissues that had, heretofore, been in a relatively hypoxic environment. Although not thoroughly studied in this context, it is safe to suppose that the cell and tissue consequences of the rapid onset perfusion are not unlike a typical ischemia reperfusion injury. The adaptive responses to this insult will, undoubtedly, involve the GSH pathway.

1.4. Brief history of the study of GSH during mammalian development

Initial investigations designed to understand the roles of GSH during early mammalian development were focused on the potential ability of GSH to function as an embryonic protectant against xenobiotics [27–31]. Numerous studies conducted in whole embryo culture have exposed rodent conceptuses to environmental pollutants, therapeutic drugs, natural toxins, and other insults and have shown the importance of GSH in the embryo and VYS as an antioxidant and protectant. Developmental toxins and teratogens such as acetaminophen [32], valproic acid [33,34], retinoic acid [33,35], thalidomide [36], phthalates (DEHP/MEHP) (unpublished observations), methanol [27], ethanol (unpublished observations), lindane [37], phenytoin [38,39], and several other agents have shown reduced toxicity in conditions of adequate and enhanced toxicity when GSH stores are depleted. Maintenance of the GSH steady state, as an adequate redox buffer, was shown to be critical for conceptal protection, especially in the tissues of the VYS, which is responsible for the majority of de novo GSH biosynthesis. As the effects of GSH modulation through mechanisms such as inhibition of synthesis (buthionine sulfoximine; BSO), Cys supplementation (N-acetylcysteine; NAC), diminished precursor supply (leupeptin, HNP inhibition), and altered GSH oxidation/reduction (GSSG-Rd, GSH Px, Trx) have continued to be studied, it became evident that the roles and functions of GSH extended far beyond its most studied protective roles [6,16,29].

Early investigations into the concept of redox regulation in growth regulation and differentiation have provided strong evidence that GSH steady state may be most important for maintaining the correct permissive intracellular environment that allows normal signaling and response to occur. This expanded role of GSH has led to additional considerations about the potential function of soluble thiols (GSH and Cys) as direct signaling molecules themselves, as well as, their indirect involvement as mediators of ROS signaling and control [4,15,40].

2. Spatial distribution of GSH in the conceptus — steady state

The functional anatomy of the organogenesis-stage rodent conceptus provides a means to separate and effectively isolate cells and tissues between discrete fluid compartments. Diffusible growth factors and other regulators of cell differentiation are secreted within tissues and into fluids across gradients to control cell-specific growth and development. Intracellular redox environments that serve as permissive regulators of signaling are determined mainly through the state of the GSH/GSSG redox couple but distinct enzyme-mediated events are required for efficient signaling [41]. The components of the GSH/soluble thiol protective systems are not in equilibrium under normal conditions but exist as a steady state where each component is constantly being added and removed from a given compartment. The overall flux of GSH and its amino acid precursors, especially Cys, is expected to be a major factor in determining the steady state levels of GSH and Cys in the various cells and tissues, therefore, important to consider in parallel with the changes in GSH.

2.1. Quantitation of glutathione and cysteine in the developing conceptus

Numerous methodologies have been applied to measure LMWTs in cells and tissues, each having their inherent advantages and disadvantages. Analytical methods to measure GSH, GSSG, Cys, cystine (CySS) and mixed disulfides are varied and it is sometimes very difficult to determine exactly what was measured (total thiol, reduced thiol, oxidized thiol etc.). Quantitation of GSH and related soluble thiols requires the direct labeling of the Cys thiol or other reactive amino acid moiety for spectrophotometric or fluorescent detection. The recent availability of commercial kits has simplified GSH measurement, where approaches range from measures of reduced GSH only to total thiol (GSH + 2GSSG and Cys + 2Cyss). The measurement of a single component can be misleading when assessing consequences of perturbations of GSH status because significant depletion of GSH of 70% or more does not necessarily change the cellular redox environment and may not lead to any immediate changes in signaling or regulation. High performance liquid chromatographic (HPLC) methods that quantify both reduced and oxidized species in the same analysis are preferred because they have much greater sensitivity and lend themselves to the direct calculation of redox potentials (E_h). Quantitation of GSSG/GSH and CySS/Cys in a single analysis using an internal standard also limits much of the variability and guesswork encountered in other methods. A detailed discussion of the advantage and utility of using E_h as the metric for comparing redox states across different tissues and species can be found elsewhere [42].

2.2. Preimplantation embryo

Glutathione concentrations in the unfertilized oocyte are relatively high when compared to other cells and tissues of prenatal and postnatal

mammalian organisms. Observed levels of 1.2 pmol/oocyte are estimated to equate to concentrations of 7.0 mM (Fig. 1). Over the course of preimplantation development through fertilization, 2, 4, 8 cell, and morula stages, GSH levels decline in a nearly linear fashion until the blastocyst stage is reached where embryos contain 0.12 pmol or concentrations of approximately 0.7 mM. These low levels of GSH are likely maintained through implantation and up until the initiation of gastrulation near GD 7 in mouse and GD 8 in rat. Oxidized thiols are maintained at a relatively low percentage of total thiol, suggesting that the early embryo is able to maintain reducing redox conditions as reduced levels decline [23].

2.3. Early postimplantation embryo — organogenesis-stage conceptus

The onset of gastrulation marks the period where the three primary germ layers (endoderm, mesoderm and ectoderm) of the embryo are established. During this time (egg cylinder stage) the definitive VYS is also being formed from trophoblast lineages. The VYS remains anchored the embryo but it becomes separated from the embryo proper by two distinct fluid compartments; yolk sac fluid (YSF) and the amniotic fluid (AF) cavity. Determinations of GSH levels beyond these early somite stages include separate measurements for embryo and VYS and on occasion, include GSH and soluble thiol flux through the fluid compartments [42]. This is important because of the distinct structural and functional contributions of each tissue and compartment in development appear to interact to maintain to soluble thiol status.

Whole VYS and embryo are the major tissue compartments most frequently assayed, although, heads, hearts, trunks, limbs and other dissected components have been measured and subjected to inter species comparisons. Histochemical analysis of tissue cryosection slices obtained from conceptuses or in primary cultures of embryonic cells and tissues, using mercury orange as the principal stain, show differential distribution of GSH and related thiols in different cells and tissue types [43]. Higher GSH concentrations produced stronger staining in ectoplacental cone, VYS and embryonic heart in GD 10 rat conceptuses with much diminished staining observed in the neuroepithelium overall and tended to have higher concentrations in the alar plate relative to the corresponding basal plate.

2.4. Steady state GSH conditions and interspecies comparisons

2.4.1. Rats

The most complete body of work characterizing the GSH system distributions and related pathways during embryogenesis is from the rat. The majority of the rat work was also conducted in rodent whole embryo culture, which preserves normal morphogenesis and functional development but does not fully replicate the complexity of intrinsic controls found in the intact uterus. The values available for comparison within and across different species requiring a degree of interpretation because the different methods used to quantify GSH, GSSG, and related compounds differ considerably, and are not amenable to absolute quantitation and/or direct comparison. Nonetheless, all species evaluated have been shown to maintain significant intracellular GSH/GSSG pools.

In rats, total GSH concentrations can range from 4.0 to 9.0 mM in the VYS and from 2.0 to 6.0 mM in the embryo proper [16,42]. These relative differences can change as a function of developmental stage. In the GD 10 rat conceptus, total GSH in embryo and VYS are relatively similar but change significantly over the next 24 h and beyond, where concentrations become relatively higher in the VYS. During this phase of development, GSH:GSSG ratios in the VYS do not generally exceed 30:1, but tend to range lower in the EMB, not to exceed 20:1. These GSH/GSSG ratios contribute to the differences in the calculated intracellular redox potentials (E_h) for EMB and VYS. Intracellular E_h values in the VYS fall into a more reducing range of −220 to −245 mV compared to a more oxidizing range of −210 to −230 in the EMB [16].

2.4.2. Mice

Interspecies comparisons between the rodent species Sprague–Dawley rats and CD-1 mice show that mice are uniformly more oxidized at similar stages of organogenesis development. This observation is drawn from data showing that in equivalently staged GD 9 mice and GD 11 rats the mice show greater overall concordance between VYS and embryo, in terms of E_h and absolute total GSH. The E_h values in GD 9 mouse VYS range from −210 to −230 mV, on average, and range from −200 to −220 mV in the EMB. These differences are more similar to the GD 10 rat and suggest that gene expression, enzymes activities, and precursor supply pathways in mice may be slightly delayed as compared to rat. In preliminary work, a redox ontogeny profile in CD-1 mice from GD 8 to GD 11 showed a linear increase in total GSH (GSH + 2× GSSG) over the four-day period. Total GSH concentrations ranged from approximately 400 μM on GD 8 to over 1700 μM on GD 11. Once again, the relative differences in concentration between embryo and VYS were minimal in the mouse. Intracellular redox potentials also showed a linear decrease over the same developmental period suggesting that tissues of the conceptus become more reducing as organogenesis moves to completion. It is of interest to note that when whole conceptuses are assessed for their redox profiles in this manner the opposite effect on E_h is observed (i.e. whole conceptuses become more oxidized with age. We speculate that this is due to the extensive oxidation of the large extracellular fluid compartments in the intact conceptus) (unpublished observations).

2.4.3. Rabbits

A systematic interspecies GSH redox profile comparison was conducted between rats and rabbits in the context of experiments to elucidate the roles of GSH and redox status in thalidomide teratogenicity. Rabbits are used as a model species for these studies because they are highly sensitive to thalidomide and produce the same types of limb defect lesions as seen in exposed human offspring. Rats and mice are resistant to thalidomide for reasons discussed below. Rat–rabbit comparisons show that the rabbit has 37% lower overall concentrations of GSH and Cys when compared to the rat on a pmol/min/mg protein basis. Embryonic concentrations of GSH from the same comparison groups show no significant differences between species. When Cys levels are compared the rat values are slightly lower in the rabbit VYS but are much lower for rabbit in the embryo (70% decrease) when compared to rat [11,44,46]. In these studies individual tissues were dissected to allow for GSH measurement in embryonic heads, limbs, trunks and whole embryos. Rabbit GSH and Cys concentrations were found to be uniformly lower in all of these comparisons.

3. GSH biosynthesis

Most cells lack the capacity to take up GSH directly from extracellular sources. The extracellular glutathionase enzyme, γ-glutamyltranspeptidase (GGT), described below, is a primary contributor of GSH precursor amino acids through the degradation, transport, and resupply of intracellular amino acid precursor pools. This is especially true for the rate-limiting precursor Cys which is generally found in low intracellular concentrations in most biological tissues. A unique feature of the rodent conceptus is the relative lack of direct amino acid transport from maternal nutrient sources across the VYS to the embryo proper [19,46]. The rodent conceptus employs the process of histiotrophic nutrition (HN) to supply the large quantities of amino acids and other precursors top support new protein synthesis, nucleic acid synthesis and many other metabolic needs. Histiotrophic nutrition pathways involve the uptake of intact proteins from maternal sources via receptor-mediated endocytosis (RME) which are captured into primary vesicles, fused with lysosomes and degraded by cysteine proteases to liberate amino acids. This is believed to be the predominant source of amino acids used for GSH synthesis. Studies conducted to understand precursor supply and GSH biosynthesis in the rat conceptus

grown in whole embryo culture showed that the direct uptake of radiolabeled [^{35}S]-Cys from culture medium accounted for less than 20% of total Cys incorporated into GSH and new proteins [21]. This percentage of direct uptake is much higher than many other amino acids that have been assessed and may be a reflection of the GGT activities found on the VYS endothelium [19,47,48]. By comparison, [^{35}S]-Met was accumulated at rates almost 3-fold those of Cys. In the early days of whole embryo culture, it was observed that Met supplementation of serum from other species could maintain optimal growth of conceptuses where the serum alone would not. It was hypothesized that Met, through the activity of the trans-sulfuration pathways associated with one carbon metabolism, could serve as a viable Cys source, as is the case in the mature liver [49–51]. Although both [^{35}S]-Cys and [^{35}S]-Met were taken up and incorporated into new proteins in the rat conceptus, no [^{35}S]-Met whatever was incorporated into newly synthesized GSH. A lack of conceptal cystathionase enzyme activity has now been confirmed through molecular and immunohistochemical means [52]. With HN uptake and supply mediated exclusively in the VYS it was of interest to observe that chemical depletion of conceptal GSH with diethyl maleate (DEM; 500 μM) caused a rapid depletion of GSH in both VYS and EMB to 25% of the starting concentration but only the VYS was able to initiate de novo GSH biosynthesis immediately after removal of the DEM [21]. Active biosynthesis did not resume in the embryo until the VYS concentrations were replete, suggesting the embryo may be dependent on GSH stores supplied through the VYS acting as the primary source of Cys and other GSH precursors.

3.1. The gamma-glutamyl cycle (GGC) in the conceptus

The biosynthesis of GSH in cells is accomplished using a compliment of six enzymes found on the plasma membrane and in the cytosol from the precursor amino acids Glu, Cys, and Gly. Extracellular GSH is selectively taken up in but a very limited number of tissues (gut and kidney proximal tubule) requiring degradation to single amino acids, in most cases, before transport into the cell. The universal facilitator of GSH breakdown and only know glutathionase enzyme is γ-glutamyltranspeptidase (GGT) which is located on the outer leaf of the cell's plasma membrane (Fig. 2) [53,54]. GGT first cleaves GSH (at the unique γ-glutamyl bond) to form free Glut and the dipeptide Cys-Gly, which is degraded to Cys and Gly for transport in to cells via traditional amino acid transporters. The Glu is quickly reunited with another amino acid (usually a Cys) and transported into the cell as γ-Glu-amino acid. Inhibition of GGT is known to elicit a reduced supply of amino acids and can result in reductions of GSH biosynthesis. Once inside the cell, precursor amino acids are enzymatically processed and passed along to enzymes responsible for GSH synthesis beginning with cyclization and decyclization reactions to release the bound amino acid and present free Glu as the first substrate precursor for GSH biosynthesis. Glu and Cys are combined in to form γ-glutamylcysteine using the enzyme glutamate-cysteine ligase (GCL). The holoenzyme functions as a heterodimer utilizing a large 72 kDa catalytic subunit (GCL*c*) and a smaller 27 kDa regulatory (GCLr) subunit, which requires ATP and Mg^{2+} as cofactors to complete catalysis. Both subunits of this enzyme are heavily regulated by induction but the holoenzyme but activity can also be regulated through non-allosteric feedback inhibition by GSH [55]. Biosynthesis is completed in the next step with the addition of Gly by glutathione synthase (GS), which also requires ATP and Mg^{2+} [55,56]. Below these various components of the GGC are discussed as they relate to development.

3.1.1. Gamma-glutamyltranspeptidase (GGT)

In the developing conceptus, GGT activity and direct amino acid transport [21] appear be present in the embryo proper and basolateral VYS [57,59] but is not found to be the predominant mechanism for import at the maternal side brush border [19]. This arrangement suggests that amino acid precursors for new GSH biosynthesis must come via other means. The alternate means identified is through the process of histiotrophic nutrition where whole maternal proteins are captured by the VYS brush border, encapsulated in vesicles, fused with lysosomes and degraded to their constituent amino acids. This process creates some interesting regulatory and metabolic issues in the conceptus where it has been shown that inhibition of the HNP function of receptor mediated endocytosis (RME) or as the inhibition of lysosomal proteolysis result reduced GSH biosynthesis and conditions of amino acid starvation. Gamma-glutamyltranspeptidase specific activities [59] are differentially expressed in VYS and embryo of the organogenesis stage conceptus. On GD 10 in the rat, specific activities range from 2.5–3.5

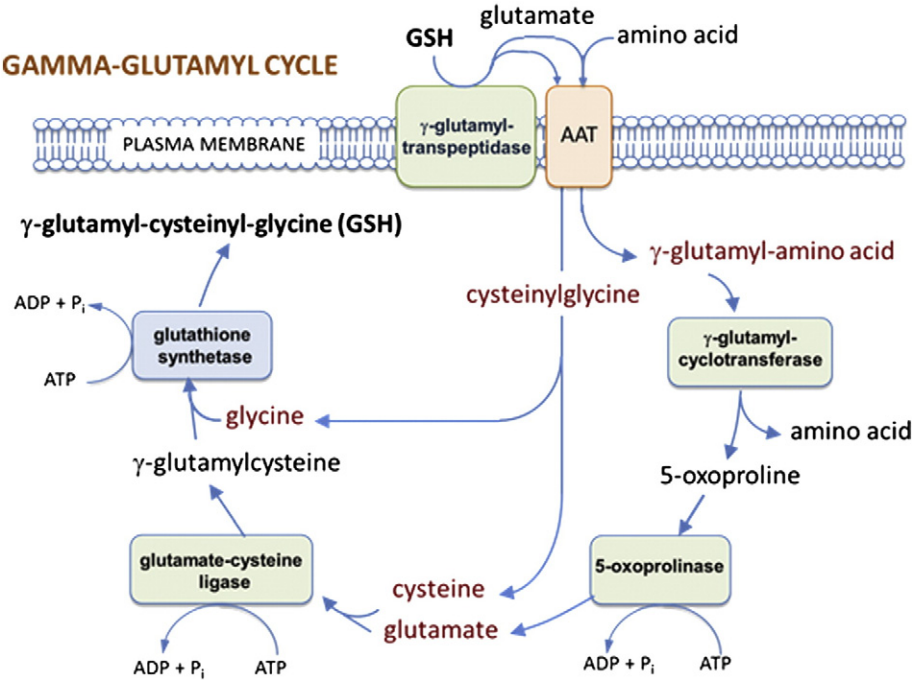

Fig. 2. The gamma-glutamyl cycle (GCC) and de novo synthesis of GSH. As GSH is usually not directly transported inside the cell, GSH breakdown into components more amenable to transport can occur. Once precursors are available intracellularly, GSH can be resynthesized to regenerate GSH via de novo synthesis enzymes (glutamate-cysteine ligase and glutathione synthetase).

nmol/min/mg protein in embryo and 13.5–16.0 nmol/min/mg protein in VYS. Specific activities increase on GD 11 to 6.0–8.0 and 19.0–23.0 nmol/min/mg protein, respectively for embryo and VYS [60]. The higher GGT activities in the VYS are supported by histochemical studies that show a significant enrichment of GGT activity on the apical (brush border) surface [57,58]. Ablation of the GGT gene has been shown to result in embryo lethality, but it is not entirely clear if the effects on GSH biosynthesis are related to this effect. Experiments in GGT inhibition using chemical inhibitors such as acivicin (AT-125) or anti-GGT antibodies show only marginal depletion of GSH in the VYS after 24 h [48]. Embryonic GSH concentrations are more significantly depleted under the identical conditions and this likely relates to the near complete dependence of the VYS on HNP for providing substrate amino acids.

The most complete characterization of amino acid transport during development has focused on the preimplantation phase of development. Several studies have described amino acid requirements and transport during the preimplantation phase of development [61,62]. Less attention has been paid to the embryo and VYS primarily because the VYS relies heavily on HNP and RME for amino acid supply.

3.1.2. 5-Oxoprolinase

5-Oxoprolinase is relevant for the regulation and modulation of GSH because it is a portal for the direct conversion of glutamate for GSH biosynthesis. This enzyme is also important in the processing of cysteine pro-drugs such as 2-oxothiazolidine-4-carboxylate (OTC), which is cleaved by 5-oxoprolinase to liberate free cysteine as a means to augment the availability of the rate-limiting precursor for GSH synthesis [63].

3.1.3. Glutamate cysteine ligase (GCLc, GCLr)

GCL activities vary temporally and spatially over the course of development. In the organogenesis stage conceptus, GCL specific activities in the GD 10 rat VYS (118.9 pmol GC/min/mg prot) are nearly double those seen in the corresponding embryo proper (60.5 pmol GC/min/mg prot). Twenty four hours later on GD 11, GCL specific activities decrease in both tissues, and the difference in specific activity between embryo and VYS increases to 3-fold [20]. GCL activity can be modulated as a function of intracellular GSH concentration through a feedback inhibition mechanism, and GCL activity is the rate limiting activity for GSH biosynthesis in the GCC.

Exposure to electrophilic compounds and oxidants can induce the expression of GCL. Diethyl maleate has been identified as one of the more potent inducers in the conceptus. On GD 11 constitutive GCLc expression in the VYS is nearly 60-fold higher than in the embryo proper. Constitutive GCLr expression is 25-fold higher in VYS compared to embryo [20]. As a consequence of this high relative expression, augmented expression due to chemical inducers is much lower in the VYS on a fold-basis compared to the embryo, suggesting a more dynamic regulatory mechanism to exist in the embryo. DEM induction with DEM elicits an 18.6-fold increase in the embryo but only by 0.29-fold in the corresponding VYS. GCLr is induced in the embryo as well, but at lower levels (8.2 fold in EMB, 0.85-fold for VYS). These results reinforce the conclusions drawn from these and related studies that suggest that, in terms of GSH biosynthesis, the VYS is operating at near optimal capacity under normal conditions and is not subject to significant induction, and the embryo can respond and react in a more dynamic manner.

3.1.4. Glutathione synthetase (GS)

Unfortunately, little is known about the induction, regulation and activity of this enzyme in the conceptus. While considered very important to GSH synthesis, GCL activities are thought to be more critical as they constitute the rate limiting reaction in GSH de novo synthesis. Still, GS study does require attention during embryogenesis.

3.2. Glutathione catalysis and glutathione-dependent enzymes

Glutathione participates in the maintenance of cellular homeostasis as a redox buffer but is also used as a cofactor, electron donor, and direct antioxidant in many critical developmental processes. Many uses lead to its net oxidation and require catalytic reduction to restore redox balance. Surprising little is known about the cellular regulation and specific catalytic mechanisms for many of the GSH-related enzymes and this is especially true for their respective roles in embryonic development [64]. In addition to the glutathionase activities of GGT and biosynthesis activities of GCL, as already discussed, several additional enzymes use or modify GSH in the conceptus in a tissue-selective manner. These include glutathione disulfide reductase (GSSG-Rd) and glutathione peroxidase (GSH-Px) and glutathione S-transferases (GST). GSSG-Rd enzymes belong to a class of selenoenzymes responsible for the reduction of GSSG and related protein-mixed disulfides which collectively represents the primary cellular mechanism for keeping GSH/GSSG ratios high and maintaining an optimal reducing steady state [64]. These enzymes require NADPH as the obligate electron donor. The GSH-Px family of enzymes is a selenoenzyme responsible for the catalytic reduction of hydrogen peroxide and other organic peroxides. Glutathione is used as its obligate electron donor and source of reducing equivalents, resulting in the net oxidation of GSH to GSSG [64].

3.2.1. Preimplantation

3.2.1.1. GSSG-Rd. Much of the evidence for GSSG-Rd activity in the preimplantation embryo is based on in vitro chemical exposure studies in conjunction with the use of selective GSSG-Rd inhibitors such as 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU, carmustine). Treatment of 2-cell to blastocyst-staged mouse embryos in vitro with the oxidant tert-butyl hydroquinone (tBHQ) resulted in the significant depletion of GSH and net increase in GSSG concentrations. The normal rapid recovery of GSH levels and accompanied by decreased GSSG following tBHQ exposure was eliminated when co-exposed to BCNU. While no direct measurements of GSSG-Rd activity were made, these results show the presence of significant activity during the preimplantation stage [65].

In an expanded analysis looking at the redox regulation and dynamics of oocytes and preimplantation embryos, Dumollard et al. showed that the maintenance of cellular nucleophilic tone and GSH reduction depended on the combined activity of GSSG-Rd and the availability of NADPH to sustain catalytic activity [66]. Additional work has addressed the roles of GSH metabolism in mammalian gametes [67].

3.2.1.2. GSH-Px. Detailed information regarding the activity and ontogeny of GSH-Px in the oocyte and early embryo is limited, for the most part, to the identification of GSH-Px transcripts [68]. A significant amount of discussion is presented to suggest that related GSH-Px activities are present and that GSH is oxidized to GSSG in the process of removing H_2O_2 and related lipid hydroperoxides, but these claims are not supported by actual activity measurements. Others have reported that GSH-Pxs are not a significant factor in the removal of H_2O_2 [69].

3.2.2. Postimplantation

3.2.2.1. GSSG-Rd. In postimplantation rodent conceptuses (rat GD 10 to GD 11), GSSG-Rd specific activities range 6–8 fold higher in the VYS (20.7–27.8 nmol NADPH oxidized/min/mg/prot) when compared to their corresponding embryo (3.3–3.4 nmol NADPH oxidized/min/mg/prot) [70]. Specific activity ontogeny profiles were measured in rat embryos from GD 9 to GD 13 and showed that the GSSG-Rd specific activity was present and consistent from GD 9 onward (Fig. 3). VYS activities were, again, significantly higher in the visceral by 4–10% when compared to its corresponding embryo. Beyond GD 11, specific activities in

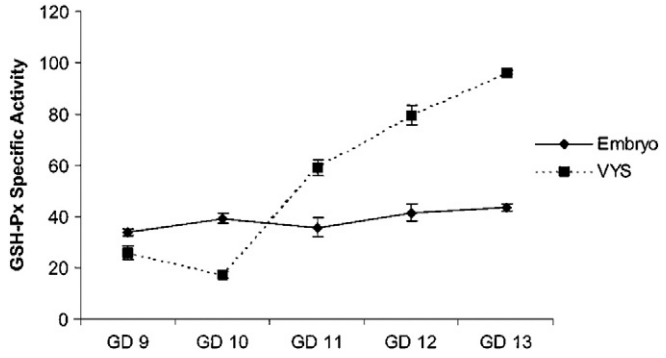

Fig. 3. Glutathione disulfide reductase specific activity in early gestation. Embryos were removed and sorted by somite number (0–12 [$n = 6$], 13–20 [$n = 8$], 21–28 [$n = 13$], 29+ [$n = 9$]) indicating GD 9–12, respectively, after which both embryo (solid line) and VYS (dotted line) enzyme activity were determined. GSSG-Rd specific activities are expressed as nmol NADPH oxidized/min/mg protein. Data are represented as means ± SEM [71].

the VYS continue to increase with age, reaching levels greater than 3-fold over GD 9 values while the embryo remained relatively constant [71].

Selective inhibition of GSSG-Rd with BCNU has been used at different stages of postimplantation development to assess the significance of GSSG reductive capacity in chemical teratogenesis. As an example, Wong and Wells [72] showed a clear exacerbation of phenytoin-induced lethality/resorption and teratogenesis in GD 12 rat embryos when the developmental toxicant was added in combination with BCNU.

3.2.2.2. GSH-Px. The hydroperoxide-eliminating activities of GSH-Px are measurable at relatively early stages in the postimplantation rodent conceptus. In the rat, GSH-Px specific activities in both the embryo and VYS were significant around 30 nmol NADPH oxidized/min/mg protein on GD 9. The pattern of higher specific activities in the embryo were reversed by GD 11 as both tissues showed increasing activities and where VYS activities exceeded those in the embryo by over 2-fold at GD 13 [71] (Fig. 4). Tissues (head, head, and trunk) within the embryo proper all increased over time at the same relative rates as shown for the intact embryo.

In the GD 10 to GD 22 chick embryo, which is comparatively more advanced in its development than the rodent at the same day of gestation, GSH-Px specific activities for the liver, the brain, and the yolk sac membrane showed ontogenies that varied across the three tissues. Liver activities were the highest of the three and continued to increase linearly with time while yolk sac membrane peaked at GD 15 and brain activities remained relatively unchanged [73].

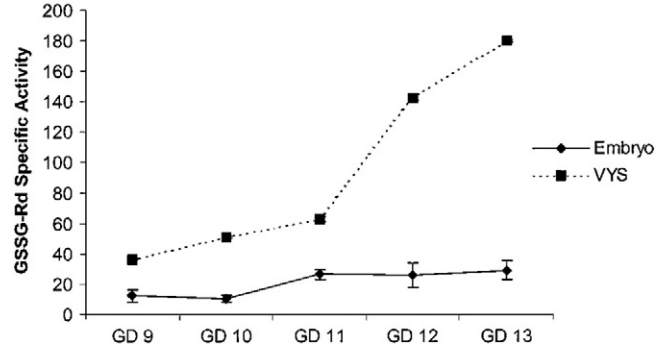

Fig. 4. Glutathione peroxidase specific activity in early gestation. Embryos were removed and sorted by somite number (0–12 [$n = 7$], 13–20 [$n = 7$], 21–28 [$n = 11$], 29+ [$n = 11$]) indicating GD 9–12, respectively, after which both embryo (solid line) and VYS (dotted line) enzyme activity were determined. GSH-Px Specific activities are expressed as nmol oxidized/min/mg protein. Data are represented as means ± SEM [71].

3.3. Glutaredoxin

Glutaredoxins (Grxs) are a class of enzyme that catalyzes the reduction of proteins or other low-molecular weight mixed disulfides with GSH [74]. Perhaps most critical to development is Grx's ability to de-S-glutathionylate proteins. Interestingly, under periods of oxidative stress, Grx can cause S-glutathionylation of various proteins using GSSG. As such, it serves as a means to regulate protein function.

During development, the role of Grxs has not been well defined. Glutaredoxin-1 (Grx1) knockout mice are phenotypically normal and viable [75], however, cells collected from knockout mice did show increased apoptosis following treatments with various oxidants and demonstrated slowed de-S-glutathionylation after H_2O_2 treatments. Conversely, mouse embryonic fibroblasts (MEFs) lacking Grx1 were more resistant to TNFα/actinomycin D treatments, suggesting that Grx1 activities may regulate/mitigate certain types of stressors better than others.

While little is known about the function of Grxs during development, Grx is present at varying degrees in various tissues throughout the organogenesis-stage mouse embryo (GD 8.5-birth) [76]. Loss of Grx activity during development is associated with high levels of apoptosis in the developing zebrafish brain, disruption of the organization of the developing vasculature and loss of normal cardiogenesis through alteration to cardiac neural crest cell migration [77–79]. Some regulatory targets of Grxs have been identified but clearly more work is needed to better understand the role and function of Grxs during development.

3.4. Glutathione S-transferases

Glutathione S-transferases (GSTs) belong to a nearly ubiquitous family of important detoxication and binding enzymes that represent the first enzymatic step in the removal of potential reactive electrophiles [80]. Lipophilic substrates bind to various GST active domains where their enzymatic activity facilitates the increased reactivity to the GSH nucleophile leading to the formation of a more water-soluble conjugate. The presence and activity of GSTs in the preimplantation embryo have been anticipated more than they have been systematically characterized and quantified [69]. Activities have been demonstrated at both oocyte and blastocyst stages in the mouse using GST-dependent confocal imaging techniques [81]. Interestingly, typical GST inducers such as polychlorinated biphenyls did not induce GST pi1 in the rabbit preimplantation embryo [82].

Glutathione S-transferase expression and catalytic activities have been characterized in several species of postimplantation conceptuses although the coverage has been surprisingly poor. Zebrafish embryos were shown to express all of the GST isoforms normally found to be expressed in adults of the same species. In these studies the transcriptional regulation and gene expression due to the pesticide Atrazine was found to vary widely across tissues and was only moderately increased [83]. In the older GD 11 chick embryonic liver, GSTs from the theta, mu, and alpha isoform classes were found to be present and inducible by acrylamide exposure [84]. Studies in the postimplantation and organogenesis-stage mouse conceptuses are sparse and rely mostly on extrapolation [69] and speculation. Several studies in embryonic gestational week 8 and fetal week 13 first trimester human conceptuses show the expression of GST alpha and pi isoforms [85,86]. These studies indicate a wide distribution of activities across several cell and tissue types indicating a robust complement of GST detoxification capacity. Results show that the GSTP1 isoform may be the most critical GST for protection of cells and tissues during this phase of human development [86].

4. The role and function of GSH in maintaining and regulating embryonic redox balance

4.1. Determination of GSH redox potential (E_h)

Glutathione, being one the largest intracellular pools of non-protein, biothiol reducing equivalents performs a critical role in regulating redox environments. Because the GSH/GSSG couple is highly responsive to the external, environmental influences, it serves to protect cellular macromolecules from oxidative damage and, due to the dynamic nature of this couple, maintains cellular redox homeostasis. Conserved, stage-specific redox states are required for normal cellular signaling and function that must coincide with related developmental periods (see section below). Prolonged redox disruption can have severe, if not lethal, consequences.

The GSH/GSSG couple is often expressed as a redox state, as being either reducing or oxidizing. This term is used ambiguously and this can be confusing, but more specifically, redox states can be defined as the half-cell reduction potential and the reducing capacity of that particular couple [2]. Quantitatively, redox states can be represented through the Nernst equation.

$$E_h = E^\circ - (RT/nF)\ \ln Q$$

Redox potentials (E_h) are measured where E° is the midpoint potential, R as the gas constant (8.314 J/K mol), T is the temperature (in Kelvin), F is the Faraday constant (9.6485×10^4/mol), n is the number of electrons transferred, and Q is the mass action expression. At room temperature (25 °C), the equation can be further simplified to:

$$E_h = E^\circ - (59.1\ \text{mV}/n)\ \log Q$$

More specifically to GSH/GSSG, at 7.0 pH E° is −240 mV, but in biological systems, pH is ~7.4 and thus, E° is calculated as −264 mV. The number of electrons transferred is 2 (n = 2). Thus, the final calculation to determine GSH/GSSG Eh is:

$$E_h = (-264) + (30) * \left(\log\left([\text{GSSG}]/[\text{GSH}]^2\right)\right)$$

Different from other redox couples (such as thioredoxin) and similar to others (such as cysteine), absolute concentrations impact the overall redox potential due to the stoichiometry of couple, where 2 GSH ⇆ GSSG. For this reason, the GSH concentration in the Nernst equation is squared. As such, ratios (GSH:GSSG) do not fully capture the nature of the GSH redox environment. An excellent example of this principle can be found in multiple publications where redox shifts occurred following treatments or during cellular differentiation [87–93] (described in greater detail below). In brief, cells that have higher overall concentrations of GSH can have identical GSH:GSSG ratios compared to cells that have less total GSH, but the E_h is considerably more reducing. For example, cells that have a total of 5 mM GSH of which 10% is oxidized as GSSG (4.5 mM GSH and 0.5 mM GSSG; a 10:1 GSH:GSSG ratio) give a redox potential of −222 mV. However, at lower overall concentrations of 1 mM GSH where 10% is oxidized as GSSG (0.9 mM GSH and 0.1 mM GSSG; also a 10:1 GSH:GSSG), redox potentials are −201 mV and are +20 mV more oxidizing, comparatively. In cases like these, redox potential measurements may provide a much more accurate means to express the redox state compared to simply providing GSH:GSSG ratios.

There exists a spectrum of views regarding the suitability of using E_h as a metric for comparing redox states across different tissues and species [2,94–96]. In the context of this review we accept the fact that cells and tissues in biological systems are not in equilibrium and that Q terms, in this case representing the absolute concentration ratios of GSH and GSSG, as used in the Nernst equation, are not intended to represent thermodynamically free concentrations for these redox couples [2]. Instead, the calculation and use of redox potentials is simply as a "readout" of perturbations in the GSH/GSSG steady state. This application is similar to what has been used by Britton Chance and many others since the early 1950s for evaluating the steady state dynamics of pyridine nucleotide (NADH and NADPH) redox states in the mitochondrial electron transfer pathway [97]. In this historical case and in current applications to GSH/GSSG redox couples in biological systems at steady state, the use of E_h provides the best correlation between perturbations and complex cellular outcomes such as proliferation, differentiation and apoptosis [96].

4.2. Factors that influence redox status

Since redox potentials are subject to both concentrations and ratio of GSH:GSSG, there may be certain periods during development where embryos/cells are more susceptible to exogenous influences. These influences can originate from numerous sources, but generally fall in to one of three subcategories: (1) environmental, (2) pharmaceutical/recreational or (3) physiological influences. Examples of environmental influences broadly include categories such as pesticides, heavy metals (mercury, arsenic, lead), industrial waste and nanoparticles [98–101]. Pharmaceutical/recreational influences include drugs (antiepileptic drugs [phenytoin, valproic acid], chemotherapeutics [thalidomide, cyclophosphamide, hydroxyurea], nutriceuticals and recreational drugs (ethanol, cocaine) [46,102–110]. Physiological influences are generally correlated with illness (hyperthermia in Rubella) or disease (hyperglycemia in diabetes) [111–113]. How these influences affect development and promote abnormal development (teratology) through alteration to cellular redox states is not entirely understood and continues to be an area of intense study and have been the subject of numerous reviews [14,15,114,115,116].

4.3. Classical and redefinition of oxidative stress

Of interest to developmental toxicology, many of the most notorious teratogens in humans are those that have been shown to induce oxidative stress [14,15]. Nearly 30 years ago, oxidative stress was originally defined as "the imbalance of oxidizing and reducing equivalents where the former predominates" [117,118]. If imbalance persists, macromolecule damage would ensue, resulting in cell death. Alternatively, a newer understanding of redox couples and redox signaling suggests that oxidative stress may be better defined as "a disruption in redox circuitry" as proposed by Jones [118,119] (see review). In brief, this definition highlights the independent nature of various redox couples, including GSH. As unique shifts to redox couples occur, specific redox signaling pathways can be activated or deactivated.

4.4. Correlation of developmental events with GSH E_h

Studies show that the progression and cessation of proliferation is reliant upon intracellular redox status with special focus on the GSH redox couple. Hutter et al. [120] showed that normal fibroblasts at low confluence (20%) had reducing GSH E_h of −220 mV. As cells became increasingly confluent and proliferation slowed due to contact inhibition, the GSH E_h increased to approximately −185 mV at 100% confluence. Using a transformed fibrosarcoma cell line where proliferation is not inhibited by contact inhibition, cellular GSH E_h did not change at differentiation degrees of confluence and was maintained at approximately −210 mV. Artificially enhancing the GSH E_h with either L-2-oxothiazolidine-4-carboxylic acid (OTC) (−10 mV) or N-acetylcysteine (NAC) (−7.8 mV) supported proliferation, where reducing conditions significantly promoted proliferation. Conversely, buthionine sulfoximine (BSO), an inhibitor of GSH de novo synthesis, treatment increased GSH E_h by +24 mV, which correlated with a decrease in proliferation of over 50%. Although specific components of the cell cycle that are redox-sensitive have not been elucidated, changes in GSH and GSSG correlated with a decrease in ROS during M

phase of the cell cycle as compared to quiescent and S phase cells, suggesting that oxidative stress and redox changes are regulating cell cycle machinery [121].

More recent advances in understanding the proliferative role of GSH is a function of subcellular localization, primarily between cytosol and nucleus, where GSH recruitment into the nucleus occurs during early proliferative events [122,123]. This hypothesis suggests that the interplay of cytosolic and nuclear concentrations of GSH promote changes to the nuclear envelope to allow cell division [124]. In terms of developmental proliferation, these approaches have not been fully explored but do present an interesting means by which GSH redox states may serve as a regulative developmental trigger.

In many models of cellular differentiation, GSH redox potentials vary significantly but show a common characteristic during differentiation: The oxidation of the GSH/GSSG redox state. In 3T3-L1 preadipocytes, undifferentiated cells contain nearly 2 mM total GSH, but following differentiation into an adipocyte, cells contain nearly 30% less GSH [88]. Changes to total GSH levels correspond to an increase in GSSG concentrations by approximately 300%. These changes constitute a +22 mV change as demonstrated by a redox shift from −238 mV to −216 mV. Shifts in the GSH redox couple during differentiation has also been observed in other cell models of differentiation for other cells types, such as myocytes (c2c12 cells) and enterocytes (HT29 cells, Caco-2 cells) [87,90].

The above examples of GSH redox changes during differentiation all utilize cell models that can differentiate into a single lineage/phenotype (i.e. preadipocytes → adipocytes). While general oxidative patterns are similar in these models, it may not best represent how redox shifts may promote or control cellular differentiation. Using human mesenchymal stem cells (hMSCs), cells can be differentiated into multiple phenotypes, including adipocytes and osteocytes, from the same progenitor [125,126]. GSH and GSSG concentrations and GSH redox potentials were determined throughout differentiation (up to 21 days) in both types of differentiating cultures [89]. Differentiation was determined via expression of markers of terminal differentiation. In undifferentiated hMSCs, total GSH concentrations were approximately 3.3 mM, and GSH E_h was −259 mV (Fig. 5). Upon 3 days of treatment with adipogenic differentiation agents, GSH E_h began to become significantly oxidized. This trend continued until day 13 of culture where GSH E_h values stabilized to −225 mV and contrary to 3T3-L1 adipogenesis, total GSH concentrations were not changed with terminal differentiation. In hMSC osteogenic cultures, GSH E_h was not significantly altered until day 10 of culture and then began to become increasingly oxidized to eventually reach the same E_h that was observed in adipogenic cultures on day 21. This observation suggests that redox regulation of differentiation through the GSH redox couple has a very unique redox signature dependent upon the terminal phenotype. Glutathione E_h values become oxidized early in adipogenic differentiation, but it is delayed in osteogenic differentiation.

Because these patterns are conserved between each phenotype, preservation of homeostatic GSH E_h becomes essential to support differentiation of each phenotype. In brief, rapid oxidation of the GSH E_h supports adipogenesis, while reduced GSH Eh followed by delayed oxidation supports an osteogenic phenotype.

These proliferative and differentiation studies help to form a more general paradigm for GSH redox state-mediated regulation of various developmental events. While every cell type is different, estimations of redox potentials that would promote each event in mammals have been suggested [119,127]. At estimated GSH E_h values of −250 mV, cells proliferate. Oxidation to levels between −220 and −200 mV favors differentiation. Further oxidation to −180 mV promotes apoptosis and oxidation to −150 mV is associated with necrosis. This paradigm is schematically presented in Fig. 6.

The hypothesis that redox states regulate development and differentiation has been largely supported by in vitro and cellular models. However, early supportive evidence in a recent publication shows that GSH E_h during zebrafish development (0–120 h postfertilization [hpf]) similarly correlates with developmental events observed in vitro [128]. At 0 hpf, GSH E_h was measured at nearly −230 mV, but over the following 18 h, GSH E_h became increasingly oxidized to −175 mV and remained in that range until 48 hpf (Fig. 7). By 72 hpf, intracellular GSH E_h had returned to more reducing conditions, −230 mV. The developmental redox ontogeny in the zebrafish GSH E_h is positively associated with very important developmental events. For example, two distinct periods of that denote periods of rapid differentiation, gastrulation and organogenesis, occur around 5.25–10 hpf and organogenesis occurs

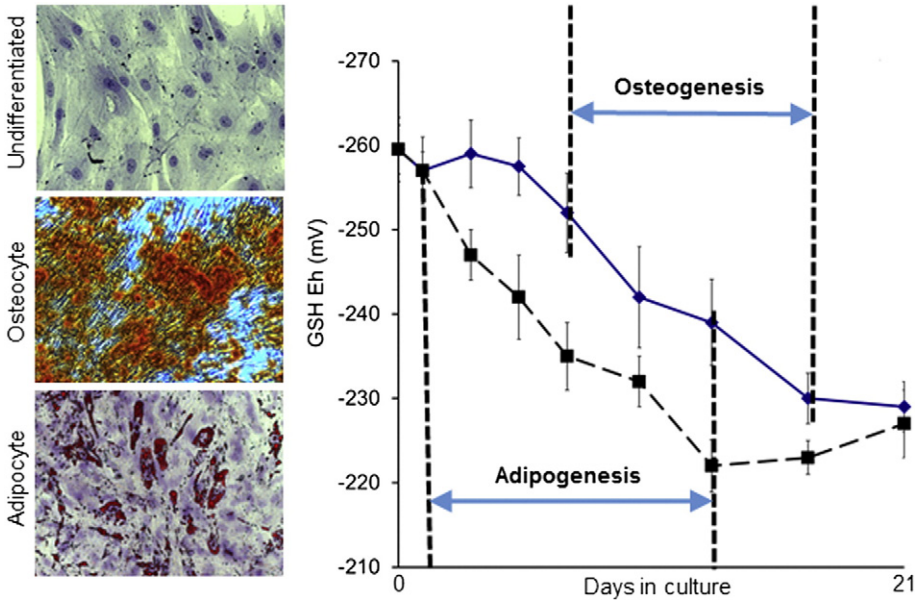

Fig. 5. Differential redox shifts during human mesenchymal stem cell (hMSC) adipogenesis and osteogenesis. hMSCs can capably differentiate into a variety of unique terminally differentiated phenotypes (left panels), including osteocytes (stained with Alizarin Red) and adipocytes (stained with Oil Red O). Adipogenesis in hMSCs is demarcated by a rapid oxidation of the GSH E_h (right panel), shifting from −260 mV to −220 mV during the first 13 days in culture. Osteogenesis also was demarcated by an oxidation in the GSH Eh but oxidation occurred starting at 7 days in culture and reached its final E_h by 17 days. These data support the redox regulation of differentiation through the GSH redox couple but also implicate a timing aspect to the redox regulation of differentiation for individual phenotypes [89].

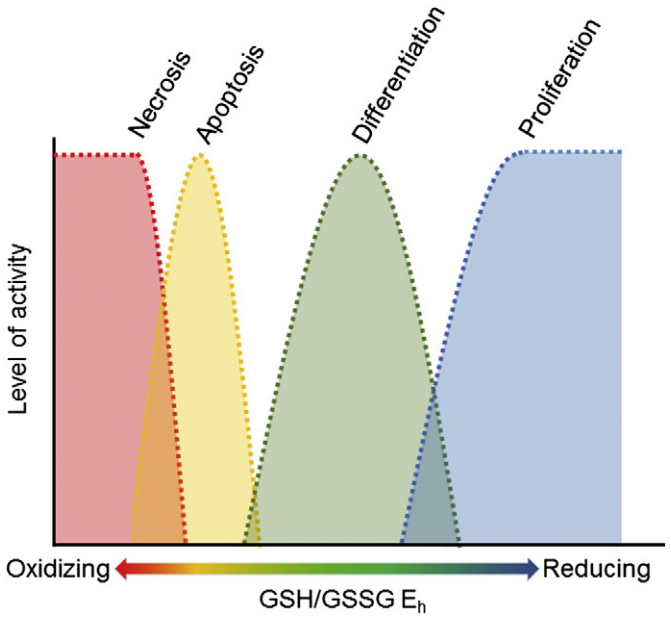

Fig. 6. Glutathione-mediated control of various cellular and developmental events. Under highly reducing GSH E_h conditions (estimated −240 to −250 mV), cellular proliferation machinery is highly active, supporting rapid and sustained cellular proliferation. As the GSH redox potential becomes more oxidized (estimated −200 to −220 mV), proliferation "slows" and cells begin to differentiate, expressing markers of terminal differentiation and performing unique cell functions. Apoptotic machinery are most active at much more oxidizing GSH E_h (estimated −180 mV) and necrosis occurs at even more oxidizing GSH E_h (estimated −150 mV) [2].

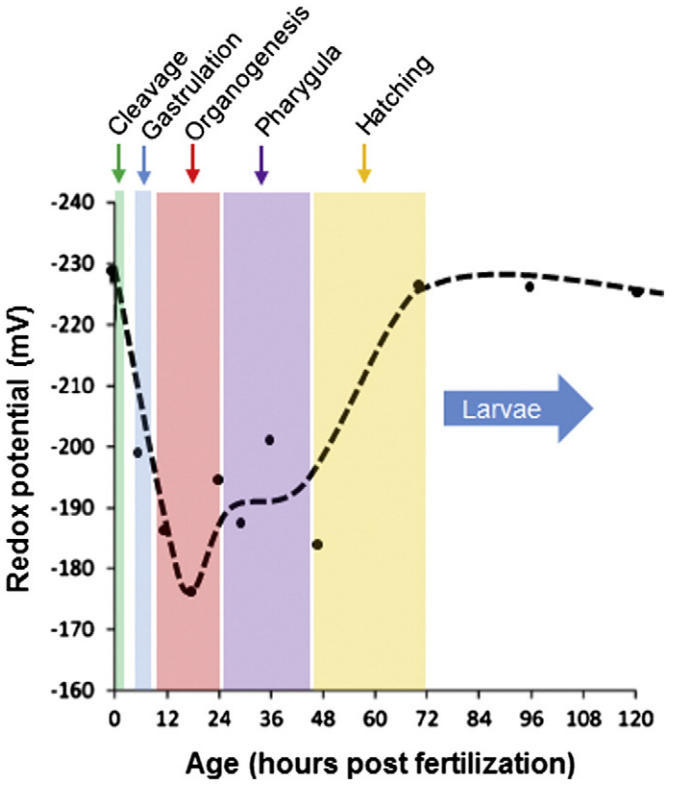

Fig. 7. Glutathione redox ontogeny during zebrafish development. Redox potential data is from Timme-Laragy et al. [128]. Important developmental events are highlighted. Interestingly, developmental periods of greatest differentiation correspond to the periods of greatest periods of oxidation. Of note, gastrulation, organogenesis (segmentation) and pharyngula periods demonstrate a substantial redox shift of approximately +30 to +55 mV. GSH concentrations increase by nearly 2.5-fold from 0 hpf to 48 hpf, with the greatest increase post 24 hpf (data not shown). In accordance with some of the in vitro models of cellular differentiation, periods of zebrafish differentiation also coincide with GSH redox shifts that are likely to support differentiative events during development.

shortly thereafter, between 10 and 24 hpf. The pharyngula period, a period of finer differentiation and morphogenesis, occurs between 24 and 48 hpf, which is also demarcated as a more oxidative period of development. These events coincide with the most oxidative GSH E_h through zebrafish development.

5. Glutathione redox states as modifiers of protein function and developmental signaling

5.1. Posttranslational modifications of proteins via S-glutathionylation

Glutathione can interact with many potential intermediates, including those involved in cell signaling. Moreover, there are numerous mechanisms by which this can occur. Under periods of oxidative stress where reactive oxygen species can directly damage and modify proteins, proteins thiols (PSH) can be oxidized to yield a protein sulfenic acid (PrOH). PrOH are reducible through reaction with reduced GSH to give water and protein S-glutathionylated adducts (Pr-SSG; see Reaction (A)); a modification that can change or disrupt protein function. Pr-SSG can also be formed through a one-electron oxidation with either protein thiol radical or GSH radical (see Reactions (B) and (C)), giving superoxide anion as a byproduct. During periods of high GSSG concentrations, disulfide exchange reactions can occur where GSSG can oxidize PSH to Pr-SSG and yield GSH (see Reaction (D)). Similarly, disulfide exchange reactions can occur between Pr-SSG and reduced proteins as well, where S-glutathionylated proteins can interact with other proteins to pass on the S-glutathionylation modification (see Reaction (E)) or a disulfide between two proteins to give reduced GSH (see Reaction (F)). The S-glutathionylation adduct can be effectively removed by reduced GSH to give PrSH and GSSG (see Reaction (G)). GSSG can then be effectively reduced to GSH through glutathione disulfide reductase using NADPH as a cofactor.

$$PrOH + GSH \rightarrow PrSSG + H_2O \quad \text{A}$$

$$PrS^{\bullet} + GS^{-} + O_2 \rightarrow PrSSG + O_2^{\bullet -} \quad \text{B}$$

$$PrS^{-} + GS^{\bullet} + O_2 \rightarrow PrSSG + O_2^{\bullet -} \quad \text{C}$$

$$PrSH + GSSG \rightarrow PrSSG + GSH \quad \text{D}$$

$$PrSH + {}'PrSSG \rightarrow PrSSG + {}'PrSH \quad \text{E}$$

$$PrSH + {}'PrSSG \rightarrow PrSS'Pr + GSH \quad \text{F}$$

$$PrSSG + GSH \rightarrow PrSH + GSSG \quad \text{G}$$

Regardless of the mechanism of the GSH system with various protein targets, modification and de-modification of redox-sensitive proteins are a critical means by which redox states can control a subset of protein and cell functions. Numerous examples of increased PrSSG formation have been reported in a variety of settings, such as during nutritional deficiency, EGF-mediated signaling and neutrophil activation [129–131]. While S-glutathionylation has not been fully examined in the context of differentiation development, there is some work demonstrating critical S-glutathionylation of signaling pathways during vascular development through modification to sirtuin 1 does indeed occur [77]. Work from our laboratories have shown that in organogenesis-stage mouse conceptuses, PrSSG levels do indeed fluctuate (unpublished observations), suggesting that GSH E_h are likely controlling

mechanisms during development. This hypothesis may serve as a basis to better understand how developmental toxicants cause dysmorphogenesis. While many developmental toxicants are capable oxidants, many do not cause cell death except at higher, extraphysiologic concentrations. This observation suggests than many developmental toxicants may not cause dysmorphogenesis through cell death, but through an alternative means, most notably through disruption of redox signaling that may control aspects of cellular proliferation and differentiation.

5.2. Alteration of enzyme activity and function

Perhaps one of the most underappreciated instances of, and perhaps the best example of, redox-sensitive signaling pathways are the kinase/phosphatase systems and the role that their redox regulation during development. Kinases that have redox regulatory elements within them include, protein kinase A (PKA), protein kinase C (PKC), MEKK1 (MAP (mitogen-activated protein kinase)/ERK (extracellular-signal-regulated kinase) kinase kinase) and creatine kinase (CK) [132–135]. In general, these kinases have cysteine residues that are sensitive to modification via S-glutathionylation, which either decreases or inhibits kinase activity completely. MEKK1 is an interesting example of regulation by S-glutathionylation. MEKK1 is involved in many different signaling cascades including those that are important in development (proliferation, differentiation and apoptosis). MEKK1 contains a cysteine residue (Cys^{1238}) in the glycine-rich loop of subdomain I of the kinase domain; a domain that is critical for binding ATP. Oxidation of Cys^{1238} appears to interfere with kinase activity [136]. Utilization of the thiol modifying agent (N-ethylmaleimide [NEM]) blocked MEKK1 activity, but in mutant MEKK1 where C1238V, NEM had no effect on MeKK1 activity. These authors further demonstrated that glutathione plays a critical role in the regulation of C^{1238} modification as well.

Phosphatases also have been shown to be redox-sensitive. These include protein tyrosine phosphatases (PTP1B), PTEN and PP2A. PP2A serves several functions in cellular proliferation and development. It has been shown to be sensitive to inactivation by H_2O_2, NEM, iodoacetamide, p-chloromercuribenzoate and GSSG [137]. Interestingly, addition of reduced GSH restores GSSG-inhibited PP2A activity, suggesting a potential reaction like described in reaction G above. Moreover, PP2A is directly related to the regulation of the NFκB signaling pathway [138] and may have implications in the dysfunction of this system during embryonic dysmorphogenesis (discussed below).

5.3. Redox compartmentation

The interplay between kinase and phosphatase activities represents a delicate balance of control to which many developmental signaling networks directly relate. However, it is difficult to rationalize how these two opposing activities interplay with each other as both redox-sensitive kinases and phosphatases are primarily deactivated by oxidizing conditions. Perhaps one level of control essential for normal signaling is redox compartmentation. The cell is a series of compartments, including nuclear, mitochondrial, endoplasmic reticular compartments. More generally, cytosolic and extracellular compartments also exist. Each of these compartments is unique in many different biochemical aspects including redox states.

Extracellular GSH pools are small by comparison to intracellular concentrations, where, in humans, extracellular GSH concentrations are estimated to be between 2 and 4 μM (vs. up to 10 mM intracellularly) [139,140] and a redox potential of approximately −150 mV (vs. −220 to −250 mV intracellularly). Because this pool is generally small it is more easily altered than in compartments with higher concentrations. During development, rodent embryos are enveloped in both the amnion and the yolk sac and bathed in their fluid. On gestational day 11, extracellular amniotic and yolk sac fluid GSH E_h is −83 and −126 mV, respectively [141]. The maintenance of extracellular redox states is important as they are link to control rates of proliferation, differentiation and susceptibility to oxidant-induced apoptosis [87,141–143]. How extracellular rates affect development *in utero* is not entirely understood and is an important consideration that warrants further study.

Other intracellular GSH pools are controlled discretely and represent unique redox GSH environments. The mitochondrial GSH E_h is approximated to be −300 mV [144,145]; a much more reducing GSH E_h than that in the cytosol (−220 to −260 mV). This is perhaps due to differences in pH. Mitochondrial pH is estimated to be approximately 7.8–8.0, whereas cytosolic is 7.4. Shifts in pH alone constitute a −24 to −36 mV shift when GSH and GSSG concentrations are applied to the Nernst equation. Still, mitochondria are considered relatively reducing.

Because these redox microenvironments have some autonomy they can be discretely regulated to manage signaling within their microdomains. In turn, compartment specific redox regulation allows for more discrete regulation of signaling networks to give more defined signaling outcomes. As such, changes to certain redox microenvironments can have very different outcomes dependent upon their location. How these discrete shifts occur in development is currently under study and may provide a key for fine tuning GSH's role during development. One particular means by which redox compartmentalized control of GSH function may be through the regulation of redox-sensitive transcription factors (see sections below).

5.4. Redox regulation of transcription factors through the GSH couple: nuclear factor Kappa B

There are many transcription factors that are sensitive to thiol modification as a means to regulate their activity. Some of these include activator protein-1 (AP1), p53, glucocorticoid receptor (GR), hypoxia inducible factor 1α and nuclear factor kappa B (NFκB) [146–148]. While all of these are of interest, this review will focus on NFκB as it has been (1) implicated in proliferation, differentiation and apoptosis, which are all developmental events, (2) shown to be regulated specifically by the GSH system, and (3) is believed to be involved in thalidomide-induced teratogenesis [36,104,149].

NFκB is a redox-sensitive transcription factor that is involved in various aspects of proliferation, differentiation and apoptosis. Simplistically, the NFκB system is composed of (1) NFκB itself, as a heterodimer of either p65–p50 or p50-cRel with the former considered to be the most predominant, and (2) inhibitory κB (IκB), an inhibitor molecule that keeps NFκB sequestered in the cytosolic space and inactive. In brief, NFκB αactivation occurs as IκB is phosphorylated by an inhibitory κB kinase (IKK), which results in a dissociation of the NFκB/IκB complex. NFκB translocates to the nucleus where it binds to DNA to affect gene regulation.

The redox regulation of NFκB has been thoroughly reviewed in depth elsewhere [146,150–152], but more specifically, GSH redox states has been shown to be intricately involved in the regulation of various aspects of NFκB signaling. Many of the components of the NFκB pathway are subject to modification by S-glutathionylation. Foremost, NFκB is in itself redox-sensitive and can be S-glutathionylated. In the p50 subunit, Cys^{62} was originally determined to be important as it is located in the DNA binding domain [153]. Subsequent work revealed that p50 exposed to oxidizing GSH E_h conditions caused a 40–70% decrease in NFκB/DNA binding [154]. Confirmation via mass spectrometry revealed that the Cys^{62} residue was indeed S-glutathionylated under these conditions and accounted for a major portion of the observed decrease in DNA binding.

Additionally, the p65 subunit is also susceptible to S-glutathionylation, where under oxidizing redox environments (high [GSSG]) or where H_2O_2 and GSH are jointly present, an increase of S-glutathionylated p65 can be detected [155]. Under oxidizing conditions, NFκB-mediated induction of ICAM-1 expression was significantly decreased and p65 was retained in the cytosol even following TNFα stimulation, suggesting that oxidizing

redox conditions inhibit nuclear translocation. While the p65 Cys residue was not identified via mass spectrometry, mutagenesis approaches revealed that Cys^{38}, Cys^{160} and Cys^{216} are likely targets for S-glutathionylation modification. It remains unclear if all residues require modification for inhibition or if each individual residue may serve to differentially regulate this translocation step in the NFκB signaling pathway.

IκB also appears to be sensitive to GSH redox states. Under oxidizing conditions (high [GSSG]), IκB is S-glutathionylated on Cys^{186} [156]. Modification of Cys^{186} results in the inhibition of phosphorylation of IκB by creatine kinase II. Additionally, IκB ubiquitination was also diminished by Cys^{186} S-glutathionylation.

Inhibitory κB kinase β-subunit (IKKβ) phosphorylates IκB and is an initial step in the activation of NFκB. Treatment with H_2O_2 reduces TNFα-induced IKKβ kinase activity. Site-directed mutagenesis of Cys^{179} showed that this residue was critical for inhibition of IKKβ kinase activity by H_2O_2 [157]. Overexpression of glutaredoxin-1 (Grx1), which catalyzes the reversible reduction of protein–glutathionyl mixed disulfides, restored IKKβ kinase activity, which supports S-glutathionylation as the primary modification in IKKβ. Conversely, Grx1 knockdown inhibited IKKβ activity and NFκB activation.

While these examples of GSH redox regulation of the NFκB pathway are convincing, it is not entirely understood how oxidizing GSH E_h may differentially regulate individual elements of the NFκB system or whether these are physiologically relevant. Clearly, more study is required to better understand how more discrete and compartmentalized changes to GSH E_h may result in alteration of signaling.

5.5. Glutathione redox dysregulation in chemical-induced teratogenesis: NFκB inhibition through thalidomide-induced oxidation of GSH pools in limb teratogenesis

Thalidomide (α-phthalimidophthalimide) was originally introduced to the public in the late 1950s as a sedative/antinausea agent. Because it was believed to have little to no side effects, it was prescribed to pregnant women to relieve morning sickness. Nearly, 4 years after it was initially introduced, thalidomide was discovered to cause serious birth defects, the most common being a limb reduction defects, termed phocomelia.

One hypothesis of the mechanism of thalidomide teratogenicity is by oxidative stress-induced alteration of the GSH redox potential and subsequent disruption of NFκB signaling [36,104]. Initial work to test this hypothesis utilized thalidomide-resistant mice and thalidomide-sensitive rabbits [158], where thalidomide caused DNA oxidation, a marker of oxidative stress, in rabbit embryos but not in mouse embryos. DNA oxidation also positively correlated with the manifestation of phocomelia in rabbit embryos. Pretreatment with a free radical trap abrogated dysmorphogenesis in rabbit newborns, suggesting that oxidative stress was central to thalidomide-induced teratogenesis. In whole embryo culture approaches, rabbit conceptuses treated with thalidomide showed a GSH depletion at lower concentrations of thalidomide than thalidomide-resistant rat conceptuses [11]. This is significant as GSH E_h are significantly more oxidizing in the rabbit limb than in a stage-similar rat limb [48], suggesting that oxidant-induced dysregulation of redox signaling through S-glutathionylation may occur more readily in the rabbit limb than then rat limb. In fact, S-glutathionylated proteins are nearly 6-fold higher in the rabbit limb compared to the rat limb, demonstrating a comparatively high degree of GSH E_h control in the rabbit limb.

One interestingly observational finding in cross-species comparisons is that rabbit limb buds have significantly less cysteine than rat limb buds, approximately 50% less [45]. Because cysteine is the rate limiting precursor to GSH de novo synthesis, sustained thalidomide-induced loss of GSH may be in part due to the inherently low cysteine concentrations in the rabbit limb, suggesting that rabbit GSH concentrations may be slower to recover to normal levels following thalidomide treatments, resulting in longer periods of GSH deficit and imbalance than in the resistant rat embryonic limb.

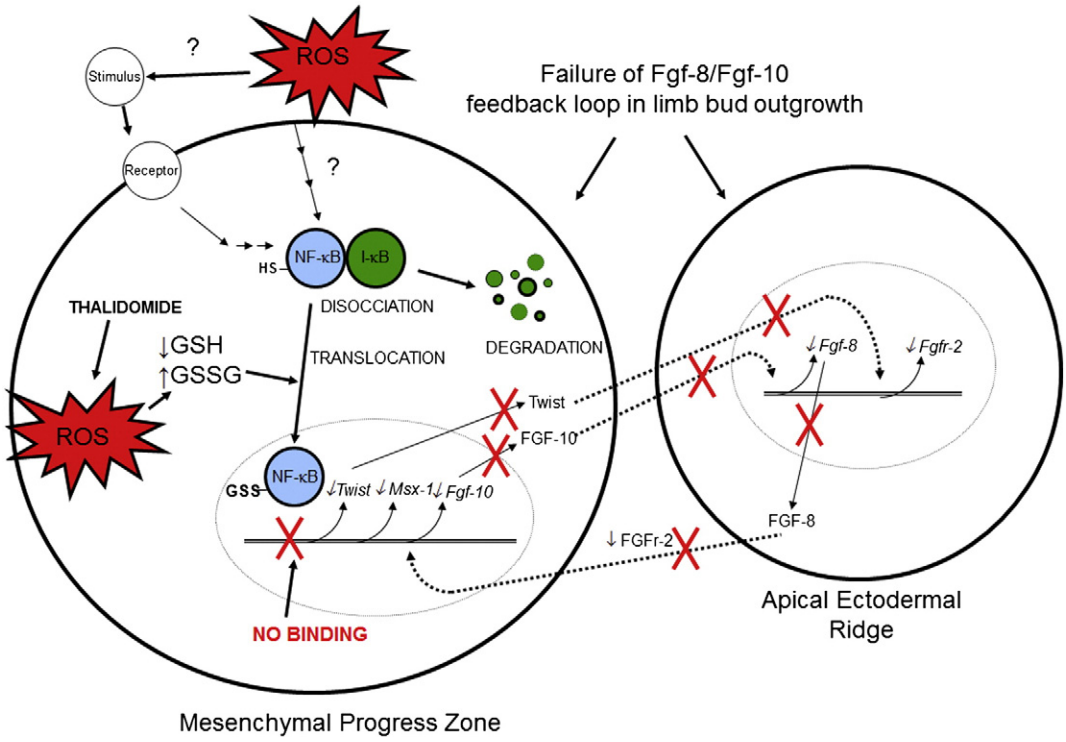

Fig. 8. As such, modification to the Cys^{62} in the p50 DNA binding domain may occur through S-glutathionylation to yield a decrease in NFκB binding. Loss of NFκB binding decreases the expression pattern of many genes, but perhaps most notably, it decreases the expression of Fgf-10. Fgf-10 from the mesenchymal progress zone (MPZ) normally induces Fgf-8 from the apical ectodermal ridge (AER) to establish a feedback loop to stimulate cell proliferation in the MPZ. Loss of NFκB signaling inhibits the institution of the Fgf-8/Fgf-10 feedback loop and limb bud outgrowth is diminished [36].

Much of limb outgrowth is an interplay between growth factors Fgf-10 and Fgf-8 in the mesenchymal progress zone (MPZ) and the apical ectodermal ridge (AER), respectively, of the early developing limb bud [159]. These growth factors constitute a positive feedback loop that stimulates outgrowth between these different limb regions. Loss of feedback results in limb bud ablation, shortening or malformation [160–162], which appears to mimic similar types of limb dysmorphogeneses cause by thalidomide in humans, including phocomelia. In systems where NFκB is inhibited, limb buds failed to develop correctly and were malformed, and the Fgf-10/Fgf-8 feedback loop was not fully established [163,164], suggesting that NFκB is fundamentally central to limb development. Thalidomide has been shown to inhibit the NFκB system in multiple mental models, including models of hepatitis, cirrhosis, cancer and inflammation [165–168], but little information was available as to how thalidomide altered NFκB activity in the limb. Using limb bud cells from rat and rabbit embryos, thalidomide inhibited phorbol ester-induced NFκB activation in the rabbit but not in the rat limb bud cell, but activation could be rescued in the rabbit limb bud cell with co-treatments with reducing agents (NAC or α-phenylbutylnitrone [αPBN]) [149]. These data suggest that NFκB is inhibited in the rabbit limb cells by thalidomide through a redox-sensitive mechanism.

In utero experimentation showed that in rabbits treated with thalidomide, the Fgf-8/Fgf-10 feedback loop was disrupted, but rats showed no such effects [149]. Pretreatment with αPBN restored the Fgf-8/Fgf-10 feedback loop, which coincides with a correction in limb abnormalities [158]. Because the NFκB pathway is critical to the initiation of, and potentially the maintenance of, limb bud outgrown through the Fgf-8/Fgf-10 feedback loop, loss of NFκB activities are likely to have devastating outcomes (Fig. 8). Current teratological data shows that thalidomide inhibits NFκB and this occurs through a redox-sensitive mechanism. Furthermore, thalidomide causes a disruption in the GSH homeostasis, and as such, findings suggest that GSH is an important mediator of limb bud NFκB activity.

6. Summary

GSH is now recognized as a molecule that is involved in multiple molecular processes, such as enzyme activation and protein folding and more broadly, cellular proliferation, differentiation and apoptosis. As many, if not all of these events are crucial during development, GSH is likely at the nexus of redox-regulation in embryogenesis, organogenesis and dysmorphogenesis. Characterization of GSH function and regulation, on both a direct or permissive basis, requires substantially more study to better develop a more profound understanding of the developmental role of GSH.

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