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Cellular Mechanisms Underlying Natural Tolerance to Low Oxygen Tension
and Extended Fasting in Elephant Seals

By

Kaitlin Allen

A dissertation submitted in partial satisfaction of the
requirements for the degree of
Doctor of Philosophy
in
Integrative Biology
in the
Graduate Division
of the
University of California, Berkeley

Committee in charge:

Professor José Pablo Vázquez-Medina, Chair
Professor Caroline Williams
Professor Peter Sudmant
Professor Robert Full

Summer 2024

Abstract

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Doctor of Philosophy in Integrative Biology

University of California, Berkeley

Professor José Pablo Vázquez-Medina, Chair

Marine mammals exhibit numerous physiological adaptations that supported their return to the ocean. At the broadest scale, these adaptations modulate organismal energetics. Chief among these are modifications to oxygen storage and utilization, as well as metabolic adaptations that enable spatial and temporal separation of foraging from energetically intensive life history stages. All marine mammals forage during apneas, necessitating a tolerance for declining blood oxygen tensions. Unlike cetaceans, pinnipeds remain partially land-dependent and are, therefore, regularly separated from their foraging grounds. The northern elephant seal (*Mirounga angustirostris*) represents a tractable model for the study of extreme hypoxia and fasting tolerance in marine mammals, and a large body of research on whole organismal responses to diving and fasting exists in this species. Still, technological constraints prohibit investigations of the dynamic cellular mechanisms critical to diving and fasting in these animals.

This dissertation identifies novel molecular and cellular mechanisms regulating hypoxia and fasting tolerance in a deep-diving marine mammal, the northern elephant seal. Chapter 1 summarizes the current understanding of intrinsic hypoxia tolerance in marine mammals, as well as the role of the energetically intensive postweaning fast in elephant seals. This chapter ultimately identifies three areas for which molecular contributions to whole organismal phenotypes are poorly defined: (1) real-time responses of the seal vasculature to hypoxia; (2) seal-specific regulation of the molecular pathways contributing to oxidative stress resistance; and (3) the role of intrinsic cellular versus systemic signals in muscle cell energetics during prolonged fasting. Chapters 2-4 address areas 1-3 by developing manipulable, proliferative cell culture systems to evaluate real-time responses to hypoxia and oxidative stress and identify cell type-specific signatures of fasting tolerance.

Chapter 2 evaluates the role of the canonical hypoxia signaling pathway in the seal arterial endothelial cell response to hypoxia. These experiments combine global transcriptional data with functional assays that identified blunted angiogenic signaling and increased glutathione synthesis as key factors in the response to hypoxia. Chapter 3 interrogates the seal endothelial cell response to reoxygenation after hypoxia and considers whether a chemical oxidant treatment recapitulates this response. Reoxygenation did not substantially alter coordinated gene expression patterns in seal cells, but chemical oxidant exposure upregulated the expression of iron-handling genes in the ferroptosis pathway and downregulated genes involved in the synthesis and metabolism of sulfur-containing amino acids. Together, these chapters utilize a previously inaccessible tissue system to identify mechanisms of intrinsic hypoxia and oxidative stress tolerance in a deep-diving mammal.

Chapter 4 examines the role of cell-autonomous versus circulating factors in seal skeletal muscle myoblast energetics. These experiments utilize cells and serum derived from elephant seal pups at early and late stages of the postweaning fast. Respirometry experiments identified both cell-autonomous and serum-borne shifts toward glycolytic ATP production with fasting progression, suggesting that metabolic regulation is substrate-specific and regulated by both intrinsic and extrinsic factors in these cells.

Together, these studies expand our understanding of marine mammal physiology within the context of a natural tolerance to energetic constraints. This work highlights the utility of primary cell culture models for connecting cellular-level changes to whole organismal function. In Chapter 5, I summarize the results and their contributions to the field.

*To my parents,
who let me ask “why?” so many times it turned into a career*

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Publications related to this work

This dissertation contains parts of or the entirety of work that has been previously published. These publications have been reformatted and/or modified to fit the format and structure of this dissertation:

Chapter 1

Allen KN, Vázquez-Medina JP. Natural Tolerance to Ischemia and Hypoxemia in Diving Mammals: A Review. *Frontiers in Physiology*. 2019;10: 1199. doi:10.3389/fphys.2019.01199.

Ensminger DC*, Salvador-Pascual A*, Arango BG*, **Allen KN***, Vázquez-Medina JP. Fasting ameliorates oxidative stress: A review of physiological strategies across life history events in wild vertebrates. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*. 2021;256: 110929. doi:10.1016/j.cbpa.2021.110929. * Equal contributions

Chapter 2

Allen KN, Torres-Velarde JM, Vazquez JM, Moreno-Santillán DD, Sudmant PH, Vázquez-Medina JP. Hypoxia exposure blunts angiogenic signaling and upregulates the antioxidant system in endothelial cells derived from elephant seals. *BMC Biology*. 2024;22. doi:10.1186/s12915-024-01892-3.

Chapter 4

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1. Introduction

1.1 Spatial independence of oxygen and nutrient availability in diving mammals

Marine mammals face many of the energetic demands associated with foraging, reproduction, and development in environments where access to oxygen is spatially and temporally separated from access to other nutrients. Northern elephant seals (*Mirounga angustirostris*), in particular, spend up to 90% of their at-sea time foraging during deep, repeated dives with limited recovery time spent breathing at the surface [1] and undertake prolonged, energetically-intensive terrestrial fasts while developing, molting, and breeding [2]. Elephant seals thus demonstrate remarkable tolerance to the stressors of diving and prolonged fasting. However, the cellular mechanisms supporting this tolerance remain difficult or impossible to assess *in vivo* in these long-lived, large-bodied marine mammals. Primary cell culture systems provide a model to study responses to diving and fasting at the cellular level [3]. Pairing these *ex vivo* systems with tissue and organismal level physiological and biochemical data supports identification of the molecular phenotypes critical to hypoxia and fasting tolerance in elephant seals.

1.2 Introduction to diving physiology

The ability to manage body oxygen stores in the face of environmental hypoxia constrains the life history of many vertebrate taxa. In humans, oxygen management is critically important in clinical settings where both acute and chronic conditions such as organ transplantation and intermittent hypoxia contribute to ischemic injuries. Diving mammals, however, experience regular fluctuations in blood flow and oxygen saturation without sustaining such injuries. To date, a range of physiological mechanisms for coping with finite oxygen availability have been identified in marine mammals (for recent comprehensive reviews on the topic, see Blix, 2018; Davis, 2014; Ponganis, 2019). The first – and perhaps most straightforward – of these mechanisms is increased mass-specific body oxygen stores which delay the onset of hypoxemia and tissue hypoxia to prolong submergence times [7–10]. Similarly, splenic contraction increases circulating oxygen levels during apnea in pinnipeds [11–14]. Populations of human breath-hold divers also demonstrate enlarged spleens and positive selection of genes implicated in spleen size (PDE10A) and regulation of vasomotor tone (BDKRB2) [15,16]. In marine mammals, these enhanced “onboard” oxygen stores are likely sufficient to support normal aerobic function in peripheral organs including the liver and kidneys during most dives [17]. Additionally, the majority of pinniped dives occur within the aerobic dive limit, defined as the dive duration beyond which post-dive blood lactate levels increase [4,18–20]. However, diving mammals experience hypoxemia during routine breath holds even within aerobic limits, readily tolerating lower arterial and venous oxygen saturations than most terrestrial mammals including humans [21–28].

The specific molecular and cellular pathways that protect marine mammals from injuries driven by fluctuations in blood flow and local oxygen tensions remain largely unexplored. In humans, hypoxemia induces cell death and tissue injury via inflammatory, necrotic, ferroptotic, and apoptotic pathways [29–34]. Limited oxygen availability during hypoxia impairs mitochondrial respiration, leading to a drop in intracellular ATP levels. Reoxygenation after hypoxia increases oxidant generation from enzymatic systems (e.g., xanthine oxidase, NADPH oxidases) and mitochondria, carrying an additional threat of oxidative injury to cells and tissues

and potentially compromising organismal health and survival (Figure 1) [35,36]. Reperfusion injuries are well documented in humans, particularly during myocardial infarction, ischemic stroke, and organ transplantation [37–39]. Thus, both hypoxemia and reduced peripheral perfusion associated with diving can potentially contribute to inflammation and oxidative stress in diving mammals. Remarkably, diving mammals appear to tolerate such conditions without injury. Therefore, understanding the mechanisms underlying this tolerance may yield insight into translational applications for human health.

1.2.1 The mammalian diving response, ischemia, and hypoxia

The mammalian diving response consists of several coordinated physiological adjustments originally considered critical in shielding the central nervous system from hypoxia during diving [40–43]. More recently, it was discovered that the diving response also maximizes the aerobic dive limit without compromising central nervous system function [4,18]. The three primary components of the diving response are apnea, bradycardia, and peripheral vasoconstriction; all three have been studied in multiple marine mammal species using a variety of experimental approaches [5,6,44,45]. In pinnipeds, apnea alone is sufficient to induce both bradycardia and vasoconstriction independent of whether the animal is diving [26,46]; bradycardia is further modulated by water temperature, the extent of facial submersion, cognition (i.e., anticipation) and exercise [18,44,45,47–49]. Peripheral perfusion and vasoconstriction have historically been difficult to measure in marine mammals. Early studies in Weddell seals indicated an extensive, profound, near-cessation of blood flow to peripheral tissues including the kidney, liver, and spleen during forced dives [50]. Comparative studies showed that seal kidneys exposed to *ex vivo* ischemia recovered both blood flow and urine production upon reperfusion, while dog kidneys did not [51]. The magnitude of the physiological response occurring in free dives is likely different from that observed during forced submersions [52,53]. Measurements of peripheral perfusion during voluntary apneas, however, have focused on skeletal muscle rather than splanchnic organs [26,54]; reduced muscle perfusion during both sleep apnea and diving permits utilization of myoglobin-bound oxygen by the muscle [24,54–56]. A lack of instrumentation has hampered direct observations of splanchnic organ perfusion during free dives, though technological developments are beginning to offset this knowledge gap. A noninvasive near-infrared-based method recently identified an overall reduction in blubber hemoglobin concentration and saturation in captive harbor seals voluntarily diving within aerobic limits [57], suggesting reduced perfusion to the periphery during routine dives.

Despite reduced cardiac output and peripheral oxygen consumption during diving, however, marine mammals deplete central oxygen stores during routine voluntary dives [23,24,28,58,59]. As a result, even continuously perfused tissues such as the brain likely experience reductions in oxygen tension as a result of diving-induced hypoxemia [57,60,61]. Elevated neuroglobin levels and selective brain cooling may supplement cerebral oxygen stores while decreasing demand during diving [62–65]. Moreover, the seal brain is capable of both producing and consuming lactate *in vivo* [66], and glucose deprivation during hypoxia does not appear to negatively impact neuronal function in seal brain slices, suggesting that the seal brain may tolerate these insults [67,68]. Recent transcriptomic analyses suggest that metabolic shifts and upregulation of several major stress response pathways may also protect the seal brain against hypoxic injury [69,70]. Thus, understanding the mechanisms that protect the marine mammal brain during diving-induced hypoxia/reoxygenation and ischemia/reperfusion events can potentially reveal new targets for pharmacological interventions in human brain injury and stroke patients.

1.2.2 Consequences of hypoxia and ischemia: oxidant generation, inflammation, and oxidative stress

The complex mechanisms underlying ischemic tissue damage have been studied extensively in biomedical models due to their clinical relevance to human conditions including organ transplantation, myocardial infarction, and stroke. Oxygen deprivation during ischemia depletes intracellular ATP; subsequently, ATP degradation products including xanthine and hypoxanthine accumulate [36]. Ischemia also dysregulates calcium levels, activating calcium-dependent proteases that cleave xanthine dehydrogenase to generate the active form of the enzyme, xanthine oxidase [71,72]. Increases in intracellular calcium during ischemic events also induce NADPH oxidase activation and promote mitochondrial superoxide radical ($O_2^{\bullet-}$) generation [73,74]. Upon reperfusion/reoxygenation, xanthine and hypoxanthine are oxidized by xanthine oxidase, generating superoxide radical and hydrogen peroxide (H_2O_2) [36,75,76]. Similarly, $O_2^{\bullet-}$ is generated by reverse electron transport at mitochondrial complex I during reperfusion [77,78]. In the vascular endothelium, $O_2^{\bullet-}$ generated by NADPH oxidases (which are activated via mechanosignaling during ischemic alterations in shear stress) contributes to tissue injury via the formation of peroxynitrite ($ONOO^-$) [79–83]. Furthermore, inflammatory molecules generated or activated by endothelial NADPH oxidase-mediated redox signaling (e.g., CAMs, selectins, NF- κ B, and the NLRP3 inflammasome) prime the vasculature for neutrophil adherence and infiltration, promoting further tissue injury during reperfusion [37,84]. In diving seals, convective oxygen transport to peripheral tissues remains sufficient to support aerobic metabolism during most dives [17,18]; however, routine hypoxemia coupled with reoxygenation upon surfacing [23–25,28,85], along with potential alterations in blood flow derived from peripheral vasoconstriction, expose the vascular endothelium to frequent fluctuations in shear and oxygen tension that resemble pathological ischemia/reperfusion events in humans.

Marine mammals do not sustain the reperfusion injuries associated with hypoxemia and ischemia/reperfusion events in humans; however, the mechanisms regulating this oxidant balance in marine mammals remain unclear. Increased purine recycling has been proposed as a mechanism to limit xanthine oxidase-derived oxidant generation in marine mammals [86], but support for this hypothesis is mixed [87–89]. Early work revealed that seal tissues accumulate hypoxanthine after simulated ischemia and are capable of generating oxidants *ex vivo* [90–92]. Similarly, circulating concentrations of xanthine and hypoxanthine increase during spontaneous terrestrial sleep apneas in elephant seals [93]. Moreover, tissue capacity to generate oxidants increases with postnatal maturation in hooded seal skeletal muscle [94], while circulating and muscle levels of xanthine oxidase increase after repetitive sleep apneas in elephant seals [93]. Together, these results suggest that seals do not avoid oxidant generation outright to cope with diving-induced ischemia/reperfusion. Of note is recent evidence showing alterations in the nitric oxide-soluble guanylyl cyclase-cGMP (NO-cGMP) pathway in peripheral tissues of Weddell seals compared to non-diving vertebrates [95] and the previously observed absence of nitric oxide in the exhalate of Weddell seals after voluntary dives [96]. Such alterations in the NO-cGMP pathway could help maintain differential perfusion during a dive while preventing the formation of $ONOO^-$ via the reaction of NO with $O_2^{\bullet-}$ generated in response to diving-induced ischemia/reperfusion [97].

1.2.3 Coping with diving-induced hypoxia and ischemia: counteracting oxidative stress and preventing inflammation

High activity and expression of antioxidant enzymes, particularly those related to the glutathione system, have been observed across diving birds and mammals. Baseline circulating and tissue antioxidant levels are higher in diving versus non-diving birds and mammals, supporting the hypothesis that a robust antioxidant defense system mitigates injury from diving-induced oxidant generation in marine vertebrates [98–105]. Whether this relationship between antioxidant levels and diving capacity holds across diving species remains unclear; interspecies comparisons of diving capacity and antioxidant levels are difficult to isolate from confounding species-specific life history factors such as fasting and maturation [99,106–108]. However, in phocid seals, the antioxidant system develops alongside diving capacity during postnatal maturation and does not decline with aging, suggesting a link between diving ability and antioxidant defenses [109–111].

1.2.4 Molecular underpinnings of hypoxia and oxidative stress tolerance in marine mammals

Recent genetic and molecular work has begun to address the underlying gene-level modifications contributing to the physiological adjustments that support diving in marine mammals. The hypoxia-inducible factor 1 (HIF-1) is considered the master regulator of the molecular response to hypoxia across taxa [112–115]. Functional HIF-1 is composed of two subunits, HIF-1 α and HIF-1 β . Under normoxic conditions, the pVHL-ubiquitin-proteasome proteolytic pathway continuously degrades HIF-1 α . During hypoxia, this degradation halts and HIF-1 α dimerizes with HIF-1 β , translocating into the nucleus where it regulates transcription of genes involved in angiogenesis, erythropoiesis, and proliferation [116,117]. Convergent substitutions in the HIF-1 α amino acid sequence across hypoxia-tolerant mammals – including cetaceans, high altitude ungulates, and subterranean rodents – suggest a critical role for HIF-1 α regulation in natural hypoxia tolerance [118]. Seals possess a single copy of the HIF-1 α gene; it is similar in sequence to terrestrial mammal HIF-1 α , though with several amino acid differences in the oxygen-dependent degradation domain [119]. Seal tissues with higher HIF-1 α protein levels show less overall protein oxidation, suggesting that HIF-1 α expression protects against oxidative stress in marine mammals [120]. Interestingly, amino acid sequence differences in cetacean HIF-1 α likely affect HIF-1 α sensitivity and responsiveness to changing oxygen conditions rather than establishing a constitutively active response [121]. Previous *in vivo* work showed marked HIF-1 α upregulation in elephant seal muscle in response to prolonged fasting and repetitive sleep apneas [93,122]. Similarly, our studies with primary vascular endothelial cells show rapid stabilization of HIF-1 α in seal cells exposed to hypoxia, but this response is decoupled from the canonical HIF-angiogenesis pathway (see Chapter 2). Together, these studies suggest a critical, but non-canonical role of HIF-1 α in mediating hypoxia tolerance in marine mammals.

1.3 Introduction to fasting in vertebrates

Elephant seals (*Mirounga* spp.) undergo prolonged terrestrial fasts during energetically intensive life stages including breeding/lactation, molt, and postnatal development. Three distinct physiological phases characterize vertebrate fasting. Phase I maintains homeostasis while relying on glycogen, lipid, and protein energy sources, though body mass and metabolic rate decline. Phase II utilizes body lipid stores for the bulk of ATP production, with an increasing dependence on

protein and further declines in body mass and metabolic rate. Both Phase I and II preserve overall homeostasis. Phase III occurs after lipid stores are depleted and relies nearly entirely on protein to fuel energetic needs. Homeostatic control is lost in this phase, which is characterized by low metabolic rates, severely reduced body mass, and cachexia. Phase III ultimately leads to starvation and death if refeeding does not occur. Fasting-tolerant vertebrates such as elephant seals generally extend Phase II, during which body mass declines but homeostasis is preserved through reliance on lipid fuel sources and sparing of body protein stores [123–125].

1.3.1 Postnatal development occurs during terrestrial fasting in elephant seals

While many fasting-tolerant vertebrates utilize strategies such as prolonged metabolic suppression and physical inactivity to minimize metabolic requirements during prolonged fasts (e.g., squirrels or bears during torpor and hibernation), marine mammals typically fast concurrently with energy-demanding processes such as breeding, molting, or migration. Elephant seal pups nurse for approximately one month, after which they are abruptly weaned and remain (fasting) on land for 2-3 months prior to departing on their first marine foraging trip [126]. During this terrestrial fast pups mobilize blubber stores to fuel an increase in mass-specific blood and muscle oxygen stores, which support extended breath hold diving [2,127]. This fast is associated with a decrease in whole body metabolic rate and an increase in metabolic suppression during rest-associated apneas [128], though the tissue-level contributions to reduced VO_2 across the postweaning fast have not been investigated. Recent work demonstrates a decline in total respiratory capacity in isolated muscle mitochondria with maturation (though adult seal muscle mitochondria maintain a high capacity for fatty acid oxidation) and overall preservation of oxidative capacity in adult female seals during the annual molt fast [129,130]. Still, the degree to which these patterns are reflected in the postnatal developmental fast remains unknown.

1.3.2 Cellular respiratory profiles can be cell-autonomous or altered by circulating factors

Two recent studies in grizzly bears, another large-bodied fasting-tolerant species, demonstrate that organismal-level changes in metabolism are reflected in cultured adipocytes [131,132]. These works confirm the utility of cell culture systems in identifying cellular-level contributions to whole organismal phenotypes. Additionally, the authors found that culturing cells isolated from bears during a given hibernation state (active season vs. hibernation) with “unmatched” serum (e.g., hibernation cells with active season serum) effectively recapitulated the respiratory phenotype observed in the season from which the serum was collected, suggesting that circulating factors can override cell-autonomous patterns in metabolic preference in this species [132]. Similarly, serum from deep diving seals blunts inflammatory signaling in response to lipopolysaccharide treatment in both seal and mouse monocytes [133]. Together, these studies indicate a role for both cell-autonomous and systemic mechanisms (circulating factors) in regulating metabolism and inflammation at the tissue level.

1.4 Areas of opportunity

Marine mammals experience diving-induced hypoxia/reoxygenation events and extended fasting without long-term detrimental effects. Physiological, biochemical, and genomic studies have begun to uncover the mechanisms underlying such extreme tolerance. Among those mechanisms, coordinated body-wide responses to delay the onset of tissue hypoxia, counteract

oxidant generation and prevent inflammation are critical. Convergent genomic changes across marine mammal lineages hint at the evolutionary underpinnings of the physiological adaptations supporting mammalian diving [134]. The increasing availability of high-quality genome references from additional species will strengthen these studies. Similarly, functional studies dissecting the cellular and molecular underpinnings that confer tolerance to hypoxia and fasting in marine mammals are beginning to be conducted [89,135,136]. *Ex vivo* systems that are amenable to physiological manipulation and molecular perturbation provide the missing link between genomic- and organismal-level investigations. Identifying the drivers of hypoxemic and fasting tolerance in marine mammals can provide a mechanistic understanding of natural tolerance to such conditions, while aiding in translation to clinical applications.

1.5 Goals for the dissertation

This dissertation addresses the above areas of opportunity by utilizing *ex vivo* and *in vitro* experimental approaches. Chapters 2 and 3 establish the first primary endothelial cell cultures from elephant seals. Chapter 2 identifies a functional decoupling of the canonical vertebrate hypoxia sensing system from downstream changes in oxygen delivery. Chapter 3 identifies iron management as a potential anti-ferroptotic strategy in elephant seal cells. Chapter 4 utilizes primary muscle cells isolated from weaned elephant seal pups to explore the role of cell-autonomous versus circulating factors in muscle cell metabolism during the postnatal developmental fast. Together, these projects develop our cellular-level understanding of hypoxia and fasting tolerance in marine mammals.

1.6 Acknowledgments

Thank you to Emily Lam and Esmeralda Garcia-Orosco for review of the hypoxia portions of this chapter. Thank you to Drs. David Ensminger and Andrea Salvador-Pascual, and Gaby Arango for comments and suggestions on the fasting portions of this chapter.

1.7 Figures

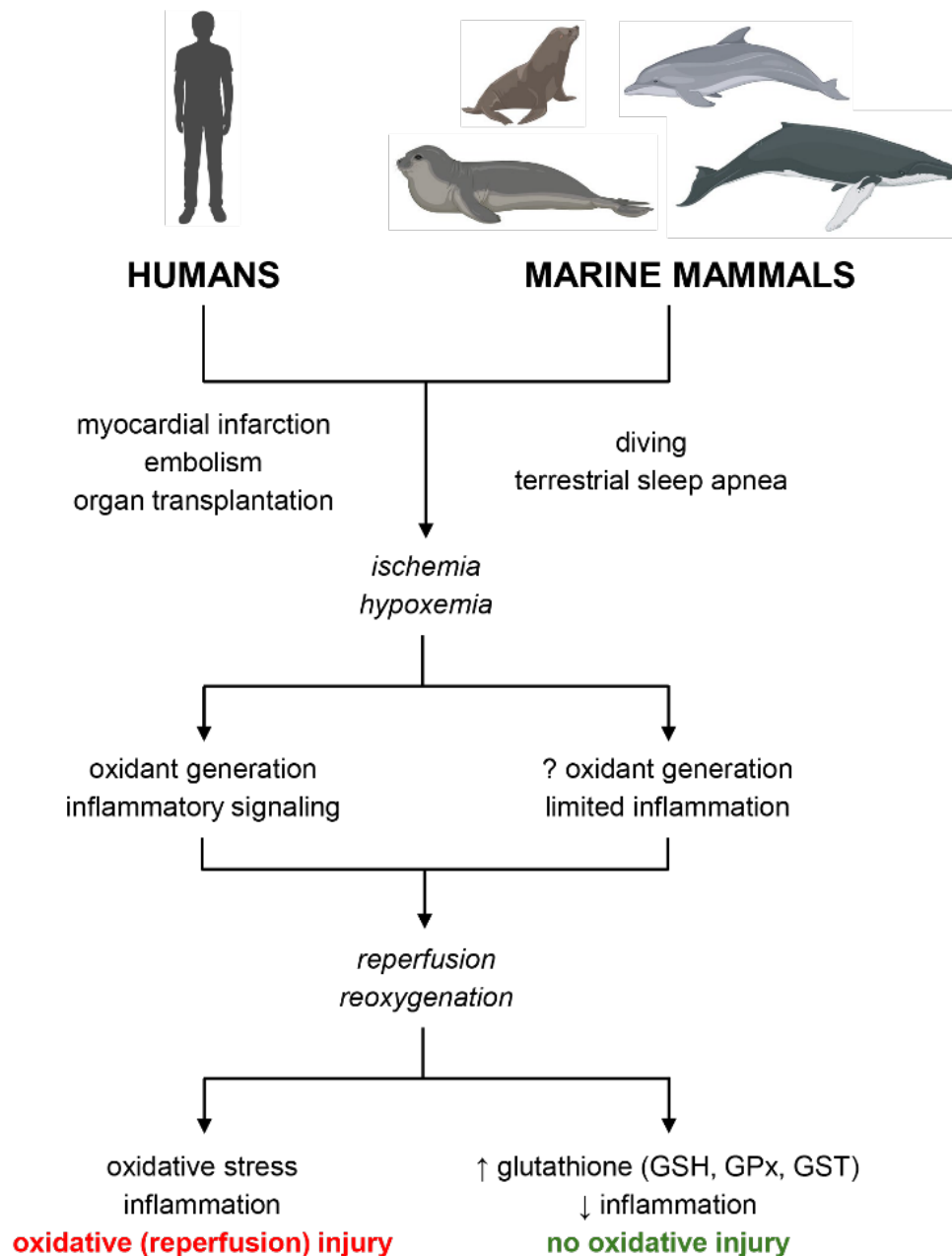


Figure 1. Biochemical mechanisms preventing reperfusion injury in diving mammals. Ischemia/reperfusion events are associated with oxidative stress and inflammation in humans but are well tolerated by diving mammals. The mechanisms protecting marine mammals against inflammatory and oxidative injury derived from diving-induced ischemia and hypoxemia are still under investigation but likely include upregulation of genes involved in antioxidant defense and hypoxia tolerance via preconditioning-like responses of the transcription factors Nrf2 and HIF-1.

2. Hypoxia exposure blunts angiogenic signaling and upregulates the antioxidant system in endothelial cells derived from elephant seals

2.1 Abstract

Elephant seals exhibit extreme hypoxemic tolerance derived from repetitive hypoxia/reoxygenation episodes they experience during diving bouts. Real-time assessment of the molecular changes underlying protection against hypoxic injury in seals remains restricted by their at-sea inaccessibility. Hence, we developed a proliferative arterial endothelial cell culture model from elephant seals and used RNA-seq, functional assays, and confocal microscopy to assess the molecular response to prolonged hypoxia.

Seal and human endothelial cells exposed to 1% O₂ for up to 6 h respond differently to acute and prolonged hypoxia. Seal cells decouple stabilization of the hypoxia-sensitive transcriptional regulator HIF-1 α from angiogenic signaling. Rapid upregulation of genes involved in glutathione (GSH) metabolism supports the maintenance of GSH pools, and intracellular succinate increases in seal but not human cells. High maximal and spare respiratory capacity in seal cells after hypoxia exposure occurs in concert with increasing mitochondrial branch length and independent from major changes in extracellular acidification rate, suggesting that seal cells recover oxidative metabolism without significant glycolytic dependency after hypoxia exposure.

We found that the glutathione antioxidant system is upregulated in seal cells during hypoxia, while this system remains static in comparable human cells. Furthermore, we found that in contrast to human cells, hypoxia exposure rapidly activates HIF-1 in seal cells, but this response is decoupled from the canonical HIF-angiogenesis pathway. These results highlight the unique mechanisms that confer extraordinary tolerance to limited oxygen availability in a champion diving mammal.

2.2 Introduction

Repeated bouts of extreme hypoxemia followed by reoxygenation characterize diving in northern elephant seals (*Mirounga angustirostris*) [24,25,60,61]. In the vasculature, hypoxia shifts the endothelium toward a pro-inflammatory state [137–139], priming vessels for increased leukocyte adhesion upon reoxygenation [140]. In addition, hypoxic changes in mitochondrial metabolism lead to succinate accumulation and subsequent mitochondrial superoxide (O₂^{•-}) generation via reverse electron transport [77,78,141], though degree of accumulation may modulate these effects [142]. Reoxygenation reintroduces oxygen to the “primed” endothelium, driving additional oxidant generation from various sources including NADPH oxidases and the electron transport chain. The resulting oxidative damage promotes endothelial dysfunction, which compromises both the barrier and vasoregulatory roles of the endothelial layer [143].

Hypoxia-induced endothelial dysfunction would catastrophically impact the dive response in marine mammals, for whom persistent peripheral vasoconstriction and fine-scale modulation of vascular tone are critical during prolonged submersion [5,50,95]. Recent work shows that nitric oxide responsiveness differs between organs and their supplying arteries in Weddell seals [95] and

that inflammatory signaling is blunted in Weddell and northern elephant seal blood [133], suggesting that diving mammals tightly regulate the inflammatory response to mitigate vascular damage during diving. Additionally, marine mammals exhibit a robust antioxidant defense system which likely counteracts the damaging effects of diving-induced oxidant generation. In particular, marine mammals possess high tissue and circulating levels of glutathione (GSH), the most abundant cellular thiol and a crucial cofactor for several antioxidant enzymes [101,102,106,110,111,144].

Besides comparative biochemical studies showing that marine mammals exhibit high antioxidant levels, recent work shows duplication events and positive selection on genes involved in GSH metabolism in diving mammals [145–147], but the dynamic regulation of the antioxidant system during hypoxia and diving remains unknown. Comprehensive assessments of antioxidant function and immunomodulation remain infeasible during diving due to technological limitations on *in vivo* studies and the inaccessibility of the animals at sea. To this end, we developed a primary cell culture system using arterial endothelial cells derived from expelled placental arteries obtained during elephant seal breeding haulouts. Arteries are generally inaccessible in living marine mammals, and arterial tissue sampling is constrained to expelled tissues or those derived from necropsy. We then conducted comparative transcriptomic and metabolic analyses to study the molecular mechanisms that drive hypoxemic tolerance in seal endothelial cells. We found that rapid hypoxic stabilization of HIF-1 α in elephant seal cells co-occurred with downregulated inflammatory signaling and a highly dynamic transcriptional response promoting vascular homeostasis alongside a blunted migratory response to hypoxia. Moreover, hypoxia increased GSH and succinate levels in seal but not human cells in coordination with enhanced respiratory capacity following hypoxia/reoxygenation. Together, these results highlight the importance of the GSH antioxidant system and mitochondrial function in protecting the vascular endothelium in a champion diving mammal exposed to extreme oxygen fluctuations.

2.3 Materials and methods

2.3.1 Tissue acquisition

Northern elephant seal (*Mirounga angustirostris*) placentae were obtained under NMFS permit # 19108 (PI: Daniel Costa, UC Santa Cruz). Whole placentae were collected from the beach at Año Nuevo Reserve (San Mateo, CA) less than 1 h after live births. The generation of cell lines was conducted under NMFS permit # 22479 (PI: José Pablo Vázquez-Medina, UC Berkeley). De-identified human placentae were donated following uncomplicated live births at Sutter Health's California Pacific Medical Center. All placentae were maintained at 4°C until dissection. All dissections were performed within 6 hours of collection.

2.3.2 Primary cell isolation and culture

Arteries were dissected from placental tissue and rinsed with ice-cold sterile Hanks' Balanced Salt Solution (HBSS; Gibco, Waltham, MA, USA). Arteries were cut open lengthwise, flushed with HBSS, and placed in a collagenase type II solution (500 U/mL in DMEM; Worthington Biochemical, Lakewood, NJ, USA) for 30 minutes in a tissue culture incubator (37°C, 5% CO₂). The collagenase solution was blocked with complete growth medium. The endothelial surface of the arteries was scraped into tissue culture-treated dishes containing complete growth medium and transferred to a tissue culture incubator. Endothelial cell islands were visible in the

culture dishes after 24 h. Growth medium was changed daily for the first 4-5 days at passage 0. Seal cells were maintained in Dulbecco's Modified Eagle Medium (DMEM; Gibco, catalog # 11885-084) supplemented with 10% fetal bovine serum (Seradigm, Avantor, Mexico), 10 mM HEPES (Gibco), 1% (1X) Antibiotic-Antimycotic (Gibco), and 4 µg/mL endothelial cell growth supplement (ECGS; Corning, Corning, NY, USA). Human cells were grown in commercial endothelial cell medium (Sciencell Research Laboratories, catalog # 1001, Carlsbad, CA, USA). Confluent cultures were cryopreserved at passage 1. Pooled stock cultures were created for human ($n=2-3$) and seal ($n=3$) cells to minimize impacts of individual variation. Northern elephant seal trophoblasts were isolated by mincing placental labyrinth in a collagenase type II solution, followed by 5 minutes in a tissue culture incubator. The collagenase solution was blocked with complete medium and passed through a 100 µm filter. The filtrate was centrifuged at 200 x g for 5 minutes at 4°C. The pellet was washed once in HBSS and resuspended in trophoblast growth medium (Sciencell Research Laboratories, catalog # 7121), then plated on tissue culture-treated dishes coated with collagen IV. Given that primary cells can lose phenotypic features with increasing passage number, we conducted all experiments in cells between passage 4-7 (and species were passage-matched within experiments) to mitigate any such changes to the extent possible. All cells were switched to the same medium formulation the day before experiments.

2.3.3 Cell characterization

The endothelial phenotype of the preparations was confirmed by DiI-acetylated LDL uptake (Invitrogen, catalog # L3484), RT-qPCR for vascular endothelial cadherin (VE-cadherin, CD144) and platelet endothelial cell adhesion molecule-1 (PECAM-1), and immunostaining for PECAM-1 (CD31; Novus Biologicals, catalog # NB100-2284, 1:100). The DiI-acetylated LDL uptake assay was conducted following manufacturer instructions. RT-qPCR was conducted using our previously published methods [135] with minor modifications to the reaction cycling conditions: 1 min at 95°C followed by 40 cycles of 20 sec at 95°C, 30 sec at 60°C. Primer sequences are listed in Table S1. Relative mRNA expression (fold change) was calculated as $2^{-\Delta\Delta C_t}$. Immunostaining was conducted following the protocol described in [148]. Tube formation was assessed by plating 10,000 cells per well in a µ-slide 3D angiogenesis glass bottom slide (Ibidi, Fitchburg, WI, USA, catalog # 81506) coated with Matrigel. All imaging was conducted using a Zeiss Axio Observer 7 inverted microscope fitted with 10X and 20X objectives and Zen software.

2.3.4 Extracellular flux assays

Oxygen consumption was measured using the Seahorse Mitochondrial Stress Test Kit (Agilent Technologies, Santa Clara, CA, USA) and an XFp Extracellular Flux Analyzer. 30,000 cells/well were seeded in fibronectin-coated miniplates. Cells were washed with serum-free assay medium (Seahorse XF DMEM pH 7.4, containing 5.56 mM glucose, 1 mM pyruvate, 4 mM L-glutamine) and incubated at 37°C in a non-CO₂ incubator for 1 h prior to assay. Oxygen consumption rates (OCR) were measured in the presence of oligomycin (1 µM), carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazide (FCCP; 1 µM for human cells, 2 µM for seal cells), and rotenone/antimycin A (0.5 µM). Optimal FCCP concentrations were determined empirically for each species. Protein content was measured in each well at the end of the assay using the Qubit Protein Assay Kit (Molecular Probes, Eugene, OR, USA). Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were normalized to protein content. Mitochondrial function was calculated according to [149] and normalized to basal respiration per well.

2.3.5 Hypoxia exposure

Cells were incubated in an InVivoO₂ physiological cell culture workstation (Baker Ruskinn, Sanford, Maine, USA) under the following conditions: 1% O₂, 5% CO₂, 37°C for 15 min, 30 min, 1 h, 2 h, 4 h and 6 h. Protein and RNA were collected within the hypoxic environment. Control cells remained in a standard tissue culture incubator (21% O₂, 37°C, 5% CO₂). Cell viability was confirmed in cells undergoing hypoxia exposure using a commercial viability/cytotoxicity kit (Thermo Fisher, catalog number: R37601). In some experiments, extracellular flux assays were conducted after hypoxia/reoxygenation treatments consisting of 1 h at 0.5% O₂ (37°C, 5% CO₂) followed by 30 min in room air (37°C, no CO₂) prior to assay.

2.3.6 Immunoblotting

Western blot was conducted using our previously published methods [150]. Briefly, cells were scraped in DPBS (Gibco) containing 1% Triton X-100 and 2% Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific, Waltham, MA, USA). Lysates were sonicated and centrifuged. Total protein content in the supernatant was determined using a BCA Rapid Gold Protein Assay (Pierce, Rockford, IL, USA). Proteins were resolved using SDS-PAGE and transferred onto nitrocellulose membranes. Membranes were blocked with Odyssey blocking buffer (LICOR, Omaha, NE, USA) and incubated overnight with an antibody against hypoxia inducible factor-1 α (HIF-1 α , Cell Signaling Technology, catalog # 36169; RRID:AB_2799095; 1:500). Proteins were visualized using IRDye 800CW secondary antibodies (LICOR) and a two-color near-infrared system (Azure c500, Azure Biosystems, Dublin, CA, USA). Membranes were stripped and reprobed with an antibody against β -actin (Cell Signaling Technology, catalog # 4967; RRID:AB_330288; 1:2,000). Intensity of individual bands was quantified using FIJI v2.1.0 and normalized to β -actin.

2.3.7 Biochemical assays and live cell imaging under hypoxic conditions

Glutathione content was measured under normoxic conditions and after exposure to 30 min and 6 h hypoxia using the GSH-Glo Assay Kit (Promega, Madison, WI, USA) according to manufacturer instructions. Luminescence was measured using a SpectraMax M3 Microplate Reader (Molecular Devices, San Jose, CA, USA). Changes in GSH levels were also measured in hypoxic cells using ThiolTracker Violet (Invitrogen, catalog # T10096). Cells were loaded with 20 μ M ThiolTracker Violet glutathione detection reagent and 100 nM SYTO 16 Green Fluorescent Nucleic Acid Stain (Invitrogen, catalog # S7578) and imaged within the hypoxic environment using an LS620 microscope (Etaluma, Carlsbad, CA, USA) fitted with a 20X objective at 0, 30 minutes, and 6 hours at 1% O₂. Fluorescence intensity was quantified in 12 random cells per field using FIJI v2.1.0.

Intracellular succinate concentration was measured in cells under normoxic conditions and after exposure to 1 and 6 h hypoxia using a commercial colorimetric assay (Sigma catalog # MAK184, St. Louis, MO, USA) according to manufacturer instructions. Absorbance at 450 nm was measured using a SpectraMax M3 Microplate Reader (Molecular Devices, San Jose, CA, USA). Succinate concentrations per well were normalized to protein content using a Pierce Rapid Gold Protein Assay (Pierce, Rockford, IL, USA).

2.3.8 Cell migration assay

Cells were plated in a 2-well silicone culture insert (Ibidi, catalog # 81176) adhered to a TC-treated 35 mm glass bottom dish and grown to confluence. At confluence, culture inserts were removed to create a “scratch.” Dishes were washed with DPBS to remove unattached cells, and media was changed. Dishes were incubated in control (normoxia) or hypoxia (1% O₂) conditions for 6 h. Live cells were imaged using a Zeiss Axio Observer 7 inverted microscope fitted with a 10X objective at 1, 2, 4, and 6 h. Cell migration was calculated as the percent difference in cell-free area in hypoxic dishes compared to control at each time point using the ScratchAssayAnalyzer function in the MiToBo FIJI plugin.

2.3.9 Mitochondrial staining and network analysis using confocal microscopy

Live cells were stained with MitoTracker Red CMXRos (Cell Signaling Technology, Danvers, MA) and NucBlue (Hoechst 33342, Invitrogen) under normoxic conditions and after 1 and 6 h hypoxia (1% O₂) followed by 30 min reoxygenation. Cells were fixed in ice cold 1:1 methanol:acetone, rinsed with PBS, and imaged in VECTASHIELD mounting media (Vector Laboratories, Burlingame, CA) using a Zeiss LSM 710 microscope fitted with a 63X objective (Plan-Apochromat 1.4 NA oil, 0.13 mm). Mitochondrial footprint and mean branch length were determined from unfiltered binarized images using the MiNA (v2.0) FIJI plugin (<https://github.com/StuartLab/MiNA>).

2.3.10 RNA-seq

Hypoxic cells were lysed in Buffer RLT without β -mercaptoethanol (Qiagen, Germantown, MD, USA). RNA extractions were carried out using an RNeasy Mini Kit (Qiagen) including on-column DNase treatment according to manufacturer instructions. DNase treatment was confirmed by a lack of genomic amplification using PCR. RNA yield was quantified using a Nanodrop 1000 Spectrophotometer (Thermo Scientific). RNA integrity number (RIN) was determined using an Agilent 2100 Bioanalyzer kit for total eukaryotic RNA (Agilent Technologies, Santa Clara, CA, USA). Samples underwent library preparation and sequencing at the UC Berkeley Functional Genomics and Vincent J. Coates Genomics Sequencing Laboratories. A KAPA RNA HyperPrep Kit (catalog # KK8541; Roche, Basel, Switzerland) was used to prepare cDNA libraries from poly(A)-captured mRNA with TruSeq adapters. Three replicates per condition were used to generate libraries, with a sequencing depth of 25M reads per sample on a NovaSeq platform (Illumina, San Diego, CA, USA).

2.3.11 Transcriptome analyses

The elephant seal (*Mirounga angustirostris*) genome was annotated as described in our previous work (https://www.dnazoo.org/assemblies/Mirounga_angustirostris) [135]. Reads were mapped to the elephant seal or human genomes (version GRCh38) using STAR aligner [151]. Transcript levels were quantified with RSEM v1.3.1 [152]. Expression levels were converted to z -scores and normalized by the mean and variance across all replicates and conditions for each gene. The median z -score was selected for each gene and used to construct a Euclidean distance matrix. Genes that changed in a concerted manner across hypoxia exposure times were clustered by k -means. Optimal clustering was selected at $k=6$ due to diminishing returns at higher k . Within each cluster, GSEA with KEGG terms was used to identify enriched biological pathways; enrichments were not assessed for “no change” clusters (seal: cluster 4; human: cluster 2). Additionally, genes

differentially expressed between control and each time point were identified using EBSeq with a false discovery rate of 5% [153]. GSEA for Reactome pathways was conducted using a summary list of genes differentially expressed at 15 min, 30 min and 1 h versus control [154]. Functional Interaction Networks (FIN) were built for the same gene lists using Cytoscape v3.8.2 [155,156]. *Cis*-regulatory analyses were conducted using iRegulon in Cytoscape [157].

2.3.12 Statistical analyses

Statistical analyses were performed using GraphPad Prism v9.3.1. Normality was evaluated using D'Agostino-Pearson or Shapiro-Wilk tests, depending on sample size. When normality assumptions were met, differences between groups with equal variance were assessed by independent two-tailed *t*-test or one-way ANOVA. When homoscedasticity assumptions were not met, differences between groups were determined by two-tailed *t*-test with Welch's correction or Welch's ANOVA. In cases where data were not normally distributed, Kruskal-Wallis tests were used. In all cases, we corrected for multiple comparisons by setting FDR to 0.05; post hoc analyses used the two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli [158]. Data are presented as mean $\pm$ s.e.m.

2.4 Results

2.4.1 Characterization of elephant seal arterial endothelial cells in primary culture

Primary endothelial cells derived from seal and human placental arteries stain positive for the endothelial marker PECAM-1 (CD31), take up acetylated low-density lipoprotein (Ac-LDL), and spontaneously form tubes when cultured on a 3D matrix (Figure 1A). CD144 (VE-cadherin) and CD31 (PECAM-1) mRNA expression is enriched in seal endothelial cells compared to placental trophoblast cells (CD144: 117-fold, $t=6.501$, $p=0.003$; CD31: 86-fold, $t=5.665$, $p=0.005$) (Figure 1B). These results support an endothelial lineage for the isolated cell cultures.

Basal oxygen consumption rates do not differ between seal and human cells, though exposure to hypoxia/reoxygenation increased basal oxygen consumption only in seal cells. Furthermore, ATP-linked respiration, maximal respiration, and spare respiratory capacity were greater in seal than in human cells at baseline and after hypoxia/reoxygenation despite hypoxia/reoxygenation-induced increases in maximal respiration and spare capacity in both species (Figure 1C). In contrast, human cells displayed greater proton leak than seal cells both at baseline and after hypoxia/reoxygenation, though leak increased in both species following hypoxia/reoxygenation (Figure 1C). While seal cells display the highest overall oxygen consumption rates after hypoxia/reoxygenation (Figure 1C), these cells also exhibit the lowest extracellular acidification rates, suggesting that glycolytic acidification of the media is not a primary component of the increased respiratory capacity (Figure 1D).

We visualized mitochondrial networks using confocal microscopy to determine whether the observed changes in oxygen consumption may be driven by morphological differences in mitochondria following hypoxia/reoxygenation. Mitochondrial footprint appeared to decline in human cells after 1 and 6 h hypoxia followed by 30 min reoxygenation, though this trend was not statistically significant (Figure 1E,G; Figure S1). Mitochondrial branch length increased transiently in human cells after 1 h hypoxia but dropped below baseline levels by 6 h ($\chi^2(3)=17.47$, $p=0.0002$; Figure 1E). In seal cells, however, hypoxia increased branch length from control to 6 h

($\chi^2(3)=9.000$, $p=0.01$; Figure 1F). These results suggest that seal cells increase mitochondrial connectivity while maintaining overall volume during hypoxia despite long-term decreases in branching and volume in human cells.

2.4.2 Hypoxia differentially regulates HIF-1 signaling in seal and human cells

We evaluated the HIF-1-mediated response to sustained hypoxia in seal and human cells. Both human and seal cells remained viable (>99%) throughout a 6 h exposure to 1% O₂ (Table S2). Hypoxia increased HIF-1 α protein levels to a maximum of ~70-fold over (normoxic) baseline abundance in both species ($p=0.005$ both species) (Figure 2A,B; Figure S2), though we observed species-specific dynamics. HIF-1 α abundance was biphasic in seal cells: an initial major peak within 15 min of hypoxia was followed by a ~45% decline from 30-120 min, with levels again increasing after 2 h. This late-stage increase co-occurred with an increase in HIF-1 α mRNA levels (Figure 2C). In contrast, HIF-1 α protein levels did not peak in human cells until 1 h in hypoxia, and abundance declined continuously following this peak. HIF-1 α mRNA levels in human cells remained relatively stable through 2 h, followed by a precipitous decline at later time points (Figure 2C). Together, these data suggest that hypoxic HIF-1 α stabilization is generally conserved despite variation in the dynamics of the response between species.

Seal and human cells displayed distinct gene expression patterns for several known HIF-1 targets (Figure 2C). Expression of factors implicated in extracellular matrix reorganization, angiogenesis and cell motility were elevated across the time course in human cells but were generally repressed or lagged behind in seal cells, including angiopoietin 2 (ANGPT2), the angiopoietin 1 receptor (TEK; TIE2), the Na⁺/H⁺ antiporter (SLC9A1; NHE1), protein phosphatase 5 catalytic subunit (PPP5C), transforming growth factor beta-3 (TGFB3), and matrix metalloprotease 14 (MMP14), while a metalloprotease inhibitor (TIMP1) showed the opposite expression pattern (Figure 2C). Placental growth factor (PGF) was downregulated in seal cells after 2 h hypoxia but displayed the opposite trend in human cells. Expression of the cellular stress protein DNA damage-inducible transcript 4 (DDIT4; REDD1) increased in seal cells at late time points, but its expression lagged that of human cells (Figure 2C). Seal cells increased expression of the first enzyme in glycogen synthesis, phosphoglucomutase 1 (PGM1), at early time points while it was generally repressed in human cells (Figure 2C). Together, these results suggest that the angiogenic signaling pathway is decoupled from HIF-1 α stabilization in seal cells exposed to hypoxia.

2.4.3 Seal cells delay the onset of pro-angiogenic transcriptional programs during hypoxia exposure

We used RNA-seq to evaluate global changes in gene expression in seal and human cells during a 6 h hypoxia exposure. We conducted *cis*-regulatory analyses with differentially expressed (DE) genes to predict transcription factors (TFs) with major roles in the response to hypoxia at each time point. Comparative analyses of the top three predicted TFs in seal and human cells after 30 min, 60 min, and 6 h in hypoxia revealed species-specific dynamics in transcriptional control: none of the nine predicted seal TFs overlapped across time points, while only six distinct TFs were predicted in human cells (Figure 3A,B). Two TFs (chromodomain helicase DNA binding protein 1, CHD1; serum response factor, SRF) were represented in both human and seal datasets. Predicted TFs in seal cells at 30 and 60 min suggest rapid metabolic (forkhead box K1, FOXK1) and inflammation-related (NF- κ B subunit RELA; NFKB1; JUN; zinc finger and BTB containing 7A,

ZBTB7A) responses to hypoxia, with a limited angiogenic signature (JUN). In contrast, the transcriptional signature in human cells at both 30 and 60 min suggests altered expression of oxygen delivery pathways via homeobox A13 (HOXA13), FOS like 1 (FOSL) and SRF, all of which regulate angiogenesis, vascular remodeling, and vasodilation [159–162]. In addition, broad changes in DNA replication and transcription may characterize the human endothelial cell response to hypoxia via churchill domain containing 1 (CHURC1) and CHD1.

Seal cells do appear to regulate expression of genes involved in oxygen delivery during hypoxia at 6 h. Two of the top three predicted TFs in seal cells at 6 h (GATA binding protein 2, GATA2; SRF) regulate angiogenesis, vascular remodeling, and vascular tone [159,163]. The third TF, Kruppel like factor 11 (KLF11) maintains vascular homeostasis and limits endothelial oxidant stress and inflammation via inhibition of NADPH oxidase 2 (NOX2) and matrix metalloproteinase 9 (MMP9) expression [164,165]. In human cells, repeated identification of HOXA13 and CHURC1 suggests a continued angiogenic signal at 6 h along with upregulation of transcription. Additionally, ELF4-mediated transcriptional changes may promote cell cycle entry during hypoxia exposure [166,167]. Cell migration data support these transcriptional data, as hypoxia accelerated migration into a cell-free gap in human but not seal cells over a 6 h exposure (Figure 3C,D; Figure S3). Together, these results suggest a highly dynamic transcriptional response to hypoxia in seal cells which alters cellular metabolism to promote endothelial homeostasis. This response stands in contrast to the consistently pro-angiogenic signal observed in human cells exposed to hypoxia.

2.4.4 The transcriptional response to hypoxia highlights species-specific differences in antioxidant gene expression

Hypoxia rapidly repressed global gene expression in both seal and human cells, with 70% of differentially expressed (DE) genes in seal and 60% of DE genes in human cells downregulated at 15 min compared to control ($p < 0.001$ for both species) (Figure 4). Gene expression in seal cells remained repressed from 30 min until 4 h ($p < 0.005$ at each time point), at which point up- versus down-regulation did not differ from 50/50 for the remainder of the experiment (4 h: $p = 0.68$; 6 h: $p = 0.36$) (Figure 4A,B). In human cells, repression was similarly maintained through 1 h ($p < 0.001$ at each time point) and shifted toward upregulation from 4–6 h (4 h: $p < 0.001$; 6 h: $p < 0.005$) (Figure 4C,D).

We then identified concerted changes in gene expression using *k*-means clustering and conducted gene set enrichment analysis (GSEA) for KEGG pathways within each cluster (Figure 5A,B; Figure S4; Figure S5). Clustering was performed for $k = 1 \dots 30$ clusters; six clusters were selected for final analysis as diminishing returns were observed beyond $k = 6$ (Figure S4). Seal cluster 6 and human cluster 3 were both enriched for HIF-1 signaling. Seal cluster 6 contained genes that gradually increased over time, while human cluster 3 genes exhibited a gradual increase in expression until 4 h, followed by a rapid decrease at 6 h. Seal cluster 5 and human cluster 6 were enriched for TNF signaling; both clusters displayed a rapid decrease in expression followed by a gradual return to baseline, suggesting that both human and seal cells downregulate pro-inflammatory pathways during the initial response to hypoxia.

Seal cluster 3 was enriched for glutathione (GSH) metabolism; this cluster contained genes for which expression peaked within 30 min of hypoxia onset. No human gene expression cluster displayed enrichment for genes involved in GSH metabolism (Figure S5). Genes from the GSH metabolism pathway present in seal cluster 3 are listed in Table 1 and include the critical GSH biosynthesis genes glutamate-cysteine ligase catalytic (GCLC) and modifier (GCLM) subunits and

glutathione synthetase (GSS) (Figure 5C). GSH levels transiently increased in human cells during hypoxia exposure but continuously increased in seal cells through 6 h in hypoxia (human: $F_{2,20}=4.034$, $p=0.04$; seal: $F_{2,21}=11.03$, $p=0.0005$) (Figure 5D). Furthermore, baseline GSH content was ~1.4-fold greater in seal compared to human cells ($t=13.26$, $p<0.001$) and remained ~3-fold higher in seal than in human cells during hypoxia exposure (2 h: 3.3-fold, $t=8.262$, $p=0.014$; 6 h: 3.1-fold, $t=6.422$, $p=0.022$) (Figure 5E), suggesting that early transcriptional changes during hypoxia exposure support increased GSH pools in seal but not human cells.

Several genes involved in mitochondrial metabolism and redox homeostasis were also present in seal cluster 3, including ornithine decarboxylase 1 (ODC1), spermidine synthase (SRM), and spermine synthase (SMS) (Table 1) [168–170]. Mitochondrial succinate accumulation during ischemia (and its associated tissue hypoxia) drives superoxide production via reverse electron transport at mitochondrial complex I upon reoxygenation [77,78], thus we measured intracellular succinate levels during hypoxia exposure. Interestingly, succinate levels increased 40% in seal but not human cells after 6 h hypoxia (seal: $F_{2,6}=19.30$, $p=0.0024$; human: $F_{2,6}=0.3164$, $p=0.74$) (Figure 5F); this relatively mild accumulation may support continued oxidative phosphorylation in seal cells after hypoxia/reoxygenation as observed in Figure 1C. Peroxiredoxin 6 (PRDX6/aiPLA₂), microsomal glutathione S-transferase 2 (MGST2), and gamma glutamyl transferase 5 (GGT5), which contribute to glutathione-dependent leukotriene synthesis [171,172] and may promote vasoconstriction in diving mammals [136], were also present in seal cluster 3 (Table 1). Together, these data suggest that in contrast to human cells, seal endothelial cells maintain cellular GSH levels, remain metabolically active, and maintain vasoconstrictive signaling during hypoxia exposure.

2.4.5 The short-term transcriptional response to hypoxia in seal cells limits inflammatory signaling

We next investigated whether short-term hypoxia (≤ 1 h) leads to differential functional enrichment in gene expression between species. We detected 42 DE genes shared between the seal and human cell response to short-term hypoxia. Generally, these shared genes displayed similar expression patterns between species (Figure 6A). Seal genes responsive to short-term hypoxia were enriched for several TGF- β signaling pathways including downregulation of TGF- β receptor signaling. In contrast, the response in human genes was dominated by pathways involved in RNA processing and transcription, ER stress, and upregulation of electron transport chain subunits (Figure 6B; Table S3). Functional interaction networks (FINs) suggested a central role for NFKB1 (p50) in the short-term hypoxia response in both species, though the expression of this subunit and its related RELB subunit were downregulated relative to control in both species (Figure 6C,D). The seal FIN also confirmed a critical role for downregulation of TGF- β signaling in seal cells (Figure 6C) as the only upregulated gene in the network was MTMR4, a TGF- β inhibitor. Interestingly, the angiogenic gene expression signature in seal cells suggested blunting of VEGF-dependent angiogenesis during short-term hypoxia (Figure 6C).

2.4.6 Pro-proliferative transcriptional signals characterize the long-term hypoxia response in seal cells

We next evaluated expression patterns in response to hypoxia exposure for ≥ 2 h. DE genes shared between seal and human cells generally displayed similar trajectories, apart from HOXA13, also predicted as coordinating the human response to hypoxia in cells (Figure 3B), for which long-

term hypoxia repressed expression in seal but stimulated expression in human cells (Figure 7A). No Reactome pathways were overrepresented in the 193 seal genes DE for 2-6 h hypoxia exposure. Human DE gene expression (333 genes) was again enriched for pathways related to tRNA and rRNA processing and respiratory electron transport (universally upregulated as during short-term exposure) as well as negative regulation of the MAPK pathway (Figure S6A; Table S4).

We then separately considered the transcriptional response to 6 h hypoxia, at which point a second increase in seal HIF-1 α protein abundance occurred alongside species-specific expression patterns for several HIF-1 targets (Figure 2A,C). GSEA revealed downregulation of several pathways related to transcription and mRNA splicing, as well as C-type lectin receptors (implicated in the immune response, cell adhesion and apoptosis [173]) in human cells at 6 h (Figure S6B). Reactome pathway enrichment analyses further supported hypoxia-induced changes in transcription including RNA polymerase II activity in human cells (Figure 7B). In contrast, seal cell gene expression at 6 h was enriched for several Smad and TGF- β signaling pathways, the VEGFA-VEGFR2 pathway, SUMOylation and SUMO E3 ligase target proteins (Figure 7B). Hypoxia-induced suppression of TGF- β signaling appeared to be regulated through increased expression of the Smad phosphatase MTMR4 and the ubiquitin ligase NEDD4L, which targets TGF- β -induced pSmad2/3 (Table S5) [174,175]. Despite the observed shift toward proliferative signaling during long-term hypoxia exposure, we did not detect changes in cell migration in seal endothelial cells in response to up to 6 h hypoxia exposure (Figure 3C,D).

2.5 Discussion

Elephant seals repeatedly experience profound hypoxemia during continuous diving [24,25]. While similar fluctuations characterize pathophysiological events in non-hypoxia adapted species [35,84], the cellular and molecular mechanisms that confer hypoxia tolerance in seals remain unclear. Here, we developed a proliferative arterial endothelial cell culture system using cells derived from elephant seals to identify key transcriptional regulators of the response to hypoxia. Our results show blunted angiogenic signaling despite rapid and sustained HIF-1 α stabilization in seal cells. Moreover, seal cells also increase GSH pools during hypoxia, which may mitigate oxidant stress upon reoxygenation despite requiring energetic input under oxygen-limited conditions. High maximal and spare respiratory capacity at baseline and after hypoxia/reoxygenation and robust mitochondrial networks in seal cells may support such demands. Together, these data suggest that seal endothelial cells modulate metabolic parameters to meet oxygen availability during hypoxia rather than attempting to increase oxygen delivery in the face of limited supply.

2.5.1 Canonical hypoxia signaling is decoupled from angiogenesis in seal cells

HIF-1 is the “master regulator” of the canonical response to hypoxia [117,176], typically promoting both angiogenesis and glycolysis. *In vivo*, *in vitro*, and *in silico* analyses of HIF-1 α structure and function indicate that marine mammal HIF-1 α stabilization is highly sensitive to hypoxia [93,119–121,177]. HIF-1 α stabilization occurred rapidly in seal endothelial cells exposed to hypoxia, and high levels of the HIF-1 α protein persisted in seal cells through a 6 h exposure. Interestingly, seal cell expression of several HIF-1 targets implicated in vascular homeostasis (ANGPT2, MMP14, SLC9A1/NHE1) [178–181] remained stable during long-term hypoxia exposure in seal cells, while expression was variable or increased in human cells. Global

transcriptional patterns supported this regulation as DE genes in seal cells predicted factors which regulate cell proliferation (FOXK1, ZBTB7A) and suppress endothelial activation (KLF11) [164,182], while human cells presented a consistently pro-angiogenic signal despite slower HIF-1 α stabilization. Accordingly, hypoxia increased gap closure rates in human but not seal cells. These results suggest that seal cells rapidly stabilize HIF-1 α but further regulate the expression of putative HIF-1 targets during hypoxia exposure.

2.5.2 Seal cells dampen inflammatory signaling throughout hypoxia exposure

Our data suggest continued regulation of inflammatory signaling in seal endothelial cells across 6 h of hypoxia exposure. Gene expression signatures early (≤ 1 h) in hypoxia exposure highlight downregulation of the TNF, NF- κ B, and TGF- β pathways in seal cells. In contrast, enrichments in human cell gene expression identified substantially fewer inflammatory pathways. Continued overrepresentation of TGF- β -related signaling pathways (including SMAD activity) coupled with increased expression of negative regulators including the dual specificity protein phosphatase MTMR4 and ubiquitin ligase NEDD4L in seal cells at 6 h, along with ZBTB7A at 30 min, suggests that tight regulation of the TGF- β signaling cascade is critical in the seal cell response to hypoxia. Our data also suggest that altered NF- κ B signaling during hypoxia exposure in seal cells may be regulated by TNF. NF- κ B activation may be either pro-inflammatory or pro-survival depending on cellular context. The activity of both NF- κ B and its inhibitor IKK β are required for hypoxic HIF-1 α accumulation and HIF-1 target gene expression [183]. Knockdown of the NF- κ B subunit RelA in endothelial cells impairs smooth muscle cell proliferation [184] and NF- κ B activity is implicated in eNOS downregulation during chronic intermittent hypoxia [185]. In Weddell seals, nitric oxide sensitivity varies across tissues and vessel beds but is generally blunted when compared to sheep [95], presumably to avoid hypoxic vasodilation during diving. Here, we found that tight regulation of NF- κ B expression in seal cells exposed to hypoxia may promote cell survival and modulate vasodilatory signaling in the endothelium. These results corroborate a generally reduced inflammatory response in marine mammal whole blood stimulated with lipopolysaccharide [133,186]. In contrast, changes to inflammatory signaling pathways occurring in human cells during short-term hypoxia exposure are supplanted by broad changes in transcriptional regulation during long-term exposure.

2.5.3 Hypoxia promotes GSH synthesis in seal cells

High tissue and circulating GSH levels, as well as high activity of many glutathione-dependent antioxidant enzymes, including glutathione peroxidases (GPX) and glutathione S-transferases (GST) [100,102,106,110,111], suggest a critical role for GSH in protecting diving mammals against oxidative stress. Recent genomic work demonstrates positive selection on several genes in the GSH metabolism pathway in marine mammals, including those we identified as enriched [145–147,187]. To date, however, the only measurement of diving GSH levels is in Weddell seals, with the observation that near-depletion of circulating GSH at the halfway point of restrained dives was followed by an increase above baseline levels within ~ 5 min of recovery [100]. Interestingly, we found that elephant seal vascular endothelial cells continue GSH synthesis during long-term hypoxia exposure alongside rapid expression increases in several GSH biosynthesis and antioxidant enzymes. Hypoxic GSH accumulation in seal cells likely counteracts oxidant generation upon reoxygenation, protecting cells against oxidative damage and cellular stress. These results track with existing *in vivo*, *in vitro*, and *in silico* evidence supporting GSH's role in maintaining redox homeostasis during diving in marine mammals

[100,102,106,110,111,146,147]. Additionally, data from non-diving hypoxia tolerant species indicate that increases in GSH prior to oxidative insult (driven by preconditioning) protect against later tissue injury [188,189], potentially justifying the “energetic cost” of producing GSH in oxygen-limited conditions. Repeated diving behavior and terrestrial apnea bouts likely contribute to a “preconditioning” effect, which protects seal cells against oxidative damage, though genomic data also support a role for evolutionary pressure in shaping GSH metabolism in both pinnipeds and cetaceans [145–147,187]. Fasting during development in elephant seals (during which time pups develop terrestrial apneas) stimulates GSH biosynthesis [110], and similar increases in GSH and related antioxidant enzymes occur with postnatal maturation in the deep-diving hooded seal [111]. In humans, breath hold training apparently eliminates a dive-associated decrease in circulating GSH after static apnea, though no increase over baseline levels is observed [190]. Therefore, it is likely that both behavioral preconditioning and underlying genomic/evolutionary changes in GSH metabolism drive hypoxic GSH upregulation in elephant seal endothelial cells.

2.5.4 Hypoxic succinate accumulation may promote mitochondrial function in seal cells

In addition to GSH biosynthesis, other components of the glutathione metabolism pathway enriched in seal cells included those related to polyamine synthesis (via ODC1, SRM, and SMS) which may further protect cells against oxidant generation upon reoxygenation [191,192]. Polyamines regulate the mitochondrial permeability transition pore via calcium flux, thus modulating activity of the pyruvate dehydrogenase complex and impacting mitochondrial respiration [193]. Hypoxic accumulation of succinate in seal cells may derive from conversion of the polyamine putrescine to succinate and sustain oxidative phosphorylation during hypoxia exposure. While excessive succinate accumulation due to reversal of succinate dehydrogenase (SDH) activity during hypoxia drives $O_2^{\bullet}$ production via complex I [78], inhibition of SDH activity by the anti-inflammatory metabolite itaconate may promote mild succinate accumulation without associated $O_2^{\bullet}$ generation [194]. Additionally, succinate competitively inhibits prolyl hydroxylases such as those responsible for HIF-1 α hydroxylation [195], supporting increased HIF-1 α sensitivity to hypoxia in seal cells. Early increases in the HIF-1 target phosphoglucomutase 1 (PGM1) in seal cells may regulate glucose metabolism during hypoxia, consistent with glycolytic repression via ZBTB7A transcriptional control [196,197]. Human cells, in contrast, upregulate several mitochondrial electron transport chain subunit components during short-term hypoxia exposure, consistent with increased mitochondrial branch length after 1 h, but overall changes in mitochondrial respiration in human cells following hypoxia/reoxygenation are milder than those in seal cells and are accompanied by higher ECAR, suggesting that human but not seal cells rely on glycolysis to support continued respiration during hypoxia.

2.6 Conclusions

In summary, we found that elephant seal endothelial cells tightly regulate inflammation and mitochondrial dynamics and blunt angiogenic signaling during hypoxia exposure. These changes occur in parallel with increased GSH synthesis, which may protect seal cells against oxidant generation during reoxygenation. Conversely, human cells signal for angiogenesis and vascular remodeling early and consistently throughout hypoxia exposure; these signals may destabilize the vasculature and increase susceptibility to inflammation and oxidative damage during reoxygenation. Here we identify candidate pathways supporting endothelial cell hypoxia tolerance in a deep diving seal. Recent genomic evidence from pinnipeds and cetaceans suggests

convergent modifications in GSH metabolism and/or HIF function in marine mammals. Further investigation via co-culture with vascular smooth muscle, peripheral blood mononuclear cells, or serum from varied diving vertebrate taxa is required to anchor these responses in the context of the sustained vasoconstriction characteristic of the diving response.

2.7 Acknowledgments

Drs. Julia María Torres-Velarde, Juan Manuel (Manny) Vazquez, Diana Moreno-Santillán, Peter Sudmant, and José Pablo Vázquez-Medina were instrumental in supporting this work. We thank Dr. Fung Lam for his assistance in obtaining human placental tissue. We thank Drs. Daniel Costa, Patrick Robinson, and Rachel Holser for collecting and sharing elephant seal placental tissue. We thank Dr. Rohit Kolara for improving the elephant seal genome annotation. The UC Berkeley QB3 Genomics facility provided sequencing service and support (RRID:SCR_022170; NIH S10 OD018174 Instrumentation Grant). Confocal imaging was conducted using resources at the UC Berkeley Molecular Imaging Center (RRID:SCR_017852), supported by the Gordon and Betty Moore Foundation. K.N.A. was supported by a National Science Foundation Graduate Research Fellowship (DGE 1752814 & 2146752) and the Berkeley Fellowship.

2.8 Tables

Table 1. Glutathione metabolism genes in seal cluster 3.

Gene symbol	Gene name
<i>idh2</i>	isocitrate dehydrogenase (NADP(+)) 2
<i>gclc</i>	glutamate-cysteine ligase catalytic subunit
<i>gclm</i>	glutamate-cysteine ligase modifier subunit
<i>gss</i>	glutathione synthetase
<i>ggt5</i>	gamma-glutamyltransferase 5
<i>gsr</i>	glutathione-disulfide reductase
<i>pgd</i>	phosphogluconate dehydrogenase
<i>prdx6</i>	peroxiredoxin 6
<i>gpx8</i>	glutathione peroxidase 8
<i>gstol</i>	glutathione <i>S</i> -transferase omega 1
<i>gstm3</i>	glutathione <i>S</i> -transferase mu 3
<i>gstk1</i>	glutathione <i>S</i> -transferase kappa 1
<i>mgst2</i>	microsomal glutathione <i>S</i> -transferase 2
<i>srm</i>	spermidine synthase
<i>sms</i>	spermine synthase
<i>odc1</i>	ornithine decarboxylase 1
<i>rrm1</i>	ribonucleotide reductase catalytic subunit M1
<i>rrm2b</i>	ribonucleotide reductase regulatory TP53 inducible subunit M2B

2.9 Figures

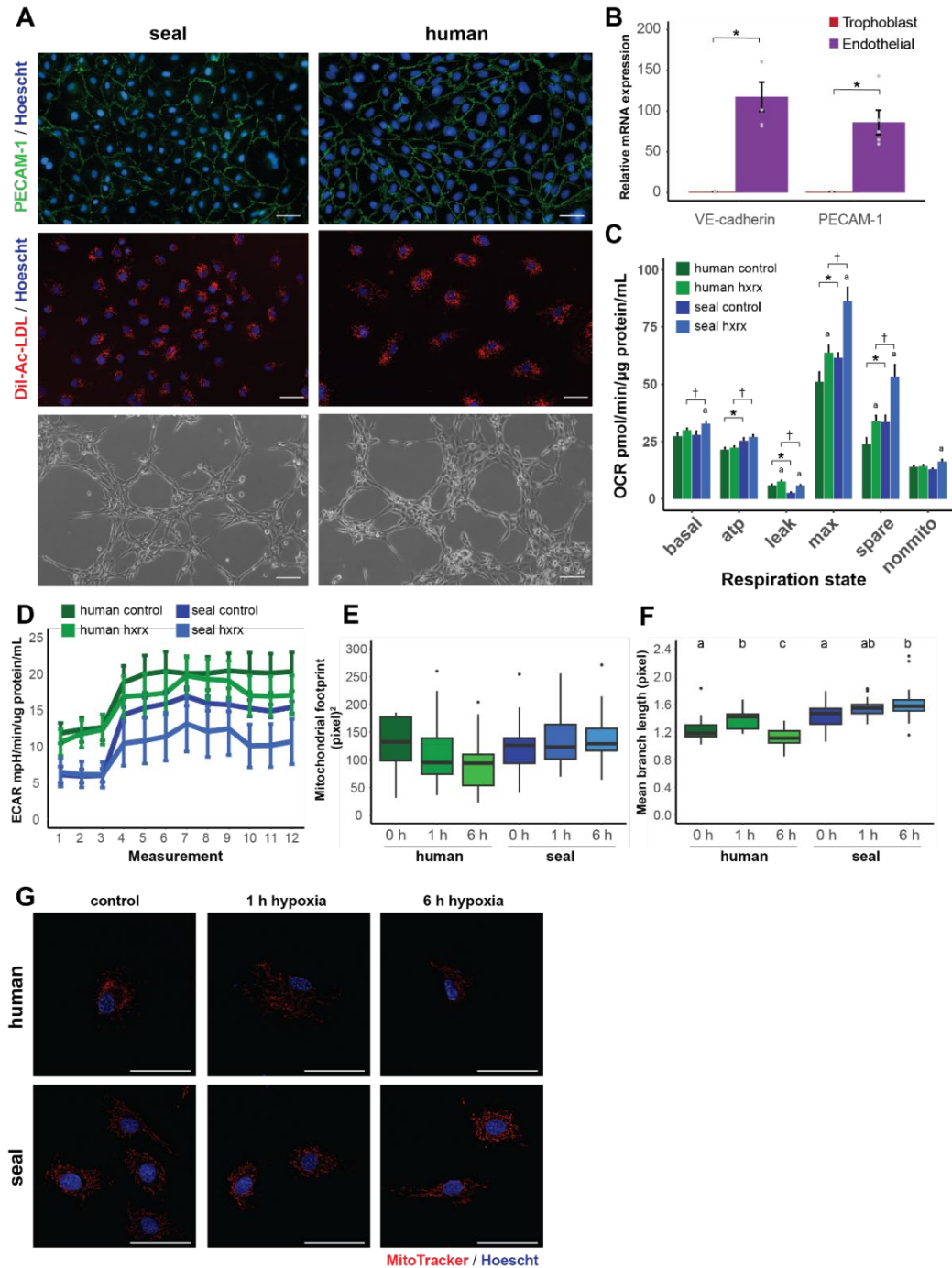


Figure 1. Primary endothelial cells express canonical endothelial markers and modulate cellular respiration in response to hypoxia/reoxygenation. (A) Top: PECAM-1 (CD31; green) staining in seal and human endothelial cells. Middle: DiI-acetylated LDL (red) uptake by seal and human cells. Nuclei (blue) in top and middle panels are stained with Hoechst. Lower: Seal and human endothelial cells spontaneously form tubes when cultured in a 3D gel matrix. (B) Relative mRNA expression levels of the endothelial markers VE-cadherin (CD144) and PECAM-1 (CD31) in seal endothelial (purple) compared to

placental trophoblast (red) cells, $n=3-5$ replicates per group. Individual points are shown as white circles with gray borders. * $p<0.05$. (C) Mitochondrial function at baseline and after hypoxia/reoxygenation in seal and human cells. Values are normalized to protein content per well. hxr, hypoxia/reoxygenation. * $p<0.05$ between species at baseline. † $p<0.05$ between species after hypoxia/reoxygenation. Lettering indicates intraspecific changes between baseline and hypoxia/reoxygenation. $n=9$ wells per group. (D) Extracellular acidification rate (ECAR) in seal and human endothelial cells at baseline and after hypoxia/reoxygenation, $n=9$ wells per group. (E, F) Mitochondrial footprint (E) and mean mitochondrial network branch length (F) in seal and human endothelial cells after hypoxia/reoxygenation. Hypoxia exposure occurred for 1 or 6 h, followed by 30 min reoxygenation. Lettering indicates intraspecific changes between baseline and hypoxia/reoxygenation. $n=13-28$ cells per group. (G) Representative images of human (top) and seal (bottom) endothelial cells stained with MitoTracker Red CMXRos after 0, 1, and 6 h hypoxia followed by 30 min reoxygenation. See Figure S1 for additional representative images. Scale bar in all images is 50 μm .

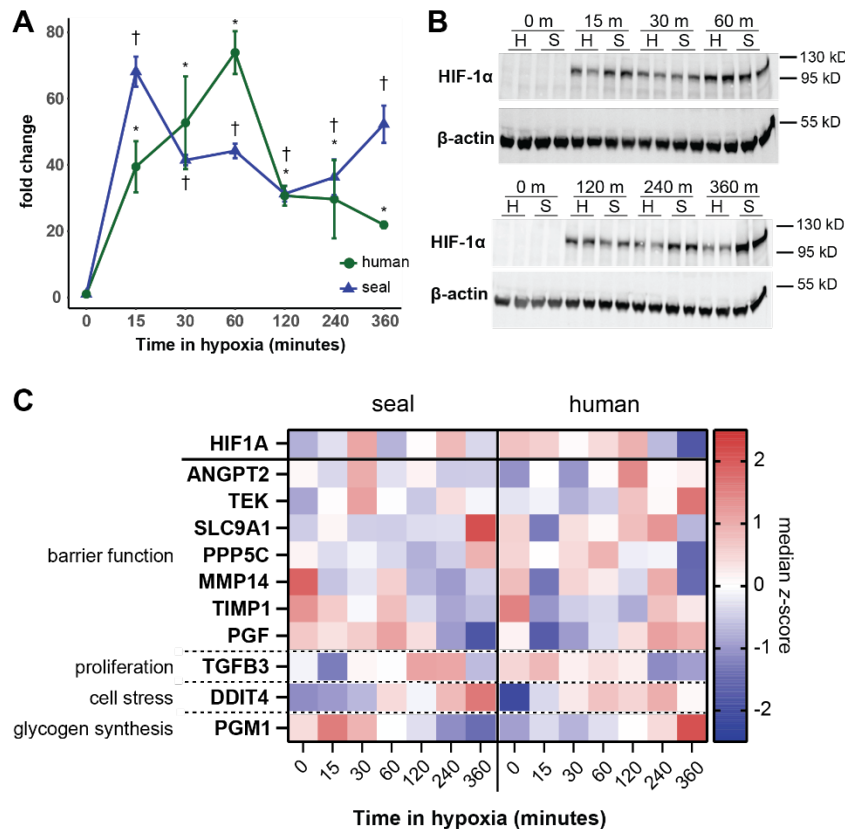


Figure 2. Hypoxia rapidly stabilizes HIF-1α in seal cells. (A) Fold change in HIF-1α protein abundance compared to normoxic baseline in endothelial cells exposed to 1% O₂ for up to 6 h, $n=3$. † $p<0.05$ versus control (seal); * $p<0.05$ versus control (human). (B) Representative western blots showing HIF-1α abundance. H, human; S, seal. Full images of gels are available in Figure S2. All samples derive from the same experiment and were distributed across four gels in total, with inter-gel controls loaded on each gel. All gels were processed in parallel. (C) Median mRNA z-scores for HIF-1α and select HIF-1 target genes in seal and human cells.

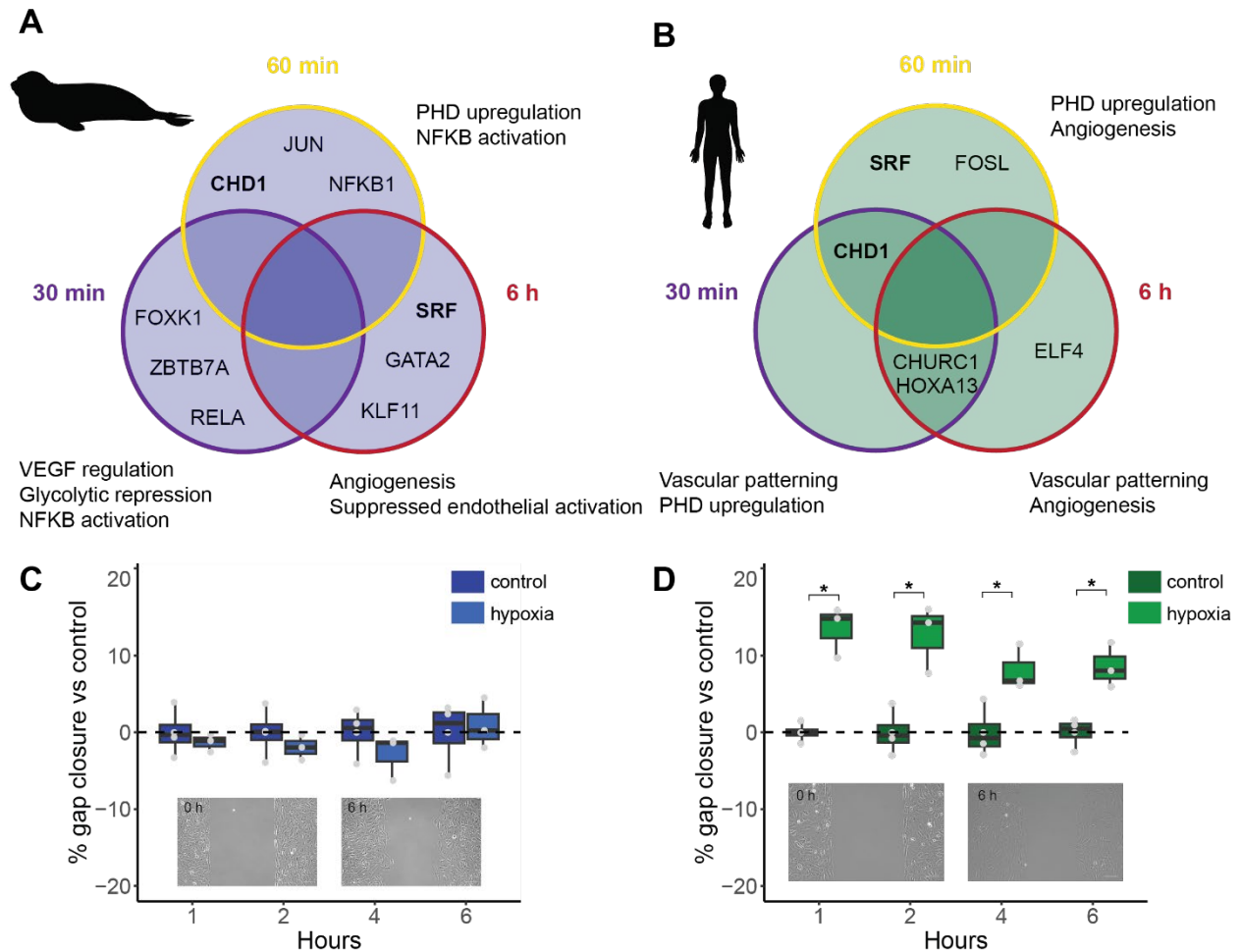


Figure 3. Seal cells delay angiogenic signaling and do not increase cell migration in response to hypoxia exposure. (A, B) Top three transcription factors predicted from genes DE at 30 min, 60 min, or 6 h versus control for (A) seal and (B) human cells. Bold text indicates factors shared between species. (C, D) Rates of gap closure in (C) seal and (D) human endothelial cells in hypoxia compared to control. Data are normalized to normoxic controls at respective time points to account for overall cell migration over time. Data are $n=4$ dishes from two independent experiments. In the boxplots, lower and upper hinges correspond to the first and third quartiles. Whiskers are $1.5 \times \text{IQR}$. Individual points are shown as gray circles. * $p < 0.05$ vs control. Inset: representative images of scratch at 0 h and after 6 h cell migration. Scale bar is 50 μm . Raw gap closure rates are available in Figure S3.

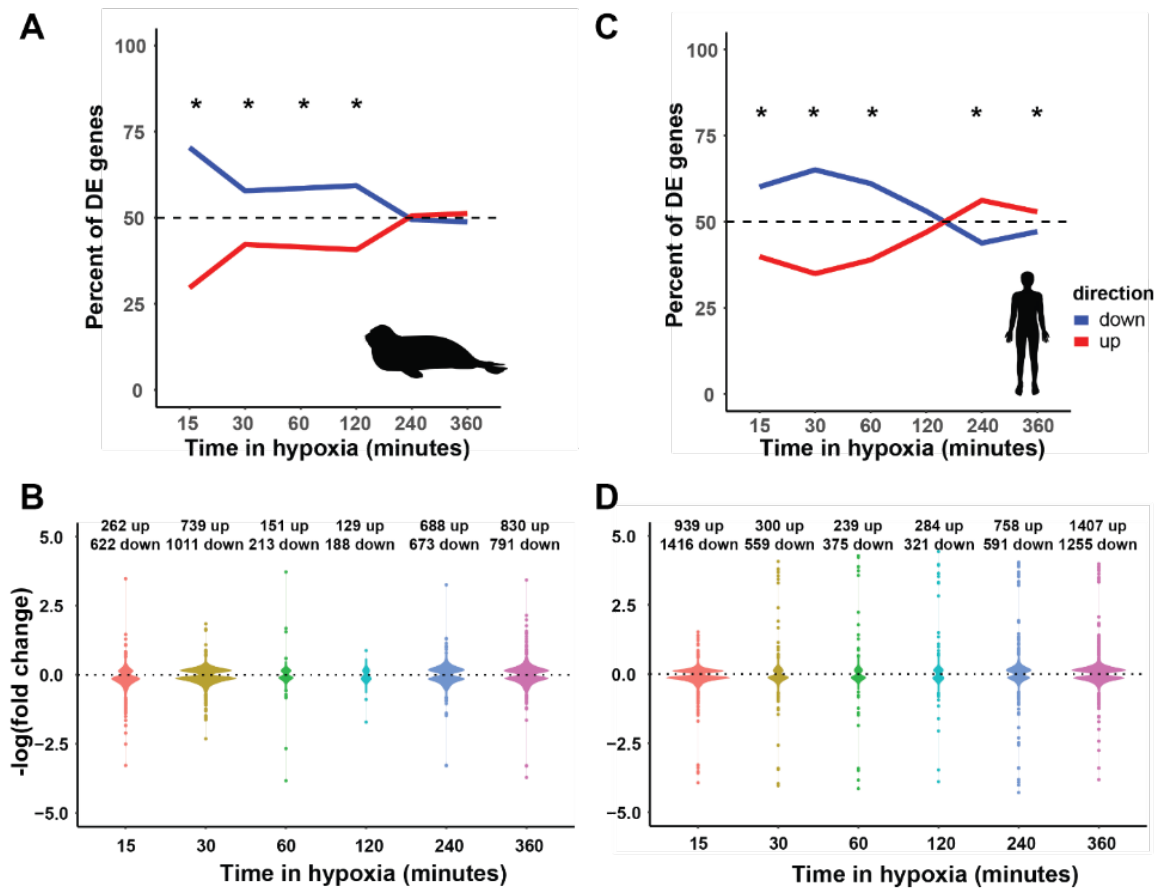


Figure 4. Seal cell gene expression equilibrates under sustained hypoxia exposure. Global differential gene expression patterns in seal (A, B) and human (C, D) cells during hypoxia compared to normoxic control. * $p < 0.05$ compared to 50/50.

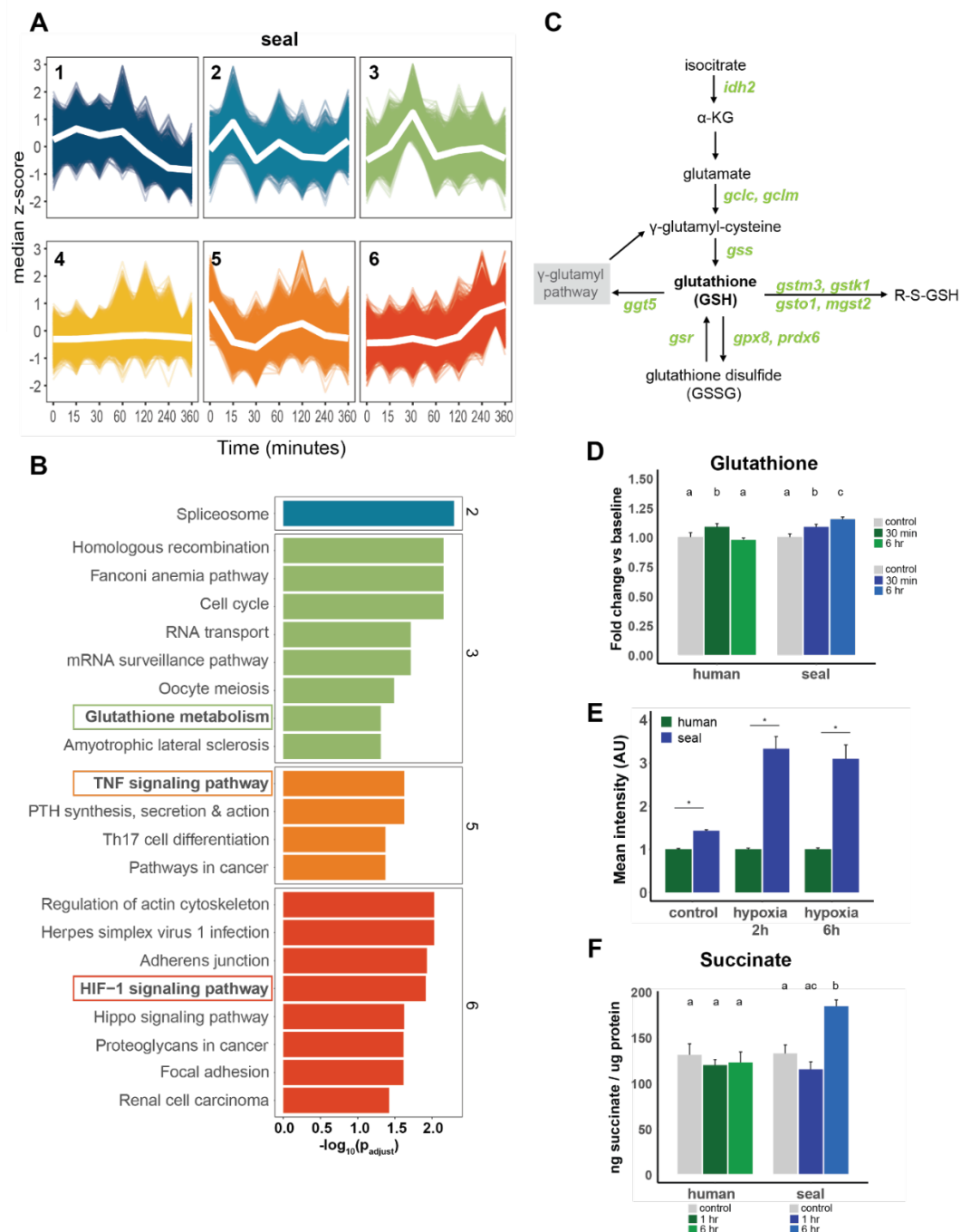


Figure 5. Concerted changes in gene expression in response to hypoxia in seal cells. (A) k -means clustering of gene expression data for seal cells exposed to 1% O_2 for up to 6 h, using $k=6$. (B) KEGG pathway enrichment for seal clusters. Numbers on the right axis correspond to seal cluster numbers. See Figure S5 for human data. (C) Diagrammatic pathway of glutathione synthesis. Green italicized gene symbols represent genes in seal cluster 3. (D) GSH content relative to each species' baseline. Lettering indicates intraspecific changes among treatments. $n=7-8$ per group. (E) Mean intensity of intracellular ThiolTracker Violet fluorescence during hypoxia exposure, using $n=4$ fields per sample and $n=3$ per group. Data are normalized to human at each time point. * $p<0.05$ between species. (F) Percent change in intracellular succinate concentration in response to hypoxia exposure, $n=3$ per group. * $p<0.05$ versus species baseline.

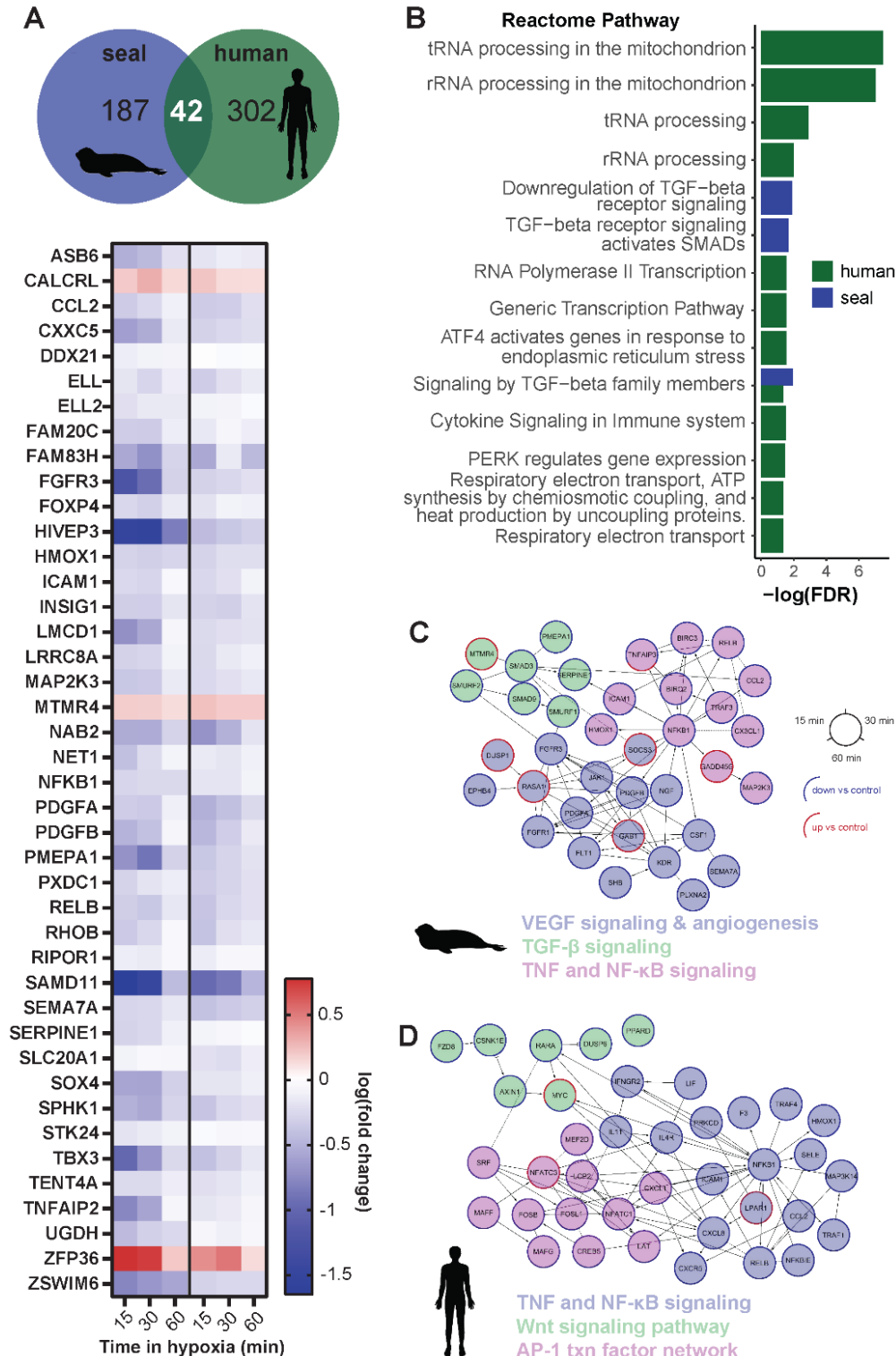


Figure 6. Short-term hypoxia modulates inflammatory signaling in seal cells. (A) Overlap between genes DE at all early time points (15 min, 30 min 60 min) versus control for seal and human cells. Heatmap shows $\log_{10}$ (fold change) for 42 DE genes shared between species. (B) Reactome pathway enrichment for genes DE at all early time points. (C,D) Functional interaction networks and select enriched pathways for seal (C) and human (D) genes DE at all early time points.

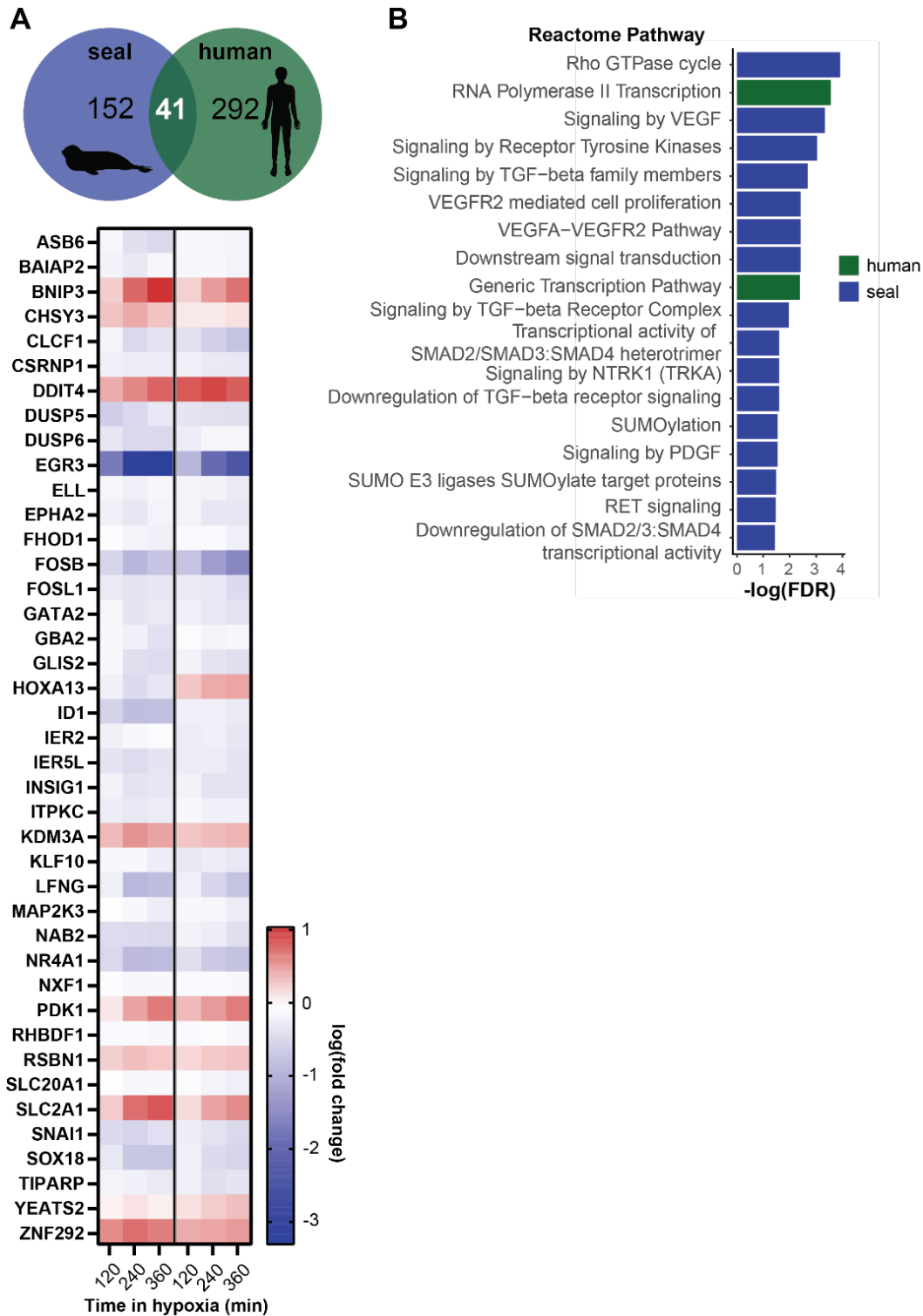


Figure 7. Long-term hypoxia modulates cell proliferation pathway expression in seal cells. (A) Overlap between genes DE at all late time points (2 h, 4 h, 6 h) versus control for seal and human cells. Heatmap shows $\log_{10}(\text{fold change})$ for 41 DE genes shared between species. (B) Reactome pathway enrichments for genes DE at 6 h versus control.

2.10 Supplementary material

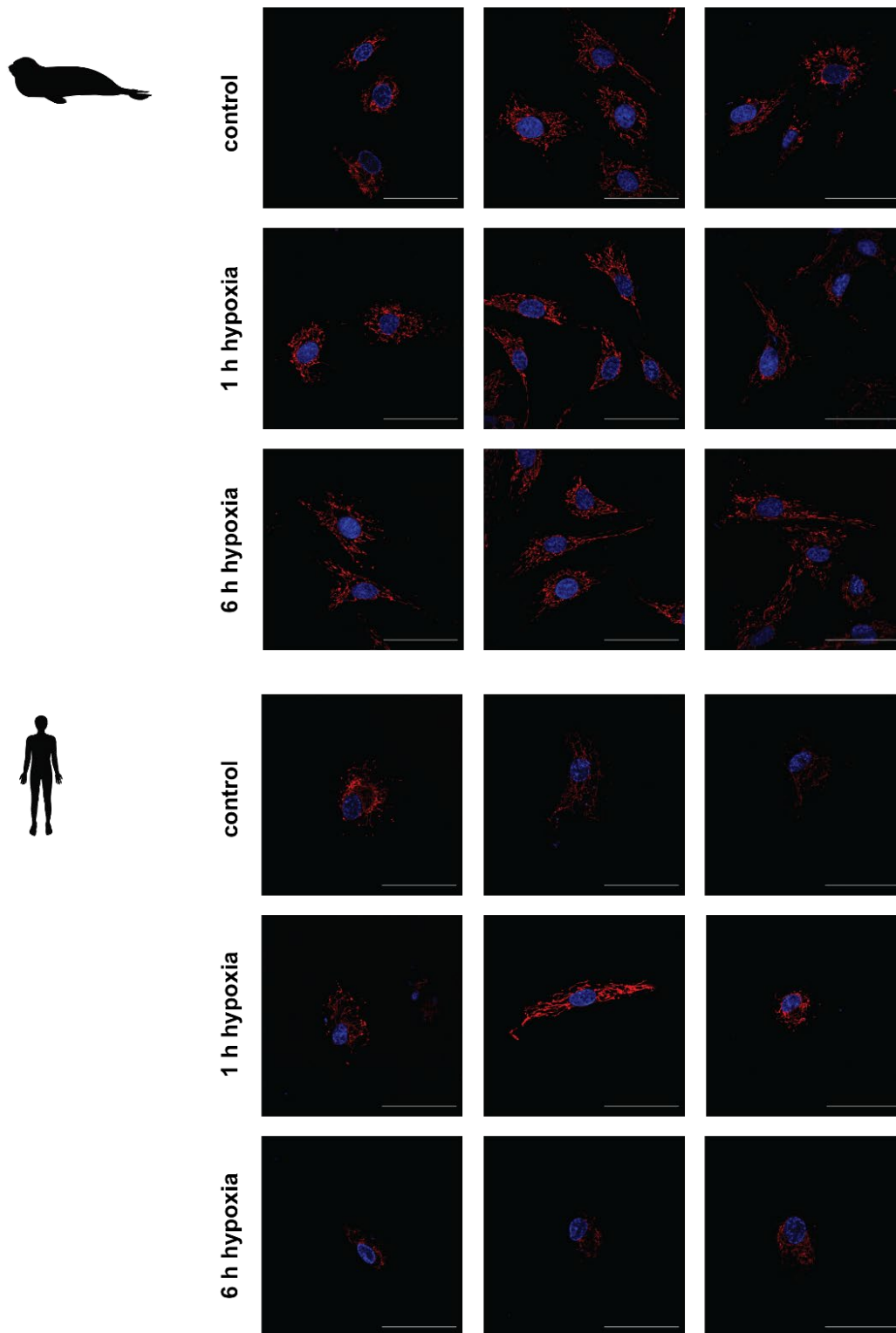
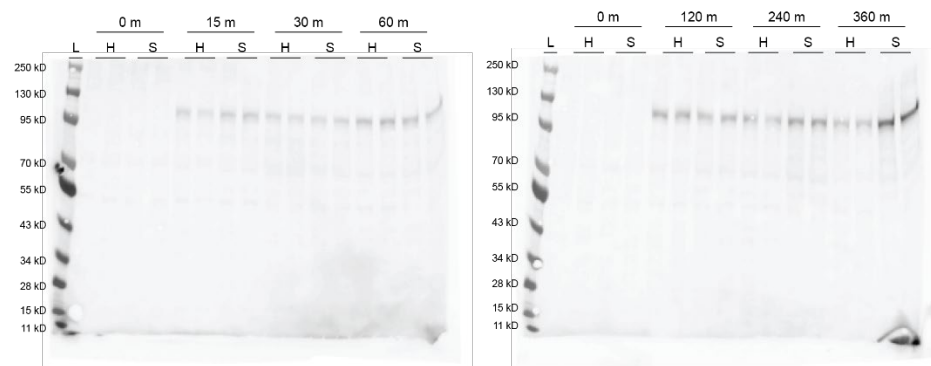


Figure S1. Representative images for mitochondrial staining after 0, 1, or 6 h hypoxia exposure followed by 30 min reoxygenation. Upper: seal. Lower: human. MitoTracker Red CMXRos (red). Hoechst (blue). Scale bar is 50 μ m.

A



B

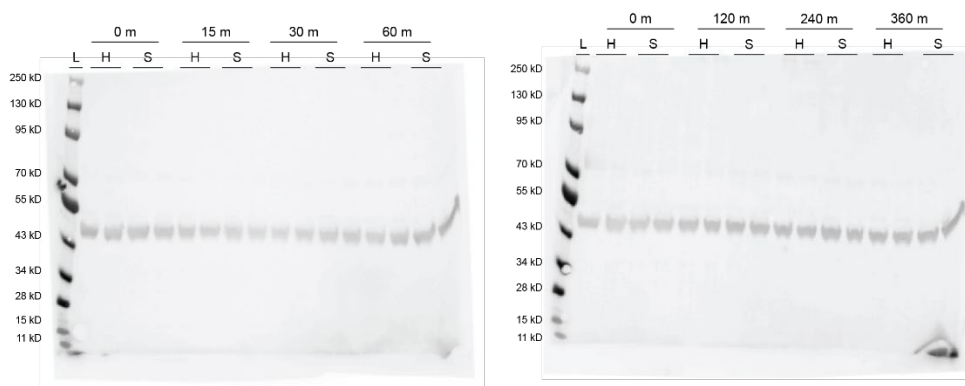


Figure S2. Uncropped western blots. (A) HIF-1α. (B) β-actin.

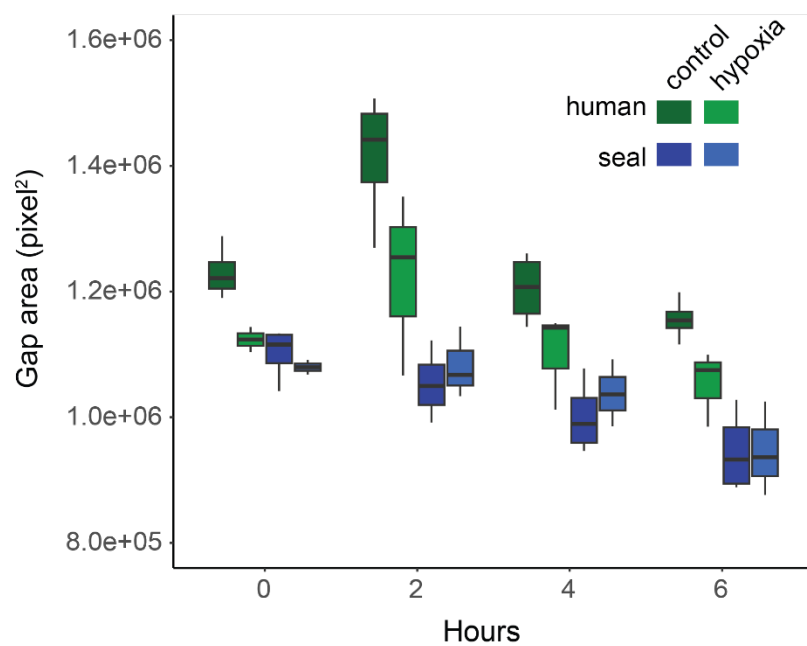


Figure S3. Raw gap area for all species and conditions during scratch assay.

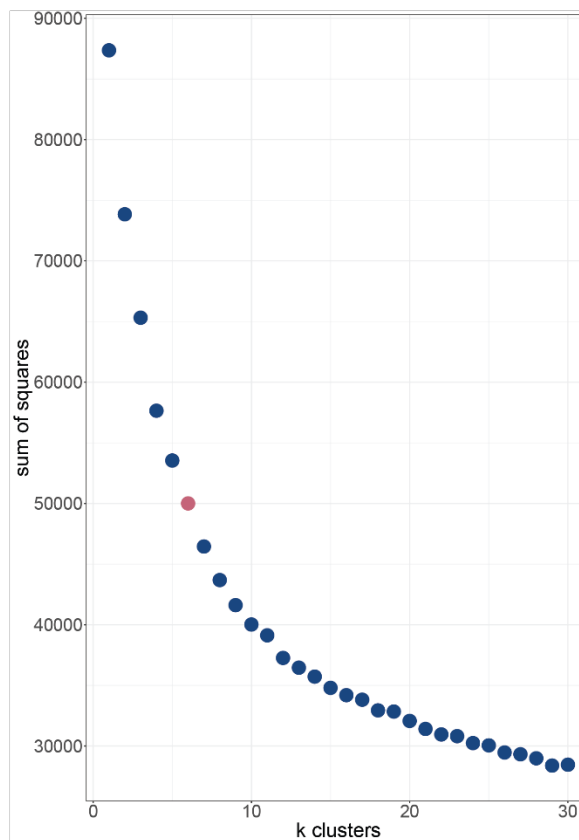
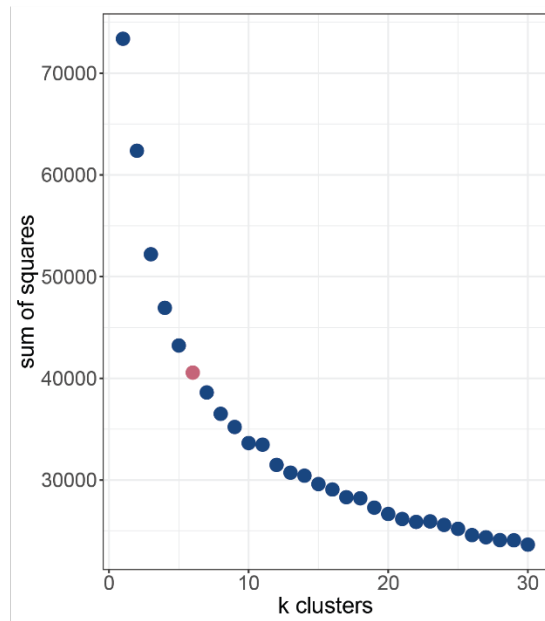


Figure S4. Total within cluster sum of squares for clustering results from $k=1...30$. Upper: seal. Lower: human. Diminishing returns were observed after 6 clusters, thus, $k=6$ (pink) was selected as the optimal clustering.

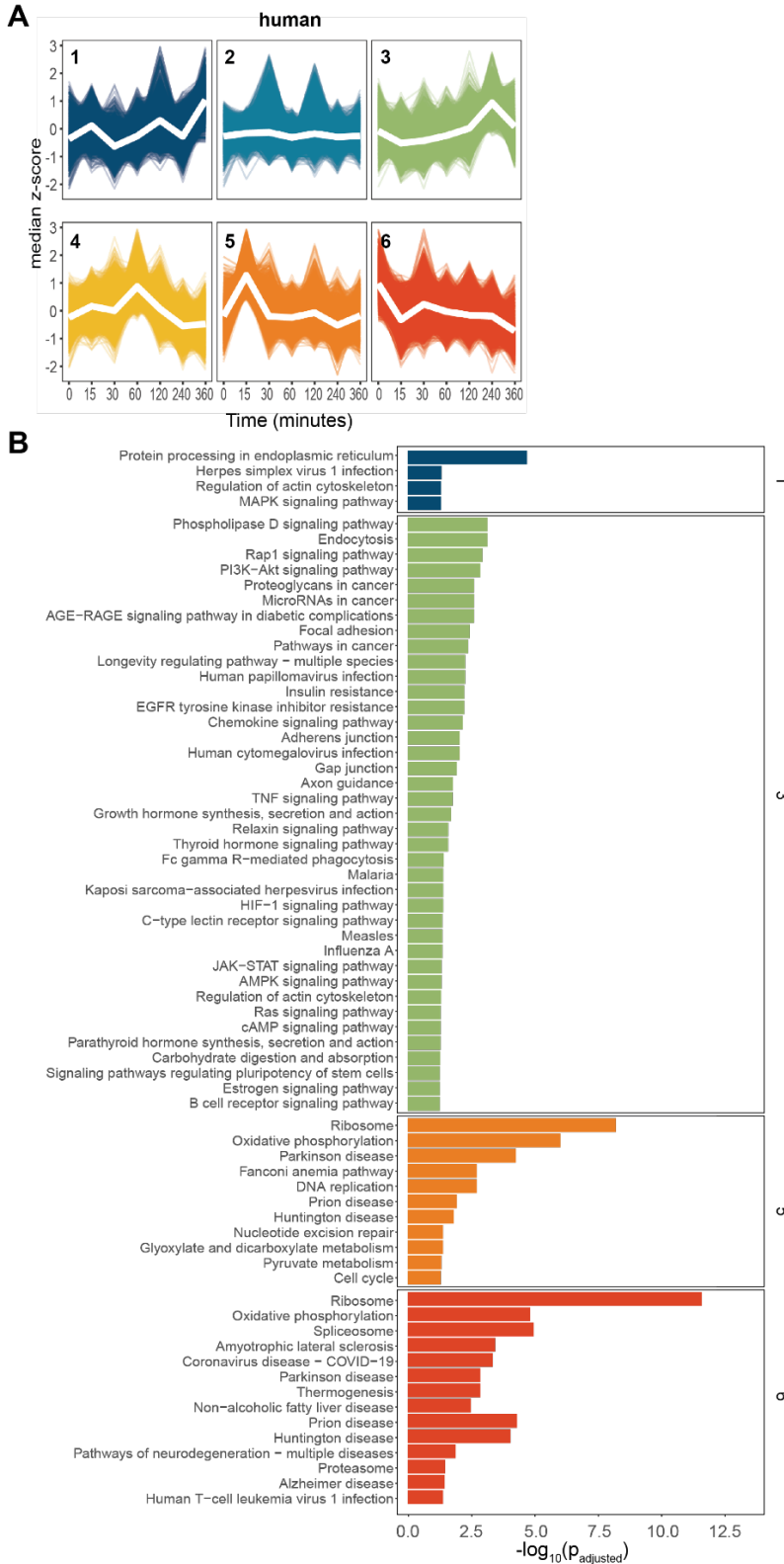


Figure S5. *k*-means clustering and KEGG pathway enrichment for human cells. *k*-means clustering of gene expression data for human cells exposed to 1% O₂ for up to 6 h, using *k*=6. (B) KEGG pathway enrichment for human clusters. Numbers on the right axis correspond to human cluster numbers.

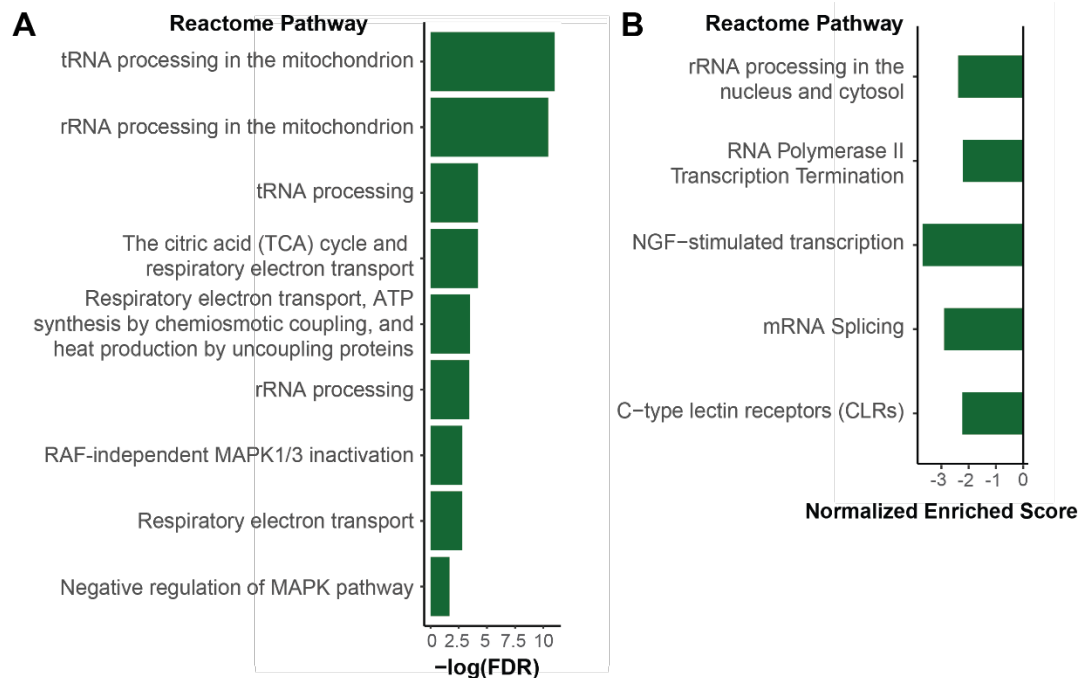


Figure S6. Long-term hypoxia exposure modulates transcription and translation in human cells. (A) Reactome pathway enrichments for genes DE at all late time points in human cells; no pathways were enriched in seal. (B) GSEA for genes DE at 6 h versus control in human cells; no pathways were enriched in seal. Normalized enriched score < 0 indicates net downregulation of the pathway.

Table S1. Primer sequences.

Target gene	Sequence 5' to 3'
<i>VE-cadherin (cd144)</i>	CCC AGA ACC GGA TGA CCA AG
	TTT CGG ATG GAG ACG CTG CT
<i>pecam-31 (cd31)</i>	CAA TAG AAG GCG GGG TCG TG
	CGT GGC TTG GCA CTG GAA GT
<i>actin</i>	CGG TCA GTT CAT GGC TGA GG
	AAG GCT CGG ACC TTC CCA AC
<i>gapdh</i>	CAA GGC TGA GAA CGG GAA GC
	ATC GGC AGA GGG AGC AGA GA

Table S2. Percent live cells after hypoxia exposure. Data are mean $\pm$ s.e.m. for 4-5 images per dish, with $n=3$ dishes per species per time. No statistically significant differences were detected between time points within each species.

	Control	30 min hypoxia	6 h hypoxia
<i>Human</i>	99.41 $\pm$ 0.34 %	99.05 $\pm$ 0.62 %	99.30 $\pm$ 0.36 %
<i>Elephant seal</i>	99.79 $\pm$ 0.05 %	99.90 $\pm$ 0.05 %	99.90 $\pm$ 0.05 %

Table S3. Fold change in expression of respiratory electron chain components in response to short-term hypoxia exposure in human cells. Fold change is expressed as a comparison to species baseline.

Gene name	15 min	30 min	60 min
<i>mt-cyb</i>	1.46	1.63	1.59
<i>mt-atp8</i>	1.96	1.82	1.88
<i>mt-co1</i>	1.90	1.76	1.74
<i>mt-co2</i>	1.67	1.77	1.70
<i>mt-nd1</i>	1.53	1.85	1.68
<i>mt-nd2</i>	1.62	1.80	1.64
<i>mt-nd4</i>	1.93	2.11	2.08
<i>mt-nd5</i>	1.57	1.77	1.80
<i>mt-nd6</i>	1.66	1.78	1.74

Table S4. Fold change in expression of respiratory electron chain components in response to long-term hypoxia exposure in human cells. Fold change is expressed as a comparison to species baseline.

Gene name	120 min	240 min	360 min
<i>dlat</i>	1.15	1.23	1.18
<i>ldha</i>	1.34	1.66	2.28
<i>mt-atp6</i>	1.67	1.84	1.62
<i>mt-atp8</i>	1.99	1.84	1.97
<i>mt-co1</i>	1.87	1.83	2.08
<i>mt-co2</i>	1.64	1.61	1.69
<i>mt-co3</i>	1.86	1.84	1.90
<i>mt-cyb</i>	1.67	1.75	1.58
<i>mt-nd1</i>	1.70	1.80	1.53
<i>mt-nd2</i>	1.68	1.72	1.52
<i>mt-nd4</i>	2.14	2.38	2.27
<i>mt-nd5</i>	1.79	2.02	2.00
<i>mt-nd6</i>	1.75	2.03	2.01
<i>pdk1</i>	2.24	3.27	4.60

Table S5. Fold change in expression of TGF- β signaling components in response to 6 h exposure in seal cells. Fold change is expressed as a comparison to species baseline.

Gene name	Fold change
<i>mtmr4</i>	2.48
<i>neddl1</i>	1.65
<i>serpine1</i>	1.38
<i>tgfbr1</i>	1.36
<i>smad2</i>	1.28
<i>itgb1</i>	1.13
<i>hdac1</i>	0.81
<i>smurf1</i>	0.76
<i>f11r</i>	0.71
<i>smurf2</i>	0.66
<i>e2f4</i>	0.62
<i>tgfbr2</i>	0.58
<i>smad3</i>	0.55
<i>ski</i>	0.54
<i>skil</i>	0.34
<i>tgif1</i>	0.28
<i>junb</i>	0.27
<i>smad7</i>	0.23

3. Oxidant stress but not hypoxia/reoxygenation modulates expression of iron and sulfur handling genes in seal endothelial cells

3.1 Abstract

Elephant seals tolerate repeated bouts of hypoxemia/reoxygenation secondary to their prolonged diving behavior. It is well accepted that these animals do not sustain excessive oxidative injury derived from these dives, but the cellular and molecular mechanisms which enable this tolerance remain undefined due to experimental limitations. We used RNA-seq and functional assays in a proliferative arterial endothelial cell culture system from elephant seals to compare the effects of hypoxia/reoxygenation with a chemical oxidant exposure.

Seal endothelial cells tolerated high doses of a chemical oxidant (*tert*-Butyl hydroperoxide, *t*-BOOH) and did not display changes in either cytotoxicity or lipid peroxidation, though human and sheep cells were susceptible to these insults. Seal cells did not alter expression or activity of the glutathione-dependent phospholipid hydroperoxidases GPX4 or PRDX6. RNA-seq revealed differential expression of ferroptosis pathway genes in seal cells with *t*-BOOH treatment, though only when compared to hypoxia/reoxygenation. Among these genes were the cellular iron exporter ferroportin 1 (SLC40A1), which displayed seal-specific upregulation with *t*-BOOH treatment. We also found that *t*-BOOH treatment downregulated genes involved in cysteine degradation when compared to (elevated) levels during hypoxia exposure.

We found that seal cells resist oxidative stress, but that this resistance apparently does not rely on phospholipid hydroperoxidase activity. Furthermore, we identified a seal-specific response in the expression of a major iron handling gene, ferroportin 1, in *t*-BOOH treatment. These results suggest that modified iron handling may protect seal cells against lipid peroxidation.

3.2 Introduction

Northern elephant seals (*Mirounga angustirostris*) are premier divers, spending up to 90% of their at-sea time submerged in prolonged, repeated dive cycles [1]. Seals utilize a suite of cardiovascular adaptations, including selective vasoconstriction [5,6,26,50], to modulate tissue oxygen consumption rates during diving. Additionally, phocid seals rely heavily on the heme-based respiratory pigments hemoglobin and myoglobin to bolster body oxygen stores 4-5 times that of most terrestrial species [7]. Despite these adaptations in both oxygen storage and utilization, northern elephant seals regularly experience arterial pO₂ values as low as 12 mmHg, corresponding to an arterial sO₂ of ~8% while diving. These hypoxemic events are followed by rapid reoxygenation during brief eupneic surface intervals [24,25]. In humans, arterial sO₂ values below 70% are an acute danger to life, and localized fluctuations in oxygen availability on par with seal values occur only during adverse cardiovascular events such as myocardial infarction and ischemic stroke [35,84]. In non-hypoxia tolerant species, some cell and tissue death occurs during hypoxia. However, the reintroduction of oxygen to the depleted system triggers the generation of reactive oxidants such as hydrogen peroxide (H₂O₂) and superoxide anion (O₂^{•-}) from mitochondrial and

enzymatic sources [35]. Thus, significant secondary injuries may occur at the resolution of the hypoxic insult.

Reactive oxidants damage lipids, DNA, and proteins, fundamentally altering cellular redox state and impacting function across molecular processes. Hypoxic iron accumulation leads to the interaction of ferrous iron (Fe^{2+}) with molecular O_2 during reoxygenation, causing peroxidation of membrane phospholipids. Additionally, iron's participation in Fenton chemistry yields the highly reactive hydroxyl radical ($\text{OH}^\bullet$), which can further damage cell membranes [198]. Excessive lipid peroxidation causes ferroptosis, a regulated form of iron-dependent cell death [199,200]. Antioxidant activity mitigates such damage, though only two enzymes can reduce phospholipid hydroperoxides, the main products of lipid peroxidation. Both such enzymes (glutathione peroxidase 4 (GPX4) and peroxiredoxin 6 (PRDX6)) utilize glutathione (GSH) in their catalytic reaction [199–201]. A large body of evidence, including our own work, implicates GSH in marine mammals' resistance to oxidative injury [99,101,102,106,110,146,147,202,203], though GSH-independent mechanisms are also known to suppress ferroptosis [204–207].

Excessive oxidant generation and altered redox states impair the vasoregulatory and barrier functions of the endothelium [137,139,143]. The highly reactive nature of most oxidants complicates *in vivo* investigation of oxidative injury in marine mammals, particularly in the vasculature. Thus, we developed primary endothelial cell cultures from northern elephant seal arteries to evaluate the response to hypoxia/reoxygenation and chemical oxidant exposure. We then conducted comparative transcriptomic and functional analyses of the molecular mechanisms contributing to oxidative stress resistance in seal endothelial cells. We found that seal endothelial cells resist lipid peroxidation despite treatment with a high dose of a chemical oxidant and that this resistance is apparently not driven by the GSH-dependent phospholipid hydroperoxidases GPX4 and PRDX6. We compared the transcriptomic response to hypoxia/reoxygenation and *t*-BOOH treatment in seal, human, and sheep endothelial cells to identify seal-specific alterations in gene expression in both conditions. Differential regulation of gene expression in the ferroptosis pathway occurred in seal cells only when comparing the response to *t*-BOOH treatment with hypoxia/reoxygenation. Moreover, this comparison identified a seal-specific increase in expression of the cellular iron exporter ferroportin 1 (SLC40A1) with *t*-BOOH treatment but not hypoxia/reoxygenation. Thus, modified iron handling rather than GSH availability appears to protect seal cells against oxidant-induced lipid peroxidation.

3.3 Materials and methods

3.3.1 Tissue acquisition, primary cell isolation, and cell culture

Northern elephant seal (*Mirounga angustirostris*) and human placental arterial endothelial cells were obtained and isolated as described in Chapter 2.3.2 [202]. Seal endothelial cells were maintained in media consisting of DMEM (low glucose, with pyruvate, with L-glutamine; Gibco catalog # 11885-084), 10% FBS (VWR Seradigm, Mexico), 10 mM HEPES, 1X MEM NEAA (Gibco, catalog # 11140-050), 4 $\mu\text{g}/\text{mL}$ endothelial cell growth supplement (ECGS; Corning, catalog # 354006), and 1X Antibiotic-Antimycotic (Gibco, catalog # 15240-062). Human endothelial cells were maintained in a commercial endothelial cell medium (Sciencell Research Laboratories, catalog # 1001, Carlsbad, CA, USA). Sheep (*Ovis aries*) placentae were obtained secondary to veterinary student training at the UC Davis School of Veterinary Medicine; placental arterial endothelial cells were isolated as described for seal and human cells in Chapter 2.3.2 and

maintained in commercial medium (Sciencell Research Laboratories). Confluent cultures were cryopreserved at passage 1. In all experiments, cells were plated in species-specific media and changed to the same media the day prior to assay.

3.3.2 Cell characterization

Seal and human endothelial cell cultures were phenotypically characterized in our previous work (Chapter 2.9, Figure 1) [202]. The endothelial phenotype of the sheep cell preparation was confirmed by immunostaining for VE-cadherin (CD144; ThermoFisher catalog # PA5-17401, RRID:AB_10987564, 1:100), as shown in Figure S1.

3.3.3 *tert*-Butyl hydroperoxide (*t*-BOOH) treatment

Seal, human, and sheep endothelial cells were exposed to increasing concentrations of *tert*-Butyl hydroperoxide (*t*-BOOH; 0, 100, 200, or 300 μ M; Alfa Aesar, catalog # A13926), an organic peroxide, for up to 2 h to determine an appropriate oxidant treatment that did not produce excessive cell death. For lipid peroxidation experiments, seal and human cells were treated with 300 μ M *t*-BOOH for 1.5 h. Subsequently, cells treated with *t*-BOOH (100 μ M) for 2 h were used for RT-qPCR, western blot, GPx activity, RNA-seq, and iron detection.

3.3.4 Cell viability, cytotoxicity, and apoptosis

Cell viability, cytotoxicity, and apoptosis were evaluated using the ApoTox-Glo Triplex Assay (Promega, catalog # G6320, Madison, WI, USA) according to manufacturer instructions. Cells were seeded in a fibronectin-coated clear bottom 96-well plate at 10,000 cells per well overnight in complete media before *t*-BOOH exposure. After exposure, cells were incubated for 30 min at 37°C with a cell-permeant substrate (GF-AFC), which fluoresces after cleavage by live-cell proteases, and a cell impermeant substrate (bis-AAF-R110), which fluoresces after cleavage by dead-cell proteases. Fluorescence was read at the following wavelengths: 400_{Ex}/505_{Em} (viability), 485_{Ex}/520_{Em} (cytotoxicity). Apoptosis was detected as luminescence resulting from caspase-3/7-mediated cleavage of the luciferase substrate aminoluciferin. Fluorescence and luminescence were measured using a SpectraMax M3 plate reader (Molecular Devices). Data are expressed as fold change from untreated control for each species.

3.3.5 Detection of lipid peroxides in live cells

Lipid peroxidation was detected in live cells using the cell-permeant dye Liperfluo (Dojindo Molecular Technologies, catalog # L248, Rockville, MD, USA) in seal and human cells treated with *t*-BOOH treatment (300 μ M for 1.5 h) as described in [208]. Cells were loaded with 5 μ M Liperfluo during the last 30 min of the treatment. Cells were counterstained with NucBlue Live ReadyProbes Reagent (Hoechst 33342). All imaging was conducted using a Zeiss Axio Observer 7 inverted microscope fitted with 10X and 20X objectives and Zeiss software. Fluorescence intensity was quantified using FIJI v2.1.0. Data are expressed as fold change from untreated control and normalized to the number of cells per field.

3.3.6 RT-qPCR

RNA was extracted using TRIzol Reagent (Invitrogen, catalog # 15596) according to manufacturer instructions and our previous work [135]. RNA pellets were resuspended in nuclease-free water and genomic DNA was removed using the TURBO DNase kit (Ambion,

catalog # AM2238) according to manufacturer instructions. cDNA was synthesized using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, catalog # 4374966) according to manufacturer instructions. RT-qPCR was conducted using our previously published methods [202] (see Chapter 2.3.3) under the following conditions: 1 min at 95°C followed by 40 cycles of 20 sec at 95°C, 30 sec at 60°C. Primer sequences are listed in Table S1. Expression levels for genes of interest were normalized to the geometric mean of 3 housekeeping genes (*gapdh*, *β-actin*, *18S*). Relative mRNA expression (fold change) between treatment and control was calculated using the $2^{-\Delta\Delta C_t}$ method.

3.3.7 Immunoblotting

Protein collection, quantification, and western blotting were conducted using our previously published methods [202] and as described in Chapter 2.3.6. Membranes were incubated overnight with antibodies against glutathione peroxidase 4 (GPX4; Abcam, catalog # 125066; RRID:AB_10973901; 1:3,000) or peroxiredoxin 6 (PRDX6; Cell Signaling Technology, catalog # 95336; RRID:AB_2800244; 1:1,000). Proteins were visualized using IRDye 800CW secondary antibodies (LICOR) and a two-color near-infrared system (Azure c500, Azure Biosystems, Dublin, CA, USA). Membranes were stripped and reprobed with an antibody against β -actin (Cell Signaling Technology, catalog # 4967; RRID:AB_330288; 1:1,000). The intensity of individual bands was quantified using FIJI v2.1.0 and normalized to β -actin.

3.3.8 Glutathione peroxidase activity assay

Total glutathione peroxidase (GPx) activity was measured using a commercial Glutathione Peroxidase Assay Kit (Cayman Chemical, catalog # 703102). Briefly, cells were homogenized on ice in lysis buffer containing 1X PBS, 1% Triton X-100, 1% Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific, catalog # 78440), and 1 mM EDTA. Crude lysates were centrifuged at 10,000 x g at 4°C for 15 min. The supernatant containing protein was stored at -80°C until use. Samples were mixed with Co-Substrate Mixture, NADPH, and cumene hydroperoxide, and absorbance was measured at 340 nm once per minute for 30 min using a SpectraMax M3 plate reader (Molecular Devices). Change in absorbance (ΔA_{340}) was calculated for each well as the slope of the linear portion of the curve of A_{340} vs. time. GPx activity was normalized to protein content per sample, which was determined using a Pierce Rapid Gold BCA Protein Assay Kit (Thermo Scientific, catalog # A53225).

3.3.9 Hypoxia/reoxygenation exposure

Hypoxia was achieved by incubating cells in an InVivoO₂ physiological cell culture workstation (Baker Ruskinn, Sanford, ME, USA) for 1 h under the following conditions: 0.5% O₂, 5% CO₂, 37°C. Reoxygenation was achieved by removing cells from the chamber and briefly opening dish lids in a tissue culture hood in room air (21% O₂) followed by a 30 min incubation in a 5% CO₂ incubator at 37°C in room air. We used a 1 h hypoxia exposure to mimic a dive duration well within physiological limits for an elephant seal but generally beyond what is observed in most pinnipeds. The 30 min reoxygenation exceeds a typical elephant seal surface interval but enables investigation of transcriptional changes in this system.

3.3.10 RNA-seq

RNA was isolated from cells exposed to: normoxia, hypoxia (1 h at 0.5% O₂), hypoxia/reoxygenation (1 h at 0.5% O₂ followed by 30 min normoxia), or *t*-BOOH (100 μM for 2 h). Cells were lysed in Buffer RLT without β-mercaptoethanol. Lysates were stored at -80°C until all samples were collected. RNA extractions were carried out using an RNeasy Mini Kit (Qiagen, Germantown, MD, USA) with on-column DNase treatment according to manufacturer instructions. DNase treatment was confirmed by a lack of genomic amplification using PCR. RNA yield was quantified using a Nanodrop 1000 Spectrophotometer (Thermo Scientific). RNA integrity number (RIN) was determined using an Agilent 2100 Bioanalyzer kit for total eukaryotic RNA (Agilent Technologies, Santa Clara, CA, USA). Samples underwent library preparation and sequencing at Novogene Corporation, Inc. Oligo(dT) bead enrichment of mRNA was followed by random fragmentation and cDNA synthesis with random hexamers and reverse transcriptase. First-strand synthesis was followed by nick-translation generation of the second strand (Illumina). Strands then underwent purification, terminal repair, and poly(A) tailing. After adapter ligation of poly(A)-captured mRNA, the library underwent size selection and PCR enrichment. Three replicates per species per condition were used to generate libraries, with a sequencing depth of 20M reads per sample on a NovaSeq platform (Illumina).

3.3.11 Transcriptome analyses

The elephant seal (*Mirounga angustirostris*) genome was annotated as described in our previous work (https://www.dnazoo.org/assemblies/Mirounga_angustirostris) [135]. Reads were mapped to the elephant seal, human (GRCh38), or sheep (ARS-UI_Ramb_v2.0) genomes using STAR aligner [151]. Annotated gene counts were as follows: elephant seal, 14,080; human, 39,943; sheep, 14,528. A total of 11,830 genes were found as commonly annotated across all three species. Transcript levels were quantified with RSEM v1.3.1 [152]. For each species, genes differentially expressed (DE) between conditions were identified using EBSeq with a false discovery rate of 5% [153]. Gene set enrichment analyses (GSEA) were conducted on summary lists of genes DE in pairwise comparisons between conditions using KEGG and Reactome pathways [154].

3.3.12 Iron content in live cells

Cellular iron content was measured semi-quantitatively using the live cell permeant dye FerroOrange (Dojindo Molecular Technologies, catalog # L374), which irreversibly fluoresces at 543_{Ex}/580_{Em} upon reaction with Fe²⁺ (ferrous iron). Cells were stained with 1 μM FerroOrange for 30 min for control, hypoxia/reoxygenation, or *t*-BOOH treatment (100 μM for 2 h). Combined fluorescence intensity of both the conditioned media and cells was measured using a SpectraMax M3 plate reader (Molecular Devices). Data are expressed as fold change from untreated control.

3.3.13 Statistical analyses

Statistical analyses were performed using GraphPad Prism v10.2.1. Normality was evaluated using D'Agostino-Pearson or Shapiro-Wilk tests, depending on sample size. All datasets met normality assumptions; differences between groups with equal variance were assessed by independent two-tailed *t*-test or one-way ANOVA. ANOVA results were corrected for multiple comparisons using Šidák's multiple comparisons test ($\alpha=0.05$). *t*-tests were corrected for multiple

comparisons using the two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli with FDR=0.05 [158]. Data are presented as mean $\pm$ s.e.m.

3.4 Results

3.4.1 Seal endothelial cells resist cell death and lipid peroxidation induced by *t*-BOOH

We measured cell viability and cytotoxicity in seal, human, and sheep cells treated with organic peroxide to study whether seal endothelial cells resist chemically-induced oxidant stress. *t*-BOOH treatment negatively impacted viability in seal, human, and sheep endothelial cells in a dose-dependent manner ($F_{11,24}=36.62$, $p<0.0001$) (Figure 1, top). This decline was least dramatic in seal cells, where viability remained above 50% of control levels until the highest *t*-BOOH dose. *t*-BOOH treatment impacted overall cell death (cytotoxicity) in a species-specific manner ($F_{11,24}=35.01$, $p<0.0001$). Remarkably, seal cells did not display changes in overall cell death (cytotoxicity) at any *t*-BOOH dose despite observed differences in viability, whereas all *t*-BOOH doses significantly increased cell death more than two-fold in both human and sheep cells (Figure 1, bottom). These results underscore the remarkable intrinsic tolerance to oxidant stress in endothelial cells derived from deep-diving elephant seals.

Caspase-3/7 activity exhibited differential responses to *t*-BOOH treatment across species ($F_{11,24}=25.40$, $p<0.0001$) (Figure S2). *t*-BOOH treatment did not increase caspase activity in seal cells, consistent with a lack of cell death (Figure 1, bottom). Caspase activity increased in human cells with 100 and 200 μ M *t*-BOOH treatment; the return to baseline at 300 μ M *t*-BOOH may suggest that other cell death pathways dominate in human cells at high *t*-BOOH doses (Figure S2). Sheep cells did not display changes in caspase activity associated with increasing *t*-BOOH dose (Figure S2) despite sharp increases in cell death (Figure 1, bottom), suggesting that apoptosis does not play a major role in *t*-BOOH-associated cell death in sheep cells at any dose. Overall, these data suggest that apoptosis does not generally drive cell death associated with chemically-induced oxidant stress (*t*-BOOH treatment) and that any such change in cell death is negligible in seal endothelial cells.

We next considered whether a high dose of *t*-BOOH (300 μ M) might induce non-lethal changes in cellular lipid peroxide levels in seal cells. Seal endothelial cells resisted any change in total lipid peroxide levels even at a high dose of *t*-BOOH, as detected by a lipid peroxidation-sensitive fluorescent dye. In contrast, lipid peroxidation levels doubled in human cells after the same treatment ($t=8.427$, $p<0.001$) (Figure 2). Together, these data indicate that seal endothelial cells remain well protected against both cell death and lipid peroxidation despite exposure to high concentrations of an organic peroxide.

3.4.2 *t*-BOOH treatment does not increase expression or abundance of phospholipid hydroperoxide glutathione peroxidases in seal endothelial cells

Glutathione peroxidases (GPx) are antioxidant enzymes that protect against oxidative damage by reducing peroxides. Among these, glutathione peroxidase 4 (GPX4) can also reduce phospholipid hydroperoxides, the main cellular products of lipid peroxidation [201]. Peroxiredoxin 6 (PRDX6) is a 1-cys peroxiredoxin that counteracts lipid peroxidation via hydrolysis through its phospholipase A₂ activity and reduction of oxidized phospholipids [209,210]. Given that seal but not human cells resist lipid peroxidation when treated with *t*-BOOH

(Figure 2), we considered whether gene expression and protein abundance of GPX4 and PRDX6 changed in response to *t*-BOOH in either species. *GPX4* gene expression declined slightly in response to *t*-BOOH treatment in both seal and human cells (~0.6-fold vs. control), though the effect was not significant in either species, perhaps due to relatively small sample sizes. Similarly, *t*-BOOH treatment significantly decreased GPX4 protein abundance in both species (seal: 0.7-fold vs. control, $t=2.832$, $p=0.025$; human: 0.8-fold vs. control, $t=2.269$, $p=0.045$) (Figure 3A; western blot available in Figure S3). In contrast, *PRDX6* gene expression increased 1.6-fold with *t*-BOOH treatment in human cells ($t=4.589$, $p=0.019$) but did not change in seal cells. In both seal and human cells, *t*-BOOH treatment ultimately decreased PRDX6 protein abundance by 0.5- and 0.6-fold, respectively (seal: $t=5.141$, $p=0.014$; human: 0.6-fold, $t=6.013$, $p=0.014$) (Figure 3B; western blot available in Figure S3), suggesting that neither GPX4 nor PRDX6 contribute to the intrinsic lipid peroxidation resistance observed in seal endothelial cells.

We then evaluated total GPx activity against cumene hydroperoxide in seal, human, and sheep cells treated with *t*-BOOH. One-way ANOVA detected differences in activity among the species and treatments ($F_{5,12}=244.4$, $p<0.0001$). A posthoc Šídák test to correct for multiple comparisons identified seal and sheep GPx activity as considerably lower than that in human cells in both control and *t*-BOOH-treated conditions ($p<0.001$ in all comparisons against human cells). GPx activity in human cells decreased 22% with *t*-BOOH treatment ($p=0.0003$), though activity remained ~2-5 times higher than in seal and sheep cells at any point. *t*-BOOH treatment did not alter GPx activity in seal or sheep cells (Figure 4). Together, these data suggest that changes in glutathione peroxidase activity or expression are unlikely to drive seal cells' resistance to lipid peroxidation.

3.4.3 Seal endothelial cells mount condition-specific transcriptional responses to hypoxia, hypoxia/reoxygenation, and *t*-BOOH treatment

Next, we considered whether global changes in gene expression could identify signatures of lipid peroxidation resistance in seal endothelial cells. We conducted RNA-seq in seal, human, and sheep cells for the following conditions: untreated control, hypoxia (0.5% O₂ for 1 h; RNA was collected before reoxygenation), hypoxia/reoxygenation (0.5% O₂ for 1 h followed by 21% O₂ for 30 min), and *t*-BOOH (100 μM for 2 h; the lowest dose tested in Figure 1). We identified genes differentially expressed (DE) for each species in each treatment compared to the untreated control. We also identified genes DE between treatments (Figure 5). In seal cells, comparison of any condition against hypoxia yielded large changes in gene expression (vs. control: 4,005 genes; vs. hypoxia/reoxygenation: 2,138 genes; vs. *t*-BOOH treatment: 6,933 genes) (Figure 5, top). A similar pattern was observed in human, but not sheep cells (Figure 5, middle and bottom).

While quantitative comparisons between up- vs. downregulation patterns must be undertaken with caution, it is apparent *t*-BOOH generally upregulated gene expression in seal cells, particularly when compared to untreated control (only 24% of DE genes were downregulated in *t*-BOOH vs. control, $p<0.0001$) or hypoxia/reoxygenation (only 23% of genes downregulated in *t*-BOOH vs. hypoxia/reoxygenation, $p<0.0001$) (Figure 5, top). In contrast, the greatest downregulation of gene expression in seal cells occurred in hypoxia/reoxygenation compared to hypoxia (63% of DE genes downregulated, $p<0.0001$) (Figure 5, top). This pattern was not observed for either human or sheep cells (Figure 5, middle and bottom). In human cells, the distribution of gene expression changes was closer to 50/50 up- vs. downregulated genes (range: 36-49% downregulated) (Figure 5, middle). Sheep cells downregulated gene expression in *t*-

BOOH treatment compared to hypoxia/reoxygenation (62% downregulated, $p<0.0001$) and generally upregulated expression during hypoxia and hypoxia/reoxygenation, regardless of the reference condition (Figure 5, bottom). In sum, we observed inverse global gene expression patterns in response to hypoxia/reoxygenation and *t*-BOOH for seal and sheep cells. In contrast, human cells did not display substantial shifts in up- vs. downregulation of gene expression under any condition.

We then compared sets of genes DE relative to control (the first 3 columns from Figure 5) to determine condition-specific changes during hypoxia, hypoxia/reoxygenation, and *t*-BOOH treatment. In seal cells, only 1% of all genes DE compared to untreated control were shared among hypoxia, hypoxia/reoxygenation, and *t*-BOOH treatment, while in human and sheep cells, the overlap was 2.4% and 9.2%, respectively (Figure 6A). In both seal and human cells, hypoxia exposure elicited the greatest condition-specific change in gene expression (seal: 88.9% of all genes DE vs. control; human: 69.8%). In contrast, hypoxia had the smallest condition-specific impact on gene expression in sheep cells (9.1% of all genes DE vs. control), where the response was dominated by hypoxia/reoxygenation (37.6%) and *t*-BOOH treatment (27%). Sheep cells also displayed considerable overlap in gene sets across treatments (5.5-8.4% of all DE genes were shared between any two treatments), whereas any two-way comparison shared less than 3% of all DE genes in seal and human cells (Figure 6A). Together, these data suggest a highly dynamic, condition-specific transcriptional response in seal cells that is attenuated in both human and sheep cells.

We also considered whether seal, human, and sheep cells shared transcriptional responses to hypoxia, hypoxia/reoxygenation, or *t*-BOOH treatment. This analysis was limited to genes that were annotated in the genomes of all three species to control for genome and annotation quality across species ($n=11,830$ genes). Overall, ~50% of seal genes DE in hypoxia compared to control were shared with the human and/or sheep response to hypoxia exposure, while human and sheep cells each shared 38% and 57% of DE genes with the remaining two species. Larger species differences in gene expression occurred for hypoxia/reoxygenation and *t*-BOOH treatment, though in both cases seal cells shared ~40% of DE genes with another species in the same condition. Conversely, sheep cells shared only 16% of DE genes with seal and/or human cells during reoxygenation, and human cells shared only 19% of DE genes with seal and/or sheep during *t*-BOOH treatment (Figure 6B). Taken together, these results show that species specificity in gene expression patterns is greatest for human and sheep cells in pro-oxidative stress conditions.

3.4.5 *t*-BOOH treatment modulates expression of oxygen-sensing pathway genes in seal endothelial cells

We next evaluated whether genes shared among species for each condition displayed similar expression patterns. We performed hierarchical clustering on the shared gene sets identified in Figure 6B (hypoxia, $n=47$; hypoxia/reoxygenation, $n=9$; *t*-BOOH, $n=28$). Expression patterns of shared genes generally concurred across species (Figure 7) but were most different in sheep cells compared with seal and human cells during hypoxia exposure (Figure 7A). In both reoxygenation and *t*-BOOH treatment, however, seal cell gene expression clustered separately from human and sheep cell expression patterns (Figure 7B, Figure 7C). The only two DE genes shared among all three species and conditions were DDIT4 (REDD1; DNA damage-inducible transcript) and VEGFA (vascular endothelial growth factor A), which were always upregulated relative to control.

We also performed gene set enrichment analysis (GSEA) and KEGG pathway enrichments for each DE gene set from Figure 5. GSEA in seal cells identified downregulation of non-integrin membrane-ECM interactions in hypoxia compared to control, while *t*-BOOH treatment downregulated steroid metabolism compared to control. Interestingly, hypoxia/reoxygenation downregulated respiratory electron transport and ATP synthesis relative to hypoxia in both seal and sheep cells (Figure 8, Figure S5). Mitochondrial fatty acid beta-oxidation was downregulated in *t*-BOOH treatment compared to hypoxia in seal and human cells (Figure 8, Figure S4). In seal cells, this downregulation was accompanied by decreases in branched-chain amino acid catabolism and ATP formation by chemiosmotic coupling, suggesting an overall dampening of mitochondrial respiration in seal cells in *t*-BOOH treatment compared to hypoxia exposure (Figure 8). Branched-chain amino acids also activate mTOR, leading to mitochondrial dysfunction and ROS production via NF- κ B activation [211]; downregulation of this pathway in seal cells may spare cells from oxidative injury during *t*-BOOH treatment. *t*-BOOH treatment also downregulated several sulfur-related pathways compared to hypoxia in seal cells, including sulfur amino acid metabolism, degradation of cysteine and homocysteine, and selenocysteine synthesis (Figure 8, Table 3). Among the 54 hit genes in all three pathways, only TXNRD1, SECISBP2, and EPRS1 were upregulated by *t*-BOOH treatment compared to hypoxia. All 13 genes within the enrichment for the degradation of cysteine and homocysteine were downregulated by *t*-BOOH treatment compared to hypoxia. These genes were involved in the methionine salvage pathway (MTAP, MRI1, ENOPH1, APIP, SUOX, MTRR), hydrogen sulfide metabolism (ETHE1, MPST), amino acid metabolism (GOT1, GOT2, ADO), redox balance (TXN2), and formation of mitochondrial iron-sulfur complexes (TST). Interestingly, 7 of these 13 genes were overexpressed (DE) in hypoxia relative to control, suggesting that the downregulated enrichment stems from both the upregulation of this pathway during hypoxia exposure and downregulation during *t*-BOOH treatment. We also observed modifications to cysteine metabolism in human and sheep cells, though the changes in these species occurred with hypoxia/reoxygenation compared to hypoxia (Figure S4, Figure S5). Cysteine is one of the three amino acids which constitute the antioxidant glutathione, and glutathione interacts with hydropersulfides to inhibit lipid peroxidation [212]. Therefore, seal-specific changes in sulfur amino acid metabolism with hypoxia exposure and *t*-BOOH treatment support a functional resistance to *t*-BOOH-induced lipid peroxidation in these cells.

Relatively few KEGG pathways were enriched in seal cells in any comparison (Figure 9, Figure S6). When comparing *t*-BOOH treatment to untreated control, we identified a seal-specific enrichment of the HIF-1 signaling pathway (Figure 9, top) (Table 1). Of the 12 HIF pathway genes supporting this enrichment, two genes (VEGFA, HMOX1) were present in the shared response to *t*-BOOH treatment across all species (Figure 7C), while two genes (HK2, SLC2A1) were shared with one other species. Eight HIF signaling pathway genes were specific to seal cells. Among these were two prolyl hydroxylases (EGLN1, EGLN3; both upregulated) which mediate hydroxylation of HIF1A and HIF2A, respectively [213]. CDKN1A and CDKN1B, which induce cell cycle arrest and may interact with the HIF ubiquitin ligase pVHL [214], were also upregulated in seal cells only. These expression patterns suggest that despite high VEGFA expression levels, the HIF response in seal cells is tightly regulated and potentially attenuated in *t*-BOOH treatment.

3.4.6 *t*-BOOH treatment, but not hypoxia/reoxygenation, upregulates expression of genes involved in ferroptosis in seal endothelial cells

Oxidative stress associated with hypoxia/reoxygenation events results from oxidant generation upon the return of oxygen to the depleted system. Whether such an oxidant “burst” occurs in the marine mammal endothelium during diving remains unaddressed. Therefore, we directly compared gene expression profiles between hypoxia/reoxygenation and *t*-BOOH (a chemical oxidant) in seal endothelial cells to assess whether chemically-induced oxidant stress recapitulates a more physiological exposure. Here, we examined genes DE in *t*-BOOH treatment relative to hypoxia/reoxygenation (Figure 5, last violin plot on all graphs).

t-BOOH treatment enriched expression of genes in the ferroptosis pathway in seal cells compared to hypoxia/reoxygenation (Figure 9, bottom) but not in any other species or pairwise comparison (Figure 9, Figure S6). Given the lack of lipid peroxidation observed in seal cells at an even higher *t*-BOOH dose than was utilized in this experiment (Figure 2), we queried the genes in this enrichment to identify the functional implications of DE genes in this pathway. The enrichment comprised six genes; three of these are involved in GSH metabolism (glutamate-cysteine ligase catalytic subunit, GCLC; glutamate-cysteine ligase modifier subunit, GCLM; sodium-independent cystine-glutamate antiporter, SLC7A11/XCT), one is involved in lipid synthesis (acyl-CoA synthetase long-chain family member 3, ACSL3), and two are involved in iron homeostasis (ferroportin 1, SLC40A1; heme oxygenase 1, HMOX1). All six genes were upregulated in *t*-BOOH treatment compared to hypoxia/reoxygenation in seal cells, but only ACSL3 and SLC40A1 displayed entirely seal-specific expression patterns across treatments (Figure 10, Table 2). ACSL3, which may contribute to lipid peroxidation resistance via the generation of relatively peroxidation-insensitive MUFAs [215–218], was expressed at high levels in control conditions, downregulated in seal cells during hypoxia exposure compared to control, and upregulated in *t*-BOOH treatment compared with both hypoxia and hypoxia/reoxygenation. ACSL3 was not DE in any comparison for either human or sheep cells. Seal cells upregulated expression of SLC40A1, the only known exporter of cellular iron, ~4-fold in *t*-BOOH treatment compared to all other conditions. SLC40A1 was never DE in sheep cells, and all instances of DE in human cells were downregulated. These results are consistent with our functional data indicating that seal cells do not exhibit exceptionally high expression, abundance, or activity of lipid hydroperoxide GSH peroxidases (Figure 3, Figure 4) and suggest that seal endothelial cells may avoid lipid peroxidation and ferroptosis by regulating cellular iron levels through GSH-independent means [205].

3.4.7 *t*-BOOH treatment modifies iron handling in seal endothelial cells relative to hypoxia/reoxygenation exposure

We used a live cell permeable dye, FerroOrange, to investigate changes in cellular iron during hypoxia/reoxygenation and *t*-BOOH treatment. FerroOrange fluoresces upon interaction with ferric iron (Fe^{2+}). Cells were exposed to either hypoxia/reoxygenation or *t*-BOOH in complete growth medium containing 10% FBS, which supplies iron via transferrin. After exposure, cells were washed to remove the growth medium and incubated with FerroOrange for 30 minutes before fluorescence detection. One-way ANOVA identified significant differences among treatment groups when considering different treatments within each species and the same treatment across species ($F_{8,196}=21.72$, $p<0.0001$) (Figure 11). *t*-BOOH treatment significantly increased FerroOrange signal compared to control in all three species (seal: 73% increase, $t=4.842$, $p<0.0001$; human: 100% increase, $t=5.778$, $p<0.0001$; sheep: 78% increase, $t=4.260$, $p=0.0005$);

the magnitude of increase did not vary across species. Hypoxia/reoxygenation decreased FerroOrange signal in human cells (~0.6-fold, $t=3.679$, $p=0.005$ vs. control). Seal and sheep cells responded to hypoxia/reoxygenation with different trends. Seal cells display a slight decrease in FerroOrange signal with hypoxia/reoxygenation, while sheep cells tended toward an increase, though neither change was statistically significant with respect to each species' control. However, FerroOrange signal during hypoxia/reoxygenation varied among species, with the highest signal occurring in sheep cells (~40% increase over control; vs. seal: $t=4.639$, $p<0.0001$; vs. human: $t=5.852$, $p<0.0001$). Despite differences in lipid peroxidation sensitivity between seal and human cells (Figure 2), we observed similar FerroOrange signal patterns between the two species across all treatments. However, the experimental design did not allow for distinguishing between intracellular and extracellular iron content, which complicates the interpretation of the observed signal. These data demonstrate that changes in cellular iron do occur in seal cells during *t*-BOOH treatment with respect to hypoxia/reoxygenation, though the exact partitioning of these changes was not identified. Together with the transcriptomic data indicating enrichment of iron and sulfur handling pathways in seal cells in *t*-BOOH treatment compared with hypoxia and hypoxia/reoxygenation, these data suggest that seal endothelial cells avoid oxidative damage by modifying iron metabolism.

3.5 Discussion

Elephant seals undergo repeated bouts of prolonged hypoxemia and brief reoxygenation secondary to their extended diving behavior. Non-hypoxia adapted species suffer oxidative tissue injuries during similar fluctuations in blood oxygen tension, including during myocardial infarction and ischemic stroke in humans. The cellular mechanisms driving resistance to hypoxia/reoxygenation injury in seals remain poorly studied due to limitations on *in vivo* experiments. Here, we utilized a proliferative endothelial cell culture system developed from seal placental arteries to compare seal cell responses to an *ex vivo* hypoxia/reoxygenation exposure and a chemical oxidant treatment. Our results show resistance to lipid peroxidation and cell death in seal cells despite treatment with high concentrations of organic peroxide. Moreover, seal cells did not display evidence of altered expression or activity of enzymes in the GSH pathway in response to *t*-BOOH treatment. In contrast, *t*-BOOH treatment downregulated the metabolism of cysteine-containing amino acids and upregulated expression of the cellular iron exporter SLC40A1 (ferroportin 1) in seal cells only, suggesting that intrinsic resistance to cellular lipid peroxidation is mediated by sulfur and iron handling.

3.5.1 Seal endothelial cells display an innate resistance to oxidative damage

Marine mammals possess robust antioxidant defenses which likely mitigate oxidative injury *in vivo* [219,220]. A large body of work in northern elephant seals suggests that GSH-related antioxidant enzymes protect tissues against oxidative injury associated with apnea-induced hypoxemia, prolonged fasting, and reproduction [93,110,202,221]. We found that seal endothelial cells maintained viability and resisted cell death and lipid peroxidation even when challenged with high doses of organic peroxide, while the same doses decreased viability and increased cytotoxicity and lipid peroxidation in comparable human and sheep cells. We considered whether the observed seal cell resistance to *t*-BOOH treatment might be driven by increased expression or activity of the phospholipid hydroperoxide GSH peroxidases GPX4 and PRDX6. Interestingly, we did not find evidence for GPX4- or PRDX6-mediated protection against oxidative injury in seal endothelial

cells, suggesting that reduction of phospholipid hydroperoxides may not be the primary line of defense in seal cells' resistance to oxidative injury.

3.5.2 Seal endothelial cells mount condition-specific responses to oxidative stress

Hypoxia, hypoxia/reoxygenation, and *t*-BOOH treatment all represent unique stressors for endothelial cells. However, the degree to which cells mount condition-specific vs. generalized stress responses to these conditions remains unknown. In both seal and human cells, hypoxia exposure elicited the largest condition-specific change in overall gene expression, with ~70-90% of genes DE vs. control under any condition occurring during hypoxia exposure. In seal cells, only 5% of all genes DE vs. control were shared among any subset of conditions, compared to 13% in human and 26% in sheep cells. These results suggest that seal cells mount condition-specific transcriptional responses to hypoxia, hypoxia/reoxygenation, and *t*-BOOH and that there is relatively little overlap between the gene sets affected by each treatment. These findings agree with our prior work indicating that the transcriptional response to hypoxia exposure in seal cells varies greatly over time [202] and suggest that seal cells' resistance to oxidative injury may rely on dynamic, regulated changes in transcription.

3.5.3 *t*-BOOH treatment downregulates metabolism of sulfur-containing amino acids in seal endothelial cells

Sulfur-containing amino acids impact the cellular redox state. Methionine, cysteine, homocysteine, and taurine contribute to cellular sulfur pools and GSH biosynthesis. Methionine possesses a hydrophobic *S*-methyl thioether side chain which interacts with phospholipid membranes. Methionine's side chain can be oxidized to yield methionine sulfoxide; selective oxidation of methionine residues may protect enzyme active sites from alteration by reactive oxidants [222–224]. At the organismal level and across taxa, however, methionine restriction extends lifespan, though this effect may depend on pentose phosphate pathway activity [225–227]. Seal endothelial cells downregulated expression of several sulfur-containing amino acid pathways during *t*-BOOH treatment compared to hypoxia exposure, including key genes in the methionine synthesis, salvage, and metabolism pathways (MTAP, MRI1, ENOPH1, APIP, SUOX, MTRR), suggesting tight regulation of sulfur-containing amino acid metabolism during *t*-BOOH exposure. ADO acts as an iron-dependent enzymatic oxygen sensor in human cells [228]; its downregulation in seal cells during *t*-BOOH compared with hypoxia may reflect a similar role in seal cells. Downregulation of GOT1 and GOT2 expression during *t*-BOOH exposure may maintain cellular α -ketoglutarate levels. In addition to its rate-limiting role in the TCA cycle, α -ketoglutarate is also a required substrate for the HIF prolyl hydroxylases, and its presence may support HIF degradation [213]. The thioredoxin antioxidant system contributes to ferroptosis resistance via the reduction of peroxiredoxins [229,230]. Simultaneous downregulation of the mitochondrial thioredoxin TXN2 and upregulation of the cytosolic thioredoxin reductase TXNRD1 suggest that *t*-BOOH treatment differentially regulates sulfur-mediated antioxidant activity compared to hypoxia exposure in seal cells; such fine-tuned regulation may protect against lipid peroxidation in these cells.

Iron-sulfur complexes form critical cofactors for the mitochondrial respiratory complexes and are stabilized by low oxygen conditions [231,232]. Downregulation of mitochondrial thiosulfate sulfurtransferase (TST) gene expression in seal cells during *t*-BOOH treatment compared to hypoxia, along with decreased expression of genes involved in mitochondrial fatty acid beta-oxidation and formation of ATP by chemiosmotic coupling, suggest that seal cells may

dampen mitochondrial respiration during *t*-BOOH exposure to avoid additional oxidant generation. Interestingly, hyperoxia destabilizes mitochondrial Fe-S cluster-containing protein complexes in human cells [233]; *t*-BOOH treatment may similarly impact Fe-S cluster stability, particularly with respect to hypoxia.

Hydrogen sulfide (H₂S) signaling also impacts mitochondrial and endothelial function. Within physiological levels, H₂S acts as an endothelial-derived relaxing factor [234], though supraphysiological H₂S levels inhibit mitochondrial complex IV activity and limit ATP production by OXPHOS [235]. Mercaptopyruvate sulfurtransferase (MPST) produces H₂S in endothelial cells [236], and MPST knockdown limits VEGF-induced angiogenesis *in vitro* [237]. Seal cells downregulated MPST expression in *t*-BOOH treatment compared with hypoxia exposure, which may modulate H₂S production, limit proliferation (despite high VEGFA expression) and maintain vasoconstriction *in vivo*. The iron-containing persulfide dioxygenase ETHE1 uses molecular O₂ to oxidize GSSH (the product of GSH and H₂S) to GSH and sulfite ion in the mitochondrial matrix [238]. Downregulation of ETHE1 in seal cells during *t*-BOOH treatment may limit H₂S catabolism, suppressing cellular respiration. Together, these data suggest tight regulation of the iron-sulfur-GSH pathways in seal endothelial cells during *t*-BOOH treatment, particularly when compared with hypoxia exposure.

3.5.4 HIF-1 signaling is tightly regulated in seal cells during *t*-BOOH treatment

The HIF-1 signaling pathway mediates the canonical cellular response to hypoxia. HIF-1 is a heterodimeric transcription factor composed of constitutively expressed α and β subunits, though the α subunit is continuously subject to proteasomal degradation under normoxia. Hypoxia inactivates the prolyl hydroxylases responsible for marking HIF-1 α for degradation, and the stabilized α subunit dimerizes with the β subunit to form the functional transcription factor [117,176,213]. Our previous work identified HIF-1 α as highly responsive to hypoxia but decoupled from canonical angiogenic signaling in seal endothelial cells [202]. The seal cell transcriptional response to *t*-BOOH, but not hypoxia or hypoxia/reoxygenation was enriched for the HIF-1 signaling pathway when compared to untreated control. Seal, human, and sheep cells all upregulated VEGFA and HMOX1 expression in response to *t*-BOOH treatment; both VEGFA and HMOX1 are HIF-1-responsive genes [113,239]. However, the HIF-1 signaling enrichment in seal cells comprised upregulation of HIF prolyl hydroxylases (EGLN1, EGLN3) [213,240] and cell cycle arrest proteins (CDKN1A, CDKN1B) [241], suggesting that *t*-BOOH induced negative feedback of the HIF-1 signaling pathway and proliferation in seal cells despite high VEGFA expression. These data are consistent with our observations that *t*-BOOH reduced viability but did not affect cytotoxicity in seal endothelial cells, and our prior work demonstrating that seal cells decouple HIF-1 signaling from angiogenesis during hypoxia exposure [202].

3.5.5 *t*-BOOH treatment modulates expression of ferroptosis-related genes in seal cells

Ferroptosis is an iron-dependent form of regulated cell death characterized by increases in lipid hydroperoxides. Iron accumulation during hypoxia increases lipid peroxidation upon interaction of Fe²⁺ with molecular O₂ during reoxygenation. We found that *t*-BOOH treatment modified the expression of genes in the ferroptosis pathway in seal cells, specifically when compared to expression levels after hypoxia/reoxygenation. Downregulation of ACSL3 expression in seal cells after *t*-BOOH treatment may sensitize cells to lipid peroxidation as ACSL3 typically generates peroxidation-insensitive MUFAs. However, high expression of ACSL3 in seal cells

under control conditions may yield constitutive protection to cell membranes. Notably, ACSL3 was never DE in human or sheep cells.

Seal endothelial cells upregulate expression of genes in the GSH pathway during a longer-but-milder hypoxia exposure, leading to hypoxic accumulation of GSH in seal but not human cells [202]. Here, we did not observe hypoxia-related changes in GSH metabolism in seal cell gene expression, though *t*-BOOH treatment upregulated expression of the cysteine transporter SLC7A11 and the GSH synthesis enzymes GCLC and GCLM in seal cells. However, this pattern was at least partially recapitulated in human and sheep cells, suggesting that *t*-BOOH-mediated increases in GSH gene expression are not seal-specific.

Heme oxygenase 1 (HMOX1) plays a dual role in ferroptosis via heme breakdown and Fe²⁺ liberation [242,243] and was the ferroptosis pathway gene most upregulated by *t*-BOOH treatment across all three species. Notably, HMOX1 was unchanged by hypoxia or hypoxia/reoxygenation in seal and human cells but responded to hypoxia/reoxygenation in sheep cells (though considerably less than during *t*-BOOH treatment). Marine mammals, including the northern elephant seal, display high carboxyhemoglobin levels and HMOX1 expression [244,245]. In this experiment, we were unable to distinguish between the pro- and anti-ferroptotic functions of HMOX1 during *t*-BOOH exposure. However, *t*-BOOH treatment upregulated the expression of SLC40A1 (ferroportin 1), the only known cellular iron exporter, in a seal-specific pattern. When paired with the observed increase in HMOX1 under the same conditions and a demonstrated resistance to *t*-BOOH-induced lipid peroxidation, this increase suggests that SLC40A1 may protect seal endothelial cells against ferroptosis by exporting iron. Hence, we measured iron levels in response to hypoxia/reoxygenation and *t*-BOOH treatment. We detected an increase in iron in *t*-BOOH treatment compared to hypoxia/reoxygenation in seal and human cells; the increase was smaller in seal than in human cells. Overall, constraints on signal detection prevented the determination of species-specific iron management strategies in cells, as we were unable to distinguish between intracellular (imported) and extracellular (exported) iron in the assay performed. The use of cell compartment-specific, highly sensitive iron assays coupled with gene manipulation strategies (e.g., SLC40A1 knockdown or knockout) could help shed light on iron handling in seal cells exposed to oxidant stress.

3.6 Conclusions

In summary, we found that seal but not human or sheep cells resist lipid peroxidation and cell death even at high doses of a chemical oxidant. Tightly regulated iron transport and modified metabolism of sulfur-containing amino acids likely drive this resistance. Interestingly, we did not find strong evidence for a direct role of GSH peroxidases in lipid peroxidation resistance in seal endothelial cells, despite a large body of work (including our own) that suggests a critical role for GSH in marine mammals' protection against oxidative injury. Additionally, hypoxia/reoxygenation did not elicit any major seal-specific gene expression signature relative to control, suggesting that this exposure does not constitute a considerable stressor for seal cells. Seal endothelial cells mounted unique and distinct transcriptional responses to hypoxia, hypoxia/reoxygenation, and *t*-BOOH treatment, indicating that tolerance to these stressors is not a "one size fits all" strategy.

3.7 Acknowledgments

We thank Alexander Li and Diamond Luong for their contributions to the experimental portions of this chapter. Drs. Diana Moreno-Santillán and Juan Manuel (Manny) Vazquez were instrumental in supporting the bioinformatics portions of this work. We thank Dr. Fung Lam for his assistance in obtaining human placental tissue. We thank Drs. Daniel Costa, Patrick Robinson, and Rachel Holser (UC Santa Cruz) for collecting and sharing elephant seal placental tissue. We thank Dr. Bret McNabb (UC Davis) for assistance in procuring sheep placental tissue.

3.8 Tables

Table 1. HIF signaling pathway genes DE in seal cells in *t*-BOOH treatment compared to control. Alternate gene symbols for select genes are listed in parentheses in the “Gene name” column.

Gene symbol	Gene name	Seal fold change	Shared by
<i>VEGFA</i>	vascular endothelial growth factor A	26.05	human, sheep
<i>HMOX1</i>	heme oxygenase 1	16.21	human, sheep
<i>HK1</i>	hexokinase 1	1.23	--
<i>HK2</i>	hexokinase 2	8.62	human
<i>CDKN1A</i>	cyclin dependent kinase inhibitor 1A	2.75	--
<i>CDKN1B</i>	cyclin dependent kinase inhibitor 1B	1.30	--
<i>EGLN1</i>	hypoxia-inducible factor prolyl hydroxylase 2	1.44	--
<i>EGLN3</i>	hypoxia-inducible factor prolyl hydroxylase 3	2.24	--
<i>LTBR</i>	lymphotoxin beta receptor (TNFRSF3)	1.24	--
<i>CUL2</i>	cullin 2	0.80	--
<i>SLC2A1</i>	solute carrier family 2 member 1 (GLUT1)	1.98	sheep
<i>SERPINE1</i>	endothelial plasminogen activator inhibitor	0.73	--

Table 2. Fold change in ferroptosis pathway genes present in the seal cell enrichment comparing *t*-BOOH treatment and hypoxia/reoxygenation. Dash denotes genes that are not DE in the given comparison. Values < 1 indicate downregulation. ctl, control; hx, hypoxia; rx, reoxygenation; tb, *t*-BOOH.

		<i>GCLC</i>	<i>GCLM</i>	<i>SLC7A11</i>	<i>ACSL3</i>	<i>SLC40A1</i>	<i>HMOX1</i>
SEAL	ctl_hx	-	-	-	0.55	-	-
	ctl_rx	-	-	-	-	-	-
	ctl_tb	1.94	-	3.35	-	3.96	16.21
	hx_rx	-	-	-	-	-	-
	hx_tb	1.98	1.52	-	1.44	3.80	12.47
	rx_tb	2.02	2.27	2.85	1.22	4.21	16.24
HUMAN	ctl_hx	-	-	0.67	-	-	-
	ctl_rx	-	0.76	0.69	-	-	-
	ctl_tb	-	1.15	-	-	0.29	2.84
	hx_rx	-	0.78	-	-	0.40	-
	hx_tb	-	1.18	1.30	-	0.25	3.63
	rx_tb	-	1.51	-	-	-	3.39
SHEEP	ctl_hx	-	-	-	-	-	-
	ctl_rx	1.79	-	-	-	-	1.20
	ctl_tb	-	2.44	9.76	-	-	15.44
	hx_rx	-	-	-	-	-	-
	hx_tb	-	2.00	14.05	-	-	12.85
	rx_tb	-	2.14	15.60	-	-	12.73

Table 3. Seal genes represented in KEGG enrichment for degradation of cysteine and homocysteine in *t*-BOOH vs. hypoxia.

Gene symbol	Gene name	Fold change (<i>t</i>-BOOH vs. hypoxia)
<i>MTRR</i>	methionine synthase reductase	0.698
<i>GOT2</i>	glutamic-oxaloacetic transaminase 2	0.665
<i>ADO</i>	2-aminoethanoethiol dioxygenase	0.637
<i>TXN2</i>	thioredoxin 2	0.625
<i>MTAP</i>	methylthioadenosine phosphorylase	0.605
<i>GOT1</i>	glutamic-oxaloacetic transaminase 1	0.603
<i>TST</i>	thiosulfate sulfurtransferase	0.580
<i>MRI1</i>	methylribose-1-phosphate isomerase 1	0.562
<i>SUOX</i>	sulfite oxidase	0.555
<i>ETHE1</i>	mitochondrial persulfide dioxygenase	0.513
<i>ENOPH1</i>	enolase-phosphatase 1	0.460
<i>MPST</i>	mercaptosulfurtransferase	0.418
<i>APIP</i>	APAF1 interacting protein	0.373

3.9 Figures

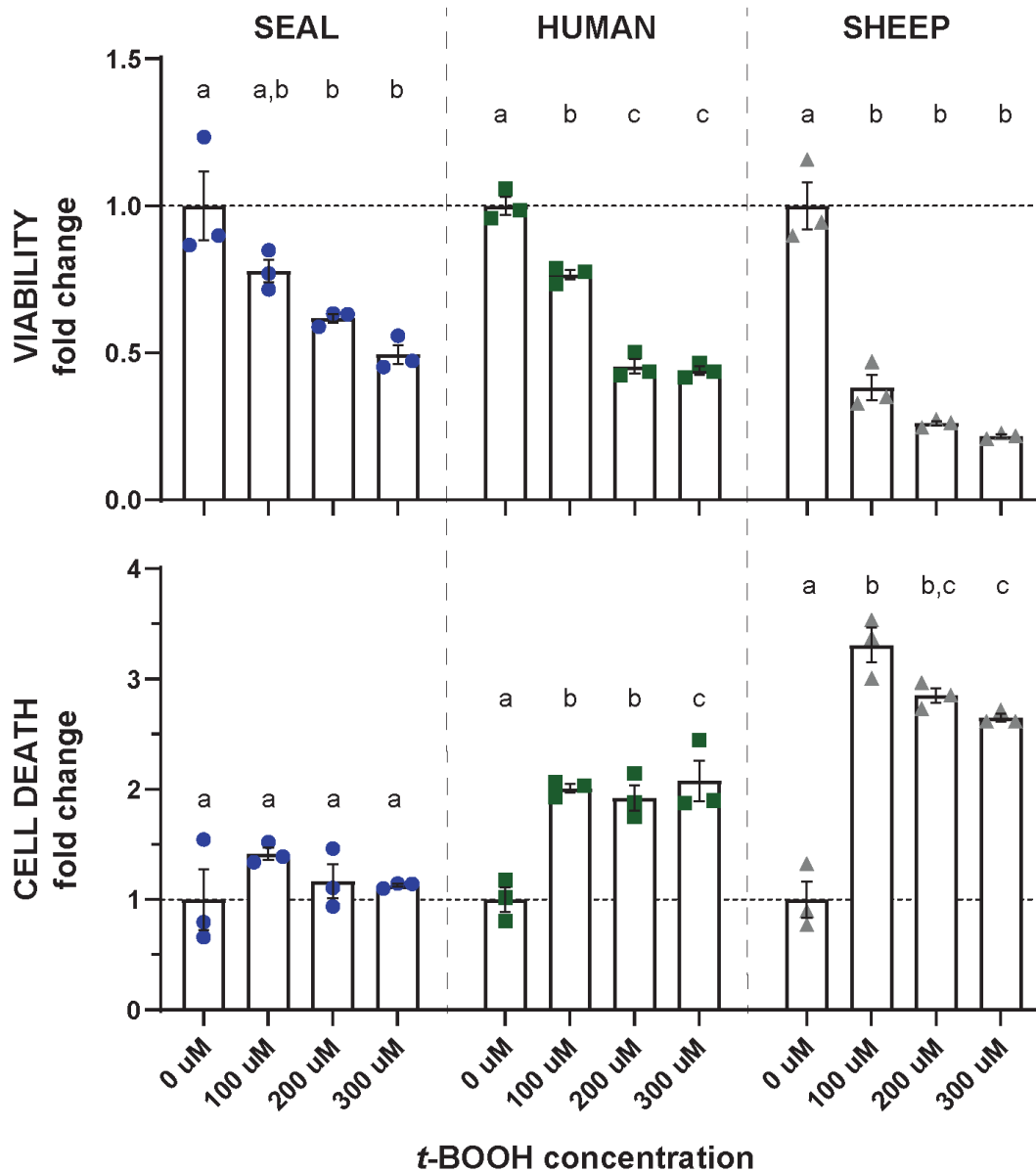


Figure 1. Seal cells resist cell death during *t*-BOOH exposure. Seal, human, and sheep endothelial cells were exposed to 0, 100, 200, or 300 µM *t*-BOOH for 2 h. Viability (top) and cell death (bottom) were measured in the same wells. Data are presented as fold change from each species' control values. Mean ± s.e.m. and individual points are plotted. *n*=3 replicates per condition. Blue, seal; green, human; gray, sheep. Different letters denote statistical significance between means (within each species). Caspase activity data from the same experiment are available in Figure S2.

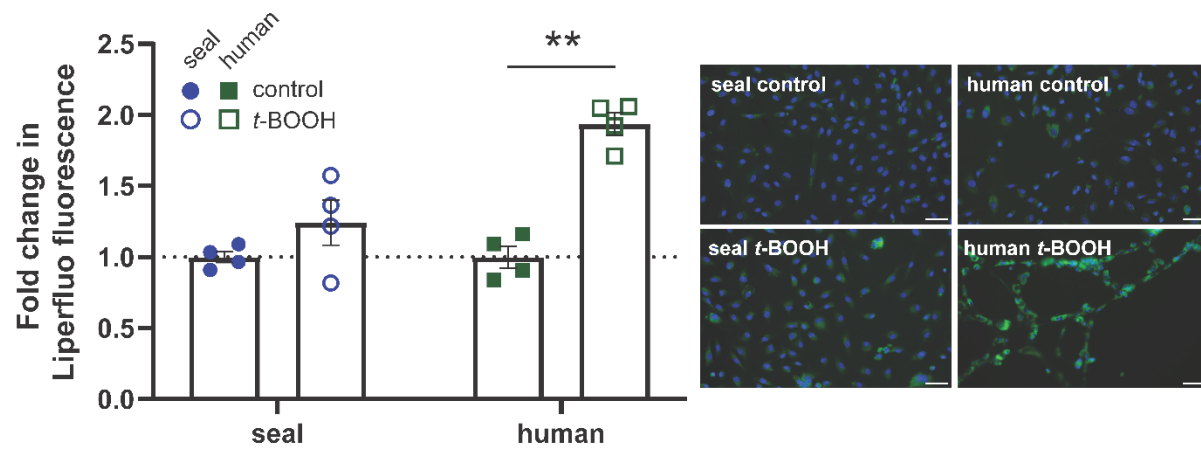


Figure 2. Seal endothelial cells resist lipid peroxidation during *t*-BOOH exposure. Fold change in Liperfluo fluorescence after treatment with 300 μ M *t*-BOOH for 1.5 h. Data are mean $\pm$ s.e.m from 5 fields each for $n=4$ replicates per condition. Blue, seal; green, human. Solid points, control; open points, *t*-BOOH. ** $p<0.001$. Representative images are included to the right of the plot. Liperfluo, green; Hoechst (nuclear), blue. Scale bar is 50 μ m.

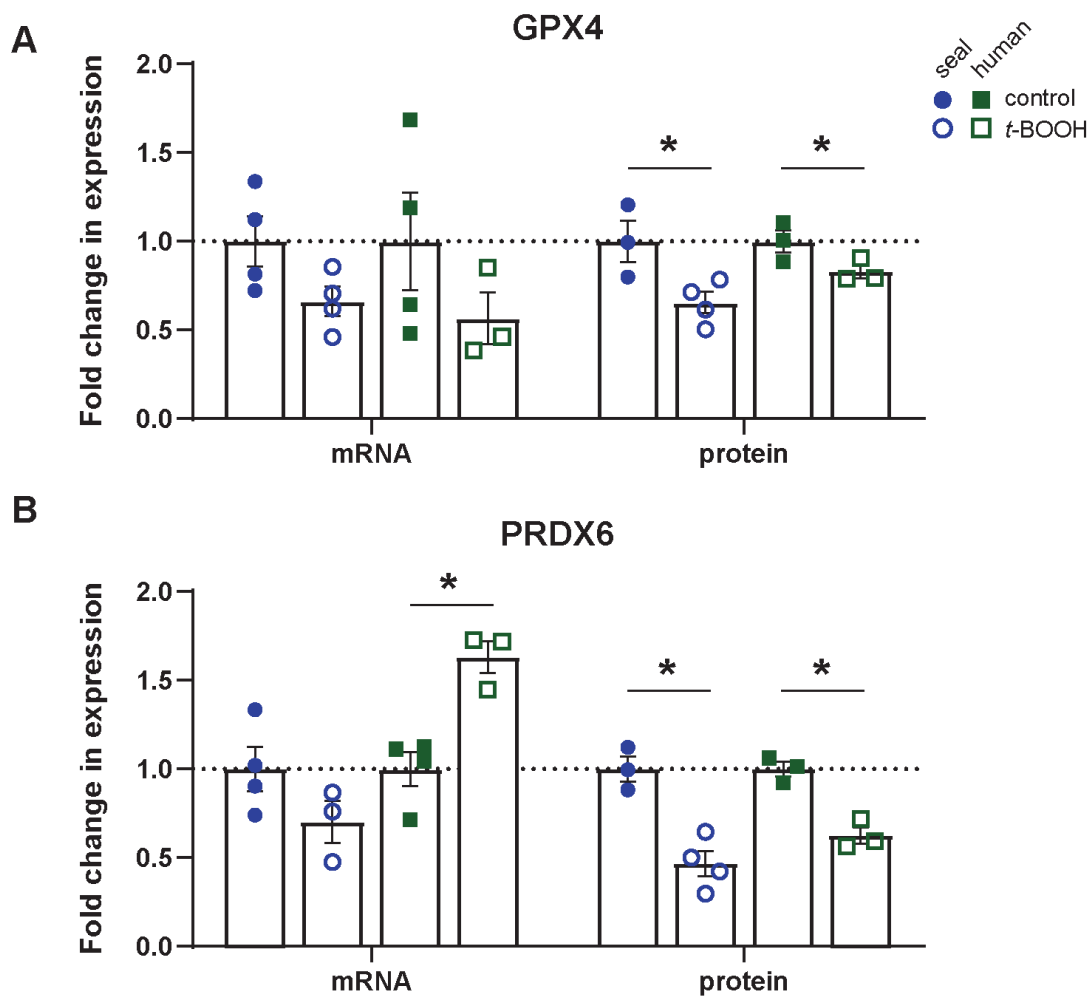


Figure 3. *t*-BOOH treatment decreases lipid hydroperoxide repair enzyme protein abundance. Fold change in GPX4 (A) and PRDX6 (B) mRNA and protein expression after treatment with 100 μ M *t*-BOOH for 2 h. Data are mean $\pm$ s.e.m. with individual points plotted. $n=3-4$ replicates per condition. Blue, seal; green, human. Solid points, control; open points, *t*-BOOH. * $p<0.05$.

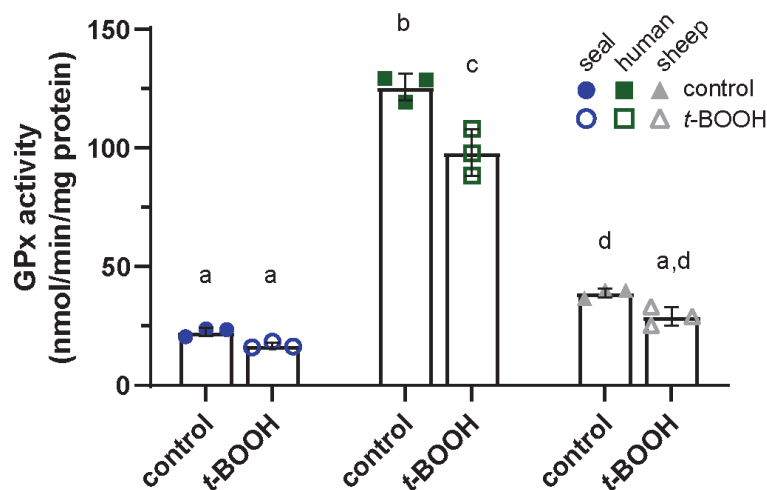


Figure 4. *t*-BOOH treatment does not affect GPx activity in seal endothelial cells. Total GPx activity after treatment with 100 μ M *t*-BOOH for 2 h. Data are mean $\pm$ s.e.m. with individual points plotted. $n=3$ replicates per condition. Blue, seal; green, human; gray, sheep. Solid points, control; open points, *t*-BOOH. Different letters denote statistical significance between means.

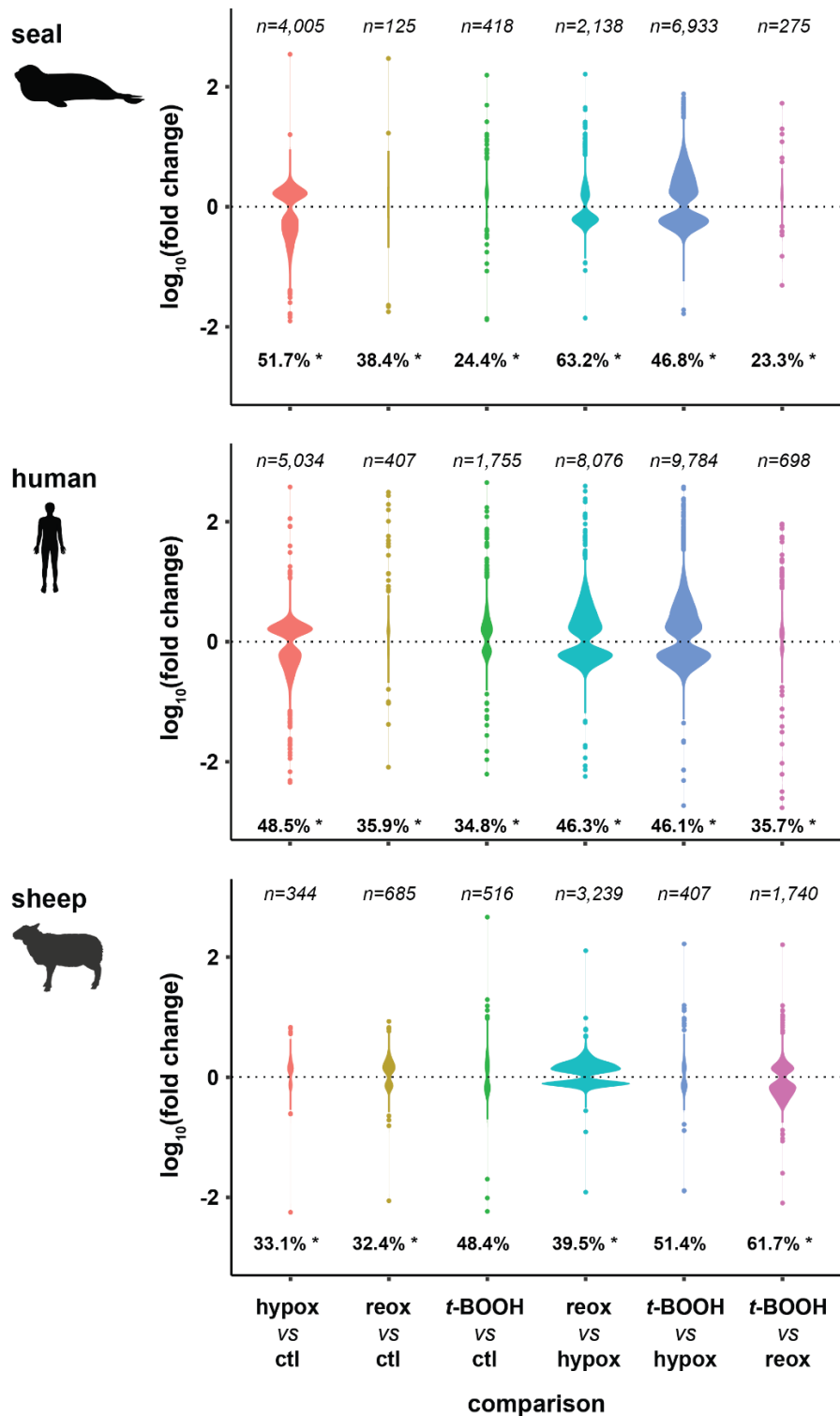
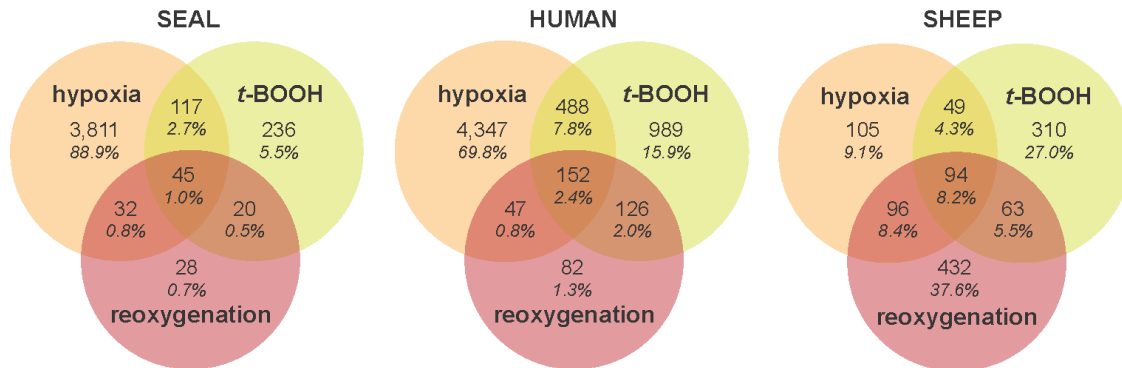


Figure 5. Summary changes in global gene expression patterns in pairwise comparisons between treatments. Italics above each plot indicate the total number of DE genes in the given comparison. Bold percentages below each plot indicate percentage of DE genes downregulated in the given comparison (e.g., 51.7% of seal genes DE in hypoxia compared to control are expressed at a lower level during hypoxia exposure than in untreated control). * $p < 0.05$ compared to 50/50.

A



B

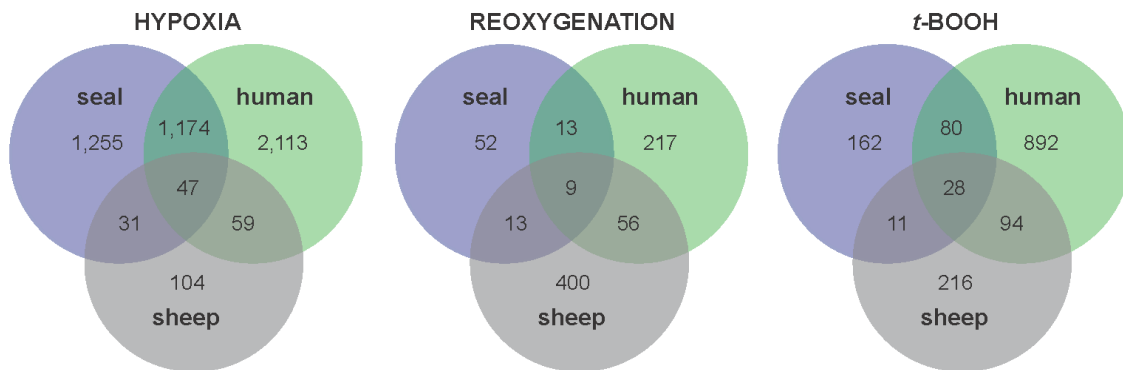


Figure 6. Hypoxia exposure dominates gene expression changes in seal and human but not sheep endothelial cells. (A) Total number of genes DE relative to untreated control in each condition, by species. Counts include all genes identified in each species, irrespective of positive identification of gene name in the annotation, or whether the gene was present in the annotation for all species. Italic percentages represent the percent of all DE genes contained within a given condition (sum=100% for each species). (B) Number of genes DE relative to untreated control in each species, by condition. Counts are constrained to those genes identified as common among the annotations for all three species ($n=11,830$ total genes).

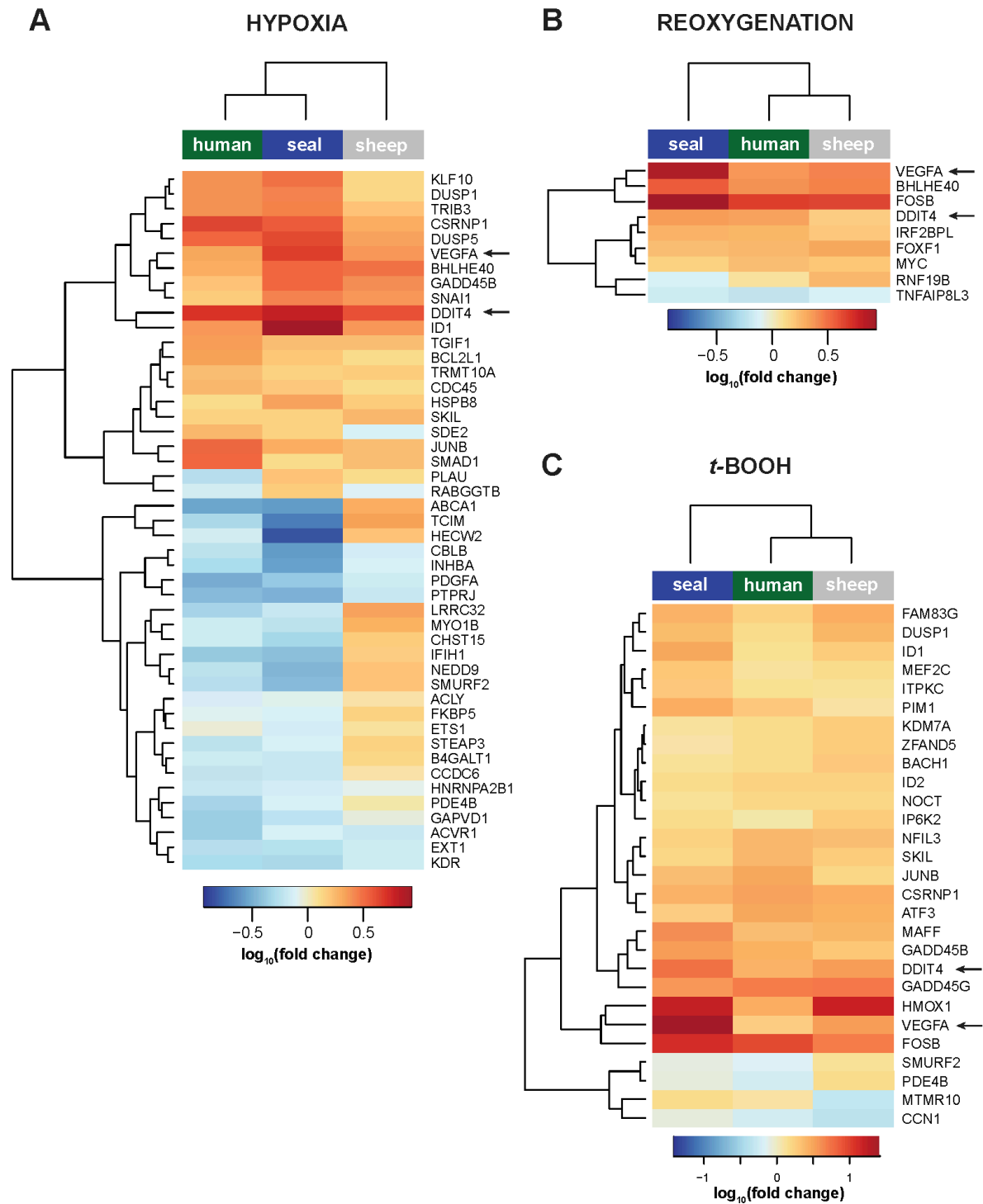


Figure 7. Genes shared between species for each condition display similar expression patterns compared to control. $\log_{10}(\text{fold change})$ in gene expression for shared gene sets for (A) hypoxia, (B) hypoxia/reoxygenation, and (C) *t*-BOOH treatment. Gene sets are drawn from center segments of Venn diagrams in Figure 6B. Arrows indicate genes present in all three sets.

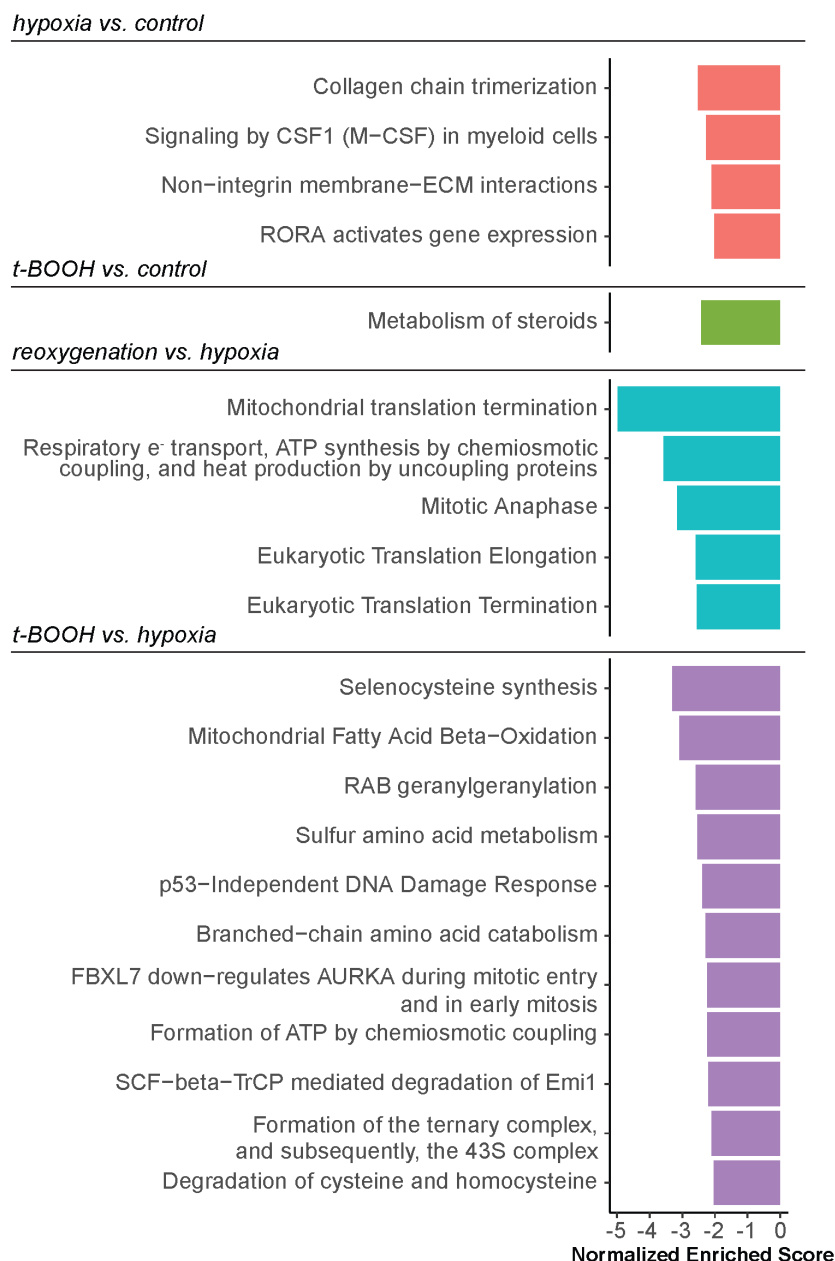


Figure 8. *t*-BOOH treatment modifies cysteine-containing amino acid metabolism in seal cells. GSEA results for seal DE genes. No pathways were enriched for reoxygenation vs. control or *t*-BOOH vs. reoxygenation. Normalized enriched score < 0 indicates net downregulation of the pathway. FDR<0.001 for all pathways. Human and sheep enrichments for the same comparisons are available in Figure S4 and Figure S5.

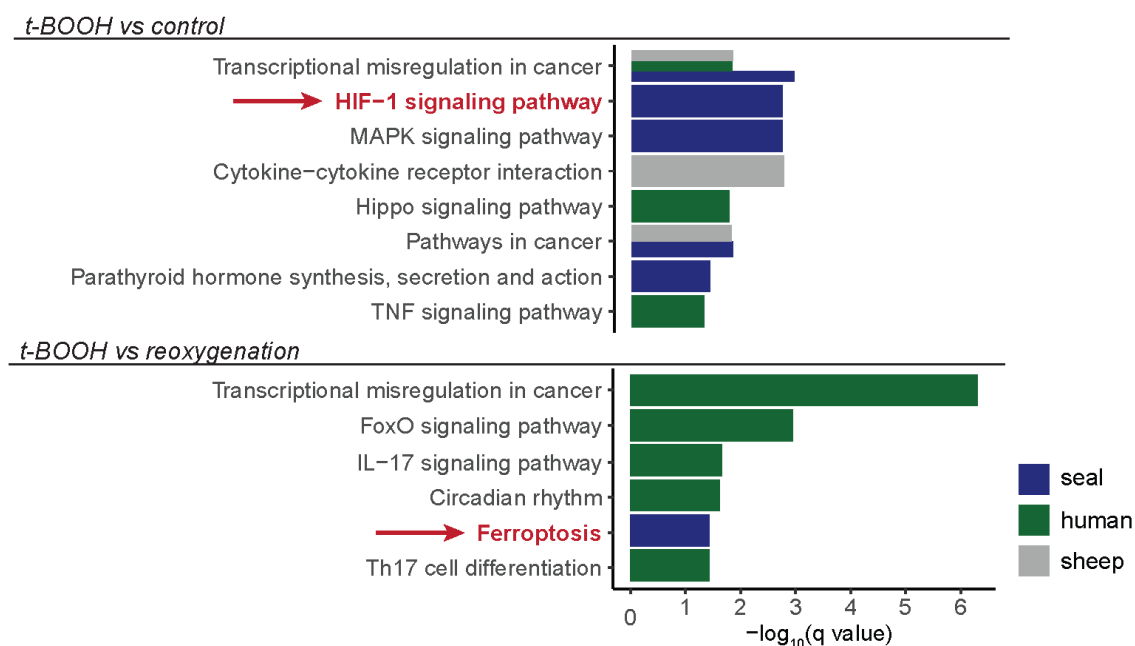


Figure 9. *t*-BOOH treatment alters HIF-1 signaling and the ferroptosis pathway in seal endothelial cells. KEGG enrichment results for genes DE in *t*-BOOH treatment compared to control (top) or hypoxia/reoxygenation (bottom). Blue, seal; green, human; gray, sheep. Complete KEGG enrichment results for all comparisons are shown in Figure S6.

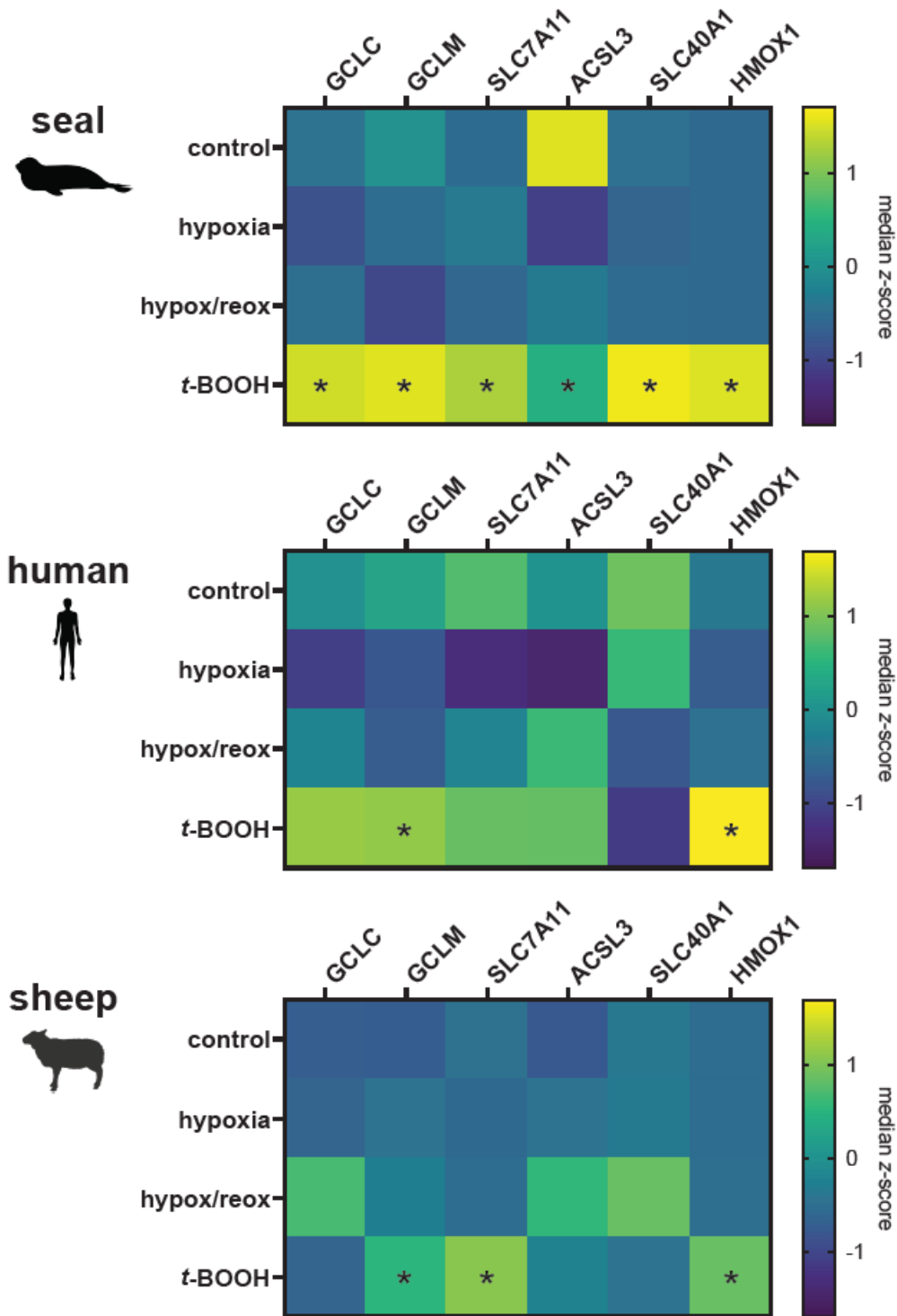


Figure 10. *t*-BOOH treatment upregulates ferroptosis pathway genes relative to hypoxia/reoxygenation in seal cells. Median *z*-scores for genes implicated in the seal ferroptosis pathway enrichment between hypoxia/reoxygenation and *t*-BOOH treatment (see Figure 9). Asterisks indicate genes from the seal ferroptosis pathway enrichment that are DE in *t*-BOOH treatment compared to hypoxia/reoxygenation for each species.

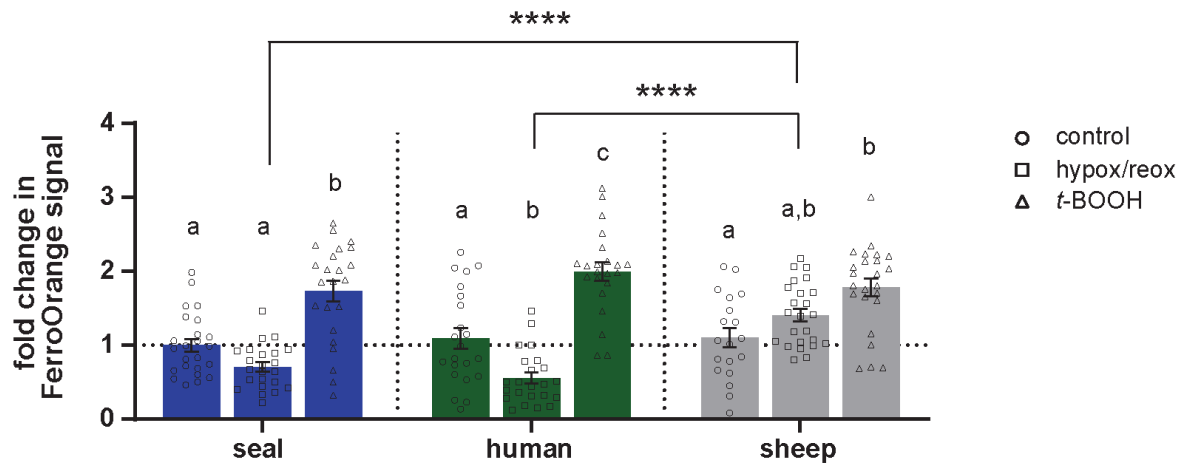


Figure 11. Hypoxia/reoxygenation and *t*-BOOH treatment do not significantly alter cellular iron uptake. Species-specific fold change in FerroOrange fluorescence in response to hypoxia/reoxygenation or *t*-BOOH treatment (100 μ M for 2 h) compared to control. Different letters denote statistically significant differences among treatments for each species. Brackets indicate differences between species within each treatment. $n=18-24$ replicates per condition. Blue, seal; green, human; gray, sheep. **** $p<0.0001$.

3.10 Supplementary material

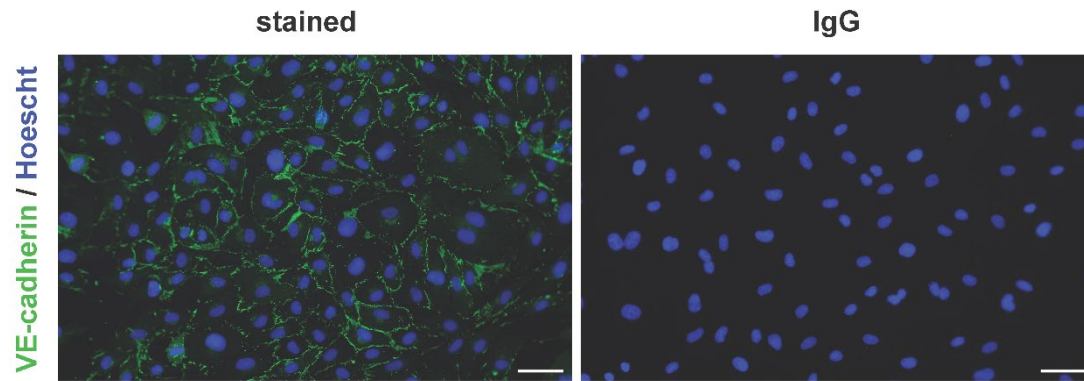


Figure S1. Primary sheep arterial endothelial cells express the canonical endothelial marker VE-cadherin (CD144). Green, VE-cadherin; blue, Hoechst (nuclear). Scale bar is 50 μ m.

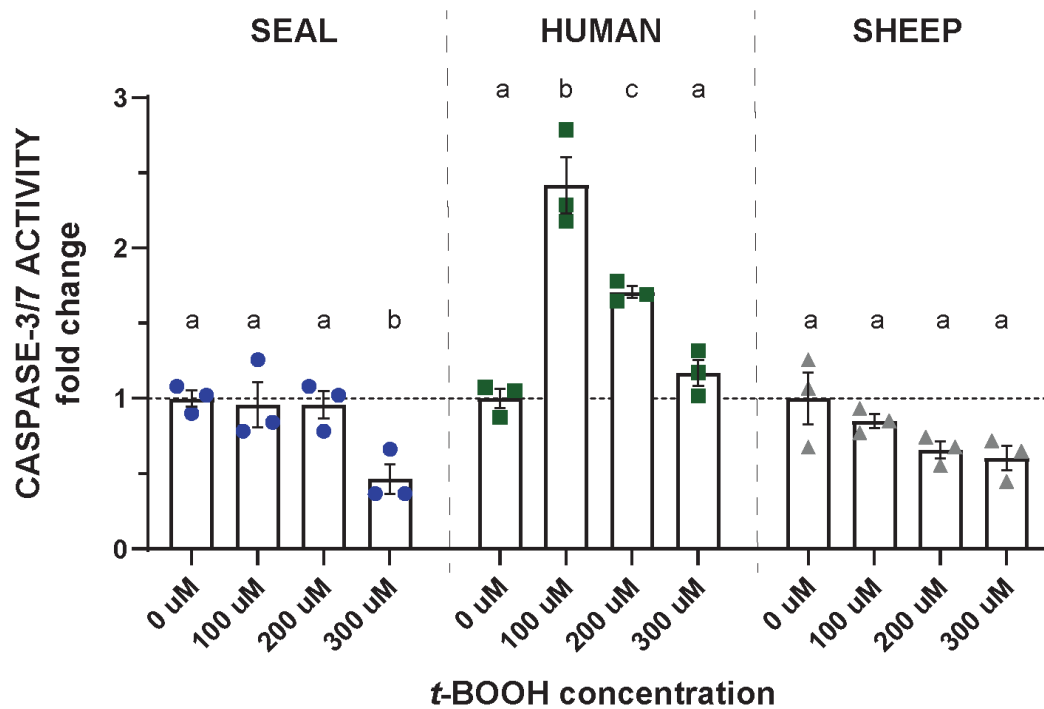


Figure S2. Caspase-3/7 activity in endothelial cells with increasing *t*-BOOH exposure. Seal, human, and sheep endothelial cells were exposed to 0, 100, 200, or 300 μ M *t*-BOOH for 2 h. Caspase activity was used as a proxy for apoptosis and measured as fold change in luminescence following addition of a luminogenic substrate containing the caspase DEVD cleavage sequence. Caspase activity was measured in the same wells as viability and cell death (see Figure 2). Data are presented as fold change from species control values. Mean $\pm$ s.e.m. and individual points are plotted. $n=3$ replicates per condition. Blue, seal; green, human; gray, sheep. Different letters denote statistical significance between means (within each species).

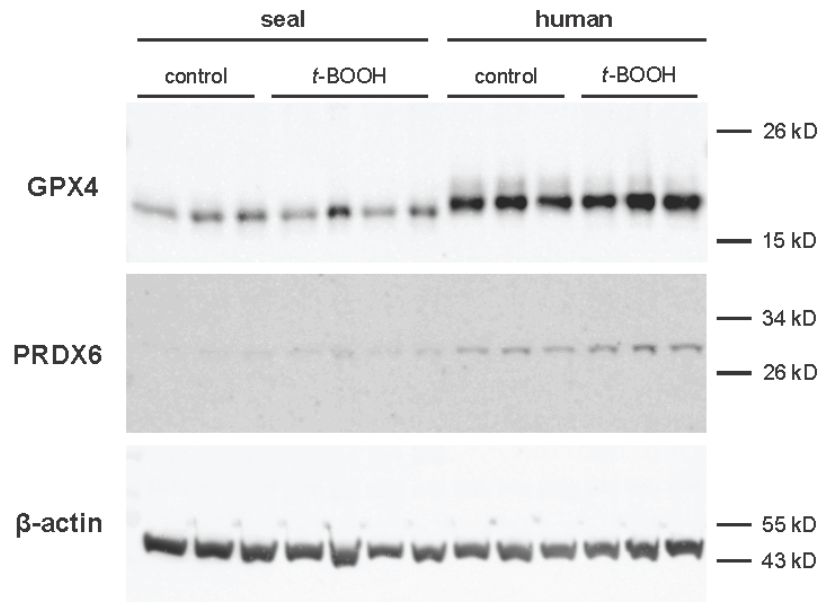


Figure S3. Western blots for the glutathione-dependent lipid hydroperoxidase enzymes GPX4 and PRDX6.



Figure S4. GSEA enrichment results for human DE genes. Comparisons are constrained to those pairwise comparisons which yielded significant results in seal cells (see Figure 8). Normalized enriched score < 0 indicates net downregulation of the pathway. FDR<0.001 for all pathways.

reoxygenation vs. hypoxia



Figure S5. GSEA enrichment results for sheep DE genes. Comparisons are constrained to those pairwise comparisons which yielded significant results in seal cells (see Figure 8). Normalized enriched score < 0 indicates net downregulation of the pathway. FDR<0.001 for all pathways.

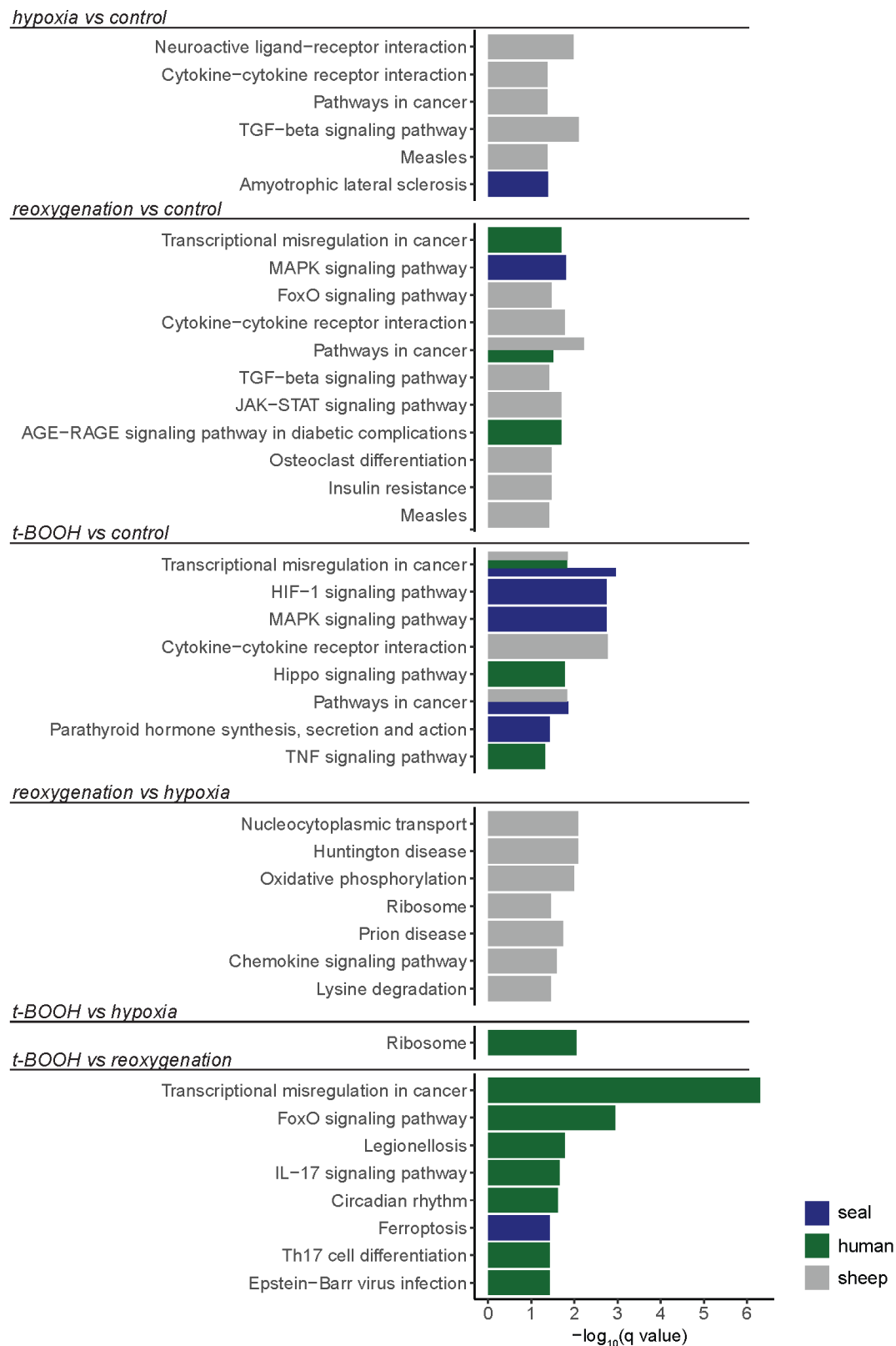


Figure S6. Complete KEGG enrichment results to accompany Figure 9. Blue, seal; green, human; gray, sheep.

Table S1. Primer sequences.

Species	Target gene	Sequence 5' to 3'
Northern elephant seal (<i>Mirounga angustirostris</i>)	<i>gapdh</i>	CAA GGC TGA GAA CGG GAA GC
		ATC GGC AGA GGG AGC AGA GA
	<i>β-actin</i>	GAC ATC CGC AAG GAC CTC TA
		GCC TCC AAT CCA CAC AGA GT
	<i>gpx4</i>	AAG TAC CGG GGC TTC GTG TG
		CCA GCG GCG AAC TCT TTG AT
	<i>prdx6</i>	GCA CCA CAG AGC TTG GCA GA
		AGG ATG GCA AGG TCC CGA TT
Human (<i>Homo sapiens</i>)	<i>gapdh</i>	CCT GCA CCA CCA ACT GCT TA
		GGC CAT CCA CAG TCT TCT GAG
	<i>β-actin</i>	TGA TGG TGG GCA TGG GTC AGA A
		TCT GGG TCA TCT TCT CGC GGT T
	<i>gpx4</i>	AGA GAT CAA AGA GTT CGC CGC
		TCT TCA TCC ACT TCC ACA GCG
	<i>prdx6</i>	CGT GTG GTG TTT GTT TTT GG
		CCA TCA CAC TAT CCC CAT CC
All	<i>18S rRNA</i>	GTA ACC CGT TGA ACC CCA TT
		CCA TCC AAT CGG TAG TAG CG

4. The postweaning fast increases glycolytic ATP production and proton leak in elephant seal muscle cells

4.1 Abstract

Several mammals fast within the context of varied life histories. Bears, ground squirrels, and bats fast while hibernating in seasons of food scarcity. Seals, in comparison, fast during energetically demanding life history stages, including postnatal development. Northern elephant seal pups fast for 2-3 months on land after weaning. During this time, the pups develop the body oxygen stores and cardiovascular control required for diving. Increases in glucocorticoid levels and decreases in insulin production and signaling during the postweaning fast likely alter muscle cell metabolism, but the extent to which any changes are cell-autonomous vs. serum-borne remains unclear. We derived proliferative skeletal muscle myoblast cultures from elephant seal pups early and late in the postweaning fast to investigate the contribution of intrinsic cellular processes vs. circulating factors in ATP production. Myoblasts derived from animals late in the fast exhibited increased glucose-dependent ATP production rates, driven by an increased glycolytic capacity in these cells. Serum from animals late in the fast shifted myoblasts toward glycolytic ATP production and increased proton leak, independent of the fasting stage from which the cells were derived. These results suggest that glycolytic contributions to ATP production are enhanced late in the postweaning fast, and that both cell-autonomous and serum-borne factors contribute to muscle metabolism in elephant seal pups.

4.2 Introduction

Fasting is a critical strategy used by many species whose foraging habits are spatially or temporally separate from other life history stages. Fasting is divided into two physiologically-controlled stages and a third (final) stage in which homeostasis is lost [125,246]. In the first phase, animals downregulate their metabolic rate while using various available energy substrates. The second phase relies predominantly on lipid stores to support energetic needs [247]. Both phases preserve overall homeostasis, though body mass declines. The third fasting phase (starvation) depletes lipid stores, and protein becomes the main energy substrate. Substantial losses in body mass occur due to catabolism of lean tissue, often leading to cachexia. Therefore, animals risk multiple organ failure if the starvation phase progresses and refeeding does not occur [125].

Fasting-tolerant vertebrates typically extend the second phase of fasting, during which body mass declines but homeostasis is preserved through reliance on lipid fuel sources and sparing of body protein stores. Many species limit metabolic demands by constraining physical activity, as occurs during hibernation, torpor, or estivation [124,125,248]. In contrast, marine mammals fast during energetically intensive life stages, including migration, reproduction, and postnatal development [2]. Northern elephant seals (*Mirounga angustirostris*) undertake annual prolonged terrestrial fasts throughout their lives, the first of which occurs after only about a month of life. Elephant seal mothers nurse their pups for one month, after which pups spend several months developing on land. This terrestrial period, known as the postweaning fast, comprises the postnatal development of extensive body oxygen stores, cardiovascular control, and antioxidant defenses required to support extended breath-hold diving, as well as molting of the neonatal lanugo coat

[126,249–251]. At the organismal level, resting metabolic rate decreases, and seals' capacity for VO_2 suppression during breath holds increases across the postweaning fast in preparation for diving [128,252]. Adiposity also declines substantially during this time [126,127,252]. Thus, muscle tissue contributes relatively more to the total body metabolic rate as the postweaning fast progresses.

Fasting decreases insulin production while increasing cortisol levels in elephant seals [253–255]. Moreover, fasting promotes adipose-specific insulin insensitivity [256]. Fasting elephant seal pups exhibit high rates of endogenous glucose production, nearly half of which derives from lactate [257]. Remarkably, only ~24% of this glucose is oxidized early in the fast, and this proportion declines to ~17% by six weeks of fasting. Overall, glucose oxidation constitutes less than 10% of the total body metabolic rate across the postweaning fast [258]. Similarly, both fasting and ontogeny decrease overall respiratory capacity while maintaining a high capacity for fatty acid oxidation and increasing leak in permeabilized muscle fibers isolated from elephant seals [129,130]. Moreover, differentiated seal skeletal muscle myotubes incubated with glucocorticoids suppress glucose-dependent oxidative phosphorylation, though glycolysis remains unchanged [135].

In adipocytes derived from grizzly bears, serum from hibernating vs. active animals differentially regulates cellular energetics. The extent to which these mechanisms are conserved across species, tissues, or fasting types (i.e., physically active vs. inactive) remains unknown. Thus, we derived proliferative primary skeletal muscle cells (myoblasts) from weaned elephant seal pups “early” (~1 week) and “late” (~8 weeks) in the postweaning fast. We used this system to evaluate cell-autonomous vs. serum-driven metabolic phenotypes associated with the postweaning fast. We found that the postweaning fast increased cell-autonomous ATP production rates in myoblasts under substrate-limited conditions, which was driven by increased glycolytic capacity. Moreover, incubating seal myoblasts with serum from animals late in the postweaning fast increased both the glycolytic contribution to ATP production and oxygen consumption associated with proton leak. Our results identified both cell-autonomous and serum-borne regulation of myoblast metabolic phenotype in elephant seals.

4.3 Materials and methods

4.3.1 Animals and sample collection

Animal handling protocols were approved by Sonoma State University and UC Berkeley Institutional Animal Care and Use Committees. All sampling was conducted under National Marine Fisheries Service Permit (NMFS) No. 19108 (PI: Daniel Costa, UC Santa Cruz). Cell cultures were developed under NMFS Permit No. 22479 (PI: José Pablo Vázquez-Medina, UC Berkeley). Samples were collected during the 2019 and 2021 seasons between February and April. In each season, nine “early” (February) and six “late” (April) fasting animals were sampled at Año Nuevo Reserve (San Mateo County, CA) (Table 1). Animals were identified as “early” in the fast by body condition and the presence of a lanugo coat. Animals were identified as “late” in the fast by (leaner) body condition and full completion of the molt. Animals were chemically immobilized with tiletamine-zolazepam and ketamine, as described previously [221]. After immobilization, blood samples were collected through a 16-gauge 3.5-inch spinal needle inserted into the extradural vein. Blood was collected in serum separator tubes (SST), allowed to clot at room temperature, and centrifuged at 1500 x g for 15 min to collect serum. Serum was transferred to

cryovials and frozen at -80°C until analysis. Muscle biopsies were collected from the flank near the hind flipper. Briefly, the skin was cleaned with alternating wipes of betadine and isopropanol. A small incision (<1.5 cm) was made with a sterile scalpel, and muscle biopsies were obtained with a sterile 6mm Miltex biopsy punch. Biopsies were rinsed with ice-cold sterile HBSS without calcium or magnesium and containing 2X Antibiotic-Antimycotic solution (Gibco). Biopsies were placed in sterile HBSS + 2X Antibiotic-Antimycotic and kept on ice until transport to the lab for myoblast isolation (~ 4 -8 h after collection). A summary of the sampling paradigm is provided in Figure 1.

4.3.2 Primary myoblast isolation and culture

Primary skeletal muscle myoblasts were isolated from elephant seal muscle biopsies during the 2019 breeding season, as previously described [135]. Briefly, muscle biopsies were rinsed in ice-cold, sterile HBSS, minced with a scalpel, and triturated with a syringe plunger on a tissue culture dish. The minced tissue was incubated in a 1 mg/mL collagenase type II solution (500 U/mL in DMEM; Worthington Biochemical, Lakewood, NJ, USA) for 30 minutes in a tissue culture incubator (37°C , 5% CO_2). The digestion mixture was passed through an 18-gauge needle 10 times, followed by a 22-gauge needle 10 times. The collagenase solution was blocked with complete growth medium, and the mixture was passed through a $100\text{ }\mu\text{m}$ filter to remove debris. The filtrate was centrifuged at $200\times g$ for 5 minutes at 4°C . The pellet was resuspended in complete myoblast growth medium consisting of Ham's F-10 Nutrient Mix (Gibco, catalog # 11550-043) supplemented with 20% fetal bovine serum (Seradigm, Avantor, Mexico), 10 mM HEPES (Gibco), and 1% (1X) Antibiotic-Antimycotic solution (Gibco) and plated on tissue culture treated dishes coated with collagen I (Sigma, catalog # C3867). Cultures were cryopreserved in 90% FBS/10% DMSO at passage 1. Primary human myoblasts were purchased from ThermoFisher (catalog # A11440, lot # 2398127) and cultured in the same media as seal cells. Experiments were conducted at passage 2-4 for all seal cells, and passage 6 for human cells.

4.3.3 Serum treatments

Serum was collected from weaned northern elephant seal pups during the 2021 breeding season, as detailed in Section 4.3.1 and Table 1. Pools of “early fasting seal serum” (EFSS; $n=8$ seals) and “late fasting seal serum” (LFSS; $n=6$ seals) were inactivated by heating at 56°C for 30 minutes. Heat-inactivated serum was filtered through a $0.22\text{ }\mu\text{m}$ filter before inclusion in culture media. Plating the cells directly in medium supplemented with seal serum induced rapid differentiation into myotubes (not shown). Thus, cells were seeded in complete medium containing 20% FBS, then switched to media containing 20% seal serum for 24 h before assay. As stated above, cells were isolated from animals born in 2019. Thus, autologous serum was never used.

4.3.4 Extracellular flux assays

Oxygen consumption and extracellular acidification rates were measured using Mito Stress Test assays on a Seahorse XFe24 Extracellular Flux Analyzer (Agilent Technologies, Santa Clara, CA, USA). Cells were cultured and seeded in collagen-coated XFe24 cell plates in complete myoblast growth medium containing 20% FBS. For experiments conducted in 20% FBS, cells were seeded at 50,000 cells/well, and the assay was run within 24 h of seeding. For experiments conducted in 20% seal serum, 25,000 cells/well were seeded overnight before switching to media containing heat-inactivated seal serum for 24 h. For all assays, cells were washed with serum-free assay medium (base: Seahorse XF DMEM pH 7.4, or Dulbecco's Modified Eagle's Medium

(Sigma, catalog # D5030); supplements: see details in Chapter 4.3.5) and incubated at 37°C in a non-CO₂ incubator for 30 min before assay. Oxygen consumption rates (OCR) and extracellular acidification rates (ECAR) were measured at baseline, and in the presence of oligomycin (1.5 µM), carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP; 2 µM), and rotenone/antimycin A (0.5 µM) or antimycin A alone (100 µM).

4.3.5 J_{ATP} and mitochondrial function calculations

ATP production rates and contributions of glycolysis and oxidative phosphorylation (OXPHOS) to ATP production were calculated from oxygen consumption and extracellular acidification rate data in assays run with assay media containing either 6 mM glucose only, or 6 mM glucose, 1 mM pyruvate, and 1 mM L-glutamine. Media buffering power and ATP production were calculated according to [259,260] (<https://github.com/gerencserlab/JATP>).

Mitochondrial function was calculated from oxygen consumption rate data in assays run with 6 mM glucose, 1 mM pyruvate, and 1 mM L-glutamine. Mitochondrial function was calculated according to [149] and normalized to baseline respiration per well.

4.3.5 Statistical analyses

We included commercially available human myoblasts as a non-seal reference in the data shown here. However, these cells were derived from a single individual. Therefore, we excluded the human cell results from statistical comparisons to seal cells, which were derived from 6 individuals per fasting state ($n=12$ seals total) and treated as separate biological replicates.

Statistical analyses were performed using GraphPad Prism v10.2.1. Normality was evaluated using Shapiro-Wilk tests. All datasets met normality assumptions; differences between groups with equal variance were assessed by independent two-tailed *t*-test or one-way ANOVA. ANOVA results were corrected for multiple comparisons using Šidák's multiple comparisons test ($\alpha=0.05$). *t*-tests were corrected for multiple comparisons using the two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli with FDR=0.05 [158]. Data are presented as mean $\pm$ s.e.m.

4.4 Results

4.4.1 The postweaning fast promotes glycolysis in seal myoblasts

Fasting progression in elephant seal pups involves substantial adjustments to organismal metabolism, but cellular-level changes are unknown. Hence, we considered whether seal muscle cells isolated from animals at the beginning and end of their postweaning fast retain different metabolic profiles despite matched culture conditions. When cultured with FBS under glucose-only conditions, myoblasts derived from late fasting pups exhibited higher rates of ATP production than those from early fasting pups ($t=2.589$, $p=0.0435$). This increase in ATP production was driven by increased glycolytic capacity; total oxidative contributions to ATP generation remained nearly constant across the fast (glycolysis: $t=3.265$, $p=0.0082$; OXPHOS: $t=0.6760$, ns) (Figure 2A). These data show that metabolic profiles are cell-autonomous in seal myoblasts under substrate-constrained conditions.

Next, we considered the impact of FBS and seal serum on seal myoblast metabolic profiles in fully supplemented conditions. One-way ANOVA identified significant differences in glycolytic

vs. oxidative contributions to ATP production among serum treatments within each cell “type” (cells derived from early vs. late fasting pups) ($F_{7,40}=6.044$, $p<0.0001$). FBS and early fasting seal serum (EFSS) yielded approximately 50/50 contributions of OXPHOS and glycolysis to ATP production rates in cells derived from early and late fasting pups, whereas late fasting seal serum (LFSS) shifted cells toward glycolysis (early cells: FBS vs. LFSS, $t=3.215$, $p=0.0154$; EFSS vs. LFSS: $t=4.239$, $p=0.0008$) (late cells: FBS vs. LFSS: $t=2.896$, $p=0.0361$) (Figure 2B). One-way ANOVA comparing serum treatments within human cells did not identify statistically significant differences among treatments (Figure S1). Together, these results demonstrate that both cells and serum from late fasting pups increase glycolytic contributions to ATP production in seal myoblasts.

4.4.2 Serum from late fasting pups increases proton leak in seal myoblasts

Baseline mitochondrial oxygen consumption comprises both ATP-linked (coupled) respiration and oxygen consumption associated with proton leak (uncoupled). One-way ANOVA identified serum-driven differences among ATP-linked and leak respiration contributions to total basal oxygen consumption rates ($F_{5,30}=5.992$, $p=0.0006$). LFSS increased oxygen consumption associated with proton leak in myoblasts from pups both early and late in the fast relative to FBS treatment (early cells: FBS vs. LFSS, $t=3.840$, $p=0.0053$; late cells: FBS vs. LFSS, $t=3.521$, $p=0.0125$) (Figure 3A). There were no differences between cell types treated with the same serum. Seal serum from either early or late fasting pups did not impact human myoblast leak contributions to basal oxygen consumption rates (Figure S2A). We were unable to determine whether basal respiration ultimately differed with cell or serum type due to inter-assay variation. Overall, these results show that serum from late fasting pups increases proton leak in seal myoblasts, regardless of the fasting state from which the cells were derived. Therefore, these results suggest that serum-borne factors contribute to muscle metabolism in elephant seal pups.

4.4.3 The spare:basal respiration ratio remains consistent during the postweaning fast

The relative contributions of basal respiration and spare respiratory capacity to maximal respiration did not vary with cell or serum type in seal myoblasts, though there was a trend toward a lesser contribution of spare respiratory capacity in seal serum-treated cells (Figure 3B). Seal serum from any fasting state decreased spare respiratory capacity contributions to maximal respiration in human myoblasts (one-way ANOVA: $F_{2,13}=7.737$, $p=0.006$; FBS vs. ESS: $t=3.552$, $p=0.011$; FBS vs. LSS: $t=3.120$, $p=0.016$) (Figure S2B). We were unable to determine whether maximal respiration ultimately differed with cell or serum type due to inter-assay variation. Our results show that serum from fasting seals generally decreases the representation of spare respiration in maximal respiration rates in both seal and human myoblasts, suggesting higher baseline energetic demands, perhaps due to increased proton leak.

4.5 Discussion

Marine mammals undertake extended terrestrial fasts during energetically demanding life stages, including reproduction, migration, molting, and postnatal development. In elephant seals, the first such fast occurs after the one-month nursing period. During the postweaning fast, glucocorticoid production increases while adipose-specific sensitivity to insulin decreases. These changes occur in parallel with the development of cardiovascular control, body oxygen stores, and antioxidant defenses, which support breath-hold diving. Skeletal muscle remains insulin sensitive,

but the degree to which cell-autonomous vs. circulating factors impact fasting muscle cell metabolism during fasting remains unknown. Therefore, we utilized a primary cell culture system derived from fasting elephant seal pups to compare intrinsic cellular and serum-driven responses in mitochondrial function to fasting. We identified fasting-associated increases in cell-autonomous ATP production rates (driven by glycolysis) under substrate-limited conditions. Moreover, serum from late fasting animals increased the glycolytic contribution to ATP production and proton leak, which uncouples oxygen consumption from ATP generation. Our results demonstrate both cell-autonomous and serum-borne shifts toward glycolytic ATP production in seal myoblasts.

4.5.1 Glycolytic contributions to ATP production increase with fasting

Fasting increases glucocorticoid levels and decreases insulin production in elephant seals [253–255]. Whole animal respiratory quotients are ~ 0.7 across the postweaning fast, indicating an overall reliance on lipid fuel sources [124,258]. However, glucocorticoids increase blood glucose levels, as does insulin insensitivity. Skeletal muscle remains insulin sensitive in fasting elephant seal pups, and glucocorticoid treatment drives glucose-dependent ATP production by glycolysis in primary elephant seal myotubes [135,256]. We found that myoblasts derived from animals late in the postweaning fast produced more ATP under glucose-only conditions than those derived from animals early in the fast, and that this increase was exclusively attributable to glycolytic ATP production. Provision of pyruvate and L-glutamine in addition to glucose yielded 50/50 contributions of glycolysis and OXPHOS to ATP production independent of the fasting stage from which cells were derived, suggesting that the observed cell-autonomous metabolic phenotype is substrate-specific. Moreover, incubating myoblasts with late (but not early) fasting seal serum shifted ATP production toward glycolysis, again independent of the fasting stage from which cells were derived. Endogenous glucose production rates decline slightly across the postweaning fast but remain high, while glucose oxidation consistently contributes to less than 10% of the whole body metabolic rate [258]. Our results in seal myoblasts are consistent with these observations at the organismal level, suggesting that oxidation is not the primary fate of glucose during the postweaning fast.

4.5.2 Circulating factors from the postweaning fast promote proton leak in seal myoblasts

Mitochondrial function studies in permeabilized muscle fibers from elephant seals demonstrate an overall reduction in OXPHOS capacity and increased proton leak associated with maturation [129], though fasting does not alter leak respiration in molting adult female seals [130]. Recent genomic and *in vitro* evidence suggests that uncoupling proteins may be under selection in marine mammals, including the putative pseudogenization of elephant seal UCP1, which ablates its thermogenic capacity [261,262]. The postweaning fast initially decreases but eventually increases uncoupling protein 2 (UCP2) mRNA expression in skeletal muscle [263], potentially elevating leak, though we did not find evidence of this in fasting pup myoblasts incubated with FBS. However, late (but not early) fasting seal serum increased leak respiration regardless of the fasting state from which the cells were derived. In grizzly bears, serum from hibernating bears blunts mitochondrial respiration and glycolysis in adipocytes, independent of whether cells were derived from active or hibernating animals. Interestingly, these changes are apparently not driven by insulin despite an overall insulin resistance associated with hibernation [131,132].

4.5.3 Limitations

Our study sample size is typical of physiological investigations in elephant seals, but ultimately represents a relatively small n . The sampling regime thus limited statistical power and precluded the inclusion of variables such as sex or known age in the analysis. Additionally, we were unable to follow the same individuals across the postweaning fast, even though we were able to detect significant effects of the fasting condition as previously reported.

4.6 Conclusions

In summary, we found that the progression of the postweaning fast increases skeletal muscle myoblast glycolytic capacity in northern elephant seal pups. Similarly, serum from animals late in the fast increased the glycolytic contribution to ATP production while also increasing leak respiration, suggesting that the postweaning fast dampens myoblasts' reliance on mitochondrial respiration to meet energetic demands.

4.7 Acknowledgments

We thank Dr. Dan Crocker (Sonoma State University) for his support in obtaining elephant seal samples for this chapter and Dr. Denis Titov (UC Berkeley NST) for facilitating access to a new Seahorse instrument.

4.8 Tables

Table 1. Summary of animal sampling.

Year	Stage	n	Sex	Serum	Cells
2019	early	8	male		x
		1	female		
	late	3	male		x
		3	female		
2021	early	5	male	x	
		2	female		
		1	unknown		
	late	3	male	x	
		3	female		

4.9 Figures

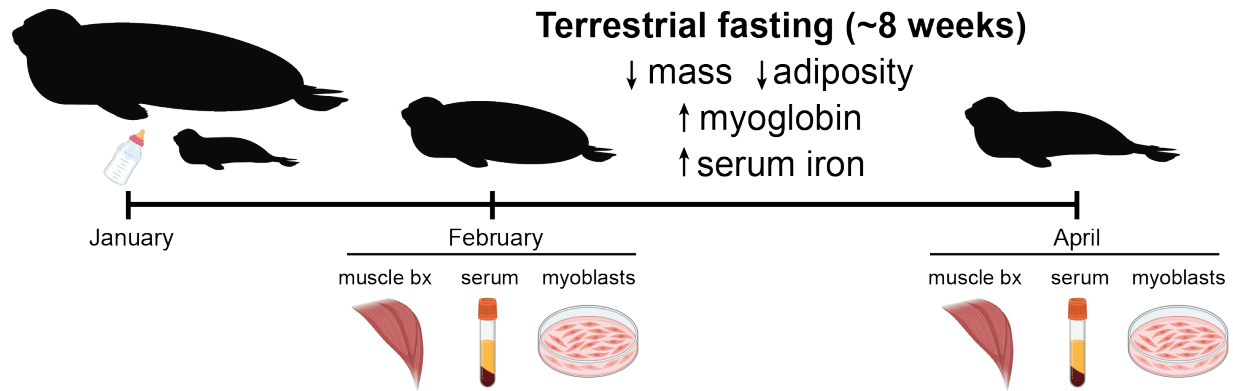
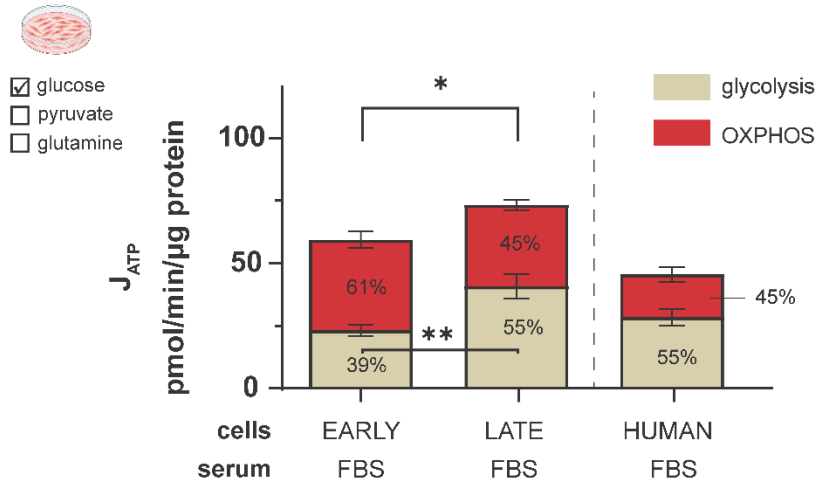


Figure 1. Field sample collection timeline. Weaned northern elephant seal pups were sampled at Año Nuevo Reserve during the 2019 and 2021 breeding seasons. Muscle samples collected in 2019 were used to generate primary myoblasts. Serum collected in 2021 was used for mitochondrial respiration assays.

A



B

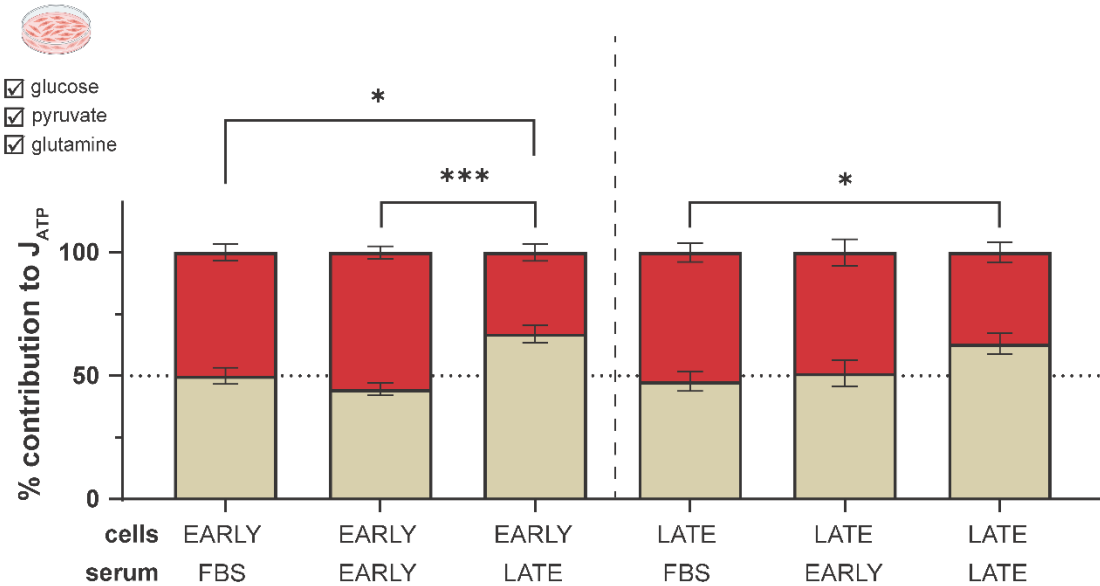


Figure 2. The postweaning fast increases the reliance of seal myoblasts on glycolysis to generate ATP. (A) Absolute ATP production rates (J_{ATP}) from glycolysis and OXPHOS in seal myoblasts grown in medium containing FBS. The assay was run with glucose (6 mM) as the only substrate. (B) Relative contributions of glycolysis and OXPHOS to ATP production rates in seal myoblasts grown in medium containing FBS, EFSS, or LFSS. The assay was run with glucose (6 mM), pyruvate (1 mM), and L-glutamine (1mM) as substrates. Beige, glycolysis; red, OXPHOS. Data are mean $\pm$ s.e.m of $n=6$ individual seals per fasting state, with $n=3-5$ technical replicates per seal. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

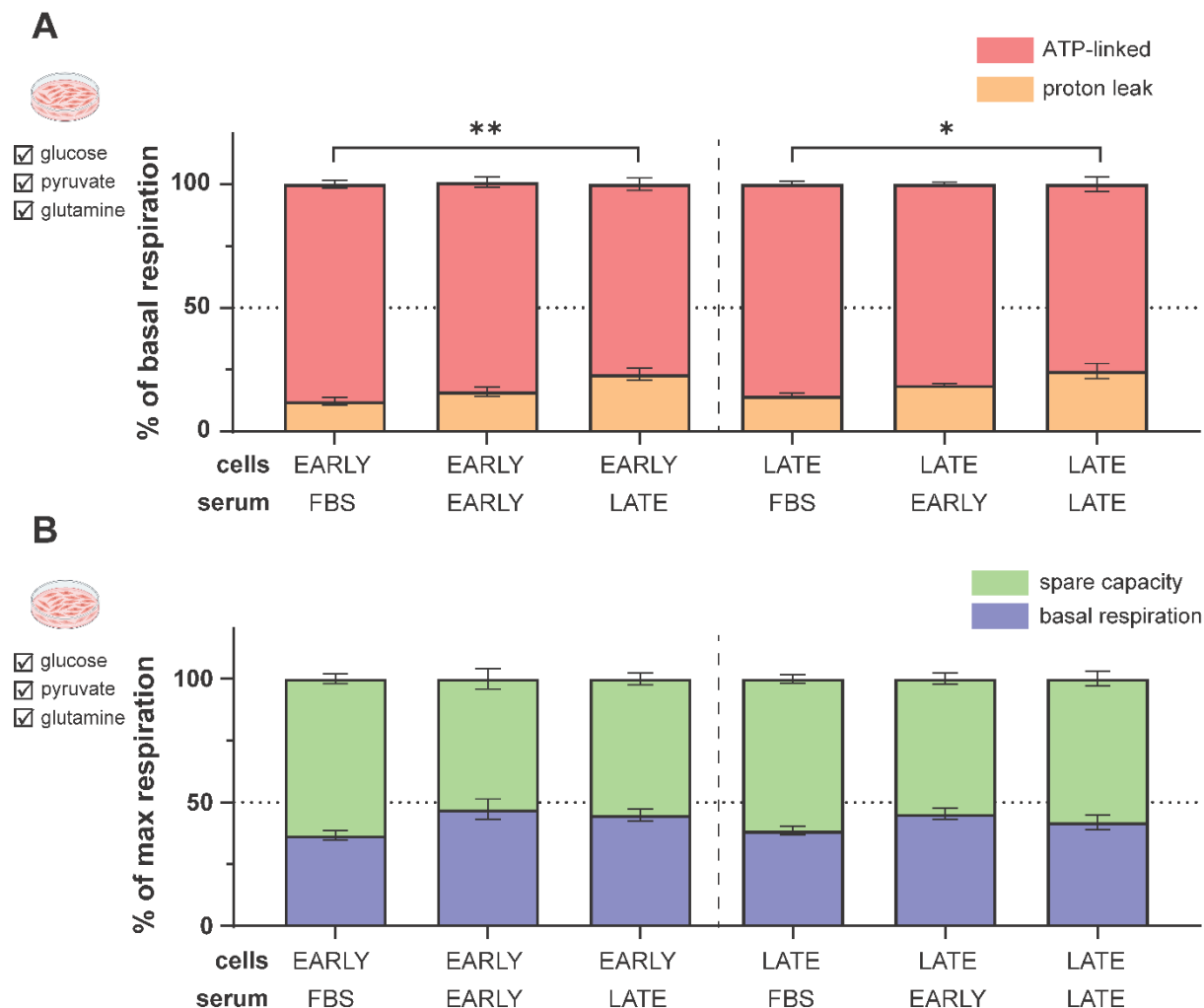


Figure 3. Late fasting seal serum increases leak in seal myoblasts. (A) Relative contributions of ATP-linked (pink) and leak-linked (orange) respiration to basal respiration in seal myoblasts grown in medium containing FBS, EFSS, or LFSS. (B) Relative contributions of basal respiration (purple) and spare respiratory capacity (green) to maximal respiration in seal myoblasts grown in medium containing FBS, EFSS, or LFSS. The assay was run with glucose (6 mM), pyruvate (1 mM), and L-glutamine (1mM) as substrates. Data are mean $\pm$ s.e.m of $n=6$ individual seals per fasting state, with $n=3-5$ technical replicates per seal. * $p<0.05$, ** $p<0.01$.

4.10 Supplementary material

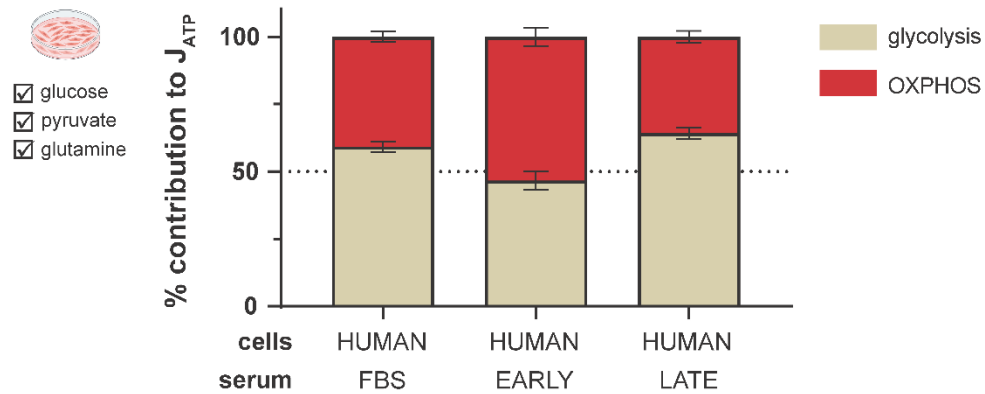


Figure S1. Fasting seal serum does not alter the relative contributions of glycolysis and OXPHOS to ATP production in human myoblasts. Relative contributions of glycolysis and OXPHOS to ATP production rates in human myoblasts grown in medium containing FBS, EFSS, or LFSS. The assay was run with glucose (6 mM), pyruvate (1 mM), and L-glutamine (1mM) as substrates. Beige, glycolysis; red, OXPHOS. Data are mean $\pm$ s.e.m of $n=5-6$ replicates in cells derived from a single individual.

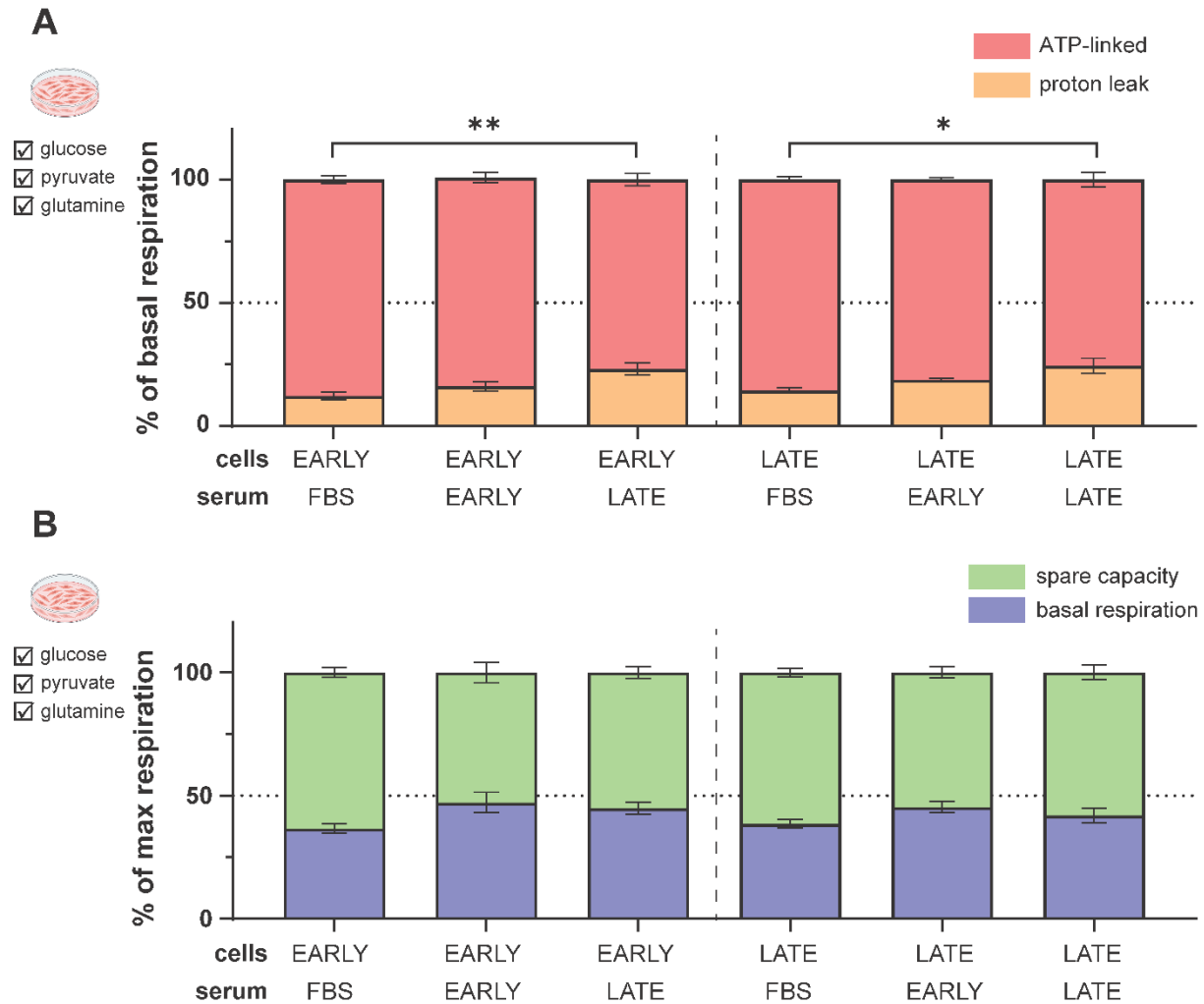


Figure S2. Fasting seal serum alters the relative contributions of basal and spare respiratory capacity to maximal respiration in human myoblasts. (A) Relative contributions of ATP-linked (pink) and leak-linked (orange) respiration to basal respiration in human myoblasts grown in medium containing FBS, EFSS, or LFSS. (B) Relative contributions of basal respiration (purple) and spare respiratory capacity (green) to maximal respiration in human myoblasts grown in medium containing FBS, EFSS, or LFSS. The assay was run with glucose (6 mM), pyruvate (1 mM), and L-glutamine (1mM) as substrates. Data are mean $\pm$ s.e.m of $n=5-6$ replicates in cells derived from a single individual. * $p<0.05$, ** $p<0.01$.

5. Conclusions

5.1 Diving mammals balance oxygen and nutrient demands with availability

Marine mammals dive to forage; thus, a significant portion of their lives is spent spatially and temporally separated from access to oxygen. Accordingly, these animals possess physiological adaptations that modulate cellular- and tissue-level energetics and support prolonged activity during breath holds. These adaptations include overall increases in body oxygen storage capacity, as well as cardiovascular and molecular changes that limit oxygen consumption rates in peripheral tissues. Still, deep-diving marine mammals must tolerate low tissue oxygen tensions while diving. Among marine mammals, pinnipeds remain partially dependent on land. While oxygen availability is not limited in the terrestrial environment, some pinniped species undertake weeks- or months-long fasts on land. Body nutrient stores and metabolic adjustments, therefore, affect organismal energetics. Decades of research on diving and fasting physiology in northern elephant seals provide an extensive body of work at the whole organismal and tissue levels. However, technological constraints on sampling continue to limit our understanding of the real-time cellular responses to hypoxia and fasting in this tolerant species.

5.2 Summary of chapters

This dissertation expands our understanding of three main facets of marine mammal physiology at the cellular level. The main findings are summarized in Figure 1 of this chapter. Chapter 2 identified a decoupling of canonical hypoxia signaling from cell proliferation in seal vasculature at both the transcriptional and functional levels. This chapter also established the glutathione antioxidant system as highly responsive to hypoxia in seal endothelial cells. Chapter 3 found that a chemical oxidant, but not a physiological hypoxia/reoxygenation exposure, upregulated the expression of genes associated with ferroptotic cell death in seal endothelial cells despite no observed changes in overall cell death under the same conditions. Seal cells specifically upregulated expression of the cellular iron exporter ferroportin 1 (SLC40A1) in response to a chemical oxidant but no other stressor, suggesting that seal cells regulate iron handling in response to oxidative stress, and that this regulation is sufficient for cytoprotection even when the applied insult exceeds physiological levels.

Chapters 2 and 3 both considered the impact of hypoxia on seal endothelial cell function, as oxygen availability critically supports oxidative metabolism and is limited during diving. However, elephant seals spend several months fasting on land during development; thus, we also considered fasting-associated changes in ATP production in muscle cells under fully oxygenated conditions. Chapter 4 identified a cell-autonomous metabolic shift toward glycolysis associated with prolonged fasting in seal pup muscle cells, though only under substrate-limited conditions. Critically, these experiments also showed that circulating (serum-borne) factors from animals early and late in the postweaning fast alter cellular energetics independent of fasting cell stage. This work points to both cell-autonomous and circulating factors as determinants of muscle cell metabolism in fasting seals.

5.3 Future directions

The experiments described here provide a first look into the dynamics of the transcriptional, functional, and metabolic cellular adaptations to hypoxia, oxidative stress, and fasting. Chapters 2 and 3 developed a novel endothelial cell culture system; this is the first such system published in the field of comparative physiology. Critical next steps include the direct measurement of iron flux through endothelial cells at baseline, during hypoxia/reoxygenation, and with *t*-BOOH exposure. Semi-quantitative iron detection methods impede baseline comparisons among species and were only partially informative here. Additionally, knockdown of candidate iron handling genes (e.g., SLC40A1) in seal cells – or overexpression of these genes in cells from lipid peroxidation-sensitive species – will shed light on the mechanisms regulating iron homeostasis in marine mammals.

Chapter 4 uses an established muscle cell culture model from our lab to tease apart cellular and circulating contributions to the fasting metabolic phenotype, which has been observed at the whole animal level for decades. While we identified both cell-autonomous and serum-driven shifts toward glycolysis associated with the postweaning fast, we lack an understanding of the factors that elicit this phenotype at both the cellular and circulating levels. Future work should distinguish between lipid and protein components of the serum as potential contributors, as well as consider cellular sensitivity (e.g., receptor expression or activity) to major metabolic hormones such as insulin and corticosteroids.

Ultimately, this dissertation develops, characterizes, and demonstrates the functional utility of two proliferative cell culture models for advancing molecular studies in marine mammal physiology. These models are readily shared across disciplines, thereby increasing access to resources in marine mammal biology without further impacting the animals themselves. Moreover, these models promote repeatability and reproducibility in research, which is often challenging in marine mammal physiology. Future work should consider tying *ex vivo* experimental results back to *in vivo* studies to bolster our understanding of marine mammal physiology across several levels of biological organization.

5.4 Figures

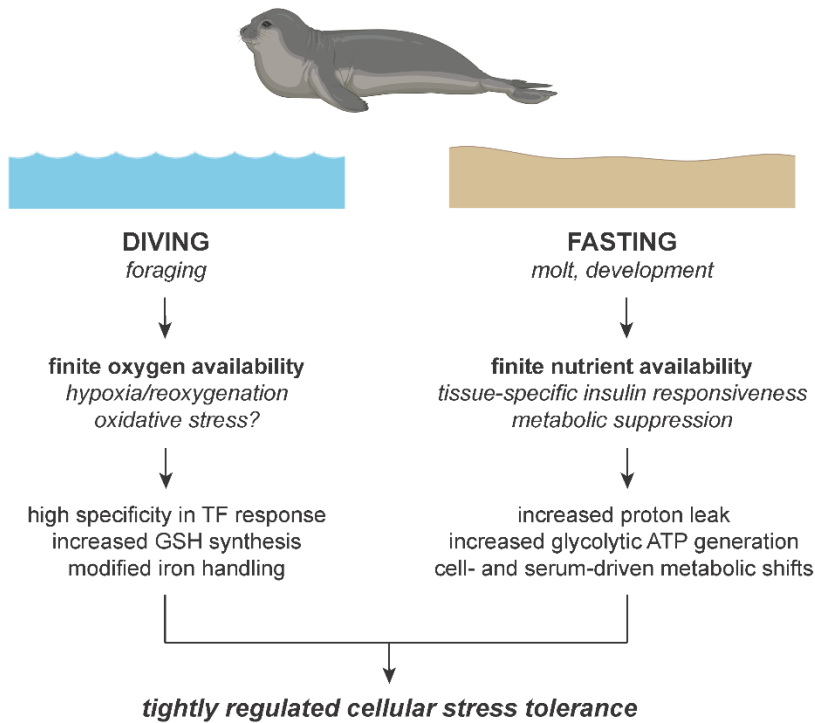


Figure 1. Summary of cellular stressors considered in primary cell culture systems in this dissertation. Elephant seals experience both finite oxygen availability (diving) and finite nutrient availability (fasting). Animals must be able to balance whole body energetic demands despite these potential constraints.

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