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SYMPOSIUM

# Functional Studies with Primary Cells Provide a System for Genome-to-Phenome Investigations in Marine Mammals

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**Synopsis** Marine mammals exhibit some of the most dramatic physiological adaptations in their clade and offer unparalleled insights into the mechanisms driving convergent evolution on relatively short time scales. Some of these adaptations, such as extreme tolerance to hypoxia and prolonged food deprivation, are uncommon among most terrestrial mammals and challenge established metabolic principles of supply and demand balance. Non-targeted omics studies are starting to uncover the genetic foundations of such adaptations, but tools for testing functional significance in these animals are currently lacking. Cellular modeling with primary cells represents a powerful approach for elucidating the molecular etiology of physiological adaptation, a critical step in accelerating genome-to-phenome studies in organisms in which transgenesis is impossible (e.g., large-bodied, long-lived, fully aquatic, federally protected species). Gene perturbation studies in primary cells can directly evaluate whether specific mutations, gene loss, or duplication confer functional advantages such as hypoxia or stress tolerance in marine mammals. Here, we summarize how genetic and pharmacological manipulation approaches in primary cells have advanced mechanistic investigations in other non-traditional mammalian species, and highlight the need for such investigations in marine mammals. We also provide key considerations for isolating, culturing, and conducting experiments with marine mammal cells under conditions that mimic *in vivo* states. We propose that primary cell culture is a critical tool for conducting functional mechanistic studies (e.g., gene knockdown, over-expression, or editing) that can provide the missing link between genome- and organismal-level understanding of physiological adaptations in marine mammals.

## Introduction

Marine mammals made multiple independent evolutionary transitions from a terrestrial to an aquatic environment. These independent reinvasions provide an ideal framework in which to study both convergent evolution and mammalian adaptation to the marine environment. A dramatic increase in the application of genomic techniques to marine mammal studies over the last decade has contributed significantly to our understanding of the genetic basis of marine mammal physiology (Cammen et al. 2016). Molecular convergence and positive selection (Xu et al. 2013; Foote et al. 2015; Zhou et al. 2015; Nery et al. 2016) as well as gene family expansion and gene loss (Sun et al. 2013; Chikina et al. 2016; ZhOu et al. 2018; Huelsmann et al. 2019; Liu et al. 2019) related to the transition from land to water are now well documented in marine mammals.

For decades, physiological investigations have highlighted some of the best-known marine mammal adaptations to the aquatic environment. In particular, hypoxia and oxidative stress tolerance are extensively characterized at the biochemical and physiological levels (Vazquez-Medina et al. 2012; Davis 2014; Blix 2018; Allen and Vazquez-Medina 2019; Ponganis 2019). Recent phylogenomic analyses are uncovering the molecular adaptations to hypoxia in marine mammals. Hemoglobin, myoglobin, and neuroglobin, all of which are critical proteins in oxygen transport and storage, are under positive selection in cetaceans (Tian et al. 2016). Similarly, positive selection and both convergent and unique

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substitutions in the amino acid sequence of hypoxia-inducible factor 1α (HIF-1α), a master transcriptional regulator of hypoxia adaptation, are present in cetaceans as well as other hypoxia-tolerant mammals, including burrowing and high altitude rodents (Zhu et al. 2018). Several metabolic gene families, including the tricarboxylic acid (TCA) cycle enzymes citrate synthase and pyruvate carboxylase, are also under positive selection in cetaceans (Tian et al. 2017). Moreover, specific amino acid changes and the expansion of lactate dehydrogenase and monocarboxylate transporter 1 (MCT1) genes in cetaceans suggest an increased ability to metabolize lactate in these animals (Yim et al. 2014; Tian et al. 2017). Recent comparative transcriptomic analyses complement the above-mentioned genomic studies to provide clues about the metabolic pathways involved in hypoxia tolerance in marine mammal tissues (Fabrizius et al. 2016; Kruger et al. 2020). Specifically, the seal brain upregulates genes involved in glucose metabolism and lactate transport (MCT1) and downregulates the pyruvate dehydrogenase complex after hypoxia/reoxygenation, suggesting increased lactate efflux under these conditions (Hoff et al. 2017). The idea that lactate is important to fuel brain metabolism during marine mammal diving was proposed four decades ago (Murphy et al. 1980) and there is mounting support for this concept (Czech-Damal et al. 2014; Hoff et al. 2016; Matsui et al. 2017). Moreover, lactate is now recognized as a primary metabolic fuel rather than a waste product (Hui et al. 2017; Brooks 2018), further suggesting that lactate is a preferred metabolic substrate used by the mammalian brain during diving.

Hypoxia/reoxygenation events associated with peripheral vasoconstriction during diving potentially expose marine mammals to increased oxidative stress (Elsner et al. 1998; Li and Jackson 2002). Physiological and biochemical studies of oxidative stress tolerance suggest that marine mammals have increased levels of endogenous antioxidants, particularly those related to the Glutathione (GSH) pathway (Vazquez-Medina et al. 2012; Allen and Vazquez-Medina 2019). Recent evolutionary genetics studies support these findings. For example, genes related to the GSH system are expanded, under positive selection, and/or have amino acid changes in cetaceans (Yim et al. 2014; Foote et al. 2015; ZhOu et al. 2018; Tian et al. 2019). Similarly, two peroxiredoxin gene families are expanded in cetacean lineages (Yim et al. 2014; ZhOu et al. 2018). Moreover, identification of an inactivating mutation in a polymerase with low fidelity for oxidative DNA damage repair in cetaceans suggests reliance on a higher fidelity polymerase, which, along with the loss of a kinase involved in lung inflammation and oxidant stress, may confer tolerance to oxidative damage (Pryor et al. 2015; Huelsmann et al. 2019). These studies provide strong support for earlier biochemical evidence of enhanced antioxidant systems in marine mammals (Vazquez-Medina et al. 2012; Allen and Vazquez-Medina 2019), but mechanistic experiments confirming these findings (e.g., gene gain or loss of function studies) are yet to be conducted.

Transcriptomics, proteomics, and metabolomics approaches are starting to provide clues about northern elephant seals' (*Mirounga angustirostris*) natural resistance to stress and prolonged food deprivation (Champagne et al. 2013; Crocker et al. 2016; Martinez et al. 2018; Deyarmin et al. 2020). Similarly, genomic and transcriptomic studies in bowhead whales (*Balaena mysticetus*) have provided insights into the extraordinary longevity and cancer resistance observed in these animals, which may be explained by a large number of gene families experiencing positive selection, duplication and loss of genes associated with DNA repair, cell cycle regulation, cancer, and aging (Keane et al. 2015). Transcriptomic studies correlating changes in global gene expression with stress, contaminant exposure, disease, and seasonality in several marine mammals have also recently described cellular pathways and genes up- or down-regulated during such conditions (Khudyakov et al. 2015b; Van Dolah et al. 2015; Morey et al. 2016; Morey et al. 2017). Work by Khudyakov et al. (2017) shows upregulation of lipolytic and adipogenic pathways in elephant seal adipose tissue during acute stress elevations (Khudyakov et al. 2017). Similarly, adipose levels of plasminogen receptor increase in ringed seals exposed to polychlorinated biphenyls (Brown et al. 2017), and toll-like receptor transcripts are elevated in the brains of harbor seals infected with phocine herpesvirus 1 (Rosales and Vega Thurber 2016). These omics analyses are contributing to the elucidation of ecologically relevant physiological pathways that characterize mammalian life in the marine environment.

Despite significant advances in the identification of pathways and candidate genes underlying physiological adaptation in marine mammals, causal hypotheses linking genome to phenome in these animals remain untested. Experimental manipulations in marine mammals are limited to the use of exogenous hormones, receptor blockers, and metabolic tracers in small numbers of captive animals or tractable pinnipeds with predictable terrestrial haul-outs and amenability to research handling

(Champagne et al. 2005; Houser et al. 2012; Crocker et al. 2014; Vazquez-Medina et al. 2013; Viscarra et al. 2013; Khudyakov et al. 2015a). Logistical concerns associated with body size, distribution ranges, reproductive rate, life span, and protected status of marine mammals make transgenesis in these animals impossible. Cellular modeling with primary cells offers a potential solution to these constraints by allowing for the direct molecular manipulation and functional testing of omics-generated hypotheses.

Primary cells provide a live *ex vivo* model for mechanistic investigations, which are crucial for a complete understanding of the diverse mammalian adaptations to aquatic life. These investigations are currently lacking in marine mammals, though cellular modeling has been applied in concert with whole organism investigations in studies of other non-traditional mammals. Thus, primary cell culture is emerging as a critical tool for conducting functional studies that provide the missing link between genome- and organismal-level understanding of physiological adaptations in wild mammals. Here, we review how functional studies with primary cells can connect genomic phenomena to whole organism physiology broadly across mammals and specifically within marine mammals.

## Cellular modeling in non-traditional mammals

Cellular modeling with primary cells has been used successfully to study the molecular underpinnings of natural resistance to extreme conditions in several unique terrestrial mammals, providing an exceptional example of a bridge between genome- and phenome-level investigations. For example, studies of longevity and cancer resistance in elephants (*Loxodonta africana*) showed that tumor protein p53 and leukemia inhibitory factor pseudogene copy expansion correspond to enhanced p53 signaling and DNA damage response in elephant skin fibroblasts and peripheral blood lymphocytes (Abegglen et al. 2015; Sulak et al. 2016; Vazquez et al. 2018). These studies provide a functional explanation for Peto's paradox, the observation that cancer rates across species are not positively correlated with the number of cells in an organism, and help explain the evolution of large body size in mammals. Similarly, a study of cold tolerance in hibernators used neurons derived from induced pluripotent stem cells (iPSCs) from 13-lined ground squirrels (*Ictidomys tridecemlineatus*) to demonstrate that these animals survive prolonged cold exposure by suppressing mitochondrial oxidant generation, which causes lysosomal membrane permeabilization and microtubule destruction in non-cold adapted species (Ou et al. 2018). Using cellular modeling with primary cells, Singhal et al. (Singhal et al. 2020) recently discovered that a variant of ATP5G1, which encodes a proton-transporting subunit of the mitochondrial ATP synthase complex, improves mitochondrial metabolism and promotes cellular resilience to metabolic stress in arctic ground squirrels (*Spermophilus parryii*).

The naked mole rat *(Heterocephalus glaber)* has been the focus of numerous investigations into its remarkable hypoxia tolerance, extended lifespan, and cancer resistance. The sequencing of the naked mole rat genome almost a decade ago provided the first molecular insights into the fascinating physiology of this animal (Kim et al. 2011). Studies using overexpression and knockdown approaches in naked mole rat primary cells then provided functional support for the mechanisms conferring cancer resistance and extended longevity, which involve enhanced DNA repair, cell cycle arrest, and suppression of oncogenic genes by high molecular weight hyaluronan (Tian et al. 2013; Tian et al. 2015; Evdokimov et al. 2018). Other studies reprogrammed naked mole rat primary fibroblasts and iPSCs to provide evidence for the genetic and epigenetic mechanisms underlying longevity and cancer resistance in these animals (Tan et al. 2017; Zhao et al. 2018).

Proteomic and transcriptomic analyses of grizzly (*Ursus arctos horribilis*) and Japanese black bear (*Ursus thibetanus japonicus*) liver, muscle, and adipose identified key regulatory genes and pathways suppressed or activated during hibernation, including decreased insulin signaling and muscle protein degradation (Jansen et al. 2019; Miyazaki et al. 2019; Mugahid et al. 2019). These studies, coupled with functional investigations in primary adipocyte cultures and adipocytes differentiated from grizzly bear adipose-derived mesenchymal stem cells (ADSCs) are contributing to our understanding of the metabolic and regulatory mechanisms that drive a natural tolerance of food deprivation and resistance to muscle atrophy during hibernation (Fink et al. 2011; Gehring et al. 2016; Rigano et al. 2017). Endocrine manipulation of the hypothalamic–pituitary–adrenal axis in elephant seals coupled to transcriptomic and proteomic analyses of muscle and adipose also provided important clues about adipogenic factors and antioxidant genes involved in the remarkable tolerance to stress and prolonged food deprivation in this species (Khudyakov et al. 2015a, 2015b; Crocker et al. 2016; Khudyakov et al. 2017; Martinez et al. 2018; Deyarmin et al.

2019, 2020). Our group is currently developing cellular models that are amenable to gene perturbation and pharmacological manipulation, and can thus provide the missing link between genome- and phenome-level analyses in elephant seals.

## Studies with marine mammal cells

Studies using cells derived from marine mammals have been conducted since the 1960s (Feltz and Fay 1966). Several cell types from multiple tissues have been obtained from various species. These include blood cells (lymphocytes, leukocytes, granulocytes, splenocytes, thymocytes, and mononuclear cells), dermal fibroblasts, skeletal muscle myoblasts, alveolar macrophages, epithelial cells (bronchial, kidney, and skin), as well as "fibroblast-like" and "epithelial-like" cells from mesentery, lung, heart, liver, brain, spleen, thyroid, urinary bladder, periorbital soft tissue, and testes (reviewed in Boroda 2017). The majority of studies conducted in marine mammal cells are ecotoxicological investigations, which are important for marine mammal conservation. These studies, however, have not functionally tested causative mechanistic hypotheses.

The genotoxic effects of methylmercury, mercury, chromium, and titanium dioxide on primary skin, testes, or lung fibroblasts isolated from belugas (*Delphinapterus leucas*), sperm whales (*Physeter macrocephalus*), bottlenose dolphins (*Tursiops truncatus*), and fin whales (*Balaenoptera physalus*) are well described (Gauthier et al. 1998; Wise et al. 2008; Mollenhauer et al. 2009; Wise et al. 2011, 2015). Similarly, the cytotoxic and genotoxic effects of various organic pollutants on cetacean primary and immortalized fibroblasts obtained from different tissues are documented extensively (Godard et al. 2006; Marsili et al. 2008; Spinsanti et al. 2008; Wise et al. 2014; FrenzilLi et al. 2014; Burkard et al. 2015; Rajput et al. 2018; Maner et al. 2019). A recent study found increased cytokine production in response to polybrominated diphenyl ethers in immortalized dolphin fibroblasts, suggesting that this contaminant activates inflammatory pathways (Rajput et al. 2018). In contrast, immortalized whale and dolphin skin fibroblasts (Rajputet al. 2018; Burkard et al. 2019), and Weddell seal (*Leptonychotes weddellii*) peripheral blood mononuclear cells (Bagchi et al. 2018) showed a blunted response to bacterial lipopolysaccharide (LPS), though dolphin leukocytes respond to LPS (Ohishi et al. 2011). These results suggest cell type- and species-specific responses to inflammatory stimuli in marine mammals. The adaptive evolution of innate immunity in marine mammals is an active research topic (Shen et al. 2012; Ishengoma and Agaba 2017; Xu et al. 2019). The development of cellular models will provide a platform to test mechanistic hypotheses generated by omics data in this area.

Comparative biochemical studies have provided information about the cellular responses associated with heavy metal toxicity resistance and hypoxia tolerance in marine mammals. For instance, elephant seal primary muscle cells mount a robust antioxidant response against heavy metal toxicity compared to human cells (Del Aguila-Vargas et al. 2020). Similarly, Weddell seal primary muscle cells display increased plasticity in myoglobin concentrations in response to hypoxia when compared to immortalized mouse muscle cells (De Miranda et al. 2012). Mechanistic studies that manipulate gene levels in marine mammal cells are, however, still lacking. Non-targeted metabolomics and lipidomics studies of polar bear (*Ursus maritimus*) plasma (Tartu et al. 2017) coupled with investigations conducted in polar bear adipocytes derived from ADSCs identified the effects of pollutants on adipogenesis and lipid metabolism in these cells; pollutants induced first wave adipogenesis but suppressed terminal differentiation, negatively impacting fat stores in this species (Routti et al. 2016). Together, these studies provide an example of the promise of using primary cell culture to bridge omics and mechanistic level investigations in marine mammals.

To the best of our knowledge, exogenous DNA has been introduced into marine mammal cells with the sole purpose of immortalization. There are no published reports of other genetic manipulations such as gene knockdown, over-expression, or editing in marine mammal cells. Marine mammal cell cultures have been immortalized by expressing simian virus large T antigen (SV40) in bowhead whale renal proximal tubular cells (Goodwin et al. 2000), and skin fibroblasts isolated from Yangtze finless porpoises (*Neophocaena phocaenoides*) (Wang et al. 2011), Indo-Pacific humpbacked dolphins (*Sousa chinensis*) (Jin et al. 2013), pygmy killer whales (*Feresa attenuata*) (Yajing et al. 2018), and spotted dolphins (*Stenella attenuata*) (Rajput et al. 2018). Immortalized humpback whale (*Megaptera novaeangliae*) fibroblasts were obtained by expression of telomerase reverse transcriptase (Burkard et al. 2019).

While primary cells closely recapitulate the physiology of their tissue of origin, they are subject to the Hayflick phenomenon (Hayflick and Moorhead 1961) and will not divide indefinitely in culture, requiring replenishment of research materials from

field sampling. Immortalization methods induce mutations, which prevent cells from entering senescence. The use of immortalized cell lines can be a useful alternative to the use of primary cells, especially when working with limited, difficult-to-obtain material collected from free-ranging animals. Immortalization, however, may irreversibly alter the cellular phenotype, particularly with regard to metabolic properties (Geraghty et al. 2014). Accordingly, confirmation that immortalized cells accurately recapitulate primary cell phenotypes is critical for drawing biologically relevant conclusions. Functional studies in immortalized cells coupled to comparative genomic analyses (Seim et al. 2013; Zhang et al. 2013) were successfully used to study mechanisms of longevity, cancer resistance, innate immunity, flight and viral dynamics in pteropid bats (Crameri et al. 2009; Biesold et al. 2011; Kühl et al. 2011; Brook et al. 2020). Thus, immortalized cells could provide an alternative platform for conducting mechanistic investigations in marine mammals.

iPSCs are a novel tool that can be used for mechanistic studies on difficult-to-obtain cell types, an advantage which is of particular interest to those studying non-model mammalian systems (Miyawaki et al. 2016; Lee et al. 2017; Lee et al. 2018; Ou et al. 2018, 2019). iPSCs are stem cells that can be differentiated into any cell type, and in which candidate genes and cellular pathways underlying relevant physiological adaptations can be manipulated using pharmacological, RNA interference (RNAi), or gene editing approaches. To the best of our knowledge, no marine mammal iPSCs have been generated, but we and others (Boroda et al. 2015) have obtained viable, metabolically active skin fibroblasts from marine mammals that could be used for this purpose or, potentially, for direct reprogramming into other cell types (Ambasudhan et al. 2011; Huang et al. 2014; Li et al. 2014; Lalit et al. 2016; Bar-Nur et al. 2018). iPSC generation and direct reprogramming methods represent a unique opportunity to study molecular- and cellular-level physiology in live, proliferating cells for tissues that are difficult or impossible to access in living marine mammals (e.g., cardiac muscle, lung, and nervous tissues).

ADSCs from the stromal vascular fraction of the blubber have been cultured from several marine mammal species including dolphins (Johnson et al. 2012; Griffeth et al. 2014), humpback whales (Hoogduijn et al. 2013), polar bears (Routti et al. 2016), and elephant seals (Louis et al. 2015). ADSCs are stem cells that can be differentiated into adipogenic, osteogenic, chondrogenic, myogenic, angiogenic, tenogenic, and periodontogenic lineages, which may not otherwise be readily obtained from living marine mammals (Mizuno 2009; Mizuno et al. 2012). Marine mammal ADSCs are, therefore, another potentially important tool for testing mechanistic hypotheses generated by omics data in a physiologically relevant context.

## Considerations for working with marine mammal cells and future directions

The application of omics tools to marine mammal research has been critical in identifying candidate genes underlying mammalian adaptations to aquatic life and cellular pathways activated or repressed during distinct physiological states. Functionally testing hypotheses generated by these approaches, however, is logistically impossible in marine mammals at the whole animal level. Cellular modeling with primary cells allows manipulation of gene levels using pharmacological or molecular tools. Therefore, the combination of whole animal multi-omics approaches and primary cell culture in marine mammals is emerging as a powerful tool to elucidate the cellular and molecular mechanisms underlying the physiological adaptations characteristic of an aquatic lifestyle (Fig. 1).

Developing cellular cultures from non-model species is challenging. It requires optimization of conditions including isolation methods, extracellular matrix and growth media composition, cell growth density, and expansion and differentiation protocols. Additionally, there are limitations to using primary cells. Unlike immortalized cells, primary cells have a finite replicative life span and will eventually undergo cellular senescence. Extensive passaging can alter the characteristics of primary cells and there may be individual variation across animals; it is therefore important to control for age and passage number when designing experiments. Finally, traditional cell culture models lack some of the metabolic regulatory mechanisms and complex spatial interactions of *in vivo* systems and 3D organoid models. Despite these limitations, primary cells recapitulate important molecular and biochemical characteristics of the *in vivo* phenotype. Moreover, this approach is the only system in which transgenesis is possible for marine mammals. Thus, cellular modeling with primary cells is a promising tool for conducting mechanistic investigations in marine mammals.

Our team has established methods for the isolation, culture, and differentiation of several primary cell types from elephant seals including myoblasts, dermal fibroblasts, vascular endothelial cells, and ADSCs (Figs. 2A, B). These cells are amenable to

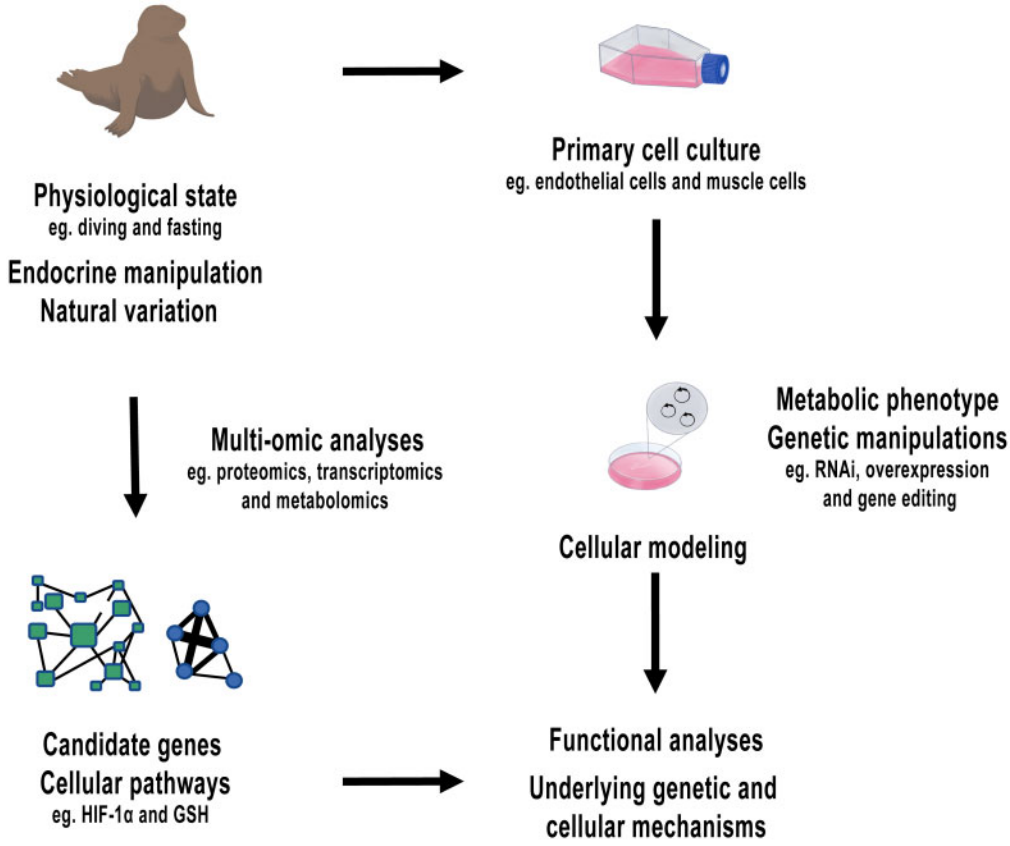


**Fig. 1.** Conceptual diagram of the proposed use of primary cells as a tool to bridge genome-to-phenome investigations in marine mammals. Whole organism physiological studies in marine mammals are limited to the study of natural variation and endocrine manipulations in select species. Primary cells retain species-specific features that can be functionally studied using gene manipulation and pharmacological approaches. Multi-omic analyses can inform these investigations by identifying candidate genes and cellular pathways (e.g., HIF-1α and GSH) implicated in adaptive physiological phenomena such as diving and fasting. Together, these studies can uncover the genetic and cellular mechanisms that underlie physiological adaptation in marine mammals.

perturbation using pharmacological, knockdown, and overexpression approaches (Figs. 2C, D). Hence, these cells provide a platform to conduct mechanistic studies that directly test hypotheses generated by omics data.

Isolation of primary cells begins with tissue collection (biopsy) in the field. Tissues are immersed in a sterile physiological solution and transported on ice to the laboratory on the day of collection. Cell isolation methods vary by cell type but generally include both mechanical and enzymatic dissociation steps. Enzymatic dissociation may utilize a variety of enzymes, including collagenase, dispase, elastase, or trypsin; enzyme selection is tissue- and cell type-specific. Dissociated tissue products are plated on tissue culture treated or otherwise coated surfaces in cell type-specific growth medium and incubated in a standard tissue culture incubator. Cell growth is visually monitored using low magnification light microscopy in the days following plating. Enrichment for specific cell types is conducted using fluorescence activated cell sorting or traditional methods such as pre-plating. Of considerable concern in primary cell culture is the avoidance of bacterial and fungal contamination. To this end, use of a HEPA-filtered biosafety cabinet is absolutely critical, as is the use of sterile technique to the extent possible during collection and isolation, and the appropriate addition of antimicrobial agents to culture media. Perhaps the most limiting constraint in marine mammal cell culture is the near-requirement that tissues be fresh; cryopreservation by any method severely reduces cell viability upon thawing, restricting opportunities for cell culture to those sampling scenarios in which equipped laboratory facilities can be reached within a day. While the above-mentioned requirements may somewhat constrain sampling opportunities for the development of marine mammal cell cultures, there remain extensive opportunities for collecting viable marine mammal tissue samples for culture.

Characterization of cellular preparations is of paramount importance to mimicking *in vivo* conditions

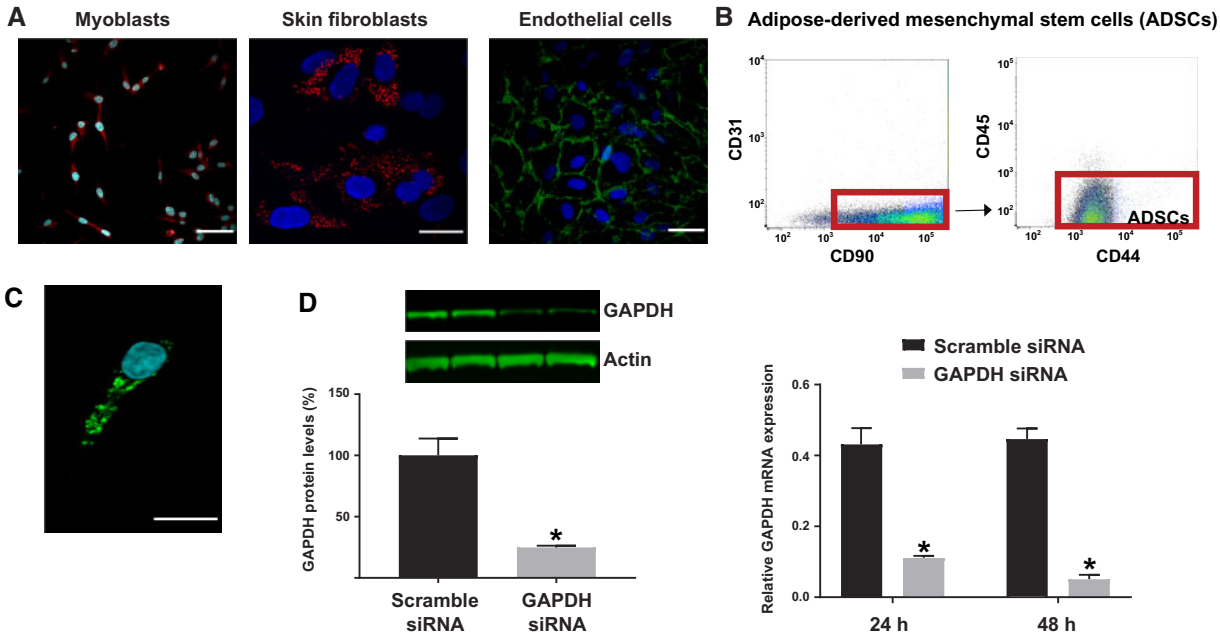


**Fig. 2.** Primary cells isolated from elephant seals offer a platform to study tissue-specific adaptive mechanisms *via* genetic manipulations. (**A**) Elephant seal cells stained with antibodies specific for each cell type: desmin (red) for myoblasts, platelet-derived growth factor receptor (red) for fibroblasts, and platelet endothelial cell adhesion molecule (CD31, green) for endothelial cells, and imaged using a confocal microscope. (**B**) Flow cytometry plots showing that elephant seal ADSCs stain positive for the mesenchymal stem cell markers CD90 and CD44, and negative for the endothelial cell marker CD31 and the lymphocyte marker CD45. (**C**) Elephant seal myoblast expressing eGFP. (**D**) Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression knockdown in elephant seal myoblasts transfected with scramble or GAPDH siRNAs (Ambion Silencer Select GAPDH siRNA, catalog # 4390849) for 24 or 48 h. GAPDH protein abundance was measured by western blot. GAPDH mRNA expression was measured by RT-qPCR using elephant seal-specific GAPDH primers (FWD 5'- CAAGGCTGAGAACGGGAAGC - 3'; RVS 5'-ATCGGCAGAGGGAGCAGAGA - 3') and actin as a housekeeping gene (FWD 5'- CGGTCAGTTCATGGCTGAGG-3'; RVS 5'- AAGGCTCGGACCTTCCCAAC - 3'). Results are expressed as mean ± SD. Differences between cells treated with scramble or GAPDH siRNA sequences were evaluated using *t*-test. $^{*}P < 0.001$.

in an *ex vivo* setting. Measuring the expression of cell type-specific genes and proteins confirms the purity of a given culture. Assessments of other cell type-specific features such as morphology and differentiation capacity further support expression analyses in confirming a preparation's phenotype. Metabolic phenotypes are also crucial for the functional characterization of cells in primary culture. In addition to basic characterizations of doubling time and senescence via traditional methods, refined analyses of oxygen consumption and extracellular acidification rates in intact cells are now possible using such technologies as Agilent's Seahorse extracellular flux analyzer (Fig. 3). These assays provide an expanded understanding of fuel preference and utilization under various conditions, a critical consideration in marine mammal physiology as these animals spend extended periods submerged or fasting as part of their life histories. Other important considerations when working with marine mammal cells include obtaining transcriptomic and epigenomic profiles.

Comparative genomic analyses recently identified the convergent loss of paraoxonase 1 function in marine mammals; this loss likely increases sensitivity to organophosphate pollutants and suggests an impaired capacity to hydrolize lipid peroxides (Meyer et al. 2018). Cellular models can help to test this hypothesis by conducting comparative overexpression and knockdown/gene editing experiments in terrestrial and marine mammal cells. Similarly, recent transcriptome analyses identified key genes regulated in response to experimental stress elevations in elephant seal skeletal muscle (Khudyakov et al. 2015a, 2015b). We are currently conducting follow-up transcriptomic and functional studies using elephant seal muscle cells to characterize the cellular response that allows these animals to adapt to energetic stress.

It is important to mention that few transcriptomic or proteomic studies have been conducted in cell culture systems compared to *in vivo* studies with marine mammals. Studies with primary cells can

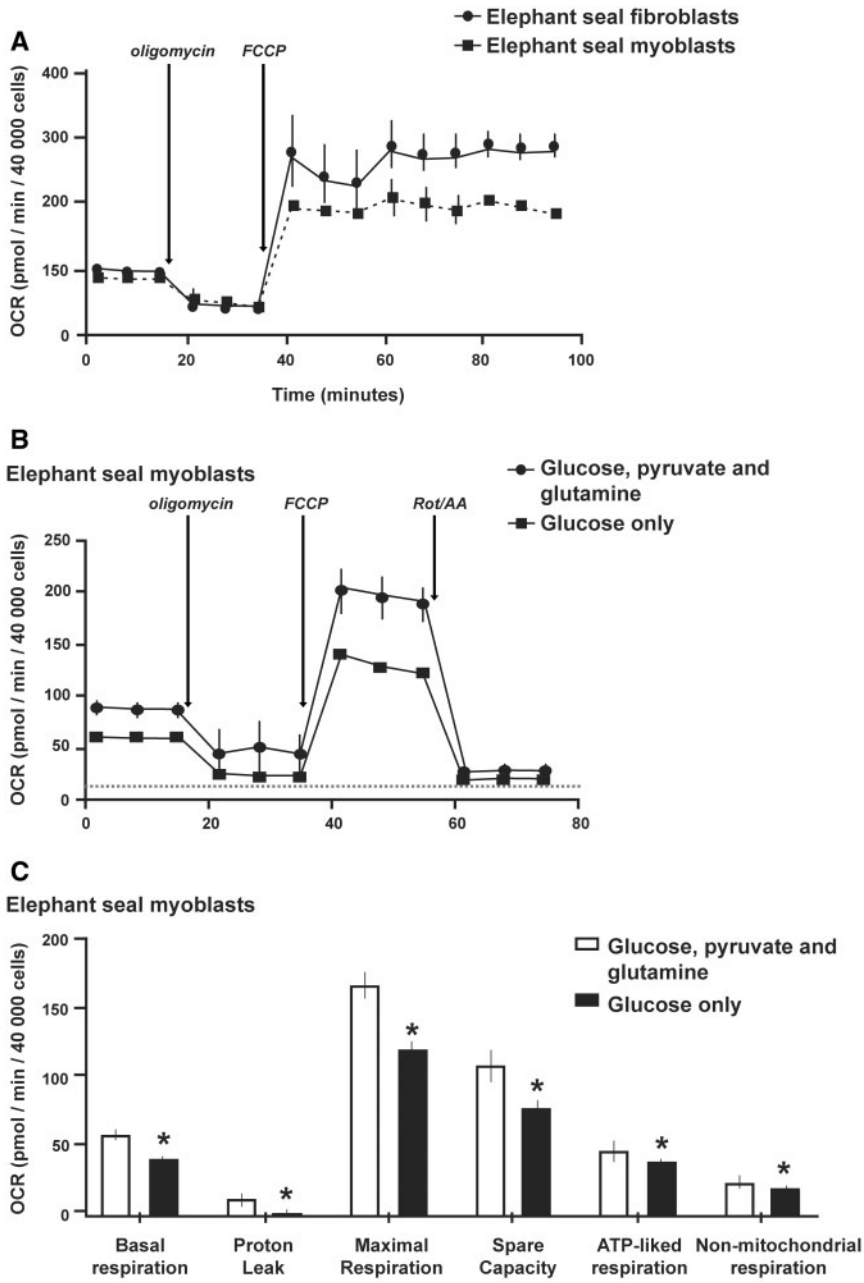


**Fig. 3.** Oxygen consumption rates (OCRs) in primary cells isolated from elephant seals. (**A**) OCR measured in skin fibroblasts and myoblasts in the presence of glucose (6 mM), pyruvate (1 mM), and L-glutamine (1 mM). Changes in OCR correspond to the addition of oligomycin (1 μM) and carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP, 2 μM). Arrows indicate the time of drug injection. (**B**) OCR in elephant seal myoblasts in the presence of glucose with or without pyruvate and L-glutamine. Changes in OCR correspond to the addition of oligomycin, FCCP, and rotenone/antimycin A (0.5 μM). (**C**) Mitochondrial function parameters in elephant seal myoblasts in the presence of glucose with or without pyruvate and L-glutamine. The dotted line indicates non-mitochondrial respiration. OCR was measured using Seahorse Cell Energy Phenotype (A) and Mito Stress kits (B) and a Seahorse Extracellular Flux Analyzer (Agilent). Values in (C) were calculated according to Divakaruni et al. (2014). Results are expressed as mean ± SEM, $n = 6$. Data were analyzed using *t*-test. $^*P < 0.05$.

highlight other potential players that are important for *in vivo* phenotypes such as circulating factors (Rigano et al. 2017). The identification of pathways involved in epigenetic regulation such as a decrease in the epigenetic transcriptional repressor SUV420H1 after experimental stress elevations in elephant seals (Khudyakov et al. 2017) adds another layer to ongoing multi-omics experiments in marine mammals. Epigenomics along with functional testing in marine mammal cells warrants further investigation.

## Conclusion

Major advancements in biomedical research including the generation of iPSCs and the development of gene editing technologies have expanded the potential for elucidating the mechanisms underlying adaptive physiological phenomena in non-traditional organisms. The application of these methods has relieved many of the technical constraints that have previously limited comparative physiology studies in non-model systems. The functional insight gained from these studies may fundamentally transform our understanding of the physiological advantages that enabled marine mammals to successfully reinvade the aquatic environment, confer tolerance to extreme conditions, and resistance to disease and aging. The application of tools that bridge genome-to-phenome investigations—primary cell culture included—are just emerging in marine mammals but have great promise for facilitating our ability to understand the gaps between whole organisms, systems, cells, and molecular level functions while accelerating the field of comparative physiology.

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