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Leptin Integrates Vertebrate Evolution: from Oxygen to the Blood-Gas Barrier

J.S. Torday^{1,*}, F.L. Powell², C.G. Farmer³, S. Orgeig⁴, H.C. Nielsen⁵, and A.J. Hall⁶

¹Department of Pediatrics, University of California-Los Angeles, CA

²Department of Medicine, University of California-San Diego, La Jolla, CA

³University of Utah, Salt Lake City, UT

⁴University of South Australia, Adelaide, Australia

⁵Department of Pediatrics, Tufts Medical Center, Boston, MA

⁶Sea Mammal Research Unit, University of St. Andrews, St. Andrews, United Kingdom

Abstract

The following are the proceedings of a symposium held at the Second International Congress for Respiratory Science in Bad Honnef, Germany. The goals of the symposium were to delineate the blood-gas barrier phenotype across vertebrate species; to delineate the interrelationship between the evolution of the blood-gas barrier, locomotion and metabolism; to introduce the selection pressures for the evolution of the surfactant system as a key to understanding the physiology of the blood-gas barrier; to introduce the lung lipofibroblast and its product, leptin, which coordinately regulates pulmonary surfactant, type IV collagen in the basement membrane and host defense, as the cell-molecular site of selection pressure for the blood-gas barrier; to drill down to the gene regulatory network(s) involved in leptin signaling and the blood-gas barrier phenotype; to extend the relationship between leptin and the blood-gas-barrier to diving mammals.

1. Introduction

The distinction between the proximate and ultimate causes of evolution is beginning to break down as we learn more about the molecular mechanisms of adaptation. Much of evolutionary investigation to date has been descriptive, whether regarding gross anatomical structure or the genes associated with these structures. The evolution of physiologic function has largely been ignored, because it is not the evolutionists criterion of being testable with fossil evidence, since there is no such record for soft tissues. But with the advent of molecular evolution, we are no longer limited to paleontologic data as the ultimate arbiter of the evolutionary process. The biologic history is embedded in the genes that determine

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*Corresponding Author: J.S. Torday, Phone: 310-222-8186, FAX: 310-222-3887, jtorday@labiomed.org.

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structure and function, allowing us to trace the emergence and contingency backwards in morphologic space and time (Torday and Rehan, 2004). The evolution of the lung represents a key adaptation by land vertebrates to atmospheric oxygen, which fluctuated between 15 and 35% over the last 500 million years, undoubtedly generating dramatic selection pressure (Berner et al., 2007) from the alternating impacts of both hyperoxia and hypoxia on physiologic systems. By understanding the diverse nature of the lung phenotype in amphibians, reptiles, mammals and birds, we set the stage for uncovering the underlying mechanisms that have resulted in such a variety of respiratory answers to the evolutionary puzzle as to how to extract oxygen from the environment and fuel metabolism. Conversely, by solving this puzzle, we gain insight into the first principles of physiology.

By further reducing the phenotype to a particular functionally critical substance, and then reverse-engineering its origins at the cell-molecular level, we can determine the cell-cell interactions that have generated the wide variety of lung forms and functions based on cell-molecular gene regulatory networks, rather than deducing their evolutionary origins based on phenotypic structural and functional homologies. Such knowledge would form the basis for understanding ultimate causation in physiology and medicine.

We began this symposium with a broad overview of the variety of vertebrate lung phenotypes that have evolved to facilitate gas exchange across the blood-gas barrier. The seeming cause-effect interrelationship between the efficiency of gas transfer across this barrier and the gross and fine structure of the lung points to underlying cell-molecular mechanisms responsible for these changes across phylogeny and ontogeny.

2. The structure and function of the blood-gas barrier (Powell, 2009)

The respiratory system is primarily responsible for oxygen and carbon dioxide exchange. Since the process of gas exchange occurs by passive diffusion across the blood-gas barrier, and the gas molecules cannot be modified, the gas-exchangers have evolved to accommodate respiration. As a result, the gas-exchanger phenotype varies by orders of magnitude among species, is extremely thin, and has an enormous surface area to promote diffusion. On the other hand, physiological demands favor a thick and strong blood-gas barrier because hydrostatic pressures in pulmonary blood vessels can reach very high levels due to exercise demands on cardiac output, increasing vascular wall stress, which can result in capillary failure and leak. By systematically determining how animals have accommodated gas exchange across the blood-gas barrier, we gain an understanding of the evolution of this structure. Generally speaking, comparative studies of the blood gas barrier have been focused on the diffusing capacity in amniotes, rather than on their biomechanics. Moreover, the diffusing capacity is commensurate with the metabolic demands across classes and species. Reptiles with active life-styles have relatively larger diffusing capacities to support relatively low oxygenation as a result of increased surface area and increased compartmentalization of the lung. As lungs have evolved, surface area increases with the increasing surface:volume ratio of the smaller chambers. The surface area of the lung also increases as a function of the complexity of lung parenchymal type, from trabecular to faveolar lungs. There are two major patterns by which lungs subdivide into smaller chambers- (1) heterogeneous partitioning of the avian respiratory system separates the

functions of gas exchange (in the constant-volume parabronchial lung that receives all of the pulmonary blood flow), and ventilation (by the air sacs that do not directly participate in gas exchange); (2) the alveoli in homogeneously partitioned mammalian lungs mediate both gas exchange and ventilation. Functionally, the gas-exchange surface area in birds is only 15% greater than similarly sized mammals, yet the thickness of the avian gas-exchange surface is less than half that of mammals, greatly increasing the diffusing capacity in birds compared to mammals. Watson et al. (Watson et al., 2008) hypothesized that the difference in thickness between the blood-gas barriers of birds and mammals results from the unique parenchymal structure of the parabronchial lung, which strengthens the pulmonary capillaries, allowing them to resist stress failure with extremely thin walls. These observations indicate that future comparative studies should consider both the biomechanical and gas exchange properties of the blood-gas barrier to understand the evolution of lungs.

This broad structure-function view of the lung phenotype can be further reduced as an organ of gas exchange, looking at it from the perspective of the evolutionary relationship between metabolism, body size and gas exchange. These insights help to further extrapolate from the lung phenotype to other organ system phenotypes.

3. Was the blood gas barrier a constraint on activity metabolism, and thereby body size of Mesozoic mammals? (Farmer et al, 2009)

Of the Late Carboniferous and Permian, synapsids were the most common and diverse large, terrestrial animals. However, synapsid diversity sharply declined in the Late Triassic and the archosaurs supplanted the synapsids in niches of large body size throughout the Jurassic and Cretaceous. During this time, synapsids evolved into tiny mammals, in the size range of mice and shrews. Was this faunal turnover a historical contingency, or were these mammalian synapsids unable to occupy ecological niches suited to large body sizes? Recently, it has been proposed that the alveolar lung arose in synapsids during the Permian, when high levels of atmospheric O₂ tipped the balance between a thin or strong blood-gas barrier in favor of strength, and that the synapsids were subsequently handicapped by fell in the Late Permian (Farmer, In press). Dr. Farmer's laboratory is this design when levels of O₂ examining the plausibility of this hypothesis with a mathematical model of diffusion of O₂ across the blood-gas barrier while red blood cells (RBCs) transit pulmonary capillaries. Because it is unclear when synapsid RBCs lost their nuclei, they are assessing levels of saturation in capillaries of sufficient diameter to allow the transit of nucleated RBCs and as well as the smaller capillaries typical of mammals. For a given transmural pressure, the capillary wall thickness must be increased for the larger capillaries to account for the increased tension in the wall (the law of Laplace), and this reduces arterial saturation. The model suggests blood flow through capillaries that are tailored to withstand the mechanical stresses of ventilation and the hydrostatic pressures of heavy exercise will not saturate under the levels of O₂ presumably present in the Triassic (12% O₂). Possibly the maximal rates of sustained activity were constrained in Mesozoic synapsids by their alveolar lungs, and synapsids were therefore restricted to small body size niches because of relaxed selection in these niches on aerobic activity metabolism. Based on cursorial adaptations in extant

terrestrial tetrapods, and on the scaling relationship of maximal rates of O₂ consumption with body size among terrestrial mammals, a body mass threshold of approximately 4–5 kg appears to exist. Below this size, terrestrial animals occupy niches where camouflage and secretive behaviors are more important than aerobic capacity. Thus, we suggest one explanation for the small body size of Mesozoic mammals is rooted in the alveolar lung morphology and its inherent constraints on the blood-gas barrier.

In order to transition from the gross structural and functional phenotypes of the lung back to its evolutionary origins, it must be reduced to a molecular phenotype on which selection pressure has acted to generate those structural and functional phenotypes. Orgeig and Daniels have traced the evolution of lung surfactant from fish to mammals and birds, demonstrating the qualitative changes in the lipid and protein components of this substance that are associated with the thinning of the blood-gas barrier. This is a critical step in the pursuit of the evolution of the lung because it reduces the phenotype to an entity that can then be further deconstructed at the cell-molecular level.

4. The surfactant system and evolution of the blood-gas barrier (Orgeig et al, 2009)

Pulmonary surfactant is a complex mixture of highly conserved lipids and proteins that forms a molecular interface at the surface of the blood-gas barrier of all vertebrate lungs. Its primary roles are to reduce alveolar surface tension and maintain alveolar stability, prevent adhesion of epithelial surfaces and increase lung compliance (Daniels et al., 2001). Interestingly, relative differences in surfactant composition, function, regulation and development between vertebrate groups have been observed. Since these differences are consistent with adaptive advantages, it has been suggested that they are due to evolutionary strategies. This is reasonable since surfactant function is determined by the physical interactions of the lipids and proteins, and any environmental force capable of altering these interactions could exert long-term selection effects on the system. Beyond the proximate effects of the surfactant itself, numerous humoral and biochemical factors regulate surfactant synthesis and secretion in both adults and during development. All these factors are under environmental control, representing molecular mechanisms through which exogenous environmental changes can be transduced to alter surfactant properties. Such environmental variables as temperature, pressure and hypoxia have exerted powerful evolutionary selection pressures that have shaped the evolution of the surfactant system in both adult and developing lungs (Orgeig and Daniels, 2009).

4.1 Temperature

The pulmonary surfactant system is composed of 90% of lipids, and lipid physicochemical properties are profoundly affected by temperature. Therefore the body temperature of an organism is critically important in determining the composition and function of the surfactant lipids. As a result, the interaction of temperature with the physicochemical properties of the lipid mixture has evolutionarily constrained the surfactant system, and has driven the evolution of surfactant lipid composition. Temperature has selected both the broad-scale evolutionary differences in surfactant lipid composition between the vertebrate

groups, and the acute changes in surfactant composition, structure and function within individuals (Lang et al., 2005; Orgeig et al., 2007). For example, those vertebrate groups with lower preferred body temperatures, i.e. fish and amphibians, have higher relative concentrations of cholesterol and unsaturated phospholipids, as these have lower phase transition temperatures, and are capable of maintaining a fluid state at these lower body temperatures (Daniels et al., 1995). However, there is an evolutionary trade-off between maintaining fluidity on the one hand, and lowering surface tension on the other (Orgeig and Daniels, 2009). Both cholesterol and unsaturated phospholipids do not readily or profoundly lower surface tension when they are compressed. Therefore, it is only the vertebrate groups with the higher preferred body temperatures, i.e. reptiles, birds and mammals, that are capable of tolerating high levels of disaturated phospholipids that have high phase transition temperatures, but are capable of dramatically reducing surface tension (Lang et al., 2005a).

At the individual level, in lizards and heterothermic mammals, including dunnarts, bats and squirrels, the reduced body temperature as animals enter torpor or hibernation correlates with an increase in cholesterol relative to the phospholipids (Daniels et al., 1990; Lang et al., 2005b). In dunnarts and bats, this compositional change is also associated with changes in surface activity as measured in vitro, such that the surfactant is more functional at the in vitro temperature that matches the body temperature of the animal from which it was isolated (Codd et al., 2002; Lopatko et al., 1998). For example, surfactant from torpid dunnarts is capable of lowering surface tension to a greater extent at 15 or 20°C than at 37°C, and vice versa for warm-active surfactant (Lopatko et al., 1998; Lopatko et al., 1999). Moreover, these compositional and functional changes are reversible upon arousal (Codd et al. 2003; Lopatko et al. 1999). It has been recently shown that these functional changes correlate with structural differences at the molecular level of the surfactant film (Orgeig et al. 2007). The molecular, biophysical and thermodynamic mechanisms underlying these differences are currently being investigated.

4.2 Pressure

Stretching of the alveolar basement membrane (reviewed by (Edwards, 2001)) and distension of the lung by fetal lung fluid (Lines et al., 1999) have profound effects on surfactant secretion and maturation, respectively. The regular compression and relaxation of the lung upon expiration and inspiration exert significant cyclical forces on the lipids of the surfactant film directly. Hence, any environmental force that alters these physical forces has the potential to alter the function of surfactant. Foot et al 2006 have recently completed a series of studies on the molecular, biochemical, biophysical and cellular adaptations of the pulmonary surfactant system of diving mammals. They hypothesized that the elevated hydrostatic pressure experienced by diving mammals has acted as a selection pressure to modify surfactant composition and function in order to cope with the repeated collapse and re-inflation of the lung that occurs during diving (Foot et al., 2006). They discovered a suite of adaptations, including alterations in the primary sequence of surfactant protein C, which is important in stabilizing the surfactant lipid film (Foot et al., 2007), and alterations in the phospholipid composition leading to a more fluid surfactant lipid mixture (Miller et al., 2006b), which demonstrated reduced surface activity, consistent with an anti-adhesive function of the surfactant designed to prevent adherence of alveolar surfaces following lung

collapse (Miller et al., 2006a). In addition, there were cellular adaptations indicating a greater resistance of alveolar type II cells derived from sealions to the stimulatory effects of high atmospheric pressure (Miller et al., 2004). It is this combination of adaptations at different organizational levels that contributes highly significantly to changes in surfactant function in diving compared with terrestrial mammals.

4.3 Hypoxia

Experimental evidence for the effects of hypoxia on the adult pulmonary surfactant system is dated and extremely scant, but suggests that there are some modifications to the surfactant lipids which result in impaired surface activity (reviewed by (Orgeig and Daniels, 2009)). On the other hand, there is some recent evidence both in egg laying amniotes and in live-bearing mammals that hypoxia exerts its selective force during the embryonic developmental period, possibly indirectly via its effect on corticosteroids. Moderate short-term hypoxia during the second half of the incubation period accelerates overall development, and up-regulates synthetic and secretory pathways of surfactant lipids in chicken embryos (Blacker et al., 2004). The mechanism likely involves plasma corticosterone, which increases in response to hypoxia during embryonic development, and administration of exogenous glucocorticoids leads to similar acceleration of surfactant lipid synthesis and secretion (Blacker et al., 2004). However, the timing of the hypoxic onslaught is important in determining the outcome for the organism, as chronic hypoxia for the entire developmental period leads to significant retardation in surfactant development with delayed hatching in a proportion of chicken embryos (unpublished data). In humans, periods of hypoxemia during development are an important contributing factor to fetal growth restriction. In the sheep model of intrauterine growth restriction, the carunclectomised sheep (Morrison, 2008), placental restriction with concomitant hypoxemia and hypoglycemia are induced by removing the majority of implantation sites before conception in order to investigate the impact of chronic hypoxemia on surfactant maturation. In this model, contrary to other more acute models of hypoxemia (Braems et al., 2000; Gagnon et al., 1999), surfactant protein and gene expression were reduced in lung tissue of growth restricted fetuses at two time-points during mid to late gestation (Orgeig et al., under review). Hence, it appears that vertebrates have developed different adaptations for short term versus chronic periods of hypoxia. Short term hypoxemia stimulates surfactant synthesis and secretion, enabling early hatching or early birth, and hence escape from the adverse environment. However, the severe shortage of substrates imposed by chronic hypoxemia necessitates delayed, or at least full-term, hatching or birth to complete the pulmonary developmental process in readiness for air-breathing.

These interactive mechanisms of surfactant regulation can now be further reduced to cell-cell signaling mechanisms that transduce such interactions to regulate surfactant. Lipofibroblasts in the alveolar interstitium produce leptin in response to the stretching of the alveolar wall, stimulating the synthesis and secretion of lung surfactant and anti-microbial peptides, promoting alveolar capillary perfusion, and stimulating the synthesis of type IV collagen. The relationship between alveolar distension, surfactant production and blood flow through the alveolus is classically recognized physiologically as Ventilation-Perfusion Matching. The regulation of type IV collagen and anti-microbial peptides by leptin provides

new portals connecting alveolar homeostasis to other tissues and organs based on the molecular evolution of epithelial barrier function.

5. Leptin signaling and the evolution of the blood-gas barrier. (Torday and Rehan, 2009)

Lipofibroblasts are found in the alveolar walls of a wide variety of mammals, including man (Rehan et al, 2006). These cells play a key role in protecting the lung against oxygen free-radicals (Torday et al, 2001), as well as regulating pulmonary surfactant production in response to the stretching of the alveolar wall (Torday et al, 2002a). Since muscle stem cells spontaneously differentiate into fat cells in 21% oxygen, but not 6% oxygen (Csete et al, 2001), we have speculated that the lipofibroblasts evolved to protect the alveolar wall against oxidant injury. Moreover, these cells produce leptin (Torday et al, 2002b), a pleiotropic hormone that regulates a variety of alveolar type II cell functions, including surfactant production (Torday et al, 2002b), antimicrobial peptides for host defense (Kanda and Watanabe, 2008), and collagen synthesis. The expression of leptin is regulated by Parathyroid Hormone-related Protein, a stretch-regulated paracrine growth factor (Torday et al, 2002b), functionally interlinking alveolar distension to surfactant production. This paracrine mechanism for alveolar homeostasis may have evolved to accommodate the metabolic drive for oxygen (Torday and Rehan, 2004) by increasing the efficiency of surfactant production, allowing for the progressive decrease in alveolar size and the concomitant increase in the blood-surface area ratio (Torday and Rehan, 2009b).

To further test the role of leptin in vertebrate lung evolution, *Xenopus* tadpole lungs were treated with leptin (Torday et al, 2009c) and observed developmental changes in lung structure and function similar to those seen in mammalian lungs, which was unexpected since the frog lung is not under the same physiologic constraints for oxygenation. Leptin stimulated lung epithelial cell surfactant phospholipid and protein expression, decreased epithelial cell height, and thinned the basement membrane, causing an overall increase in air space diameter. All of these observed effects of leptin on lung development are molecularly homologous to processes that have occurred both phylogenetically and developmentally during vertebrate lung evolution from fish (swim bladder) to man. We have demonstrated the integrating effects of the environmentally-sensitive (both internal and external), pleiotropic hormone leptin on the development of the *Xenopus laevis* tadpole lung in transition from water to land. It provides an evolutionary-developmental integrating mechanism for gene regulatory networks both within the lung, and between organs, from molecular oxygen, to cells, to complex physiologic traits (Torday and Rehan, 2009b).

The central purpose of this symposium was to show that reducing the lung phenotype to its functional components would lead to its molecular origins. Leptin signals to integrate key lung epithelial barrier components such type IV collagen and surfactant through the Epidermal Growth Factor receptor to linking of ErbB dimers, providing a way of mechanistically interrelating phylogenetic and ontogenetic phenotypic changes in the epithelial barrier. These molecular links provide a way of determining if those changes are causal.

6. ErbB and Leptin Pathways in Blood Gas Barrier Development and Homeostasis: Model of Evolutionary Cooperativity (Nielsen et al, 2009)

Epidermal growth factor receptor (EGF-R) is one of the four mammalian ErbB receptors. The EGF-R and its ligands have important developmental roles in the maturation of skin barrier function, the maturation and function of the gut villous epithelial barrier, and maturation of breast glandular epithelial function. Thus it is not surprising that EGF-R and other ErbB receptors also function in the development and regulation of function of the alveolar blood gas barrier. In this function they act, at least in part, through an “inside-out” process involving fibroblast-type II cell communication. The four ErbB receptors are membrane-associated tyrosine kinase receptors that act as receptor homo- and heterodimers in response to ligands of EGF-R, ErbB3 and ErbB4 (ErbB2 has no known ligand). Studies show the presence and activity of each ErbB receptor in developing lung fibroblasts and type II cells. The activity of protease enzymes TACE (Tumor Necrosis Activating Factor) and gamma secretase are important in the function of EGF-R and ErbB4 in promoting and maintaining alveolar type II cell function. TACE activity releases ErbB ligands such as TGF alpha, heparin-binding EGF, and Neuregulin (NRG) that are important for epithelial barrier maturation in multiple systems, including the lung. In the developing lung, TACE activity in fibroblasts releases NRG, the primary ligand of ErbB4. Upon NRG binding to ErbB4 in type II cells TACE and gamma secretase act in concert to induce receptor nuclear transport and gene regulation. The two step cleavage of ErbB4 by TACE and gamma secretase at the intracellular membrane releases the intracellular fragment which traffics to the nucleus with chaperone proteins YAP and STAT5. TACE, gamma secretase, and YAP all show developmental regulation in concert with the development of type II cell for the development and maintenance of the alveolar space and the alveolar type II cell barrier function. Deletion of ErbB4 interferes with the innate pulmonary immune system, triggering an inflammatory response. Mice with pulmonary deletion of ErbB4 exhibit decreased surfactant protein D (SP-D) expression, reduced saccular size, increased saccular wall thickness and volume density, and increased number of neutrophils and macrophages. These changes begin in late fetal development and persist into adulthood.

Crosstalk between the leptin receptor and ErbB signaling mechanisms have been described in aortic smooth muscle cells and in epithelial cells in kidney, intestine and breast cancer. At least some of these actions involve stimulation of TACE by leptin signaling. Several pieces of evidence suggest that ErbB receptors and leptin pathways are closely involved through cross talk to establish and maintain alveolar epithelial barrier function. For example, deletion of the PTHrP receptor, of TACE, and of gamma secretase each exhibit similar gross pulmonary phenotypes with reduced saccular development and neonatal mortality from respiratory insufficiency. Activation of the B isoform of the leptin receptor is capable of directly activating several signaling proteins which are also important for ErbB signaling for lung alveolar barrier maturation and function, including phospholipase C gamma, STAT5, and PI3kinase. Study of the potential crosstalk between leptin signaling and ErbB signaling in lung epithelial barrier function is likely to be a richly fruitful endeavor.

Overall, consideration of the roles of the ErbB receptor signaling system and leptin signaling in lung epithelial development and function suggests specific principles for the evolution of developmental mechanisms. Evolution, whether at the level of molecular ontogenetic mechanisms, physiological processes or adult phenotypes is not parsimonious. Rather, it co-opts and modifies pre-existing pathways in response to new stresses. This strategy allows compensation for, and reinforcement of developmental events that are critical for survival. Thus, the lack of lethality in a mouse knockout model of a particular molecule may indicate the developmental importance of that molecule, rather than its non-importance.

Diving mammals are known to have evolved from land vertebrates, as has the composition of their surfactant. Based on the mechanistic interrelationship between the stretching of the alveolar wall, its cytoarchitecture and surfactant composition, adaptation of the lung for breathing in seals and whales is predicted to be the result of molecular modifications to the lung epithelial barrier of land vertebrates. Therefore, the respiratory adaptation of diving mammals provides a strong predictive model for the hypothetical role of leptin signaling in facilitating vertebrate gas exchange.

7. Leptin's Role in the Evolution of the Blood-Gas Barrier in Diving Mammals (Hall et al, 2009)

Seals and cetaceans are highly adapted to a life in the ocean. They have evolved many exquisite methods for exploiting prey in the marine environment, foraging for sometimes very long periods at depth whilst returning to the surface on occasion to unload CO₂ and load O₂. While this pattern is constant across all marine mammals, there are various ways in which different Groups have modified their respiratory physiology to cope with the restrictions that the need to breathe air but maximize time underwater impose. There are a number of morphological differences in the terminal airways of the lung among the different marine mammals, but all appear to have cartilage reinforcements or thickened muscles in some portion of the terminal and respiratory bronchioles (Kooyman, 1973). Under hydrostatic pressure the lungs collapse and air is forced into the reinforced upper airways of the bronchial tree, nitrogen is isolated from the region of gas exchange and tissue nitrogen accumulation is avoided. In addition, there are differences in diving lung volume among various clades (Kooyman, 2006). Unlike the otariids (eared seals) and the cetaceans (whales, dolphins and porpoises), the phocids or true seals dive on expiration and collapse their lungs during the initial period of the dive. To facilitate this they have compliant chest walls and reinforcements in the lungs allowing the alveoli to collapse in an ordered and regulated way. Surfactant appears to play a key role in this collapse, particularly as an anti-adhesive and in the prevention of atelectasis (Miller et al., 2006a). Since pulmonary surfactant integrity must be maintained during dives for reinflation of the lungs at the surface, enhanced surfactant synthesis is likely to be essential in the phocids since there is little compelling evidence for radical differences in surfactant composition between seals and terrestrial mammals (Miller et al., 2006b).

Leptin plays a central role in the stretch-induced surfactant production pathway. Mechanostimulation of the alveolus induces a paracrine signaling loop in which leptin, secreted from lipofibroblasts (LF), and parathyroid hormone-related protein (PTHrP) from

alveolar type II (ATII) cells, promote surfactant synthesis (Torday and Rehan, 2002). Stretching LFs and ATII cells stimulates PTHrP binding to its LF receptor and increases leptin stimulation of surfactant phospholipid synthesis. Thus the synergistic effects of leptin and stretch could be vital to meet the likely rapid surfactant turnover demands in these animals.

By cloning and sequencing leptin mRNA from two phocid seals (grey *Halichoerus grypus* and harbour *Phoca vitulina*), the Hall laboratory found that phocine leptin contained substitutions in regions normally highly conserved between widely distinct vertebrate groups (Hammond et al., 2005). Subsequently, they cloned and sequenced leptin mRNA from another phocid, the Weddell seal (*Leptonychotes weddellii*), an otariid (California sea lion *Zalophus californianus*) and a small cetacean (harbour porpoise *Phocoena phocoena*) and compared them with known vertebrate leptin sequences. They discovered that the leptin gene sequence has been conserved throughout mammalian phyla, with the notable exception of phocid seals. The leptin sequence from the phocids contains several non-synonymous substitutions within regions that are conserved in all other mammals, including the porpoise and sea lion. Comparing the marine mammal protein sequences to the horse (because it is about equally related to the caniforms and the cetartiodactyls), they found higher identity between the artiodactyls, porpoise and sea lion (>87%) but much lower (<71%) identity with phocid seals. A resulting phylogenetic analysis using maximum likelihood found significant evidence of positive selection pressure along the branch leading to the phocid clade, highlighting the potential functional significance of these changes in the leptin protein. A novel finding of this analysis is that the majority of sites under selection are predicted to occur on exposed surfaces of the leptin molecule, in the A-B loop and the E helix, and likely modify leptin function. The Hall laboratory recently cloned the grey seal LEPR (long-form) receptor and found it was conserved over the entire cDNA, including the critical leptin binding residues, compared to other mammals. Thus, the unique substitutions in phocid leptin are unlikely to alter its receptor affinity. They hypothesize that the unique and focussed selection pressure on the phocid leptin gene was created by a specific need and increased demand for pulmonary surfactant in these species while maintaining its major role in energy regulation.

Conclusion

Like Theseus, we need a string to lead us through the Labyrinth of evolution. If we want to understand the evolution of lungs it is not adequate to consider evolution as “tinkering” (Jacob, 1977), because then we cannot progress in determining the evolutionary puzzle in any greater detail. Evolution, whether at the level of molecular ontogenetic mechanisms, physiological processes or adult phenotypes is not parsimonious. Rather, it co-opts and modifies pre-existing pathways in response to new stresses. By focusing on the molecular aspects of surfactant in relation to lung phenotypes, we begin to see the genetic connections to other molecular phenotypes, which may or may not be relevant to the gas exchange function of the lung. This is critical to our deciphering of evolutionary strategy, because present day adaptations are derived from homologies that facilitated the advent of structures and functions that were relevant to earlier phenotypes. If we are to understand the origins of respiratory physiological processes and their implications for lung structure, we must pursue

this line of investigation. This functional evo-devo approach (Breuker, 2006) is difficult to transform into hard facts, but in the long run will lead to predictive science and medicine.

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