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REVIEW

Vitamin D: calcium and bone homeostasis during evolution

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Vitamin D_3 is already found early in the evolution of life but essentially as inactive end products of the photochemical reaction of 7-dehydrocholestol with ultraviolet light B. A full vitamin D (refers to vitamin D_2 and D_3) endocrine system, characterized by a specific VDR (vitamin D receptor, member of the nuclear receptor family), specific vitamin D metabolizing CYP450 enzymes regulated by calciotropic hormones and a dedicated plasma transport-protein is only found in vertebrates. In the earliest vertebrates (lamprey), vitamin D metabolism and VDR may well have originated from a duplication of a common PRX/VDR ancestor gene as part of a xenobiotic detoxification pathway. The vitamin D endocrine system, however, subsequently became an important regulator of calcium supply for an extensive calcified skeleton. Vitamin D is essential for normal calcium and bone homeostasis as shown by rickets in vitamin D-deficient growing amphibians, reptiles, birds and mammals. From amphibians onward, bone is gradually more dynamic with regulated bone resorption, mainly by combined action of PTH and 1α,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) on the generation and function of multinucleated osteoclasts. Therefore, bone functions as a large internal calcium reservoir, under the control of osteoclasts. Osteocytes also display a remarkable spectrum of activities, including mechanical sensing and regulating mineral homeostasis, but also have an important role in global nutritional and energy homeostasis. Mineralization from reptiles onward is under the control of well-regulated SIBLING proteins and associated enzymes, nearly all under the control of $1,25(OH)_2D_3$. The vitamin D story thus started as inert molecule but gained an essential role for calcium and bone homeostasis in terrestrial animals to cope with the challenge of higher gravity and calcium-poor environment.

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Introduction

Vitamin D (refers to vitamin D_2 and D_3) has well-known skeletal actions in mammals targeting several calcium-transporting or calcium-sensing tissues, whereby vitamin D deficiency causes rickets (reviewed by Pettifor and Prentice[1]) and teeth malformations.[2,3] It may also have many extraskeletal effects by regulating a large number of genes. The concentration of ionized calcium in extracellular and intracellular compartments is very tightly regulated in vertebrates as well as in invertebrates, whereas an endoskeleton structure is a typical hallmark of terrestrial vertebrates (tetrapods), preceded earlier in evolution in fish jaw bones and the skeleton of bony fish. Vitamin D has clear effects on calcium and bone homeostasis of mammals and birds, as outlined in the other chapters of the present special issue on 'vitamin D and bone', but its role earlier in evolution is less well understood.[4–10] In this chapter, we will examine the evolutionary origin of vitamin D and its spectrum of activities.

Vitamin D During Early Evolution of Life

The vitamin D_3 molecule originated early in the evolution of life as the end product of the photochemical conversion of 7-dehydrocholesterol by ultraviolet light B (UVB). The cholesterol synthesis pathway is a very early phenomenon in life (in fact found in all eukaryotes) and starts from squalene and lanosterol. Squalene is an isoprenoid polymer that is already present in rocks preceding the presence of life and it can spontaneously rearrange to form lanosterol. Lanosterol is the starting point of biochemical steroid synthesis into cholesterol (or the equivalent ergosterol in fungi and sitosterol in plants). This synthesis requires a large number of enzymes involving P450-like structures requiring molecular O_2 to oxidize

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cholesterol precursors. This synthetic pathway is highly conserved during evolution.[11,12] Cholesterol is important for membrane function regulating endo- and exocytosis[11,12] and vitamin D may well have acquired such a function early in the evolution of unicellular eukaryocytes. Indeed, the photochemical reaction resulting in vitamin D is considered[13,14] to be a highly efficient protection of life in early marine organisms against DNA damage induced by UVB. Such damage was a more critical problem a few billion years ago than today because of low O_2 and thus also of low or absent ozone layers of the atmosphere. Of course these organisms need(ed) access to sunlight for their photosynthesis using different wavelengths than the ones responsible for UVB damage and vitamin D synthesis. Vitamin D is therefore regularly found in phytoplankton[5,13,15,16] as well as in zooplankton.[17] Plankton is a major part of the food chain of many fish, and their vitamin D content may be about 0.08–0.27%.[15] This high nutritional supply is therefore considered to be the main reason why fish (liver) has such a high vitamin D content, especially as fish like cod are deep water fish.[13,16,17] Others, however, found that oral cholecalciferol is rapidly metabolized in fish so that this food chain origin of massive vitamin D accumulation is questionable (D Fraser, personal communication). Moreover, vitamin D can be formed in the skin of rainbow trout by the action of visible light—in the range of 440–480 nm. This is blue light and of course blue light is the wavelength that has the deepest penetrating power into water (D Fraser, personal communication).

Ergosterol rather than cholesterol is found in many but not all algae[18] with higher concentration when grown in tropical waters than in more Northern areas. Yeast also generates vitamin D_2 when ergosterol is exposed to UVB. This characteristic of yeast has been exploited by Steenbock *et al.*[19] to produce commercial quantities of vitamin D_2 used to combat rickets and vitamin D deficiency but this photochemical production is unlikely to have had an important role in evolution as the life forms that produce ergosterol are usually living in the dark and are not exposed to UVB.

Vitamin D_2 and D_3 are usually considered as inactive end products generated by a photochemical reaction, but exposure of algae to vitamin D_3 (at 10^{-8} M) has growth promoting activity.[20,21] So these compounds may well have important membrane effects for cell function of unicellular organisms, similar to the importance of cholesterol for membrane functions, but this biological effect does not involve vitamin D metabolism and action as in higher vertebrates.[22]

Many higher plants also produce vitamin D either as provitamins, vitamins or related compounds.[23–25] Both vitamin D_2 and vitamin D_3 have been identified in UVB exposed leaves of tomato plants.[18] Solanum glaucophyllum cells are able to synthesize even 1a,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), as glycoside, and sometimes this concentration is high enough to poison grazing animals.[25,26] The molecular mechanism of vitamin D in plants and yeast, seems to be related to membrane effects on ATPase activity and proton pumping.[27]

Little is known about vitamin D during further evolution of invertebrates. Snails do have circulating 25OHD and more polar metabolites but apparently not $1,25(OH)_2D_3$ itself. Moreover, they react to vitamin D supplementation and show increased mineral deposition in their exoskeleton.[28,29] Some crustaceans have a high concentration of $1,25(OH)_2D_3$ as measured by VDR receptor (vitamin D receptor, member of the nuclear receptor family) binding after extraction and extensive high-performance liquid chromatography purification[30,31] and the concentration depends on their molt cyclus.

In conclusion, before the start of vertebrates vitamin D_3 and D_2 already existed for millions of years first as an inactive end product of a photochemical reaction between UVB and 7-dehydrocholesterol or ergosterol. More polar metabolites are found several times during further evolution of life, and vitamin D or its polar metabolites show biological activity in yeast, plants and some invertebrates. However, the full vitamin D endocrine system as described below in higher vertebrates is not found in invertebrates.

Origin of the Vitamin D Endocrine System in Vertebrates

The full vitamin D system as we know it in mammals and birds comprise a combined presence of (1) a specific nuclear receptor (NR), belonging to the class of NRs, (2) vitamin D metabolizing enzymes belonging to the CYP P450 family, (3) a specific transport system with vitamin D-binding protein (DBP) being a high affinity–high capacity extracellular transport system. In addition, (4) phosphate-regulating hormones such as fibroblast growth factor 23 (FGF23) have major effects on vitamin D metabolism or action, beside their effect on phosphate homeostasis and mineral deposition. Finally, the vitamin D endocrine system uses a very complex intracellular signaling system of gene transcription, whereby several hundreds of genes are up or downregulated by the vitamin D hormone. Most if not all of these components, however, are not found in cartilaginous fish or earlier in the evolution of life. Thus, the vitamin D endocrine system found its origin somewhere between the evolution of fish and terrestrial tetrapods, and this is most likely due to several gene duplications.

Origin of VDR. The human NR family counts 48 genes and these NRs have been fairly well conserved during the evolution of terrestrial animals[32,33] but are absent in unicellular eukaryotes. The origin of the NR family according to one hypothesis is that the DNA-binding domain and the ligand binding domain of all NRs is a chimeric recombination of genes/proteins already present early in evolution as LIM and Pex11p, respectively.[34] Phylogenetic analysis revealed that VDR originated from gene duplication and has the closed similarity with other members of the NRII family, PXR and LXR,[32,35] whereby all three NRs probably have arisen from a single ancestral gene in the genome of chordate invertebrates such as found in *Ciona intestinalis* (sea squirt)[36] or *Branchiostoma floridae* (American amphioxus).[35] An older NR in the nematode *C. elegans*, NHR-8, is closely related to LXR and VDR, regulates cholesterol and bile acid metabolism and is also structurally related to DAF-12, which has an important role in metabolism and longevity. A genuine VDR is absent in non-chordate species whereas the most closely (30% amino-acid identity) related NR in insects and crustaceans is the ecdysone receptor, which also accommodates a steroid hormone with cholesterol-like side chain.[37]

A VDR with high ligand selectivity for $1,25(OH)_2D_3$ and related metabolites is found in nearly all vertebrates, including mammals (for example, human and mouse), reptiles, birds (for example, chicken and quail), amphibians (for example, frog)

and fish such as bony fish (for example, zebrafish) and even lamprey (an member of early agnatha or jawless fish).[36] VDR is thus present in primitive cartilaginous fish even before the origin of calcified jaws and cranium, such as in Lamprey.[38] However, no PXR gene is found in lamprey, suggesting that VDR may be the original NRII gene descending from the common PXR/VDR from earlier invertebrates, or alternatively that PXR has been lost during the evolution of fish.

Fish species can be broadly classified into basic (for example, zebrafish and salmon) and late teleosts (pufferfish and medaka), which have a different bone structure (acellular and cellular bone in late versus basic teleosts, respectively, see below). The teleosts display two paralogous VDRs (α and β) with different functionality in different fish.[39–41] These fish VDRs have about 70% homology with VDRs from higher vertebrates. The zebrafish VDR is structurally and functionally very similar to that of mammals and in fact the zebrafish VDR-ligand binding domain was used to cocrystalize with $1,25(OH)_2D_3$ and define its ligand binding pocket.[42] VDR is widely expressed in nearly all zebrafish tissues—not only related to calcium transport such as gills, kidney and intestine but also in bone and many endocrine tissues, brain and other tissues—starting already early in its embryonic development.[39,43] Such widespread distribution of VDR was also found in other fish such as rainbow trout.[44] $1,25(OH)_2D_3$ also regulates the calcium-transporting transient receptor protein (TRP) genes and thereby increases calcium influx and the calcium content of zebrafish embryos[45,46] and rainbow trout.[44] The important role of TPR channels for calcium and bone homeostasis was also clearly demonstrated by severely delayed bone formation in zebrafish with a loss-of-function mutation in the single orthologue of TRPV5 and 6, trpv5/6 gene.[47] $1,25(OH)_2D_3$ also upregulates CYP24A1 (24R-hydroxylase) and downregulates CYP27B1 (1α-hydroxylase), demonstrating a feedback system.

In late or advanced teleost fish, VDR is also present as demonstrated in the freshwater medaka (*Ozyrias latipes*)[41] and green spotted pufferfish (*Tetraodon nigrividis*).[48] These teleosts have an acellular postcranial bone (lack of osteocytes) and no multinucleated osteoclasts[49] (see discussion below). Vitamin D_3 or its metabolites had no effects on the advanced teleost tilapia (*Oreochromis mossambicus*[15]). It is thus clear that the vitamin D endocrine system, as we know it in mammals and birds, started during the evolution of fish, as polar metabolites of vitamin D and the widespread presence of VDR are fairly universal in fish. The function of this new NR is, however, less clear as in the agnathan lamprey it does not seem to be involved in calcium homeostasis, whereas vitamin D has clear positive effects on calcium absorption (gill or intestine) and bone mineralization in early bone fish such as early teleosts. Such effects are less obvious in late teleosts with acellular postcranial bone characterized by low remodeling, whereas early teleosts have a bone structure with cellular bone and multinucleated osteoclasts.

Origin of vitamin D metabolism by specific CYP genes. Enzymes capable of metabolizing vitamin D into more polar metabolites already emerged early in the evolution of life as shown in plants, snails and crustacean (see above) but it is unknown whether this involves P450/CYP proteins. Several bacteria express CYP enzymes capable to produce $1,25(OH)_2D_3$ with high efficacy but the relation of these CYP107 family to vertebrate vitamin D metabolizing enzymes is unknown.[50]

The vitamin D hormone $1,25(OH)_2D_3$ is already found in plasma of cartilaginous fish such as shark and lamprey[51] in concentrations similar to that in human serum. Some data also suggest that fish kidney homogenates can generate $1,25(OH)_2D_3$[52] but this has not been uniformly confirmed.[53] $1,25(OH)_2D_3$ is, however, clearly present in serum of salmon. Starvation during migration of salmon (*Salmo salar*) from seawater to fresh water is associated with marked bone demineralization. During the reverse seawards migration of salmons from fresh water to sea water, low $1,25(OH)_2D_3$ levels increase several fold whereas VDR concentration in gill and kidney decreases by 50%. This indicates a clear physiologic response but it is unclear what role $1,25(OH)_2D_3$ had in the calcium and bone homeostasis.[54] There are several P450 enzymes with highly selective activity for vitamin D metabolism such as CYP2R1 (25-hydroxylation of vitamin D), CYP27B1 (1a-hydroxylation of 25OHD) and CYP24A1 (24R-hydroxylation of 25OHD) but the evolutionary origin of these CYP genes in chordates and early vertebrates is not well defined. Zebrafish, however, clearly express CYP27B1 and CYP24A1.[45] Similarly, serum levels of $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$ varied in opposite direction when rainbow trout was exposed to different salinity.[55]

Enzymes capable of metabolizing vitamin D into more polar metabolites already emerged early in the evolution of life as shown in plants, snails and crustaceans (see above) but it is unknown whether this involves CYP P450 proteins. Several bacteria express CYP enzymes capable of producing $1,25(OH)_2D_3$ with high efficacy but the relation of these CYP107 family to vertebrate vitamin D metabolizing enzymes is unknown.[50]

Therefore, it is fairly obvious that the enzymes responsible for the generation of polar metabolites of vitamin D in higher vertebrates are already functional in bony fish and even in cartilaginous fish.

Origin of specific transport system for vitamin D metabolites. The vitamin D endocrine system of mammals and birds is also characterized by a specific serum transport protein, DBP. The evolutionary origin of this protein is complex. The family counts four members: albumin, DBP, a-fetoprotein and afamin. From the homology of these genes/proteins, an ancestral gene is supposed to split into the DBP/Gc and the albumin/aFP/afamin family about 570–880 million years ago (Mya) whereas the second gene duplication occurred around 360–410 Mya at the time of the divergence of amphibians and reptiles.[56,57] In zebrafish only, one gene is found and has Gc rather than albumin characteristics, although it does not have a vitamin D binding cleft as in avian or mammalian DBP.[56] A study of a very large number of animals ($n = 130$) by Hay and Watson[58], using binding characteristics of sera, revealed that all bony fish, birds and mammals use a specific 25OHD binding protein for transport of vitamin D and its metabolites whereas cartilaginous fish and amphibia use lipoproteins. Later on, gene and protein analysis refined this analysis. The presence of DBP in some cartilaginous fish (*Cyprinus carpio*) but not in others (*Tilapia nilotica*) suggest that DBP originated during the evolution of fish, in concordance with its evolutionary homology pattern.[59] Unlike the initial report by Hay and Watson[58],

amphibia (five species) all had a DBP-like serum protein with high affinity for 25OHD[59] albeit without actin binding properties. Therefore, it seems that, based on binding properties and genetic analysis, a specific transport system for vitamin D and its metabolites started during the evolution of fish and is then retained in all higher vertebrates. DBP null humans have not yet been found although DBP null mice are viable. DBP is smaller in size than albumin and therefore partially filtered in the renal glomeruli but the megalin–cubulin receptor complex of renal epithelium allows the cargo reuptake of a large number of proteins and metabolites (including DBP and 25OHD).[60] This mechanism prevents the renal loss of 25OHD while facilitating the supply of 25OHD for renal metabolism into $1,25(OH)_2D_3$ (reviewed in Bouillon[9]). This endocytic receptor system is already operational in zebrafish[61] but its role in vitamin D metabolism in fish is not yet further explored. Remarkably, the same megalin receptor mechanism is also important for melanin pigment—an important competitor for UVB synthesis in general—handling in the cuticle of drosophila[62] but this phenomenon has not been studied in relation to vitamin D metabolism.

Origin and evolution of phosphaturic hormones. Fibroblast growth factors are proteins with a wide variety of functions. There are 22 members in the human genome, further classified as canonical, intracellular and hormone-like FGFs. Hormone-like FGFs, such as FGF15/19, 21 and 23, are vertebrate specific[63] and originate from gene duplications early in vertebrate evolution.[64] FGF23 is a major phosphaturic hormone, and, in collaboration with aKlotho, decreases renal phosphate reabsorption and production of $1,25(OH)_2D_3$ in the kidney and other tissues whereas it enhances the degradation of 25OHD and $1,25(OH)_2D_3$ by upregulation of CYP24A1. FGF23 is mainly produced by osteocytes and $1,25(OH)_2D_3$ is a major stimulus of the synthesis of FGF23. The zebrafish shows already a full FGF23 system but FGF23 is mainly expressed in the corpuscles of stannius located on the dorsal surface of the pronephon and mesonephron (and not in osteocytes as in mammals). Corpuscles of stannius is also responsible for the secretion of stanniocalcin, a major hypocalcemic hormone of fish. Zebrafish also express the FGF23 coreceptor aKlotho.[65]

From these observations, it is clear that while invertebrates do not express the FGF23 phosphaturic hormone, it is fully expressed in zebrafish but its activity on calcium and bone homeostasis in the early evolution of vertebrates needs further study.

Origin and evolution of regulation of bone mineralization. SIBLINGs are a group of proteins, including DMP1, MEPE, osteopontin and bone sialoprotein, produced by osteocytes, and have an essential role in deposition of calcium in extracellular matrix of bone and teeth. One of these proteins, ovocleidin-116, first appeared in dinosaurs and was preserved in modern birds and reptiles. It is essential for egg shell calcification,[66] a highly efficient mechanism for more successful reproduction for early terrestrial animals. Such SIBLING proteins seem to be absent even in bony fish[66] but are essential for bone and teeth mineralization in higher animals.[67] In essence SIBLING proteins and several endopeptidases, including PHEX, interact to generate ASARM peptides that regulate FGF23, phosphate metabolism, bone mineralization, osteoclastogenesis and fat energy metabolism.[66,67] Such proteins seem to be absent in fish and this is no surprise as most bony fish lack osteocytes.[66,67]

The Role of Vitamin D During Vertebrate Evolution

Although VDR and CYPs and polar vitamin D metabolites are clearly present in early fish such as lamprey, the vitamin D endocrine system is unlikely to regulate their calcium homeostasis as they do not have a calcified cartilage nor have cranial or postcranial bones. However, activation of lamprey VDR by $1,25(OH)_2D_3$ is able to regulate CYP3A4, a gene involved in xenobiotic detoxification, in line with VDR/PXR action earlier in evolution. It is possible that this is in fact the initial reason for the conversion of vitamin D into more polar metabolites with better clearance than the fat-soluble vitamin D, obtained in large amounts from plankton.[15] Little is known about the effects of vitamin D deficiency on calcium or bone homeostasis in basic or late teleosts but vitamin D metabolites are unlikely to have had a major role in calcium homeostasis of fish living permanently in a seawater environment. Gilthead sea bream (*Sparus aureus*) fed a vitamin D-deficient diet for 22 weeks were found to have undetectable serum $1,25(OH)_2D_3$ levels but still remained normocalcemic albeit with reduced growth and calcium fluxes.[68] Similarly, roar fry (*Labeo rohita*) maintained on a vitamin D-deficient diet for 240 days survive and grow normally and are able to maintain a normal total body calcium and phosphate content albeit with a small decrease in total body weight.[69] Administration of vitamin D or $1,25(OH)_2D_3$ to Tilapia mosambica did not change their serum calcium or phosphate concentration nor did it change their highly efficient intestinal calcium absorption.[15] Other studies in several species of fish, however, demonstrate clear effects of $1,25(OH)_2D_3$ therapy. Injection of $1,25(OH)_2D_3$ into mature female European eel stimulated bone formation and reduced bone resorption.[70] $1,25(OH)_2D_3$ injection increased plasma calcium levels in cod (*Gadus morhua*)[71] and carp (*Cyprinus carpio*).[72] $1,25(OH)_2D_3$ also enhanced cartilage calcification in zebrafish.[73] A whole transcriptome analysis after $1,25(OH)_2D_3$ administration in zebrafish revealed an increasing number of genes being regulated by $1,25(OH)_2D_3$ over a time span of days, with only four genes at 2 days after fertilization increasing to >1000 genes being regulated by $1,25(OH)_2D_3$ on day 7 (or about 10% of the whole genome).[73] Some of these genes were related to calcium homeostasis as in mammals (RANK and CYP24A1) but most genes had a more general spectrum of activities such as lipid metabolism (a.o. upregulation of leptin and PPARs and genes for adipocyte differentiation), bile acid metabolism and immune system.[73]

From these studies, it seems clear that the vitamin D endocrine system in zebrafish is operational and has positive effects on calcium homeostasis. In many other fish no biological effects of vitamin D deficiency or therapy were observed. This probably indicates that the vitamin D endocrine system started during the evolution of fish maybe first as a catabolic degradation and later on as calcium conserving mechanism. The role of vitamin D endocrine system on bone resorption is, however, not fully evaluated but seems unlikely. Whether the presence of VDR in non-calcium-transporting tissues has functional implications is not really known nor studied.

Vitamin D deficiency in amphibians provokes rickets as shown already a long time ago in elegant studies of *Xenopus laevis*.[74] This has also been clearly confirmed in reptiles such as lizards.[75]

Vitamin D deficiency in birds (for example, chick or gallus domesticus), and quail[76] creates a dual problem as vitamin D is essential for calcium absorption and bone mineralization but also essential for egg shell calcification. Indeed, in animals laying hard-shelled eggs, there is a need for rapid calcium supply to the uterus for deposition of calcium. This is anticipated by high intestinal vitamin D-dependent calcium absorption with temporary calcium deposition in medullary bone (reviewed in Jonchere *et al.*[77]). The mechanisms involved in calcium transport of chick uterus are very similar to what happens in the intestine but with different polarity.[77] The vitamin D endocrine system is highly involved in these processes of intestinal and uterine calcium transport and therefore serum $1,25(OH)_2D_3$ markedly fluctuates in line with the ovulatory cycle of birds.[78,79] The vitamin D endocrine system is also essential for egg shell decalcification necessary for embryonic bone formation and for hatching.[80–82]

Vitamin D is also essential for bone homeostasis in mammals and the existence of vitamin D was first demonstrated in dogs (reviewed in DeLuca[10] and later on in numerous other species, including rodents and monkeys. This does not imply that vitamin D requirements are the same in all species. Indeed in the feline species, owing to the low levels of 7-dehydrocholesterol in the skin, UVB cannot produce vitamin D itself, making vitamin D a true vitamin in these species.[83] Horses and some ruminants (for example, sheep and goats) have a high, largely vitamin D independent, intestinal calcium absorption so that their requirements for vitamin D are lower.[84,85] Other species are more sensitive to vitamin D deficiency especially when removed from their sun-rich environment. Llamas with their thick hair and dark skin living at high altitude, and New World monkeys living in tree tops are used to high sun exposure and are especially sensitive to vitamin D deficiency when they are no longer exposed to intensive sunlight.[86]

Vitamin D probably had a major role in human evolution out of Africa, as decreasing access to UVB was probably a major drive in selecting genes responsible for skin (de)pigmentation.[87,88] Indeed the dark skin of early humans protected them against UV-induced destruction of folic acid and avoided excess production of vitamin D, whereas a fairer skin allows a greater efficacy of UVB for vitamin D synthesis.

The Origin of the Vitamin D Endocrine System in Vertebrates: A Plausible Hypothesis

Life originated in sea water near volcanic craters about 3.5 billion years ago and the ionic concentration of the oceans remained very stable over time. The calcium concentration of sea water is about 10 mM whereas its concentration in extracellular fluids is about 2.5 mM in fresh water fish and tetrapods. Serum calcium concentration is very tightly regulated in all animals as ionized calcium has a vital role for nerve and muscular function (including cardiomyocytes), coagulation, and tooth and bone formation. It is also vital for reproduction as meeting the calcium requirements for egg shell in birds or for fetal and postnatal development is essential for species survival. Moreover, the intracellular ionized calcium concentration is 100- to 1000-fold lower than in extracellular fluid and this gradient is of vital importance for cell function and survival.

The phosphate concentration of the ocean is much lower in seawater than in body fluids and again phosphate is vital for a large number of cellular functions such as handling and storage of energy, second signaling such as phosphorylation of proteins by kinases, and building important structures such as DNA/RNA, membranes and bone. Therefore, during the evolution of life in seawater calcium was plenty available (or in excess) whereas phosphate supply was limited. When conquering the earth amphibians and all tetrapods were confronted with a milieu poor in calcium and rather rich in phosphate. No wonder that this required major evolutionary adaptations to conquer successfully the massive land area. Terrestrial animals have to cope with a sixfold higher gravity in comparison with marine animals so that a solid bone structure is needed for support of muscles and for total body mobility; this requires a highly efficient system for intestinal calcium absorption. Second, it is logical to avoid excess weight by building bones with maximal strength while minimizing weight, whereas this did not pose a problem for bony fish living in a low gravity water milieu; this requires a system of flexible bone remodeling. In addition, maintaining a normal serum calcium homeostasis is greatly facilitated by an internal reservoir of calcium to cope with the vagaries of variable dietary calcium supply; this is again possible by a flexible and highly regulated turnover of bone.

Having a solid bone was a successful solution during the evolution of fish, first starting with a bony structure for teeth implantation as a competitive advantage for access to food. This required a flexible bone structure to allow lifelong growth and teeth replacement. The solution was found by a combination of bone modeling (mostly by membraneous bone formation), and remodeling using osteoclast-like cells. This was followed by calcification of a large number of cranial bones, again by primary membranous bone formation as to protect the brain structures. Later on postcranial solid bones provided support for muscle and have been an enormously successful event as bony teleost fish represent by far the largest number of vertebrate species (about 23 000 or more than twice the number of bird species).

Early teleosts have a dynamic cellular bone structure with osteocytes and osteoclasts, which allow lifelong bone remodeling much like the calcified jaw structures in earlier fish species. Late teleosts, however, do not have cellular bones as osteocytes are lacking. They also do not have multinucleated osteoclasts but instead have superficial monocytic cells (although they express TRAP, V-ATPase and cathepsin K[89]) that allow minimal bone remodeling. Therefore, their bone structures are solid instead of hollow and, in the absence of real resorption, do not represent a calcium reservoir for maintaining serum calcium homeostasis. With evolutionary hindsight, this seems logical as bony fish living in a calcium-rich oceans do not need a large internal calcium reservoir and a solid bone mass does not create a weight handicap in a low gravity milieu.

Moving to a terrestrial milieu, however, made a large calcium reservoir very useful to cover calcium supply during starvation or periods of deprivation of nutritional calcium supply (**Figure 1**). Moreover, excess weight of solid bones can better be replaced by a lighter bone structure with flexible adaptation to local strength requirements. This requires mechanical sensors, dynamic bone remodeling units as well as hormonal signaling system for integration of calcium and bone homeostasis.

Three hormones are vital for calcium and phosphate homeostasis in mammals: parathyroid hormone and the vitamin D endocrine system are the main regulators of calcium homeostasis, and the phosphatonin FGF23 is the dominant factor regulating serum phosphate homeostasis. Of course all three factors coordinate the overall homeostasis of these two ions.

Remarkably, all these three hormones are only found in vertebrates. The full hormonal vitamin D system and FGF23 first appeared in fish. The parathyroid glands, however, are only startedin amphibians, but nevertheless two PTH genes and one PTrP gene were already identified in cartilaginous (the elephant shark[90]) and bony fish (*Takifugu rubripes*[91]). The overall gene structure of these fish PTH family members closely resembles that of higher vertrebrate species. Receptors for PTH and PTHrP seem to predate vertebrate radiation[92] but probably related to PTHrP and not to PTH actions. PTH genes probably originated from a common ancestor with PTHrP gene by gene duplication in teleost fish.[92] Fish PTH(1-34) can stimulate cAMP generation *in vitro* in human osteoblast-like cells and stimulates calcium resorption from fish scales.[93]

The primary role of these key hormones is to maintain serum or extracellular calcium homeostasis rather than bone homeostasis as discussed elsewhere in this issue and in several other reviews[94,95]. From an evolutionary standpoint, this seems fairly logical as a normal serum calcium concentration, maintained within a narrow range, is essential for nerve and muscle function (including cardiac muscle), and blood coagulation and thus is more essential for immediate survival than bone homeostasis. Therefore, as described elsewhere in this special issue,[94–96] in times of stress (such as calcium or phosphate deficiency) bone mass will be sacrificed in favor of serum calcium and phosphate homeostasis, with the 'evolutionary hope' that normal access to these ions will later on allow restoration of normal bone homeostasis. The morphology of fish bone is very different from that of higher vertebrates (**Table 1**). There is no special relationship between bone marrow and bone in fish as is the case in mammals whereby bone marrow cells are at the origin of osteoclasts. Dentigerous bones are remodeled throughout life to allow lifelong resorption and replacement of teeth, but postcranial bones are usually metabolically inactive and do not show remodeling. The lack of osteocytes in mature teleosts also implies that the mechanosensing system is lacking in fish. Above all, the calcium reservoir of bone in fish does not represent a major factor in calcium homeostasis even in situations of calcium stress.[49]

In contrast to the calcium and phosphate metabolism of mammals, marine animals faced and still face an environment totally different from that of terrestrial animals (**Table 2**). To cope with a high calcium concentration of the ocean, they needed potent hypocalcemic hormones and stanniocalcin is the key factor in fish.[97] This peptide hormone is produced by endocrine structures situated close to the kidney and inhibits calcium influx by gills by downregulation of calcium channels with a TRP-like structure. Removal of this endocrine structure causes hypercalcemia in fish. It is therefore remarkable but logical that later, during tetrapod evolution, the same type of calcium channel (TRPV5 and V6) is positively regulated by the vitamin D hormone.[98] The stanniocalcin gene is also found in mammals but its gene products do not regulate their calcium homeostasis but probably act as paracrine growth factors. This conclusion is

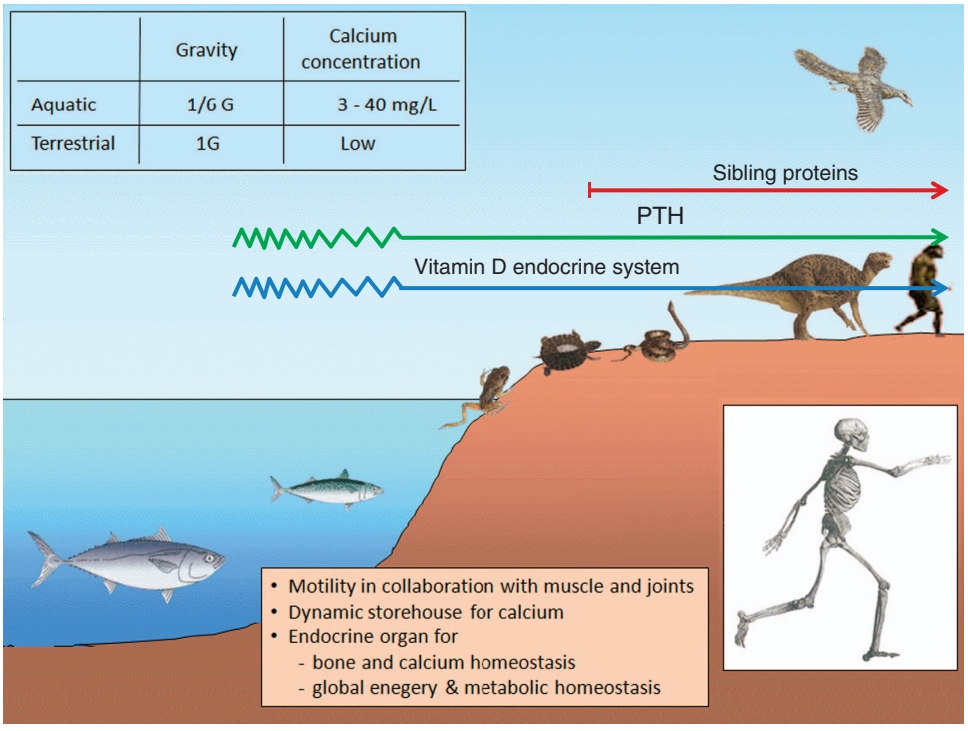

Figure 1 Evolution of calcium homeostasis in vertebrates. The full vitamin D endocrine system (metabolism by specific CYPs, transport by specific plasma protein, a specific NR and a large number of genes regulated by its action) first appeared in fish but its role in overall systemic calcium and bone homeostasis is only evident from amphibian onward. PTH genes appeared separate from the PTHrP gene during evolution of fish but a role for PTH in overall systemic calcium and bone homeostasis is only evident from amphibian onward. ⋀⋀⋀ indicates the presence of the hormonal system but yet without full role as calciotropic hormone.

Table 1 Calcium-regulating hormones during the evolution of vertebrates

Species	*Hard tissues*	*Vitamin D endocrine system*	*PTH*	*Stanniocalcin*	*Calcitonin*	*FGF23*
Cartilaginous fish	Cartilage	Partial	(Yes)[a]	Yes	Yes(?)	Yes(?)
Bony fish	Cartilage/teeth/bone	Yes	(Yes)	Yes	Yes	Yes
Amphibians	Idem	Yes	Yes	(+)	Yes	Yes
Reptiles	Idem	Yes	Yes	(+)	Yes	Yes
Birds	Idem	Yes	Yes	(+)	Yes	Yes
Mammals	Idem	Yes	Yes	(+)	Yes	Yes

Abbreviations: FGF23, fibroblast growth factor 23; (+), stanniocalcin is present as a gene but not as a hormone. As shown in this table, the vitamin D endocrine system and FGF23 started early during the evolution of vertebrates, whereas PTH only emerged in amphibians. Stanniocalcin lost is hormonal function in terrestrial animals. SIBLING proteins are hormone-dependent factors regulating mineral deposition in bone but are only found from reptiles onward.
[a]Fish do not have parathyroid glands but PTH genes are present in their genome and are expressed in very many tissues; the functionality of PTH in fish is unclear but PTH is unlikely to be a major calciotropic hormone.

Table 2 Comparison of bone structure of teleosts and mammals

	Late teleosts	*Mammals*
Bone marrow in contact with bone	No	Yes
Osteocytes	No	Yes
Osteoclasts	Monocytic	Multinucleated
Bone remodeling	Minimal	Continuous and regulated to maintain bone strength and calcium homeostasis
Bone modeling	Life long	Life long
General bone structure	Solid	Hollow and adaptive to loading

based on data obtained in mice with transgenic over or underexpression of stanniocalcin.[97,99]

Calcitonin (CT) is certainly expressed in fish, and fish CT is even more potent in humans than mammalian CT. The physiologic role of CT in fish is, however, disputed as some studies could not reveal an effect on calcium homeostasis whereas others found the expected hypocalcemic effects. This effect is mediated by lowering calcium influx in the gill and causes especially acute changes in serum calcium in salmon, eel, carp and goldfish.[100] Others found no effect of CT (nor of PTH) in cartilaginous fish, so that it seems likely that CT became a true hormone in bony fish. The CT gene (and its gene products such as CT and CGRP) and four CT receptors are conserved from fish to mammals.[101] Overexpression or knock out of the CT gene decreases or increases the epithelial calcium channel (TRP), respectively, in zebrafish, indicating the physiological role in this species.[101] Similarly, exposure to high calcium medium increased CT and downregulated TRP channel expression. CT has (acute) hypocalcemic effects in frogs, birds and mammals but the effect is not found in all species (for example, absent in some reptiles) or only transient. Therefore, the role of CT in calcium and bone physiology is far less important than the other major calciotropic hormones (vitamin D hormone, PTH and FGF23). CT may, however, have a greater role than presently accepted in situations of calcium stress such as during pregnancy or lactation in mammals[100] or during egg shell calcification in birds.[102]

Parathyroid glands first appeared during evolution in amphibians but PTH genes are widely expressed in most tissues of fish.[90] PTH receptors are older and were already used for signaling of PTHrP, which functions as a major paracrine factor[103] and can also act as a hypercalcemic factor in early vertebrates. PTHrP is already present in cartilaginous sharks and rays[104] but a pituitary factor, probably prolactin or somatolactin, is the major hypercalcemic factor in fish. PTH probably has a minor role in systemic calcium or bone homeostasis in fish apart from its effect on mineral resorption from scales.[93] PTH is, however, active in amphibians, reptiles and birds, very much as in mammals.[100] Indeed in all these species, parathyroidectomy results in hypocalcemia, tetany and potentially death. Parathyroid hormone is a major stimulator of the formation and function of multinucleated osteoclasts and this happens in collaboration with the active form of vitamin D and other stimuli.[100] It is also a potent stimulator of renal synthesis of $1,25(OH)_2D_3$, which then stimulates active calcium absorption in the intestine.

Summary and Conclusions

A description of the evolutionary importance of vitamin D is by definition problematic as only data available from presently living organisms are available. Moreover, there are no systematic comparative studies on the vitamin D endocrine system of invertebrates or vertebrates. There are also no comprehensive comparative studies on general calcium and bone homeostasis and their hormonal regulation. Therefore, the present review is essentially a description of the best plausible scenario of the evolution of vitamin D based on partial data sporadically generated in different species.

Vitamin D_3 and D_2 are molecules already found early in the evolution of life but essentially as inactive end products of the photochemical reaction of 7-dehydrocholestol or the ergosterol equivalent in plankton. This photochemical reaction is maintained throughout life in all cells if exposed to short waves of UVB and if sufficient 7-dehydrocholestol is present. In some species, the concentration of 7-dehydrocholestol is so low that they cannot produce vitamin D (for example, feline species such as cats and lions, and maybe to some extent also in dogs). The same is true for animals with virtually no exposure to UVB such as some subterrestrial or nocturnal animals (for example, mole rats). Vitamin D or some more polar metabolites gained biological activity (growth and cell survival) in some invertebrate species (for example, yeast or snails). A full vitamin D endocrine system, characterized by a specific VDR, specific vitamin D metabolizing CYP P450 enzymes regulated by calciotropic

hormones and a dedicated plasma transport protein is only found in vertebrates. In the earliest vertebrates, vitamin D metabolism and VDR may well have originated as a duplication of a common PRX/VDR ancestor gene whereby vitamin D metabolism was a solution for catabolism of excessively available nutritional vitamin D as part of a xenobiotic detoxificating/catabolic pathway (for example, in lamprey and some other fish). The vitamin D endocrine system, however, subsequently became an important regulator of calcium supply when the calcium requirements increased substantially by the development of an extensive calcified skeleton and even more so by moving from a calcium-rich ocean to a calcium-poor terrestrial environment. From amphibian onward, vitamin D is essential for normal calcium and bone as shown by rickets in vitamin D-deficient growing amphibians, reptiles, birds and mammals. In teleostosts, bone remodeling is still absent or minimal and bone is therefore not yet functioning as a calcium reservoir for times of nutritional calcium deficiency. From amphibians onward, bone is gradually more dynamic with regulated bone resorption (mainly by combined action of PTH and $1,25(OH)_2D_3$ on the generation and function of multinucleated osteoclast; reviewed in Suda *et al.*[105]).

The hormonal regulation of serum calcium and bone homeostasis in vertebrates thus went through a radical overhaul when moving from fish to terrestrial tetrapods. Serum calcium homeostasis in marine animals was tightly regulated as in higher animals but mainly required potent hypocalcemic hormones such as stanniocalcin with CT as second fiddle. Hypercalcemic factors, such as PTHrP and the hypophysial prolactin/growth hormone, had a minor role for calcium homeostasis. Terrestrial animals needed primarily hypercalcemic hormones to allow better nutritional absorption of calcium and the vitamin D hormone was and is thereby essential from amphibian onward. A large internal reservoir of calcium was hardly important for animals living in a calcium-rich ocean but gained a strategic survival benefit in tetrapods. The use of an internal calcium reservoir for maintaining serum calcium is not totally novel in tetrapods, as calcium reservoirs in scales and jaw bones were already used in fish without calcified postcranial bones. Similarly, the exoskeleton of crustaceans and snails can be resorbed for reuse during molting. In these cases, resorption of calcium (carbonate) reservoirs used mechanisms different from the well-regulated bone turnover in higher vertebrates. Bone or bone-like tissues only became gradually important during the evolution of fish, first for the generation of teeth and jaws with a bone structure, whereas the rest of the body was mainly a cartilaginous skeleton. Basal teleost fish have (cranial) bones with osteocytes but the large family of late teleosts has acellular bones (essentially solid bone without osteocytes and without remodeling), whether or not they remained in the calcium-rich ocean or in calcium-poor freshwater.

The effective use of bone as calcium reservoir required a dynamic bone turnover with specialized hormones and specialized bone cells. This coincides in evolution with the creation of specialized bone cells, osteocytes, with a remarkable spectrum of activities[106] including mechanical sensing, production of hormones (for example, FGF23 and sclerostin) and able to either act as osteoblasts or display osteolytic activities. These remarkable osteocytes not only regulate mineral homeostasis but also have an important role in global nutritional and energy homeostasis,[107] and the vitamin D endocrine system participates in this process.[108] Later on, mineral deposition is also better fine-tuned by osteocytes and osteoblasts. Indeed, mineralization from reptiles onward is under the control of well-regulated SIBLING proteins and associated enzymes (reviewed in Rowe[66] and Lieben *et al.*[109]). Exposure to greater phosphate supply in terrestrial animals generated the requirement of potent phosphaturic hormones such as FGF23, acting in close collaboration with PTH and $1,25(OH)_2D_3$ to regulate not only phosphate but also calcium homeostasis.

The vitamin D story thus started early in the evolution of life as an inert molecule as end product of a photochemical reaction. During the early evolution of vertebrates it gained a second life as the substrate for a ligand of a new NR, essential for normal calcium and bone homeostasis of terrestrial animals. In the recent history of humans and owing to differences in life style and life expectancy, vitamin D became even one of the most frequently used 'drugs' to compensate for less exposure to UVB.

Conflict of Interest

The authors declare no conflict of interest.

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