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Premature aging in vitamin D receptor mutant mice☆

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ABSTRACT

Hypervitaminosis vitamin D_3 has been recently implicated in premature aging through the regulation of 1alpha hydroxylase expression by klotho and fibroblast growth factor-23 (Fgf-23). Here we examined whether the lack of hormonal function of vitamin D_3 in mice is linked to aging phenomena. For this, we used vitamin D_3 receptor (VDR) "Tokyo" knockout (KO) mice (fed with a special rescue diet) and analyzed their growth, skin and cerebellar morphology, as well as overall motor performance. We also studied the expression of aging-related genes, such as Fgf-23, nuclear factor kappaB (NF-kappaB), p53, insulin like growth factor 1 (IGF1) and IGF1 receptor (IGF1R), in liver, as well as klotho in liver, kidney and prostate tissues. Overall, VDR KO mice showed several aging related phenotypes, including poorer survival, early alopecia, thickened skin, enlarged sebaceous glands and development of epidermal cysts. There was no difference either in the structure of cerebellum or in the number of Purkinje cells. Unlike the wildtype controls, VDR KO mice lose their ability to swim after 6 months of age. Expression of all the genes was lower in old VDR KO mice, but only NF-kappaB, Fgf-23, p53 and IGF1R were significantly lower. Since the phenotype of aged VDR knockout mice is similar to mouse models with hypervitaminosis D_3, our study suggests that VDR genetic ablation promotes premature aging in mice, and that vitamin D_3 homeostasis regulates physiological aging.

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1. Introduction

Vitamin D has an essential role in calcium homeostasis [1], and its target organs include bone, kidney, intestine and parathyroid glands [2]. In addition to the classic functions on regulation of calcium and bone homeostasis, vitamin D_3 has been shown to influence a variety of other systems, including cell proliferation and cancer, immunology, glucose homeostasis and cardiovascular system [3]. The bioactive metabolite of vitamin D, $1\alpha,25(OH)_2D_3$ (calcitriol), is a steroid hormone that also plays an important role in the nervous system [3–5]. The effects of calcitriol are mediated through its interaction with a high-affinity nuclear vitamin D receptor (VDR), a member of the nuclear receptors superfamily of ligand-activated transcription factors [6–8]. VDRs are widespread in the brain and the spinal cord including the areas involved in the regulation of motor activity and behaviour [9–12]. Collectively, this implies a possible role of calcitriol and VDR in the regulation of behaviour [13–19].

Aging is considered to be controlled by multiple genes and environmental factors [20–22], and vitamin D_3 may be one of those factors [23,24]. For example, high levels of $1,25(OH)_2D_3$ has also been shown to lead to early aging [23], whereas animal data show premature aging-like features caused by hypervitaminosis D_3 in klotho, a putative anti-aging gene, and fibroblast growth factor 23 (Fgf-23) mutant mice [25,26].

Elderly populations suffer from vitamin D_3 deficiency that leads to impaired bone mineralization [23]. In humans, VDR gene variants have also been linked to changes in cognitive function and depressive symptoms in old age, whereas vitamin D_3 insufficiency increases age-related diseases and mortality [23,27–34]. Both hypervitaminosis and hypovitaminosis D_3, as well as impaired VDR function and hypoparathyroidism, have been suggested to cause sensorineural hearing loss [35–40].

Animal model lacking functional VDR, such as the VDR knockout (KO) mice, have proven to be a useful tool in studying functions of vitamin D in different physiological processes. Solving the problem of survival of the VDR KO mice by feeding a special rescue diet opened up possibilities to study the role of vitamin D_3 deficiency in aging [41]. Our preliminary studies suggested that most of the phenotypical features shown in the VDR KO mice might be related to premature aging [3,18,42–44] In the present study, we analyzed

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aging in VDR KO mice by measuring survival, weight gain, skin and cerebellum morphology, swimming and expression of different aging-related genes.

2. Materials and methods

2.1. Experimental animals

VDR KO mice were initially generated at the University of Tokyo (Japan) and provided by Professor Kato [44]. Mice used were littermates on 129S1 and NMRI genetic background produced in Tampere University (Finland) by heterozygous crosses. Subjects were 27 KO female, 25 KO male, 21 WT female and 34 WT male mice on 129S1 background, and 47 KO female, 23 KO male, 65 WT female and 129WT male mice on NMRI background (1.5–24 months old). Mice were maintained in a virus/parasite-free facility under controlled temperature (22 ± 2 °C) and humidity (60%) with 12:12-h light-dark cycle. The animals were housed in plastic cages with food and water available *ad libitum*. To normalize mineral homeostasis and prolong lifespan, the VDR KO mice were fed a special rescue diet containing 2% calcium, 1.25% phosphorus and 20% lactose (Lactamin AB, Kimstad, Sweden)[45]. This special diet has been shown to normalize serum calcium, phosphorous and parathyroid hormone as well as skeletal phenotype of VDR KO mice [46,47]. Genotyping of the mice was confirmed by PCR on DNA prepared from tail tissue. Four primers were used to amplify a 166 bp VDR (forward, 5′-CTG CTC TTC TTA CAG GGA TGG-3′ and reverse, 5′-GAC TCA CCT GAA GAA ACC CTT G-3′) and 400 bp Neo (forward, 5′-ATC TTC TGT CAT CTC ACC TTG C-3′and reverse, 5′-CAA GCT CTT CAG CAA TAT CAC G-3′) band from the targeted allele. All animal experiments were approved by the Ethical Committee of the University of Tampere. Animal care and experimental procedures were conducted according to the European legislation.

2.2. Macroscopic phenotype assessment

The weight of mice (27 KO + 28 WT mice from the strain 129S1 and 11 KO + 18 WT mice from the strain NMRI) was measured at different ages (6.5–14.5 mo for NMRI and 3–17 mo for 129S1). Survival of additional 14 VDR KO and 11 WT mice (NMRI), not included otherwise to this study, was recorded as age when the animal died naturally or was euthanized due to health problems.

2.3. Motor phenotype assessment

Motor abilities of the mice (26 KO + 25 WT mice from the strain 129S1 and 36 KO + 41 WT mice from the strain NMRI) were measured by assessing swimming abilities. A glass cylinder (20 cm in diameter) was filled with warm water (22–25 °C) to a depth of 20 cm. The mice were individually lowered into the water, and their ability to swim was assessed for 3 min. Normal swimming behaviour was recorded when the animal kept a horizontal body position, with its nose above the surface. Abnormal swimming was recorded when the animal's position was vertical, with its nose pointing upward. Sinking episodes were scored when the position of the mouse was vertical and its nose went below the surface of the water. In cases of sinking, the animal was immediately rescued. The swimming ability was scored as 1 for normal swimming, 0.5 for slightly vertical swimming position and 0 for abnormal swimming and/or sinking. The water was changed between trials.

2.4. Histological analyses

Five KO and 5 WT 129S1 mice were euthanized with carbon monoxide, and a part of the back skin was shaved. A piece of the skin

Table 1
Primers used in the RT-PCR experiments.

Gene	Primer sequence
mβ-actin	5′-GCTTCTTTGCAGCTCCTTCGT-3′
	5′-CCAGCGCAGCGATATCG-3′
mNF-κB	5′-TGGCCGTGGAGTACGACAA-3′
	5′-GCATCACCCTCCAGAAGCA-3′
mFgf-23	5′-ACTTGTCGCAGAAGCATC-3′
	5′-GTGGGGGAACAGTGTAGAA-3′
mp53	5′-AGAGACCGCCGTACAGAAGA-3′
	5′-CTGTAGCATGGGCATCCTTT-3′
IGF-I[a]	5′-TGGATGCTCTTCAGTTCGTG-3′
	5′-GTCTTGGGCATGTCAGTGTG-3′
IGF-IR	5′-CGAGCTTCCTGTGAAAGTGATGT-3′
	5′-CACGTTATGATGATTCGGTTCTTC-3′
mKLm[b]	5′-GGACATTTCCCTGTGACTTTGC-3′
	5′-AGAGAGAGTAGTGTCCACTTGAACGT-3′

[a] Nagata et al. Pediatr. Surg. Int. 2007. IGF-I: NM_010512.
[b] Mouse klotho membrane protein NM_013823.

was cut and fixed for 24 h at room temperature in 4% paraformaldehyde in 1× PBS, and kept in 70% ethanol at 4 °C until embedded in paraffin. 5 μm sections were cut with a microtome and the slides were stained with hematoxylin and eosin (HE). The stained slides were observed under a Nikon light microscope. For the brain samples, animals (5 KO and 5 WT NMRI mice) were anesthetized, and perfusion fixed with a mixture of saline and heparin for removal of the blood following 4% paraformaldehyde in 1× PBS. Brains were removed, fixed for 24 h at room temperature in the same fixative solution and kept in 70% ethanol at 4 °C until embedded in paraffin. The cerebellum was detached and embedded. Frontal 5 μm sections were cut with a microtome and samples were taken every 50 μm. Sections were stained with hematoxylin-eosin (HE), observed under a Nikon light microscope, and photographed. Purkinje cells were counted from the 2nd or 3th cerebellar lobule and from both side flocculus as a number of cells per 500 μm, approximately 2 mm from the beginning of the cerebellum.

2.5. Quantitative real-time PCR

To study the expression of genes that are related to both aging and vitamin D, liver tissue samples from 9 KO + 14 WT mice from the strain NMRI and 14 KO + 16 WT mice from the strain 129S1, kidney from 13 KO + 14 WT mice from 129S1 strain and prostate from 11 KO + 10 WT mice from strain 129S1 were taken to Eurozol®-solution (Euroclone S.p.A., Milano, Italy) and homogenized. Total RNA was isolated by following the manufacturer's instructions. RNA amounts were quantified by measuring the absorbance at 260 nm. The cDNA was synthesized from the total RNA by reverse transcription PCR using a High Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA, USA). The reaction was performed at 37 °C for 2 h. Samples were stored at −20 °C prior to the real-time PCR reaction. Expression of mRNA in the tissues was detected using real-time PCR. Target cDNA was amplified by PCR for 40 cycles (1 cycle: 95 °C for 15 s, 60 °C for 1 min) in ABI PRISM® 7000 SDS using SYBR® Green solution (Applied Biosystems, Foster City, CA, USA) and 20 ng of template. The primer sequences used in this study are described in Table 1. To estimate the quality of RNA and to calculate the relative expression ratio the β-actin, cDNA in each sample was also amplified by PCR using β-actin primers.

3. Results

3.1. Premature aging-like phenotype of the VDR KO mouse

VDR KO mice exhibit multiple features resembling premature aging. The mean survival age for rescue diet fed VDR KO mice was

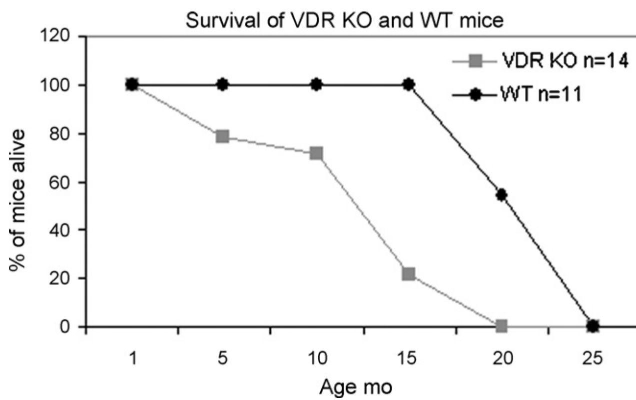

Fig. 1. Survival curve of the VDR KO and WT mice (14 KO + 11 WT mice from strain NMRI). Presented as a % of mice alive at the time.

10.6 months (min 2 and max 17 months, $n = 14$), whereas the mean age for the WT mice was 20.5 months (min 18 and max 24 months, $n = 11$) (Fig. 1). Survival age was recorded when mice either died or were euthanized because of problems in health (NMRI strain). KO mice were smaller in size, showed wrinkled skin, and developed alopecia (Fig. 2). Weight of the VDR KO mice was smaller than that of WT controls from 7 months on (Fig. 3). The difference in weight between genotypes was greater in the 129S1 strain than in the NMRI strain ($p < 0.0001$ vs. $p < 0.025$, *U*-test, respectively).

Light microscopic examination of the skin of 8–9 month old VDR KO mice revealed alopecia, enlarged sebaceous glands, formation of dermal cysts, thinner layer of the subcutaneous fat and thickening of the skin. Abnormalities detected in the VDR KO mice were not present in the skin of 9–9.5-month-old control mice (Fig. 4).

3.2. Cerebellum and Purkinje cells

The light microscopic analysis did not reveal any abnormalities in the structure of the cerebellum. There were no differences in the number of Purkinje cells between VDR KO and WT mice in the cerebellar lobules (23.4 ± 5.55 and 17.2 ± 4.60 cells per 500 μm) nor in the flocculus (12.0 ± 1.47 and 14.3 ± 1.48 cells per 500 μm respectively, mean ± S.D.) (Fig. 5).

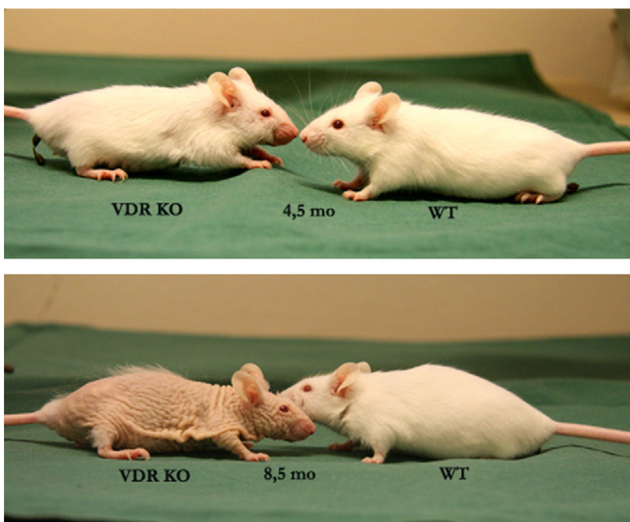

Fig. 2. Phenotype of VDR knockout mouse (KO) compared to wildtype littermate (WT; NMRI background strain) at the age of 4.5 (top) and 8.5 (bottom) months.

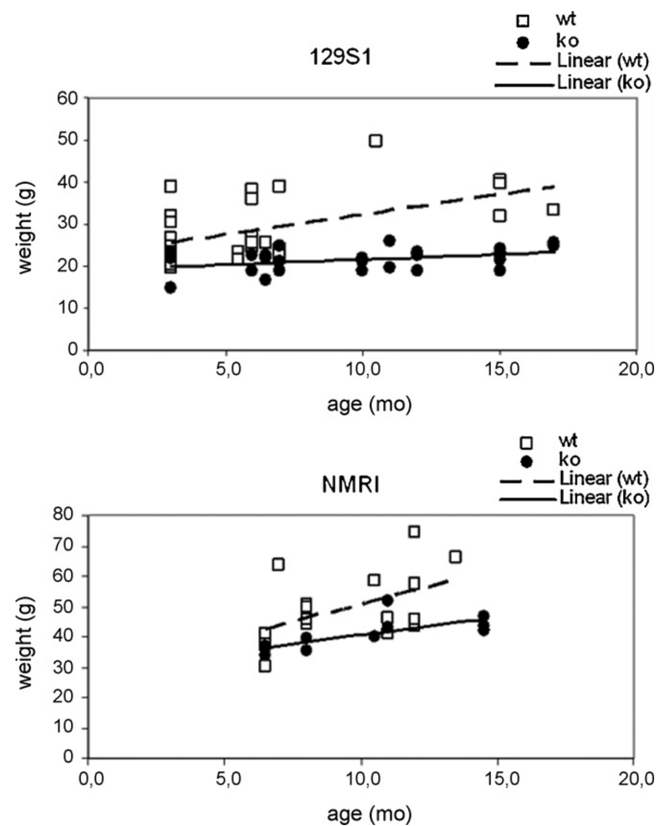

Fig. 3. Body weight of VDR knockout (KO) and wildtype (WT) mice relative to age (129S1 and NMRI background strains). 129S1 KO $n = 27$ and WT $n = 28$, NMRI KO $n = 11$ and WT $n = 18$.

3.3. Swimming test

The VDR KO mice from both strains were unable to swim after 6 months of age; the mice were unable to float or keep horizontal swimming position and sank as a result. In contrast, WT control mice of all ages showed normal swimming position without any sinking (Fig. 6).

3.4. Expression of NF-κB, Fgf-23, p53, IGFI, IGFIR and Klotho

As can be seen in Fig. 7, expressions of all the six aging related genes were lower in old VDR KO mice. NF-κB was significantly lower in old NMRI VDR KO mice, but there was no difference between the genotypes in young 129S1 mice. Fgf-23 expression was generally lower in VDR KO mice in both strains, but significantly different only in the old mice. In the NMRI mice, the expression of p53 was significantly lower in VDR KO mice compared to control mice. There was no difference in the young 129S1 mice. Expression of the Klotho gene was lower in old VDR KO mice although the difference was not significant, there were no difference in the expression levels in young mice. Expression on IGFI was unaltered in VDR KO mice in both strains. IGFIR expression was reduced in VDR KO mice in NMRI strain (Fig. 7.), but there was no difference between the genotypes in young 129S1 mice.

4. Discussion

Vitamin D_3 has been linked to aging in human and animal studies [23,34]. Premature aging-like phenotype has been reported in mouse models that express high levels of vitamin D_3. Several studies have shown that vitamin D_3 insufficiency increases age-related diseases and mortality [23,27–33]. This study shows

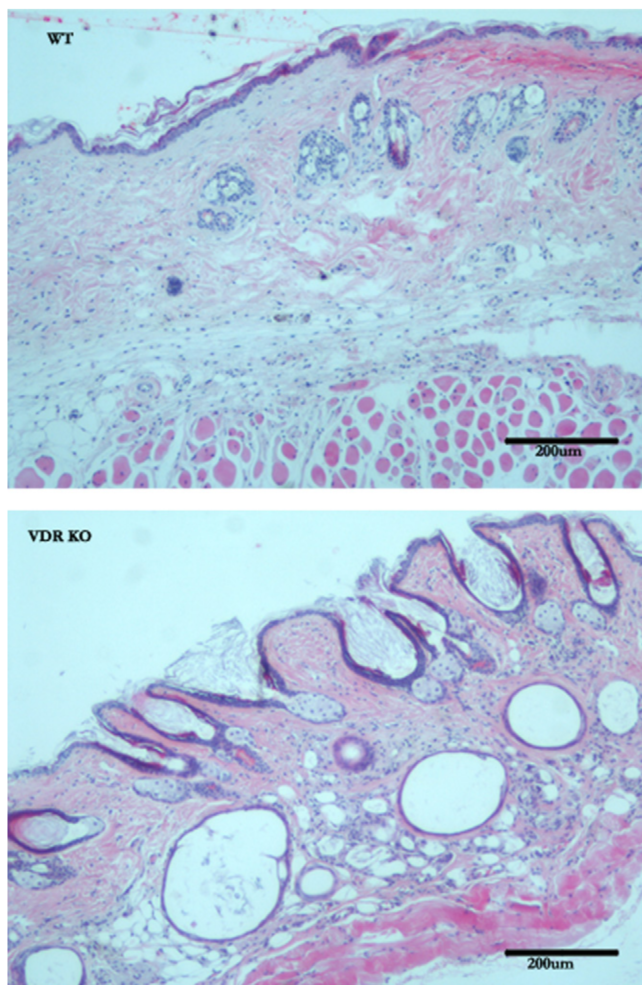

Fig. 4. Skin morphology of a representative 8-months old VDR knockout mouse (KO) compared to a wildtype (WT) mouse. Similar phenotypes were observed in 5 KO mice and 5 WT mice in this study 129S1 background strain.

that the phenotype of VDR KO mice resembles premature aging and is similar to those described in mouse models of hypervitaminosis D_3 [26,48–50]. Our group has already demonstrated that VDR KO mice suffer from thalamic calcification, progressive hearing loss, and vestibular problems [43,47,51]. Reports by other groups reveal that VDR KO mice have growth retardation, reduced life span, atrophy of the reproductive organs and infertility, muscle atrophy, immunological deficiency, osteoporosis and sensitivity to cancer [3,44,52–54]. It has also been reported by several groups that VDR deletion in mice causes hypertension, cardiac hypertrophy and increased thrombogenicity despite the use of a rescue diet [3]. All similar features are observed during human aging [55–58].

Here we show that VDR KO mice have shorter life span and are smaller than WT controls even when fed with the rescue diet. It might be that those VDR KO mice that died before the age of 3 months where suffering from hypocalcemia despite the rescue diet. However, the maximum age still remained lower than that of control mice's, which might be because of other aging related diseases. A study by Amling et al. revealed that the growth of VDR KO mice is restored by preserving normal mineral ion homeostasis [45]. In the present study the size of the animals was analyzed only by weight, and the difference detected might be partially due to reduced volume of body fat in the VDR KO mice [59]. In this study, the subcutaneous fat was reduced in the aged VDR KO mice (Fig. 4). Recently, Narvaez et al. hypothesized that vitamin D_3 signalling may

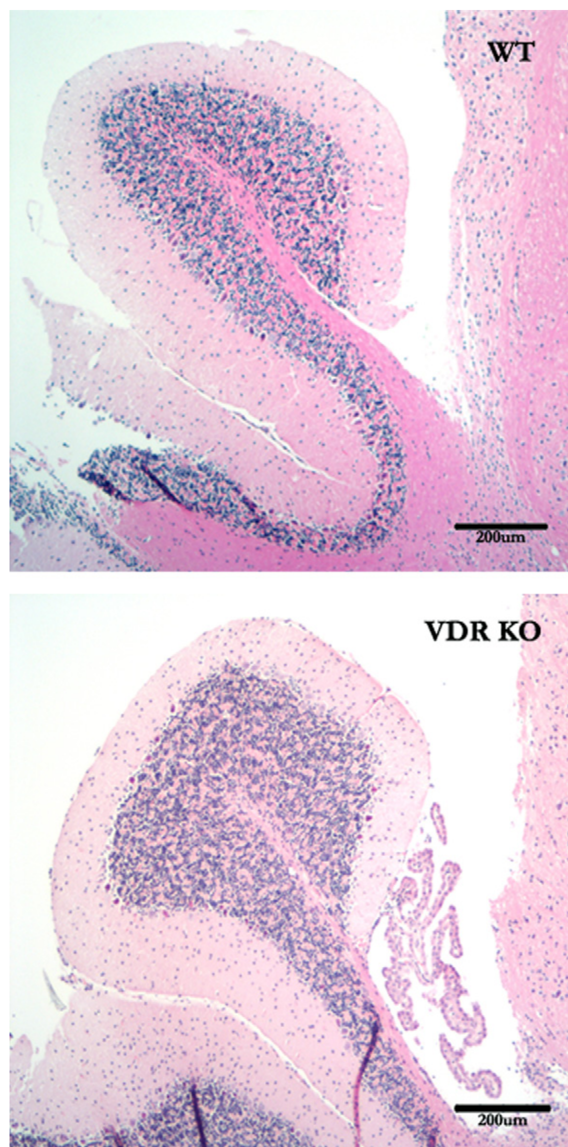

Fig. 5. Cerebellum flocculus morphology of a representative 11.5 months old VDR knockout mouse (KO) compared to a 12 months old wildtype mouse (WT) mouse. NMRI background strain. Similar phenotypes were observed in 5 KO mice and 5 WT mice in this study.

become important in the control of adipogenesis during the aging process [59]. We found that the difference in weight between genotypes was greater in 129S1 strain, which suggests there are some strain specific differences in the growth and adipogenesis. It seems that the NMRI strain has a better ability to compensate for some of the effects caused by the VDR mutation. It might be that as the NMRI is an outbred strain it remains bigger and retains an enhanced ability for survival because of higher heterozygosis.

The skin of the VDR KO mice is highly wrinkled (Fig. 2) presumably because of reduced subcutaneous fat which is similar in aging human skin [60].

It is well known that calcium regulation is linked to aging in the brain, whereas vitamin D_3 treatment reduces calcium-mediated biomarkers of aging in the brain [61–64], while VDR KO mice display severe thalamic calcification [43]. Aging is also the major risk factor in many neurodegenerative disorders [65–67]. Brain atrophy is considered a sign of aging in the brain [68]. It was shown by Féron et al. that developmental vitamin D_3 deficiency leads to differences in brain length/width ratio in prenatal rats, as well

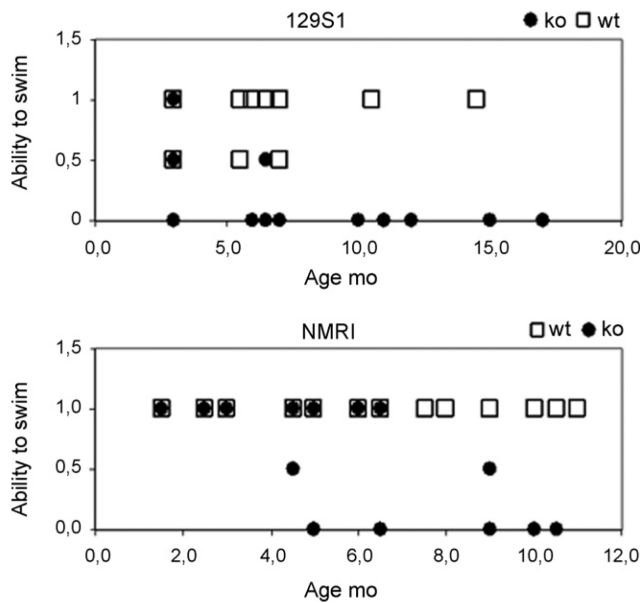

Fig. 6. Swimming ability of VDR knockout (KO) and wildtype (WT) mice compared at different ages: above – in 129S1 strain (KO $n = 26$, WT $n = 25$); below – in NMRI strain (KO $n = 36$, WT $n = 41$, from which 10 were retested after 5 months). In both strains, KO mice lost their ability to swim after 6 months.

as increased lateral ventricles size in adult rats [69]. Our studies showed that VDR KO mice have vestibular problems [51]. In addition to the vestibular organs, cerebellum also has an important role in the modulation of movement [70]. Although the morphological analysis of the cerebellum of VDR KO mice did not reveal any changes when compared to controls, and the number of Purkinje cells was also normal (Fig. 5), motor phenotype was robustly affected in our VDR KO mice. Swimming ability of the VDR KO mice has been studied by several groups [18,51,71]. Here we show that swimming ability of the VDR KO mice is poor after 6 months of age, regardless of the background strain used. This implies that the loss of swimming ability, whether it is caused by vestibular or motor problems, presumed to be caused by the VDR mutation.

The expression of aging-related genes was also studied in our VDR KO mice. Nuclear factor kappaB (NF-kappaB) regulates expression of genes involved in immune system response, inflammation and apoptosis [72]. In a study by Adam et al., it was identified to be a motif most strongly associated with mammalian aging [73]. In a recent study it was also shown that vitamin D_3 decreases NF-kappaB activity [74]. Here we only showed that in old VDR KO mice (NMRI strain), the expression of NF-kappaB was decreased when compared to the expression level of control mice, suggesting that it is regulated by vitamin D.

Fgf-23 is secreted from bone and it acts on kidney to suppress renal phosphate reabsorption and vitamin D synthesis [75]. Premature aging-like phenotypes of Fgf-23 and klotho-deficient mice and their reversal by vitamin D_3 restriction suggest that vitamin D_3 is primarily responsible for the aging [26,49,75]. Since hormonal forms of vitamin D_3 stimulate Fgf-23 expression, the low expression of Fgf-23 in VDR KO mice might be due to the lack of vitamin D_3 action.

p53 is a tumor suppressor protein that induces cell growth arrest and apoptosis [76]. Activity of p53 is also responsive for cellular and replicative senescence [77]. It has been shown that VDR is regulated by the p53 family and the expression of VDR is induced by DNA

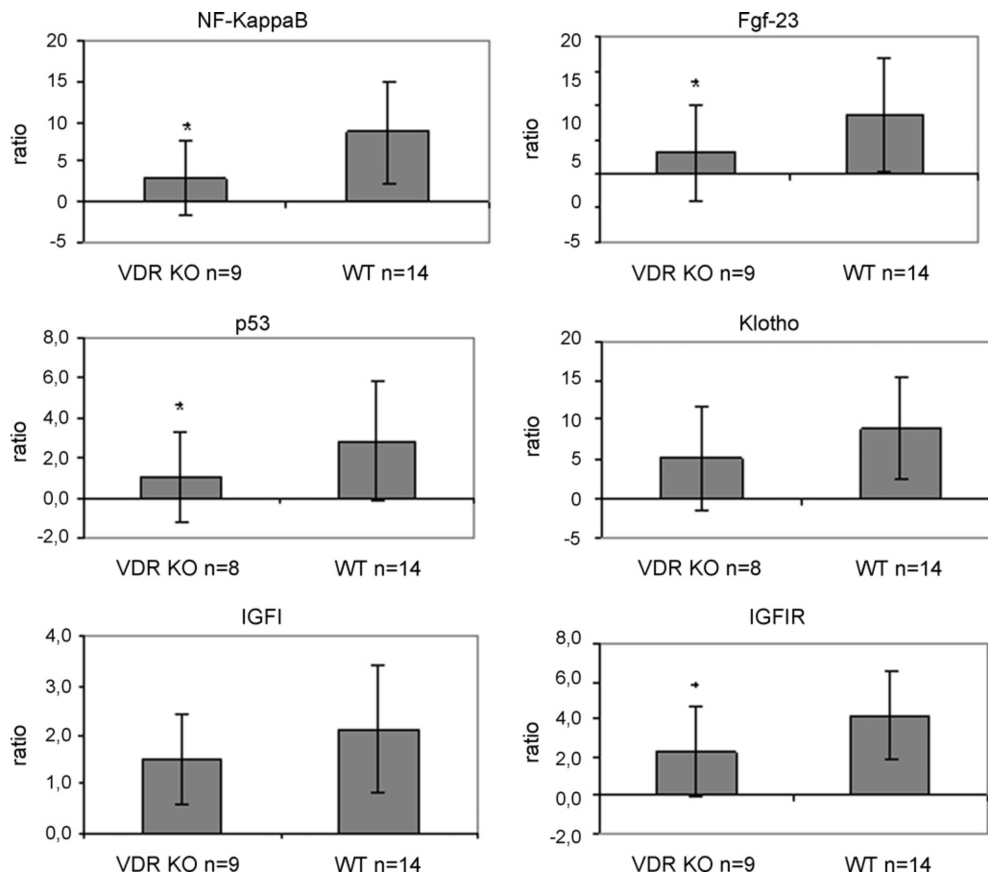

Fig. 7. Relative expression of NF-kappaB, Fgf-23, p53, IGFI, IGFIR were analyzed by real time RT-PCR from mRNA in livers and klotho mRNA in kidneys, liver and prostates of VDR knockout (KO) and wildtype (WT) control mice of NMRI (8–12 months old) and 129S1 strains (3–5 months old). *$P < 0.05$, Mann–Whitney U-test.

damage [78,79]. Our results showed that expression of p53 was decreased in aged VDR KO mice, which further confirms the findings that vitamin D_3 is able to upregulate p53 (Fig. 6). The lower level of p53 expression in VDR KO mice might also explain the enhanced susceptibility of VDR inactivated murine skin to UV-induced cancer [54].

Klotho over expression extends life span and its deficiency causes aging-like phenotype, as showed in several studies [25,49,50,80]. The anti-aging properties of klotho are mediated through inhibition of insulin and IGFI signalling [50]. In our VDR KO mice, the expression of klotho was lower in the old mice but there was no difference in the expression levels in liver, kidney or prostate in young mice.

It has been shown that IGFI deficiency increases life span [48,81] and IGFI is regulated by vitamin D_3, and IGF binding proteins (IGFBP) are vitamin D target genes [82,83]. In this study there was no difference between the genotypes in the expression of IGFI, but expression of IGFIR was significantly reduced in old NMRI VDR KO mice compared to controls suggesting a lower IGFI activity. This study shows that young VDR KO mice lack almost all the aging like phenotype seen in old VDR KO mice. The aging-related genes, described here, are regulated by many other factors besides vitamin D and it might explain why we don't see the effect on younger animals. Our results, when combined with the knowledge obtained from prior studies, suggest that VDR KO mice show similar symptoms of premature aging that have been linked to abnormal vitamin D system.

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