

Distribution of vitamin D3 receptor in the epididymal region of roosters (*Gallus domesticus*) is cell and segment specific

Rubem A.P. Dornas^{a,1}, André G. Oliveira^{a,1}, Evanguedes Kalapothakis^b,
Rex A. Hess^c, Germán A.B. Mahecha^a, Cleida A. Oliveira^{a,*}

^a Department of Morphology, Federal University of Minas Gerais, Av. Antônio Carlos 6627, Cx. Postal 486, CEP 31270-901, Belo Horizonte, MG–Brazil

^b Department of General Biology, Federal University of Minas Gerais, Av. Antonio Carlos 6627, Cx. Postal 486, CEP 31270-901, Belo Horizonte, MG–Brazil

^c Department of Veterinary Biosciences, University of Illinois, 2001 S. Lincoln, Urbana, IL 61802, USA

Received 17 August 2006; revised 5 October 2006; accepted 22 October 2006

Available online 6 December 2006

Abstract

Vitamin D3 is a steroid hormone well known by its role in maintaining calcium homeostasis, however this hormone may also participate in other biological functions, including control of reproductive processes. The vitamin D3 action is mediated by the vitamin D3 receptor (VDR). VDR is widely distributed in the rodent reproductive tract, however the occurrence of VDR and the role of the vitamin D3 in the avian reproductive tract remain unknown. The aim of the present study was to investigate the expression and cellular distribution of VDR in the epididymal region of roosters. VDR expression was investigated by Western blotting analysis and the tissue distribution of the receptor was determined by immunohistochemistry. The Western blotting assay revealed a major VDR protein band of 61 kDa in the epididymal region of rooster. Nuclear VDR expression was found in all segments of the epididymal region, namely rete testis, efferent ductules, connecting ducts and epididymal ducts. Nonciliated cells of the distal efferent ductules showed the highest levels of VDR expression, followed by the proximal efferent ductules and rete testis. The connecting and epididymal ducts showed less intense VDR immunostaining. The differential VDR expression in the epididymal region segments reveals that several extratesticular ducts may be target for vitamin D3 action and suggests that vitamin D3 may have a regional-specific function, such as calcium transport, that is modulated through VDR activity.

© 2006 Elsevier Inc. All rights reserved.

Keywords: Vitamin D3 Receptor; Efferent ductules; Epididymis; Roosters

1. Introduction

Vitamin D receptor (VDR) is a member of the steroid/thyroid hormone super-family of nuclear receptor, which mediates the effects of the vitamin D3 (1,25-(OH)₂D₃). Vitamin D3 is a steroid hormone well known by its role in maintaining calcium homeostasis (Bennett et al., 2006; Hoenderop et al., 2005; Lips, 2006; Walters, 1992). However, this hormone also participates in other important biological functions, including modulation of cell proliferation

and cell differentiation (Walters, 1992; Lips, 2006), as well as control of reproductive processes (Kwieceński et al., 1989; Stumpf and Denny, 1989).

VDR is widely distributed in the female and male reproductive tissue of rodents, indicating that this hormone likely plays an important local role (Johnson et al., 1996; Kinuta et al., 2000; Levy et al. (1985a,b); Merke et al., 1983; Schleicher et al., 1989). Indeed, deficiency in vitamin D3 has been shown to cause reduction in male and female fertility (Kwieceński et al., 1989; Stumpf and Denny, 1989). In the male, genetic disruption of VDR (VDR-KO) resulted in testicular effects, as seen by a transient increase in testicular weight, dilation of seminiferous tubules lumen and reduction in epithelium, resulting in decreased spermatogenesis

* Corresponding author. Fax: +55 31 3499 2771.

E-mail address: cleida@icb.ufmg.br (C.A. Oliveira).

¹ These two authors contributed equally to carry out the investigation.

(Kinuta et al., 2000). These effects culminated in atrophy of the tubules in older animals. Similar to rodents, lack of vitamin D3 resulted in testicular degeneration and incomplete spermatogenesis in chicken (Kurtul, 2002). A role for vitamin D3 in the avian testis has also been indirectly demonstrated by its effect on the modulation of the expression of Calbindin-D28k, a molecular marker for vitamin D action (Inpanbutr et al., 1996). However, information about vitamin D effects in extratesticular organs as well as information about the occurrence of VDR in the testis and all other segments of the male genital system of birds is still lacking.

Contrasting to mammals, the male genital tract of avian species is characterized by the occurrence of prominent efferent ductules and a short and non-differentiated epididymal duct (Aire, 1979; Clulow and Jones, 2004), which in conjunction with the extratesticular rete testis composes the epididymal region. The prominence of the efferent ductules points out that this segment may be of high importance for avian reproduction. The avian efferent ductules, similar to those of mammals, play an important role reabsorbing more than 90% of the fluid coming from the testis (Clulow and Jones, 1988). On the other hand, differing from mammals, avian efferent ductules are also responsible for significant reabsorption of calcium (Clulow and Jones, 2004). The molecular mechanism underlying the fluid and calcium reabsorption in the bird male tract is still not determined. Considering that transcellular calcium movement in other calcium-transporting epithelia, such as intestine and kidney, depends on vitamin D3 and a functional VDR (Hoenderop et al., 2000; Li et al., 2001; Van Cromphaut et al., 2001), the presence of VDR in the avian efferent ductules would be predicted. Therefore, the aim of the present study was to investigate the cellular distribution of the VDR in the extratesticular ducts of the rooster, emphasizing the efferent ductules and epididymal duct.

2. Materials and methods

2.1. Animals

The investigation was performed on epididymal region of 08 crossbreed roosters (*Gallus domesticus*), obtained from domestic and commercial sources and housed in the facilities of the Federal University of Minas Gerais, Brazil. The principles of research involving animals followed those expressed in the 'Princípios éticos para o uso de animais em experimentação', advocated by the 'Comitê de ética em pesquisa', published by the Federal University of Minas Gerais—UFMG (<http://www.ufmg.br/coep/cetea.html>).

2.2. Tissue preparation

The roosters were weighted, anesthetized (i.p. sodium pentobarbital 50 mg/kg body weight), and perfused intracardially with 10% neutral buffer formalin. After fixation, the epididymal regions were isolated from the testis and fragments of tissue were embedded in paraffin, sectioned at 5.0 μ m and used for immunohistochemistry.

2.3. Immunohistochemistry

Vitamin D receptor (VDR) expression was localized in the epididymal region by using immunohistochemistry, following previous protocol (Oliveira et al., 2004). Staining was performed in two different sets to confirm

the results. Sections were dewaxed in xylene, rehydrated through a graded series of ethanol, washed in distilled water and phosphate buffer saline (PBS) and then blocked for endogenous peroxidase by incubation with 0.6% H₂O₂ in methanol for 30 min. The sections were subjected to antigen retrieval procedure by microwaving in 0.01 M sodium citrate buffer pH 6.0. After washing in PBS, the avidin–biotin non-specific binding was blocked using the Vector blocking kit (Vector Laboratories, Burlingame, CA). Additional washing in PBS was performed before the next 1 h incubation in 10% normal rabbit serum. The sections were incubated for 40 h at 4 °C with the diluted (1:50) primary rat anti-chicken VDR antibody (Labvision Co., Fremont, CA). For negative control, the sections received PBS in place of the primary antibody. After washing in PBS, the sections were exposed to 10% normal rabbit serum for 1 h before incubation with a rabbit anti-rat biotinylated secondary antibody (Dako, Carpinteria, CA), used at 1:50 dilution. The sections were then incubated with avidin–biotin complex (Vectastain Elite ABC kit—Vector Laboratories, Burlingame, CA) for 30 min. To visualize the immunoreaction, sections were immersed in 0.05% of 3,3' diaminobenzidine containing 0.01% H₂O₂ in 0.05 M Tris–HCl buffer, pH 7.6. The reaction was monitored microscopically and stopped by immersion in distilled water, as soon as a brown color staining was visualized. Sections were lightly counterstained with Mayer's hematoxylin, dehydrated in ethanol, cleared in xylene and mounted.

2.4. Image analysis

VDR immunostaining intensity was quantified by computer-assisted image analysis, based on previously reported protocols (Oliveira et al., 2003; Zhu et al., 2000). Images from 05 different areas of the proximal and distal efferent ductules as well as connecting and epididymal ducts of each animal were taken by using a Nikon Eclipse E600 microscope (Nikon Co., Melville, NY). Digital images were processed with Adobe Photoshop (Adobe Systems, Mountain View, CA), converted to the grayscale mode and inverted. The images were then exported to Image-Tool software (University of Texas Health Sciences Center, San Antonio, TX), for quantitative analysis. For this proposal, 25 nuclei of nonciliated cells of the efferent ductules and principal cells of the connecting and epididymal ducts, all positive to VDR immunostaining, were traced, measured and pixel intensity was determined for the traced areas. Due to difficulties to clearly delimitate the nuclei of the epithelial cells of the rete testis after image processing, the quantification of the immunostaining in this segment was not performed. Background intensity was determined by tracing an unlabeled area adjacent to the measured cells. Final pixel intensity was calculated by subtracting the values detected in labeled nuclei from the background. Data were expressed as mean \pm SEM.

2.5. Statistical analysis

Differences in VDR expression among segments of the epididymal region were analyzed by using the multiple variance analyses (ANOVA). The post-hoc Tukey test was used for multiple comparisons between segments. Differences were considered significant at $P \leq 0.05$.

2.6. Western blotting

Western blotting analyses were performed to confirm the specificity of the antibody used. Rooster duodenum was used as a positive control. For this purpose, duodenum and epididymal regions from roosters perfused with a 0.75% saline solution were dissected out and rinsed vigorously in PBS before freeze in liquid nitrogen. Frozen fragments (200 mg) were macerated in dry ice and resuspended in 1.5 ml of sample buffer under reducing conditions. After boiling for 5 min, the samples were subjected to continuous electrophoresis using 10% SDS–PAGE (sodium dodecyl sulphate–polyacrylamide gel electrophoresis). The separated proteins were transferred to nitrocellulose membrane and blocked with 10% normal rabbit serum for 1 h at room temperature. The membrane was incubated with rat anti-chicken monoclonal antibody against VDR (Labvision Co., Fremont, CA) diluted 1:500 for 1 h. After washing with PBS–tween 0.05%, the

blot was incubated in a biotinylated secondary antibody rabbit anti-rat (Dako, Carpinteria, CA), used at 1:6000 dilution. The membrane was then incubated with the avidin–biotin complex (Vectastain Elite ABC kit—Vector Laboratories, Burlingame, CA) for 30 min. After several washes, the reaction was developed by the addition of 0.1% 3,3'-diaminobenzidine in PBS containing 0.05% chloronaphthol, 16.6% methanol and 0.04% H₂O₂. The reaction was stopped with deionized water.

3. Results

The rooster epididymal region contains the rete testis, proximal and distal efferent ductules, connecting ducts and epididymal duct. All these segments showed positivity for VDR immunostaining (Fig. 1). The immunoreactivity was noted as a nuclear staining seen in the epithelium and some cells of the connective tissue. The intensity of the epithelial staining was different depending on the segment considered: stronger positivity (measured in pixels) was found in the distal efferent ductules followed by the proximal efferent ductules and epididymal duct as well as the connecting ducts (Table 1 and Fig. 2). The intensity of staining for the connecting ducts was not statistically different when compared to the epididy-

mal ducts. In contrast to the epithelium, the staining of the stromal cells showed similar intensity throughout the excurrent ducts. Vascular endothelium also showed positivity for VDR. No immunolabeling was observed in control sections treated with PBS in place of the primary antibody (Fig. 1).

3.1. Rete testis

The cuboidal epithelial cells lining the rete testis were moderately immunopositive for VDR (Table 1). Cells morphologically similar to macrophages (Aire and Malmqvist, 1979), which were present in the lumen of the rete, were also positive for VDR (Fig. 1B).

3.2. Efferent ductules

In both proximal and distal segments of the efferent ductules, nuclei of the epithelial nonciliated cells were always positive for VDR (Fig. 1C and D). However, the nonciliated cells of the distal ductules exhibited more intense staining for the receptor (Fig. 1D and Table 1). On the other

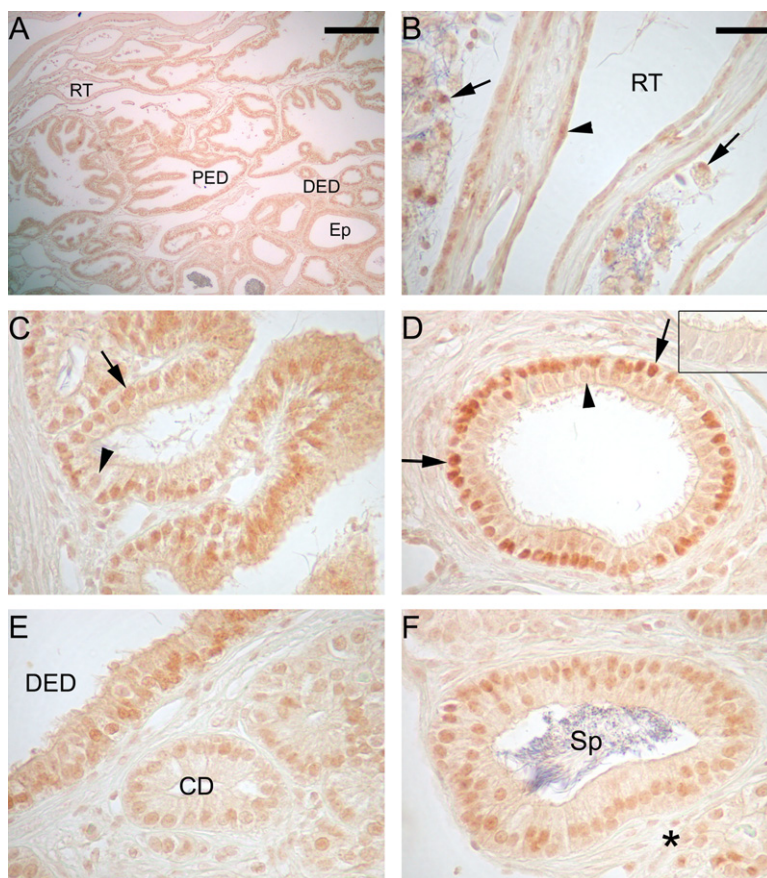


Fig. 1. Expression of VDR in the epididymal region of roosters. (A) General view of the epididymal region showing positive immunostaining for VDR in different segments of the excurrent ducts. RT; rete testis, PED; proximal efferent ductule; DED; distal efferent ductule, Ep; epididymal duct. (B) Extratesticular rete testis (RT) showing moderate immunostaining in the nuclei of epithelial cells (arrowhead), as well as positive macrophage-like cells (arrows) in the lumen. (C) Proximal efferent ductule has moderately positive staining in the nonciliated cells (arrow), in contrast to ciliated cells (arrowhead), which were negative or weakly stained. (D) Distal efferent ductule has the strongest nuclear immunostaining in the nonciliated cells (arrow). Ciliated cells (arrowhead) are negative. The smaller insert represents the negative control. (E) Connecting ducts (CD) showed discrete reactivity to VDR. (F) In the epididymal duct, weak VDR immunostaining was detected in the epithelium as well as connective tissue (*). Spermatozoa (Sp) were found in the lumen of the duct. Bar in A = 200 μ m. Bar in B (the same for C–F) = 20 μ m.

Table 1
Immunostaining intensity for VDR in the ducts composing the epididymal region of the roosters

	VDR
<i>Rete testis</i>	
Epithelial cells	++
Luminal macrophages	+++
Stromal cells	+/-
<i>Proximal efferent ductules</i>	
Epithelial nonciliated cells	++
Epithelial ciliated cells	+/-
Stromal cells	+/-
<i>Distal efferent ductules</i>	
Epithelial nonciliated cells	+++
Epithelial ciliated cells	+/-
Stromal cells	+/-
<i>Connecting and epididymal ducts</i>	
Epithelial principal cells	+
Epithelial basal cells	+
Stromal cells	+/-

Score were as follows: -, negative; +/-, intermittent staining; +, weak staining; ++, moderate staining; +++, strong staining.

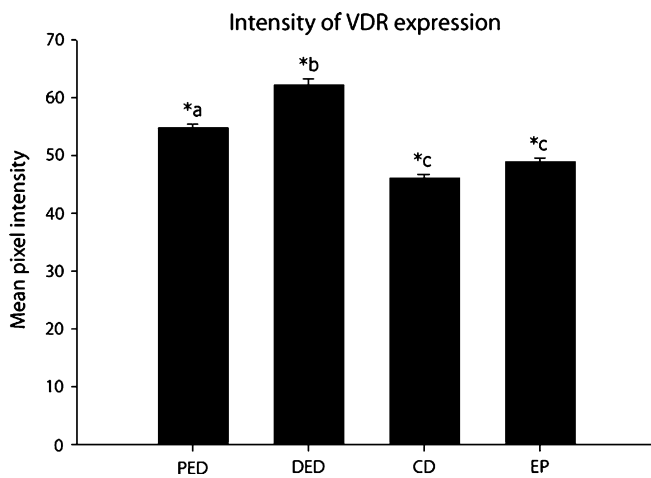


Fig. 2. Quantification of immunohistochemistry for VDR in the extratesticular ducts of roosters. *a, *b, and *c means difference statistically significant ($P \leq 0.05$) between the segments analyzed; Values represent mean \pm SEM; $n = 04$. PED, proximal efferent ductules; DED, distal efferent ductules; CD, connecting duct; EP, epididymal duct.

hand, the ciliated cells were weakly stained or negative at all (Fig. 1C and D, Table 1).

3.3. Connecting duct and epididymal duct

In the connecting and epididymal ducts, the basal and principal cells appeared weakly stained for VDR (Fig. 1E and F). There appeared to be no difference in staining intensity between both epithelial cell types (Table 1).

3.4. Western blotting analysis

In the epididymal region, it was detected a major band of approximately 61 kDa (Fig. 3), while the duodenum presented

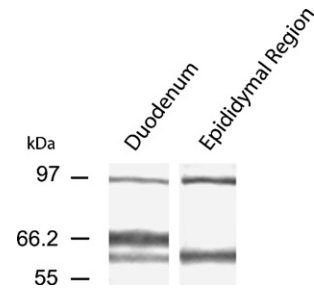


Fig. 3. Western blotting analysis of VDR expression in duodenum and epididymal region of roosters; $n = 04$.

other positive band of about 65 kDa. In both duodenum and epididymis, distinct minor band of 97 kDa was also observed.

4. Discussion

VDR was shown to be widely distributed in the epididymal region of roosters, with specific regional and cellular expressions along the reproductive tract.

The Western blotting pattern observed for VDR in this study is in agreement with previous data in hen's oviduct (Yoshimura et al., 1997). In the duodenum it was detected a doublet band of 61 and 65 kDa, which may correspond to the 58.6 and 60.3 kDa isoforms previously described in avian tissues (Elaroussi et al., 1994; Lu et al., 1997). However, only a 61 kDa protein was detected in the epididymal region, suggesting that only the low molecular weight isoform may be expressed and/or active in the epididymal region. Since VDR performs a wide variety of functions, it has been speculated that VDR isoforms may exist in different tissues with different roles (Lu et al., 1997). This possibility warrants further investigation.

The most intense immunostaining for VDR was found in the efferent ductule epithelium. These data are in broad agreement with those previously described for the rat, in which testicular Sertoli cells and efferent ductules were found to have the most intense binding sites for [3 H]-1,25-(OH) $_2$ D $_3$ (Stumpf and O'Brien, 1987). The exact role of vitamin D $_3$ in the efferent ductules is not known, but it has been suggested that it may participate in the modulation of fluid reabsorption (Johnson et al., 1996). It was already demonstrated that avian efferent ductules are also involved in fluid reabsorption (Bahr et al., 2006; Clulow and Jones (1988, 2004); Zaniboni et al., 2004), with the rate of reabsorption being even greater in birds than in rats (Clulow and Jones, 1988). So, it is possible that vitamin D acting through VDR is participating in the regulation of this important physiological function that increases sperm concentration for storage in the avian epididymal and deferens ducts. On the other hand, differing from rats, there is significant reabsorption of calcium along the avian efferent ductules (Clulow and Jones, 2004). Therefore, other possible explanation for higher VDR expression in these ductules may be related to modulation of the transepithelial calcium transport.

VDR expression was less abundant in the rooster epididymal duct. This finding is in agreement with data obtained in

rat in which the epididymis also presented low level of binding to [^3H]-1,25-(OH) $_2\text{D}_3$ (Stumpf and O'Brien, 1987; Walters, 1984). Connecting ducts presented similar expression of VDR when compared to the epididymal duct, corroborating previous interpretation that together they form one functional unit, as shown by several parameters, including estimative of fluid reabsorption and spermatozoa transit times (Clulow and Jones, 1988), as well as expression of some ion transporters (Bahr et al., 2006). There is little net fluid transport in the epididymal duct, but this region appears to be a site where the spermatozoa acquire their mature pattern of motility (Clulow and Jones, 1988). The exact physiological role of vitamin D $_3$ in this segment remains to be established.

In conclusion, VDR is widely but differentially expressed along the male genital tract of roosters, with higher levels of the receptor being found in the efferent ductules. This data suggest that several extratesticular ducts may be target for vitamin D $_3$ action and that this hormone may have a regional-specific function, such as calcium transport, that is modulated through VDR activity.

Acknowledgments

This work was partially supported by the Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAP-EMIG) and the Brazilian National Council of Scientific and Technological Development (CNPq). André G. Oliveira was the recipient of a scholarship from CNPq and Rubem A. P. Dornas was the recipient of a scholarship from the FAPEMIG/PROBIC. We thank Mr. Saul Oliveira and Mrs. Conceição Oliveira for kindly donate some of the samples used in the study.

References

- Aire, T.A., 1979. Micro-stereological study of the avian epididymal region. *J. Anat.* 129, 703–706.
- Aire, T.A., Malmqvist, M., 1979. Macrophages in the excurrent ducts of the testes of normal domestic fowl (*Gallus domesticus*). *Anat. Histol. Embryol.* 8, 172–176.
- Bahr, J.M., Dalponte, M., Janssen, S., Bunick, D., Nakai, M., 2006. Ion transporters for fluid reabsorption in the rooster (*Gallus domesticus*) epididymal region. *Anim. Reprod. Sci.*
- Bennett, A.K., Hester, P.Y., Spurlock, D.E., 2006. Polymorphisms in vitamin D receptor, osteopontin, insulin-like growth factor 1 and insulin, and their associations with bone, egg and growth traits in a layer–broiler cross in chickens. *Anim. Genet.* 37, 283–286.
- Clulow, J., Jones, R.C., 1988. Studies of fluid and spermatozoal transport in the extratesticular genital ducts of the Japanese quail. *J. Anat.* 157, 1–11.
- Clulow, J., Jones, R.C., 2004. Composition of luminal fluid secreted by the seminiferous tubules and after reabsorption by the extratesticular ducts of the Japanese quail, *Coturnix coturnix japonica*. *Biol. Reprod.* 71, 1508–1516.
- Elaroussi, M.A., Prah, J.M., DeLuca, H.F., 1994. The avian vitamin D receptors: primary structures and their origins. *Proc. Natl. Acad. Sci. USA* 91, 11596–11600.
- Hoenderop, J.G., Nilius, B., Bindels, R.J., 2005. Calcium absorption across epithelia. *Physiol. Rev.* 85, 373–422.
- Hoenderop, J.G., Willems, P.H., Bindels, R.J., 2000. Toward a comprehensive molecular model of active calcium reabsorption. *Am. J. Physiol. Renal. Physiol.* 278, F352–F360.
- Inpanbutr, N., Reiswig, J.D., Bacon, W.L., Slemmons, R.D., Iacopino, A.M., 1996. Effect of vitamin D on testicular CaBP28K expression and serum testosterone in chickens. *Biol. Reprod.* 54, 242–248.
- Johnson, J.A., Grande, J.P., Roche, P.C., Kumar, R., 1996. Immunohistochemical detection and distribution of the 1,25-dihydroxyvitamin D $_3$ receptor in rat reproductive tissues. *Histochem. Cell Biol.* 105, 7–15.
- Kinuta, K., Tanaka, H., Moriwake, T., Aya, K., Kato, S., Seino, Y., 2000. Vitamin D is an important factor in estrogen biosynthesis of both female and male gonads. *Endocrinology* 141, 1317–1324.
- Kurtul, I., 2002. Effects of vitamin-D depletion on chicken testes. *Isr. J. Vet. Med.*, 57.
- Kwecinski, G.G., Petrie, G.I., DeLuca, H.F., 1989. Vitamin D is necessary for reproductive functions of the male rat. *J. Nutr.* 119, 741–744.
- Levy, F.O., Eikvar, L., Jutte, N.H., Cervenka, J., Yoganathan, T., Hansson, V., 1985a. Appearance of the rat testicular receptor for calcitriol (1,25-dihydroxyvitamin D $_3$) during development. *J. Steroid. Biochem.* 23, 51–56.
- Levy, F.O., Eikvar, L., Jutte, N.H., Froysa, A., Tvermyr, S.M., Hansson, V., 1985b. Properties and compartmentalization of the testicular receptor for 1,25-dihydroxyvitamin D $_3$. *J. Steroid. Biochem.* 22, 453–460.
- Li, Y.C., Bolt, M.J., Cao, L.P., Sitrin, M.D., 2001. Effects of vitamin D receptor inactivation on the expression of calbindins and calcium metabolism. *Am. J. Physiol. Endocrinol. Metab.* 281, E558–E564.
- Lips, P., 2006. Vitamin D physiology. *Prog. Biophys. Mol. Biol.* 92, 4–8.
- Lu, Z., Hanson, K., DeLuca, H.F., 1997. Cloning and origin of the two forms of chicken vitamin D receptor. *Arch. Biochem. Biophys.* 339, 99–106.
- Merke, J., Kreusser, W., Bier, B., Ritz, E., 1983. Demonstration and characterisation of a testicular receptor for 1,25-dihydroxycholecalciferol in the rat. *Eur. J. Biochem.* 130, 303–308.
- Oliveira, C.A., Mahecha, G.A., Carnes, K., Prins, G.S., Saunders, P.T., Franca, L.R., Hess, R.A., 2004. Differential hormonal regulation of estrogen receptors ERalpha and ERbeta and androgen receptor expression in rat efferent ductules. *Reproduction* 128, 73–86.
- Oliveira, C.A., Nie, R., Carnes, K., Franca, L.R., Prins, G.S., Saunders, P.T., Hess, R.A., 2003. The antiestrogen ICI 182,780 decreases the expression of estrogen receptor-alpha but has no effect on estrogen receptor-beta and androgen receptor in rat efferent ductules. *Reprod. Biol. Endocrinol.* 1, 75.
- Schleicher, G., Privette, T.H., Stumpf, W.E., 1989. Distribution of soltritol [1,25(OH) $_2$ -vitamin D $_3$] binding sites in male sex organs of the mouse: an autoradiographic study. *J. Histochem. Cytochem.* 37, 1083–1086.
- Stumpf, W.E., Denny, M.E., 1989. Vitamin D (soltritol), light, and reproduction. *Am. J. Obstet. Gynecol.* 161, 1375–1384.
- Stumpf, W.E., O'Brien, L.P., 1987. 1,25 (OH) $_2$ vitamin D $_3$ sites of action in the brain. An autoradiographic study. *Histochemistry* 87, 393–406.
- Van Cromphaut, S.J., Dewerchin, M., Hoenderop, J.G., Stockmans, I., Van Herck, E., Kato, S., Bindels, R.J., Collen, D., Carmeliet, P., Bouillon, R., Carmeliet, G., 2001. Duodenal calcium absorption in vitamin D receptor-knockout mice: functional and molecular aspects. *Proc. Natl. Acad. Sci. USA* 98, 13324–13329.
- Walters, M.R., 1984. 1,25-Dihydroxyvitamin D $_3$ receptors in the seminiferous tubules of the rat testis increase at puberty. *Endocrinology* 114, 2167–2174.
- Walters, M.R., 1992. Newly identified actions of the vitamin D endocrine system. *Endocr. Rev.* 13, 719–764.
- Yoshimura, Y., Ohira, H., Tamura, T., 1997. Immunocytochemical localization of vitamin D receptors in the shell gland of immature, laying, and molting hens. *Gen. Comp. Endocrinol.* 108, 282–289.
- Zaniboni, L., Akuffo, V., Bakst, M.R., 2004. Aquaporins are observed in the duct epithelia of the epididymal region of the large white turkey. *Poult. Sci.* 83, 1917–1920.
- Zhu, L.J., Hardy, M.P., Inigo, I.V., Huhtaniemi, I., Bardin, C.W., Moo-Young, A.J., 2000. Effects of androgen on androgen receptor expression in rat testicular and epididymal cells: a quantitative immunohistochemical study. *Biol. Reprod.* 63, 368–376.