Dissertation

VITAMIN D AND TESTOSTERONE- IS THERE A RELATIONSHIP?

submitted by

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Declaration of authenticity

I hereby declare that this dissertation is my own original work and that I have fully acknowledged by name all of those individuals and organisations that have contributed to the research for this dissertation. Due acknowledgement has been made in the text to all other material used. Throughout this dissertation and in all related publications I followed the guidelines of “Good Scientific Practice”.

Graz, June 2014

Dedicated to Mario

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Abbreviations

$1,25(OH)_2D$	1α25-dihydroxyvitamin D3
25(OH)D	25-hydroxyvitamin D3
AMH	Anti-Müllerian hormone
ABP	Androgen binding protein
ALC	Adult Leydig cell
AR	Androgen receptor
Ca^{2+}	Calcium ion
cAMP	Cyclic adenosin-monophosphate
CK	Cytokeratin
cOC	Carboxylated osteocalcin
CREB	cAMP response element-binding protein
CYP2R1	Vitamin D 25-hydroxylase
CYP3A3	Cytochrome P_{450} 3A4
CYP11A1	Cholesterol side-chain cleavage enzyme
CYP19A1	Aromatase
CYP17A1	Steroid 17-alpha-monooxygenase
CYP24A1	24-hyroxylase
DAPI	4′,6-Diamidin-2-phenylindol
dNTP	Deoxy-nucleoside triphosphate
ESR1	Estrogen receptor 1
FBS	Fetal bovine serum
FSH	Follicle stimulating hormone
FSHR	Follicle stimulating hormone receptor
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GDNF	Glial cell-line derived neutrophic factor
HSD3b	3b-hydroxysteroid dehydrogenase
HSD11B1	11β-hydroxysteroid dehydrogenase type 1
IgG	Immunglobuline G
IGF-1	Insulin-like growth factor 1

ILC	Immature Leydig cell
IL1	Interleukin 1
IPA	Ingenuity Pathway analysis
LC	Leydig cell
LH	Luteinizing hormone
LHCGR	Luteinizing hormone/choriongonadotropin receptor
LIF	Leukemia inhibitory factor
NC	Negative control
OC	Osteocalcin fragment 1-49
PCR	Polymerase chain reaction
PLC	Precursor Leydig cell
PS	Penicillin/Streptomycin
RIN	RNA integrity number
RT-PCR	Reverse-Transcriptase PCR
RT-qPCR	Real Time quantitative PCR
SSC	Spermatogonial stem cell
SHBG	Sex-hormone binding globulin
SLC	Stem Leydig cell
SRD5A2	steroid reductase 5A2
StAR	Steroid acute regulatory protein
TGFa/b	Transforming growth factor **α/**ß
ucOC	Undercarboxylated osteocalcin
VDR	Vitamin D receptor
VDRE	Vitamin D response element
WST-1	Water-soluble tetrazolium salt 1

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1. Zusammenfassung

Einleitung: Vitamin D ist ein vielseitiges Hormon und spielt eine wesentliche Rolle in der Calcium-Homöostase und im Knochen- und Mineralstoffwechsel. In unserer klinischen Studien konnten wir zeigen, dass Serum Vitamin D und Serum Testosteron-Werte bei Männern saisonal assoziiert sind. Basierend auf Studien in Nagetiermodellen ist ein Zusammenhang zwischen Vitamin D und testikulärer Funktion, sowie der Aufrechterhaltung der Spermatogenese bekannt.
Dem Knochen als endokrines Organ und speziell dem Peptidhormon Osteocalcin (OC), das in den Osteoblasten im Knochen gebildet wird und vorwiegend als Marker des Knochenaufbaus dient, kann nach neuen Erkenntnissen ebenso eine Rolle im Androgenstoffwechsel zugeschrieben werden.

Methoden: Für unsere Voruntersuchungen wurden tierische Testes herangezogen und ein Testes-Zellisolierungsprotokoll etabliert. Humane testikuläre Zellen wurden aus Spendergewebe, das einerseits von orchidektomierten Organspendern der Klinischen Abteilung für Transplantationschirurgie, andererseits von Teilorchidektomierten Hodenkrebs-Patienten der Universitätsklinik für Urologie stammte, jeweils gesunde Testes-Explantate, isoliert und für einige Wochen in Kultur gezüchtet. Ntera2/d1 (NT2/d1), humane, testikuläre Keimbahnkrebszellen, wurden für Zellviabilitätsversuche (WST-1-Zellproliferationsassay) unter der Zugabe von 1,25$(OH)_2$D herangezogen. Zelluläre Effekte von Vitamin D, aber auch von LH (Luteotropes Hormon), IGF-1 (Insulin-like Wachstumsfaktor 1), Testosteron und OC wurden auf mRNA Ebene in humanen testikulären Zellen analysiert. Testosteron und DHT (Dihydrotestosteron) wurden im Zellkulturüberstand nach 1,25$(OH)_2$D- bzw. LH- und OC-Behandlung mit Enzyme-Linked-Immunosorbent-Assay (ELISA) gemessen. Die Genexpression wurde basal und nach Zugabe von 1,25$(OH)_2$D mittels Microarray und quantitativer RT-PCR (TaqMan assays) analysiert und der Vitamin D-Rezeptor (VDR) auf Proteinebene mittels Immuncytochemie nachgewiesen.

Ergebnisse: Vorversuche, unter anderem in Schweinehodenzellen, zeigten eine dosisabhängige Steigerung der mRNA-Expression ausgewählter Vitamin D- und Androgenstoffwechsel-Gene nach 1,25$(OH)_2$D Zugabe.

Unsere Untersuchungen in humanen testikulären Zellen zeigten einen Anstieg der Genexpression relevanter Androgenstoffwechselgene nach Zusatz von supraphysiologischen Dosen an 1,25$(OH)_2$D. Die Microarray-Analyse zeigte 63 signifikant regulierte Gene nach Vitamin D-Behandlung, davon waren 57 hochreguliert und 7 niederreguliert. Weiters konnte gezeigt werden, dass 1,25$(OH)_2$D supraphysiologisch die Zellproliferation in gesunden, humanen testikulären Zellen erhöhte, jedoch keine signifikanten Effekte in Hodenkrebszellen NT2/d1 zeigte.

Erhöhte Testosteronkonzentrationen nach 1,25$(OH)_2$D-Gabe konnten im Zellkulturüberstand gemessen werden. Auch die Zugabe von Osteocalcin erhöhte die Testosteron-, sowie DHT-Konzentrationen in den primären testikulären Zellen dosisabhängig.

Schlussfolgerung: Wir können erstmals eine reproduzierbare Beteiligung von Vitamin D und OC an der Androgensynthese in primären humanen Testeszellen nachweisen. Vermutlich sind die Aktivierung des VDR und folglich der Vitamin D response elements (VDREs) in den Zielgenen wichtige Initiatoren der Androgen-Transkription. Weiters wird vermutet, dass auch durch die Bindung von OC an Leydigzell-spezifische Rezeptoren, die Transkription ausgewählter Gene des Steroidstoffwechsels initiiert wird.

Diese Ergebnisse unterstützen neueste Forschungsansätze, wonach der Knochen als endokrines Organ und speziell zirkulierendes OC und Vitamin D wichtige Faktoren der männlichen Fertilität sind. Details der Signalwege von Vitamin D und OC in der Androgensynthese bleiben zu klären. Vitamin D und OC könnten in Zukunft als wichtige Biomarker in der männlichen Fertilität berücksichtigt werden.

2. Abstract

Background: Vitamin D is well known for its function in calcium and bone homeostasis. We have already published based on clinical data that vitamin D and testosterone serum levels in men are saisonal associated. In addition, signalling through the vitamin D receptor (VDR) seems to be of high importance for testicular function, such as spermatogenesis and the synthesis of androgens in rodent models.

The bone derived hormone osteocalcin (OC) has been shown to influence male testosterone production by inducing the transcription and synthesis of androgenic genes.

Methods: We have performed pre-experiments using animal testis tissue and established a protocol for the isolation of testicular cells.

Human testicular primary cells were isolated from human testis tissue, which was derived either from organ donors at the Division of Transplantation Surgery, or from semi-orchidectomized patients due to small testicular cancer at the Department of Urology, in both cases healthy testis explants. Cells were cultured for several weeks to test various stimuli and dosages of vitamin D effects. The testicular carcinoma cell line Ntera2/d1 (NT2/d1) was used to test cell viability.

RNA microarrays were performed to screen for vitamin D effects in human testicular cells on transcript level.

Effects of 1,25$(OH)_2$D, luteinizing hormone (LH), insulin-like growth factor 1 (IGF-1), OC, as well as testosterone on gene expression of selected genes of the androgen and vitamin D synthesis pathway, respectively, were analyzed using real time quantitative PCR (RT-qPCR).

Testosterone and dihydrotestosterone in the cell culture supernatants were analyzed using testosterone enzyme-linked immunosorbent assays (ELISAs). The expression of VDR in human testicular cells was shown by immunocytochemistry.

Results:

In pre-studies using porcine testicular cells, we have shown a dose-dependent effect of 25(OH)D and 1,25$(OH)_2$D on mRNA level of selected genes.

Our studies in human testicular cell cultures have shown that supraphysiological doses of 1,25$(OH)_2$D (100 nM) led to significant alterations in gene expression of reproductive genes.

Microarray analysis revealed sixty three genes in testicular cells to be differentially expressed after 1,25$(OH)_2$D treatment, resulting in 56 upregulated and 7 down-regulated mRNA transcripts.

Supraphysiologic doses of 1,25$(OH)_2$D significantly increased cell proliferation and viability and testosterone concentrations in human testicular cells as measured by WST-1 cell proliferation assay and ELISA, respectively. In testicular carcinoma cells NT2/d1, cell proliferation was not affected by vitamin D. Physiologic doses of osteocalcin increased testosterone and dihydrotestosterone concentrations dose-dependently in testicular primary cells as well as mRNA levels of genes related to the androgen synthesis and metabolism pathway, compared to stimulation with several concentrations of LH.

Conclusions: Vitamin D might play a direct role in male steroidogenesis in vitro, probably by the activation of VDR and the induction of vitamin D response elements (VDREs) in the promotor region of target genes that might increase gene expression of genes involved in male androgen synthesis and testosterone production.

We could further show activation of gene expression and testosterone production by OC, assuming that vitamin D might act indirectly through bone to induce male steroidogenesis.

Signal transduction pathways of vitamin D as well as osteocalcin need to be further claryfied, VitD and OC might be considered as important biomarkers in male fertility.

3. Introduction

3.1. Vitamin D

Vitamin D is a fat-soluble vitamin and a steroid hormone as well. It can be produced from 7-dehydrocholesterol after exposure to sunlight (UVB radiation) and consecutive conversion of the precursor to vitamin D (cholecalciferol). Although 80-90% of vitamin D production derives from sunlight-induced (ultraviolet B radiation, wavelength 290-315nm) production in the skin there is also a small amount that can be derived from diet or supplements. Vitamin D from the diet can either be derived as vitamin D2 (ergocalciferol) from plants and fungi or in form of vitamin D3 (cholecalciferol) by uptake of fatty fish or cod-liver oil (1).

In the liver, cholecalciferol is hydroxylated to 25-hydroxyvitamin D (25(OH)D, or calcidiol), by the enzyme 25-hydroxylase (encoded by *CYP27A1*). Although the liver contains nearly all of the P_{450} enzymes with 25-hydroxylase activity, a physiological form of CYP2R1 is present in peripheral tissues, among them in testes, especially in Leydig cells (2).

Another enzymatic conversion occurs in the kidney, but also in nearly all human cells, where 25-hydroxycholecalciferol serves as a substrate for the enzyme 1-alpha-hydroxylase (*CYP27B1*), which converts it to the biologically most active form, 1α,25$(OH)_2$-Cholecalciferol (1,25$(OH)_2$D, or Calcitriol) (3).

When transported in the circulation, 1,25$(OH)_2$D is preferably bound to a vitamin D-binding protein (VDBP) encoded by the *GC* gene and acts mainly through binding to its nuclear receptor, the vitamin D receptor (VDR). Hetero-dimers with the retinoid X-receptor (RXR) are formed, which in turn bind to vitamin D responsive elements (VDREs) in the region of several target genes (4). VDREs are present in hundreds of genes with effects on cell proliferation, cell growth and differentiation, or apoptosis (5).

A simplified, schematic overview of the vitamin D synthesis pathway is shown in Figure 1.

Vitamin D from the diet and skin that is metabolized to 25(OH)D is used in clinical practice to determine a patient's serum vitamin D status as vitamin D sufficient (25(OH)D ≥ 30 ng/ml), vitamin D insufficient (25(OH)D 20-29 ng/ml) and vitamin D deficient (25(OH)D< 20 ng/ml) (1). Summarized, serum concentrations of 25(OH)D are the main validated biomarker of vitamin D status, equivalent to the pool of vitamin D in the respective body, because 25(OH)D is 10^3 times more concentrated than $1,25(OH)_2D$ and is thought to have considerable functions (1, 6).

Both forms of vitamin D, 25(OH)D as well as $1,25(OH)_2D$, can be further converted to vitamin D metabolites known as $24,25(OH)_2D$ or $1\alpha,24,25(OH)_2D$ by the enzyme 1,25-dihydroxyvitamin D 24-hydroxylase (CYP24A1) (7).

If vitamin D metabolites exhibit also biological actions like their precursor do, has to be further clarified (8).

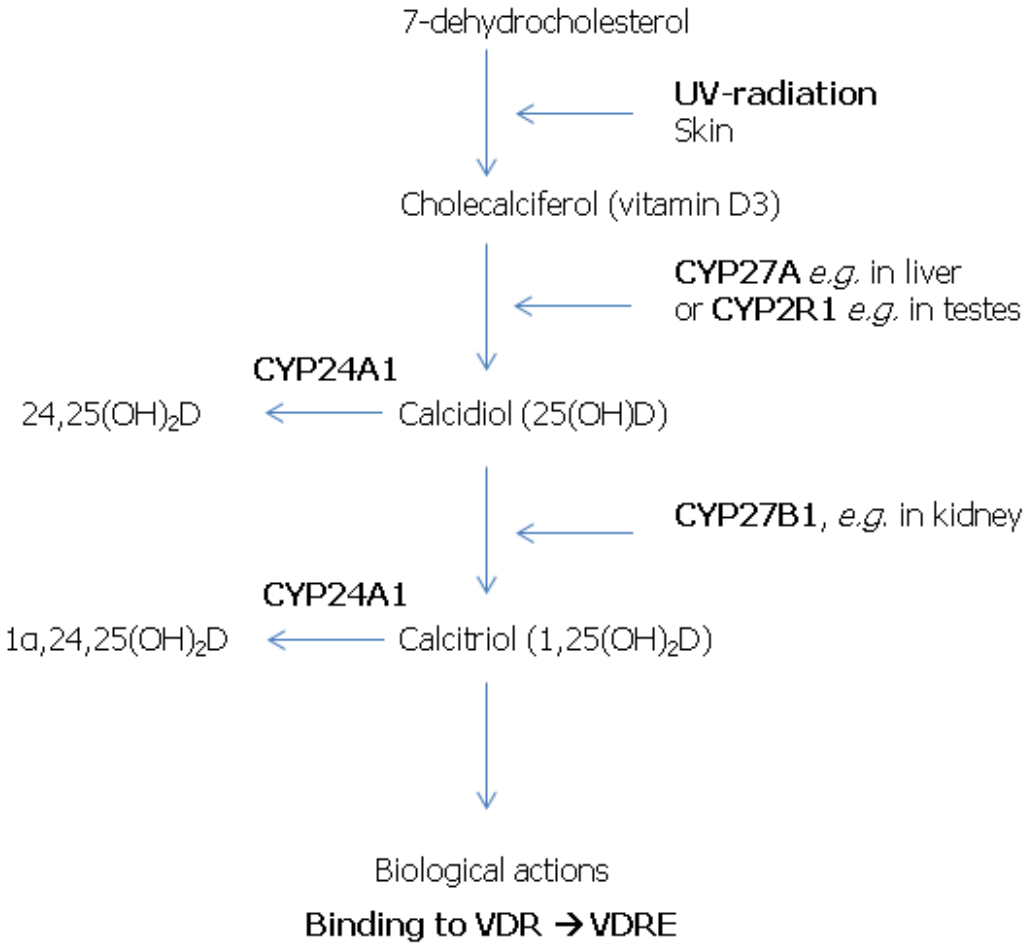

Figure 1. Simplified, schematic overview of the synthesis and metabolism of vitamin D (modified from Morris, HA. et al., Clin Biochem Rev., 2005 (9). CYP27A1, CYP2R1 (vitamin D-25-hydroxylase), CYP27B1 (25-hydroxyvitamin D-1alpha-hydroxylase), CYP24A1 (1,25-hydroxyvitamin D-24-hydroxylase), VDR (vitamin D receptor), VDRE (vitamin D responsive element).

3.2. Production and action of steroid hormones

3.2.1. Steroid hormone synthesis

All steroid hormones have one precursor hormone in common, which is cholesterol. Cholesterol can be taken up by diet or can be synthesized from acetyl CoA. A number of enzymes regulate the production of steroid hormones; the majority of them is belonging to the cytochrome P450 superfamily (CYPs), to hydroxysteroid dehydrogenases (HSDs), and to steroid reductases (10).

There is a number of physiological functions in which steroid hormones exert their functions besides their role in reproductive system and development of sexual characteristics (11). It is known that they are associated with immunological responses, influence the water and salt balance and influence the protein and carbohydrate homeostasis (12-14).

Biologically active steroid hormones can be synthesized in brain, placenta, adrenal gland, ovary, testis and adipose tissue (15).

3.2.2. Steroidogenesis in the testis

In men, the role of the testis is to produce fertile sperm and steroid hormones for reproductive function (15).

The development from spermatogonial cells into mature sperm cells is stimulated by follicle stimulating hormone (FSH), secreted by the pituitary. FSH binds to FSH receptors (FSHR) presented on Sertoli cells, resulting in an activation of several factors required for spermatid production (15).

Male steroidogenesis is induced by LH (Luteinizing hormone) through the activation of cyclic adenosine monophosphate (cAMP) production followed by an increase in intracellular concentrations of calcium ions (Ca^{2+}) in Leydig cells (16, 17).

The secretion of both gonadotropins, FSH and LH, by the pituitary is necessary for the induction of testosterone synthesis. In turn, testosterone acts in a feedback mechanism by stimulating the hormone secretion by the pituitary, with FSH as key stimulus for spermatogenesis (18).

3.2.3. Synthesis of testosterone and its function

Testosterone is a steroid hormone that is belonging to the family of androgens. It is derived from cholesterol as well, followed by a cascade of androgen precursor. A summary of the steroids and the enzyme-catalysed steps is shown in Figure 2.

As main androgen in circulation testosterone is obligatory to support spermatogenesis, differentiation of male internal and external reproductive organs and to induce male sex characteristics and sexual behavior (19). It is necessary for male fertility and functional reproduction, but *e.g.* also for bone metabolism as it is an important factor in the prevention of male osteoporosis (20).

Testosterone can be converted to estrogens by the enzyme aromatase (CYP19A1) or to the more active form DHT (dihydrotestosterone) by the enzyme 5α-reductase (SRD5a) that exists in two isoforms, namely SRD5α1 and SRD5α2 (21, 22). Testosterone and DHT are the only naturally occurring steroids that activate the AR (androgen receptor) with similar high-equilibrium binding affinity (23). Direct binding of both steroids initiates an androgen-dependent gene activation leading to an increased transcription of specific genes important for spermatogenesis and male fertility in general (24).

Other androgens, like androstanediol or DHEA (dehydroepiandrosterone) may also bind to the AR, but the binding of them does not or only slighlty activate the AR to induce downstream signaling effects (25).

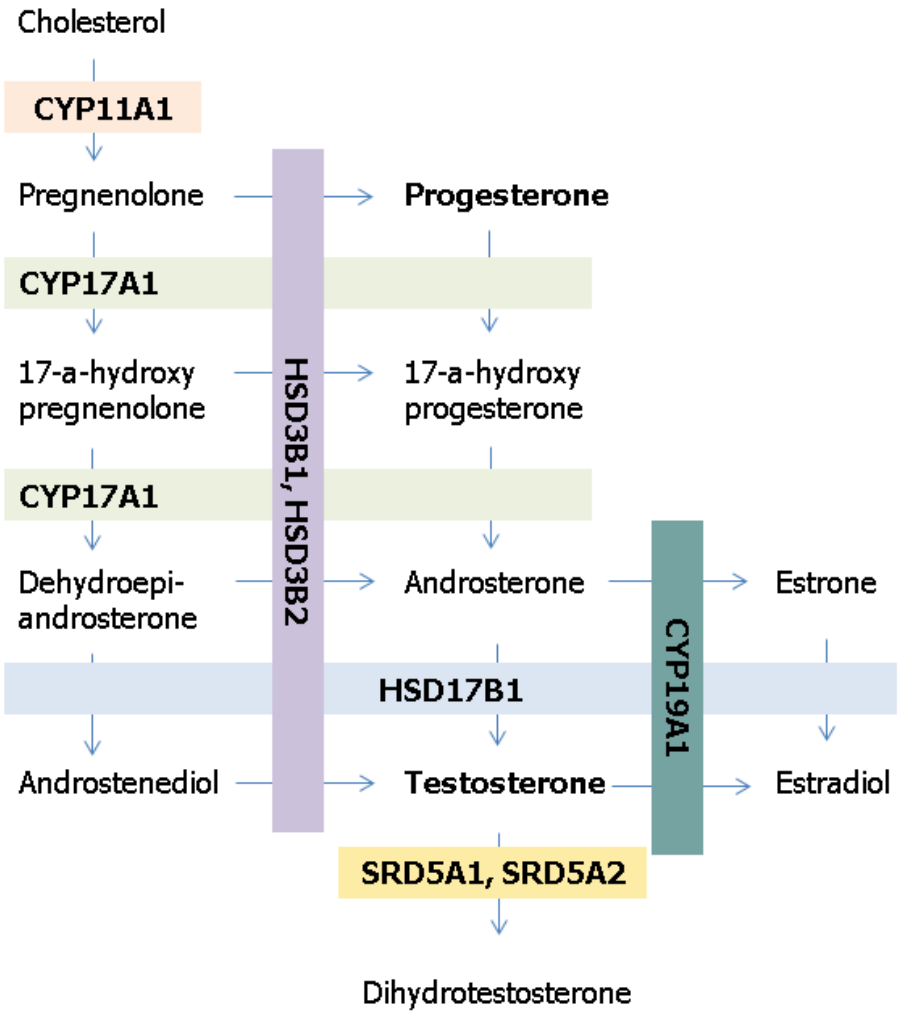

Figure 2. Simplified and schematic overview of the steroidogenesis pathway, modified from Nacusi LP. and Tindall DJ., Nat Rev Urol., 2011 (26). CYP11A1 (cholesterol side-chain cleavage enzyme), CYP17A1 (steroid 17α-monooxygenase), HSD3B1 (3β-hydroxysteroid dehydrogenase 1), HSD3B2 (3β-hydroxysteroid dehydrogenase 2), HSD17B1 (17β-hydroxysteroid dehydrogenase 1), CYP19A1 (aromatase), SRD5A1/A2 (steroid 5α-reductase 1/2).

3.3. Osteocalcin

Osteocalcin (OC) is a peptide hormone, a 49-aminoacid protein, produced by osteoblasts in the bone that has been recognized as a by-product of bone metabolism during two decades and functioned as a marker for bone turnover (27).

Osteocalcin undergoes post-translational modifications that further could differentially affect its bioavailability and bioactivity. Post-translational modification is done by vitamin K-dependent carboxylation resulting in carboxylated osteocalcin (cOC), as well as uncarboxylated or undercarboxylated osteocalcin (ucOC) (28).

Recently, uOC has shown to increase β-cell proliferation in the pancreas (29) as well as insulin sensitivity in tissues like muscle and adipose tissue (29, 30). In the literature different reports suggest that ucOC, but not cOC is responsible for metabolic actions of osteocalcin. Controversies of both isoforms that question if ucOC and cOC exert differential effects are still existent, but in general bone might function as an endocrine organ (29, 31).

The newly discovered endocrine action of bone has further suggested a feedback loop between the gonads and the skeleton. Clarke and Khosla have shown that androgens have beneficial effects on bone (32). Another feedback loop might suggests that OC has an influence on male testosterone biosynthesis (33). Oury *et al.* have shown that the testes are indeed a target organ for OC (34).

Summarized, latest studies have shown a crosstalk between testes and bone via OC in which OC has been discovered to influence testosterone production by the testis to regulate male fertility (30, 35) and vice versa, androgens have shown to have beneficial effects on bone (32).

3.4. Influences of vitamin D, the skeleton, and male androgen synthesis

Leydig cells express the VDR and vitamin D metabolizing enzymes (36) and have the ability to secrete INSL3 that is known to have a role in osteoblast function (37, 38). 1,25$(OH)_2$D is able to activate osteoblastic synthesis of osteocalcin by transcriptional activation of the human osteocalcin gene (39-41).

Besides the classical steroid hormones and factors known to be involved in functional spermatogenesis, namely IGF-1 (insulin-like growth factor-1), TGFα/β (transforming growth factor α and ß), IL1 (interleukin 1), TH (thyroid hormone) and AMH (anti-mullerian hormone), there is evidence that vitamin D, beyond his traditional role in calcium homeostasis and function as a regulator of bone health, may also modulate reproductive processes in women and men (42).

Our study group has shown significant associations of 25(OH) vitamin D levels and testosterone levels and further a similar seasonal association of both hormones with a common depth point in March and peak levels in August in a clinical study, which included 2299 men (43). The seasonal variation of vitamin D paralleled by testosterone in men has been shown also by others (44-46) assuming that there might be a relationship of vitamin D and testosterone.

Our group could show that daily supplementation with 83 µg (3,332 IU) of vitamin D was able to increase testosterone levels in a cohort of healthy overweight men undergoing a weight reduction program. In this randomized controlled trial, total, bioactive, as well free testosterone levels were significantly increased in the vitamin D supplemented group in contrast to the placebo group (47).

Based on rodent studies, vitamin D deficient male rats have shown a reduction in successful matings and overall fertility (48), further an incomplete spermatogenesis and degenerative changes within the testes, compared to controls (49).

The skeleton is usually viewed to be a tissue responsible for mechanical and scaffolding functions and to be a recipient of hormonal input. Vitamin D plays an essential role in maintaining a healthy and mineralized skeleton (50) as it is well known that vitamin D deficiency is associated with rickets in children (51) and osteomalacia in adults (52).

An OC induced testosterone production in Leydig cells has been shown in male murine models, but OC has failed to have an influence on estrogen or testosterone synthesis in the ovaries (30, 34). Responsible for the OC induced testosterone production in male Leydig cells is a G-protein coupled receptor (GPCR) present on the surface of Leydig cells, known as GPRC6A that belongs to the family of calcium sensing receptors that is not expressed in the ovaries (53, 54). Activation of the GPRC6A on Leydig cells lead to an intracellular signaling event triggered through an adenylat cyclase and the production of cAMP (cyclic adenosine monophosphate). CREB (cAMP response element-binding protein) might be a possible transcriptional effector of OC regulation of testosterone biosynthesis by favoring the expression of key enzymes involved in testosterone biosynthesis (55).

In a clinical study ucOC and ucOC/total OC ratio were positively associated with serum free testosterone levels in men with Type 2 diabetes and ucOC/total OC ratio was negatively associated with LH. This phenomenon might be explained by a feedback loop of the gonads, where a reduction in ucOC leads further to reduced testosterone levels that in turn raised LH (56).

3.5. Testicular cells

3.5.1. Leydig cells

Adult, mature Leydig cells (ALCs) of the testis are responsible for the production of the male sex hormone testosterone, but they first need to develop from stem LCs to progenitor LCs (PLCs) and further to immature LCs (ILCs) until they are finally developed to ALCs (57). The differentiation of human Leydig cells from mesenchymal cells or fibroblast-like precursor cells is hormonally regulated (58).

We assume that the development of human Leydig cells is similar to that of rodents, in which Leydig cell development can be characterized in the four stages as displayed in Figure 3: Stem Leydig cells (SLC), progenitor Leydig cells (PLC), immature Leydig cells (ILC) and adult Leydig cells (ALC) (59).

At postnatal day 7, the testicular interstitium includes spindle-shaped SLC that do not express any of the Leydig cell-specific markers like HSD3B (hydroxysteroid dehydrogenase 3b) or LHR (luteinizing hormone receptor), assuming that these cells did not enter the Leydig cell lineage (59).

Around postnatal day 14, as a direct result of proliferation of SLC in the testicular interstitium, PLC are generated which are HSD3B positive. PLC are small, spindle-shaped cells and are members of the Leydig cell lineage due to their expression of Leydig cell markers including HSD3B, LHR and their production of androgen, mainly androsterone (60).

PLCs develop, enlarge, become round and acquire numerous lipid inclusions until postnatal day 28 when they transform into ILCs. The smooth endoplasmic reticulum (sER) expands during the development from PLCs to ILCs concurrent with the levels of HSD3B (61), CYP17A1 (steroid-17α-hydroxylase/17,20 lyase), CYP11A1 (cholesterol side-chain cleavage enzyme) (60).

From day 28 to day 56 the ILC population doubles only once and ILCs differentiate into ALCs, leading to an increase in testosterone production. Compared to ILCs the ALCs have fewer and smaller lipid inclusions and a greater abundance to sER (62). Normally ALCs do not proliferate but they are able to regenerate (63).

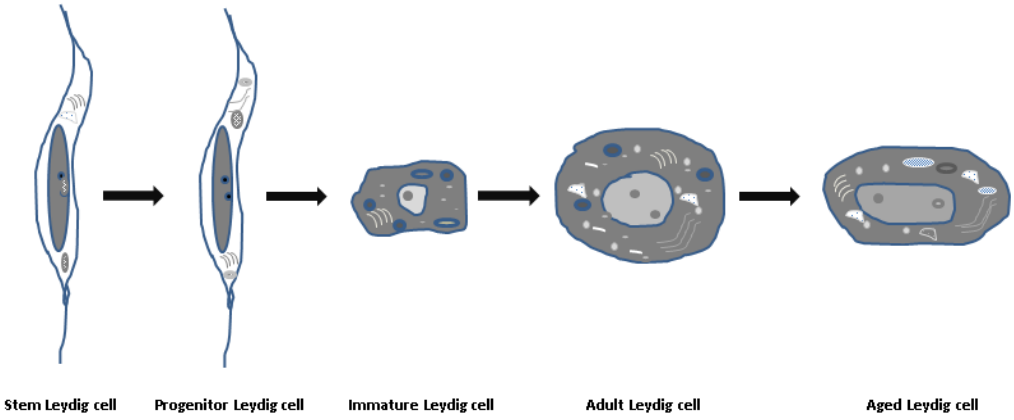

Figure 3. Schematic overview of the different stages of Leydig cell development (Modified from Chen et al (2010) (64).

3.5.2. Sertoli cells

Sertoli cells are somatic cells within testes that are essential for spermatogenesis, as they facilitate the progression of germ cells to spermatozoa by controlling the environmental milieu within the seminiferous tubules. Besides the role of Sertoli cells in testis formation, they provide factors important for functional spermatogenesis, like junction complexes and barriers as well as nutrients (65). The physical and metabolic support of Sertoli cells is necessary for germ cell differentiation, meiosis and transformation into spermatozoa (66).

Sertoli cells are very often called “nurse” cells, because of their function to nourish sperm cells during their development and secrete lots of factors, like AMH

(anti-Müllerian hormone), inhibin and activin, ABP (androgen binding protein), estradiol or the GDNF (glial cell-line-derived neutrophic factor).

Spermatogenesis is mainly regulated by the action of testosterone and FSH on Sertoli cells (65). Furthermore, the action of FSH is demonstrated biochemically by an increase in cAMP levels, increased protein synthesis and increased estradiol production (67).

After an initial formation of the embryonic testes, in which Sertoli cells sequester germ cells (gonocytes) inside of newly formed seminiferous tubules, Sertoli cells and germ cells undergo rapid proliferation. Mentionable at this point is the fact that only immature Sertoli cells in the peripubertal period have the ability to proliferate.

The final number of Sertoli cells is determined before adulthood (66). The number of Sertoli cells in the adult testes determines the size of the testis as well as the daily sperm production (68).

3.5.3. Testicular germ cells and stem cells

Germ cells develop to spermatogonia, spermatocytes, spermatids and spermatozoons through the process of spermatogenesis. The process of spermatogenesis starts with self-renewal and differentiation of spermatogonial stem cells (SSC) (69). Sertoli cells in the seminiferous tubules regulate the self-renewal division of SSC by secreting GDNF (70). Conrad et *al.* assumed that although cells of the germline are restricted to generate gamets, they have the ability to give rise to cells that are pluripotent (71).

The pluripotency of human stem cells is proved by the ability of cells to form teratoma and by the confirmation of transcription factors and factors associated to

pluripotency by microarray assays. Teratomas are tumors containing cells of all three germ layers (72).

Pluripotency factors that are expressed in adult human testicular germ line stem cells are STAT3, CD9, KLF4, OTEX, VASA, POU6F1, DAZL, OCT4, NANOG, SOX2, e-cadherin and GDF3 (71).

The ability of mesenchymal cells from testes is of high interest concerning to a possible restoration of spermatogenesis, *e.g.* in patients after oncotherapy, as well as studies that focus on the establishment of other cell types from these pluripotent cells (73).

4. Hypothesis of the thesis

4.1. First hypothesis

We hypothesize that vitamin D might act in a direct way on male steroidogenesis by binding to vitamin D receptors (VDR) present on testicular cells. The activated VDR further might act as transcription factor that binds to VDREs in the promotor region of target genes, thereby inducing gene expression, *e.g.* genes involved in androgen synthesis.

4.2. Second hypothesis

We assume that vitamin D might also be involved indirectly in male steroidogenesis via the skeleton. We suggest that osteocalcin might induce gene transcription of several genes involved in androgen synthesis and testosterone production in human testicular cells.

5. Goal of the thesis

We want to identify genes and pathways of male steroidogenesis that are directly and indirectly affected by 1,25$(OH)_2$D using a human testicular cell culture model.

As a second goal, we want to identify indirect effects of vitamin D via the bone derived hormone osteocalcin on male steroidogenesis and on *in vitro* testosterone production in human testicular cells.

At the moment, no human testicular cell line is commercially available and experiments are restricted to animal models. We think that the establishment of a human testicular cell line, including testosterone-producing Leydig cells and growth- and co-factor donating Sertoli cells, is of high interest for the scientific community.

6. Reagents and Materials

6.1. Cell culture reagents

1,25$(OH)_2$D (Sigma-Aldrich, St. Louis, MO)

25(OH)D (Sigma-Aldrich, St. Louis, MO)

Accutase® solution (Sigma-Aldrich, St. Louis, MO)

Amphotericin B solution (0,25mg/ml) (Sigma-Aldrich, St. Louis, MO)

Calciol (Sigma-Aldrich, St. Louis, MO)

Collagenase I (Sigma-Aldrich, St. Louis, MO)

Collagenase B (Roche Diagnostics GmbH, Vienna, Austria)

Dimethylsulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO)

DME/F12 medium GlutaMax, 1:1 (Gibco®, life technologies, Carlsbad, CA)

DNase 1 (Sigma-Aldrich, St. Louis, MO)

EDTA (2mM) (Sigma-Aldrich, St. Louis, MO)

Ethanol (ROTH, Karlsruhe, Germany)

FBS (PAA, Pasching, Austria)

Gelatin powder (Sigma-Aldrich, St. Louis, MO)

Glycine (1M) (Sigma-Aldrich, St. Louis, MO)

HEPES (Gibco®, life technologies, Carlsbad, CA)

IGF-1 (Sigma-Aldrich, St. Louis, MO)

Insulin solution (Sigma-Aldrich, St. Louis, MO)

Luteinizing hormone from human pituitary (Sigma-Aldrich, St. Louis, MO)

Non-essential amino acids (100X) (Gibco®, life technologies, Carlsbad, CA)

Osteocalcin fragment 1-49 (Sigma-Aldrich, St. Louis, MO)

PBS (pH 7,2-7,3) (Apotheke LKH- Univ.-Klinikum Graz)

Penicillin/Streptomycin solution (10,000 U/10mg/ml) (Sigma-Aldrich, St. Louis, MO)

Percoll (GE Healthcare Bio-Sciences AB, Uppsala, Sweden)

Soy trypsin inhibitor (Sigma-Aldrich, St. Louis, MO)

Transferrin (Sigma-Aldrich, St. Louis, MO)

Trypsin/EDTA (Gibco®, life technologies, Carlsbad, CA)

Vitamin solution (100X) (Gibco®, life technologies, Carlsbad, CA)

6.2. Cell culture expendable materials

CELLSTAR® cell culture flasks with filter cap (25 cm², 75 cm² and 175 cm²) (Greiner Bio-One GmbH, Frickenhausen, Germany)

Cell strainer (75 µm, 100 µm) (BD Biosciences, New Jersey, USA)

Cryo tubes (2ml) (Greiner bio-one, Frickenhausen, Germany)

Disposable serological pipettes (2ml, 5ml, 10ml 25ml) (Corning Incorp., NY, USA)

Gauge needles (Sterican, B. Braun Melsungen AG, Germany)

Sterile syringe filter (0,2 µm) (Sartorius AG, Göttingen, Germany)

6.3. Human testicular carcinoma cell line *Ntera2/d1*

Ntera2/d1 (NT2/d1) cells were testis pluripotent embryonal carcinoma cells, derived from a lung metastasis and were purchased from ATCC (ATCC, Lot-Nr: 59368198). The adherent and epithelial-like growing cells were cultured in DME media, supplemented with 2 mM Glutamin and 10% FBS at 37°C and 5% CO_2 and splitted with Accutase as soon as they became confluent.

6.4. Reagents and test kits for genetic analysis

Bioanalyser RNA analysis kit (Agilent Technologies, Santa Clara, CA)

Chloroform (ROTH, Karlsruhe, Germany)

GeneChip Human 1.0 ST array (Affymetrix, Santa Clara, CA)

High Capacity RNA to cDNA Kit (Applied Biosystems, Foster City, CA)

Isopropanol (ROTH)

LightCycler® 480 SYBR Green I Mastermix (Roche, Mannheim, Germany)

RNeasy Mini Kit (Quiagen, Hilden, Germany)

TaqMan® gene expression assays (Invitrogen™, life technologies) (**Table 1**)

TriReagent (Ambion®, life technologies)

UltraPure™ DNase/RNase-Free Distilled Water (Invitrogen™, life technologies)

WT Expression Kit for Affymetrix GeneChip whole transcript (WT) expression arrays (Ambion®, life technologies)

WT Terminal Labeling Kit (Affymetrix, Ambion®, life technologies)

6.5. ELISAs and cell proliferation reagents

5a-dihydrotestosterone ELISA (IBL International, Hamburg, Germany)

Testosterone ELISA (Demeditech, Kiel-Wellsee, Germany)

Testosterone positive control (Lyphocheck^{TM} Immunoassay Plus Control Level 1 and 3, Biorad, Vienna, Austria)

Trypan-blue solution (0,4%) (Sigma-Aldrich, St. Louis, MO)

WST-1 cell proliferation reagent (Roche Diagnostics, Vienna, Austria)

Table 1. TaqMan® gene expression assays

	gene symbol	Amplicon length	TaqMan® Assay
Housekeeping genes			
Glycerinaldehyd-3-phosphat-dehydrogenase	GAPDH	122 bp	Hs99999905_m1
beta Actin	ACTB	171 bp	Hs99999903_m1
18S rRNA	18S	187 bp	Hs99999901_s1
Steroid metabolism			
Estrogen-Receptor alpha	ESR1	62 bp	Hs00174860_m1
Estrogen-Receptor beta	ESR2	63 bp	Hs00230957_m1
Cytochrome P_{450} 3A4	CYP3A4	92 bp	Hs00430021_m1
Steroid 17-alpha-monooxygenase	CYP17A1	72 bp	Hs01124136_m1
Androgen-Receptor	AR	72 bp	Hs00171172_m1
Aromatase	CYP19A1	105 bp	Hs00903413_m1
Steroid 5 alpha reductase 1	SRD5A1	120 bp	Hs00602694_m1
Steroid 5 alpha reductase 2	SRD5A2	83 bp	Hs00165843_m1
Hydroxysteroid 17β-dehydrogenase	HSD17B1	124 bp	Hs00166219_g1
Cholesterol side-chain cleavage enyzme	CYP11A1	77 bp	Hs00167984_m1
Hydroxysteroid-dehydrogenase 3β1	HSD3B1	112 bp	Hs04194787_g1
Hydroxysteroid-dehydrogenase 3β2	HSD3B2	136 bp	Hs00605123_m1
Luteinizing hormone Receptor	LHCGR	105 bp	Hs00896336_m1
Follicle stimulating hormone Receptor	FSHR	79 bp	Hs00174865_m1
Steroid acute regulatory protein	STAR	78 bp	Hs00986559_g1
11β-Hydroxylasen	CYP11B1	64 bp	Hs00357016_g1
Vitamin D metabolism			
VDR	VDR	62 bp	Hs00172113_m1
Vitamin D 24-Hydroxylase	CYP24A1	123 bp	Hs00167999_m1
25-hydroxy steroid-dehydrogenase	CYP2R1	79 bp	Hs01379776_m1
1,25-Hydroxylase	CYP27B1	60 bp	Hs00168017_m1
Vitamin D-25-Hydroxylase	CYP27A1	80 bp	Hs01026016_m1
Microarray			
Insulin-like growth factor 1	IGF-1	68 bp	Hs01547656_m1
Alkaline phosphatase	ALPL	79 bp	Hs01029144_m1
CD 14 molecule	CD14	140 bp	Hs02621496_s1
Methylenetetrahydrofolate reductase	MTHFR	95 bp	Hs00195560_m1
Solute carrier family 1 member 1	SLC1A1	76 bp	Hs00188172_m1
Kruppel-like factor 4	KLF4	110 bp	Hs00358836_m1
Dipeptidyl-peptidase 4	DPP4	90 bp	Hs00175210_m1
Calponin-like transmembrane domain protein	CLMN	66 bp	Hs00226865_m1
Transmembrane protein 37	TMEM37	90 bp	Hs01931464_s1
Six-transmembrane epithelial antigen of prostate 4	STEAP4	75 bp	Hs01026584_m1

6.6. Reagents/material for immunocytochemistry

Antibody diluent (Dako North America, Inc., CA)

Blocking buffer:
5 % normal goat serum (Dako), 3% Triton™X-100 (Sigma-Aldrich, St. Louis, MO)

Chamber glass slides (Lab-Tek™ II Chamber slide™ System, Nalge Nunc International Corp., Naperville, IL)

Cytokeratin antibody (Polyclonal Rabbit Anti-Cow Cytokeratin Wide Spectrum Screening, Dako)

DAPI (Vectashield Hard Set Mounting Medium with DAPI, Vector Laboratories, Burlingame, CA)

EnVision™ FLEX Target Retrieval Solution (Dako North America, Inc., CA)

Epiandrosterone (MP Biomedicals, LLC, Solon, OH)

Fixation buffer:
3,7% paraformaldehyde and 0,18 % Triton™X-100 (Sigma-Aldrich, St. Louis, MO)

IgG control antibody (FLEX, Negative control Mouse Cocktail of mouse IgG1, IgG2a, IgG2b, IgG3 and IgM, ready-to use, Dako)

Labeled antibodies:
Alexa Fluor® 555 Rabbit Anti-Goat IgG (H+L) (Invitrogen™, life technologies, Carlsbad, CA)
Goat Anti-Mouse IgG (H+L) Dylight™ 488 Conjugated (Thermo Scientific, Rockford, IL)

Nitroblue tetrazolium chloride (NBT, Sigma-Aldrich, St. Louis, MO)

Nicotinamide adenine dinucleotide (βNAD, Sigma-Aldrich, St. Louis, MO)

PBS pH 7,2-7,3 (Apotheke LKH- Univ.-Klinikum Graz)

Vitamin D receptor antibody (VDR (D-6), Santa Cruz Biotechnology Inc., Santa Cruz, CA)

6.6.1. Breast cancer cells *MDA-MB231* and *SKBR3*

The human breast cancer cell lines *MDA-MB231* and *SKBR3* were a gift from Dr. Nadja Dandachi at the Division of oncology at the Medical University of Graz. We got the breast cancer cells in form of frozen cytospins for the use in immunocyto-chemistry.

7. Methods

7.1. Pre-experiments

7.1.1. Cell isolation protocol for testicular cells

Feline and canine testes were obtained after routine castration in collaboration with the veterinarian practice of Dr. Tritthart, Graz, Austria.

In collaboration with the veterinarian Dr. Bubalo, we have obtained rat and ovine testes from the Institute of biomedical research (Hahnhof) at the medical university of Graz as well as porcine testes after routine castration from a pig farm in Croatia.

Testes were stored in cold PBS supplemented with 5% Penicil-lin/Streptomycin solution and transported on ice to our laboratory. Small parts of the animal testes (300g tissue) were either step-wise frozen (15 min at 4°C→ 20 min at -20°C→ 24 h at -80°C→ for long term freezing at -170°C in liquid nitrogen)

in DMEM/F12 supplemented with 20% FBS and 10% DMSO, or they were immediately prepared for cell isolation.

Isolation of testicular cells was performed according to the method described previously (74) with little modifications. The epididymis and tunica albuginea of the testis were removed and testes explants (~ 500 g) immediately digested mechanically as small as possible using small surgery scissors. The mechanical dissected tissue parts were digested for 90 min at 37°C in 50 ml 15mM $NaHCO_3$, 15 mM Hepes buffered Ham's F-12 medium and Dulbecco's modified Eagle medium containing 2 mg/ml collagenase B, 10 µg/ml DNase, 1µg/ml soy trypsin inhibitor in a constant shaking waterbath (80 rpm). Digested tissue fragments were filtered through a mesh filter (150 µm), cells centrifuged (5 min, 200 *g*) and resuspended in fresh F-12/DME medium. Interstitial cells were separated from seminiferous tubule fragments by unit gravity sedimentations (2 times for 15 min). Interstitial cells in both supernatants were combined and Leydig-cell enriched fractions prepared by unit gravity sedimentation for 15 min followed by centrifugation (5 min, 200 *g*). Interstitial cells were resuspended in 3 ml fresh F-12/DME medium and applied to a 4-layer discontinuous Percoll gradient (21, 26, 34 and 60% in F-12/DME). Centrifugation occurred at 1500 *g* for 30 min. Cells were collected from the cell band between 34 and 60% Percoll (~ 10 ml), diluted with 20 ml fresh medium and centrifuged (10 min, 200 *g*). Cells including Leydig cells were washed 2 times in PBS (5 min, 300 *g*) to get rid of the Percoll solution and seeded into culture flasks (75 cm^2).

To remove peritubular myoid cells, sedimented tubular fragments from the first two unit gravity sedimentations were suspended in 2 volumes of Ca^{2+} and Mg^{2+} free PBS with 1 M glycine and 2 mM EDTA and further digested by constant up and down pipetting for 5-10 min. Contaminating germ cells were tried to remove as follows: A digestion of the cell suspension in F-12/DME supplemented with 2 mg/ml trypsin and 2,5 mM EDTA at 37°C for 10 min was done. Afterwards the suspension was pressed through a sterile syringe needle (0,40 mm). Germ cells remaining in the supernatants after adjacent sedimentations (2 x 15 min)

were removed and Sertoli-enriched fractions were seeded into cell culture flasks (25 cm^2).

7.1.2. 25(OH)D and 1,25$(OH)_2$D dose response

Porcine testicular cells were treated with several concentrations of 25(OH)D (50, 100, 200 and 500 ng/ml) or 1,25$(OH)_2$D (0,1, 0,5, 10 and 100 nM) for 16 h. Gene expression levels were analyzed by RT-qPCR using the LightCycler® 480 SYBR Green I Mastermix and porcine primer pairs as summarized in Table 4. Sample preparation for the PCR mix as well as the temperature protocol for the LightCycler® are summarized in Table 2 and Table 3, respectively. 15 µl RT-qPCR-mastermix were mixed with 5 µl cDNA (2 µl cDNA and 3 µl pure water), in total 20 µl per PCR reaction.

Table 2. Preparation of RT-qPCR samples using LightCycler® 480 SYBR Green I mastermix

Reagent	Volume (µl)
SYBR Green I Master Mix (2 x conc.)	10
Primer forward (20µM)	1
Primer reversed (20µM)	1
Nuclease-free water	3
Total	**15**

Table 3. Temperature protocol for RT-qPCR using LightCycler® 480 SYBR Green I mastermix

Step	Time	Temperature (°C)	Cycles
Pre-incubation	10 min	95	1
	10 sec	95	
Amplification	10 sec	60	45
	20 sec	72	
	10 sec	95	
Melting curve	60 sec	70	1
		95	
Cooling	10 sec	40	1

Table 4. Porcine primer pairs for RT-qPCR using LightCycler® 480 SYBR Green I Mastermix

Gene	Forward (F) and reverse (R) primer sequences	bp	Tm (°C)
GAPDH	F-CCCTGAGACACGATGGTGAA R-GGTTCACGCCCATCACAAAC	415	59/60
VDR	F-CCTTCCAGCCCGAGTGCAGC R-CGGAACAGCGCAGACTGGCA	550	65/66
CYP24A1	F-CCCCCGTCGAGCTGCACAAG R-ACGCCAAACGGAAGGTGGGC	607	65/66
HSD3B	F-TCTCCAGGTTCGCCCGCTCA R-GAATGGGCTCCCCTCCCCGT	674	65/66
CYP19	F-TGGACCTCGTCATGCGAAAA R-AGCTAGCAAAGATGGGTGGT	475	59/60
CYP2R1	F-TTGCTGCTCTTCGCCCTCGG R-GAAGACTGAGGCACTGGCGGC	624	65
CYP17A1	F-CCACACGGAATGGAGAGTCC R-TCGCCAATGCTGGAGTCAAT	676	60

7.2. Ethical approval

Human testes were obtained either from testicular cancer patients at the Department of Urology, without anti-androgenic treatement, or from organ donors at the Department of Surgery, Division of Transplant Surgery, in total seven samples from patients aged 35-81 years. The local ethical committee of the Medical University of Graz, Austria, approved the protocol for both institutions and the positive votum was deposited with the ethical identification number “21-176 ex 09/10. Patients at the Department of Urology gave their informed consent. The ethical votum is updated annually by interim reports to the ethical committee.

Pieces of the healthy part of the testes (5 mm^3) from urological patients, or parts of testes (3 cm^3- 5 cm^3) from organ donors at the Division of Transplant Surgery were transported in a PBS solution containing 100 µg/ml Penicillin, 100 U Streptomycin and 2,5 µg/ml Amphotericin on ice and immediately processed.

7.3. Isolation of human testicular cells

For the isolation of human testicular cells we have used the protocol established with animal testicular tissue (see **7.1.1**.) with some minor modifications: When human tissue was limited in its amount and size, we did not remove peritubular myoid cells, as well as germ cells by additional steps assuming that we would have lost precious cells with every additional step.

7.4. Inverse phase microscopy

We used the Olympus inverted microscope (Olympus IX53, Tokyo, Japan) for routine microscopy, which included standard routine cell growth observations as well as cell counting using the hemocytometer (Neubauer proofed chamber, Marienfeld-Superior, Lauda-Königshofen, Germany).

For fluorescence microscopy, the Olympus microscope BX51 (Olympus Optical CO. (Europe) GmbH, Hamburg, Germany) with incorporated digital camera was used. A CCD camera was connected to the computer to observe the morphology of the cell cultures on the monitor using the "cellSens" digital imaging software (cellSens Standard version 1.8.1, Olympus).

7.5. Immuno- and cytochemical staining

7.5.1. HSD3ß staining

Human Leydig cells were histochemically identified by 3β-hydroxysteroid-dehydrogenase staining as described previously (75) with some modifications: 20 µl of interstitial cell suspensions were allowed to grow on chamber glass slides overnight at 37°C in a humidified atmosphere at 5% CO_2.

On the next day, the staining solution was prepared as follows: In one 1,5 ml Eppendorf tube 80 µl (0,2 mg/ml) nitroblue tetrazolium chloride and 20 µl (1 mg/ml) β-nicotinamide adenine dinucleotide, in another 1,5 ml Eppendorf tube 96 µl (0,12 mg/ml) Epiandrosterone and 4 µl PBS were mixed separately and then both tube contents combined. The cell supernatant in the chamber slides was re-

moved, cells washed 2 times with PBS and medium replaced with staining solution.

After 6 h, cells positive for HSD3β were stained purple to blue and were observed by the Olympus BX51 microscope. Pictures have been made and digital images handeled using the ImageJ software (ImageJ, version 1.46r, National Health Institute, USA).

7.5.2. Nuclear staining

20-30 µl of testicular cell suspension were seeded on chamber slides and incubated overnight at 37°C. When cells got adherent to the slide, they were washed 2 times with PBS and then covered with 5 drops of mounting media including DAPI. Cells were immediately capable for their observation using the inverted phase microscope.

7.5.3. VDR staining

We have seeded 3 x 10^5 primary testicular cells on each chamber in a chamber slide and treated them with 100 nM 1,25$(OH)_2$D overnight. *MDA-MB231* breast cancer cells were used as VDR-negative control according to Cordes et *al.* (76) and the breast cancer cell line *SKBR3* functioned as VDR-positive control.

Cells were further fixed with fixation buffer for 30 min at 37°C. After 3 times washing in PBS for 5 min, cells were permeabilized for 20 min in retrieval buffer at 85°C. After three washing steps, cells were blocked with blocking buffer for 30 min at room temperature. The blocking buffer was discarded and cells immediately

treated with VDR antibody, Cytokeratin antibody and IgG control overnight in a humidified chamber at 4°C in the dark.

The used antibodies and buffers are summarized in Table 5. After three times washing we have treated cells with labeled antibodies for 1 h in a humidified chamber in the dark at room temperature. After three final washing steps cells 15 µl of mounting media including DAPI were added and cells covered with a cover slip. Labeled cells were immediately ready for observation using the Olympus fluorescent microscope BX51 and captured images were analyzed using the Olympus cellSens® software.

Table 5. Primary antibodies used for immunocytochemistry.

Antibody	Species	Dilution	Fixation	Company
VDR-D6	mouse	1:100	3,7% paraformaldehyde, 0,18 % Triton	Santa Cruz, sc-13133
CK	rabbit	1:300	3,7% paraformaldehyde, 0,18 % Triton	Dako, Z0622
IgG control	mouse	ready-to-use	3,7% paraformaldehyde, 0,18 % Triton	Dako, IR750

7.6. Cell proliferation and viability

The WST-1 cell proliferation reagent was used to quantify viability and proliferation in human primary testicular cells as well as human testiclar carcinoma cells NT2/d1.

The method is based on the principle that mitochondrial dehydrogenases in viable cells are able to convert WST-1, a tetrazolium salt, into formazan, which

leads to a change of absorbance measurable with a plate reader at 450- 650 nm of wavelength.

7.6.1. Cell viability in testicular carcinoma cells

We have spread an amount of 1x 10^6 cells/ml (*NT2/d1*) in 96 well plates. Cells were incubated for 24 h in a humidified atmosphere at 37°C with 5% CO_2.

The next day cells were adherent and the supernatant was removed and replaced with fresh medium including hormones, 25(OH)D or 1,25$(OH)_2$D, respectively, as summarized in Table 6. Cells treated with vehicle, which was ethanol, the solvent for vitamin D, were used for control. Cell proliferation was analyzed after 24, 48 and 72 h of vitamin D treatment in *NT2/d1* cells.

For the cell proliferation measurement after each time point of incubation 10 µl WST-1 cell proliferation reagent were added for 2 h and the absorbance measured with a spectroscopic plate reader (SPECTROstar Omega, BMG Labtech, Ortenberg, Germany) at 490 nm.

All samples were tested in 3 replicates and the whole experiments repeated at least twice.

7.6.2. Cell viability in human testicular cells

Human testicular primary cells were seeded into 96-well plates with a cell density of 2x 10^5 cells/ml. After 24 h and adherence to the plates, we have replaced the medium with medium that included 10, 50 or 100 nM 1,25$(OH)_2$D, as summarized in Table 6.

In another experiment we have replaced the cell supernatant with medium supplemented with different concentrations of testosterone (1, 5, 10, 20, 50 ng/ml) to the cells.

Cell viability was analyzed 24, 48 and 72 h after hormonal treatment according to the manufacturer's protocol and compared to controls.
All samples were tested in 3 replicates and the experiments repeated twice.

Table 6. Overview of the experimental setups used for cell proliferation and viability tests

Hormone/Treatment	Concentrations	Incubation	Cell number/ well format	Annotations
Human testicular primary cells				
1,25$(OH)_2$D	10 50 100 *nM*	24 h	2 x 10^5/ 96-well plate	Ethanol served as control
Testosterone	1 5 10 20 50 *ng/ml*	24 h	2 x 10^5/ 96-well plate	Ethanol served as control
Human testicular carcinoma cells NT2/d1				
1,25$(OH)_2$D	100 nM	24 h	1 x 10^6/ 96-well plate	Ethanol served as control
25(OH)D	100 nM	24 h		

7.6.3. Cell counting using trypan blue

We have determined the viability of freshly isolated testicular cells with the trypan blue solution and have used the uptake of trypan blue by the cells as marker for cell death.

25 µl cell suspension and 25 µl of trypan blue solution were mixed in a 1,5 ml reaction tube and 20 µl of this suspension were transferred to a hemocytometer (Neubauer chamber) and covered with a cover slip.

Cells have been counted by hand using the Olympus IX53 microscope. The number of blue-stained cells was expressed as percentage of the total number of cells counted per coverslip, giving information about the non-viable cells.

7.7. Immunological assays

7.7.1. Testosterone ELISA

Testosterone in the culture media of cultured testicular cells was assayed using a commercial immunoassay (Demeditech, Kiel-Wellsee, Germany) according to the manufacturer's recommendations.

Two samples with defined testosterone concentrations were used as positive control: Lyphocheck control Level 1 with 1,41 ng/ml (range 0,820-2,00) and Lyphochek control Level 3 with 12, 0 ng/ml (range 6.97-17,1).

ELISA plates (96-well plates) were analyzed using the Gladiator (IASON, Graz, Austria). After standard curve interpolation the concentrations of the sam-

ples were expressed in nanograms per milliliter (ng/ml). All samples were assayed in duplicates from at least 3 separate experiments.

7.7.2. 5α-DHT ELISA

5αDHT (5α-dihydrotestosterone) was measured from the cells supernatants after addition of the hormones using a commercial ELISA (5α-DHT-ELISA, IBL International, Hamburg, Germany) according to the manufacturer's recommendations.

7.8. Setups for androgen measurements

7.8.1. Responses on 1,25$(OH)_2$D compared to LH

Human primary testicular cells were treated either with vehicle (Ethanol) or 100 nM 1,25$(OH)_2$D and testosterone measured in the cell supernatant after 24 h.

In another experiment we added 5 IU/l LH alone or in combination with 100 ng/ml IGF-1, with or without additionally 100 nM 1,25$(OH)_2$D to primary testicular cells and analyzed testosterone concentrations after 24 h.

7.8.2. Dose-response effects of LH and osteocalcin

Dose-dependent effects of different concentrations of LH (0,5, 1, 5, and 10 IU/l) and osteocalcin (0.05, 0,5, 1, 5, 10, 30, and 50 ng/ml) on testosterone delivery in humane testicular cells. Hormones were added for 24 h at 37°C in a humidified atmosphere with 5% CO_2 until they were analysed.

7.8.3. Responses on osteocalcin compared to LH

To analyse effects of osteocalcin compared to LH on testosterone synthesis, we added either different concentrations of LH (0,5 or 5 IU/l), or osteocalcin (1, 10, 30 ng/ml) alone, or both hormones together (5 IU/l LH + 10 ng/ml OC and 5 IU/l LH + 30 ng/ml OC) to primary testicular cells. Testosterone levels were analyzed from the cell supernatants after 24 h.

All hormonal treatments for the separate setups and experiments are summarized in Table 7.

Table 7. Experimental desing for determination of testosterone concentrations by ELISA

Hormone/Treatment	Concentrations	Incubation
Experiment 1		
$1,25(OH)_2D$	100 nM	24 h
Experiment 2		
$1,25(OH)_2D$	100 nM	24 h
LH	5 IU/l	
IGF-1	100 ng/ml	
Experiment 3		
LH	0,5 IU/l 1 IU/l 5 IU/l 10 IU/l	24 h
Osteocalcin	0,05 ng/ml 0,5 ng/ml 1 ng/ml 5 ng/ml 10 ng/ml 30 ng/ml 50 ng/ml	
Experiment 4		
LH	0,5 IU/l 5 IU/l	24 h
Osteocalcin	1 ng/ml 10 ng/ml 30 ng/ml	

7.9. Gene expression analysis

7.9.1. RNA isolation

Total RNA was extracted from isolated testicular cells (1x 10^6 cells) in TriReagent, followed by phase separation using chloroform and RNA precipitation using isopropanol. The concentration of isolated RNA was determined using the Nanodrop spectrophotomer (NanoDrop® Spectrophotometer ND-1000, peQLab Biotechnology GmbH, Erlangen, Germany).

The NanoDrop® was used to receive information about the RNA quality: *e.g.* if the 260/230 ratio, which should be ~2- 2,2, or the 260/280 ratio, which should be

~2 for RNA, was too low, that might have indicated the presence of protein, phenol or other contaminants.

If RNA was not of acceptable quality it was cleaned-up using the RNeasy Mini Kit (Quiagen, Hilden, Germany) according to the manufacturer's instructions.

RNA integrity in samples used for the microarray analysis was additionally assessed using the Bioanalyser. When RNA samples were of acceptable quality they could have been used for microarrays.

7.9.2. Reverse transcriptase PCR (RT-PCR)

1000 ng of each RNA preparation were reverse-transcribed using the High Capacity RNA to cDNA Kit following the manufacturer's instructions. 10 µl of prepared 2x-master mix (Table 8) were combined with 10 µl of RNA (1000 ng RNA + pure water), in total 20 µl.

The program for the Thermocycler-PTC 200 (MJ Research, Quebec, Canada) was: 10 min at 25°C → 120 min at 37°C → 5 min at 85°C, followed by a final cooling.

Table 8. Preparation of the 2x-mastermix for cDNA synthesis by RT-PCR

Reagent	Volume (µl)
10x RT-Puffer	2
25x dNTP Mix (100mM)	0,8
10x RT Random Primers	2
MultiScribe Reverse Transcriptase	1
Nuclease-free water	4,2
Total	**10**

7.9.3. RNA microarray analysis

7.9.3.1. Hybridization and analysis

RNA was isolated as described in **7.9.1.** and RNA integrity assessed using the Bioanalyser (Agilent Technologies, Santa Clara, CA).

Hybridizations and analysis were done at the Division Core Facility Molecular biology at the Centre of Medical Research (ZMF) at the Medical University of Graz.

Total RNA was labeled using the Ambion WT Expression Kit for Affymetrix GeneChip whole transcript (WT) expression arrays and hybridized to GeneChip Human 1.0 ST arrays as described in the manufacturer's manual.

250 ng of total RNA for each sample were reverse transcribed to double strand cDNA using engineered primers containing a T7 promoter sequence. Anti-sense RNA was generated through in vitro transcription using T7 RNA polymerase and cDNA as template. Random hexamers were used to generate single-stranded cDNA and dUTPs were incorporated at the same step in order to fragment later in the protocol. After hydrolyzation of the cRNA with RNase H, the sense strand of cDNA was purified using nucleic acid binding beads according to the manual and subsequently fragmented by incubation with UDG (uracil DNA glycosylase) and APE 1 (apurinic/apyrimidic endonuclease 1) which recognize the unnatural dUTP residues (WT Terminal Labeling Kit, Affymetrix). A terminal labeling occured with TdT (terminal deoxynucleotidyl transferase) followed by an incubation time of the labeled sense strand DNA for 16h at 45°C with rotation in a hybridization oven.

The Affymetrix Genechip® fluidics station 450 was used for washing and staining (GeneChip® HT hybridization, wash and stain kit, Affymetrix) according to the manual (protocol on fluidics station: FS450_0007).

Labeling and hybridization controls were evaluated with the expression console EC 1.3 and arrays were scanned with the Affymetrix GeneChip scanner GCS3000.

7.9.3.2. Assessment of microarray data

The analysis of microarrays has been performed for seven testicular cell samples that were available in control and 1,25$(OH)_2$D treated conditions. The “CEL files” were imported into Partek Genomic Suite software (software v6.6, Partek Inc, St. Louis, MO) and robust multi-chip average (RMA) normalized, including background correction, quantile normalization across all arrays and median polished summarization based on log transformed expression values.

For statistical analysis, a 3-way ANOVA was performed comparing 1,25$(OH)_2$D-treated with control samples over all patient samples obtained from both institutes. Genes with FDR5% and fold change of at least 1,5 or -1,50, respectively, were considered to be significantly affected by 1,25$(OH)_2$D.

Genes that have passed the determined filter criteria were further analyzed with the Ingenuity IPA (**I**nteractive **P**athway **A**nalysis of complex 'omics data) software (version 17199142, Ingenuity systems, Redwood City, CA). IPA categorized the top-regulated genes, among other things, in functions and/or diseases, networks and canonical metabolic and signaling pathways.

7.9.3.3. Confirmation of top genes by RT-qPCR

The messenger RNA (mRNA) expression of twelve significantly 1,25$(OH)_2$D regulated genes (based on microarray analysis) was confirmed by RT-qPCR (real time quantitative polymerase chain reaction).

RT-qPCR was performed on the LightCycler® 480 Instrument II using TaqMan® gene expression assays for alkaline phosphatase, liver/bone/kidney (*ALPL*), CD14 molecule (*CD14*), calmin, or calponin-like transmembrane domain protein (*CLMN*), aromatase (*CYP19A1*), vitamin D_3-24-hydroxylase (*CYP24A1*), dipeptidyl-peptidase 4 (*DPP4*), insulin-like growth factor 1 (*IGF-1*), krüppel-like factor 4 (*KLF4*), methylene-tetrahydrofolate reductase (NAD(P)H) (*MTHFR*), solute carrier family 1 member 1 (*SLC1A1*), six-transmembrane epithelial antigen of prostate 4 (*STEAP4*) and transmembrane protein 37 (*TMEM37*) (TaqMan® gene expression assays are summarized in Table 1 in Reagents and Materials).
For TaqMan® gene expression assay validations we have used pooled cDNA from the samples and determined the linear range of the assays.

The temperature protocol for the LightCycler® 480 Instrument II is displayed in Table 9.

7.9.4. Gene expression of human testicular cells after hormonal treatment

7.9.4.1. Gene expression at baseline

Relative gene expression of the VDR and vitamin D metabolizing enzymes (CYP27A1, CYP27B1, CYP2R1, CYP24A1) as well as of selected genes of the androgen metabolism (*AR, LHCGR, CYP11A1, HSD3B1, HSD3B2, CYP17A1, HSD17B1, CYP3A4, CYP19A1, SRD5A2, GPRC6A, ESR1, ESR2*) were analyzed in isolated testicular primary cells at baseline using TaqMan® gene expressions assays (Table 1) and the LightCycler® 480 Instrument II (Roche Diagnostics GmbH, Vienna, Austria). We have used the same temperature protocol for the LightCycler® as mentioned in **7.9.3.3.** and summarized in Table 9.

Table 9. TaqMan Run Protocol for RT-qPCR analysis using TaqMan® gene expressions assays

Step	Time	Temperature (°C)	Cycles
Pre-incubation	2 min	50	1
Denaturation	10 min	95	1
Amplification	15 sec	95	50
	30 sec	60	
Cooling	10 sec	40	1

7.9.4.2. Effects of 1,25(OH)$_2$D on gene expression

The transcript level of selected genes of the androgen synthesis pathway was analyzed after addition of 5 IU/l LH alone, or in combination with 100 ng/ml IGF-1 with or without addition of 100 nM 1,25(OH)$_2$D to testicular cells, independent of the microarray experiment by RT-qPCR using TaqMan® gene expressions assays (Table 1).

The selected genes were 3ß-hydroxysteroid dehydrogenase 2 (HSD3B2), Cholesterol monooxygenase (CYP11A1), aromatase (CYP19A1), cytochrome P450 3A4 (CYP3A4), estrogen receptor α (ESR1) and steroid 5α reductase 2 (SRD5A2).

The mRNA expression of the selected genes was analyzed in triplets and normalized against an internal control, which was the housekeeping gene *GAPDH*.

The relative quantification of gene expression and the calculation of fold changes was done using the ΔΔCt method according to Pfaffl (77).

7.9.4.3. Effects of osteocalcin on gene expression

The transcript level of selected genes of the androgen synthesis and metabolism pathway was analyzed after addition of 1 or 5 IU/l LH, or 10 or 30 ng/ml osteocalcin (OC) alone as well as after combined addition of 5 IU/l LH and 10 ng/ml OC, or 30 ng/ml OC to testicular cells using again RT-qPCR and TaqMan® gene expression assays.

For this experiment the following genes, which were belonging to the androgen synthesis and metabolism pathway, were selected: androgen receptor (AR), luteinizing hormone/choriongonadotropin receptor (LHCGR), cholesterol monooxygenase (CYP11A1), 3β-hydroxysteroi dehydrogenase 1 (HSD3B1), 3β-hydroxysteroid dehydrogenase 2 (HSD3B2), steroid 17α-monooxygenase (CYP17A1), 17β hydroxysteroid dehydrogenase 1 (HSD17B1), cytochrome P450 3A4 (CYP3A4), and aromatase (CYP19A1).

The expression of mRNA of the selected genes was analyzed in triplets and normalized against an internal control, which was the housekeeping gene *GAPDH*. Relative quantifications of gene expressions were calculated as mentioned previously.

7.10. Statistical analysis

Statistical analysis was carried out using GraphPad (Version 5, GraphPad Software Inc., San Diego, CA, USA). One-way analysis of variance (ANOVA) followed by Dunnett post analysis was used for statistical differences (> 2 groups).

Microarrays were analyzed using Partek Genomic Suite software (software v6.6, Partek Inc, St. Louis, MO). Data ("CEL files") received from microarray scans was robust multi-chip average (RMA) normalized, which included background correction, quantile normalization across all arrays and median polished summarization based on log transformed expression values. Afterwards we used three-way ANOVA, comparing 1,25$(OH)_2$D-treated with control samples over all patient samples (from both institutes).

Mean values after confirmation of selected genes by RT-qPCR were compared by *t* test (2 groups) and a *Pearson* correlation was calculated comparing microarray and RT-qPCR. Differences were regarded as statistically significant at $P < 0.05$. Values are presented as mean ± SD.

8. Results

8.1. Pre-experiments

8.1.1. Feline, rodent and porcine testicular cells

Testicular cells were isolated from cat, rat and pig testes for the establishment of a cell isolation protocol for the use of human testes tissue. Animal testicular cells were adherent growing and cultured for several weeks where they were viable for ~ 9-10 passages until they became necrotic without special cell culture conditions. A mixture of several cell types was observable in all cell cultures obtained from all three animals using the inverted phase microscope. Epitheloid growing Leydig and Sertoli cells, as well as spindle shaped peritubular myoid cells were growing together with small, round germ cells that were growing in suspension (Figure 4).

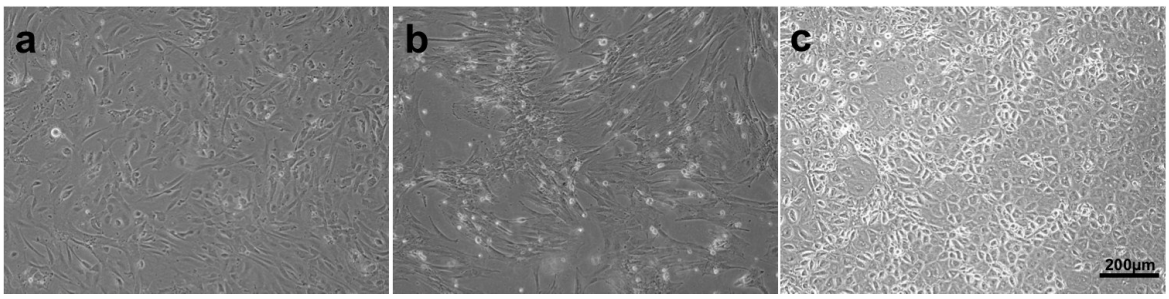

Figure 4. Isolated, animal testicular cells at passage 3-4. Confluent, epitheloid cells are shown. **a** Feline testicular primary cells. **b** Rodent testicular cells. **c** Porcine testicular cells. bar=200 µm

8.1.2. Dose-response of vitamin D forms

We have analyzed the gene expression of vitamin D receptor (VDR), Vitamin D 25-hydroxylase (CYP2R1), 1,25-dihydroxyvitamin D_3 24-hydroxylase (CYP24A1), steroid 17α-dehydrogenase (CYP17A1), 3β-hydroxysteroid dehydrogenase/Δ-5-4 isomerase (HSD3B), and aromatase (CYP19A1) by RT-qPCR after the addition of several concentrations of 25(OH)D or $1,25(OH)_2D$ to porcine testicular cells.

Both vitamin D metabolites have shown effects on mRNA expression of selected genes in porcine testicular cells dose dependently. 200 ng/ml of 25(OH)D has shown the highest increase on mRNA level of the selected genes, compared to baseline, with fold changes up to 3 (CYP24A1) (Figure 5). Addition of 10 nM of $1,25(OH)_2D$ has shown highest effects on mRNA level of selected genes, reaching fold changes up to 3,42 (HSD3B2) (Figure 6).

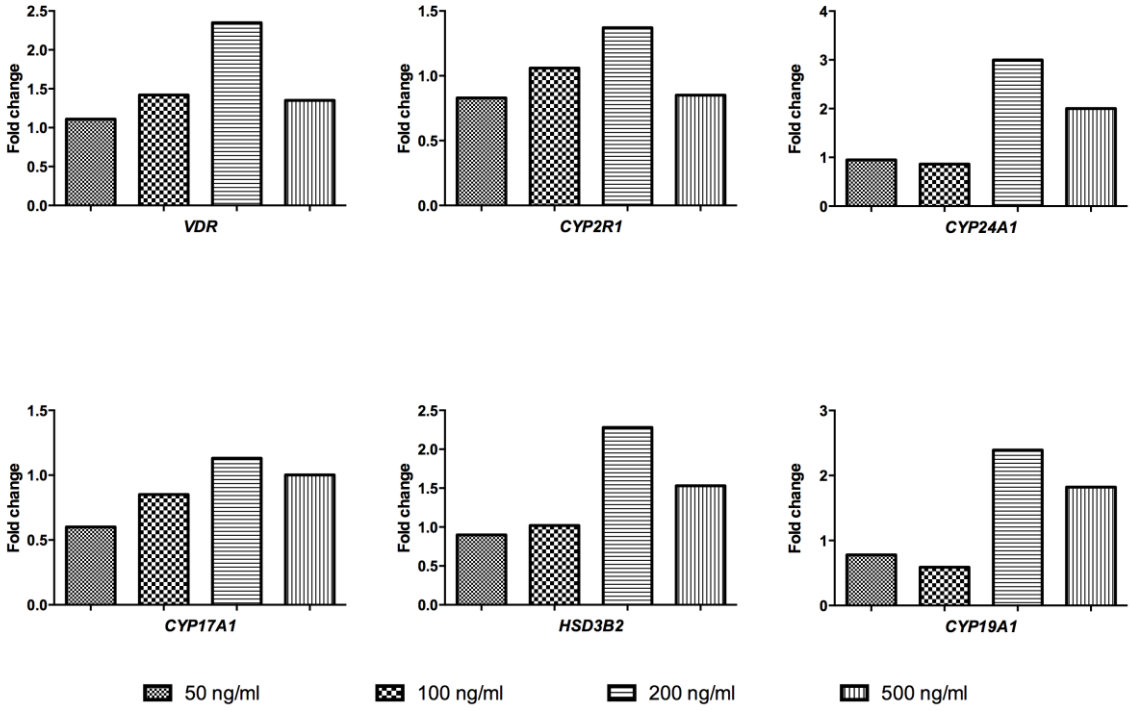

Figure 5. Dose dependent expression of selected genes after addition of 25(OH)D to human testicular primary cells. CYP17A1 (Steroid 17-alpha-monooxygenase), VDR (vitamin D receptor), CYP24A1 (1,25-dihydroxyvitamin D3 24-hydroxylase), CYP2R1 (Vitamin D 25-hydroxylase), HSD3B2 (3β-hydroxysteroid dehydrogenase/Δ-5-4 isomerase 2) and CYP19A1 (Aromatase).

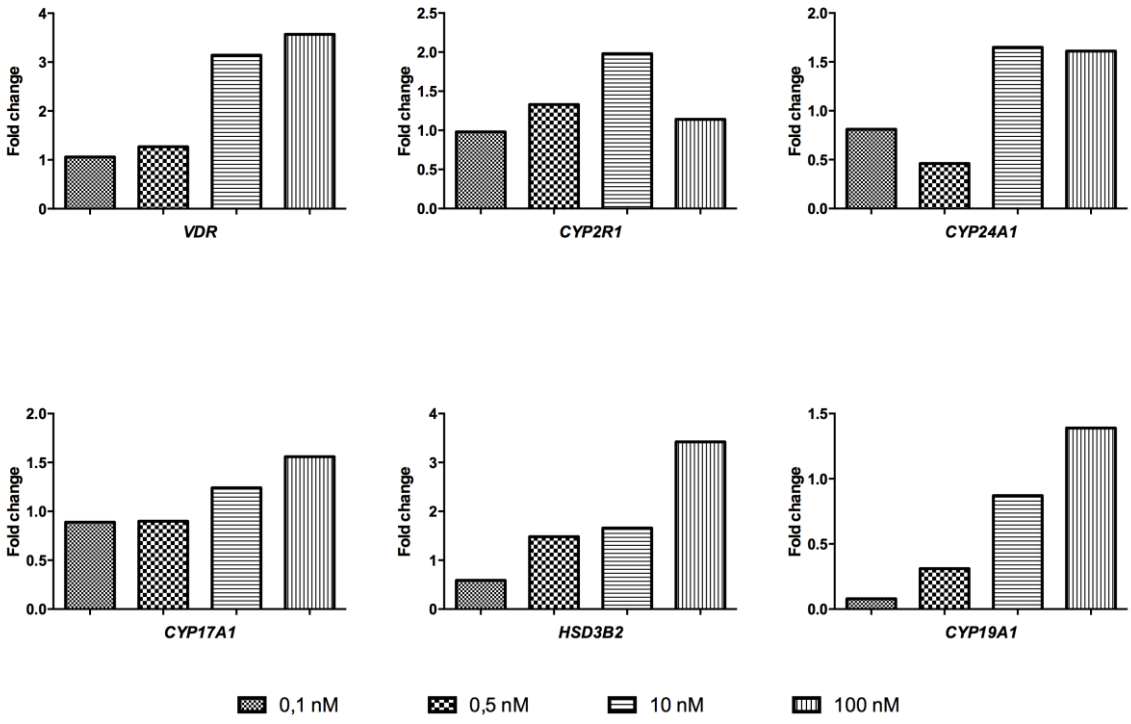

Figure 6. Dose dependent expression of selected genes after addition of 1,25(OH)2D to human testicular primary cells. CYP17A1 (Steroid 17-alpha-monooxygenase), VDR (vitamin D receptor), CYP24A1 (1,25-dihydroxyvitamin D3 24-hydroxylase), CYP2R1 (Vitamin D 25-hydroxylase), HSD3B2 (3β-hydroxysteroid dehydrogenase/Δ-5-4 isomerase 2) and CYP19A1 (Aromatase).

8.2. Human primary testicular cells

8.2.1. Morphological characterization

Testicular cells were isolated and cultured from healthy testes tissue biopsy samples of four brain dead organ donors from the Department of Transplantation Surgery and of three men who underwent orchiectomy after the diagnosis of small testicular cancer at the department of Urology (Table 11).

Cells adhered on the bottom of the cell culture flask 16 hours after isolation. We have identified testicular Leydig, Sertoli as well as peritubular myoid cells (tes-

ticular fibroblasts) based on their morphology and floating germ cells in the supernatants of the cell cultures.

Leydig cells initially appeared spindle shaped and changed their morphology into epitheloid appearance when finally differentiated into adult Leydig cells. aLCs could further be identified by a high active cytoplasm with enriched crystal-like structures, so called Reinke' crystals (Figure 7c). Larger, flat growing and often tree-like-branched cells were indicated as human Sertoli cells, additionally characterized by their typical included phagosomes (Figure 7d).

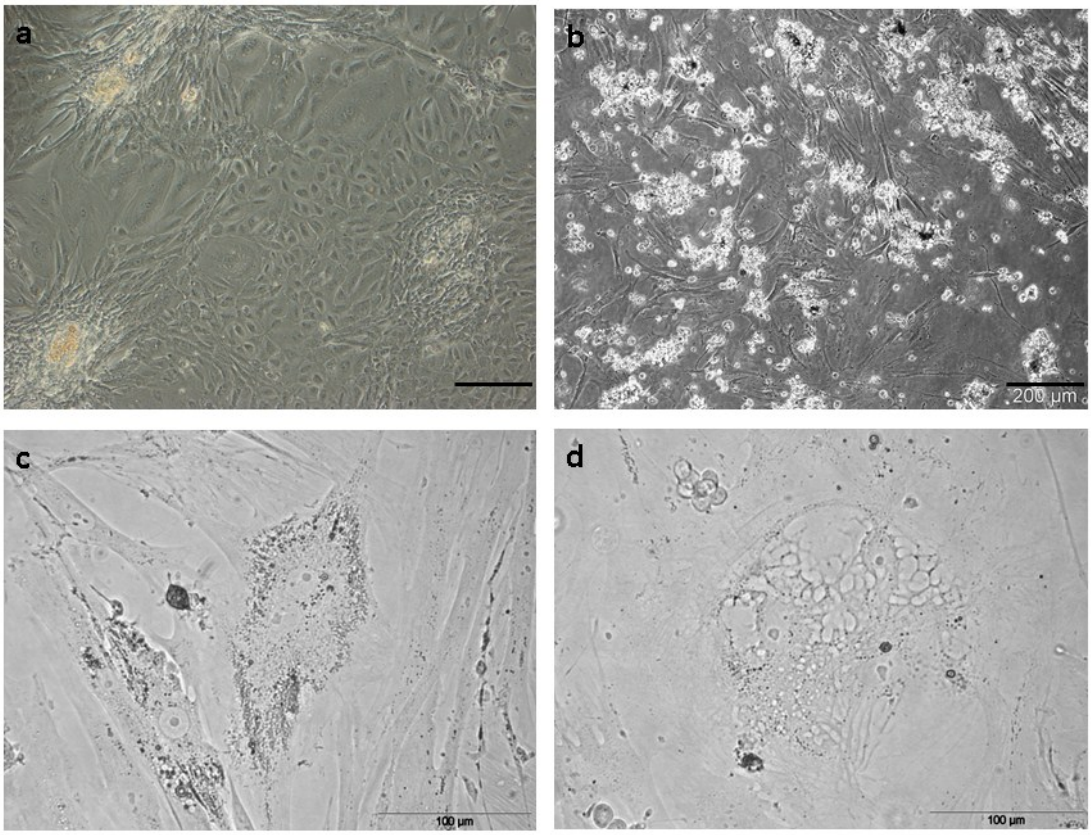

Figure 7. Testicular primary cells isolated from human testes tissue. **a** Confluent, mixed testicular primary cell culture cultured for 12 days. (bar=200µm). **b** Around day 6 round germcell-clusters appeared in suspension in the cell supernatants. (bar=200µm). **c** Magnified Leydig cells show highly granulated cytoplasm including so-called Reinke' crystals (600x magnified, bar=100µm) **d** Sertoli cell with big phagosomes in the cytoplasm (bar=100µm).

Table 10. Patients included in the study.

Patient	Age (years)	Source
HTTR #1	75	Transplantation surgery
HTTR #2	40	Transplantation surgery
HTTR #3	72	Transplantation surgery
HTTR #4	81	Transplantation surgery
URO #2	35	Urology
URO #3	37	Urology
URO #4	42	Urology

8.2.2. HSD3β staining of testicular Leydig cells

Leydig cells were additionally characterized by cytochemical staining of HSD3β (Figure 8). Within the testes HSD3β is exclusively expressed in Leydig cells and therefore used as a Leydig cell marker, although the number of identified Leydig cells varied in the different cell cultures. Highly dense HSD3β-staining has been observed in the nuclear membranes within the cells, but HSD3β was also observed in the cytoplasm.

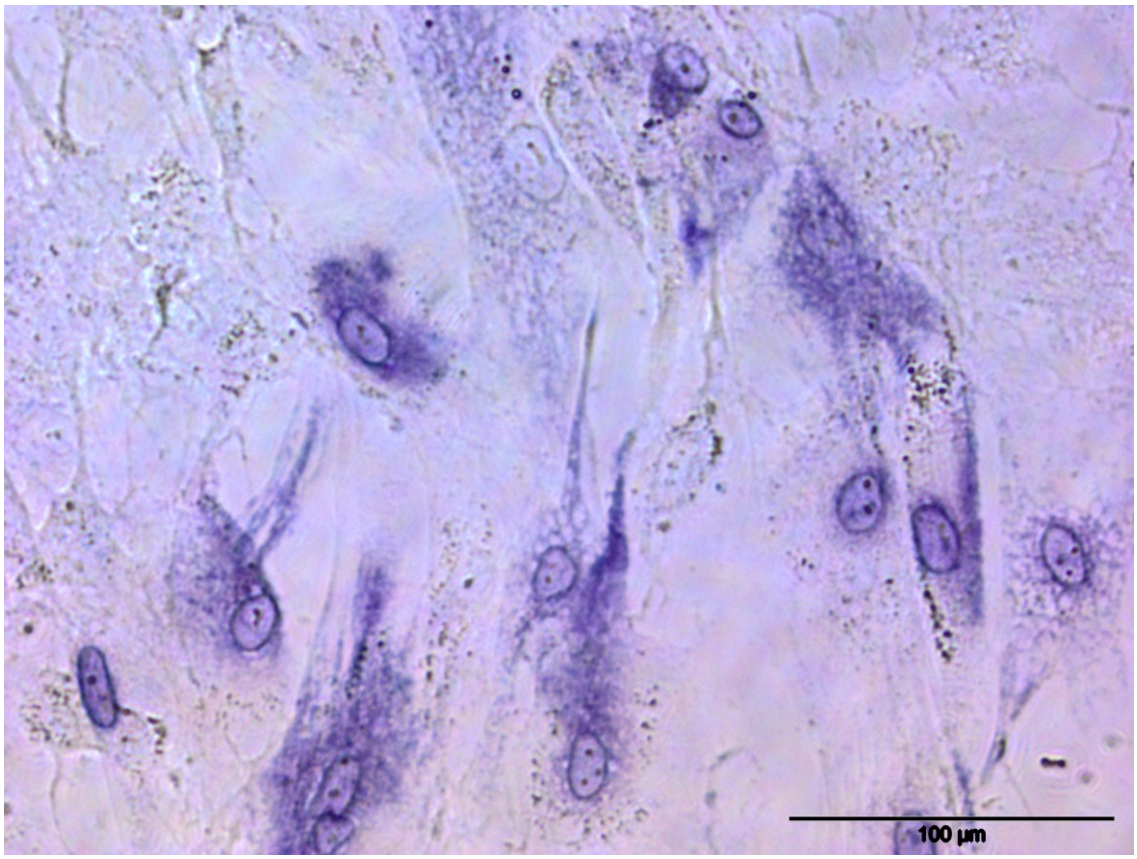

Figure 8. HSD3β staining of human testicular primary cells. Positive stained Leydig cells are shown in purple. Highly dense staining has been shown in the nuclear membranes (bar=100 µm).

8.2.3. Nuclear staining in testicular cells

As shown in Figure 9, nuclei of testicular cells have benn stained blue using DAPI. We have observed that the majority of testicular cell nuclei showed a round to oval appearance. Even nucleoli could be shown. Overall, nuclei have shown a high density.

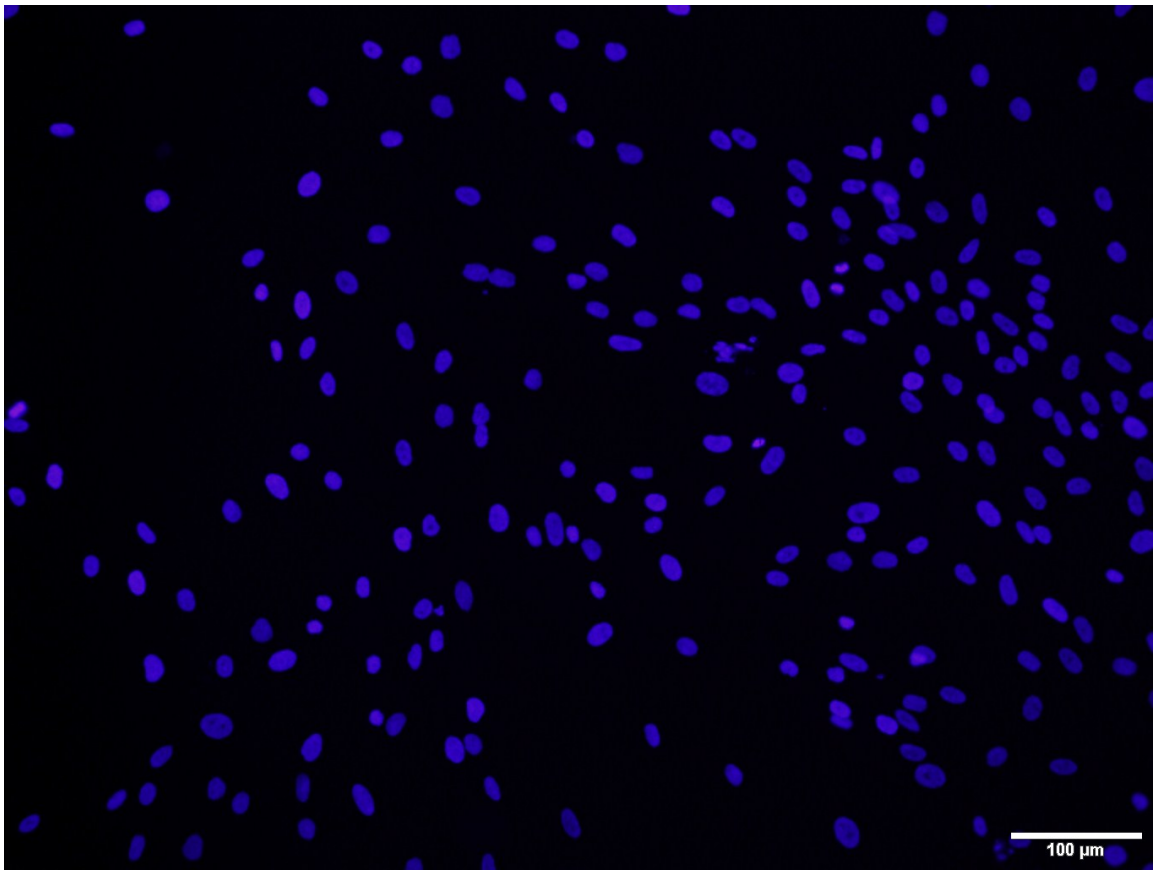

Figure 9. DAPI-stained, testicular cell nuclei analyzed by immunofluorescence microscopy. Round, blue spots indicate nuclei from healthy testicular cells. (bar=100 µm).

8.2.4. Immunocytochemical staining of VDR

Staining of the VDR by immunocytochemical methods has shown positive staining in the breast cancer cell line *SKBR3*, used as positive control. In *SKBR3* cells VDR expression was observed in the cytoplasmic compartment. We have further observed a weak signal in *MDA-MB231* breast cancer cells, which we have used as negative control, but the signal was significantly lower than in positive cells.

In human, testicular, primary cells, VDR expression signal was strong after treatment with 100 nM 1,25$(OH)_2$D. After addition of 1,25$(OH)_2$D VDR expression

was observed mainly in the cell's nuclei, compared to un-stimulated testicular cells, where cytosolic VDR expression has been observed (Figure 10).

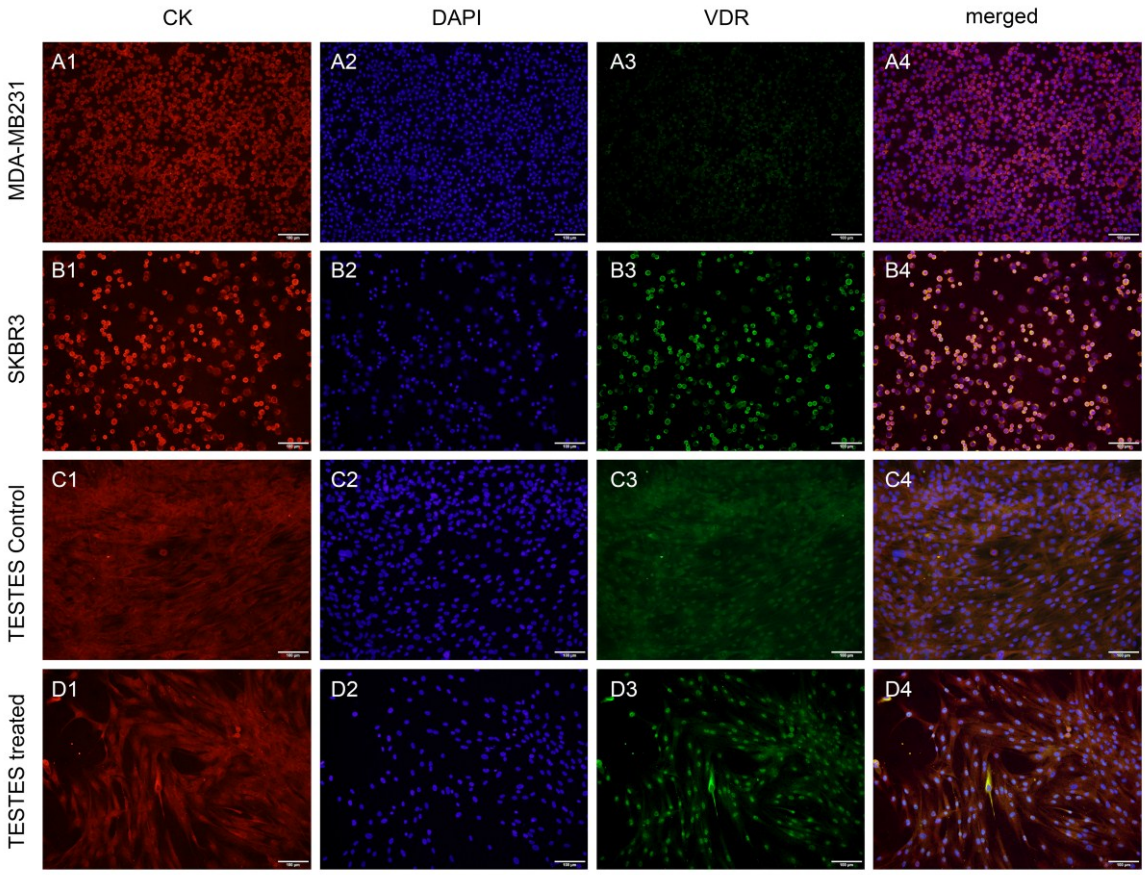

Figure 10. Basal and 1,25$(OH)_2$D stimulated VDR expression in human primary testicular cells. Red fluorescence (A1, B1, C1, D1) is labeled cytoplasm (CK), blue fluorescence (A2, B2, C2, D2) shows labeled nuclei (DAPI) and green fluorescence (A3, B3, C3, D3) shows labeled VDR. The merged images demonstrate that human testicular primary cells treated with 100 nM 1,25$(OH)_2$D display predominantly nuclear localization of VDR (D4), compared to unstimulated testicular cells as well as breast cancer control cells that show VDR expression predominantly in the cytosolic compartment (A4, B4, C4). Magnification: 200x.

8.3. Cell viability and proliferation

8.3.1. Cell viability of human testicular cells

Analysis of cell viability immediately after testicular cell isolation using trypan blue solution has shown ~ 90% viable cells as counted using the hemocytometer. The remaining cells were probably stressed and damaged during the cell isolation procedure.

8.3.2. Effects of 25(OH)D and 1,25(OH)$_2$D on cell proliferation in *NT2/d1* cells

In the testicular carcinoma cell line *NT2/d1* addition of 100 nM 25(OH)D as well as 100 nM 1,25(OH)$_2$D did not change cell proliferation with both tested concentrations and during the observed period of time as displayed in Figure 11.

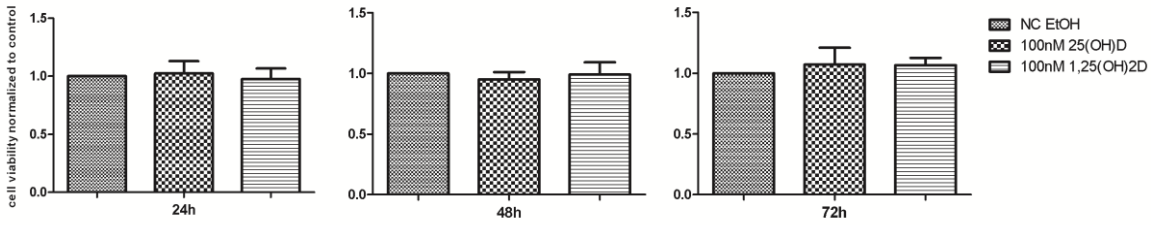

Figure 11. Cell proliferation and viability of human testicular carcinoma cells NT2/d1. Human testicular carcinoma cells were treated with either 100 nM 25(OH)D or 1,25(OH)$_2$D for 24, 48 and 72 h in humidified atmosphere at 37°C and 5% CO_2. Cell proliferaiton was analyzed using the WST-1 cell proliferation test. Addition of both vitamin D forms did not affect cell proliferation in the used concentrations within the selected time period in NT2/d1 cells.

8.3.3. 1,25(OH)$_2$D and cell proliferation in healthy testicular cells

Human primary testicular cells were treated with several concentrations of 1,25(OH)$_2$D and cell proliferation analyzed after 24, 48 and 72 h. All tested concentrations of 1,25(OH)$_2$D increased cell proliferation at all three measured time points. Significant increases were observed after addition of 10 nM of 1,25(OH)$_2$D for 48 h (P< 0.05) as well as after addition of 50 nM 1,25(OH)$_2$D for 72 h (P< 0.01).

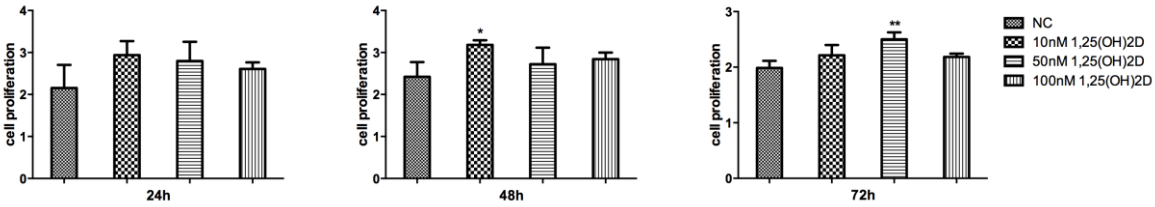

Figure 12. Cell proliferation of human testicular cells after addition of 1,25(OH)$_2$D for 24, 48 and 72 h. Treatment with 1,25(OH)$_2$D increased cell proliferation significantly after addition of 10 nM for 48 h and 50 nM for 72 h. ** P<0.01.; * P< 0.05.

8.3.4. Testosterone and cell proliferation in healthy testicular cells

Addition of testosterone for 24, 48 and 72 h increased cell proliferation in human primary testicular cells. A significant increase in cell proliferation was seen after 24 h, when 1 ng/ml (P<0.001), 5 ng/ml (P<0.05) and 10 ng/ml (P<0.01) of testosterone were added, compared to control cells (treated with vehicle).

Addition of 1 ng/ml testosterone did also significantly increase cell proliferation (P<0.001) after 48 h.

Although cell proliferation was increased after 72 h by the tested testosterone concentrations as well, no significances could be observed anymore.

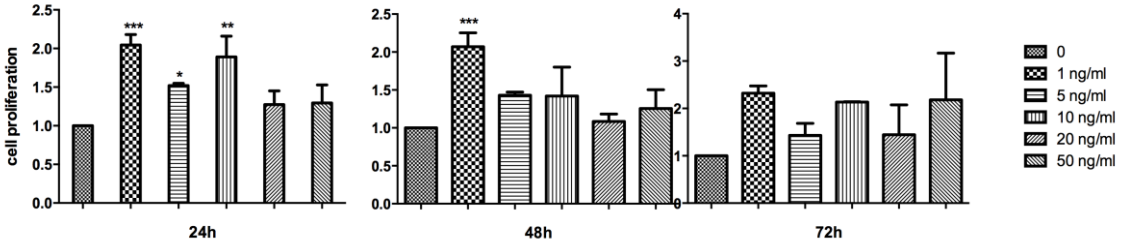

Figure 13. Cell proliferation of human testicular cells after addition of different concentrations of testosterone for 24, 48 and 72 h. 1 ng/ml of testosterone significantly increased cell proliferation after 24 and 48 h (P<0.001). 5 ng/ml (P<0.05) and 10 ng/ml (P<0.01) of testosterone have also shown to increase cell proliferation when treated for 24 h. *** P<0.001, ** P<0.01, * P< 0.05.

8.4. Analysis of testosterone secretion

8.4.1. Effects of 1,25(OH)$_2$D on testosterone delivery

We have investigated that addition of 100 nM 1,25(OH)$_2$D increased testosterone production significantly (P< 0.01) compared to control cells (Figure 14A).

Testosterone production have been further analyzed after 24 h of addition of 5 IU/l LH alone or together with 100 ng/ml IGF-1, respectively, which did slightly increase testosterone production *in vitro,* but without significance.

By contrast, when we have measured testosterone concentrations after addition of LH, IGF-1 in combination with 100 nM 1,25(OH)$_2$D in parallel we could show that this combination led to a significant increase (P< 0.05) in testosterone delivery (Figure 14B).

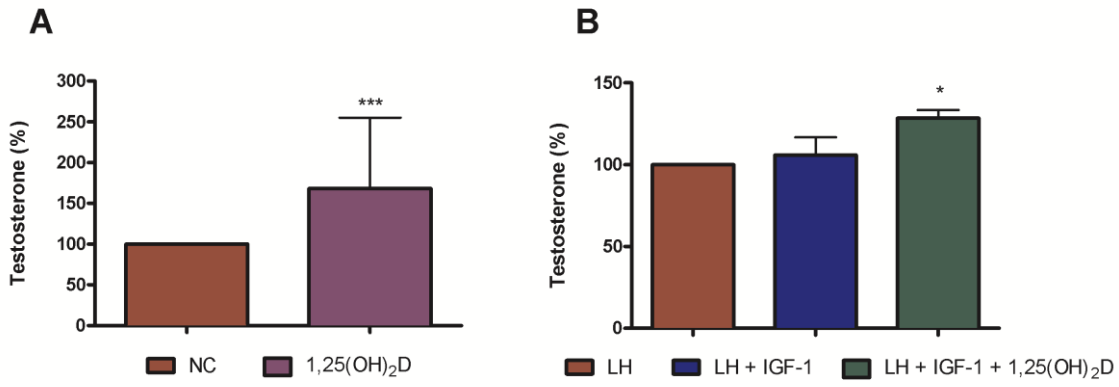

Figure 14. Testosterone synthesis in human testicular cell cultures. **A** Addition of 100 nM 1,25(OH)$_2$D for 24 h significantly increased testosterone production compared to treatment with vehicle (ethanol). **B** The combined addition of LH and IGF-1 together with 1,25(OH)$_2$D has shown to significantly increase testosterone production as compared to addition of LH alone. Results are represented as means ± SD. *** P< 0.001, * P<0.05. NC (negative control).

8.4.2. Effects of LH and osteocalcin on testosterone and DHT secretion

Testosterone and DHT concentrations from the cell supernatants of human primary testicular cells were measured 24 h after addition of LH and osteocalcin in different dosages.

Addition of OC, but not LH, has shown a dose-dependent increase in testosterone as well as DHT delivery of human testicular cells *in vitro* (Figure 16).

The Pearson correlation, shown in Figure 15, has shown a positive correlation of testosterone and DHT delivery in testicular primary cells (r^2= 0,8059, *P*<0.0001).

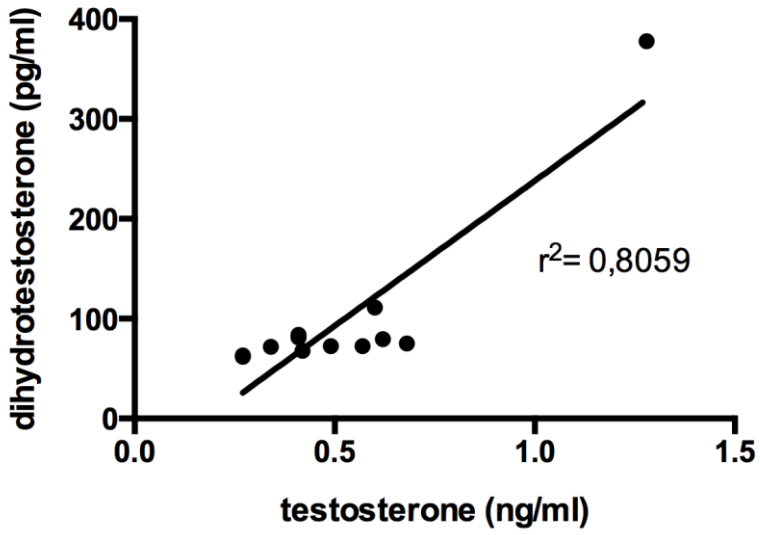

Figure 15. Pearson correlation. Positive correlations of testosterone-values and DHT-values have been shown in human primary testicular cells after addition of LH and OC (r^2=0,8059, P<0.001).

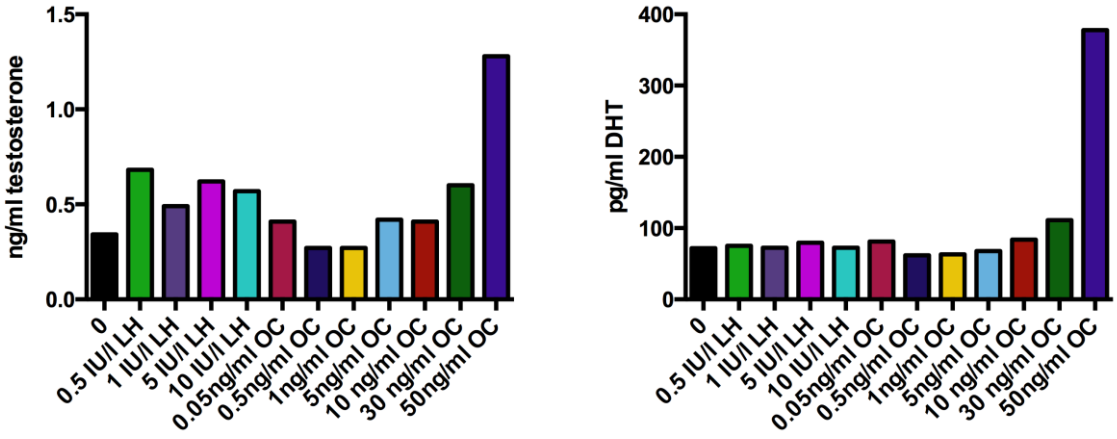

Figure 16. Analysis of testosterone and DHT concentrations from supernatants of human testicular cells. Testosterone and DHT have been measured both by ELISA. OC has shown to increase testosterone as well as DHT delivery of human testicular cells dose-dependently. By contrast, different concentrations of LH have increased testosterone levels, but did not change DHT concentrations.

8.4.3. Effect of osteocalcin on testosterone delivery

We have shown that treatment of human testicular cells for 24 h with 10 ng/ml OC, which were the physiologic serum concentrations for OC, significantly increased testosterone synthesis (P<0.05) compared to addition of several concentrations of LH, which is known stimulate male testosterone synthesis *per se*.

The combined treatment of testicular cells with LH together with osteocalcin for 24 h, led to significantly decreased testosterone concentrations (P<0.001).

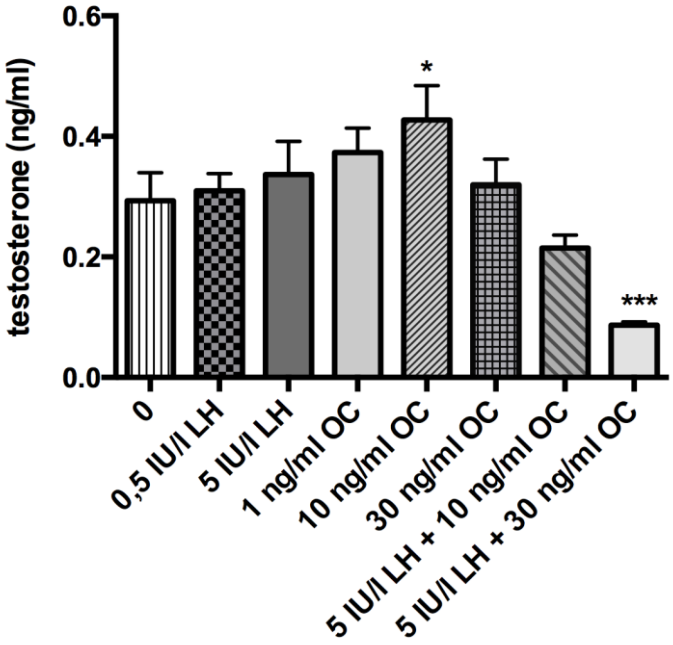

Figure 17. Analysis of testosterone concentrations from supernatants of human testicular cells. Testosterone concentrations were measured by ELISA. Addition of LH has shown a dose-dependent increase of testosterone delivery. When OC was added testosterone secretion was significantly increased. By contrast, a combined addition of LH with OC has shwon to significntla decrease testosterone delivery in human testicular cells when added in the used concentrations. * P<0.05; *** P<0,001. LH (luteinizing hormone), OC (osteocalcin).

8.5. Microarray analysis

8.5.1. RNA integrity and quality

After careful purification of each RNA sample using the RNeasy mini kit RNA integrity and quality was checked using the Bioanalyzer. Samples were loaded on the chip and the applied components were separated electrophoretical (Figure 18).

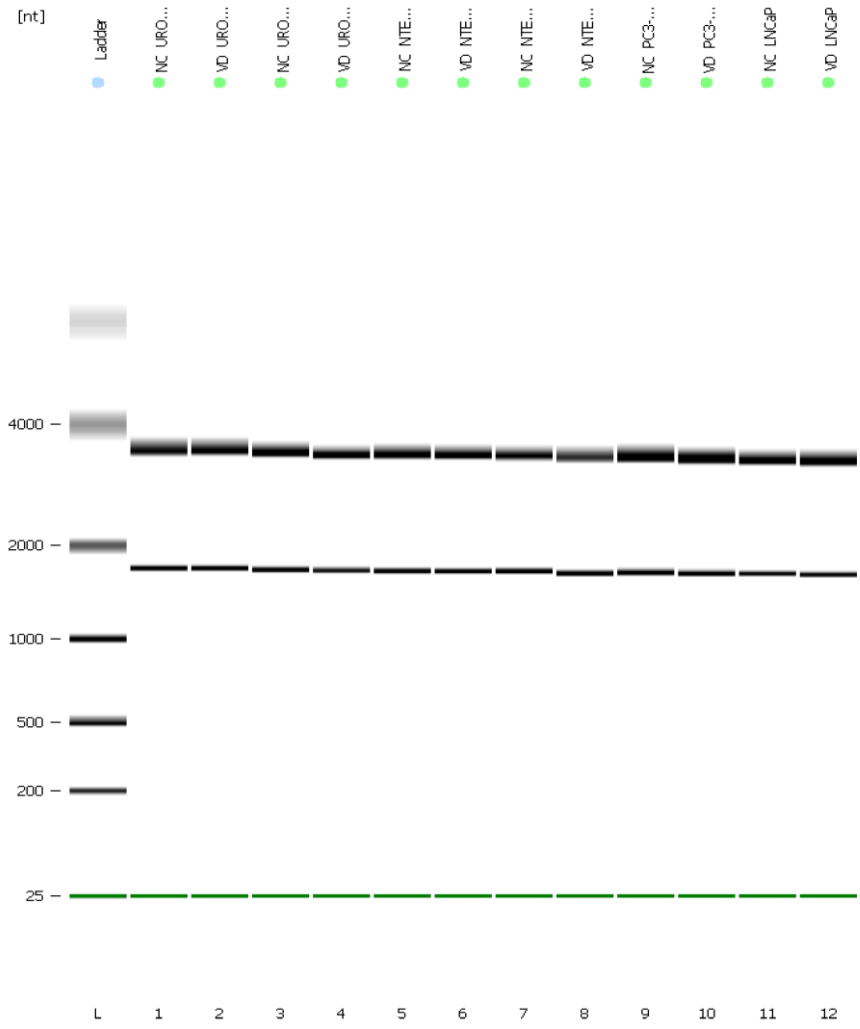

Figure 18. Electrophoretic analyzed RNAs using the Bioanalyzer. Isolated RNA from seven patients in control and 1,25$(OH)_2$D treated condition were applied on a Bioanalyzer chip (Agilent Technologies) and RNA quality analyzed. Data from the elctropherogram has shown RIN-values between 9 and 10, which identified highly qualitative RNA.

8.5.2. Microarray gene chips

Microarray gene chips were scanned by the GCS3000 and have shown very intense arrays. Strong signals were consistent in all 14 arrays (Figure 19).

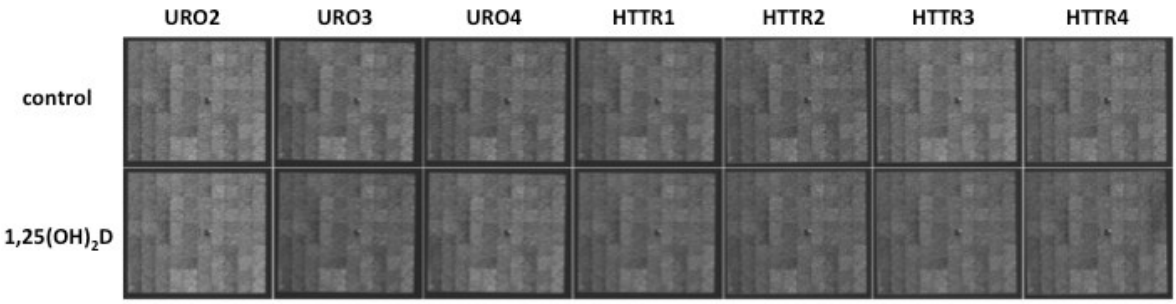

Figure 19. Microarray gene chips. Scanning using the Affymetrix GeneChip scanner GCS3000 has shown strong signals in all 14 gene chips.

8.5.3. Top 1,25(OH)$_2$D regulated genes

After calculation of 3-way ANOVA and based on filter criteria for fold change ≥ 1.5 and *P*-values of $P \leq 0.05$ we could observe 63 genes that have significantly changed by 1,25(OH)$_2$D treatment, thereof 56 genes were significantly up and 7 genes significantly downregulated by 1,25(OH)$_2$D. The 20 top up and 3 top down-regulated genes are summarized in Table 12.

Table 11. Top 1,25$(OH)_2$D up and downregulated genes in human testicular cells

Gene symbol	Gene title	Fold-Change	P-value FDR 5% cut off
TOP upregulated genes			
CILP	cartilage intermediate layer protein, nucleotide pyrophosphohydrolase	3,83	0,001
EFTUD1	elongation factor Tu GTP-binding domain-containing protein 1 pseudogene	3,00	0,000
EFTUD1	elongation factor Tu GTP binding domain containing 1	2,74	8,8E-05
SLC22A3	solute carrier family 22, member 3	2,70	0,005
SULT1C2	sulfotransferase family, cytosolic, 1C, member 2	2,51	0,003
TREM1	triggering receptor expressed on myeloid cells	2,42	0,016
STEAP4	STEAP family member 4	2,33	0,019
MAPK13	mitogen-activated protein kinase	2,28	0,001
DAB1	disabled homolog 1 (Drosophila)	2,14	0,001
OSR2	odd-skipped related 2 (Drosophila)	2,13	0,000
THBD	thrombomodulin	2,00	0,002
KCNK3	potassium channel, subfamily K, member 3	1,98	0,000
IGF1	insulin-like growth factor 1 (somatomedin C)	1,96	0,040
RAB9B	RAB9B, member RAS oncogene family	1,92	0,007
CLMN	calmin (calponin-like, transmembrane)	1,92	0,010
FBP1	fructose-1,6-bisphosphatase 1	1,90	0,003
CD14	CD14 molecule	1,89	0,018
CD226	CD226 molecule	1,88	0,048
AVIL	advillin	1,87	0,002
TOP downregulated genes			
MPEG1	macrophage expressed 1	-1,81	0,027
HAS2	hyaluronan synthase 2	-1,52	0,001
MIR221	microRNA 221	-1,52	0,016

8.5.4. Confirmation of top regulated genes

From the 63 significantly regulated genes obtained from microarray data, twelve genes have matched to keywords like “male steroidogenesis”, “testis”, “testosterone”, “vitamin D” and “male fertility” due to literature research using *PubMed* search.

A clustered heat-map has been created for all 12 genes that have fitted best to the before mentioned keywords, including all 7 patients in control and 1,25$(OH)_2$D treated condition (Figure 20).

We have validated the expression patterns of these twelve genes using semi-quantitative qPCR analysis. *CYP24A1*, *IGF-1*, *ALPL*, *MTHFR*, *CYP19A1*, *STEAP4*, *DPP4*, *KLF4*, *CD14*, *SLC1A1*, *TMEM37* and *CLMN* have shown significant upregulations in 1,25$(OH)_2$D treated samples compared to control samples (fold changes >1.5 and *P*-values <0.05) and have further matched to microarray results, although fold changes appeared higher using the RT-qPCR method (Table 13, Figure 21A).

Nevertheless, the Pearson correlation has shown that the results obtained from both methods were correlating to each other (Pearson r=0.7469) (Figure 21B).

CYP24A1 appeared at the top of the differentially expressed genes being upregulated more than 120 fold after 1,25$(OH)_2$D treatment, as analyzed by microarray as well as 3475 fold when analyzed using RT-qPCR (displayed as $\log_2$ values in the graphs).

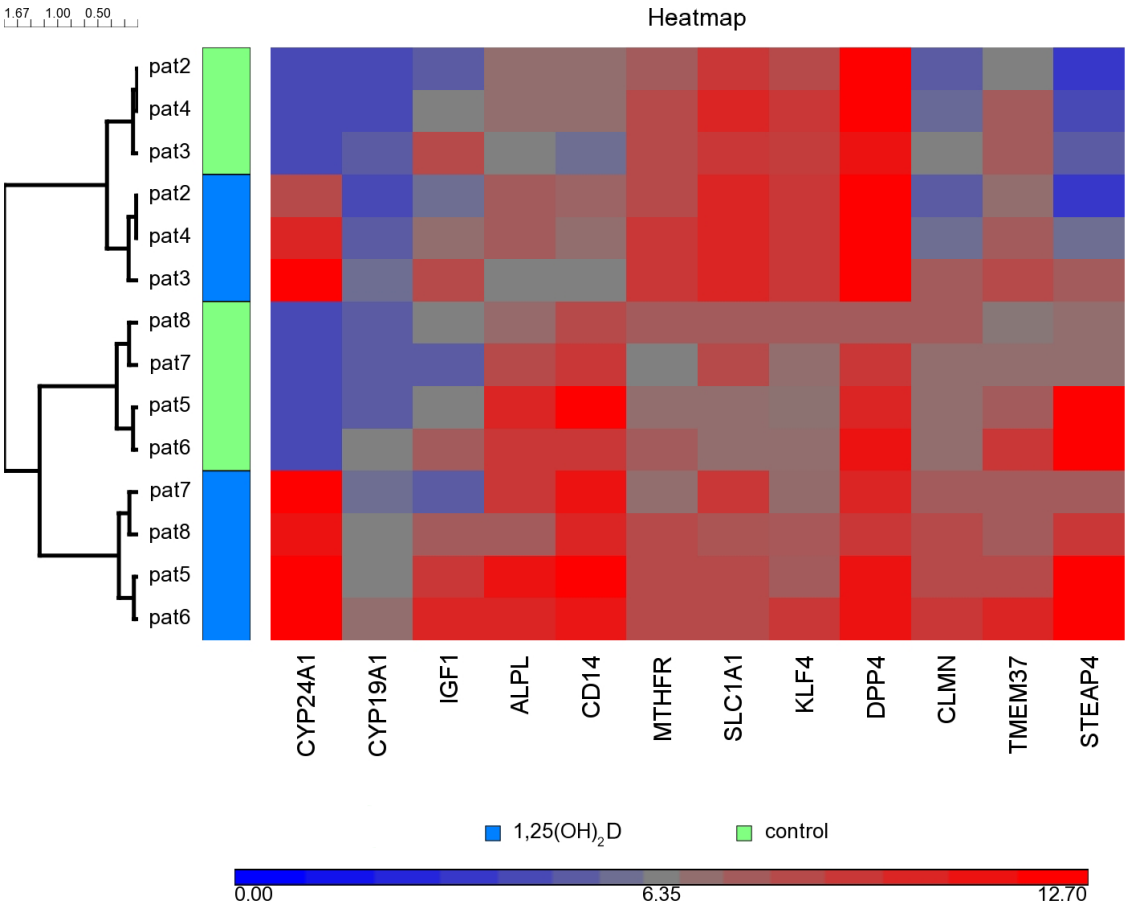

Figure 20. Hierarchical clustering of human testicular cells exposed to 100 nM 1,25(OH)$_2$D. Testicular cells were isolated from 7 patient samples (pat2, pat3, pat4, pat5, pat6, pat7, pat8) and cultured with 100 nM 1,25(OH)$_2$D for 24h. Gene expression was evaluated by micorarray and the data processed doing a RMA normalization (FRD5%) (Partek Software v.6.6). The colored barand values below indicate gene expression in target samples (i.e., red, more expressed and blue, less expressed). The patients are clustered on the left side, control samples are indicated by green boxes and 1,25(OH)$_2$D treated samples by blue boxes. On the bottom the twelve significantly upregulated genes (confirmed by RT-qPCR) that we clustered in this heatmap are named. The results showed 1,25(OH)$_2$D to differentially affect the expression of the selected genes dependent on the patient.

Table 12. Comparison of microarray and RT-qPCR data for significantly 1,25(OH)$_2$D regulated genes

Gene	Summary of functions	Affymetrix		RT-qPCR	
		Fold change	P-value (Partek)	Fold change	P-value (t-test)
ALPL	Bone metabolism	1,52	0,010	2,27	0,011
CD14	Mediator of the innate immune response	1,89	0,018	3,52	0,006
CLMN	Actin binding	1,92	0,010	2,57	0,026
CYP19A1	Steroid metabolism, estrogen biosynthesis	1,54	0,007	2,67	0,008
CYP24A1	Calcium homeostasis, Vit. D metabolism	122,19	9,6E-06	3475	0,036
DPP4	T-cell activation	1,65	0,004	2,12	0,036
IGF-1	Insulin regulation	1,96	0,040	3,24	0,070
KLF-4	Embryonic development	1,51	0,025	2,26	0,125
MTHFR	Aminoacid catalyzation	1,63	0,000	1,89	0,010
SLC1A1	Membrane transporter	1,74	0,001	1,9	0,030
STEAP4	Adipocyte development and metabolism	2,33	0,019	4,22	0,027
TMEM37	Calcium channel activity	1,70	0,001	2,89	0,030

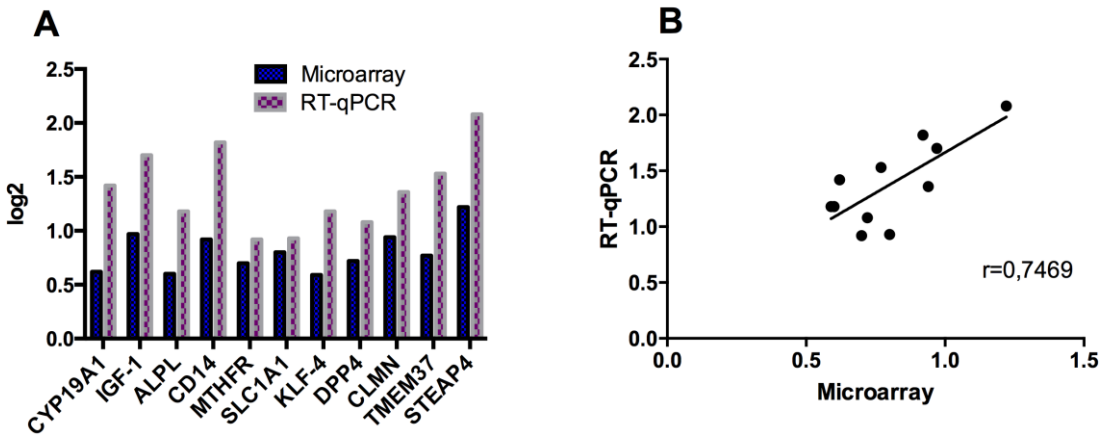

Figure 21. Effect of 1,25(OH)$_2$D on expression of twelve selected genes in human testicular cells. **A** Testicular cells were isolated from 7 patients and treated with 100 nM 1,25(OH)$_2$D or vehicle (control) for 24 h and analyzed by RT-qPCR. Relative expression was calculated as 2^{-ddCt}, using GAPDH as internal control and the average value of the target gene in control samples, as reference. For clarity reasons we have mapped the relative expressions as log2 mean values ±SD and left out the expression of CYP24A1 (log2>7). **B** The graph shows the correlation of both methods (microarray *vs* RT-qPCR) compared to each other.

8.5.5. Classification of 1,25(OH)$_2$D regulated genes into biological functions/diseases and networks

The 63 top genes were analyzed for functional annotations and biological relevance using IPA, where regulated genes were classified into pathways and functions/diseases.

The five top biological functions affected by 1,25(OH)$_2$D treatment in testicular cells (ranked by significance; *P*-value) were “protein synthesis”, “skeletal and muscular system development and function”, “connective tissue disorders”, “inflammation” and “skeletal and muscular disorders”.

The 20 genes, which were associated with the five top biological functions, were in large part also assigned to biological functions and/or diseases regarding to “reproductive system disease/reproductive system development and function“,

„endocrine system disorders/endocrine system development and function“ and „vitamin and mineral metabolism“ (Table 14).

The top canonical metabolic and signaling pathways affected by 1,25$(OH)_2$D treatment and identified by IPA analysis in our data set are summarized in Figure 22. We have found most of the genes belonging to the „VITAMIN DR/RXR activation“ as the pathway most affected by 1,25$(OH)_2$D treatment.

Interestingly, when searching for top categories ranked by IPA, most of the 63 genes significantly regulated by 1,25$(OH)_2$D were identified belonging to „small molecule biochemistry“ (54 genes), „skeletal and muscular system development and function“ (45 genes), „tissue development“ (45 genes), „cancer“ (39 genes) and „cellular development“ (38 genes) (*data not shown*).

IPA also calculated networks of differentially expressed genes in human testicular cells after 1,25$(OH)_2$D treatment. Thereby differentially expressed genes are overlaid onto a global molecular network developed from information contained in the Ingenuity Pathways knowledge base. IPA generated gene networks from our data (Figures 24-27). Interestingly, the majority of 1,25$(OH)_2$D regulated genes, which were mapped by IPA, play relevant roles in „protein synthesis“, „inflammatory response“ and „cell cycle”, or “dermatological diseases and conditions”, “inflammatory disease” and “cell signaling”, or “cancer”, “cellular development”, “hematological system development and function”, or “drug metabolism”, “lipid metabolism” and “molecular transport” in these networks.

Using the information from the Ingenuity Pathways Knowledge Base, upstream regulators specifically induced by 1,25$(OH)_2$D were calculated and a gene network of the top five 1,25$(OH)_2$D „activated“ („vitamin D_3-VITAMIN DR-RXR“, „SOX11“, „prostaglandin E2“, „decitabine“ and „lipopolysaccharide“) and three „inhibited“ upstream regulators („LY2940022“, „gentamicin“ and „CD3“, miRNAs were excluded in our analysis) were generated (Figure 23).

Table 13. Top biological functions of 1,25$(OH)_2$D regulated genes in human testicular cells

Biological functions and/or diseases- IPA	Gene symbol
Reproductive system development and function	
size of testis, mating behaviour	*CYP19A1, IGF1*
aromatase deficiency, size of reproductive tract, formation of Sertoli cells, conversion of androgen, metabolism of testosterone	*CYP19A1*
delay in initiation of development of Leydig cells, formation of efferent ductule and vas deferens, proliferation of Leydig precursor cells	*IGF1*
Endocrine system development and function/ endocrine system disorders	
diabetes mellitus, insulin resistance, glucose tolerance	*ADAMTS9, CD226, CYP19A1, DAB1, DPP4, IGF1, KLF4, LGALS9, mir-221, MPEG1*
concentration of hormone	*CYP19A1, DPP4, IGF1, KCNK3, MAPK13, STEAP4*
synthesis of hormone	*CD14, CYP19A1, IGF1*
synthesis and concentration of beta-estradiol	*CYP19A1, IGF1*
infantile hypercalcemia	*CYP24A1*
stimulation of somatotrophs	*IGF1*
metabolism of testosterone and concentration of dihydrotestosterone	*CYP19A1*
Vitamin and mineral metabolism	
concentration of 1-alpha, 25-dihydroxy vitamin D3	*CYP24A1, IGF1*
quantity of vitamin	*CYP24A1, IGF1, MTHFR*
catabolism of 1-alpha, 25-dihydroxy vitamin D3, activation of cholecalciferol	*CYP24A1*
synthesis of beta-estradiol	*CYP19A1, IGF1*
quantity of mineral	*CD14, FAM20C*
synthesis of steroid	*CYP19A1, CYP24A1, IGF1, KCNK3*

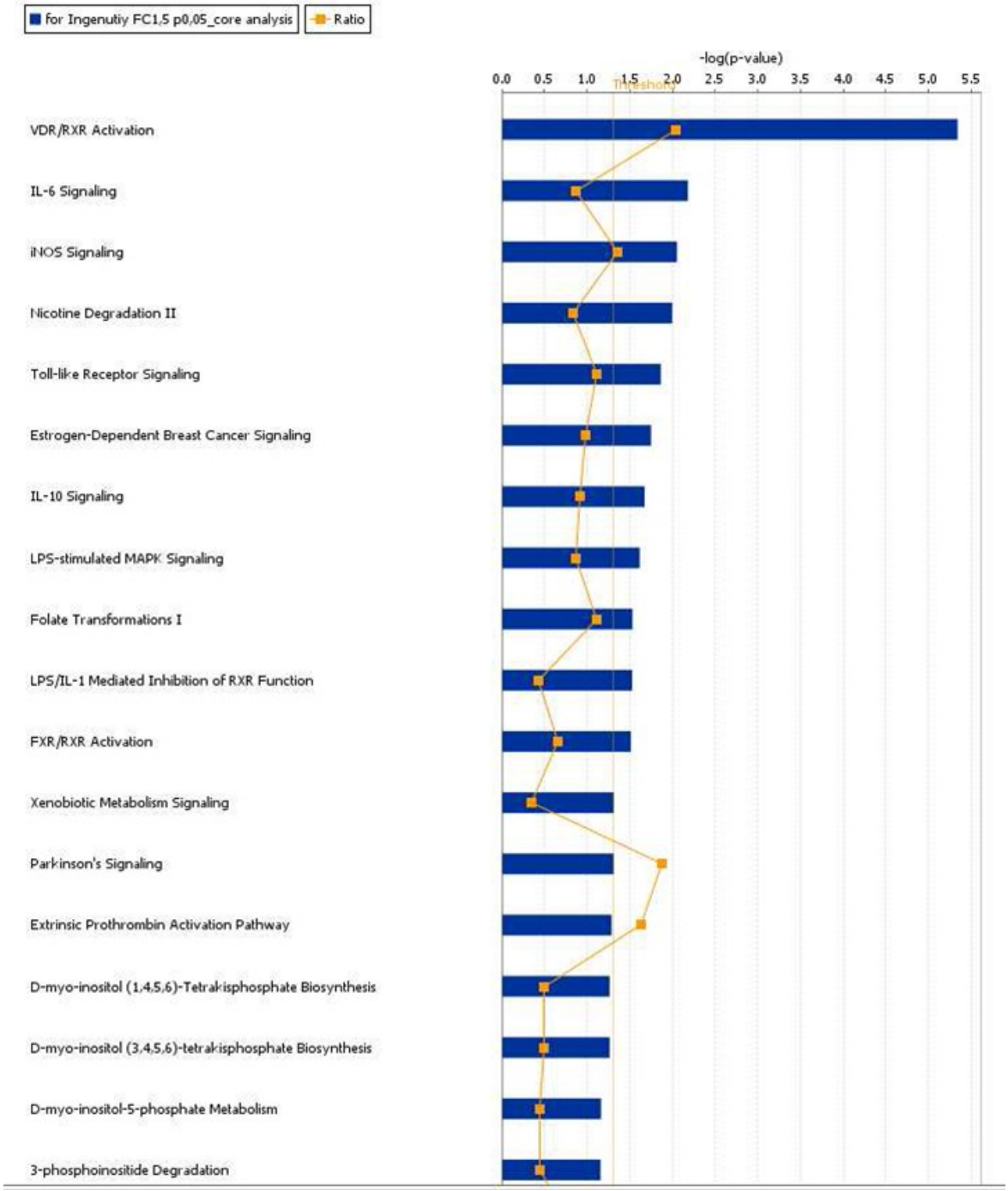

Figure 22. Top canonical pathways generated by IPA constructed from genes exclusively regulated by $1,25(OH)_2D$.

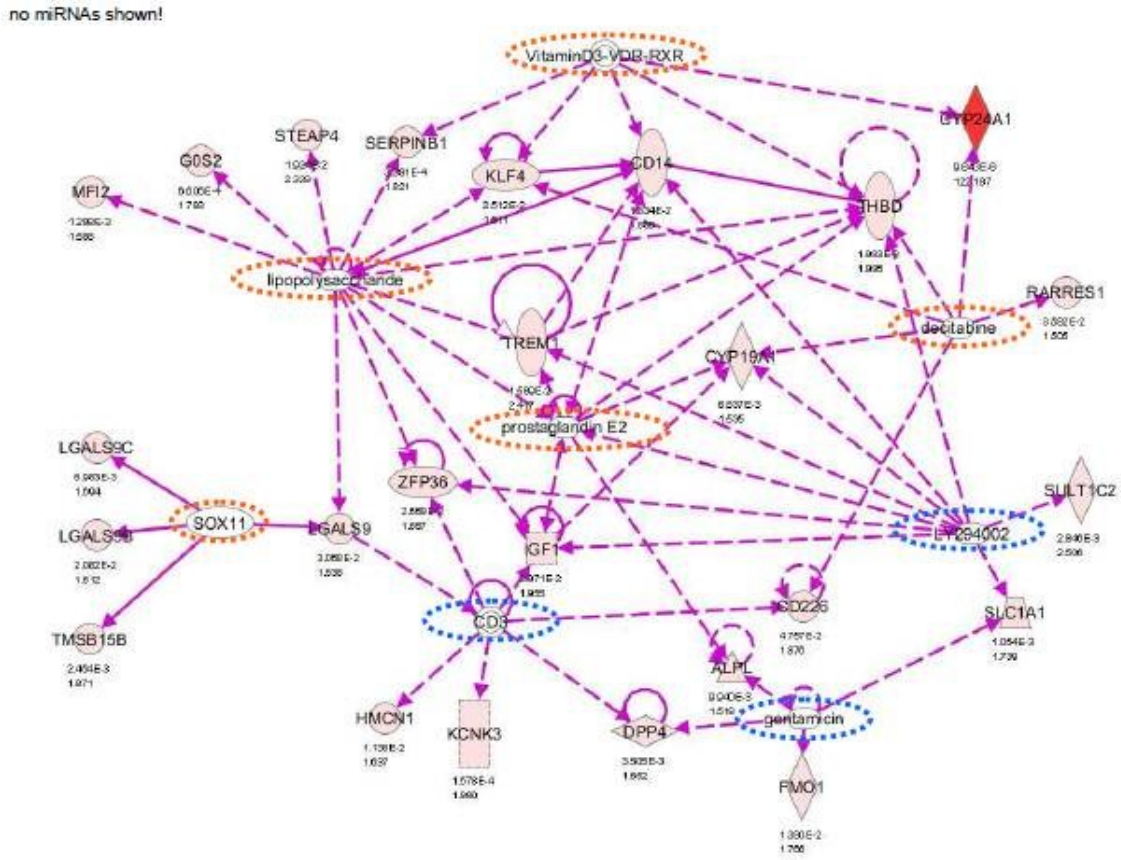

Figure 23. Graphic network representation of the top 1,25(OH)$_2$D regulated up stream regulators identified by IPA. Red dashed circles indicate activated and blue dashed circles inhibited upstream regulators. Indirect and direct biological relationships of represented genes are shown as dashed lines or solid lines, respectively.

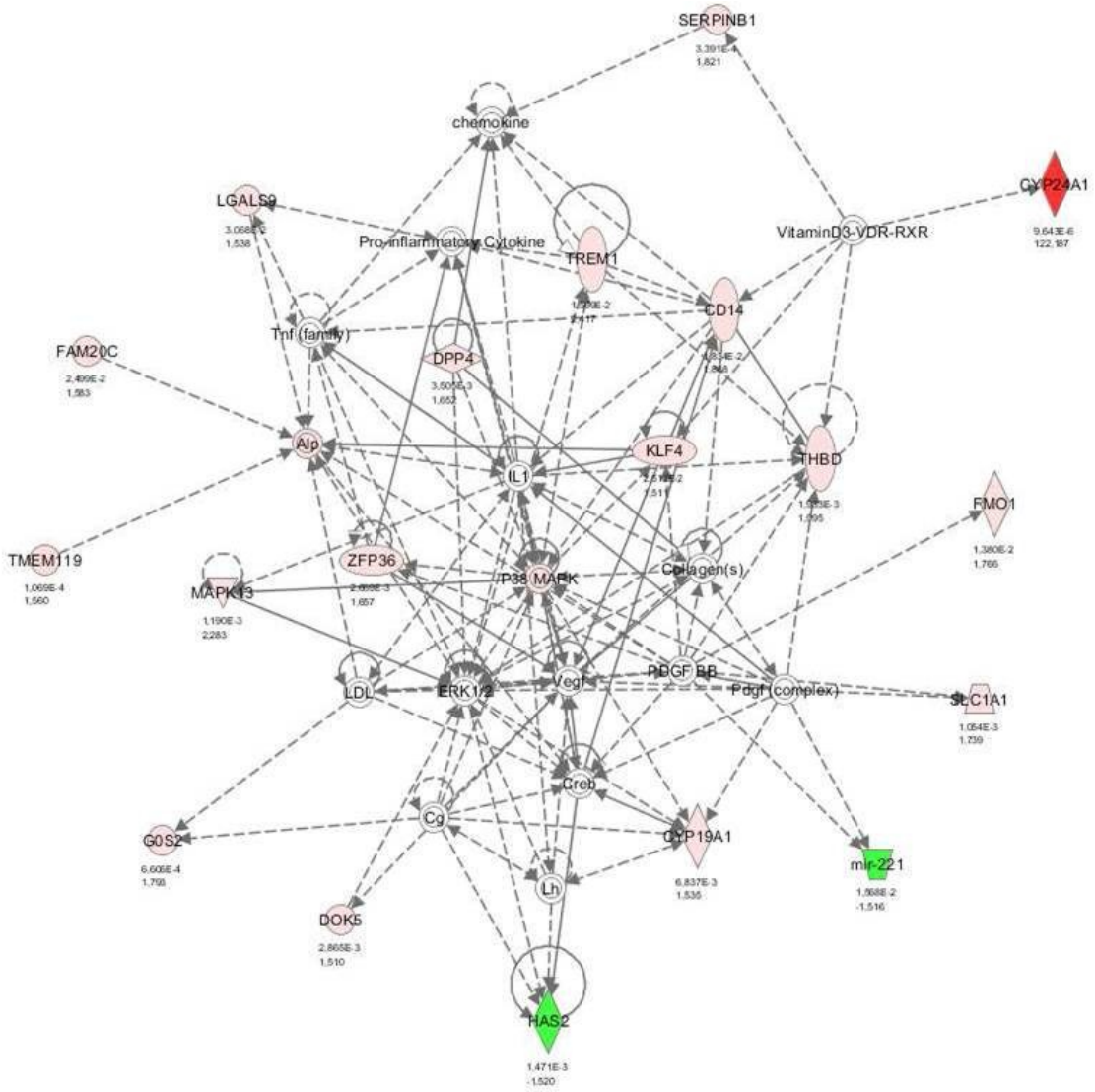

Figure 24. Diagram of the top associated network generated for genes regulated by 1,25$(OH)_2$D in testicular cells belonging to the top functions as analyzed by IPA. The network shows genes regulated by 1,25$(OH)_2$D in „protein synthesis“, „inflammatory response“ and „cell cycle“. Expression levels are shown by colors and intensities (the more intense the color the higher the expression level). Green indicates gene downregulation and pink to red indicate gene upregulation. Upregulated (CYP24A1, SERPINB1, TREM1, CD14, KLF4, THBD, FMO1, SLC1A1, CYP19A1, DOK5, LGALS9, DPP4, FAM20C, MAPK13, GOS2, ZFP36, TMEM119), downregulated (mir-221, HAS2) and not significant regulated genes are displayed in the diagram with their interactions.

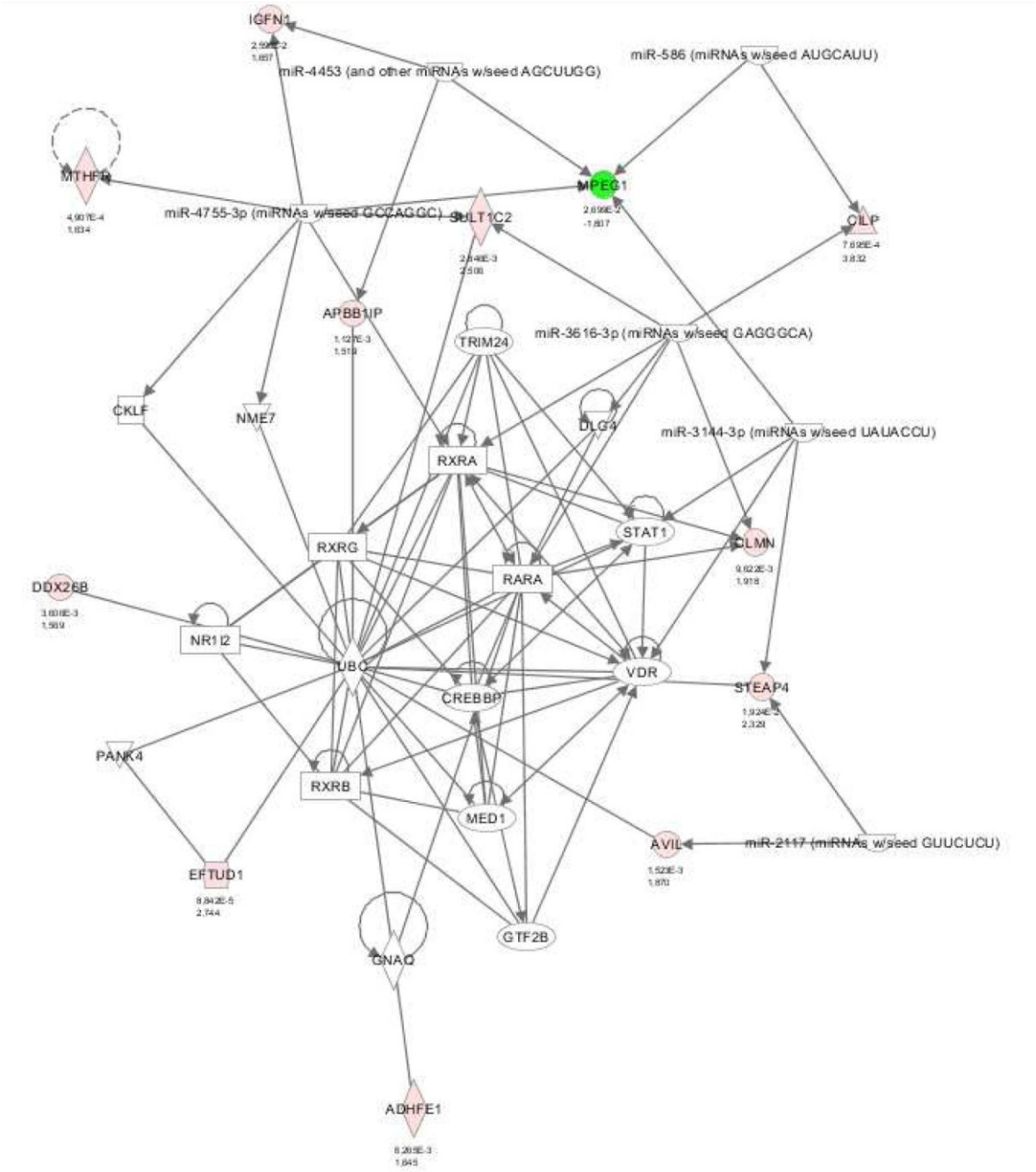

Figure 25. Graphic representation of the most relevant gene networks identified by IPA in our data set with their biological functions. Genes or gene products are displayed as nodes. Direct (solid lines) and indirect (dashed lines) biological relationships between two nodes are represented as edges. The network shows genes regulated by 1,25$(OH)_2$D in “dermatological diseases and conditions”, “inflammatory disease” and “cell signaling”. Expression levels are shown by colors and intensities (the more intense the color the higher the expression level). Green indicates gene downregulation and pink to red indicate gene upregulation. Upregulated (MTHFR, IGFN1, APBB1IP, SULT1C2, CILP, CLMN, DOX26B, EFTUD1, STEAP4, AVIL, ADHFE1), downregulated (MPEG1) and not significant regulated genes are displayed in the diagram with their interactions.

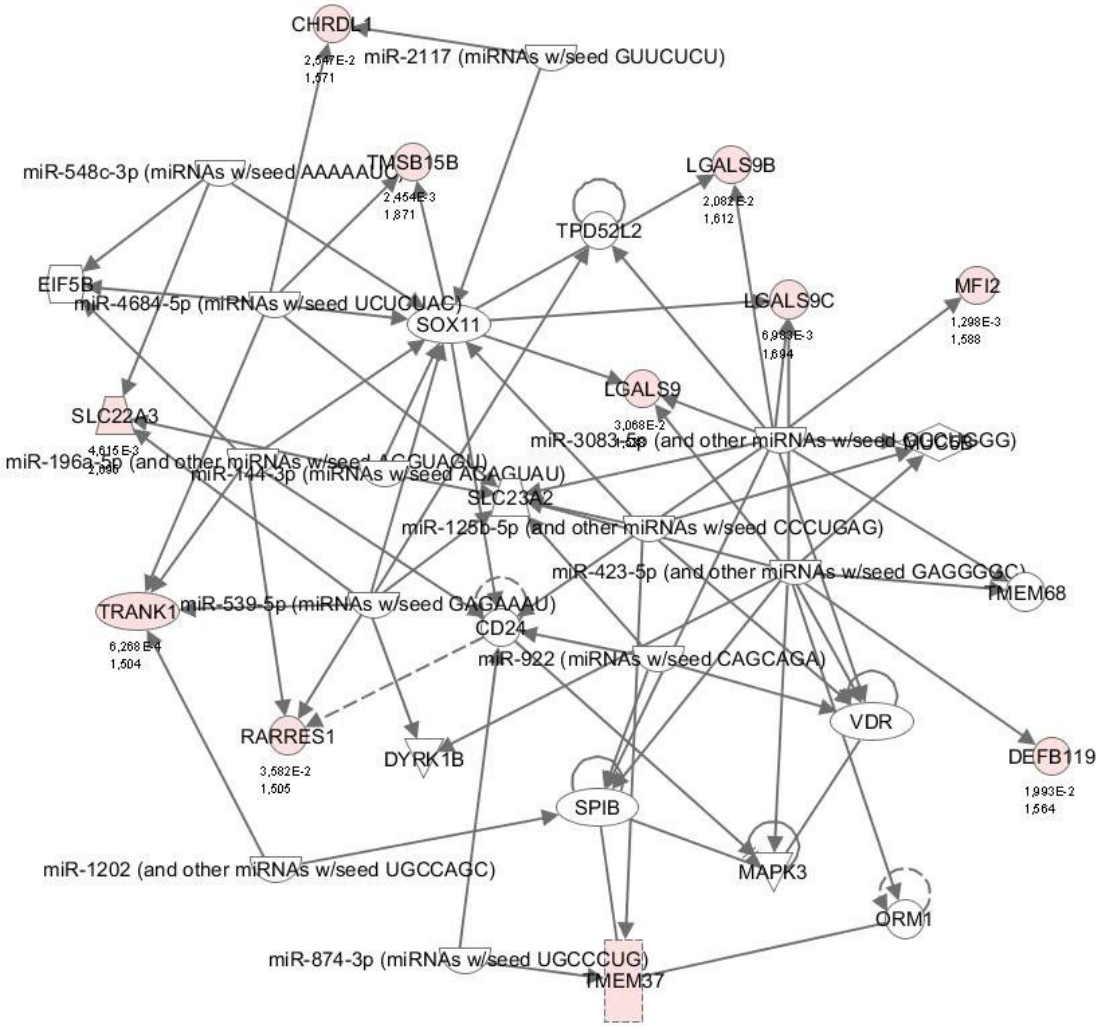

Figure 26. Graphic representation of the most relevant gene networks identified by IPA in our data set with their biological functions. Genes or gene products are displayed as nodes. Direct (solid lines) and indirect (dashed lines) biological relationships between two nodes are represented as edges. The network shows genes regulated by 1,25(OH)$_2$D in “cancer”, “cellular development”, “hematological system development and function”. Expression levels are shown by colors and intensities (the more intense the color the higher the expression level). Upregulated (CHRDL1, TMEM37, DEFB119, TRANK1, SLC22A3, LGALS9, MFI2, TMSB15B, LGALS9C, LGALS9B, RARRES1) and not significant regulated genes are displayed in the diagram with their interactions.

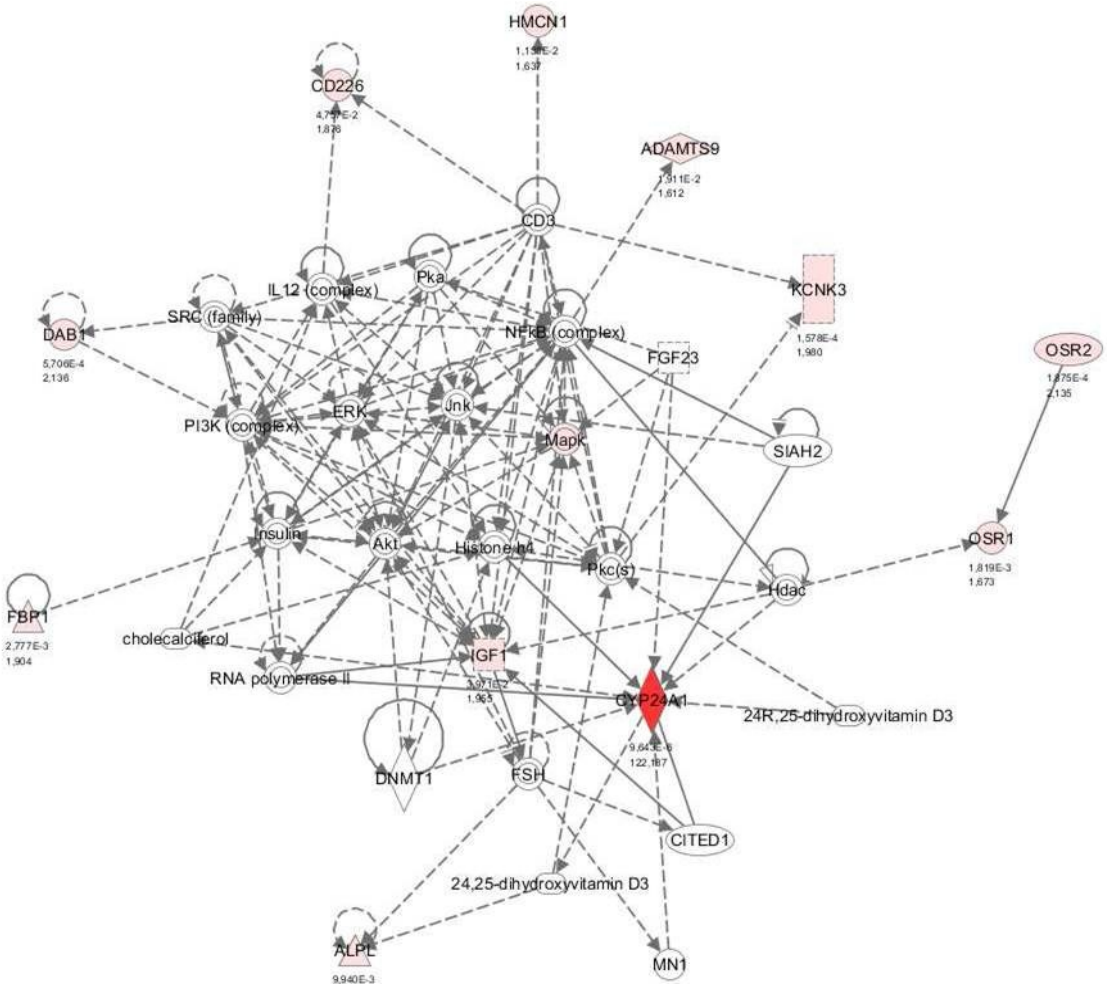

Figure 27. Graphic representation of the most relevant gene networks identified by IPA in our data set with their biological functions. Genes or gene products are displayed as nodes. Direct (solid lines) and indirect (dashed lines) biological relationships between two nodes are represented as edges. The network shows genes regulated by 1,25$(OH)_2$D in “drug metabolism”, “lipid metabolism” and “molecular transport”. Expression levels are shown by colors and intensities (the more intense the color the higher the expression level). Upregulated (CYP24A1, IGF-1, FBP1, ALPL, OSR1, OSR2, DAB1, KCNK3, MAPK, ADAMTS9, CD226, HMCN1) and not significant regulated genes are displayed in the diagram with their interactions.

8.5.6. Gene regulation of the cholesterol and androgen metabolism

Microarrays from cell samples of seven donors have identified significantly (P<0.05) 1,25$(OH)_2$D upregulated genes that refer to the androgen metabolism pathway as summarized in Table 15. Expression levels between the samples have varied depending on the source of material. Testicular cells isolated from samples from the Transplantation Surgery have shown higher fold changes for most of the genes.

Table 14. List of genes belonging to the androgen metabolism pathway affected by 1,25$(OH)_2$D

symbol	Gene title	URO		Transplant		Over all	
		Fold Change	*P*-value	Fold Change	*P*-value	Fold Change	*P*-value
ABCA1	ATP-binding cassette, sub-family A (ABC1), member 1	1,24	0.001	**1,60**	0.006	1,43	0.001
CYP2B7P1	Cytochrome P450, Family 2, Subfamily B, Polypeptide 7 Pseudogene 1	1,14	0.214	**1,62**	0.036	1,40	0.019
CYP7B1	25-Hydroxycholesterol 7-Alpha-Hydroxylase	1,19	0.476	**1,70**	0.018	1,46	0.020
DAZL	Deleted in azoospermia-like	1,12	0.419	**1,66**	0.264	1,40	0.169
HPGD	15-Hydroxyprostaglandin Dehydrogenase	**1,75**	0.039	1,30	0.033	1,48	0.003

8.5.7. Regulation of genes involved in calcium signaling pathways

Microarray analysis has further detected a couple of significantly 1,25(OH)$_2$D regulated genes that were on the one hand associated with calcium metabolism, on the other hand involved in calcium signaling processes, as shown in Table 16. Most of these genes were significantly downregulated as indicated by "-".

Table 15. 1,25(OH)$_2$D regulated genes involved in Ca^{2+} metabolism and signaling pathways.

Gene symbol	Gene title	Fold change	*P*-value
S100A8	S100 calcium binding protein A8	-1,96	0.034
C2CD4A	C2 calcium-dependent domain containing 4A	-1,32	0.015
CACNB4	calcium channel, voltage-dependent, β4 subunit	-1,29	0.044
CACNG1	calcium channel, voltage-dependent, γ subunit 1	-1,16	0.009
CACNA2D4	calcium channel, voltage-dependent, α2/δ subunit 4	-1,15	0.048
CALCOCO1	calcium binding and coiled-coil domain 1	1,25	0.000
CACNB2	calcium channel, voltage-dependent, β2 subunit	1,21	0.010

8.5.8. 1,25(OH)$_2$D regulated genes involved in immunological signaling

We have found a significant number of genes with immune functions that were significantly regulated in human testicular cells after treatment with 1,25(OH)$_2$D. Significantly (P<0.05) up and down regulated genes are shown in Table 17. Downregulated genes are indicated by a "-".

However, we had again to consider that depending on source (urological or transplantation patients) and the size of testes material, different expression levels have been analyzed in the selected genes. Nevertheless, a clearly interplay between genes with roles in immune functions and in vitamin D metabolism has been shown (gene expression levels “over all”).

Table 16. Significantly 1,25(OH)$_2$D regulated genes involved in immunological processes

symbol	Gene title	URO		Transplant		Over all	
		Fold Change	*P*-value	Fold Change	*P*-value	Fold Change	*P*-value
LILRB4	leukocyte immunoglobulin-like receptor, subfamily B 4	1,10	0.029	**1,88**	0.053	1,49	0.040
CCRL1	chemokine receptor-like 1	1,27	0.480	**1,39**	0.056	1,34	0.054
IGFN1	immunoglobulin-like and fibronectin type III domain containing 1	1,23	0.367	**2,07**	0.046	1,66	0.026
IL1RAP	interleukin 1 receptor accessory protein	-1,01	0.937	**1,30**	0.026	1,16	0.072
IL2RA	interleukin 2 receptor, alpha	1,12	0.000	**1,24**	0.115	1,19	0.022
IL1RL1	interleukin 1 receptor-like 1	**1,43**	0.201	1,18	0.415	1,28	0.093
IL17RB	interleukin 17 receptor B	-1,01	0.930	**1,93**	0.106	1,45	0.120
IGFBP3	insulin-like growth factor binding protein 3	1,20	0.091	**1,45**	0.081	1,34	0.017
CCL11	chemokine ligand 11	-1,13	0.523	**-2,28**	0.094	-1,69	0.071
DOCK11	dedicator of cytokinesis 11	1,24	0.103	**1,35**	0.004	1,30	0.000

8.5.9. Regulation of genes associated with vitamin D and nuclear receptor signaling

Treatment with 1,25(OH)$_2$D has further shown significantly up and down regulated genes that were involved in vitamin D and nuclear receptor signaling in human testicular cells (Table 18). Again, downregulated genes are indicated by a "**-**".

Table 17. 1,25$(OH)_2$D regulated genes involved in vitamin D and nuclear receptor signaling

Gene symbol	Gene title	Fold change	*P*-value
Genes involved in vitamin D and nuclear receptor signaling			
MED29	mediator complex subunit 29	1,13	0.029
NRIP1	nuclear receptor interacting protein 1	1,20	0.015
NR2F1	nuclear receptor subfamily 2F, member 1	-1,34	0.046
NCOR2	nuclear receptor corepressor 2	-1,18	0.017
ABHD2	abhydrolase domain containing 2	-1,11	0.046
FOSL2	FOS-like antigen 2	1,27	0.002

8.5.10. 1,25$(OH)_2$D regulated genes involved in MAPK and MEK/ERK signaling

Microarray analysis has revealed significantly up and down regulated genes in human testicular cells after 1,25$(OH)_2$D addition that belong to MAPK and MEK/ERK signaling pathways (Table 19). We have indicated down-regulated genes by "-". MAPK10 seemed to be highly significantly up-regulated by 1,25$(OH)_2$D in testicular cells.

Table 18. 1,25(OH)$_2$D regulated genes involved in MAPK and MEK/ERK signaling

Gene symbol	Gene title	Fold change	*P*-value
Genes associated/belonging to MAPK and MEK/ERK signaling			
MAPK13	mitogen-activated protein kinase 13	-1,26	0.006
MAPK10	mitogen-activated protein kinase 10	2,28	0.001
MAPK14	mitogen-activated protein kinase 14	1,14	0.004
SMAP2	small ArfGAP2	1,11	0.004
MAP2K3	mitogen-activated protein kinase kinase 3	-1,12	0.034

8.6. Gene expression analysis using RT-qPCR

8.6.1. Gene expression at baseline

Gene expression analysis has shown that VITAMIN DR and vitamin D metabolizing enzymes as well as genes of the androgen synthesis and metabolism pathway were expressed at baseline in human primary testicular cells.

As shown in Figure 28, we have analyzed the expression of vitamin D receptor (VDR), cytochrome P450 3A4 (CYP3A4), vitamin D 25-hydroxylase (CYP2R1), sterol 27-hydroxylase (CYP27A1), 1α-hydroxylase (CYP27B1), 1,25-dihydroxyvitamin D_3 24-hydroxylase (CYP24A1), steroid 5a-reductase 2 (SRD5A2), 3ß-hydroxysteroid dehydrogenase (HSD3b), Estradiol 17-beta-dehydrogenase 1 (HSD17B1), G-protein coupled receptor C6A (GPRC6A), estrogen receptor α and β (ESR1/2), aromatase (CYP19A1), steroid 17α-monooxygenase (CYP17A1) and androgen receptor (AR) in human testicular cells at baseline. The ΔCt method was used to analyze a relative gene expression compared to the internal control ß-Actin.

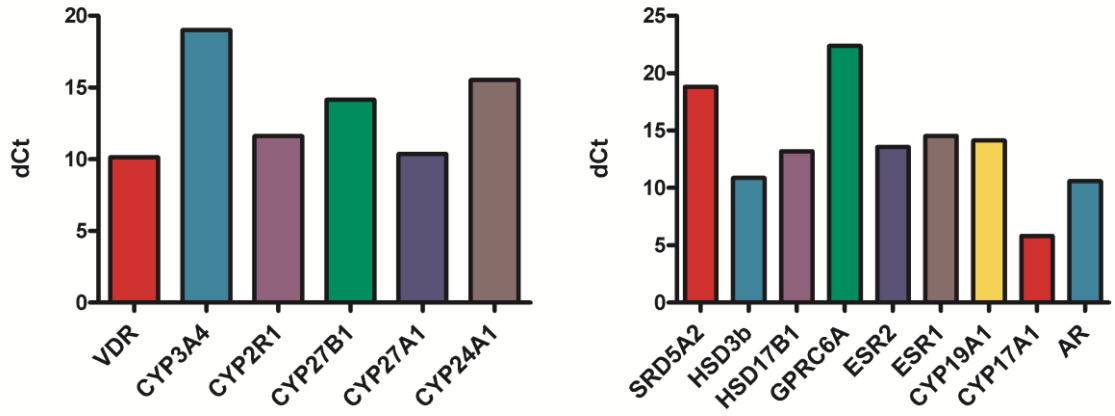

Figure 28. Analysis of mRNA expression at baseline in human testicular primary cells. dCt-values of selected genes of the vitamin D as well as androgen synthesis and metabolism pathway were analyzed using ß-Actin as internal control, which was substracted from Ct-values of the selected genes. mRNA levels of vitamin D receptor (VDR), cytochrome P450 3A4 (CYP3A4), vitamin D 25-hydroxylase (CYP2R1), sterol 27-hydroxylase (CYP27A1), 1a-hydroxylase (CYP27B1), 1,25-dihydroxyvitamin D3 24-hydroxylase (CYP24A1), steroid 5a-reductase 2 (SRD5A2), 3β-hydroxysteroid dehydrogenase (HSD3b), estradiol 17β-dehydrogenase 1 (HSD17B1), G-protein coupled receptor C 6A (GPRC6A), estrogen receptor α and β (ESR1/2), aromatase (CYP19A1), steroid 17α-monooxygenase (CYP17A1) and androgen receptor (AR) are shown.

8.6.1. Gene expression after addition of 1,25(OH)$_2$D

1,25(OH)$_2$D has shown significant up and downregulations of selected genes of the androgen metabolism, which we were not able to analyse with the Affymetrix GeneChip Human 1.0 ST array.

As analyzed by RT-qPCR, treatment of testicular cells with 100 nM 1,25(OH)$_2$D in addition to 5 IU/l LH and 100 ng/ml IGF-1 increased mRNA expression of *HSD3B2, CYP11A1, CYP19A1, CYP3A4, ESR1* and *SRD5A1* (Figure 29) significantly compared to addition of LH or LH and IGF-1 alone.

When we have tested physiologic serum concentrations of 1,25(OH)$_2$D (50-500 pM) in human testicular cells, positive effects on mRNA expression of the selected genes concerning to the androgen biosynthesis pathway in human testicular

cells (Figure 30) were diminished, except for the *HSD3B1* expression, which has shown to be upreglated when treated with a combination of 5 IU/l LH, 100 ng/ml IGF-1 and 100 pM 1,25(OH)$_2$D.

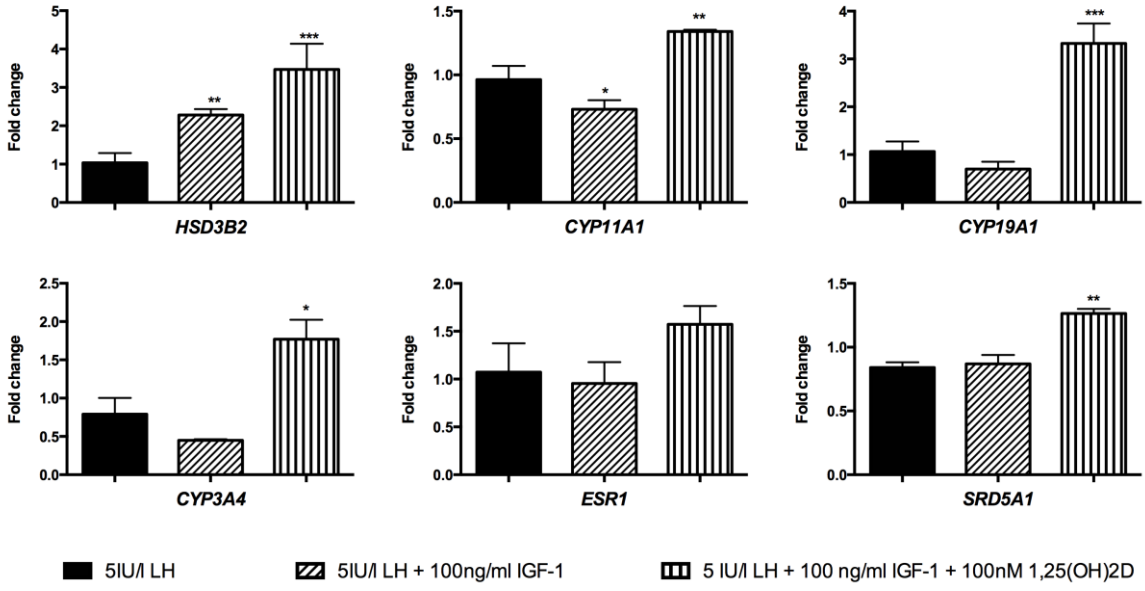

Figure 29. Gene expression of selected genes of cholesterol homeostasis as well as androgen synthesis and steroid metabolism genes. Addition of 1,25(OH)$_2$D in combination with LH and IGF-1 increased gene expression of HSD3B2, CYP11A1, CYP19A1, CYP3A4 and SRD5A1 significantly. HSD3B2 (3β-hydroxysteroid dehydrogenase), CYP11A1 (cholesterol monooxygenase), CYP19A1 (aromatase), CYP3A4 (cytochrome P450 3A4), ESR1 (estrogen receptor α), and SRD5A1 (steroid 5α-reductase). LH (luteinizing hormone), IGF-1 (insulin like growth factor 1), 1,25(OH)2D (1,25-dihydroxyvitamin D). * P<0.05, ** P< 0.01, *** P<0.001.

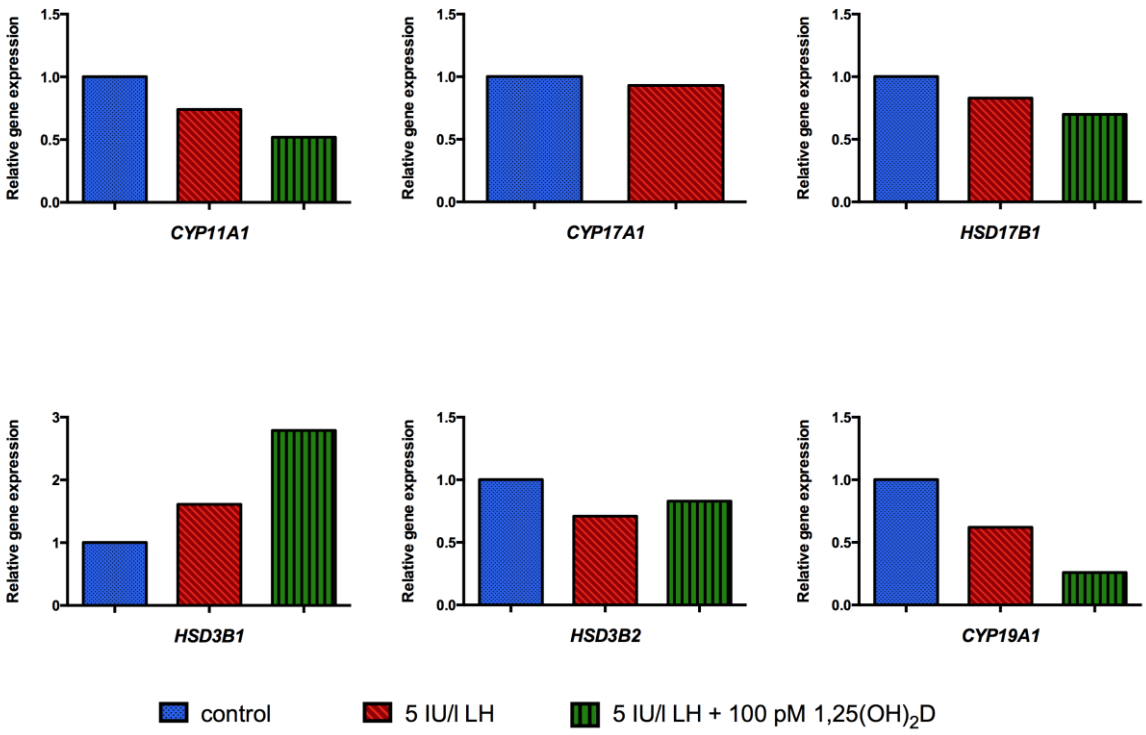

Figure 30. Relative gene expression of selected genes of the androgen synthesis and steroid metabolism pathway. Addition of physiologic doses of $1,25(OH)_2D$ did not increase mRNA expression of CYP11A1, HSD17B1, HSD3B2 or CYP19A1 in testicular cells, only HSD3B1 was increased after addition of 100 pM $1,25(OH)_2D$. CYP11A1 (cholesterol monooxygenase), CYP17A1 (steroid 17α-monooxygenase), HSD17B1 (17β-hydroxysteroid dehydrogenase 1), HSD3B1 (3ß-hydroxysteroid dehydrogenase 1), HSD3B2 (3ß-hydroxysteroid dehydrogenase 2), and CYP19A1 (aromatase). LH (luteinizing hormone), $1,25(OH)_2D$ (1,25-dihydroxyvitamin D).

8.6.2. Gene expression after addition of OC

We have analyzed the gene expression of selected genes of the cholesterol homeostasis as well as androgen synthesis and metabolism pathway in human testicular cells. The mRNA levels have been analyzed after addition of different concentration of LH or OC separately or when added in combination.

As shown in Figure 31, OC increased gene expression of steroid acute regulatory protein (*StAR*), cholesterol monooxygenase (*CYP11A1*) and steroid 17-α-monooxygenase (*CYP17A1*). The mRNA of StAR was significantly increased after

addition of 30 ng/ml OC. 10 ng/ml of OC significantly increased *CYP11A1* gene expression in human testicular cells. However, when 10 or 30 ng/ml OC were added together with 5 IU/l LH, the increasing effect of OC on mRNA of the selected genes was diminished.

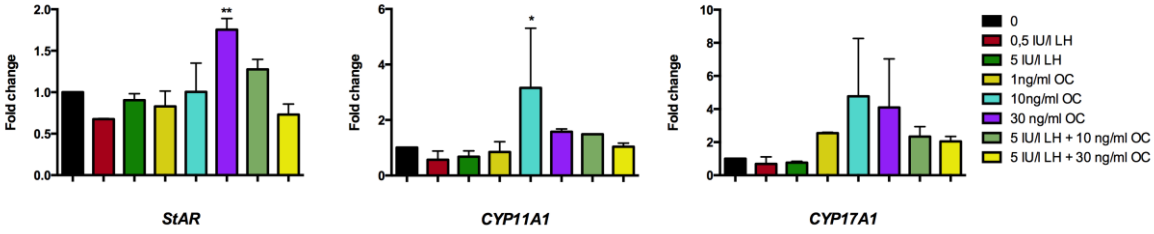

Figure 31. Relative gene expression of selected genes of the cholesterol homeostasis as well as androgen synthesis pathway. Addition of OC increased gene expression of StAR, CYP11A1 and CYP17A1 dose dependently. After combined addition of LH with OC, upregulating effects of OC were diminished. StAR (steroid acute regulatory protein), CYP11A1 (cholesterol monooxygenase), and CYP17A1 (steroid 17α-monooxygenase). LH (luteinizing hormone), IGF-1 (insulin like growth factor 1), OC (osteocalcin). * P<0.05, ** P< 0.01.

Further, RT-qPCR analysis has shown that treatment with OC in addition to 1,25$(OH)_2$D led to synergistic effects on mRNA level of selected genes. Based on RT-qPCR results we analysed a significantly increased gene expression of *LHCGR* (P<0.001), *CYP11A1* (P<0.001), *HSD3B2* (P<0.001), *CYP3A4* (P<0.01), and *CYP19A1* (P<0.001) (Figure 32). Addition of LH alone (blue bar) did not significantly change mRNA levels in human testicular cells compared to control.

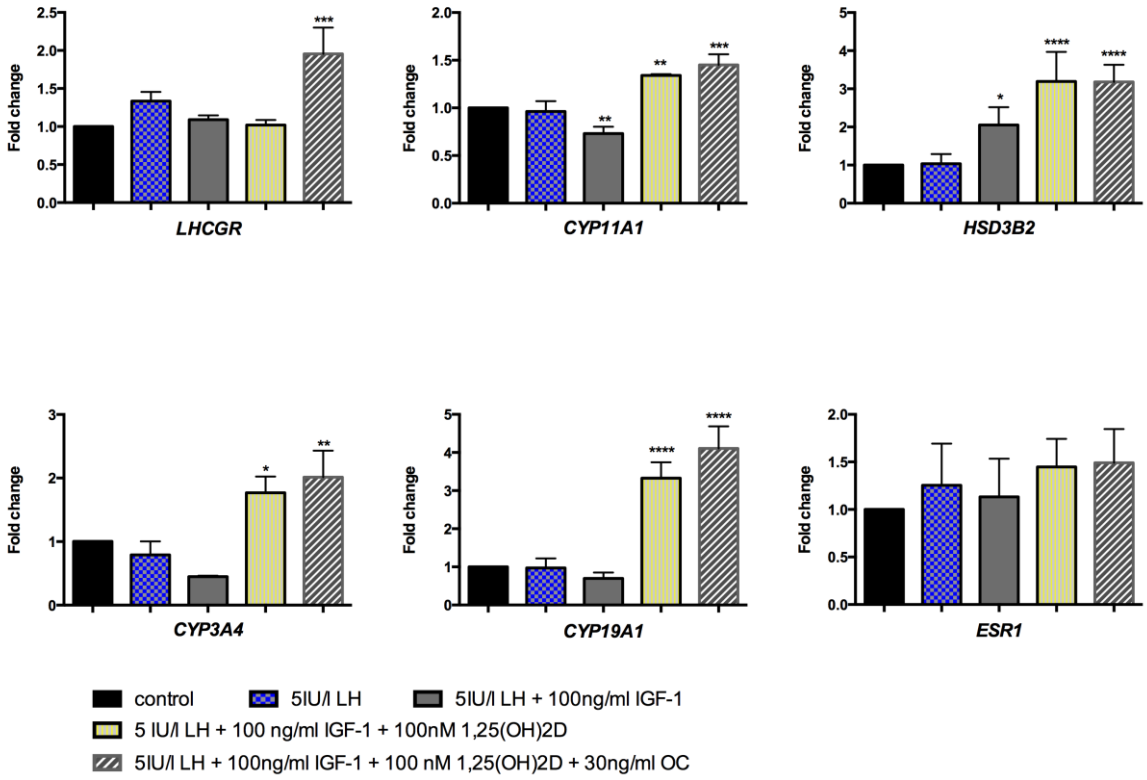

Figure 32. Gene expression of selected genes of the cholesterol homeostasis as well as androgen synthesis and steroid metabolism pathway. Addition of 1,25$(OH)_2$D in combination with LH and IGF-1 increased gene expression of CYP11A1, HSD3B2, CYP3A4, CYP19A1 significantly. The increasing effect on gene expression of the selected genes was additionally increased after the combined addition of LH, IGF-1, 1,25$(OH)_2$D together with OC, which increased mRNA of LHCGR, CYP11A1, HSD3B2, CYP3A4, CYP19A1 most significantly. LHCGR (luteinizing hormone/choriogonadotropin receptor), CYP11A1 (cholesterol monooxygenase), HSD3B2 (3β-hydroxysteroid dehydrogenase 2), CYP3A4 (cytochrome P_{450} 3A4), CYP19A1 (aromatase), ESR1 (estrogen receptor α). LH (luteinizing hormone), IGF-1 (insulin like growth factor 1), 1,25$(OH)_2$D (1,25-dihydroxyvitamin D), OC (osteocalcin). * P<0.05, ** P< 0.01, *** P<0.001.

9. Discussion

Our results provide evidence that vitamin D might be associated with male androgenesis as we have shown that it increased *in vitro* testosterone production in human primary testicular cells. Microarray analysis has shown a number of significantly up-regulated genes by 1,25$(OH)_2$D related to cholesterol homeostasis and steroid metabolism that have been proved by real time quantitative polymerase chain reaction (RT-qPCR).

In parallel, we have shown that the bone derived polypeptide osteocalcin could be play a role in male steroidogeneis as it stimulated testosterone and DHT levels in human testicular cells dose-dependently and significantly increased mRNA expression of several genes related to androgen synthesis and metabolism.

Based on our results, we suggest that there exists on the one hand a direct association of vitamin D with male testosterone synthesis and on the other hand an indirect way via the activation of the osteoblast secreted hormone osteocalcin that is able to stimulate androgenesis in men.

We assume that binding of 1,25$(OH)_2$D to its cognate vitamin D receptor (VDR) leads to its activation as nuclear receptor. We were able to document the expression of VDR in human testicular cells by immunocytochemistry as well as by RT-qPCR on mRNA level.

Umesono et *al*. (78) have shown that VDR-RXR heterodimers tend to prefer classical structures of a VDRE, like the DR3-type, containing two conserved hexameric half-sites separated by a three nucleotide spacer. Once bound to a VDRE, that every VDR target gene should contain at least once, the VDR-RXR complex induces transcription. Coactivators, *e.g.* with histone acetyl transferase (HAT) activity, or like the steroid receptor coactivator-1 (SRC-1), CoA and CoR proteins are recruited together with the DNA binding domain of the VDR as well as other components of the RNA polymerase promoter complex and build the transcription

complex. As we have identified in our study most of the significantly 1,25$(OH)_2$D regulated genes were belonging to VDR-RXR signaling, we suggest that VDREs are expressed in the promotor region of the target genes that we have analyzed, which could be regulated by 1,25$(OH)_2$D and further be responsible for the increasing effect of vitamin D on steroidogenic gene expression.

Wang et *al.* found 11 VDREs in genes that were induced over 10-fold in squamous cell carcinoma cells (SCC25), like *CD14* or *SerpinB1* that we have identified as significantly induced genes in our testicular cells as well (79).

Based on ChIP-seq defined genome-wide mapping Ramagopalan et *al*. have shown a huge number of genes, which were influenced by vitamin D stimulation. 2776 VDR binding sites could be identified after stimulation with 1,25$(OH)_2$D. Further, a number of gene loci were highlighted in that a role of vitamin D would have not been proposed (80). Using the application of ChIP-chip assays enabled the identification and characterization of a number of VDR target genes. Zella et *al*. have used the method to analyze the VDR as well as Meyer et *al*. for the analysis of the *1,25-dihydroxyvitamin D_3 24-hydroxylase* (*CYP24A1)* (81, 82). In summary, ChIP based, microarray and deep sequencing techniques have opened a large insight in how the "hormone" 1,25$(OH)_2$D controls the expression of several genes. However, much more needs to be discovered to understand how vitamin D modulates gene expression in detail.

A number of VDR binding sites have been discovered in the promotor region of several genes in the past, such as in the *CD226* gene, coding for a protein strongly associated with autoimmune disease susceptibility and that we have shown, besides others, to be one of our most significantly 1,25$(OH)_2$D up-regulated gene.

Blomberg Jensen et al. have shown the expression of VDR and vitamin D metabolizing enyzmes in human normal kidney at baseline level (83). Besides their cellular expression in human kidney, the same genes encoding for enzymes, such as CYP2R1, CYP27A1, CYP27B1, and CYP24A1 were expressed in the male reproductive tract as well (36). According to Blomberg Jensen et al. we can

confirm the expression of the VDR and vitamin D metabolizing enzymes in human testicular cells as well, suggesting that there must be a function for vitamin D in the male reproductive tract.

Stimulation with vitamin D significantly induced the steady state of mRNA expression of *CYP24A1*, which was low in the absence of ligand, but was induced trough stimulation with $1,25(OH)_2D$ up to 120-fold or 3000-fold, as shown by microarray analysis and RT-qPCR, respectively, according to the results of Väisänen et *al*. (84). *CYP24A1* belongs to the metabolic pathway of vitamin D and appears at the top of the $1,25(OH)_2D$ influenced genes.

As we have added supraphysiolgical serum concentrations of $1,25(OH)_2D$ to the cells, we assume that a “too-much” of vitamin D could lead to a defense of the cell to avoid an intoxication. In fact, testicular cells increased the gene expression of the vitamin D depletion enzyme *CYP24A1* leading to inactivation and conversion into vitamin D metabolites. Wang et *al.* (79) also identified in their *in silico* as well as microarray based studies *CYP24A1* as the most $1,25(OH)_2D$ induced gene.

On the other hand, $1,25(OH)_2D$ increased the conversion of testosterone to estrogen by increasing the gene expression of CYP19A1 testicular cells. Our results are in accordance to Lundquist and colleagues (85) who have shown an increased aromatase expression by $1,25(OH)_2D$ in *LNCaP* cells.

Milani et *al.* have shown several up-regulated genes (*CYP24A1, CLMN, DPP4, EFTUD1, SERPINB1, BMP6, CD14, FAM20C, THBD, CILP, CYP19A1, KCNK3* and *SLC1A1*) after $1,25(OH)_2D$ treatment (10-100nM) as tested by microarrays in breast cancer cultures and derived fibroblasts, squamous carcinoma, immortalized prostate, lymphoblastoid cell lines and in carotid artery smooth muscle cells (86), according to our results.

To the best of our knowledge we were able to show for the first time in human primary testicular cells that treatment with $1,25(OH)_2D$ led to changes in the gene expression of genes related to bone and androgen metabolism. In our functional studies, we have shown that vitamin D was able to influence gene expres-

sion profiles of several other candidate genes involved in bone as well as steroid-biosynthesis and metabolism. The gene encoding for the cholesterol monooxygenase (*CYP11A1*), an enzyme responsible for the catalyzation of cholesterol to pregnenolone, was highly upregulated by 1,25$(OH)_2$D followed by genes belonging to the androgen biosynthesis pathway, such as *CYP17A1*, *HSD3B*, *HSD17B1*, and *SRD5A2*. These upregulations by vitamin D strengthen our hypothesis that there might be a role for VDR also in the male reproductive system that is based on several animal studies that reflect the importance of vitamin D in reproduction. Yagishita et *al.* have also shown that there exists a cooperative regulation of steroidogenic gene expression by vitamin D by that vitamin D enhanced neurosteroid biosynthesis in human glioma cells and up-regulated *HSD3B* and *CYP11A1* (87). We were the first to show this up-regulation of steroidogenic gene expressions in healthy human testicular cells (88).

The gene expression profiles in our testicular cells treated with 100 nM 1,25$(OH)_2$D were determined by Affymetrix Human 1.0 ST GeneChip arrays that covered more than 33 000 transcripts to analyze the expression level of more than 22 000 well-characterized human genes. The analysis has shown further androgenic and androgen induced genes to be significantly up-regulated by 1,25$(OH)_2$D (*ABCA1, HPGD, CYP7B1, AIG1*) which we assume to be direct, or indirect VDR target genes. *ABCA1* is known to be mainly expressed in Sertoli cells and might influence male fertility as shown by *ABCA1* knockout mice that showed reduced intra-testicular testosterone levels as well as reduced sperm counts compared with wild type animals. Absence of *ABCA1* led further to a decline in lipids including high-density lipoprotein cholesterol (HDL-C) from Leydig cells that is the primary source of cholesterol for steroidogenic tissue (89). Taking into account the importance of cholesterol as an essential androgen precursor, an up-regulation of *ABCA1* by vitamin D might maintain Leydig cell testosterone synthesis by induction of enzymes that are important for the conversion of androgens that function as testosterone precursor.

Despite vitamin D actions on androgen production and metabolism we were able to show other biological functions and physiological pathways where vitamin

D might be involved or at least associated. Microarray analysis depicted a number of genes related to inflammatory response and immunological functions, like CD14, IGF-1, CD226, KLF-4, TREM1, LGALS9, or THBD. According to these findings we probably keep vitamin D in our eyes regarding to functions in the human immune system. Our findings are consistent with previous studies suggesting an important role for vitamin D in the modulation of the adaptive as well as innate immune responses (90, 91).

Besides the analysed direct effects of vitamin D on gene expression of steroidogenic target genes and testosterone production, we suggest that vitamin D additionally may influence male testosterone synthesis indirectly, based on our second hypothesis.

According to the literature (30, 35, 92) an involvement of bone regulation in the steroidogenic pathway of men is imaginable. A link from vitamin D to bone and from bone to steroidogenesis is given by the fact that 1,25$(OH)_2$D also regulates gene expression in human primary osteoblasts (93). Further, a number of effects of vitamin D have been described in human osteoblastic cell lines as well as the detection of VDR expression in osteoblasts (94). In our study, we observed a number of significantly 1,25$(OH)_2$D up-regulated genes related to bone metabolism in testicular cells by microarray analysis, such as *insulin-like growth factor 1* (*IGF-1*) or *insulin-like growth factor binding protein 3* (*IGFBP3*). A clinical study performed with 46 vitamin D-deficient children suffering from rickets has shown that supplementation with vitamin D resulted in an increase in circulating IGF-1-levels, but a decrease in PTH-levels (95), confirming our results on cellular level. PTH and related hormones are able to increase the production of 1,25$(OH)_2$D enhancing the absorption of Ca^{2+} by the intestine (96). In turn, we have identified *parathyroid hormone-related protein precursor* (*PTHrP*), a member of the *PTH* family to be down-regulated by 1,25$(OH)_2$D (Fold change -1,38, *P*-value 0,03239) in our experiments. The repressive effect of 1,25$(OH)_2$D on *PTHrP* mRNA expression could be explained by a VDRE that acts negatively in the human *PTH* gene (97). On the other hand, the high up-regulation of *IGF-1* by 1,25$(OH)_2$D might act in a way negatively by acting as repressor on *PTHrP* mRNA expression, although

physiological doses of IGF-1 alone did neither affect mRNA expression of androgenic genes, nor testosterone secretion in testicular primary cells.

Oury et *al*. showed that the skeleton might play a role in male reproduction and fertility on the basis of the involvement of OC a which might induce testosterone production in men by binding to a G-protein coupled receptor (GPRC6A) belonging to the family of calcium sensing receptors that is especially expressed on Leydig cells within the testis (92). The same group found that OC treatment of mouse Leydig cells led to increased cAMP production. CREB, a cellular transcription factor, might mediate the effects of OC on Leydig cells.

We have analyzed that *StAR*, *CYP11A1*, and *CYP17A1* were upregulated in human testicular cells by the treatment with OC. Indeed, Zhang et *al.* have shown that CREB was able to bind in the promotor region of *CYP11A1*, *HSD3b*, and *StAR* (98), suggesting that OC might regulate the steroidogenic gene expression in men through cAMP-protein kinase-CREB signaling pathway.

In another experiment we have shown that the combined addition of vitamin D with OC led to increased mRNA expressions of *LHCGR*, *CYP11A1*, *HSD3B2*, *ESR1*, *CYP3A4*, and *CYP19A1*, which led us to the suggestion that vitamin D and OC might function both on male steroidogenesis leading to a synergistic effect.

Based on microarray analysis we have shown that the mRNAs encoding for *ALPL, CD14, CYP19A1, FAM20C* and *IGF1*, all proteins that are linked or involved in bone metabolism and relevant for bone mineral density, were significantly uregulated confirming the effect of vitamin D on bone metabolism.

Recent findings based on estrogen receptor knockout (ER KO) studies suggest that at least part of the androgen action on bone is dependent on aromatization of androgens into estrogens (99) confirming the high up-regulation of aromatase (*CYP19A1)* gene expression in our study, although the involvement of testosterone and estrogen in bone metabolism in men has still to be researched.

Studies have shown that there might exist a pancreas-bone-testis-axis in males via osteocalcin-stimulated testosterone biosynthesis, which is positively

regulated by insulin signaling in osteoblasts. The osteoblast action in turn might be regulated by vitamin D (30).

The present genome-wide microarray analysis in testicular cells revealed an overview of the 1,25$(OH)_2$D influenced regulatory and metabolic pathways and we have shown 1,25$(OH)_2$D to increase testosterone-secretion in testicular cells. Effects of vitamin D on bone metabolism could be shown, as vitamin D increased a number of bone related genes. Vice versa, OC has shown to be associated with male steroidogenesis as well, as OC treatment led to increased androgenic mRNA expression and to a dose-dependent increase in testosterone as well as DHT levels of human testicular cells *in vitro*.

Strengths of our study are that we were, to the best of our knowledge, the first, who studied the effects of vitamin D on male androgen metabolism in a human testis cell culture model. To date, most of the studies on male steroidogenesis were performed using rodent models (100). Human mixed cultures of testicular cells function as a better model than other human cell lines, because the behavior of primary testicular cells is much more close-to-nature than cell lines.

However, our study has several limitations based on the difficulty to aquire human testis samples: There was a number of seven patients in our study and age of the patients differed as well as the amount of the available basic raw material, which led to limited cell counts achieved from urological samples in contrast to higher available material and therefore more isolated cells from transplantational samples. Supraphysiologic doses of 1,25$(OH)_2$D were used in our experiments based on literature (86, 93, 101). Furthermore we performed pre-studies that have shown no effects of 1,25$(OH)_2$D when used in physiologic concentrations. We had to consider that RT-qPCR has shown to be a more sensitive method for quantification of mRNA expression than using microarrays, which have shown lower expression rates over all analyzed genes and therefore we have used microarrays mostly as screen and RT-qPCR for the relative quantification of gene expressions.

In the past, the clinical significance of vitamin D in the endocrine system was highlighted, because a poor vitamin D status has been associated with an in-

creased risk for severe diseases including cancer, diabetes, hypertension, autoimmune diseases, musculoskeletal diseases, cardiovascular diseases and also all-cause mortality (102-104).

We assume based on our results that vitamin D is associated with the regulation of male androgen biosynthesis linked to male reproduction. However, we do not know yet about the molecular basis by which vitamin D exerts its effects, which has to be further elucidated.

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11. Appendix

ORIGINAL ARTICLE
Endocrine Research

Testicular synthesis and vitamin D action

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Context: The vitamin D system has pleiotropic effects not only in bone metabolism. Its role in testicular steroidogenesis is new and deserves intensive research.

Objective: We hypothesize that vitamin D, especially $1,25(OH)_2D_3$ (calcitriol) induces male steroidogenesis and intend to identify its impact on genes and pathways in testicular androgen regulation.

Methods: Human adult primary testicular cells were isolated, treated with $1,25(OH)_2D_3$ and their gene expression levels profiled by microarray analysis. Highly regulated genes were confirmed by real time quantitative PCR (RT qPCR). In addition, effects of $1,25(OH)_2D_3$ in combination with luteinizing hormone (LH) and insulin-like growth factor 1 (IGF-1) on gene expression level of androgens were assessed. Testosterone levels in the culture media were determined by high-resolution ELISA. The expression of vitamin D receptor (VDR) was confirmed at baseline and after $1,25(OH)_2D_3$ stimulation using immunocytochemistry.

Results: Microarrays depicted sixty-three genes significantly regulated by $1,25(OH)_2D_3$, including genes related to male androgen and vitamin D metabolism, mainly triggered by VDR/RXR receptor activation. $1,25(OH)_2D_3$ led to significant changes in the expression profiles of reproductive genes and significantly increased testosterone synthesis in human testicular cell cultures.

Conclusions: Data from our human primary testicular cell culture model suggest that vitamin D plays a major role in male steroidogenesis *in vitro*.

Vitamin D might modulate reproductive processes in women and men (1) beyond its effects in mineral metabolism. The vitamin D receptor (VDR) and vitamin D metabolizing enzymes are expressed in reproductive tissues of women and men (2–4).

In a clinical study including 2299 men, significant associations of 25-hydroxyvitamin D (25(OH)D) and testosterone levels and a similar seasonal variation of both hormones have been identified (5). This was recently confirmed (6–8) suggesting a relationship of vitamin D and testosterone.

In animal models, vitamin D deficient male rats have shown a reduction in successful matings (9), an incomplete spermatogenesis, and degenerative changes within the testes compared to controls (10). Studies in rodents have confirmed the expression of VDR in cells of the genital tract, such as spermatogonia, suggesting a role of vitamin D in spermatogenesis and sperm maturation in rats. Vice versa, vitamin D receptor null mutant mice have shown gonadal insufficiency and fertility was reduced in male rats under vitamin D deficient diet (9, 11).

Vitamin D forms like vitamin D2 (eg, ergocalciferol from plant sources or supplementation) or vitamin D3 (from human skin or animal sources) have pleiotropic effects and are converted to the active form calcitriol ($1,25(OH)_2D_3$). Based on clinical studies, vitamin D3 has shown to more effectively increase $25(OH)D_3$ serum levels than vitamin D2 (12).

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Abbreviations:

$1,25(OH)_2D_3$ acts mainly through binding to its nuclear receptor, the vitamin D receptor (VDR). The VDR interacts with the retinoid X receptor (RXR), forming a VDR-RXR heterocomplex, by which VDR acts as transcription factor able to bind to VDREs in the promoter region of target genes (13).

Testosterone is obligatory to support spermatogenesis, as it is the main androgen in circulation. It is produced by Leydig cells in the testis and necessary for differentiation of male internal and external reproductive organs and to induce male sex characteristics and sexual behavior.

Steroidogenesis in men is induced by luteinizing hormone (LH) through the activation of cyclic AMP production (cAMP) followed by an increase in intracellular concentrations of calcium ions (Ca^{2+}) in Leydig cells (14, 15). The secretion of gonadotropins by the pituitary gland, namely follicle stimulating hormone (FSH) and LH, contributes to a balanced endocrine interplay for a complete and functional germ cell development (16).

Other studies, mainly in animals, have demonstrated an increase in testosterone synthesis by a combination of LH with IGF-1 (17, 18).

The present study intended to identify the impact of vitamin D, especially $1,25(OH)_2D_3$, in human testicular primary cells on gene expression of relevant genes in male steroidogenesis as well as on testosterone secretion. Our goal was to identify effects of $1,25(OH)_2D_3$ on male steroidogenesis, which until now have not been studied in a human adult primary testicular cell culture model.

Materials and Methods

Sources of testis tissue

Testis tissue was obtained either from brain-dead organ donors at the Department of Surgery, Division of Transplantation Surgery, or from healthy testicular tissue of planned orchiectomy patients at the Department of Urology, without prior antiandrogenic treatment, yielding seven samples from patients aged 35–81 years. The protocol was approved by the ethical committee of the Medical University of Graz, Austria. Testis tissue was transported on ice in a phosphate buffered saline solution (PBS, PAA Laboratories, Pasching, Austria) containing 100 μg/ml Penicillin, 100 U Streptomycin and 2,5 μg/ml Amphotericin B (all from Sigma-Aldrich, St. Louis, MO) and immediately processed.

Isolation of human testicular cells and validation of cell viability

Isolation of testicular cells from donor testis samples was performed according to the method described previously (19) with some modifications: Small testis explants (0,5–0,8 cm^3) were digested for 60 minutes at 37°C in Ham's F-12 medium and Dulbecco's modified Eagle medium (1:1, F-12/DMEM, Life Technologies, Carlsbad, CA) containing 2 mg/ml collagenase B (Roche, Vienna, Austria), 10 μg/ml deoxyribonuclease (DNase) 1 (DNase 1, Sigma-Aldrich), 1 μg/ml soy trypsin inhibitor (Sigma-Aldrich) in a constantly shaking water bath. After Percoll (Percoll, GE Healthcare, Uppsala, Sweden) gradient centrifugation cells were collected, washed in PBS (2 × 5 minutes, 300 *g*) and cell viability determined using trypan blue solution (0,4%, Sigma-Aldrich) and a hemocytometer (Neubauer chamber) using the uptake of trypan blue by the cells as marker for cell death.

Culture of testicular cells and hormonal treatment

Isolated testicular cells were cultured in F-12/DME GlutMAX medium (1:1, Life Technologies) supplemented with 10% fetal bovine serum (FBS, PAA Laboratories), 100 μg/ml Penicillin, 100 U Streptomycin, 2,5 μg/ml Amphotericin B and 15 mM HEPES solution (Life Technologies) in an atmosphere of 37°C and 5% CO_2.

Hormonal treatments, cell numbers, and well-formats for all experiments are summarized in Supplemental Table 1. Cells were stored in TriReagent (Ambion, Life Technologies) at −80°C for RNA isolation. Testosterone concentrations of testicular cells were analyzed at baseline and after addition of hormones in the testicular cell supernatants.

Cytochemical HSD3β staining

Hydroxysteroid dehydrogenase 3β (HSD3β) staining was used to identify human Leydig cells. 20 μl of interstitial cell suspensions were allowed to grow on chamber glass slides overnight at 37°C in a humidified atmosphere at 5% CO_2. Cells were stained with staining solution (0,2 mg/ml nitroblue tetrazolium chloride (NBT, Sigma-Aldrich), 1 mg/ml β-nicotinamide adenine dinucleotide (NAD) (βNAD, Sigma-Aldrich), 0,12 mg/ml epiandrosterone (MP Biomedicals, LLC, Solon, OH) and 4 μl PBS). Leydig cell numbers were determined semiquantitatively by counting the amount of stained cells.

Immunocytochemical staining for VDR

Primary testicular cells were seeded into chamber slides (Lab-Tek™ II Chamber slide™ System, Nalge Nunc International Corp., Naperville, IL) and treated with 100 nM $1,25(OH)_2D_3$ overnight. *MDA-MB231* breast cancer cells were used as a VDR-negative control according to Cordes et *al.* (20) and the breast cancer cell line *SKBR3* served as a VDR-positive control. Cells were fixed with fixation buffer (3,7% paraformaldehyde, 0,18% Triton) for 30 minutes at 37°C. Permeabilization of cells was done for 20 minutes in retrieval buffer (EnVision™ FLEX Target Retrieval Solution, High pH, 50x, Dako North America, Inc., CA) at 85°C followed by blocking with 5% normal goat serum (Dako) and 3% Triton™X-100 (Sigma-Aldrich) for 30 minutes at room temperature. Cells were treated with VDR (Santa Cruz Biotechnology Inc., Santa Cruz, CA), Cytokeratin (Dako) and IgG control antibody (Dako) overnight in a humidified chamber at 4°C in the dark. Antibody dilutions are listed in Supplemental Table 2. Labeling occurred with Alexa Fluor® 555 Rabbit Anti-Goat IgG (H+L) (Life Technologies) and Goat Anti-Mouse IgG (H+L) Dylight™ 488 Conjugated (Thermo Scientific, Rockford, IL) for 1 hour in a humidified chamber in the dark at room temperature. 15 μl of mounting medium including 4′,6-diamidino-2-phenylindole (DAPI) (Vectashield Hard Set Mounting Medium with DAPI, Vector Laboratories, Burlingame, CA) was added and cells covered. Labeled cells were observed using the Olympus fluorescent microscope BX51 (Olympus, Tokyo,

Japan). Captured images were analyzed using cellSens® software (cellSens Standard version 1.8.1, Olympus) and processed by ImageJ software (ImageJ version 1.47, National Institutes of Health, USA).

Testosterone measurement

In the culture media of human adult testicular cell cultures testosterone was measured using a commercially available high sensitive immunoassay (Testosterone ELISA, Demeditech #DE1559, Kiel-Wellsee, Germany) according to the manufacturer's recommendations. Lyphocheck™ Immunoassay Plus Control level 1 and 3 with defined testosterone concentrations (1,41 ng/ml (range 0,82–2,00) and 12,00 ng/ml (range 6,97–17,10), respectively) were used as positive controls (Biorad, Vienna, Austria). The plate was analyzed using the Gladiator equipment (IASON, Graz, Austria). After standard curve interpolation, the concentrations of the samples were expressed in nanograms per milliliter (ng/ml). All samples were assayed in duplicates from at least 3 independent experiments.

RNA isolation

Total RNA was extracted from isolated testicular cells (1×10^6 cells) in TriReagent (Ambion, Life Technologies), followed by phase separation using chloroform and RNA precipitation using isopropanol. RNA was purified using the RNeasy Mini Kit (Quiagen, Hilden, Germany) according to the manufacturer's instructions. The concentration of isolated RNA was determined using the Nanodrop spectrophotomer (NanoDrop® Spectrophotometer ND-1000, peQLab Biotechnology GmbH, Erlangen, Germany) and RNA integrity assessed using the Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). All RNA samples were of acceptable quality (RIN of 9,2 –10).

mRNA microarrays of testes cell samples

Hybridizations and analyses were done at the Core Facility "Molecular Biology" at the Center for Medical Research (ZMF) of the Medical University of Graz.

250 ng of total RNA was labeled using the Ambion WT Expression Kit for Affymetrix GeneChip whole transcript (WT) expression arrays (Life Technologies) and hybridized to GeneChip Human 1.0 ST arrays as described in the manufacturer's manual (Affymetrix, Santa Clara, CA). The Affymetrix Genechip® fluidics station 450 was used for washing and staining (GeneChip® HT hybridization, wash and stain kit, Affymetrix). Labeling and hybridization controls were evaluated with the expression console EC 1.3 and arrays were scanned with the Affymetrix GeneChip scanner GCS3000.

Data analysis of microarrays

The analysis of microarrays was performed for seven testicular cell samples that were available in control and $1,25(OH)_2D_3$ treated conditions. The "CEL files" were imported into Partek Genomic Suite software (software v6.6, Partek Inc, St. Louis, MO) and robust multichip average (RMA) normalized, including background correction, quantile normalization across all arrays, and median polished summarization based on log transformed expression values. Genes that passed the determined filter criteria after three-way ANOVA were further analyzed with Ingenuity IPA (Interactive Pathway Analysis of complex 'omics data) software (version 17199142, Ingenuity systems, Redwood City, CA). IPA categorized the top-regulated genes, among other things, into functions and/or diseases, networks, and canonical metabolic and signaling pathways.

RT-PCR and Quantitative Real-time PCR

1000 ng of each RNA preparation were reverse-transcribed using the High Capacity RNA to cDNA Kit (Applied Biosystems, Life Technologies) following the manufacturer's instructions. Relative gene expressions of VDR and vitamin D metabolizing enzymes (CYP2R1, CYP27A1, CYP27B1, CYP24A1) were analyzed at baseline in our pre-experiments.

The messenger RNA (mRNA) expression of twelve significantly $1,25(OH)_2D_3$ regulated genes (based on microarrays) was confirmed by real time quantitative polymerase chain reaction (PCR) (RT-qPCR). RT-qPCR was performed on the Light Cycler (Roche Diagnostics GmbH, Vienna, Austria) using TaqMan® gene expression assays for alkaline phosphatase, liver/bone/kidney (*ALPL*), CD14 molecule (*CD14*), calmin, or calponin-like transmembrane domain protein (*CLMN*), aromatase (*CYP19A1*), vitamin D_3-24-hydroxylase (*CYP24A1*), dipeptidyl-peptidase 4 (*DPP4*), insulin-like growth factor 1 (*IGF-1*), krüppel-like factor 4 (*KLF4*), methylene-tetrahydrofolate reductase (NAD(P)H) (*MTHFR*), solute carrier family 1 member 1 (*SLC1A1*), six-transmembrane epithelial antigen of prostate 4 (*STEAP4*) and transmembrane protein 37 (*TMEM37*) (Life Technologies). TaqMan® gene expression assays were validated and the linear range of the assays determined.

Further we analyzed selected genes of cholesterol homeostasis as well as steroid metabolism (*CYP11A1, HSD3B2, CYP19A1, CYP3A4, ESR1* and *SRD5A1),* which were not entirely available from Affymetrix GeneChip Human 1.0 ST arrays. The relative gene expression was analyzed after addition of LH alone, or in combination with IGF-1 and $1,25(OH)_2D_3$ to testicular cells, independent of the microarray experiment using TaqMan® gene expression assays (Life Technologies). Expression of mRNA of the selected genes was analyzed in triplets and normalized against an internal control, which was the housekeeping gene *GAPDH*. The relative quantification of gene expression and the calculation of fold changes was done using the ΔΔCt method according to Pfaffl (21).

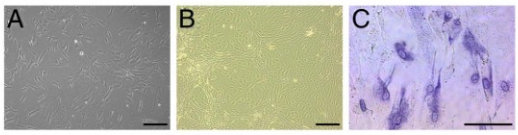

Figure 1. Testicular primary cells isolated from human testes tissue. A Testicular cells show adherence after 16 hours in culture. Bar = 100 μm. B Testicular primary cells become confluent when cultured for 10 days and include different cell types. Bar = 100 μm. C Leydig cells are detected by HSD3β staining (purple) (600x magnified). Bar = 200 μm.

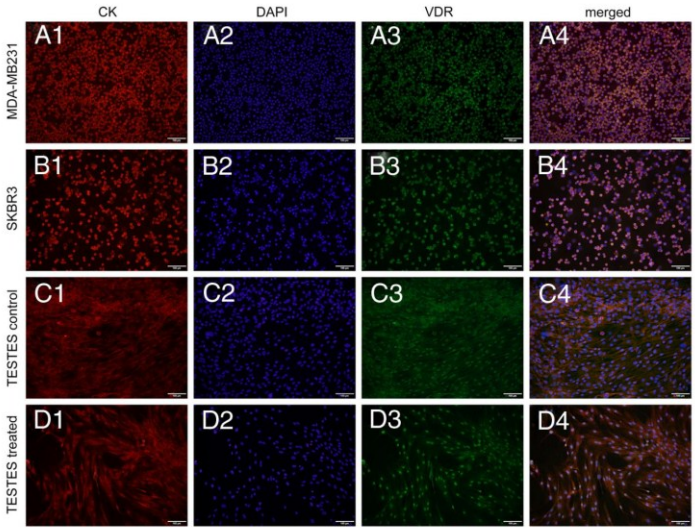

Figure 2. Basal and $1,25(OH)_2D_3$ stimulated VDR expression in human primary testicular cells. Red fluorescence (A1, B1, C1, D1) detects labeled cytoplasm (CK), blue fluorescence (A2, B2, C2, D2) shows labeled nuclei (DAPI) and green fluorescence (A3, B3, C3, D3) shows labeled VDR. The merged images demonstrate that human testicular primary cells treated with 100 nM $1,25(OH)_2D_3$ (TESTES treated) display predominantly nuclear localization of VDR (D4), compared to unstimulated testicular cells (TESTES control) as well as breast cancer control cells (*MDA-MB231*, *SKBR3*) that show VDR expression predominantly in the cytosolic compartment (A4, B4, C4). CK = cytokeratin, DAPI = 4′,6-diamidino-2-phenylindole,) VDR= vitamin D receptor. Bar = 100 μm.

Statistical analysis

Statistical analysis was carried out using GraphPad (Version 5, GraphPad Software Inc., San Diego, CA, USA) for nonparametric analysis. One-way analysis of variance (ANOVA) followed by Dunnett post analysis was used for statistical differences (> 2 compared groups).

Microarrays were analyzed after RMA normalization using three-way ANOVA, comparing $1,25(OH)_2D_3$-treated with control samples over all patient samples. Genes with FDR 5% and fold change of at least 1,5 or –1,5, respectively, were considered to be significantly. The *Pearson correlation* was calculated comparing microarray and RT-qPCR techniques. Differences were regarded as statistically significant at $P < .05$. Values are presented as mean ± SD, except when otherwise noted.

Results

Isolation, identification and cell viability of human adult primary testicular cells

Analysis of cell viability immediately after cell isolation using trypan blue (0,4% trypan blue solution, Sigma-Aldrich) showed over 90% viable cells. Isolated cells became adherent after 16 hours in culture (Figure 1A). Confluent monolayer with different cell types could be observed by light microscopic examination after 10 days in culture (Figure 1B). Leydig cells were identified by cytochemical staining of HSD3β (Figure 1C). Leydig cell numbers varied between testicular cell cultures by about 50%, as analyzed semiquantitatively.

Detection of VDR in testicular cells on protein level

Besides the detection of VDR and vitamin D metabolizing enzymes on mRNA level at baseline (Figure 5), we investigated the expression of VDR in testicular cells at baseline and after stimulation with $1,25(OH)_2D_3$ on protein level by immunocytochemistry (Figure 2). At baseline, VDR was moderately expressed predominantly in the cytosolic compartment in testicular cells. Addition of 100 nM $1,25(OH)_2D_3$ led to a shift of VDR expression from the cytoplasm to the nucleus, where VDR was predominantly expressed. In control cells, namely breast cancer cells *SKBR3*, a strong signal for VDR was determined in the cytosolic compartment and *MDA-MB231*, supposed to be VDR-negative, displayed moderate cytosolic VDR expression at baseline.

$1,25(OH)_2D_3$ treatment increased testosterone levels

We demonstrate a significant increase in testosterone production after exclusive addition of 100 nM $1,25(OH)_2D_3$ ($P < .001$) compared to control cells treated with vehicle (ethanol) (Figure 3A).

Addition of 5 IU/l LH, 100 ng/ml IGF-1 together with 100 nM $1,25(OH)_2D_3$ significantly increased testosterone levels ($P < .05$) (Figure 3B), compared to treatment with LH or LH and IGF-1 only.

Up-regulation of androgen-related genes and genes involved in reproductive and endocrine functions by $1,25(OH)_2D_3$

Treatment of testicular cells with 100 nM $1,25(OH)_2D_3$ in addition to 5 IU/l LH and 100 ng/ml IGF-1 increased mRNA expression of *HSD3B2*, *CYP11A1*, *CYP19A1*, *CYP3A4*, *ESR1* and *SRD5A1* (Figure 4) when compared to addition of LH alone. Physiological serum concentrations of $1,25(OH)_2D_3$ (50- 500 pM) did not show significant effects in our testicular cells on mRNA level of genes affecting the androgen biosynthesis pathway (*data not shown*).

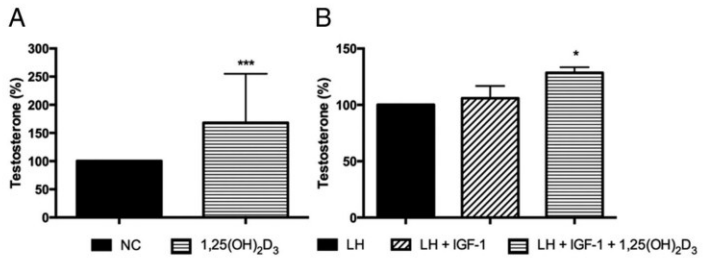

Figure 3. Testosterone synthesis in human primary testicular cell cultures. A Addition of 100 nM $1,25(OH)_2D_3$ for 24 hours significantly increased testosterone production compared to control ($P < .001$, by Wilcoxon signed rank test). Testosterone concentrations are displayed as percentage normalized to control. B Addition of $1,25(OH)_2D_3$ (100 nM) in combination with LH (5 IU/l) and IGF-1 (100 ng/ml) has shown to significantly increase testosterone production compared to addition of LH or LH + IGF-1 alone ($P < .05$, by one-way ANOVA). Testosterone concentrations are displayed as percentage and normalized to LH. Results are represented as means ± SD. * $P < .05$, *** $P < .001$. NC= control (vehicle), LH= Luteinizing hormone, IGF-1= insulin-like growth factor 1.

Using microarrays, we were able to detect a significant $1,25(OH)_2D_3$-induced up-regulation of genes related to androgen metabolism (P-value < 0.05). Based on the filter criteria we observed 63 genes that were significantly changed by $1,25(OH)_2D_3$ treatment, of which 51 genes were mapped by IPA.

The microarray data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO series accession number GSE55207.

Significantly regulated genes were stratified by IPA regarding to biological relevance with functional annotations, pathways, and functions/diseases. The top five biological functions affected by $1,25(OH)_2D_3$ treatment in testicular cells (ranked by significance; P-value) were "protein synthesis", "skeletal and muscular system development and function", "connective tissue disorders", "inflammation" and "skeletal and muscular disorders" (*data not shown*). Most of the genes are associated to the IPA ranked top five "biological functions" including "reproductive" and "endocrine systems" and "mineral metabolism" (Supplemental Table 3). Most the $1,25(OH)_2D_3$ affected genes were found in the canonical pathway VDR/RXR activation" (*data not shown*).

From the 63 significantly up-regulated genes obtained from microarray data twelve genes matched keywords like "male steroidogenesis", "testis", "testosterone", "vitamin D" upon literature research using *PubMed*. We have validated the expression patterns of these twelve genes using semiquantitative qPCR analysis. *ALPL*, *CD14*, *CLMN*, *CYP19A1*, *CYP24A1*, *DPP4*, *IGF-1*, *KLF4*, *MTHFR*, *SLC1A1*, *STEAP4* and *TMEM37* have shown significant up-regulations in the $1,25(OH)_2D_3$ treated samples compared to control samples (fold changes > 1,5

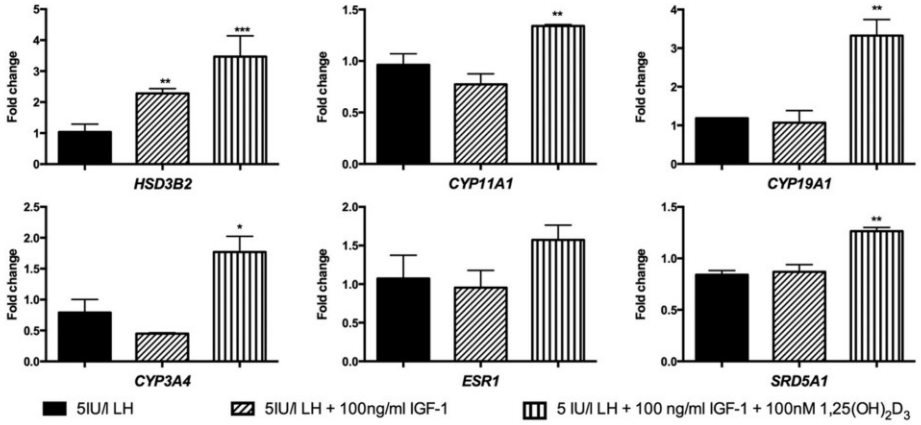

Figure 4. Mean values ± SD of fold changes for mRNA levels of genes of the androgen synthesis and metabolisms pathway in human testicular primary cells (n = 4–6), determined by RT-qPCR using TaqMan® assays. Testicular primary cells were treated for 24 hours with vehicle (control) or hormones: Either 5 IU/l LH (LH) alone, or LH in combination with 100 ng/ml insulin-like growth factor 1 (IGF-1), or LH with IGF-1 and 100 nM $1,25(OH)_2D_3$. $1,25(OH)_2D_3$ induced mRNA expression of selected genes of the androgen biosynthesis pathway by combined addition with LH and IGF-1. Fold changes of selected genes were compared to fold changes obtained by LH treatment alone: *3beta-hydroxysteroid dehydrogenase II* (*HSD3B2*), *cholesterol side-chain cleavage enzyme* (*CYP11A1*), *Steroid 5-α-reductase* (*SRD5A1*), *aromatase* (*CYP19A1*), *Cytochrome P450 3A4 (CYP3A4)*, and *estrogen receptor 1 (ESR1)* mRNA expression were increased after addition of $1,25(OH)_2D_3$. Fold changes were calculated using the ddCt-method and adjacent 2^{-ddCt}. Statistical analysis was performed by one-way-ANOVA and Dunnett's multiple comparison test. * $P < .05$; ** $P < .01$; *** $P < .001$.

and *P*-values < 0.05) (Table 1) Fold changes obtained by RT-qPCR correlated with microarray results (Pearson r = 0.7469; $P < .01$).

Discussion

Our results provide evidence that $1,25(OH)_2D_3$ is directly involved in human male testicular androgenesis. In primary human testicular cells we show that $1,25(OH)_2D_3$ increases testosterone production and mRNA expression of enzymes involved in androgen production and their precursors. We are the first to show the up-regulation of steroidogenic gene expression in healthy human testicular cells, which has previously only been shown in animal cells in vitro (22–24). Human mixed cultures of testicular cells serve as a better model than human cell lines, because the behavior of primary testicular cells might be more physiological than that of cancer cell lines. In addition, the present genome-wide microarray analysis in primary human adult testicular cells treated with $1,25(OH)_2D_3$ revealed an up-regulation of genes involved in cell-to-cell signaling and cellular development, as well as pathways requiring the activation of the VDR/RXR.

In clinical studies, daily supplementation with 83 μg

Table 1. Comparison of results obtained with microarray and RT-qPCR techniques for the selected genes. Fold changes are means ± SD of 7 patients (*P*-value <0.05, by *t* test)

Gene	Summary of functions	microarray		RT-qPCR	
		Fold change	*P*-value (Partek)	Fold change	*P*-value (*t* test)
ALPL	Bone metabolism	1,52	0.010	2,27	0.011
CD14	Mediator of innate immune response	1,89	0.018	3,52	0.006
CLMN	Actin binding	1,92	0.010	2,57	0.026
CYP19A1	Steroid metabolism, estrogen biosynthesis	1,54	0.007	2,67	0.008
CYP24A1	Calcium homeostasis, Vitamin D metabolism	122,19	9.6E-06	3475	0.036
DPP4	T-cell activation	1,65	0.004	2,12	0.036
IGF-1	Insulin regulation	1,96	0.040	3,24	0.070
KLF-4	Embryonic development	1,51	0.025	2,26	0.125
MTHFR	Aminoacid catalyzation	1,63	0.000	1,89	0.010
SLC1A1	Membrane transporter	1,74	0.001	1,90	0.030
STEAP4	Adipocyte development and metabolism	2,33	0.019	4,22	0.027
TMEM37	Calcium channel activity	1,70	0.001	2,89	0.030

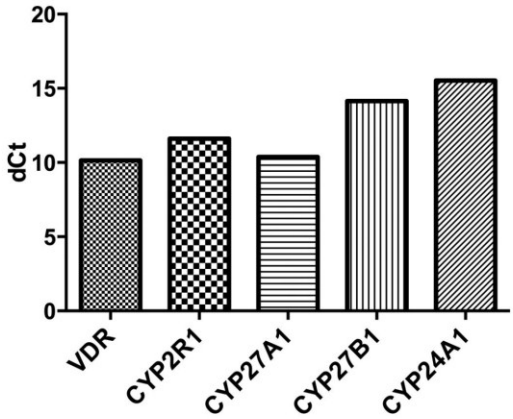

Figure 5. Relative gene expression of VDR and vitamin D metabolizing enzymes in human testicular primary cells at baseline. *GAPDH* was used as internal control and substracted from C_t-values of the selected genes. VDR= vitamin D receptor, CYP2R1= vitamin D 25-hydroxylase, CYP27A1= sterol 27-hydroxylase, CYP27B1 = 1a-hydroxylase, CYP24A1= vitamin D_3-24-hydroxylase. The relative gene expression is displayed as dC_t-value for the selected genes.

(3332 IU) vitamin D was able to increase testosterone levels in a cohort of healthy overweight men undergoing a weight reduction program (25). In this randomized controlled trial (RCT), total testosterone, bioactive testosterone, and free testosterone levels were significantly increased in the vitamin D supplemented group compared to the placebo group. However, a study by Jorde et al found no testosterone increasing effect in 282 subjects receiving either high dose vitamin D or placebo (8). We need to keep in mind that in both clinical studies vitamin D was applied in supraphysiologic doses, as in our experiments, based on the literature (26) and on our prestudies that showed no effect of $1,25(OH)_2D_3$ when used in physiological serum concentrations.

In our study, addition of $1,25(OH)_2D_3$ up-regulated the gene expression of steroidogenic enzymes involved in the pathway from cholesterol to active steroid hormones and their metabolism (CYP11A1, HSD3B2, CYP19A1, CYP3A4 and SRD5A1) (27). We assume that VDREs in the promoter regions of the selected genes are recognized by activated VDRs that act as inducer of gene expression and that we detected predominantly in the nucleus when $1,25(OH)_2D_3$ is available as ligand.

Secreted testosterone can either be converted to estrogens by the enzyme aromatase (*CYP19A1*), known to be expressed in Leydig cells within the adult mammalian testes (28), or to the more active androgen dihydrotestosterone (DHT) by steroid 5-α-reductase (*SRD5A1*). Estrogens are necessary for normal male reproduction, as they are potential regulators of spermatogenesis in numerous species including human (29). We assume that *CYP19A1* gene expression in testes might also be regulated by $1,25(OH)_2D_3$ as VDREs were detected in the promoter region of *CYP19A1* in breast and bone (11, 30). However, *SRD5A1* is up-regulated too. We speculate that *SRD5A1* and *CYP19A1* expression might be dependent on the applied or available vitamin D concentration and further by the requirement of the testes for either estrogen or androgens, which should be in balance in healthy humans.

There is evidence for a VDR expression in the human pituitary, suggesting that $1,25(OH)_2D_3$ might have regulatory functions in the gene expression and hormone secretion of pituitary cells (31). We suggest a synergistic effect of LH and $1,25(OH)_2D_3$ as we were able to show that addition of $1,25(OH)_2D_3$ together with LH significantly increases testosterone synthesis in vitro when compared to addition of LH alone, which is known to stimulate male testosterone secretion per se (32).

Besides the stimulatory effects of $1,25(OH)_2D_3$ on mRNA levels of androgenic genes, we observed other significantly up-regulated genes by microarray, eg, *alkaline phosphatase* (*ALPL*) a marker for bone turnover (33), *CD14 molecule* (*CD14*), a component of the innate immune system (34), or *krüppel like factor 4* (*KLF-4*), involved in Sertoli cell differentiation (35) and we show *IGF-1* to be significantly up-regulated after $1,25(OH)_2D_3$ addition in testicular cells. Based on cross-sectional studies in community-based cohorts, positive correlations of vitamin D and IGF-1 have been observed (36, 37). Ameri et *al.* showed that vitamin D increased circulating IGF-1 levels in adults and speculated that a better vitamin D status can favor normalization of IGF-1-levels in adults with treated growth hormone (GH) deficiency (GHD) (38).

Based on our microarray results, $1,25(OH)_2D_3$ influences several physiological processes, including immune functions, insulin regulation, and bone metabolism, which need to be further elucidated.

However, our study has several limitations based on the difficulty to collect human testes samples. In our seven samples, the age of the patients differed as well as the amount of the available tissue material, which potentially led to different cell counts and might have influenced the results. There is some evidence that aging might reduce the conversion of cholesterol to testosterone resulting from reductions in steroidogenic enzyme activities (39). Semiquantitative counting of Leydig cells after HSD3b staining revealed different amounts of Leydig cells between the cultures, which varied by about 50%. This variability might have led to decreased effects of LH in our cultures with potentially different amounts of secreted testosterone. There is evidence that administration of LH fails to stimulate old Leydig cells to produce high testosterone levels, as shown in studies in old rats (40). However, our results were concordant for all probands and samples despite different amounts of Leydig cells.

In general, we had to consider that RT-qPCR has shown to be a more sensitive method for quantification of mRNA expression than using microarrays, which have shown lower expression rates over all analyzed genes. Therefore we used microarrays mostly as a screening method and RT-qPCR for the relative quantification of gene expressions. This might be an advantage for the detection of candidate gene changes.

In conclusion, we assume, based on our results from human primary testicular cells and as confirmed by clinical studies that vitamin D is involved in the regulation of male androgen metabolism. Taking into account the potential association of vitamin D with semen quality and androgen status in men and the up-regulation of steroidogenic genes by $1,25(OH)_2D_3$ in our human testicular primary cell culture model, vitamin D, within physiological limits, might open up an important treatment option in male testosterone deficiency in the future.

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