Aus der Medizinischen Klinik des St. Hedwig Krankenhauses
Akademisches Lehrkrankenhaus
der Medizinischen Fakultät Charité –Universitätsmedizin Berlin

DISSERTATION

1, 25 dihydroxyvitamin D3 inhibits the proliferation of thyroid anaplastic cancer stem-like cells via cell cycle arrest without affecting apoptosis

zur Erlangung des akademischen Grades
Doctor medicinae (Dr. med.)

vorgelegt der Medizinischen Fakultät
Charité – Universitätsmedizin Berlin

von

Wen Peng

aus Jiangsu, China

Gutachter: 1. Prof. Dr. Med. K.-M. Derwahl

2. Prof. Dr. H. M önig

3. Priv.-Doz. Dr. Med. S. Diederich

Datum der Promotion: 25. 10. 2013

CONTENTS

Abbreviations

ABCG2	ATP-binding cassette transporter G2
ALDH1	aldehyde dehydrogenase 1 family
AML	acute myeloid leukemia
bFGF	basic fibroblast growth factor
BRCA1	breast cancer type 1
cDNA	complementary deoxyribonucleic acid
CSCs	cancer stem cells
DBP	vitamin D binding protein
DMEM	dulbecco's modified eagle medium
DMSO	dimethyl sulfoxide
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
FACS	fluorescence-activated cell sorting
FCS	fetal calf serum
HBSS	Hank's balanced salt solution
HNF4	hepatocyte nuclear factor 4
HTh74 R	HTh74 doxorubicin-resistant subline
MDR1	multidrug resistance transporter 1
MTD	maximum tolerated dose
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
Oct4	octamer-binding transcription factor 4
PI	propidium iodine
qPCR	quantitative polymerase chain reaction
RNA	messenger ribonucleic acid
rRNA	ribosomal ribonucleic acid
RT-PCR	reverse transcription-polymerase chain reaction
SAHA	suberoylanilide hydroxamic acid
SCC	squamous cell carcinoma
SD	standard error
SFE	sphere forming efficiency
SOX2	SRY (sex determining region Y)-box 2

SP	side populations
TBE	tris-borate EDTA
Tg	thyroglobulin
TSH	thyroid stimulating hormone
TTF1	thyroid transcription factor 1
UV	ultraviolet
VDR	vitamin D receptor

1. Introduction

1.1 Vitamin D synthesis and metabolism

Vitamin D is not a true vitamin, but a classical hormone. Its synthesis involves a multistep process that begins in the skin from 7-dehydrocholesterol. Ultraviolet light of the appropriate wavelength (270-300 nm) catalyzes the conversion of 7-dehydrocholesterol to cholecalciferol (vitamin D3). Vitamin D3 is further hydroxylated in the liver, predominantly by *CYP2R1*, to 25α hydroxylcholecalciferol (25(OH)D3) and then 1α-hydroxylated in the kidney by *CYP27B1* to 1,25 dihydroxycholecalciferol (1,25(OH)2D3) or calcitriol (Fig. 1.1) (1).

Calcitriol is the bioactive form of vitamin D. It exerts its biologic functions by binding to a nuclear receptor, and then regulates transcription of DNA into RNA, which is so-called genomic pathway of vitamin D. On the other hand, calcitriol is capable of affecting transcription via cross talk with other signalling pathways, which is the non-genomic pathway of vitamin D. Vitamin D action is mainly limited by catabolism, with the enzyme of 24-hydroxylase (*CYP24A1*), which catabolises the transformation to 1,24,25(OH)3 D3. This compound has a substantially lower affinity for the vitamin D receptor (VDR), and then is further metabolized to excreted products (2).

1.2 Antitumor effects of vitamin D

A considerable amount of data on antitumor effects of vitamin D compounds has been obtained in both epidemiological and *in vitro* studies. In epidemiological studies, on the one hand, risk factors which may lead to "low" vitamin D levels (e.g. geography, lifestyle/activity, history of sun exposure) have been associated with higher frequency of several types of cancers (e.g. colon, breast, prostate) in different populations (3-6); on the other hand, serum calcitriol levels or sufficient vitamin D supplementation was shown to be associated with reduced risk of different cancers, including colon, breast, ovary and pancreas carcinomas(6). Meanwhile numerous *in vitro* studies have drawn the association of exposure of tumor cells to high concentrations of vitamin D with an inhibited proliferation (2, 7-9). In some cases, vitamin D is even able to induce the differentiation, which has been observed in colon, breast, prostate, squamous cell carcinoma, osteosarcoma and myeloid leukemia cancer cells (10, 11). Vitamin D compounds are capable of inhibiting the proliferation or even killing cancer cells *in vitro* and *in vivo* and more recently, the ability of vitamin D to potentiate antitumor actions of some cytotoxic agents has been reported, including taxanes, paclitaxel, platimum etc (2, 12, 13). Anticancer effects of

calcitriol or its analogues were observed in various cancers, such as squamous cell carcinoma (SCC) (14), human carcinomas arising in the prostate, lung, ovary, breast, bladder, pancreas (15-21) as well as in thyroid cancer cells (22-27).

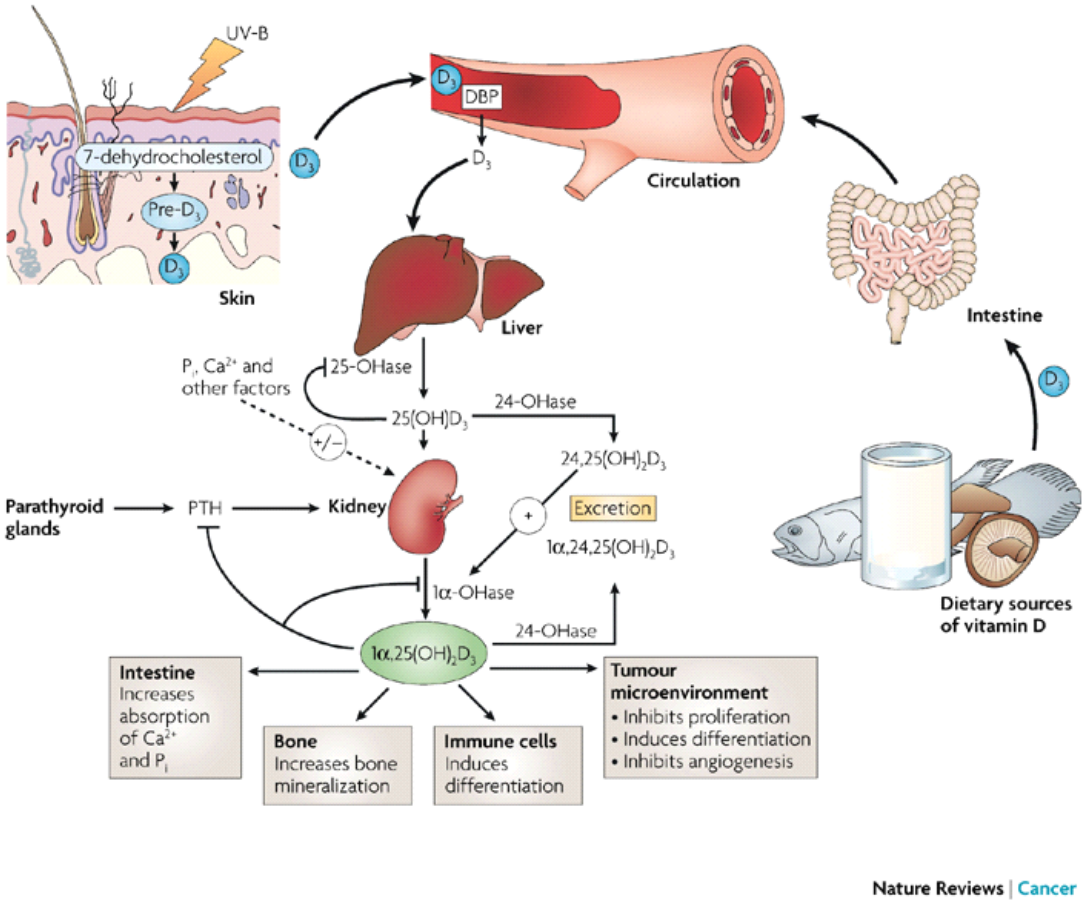

Fig.1.1 Vitamin D synthesis process. Photochemical synthesis of vitamin D3 (cholecalciferol, D3) occurs cutaneously where pro-vitamin D3 (7-dehydrocholesterol) is converted to pre-vitamin D3 (pre-D3) in response to ultraviolet B (sunlight) exposure. Vitamin D3, obtained from the isomerization of pre-vitamin D3 in the epidermal basal layers or intestinal absorption of natural and fortified foods and supplements, binds to vitamin D-binding protein (DBP) in the bloodstream, and is transported to the liver. D3 is hydroxylated by liver 25-hydroxylases (25-OHase). The resultant 25-hydroxycholecalciferol (25(OH)D3) is 1α-hydroxylated in the kidney by 25-hydroxyvitamin D3-1α-hydroxylase (1α-OHase). This yields the active secosteroid 1α,25(OH)2D3 (calcitriol), which has different effects on various target tissues. The synthesis of 1α,25(OH)2D3 from 25(OH)D3 is stimulated by parathyroid hormone (PTH) and suppressed by Ca^{2+}, Pi and 1α,25(OH)2D3 itself. The rate-limiting step in catabolism is the degradation of 25(OH)D3 and 1α,25(OH)2D3 to 24,25(OH)D3 and 1α,24,25(OH)2D3, respectively, which occurs through 24-hydroxylation by 25-hydroxyvitamin D 24-hydroxylase (24-OHase), encoded by the *CYP24A1* gene.

24,25(OH)D3 and 1α,24,25(OH)2D3 are consequently excreted. The main effects of 1α,25(OH)2D3 on various target tissues are highlighted above (Taken from: Vitamin D signalling pathways in cancer: potential for anticancer therapeutics. Nat Rev Cancer. 2007;7(9):684-700.) (1).

An epidemiological study from Poland showed that decreased 1,25 dihydroxyvitamin D3 concentration in peripheral blood serum was related with thyroid cancer (28), while research in cell lines demonstrated the inhibitory effects of calcitriol and its analogue on the thyroid cancer cells. When vitamin D was combined with other cytotoxic drugs such as paclitaxel or suberoylanilide hydroxamic acid (SAHA), it also potentiated the anticancer effects of these drugs (26). Furthermore, VDR polymorphisms have been associated with thyroid cancers, which may modify the risk of cancers (29, 30).The mechanism of anticancer effects varies a lot depending on different cancer types. But in general, regulation of proliferation, differentiation, apoptosis and angiogenesis are involved (2, 11).

1.3 Cancer stem cells (CSCs) hypothesis and evidence of thyroid CSCs

Two conflicting models were described to explain tumor formation: the clonal evolution model (stochastic model) and the cancer stem cell model (hierarchy model) (31). The clonal evolution model hypotheses that a substantial proportion of the tumor cells are able to sustain the tumor growth, and the probability to initiate a new tumor for each carcinoma cell is the same. In contrast, the cancer stem cell model suggests that only a small proportion of cells in a tumor have the ability to initiate tumor growth. Based on the two cancer-formation theories, the principles for cancer treatment may differ from each other. With the clonal evolution model, anticancer therapy has to be targeted on every carcinoma cell in the tumor; while with the cancer stem cell model, the small subset of cancers which are responsible for initiating a new tumor may become the main target (31-33) (Fig.1.2). This small group of cancer cells, which promote the tumor initiation, and which contribute to chemotherapy resistance, with a high tendency to metastasis and relapse, were identified as CSCs or cancer stem-like cells (31, 32).

Regarding the finding of cancer stem-like cells, they were first identified and described in acute myeloid leukemia (AML), with the cell surface markers CD34+, CD38- (34, 35). After that, more and more evidence about the existence of cancer stem cells was observed in other solid tumors, including in the brain, ovary, pancreatic, prostate, colon and breast tumors (3, 36-42).

Until recently, several studies on adult thyroid stem cells and thyroid CSCs were published (43-53). In our laboratory, thyroid adult stem cells were developed from human thyroid tissues (44, 45). A few stem cell markers or endodermal markers, such as Oct4, GATA4, HNF4α, were expressed in these stem/progenitor cells (44). Adult thyroid stem-like cells have also been characterized in mouse thyroid cells (54). The existence of thyroid CSCs or cancer stem-like cells derived from anaplastic thyroid cancer cell lines have also been characterized, both in our lab and by others (47, 48, 50, 52, 53).

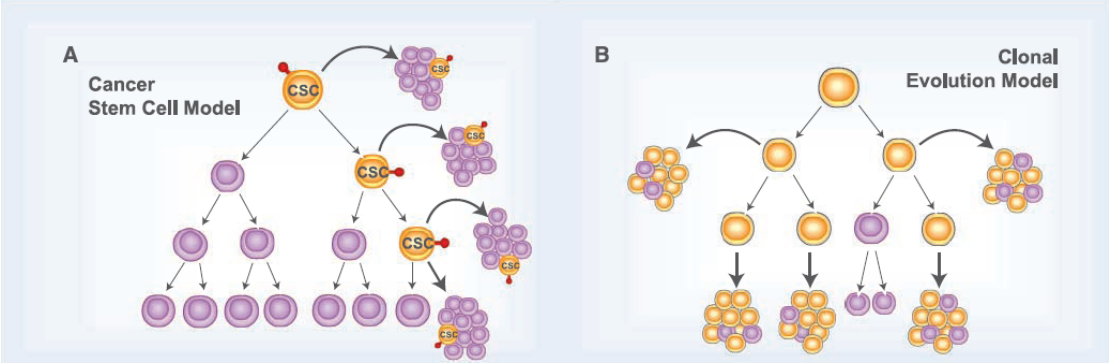

Fig.1.2 Models for the nature of sustained tumor growth. A. in the CSC model (hierarchy model), only the CSC, which can be isolated prospectively by surface markers, possesses self-renewal activity and, hence, represents the only relevant target for therapy. B. in the clonal evolution model (stochastic model), a substantial proportion of the tumor cells can sustain its growth, and hence, therapy must attempt to eliminate all the cells. (Taken from: Adams JM, Strasser A. Is tumor growth sustained by rare cancer stem cells or dominant clones? Cancer Res. 2008;68(11):4018-21.)(32)

1.4 Cancer spheres possess characteristics of cancer stem-like cells

It is hypothesized that CSCs or cancer stem-like cells may play an pivotal role in many steps of tumors, including the tumor initiation, progression, metastasis, and therapeutic resistance (31-33). CSCs may possess some characteristics, including high expression of drug transporters, relative cell-cycle quiescence, high function of DNA repair machinery, and resistance to apoptosis , which may lead to resistance to chemotherapy and radiotherapy (55-57). In terms of the isolating and sorting of CSCs, since CSCs and adult stem cells share some common features, including the ability to self-renew and differentiation and the expression of some stem cell markers (pluripotent or multidrug-resistance or endodermal gene marker Oct4, ABCG2, HNF4α etc. and cell surface marker CD133, CD24, CD34, CD38, CD44, ALDH1 etc.) (31, 35, 44, 45, 49-51, 53, 58-62), the techniques used for identifying and isolating adult stem cells and CSCs are similar.

Flowcytometry with specific cell surface markers is widely used to isolating adult stem cells and CSCs. Different cell surface markers were identified for stem cells from different tissue or cell origin. It has been suggested that integrin a2/b1, CD44, or CD133 are molecular markers for human prostate stem cells (63), whereas CD44+/ $CD242^{/low}$ is carried out as the breast cancer stem cell markers (62, 64).

Side population sorting with flowcytometry is another way to access the stem/progenitor cells after side population stem cells were first isolated in hematopoietic stem cells, which is capable to efflux Hoechst 33342 actively (47, 48, 65-69). However, either with cell surface marker sorting or side population isolating, only a low yield of cells are isolated with flowcytometry (66, 67, 70). Therefore, stem cell sorting system with flowcytometry is not able to provide sufficient stem/progenitor cells for further research. In addition, cell damage during cell labelling and sorting is another disadvantages (70). How to isolate and further amplify CSCs for further research, is still a question which need to be answered.

To solve this problem, 3-dimensional sphere culture system in serum-free medium with grow factors are suggested to isolate and amplify CSCs/progenitor cells or cancer stem-like cells (71-74). Based on the stem cell theory, in this 3-dimensional sphere culture system, most cells die, and only the cancer stem cells survive, which may give rise to the progenitor cells, and then form the spheres (45, 47, 58). First developed in neuro stem cell isolation, this sphere culture system now is expanded to various cell origins especially for different cancer cells, including prostate cancer, breast cancer, lung cancer, squamous cell carcinoma, and thyroid carcinoma (31, 47, 64, 74-76). In anaplastic thyroid cancer cell lines, thyroid cancer stem-like cells were obtained via the sphere culture system as well (47). Therefore, tumor sphere culture provides an efficient approach to amplifying cancer stem-like cells including thyroid cancer stem-like cells.

In our laboratory, we have used Hoechst staining to identify and characterize thyroid CSCs. Meanwhile, a doxorubicin-resistant HTh74 sub-line (HTh74R) was developed and maintained in the medium containing 0.50μg/ml doxorubicin for more than 6 months (47). Our previous studies found that, the SP fraction in HTh74R was as high as 70%, compared with that in HTh74, which was less than 1%. Side population, which showed expression of stem cell marker Oct4 and significantly higher expression of ABC transporter gene, ABCG2 and MDR1, can also be enriched in serum-free medium containing mitogens (47).Taken together, in thyroid anaplastic cell line HTh74 and HTh74R cells, cancer spheres cultured in serum-free medium supplemented

with adequate mitogens possess the characteristics of stem-like cells, which can be used for stem cell research.

As mentioned above, vitamin D compound exhibits an inhibitory effect on many tumors, and may induce the differentiation of many tumors. However, the effects of vitamin D compound on cancer stem cells, or more specifically, on thyroid cancer stem cells are still unknown.

1.5 Aim of the present study

The aim of the present work is (1) to determine whether vitamin D binding site exists in thyroid cancer stem-like cells; (2) to explore whether vitamin D may inhibit the growth of thyroid CSCs or cancer stem-like cells; (3) to illustrate whether cell cycle arrest and enhanced apoptosis are involved in the growth arrest by vitamin D; (4) to investigate whether vitamin D may promote the differentiation of thyroid cancer stem-like cells.

2. Materials and methods

2.1 Cell culture

2.1.1 Cell lines

Thyroid cancer cell lines were stored in liquid nitrogen at -196℃. For the experiment, cells were placed in 37℃ water bath and shaken gently for 1-2 min and then immediately suspended in pre-warmed culture media.

The HTh74 cell line, an anaplastic thyroid cancer cell line, was developed and kindly provided by Prof. Nils-Erik Heldin (Uppsala University, Uppsala, Sweden) (57).

A doxorubicin-resistant HTh74 sub-line (HTh74R) was developed in our lab. HTh74 cell line was exposed continuously to doxorubicin (Sigma, Germany), with the starting concentration of 0.01μg/ml and then increasing in a stepwise manner to 0.5μg/ml. HTh74R subline was maintained in the medium with 0.5μg/ml doxorubicin for more than 6 months (47).

2.1.2 Culture conditions

Cell culture conditions were as described by Zheng before (47). Briefly, anaplastic thyroid carcinoma cell line HTh74 cell line was grown in Ham's F-12 medium with L-glutamine (Invitrogen, Germany), with 10% fetal calf serum (FCS, v/v) (Invitrogen, Germany), 1% non-essential amino acids (MEM, v/v) (Invitrogen, Germany), 100U/ml penicillin, 100μg/ml streptomycin, 2.5μg/ml amphotericin B (Roche, Germany). For HTh74R cell line, 0.5μg/ml doxorubicin (Sigma, USA) was kept in the culture medium (47).

For most of the experiments, cells were cultured in 100mm or 60mm plastic culture dishes and kept in a humidified incubator at 37℃ under 5% CO2, with a medium change every 3-4 days. Cell passaging was performed when a 70-80% of cell confluency was reached (77).

2.1.3 Cell counting

Cell counting was performed mainly as described in Zheng´s dissertation (77), with a hemacytometer. Cultured monolayer cells were trysinized to detach, and then neutralized with volume-equivalent HBSS. After centrifugation, a diluted cell suspension was placed on both counting chambers of the hemacytometer. The number of the loaded cells were counted under a

microscope at 100 ×magnification. Depending on the dilution, the total cell number of the original cell suspension was calculated (77).

2.1.4 Tumor sphere culture

Tumor spheres were cultured as previously described (47). Specifically, HTh74 and HTh74R spheres were generated by seeding HTh74 and HTh74R cells (1×10^4cells/ml) into serum-free DMEM/F12 (1:1, v/v) medium containing B27 (1:50 dilution, Invitrogen), bFGF (20ng/ml, Invitrogen) and EGF (20ng/ml, Invitrogen). Every 2-3 days, B27, bFGF and EGF were supplemented (47). After 5-7 days culture, tumor spheres, which were enriched in cancer stem-like cells, were grown up for further use.

2.2 RNA isolation, reverse transcription, polymerase chain reaction (RT-PCR)

2.2.1 RNA isolation and reverse transcription

Total RNA isolation was extracted from cultured cells by using the RNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's specifications. Reverse transcription was performed with iScript™ cDNA Synthesis Kit (Bio-Rad, Germany). 1µg RNA (as calculated by spectrophotometry), 4µl 5×iScript reaction mix, 1µl iScript reverse transcriptase, and RNase-free water were added to a final volume of 20µl. The reaction mix was heated to 25℃ for 5min, 42℃ for 30min, 85℃ for 5min and held at 4℃. cDNA samples were stored at -20℃.

2.2.2 Polymerase chain reaction (RT-PCR)

Hot Start method was adopted for the polymerase chain reaction, which was clearly described in previous work (77). Cycling conditions were followed as: 95℃ for 30 sec (denaturation), 58℃ for 30 sec (annealing, for both Vitamin D receptor and internal control) and 72℃ for 1 min (extension), followed by a final extension at 72℃ for 10 min and terminated at 4℃. ß-actin was used as an internal control. Primer pair sequences, annealing temperature and product sizes are listed in Table 1.

PCR products were separated on 2% (w/v) agarose gels with 0.5µg/ml ethidium bromide. Gels were run at a voltage of 90V in TBE running buffer for 60 min. A UV-transilluminator at 312 nm was used for the visualisation of the product bands (77).

Table 1. Primer sequences, annealing temperatures and product sizes for RT-PCR and qPCR

Target gene	Primer sequences	Annealing temperature(℃)	Expected size (bp)
VDR	S: 5'-CTGACCCTGGAGACTTTGAC-3'	60℃	277
	AS: 5'-TTCCTCTGCACTTCCTCATC-3'		
ß-actin	S: 5'-ACCAACTGGGACGACATGGAGAAA-3'	58℃	192
	AS: 5'-TAGCACAGCCTGGATAGCAACGTA-3'		
NIS*	S: 5'-TCTCTCAGTCAACGCCTCT -3'	58℃	298
	AS: 5'-ATCCAGGATGGCCACTTCTT -3'		
18s rRNA*	S: 5'-CTCAACACGGGAAACCTCAC-3'	58℃	110
	AS: 5'-CGCTCCACCAACTAAGAACG-3'		

S: sense primer; AS: antisense primer

* The primers were used for qPCR

2.3 Cell viability assay

Cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide (MTT) assay (Sigma, St. Louis, MO). Cells ($2\text{-}5\times10^3$) were seeded in 96-well plates. After 24 hours, they were treated with different doses of calcitriol (Sigma-Aldrich, St. Louis, MO) or volume equivalent vehicle (DMSO) for 24, 48 or 72 hours. The concentrations of vehicle in the cell medium were not higher than 0.1%. MTT assay was performed by incubating the cells with 0.5mg/ml MTT for 4 h at 37℃ in 5% CO2. The formazan product was dissolved in DMSO and absorbance was read at 490 nm. All experiments were repeated at least three times in quadruplicate.

2.4 Sphere formation experiment

HTh74 and HTh74R spheres were generated by seeding HTh74 and HTh74R cells (1×10^4cells/ml) into serum-free DMEM/F12 (1:1, v/v) medium containing B27 (1:50 dilution, Invitrogen), bFGF (20ng/ml, Invitrogen) and EGF (20ng/ml, Invitrogen) in 6 cm dishes, which was as described previously (47). Calcitriol (100nM) and vehicle (0.1% DMSO) were added at the same day (day0). Every 2-3 days, B27, bFGF and EGF were added. On day 5, cancer sphere numbers in each dish were counted. Sphere-forming efficiency (SFE) was calculated as the number of sphere-like structures (large diameter>30μm) formed in 5 days divided by the original number of cells seeded and expressed as percentage means±SD. Four independent experiments were performed.

2.5 Secondary passaged thyroid cancer stem-like cells

For the establishing of secondary generation of thyroid cancer stem/progenitor cells, tumor spheres derived from HTh74 and HTh74R were collected and dissociated enzymatically (15 min in 0.05% trypsin, 0.53nM EDTA-4Na at 37°C) and mechanically into single cells. 1-5×10^4 of these single cells, which are enriched with thyroid cancer stem cells and progenitor cells (47), were seeded in a 6cm dish as monolayer in serum-free DMEN/F12 medium. Cells termed thyroid cancer stem-like cells underwent starvation and adhesion for 24h. Then B27, bFGF and EGF were added, together with 100nM calcitriol or vehicle. The concentration of vehicle was controlled not to exceed 0.1%. After another 72h incubation, photos were taken, and the thyroid cancer stem-like cells with calcitriol or vehicle incubation were collected for cell cycle analysis, cell apoptotic assay, and quantitative real-time PCR for differentiation marker.

2.6 Cell cycle analysis and apoptosis analysis with flow cytometry

Cell cycle arrest and apoptotic assay with flow cytometry were performed as described before (52, 78). Thyroid cancer stem-like cells were harvested after 72h incubation with 100nM calcitriol or vehicle.

For cell cycle analysis, thyroid cancer stem-like cells were collected and washed in PBS, fixed in 70% ethanol for 1 h at 4°C and then stored for up to 2 weeks at -20°C. Before analysis, these cells were washed again and centrifugated with cold PBS, and then suspended in 1 ml PI staining solution (PBS with 200 μg/ml RNase A, 50 μg/ml PI and 0.1% Triton X-100). Cells underwent incubation at room temperature for 30 min before analysed on a FACS LSRII flow cytometer (BD, Heidelberg, Germany) (78).

For apoptotic assay, double staining with fluorescein isothiocyanate (FITC)-conjugated annexin V and PI was used based on the manufacturer's specifications (BD PharmingenTM). Briefly, thyroid cancer stem-like cells with calcitriol or vehicle incubation for 72h were collected and resuspended in 1 × Binding Buffer. 5 μl FITC-conjugated annexin V (50 μg/ml) and 10 μl PI solution (100 μlg/ml) were added to each 500 μl cell suspension. After 10 min light-protected incubation at room temperature, cell apoptosis analysis was performed with FACS LSRII flow cytometer (BD, Heidelberg, Germany) (52, 78).

The numbers of viable (annexin V-/PI-) and apoptotic (annexin V+/PI-) cells and the proportion of cells in different cell cycle phases were calculated with the FACSDiva software (BD) (52).

2.7 Quantitative real-time PCR

Real-time quantitative RT-PCR was performed with iQReal time PCR detector system (Bio-Rad, CA, USA). SYBR Green reactions were carried out with iQ™ SYBR@ Green Supermix (Bio-Rad, Germany).

Real-time PCR was performed following manufacture's instructions in a 96 well plate. In brief, for each 25µl reaction, 12.5µl 1×iQ SYBR Green Supermix, 2µl cDNA (100ng), 300nM of sense and antisense primers, and a corresponding volume of sterile water were included. The reactions underwent an initial enzyme activation at 95℃ for 15 min, followed by 50 cycles at 95℃ for 15s, 58℃ for 30s, and 72℃ for 30s. This procedure was performed on each sample in duplicate and 3 independent sets of RNA were prepared.

The CT values in real time PCR were analyzed with the $(2\times efficiency)^{-\triangle\triangle CT}$ method and normalized by the value of the internal control 18s rRNA. Results were tabulated as mean±SD. Primer sequences, product sizes, and annealing temperature were listed in Table 1.

2.8 Statistical analysis

Statistical analysis was performed with SPSS 13.0 software. Results are expressed as means±SD of three independent experiments, except when otherwise stated. Significance was defined at the 0.05 level.

2.9 Experimental equipment

2.9.1 Apparatus

FACS Calibur equipment	Becton-Dickinson Bioscience
Laminar flow cabinet	Heraeus, Laminair HB 2448
Cell culture incubator	Heraeus
Microscope	Nikon, TMS
Digital camera	Sony DSC-W7
Water bath	Kotterman Labortechnik
UV Illuminator	Bachofer Laboratoriumsgeräte
Thermocycler	Biometra, Trio-Thermoblock
Spectrophotometer	Pharmacia, Ultrospec II
Autoclave	H+V Varioklav

Shake incubator	Infors HT
Voltage generators	Consort, Electrophoresis power supply, E455
Homogeniser	Eppendorf Thermomixer 5436
Centrifuges	Hettich Mikro 200R
Heating oven	Memmert
Pipettes and tips	Eppendorf
Plastic centrifuge tubes	Sarstedt
Falcon tubes	Becton Dickinson
Plastic culture dishes and plates	Sarstedt
Elisa machine	Biotek

2.9.2 Software

FACSDiva software (BD)	Flow cytometry analysis
Picasa 3	Figure preparation
Microsoft office excel	Figure preparation
SPSS 13.0	Figure preparation

3. Results

3.1 VDR mRNA expression in HTh74 cells, HTh74R cells and derived cancer spheres

VDR expression was detected in the human anaplastic thyroid cancer cell line HTh74, doxorubicin resistant HTh74 cell line HTh74R, as well as in the derived tumor spheres (HTh74 SP and HTh74R SP). The amplified sequences displayed the expected size of 277bp. ß-actin was detected as a control (Fig. 3.1).

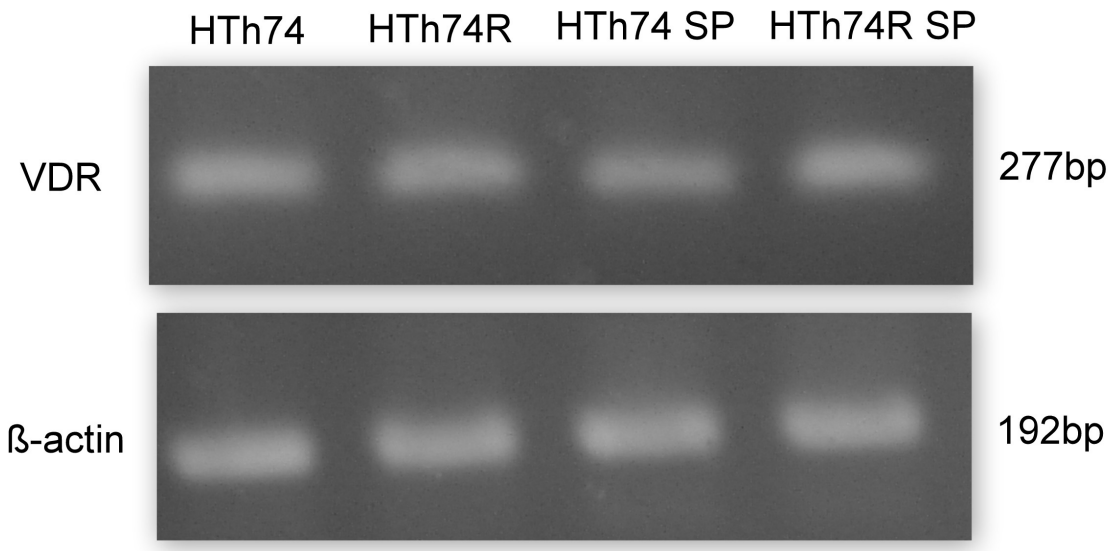

Figure 3.1 mRNA expression of VDR gene in HTh74, HTh74R, HTh74 SP and HTh74R SP. The expected size of vitamin D receptor was 277bp. ß-actin was used as a control with an expected size of 192bp.

3.2 Calcitriol inhibited thyroid anaplastic cancer cell proliferation

By cell viability assay the effect of calcitriol on thyroid anaplastic cancer cells HTh74 and its doxorubicin-resistant subline HTh74R was investigated. As shown in Fig 3.2, calcitriol significantly inhibited the growth of HTh74 and HTh74R cell lines in a dose and time-dependent manner, with a maximal growth inhibition at 72h with a concentration of 100nM. The growth inhibitory effect on doxorubicin-resistant HTh74R cells that are enriched with CSCs and progenitor cells was more pronounced than on HTh74 cells. After 72h incubation with 100nM calcitriol, a maximal 13% inhibition was observed in HTh74 (P<0.05), in contrast to a 36% inhibitory effect in HTh74R (P<0.01).

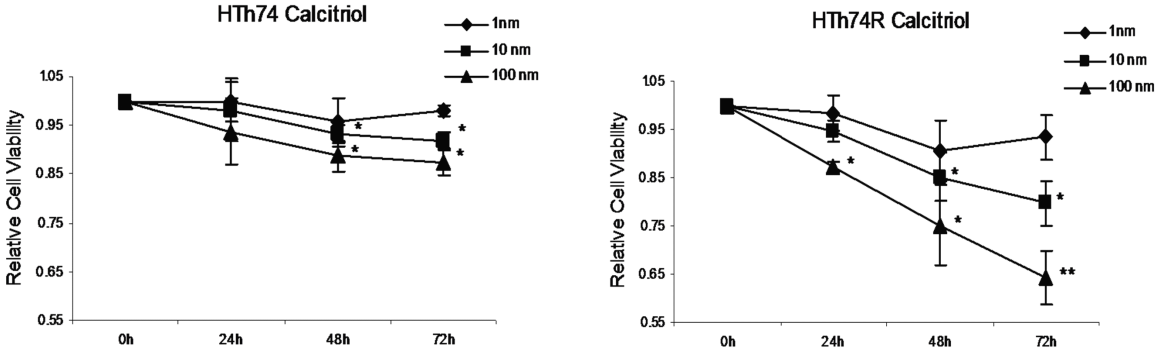

Figure 3.2 Effects of calcitriol on thyroid cancer cell growth using viability assay. HTh74 and HTh74R cells were treated for 24h, 48h and 72h with 1nM, 10nM and 100nM calcitriol or vehicle (control). Three independent experiments were performed. **P*<0.05, ***P*<0.01 compared to control (100%).

3.3 Calcitriol reduced sphere formation efficiency (SFE) in anaplastic thyroid cancer cell lines

The effects of calcitriol on sphere formation in HTh74 cells and HTh74R cells were analyzed by sphere formation assay. After 120h incubation with 100nM calcitriol or vehicle, sphere formation efficiency (SFE) was significantly lower in the intervention group than that in the vehicle group in both cell lines, with a reduction of 64% and 61% in HTh74 and HTh74R, respectively. Meanwhile, the sphere size in calcitriol group was reduced (Fig 3.3 A,B).

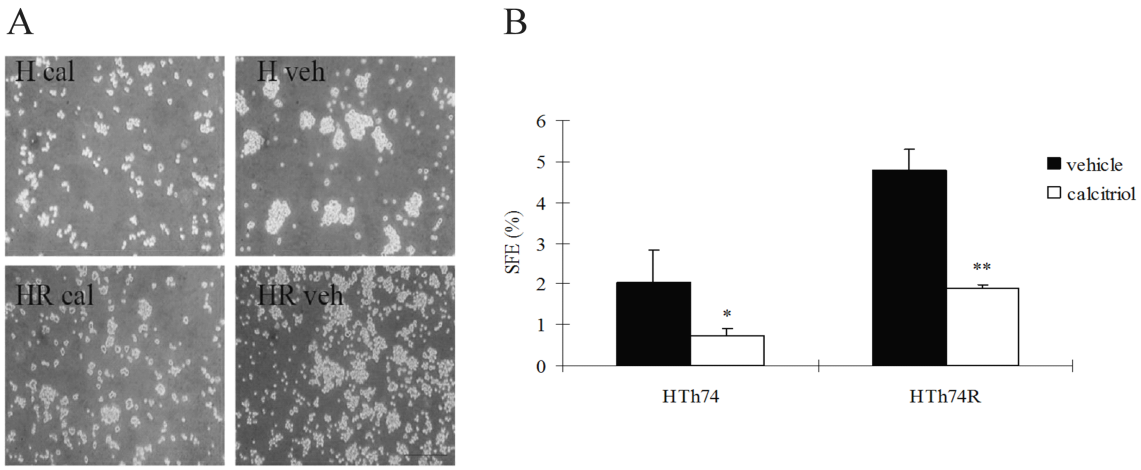

Figure 3.3 Effects of calcitriol on cancer sphere formation efficiency (SFE). A. After 120h incubation with 100nM calcitriol or volume equivalent vehicle, in both HTh74 and HTh74R cell lines, sphere density was lower, accompanied by smaller sphere size in calcitriol group (10× magnifications, scale bar:100μm). H: HTh74 cells; HR: HTh74 resistant cells; cal: treated with calcitriol; veh: treated with

vehicle. B. SFE was calculated as the number of sphere-like structures (large diameter>30μm) formed in 5 days divided by the original number of cells seeded and expressed as percentage (means±SD). Four independent experiments were performed. After 120 hours incubation with calcitriol, thyroid cancer sphere formation, which is a hallmark of cancer stem cells, was significantly decreased. Inhibition of sphere formation was observed both in HTh74 and HTh74R cell lines. *P<0.05, **P<0.01.

3.4 Calcitriol induced G2/M phase arrest in thyroid anaplastic cancer stem-like cells

Cell cycle arrest analysis in HTh74 stem-like cells with flow cytometry showed a significant increased fraction of G2/M phase (from 18% to 25%), accompanied by decreased cells in G1 phase (from 70% to 57%). In HTh74R stem-like cells, similar results in cell cycle arrest were observed (G2/M from 22% to 28%, G1 from 64% to 57%) (Fig 3.4.1).

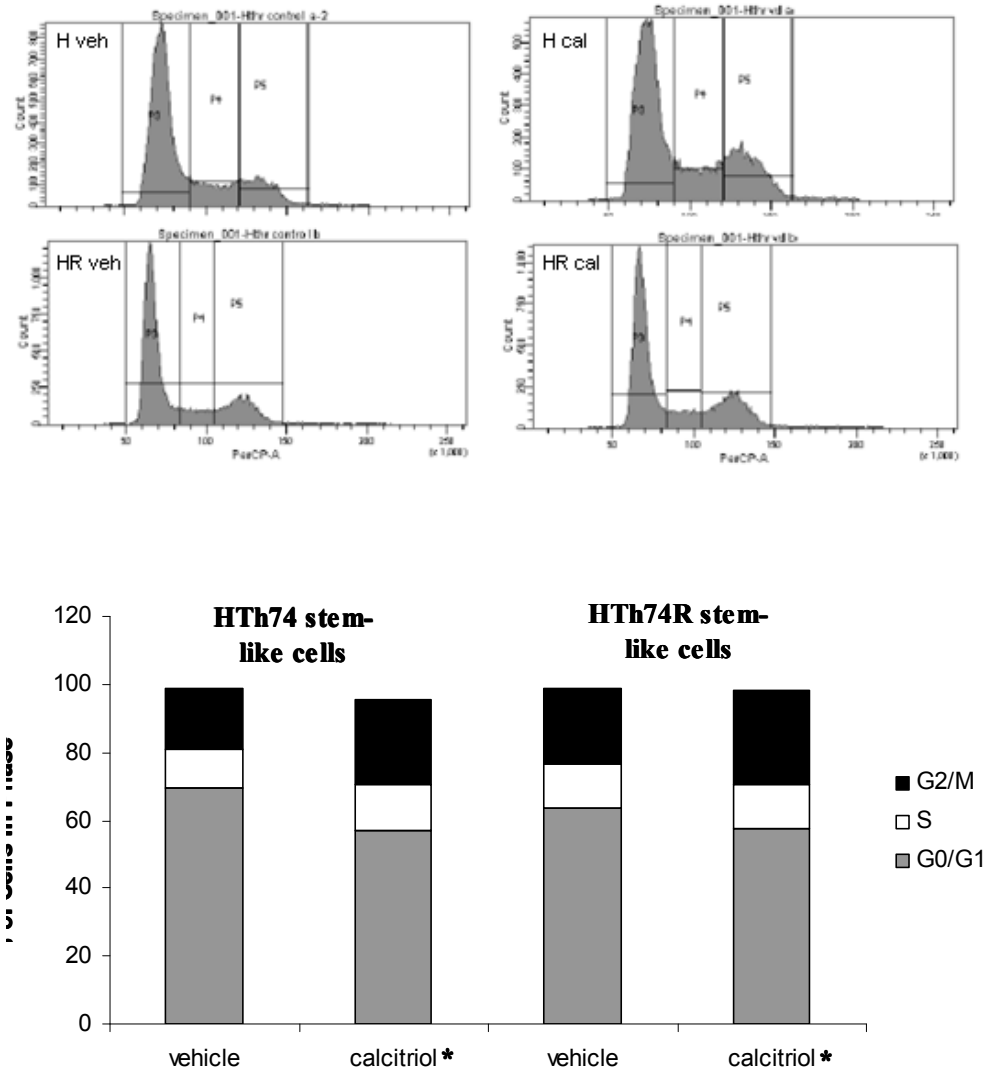

Figure 3.4.1 Effects of calcitriol on cancer stem-like cell cycle arrest. After incubation with 100nM calcitriol or vehicle for 72 hours, HTh74 and HTh74R stem-like cells were used for cell cycle analysis

with flow cytometry. In HTh74 stem-like cells, an increased fraction of G2/M phase accompanied by a decrease of cells in G1 phase was observed. A comparable result was also observed in HTh74R stem-like cells. H: HTh74 stem-like cells; HR: HTh74R stem-like cells; cal: treated with calcitriol; veh: treated with vehicle. Four independent experiments were performed. *P<0.05 *vs.* vehicle.

3.5 Calcitriol did not enhance apoptosis in thyroid anaplastic cancer stem-like cells

No significant enhanced apoptosis was observed in HTh74 stem-like cells after 100nM calcitriol incubation for 72 hours. Meanwhile, HTh74R stem-like cells were also resistant to the apoptosis-inducing effects of calcitriol (Fig 3.5.1).

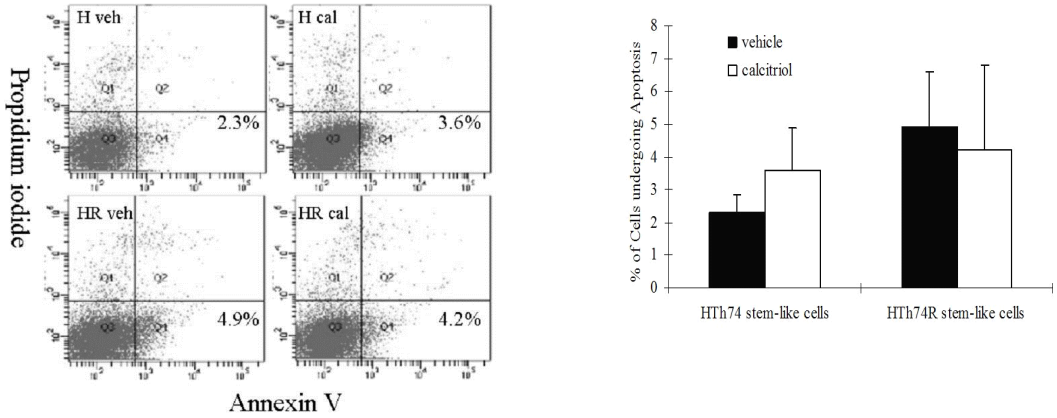

Figure 3.5.1. Effects of calcitriol on cancer stem-like cell apoptosis. After incubation with 100nM calcitriol or vehicle for 72 hours, HTh74 and HTh74R stem-like cells were used for apoptosis analysis with flow cytometry. No significant apoptosis-inducing effects were observed in either HTh74 stem-like cells or HTh74R stem-like cells after calcitriol intervention. H: HTh74 stem-like cells; HR: HTh74 resistant stem-like cells; cal: treated with calcitriol; veh: treated with vehicle. Three independent experiments were performed.

3.6 Calcitriol induced a differentiation-like morphologic change in anaplastic cancer stem-like cells

The HTh74 stem-like cells treated with calcitriol showed a wild-type HTh74 cell-like shape, with larger cell volume and a close adhesion to the dishes, whereas the cells with vehicle incubation had a round shape with smaller cell volume, and loose attachment to the dishes, which were characteristics of undifferentiated cells. The cells in the vehicle group may be easily detached without trypsin incubation. In HTh74R stem-like cells, a similar morphological change was observed (Fig 3.6.1).

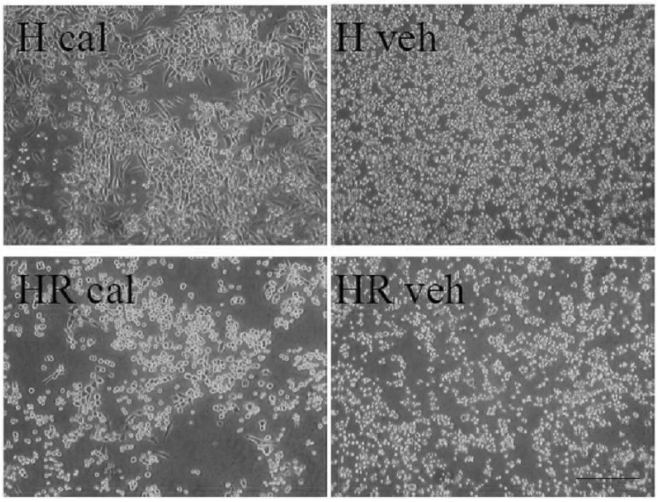

Figure 3.6.1 Morphological features of thyroid cancer stem-like cells after 72 hours incubation with calcitriol or vehicle. With 72 hours incubation with calcitriol, HTh74 stem-like cells showed a wild-type HTh74 cell-like shape, with larger cell volume and a close adhesion to the dishes, whereas the cells with vehicle incubation showed a round shape with smaller cell volume, and loose attachment to the dishes, which were characteristics of undifferentiated cells. In HTh74R stem-like cells, a similar morphological change was observed (10× magnifications, scale bar:100μm). H: HTh74 stem-like cells; HR: HTh74 resistant stem-like cells; cal: treated with calcitriol; veh: treated with vehicle.

To further explore the putative differentiation-inducing effect of calcitriol on thyroid cancer stem-like cells, mRNA relative expression of NIS gene was detected with quantitative real-time PCR. Neither HTh74 nor HTh74R stem-like cells showed an increased expression of NIS after calcitriol intervention compared with vehicle group (Fig. 3.6.2).

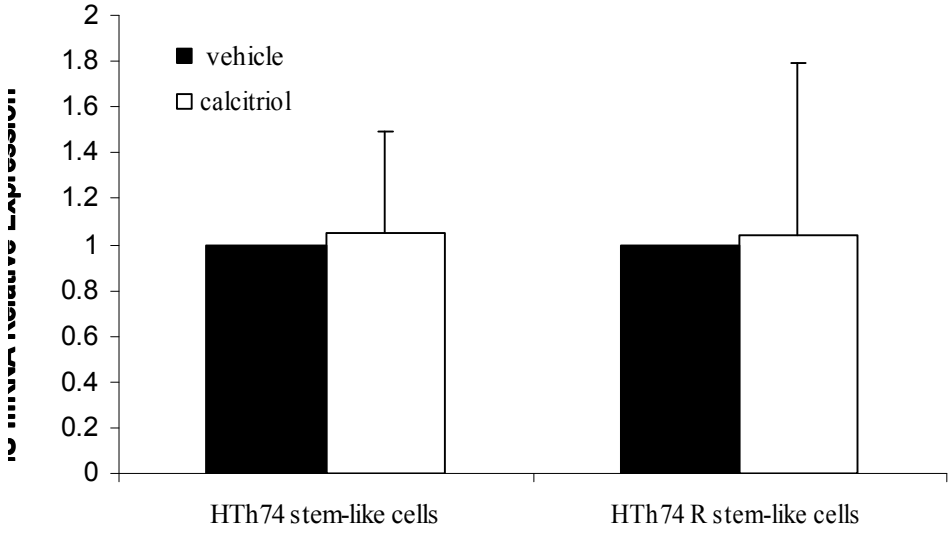

Figure 3.6.2 NIS mRNA relative expression after 72 hour incubation with vehicle or 100nM calcitriol in HTh74 and HTh74R stem-like cells. The Ct values of the real-time PCR were calculated by the $(2\times\text{efficiency})^{-\Delta\Delta Ct}$ method, and normalized by the value of the internal control 18s rRNA. Data are presented as mean of fold change ± SD *vs.* control and were derived from three independent experiments.

4. Discussion

A number of epidemiological studies have correlated vitamin D status with various cancer risks, including prostate cancer, breast cancer, colon cancer (3-5). Schwartz and Hanchett confirmed the inverse correlation between UV radiation, which is the major source of vitamin D, and prostate cancer mortality at the country level using three-dimensional mapping (79). In a prospective study, McCullough *et al.* reported an inverse association of dietary vitamin D with breast cancer risk in an analysis restricted to postmenopausal breast cancer cases (80). In a population-based case control study, the risk of getting colon cancer was decreased three-fold in people with a serum 25(OH)D concentration of 20 ng/ml or more . These epidemiological studies suggested a protective effect of vitamin D against cancers, despite possible inaccuracy of vitamin D value evaluation.

Data available *in vitro* and *in vivo* studies are consistent with the reports in epidemiological investigations. Vitamin D and its analogues inhibit the proliferation or even kill the cancer cells from a wide range of tissue origins. In some settings, vitamin D compound may even induce the differentiation of cancer cells (7-10). Getzenberg and colleagues demonstrated profound *in vitro* and *in vivo* antiproliferative and differentiating effects of calcitriol and its analogue on prostate carcinoma (15). Nakagawa *et al.* showed that 1,25(OH)2D3 inhibited the metastatic growth of lung cancer cells in a defined animal model (16). Colston and co-workers illustrated vitamin D and its analogues inhibited the proliferation of breast cancer cells in both *in vitro* and *in vivo* environments (18).

All these studies suggest that vitamin D compound may play a role in the pathogenesis, progression and therapy of various cancers (2). However, to our knowledge, data on the effects of vitamin D compound on cancer stem cells are still missing.

The present research replicated the significant growth arrest by calcitriol on HTh74 wild type cells, as previously shown by others (25, 26), and for the first time, found an almost 3 times greater antiproliferative effect on a doxorubicin resistant HTh74 cell line HTh74R, which is enriched with CSCs and cancer progenitor cells (47). These data imply a correlation between vitamin D compound and thyroid CSCs/cancer progenitor cells.

Many currently cytotoxic drugs are capable of reducing tumor size but usually only transiently. This phenomenon may due to the failure to eradicate CSCs/progenitor cells that are more resistant to chemotherapies (31, 47, 58). Failure in killing CSCs may result in cancer therapy resistance, thus lead to treatment failure. Therefore, targeting CSCs may be an essential part of a successful cancer therapy and become the key for overcoming drug resistance.

Our group has demonstrated that HTh74R cells possess a significantly higher Oct4, ABCG2 and MDR1 mRNA expression than HTh74 cells, as well as a higher fraction of side population (SP) cells (47). The expression of Oct4 is one of the characteristics of adult and embryonic stem cells (44, 45, 81). ABCG2 and MDR1 genes encode proteins which are included in the superfamily of ATP-binding cassette (ABC) transporters, which are able to transfer the Hoechst dye out of the CSCs, otherwise Hoechst dye may bind to the DNA in other cells (47, 82). In addition to the higher expression of stem cell marker and ABC transporters, the resistance cell line consists of a 70% side population fraction enriched with Oct4-positive cancer stem-like cells, which is significantly higher than that in wild type HTh74 cell line (47). The gene expression profile and higher fraction of side population cells in resistant cell line indicate that HTh74R cells possess characteristics of the CSCs/cancer progenitor cells compared with wild-type HTh74 cells. Therefore, the pronounced growth arrest of HTh74R cells may imply calcitriol's potential role in thyroid CSCs.

Sphere formation assay has been widely used to identify stem cells, which is based on the self-renew ability and the capacity to differentiate at the single cell level *in vitro*(83). Meanwhile, some common characteristics, such as self-renewal, slow-cycling and ability to differentiate, are shared by CSCs and adult stem cells , which both may give rise to progenitor cells (47, 58, 84). In the non-adhesive cell culture system, self-proliferation of stem cells gives rise to tumor spheres, which are enriched with CSCs/cancer progenitor cells or cancer stem-like cells. Therefore, sphere formation is a hallmark of cancer stem cells including thyroid cancer stem cells (47, 48, 52).

Expression of stem/progenitor cell surface markers (e.g. CD44, CD133, ALDH-1) and stem cell genes (e.g. Oct4, SOX2, NANOG) has been detected in spheres derived from oral squamous carcinoma cells, breast cancer cells and colorectal cancer cells (41, 71-73). Meanwhile, sphere formation in serum-free medium with growth factors could be a efficient method to isolate and

amplify CSCs/progenitor cells (71-74, 85, 86). In thyroid anaplastic cancer cells, our group confirmed the mRNA expression or higher expression of Oct4, ABCG2 and MDR1 in tumor spheres derived from HTh74 cell line, thereby identifying the stem-cell like features of HTh74 tumor spheres (47).

Stem cell marker expression in thyroid cancer spheres indicates that sphere formation assay is an approach to identifying and evaluating stem-like cells. On the other hand, sphere formation is an efficient method to amplify stem-like cells.

The current study revealed that after 120 hours 100nM calcitriol incubation, sphere formation efficiency (SFE) in HTh74 cell line was significantly decreased by more than 50% compared with the vehicle group. In HTh74R cell line, a similar result was obtained. These data demonstrate the decreased fraction of stem cells after calcitriol intervention. On the other hand, the size of cancer spheres formed in the calcitriol group was reduced, indicating a decreased number of daughter cells arising from each stem cell. This sphere formation assay suggests an anti-thyroid CSCs effect of calcitrol, which corresponds to recent reports on the inhibitive effect of calcitriol on stem cells/ progenitor cells from prostate origin or mesenchymal multipotent cells (87-89). These findings provide a broad insight into calcitriol, which not only decreases the proliferation of cancer cells, but also exerts an inhibitive effect on CSCs or cancer stem-like cells.

To explore the mechanism of the anti-proliferative effect of calcitriol on cancer stem-like cells, cell cycle and apoptotic analysis were performed with flow cytometry. In the cell cycle arrest analysis, an increased G2/M fraction was observed in both HTh74 and HTh74R stem-like cells at 72 hours. In contrast, G0/G1 phase arrest in wild type HTh74 cell line was found by others (26). This is consistent with similar findings from prostate origin (88, 90). In Maund and colleagues' study, a delayed G2/M progression was seen in prostate stem/ progenitor cells after 72 hours calcitriol incubation (88), whereas a persistent G1 accumulation was detected in prostate cancer cells (90). These data demonstrate that cell cycle arrest constitutes one of the mechanisms of the growth arrest induced by calcitriol in cancer stem-like cells. Calcitriol may act in a different way on cell cycle progression in thyroid cancer stem-like cells than in wild type thyroid cancer cells.

Apoptotic analysis showed no significant apoptosis-inducing effect of calcitriol on thyroid anaplastic cancer stem-like cells. Calcitriol inhibited cell proliferation by inducing cell cycle arrest without affecting apoptosis. This is consistent with the study on mesenchymal multipotent

cells, which harbor the characteristics of stem cells/progenitor cells (87). Moreover, apoptosis resistance is one of the biological features of cancer stem cells (55, 56). It was found that apoptosis was not affected by calcitriol in various thyroid cancer cell lines (24), which corresponds to the present results.

In addition, a differentiation-inducing effect of calcitriol is widely reported as well. In some cases, vitamin D may directly induce or potentiate the differentiation in cancer therapy. Such observation has been obtained in colon, breast, prostate, squamous cell carcinoma, osteosarcoma and myeloid leukemia cancer cells (11). In thyroid cancer cell lines, differentiation marker mRNA expression was shown to be increased after calcitriol intervention by different research groups (26, 91). Clinckspoor and colleagues observed a modest increase in NIS and thyroglobulin mRNA expression after calcitriol treatment in anaplastic thyroid cancer cell lines (26). Meanwhile, Akagi *et al.* demonstrated an enhanced NIS expression level after mono-treatment of calcitriol, and a further increased NIS expression after co-treatment with calcitriol and SAHA (91).

Vitamin D may induce the cell differentiation in stem cells and CSCs as well. In human mesenchymal stem cells, vitamin D accelerated differentiation-related gene expression and osteoblast differentiation (92). Vitamin D may direct or synergistically stimulate the osteogenic differentiation in mesenchymal stem cells or adipose stem cells (93-95). A Gemini vitamin D analog repressed the CD44 expression in breast cancer cells both *in vitro* and in xenograft tumors, suggesting a prodifferentiation role on breast cancer stem cells (96) .

In the present study, a morphologic change of anaplastic cancer stem-like cells was observed after calcitriol intervention. The cells treated with calcitriol presented a wild-type cancer cell-like shape and a close adhesion to the dishes. In contrast, the cells with vehicle incubation demonstrated the characteristics of undifferentiated cells without close adhesion. These morphologic changes indicate that calcitriol may play a role in the adhesion of thyroid cancer stem-like cells, which is a key factor in the differentiation of stem cells. It is believed that adhesion may confer cell tensional integrity (tensegrity) and repression of apoptotic signals to the cells, whereas detachment works in the opposite way (45, 97, 98). Regarding the thyroid differentiation marker NIS mRNA expression, it did not differ between calcitriol and vehicle groups. Since loss of NIS expression and iodide uptake ability are characteristics of anaplastic

cancers, which consequently lead to the failure of radioiodide treatment (99), this may explain the real-time PCR results about NIS expression.

The putative side effect of high dose vitamin D on the calcium metabolism is of most concern when vitamin D compounds are used as anticancer agents. The anticancer effect of vitamin D was dose-dependent in *in vitro* studies, and the most frequently used doses were 50-100nM (25, 100-102). In a recent study of thyroid cancer cell lines, the maximum calcitriol level was as high as 1000nM (26), which was even ten times higher than the maximum concentration used in the present study. Nevertheless, the minimum effective concentration varies depending on cancer types. In very sensitive cancer cell lines, even 1nM concentration calcitriol was capable of inhibiting cell growth (25). On the other hand, the tolerated vitamin D concentration may be higher than our expectation. Preclinical studies have indicated that calcitriol therapy with high doses is safe up to at least 100mcg weekly intravenously and perhaps higher doses are possible with the oral formulation, although the MTD and an optimal phase II dose have not been defined (2).

Regarding the therapeutic applications of vitamin D compounds in cancers, phase I studies have been carried out and the toxicity of vitamin D has been evaluated (2). The most discussed vitamin D compound is calcitriol, when calcitriol was used as either a single agent or in combination studies. Calcitriol was clearly demonstrated a potentiating ability to the antitumor effects of a wide range of traditional chemotherapys in some preclinical studies, including taxanes, paclitaxel, platinum analogues, etc (2, 12, 13). All the preclinical data support potential application of vitamin D compound in cancer therapy.

For the clinical use of calcitriol as anticancer or adjacent anticancer compound, it is essential to further clarify the mechanism of the anticancer effects of vitamin D in various cancers. The MTD and optimal doses have to be defined as well when vitamin D compound is used as a single agent and in combination with cytotoxic agents. Furthermore, vitamin D analogues without or with less toxicity have to be developed.

In summary, this study presented *in vitro* antiproliferative effects of calcitriol in anaplastic thyroid cancer stem-like cells. A G2/M cell cycle arrest without enhanced cell apoptosis was involved in the growth arrest. Pro-differentiation effects of calcitriol on thyroid cancer stem-like cells were implied by cell morphological changes. It might be essential to further uncover the

underlying mechanism of the growth arrest, and to clarify the putative differentiation-inducing effect on thyroid CSCs.

5. Summary

Failure of traditional cancer therapy to eradicate anaplastic thyroid cancers is mainly due to resistance of cancer stem cells (CSCs), which are responsible for cancer initiation, progression, metastasis and drug resistance (47). Anticancer effects of vitamin D compounds have been indicated by considerable data, including on thyroid anaplastic cancer cells (26, 91). However, there is still a lack of understanding of the effects of vitamin D on thyroid CSCs or cancer stem-like cells.

To uncover the effects of vitamin D on thyroid anaplastic cancer stem-like cells, a sphere formation assay was performed in HTh74 cells and a derived doxorubicin resistant cell line HTh74R. Cell cycle arrest and apoptotic analyses were employed in thyroid cancer stem-like cells derived from both cell lines with flow cytometry. Furthermore, the putative pro-differentiation effects of vitamin D were explored by morphological characteristics and an investigation of mRNA expression profile.

The present study demonstrated the antiproliferative effects of calcitriol on anaplastic thyroid cancer cell line HTh74 and its derived cancer stem-like cells. Sphere formation from HTh74 cells was significantly suppressed by calcitriol intervention. G2/M cell cycle arrest without enhanced apoptosis was involved in the mechanism of growth arrest of HTh74 stem-like cells. Calcitriol showed a similar inhibitive effect in the doxorubicin resistant HTh74 cell line HTh74R and its derived cancer stem-like cells. In addition, cell morphological changes implied a pro-differentiation role of calcitriol on thyroid anaplastic stem-like cells, despite the absence of the gene expression profile changes.

In conclusion, the *in vitro* antiproliferative effect of calcitriol in anaplastic thyroid cancer stem-like cells was illustrated. Further studies are necessary to explore the underlying mechanism for the effect of growth arrest and to uncover the differentiation-inducing effect of calcitriol on thyroid CSCs.

Zusammenfassung in deutscher Sprache

Ein wesentlicher Grund für das Versagen der traditionellen Tumortherapie in der Behandlung anaplastischer Schilddrüsenkarzinome ist die Resistenz gegenüber so genannten cancer stem cells (CSC). Diese sind verantwortlich für die Tumorinitiierung, -progression, die Metastasierung und die Resistenz gegenüber Chemotherapeutika (47). Die Wirksamkeit von Vitamin D-Präparaten in der Tumortherapie wurde durch zahlreiche Untersuchungen, einschließlich Untersuchungen an Schilddrüsenkarzinomzellen, belegt (26, 91). Unbekannt ist jedoch die Wirkung von Vitamin D auf Schilddrüsen-CSCs oder -carcinomstammzell-ähnliche Zellen.

Um die Wirksamkeit von Vitamin D auf Stammzell-ähnliche Zellen von anaplastischen Schilddrüsenkarzinomen zu untersuchen, wurde ein so genannter Sphere-Formation-Assay an HTh74-Zellen und der Doxorubicin-resistenten Zelllinie HTh74R durchgeführt. Zellzyklusanalysen und Apoptoseuntersuchungen wurden in diesen Stammzell-ähnlichen aus beiden Zelllinien isolierten Zellen mit Flow-Zytometrie-Methoden durchgeführt. Ferner wurde ein möglicher Differenzierungseffekt von Vitamin D durch Nachweis morphologischer Veränderungen und durch Untersuchungen von mRNA-Expressionsprofilen untersucht.

Die vorliegende Studie zeigte einen antiproliferativen Effekt des Vitamin-D-Präparates Calcitriol auf die anaplastische Schilddrüsenkarzinomzelllinie HTh74 und die von dieser Zelllinie abgeleiteten Carcinomstammzell-ähnlichen Zellen. Die Bildung von Spheren aus HTh74-Zellen wurde signifikant unterdrückt durch die Gabe von Calcitriol. Es fand sich ein G2/M-Zellzyklusarrest; eine gesteigerte Apoptose war in der Wachstumshemmung der HTh74-Stammzell-ähnlichen Zellen jedoch nicht nachweisbar. Calcitriol zeigte ähnliche wachstumshemmende Effekte in der Doxorubicin-resistenten HTh74-Zelllinie HTh74R. Veränderung der Zellmorphologie deuteten auf einen positiven Effekt von Calcitriol auf die Differenzierung der von anaplastischen Schilddrüsenkarzinomen abgeleiteten Carcinomstammzell-ähnlichen Zellen hin, obwohl Veränderungen der Gen-Expressionsprofile nicht nachweisbar waren.

Zusammenfassend wurde in vitro ein antiproliferativer Effekt von Calcitriol auf die Stammzell-ähnlichen Zellen anaplastischer Schilddrüsenkarzinome nachgewiesen.

Weitere Studien sind notwendig, um die zugrunde liegenden Mechanismen der Wachstumshemmung und den Differenzierungs-induzierenden Effekt von Calcitriol auf Schilddrüsenkarzinom-Stammzellen aufzuklären.

6. Reference

1. Deeb KK, Trump DL, Johnson CS. Vitamin D signalling pathways in cancer: potential for anticancer therapeutics. Nat Rev Cancer 2007;7(9):684-700.

2. Trump DL, Deeb KK, Johnson CS. Vitamin D: considerations in the continued development as an agent for cancer prevention and therapy. Cancer J 2010;16(1):1-9.

3. Singh SK, Hawkins C, Clarke ID, Squire JA, Bayani J, Hide T, et al. Identification of human brain tumour initiating cells. Nature 2004;432(7015):396-401.

4. Schwartz GG, Skinner HG. Vitamin D status and cancer: new insights. Curr Opin Clin Nutr Metab Care 2007;10(1):6-11.

5. Garland CF, Comstock GW, Garland FC, Helsing KJ, Shaw EK, Gorham ED. Serum 25-hydroxyvitamin D and colon cancer: eight-year prospective study. Lancet 1989;2(8673):1176-8.

6. Grant WB, Mohr SB. Ecological studies of ultraviolet B, vitamin D and cancer since 2000. Ann Epidemiol 2009;19(7):446-54.

7. Rubin D, Levij IS. Suppression by vitamins D2 and D3 of hamster cheek pouch carcinoma induced with 9,10-dimethyl-1,2-benzanthracene with a discussion of the role of intracellular calcium in the development of tumors. Pathol Microbiol (Basel) 1973;39(6):446-60.

8. Eisman JA, Martin TJ, MacIntyre I, Frampton RJ, Moseley JM, Whitehead R. 1,25-Dihydroxyvitamin D3 receptor in a cultured human breast cancer cell line (MCF 7 cells). Biochem Biophys Res Commun 1980;93(1):9-15.

9. Eisman JA, Macintyre I, Martin TJ, Frampton RJ, King RJ. Normal and malignant breast tissue is a target organ for 1,25-(0H)2 vitamin D3. Clin Endocrinol (Oxf) 1980;13(3):267-72.

10. Abe E, Miyaura C, Sakagami H, Takeda M, Konno K, Yamazaki T, et al. Differentiation of mouse myeloid leukemia cells induced by 1 alpha,25-dihydroxyvitamin D3. Proc Natl Acad Sci U S A 1981;78(8):4990-4.

11. Gocek E, Studzinski GP. Vitamin D and differentiation in cancer. Crit Rev Clin Lab Sci 2009;46(4):190-209.

12. Osborn JL, Schwartz GG, Smith DC, Bahnson R, Day R, Trump DL. Phase II trial of oral 1,25-dihydroxyvitamin D (calcitriol) in hormone refractory prostate cancer. Urol Oncol 1995;1(5):195-8.

13. Gallagher JC, Goldgar D. Treatment of postmenopausal osteoporosis with high doses of synthetic calcitriol. A randomized controlled study. Ann Intern Med 1990;113(9):649-55.

14. McGuire TF, Trump DL, Johnson CS. Vitamin D(3)-induced apoptosis of murine squamous cell carcinoma cells. Selective induction of caspase-dependent MEK cleavage and up-regulation of MEKK-1. J Biol Chem 2001;276(28):26365-73.

15. Getzenberg RH, Light BW, Lapco PE, Konety BR, Nangia AK, Acierno JS, et al. Vitamin D inhibition of prostate adenocarcinoma growth and metastasis in the Dunning rat prostate model system. Urology 1997;50(6):999-1006.

16. Nakagawa K, Kawaura A, Kato S, Takeda E, Okano T. 1 alpha,25-Dihydroxyvitamin D(3) is a preventive factor in the metastasis of lung cancer. Carcinogenesis 2005;26(2):429-40.

17. Zhang X, Jiang F, Li P, Li C, Ma Q, Nicosia SV, et al. Growth suppression of ovarian cancer xenografts in nude mice by vitamin D analogue EB1089. Clin Cancer Res 2005;11(1):323-8.

18. Colston KW, Chander SK, Mackay AG, Coombes RC. Effects of synthetic vitamin D analogues on breast cancer cell proliferation in vivo and in vitro. Biochem Pharmacol 1992;44(4):693-702.

19. Colston KW, James SY, Ofori-Kuragu EA, Binderup L, Grant AG. Vitamin D receptors and anti-proliferative effects of vitamin D derivatives in human pancreatic carcinoma cells in vivo and in vitro. Br J Cancer 1997;76(8):1017-20.

20. Welsh J, Wietzke JA, Zinser GM, Byrne B, Smith K, Narvaez CJ. Vitamin D-3 receptor as a target for breast cancer prevention. J Nutr 2003;133(7 Suppl):2425S-2433S.

21. Ma Y, Yu WD, Trump DL, Johnson CS. 1,25D3 enhances antitumor activity of gemcitabine and cisplatin in human bladder cancer models. Cancer 2010;116(13):3294-303.

22. Okano K, Usa T, Ohtsuru A, Tsukazaki T, Miyazaki Y, Yonekura A, et al. Effect of 22-oxa-1,25-dihydroxyvitamin D3 on human thyroid cancer cell growth. Endocr J 1999;46(2):243-52.

23. Suzuki S, Takenoshita S, Furukawa H, Tsuchiya A. Antineoplastic activity of 1,25(OH)2D3 and its analogue 22-oxacalcitriol against human anaplastic thyroid carcinoma cell lines in vitro. Int J Mol Med 1999;4(6):611-4.

24. Liu W, Asa SL, Fantus IG, Walfish PG, Ezzat S. Vitamin D arrests thyroid carcinoma cell growth and induces p27 dephosphorylation and accumulation through PTEN/akt-dependent and -independent pathways. Am J Pathol 2002;160(2):511-9.

25. Sharma V, Fretwell D, Crees Z, Kerege A, Klopper JP. Thyroid cancer resistance to vitamin D receptor activation is associated with 24-hydroxylase levels but not the ff FokI polymorphism. Thyroid 2010;20(10):1103-11.

26. Clinckspoor I, Verlinden L, Overbergh L, Korch C, Bouillon R, Mathieu C, et al. 1,25-dihydroxyvitamin D3 and a superagonistic analog in combination with paclitaxel or suberoylanilide hydroxamic acid have potent antiproliferative effects on anaplastic thyroid cancer. J Steroid Biochem Mol Biol 2011;124(1-2):1-9.

27. Bennett RG, Wakeley SE, Hamel FG, High RR, Korch C, Goldner WS. Gene expression of vitamin D metabolic enzymes at baseline and in response to vitamin D treatment in thyroid cancer cell lines. Oncology 2012;83(5):264-72.

28. Stepien T, Krupinski R, Sopinski J, Kuzdak K, Komorowski J, Lawnicka H, et al. Decreased 1-25 dihydroxyvitamin D3 concentration in peripheral blood serum of patients with thyroid cancer. Arch Med Res 2010;41(3):190-4.

29. Kostner K, Denzer N, Muller CS, Klein R, Tilgen W, Reichrath J. The relevance of vitamin D receptor (VDR) gene polymorphisms for cancer: a review of the literature. Anticancer Res 2009;29(9):3511-36.

30. Penna-Martinez M, Ramos-Lopez E, Stern J, Hinsch N, Hansmann ML, Selkinski I, et al. Vitamin D receptor polymorphisms in differentiated thyroid carcinoma. Thyroid 2009;19(6):623-8.

31. Reya T, Morrison SJ, Clarke MF, Weissman IL. Stem cells, cancer, and cancer stem cells. Nature 2001;414(6859):105-11.

32. Adams JM, Strasser A. Is tumor growth sustained by rare cancer stem cells or dominant clones? Cancer Res 2008;68(11):4018-21.

33. Adams JM, Kelly PN, Dakic A, Carotta S, Nutt SL, Strasser A. Role of "cancer stem cells" and cell survival in tumor development and maintenance. Cold Spring Harb Symp Quant Biol 2008;73:451-9.

34. Lapidot T, Sirard C, Vormoor J, Murdoch B, Hoang T, Caceres-Cortes J, et al. A cell initiating human acute myeloid leukaemia after transplantation into SCID mice. Nature 1994;367(6464):645-8.

35. Bonnet D, Dick JE. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. Nat Med 1997;3(7):730-7.

36. Szotek PP, Pieretti-Vanmarcke R, Masiakos PT, Dinulescu DM, Connolly D, Foster R, et al. Ovarian cancer side population defines cells with stem cell-like characteristics and Mullerian Inhibiting Substance responsiveness. Proc Natl Acad Sci U S A 2006;103(30):11154-9.

37. Li C, Heidt DG, Dalerba P, Burant CF, Zhang L, Adsay V, et al. Identification of pancreatic cancer stem cells. Cancer Res 2007;67(3):1030-7.

38. Hermann PC, Huber SL, Herrler T, Aicher A, Ellwart JW, Guba M, et al. Distinct populations of cancer stem cells determine tumor growth and metastatic activity in human pancreatic cancer. Cell Stem Cell 2007;1(3):313-23.

39. Collins AT, Berry PA, Hyde C, Stower MJ, Maitland NJ. Prospective identification of tumorigenic prostate cancer stem cells. Cancer Res 2005;65(23):10946-51.

40. Patrawala L, Calhoun T, Schneider-Broussard R, Li H, Bhatia B, Tang S, et al. Highly purified CD44+ prostate cancer cells from xenograft human tumors are enriched in tumorigenic and metastatic progenitor cells. Oncogene 2006;25(12):1696-708.

41. O'Brien CA, Pollett A, Gallinger S, Dick JE. A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. Nature 2007;445(7123):106-10.

42. Li HZ, Yi TB, Wu ZY. Suspension culture combined with anticancer regimens for screening breast cancer stem cells. Med Hypotheses 2007;68(5):988-90.

43. Thomas D, Friedman S, Lin RY. Thyroid stem cells: lessons from normal development and thyroid cancer. Endocr Relat Cancer 2008;15(1):51-8.

44. Thomas T, Nowka K, Lan L, Derwahl M. Expression of endoderm stem cell markers: evidence for the presence of adult stem cells in human thyroid glands. Thyroid 2006;16(6):537-44.

45. Lan L, Cui D, Nowka K, Derwahl M. Stem cells derived from goiters in adults form spheres in response to intense growth stimulation and require thyrotropin for differentiation into thyrocytes. J Clin Endocrinol Metab 2007;92(9):3681-8.

46. Fierabracci A, Puglisi MA, Giuliani L, Mattarocci S, Gallinella-Muzi M. Identification of an adult stem/progenitor cell-like population in the human thyroid. J Endocrinol 2008;198(3):471-87.

47. Zheng X, Cui D, Xu S, Brabant G, Derwahl M. Doxorubicin fails to eradicate cancer stem cells derived from anaplastic thyroid carcinoma cells: characterization of resistant cells. Int J Oncol 2010;37(2):307-15.

48. Mitsutake N, Iwao A, Nagai K, Namba H, Ohtsuru A, Saenko V, et al. Characterization of side population in thyroid cancer cell lines: cancer stem-like cells are enriched partly but not exclusively. Endocrinology 2007;148(4):1797-803.

49. Friedman S, Lu M, Schultz A, Thomas D, Lin RY. CD133+ anaplastic thyroid cancer cells initiate tumors in immunodeficient mice and are regulated by thyrotropin. PLoS One 2009;4(4):e5395.

50. Zito G, Richiusa P, Bommarito A, Carissimi E, Russo L, Coppola A, et al. In vitro identification and characterization of CD133(pos) cancer stem-like cells in anaplastic thyroid carcinoma cell lines. PLoS One 2008;3(10):e3544.

51. Klonisch T, Hoang-Vu C, Hombach-Klonisch S. Thyroid stem cells and cancer. Thyroid 2009;19(12):1303-15.

52. Chen G, Xu S, Renko K, Derwahl M. Metformin inhibits growth of thyroid carcinoma cells, suppresses self-renewal of derived cancer stem cells, and potentiates the effect of chemotherapeutic agents. J Clin Endocrinol Metab 2012;97(4):E510-20.

53. Ke CC, Liu RS, Yang AH, Liu CS, Chi CW, Tseng LM, et al. CD133-expressing thyroid cancer cells are undifferentiated, radioresistant and survive radioiodide therapy. Eur J Nucl Med Mol Imaging 2013;40(1):61-71.

54. Hoshi N, Kusakabe T, Taylor BJ, Kimura S. Side population cells in the mouse thyroid exhibit stem/progenitor cell-like characteristics. Endocrinology 2007;148(9):4251-8.

55. Dean M, Fojo T, Bates S. Tumour stem cells and drug resistance. Nat Rev Cancer 2005;5(4):275-84.

56. Jordan CT, Guzman ML. Mechanisms controlling pathogenesis and survival of leukemic stem cells. Oncogene 2004;23(43):7178-87.

57. Guzman ML, Jordan CT. Considerations for targeting malignant stem cells in leukemia. Cancer Control 2004;11(2):97-104.

58. Derwahl M. Linking stem cells to thyroid cancer. J Clin Endocrinol Metab 2011;96(3):610-3.

59. de Beca FF, Caetano P, Gerhard R, Alvarenga CA, Gomes M, Paredes J, et al. Cancer stem cells markers CD44, CD24 and ALDH1 in breast cancer special histological types. J Clin Pathol 2012.

60. Schwartz T, Stark A, Pang J, Awuah B, Kleer CG, Quayson S, et al. Expression of aldehyde dehydrogenase 1 as a marker of mammary stem cells in benign and malignant breast lesions of Ghanaian women. Cancer 2012.

61. Lohberger B, Rinner B, Stuendl N, Absenger M, Liegl-Atzwanger B, Walzer SM, et al. Aldehyde dehydrogenase 1, a potential marker for cancer stem cells in human sarcoma. PLoS One 2012;7(8):e43664.

62. Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF. Prospective identification of tumorigenic breast cancer cells. Proc Natl Acad Sci U S A 2003;100(7):3983-8.

63. Leong KG, Wang BE, Johnson L, Gao WQ. Generation of a prostate from a single adult stem cell. Nature 2008;456(7223):804-8.

64. Jung JW, Park SB, Lee SJ, Seo MS, Trosko JE, Kang KS. Metformin represses self-renewal of the human breast carcinoma stem cells via inhibition of estrogen receptor-mediated OCT4 expression. PLoS One 2011;6(11):e28068.

65. Goodell MA, Brose K, Paradis G, Conner AS, Mulligan RC. Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. J Exp Med 1996;183(4):1797-806.

66. Yanamoto S, Kawasaki G, Yamada S, Yoshitomi I, Kawano T, Yonezawa H, et al. Isolation and characterization of cancer stem-like side population cells in human oral cancer cells. Oral Oncol 2011;47(9):855-60.

67. Song J, Chang I, Chen Z, Kang M, Wang CY. Characterization of side populations in HNSCC: highly invasive, chemoresistant and abnormal Wnt signaling. PLoS One 2010;5(7):e11456.

68. Lopez J, Poitevin A, Mendoza-Martinez V, Perez-Plasencia C, Garcia-Carranca A. Cancer-initiating cells derived from established cervical cell lines exhibit stem-cell markers and increased radioresistance. BMC Cancer 2012;12:48.

69. Nakatsugawa M, Takahashi A, Hirohashi Y, Torigoe T, Inoda S, Murase M, et al. SOX2 is overexpressed in stem-like cells of human lung adenocarcinoma and augments the tumorigenicity. Lab Invest 2011;91(12):1796-804.

70. Hu WY, Shi GB, Hu DP, Nelles JL, Prins GS. Actions of estrogens and endocrine disrupting chemicals on human prostate stem/progenitor cells and prostate cancer risk. Mol Cell Endocrinol 2012;354(1-2):63-73.

71. Chiou SH, Yu CC, Huang CY, Lin SC, Liu CJ, Tsai TH, et al. Positive correlations of Oct-4 and Nanog in oral cancer stem-like cells and high-grade oral squamous cell carcinoma. Clin Cancer Res 2008;14(13):4085-95.

72. Lee J, Kotliarova S, Kotliarov Y, Li A, Su Q, Donin NM, et al. Tumor stem cells derived from glioblastomas cultured in bFGF and EGF more closely mirror the phenotype and genotype of primary tumors than do serum-cultured cell lines. Cancer Cell 2006;9(5):391-403.

73. Zhong Y, Guan K, Guo S, Zhou C, Wang D, Ma W, et al. Spheres derived from the human SK-RC-42 renal cell carcinoma cell line are enriched in cancer stem cells. Cancer Lett 2010;299(2):150-60.

74. Chen SF, Chang YC, Nieh S, Liu CL, Yang CY, Lin YS. Nonadhesive culture system as a model of rapid sphere formation with cancer stem cell properties. PLoS One 2012;7(2):e31864.

75. Rybak AP, He L, Kapoor A, Cutz JC, Tang D. Characterization of sphere-propagating cells with stem-like properties from DU145 prostate cancer cells. Biochim Biophys Acta 2011;1813(5):683-94.

76. Morrison BJ, Steel JC, Morris JC. Sphere culture of murine lung cancer cell lines are enriched with cancer initiating cells. PLoS One 2012;7(11):e49752.

77. Zheng X. Doxorubicin fails to eradicate cancer stem cells derived from anaplastic thyroid carcinoma cells. Berlin: Universitätsmedizin Berlin; 2010.

78. Chen G. Effect of metformin on growth of differentiated thyroid cells and thyroid carcinoma cells and their derived cancer stem cells. Berlin: Charité - Universitätsmedizin Berlin; 2012.

79. Schwartz GG, Hanchette CL. UV, latitude, and spatial trends in prostate cancer mortality: all sunlight is not the same (United States). Cancer Causes Control 2006;17(8):1091-101.

80. McCullough ML, Rodriguez C, Diver WR, Feigelson HS, Stevens VL, Thun MJ, et al. Dairy, calcium, and vitamin D intake and postmenopausal breast cancer risk in the Cancer Prevention Study II Nutrition Cohort. Cancer Epidemiol Biomarkers Prev 2005;14(12):2898-904.

81. Tai MH, Chang CC, Kiupel M, Webster JD, Olson LK, Trosko JE. Oct4 expression in adult human stem cells: evidence in support of the stem cell theory of carcinogenesis. Carcinogenesis 2005;26(2):495-502.

82. Feuring-Buske M, Hogge DE. Hoechst 33342 efflux identifies a subpopulation of cytogenetically normal CD34(+)CD38(-) progenitor cells from patients with acute myeloid leukemia. Blood 2001;97(12):3882-9.

83. Pastrana E, Silva-Vargas V, Doetsch F. Eyes wide open: a critical review of sphere-formation as an assay for stem cells. Cell Stem Cell 2011;8(5):486-98.

84. Li L, Neaves WB. Normal stem cells and cancer stem cells: the niche matters. Cancer Res 2006;66(9):4553-7.

85. Tsai LL, Yu CC, Chang YC, Yu CH, Chou MY. Markedly increased Oct4 and Nanog expression correlates with cisplatin resistance in oral squamous cell carcinoma. J Oral Pathol Med 2011;40(8):621-8.

86. Hueng DY, Sytwu HK, Huang SM, Chang C, Ma HI. Isolation and characterization of tumor stem-like cells from human meningiomas. J Neurooncol 2011;104(1):45-53.

87. Artaza JN, Sirad F, Ferrini MG, Norris KC. 1,25(OH)2vitamin D3 inhibits cell proliferation by promoting cell cycle arrest without inducing apoptosis and modifies cell morphology of mesenchymal multipotent cells. J Steroid Biochem Mol Biol 2010;119(1-2):73-83.

88. Maund SL, Barclay WW, Hover LD, Axanova LS, Sui G, Hipp JD, et al. Interleukin-1alpha mediates the antiproliferative effects of 1,25-dihydroxyvitamin D3 in prostate progenitor/stem cells. Cancer Res 2011;71(15):5276-86.

89. Li J, Fleet JC, Teegarden D. Activation of rapid signaling pathways does not contribute to 1 alpha,25-dihydroxyvitamin D3-induced growth inhibition of mouse prostate epithelial progenitor cells. J Cell Biochem 2009;107(5):1031-6.

90. Zhuang SH, Burnstein KL. Antiproliferative effect of 1alpha,25-dihydroxyvitamin D3 in human prostate cancer cell line LNCaP involves reduction of cyclin-dependent kinase 2 activity and persistent G1 accumulation. Endocrinology 1998;139(3):1197-207.

91. Akagi T, Luong QT, Gui D, Said J, Selektar J, Yung A, et al. Induction of sodium iodide symporter gene and molecular characterisation of HNF3 beta/FoxA2, TTF-1 and C/EBP beta in thyroid carcinoma cells. Br J Cancer 2008;99(5):781-8.

92. Piek E, Sleumer LS, van Someren EP, Heuver L, de Haan JR, de Grijs I, et al. Osteo-transcriptomics of human mesenchymal stem cells: accelerated gene expression and osteoblast differentiation induced by vitamin D reveals c-MYC as an enhancer of BMP2-induced osteogenesis. Bone 2010;46(3):613-27.

93. Song I, Kim BS, Kim CS, Im GI. Effects of BMP-2 and vitamin D3 on the osteogenic differentiation of adipose stem cells. Biochem Biophys Res Commun 2011;408(1):126-31.

94. Gigante A, Torcianti M, Boldrini E, Manzotti S, Falcone G, Greco F, et al. Vitamin K and D association stimulates in vitro osteoblast differentiation of fracture site derived human mesenchymal stem cells. J Biol Regul Homeost Agents 2008;22(1):35-44.

95. Feng Y, Sun Y, Jia W, Zhang C. Platelet-rich plasma and 1,25(OH)2 vitamin D3 synergistically stimulate osteogenic differentiation of adult human mesenchymal stem cells. Biotechnol Lett 2010;32(5):635-42.

96. So JY, Lee HJ, Smolarek AK, Paul S, Wang CX, Maehr H, et al. A novel Gemini vitamin D analog represses the expression of a stem cell marker CD44 in breast cancer. Mol Pharmacol 2011;79(3):360-7.

97. Edwards GM, Wilford FH, Liu X, Hennighausen L, Djiane J, Streuli CH. Regulation of mammary differentiation by extracellular matrix involves protein-tyrosine phosphatases. J Biol Chem 1998;273(16):9495-500.

98. Di Matola T, Mueller F, Fenzi G, Rossi G, Bifulco M, Marzano LA, et al. Serum withdrawal-induced apoptosis in thyroid cells is caused by loss of fibronectin-integrin interaction. J Clin Endocrinol Metab 2000;85(3):1188-93.

99. Furuya F, Shimura H, Suzuki H, Taki K, Ohta K, Haraguchi K, et al. Histone deacetylase inhibitors restore radioiodide uptake and retention in poorly differentiated and anaplastic thyroid cancer cells by expression of the sodium/iodide symporter thyroperoxidase and thyroglobulin. Endocrinology 2004;145(6):2865-75.

100. Flores O, Burnstein KL. GADD45gamma: a new vitamin D-regulated gene that is antiproliferative in prostate cancer cells. Endocrinology 2010;151(10):4654-64.

101. Rohan JN, Weigel NL. 1Alpha,25-dihydroxyvitamin D3 reduces c-Myc expression, inhibiting proliferation and causing G1 accumulation in C4-2 prostate cancer cells. Endocrinology 2009;150(5):2046-54.

102. Kovalenko PL, Zhang Z, Cui M, Clinton SK, Fleet JC. 1,25 dihydroxyvitamin D-mediated orchestration of anticancer, transcript-level effects in the immortalized, non-transformed prostate epithelial cell line, RWPE1. BMC Genomics 2010;11:26.

Acknowledgements

This work was planned, performed and completed in the laboratory of Experimental Endocrinology of St. Hedwig Hospital in Berlin. I would like to express my sincere gratitude to the following people for their invaluable help:

to Prof. Dr. Karl-Michael Derwahl, “Doktorvater” and supervisor of this research project. It is due to his drive, his ingenious ideas and his constant motivation that I succeeded with this work. Without his encouragement and support I would never have managed to overcome all the difficulties of being a research fellow working and living in Germany and for this I am truly indebted to him.

to Diana, Tini, for their excellent technical support, for teaching me all the lab methods and protocols, for always being there for me, and most importantly, for the great time we had together.

to Guofang, for her great support, in research as well as in life, that I can adapt to the complete new environment in Germany.

to Shuhang and Xuqin, for their previous excellent work and support.

to Prof. Köhrle and Kostja in the Institue of Experimental Endocrinology, Charite, for their expert advice and discussions, and for the technical support.

to all the members of GK1208, for the excellent organization of each seminar and the good time we had together.

to my parents, my family, for always being with me and supporting me. They are my greatest motivation that I can keep on going ahead.

Erklärung

„Ich, Wen Peng, erkläre, dass ich die vorgelegte Dissertation mit dem Thema: ‚1, 25 dihydroxyvitamin D3 inhibits the proliferation of thyroid anaplastic cancer stem-like cells via cell cycle arrest without affecting apoptosis‘ selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt, ohne die (unzulässige) Hilfe Dritter verfasst und auch in Teilen keine Kopien anderer Arbeiten dargestellt habe.“

Datum Unterschrift

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.