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DISSERTATION

17ß-estradiol stimulates growth and inhibits function of thyroid stem/progenitor cells: a clue to understand the higher prevalence of thyroid nodules in females

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To my parents

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Abbreviations

ABCG2	ATP-binding cassette transporter G2
ABCP	ATP-Binding Cassette Gene
BCRP	breast cancer resistance protein
bFGF	basic fibroblast growth factor
CSCs	cancer stem cells
cDNA	complementary deoxyribonucleic acid
℃	degree Celsius
DMEM	Dulbecco's modified Eagle medium
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotide triphosphates
DPN	2,3-Bis(4-hydroxyphenyl)propionitrile, ERβ-selective agonist
E_2	17β-estradiol
EB	embryoid body
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
ERK	extracellular signal-regulated kinase
ERRs	estrogen related receptors
ERs	estrogen receptors
ESCs	embryonic stem cells
FACS	fluorescence-activated cell sorting
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
FNAB	fine-needle aspiration biopsy
FTC	follicular thyroid cancer
×g	G-Force (unit of measurement of rotation speed of a centrifuge)
HBSS	Hank's balanced salt solution

HNF4	heptocyte nuclear factor 4
ICM	inner cell mass
MEM	non-essential amino acids
$MgCl_2$	magnesium chloride
M-MLV-RT	murine Moloney leukemia virus reverse transcriptase
ml	milliliter
mRNA	messenger ribonucleic acid
Ng	nanogram
nM	nanomole per liter solution (unit of concentration)
NIS	sodium iodide symporter
Oct4	octamer transcription factor-4
PAX8	paired box gene 8
PBS	phosphate buffered saline
PCR	polymerase chain reaction
*p*ERK	phospho-extracellular signal-regulated kinase
PH	potentia hydrogenii (negative decimal logarithm of hydrogen-ion concentration)
PI	propidium iodide
poly-HEMA	poly(2-hydroxyethyl methacrylate), poly(2-HEMA)
PPT	1,3,5-Tris(4-hydroxyphenyl)-4-propyl-1H-pyrazole, ERα-selective agonist
rpm	revolutions per minute
RT	reverse transcription
SP	side population
TBE	Tris-borate EDTA
TBS	Tris buffered saline
Tg	thyroglobulin
TPO	thyroid peroxidase

TSH	thyroid stimulating hormone
TSHr	thyroid stimulating hormone receptor
UV	ultraviolet
µg	microgram
Ml	microliter
µM	micromole per liter solution (unit of concentration)

1. Introduction

The incidence of thyroid nodules which is a hallmark of human thyroid glands has been rising worldwide (1). About 4 to 7 percent of the population may have palpable thyroid nodules, however, 19 to 67 percent have incidental nodules on ultrasonography (2). In fact, 20 to more than 50 percent of the normal population develop one or more thyroid nodules during their life-time (3). There is also some evidence that thyroid nodules are present in up to 30 percent of the German population (4). The majority of patients with thyroid nodules are euthyroid and asymptomatic; only less than 1 percent of nodules causes hyperthyroidism or thyrotoxicosis. Most palpable and non-palpable thyroid nodules are benign, however, 5 percent may show features of thyroid cancer (5).

Fine-needle aspiration biopsy (FNAB) which is obtained in all suspicious cases, is very useful to classify thyroid nodules as benign (69%), suspicious (10%), malignant (4%) and nondiagnostic nodules (17%) (6). Although many efforts have been made so far, there are still no appropriate clinical approaches to clearly distinguish benign and malignant nodules in these patients. Our limited knowledge of the molecular background and etiology of thyroid nodules might be an important reason.

Although the etiology of thyroid nodules is not yet fully known, there are many pathogenetic factors that are relevant for the pathogenesis of thyroid nodules and tumors, such as abnormal iodide intake, ionizing radiation exposure, mutagenesis, aging, gender, over-expression of growth factors and their related receptors (7, 8). Thyroid nodules are 4 times more prevalent in women than in men (9). Some studies found that the incidence of thyroid nodules increased by 2 percent yearly if the population is exposed to ionizing radiation (3). It seems that the development of thyroid nodules is a multi-factorial process, and a comprehensive concept of the pathogenesis of thyroid nodules and nodular goiters is still missing.

Classical theory believed that differentiated follicular thyroid cells might be altered by a

sequence of molecular aberrations achieved during cell cycling as the primary source of thyroid tumourigenesis (10). However, in normal thyroid tissue the proliferation rate is very low (11). Previous studies demonstrated that human thyrocytes divide only about five times during adulthood which corresponds to a turnover time of about 8.5 years for the follicular thyroid cell (12). Therefore, the molecular mechanism of nodule and tumor initiation in resting thyroid tissue, which is different from tissues with high cell turnover and higher sensitivity to mutagenesis such as the colon, has still to be elucidated.

According to classical theory of pathogenic mechanisms in thyroid cancer, thyroid cancer cells originate from the sequential accumulation of genetic alterations during the life cycle of well-differentiated premalignant thyrocytes. The mutation of tumor oncogenes and suppressor genes plays an important role in thyroid carcinogenesis. Some oncogenes such as RET proto-oncogenes and BRAF may have the potential to activate genetic alternations in the mediators of signal transduction pathway and thus sustain tumor development and/or progression (13). The multistep model of thyroid cancer suggests a step-wise dedifferentiation process from the normal differentiated thyroid follicular cell to a papillary or a follicular and finally a anaplastic thyroid carcinoma. Although these mutations activate cell cycle checkpoints that curtail hyperproliferation, there are instances in which cells escape these checkpoints and develop into cancer (14). The rather low proliferation rate of adult thyrocytes limits the accumulation of mutations and other genetic or epigenetic changes, a prerequisite of cell transformation. On the other hand, the oncofetal markers have been detected in thyroid carcinomas and fetal cell remnants within the thyroid gland (15). Furthermore, there is also much evidence showing that most thyroid nodules appear to be of a clonal origin (4), which indicates that these nodules may be derived from a single cell and naturally occurring clonal cell patch.

1.1 Adult stem and progenitor cells in thyroid tissue

Stem cells are cells that are capable of self-renewal and differentiation into many different specialized cell types. Stem cells found in almost all multicellular organisms are broadly classified as embryonic stem cells (ESCs), fetal stem cells, and adult stem cells. ESCs are the

most versatile type of stem cells, since they have the ability to differentiate into all cells of the adult body (16). An embryonic stem cell is derived from inner cell mass (ICM), which is part of the early (d4 to d5) embryo called the blastocyst and comes from the first entity of life, the fertilized egg (17). ICM cells are no longer totipotent, however, they may retain the ability to develop into all cell types of the embryo proper (pluripotency, Fig 1.1). When all the cells move down the stem cell hierarchy, they begin to lose pluripotent capabilities and become more specialized in structure and function (14, 17).

Adult stem cells are undifferentiated cells as well, but they are found among differentiated cells in a tissue or organ. They have the ability to self-renew or differentiate to yield some or all of the major specialized cell types of the tissue or organ (18). Therefore, the main differences between embryonic stem cells and adult stem cells are their location and potency. Embryonic stem cells are located in blastocysts, whereas adult stem cells are located in adult tissues. In fact, adult stem cells were found in many tissues and organs, such as bone marrow, skeletal muscle, adipose tissue, pituitary, the central nervous and system, etc (19, 20). Adult stem cells are also basically limited in their differentiation potential, and only differentiate into cell types of their original tissue. Their primary role in a living organism is to maintain and repair the tissue damaged by disease or injury. By asymmetric cell division the adult stem cells can self-renew and produce other progenitor cell which are partly differentiated and further divide and give rise to differentiated cells, thus providing a simple method for tissue homeostasis (21).

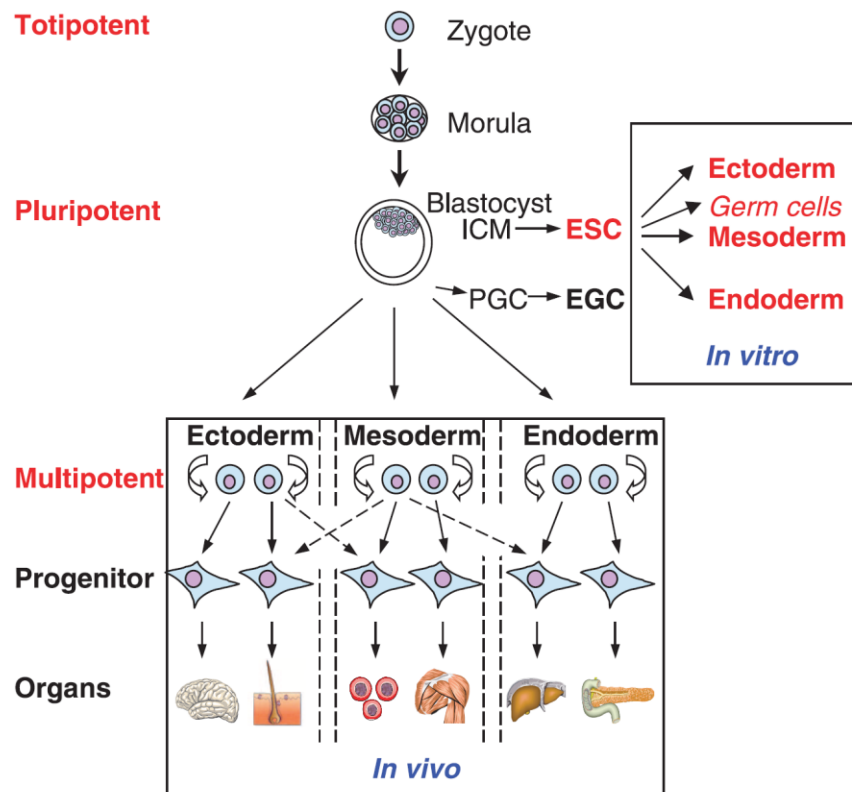

Fig 1.1 The schematic hierarchy of stem cells. Zygote and early cell division stages (blastomeres) to the morula stage are defined as totipotent, because they can generate a complex organism. At the blastocyst stage, only the cells of the inner cell mass (ICM) retain the capacity to build up all three primary germ layers, the endoderm, mesoderm, and ectoderm as well as the primordial germ cells (PGC), the founder cells of male and female gametes. In adult tissues, multipotent stem and progenitor cells exist in tissues and organs to replace lost or injured cells. At present, it is not known to what extent adult stem cells may also develop (transdifferentiate) into cells of other lineages or what factors could enhance their differentiation capability (dashed lines). Embryonic stem (ES) cells, derived from the ICM, have the developmental capacity to differentiate in vitro into cells of all somatic cell lineages as well as into male and female germ cells.

[Taken from: Wobus AM, Boheler KR. Embryonic stem cells: prospects for developmental biology and cell therapy. Physiol Rev, 2005, 85:635-678(17).]

In order to study early thyroid cell proliferation, a novel ESC-based approach was successfully established in vitro (22, 23). After two weeks of treatment with TSH, cultures of EB-derived adherent cell populations contained thyrocyte-like cells with the appearance of a set of genes (PAX8, NIS, TSHR, TPO and TG) (23). However, the thyroid-like cell progenitors generated by

TSH-insulin/insulin-like factor-I treatment were usually transient, variable and too low in cell number for further functional studies (15).

Thyroid follicular cells are derived from the endoderm, thus the morphogenesis of thyroid is similar to many endoderm-derived organs (24). The adult thyroid gland has been thought to be another source of stem/progenitor cells. Adult stem cells were indeed detected as single cells or groups of two or three cells dispersed throughout human thyroid gland (25). By means of RT-PCR, flow cytometry and immunofluorescence, stem cell marker Oct4 and endodermal marker GATA4 and HNF4α were found to be expressed in primary culture isolated from human goiter, thus indicating the presence of adult stem and precursor/progenitor cells of endodermal origin in human thyroid gland. Oct4, a transcription factor expressed in the developing endoderm, plays an important role in maintaining the pluripotency of ICM cells and ESCs (26). Therefore, the expressions of stem cell markers reinforce the hypothesis that a subpopulation of pluripotent stem cells is present in human thyroid goiter (14).

Taking advantages of the fact that both of ESCs and adult stem cells express ABCG2 transporters, Lan et al. from our group successfully isolated stem cells as a side population (27) by FACS from a non-side population fraction which comprises differentiated cells and endodermal marker-positive cells (28). ABCG2 (or breast cancer resistance protein, BCRP, ABCP) is a member of the ABC transporter family, and it has the ability to use the hydrolysis of ATP to pump toxin from cells, such as Hoechst 33342 (29). The expressed genes related to stem cell pluripotency were identified by RT-PCT in SP cells (28, 30). The side population, whose ABCG2 transporters could be inhibited by verapamil, represents a stem cell enriched population with low degree of cellular differentiation (31, 32). These SP cells were also found in normal mouse thyroid through the use of Hoechst 33342 dye (30).

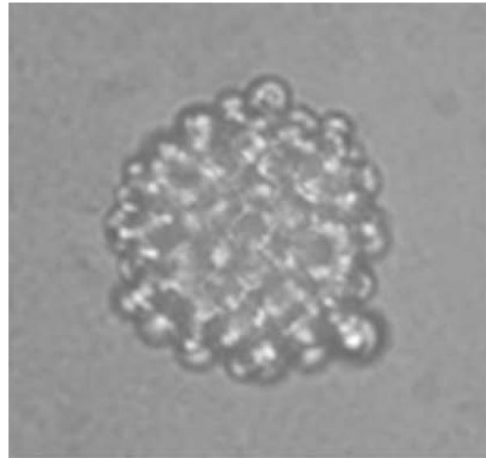

Fig 1.2 Thyrosphere derived from nodular goiter in response to intense growth stimulation. Under intense growth stimulation with EGF and bFGF, a small number of cells grew out from human thyroid cell cultures as floating spheres after 5 d of culture.

[Taken from: 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. The Journal of clinical endocrinology and metabolism, 2007, 92:3681-3688(28).]

Stem cell self-renewal and differentiation in vivo are controlled by the stem cell's microenvironment or niche that physically hosts the stem cells and perpetually maintains the pool of slowly dividing stem cells (33). Malnutrition (serum deprivation) of cell cultures in vitro is believed to alternate the normal niche control and thus result in an outgrowth of stem cells. In a complementary approach that does not allow adherence to a substratum, most differentiated thyrocytes derived from nodular goiters did not grow under the condition with medium containing growth factor (EGF and bFGF) but no TSH and serum. However, a small number of cells grew after 5-7d culture as floating spherical colonies, termed thyrospheres or spheroids (Fig 1.2) (28, 34). The percentage of SP cells was increased from 0.1% in primary thyroid culture to 5% in growth factor-stimulated spheres as revealed by FACS (28). Gene profile of thyrospheres showed high expression of stem cell markers Oct4 and ABCG2 as well as endodermal marker GATA4, but no expression of the thyroid differentiation markers PAX8, Tg, NIS, TSHr and TPO.

These adult stem cells derived from goiters were proved to have an intrinsic ability to generate

differentiated thyroidal cells and the potential to produce progenitor cells. Lan et al. dispersed the thyrospheres, allowed the cells to grow as a monolayer, and then induced stem cells to differentiate with TSH in serum-enriched medium (28). The differentiation markers of thyroid follicular cells such as PAX8, Tg, NIS TSHR and TPO were detectable after 21d culture. Iodide uptake was shown after the thyrosphere cells were embedded in collagen.

Recently, cancer stem cells (CSCs) were isolated from anaplastic thyroid cancer (ATC) cell lines by Zheng and co-workers from our group (35). CSCs sorted as SP by FACS using Hoechst 33342 dye expressed Oct4. As a characteristic marker for adult and embryonic stem cells, Oct4 is also expressed in some human tumors and some cancer cell lines but not in normal differentiated cells (25, 28, 36). So far, cancer stem cells have been proved to be present in some thyroid carcinoma cell lines (25, 27, 37, 38). Due to many properties shared with embryonic cells, such as pluripotency, undifferentiated state as well as self-renewal, the stem cells are widely believed to be involved in the pathogenesis of human thyroid tumors (14, 37, 39-41).

1.2 Thyroid nodules and nodular goiter: a stem cell disease?

Molecular biology research on the pathogenesis of nodular goiters revealed that not only thyroid adenomas but also many thyroid nodules are clonal in origin and thus are true benign tumors (4, 42-46). The normal thyroid epithelium is thought to be organized into large stem cell-derived monoclonal patches, and monoclonality in neoplastic and hyperplastic lesions may just be a reflection of normal thyroid epithelium clonal composition (47). In addition, some thyroid cells grow autonomously with a higher growth potential after transplantation of nodular goiter tissues on a nude mouse (48). These rapidly and autonomously replicating cells were hypothesized to initiate nodule formation in human multinodular goiters.

Only less than 1% cells in adult nodular goiters are stem or progenitor cells (25, 28). The multipotentiality and self-renewal ability of these stem cells are controlled by stem cell niches providing a microenvironment composed of cellular structures or extracellular matrix in which undifferentiated stem cells are maintained (49, 50). Growth factors are potent stimulators of

many types of adult stem cell growth. When stimulated with EGF and basic FGF, adult stem and progenitor cells in thyroid cell cultures gradually escaped niche control and thus grew as three-dimensional thyrospheres in suspension culture in vitro (28). These stem cell-derived spheres were composed of 5% stem cells and 95% progenitor cells. When TSH-enriched medium was added, proliferation rate of progenitor cells slowed down and the differentiation process was initiated (28).

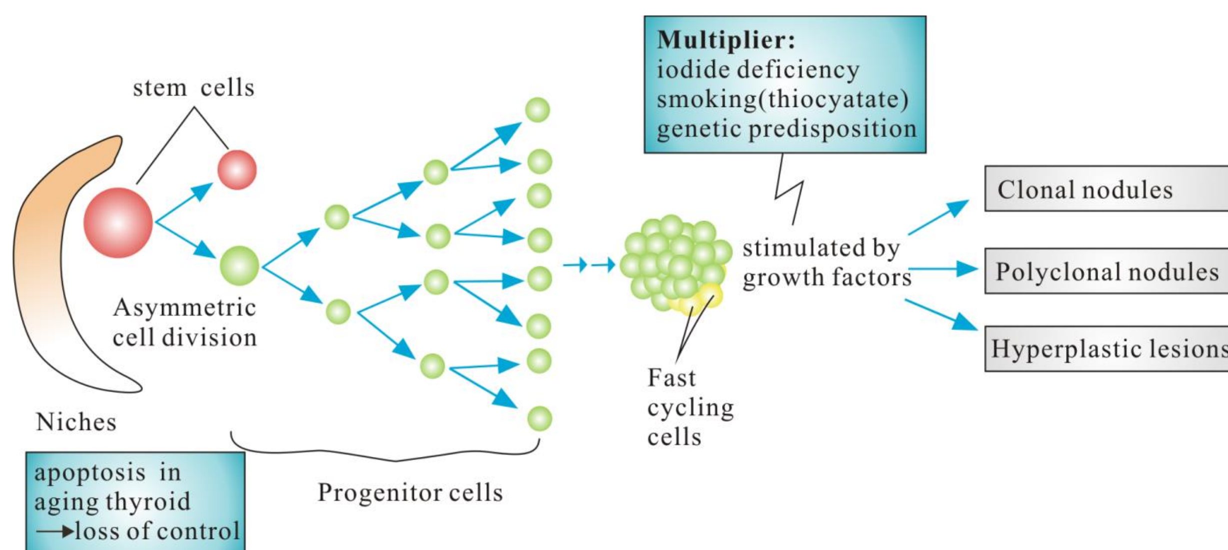

Figure 1.3 Thyroid nodules and nodular goiter as a stem cell disease. Apoptosis, operative in ageing thyroids, may limit growth control by niches. In turn, an essentially quiescent stem cell may proliferate to give birth to a daughter stem cell and a progenitor cell (asymmetric cell division). Under the influence of locally expressed growth factors, one of these cells (or different cells) with a higher than average growth rate may grow out to form nodules or hyperplastic lesions, whereas unstimulated cells differentiate into normal thyroid cells as shown in vitro. Progenitor cells that do not achieve full differentiation may be the origin of a non-functioning nodule or adenoma.

[Taken from: Derwahl M. Thyroid nodules and nodular goiter: a stem cell disease? Hot Thyroidology. http://www.hotthyroidology.com/editorial_180.html (51).]

Epidemiologic studies demonstrated that thyroid nodular transformation increases with age whereas the goiter size decreases (51, 52). In addition, there is some experimental evidence that

apoptosis of thyrocytes is a main factor of cell loss during goiter formation and involution (53). Hypofunction, destruction and necrosis of normal thyroid tissue has been demonstrated in goiter tissues by previous histological and immunohistochemical studies (54). Apoptosis of thyrocytes appears to be a prerequisite for thyrosphere formation and therefore the proliferation of stem and progenitor cells in vitro (28).

When the niches are altered by apoptosis, operative in ageing thyroids, a primitive quiescent stem cell may proliferate to give birth to a daughter stem cell and a progenitor cell by asymmetric cell division (Fig 1.3). One of these cells with fast cycling may overgrow, whereas other cells differentiate into normal thyroid cells (28). Under the influence of locally expressed growth factors in vivo, the progenitor cells containing fast-cycling cells with a higher than average growth rate may grow out to form nodules or hyperplastic lesions. Progenitor cells not achieving full differentiation may be the origin of a non-functioning nodule or adenoma (51). Therefore, intense stimulation of thyroid stem cells by these growth factors in vitro may correspond to processes of nodular transformation in vivo that last for years or even decades (28).

1.3 Estrogen, stem cells and thyroid nodules

Estrogens are a group of steroid compounds, named for their importance in the estrous cycle, and functioning as the primary female sex hormones. Estrogens are produced primarily by developing follicles in the ovaries, the corpus luteum, and the placenta. Some estrogens are also produced in smaller amounts by other tissues such as the liver, fat, adrenal glands, and the breast (55). These secondary sources of estrogens are especially important in postmenopausal women. Recently, the human thyroid gland was proved to have the potential for both estrogen synthesis and intracine or paracrine estrogen responsiveness (56). 17β-estradiol (E_2), which is produced by ovary and the predominant sex hormone present in females, represents the major estrogen in humans among three estrogens, and has the highest affinity for estrogen receptors in the largest quantity (57).

Steroid hormones play an important role in physiological processes including reproduction, differentiation, development and homeostasis (58). E_2 does not only have a critical impact on reproductive and sexual functioning but also regulates many physiological processes including cell growth and development of other organs including the heart, bones, brain, liver, and thyroid (59-63). Sex-based differences in the incidence of hypertensive heart disease and coronary artery disease, the development of atherosclerosis, and cardiac remodeling after myocardial infarction suggested that estrogen has direct effects on the myocardium, endothelium, and vascular smooth muscle (64). Estrogen also elicits a selective enhancement of the growth and differentiation of axons and dendrites (neurites) in the developing brain, showing its neural actions in the central nervous system (CNS) (65, 66). 17β-estradiol has been proved to promote tumor development, such as breast and ovarian cancer (67, 68).

As shown in Fig 1.4, estrogen appears to have different effects on proliferation, differentiation, and migration of stem and progenitor cells (69). E_2 is able to induce the proliferation of ESCs via estrogen receptors, since the increased [^{3}H] thymidine incorporation by E_2 is blocked using tamoxifen, an estrogen antagonist (70). In addition to regulation of cell cycle progression, estrogen rapidly increases mRNA expression of the protoongenes c-fos. c-jun, and c-myc in mouse ESCs (70). Like some other growth factors such as growth hormone (71) (72), epidermal growth factor (73, 74), and basic fibroblast growth factor (bFGF) (74, 75), estrogen also acts as a promotor for the proliferation of adult neural stem/progenitor cells (76, 77). Interestingly, estrogen is also able to increase the ratio of dopaminergic neurons derived from neural stem cells (NSCs) in vitro, suggesting the effects of estrogen on differentiation of neural stem cells (78). Effects of estrogen on proliferation and differentiation of neural stem cells provide new useful strategies for the treatment of Parkinson’s disease (76, 78). By decreasing the rate of apoptosis, E_2 also increases the number of endothelial progenitor cells (EPCs) (69). By enhancing the proliferation of EPCs, E_2 may help through repair and regeneration to compensate for damaged injured vessels, or ischemic myocardial tissues (69, 79). Taken together, estrogen modifies the functions of different stem cell and thus plays further roles in the physiological and pathological process in the human body.

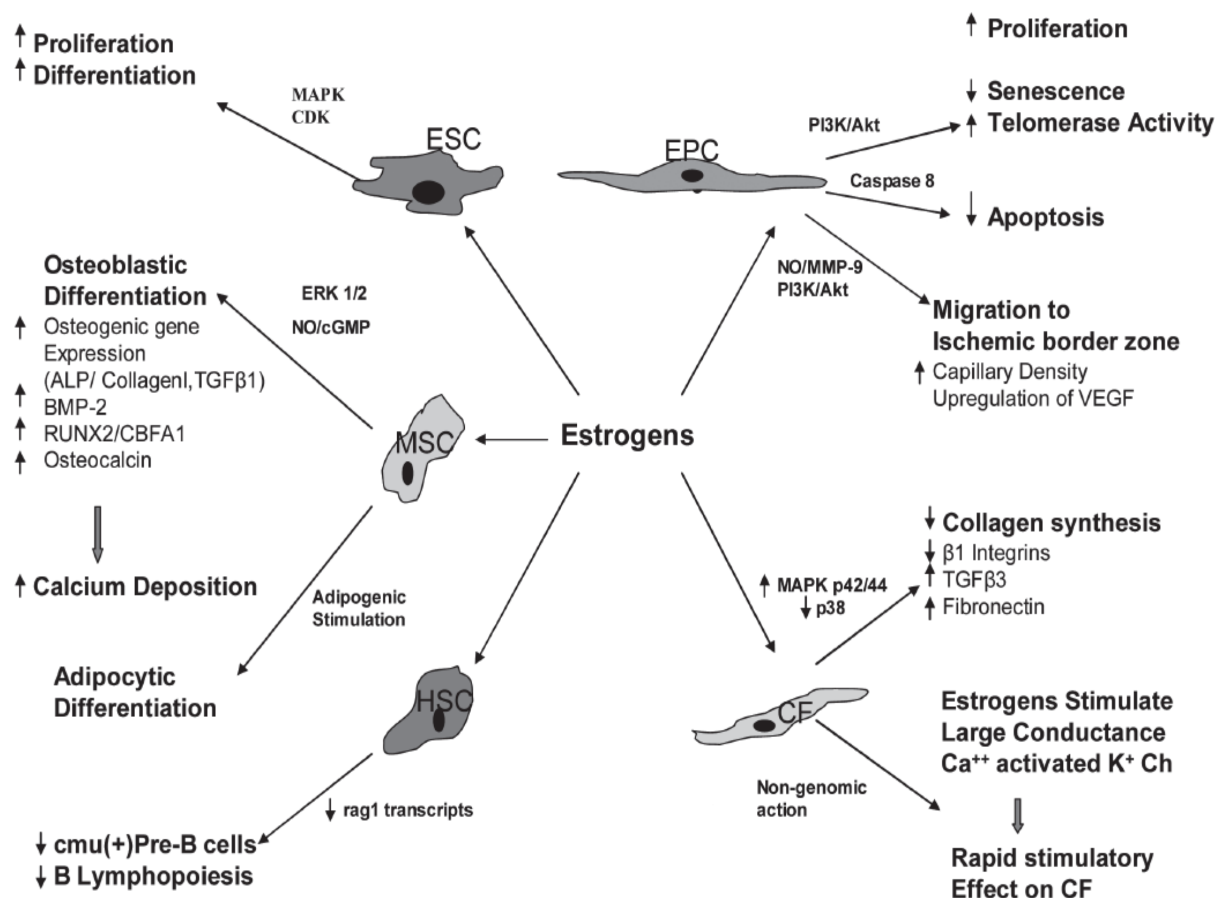

Fig 1.4 Effects of estrogens on various stem and progenitor cells. Estrogens exert immense influences on the functions of stem and progenitor cells, with examples illustrating that estrogens promote proliferation of ESCs stem cells by activating the MAPK cascade as well as cyclin-dependent kinases, with associated increases in cyclins D1. ESC (embryonic stem cell); EPC (endothelial progenitor cell); MSC (mesenchymal stem cell); HSC (hematopoietic stem cell); CF (cardiac fibroblast); BMP (bone matrix protein); RUNX2/CBFA1 (runt-related transcription factor 2/core-binding factor alpha).
[Taken from: Ray R, Novotny NM, Crisostomo PR, et al. Sex steroids and stem cell function. Mol Med, 2008, 14:493-501(69).]

Estrogens exert immense influences on myocardial remodeling following an ischemic insult, partially through paracrine growth hormone production by bone marrow mesenchymal stem cells (MSCs) and endothelial progenitor cells. Estrogens also facilitate the mobilization of endothelial progenitor cells to the ischemic myocardium and enhance neovascularization at the ischemic

border zone. Moreover, estrogens limit pathological myocardial remodeling through the inhibitory effects on the proliferation of the cardiac fibroblasts.

Epidemiologic studies found that thyroid nodules including thyroid cancer are more frequent in women than men (80-83). Epidemiological studies indicate that the gender difference in the susceptibility to this thyroid carcinomas becomes apparent after sexual maturity (84). The studies on the role of exogenous hormone also implied a role of female hormones on thyroid cancer promotion since oral contraceptives moderately increased risks of developing thyroid cancers (84, 85). Therefore, hormonal changes related to the menstrual cycle, pregnancy, menopause, and hormone use have been suggested as etiologic factors of thyroid cancer (83). The difference in incidence of thyroid diseases between genders also suggests that estrogens play an important role in the growth and progression of thyroid tumors.

E_2 has been clearly implicated to have potential to promote the proliferation of human thyroid cells isolated from thyroid goiter nodules (60), and FRTL-5 cells derived from Fischer rat thyroid (86). Moreover, E_2 also increases the growth of papillary and follicular thyroid carcinoma cells (87), Hurthle thyroid carcinoma cells (60), and anaplastic thyroid carcinoma cells (88). Animal experiments agreed with these data of both epidemiological and cell experimental researches. Ovariectomy decreased the incidence of thyroid tumor when compared with ovary intact rats with the highest E2 level, while estradiol/testosterone supplementation increased the incidence (89).

In the last two decades, many studies have been performed to elucidate the molecular mechanisms of estrogen in tumor pathogenesis. Cell cycle related to the growth and progression was recognized to be regulated by estrogen, thus inducing the cellular proliferation of normal or tumor cells (90). Cyclin D1-regulating G1/S transmition in the cell cycle was significantly increased in protein and mRNA levels by E_2 stimulation of human thyroid cells and carcinoma cells (60, 87). E_2 regulates the cell cycle via specific estrogen receptors α and β (ER-α and ER-β) encoded by separate genes, ER-α gene (ESR1) and ER-β gene (ESR2), respectively (91), explaining why the estrogen receptor inhibitors have the potential to prevent the effects of

estrogen (86, 87). Both receptors are coexpressed in thyroid cells derived from goiter (60), rat thyroid cells (86), and thyroid tumor cells (60, 87). ER-α and ER-β may regulate distinct cellular pathways despite sharing a similar mechanism of action and domain structures. In addition to likely mechanisms, estrogens may also generate a direct genotoxic effects to increase mutation rates, and contribute to chromosomal nondisjunction by impairing the formation of mitotic spindles (84).

A growing body of evidence has demonstrated that ERs are expressed in undifferentiated human ESCs and embryoid bodies (36), suggesting the potency of estrogen to influence the differentiation of hESCs (70, 92). Nevertheless, it is still unclear whether these estrogen receptors are expressed in thyroid adult stem cells, or whether they play an important role in estrogen action. Since estrogen is a potent stimulator of many types of stem cell growth, estrogen is speculated also to promote the growth of thyroid stem cells. In addition, it is still unclear whether estrogen modifies the functions of adult thyroid stem/progenitor cells during their proliferation and differentiation process, and if it induces outgrowth of stem cells and aberrant differentiation to transform thyroid nodules.

1.4 Aim of the present study

A higher prevalence of thyroid nodules in women than in men demonstrates that estrogen plays an important role in the pathogenesis of thyroid nodules. Effects of this steroid hormone on thyroid cells have been described more recently, however, the mechanism behind this is still unknown. Ongoing advances in thyroid stem cell research have opened new avenues for research.

Therefore, the aim of the present work was (1) to investigate whether 17β-estradiol has the potential to promote the proliferation of human thyroid stem/progenitor cells by determining bromodeoxyuridine (BrdU) incorporation and thyrosphere formation after passaged in to secondary generation, (2) to analyze estrogen receptor expression in human thyroid stem and progenitor cells by conventional real-time RT-PCR and immunofluorescence staining, (3) to clarify whether 17β-estradiol stimulates cell cycle progression by induction of cyclin D1 gene

expression, (4) to observe the influence of 17β-estradiol on TSH-induced differentiation of adult thyroid stem and progenitor cells into thyrocytes.

2. Materials and Methods

2.1 Cell culture

2.1.1 Profile of patients

Thyroid tissues provided by the Endocrine Surgery Department of St. Hedwig Krankenhaus in Berlin were obtained from 54 patients with nodular goiters undergoing thyroidectomy. The mean age of the patients was 52.65±13.46 years. Malignancy was ruled out in all cases by means of fine needle aspiration biopsy and cytological examination prior to the operation, rapid excision biopsy during the operation, and histological examination of tissue after surgery. Informed consent was obtained from all patients prior to the operation. The study was approved by the ethics committee of Charité, University Medicine Berlin.

2.1.2 Preparation of primary cultures

Primary cultures of human thyrocytes and thyrospheres isolated from nodular goiter tissues were established as described previously (28). Briefly, thyroid tissue specimens were separated by the surgeon from surrounding tissue in operating room, placed in sterile transport tubes containing 20 ml transport medium (HBSS solution with 100 U/ml penicillin and 100 μg/ml streptomycin), placed on ice and transported to the laboratory. Then all work with cell cultures was performed in a laminar flow cabinet.

2.1.3 Primary culture of thyrocytes

Preparation of primary thyroid cultures from human nodule tissue was performed as described previously (28). Employing sterile techniques, macroscopically visible capsule material and other connective tissues were removed using scalpel and tweezers. In order to increase surface area, the remaining tissue was grossly chopped with a scalpel, to facilitate subsequent dispartment in follicles. Enzyme digestion was carried out in fresh HBSS containing 5 mg/ml Collagenase A (Roche, Mannheim, Germany) and 30 mg/ml dispase II (Roche, Mannheim, Germany) at 37 ℃ for 1.5 h in a shaking bath with slow swirling motion at about 80 rpm. After

90 min, the digest was filtered through a sterile mesh sieve, leaving behind the undigested tissue fragments. The flow-through was centrifuged for 5 min at 310 ×g. The supernatant was returned to the remaining tissue to continue digestion, while the thyroid cell pellet was re-suspended in pre-warmed (37 ℃) primary cell culture medium, put in 100 mm cell culture dishes (Sarstedt) and placed in a humidified incubator at 37 ℃, with a 5% CO_2 atmosphere for 20 min. This was the pre-plating step which helps to reduce fibroblast contamination since fibroblasts adhere to the dish surface more readily than thyrocytes. After 20 min, the medium with unattached cells was transferred to another plastic dish and placed in the incubator for culture. The digestion procedure for undigested tissue fragments was repeated several times until only white connective tissue remained.

2.1.4 Primary culture of thyrospheres

In the approach for prospective enrichment of thyroid stem cells, a new culture system for non-adherent sphere formation was performed as described previously (28).

Primary thyroid cells were prepared as described above and cell suspension was sieved through a 30 µm strainer, centrifuged and re-suspended in growth factor-enriched medium: serum-free DMEM/Ham's F-12 (1:1) containing B-27 (1:50), 20 ng/ml EGF (Invitrogen, Karlsruhe, Germany), and 20 ng/ml bFGF (Invitrogen, Karlsruhe, Germany). Single cellularity was confirmed under microscope. Cells were cultured in 100 cm Poly (2-hydroxyethyl methacrylate, 2-HEMA)-coated dishes at 10,000 viable cells/ml in a 37 ℃, 5% CO_2 culture incubator. Every 2-3 d, B27, bFGF and EGF were added. Under these conditions, most primary thyrocytes died, and only a small number of cells survived, proliferated and formed floating spheres following 5-8 d of culture, which were termed 'thyrospheres'.

During the sphere formation, the sphere size in every dish was monitored. Sphere cells were prepared for RNA isolation, passaged for secondary generation, and stimulated with estrogen.

2.1.5 Secondary generation of thyroid stem/progenitor cells

Adult primary thyrospheres were usually cultured for 5-7 d. In order to avoid differentiation in situ and difficult dissociation of thyrospheres, the suspension culture was monitored every day to ensure that thyrospheres are not allowed to grow too large. When the thyrospheres were ready for passaging, medium with suspended cells was transferred into an appropriately sized sterile tissue culture tube. When few cells remained attached to the substrate, a stream of medium was shot to detach them. After spinning at 110 g (800 rpm) for 5 min, 100% of the supernatant was essentially removed and the cells were resuspended in 1 ml of trypsin-EDTA (Invitrogen, Germany), and then incubated at 37 ℃ with shaking for 15 min. An equal volume of serum was added into tube to inhibit trypsinization, mixed well, and then centrifuged at 110 g for 5 min. After 100% of the supernatant was essentially removed, cells were resuspended by the addition of 1 ml of DMEM/F-12(1:1). Then cells were dissociated mechanically once or twice using a 1000 µl tip. The tip was placed at the bottom of the tube so as to restrict the flow of cells by ~50% and continued trituration which was repeated three times. After counting of cell numbers, cells were seeded for the next culture passage into poly-L-ornithine and fibronectin-coating (Sigma-Aldrich, Steinheim, Germany) dishes with DMEM/F-12(1:1) and mitogens (EGF, bFGF and B27) at a density of 10^5 cells/ml.

2.1.6 Culture conditions

For most experiments, cells were grown as spheres or monolayers in 100 mm plastic culture dishes and kept in a humidified incubator at 37 ℃ in 5% CO_2, supplemented with mitogens or medium change each 2-3 d. Upon reaching the desired confluency of 70-80% in monolayer culture or sphere formation for 5-7 d, cells were passaged after trypsinization using 0.05% trypsin, 0.53 mM EDTA-4Na.

Primary thyroid cells were cultured in Ham's F-12 medium (Gibco, Karlsruhe, Germany) with L-glutamine, supplemented with 10 % fetal calf serum (FCS) (Gibco, Karlsruhe, Germany), 1% MEM(v/v) (Gibco, Karlsruhe, Germany), 5 mU/ml TSH (from bovine pituitary, Sigma, Steinheim, Germany), five hormones or growth factors (H5-mix), 100 U/ml penicillin, 100 µg/ml

streptomycin (Roche, Mannheim, Germany), and 2.5 μg/ml amphotericin B (Bristol-Meyer Squibb, Germany).

Primary thyrospheres were culture in DMEM/F-12 medium supplemented with B-27 (1:50), 20 ng/ml EGF, and 20 ng/ml bFGF, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2.5 μg/ml amphotericin B. The culture dishes were coated with poly-HEMA in advance.

Monolayer cultures of thyroid stem/progenitor cells were grown in DMEM/F-12 (Invitrogen, Germany) supplemented with B-27 (1:50), 20 ng/ml EGF, and 20 ng/ml bFGF, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2.5 μg/ml amphotericin B. The culture dishes were coated with poly-L-ornithine/fibronectin for cell attachment before use.

H5-mix:

10 ng/ml glycyl-histidyl-lysine (Sigma-Aldrich, Steinheim, Germany)

10 μg/ml insulin (human, recombinant, Aventis Pharma, Frankfurt, Germany)

10 ng/ml somatostatin (Sigma-Aldrich, Steinheim, Germany)

5 μg/ml transferrin (Sigma-Aldrich, Steinheim, Germany)

3.2 ng/ml hydrocortisone (Sigma-Aldrich, Steinheim, Germany)

2.1.7 Cell counting

Following trypsinization and neutralization protocol for cell cultures, a uniform cell suspension was obtained and placed in a centrifuge tube. A 1:2 diluted cell suspension in trypan blue (Sigma-Aldrich, Steinheim, Germany) was prepared and loaded into both counting chambers of the hemacytometer and the coverslip was placed over them. The cells were counted under a microscope at 100 × magnification. Cell numbers (total and viable: Trypan blue-unlabeled) overlaying four × 1 mm^2 areas of the counting chamber were determined. Then the total (or viable) cell number of the original cell suspension was calculated as follows:

Total (or viable) cells recovered =

Cells/ mm^2 divided by dilution (1/2), and multiplied by 10^4 and total volume of cell suspension

2.2 RNA extraction and reverse transcription PCR (RT-PCR)

2.2.1 Total RNA isolation

Total RNA was extracted from cultured cells by using the RNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's specifications. Briefly, after aspiration of the culture medium, 350 µl buffer RLT, supplemented with β-mercaptoethanol (β-ME, add 10 µl β-ME per 1 ml buffer RLT), was added to the monolayer cultures. Cell lysates were then collected using a rubber policeman, pipetted in QIA Shredder microcentrifuge tubes (Qiagen, Hilden, Germany) and centrifuged for 2 min at maximum speed for homogenization. Equal volumes of 70 % ethanol were added to the homogenized lysate and mixed by pipetting. Samples were then applied to RNeasy mini columns placed in 2 ml collection tubes and centrifuged for 15 sec at 8000 ×g. Flow-through was discarded and 700 µl of RW1 buffer was added to the RNeasy columns and centrifuged for 15 sec at 8000 ×g to wash the columns. Flow-through and collection tubes were discarded and the RNeasy columns were transferred into new 2 ml collection tubes. 500 µl of buffer RPE was pipetted onto the RNeasy columns which were then centrifuged again for 15 sec at 8000 ×g to wash. Flow-through was discarded and another 500 µl buffer RPE was added to the RNeasy columns. Tubes were then centrifuged again for 2 min at 8000 ×g to dry the RNeasy silica-gel membrane. For elution, the RNeasy columns were transferred to new 1.5 ml collection tubes and 30 µl of RNase-free water was pipetted directly onto the RNeasy silica-gel membrane. Tubes were closed gently and centrifuged for 1 min at 8000 ×g. The resulting RNA was either stored at - 20 ℃ or used directly for spectrophotometry and RT-reaction (see below).
For spectrophotometry analysis, 2 µl of RNA was diluted 1:500 in RNase-free water and absorption was calculated at OD260. For each probe, the mean value of at least two independent readings was adopted as the result.

DNase I working solution:

DNase I stock solution	10 µl
buffer RDD	70 µl

2.2.2 Reverse transcription reaction

For reverse transcription, 1 µl of Oligo(93)$_{12-18}$ Primer (0.5 µg/ul) (Invitrogen, Germany) was added to the volume of mRNA solution containing 1 µg of mRNA (as calculated by spectrophotometry). RNase-free water was added to a final volume of 16.75 µl and the mix was heated to 70 ℃ for 5 min. Probes were placed on ice and the following reagents were added consecutively: 5 µl of M-MLV 5× reaction buffer, 1.25 µl of dNTP nucleotides (10 mM, Roche, Mannheim, Germany), 1 µl (25 Units) of RNAGuard RNase inhibitor (Invitrogen, Germany) and 1 µl (200 Units) of M-MLV reverse transcriptase (Promega, Mannheim, Germany). The mixture was warmed to 42 ℃ for 60 min, 95 ℃ for 5 min and the reaction was terminated at 0 ℃. cDNA samples were stored at -20 ℃.

2.2.3 Primer preparation

All primers were obtained in powder form from Invitrogen Inc. Upon delivery, primers were diluted in RNase-free water and concentration was 5 pmol/µl for conventional RT-PCR and 10 pmol/µl for quantitative real time PCR, aliquoted and stored at -20 ℃.

2.2.4 Polymerase chain reaction

For PCR amplification the hot start method was performed. In brief, 4 µl of complementary DNA was added to a 45.5 µl master mix containing 5 µl 10× reaction buffer, 1.5 mM MgCl2, 1 µl dNTPs (10 mM) and 30 pmol of sense and antisense primers. Negative controls without template cDNA were included in all cases to exclude carry-over contamination with genomic DNA. 50 µl of mineral oil was added to each reaction tube, the lids were closed and the mixture was preheated to 95 ℃ for 10 min before adding Taq polymerase (Invitrogen, Karlsruhe, Germany) to reduce non-specific annealing and primer elongation events. Probes were then cooled to 80ºC and 0.5 µl of Taq DNA polymerase (5 U/µl) was added to each reaction tube. Using a thermocycler, cycling conditions were as follows: at 95 ℃ for 30 sec (initial denaturation), 52-63 ℃ for 30 sec (primer specific, annealing) and 72 ℃ for 1 min (extension), followed by a final extension at 72 ℃ for 10 min and termination at 4 ℃. The number of cycles used was determined to be in the log-linear phase of the amplification reaction. In all PCR

analyses, β-actin served as an internal control. Primer pair sequences, product lengths and annealing temperatures were as follows:

Table 2.1 Primer sequences, annealing temperatures, cycles and product sizes for RT-PCR

Target gene	Primer sequences#	Annealing Temp	Cycles	Expected size (bp)
GAPDH	S: 5'-GAAGGTGAAGGTCGGAGTC-3' AS: 5'-GAAGATGGTGATGGGATTTC-3'	58	26	226
ER-α	S: 5'-CCACTCAACAGCGTGTCTC-3' AS: 5'-GGCAGATTCCATAGCCATAC-3'	58	36	243
ER-β	S: 5'-CGCCAGTTATCACATCTGTATG-3' AS: 5'-CCACTAACCTTCCTTTTCAGTG-3'	58	36	112
NIS	S: 5'-TCTCTCAGTCAACGCCTCT-3' AS: 5'-ATCCAGGATGGCCACTTCTT-3'	58	36	298
Tg	S: 5'-GAGCCCTACCTCTTCTGGCA-3' AS: 5'-ATCCAGGATGGCCACTTCTT-3'	58	36	324
TSHR	S: 5'-AGCCACTGCTGTGCTTTTAAG-3' AS: 5'-CCAAAACCAATGATCTCATCC-3'	58	36	131
TPO	S: 5'-GTCTGTCAGGCTGGTTATGG-3' AS: 5'-CAATCACTCCGCTTGTTGGC-3'	58	36	242
PAX8	S: 5'-TTTGCTTGGCTCTTTCTACACCTC-3' AS: 5'-GAATGTCTGTTTTAAGCTCCCTGG-3'	58	36	205
GATA4*	S: 5'-ACAAGATGAACGGCATCAAC-3' AS: 5'-CGTGGAGCTTCATGTAGAGG-3'	58		174
GATA4	S: 5'-CTCCTTCAGGCAGTGAGAGC-3' AS: 5'-GAGATGCAGTGTGCTCGTGC-3'	58	36	575
OCT4	S: 5'-GACAACAATGAGAACCTTCAGGAG-3' AS: 5'-CTGGCGCCGGTTACAGAACCA-3'	55	30	216
CyclinD1	S: 5'-ACAAACAGATCATCCGCAAACAC-3' AS: 5'-TGTTGGGGCTCCTCAGGTTC-3'	58	30	144

S: sense primer; AS: antisense primer

* The primers were used for qPCR

2.2.5 Agarose gel electrophoresis

PCR products were separated on 1.5% or 2% (w/v) agarose gels (2% for expected fragment sizes of 250 bp or lower) containing 0.5 µg/ml ethidium bromide. Gels were run at a voltage of 90 V in TBE running buffer for 60 min. Bands were visualized on a UV-transilluminator at 312 nm.

TBE Buffer (20X):

1.8 M Tris-base

1.8 M boric acid

25 mM EDTA-$Na_2.2H_2O$

PH was adjusted to 8.3

2.3 Real-time RT-PCR

Real-time quantitative RT-PCR was performed with iCycler iQ Real time PCR detector system (Bio-Rad, CA, USA). SYBR Green reactions were performed using ABsolute™ QPCR SYBR Green Fluorescein Mix (Applied Thermo Fisher scientific Inc). The PCR reaction was performed in a 96 well plate. Cycling conditions were as follows: initial enzyme activation at 95 ℃ for 15 min, followed by 50 cycles at 95 ℃ for 15 s; 58 ℃ for 30 s; 72 ℃ for 30 s. Relative expression levels of each gene in real time were analyzed using the 2-ΔΔCT method and normalized to the expression of the housekeeping gene 18s rRNA. Each sample was replicated twice from 3 independent sets of RNA preparations. Results are tabulated as mean ± SEM of 3 independent experiments. The Ct values of the real-time PCR were calculated by the $(2*\text{efficiency})^{\Delta\Delta Ct}$ method and normalized by the value of the internal control 18s rRNA. Data were presented as mean of fold change ±SEM vs control and derived from at least three independent experiments.

2.4 BrdU incorporation

Human thyroid stem/progenitor cells proliferation was initially evaluated by measuring the

incorporation of BrdU using cell proliferation ELISA, BrdU colorimetric kits purchased from Roche to determine DNA synthesis. After culturing 5-7 d in suspension condition, thyrosphere cells were dissociated into single cells by incubation for 15 min with trypsin and then plating by 8,000-10,000 cells per well into 96-well microtiter plates pre-coated with poly-L-ornithine and fibronectin. Cells were allowed to adhere overnight and starved of growth factor (EGF, bFGF, phenol red, et al) 24 h before E_2 stimulation. Subsequently, the cells were treated with E_2 for 24 h, 10 µl of 100 µM BrdU labeling solution was added into 100 µl/well medium and the cells were reincubated for an additional 4 h at 37 ℃. After removal of the labeling medium and drying of the labeled cells, the dry cells were stored up to one week at +2 to +8 ℃. The cells were incubated with 200 µl/well FixDenat for 30 min and then with anti-BrdU-POD working solution for approx. 90 min at room temperature. After the cells were developed 15 min with 100 µl substrate solution, 25 µl 1 M H_2SO_4 was added into each well. The plate was measured immediately within 5 min in an ELISA reader at 450 nm with 690 nm for the reference wavelength.

2.5 Immunofluorescence staining

Before immunofluorescence staining, coverslips were coated with poly-L-lysine (Sigma, USA). Poly-L-Lysine was diluted to 0.1 mg/ml with sterile deionized water before coating slides. Poly-L-Lysine solution was aspirated 0.5 ml/25 cm^2 to the coverslips. The coverslips were rocked gently to ensure even coating. After 5 minutes, the solution was removed by aspiration and the surface was thoroughly rinsed with sterile deionized water 3 times. There was at least 2 hours of time allotted for drying before introducing cells and medium.

Thyrospheres were trypsinized into single cells and passaged to second generation by monolayer culture. Briefly, the cells were plated onto the coverslips and grown in culture medium containing growth factors (EGF, bFGF and B-27). After 24 h adhesion cells were rinsed in PBS, followed by fixation in pure methanol at -30 ℃ for 5 min. The fixed cells were permeabilized by 0.2% Triton X-100 for 10 min at RT(room temperature), and then blocked by normal goat serum (10%) for 90 min at RT. Coverslips were incubated with the monoclonal anti-ERα (1:50) and

polyclonal anti-ERβ (1:100) antibodies at RT for 1 h and then 4 ℃ overnight. Unbound antibodies were removed by rinsing in washing buffer (PBS containing 0.1% Tween-20 and 1%BSA), followed by incubation for 60 min at RT with FITC-conjugated secondary antibody as secondary antibodies in dark (1:400, Santa Cruz) with gentle shaking. FITC-labeled cells were analyzed by fluorescence Zeiss microscope using standard fluorescent filters (excitation 488 nm).

2.6 Experimental equipment

2.6.1 Apparatus

Laminar flow cabinet	Heraeus, Laminair HB 2448
Cell culture incubator	Heraeus
Phase contrast microscope	Nikon, TMS
Fluorescence microscope	Zeiss
Digital camera	Sony DSC-W7
Water bath	Kotterman Labortechnik
UV Illuminator	Bachofer Laboratoriumsgeräte
UV camera	Polaroid MP4 Land Camera with Polaroid 545 4X5 film holder
Scanner	CanoScan 5000
Thermocycler	Biometra, Trio-Thermoblock
Spectrophotometer	Pharmacia, Ultrospec II
Autoclave	H+P Varioklav
Shake incubator	Infors HT
Precision scale	Advenurer OHAUS
Voltage generators	Consort, Electrophoresis power supply, E455
Homogenisator	Eppendorf Thermomixer 5436
Centrifuges	Hettich Mikro 200R

	Beckmann Microfuge E Hettich Rotina 46R
Heating oven	Memmert
Pipets and tips	Eppendorf
Plastic centrifuge tubes (1.5, 15 and 50 ml)	Sarstedt
Falcon tubes	Becton Dickinson
Plastic culture dishes and plates	Sarstedt
Polystyrene slide flasks	Nunc (Wiesbaden, Germany)
Elisa machine	Nunc (Wiesbaden, Germany)
iCycle	BIORAD (CA, USA)

2.6.2 Software

Biology Workbench version 3.2	DNA and mRNA sequence analysis
Adobe photoshop version 7.0	Figure preparation
BIORAD IQ5	Analysis of qPCR dates
Image-Pro Plus 6.0	Figure preparation
GraphPad Prism 4.0	Figure preparation

3. Results

3.1 Primary, secondary passaged thyroid stem/progenitor cells culture

Primary sphere culture of thyroid stem/progenitor cells isolated from nodules and adenomas was established as described in *Materials and Methods*. Single cells of thyroid cells derived from thyroid goiters grew under special culture conditions enriched in growth factors EGF and bFGF and not allowed to attach to the substratum. Only a few populations of cells formed spherical colonies which were termed “thyrospheres”.

These spheres were bright, smooth-edged, and compact and were clearly different from irregular clumps of cells under the microscope (Fig. 3.1 A and B). Many thyrospheres were very large and grew very slowly after 7 days culture. As a rule of thumb, most spheres became difficult to dissociate and eventually began to differentiate *in situ* after 5-7 days floating culture. The cells of the central portion of spheres stopped growing and started differentiation because of malnutrition and continuous stimulation of growth factors (94).

Poly-L-ornithine/fibronectin coating is able to render dispersed thyroid stem/progenitor cells more adhesively to grow in the presence of growth factors and absence of serum with basal DMEM/F12 medium after 24 hours (Fig 3.1, D). Most of cells were attached at 1 hour after passage (Fig. 3.1 C). Analysis of expression profiles by semi quantitative RT-PCR revealed that the expression of Oct4 as a stem cell marker was increased in primary thyrospheres and secondary passaged thyroid stem/progenitor cells, corresponding to the increase in ABCG2-positive side population from 0.1% in primary thyrocytes to 5% in thyrospheres under intensive culture with growth factors (28).

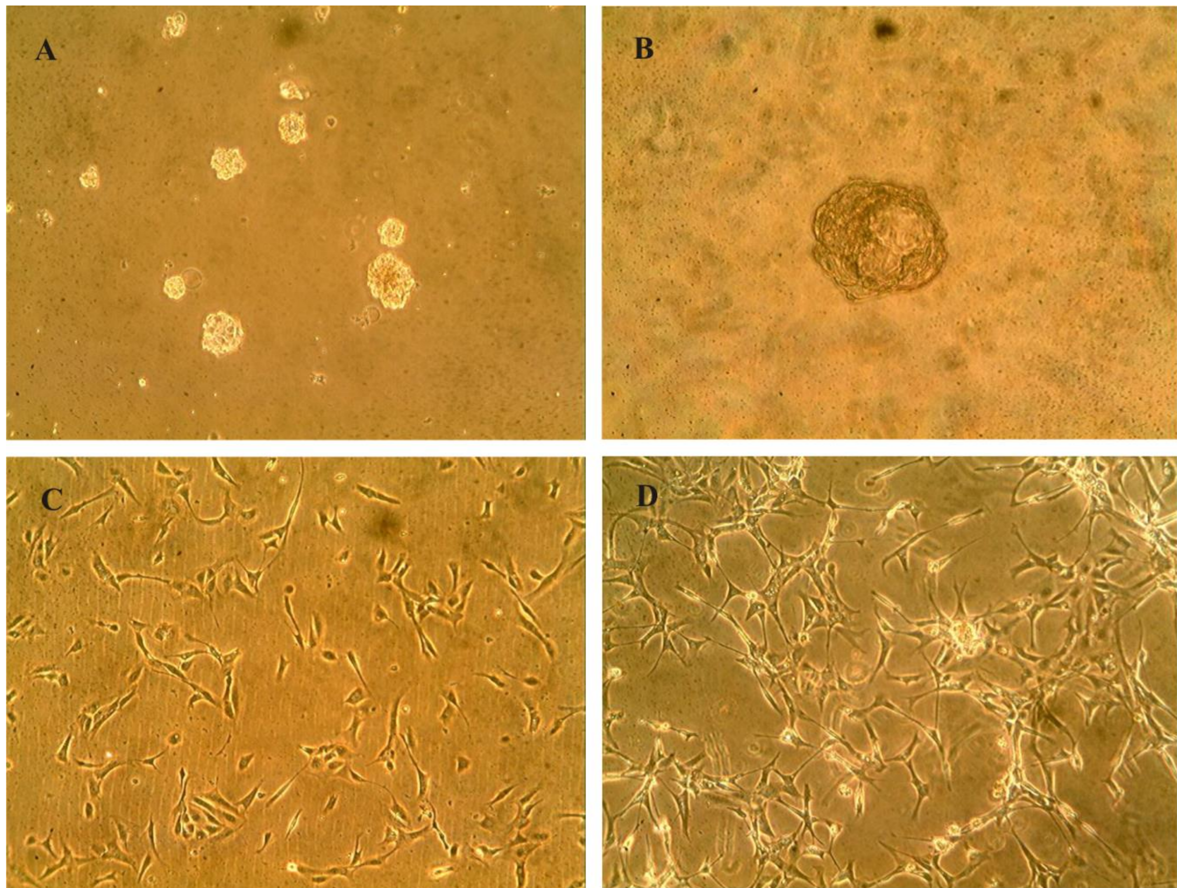

Fig 3.1 Cell culture and characterization of human thyroid stem/progenitor cells. Human thyroid stem/progenitor cells were cultured in poly-Hema or poly-L-orithine and fibronectin-coated dishes or plates to form floating spheres or monolayer cells respectively. (A) Floating thyrospheres in culture, 10×, (35) (B) Representative morphologic photo of thyrosphere, 20×, (C) Monolayer of thyroid stem/progenitor cells 24 h after trypsinization and passaging, 10×, (D) Monolayer of thyroid stem/progenitor cells 3 d after passaging, 10×.

To verify the new expansion approach of thyroid stem cells, the expression of Na^+/I^- symporter as a differentiation marker of thyroid follicular cells was analyzed by RT-PCR. NIS was undetectable in thyrospheres or in secondary passaged thyroid stem/progenitor cells. Our previous data indicated that not all differentiation markers (TSHR, NIS, Tg and TPO mRNA) and thyroid transcription factor PAX8 were expressed in thyroid spheres, while both stem cell markers (Oct-4 and ABCG2 mRNA) and endodermal progenitor cell markers(GATA4 and HNF-4) were present in secondary thyrospheres. The expression pattern confirmed their thyroid

stem/progenitor phenotype under the suspending culture conditions. The increased Oct4 expression and negative NIS expression also confirmed the thyroid stem/progenitor phenotype of secondary passaged cells under the given culture conditions.

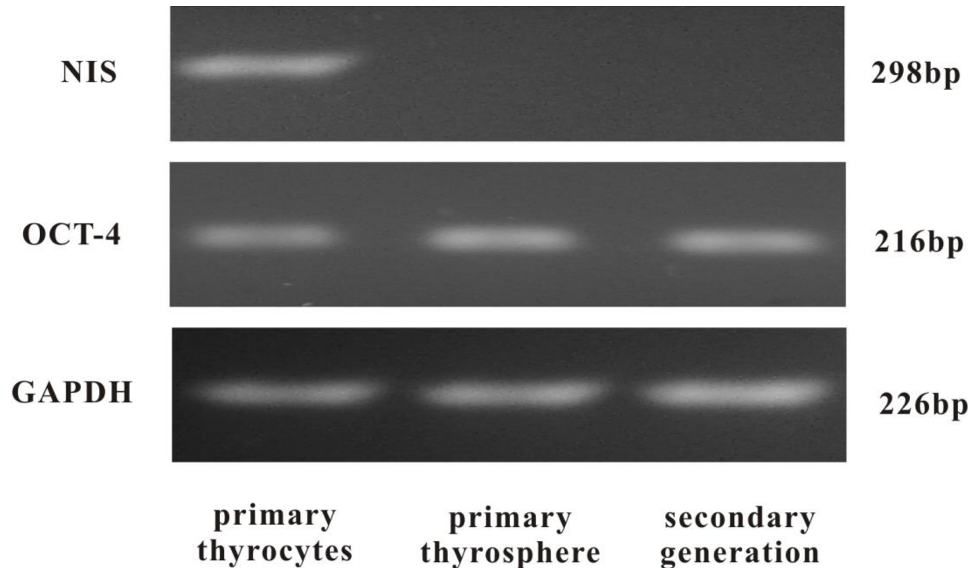

Fig 3.2 RT-PCR was performed using isolated mRNA from primary thyrocytes, thyrospheres and secondary generation of human thyroid stem/progenitor cells. A representative ethidium bromide agarose gel predicts the single band of each RT-PCR product from primary thyrocytes, primary thyrospheres and secondary generation of human thyroid stem/progenitor cells with primers specific to human NIS and Oct-4, and GAPDH as an internal control.

3.2 ER-α and ER-β were expressed in thyroid stem/progenitor cells and thyrocytes derived from thyroid nodules

To determine the expression of ER subtypes in thyroid stem/progenitor cells, conventional RT-PCR and quantitative real-time PCR were performed. MCF-7 human breast cancer cells were used as a positive control since both ER-α and ER-β are present in these cells. Total RNA was isolated from MCF-7 cells, primary thyrospheres and primary thyrocytes, and GAPDH was used as an internal control in PCR. ER-α and ER-β with the expected sizes of 243 bp for ER-α and 112 bp for ER-β were detected in all three kinds of cells. As a positive control, the expression of

ER-α was extremely high in MCF-7 cells, much higher than in thyrospheres and in thyrocytes (Fig. 3.3). Quantitative PCR analysis revealed a significant difference in expression of ER-α mRNA between primary thyrocytes and thyrospheres (1.10±0.35 *vs* 8.85±0.81，$P<0.001$)(Fig. 3.4). In contrast, the expression of ER-β mRNA in primary thyrospheres was not significantly different from that in primary thyrocytes (2.54±0.90 *vs* 1.05±0.25, $P>0.05$). To visually observe expression of ER isoforms in individual cells, immunofluorescence staining was performed using specific antibodies. ER-α and ER-β were detectable in the cytoplasm and nucleus, with ER-α enriched in the nucleus and ER-β in the cytoplasm (Fig. 3.5 A-F).

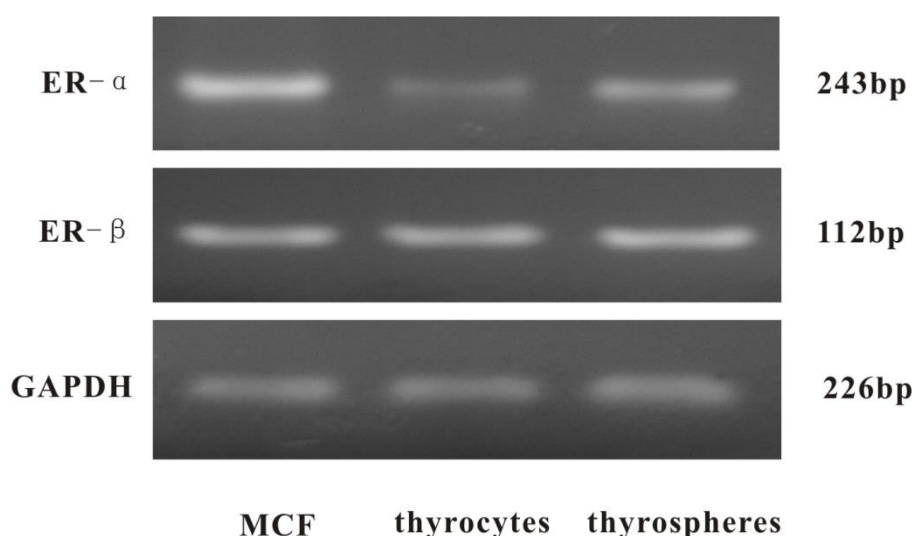

Fig. 3.3 Expression of estrogen receptors-α and -β in human thyroid stem/progenitor cells. The expressions of ER-α and ER-β at mRNA level in human thyroid stem/progenitor cells were determined by RT-PCR. A representative ethidium bromide agarose gel predicts the single band of each RT-PCR product, and the accurate size of the PCR product, ER-α (243 bp) and ER-β (112 bp).

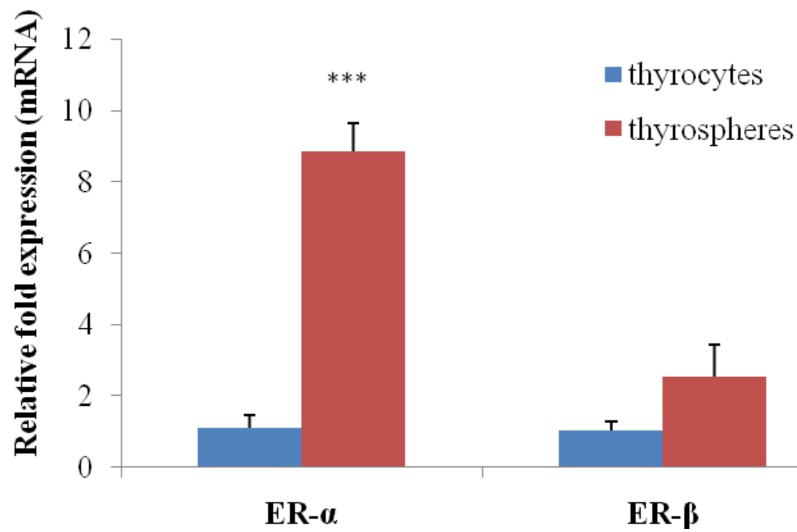

Fig. 3.4 Expression of estrogen receptors-α and -β in human thyroid stem/progenitor cells. The expressions of ER-α and ER-β at mRNA level in human thyroid stem/progenitor cells were determined by quantitative real-time PCR using specific primers for the ligand binding domain of human ER-α and ER-β. The Ct value of the real-time RT-PCR was calculated by the $(2* \text{efficiency})^{-\triangle\triangle Ct}$ method, and normalized by the value of the internal control GAPDH. Data were presented as mean of fold change ± SEM *vs* control and are derived from 3 independent experiments(***, $P < 0.01$).

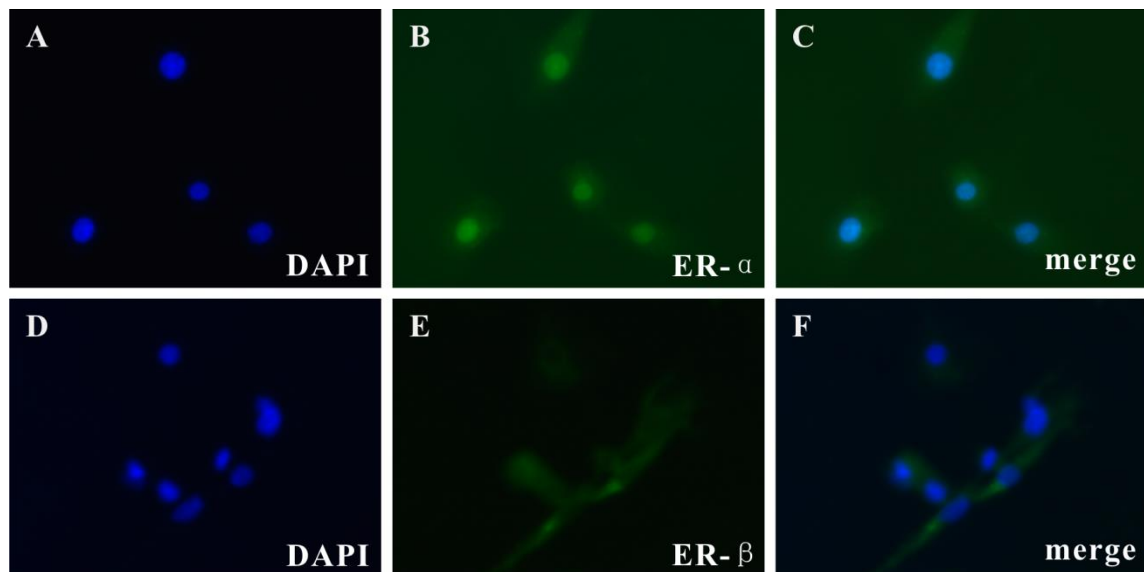

Fig 3.5 Immunofluorescence staining using specific primary antibodies. Localizations of ER-α and ER-β in

human thyroid stem/progenitor cells were determined by immunofluorescence staining using antibodies as described in *Materials and Methods*. In human thyroid stem/progenitor cells, ER-α and ER-β were observed respectively. Most of ER-α was localized to cytoplasm, and ER-β in the nucleus. (A-C), ER-α, (D-F) ER-β in progenitor/stem cells.

3.3 Estrogen promotes proliferation of thyroid stem/progenitor cells

3.3.1 Estrogen increases BrdU incorporation in human thyroid progenitor/stem cells

Proliferative capacity of human thyroid progenitor-stem cells was quantitatively determined by BrdU colorimetric ELISA. To eliminate influence of growth factors and phenol red in the culture medium, the cells were incubated in phenol red-free basal medium for 24 h before stimulation with E_2. BrdU ELISA was performed after cells were exposed to various concentrations of E_2 for 24 h in phenol red-free basal medium. Dose-response analysis showed that a range of E_2 concentrations from 0.1 nM to 10 nM resulted in statistically significant increase of BrdU incorporations. The decrement in BrdU incorporation at 10 nM and 100 nM indicates that the efficacy of E_2 on proliferation is dose-sensitive, with E_2 maximally effective at 1 nM (167.20±4.07% *vs* control, $P<0.01$).

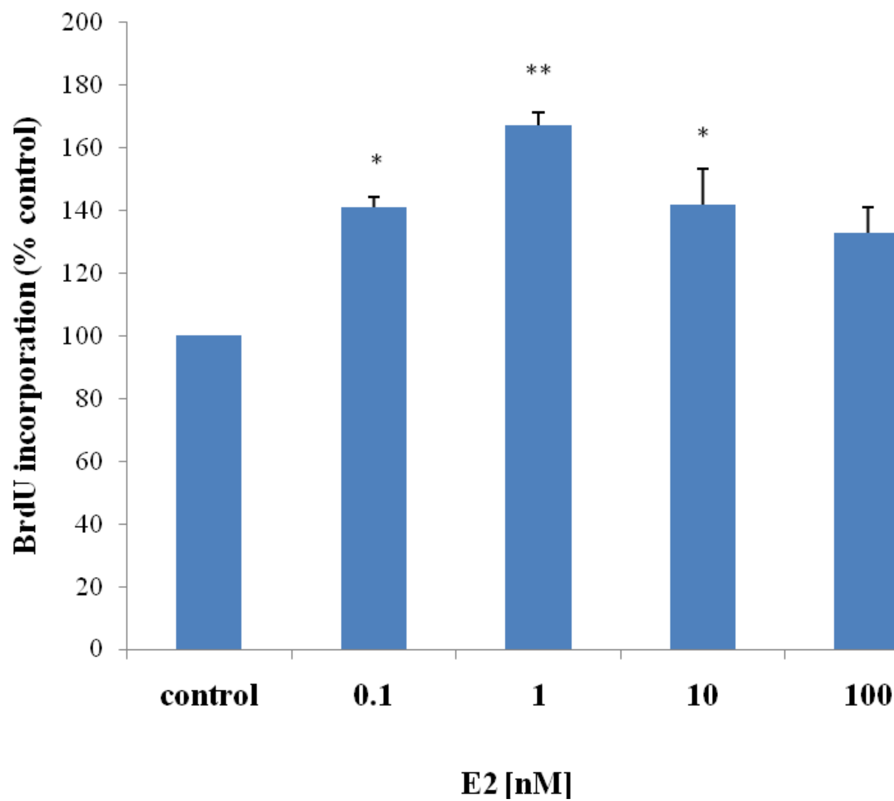

Fig. 3.6 Effects of E_2 on BrdU incorporation. After being dissociated into single cells and seeded into 96-well microtiter plates pre-coated with poly-L-ornithine/fibronectin, human thyroid progenitor/stem cells were incubated under starvation condition (absence of EGF, bFGF and B27 supplement) for 24 hours. The cells were incubated in the presence of various concentrations of E_2 in basal medium for an additional 24 h. Control cells were incubated with basal medium. Cells were incubated with BrdU labeling medium for 4 hours, and BrdU incorporation was measured by BrdU ELISA. Data were derived from 3 independent experiments conducted with four to six replicates. Results were plotted as percentage of vehicle control (mean ± SEM, *, $P<0.05$, **, $P<0.01$).

3.3.2 Estrogen has the potential to maintain the formation of thyrospheres

Human thyroid progenitor cells were dissociated into single cells and stimulated with 20 ng/ml EGF, 20 ng/ml bFGF or 1 nM E_2. During the next 6 days, the 'thyrospheres' were reformed from single cells in culture. Our previous study demonstrated that thyrospheres developed from individual cells and not by multicellular aggregation (28). After being passaged, a small number of cells grew out and reformed spheres under growth stimulation. E_2 was capable of reforming new spheres *in vitro*, although the sphere size was smaller than those in the presence of EGF and bFGF (Fig. 3.7).

3.4 ER-α in stem/progenitor cells was up-regulated by E_2 stimulation

As demonstrated above, ER-α and ER-β were shown to be co-expressed in thyroid progenitor/stem cells by RT-PCR and immunofluorescence staining. To further investigate the effect of E_2 on estrogen receptors, quantitative PCR was conducted. Thyroid progenitor/stem cells were introduced to 1 nM E_2 for 24 h. Then RNA was isolated for quantitative real-time PCR. As shown in Fig. 3.8, level of ER-α was significantly increased by E_2 (4.9 ± 0.32 *vs* control), while ER-β was not.

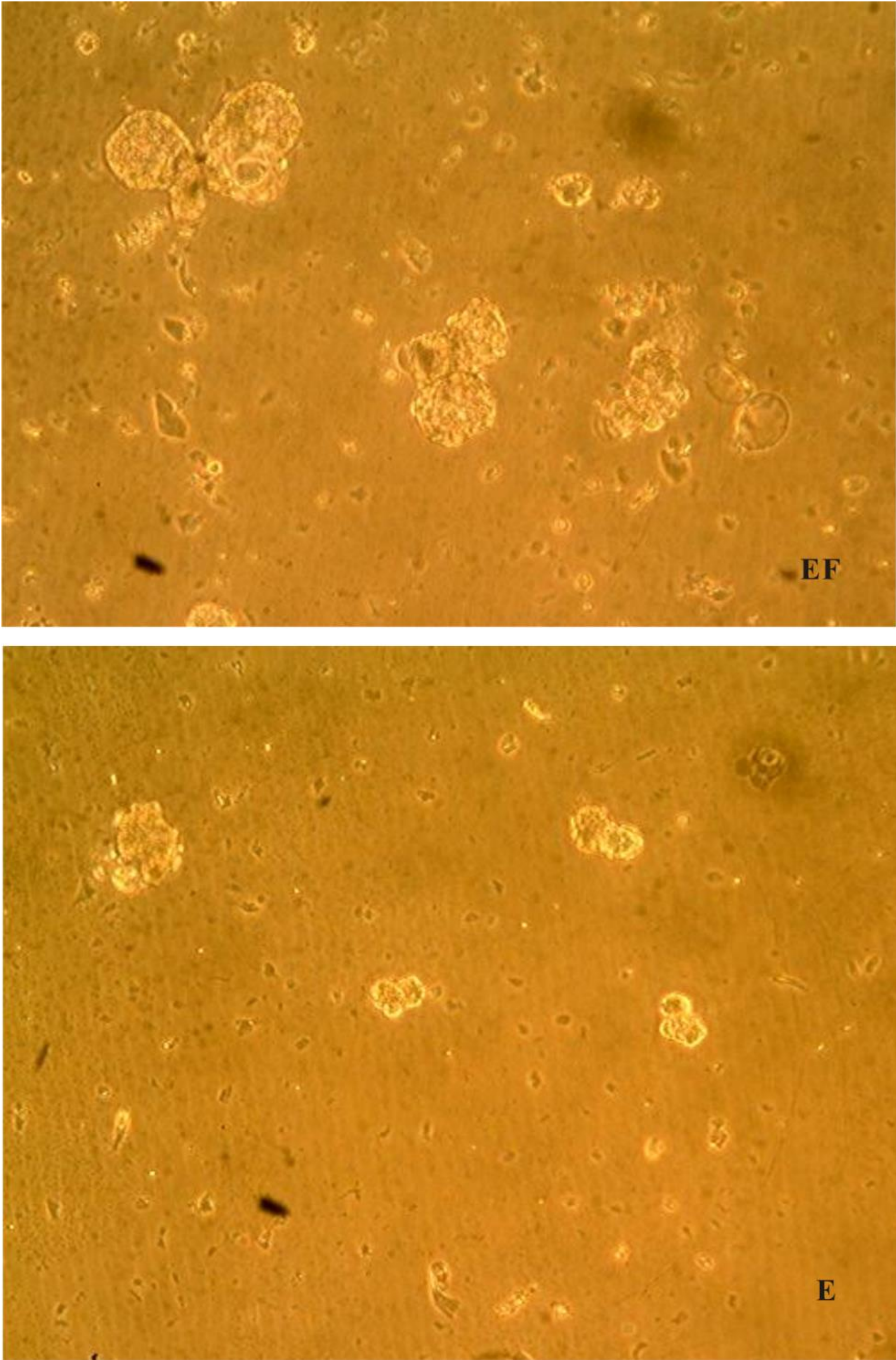

Fig. 3.7 Thyrospheres were dissociated and seeded in defined media including EGF and bFGF or in similar media in which these growth factors had been replaced with E_2 (E), 10×.

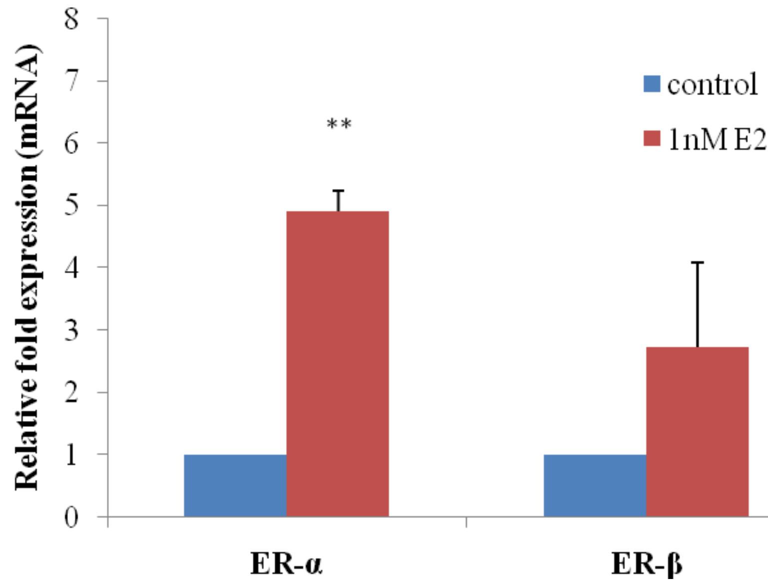

Fig. 3.8 Expression of ER-α and ER-β in thyroid progenitor/stem cells by estrogen stimulation. After cells were incubated with 1 nM E_2 for 24 h, real-time PCR was performed to determine expressions of ER-α and ER-β. The Ct values were calculated by the $(2^* \text{ efficiency})^{-\triangle\triangle Ct}$ method and normalized by the value of the internal control GAPDH. Dates were presented as mean of fold change ± SEM *vs* control and derived from 3 independent experiments, **, $P < 0.01$.

3.5 Cyclin D1 expression in response to E_2 treatment

Cyclin D is a member of the cyclin protein family that is involved in regulating cell cycle progression. The synthesis of cyclin D is initiated during G1 and promotes progression through the G1-S phase of the cell cycle.

There is a great deal of evidence supporting the view that E_2 is able to increase expression of cyclin D1 protein and mRNA level in thyroid cancer cells and human thyrocytes (60, 95). The expression levels of cyclin D1 mRNA were examined after E_2 treatment at various times. Analysis using conventional RT-PCR revealed that mRNA expression of cyclin D1 was increased from 3 h to 24 h after E_2 stimulation (Fig 3.8), indicating that E_2 treatment upregulates cyclin D1 and thus induces growth of human thyroid stem/progenitor cells.

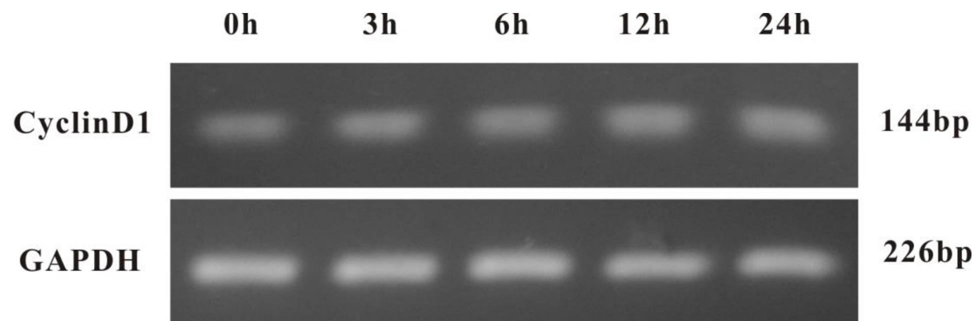

Fig 3.9 Upregulation of cyclin D1 mRNA levels induced by E_2 treatment in thyroid stem/progenitor cells. The expressions of cyclin D1 at mRNA level were determined by RT-PCR. A representative ethidium bromide agarose gel predicts the single band of each RT-PCR product and the accurate size of the PCR product, cyclin D1 (144 bp).

3.6 Effects of E_2 on differentiation of thyroid progenitor cells

3.6.1 Estrogen decreases differentiated markers during differentiation

Discoveries in recent years have suggested that adult stem cells have the ability to differentiate into different types of cell under specific differentiated conditions. To observe the different effects of E_2 and TSH on differentiation of human thyroid progenitor/stem cells, the cells were cultured in basal medium containing 10% serum for 3d, and then exposed to TSH or/and 1 nM E_2 for additional 3 d. Morphology of the cells showed fibroblast-like aspect of progenitor/stem cells when treated for 3 days with 1 nM E_2 (Fig. 3.10). Cells were smaller when treated with E_2 for 3 days, even when TSH was present in the basal medium.

After the secondary passaged cells were treated with or without E_2 for 3 d or 18 d, total RNA was isolated, and cDNA was amplified by RT. Conventional or real-time qPCR was performed to determine gene expressions. Under the influence of TSH in serum-enriched medium, these thyrosphere-derived cells expressed thyroid differentiated markers at an early stage (PAX8, Tg, NIS, TSHr, and TPO) (6 d) (Fig. 3.11) and late stage (21 d) (Fig.3.12). In response to E_2

stimulation, expression levels of some differentiation markers (TSHr, NIS and TPO) were significantly decreased at the early stage as revealed by quantitative PCR analysis. On the other hand, GATA4, an endodermal marker, was significantly upregulated (2.15±0.43% *vs.* control, $P<0.05$). After treatment with E_2 for 21 d, RT-PCR was performed to determine these markers. Corresponding to gene expression in the early state, NIS expression was significantly decreased, while a significantly higher GATA4 expression was observed after E_2 treatment at the late stage (Fig. 3.12).

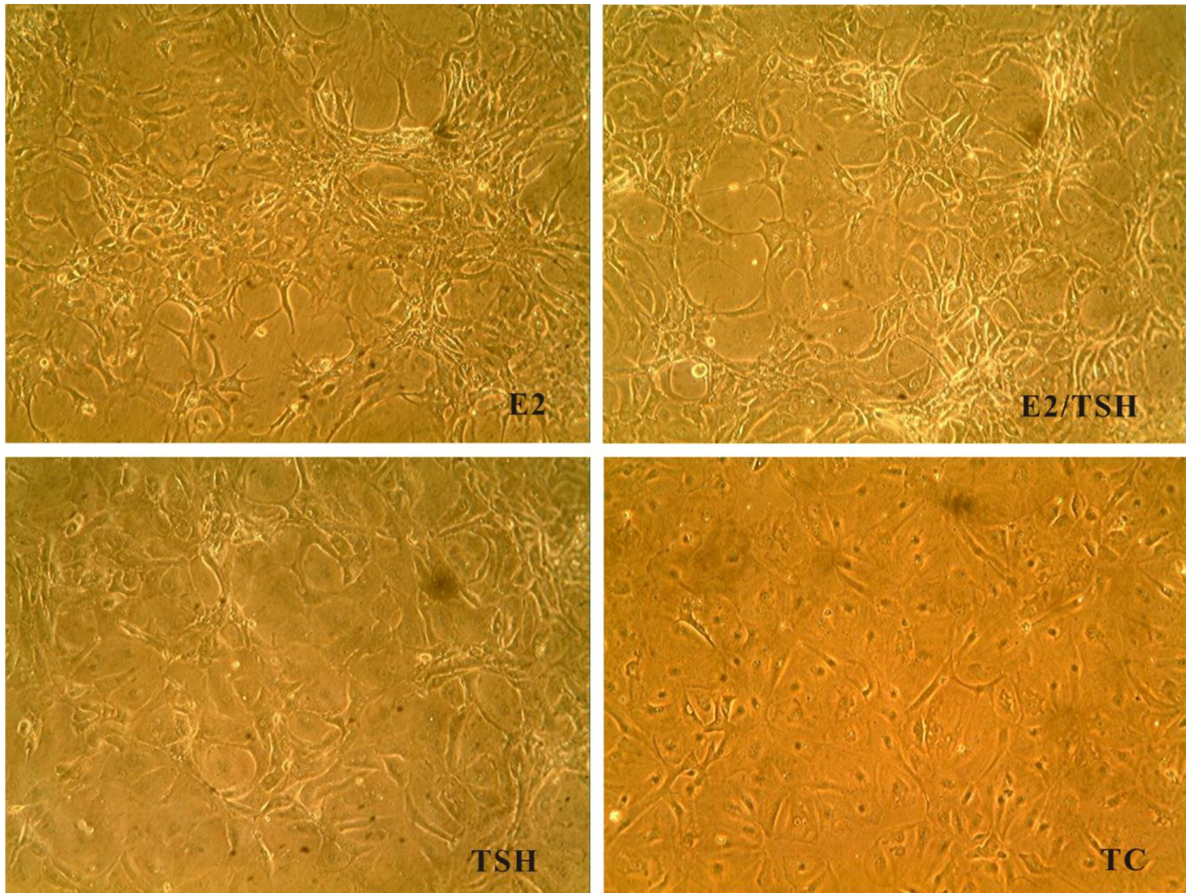

Fig. 3.10 Morphology of human thyroid progenitor/stem cells and thyrocytes. Phase contrast microscopy of living cells(×10). TC, primary human thyrocytes.

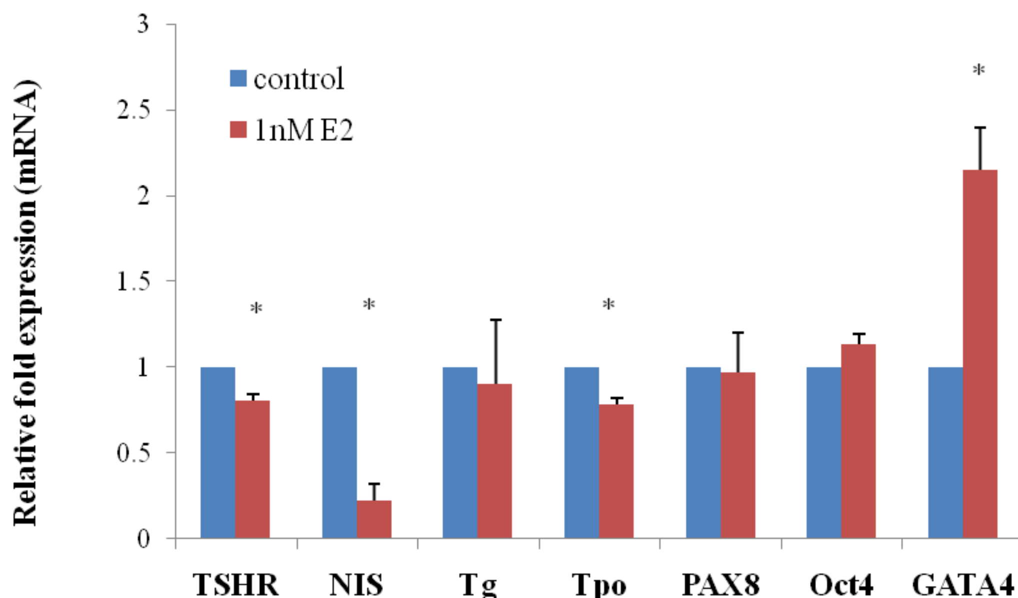

Fig. 3.11 Effects of E_2 on TSH-induced differentiation of human thyroid stem/progenitor cells after 6 d. The Ct values were calculated by the $(2^{*} \text{efficiency})^{-\triangle\triangle Ct}$ method, and normalized by the value of the internal control GAPDH. The effect of estrogen on differentiation had been repeated in 3 independent experiments. Dates were presented as mean of fold change ±SEM *vs* control and derived from 3 independent experiments, *, $P < 0.05$.

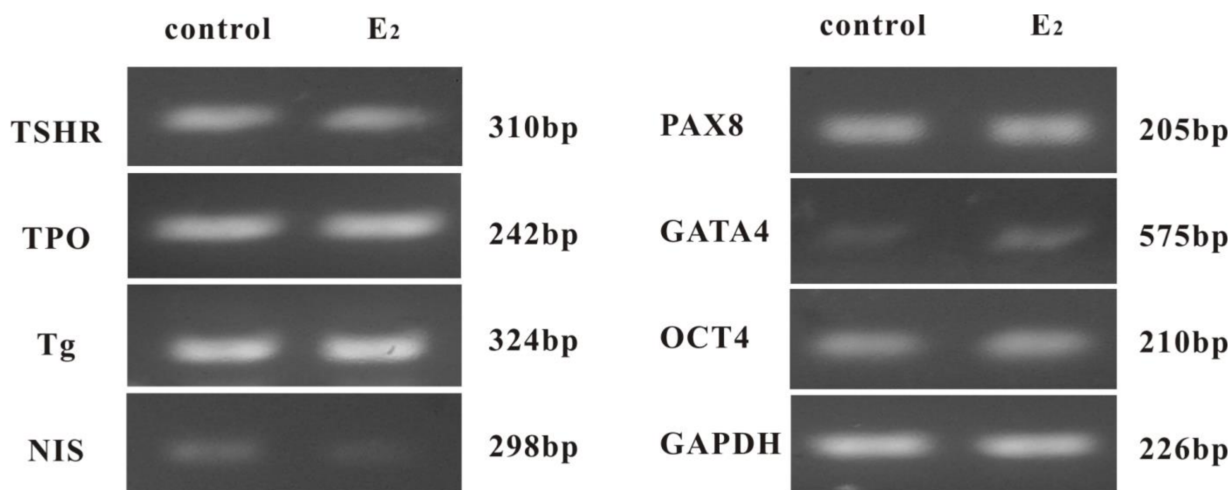

Fig 3.12 Effects of E_2 on TSH-induced differentiation of human thyroid stem/progenitor cells after 21 d stimulation.

3.6.2 E_2 inhibits NIS expression in a dose-dependent manner

The balance between self-renewal and differentiation of stem cells is the key to the regulation of stem cell development. As described above, E_2 has the ability to promote the growth of human adult thyroid stem/progenitor cells. On the other hand, E2 decreased the differentiation marker NIS in the early stage and late stage. To analyze the differential effects of E_2 on growth and differentiation, the thyroid stem/progenitor cells were exposed to various concentration of E_2 for 21 d. RT-PCR was performed to determine the expression of NIS mRNA. NIS was significantly decreased by E_2 stimulation within a physiological range (0.1 nM to 10 nM). 1nM E2 obtained the maximal inhibitory effect on NIS expression, corresponding to the potential of E_2 to induce the proliferation of adult stem cells (Fig. 3.13).

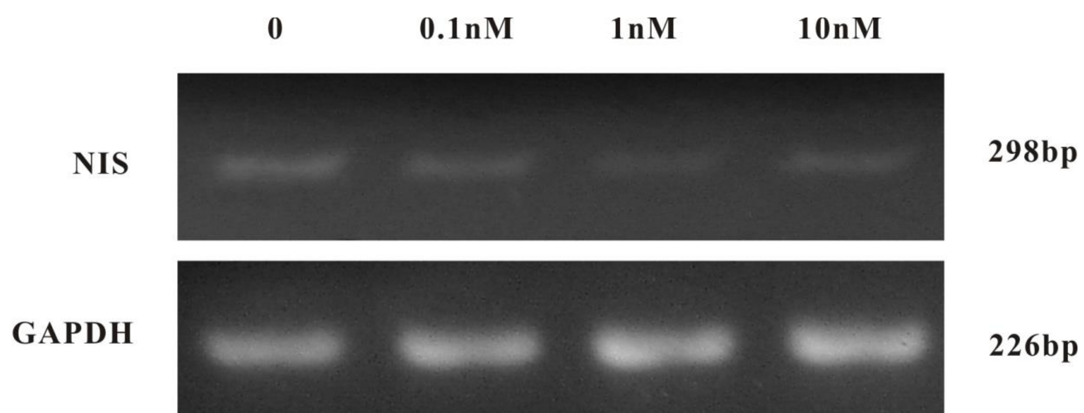

Fig. 3.13 Dose sensitivity of E_2 on NIS expression in differentiation condition. RT-PCR showed expression of NIS mRNAs in thyroid progenitor/stem cells which were treated with serum/TSH and different concentrations of E_2.

3.6.3 Estrogen inhibited TSH-induced expression of sodium/iodide symporter

To evaluate the effects of E_2 on TSH-stimulated expression of sodium/iodide symporter, secondary generated thyroid progenitor/stem cells were exposed to E_2 or/and TSH. As shown in Fig. 3.14, TSH significantly increased expression of NIS mRNA (1.98±0.25 *vs* control, $P<0.01$), whereas TSH-stimulated expression level was significantly suppressed by E_2 (0.89±0.04 *vs*

2.0±0.25).

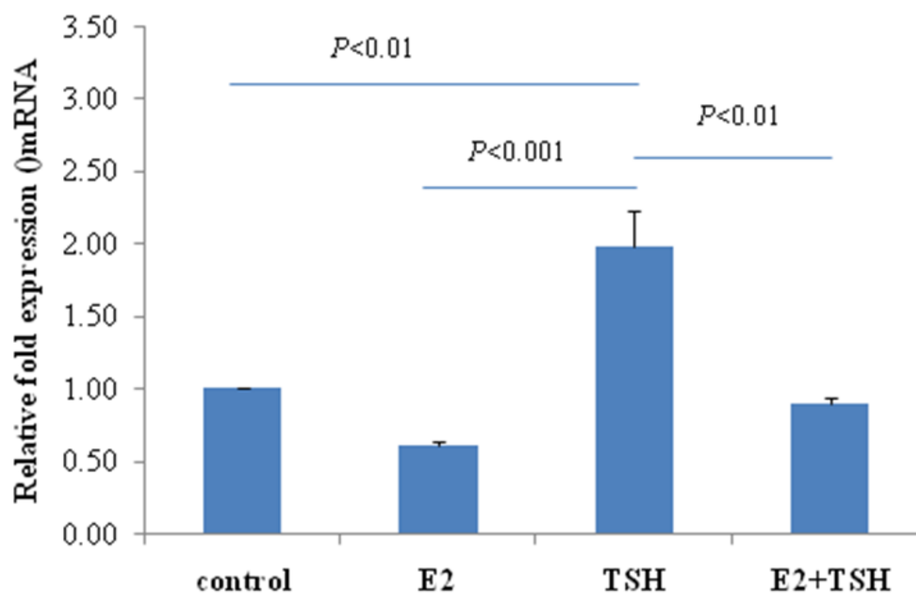

Fig. 3.14 The suppressive effect of E_2 on expression of NIS mRNA. The Ct values were calculated by the (2* efficiency)-ΔΔCt method, and normalized by the value of the internal control GAPDH. Dates were presented as mean of fold change ± SEM vs control and derived from 4 independent experiments. Statistical analysis was performed with nonparametric ANOVA, *, P＜0.05, **, P<0.01.

4. Discussion

Nodular disorders of the thyroid gland are relatively common among adults living in Germany. Using high-resolution ultrasound, a substantially higher prevalence of thyroid nodules (68%) than previously reported was found (7). Nodules arising in hyperplastic endocrine glands may be polyclonal or clonal in origin, and in both lesions a wide range of functional and morphological heterogeneity may emerge secondarily (54). According to recent studies, most nodules, including benign adenoma and papillary thyroid carcinoma, are of clonal origin, considering the clonality as the most important single characteristic of thyroid neoplasias (45, 46, 95, 96). In most cases, the spontaneous proliferation of a benign neoplasia could be the underlying mechanism for autonomous growth of thyroid lesions (1). However, the causes of spontaneous neoplasia growth in human thyroids are still unclear, since the human thyroid cells have been estimated to divide only 5 times during adulthood, but cell division is a prerequisite to gaining molecular alterations that lead to neoplastic growth.

In recent years, stem cell research has opened new pathways for understanding many of the physiological mechanisms controlling basic biological processes as well as disease mechanisms. Adult stem cells which correspond to remnants of the fetal thyroid are a putative source of thyroid neoplasia (25). Our group identified properties of human thyroid stem/progenitor cells derived from goiter, including a capacity of self-renewal and the ability to differentiate into more than one cell type (25, 28). The cancer stem cells that may originate from normal stem cells undergoing an aberrant differentiation were also isolated and identified from anaplastic thyroid carcinomas cell lines (35). Some markers for pluripotent embryonic and adult stem cells, such as Oct4 and ABCG2, are expressed in both stem cells derivation from normal thyroid tissues and anaplastic thyroid carcinoma cell lines. Moreover, some other studies confirmed the presence of stem cells in human thyroid and various thyroid cancer cells (34, 37).

The initial doubling time of stem cells was about 12 h, which is much shorter than that of normal human thyrocytes (28). As reported previously, in a non-adherent three-dimensional culture, these stem cells are able to form spheres in a medium enriched with EGF and bFGF (28, 34). The

average cell counts in each sphere increased significantly during the first several days in culture. Recent studies have found that adult neural stem cells transform into tumorigenic cell lines or cancer stem cells after expansion *in vitro* (97). This suggests that expansions of these stem cells for therapeutic strategies could also harbor the risks for amplifying progenitor cells with acquired genetic abnormalities and therefore induction of tumors after transplantation (97-99). In response to intensive growth factors stimulation, stem cells can outgrow as thyrospheres, thus revealing new clues for the relationship between stem cells and neoplastic thyroid diseases (100).

It has been suggested that growth factors play an important role in the pathogenesis of human thyroid goiters and carcinomas (101, 102). Expression of growth factor receptors is increased in human goiters, and bFGF is found to inhibit thyroid function measured by ^{125}I uptake in primary human thyrocytes (103). EGF enhances migration and invasiveness of thyroid cancer, and is also able to induce thyroid cell proliferation with the loss of thyroid-specific functions, including iodide uptake (104). Moreover, after human thyrocytes were treated with EGF and serum, the evolution showed pattern of stimulated cells converges to the pattern of PTCs in which the cells have a chronically activated MAPK pathway (105). This evidence suggests that growth factors play an important role in the regulation of growth and function of thyroid.

Estrogens are potential mutagens in a number of target tissues including the thyroid gland where they play a pivotal role in the growth regulation of human thyroid cells and tumors (60). Several lines of evidence support the concept that the EGFR-MAPK signaling axis is a common pathway that is regulated by estrogen. Estrogen triggers rapid yet transient activation of the MAPKs, extracellular signal-regulated kinase Erk-1 and Erk-2. Coordinated signaling between growth factor receptors and estrogen receptors is required for controlled growth of normal mammary epithelium. EGF-related ligands enhance ER transcriptional activity (106, 107), and this has been shown to result from MAPK-mediated phosphorylation of serine 118 within the activation function II (ATF-II) domain of the ER (108). Interestingly, estrogen was proved to induce human neural progenitor cell proliferation mediated by ER-activated expression of pERK (76). Estrogen stimulates proliferation of mouse embryonic stem cells, and this action is mediated by MAPKs, CDKs, or protooncogenes (70).

In the floating culture system, the average cell count in each sphere and sphere size reached a plateau after the first 5 days of cell growth (28). This is explained by the fact that with increasing size of spheres, proliferation rate slows down; many stem and progenitor cells stop growing and start differentiation. The central portion may contain degenerating cells that probably die from worsened nutrition conditions in large spheres (94). Fierabracci reported that the ‘spheroids’ were passaged into secondary generation after being mechanically dissociated into single cells and then resuspended in the fresh medium with growth factors (EGF and bFGF) in new tissue culture plates (34). After the next 7 days, these cells were reformed into ‘spheroids’, hampering the long-term stimulation of estrogen to each cell. In an alternative approach, poly-L-ornithine and fibronectin were used to coat cell culture dishes to promote attachment and expansion of stem cells (109-112).

In the present study, poly-L-ornithine and fibronectin coating also induced dispersed thyroid stem/progenitor cells to attach and grow in the presence of growth factors and the absence of serum. The expression of Na^{+}/I^{-} symporter (15) (15) as a differentiation marker of thyroid follicular cells was undetectable in thyrospheres and secondary passaged thyroid stem/progenitor cells. NIS is an integral plasma membrane glycoprotein of the thyroid gland, which mediates the active transport of iodine into the thyroid follicular cells as the crucial first step for thyroid hormone biosynthesis (113). When differentiation was induced by stimulation with TSH for 21 d, thyrosphere-derived cells expressed NIS but lost expression of stem cell marker Oct4 (28). Therefore, the increased Oct4 expression and negative NIS expression can be helpful in identifying the thyroid stem/progenitor phenotype of secondary passaged cells under the new culture conditions.

Estrogen was reported to promote growth of various cells including normal human thyrocytes, thyroid cancer cells, and stem cells. The secondary generation of thyroid stem/progenitor cells was also induced to proliferate in response to E_2 stimulation in a dose-sensitive manner (Fig. 3.6). E_2 was maximally effective in inducing BrdU-incorporation at 1 nM. Thyroid stem cells generated new spheres when cultured with 1 nM E_2 in the absence of EGF and bFGF, although

the size was smaller than that of spheres in the presence of EGF and bFGF (Fig. 3.7). The smaller size may indicate that E_2 has a different potential to maintain growth of stem cells *in vitro* than EGF and bFGF (76).

Our previous study found that 10 nM E_2 is able to significantly increase the percentage of BrdU-labeled cells in thyroid carcinoma cells and thyrocytes derived from nodules (60). Furthermore, FRTL-5 cell growth was maximally induced only when 100nM E_2 was used (86). The study showed that 100 nM E2 was also the maximally effective dose for proliferation of human neural stem/progenitor cells (76). These data suggest that different types of cells have different sensitivity to E_2 stimulation. In females, the serum estrogen level is lower than 0.28 nM in the follicular phase and 1.1 nM in the luteal phase in premenopausal women, but it may increase to 150 nM in the third trimester of pregnancy (114). These dates suggest that the fluctuation of serum estrogen level may regulate the growth of various types of cells in human thyroid, particularly thyroid stem/progenitor cells, which may explain why females are more prone to develop thyroid neoplasias (5).

The present work demonstrated that ER-α and ER-β mRNAs and protein are co-expressed in thyroid stem/progenitor cells as revealed by RT-PCR, quantitative PCR and immunofluorescence staining (Fig. 3.3, Fig. 3.5). In contrast to ER-β, ER-α mRNA expression level in thyroid stem/progenitor cells was significantly higher than that in normal thyrocytes (Fig. 3.3, Fig. 3.4). In previous studies, both ER-α and ER-β have been identified in human thyrocytes derived from goiter, human thyroid carcinoma cell lines, and cell lines derived from rat primary thyroid tumors (60, 86-88, 115-117).

ER-α and ER-β have distinct transcriptional abilities and may regulate distinct cellular pathways although both of them share similar mechanisms of action (118). Particularly when ERs are co-expressed, ER-α participates in the initiation and progression of neoplasia, while ER-β may exhibit an inhibitory action on ER-α mediated gene expression (85, 119). ER-α mRNA has been reported to be upregulated during carcinogenesis of breast cancers, whereas ER-β mRNA is downregulated (120). ER-α mRNA expression levels are significantly higher in follicular

adenomas than in follicular carcinomas, accordingly, ratios of ER-α to ER-β mRNA levels are significantly higher in follicular adenomas compared to follicular carcinomas and normal thyroid tissues (117). Recently, increasing evidence supported that ER-α and ER-β exert differential roles in the apoptosis of thyroid cancer cells. The apoptosis of thyroid papillary carcinoma cells (KAT5), follicular thyroid carcinoma cells (FRO) and anaplastic carcinoma cells (ARO) is positively associated with ER-β but negatively with ER-α (88). In general, ER-α functions as a promoter for growth of benign or malignant thyroid tumors, whereas ER-β as a suppressor, indicating that the imbalance between ER-α and ER-β may contribute to thyroid carcinogenesis (Fig. 4.1) (84).

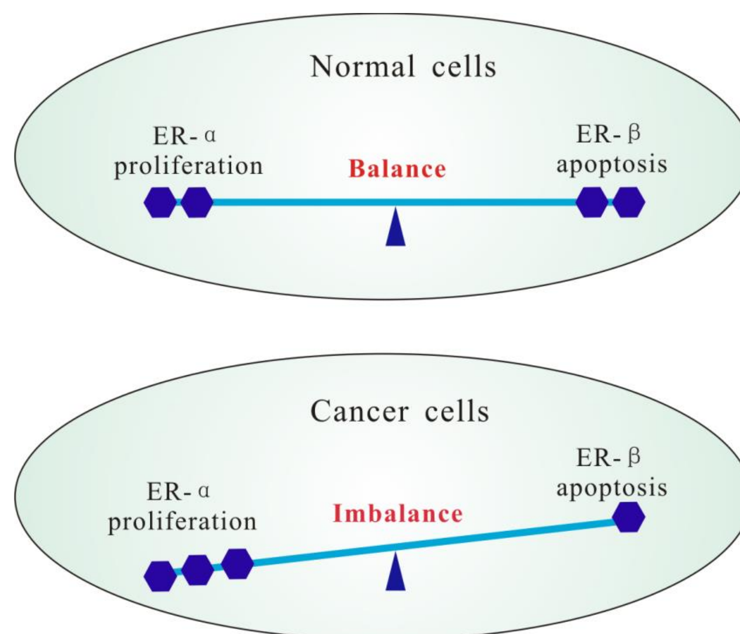

Fig. 4.1 Schematic illustration of ER-α and ER-β balance shift and tumor development.

[Taken from: Chen GG, Vlantis AC, Zeng Q, et al. Regulation of cell growth by estrogen signaling and potential targets in thyroid cancer. Curr Cancer Drug Targets, 2008, 8:367-377(84).]

More recently, ERs also have been simultaneously detected by RT-PCR and by western blot in embryonic stem cells and adult stem cells (70, 77, 78, 92, 112). ER-α and ER-β are observed to be co-expressed in mouse ES cells, and both of them are upregulated by estrogen treatment (70), while ER-β was the predominant ER in human neural stem/progenitor cells (76). In the present

study, however, only ER-α was upregulated by E_2 treatment of human thyroid stem/progenitor cells in vitro (Fig. 3.8). The higher expression of ER-α after E_2 stimulation may suggest that estrogen regulates the growth of stem cells via ER-α.

The cell growth promoted by estrogen was reported to be associated with an increased expression of cyclin D1. Overexpression of cyclin D1 plays important roles in the development of many human cancers including parathyroid adenoma (121), breast cancer (122), colon cancer (123), lung cancer (124), pancreatic carcinoma (125), and pituitary tumors (126). Cyclin D1 encodes the regulatory subunit of a holoenzyme that phosphorylates and inactivates the retinoblastoma protein, and thus promotes progression through the G1-S phase of the cell cycle (127). Some studies found that cyclin D1 is overexpressed in cold thyroid nodules (CTNs) and papillary thyroid carcinomas associated with poor prognosis (128-130). Estrogen is believed to activate cyclin D1 gene expression via ER-α, while it inhibits cyclin D1 expression via ER-ß (119). Previous research in mammary cancer cells demonstrated that cyclin D1 is an important target gene through which estrogen-complexed ER-α mediates its proliferation (119).

Our previous study also demonstrated that the growth stimulatory effect of E_2 on benign and malignant thyroid cells was associated with an increased expression of cyclin D1 (60). In the present study, the expression level of cyclin D1 mRNA was increased in thyroid stem/progenitor cells after stimulation with 1 nM E_2 (Fig. 3.9), demonstrating that cyclin D1 is also involved in cellular proliferation of thyroid stem/progenitor cells. Cyclin D1 mRNA expression was increased 3 h after E_2 stimulation of thyroid stem/progenitor cells. Stimulation of E_2 to G0/G1-arrested cells resulted in induction of cyclin D1 mRNA within 1-3 h and a three- to five fold increase in synthesis and steady-state levels of cyclin D1 protein within 3-6 h (131).

An important characteristic of a stem cell is its pluripotency or multipotency, which implies that adult stem cells have the remarkable potential to develop into different cell types in vivo or vitro (132). In response to serum and TSH, the thyrosphere-derived cells are able to produce thyroid-like cells with expression of thyroid differentiation makers PAX8, Tg, NIS, TSHr, and TPO (28, 34). After being embedded into collagen in a 3D culture system, these differentiated

cells formed thyroid follicular-like structures that displayed TSH-dependent ^{125}iodide uptake (28). In addition, thyrospheres may also develop into cells expressing neuronal marker β-tubulin III when they are co-cultured with the neuroblastoma cell line, and differentiate towards to adipogenic lineage, confirming their pluripotency (34).

Stem cells are faced with a uniquely difficult task: to avoid cell cycle exit and differentiation, and to avoid uncontrolled proliferation or even tumor formation (133, 134). Maintaining the balance between self-renewal and differentiation is an important issue in stem cell and cancer biology, and it allows stem cells to undergo proliferation only to sustain tissue regeneration or repair (135). During embryonic development, various niche factors act on embryonic stem cells to alter gene expression, and induce their proliferation or differentiation for the development of the fetus (136). Therefore, the better understanding of balance between self-renewal vs. differentiation of stem cells might be the significant basis for understanding the thyroid organogenesis, thyroid cancer biology, and in designing further stem cell research.

Our current study demonstrates that E_2 has the potential to induce the proliferation of adult thyroid stem cells. Thyrosphere reformation in the presence of E_2 suggests the ability of E_2 to promote the division of stem cells *in vitro* as well. It is very crucial to investigate the influence of E_2 on the differentiation of adult thyroid stem cells that is the other end of the rope.

Thyroid stimulating hormone (TSH) is the primary hormonal regulator of thyroid function and stimulates iodide accumulation in the thyroid (137). The TSH is able to stimulate NIS transcription and biosynthesis, and it is also required for targeting NIS to and/or retaining it at the plasma membrane (113), whereas E_2 blunts TSH-induced expression of NIS in adult thyrosphere-derived cells (Fig. 3.14).

TSH was reported to have the potential to induce ES cells to differentiate into thyroid cells (23). Under the influence of TSH and serum, adult thyroid stem cells are also induced to differentiate into thyrocytes that expressed PAX8, TG, NIS, TSHr, and TPO, which is consistent with our previous study. Notably, the gene profile of thyrosphere-derived cells showed that GATA4, an

endodermal marker, is upregualted by E_2 treatment but not by Oct4 or differentiation markers (Fig. 3.11). As demonstrated by quantitative analysis, only NIS, TSHR, TPO and GATA4 are significantly regulated by E_2 stimulation in the early stage of differentiation process (Fig. 3.12).

Thyroid follicular cells have also been proved to contain functional ERs that enhance cell growth and inhibit expression of NIS (86, 138). Estradiol is able to affect thyroid cells derived from goiters directly (60). Compared with normal thyrocytes, stem cells display a much higher proliferation rate on growth stimulation (28). Under intensive stimulation with estrogen, asymmetric cell division gains more progenitor cells (Fig. 1.4, Fig. 4.3). On the other hand, clinical and experimental data demonstrated low thyroid hormone synthesis and significantly reduced NIS expression in thyroid nodules (139, 140). Therefore, progenitor cells characterized by lower NIS expression may suggest a putative role of the offspring of stem cells in chronic estrogen-stimulated nodular transformation of the thyroid in females who develop more nodules than man (60).

New data also support the link between stem cells and tumors. Tumors may originate from transformed stem cells. Continuous and longtime expansion of precursor cells in culture was thought to harbor the risk of spontaneous transformation which is likely to result in tumor formation in graft recipients (97). The spontaneous immortalization of adult neural stem cells leads to development of an immortal clonal population with a potential to produce high-grade and invasive brain tumors in immunodeficient mice (97, 99). Cancer stem-like cell lines were further generated from adult neural precursors following expansion in a culture assay adapted to brain stem and progenitor cells (98). Similar signaling pathways for growth may regulate self-renewal in stem cells and cancer cells, and cancer cells may include cancer stem cells that drive tumorigenesis (141). Both stem cells and cancer stem cells (CSCs) have the ability of asymmetric cell division, i.e. they might generate daughter cells containing a self-copy (self-renewal) and progenitor cells. CSCs are defined as transformed cells that are thought to share several characteristics with normal stem cells.

Does thyroid tumor arise from oncogenic transformation of stem cells into CSCs? Our recent

study successfully demonstrated that CSCs derived from anaplastic thyroid carcinoma cell lines expressed ABCG2 and MDR1 transporters of the ABC gene family, which enabled the exclusion of the Hoechst dye from CSC that otherwise binds to the DNA in non-stem cells (35). This supports the hypothesis that a small population of tumor cells termed CSCs can be regarded as the origin of thyroid cancer.

More evidences are required to confirm the transformation of thyroid stem cells to CSCs and the mechanisms. So far, several possible mechanisms have been suggested, including DNA double-stranded breaking (99), aberrantly activated Notch signaling (97), constitutive and excessive activation of the PDGFRα pathway (98, 142). Other signaling pathways associated with oncogenesis, such as Sonic hedgehog (Shh) and Wnt signaling pathways, may also play an important role in regulating stem cell self-renewal and its transformation (141). The crosstalk and interrelationship between stem cell and cancer stem cell will focus light on new potential research into stem cells and thyroid diseases.

Based on current knowledge, a schematic diagram has been drawn for the role of stem cells in the physiological and pathological development of the thyroid (Fig. 4.2). The essentially quiescent stem cell may proliferate to give birth to a daughter stem cell and a progenitor cell by asymmetric cell division. Under normal conditions, stem cells stimulated by TSH and serum undergo differentiation into normal and functional thyroid cells. However, iodide deficiency, accumulated mutation, apoptosis of thyroid cells, operative in ageing thyroids, higher level of estrogen, and other locally expressed growth factors might cause the alterations of niches. One possibility is that thyroid stem cells undergo aberrant differentiation to promote the growth of the partly differentiated progenitor cells and thus form thyroid nodules (51). Another possibility is that transformation of stem cells to cancer stem cells is initiated, resulting in the development of undifferentiated, poorly differentiated or differentiated thyroid cancers.

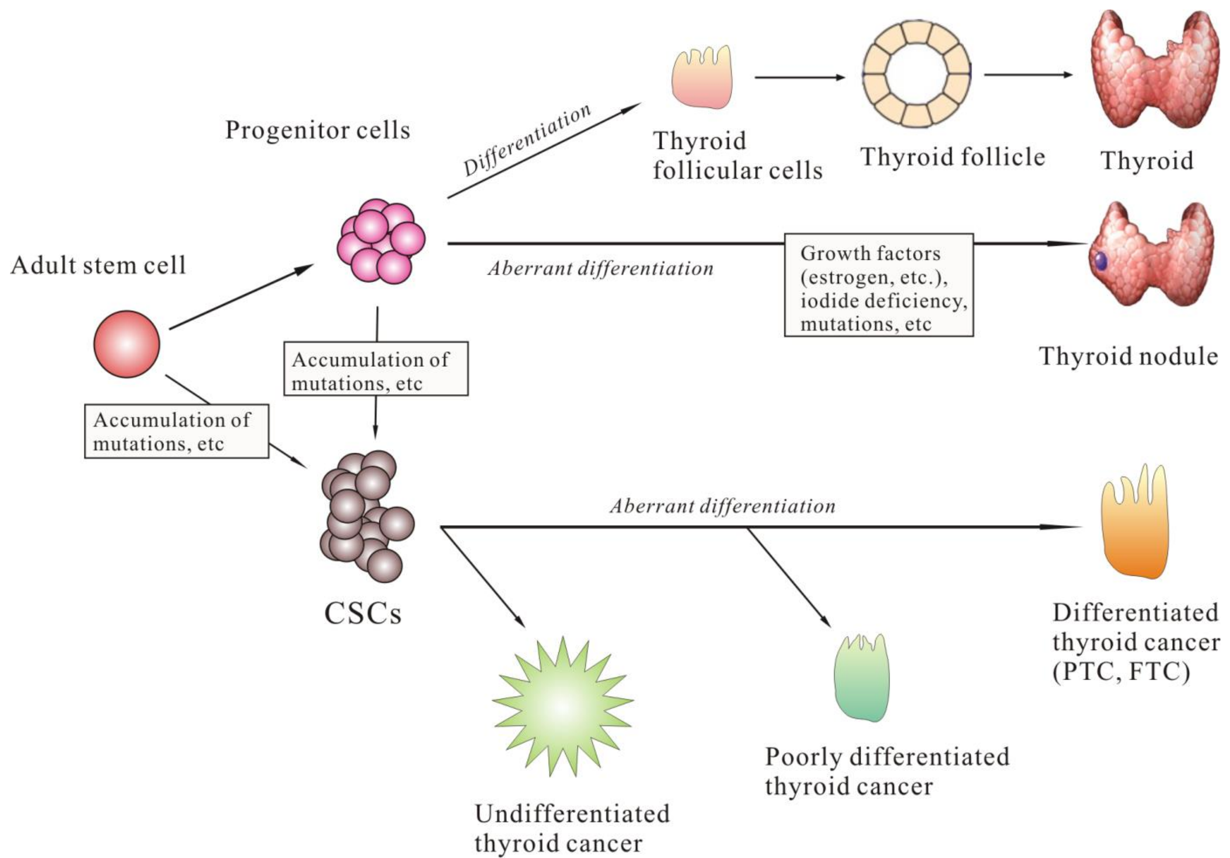

Fig. 4.2 Stem cell hypothesis for thyroid diseases. CSCs, cancer stem cells, PTC, papillary thyroid carcinoma, FTC, follicular thyroid carcinoma.

Our current study paves a path for further research into the putative link between stem cells and thyroid nodular diseases. Aberrant differentiated thyroid stem cells under estrogen stimulation display an increasing ratio of ER-α to ER-β, upregulation of cyclin D1, lower expression of NIS, and number of progenitor cells with not fully-achieving differentiation. Further studies are required to investigate the gene profiles among aberrantly differentiated stem cells, benign and malignant thyroid nodules. In addition, the molecular and cellular events *in vivo* that may occur in the tumorigenesis of adult stem cells need to be studied. This may suggest a link between stem cell and thyroid nodules or tumors.

5. Summary

Nodular disease of the thyroid gland has been rising worldwide. Its prevalence is 4 times higher in women than in men. Both epidemiological and experimental cell research has demonstrated that estrogen plays an important role in the pathogenesis of thyroid nodules. However, a comprehensive understanding of the relevance of estrogen in pathogenesis of thyroid nodular disease is still missing. Ongoing advances in stem cell research have opened new avenues for research. The purpose of my current study was to determine whether estrogen has the potential to promote the proliferation of human thyroid stem/progenitor cells, clarify the intracellular events involved in E_2-induced stem/progenitor cells proliferation and analyze the influence of E_2 on TSH-induced differentiation of progenitor cells into thyrocytes.

Intense growth stimulation of stem cells resulted in the formation of spheres that consist of highly proliferating stem and progenitor cells. After passaged into secondary generation and starved from growth factors, thyroid stem/progenitor cells proliferated in response to E_2 stimulation in a dose-dependent manner. 1 nM E_2 was maximally effective to induce BrdU incorporation and also to generate new thyrospheres in the absence of other growth factors. The results further demonstrated that ER-α and ER-β were co-expressed in thyroid stem/progenitor cells, and regulate distinct cellular pathways. Only ER-α expression was significantly upregulated by E_2 stimulation. The cell growth promoted by estrogen was associated with an increased expression of cyclin D1 that promotes progression through the G1-S phase of the cell cycle. Under the influence of TSH, adult thyroid stem cells were induced to differentiate into thyrocytes (expression differentiation marker as NIS, thyroglobulin, etc.). By estrogen stimulation, NIS was simultaneously decreased significantly.

The stimulation effect on growth and inhibitory effect on NIS expression of estrogen reminds of cell clone with a higher than average growth potential and a lower NIS expression as the origin of non-functional thyroid nodules in females. The results demonstrate the molecular cascade and related cell biology events involved in estrogen-effected proliferation and differentiation of thyroid stem cells in vitro, and suggest a link between stem cells and the pathogenesis of thyroid

nodules. Further studies are required to investigate the gene profiles among aberrantly-differentiated stem cells and benign and malignant thyroid nodules.

ZUSAMMENFASSUNG IN DEUTSCHER SPRACHE

Die Entwicklung von Schilddrüsenknoten nimmt weltweit zu. Frauen sind 4 mal häufiger betroffen als Männer. Epidemiologische und zellbiologische Studien haben gezeigt, dass Östrogene in der Pathogenese von Schilddrüsenknoten eine wichtige Rolle spielen. Die Bedeutung von Östrogen in der Pathogenese von Schilddrüsenknoten ist jedoch in ihren Einzelheiten noch unbekannt. Die Fortschritte der Stammzellforschung haben auch auf diesem Gebiet neue Wege eröffnet.

Ziel meiner gegenwärtigen Arbeit war es:
1. zu analysieren, ob Östrogene die Proliferation menschlicher Schilddrüsenstamm-/progenitorzellen stimulieren,
2. die intrazellulären Veränderungen, die in der Östrogen-induzierten Stamm-/Progenitorzellproliferation involviert sind, aufzuklären und
3. den Einfluss von Östrogenen auf die TSH-induzierte Differenzierung von Progenitorzellen in Schilddrüsenzellen zu analysieren.

Eine intensive Stimulation von Stammzellen führte zur Bildung von sogenannten „spheres", die sich aus stark proliferierenden Stamm- und Progenitorzellen zusammensetzen. Nach Passage dieser Zellen in eine zweite Generation und nach Entzug von Wachstumsfaktoren proliferierten diese Schilddrüsenstamm- und progenitorzellen nach Stimulation mit Östrogen dosisabhängig. Mit 1 nM E_2 wurde der maximaler Einbau im BrdU-ELISA erreicht und gleichzeitig in Abwesenheit von Wachstumsfaktoren die Entstehung neuer „thyrospheres" induziert. Weiter wurde gezeigt, dass ERα und ERß in Schilddrüsenstammzellen und -progenitorzellen co-exprimiert werden und unterschiedliche Signalwege aktivieren. Allerdings wurde nur die ER-Alpha-Expression signifikant durch eine E_2-Stimulation hoch reguliert.

Die Stimulation des Zellwachstums durch E_2 war assoziiert mit einer vermehrten Expression von Cyclin D_1, das für die Progression durch die G_1 bis S-Phase des Zellzyklus verantwortlich ist.
Unter dem Einfluss von TSH wurde die Differenzierung adulter Schilddrüsenstammzellen in

reife Thyreozyten induziert mit vermehrter Expression von Differenzierungsmarkern (u. a. NIS). Durch Östrogenstimulation wurde dagegen die NIS-Expression signifikant gehemmt.

Die wachstumsstimulierende Wirkung von E_2 und ihr hemmender Effekt auf die NIS-Expression erinnern an Zellklone mit einem überdurchschnittlichen Wachstumspotential und einer verminderten NIS-Expression, die als Ursprung nicht-speichernder Schilddrüsenknoten diskutiert werden. Die Ergebnisse dieser Arbeit zeigen die molekularen und zellbiologischen Veränderungen, die E_2 auf die Proliferation und die Differenzierung von Schilddrüsenstammzellen in vitro ausübt und lassen eine Verbindung zwischen den Stammzellen und der Pathogenese von Schilddrüsenknoten vermuten. Weitere Studien sind erforderlich, um die Genprofile aberrant differenzierter Stammzellen und benigner und maligner Schilddrüsenknoten miteinander zu vergleichen.

6. References

1. **Derwahl M, Studer H** 2002 Hyperplasia versus adenoma in endocrine tissues: are they different? Trends Endocrinol Metab 13:23-28
2. **Mazzaferri EL** 1992 Thyroid cancer in thyroid nodules: finding a needle in the haystack. Am J Med 93:359-362
3. **Tan GH, Gharib H** 1997 Thyroid incidentalomas: management approaches to nonpalpable nodules discovered incidentally on thyroid imaging. Ann Intern Med 126:226-231
4. **Hoermann R, Quadbeck B** 1998 Course of thyroid nodules. Exp Clin Endocrinol Diabetes 106 Suppl 4:S27-28
5. **Ghassi D, Donato A** 2009 Evaluation of the thyroid nodule. Postgrad Med J 85:190-195
6. **Hegedus L, Bonnema SJ, Bennedbaek FN** 2003 Management of simple nodular goiter: current status and future perspectives. Endocr Rev 24:102-132
7. **Guth S, Theune U, Aberle J, Galach A, Bamberger CM** 2009 Very high prevalence of thyroid nodules detected by high frequency (13 MHz) ultrasound examination. Eur J Clin Invest 39:699-706
8. **Teng W, Shan Z, Teng X, Guan H, Li Y, Teng D, Jin Y, Yu X, Fan C, Chong W, Yang F, Dai H, Yu Y, Li J, Chen Y, Zhao D, Shi X, Hu F, Mao J, Gu X, Yang R, Tong Y, Wang W, Gao T, Li C** 2006 Effect of iodine intake on thyroid diseases in China. N Engl J Med 354:2783-2793
9. **Mazzaferri EL** 1993 Management of a solitary thyroid nodule. N Engl J Med 328:553-559
10. **Farid NR, Shi Y, Zou M** 1994 Molecular basis of thyroid cancer. Endocr Rev 15:202-232
11. **Krohn K, Stricker I, Emmrich P, Paschke R** 2003 Cold thyroid nodules show a marked increase in proliferation markers. Thyroid 13:569-575
12. **Coclet J, Foureau F, Ketelbant P, Galand P, Dumont JE** 1989 Cell population kinetics in dog and human adult thyroid. Clin Endocrinol (Oxf) 31:655-665
13. **Romagnoli S, Moretti S, Voce P, Puxeddu E** 2009 Targeted molecular therapies in

thyroid carcinoma. Arq Bras Endocrinol Metabol 53:1061-1073

14. **Thomas D, Friedman S, Lin RY** 2008 Thyroid stem cells: lessons from normal development and thyroid cancer. Endocr Relat Cancer 15:51-58
15. **Klonisch T, Hoang-Vu C, Hombach-Klonisch S** 2009 Thyroid stem cells and cancer. Thyroid 19:1303-1315
16. **Johnson BV, Shindo N, Rathjen PD, Rathjen J, Keough RA** 2008 Understanding pluripotency--how embryonic stem cells keep their options open. Mol Hum Reprod 14:513-520
17. **Wobus AM, Boheler KR** 2005 Embryonic stem cells: prospects for developmental biology and cell therapy. Physiol Rev 85:635-678
18. **Lowry WE, Richter L** 2007 Signaling in adult stem cells. Front Biosci 12:3911-3927
19. **Barrilleaux B, Phinney DG, Prockop DJ, O'Connor KC** 2006 Review: ex vivo engineering of living tissues with adult stem cells. Tissue Eng 12:3007-3019
20. **Okano H, Sawamoto K** 2008 Neural stem cells: involvement in adult neurogenesis and CNS repair. Philos Trans R Soc Lond B Biol Sci 363:2111-2122
21. **Yamashita YM, Yuan H, Cheng J, Hunt AJ** 2010 Polarity in stem cell division: asymmetric stem cell division in tissue homeostasis. Cold Spring Harb Perspect Biol 2:a001313
22. **Arufe MC, Lu M, Kubo A, Keller G, Davies TF, Lin RY** 2006 Directed differentiation of mouse embryonic stem cells into thyroid follicular cells. Endocrinology 147:3007-3015
23. **Lin RY, Kubo A, Keller GM, Davies TF** 2003 Committing embryonic stem cells to differentiate into thyrocyte-like cells in vitro. Endocrinology 144:2644-2649
24. **De Felice M, Di Lauro R** 2004 Thyroid development and its disorders: genetics and molecular mechanisms. Endocr Rev 25:722-746
25. **Thomas T, Nowka K, Lan L, Derwahl M** 2006 Expression of endoderm stem cell markers: evidence for the presence of adult stem cells in human thyroid glands. Thyroid 16:537-544
26. **Rodriguez RT, Velkey JM, Lutzko C, Seerke R, Kohn DB, O'Shea KS, Firpo MT** 2007 Manipulation of OCT4 levels in human embryonic stem cells results in induction of

differential cell types. Exp Biol Med (Maywood) 232:1368-1380

27. **Todaro M, Iovino F, Eterno V, Cammareri P, Gambara G, Espina V, Gulotta G, Dieli F, Giordano S, De Maria R, Stassi G** 2010 Tumorigenic and metastatic activity of human thyroid cancer stem cells. Cancer Res 70:8874-8885
28. **Lan L, Cui D, Nowka K, Derwahl M** 2007 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 92:3681-3688
29. **Yin L, Castagnino P, Assoian RK** 2008 ABCG2 expression and side population abundance regulated by a transforming growth factor beta-directed epithelial-mesenchymal transition. Cancer Res 68:800-807
30. **Hoshi N, Kusakabe T, Taylor BJ, Kimura S** 2007 Side population cells in the mouse thyroid exhibit stem/progenitor cell-like characteristics. Endocrinology 148:4251-4258
31. **Bhattacharya S, Das A, Mallya K, Ahmad I** 2007 Maintenance of retinal stem cells by Abcg2 is regulated by notch signaling. J Cell Sci 120:2652-2662
32. **Meissner K, Heydrich B, Jedlitschky G, Meyer Zu Schwabedissen H, Mosyagin I, Dazert P, Eckel L, Vogelgesang S, Warzok RW, Bohm M, Lehmann C, Wendt M, Cascorbi I, Kroemer HK** 2006 The ATP-binding cassette transporter ABCG2 (BCRP), a marker for side population stem cells, is expressed in human heart. J Histochem Cytochem 54:215-221
33. **Kobel S, Lutolf M** 2010 High-throughput methods to define complex stem cell niches. Biotechniques 48:ix-xxii
34. **Fierabracci A, Puglisi MA, Giuliani L, Mattarocci S, Gallinella-Muzi M** 2008 Identification of an adult stem/progenitor cell-like population in the human thyroid. J Endocrinol 198:471-487
35. **Zheng X, Cui D, Xu S, Brabant G, Derwahl M** 2010 Doxorubicin fails to eradicate cancer stem cells derived from anaplastic thyroid carcinoma cells: characterization of resistant cells. Int J Oncol 37:307-315
36. **Tai MH, Chang CC, Kiupel M, Webster JD, Olson LK, Trosko JE** 2005 Oct4 expression in adult human stem cells: evidence in support of the stem cell theory of carcinogenesis. Carcinogenesis 26:495-502

37. **Mitsutake N, Iwao A, Nagai K, Namba H, Ohtsuru A, Saenko V, Yamashita S** 2007 Characterization of side population in thyroid cancer cell lines: cancer stem-like cells are enriched partly but not exclusively. Endocrinology 148:1797-1803

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

39. **Takano T** 2007 Fetal cell carcinogenesis of the thyroid: theory and practice. Semin Cancer Biol 17:233-240

40. **Derwahl M** 2011 Linking stem cells to thyroid cancer. J Clin Endocrinol Metab 96:610-613

41. **Gibelli B, El-Fattah A, Giugliano G, Proh M, Grosso E** 2009 Thyroid stem cells--danger or resource? Acta Otorhinolaryngol Ital 29:290-295

42. **Derwahl M** 1996 Molecular aspects of the pathogenesis of nodular goiters, thyroid nodules and adenomas. Exp Clin Endocrinol Diabetes 104 Suppl 4:32-35

43. **Salabe GB** 1990 The clonal origin of thyroid nodules in euthyroid nodular goiter. Thyroidology 2:129-130

44. **Kopp P, Kimura ET, Aeschimann S, Oestreicher M, Tobler A, Fey MF, Studer H** 1994 Polyclonal and monoclonal thyroid nodules coexist within human multinodular goiters. J Clin Endocrinol Metab 79:134-139

45. **Thomas GA, Williams D, Williams ED** 1989 The clonal origin of thyroid nodules and adenomas. Am J Pathol 134:141-147

46. **Aeschimann S, Kopp PA, Kimura ET, Zbaeren J, Tobler A, Fey MF, Studer H** 1993 Morphological and functional polymorphism within clonal thyroid nodules. J Clin Endocrinol Metab 77:846-851

47. **Jovanovic L, Delahunt B, McIver B, Eberhardt NL, Grebe SK** 2003 Thyroid gland clonality revisited: the embryonal patch size of the normal human thyroid gland is very large, suggesting X-chromosome inactivation tumor clonality studies of thyroid tumors have to be interpreted with caution. J Clin Endocrinol Metab 88:3284-3291

48. **Peter HJ, Gerber H, Studer H, Smeds S** 1985 Pathogenesis of heterogeneity in human multinodular goiter. A study on growth and function of thyroid tissue transplanted onto

nude mice. J Clin Invest 76:1992-2002

49. **Zhang J, Li L** 2008 Stem cell niche: microenvironment and beyond. J Biol Chem 283:9499-9503
50. **Li L, Neaves WB** 2006 Normal stem cells and cancer stem cells: the niche matters. Cancer Res 66:4553-4557
51. **Derwahl M** Thyroid nodules and nodular goiter: a stem cell disease? Hot Thyroidology
52. **Berghout A, Wiersinga WM, Smits NJ, Touber JL** 1990 Interrelationships between age, thyroid volume, thyroid nodularity, and thyroid function in patients with sporadic nontoxic goiter. Am J Med 89:602-608
53. **Tamura M, Kimura H, Koji T, Tominaga T, Ashizawa K, Kiriyama T, Yokoyama N, Yoshimura T, Eguchi K, Nakane PK, Nagataki S** 1998 Role of apoptosis of thyrocytes in a rat model of goiter. A possible involvement of Fas system. Endocrinology 139:3646-3653
54. **Studer H, Derwahl M** 1995 Mechanisms of nonneoplastic endocrine hyperplasia--a changing concept: a review focused on the thyroid gland. Endocr Rev 16:411-426
55. **Purohit A, Reed MJ** 2002 Regulation of estrogen synthesis in postmenopausal women. Steroids 67:979-983
56. **Dalla Valle L, Ramina A, Vianello S, Fassina A, Belvedere P, Colombo L** 1998 Potential for estrogen synthesis and action in human normal and neoplastic thyroid tissues. J Clin Endocrinol Metab 83:3702-3709
57. **Adeoya-Osiguwa SA, Markoulaki S, Pocock V, Milligan SR, Fraser LR** 2003 17beta-Estradiol and environmental estrogens significantly affect mammalian sperm function. Hum Reprod 18:100-107
58. **Baker ME** 2003 Evolution of adrenal and sex steroid action in vertebrates: a ligand-based mechanism for complexity. Bioessays 25:396-400
59. **Windahl SH, Lagerquist MK, Andersson N, Jochems C, Kallkopf A, Hakansson C, Inzunza J, Gustafsson JA, van der Saag PT, Carlsten H, Pettersson K, Ohlsson C** 2007 Identification of target cells for the genomic effects of estrogens in bone. Endocrinology 148:5688-5695
60. **Manole D, Schildknecht B, Gosnell B, Adams E, Derwahl M** 2001 Estrogen promotes

growth of human thyroid tumor cells by different molecular mechanisms. J Clin Endocrinol Metab 86:1072-1077

61. **McCarthy MM** 2008 Estradiol and the developing brain. Physiol Rev 88:91-124
62. **Lee AJ, Mills LH, Kosh JW, Conney AH, Zhu BT** 2002 NADPH-dependent metabolism of estrone by human liver microsomes. J Pharmacol Exp Ther 300:838-849
63. **Deroo BJ, Korach KS** 2006 Estrogen receptors and human disease. J Clin Invest 116:561-570
64. **Ho KJ, Liao JK** 2002 Nonnuclear actions of estrogen. Arterioscler Thromb Vasc Biol 22:1952-1961
65. **McEwen BS, Alves SE** 1999 Estrogen actions in the central nervous system. Endocr Rev 20:279-307
66. **Toran-Allerand CD, Singh M, Setalo G, Jr.** 1999 Novel mechanisms of estrogen action in the brain: new players in an old story. Front Neuroendocrinol 20:97-121
67. **Huseby RA, Maloney TM, McGrath CM** 1984 Evidence for a direct growth-stimulating effect of estradiol on human MCF-7 cells in vivo. Cancer Res 44:2654-2659
68. **Stopper H, Schmitt E, Gregor C, Mueller SO, Fischer WH** 2003 Increased cell proliferation is associated with genomic instability: elevated micronuclei frequencies in estradiol-treated human ovarian cancer cells. Mutagenesis 18:243-247
69. **Ray R, Novotny NM, Crisostomo PR, Lahm T, Abarbanell A, Meldrum DR** 2008 Sex steroids and stem cell function. Mol Med 14:493-501
70. **Han HJ, Heo JS, Lee YJ** 2006 Estradiol-17beta stimulates proliferation of mouse embryonic stem cells: involvement of MAPKs and CDKs as well as protooncogenes. Am J Physiol Cell Physiol 290:C1067-1075
71. **Wright JL, Stanford JL** 2009 Metformin use and prostate cancer in Caucasian men: results from a population-based case-control study. Cancer Causes Control 20:1617-1622
72. **McLenachan S, Lum MG, Waters MJ, Turnley AM** 2009 Growth hormone promotes proliferation of adult neurosphere cultures. Growth Horm IGF Res 19:212-218
73. **Egloff AM, Rothstein ME, Seethala R, Siegfried JM, Grandis JR, Stabile LP** 2009 Cross-talk between estrogen receptor and epidermal growth factor receptor in head and

neck squamous cell carcinoma. Clin Cancer Res 15:6529-6540

74. **Tropepe V, Sibilia M, Ciruna BG, Rossant J, Wagner EF, van der Kooy D** 1999 Distinct neural stem cells proliferate in response to EGF and FGF in the developing mouse telencephalon. Dev Biol 208:166-188

75. **Kosaka N, Kodama M, Sasaki H, Yamamoto Y, Takeshita F, Takahama Y, Sakamoto H, Kato T, Terada M, Ochiya T** 2006 FGF-4 regulates neural progenitor cell proliferation and neuronal differentiation. FASEB J 20:1484-1485

76. **Wang JM, Liu L, Brinton RD** 2008 Estradiol-17beta-induced human neural progenitor cell proliferation is mediated by an estrogen receptor beta-phosphorylated extracellularly regulated kinase pathway. Endocrinology 149:208-218

77. **Okada M, Murase K, Makino A, Nakajima M, Kaku T, Furukawa S, Furukawa Y** 2008 Effects of estrogens on proliferation and differentiation of neural stem/progenitor cells. Biomed Res 29:163-170

78. **Kishi Y, Takahashi J, Koyanagi M, Morizane A, Okamoto Y, Horiguchi S, Tashiro K, Honjo T, Fujii S, Hashimoto N** 2005 Estrogen promotes differentiation and survival of dopaminergic neurons derived from human neural stem cells. J Neurosci Res 79:279-286

79. **Masuda H, Kalka C, Takahashi T, Yoshida M, Wada M, Kobori M, Itoh R, Iwaguro H, Eguchi M, Iwami Y, Tanaka R, Nakagawa Y, Sugimoto A, Ninomiya S, Hayashi S, Kato S, Asahara T** 2007 Estrogen-mediated endothelial progenitor cell biology and kinetics for physiological postnatal vasculogenesis. Circ Res 101:598-606

80. **Enewold L, Zhu K, Ron E, Marrogi AJ, Stojadinovic A, Peoples GE, Devesa SS** 2009 Rising thyroid cancer incidence in the United States by demographic and tumor characteristics, 1980-2005. Cancer Epidemiol Biomarkers Prev 18:784-791

81. **Duan Y, Wang X, Peng W, Feng Y, Tang W, Wu X, Mao X, Bo R, Li W, Chen J, Qin Y, Liu C** 2009 Gender-specific associations between subclinical hypothyroidism and blood pressure in Chinese adults. Endocrine 36:438-444

82. **Canchola AJ, Horn-Ross PL, Purdie DM** 2006 Risk of second primary malignancies in women with papillary thyroid cancer. Am J Epidemiol 163:521-527

83. **Truong T, Orsi L, Dubourdieu D, Rougier Y, Hemon D, Guenel P** 2005 Role of goiter and of menstrual and reproductive factors in thyroid cancer: a population-based

case-control study in New Caledonia (South Pacific), a very high incidence area. Am J Epidemiol 161:1056-1065

84. **Chen GG, Vlantis AC, Zeng Q, van Hasselt CA** 2008 Regulation of cell growth by estrogen signaling and potential targets in thyroid cancer. Curr Cancer Drug Targets 8:367-377

85. **Paech K, Webb P, Kuiper GG, Nilsson S, Gustafsson J, Kushner PJ, Scanlan TS** 1997 Differential ligand activation of estrogen receptors ERalpha and ERbeta at AP1 sites. Science 277:1508-1510

86. **Furlanetto TW, Nguyen LQ, Jameson JL** 1999 Estradiol increases proliferation and down-regulates the sodium/iodide symporter gene in FRTL-5 cells. Endocrinology 140:5705-5711

87. **Kumar A, Klinge CM, Goldstein RE** 2010 Estradiol-induced proliferation of papillary and follicular thyroid cancer cells is mediated by estrogen receptors alpha and beta. Int J Oncol 36:1067-1080

88. **Zeng Q, Chen GG, Vlantis AC, van Hasselt CA** 2007 Oestrogen mediates the growth of human thyroid carcinoma cells via an oestrogen receptor-ERK pathway. Cell Prolif 40:921-935

89. **Thiruvengadam A, Govindarajulu P, Aruldhas MM** 2003 Modulatory effect of estradiol and testosterone on the development of N-nitrosodiisopropanolamine induced thyroid tumors in female rats. Endocr Res 29:43-51

90. **Watanabe J, Kamata Y, Seo N, Okayasu I, Kuramoto H** 2007 Stimulatory effect of estrogen on the growth of endometrial cancer cells is regulated by cell-cycle regulators. J Steroid Biochem Mol Biol 107:163-171

91. **Heldring N, Pike A, Andersson S, Matthews J, Cheng G, Hartman J, Tujague M, Strom A, Treuter E, Warner M, Gustafsson JA** 2007 Estrogen receptors: how do they signal and what are their targets. Physiol Rev 87:905-931

92. **Xie CQ, Jeong Y, Fu M, Bookout AL, Garcia-Barrio MT, Sun T, Kim BH, Xie Y, Root S, Zhang J, Xu RH, Chen YE, Mangelsdorf DJ** 2009 Expression profiling of nuclear receptors in human and mouse embryonic stem cells. Mol Endocrinol 23:724-733

93. **Vincent BH, Montufar-Solis D, Teng BB, Amendt BA, Schaefer J, Klein JR** 2009

Bone marrow cells produce a novel TSHbeta splice variant that is upregulated in the thyroid following systemic virus infection. Genes Immun 10:18-26

94. **Mokry J, Subrtova D, Nemecek S** 1995 Cultivation of neural EGF-responsive precursor cells. Sb Ved Pr Lek Fak Karlovy Univerzity Hradci Kralove 38:167-174

95. **McCarthy RP, Wang M, Jones TD, Strate RW, Cheng L** 2006 Molecular evidence for the same clonal origin of multifocal papillary thyroid carcinomas. Clin Cancer Res 12:2414-2418

96. **Krohn K, Fuhrer D, Holzapfel HP, Paschke R** 1998 Clonal origin of toxic thyroid nodules with constitutively activating thyrotropin receptor mutations. J Clin Endocrinol Metab 83:130-134

97. **Casalbore P, Budoni M, Ricci-Vitiani L, Cenciarelli C, Petrucci G, Milazzo L, Montano N, Tabolacci E, Maira G, Larocca LM, Pallini R** 2009 Tumorigenic potential of olfactory bulb-derived human adult neural stem cells associates with activation of TERT and NOTCH1. PLoS One 4:e4434

98. **Siebzehnrubl FA, Jeske I, Muller D, Buslei R, Coras R, Hahnen E, Huttner HB, Corbeil D, Kaesbauer J, Appl T, von Horsten S, Blumcke I** 2009 Spontaneous in vitro transformation of adult neural precursors into stem-like cancer cells. Brain Pathol 19:399-408

99. **Shiras A, Chettiar ST, Shepal V, Rajendran G, Prasad GR, Shastry P** 2007 Spontaneous transformation of human adult nontumorigenic stem cells to cancer stem cells is driven by genomic instability in a human model of glioblastoma. Stem Cells 25:1478-1489

100. **Bidey SP, Hill DJ, Eggo MC** 1999 Growth factors and goitrogenesis. J Endocrinol 160:321-332

101. **Kanamori A, Abe Y, Yajima Y, Manabe Y, Ito K** 1989 Epidermal growth factor receptors in plasma membranes of normal and diseased human thyroid glands. J Clin Endocrinol Metab 68:899-903

102. **Brzezinski J, Lewinski A** 1998 Increased plasma concentration of epidermal growth factor in female patients with non-toxic nodular goitre. Eur J Endocrinol 138:388-393

103. **Cocks HC, Thompson S, Turner FE, Logan A, Franklyn JA, Watkinson JC, Eggo**

MC 2003 Role and regulation of the fibroblast growth factor axis in human thyroid follicular cells. Am J Physiol Endocrinol Metab 285:E460-469

104. **Mincione G, Di Marcantonio MC, Tarantelli C, D'Inzeo S, Nicolussi A, Nardi F, Donini CF, Coppa A** 2011 EGF and TGF-beta1 Effects on Thyroid Function. J Thyroid Res 2011:431718

105. **Hebrant A, van Staveren WC, Delys L, Solis DW, Bogdanova T, Andry G, Roger P, Dumont JE, Libert F, Maenhaut C** 2007 Long-term EGF/serum-treated human thyrocytes mimic papillary thyroid carcinomas with regard to gene expression. Exp Cell Res 313:3276-3284

106. **Filardo EJ** 2002 Epidermal growth factor receptor (EGFR) transactivation by estrogen via the G-protein-coupled receptor, GPR30: a novel signaling pathway with potential significance for breast cancer. J Steroid Biochem Mol Biol 80:231-238

107. **Ignar-Trowbridge DM, Nelson KG, Bidwell MC, Curtis SW, Washburn TF, McLachlan JA, Korach KS** 1992 Coupling of dual signaling pathways: epidermal growth factor action involves the estrogen receptor. Proc Natl Acad Sci U S A 89:4658-4662

108. **Lee AV, Cui X, Oesterreich S** 2001 Cross-talk among estrogen receptor, epidermal growth factor, and insulin-like growth factor signaling in breast cancer. Clin Cancer Res 7:4429s-4435s; discussion 4411s-4412s

109. **Hazel T, Muller T** 2001 Culture of neuroepithelial stem cells. Curr Protoc Neurosci Chapter 3:Unit 3 1

110. **Lendahl U, Zimmerman LB, McKay RD** 1990 CNS stem cells express a new class of intermediate filament protein. Cell 60:585-595

111. **Kawaguchi J, Nichols J, Gierl MS, Faial T, Smith A** 2010 Isolation and propagation of enteric neural crest progenitor cells from mouse embryonic stem cells and embryos. Development 137:693-704

112. **Murashov AK, Pak ES, Hendricks WA, Tatko LM** 2004 17beta-Estradiol enhances neuronal differentiation of mouse embryonic stem cells. FEBS Lett 569:165-168

113. **Dohan O, De la Vieja A, Paroder V, Riedel C, Artani M, Reed M, Ginter CS, Carrasco N** 2003 The sodium/iodide Symporter (NIS): characterization, regulation, and

medical significance. Endocr Rev 24:48-77

114. **Clarke R, Leonessa F, Welch JN, Skaar TC** 2001 Cellular and molecular pharmacology of antiestrogen action and resistance. Pharmacol Rev 53:25-71

115. **Zeng Q, Chen G, Vlantis A, Tse G, van Hasselt C** 2008 The contributions of oestrogen receptor isoforms to the development of papillary and anaplastic thyroid carcinomas. J Pathol 214:425-433

116. **Cho MA, Lee MK, Nam KH, Chung WY, Park CS, Lee JH, Noh T, Yang WI, Rhee Y, Lim SK, Lee HC, Lee EJ** 2007 Expression and role of estrogen receptor alpha and beta in medullary thyroid carcinoma: different roles in cancer growth and apoptosis. J Endocrinol 195:255-263

117. **Egawa C, Miyoshi Y, Iwao K, Shiba E, Noguchi S** 2001 Quantitative analysis of estrogen receptor-alpha and -beta messenger RNA expression in normal and malignant thyroid tissues by real-time polymerase chain reaction. Oncology 61:293-298

118. **Matthews J, Gustafsson JA** 2003 Estrogen signaling: a subtle balance between ER alpha and ER beta. Mol Interv 3:281-292

119. **Liu MM, Albanese C, Anderson CM, Hilty K, Webb P, Uht RM, Price RH, Jr., Pestell RG, Kushner PJ** 2002 Opposing action of estrogen receptors alpha and beta on cyclin D1 gene expression. J Biol Chem 277:24353-24360

120. **Leygue E, Dotzlaw H, Watson PH, Murphy LC** 1998 Altered estrogen receptor alpha and beta messenger RNA expression during human breast tumorigenesis. Cancer Res 58:3197-3201

121. **Hsi ED, Zukerberg LR, Yang WI, Arnold A** 1996 Cyclin D1/PRAD1 expression in parathyroid adenomas: an immunohistochemical study. J Clin Endocrinol Metab 81:1736-1739

122. **Gillett C, Fantl V, Smith R, Fisher C, Bartek J, Dickson C, Barnes D, Peters G** 1994 Amplification and overexpression of cyclin D1 in breast cancer detected by immunohistochemical staining. Cancer Res 54:1812-1817

123. **Lynch J, Keller M, Guo RJ, Yang D, Traber P** 2003 Cdx1 inhibits the proliferation of human colon cancer cells by reducing cyclin D1 gene expression. Oncogene 22:6395-6407

124. **Keum JS, Kong G, Yang SC, Shin DH, Park SS, Lee JH, Lee JD** 1999 Cyclin D1 overexpression is an indicator of poor prognosis in resectable non-small cell lung cancer. Br J Cancer 81:127-132

125. **Gansauge S, Gansauge F, Ramadani M, Stobbe H, Rau B, Harada N, Beger HG** 1997 Overexpression of cyclin D1 in human pancreatic carcinoma is associated with poor prognosis. Cancer Res 57:1634-1637

126. **Hibberts NA, Simpson DJ, Bicknell JE, Broome JC, Hoban PR, Clayton RN, Farrell WE** 1999 Analysis of cyclin D1 (CCND1) allelic imbalance and overexpression in sporadic human pituitary tumors. Clin Cancer Res 5:2133-2139

127. **Fu M, Wang C, Li Z, Sakamaki T, Pestell RG** 2004 Minireview: Cyclin D1: normal and abnormal functions. Endocrinology 145:5439-5447

128. **Khoo ML, Ezzat S, Freeman JL, Asa SL** 2002 Cyclin D1 protein expression predicts metastatic behavior in thyroid papillary microcarcinomas but is not associated with gene amplification. J Clin Endocrinol Metab 87:1810-1813

129. **Varkondi E, Gyory F, Nagy A, Kiss I, Ember I, Kozma L** 2005 Oncogene amplification and overexpression of oncoproteins in thyroid papillary cancer. In Vivo 19:465-470

130. **Eszlinger M, Krohn K, Berger K, Lauter J, Kropf S, Beck M, Fuhrer D, Paschke R** 2005 Gene expression analysis reveals evidence for increased expression of cell cycle-associated genes and Gq-protein-protein kinase C signaling in cold thyroid nodules. J Clin Endocrinol Metab 90:1163-1170

131. **Foster JS, Henley DC, Ahamed S, Wimalasena J** 2001 Estrogens and cell-cycle regulation in breast cancer. Trends Endocrinol Metab 12:320-327

132. **Blau HM, Brazelton TR, Weimann JM** 2001 The evolving concept of a stem cell: entity or function? Cell 105:829-841

133. **Doerflinger RM** 2008 The problem of deception in embryonic stem cell research. Cell Prolif 41 Suppl 1:65-70

134. **Chang YJ, Weng CL, Sun LX, Zhao YT** 2012 Allogeneic bone marrow transplantation compared to peripheral blood stem cell transplantation for the treatment of hematologic malignancies: a meta-analysis based on time-to-event data from randomized controlled

trials. Ann Hematol 91:427-437

135. **He XC, Zhang J, Li L** 2005 Cellular and molecular regulation of hematopoietic and intestinal stem cell behavior. Ann N Y Acad Sci 1049:28-38
136. **Xie T, Li L** 2007 Stem cells and their niche: an inseparable relationship. Development 134:2001-2006
137. **Vassart G, Dumont JE** 1992 The thyrotropin receptor and the regulation of thyrocyte function and growth. Endocr Rev 13:596-611
138. **Furlanetto TW, Nunes RB, Jr., Sopelsa AM, Maciel RM** 2001 Estradiol decreases iodide uptake by rat thyroid follicular FRTL-5 cells. Braz J Med Biol Res 34:259-263
139. **Paschke R, Neumann S** 2001 Sodium/iodide symporter mRNA expression in cold thyroid nodules. Exp Clin Endocrinol Diabetes 109:45-46
140. **Russo D, Bulotta S, Bruno R, Arturi F, Giannasio P, Derwahl M, Bidart JM, Schlumberger M, Filetti S** 2001 Sodium/iodide symporter (NIS) and pendrin are expressed differently in hot and cold nodules of thyroid toxic multinodular goiter. Eur J Endocrinol 145:591-597
141. **Reya T, Morrison SJ, Clarke MF, Weissman IL** 2001 Stem cells, cancer, and cancer stem cells. Nature 414:105-111
142. **Jackson EL, Garcia-Verdugo JM, Gil-Perotin S, Roy M, Quinones-Hinojosa A, VandenBerg S, Alvarez-Buylla A** 2006 PDGFR alpha-positive B cells are neural stem cells in the adult SVZ that form glioma-like growths in response to increased PDGF signaling. Neuron 51:187-199

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… My parents, for they are my life.

Erklärung

„Ich, Shuhang Xu, erkläre, dass ich die vorgelegte Dissertationsschrift mit dem Thema: ‚17ß-estradiol stimulates growth and inhibits function of thyroid stem/progenitor cells: a clue to understand the higher prevalence of thyroid nodules in females’ 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

Curriculum Vitae

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