2. Materials and Methods

2.1 Cell culture

2.1.1 Patient profile

Thyroid tissues were obtained from 30 patients with nodular goiters after thyroidectomy, provided by the Endocrine Surgery Department of the St. Hedwig Clinic in Berlin. 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 the Charité, Humboldt University of Berlin. The patient group consisted of 30 patients, 17 female (57 %) and 13 male (43 %). The mean age of the patients was 58.39 years, with a range of 51-78 years. The mean age of female patients was 57.76 years, that of male patients 59.11 years.

2.1.2 Preparation of primary thyroid cell cultures

Preparation of primary thyroid cultures from human nodule tissue was performed as we described previously (96). Firstly, in the operating room, tissue specimens were separated by the surgeon from surrounding tissue, put in sterile transport tubes containing 20 ml transport medium (HBSS solution with 100 U/ml penicillin, 100 µg/ml streptomycin, and 2.5 µg/ml amphotericin B), placed on ice and transported to the laboratory. Then all work with cell cultures was performed in a laminar flow cabinet. Employing sterile techniques, macroscopically visible capsule material and other connective tissues were removed using scalpel and tweezers, and 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 20 ml fresh HBSS containing 250 µl Collagenase A (stock: 5 mg/ml, Roche, Mannheim, Germany) and 250 µl dispase II (stock: 30 mg/ml) at 37°C for 1.5 h in a shaker 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°C) primary cell culture medium, put in 100 mm cell

culture dishes (Sarstedt) and placed in a humidified incubator at 37°C, with a 5 % CO2 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.

2.1.3 Cell lines

All cell lines were stored in liquid nitrogen at -196°C. For experiment, cells were transferred to a pre-warmed (37°C) water bath to thaw (generally 1-2 min) and then suspended in nutrient medium in culture dishes.

Cell lines used in our research included:

- The FRTL-5 cell line, which is a stable thyroid cell line derived from the thyroid of Fisher rats under defined culture conditions, has been widely adopted as a model for the study of differentiated thyroid cell function. It was obtained from American Type Culture Collection (Bethesda, MD).

- The Hela human cervix carcinoma cell line, which was established in 1952 from a very aggressive cervix carcinoma of a female patient named Henrietta Laks (the cell line has been named after her initials, He and La) (97), has been extensively used to investigate the mechanisms of carcinogenesis. It was kindly provided by Professor Dr Schaefer, Berlin.

2.1.4 Culture conditions

For most experiments, cells were grown as monolayers in 100 mm plastic culture dishes and kept in a humidified incubator at 37°C in 5 % CO2, with a medium change each 3-4 d. Upon reaching the desired confluency of 70-80 %, 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).

Monolayers of FRTL-5 cells were grown in Ham's F-12 medium supplemented with 5 % FCS, 1 % MEM (v/v), 5 mU/mL TSH, H5-mix, 100 U/ml penicillin, 100 µg/ml streptomycin, and 2.5 µg/ml amphotericin B.

Monolayer cultures of Hela cells were grown in DMEM (Gibco, Karlsruhe, Germany) supplemented with 10 % FCS, 1 % MEM (v/v), 100 U/ml penicillin, 100 µg/ml streptomycin, and 2.5 µg/ml amphotericin B.

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)

2.1.5 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 in 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. And then the total (or viable) cell number of the original cell suspension can be 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 Flow cytometry and fluorescence-activated cell sorting (FACS)

2.2.1 Flow cytometry

To identify the protein expression of ABCG2 transporter in cells isolated from human thyroid nodules, indirect immunofluorescent staining was performed. Monolayer cultured thyrocytes of 2^{nd} passage were dissociated with 0.05 % trypsin, 0.53 mM EDTA-4Na at 37^oC for 5 min and collected by centrifugation at 310 ×g for 5 min. Cells were then washed in PBS and incubated with ABCG2 mAb (1 : 100, Santa Cruz Biotechnology, USA) for 30 min on ice, which reacts with the extracellular portion of the human ABCG2 protein and has been used for flow cytometry analysis of living cells such as acute myeloid leukemia cells (98), cell lines overexpressing ABCG2 (99), and human limbal epithelial cells (100). Cells were then pelleted at 310 ×g for 5 min and washed twice with PBS. This step was followed by incubation with a FITC-conjugated donkey anti-mouse IgG antibody (1 : 100, Santa Cruz Biotechnology, USA) for 30 min on ice in the dark. Cells were centrifuged again at 310 ×g for 5 min and the same washing procedure was followed. Supernatant was then pipetted off and the pellet was re-suspended in 200 µl of PBS in a Falcon tube for analysis. Propidium iodide (PI) (Santa Cruz Biotechnology, USA) was added at a final concentration of 2 µg/ml immediately prior to analysis for identification of dead cells.

For flow cytometry after indirect immunofluorescence labelling, cells were gated on forward and side scatter and PI positive cells were excluded. Viable (PI-negative) cells were analyzed with a Flow cytometer in the facilities of the German Rheumatology Research Center, Berlin. Cellquest software was used for data analysis. During all staining and analyzing procedures, the cells were kept in the dark on ice.

2.2.2 FACS and side population

To isolate the thyroid side population fraction, FACS was performed using the Hoechst 33342 staining method as outlined by Goodell *et al.* (54). The ability of FACS to discriminate Hoechst

SP cells is based on the differential efflux of Hoechst 33342 by a transmembrane transporter, ABCG2. This is an active biological process, and optimal resolution of the profile is obtained, great attention being paid to the staining conditions. The cell concentration, Hoechst dye concentration, staining time, and staining temperature are all critical. And it is also very important to keep the sample protected from light throughout the staining procedure and during analysis.

First, the water bath was kept at precisely 37°C (checked with a thermometer), and DMEM+ medium (see below) was pre-warmed while preparing the cultured thyroid cells. Then primary cultured thyroid cells of 2nd passage were trypsinized from culture dishes, washed, counted accurately, and suspended at 10^6 cells per ml in pre-warmed DMEM+. Cells were mixed well and pre-incubated in a 1.5-ml Eppendorf tube at 37°C for 10 min. Then the cells were labeled in DMEM+ medium with 5 µg/ml (a 200 × dilution of the stock) Hoechst 33342 dye at 37°C for 120 min exactly, either alone or in combination with 50 µM verapamil (Sigma, USA), which is a potent inhibitor of ABC membrane transporters, and traditionally used as a guiding parameter to determine where to set the boundary between SP and non-SP cells (54). The top level of the cell suspension was totally submerged under water in the bath to ensure that the temperature of the cells was maintained at 37^{o}C. Tubes were gently inverted every 30 min during the incubation to discourage cell settling and clumping. After 120-min incubation, the cells were spun down at 310 ×g for 5 min at 4°C (in a precooled rotor) and re-suspended in 200 µl cold HBSS+.

When the staining process was over, the cells were maintained at 4°C in order to prevent further dye efflux until sorting. In order to remove cellular aggregates, cells were filtered through a 30 µm porous polyamide mesh prior to analysis. Cells were counterstained with 2 µg/ml propidium iodide (PI) for dead cell discrimination immediately prior to sorting. This was not required to see the SP cells, but helped to exclude the dead cells from the profile.

A 350-nm UV laser was used to excite Hoechst 33342 and PI. Analysis was performed on a FACS Calibur Equipment (Becton-Dickinson Biosciences, Heidelberg, Germany) by using a dual-wavelength analysis (blue, 424-444 nm; red, 675 nm) in the facilities of the German Rheumatology Research Center, Berlin. Dead and dying cells (<15 %) were excluded from the analysis on the basis of PI uptake (fluorescence at 564-606 nm), and only data for viable cells were analyzed for Hoechst labeling using WinMDI software (DeNovo Software, Toronto,

Canada). All of the parameters were collected using linear amplification in list mode and displayed in a Hoechst blue versus Hoechst red dotplot.

The side population was identified and selected by gating on the characteristic fluorescence emission profile (low labeling, Fig. 1.4). In order to select a most specific and homogeneous SP from primary thyroid cells, the SP gate for cell collection included only verapamil-sensitive cells with the lowest Hoechst incorporation. A comparable quantity of non-SP cells was also collected. During all staining and sorting procedures, the cells were kept in the dark on ice. Sorted SP and non-SP cell populations were recovered in media (as indicated below) for culture and pelleted for RNA isolation or cytospun onto slides for cytological analysis.

Reagent solutions:

Hoechst 33342: Obtained from Sigma (called Bis-Benzimide) as a powder. Re-suspended at 1 mg/ml (stock concentration) in water, filter sterilized, and frozen in small aliquots at - 20°C. It was allowed to thaw at room temperature protected from light before use.

Verapamil: Obtained from Sigma. Re-suspended at 50 mM (stock solution) in water, sterilized by autoclaving and frozen in aliquots - 20°C. It was allowed to thaw at room temperature protected from light and diluted at 50 µM (work solution) for use.

HBSS+: Hanks Balanced Salt Solution (Gibco, Karlsruhe, Germany) with 2 % FCS and 10 mM HEPES buffer.

DMEM+: DMEM with 2 % FCS and 10 mM HEPES buffer.

Propidium iodide: Obtained from Sigma. Frozen stock is at 10 mg/ml in water. Working stock (covered with aluminum foil and kept in the refrigerator) was 200 µg/ml in PBS. Final concentration of PI in samples was 2 µg/ml.

2.3 Cell culture of SP and non-SP

Comparable numbers of SP and non-SP cells were collected and first recovered in Ham's F12 medium supplemented with penicillin-streptomycin and amphotericin B after FACS. Cells were then centrifuged and seeded in different culture conditions as indicated below.

1) Cells were cultured in medium for primary thyroid cells as described in 2.1.4.
2) Cells were cultured in medium for primary thyroid cells in culture dishes coated with Matrigel (Sigma-Aldrich, Saint Louis, USA, details see below).

Matrigel preparation and coating:

Matrigel was purchased from Sigma and stored at - 20^{o}C. Before use gel was thawed overnight at 4^{o}C and then diluted up to twofold with cold (4°C) DMEM medium. For a 24-well plate, 200-250 µl of Matrigel was evenly dispensed to each well using pipets that were pre-cooled to 4°C. It gelled as a thin layer (about 0.5 mm) within 5 min at 20°C and then be ready for cell culture. For prolonged manipulations, work was always conducted on ice.

Comparable numbers (about 500-1000) of SP and non-SP cells separated from FACS were then plated on the top of the thin Matrigel layer in each well.

3) Cells were cultured under intense growth stimulation with EGF and bFGF (as described in 2.5.1).

4) Co-cultures of SP and non-SP cells:
After FACS, SP and non-SP cells were also co-cultured for 3 weeks in regular culture conditions. Immediately after FACS (termed as d 0), and then on d 7, d 14 and d 21, total RNA of cultured cells was extracted, and RT-PCR was performed for gene expression analysis of stem cell marker Oct4 and endodermal progenitor cell marker GATA4 as described in 2.7.

2.4 Cytospin and Giemsa staining

Comparable numbers (about 500-1000) of SP and non-SP gated cells were collected in PBS, and cytospun onto clean positively charged slides using a Micro-cytospin Centrifuge. The resulting slides were fixed in 10 % buffered formalin (equivalent of 4 % buffered paraformaldehyde) for 1 min at room temperature, and then slowly frozen in 100 % methanol at 80^{o}C overnight as described previously (101).

Cytospins of SP and non-SP fractions were Giemsa stained for 5 min and then photographed microscopically at 100 × and 400 × magnifications for image analysis.

2.5 Suspension culture system

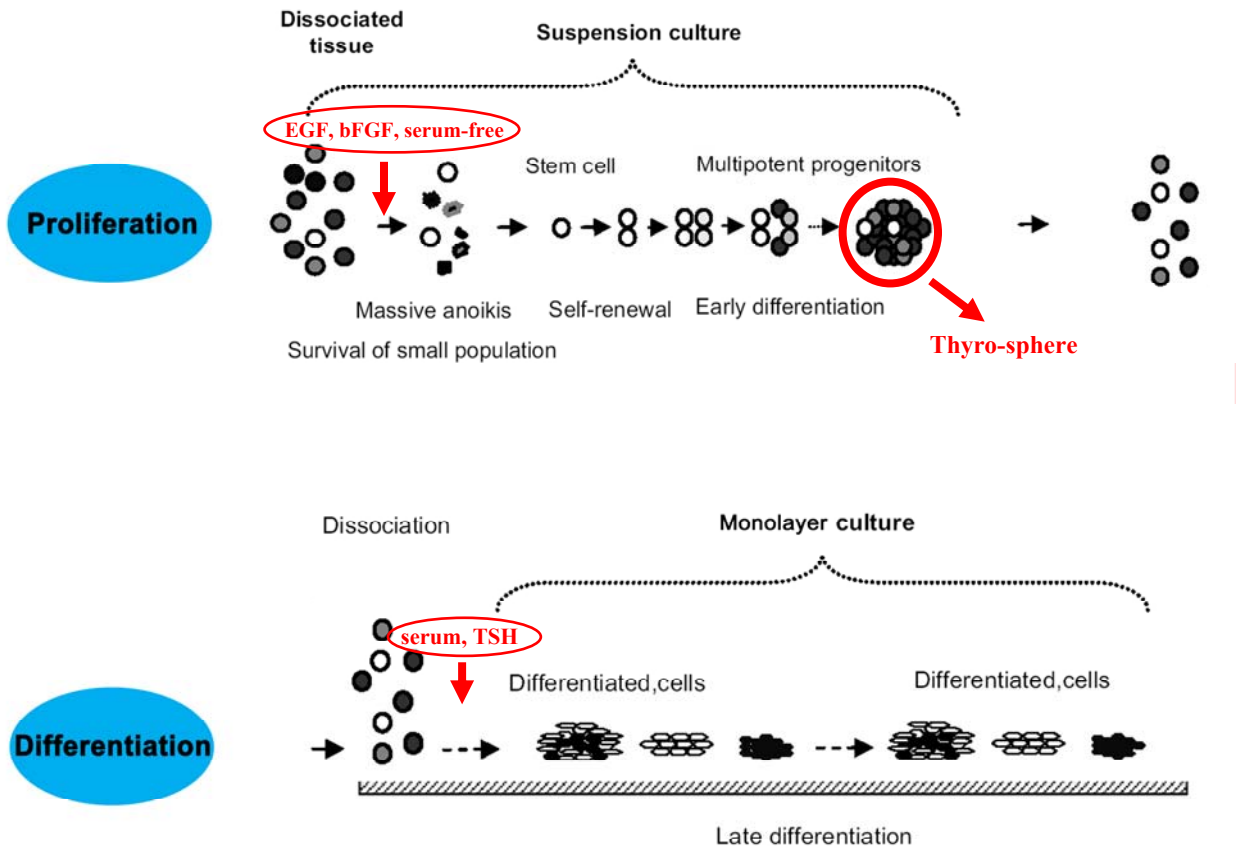

Figure 2.1 Schematic outline of the experimental strategy in stem cell proliferation and differentiation. [modified from: Dontu G, Breast Cancer Research, 2004, 6: R605-R615]

2.5.1 Sphere culture

In a complementary approach for prospective enrichment of stem cells, a new culture system for non-adherent sphere formation was performed, as shown in the upper panel of Fig. 2.1.

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 mm dishes at 10,000 viable cells/ml in a 37^{o}C, 5 % CO2 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 'thyro-spheres'.

During the sphere formation, the sphere numbers in every dish and the average cell counts in every sphere were analyzed. Sphere cells were collected for RNA isolation and detected for gene expression analysis. In addition, sphere cells were stained with Hoechst 33342 and sorted by FACS for further characterization.

To investigate the optimal culture condition for thyro-sphere formation, primary thyroid cells were prepared as described above, but cultured either in growth factor-enriched medium plus 10 % FCS, or in growth factor-enriched medium plus 5 mU/ml TSH.

2.5.2 Cell differentiation

To determine whether sphere cells have retained the capacity to differentiate into thyroid cells, a differentiation experiment was performed as outlined in the lower panel of Fig. 2.1. All thyro-spheres were collected when the sphere sizes reached a plateau, purified by being centrifugated twice at 200 ×g for 5 min, and dissociated into single cells enzymatically (15 min in 0.05 % trypsin, 0.53 mM EDTA-4Na at 37^{o}C). An aliquot of the dispersed single cell suspension was loaded in a hemacytometer and counted. The sphere differentiation induction was then carried out using a two-step protocol. In the first step, cell differentiation was initiated for 3 d in a growth factor-withdrawal Ham's F12 medium supplemented with 10 % FCS at a density of 1,000 cells/ml to promote the cell adhesion. Within 24 h of plating, almost all cells

attached. In the second step, 5 mU/ml TSH was added to the culture medium, and the cells were then maintained as monolayer in culture for subsequent investigations, with a medium change each 3-4 d. During the experiment, cells were harvested at d 0, 3, 6, 9, 15 and 21 for RNA isolation and the time course of gene expression profiles was then analyzed by RT-PCR.

2.6 BrdU incorporation assay using immunofluorescent staining

To examine whether thyro-spheres developed by cell division as opposed to cellular aggregation, BrdU incorporation was performed with 5-bromo-2´-deoxy-uridine labeling and detection kit I (Roche) according to the manufacture's instructions.

Briefly, the thymidine analog BrdU labeling medium (final concentration: 10 μM) was added to the cell culture medium 1 d after seeding. On d 5, the spheres were washed and spun down (500 ×g, 10 min) onto a clean glass slide with a centrifuge. Then spheres were fixed with ethanol fixative (see below, pH 2.0) for 20 min at room temperature and washed once with washing buffer. After the peripheral zone of the area to be stained was carefully dried, the cells were covered with a sufficient amount of Anti-BrdU working solution (1 : 10 diluted from the supplied stock) and incubated for 30 min at 37°C in a humid atmosphere. Then the glass slides were washed 3 times and covered with a sufficient amount of Anti-mouse-FITC working solution (1 : 10 diluted from the supplied stock) for 30 min incubation at 37°C in a humid atmosphere in the dark. Subsequently, the slides were washed 3 times again and mounted in Vectashield (Vector) after being air-dried. For evaluation by fluorescence microscopy an excitation wavelength in the range of 450–500 nm (*e.g.*, 488 nm) and detection in the range of 515-565 nm were used.

The growth rates of sphere cells that underwent intense growth stimulation and differentiation conditions were analyzed as follows: following 5 d of sphere formation (d 1-5), spheres were transferred to differentiating conditions with both serum and TSH and cultured for another 5 d (d 6-10). During the sphere formation and early differentiation, BrdU (10 μM) was added to the respective culture media 12 h before each assay. Growth rates were then estimated by counting the percentage of BrdU-labeled cells. For each experiment at least 300 nuclei were counted.

Ethanol fixative:

100 % ethanol 140 ml
Glycine 0.75 g
Distilled water 60 ml
PH was adjusted to 2.0, stored at 4ºC.

2.7 Reverse transcription and polymerase chain reaction (RT-PCR)

2.7.1 Total RNA isolation

For cultured cells, total RNA was extracted using the RNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's specifications. In brief, 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 - 20ºC or used directly for spectrophotometry and RT-reaction (see below).

For FACS-sorted cells, total RNA was extracted using the RNeasy Micro Kit (Qiagen, Hilden, Germany) according to the manufacturer's specifications. Briefly, after cell sorting, SP cells and the equal number of non-SP cells were separately collected into the sterilized Falcon tube

containing 75 µl buffer RLT supplemented with β-ME and vortexed for lysis. Then 5 µl of carrier RNA (4 ng/µl) was added to each tube. Cell lysates were pipetted onto QIAShredder spin column and centrifuged for 2 min at maximum speed at room temperature for homogenization. 75 µl of 70 % ethanol was added to the homogenized lysate and mixed by pipetting. Samples were then applied to RNeasy MinElute spin columns placed in 2 ml collection tubes and centrifuged for 15 sec at 8000 ×g. Flow-through was discarded and 80 µl of DNase I incubation mix was directly added to the RNeasy MinElute Silica-gel membrane. The tubes were placed on the benchtop at room temperature for 15 min, 350 µl of RW1 buffer was then added and centrifuged for 15 sec at 8000 ×g to wash the columns. Flow-through and collection tubes were discarded and the RNeasy MinElute spin 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 500 µl of 80 % ethanol was added to the RNeasy columns. Tubes were then centrifuged again for 2 min at 8000 ×g to dry the silica-gel membrane. The RNeasy columns were then transferred into a new 2 ml collection tube and centrifuged for 5 min at 8000 ×g with the caps of the spin columns open. Finally, for elution, the RNeasy columns were transferred to new 1.5 ml collection tubes and 14 µl of RNase-free water was pipetted directly onto the RNeasy MinElute silica-gel membrane. Tubes were closed gently and centrifuged for 1 min at 8000 ×g. The resulting RNA was either stored at - 20ºC 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.

carrier RNA stock solution:

carrier RNA	310 µg
RNase-free water	1 ml

Stored at - 20 ºC.

carrier RNA working solution (Solution B):

Solution A	carrier RNA stock solution	5 µl
	buffer RLT supplemented with β-ME	34 µl

Solution B	solution A	6 µl
	buffer RLT supplemented with β-ME	54 µl

DNase I working solution:

DNase I stock solution	10 µl
buffer RDD	70 µl

2.7.2 Reverse Transcription reaction

For reverse transcription, 2 µl of Oligo- p(dT)15 primer was added to the RNA solution containing 1 µg of RNA (as calculated by spectrophotometry). RNase-free water was added to a final volume of 16.75 µl and the mix was heated to 70ºC for 5 min. Probes were placed on ice and the following reagents were added consecutively: 5 µl M-MLV 5 × Reaction buffer, 1.25 µl 10 mM dNTP nucleotides (Roche, Mannheim, Germany), 1 µl of RNAGuard RNase inhibitor (Amersham, Aylesbury, UK, 25 Units) and 1 µl M-MLV Reverse Transcriptase (Promega, Mannheim, Germany, 200 Units). The mixture was warmed to 42ºC for 60 min, 95ºC for 5 min and the reaction was terminated at 0ºC.

2.7.3 Primer preparation

All primers were obtained in powder form from Invitrogen Inc. Upon delivery, primers were diluted in 1 ml of RNAse free water and concentration was calculated with spectrophotometry as described above. Primers were then diluted with RNAse free water to a concentration of 4 pmol/µl, and stored at - 20ºC.

2.7.4 Polymerase chain reaction (PCR)

For PCR amplification the Hot Start method was used. In brief, 4 µl of complementary DNA was added to a 45.5 µl master mix containing 5 µl 10 × reaction buffers, 1.5 mmol/l $MgCl_2$, 1 µl dNTPs and 30 pmol of sense and antisense primers. Negative controls without template cDNA were included in all cases to preclude carry-over contamination with genomic DNA. Three drops of mineral oil were added to cover the reaction solution, the lids were closed and the mixture was preheated to 95ºC 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, reactions were carried out at 95°C for 30 sec (initial denaturation), 53-59°C for 30 sec (annealing) and 72°C for 1 min (extension), followed by a final extension at 72°C for 10 min and termination at 4°C. For most detected genes, 35 amplification cycles were performed, with the exception of ß-actin (30 cycles). All cDNAs were amplified in the log-linear phase of PCR by 30-35 cycles. In all PCR analyses, β-actin served as an internal control. Primer pair sequences, product lengths and annealing temperatures are shown in table 2.1.

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

Target gene	Primer sequences [#]	Annealing Temp. (^{o}C)	Expected size (bp)
ABCG2 (human)	S:5'-AGTTCCATGGCACTGGCCATA-3' AS: 5'-TCAGGTAGGCAATTGTGAGG-3'	53	379
ABCG2 (rat)	S: 5'-AGTTCCATGGCCCTGGCCATA-3' AS: 5'-TCAGGTAGGCAATTGTGAGG-3'	53	385
Oct4	S: 5'-GACAACAATGAGAACCTTCAGGAG -3' AS: 5'-CTGGCGCCGGTTACAGAACCA-3'	55	216
GATA4	S: 5'-CTCCTTCAGGCAGTGAGAGC-3' AS: 5'-GAGATGCAGTGTGCTCGTGC- 3'	58	575
HNF4	S: 5'-TCTCATGTTGAAGCCACTGC-3' AS: 5'-GGTTTGTTTTCTCGGGTTGA-3'	57	505
PAX8	S: 5'-TCCACCCCTTCCTCTTTATCT-3' AS: 5'- AGTCCTCCTGTTGCTCAGTCG-3'	58	441
TG	S: 5'- AGTCCTAAGTCCCCT GATGC-3' AS: 5'- CAAGGGAGACGTCGAGT GT-3'	55	280
NIS	S: 5'-TCTCTCAGTCAACGCCTCT-3' AS: 5'-ATCCAGGATGGCCACTTCTT-3'	58	299
TSHR	S: 5'-AATCCCTGTGAATGCTTTTC-3' AS: 5'-ACTCAAGGAAAGTGGAAGTT-3'	55	310
TPO	S: 5'-GTCTGTCAGGCTGGTTATGG-3' AS: 5'-CAATCACTCCGCTTGTTGGC-3'	57	242
β-actin	S: 5'-CCCAGGACCAGGGC GTGAT -3' AS: 5'-TCAAACATGATCTGGGTCAT-3'	59	280

[#] **S**: sense primer; **AS**: antisense primerr

2.7.5 Gel electrophoresis

For visualization of PCR products, agarose gel electrophoresis was performed. Agarose gels were prepared at a concentration of 1.5 % or 2 % (2 % for expected fragment sizes of 250 bp or lower) with 5 % (v/v) ethidium bromide. Then PCR products were separated by gel electrophoresis at 95 V for about 60 min (TBE running buffer). Bands were visualized on a UV-transilluminator at 312 nm, and images were documented and analyzed using Image J software.

TBE buffer (20 ×):

1.8 M Tris-base
1.8 M Boric acid
25 mM EDTA-$Na_2.2H_2O$
PH was adjusted to 8.3

2.8 Iodide uptake assay

To induce the differentiation of thyro-sphere cells into thyroid cells, sphere-derived cells were cultured in differentiating conditions for 21 d and then harvested for following experiments: Cells were transferred on to 60 × 15 mm dishes coated with 0.75 % (w/v) agarose at a density of 2.5×10^6 cells in 5 ml DMEM supplemented with 10 % FCS and cultured over night at 37°C in the incubator. The cell aggregates developed in the culture were then centrifuged at 310 ×g for 5min and then suspended in an ice-cold neutralized collagen solution (102) at a concentration of 2×10^3 cells/20 µl collagen solution. Then the cell suspension was plated as 20 µl drops in 24-well plates and allowed to gel in an incubator at 37°C for 30 min. Serum-free medium (0.5ml, DMEM containing 5 mU/ml TSH, insulin 0.3 IE /ml) was added to each well. To measure the iodide uptake activity of follicles developed in collagen after 3-4 d of culture, cells were washed with HBSS and then incubated in the same buffer containing ^{125}I (0.1 µCi) and NaI (0.5 µM) for 40 min at 37°C in a humidified atmosphere. At the end of the incubation, the medium was removed and the collagen gels were washed four times with ice-cold HBSS. The collagen gels were transferred to tubes and the radioactivity was measured in a γ-counter as previously described (103).

Parallel wells of cells were incubated in the absence or presence of sodium perchlorate. $^{125}I^-$uptake of thyro-sphere cells that were maintained in the absence of TSH and serum for 21 d and of normal cultured thyrocytes isolated from the same patients was also measured. FRTL-5 cells were employed as a positive control. ^{125}I-uptake was expressed as cpm/well. The values represented the mean ± SD of three independent experiments performed in triplicate.

Agarose-coated dish preparation:

Agarose (Sigma, Steinheim, Germany), at a concentration of 0.75 % (w/v) in water, was heated for 5 min at 120°C; while it was still hot, 1.5 ml (for a 60 × 15 mm dish) of the solution was added to each dish. The dish was left at room temperature until the agarose solidified. Dishes were washed with medium before use.

Collagen gel preparation:

Collagen VII from rat tail (Sigma, Steinheim, Germany), was solubilized for about 8 h at 4°C in a sterile 0.1 % (v/v) acetic acid solution (diluted with sterile water) in a shaker. The final concentration was 5 mg/ml. Then a pH of 7.2 and a convenient ionic strength were obtained by mixing 8 volumes of the ice-cold collagen solution with 2 volumes of a 1 : 1 mixture of a 10 × concentrated Minimum Essential Amino Acids (Invitrogen, Karlsruhe, Germany) with 0.125 N NaOH in 0.26 M $NaHCO_3$.

2.9 Experimental equipment

2.9.1 Apparatus

FACS calibur Equipment	Becton-Dickinson biosciences
Laminar flow cabinet	Heraeus, Laminair HB 2448
Cell culture incubator	Heraeus
Phase contrast microscope	Nikon, TMS
Biological microscope	JNOEC, XS-402
Fluorescence microscope	JNOEC

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
Polysterene slide flasks	Nunc

2.9.2 Software

Biology Workbench version 3.2	DNA and mRNA sequence analysis
Cellquest	flow cytometry analysis
WinMDI version 2.8	FACS analysis
Adobe photoshop version 7.0	figure preparation
Image J version 1.34s	gel densitometry and cell morphology analysis (freely available at http://rsb.info.nih.gov/ij/)