2 Materials and Methods

2.1 Cell culture

2.1.1 Preparation of primary thyroid cell cultures

The Rapoport protocol for obtaining primary thyroid cultures from human goiter tissue was first described in 1982 (98). For the present work, a modified version of the protocol was used (99).

All work with cell cultures was done in a laminar flow cabinet under sterile conditions. Primary thyroid cells were isolated from human thyroid tissue provided by the Endocrine Surgery Department of the St.Hedwig Clinic, in Berlin. Tissue samples were collected from patients undergoing surgery for non-malignant goiter. Informed consent was obtained from all patients prior to the operation. The study was approved by the ethics comitee of the Charite University Hospital. Tissue stemming from nodular thyroids was classified either as nodular or paranodular, according to intraoperative surgical assessment.

In the operating room, tissue pieces were separated by the surgeon from surrounding tissue, put in sterile transport tubes filled with 20 ml transport medium (HBSS solution enriched with 100 U/mL penicillin, 100 µg/mL streptomycin, and 2.5 µg/mL amphotericin), placed on ice and transported to the laboratory.

Under the laminar flow cabinet, macroscopically visible connective tissue was removed with scalpel and tweezers and the remaining tissue was grossly chopped with a scalpel, to facilitate the subsequent dispartment in follicles. For digestion in follicles, the thyroid tissue pieces were suspended in 20 ml of transport medium enriched with 1.25 mg/ml Collagenase Type A and 10 mg/ml Dispase II, and placed in an incubation shaker at a temperature of 37°C at about 80 rpm.

After 90 minutes, dissolved material was filtered through a sterile, coarsely meshed sieve and centrifuged for 5 minutes at 1200*g. The supernatant was poured back to the remaining tissue pieces and digested again at 37°C at 80 rpm. The thyroid cell sediment was suspended in 10 ml of warm (37°C) primary culture medium (s. 3.1.3 Culture conditions-cell passaging), put in cell culture dishes and placed in an incubator (37° C, 5% CO2). This procedure was repeated several times, until all thyroid tissue was fully dissolved.

2.1.2 Thyroid cell lines

All thyroid cell lines were stored in liquid nitrogen at a temperature of -196 °C. For defrosting, cells were placed in warm (37° C) water bath for 5-8 minutes and then suspended in nutrient mixture and placed in culture dishes.

Hth74 is a thyroid anaplastic carcinoma cell line, which has been isolated from thyroid carcinoma tissue of a 73 year old woman (100).

HTC cells are defined as a human thyroid carcinoma cell line derived from a follicular thyroid carcinoma (101).

The HeLa cell line 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) (102). The patient died a few months after the diagnosis, the cell line is however immortal and has been extensively used to investigate the mechanisms of carcinogenesis. The isolated tumor cells are highly undifferentiated, very invasive and display an increased proliferation rate (103) .

The FRTL5 cell line is a stable thyroid cell line derived from the thyroid of Fischer rats under defined culture conditions and has been widely adopted as a model for the study of differentiated thyroid cell function (104, 105) .

2.1.3 Culture conditions and media

All cells were grown as monolayers in 150 or 200 mm plastic culture dishes and kept in a humidified incubator at 37° C in 5% CO2, with a medium change each 3–4 days. Primary thyrocyte cultures were cultured in Ham's F-12 medium with L-glutamine, supplemented with 10% FCS, MEM, five hormones or growth factors (H5-mix: 10 ng/mL glycyl-histidyl-lysine, 10 μg/mL insulin, 10 ng/mL somatostatin, 5 μg/mL transferrin; 3.2 ng/mL hydrocortisone), 100 U/mL penicillin,100 μg/mL streptomycin, and 2.5 μg/mL amphotericin, with or without 5 mU/mL TSH, as indicated.

Monolayers of FRTL5 cells were grown in Ham's F-12 medium supplemented with 5% FBS, 1% MEM v/v, 5 mU/mL TSH, 100 U/mL penicillin,100 μg/mL streptomycin, and 2.5 μg/mL amphotericin.

Monolayer cultures of the HTh74 and the HTC human thyroid carcinoma cell lines were grown in F-12 medium supplemented with 10% FCS, 1% MEM v/v, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2.5 μg/mL amphotericin.

Monolayer cultures of the HeLa human cervix carcinoma cell line were grown in DMEM medium supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, and 2.5 μg/mL amphotericin.

2.1.4 Cell passaging

Cells were passaged upon reaching the desired confluency of 60-80%. For passaging, the medium was aspirated and the cultures were washed briefly with HBSS. After removal of the HBSS solution, trypsin (1X) was added (7 mL for 200 mm dishes, 5 mL for 150 mm dishes) and the cultures were placed in the incubator at a temperature of 37º C (5 min for primary cultures, 6 min for FRTL5, 3 min for all carcinoma lines). Adherent cells were then gently dissociated from the plastic culture disk surface using light pippeting and placed in 50 mL centrifuge tubes filled with 15 mL of F12 medium supplemented with 10% FCS, to block trypsin activity. After centrifugation at 1000 rpm at 4º C for 5 min, supernatant was aspirated and the remaining pellet was resuspended in new culture dishes filled with warm (37º) culture medium.

2.1.5 Trypan blue exclusion

Trypan Blue is a dye used for distinguishing viable from non-viable cells. The basic principle of the method is that trypan blue only interacts with the cells if the membrane is damaged. Therefore, trypan blue only dyes non-viable cells while viable cells are excluded (106). For application of the protocol, cell suspensions were obtained following the trypsinization/trypsin neutralization protocol described above for normal cell passage. In a 1.5 ml tube, 15 µl of trypan blue solution were combined with 15 µl of cell suspension (previously lightly vortexed to ensure uniform distribution of the cells in the medium). The ingredients were further pipetted up and down several times and 10 µl of the mixture was loaded in each of the two counting chambers of a hemacytometer (Neubauer cell counting chamber) by placing the pipette at the edge of the coverslip and allowing the suspension to fill the space by capillary action. The four 1x1mm counting areas of the hemacytometer were then observed under a microscope and the number of cells (total and viable) per counting area was determined. Non viable cells appeared as blue, viable cells were clear in a bluish background.

For calculation of the cell concentration the following formula was used (shown with an example):

Total (or viable) cells counted in 4 mm^2	Divided by 4 = cells per mm^2	Divided by dilution	= cells/10^{-4}	x 10^4= cells/ml	x total volume of cell suspension= Total (or viable) cells recovered
101 (92)	25 (23)	1/2	50 (46)	5.0 (4.6)x10^5 cells/ml	2.0 (1.84) x 10^6 cells

2.2 Stimulation experiments

2.2.1 Stimulation with xanthosine

In order to test the theory of Suppression of Assymetric Cell Kinetics (SACK), cells were treated with xanthosine, a purine nucleoside which influences the cell cycle by promoting guanine ribonucleotide biosynthesis and has been used to reversibly convert cells from asymmetric cell kinetics to symmetric cell kinetics (97). Primary thyrocyte cultures were treated with different concentrations of xanthosine (100 μM, 200 μM or 400 μM). Xanthosine was purchased in powder form from Sigma Aldrich Chemie (No x-0750) and was diluted in 125 mM NaOH as a stock solution (personal communication with the authors). Final NaOH concentration in the media was kept in the range of 1.25 mM or lower. Cells were kept under the same conditions as all other cultures with a medium change every 3-4 days. Primary thyrocyte cultures originating from the same patient(s) and in the same passage number were treated further with H5+TSH medium and served as controls. mRNA isolation was performed at designated timepoints (48h, 5 days, 7 days, 14 days, 30 days) and RT-PCR for detection of stem cell marker expression was performed. All experiments were repeated at least three times, with three different cultures at different passages.

In an attempt to achieve a pure stem cell culture, as described in Lee et al, "Clonal Expansion of Adult Rat Hepatic Stem Cell Lines by Suppression of Asymmetric Cell Kinetics (97) , cells obtained directly after tissue digestion were suspended in normal culture medium (H5+TSH supplemented with 10% FCS) and plated in 150 mm culture dishes. After 48 hours the medium was aspirated and adherent cells were removed with trypsin, combined in 20 mL of the medium and pelleted at 1200 rpm for 5 min at 4ºC. Cells were then resuspended in 20 mL of medium, and the number of viable cells was determined by trypan blue exclusion using a Neubauer cell counter chamber (see paragraph 2.1.5). Cells were diluted to a density of 20 viable cells per milliliter in the same culture medium containing either 200 μM or 400 μM Xs. Aliquots of 100 μL of the diluted cells were pippeted into 96-well culture plates (i.e., giving an expected average of two cells per well). Cells were kept under the same conditions as all other cultures with a medium change every 3-4 days. Wells were observed daily for the formation of single epithelioid cell colonies by phase microscopy over a period of one month.

2.2.2 Stimulation with TSH

For TSH stimulation experiments, cells were randomized in two groups (high dose TSH and normal dose TSH controls). The first group was treated with a very high concentration of TSH (200 mU/ml) while the second group was treated further with normal H5+TSH medium (containing 5 mU/ml TSH). For stimulation times and outcome see Results, paragraph 3.9.

2.3 Experiments with nucleic acids (mRNA/cDNA)

2.3.1 Isolation of total mRNA

Total RNA was extracted using the Qiagen RNeasy isolation kit according to the manufacturer's specifications. In brief, after aspiration of the culture medium, buffer RLT, supplemented with ß-mercaptoethanol, was added to the monolayer cultures to lyse the cells (350µL for 5cm dishes, 600µL for 10 cm dishes). Cell lysates were then collected using a rubber policeman, pippeted in QIA Schredder microcentrifuge tubes and centrifuged for 2 min at maximum speed for homogenization. Equal volumes of 70% ethanol were added to the homogenized lysate and mixed by pippeting. Samples were then applied to RNeasy mini columns placed in 2 mL collection tubes and centrifuged for 15 sec at 10000 rpm. Flow through was discarded and 700 µL of RW1 Buffer was added to the RNeasy columns and centrifuged for 15 sec at 10000 rpm to wash the columns. Flow-through and collection tubes were discarded and the RNeasy columns were transferred into new 2mL collection tubes. 500µL of Buffer RPE were pippeted onto the RNeasy columns which were then centrifuged again for 15 sec at 10000 rpm to wash. Flow-through was discarded and another 500 µL Buffer RPE were added to the RNeasy columns. Tubes were then centrifuged again for 2 min at 10000 rpm 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 were pippeted directly onto the RNeasy silica-gel membrane. Tubes were closed gently and centrifuged for 1 min at 10000 rpm. The resulting RNA yield was either stored at -80º or used directly for spectrophotometry and RT-reaction (see below)

2.3.2 Calculation of mRNA concentration with spectrophotometry

For spectrophotometry, 2 µL of mRNA were diluted 1:500 in RNase free water, pipetted in a quartz cuvette and absorption was calculated at a wavelength of 260 nm. Prior to calculations, the spectrophotometer was calibrated to zero using salt-free water as a test probe.

The calculation of the mRNA concentrations was based on the fact that an RNA concentration of 40 mg/ml corresponds to an optical density of 1.0 (106). For each probe, the mean value of at least two independent readings was adopted as the result.

2.3.3 Reverse Transcription

RNA is not suitable as a substrate for the polymerase chain reaction (106), therefore the complementary DNA sequences (cDNA) were synthesized using reverse transcription.

For reverse transcription, 2 μL of Oligo- p(dT)15 primers were 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 at 70º C for 5 minutes. This led to the disintegration of the secondary mRNA structure and to the binding of the random hexamer primers (Oligo-p(dT)15). Probes were placed on ice and the following reagents were added consecutively: 5μL of M-MLV 5X Reaction Buffer, 1.25 μL of dNTP nucleotides, 1μL of RNAGuard RNase inhibitor (25 Units) and 1 μL of M-MLV Reverse Transcriptase (200 Units). For cDNA synthesis, the mixture was warmed at 42º C for 60 minutes. The reverse transcriptase enzyme was denaturized by a subsequent heating at 95º C for 5 minutes and the reaction was terminated at 0º C (106).

2.3.4 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, aliquoted and stored at -30º C.

2.3.5 Polymerase Chain Reaction (PCR)

The polymerase chain reaction (PCR) was developed by Kary Mullis in 1984 as a method for the amplification of specific DNA sequences (107). The method is based on the use of oligonucleotides, called primers, which are complementary to the flanking regions of the DNA sequence, which is to be amplified. The elongation of the primers is catalyzed by the thermostable Taq-DNA-polymerase. The amplification is performed in cycles. During each cycle the two complementary DNA strands are firstly separated by a short heating step after which the probes are cooled to allow primer hybridization (annealing). Subsequently, DNA is enzymatically synthesized at a temperature of 72°. The main principle is that the DNA product of each cycle serves as the template for the next cycle so that theoretically the amount of the

reaction product after each cycle doubles. This leads to an exponential increase of the quantity of the amplified DNA sequence (1).

In the present work, the Hot Start PCR method was used as described in Manole et al (108). In brief, 4 μL of complementary DNA were added to a 45.5 μL master mix containing 5 μL 10X reaction buffer, 1 μL dNTPs, $MgCl_2$ and 50 pmol of sense and antisense primers. The concentration of $MgCl_2$ was varied according to the marker under detection: Increasing the concentration of $MgCl_2$ in the reaction mix increases the sensitivity (and lowers the specificity) of the method, while decreasing it increases the specificity (and lowers the sensitivity) (106). The final $MgCl_2$ concentration used for each marker is described in Results, paragraph 3.2. Negative controls without template cDNA were included in all cases to rule out carry-over contamination with genomic DNA. Three drops of mineral oil were added to each reaction tube, the lids were closed and the mixture was preheated to 95º C for ten minutes before the addition of Taq polymerase to reduce non-specific annealing and primer elongation events (the Hot Start principle). Probes were then cooled to 80º C 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), 55-61° C for 30 sec (primer annealing) and 72° C for 1 min (primer extension), followed by a final extension at 72° C for 10 min and termination at 4° C. For most genes under detection, 35 amplification cycles were performed, with the exception of ß-actin, were 25 cycles were used. Primer pair sequences, cDNA fragment sizes and annealing temperatures are shown in Table 2.1. PCR products were either visualized directly by agarose gel electrophoresis or stored at +4ºC.

2.3.6 Agarose gel electrophoresis

Agarose gels were prepared by mixing TBE solution (see materials) with agarose powder at a concentration of 1.5% or 2 % (2% for expected fragment sizes of 250 bp or lower). The solution was heated in a microwave oven for 3 minutes and ethidium bromide was added at a concentration of 5% v/v. The solution was heated again for another $1^{1/2}$ min and poured into a PCR chamber, a PCR gel comb was placed and gels were left to harden at room temperature. Gels were then immersed in TBE and 5 μL of each PCR probe mixed with 1 μL of gel loading dye (Orange G buffer) were loaded in each comb hole. Gels were run at a voltage of 90^{-} V for 45 min. Results were visualized under a UV illuminator and images were captured on p53 Polaroid film using a Polaroid MP4 Land Camera. Photographs were scanned using CanoScan 5000 equipment and software. For densitometry, scanned gel images were analysed with the ImageJ picture processing program (for results see Appendix).

Detected marker (Pubmed Accession No)	Primer sequences	Temp. profile for PCR	Amplified fragment size	Reference
ß-actin (NM 001101)	ß-actin sense: 5'-CCCAGGCACCAGGGCGTGAT -3' ß-actin antisense 5'-TCAAACATGATCTGGGTCAT-3'	30'' 95°C 30'' 59ºC 60'' 72°C 25 cycles	250 bp	(108)
GATA-4 (NM 002052)	GATA-4 sense: 5'-CTCCTTCAGGCAGTGAGAGC-3' GATA-4 antisense: 5'-GAGATGCAGTGTGCTCGTGC- 3'	30'' 95°C 30'' 58ºC 60'' 72°C 35 cycles	575 bp	(59)
HNF4α (X87870)	HNF4α sense: 5'-TCTCATGTTGAAGCCACTGC-3' HNF4α antisense 5'-GGTTTGTTTTCTCGGGTTGA-3'	30'' 95°C 30'' 57ºC 60'' 72°C 35 cycles	501 bp	(90)
Oct4 (NM 203289 & NM 002701) (two isoforms)	Oct4 sense: 5'-GACAACAATGAGAACCTTCAGGAGA-3' Oct4 antisense: 5'-CTGGCGCCGGTTACAGAACCA-3'	30'' 95°C 30'' 55ºC 60'' 72°C 35 cycles	215 bp	(43)
Tg (NM 003235)	Tg sense 5'- AGTCCTAAGTCCCCTGAT GC-3' Tg antisense: 5'- CAAAGGGAGACGTCGAGTGT-3'	30'' 95°C 30'' 55ºC 60'' 72°C 35 cycles	280 bp	(99)
Rat Oct4 (NM 001009178)	Rat Oct4 sense 5'- GAC AACAACGAGAACCTTCAGGAGA-3' Rat Oct4 antisense 5'- CTGGCGCCGGTTACAGAACCA -3'	30'' 95°C 30'' 55ºC 60'' 72°C 35 cycles	215 bp	Custom design
AFP (NM 001134)	AFP sense 5'-AGAACCTGTCACAAGCTGTG-3' AFP antisense 5'-GACAGCAAGCTGAGGATGTC-3'	30'' 95°C 30'' 56°C 60'' 72°C 35 cycles	680 bp	(89)
p63 (NM 003722)	p63 sense 5'-CGCTTCGTACCATCACCGTTCT-3' p63 antisense 5'-CCTGGACGTATTCCACTGAACT-3'	55ºC	550 bp	(109)

Table 2.1: Markers detected by PCR: primer sequences, temperature profiles, sizes of amplified fragments and literature sources.

2.3.7 Extraction and purification of DNA from agarose gels

PCR products were extracted from agarose gels using the QIAquick Gel Extraction Kit as described in the manufacturer's protocol. In brief, 10 μL of each cDNA probe (mixed with 2 μL of Orange G loading dye) were loaded on agarose gels and fragments were separated using electrophoresis as described above. Under UV light, DNA bands were excised using a dean, sharp scalpel and placed in previously weighed 1.5 ml tubes. The sizes of the gel slices were minimized by removing excess agarose. Filled tubes were weighed again and the pure weights of the gel slices were calculated by subtraction. Three volumes of Buffer QG were added to 1 volume of gel (100 mg ~ 100 μl) and the tubes were incubated at 50ºC for 10 min, until gel slices had completely dissolved. To help dissolve gel, tubes were vortexed every 2–3 min during the incubation. After gel slices had dissolved completely, as indicated by the yellow color of the mixture, 1 gel volume of isopropanol was added and samples were mixed again. To bind DNA, the samples were applied to QIAquick spin columns placed in 2 ml collection tubes, and centrifuged for 1 min. Flow-through was discarded and QIAquick columns were placed back in the same collection tubes. To wash, 0.75 ml of Buffer PE were added to QIAquick columns which were centrifuged again for 1 min. Flow-through was discarded and the QIAquick columns were centrifuged for an additional 1 min at 13,000 rpm. For elution, QIAquick columns were placed into clean 1.5 ml microcentrifuge tubes. 50 μl of H_2O were added to the center of the QIAquick membrane and columns were centrifuged for 1 min. The resulting DNA yield was either used directly for enzymatic restriction or stored at +4ºC.

2.3.8 DNA restriction

For validation of PCR products, DNA restriction experiments were performed. Restriction enzymes are endonucleases that recognize specific base sequences of a DNA double helix and hydrolyse in specific positions. This leads to the cleavage of the initial DNA sequence in two or more pieces (110).

The reaction mixtures contained 16 μl of purified DNA, mixed with 2 μl of restriction enzyme and 2 μl of corresponding buffer. The reactions were perfomed by incubation at 37ºC for 2h. Products were separated in 2% agarose gels and visualized under UV light as described above. For choice of enzymes and restriction sites see Table 3.2 in Results.

2.4 In-situ immunostaining

For in-situ immunostaining, an antibody is used to link a cellular antigen specifically to a stain that can be more readily seen with a microscope (111) . The first step of the procedure includes treatment of the cells with a fixative. Fixatives are needed to stabilize cells thereby protecting them from the rigors of subsequent processing and staining techniques. Subsequently, the cell membranes have to be made permeable so that the antigens become accessible to the antibodies. In a further preparatory step, a blocking reagent has to be applied (e.g. pig or goat serum), to avoid unspecific background staining through binding of the antibody with e.g. collagen or connective tissue elements (111) .

The staining method is based on the use of a primary antibody, which can specifically bind to the antigen under detection (in this case the stem cell markers). A secondary antibody is then added, which is generated against the immunoglobulins of the primary antibody source, e.g., if the primary antibody is raised in rabbit, then the secondary antibody could be goat anti-rabbit. After the binding of primary and secondary antibody, a third antibody is added which is coupled to a detection system (112). In this case, a colorimetric (enzyme-mediated) detection method was used: The third antibody was conjugated to the enzyme extravidin-alkaline phosphatase. Cells were then covered with Fast Red Naphthol for the designated length of time. Naphthol AS acts as the substrate for alkaline phosphatase and the Fast Red chromogen precipitates at the enzymatic sites producing a vibrant red/pink color. Cells were then counterstained with Mayer's Haemalaun, which dyed all non-stained cells in a deep blue color.

Detection of antigens in tissues is known as immunohistochemistry, while detection in cultured cells is generally termed immunocytochemistry (111).

2.4.1 Immunocytochemistry

For immunocytochemistry, primary cultures and different cell lines were grown in polysterene chamber slides under the same conditions as described above. Upon reaching the desired confluency, the medium was aspirated and the slide flask casket was carefully broken open. Cells were fixed and permeabilized for 5 min with a mixture of 50%-50% ethanol-methanol which had been previously cooled to -20°C. After washing with running water for 10 min, ethanol-methanol fixed cells were treated with 15% acetic acid to block internal alkaline phosphatase activity. Cells were then washed again with running water for 45 min and TBS (see Materials) for 3 min and blocked for 30 min with TBS containing 10% normal pig serum. Cells

were then incubated with primary antibody diluted in TBS containing 0.5% bovine serum albumin overnight at +4°C. The next day, primary antibodies were removed and the cells were washed four times with TBS and incubated for 2 h with secondary anti-rabbit or anti-mouse antibodies, diluted to 1:500 in TBS with 0.5% BSA. After washing three times with TBS, cells were incubated with an extravidin-alkaline phosphatase conjugated antibody at a dilution of 1:500 in TBS with 0.5% BSA. Cells were exposed to Fast Red Naphthol substrate solution for 5 minutes and after washing for 10 min with running water they were counterstained with Mayer's Hemalaun for 5 min and washed again for 10 min with running water before final embedding with Kayser's glycerine.

2.4.2 Immunohistochemistry

For immunohistochemistry, serial sections of formalin-fixed, paraffin-embedded tissues derived from 6 different nodular goiters were kindly provided by Professor Schneider (Institute of Pathology, Klinikum Buch, Berlin, Germany). Because the substrate in this case was tissue rather than cultured cells, an antigen retrieval protocol was used, to unmask the antigens under detection. In brief, after warming at 60º C for 30 min, sections were deparaffinized by xylene (3 x 5min), rehydrated through a series of ethanol washes (2x3 min 100% ethanol, 1x3min 80% ethanol, 1x3min 60% ethanol) and rinsed in water. Sections were then fixed, permeabilized, blocked and subjected to antigen detection with an extravidin-alkaline phosphatase system, as described above for immunocytochemistry.

2.4.3 Microscopy

Slides were examined at 40X, 100X, 200X 400X and 630X magnifications using an Opton Standard 16 halogen microscope interfaced with an Opton MC6 camera. Images were caught on Kodak Ektachrome 160 colour film and scanned using Canon equipment and software.

2.5 Flow cytometry and fluorescence-activated cell sorting (FACS)

To characterize cells with specific stem cell markers on the protein level, flow cytometry after indirect immunofluorescence staining was performed. Fluorescence activated cell sorting (FACS) further enabled the isolation of stem-cell-marker positive cell populations. FACS is the most widely used method for selectively isolating cells based on their marker expression profiles

and further cultivating them. This method has already been successfully applied in a number of reports of adult stem cell isolation from differentiated tissues (113, 114)

Flow cytometry is defined as the measurement of the cellular and fluorescent properties of cells in suspension as they pass by a laser or other light source (110). The measurements are represented by changes in light scattered, light absorbed, and light emitted by a cell as it passes by fixed detectors directed off the light source. From these measurements, specific populations and subsets within them are defined and even isolated physically using a dedicated cell sorter typically by manipulating cell charge. Prior to this procedure, the cells have to be labelled using a specific primary antibody (in this case against the stem cell markers) and subsequently a secondary antibody conjugated to a fluorochrome. As the cells pass through the cytometer, all light signals, whether from fluorescently labelled cells or from the beam scattered by unlabeled cells, are transferred to a computer and transformed into digital signals. These signals can then be displayed as histogram graphs or, as is the case in these experiments, as two-dimensional graphs called dot-plots. In these diagrams, one parameter is plotted against another in an X versus Y axis display (see Results, figure 3.26).

For application of the protocol, the cell cultures were grown as described above, trypsinised and washed in PBS. According to the manufacturer's protocol, the recommended cell concentration was $1x10^6$ cells per ml PBS. Because the antigens under detection were all transcription factors localised in the nucleus, cells were permeabilized by alcohol. Cells were subsequently incubated with an unconjugated primary antibody in a microcentrifuge tube for 30 minutes on ice. Cells were then centrifuged at 1000 rpm for 5 min and washed twice with PBS. Secondary antibody (anti-mouse or anti-rabbit) conjugated with FITC, PE or APC fluorochrome was then added, cells were centrifuged again at 1000 rpm for 5 min and the same washing procedure was followed. Supernatant was then pipetted off and the pellet was resuspended in 200µl PBS in a Falcon tube. The suspension was then filtered through a 30µM filter. Propidium iodide (PI) at a final concentration of 0,1µg/ml was added 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. The analysis was performed with FACS Calibur Equipment (Becton- Dickinson Biosciences, Heidelberg, Germany) in the facilities of the German Rheumatology Research Center, Berlin. Cellquest software was used for data analysis.

2.6 Materials

2.6.1 Sterilisation methods

Most of the plastic materials and solutions used were sterilised in a high pressure, steam-saturated autoclave at a temperature of 121° C and stored under sterile conditions prior to use.

Glass vessels and reusable metal instruments were heated to 200°C in a heating oven for at least two hours.

To avoid contamination with microorganisms, all cell culture solutions and media were filtered through a 0,22 µm cellulose acetate filter (Corning 430769) prior to use. FCS was pre-filtered through a 0,8 µm filter (Schleicher and Schuell) to avoid obstruction of the 0,22 µm filter.

2.6.2 General Solutions

TBS: 109 g/l Tris
55,6 g/l boric acid
9,3 g/l EDTA-Na_2· 2 H_2O

PBS: 120 mM NaCl
2,7 mM KCL
10 mM NaH_2PO_4/Na_2HPO_4

TBE: 10 mM Tris, pH: 7,6
150 mM NaCl

2.6.3 Enzymes and chemicals

Enzymes	
Collagenase A	Roche (Mannheim, Germany)
Dispase	Roche (Mannheim, Germany)
Restriction Endonucleases and Buffers	Roche (Mannheim Germany)
Chemicals	
50 bp DNA ladder	Invitrogen Inc (Karlsruhe, Germany)
TrackIt Cyan/Orange gel loading buffer	Invitrogen Inc (Karlsruhe, Germany)

Trypan Blue solution (0,4%)	Sigma Aldrich (Steinheim, Germany)

Materials for Reverse Transcription and PCR

Taq Polymerase and 5X PCR Buffer	Invitrogen Inc (Karlsruhe, Germany)
MgSO4	Invitrogen Inc (Karlsruhe, Germany)
Reverse Transcriptase and 10X Reaction Buffer	Promega Corp (Mannheim, Germany)
RNAGuard RNase Inhibitor	Amersham Biosciences (Aylesbury, UK)
Primers	Invitrogen Inc (Karlsruhe, Germany)
dNTP mononucleotides	Roche (Mannheim, Germany)
p(dT)15 oligos	Roche (Mannheim, Germany)

Cell culture materials

Media:

Coon's modified Ham F12	Gibco Invitrogen (Karlsruhe, Germany)
Hank's balanced salt solution (HBSS)	Gibco Invitrogen (Karlsruhe, Germany)
Dulbeccos modified Eagle medium (DMEM)	Gibco Invitrogen (Karlsruhe, Germany)
RPMI 1640	Gibco Invitrogen (Karlsruhe, Germany)
Fetal Calf Serum (FCS)	Gibco Invitrogen (Karlsruhe, Germany)

Hormones:

Apo-Transferrin	Sigma-Aldrich (Steinheim,Germany)
Glycyl-Histidyl-Lysine	Sigma-Aldrich (Steinheim,Germany)
Hydrocortison	Sigma-Aldrich (Steinheim,Germany)
Somatostatin	Sigma-Aldrich (Steinheim,Germany)
TSH from Bovine Pituitary	Sigma-Aldrich (Steinheim,Germany)
Insulin (Insuman Rapid)	Aventis Pharma (Frankfurt, Germany)

Aminoacids :

MEM non-essential aminoacids (100x)	Gibco Invitrogen (Karlsruhe, Germany)

Antibiotica:

Penicillin/Streptomycin	Roche (Mannheim, Germany)
Amphotericin B	Bristol-Meyers Squibb (Germany)

Stimulation Factors

Xanthosine (x-0750)	Sigma-Aldrich (Steinheim, Germany)

2.6.4 Ready-Kits

RNeasy Mini-Kit (mRNA isolation)	Qiagen (Hilden, Germany)
QIAshredder	Qiagen (Hilden, Germany)
Qiaquick Gel Extraction Kit	Qiagen (Hilden, Germany)

2.6.5 Antibodies

Primary antibodies

GATA-4 rabbit polyclonal	Santa Cruz (sc-9053)
HNF4α goat polyclonal	Santa Cruz (sc-6556)
HNF4α rabbit polyclonal	Santa Cruz (sc-8987)
Oct4 mouse monoclonal	Santa Cruz (sc-5279)
ERK-1 rabbit polyclonal	Santa Cruz (sc-94)
Tg goat polyclonal	Santa Cruz (sc-7835)

Secondary antibodies

Donkey anti-mouse	DAKO A/S Denmark (E0433)
Donkey anti-rabbit	DAKO A/S Denmark (E353)

Fluorochrome conjugated secondary antibodies

Goat anti-mouse IgG-FITC	Santa Cruz (sc-2010)
Donkey anti-rabbit IgG-FITC	Santa Cruz (sc-2090)
Donkey anti-rabbit IgG-PE	Santa Cruz (sc-3475)
Donkey anti-goat IgG-F(ab')-APC	Santa Cruz (sc-3860)
Goat anti-mouse IgG-FITC	Santa Cruz (sc-2010)
Donkey anti-goat IgG-FITC	Santa Cruz (sc-2024)

Extravidin-alkaline phosphatase conjugated antibody	Sigma Chemicals (St Luis,MO) (E-2636)

2.6.6 Materials for immunocytochemistry, immunohistochemistry and flow cytometry

Bovine Serum Albumin (BSA)	Sigma-Aldrich (Steinheim, Germany)
Mayers Hemalaun	Omikron GmbH (Berlin, Germany)
Fast Red Napthol tablets	Sigma Chemicals (St Louis, MO)
Propidium Iodide	Santa Cruz

All basic chemicals, not listed here in detail, where purchased either as powders or stock solutions from the companies Sigma, Merck, Biomol and Boehringer Mannheim. Powders were diluted with sterile, salt-free water prior to use. Materials used for mRNA experiments were diluted in sterile RNAse-free water (Qiagen RNEasy Minikit).

2.6.7 Equipment

Laminar flow cabinet	Heraeus, Laminair HB 2448
Cell culture incubator	Heraeus Instruments
Phase contrast microscope	Nikon, TMS
Halogen microscope	Opton Standard 16
Halogen microscope camera	Opton MC6 with Karl Zeiss Lens
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	Gibco BRL, EPS ST 606 T Pahrmacia, ECPS 3000/150 Consort, Microcomputer electrophoresis power supply
Homogenisator	Eppendorf Thermomixer 5436

Centrifuges	Hettich Mikro 200R
	Beckmann Microfuge E
	Hettich Rotina 46R
Heating Oven	Memmert
Pippetes and tips	Eppendorf
Plastic centrifuge tubes (1.5, 15 and 50 ml)	Sarstedt
Falcon Tubes	Becton Dickinson

Plastic culture materials

100 X 20 mm tissue culture dishes	Sarstedt
150 X 20 mm tissue culture dishes	Sarstedt
24-well tissue culture plates	Sarstedt
96-well tissue culture plates	Sarstedt
Polysterene slide flasks	Nunc (Wiesbaden, Germany)
	(Cat. No 170920)

Software

SPSS Vers. 11.5	Statistical analysis
Adobe Photoshop Vers 6.0	Preparation of pictures and figures
Biology Workbench Vers. 3.2	DNA sequence analysis and restriction
	enzyme site calculation
Cellquest	Flow cytometry analysis
Image J	Gel Densitometry

FACS Equipment

FACS Calibur Equipment	Becton-Dickinson Biosciences

(located in the facilities of the German Rheumatology Center, Berlin)