3 RESULTS

3.1 Patient group

Thyroid tissues were obtained from 32 patients with nodular goiters after thyroidectomy. Malignancy was ruled out in all cases by means of cytological examination prior to the operation and/or rapid excision biopsy during the operation. The patient group consisted of 32 patients, 18 females (56,25 %) and 14 males (43,75%). The mean age of the patients was 56.91 years. The mean age of female patients was 56.73 years and of male patients 57.25 years. The youngest patient was a 33 year old female, the oldest a 71 year old female.

3.2 Detection of stem cell marker RNA by RT-PCR

After total mRNA isolation, the expression of stem cell marker mRNA in primary thyroid cell culture was investigated using the RT-PCR method. The results are summarized in table 3.1:

Marker	**No of patients tested**			**No of positive cultures (% of total)**		
	Males	Females	Total	Males	Females	Total
Oct4	14	18	32	14 (100%)	18 (100%)	32 (100%)
GATA-4	14	18	32	14 (100%)	18 (100%)	32 (100%)
HNF4α	14	18	32	14 (100%)	18 (100%)	32 (100%)
p63	14	18	32	14 (100%)	18 (100%)	32 (100%)
AFP	14	18	32	0 (0%)	1 (5.5%)	1 (3.125%)
Tg	14	18	32	14 (100%)	18 (100%)	32 (100%)

Table 3.1: Summary of PCR results regarding the expression of stem cell marker mRNA in primary thyrocyte cultures obtained from human goitres.

Oct4

Expression of Oct4 mRNA could be detected in all of the primary cultures tested. The amplified sequence displayed the expected size of 215 base pairs (bp) in all cases. Specific signal could be achieved using an annealing temperature of 55° and a $MgCl_2$ concentration of 1.5 mM.

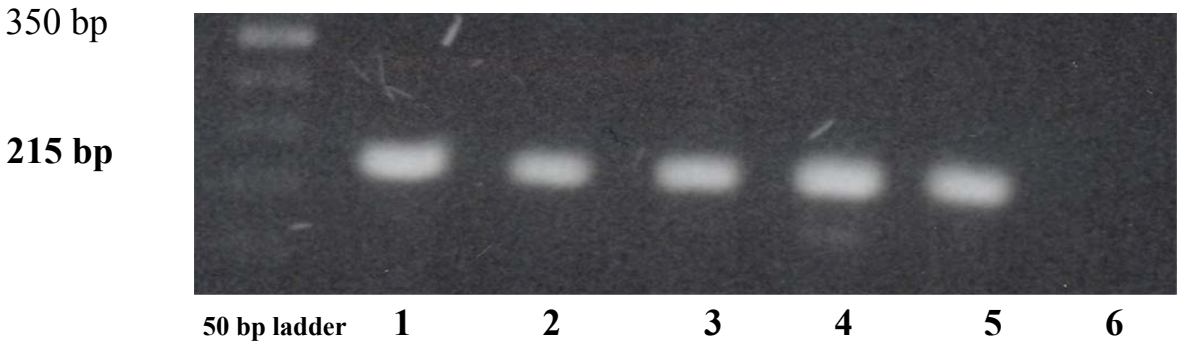

Figure 3.1: Oct4 mRNA expression in five different primary thyrocyte cultures (lanes 1-5). Lane 6 shows a no-template contol.

HNF4α

Using HNF4α specific primers, positive signal could be observed in all of the primary cell cultures. The amplified sequences displayed the expected size of 501 bp. Specific signal was achieved by using an annealing temperature of 57° and a $MgCl_2$ concentration of 1,0 mM.

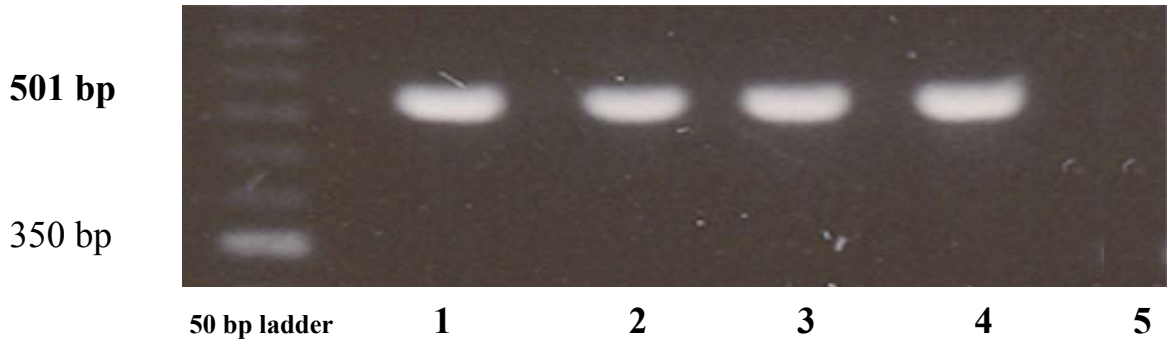

Figure 3.2: HNF4α mRNA expression in four (n=4) different primary thyrocyte cultures (lanes 1-4). Lane 5 shows a negative no-template control.

GATA-4

GATA-4 mRNA expression was detectable in all of the primary cultures tested. Using a $MgCl_2$ concentration of 1.0 mM and an annealing temperature of 58° a specific signal for GATA-4 could be achieved in all cases. The expected fragment size was 575 bp. Figure 3.3 displays a representative sample where four (n=4) different primary thyrocyte cultures have been tested.

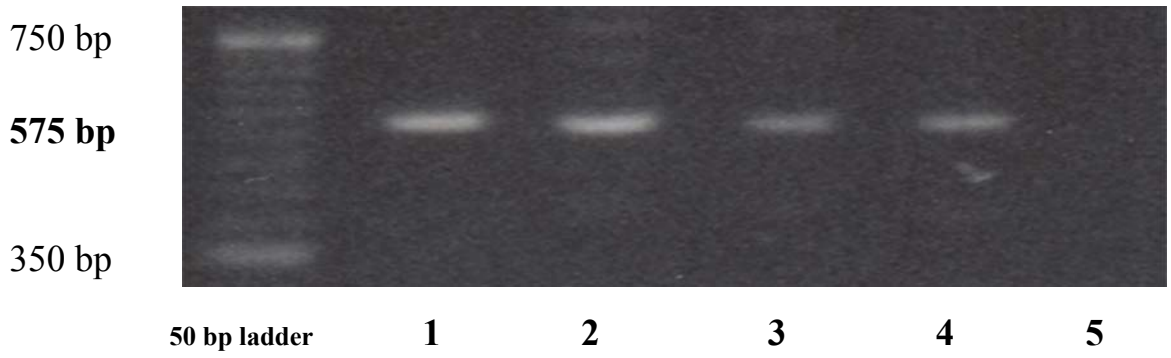

Figure 3.3: GATA-4 m-RNA expression in four (n=4) different primary thyrocyte cultures (lanes 1-4) . Lane 5 shows a no-template control.

p63

Positive p63 signal could be observed in all of the primary thyroid cultures. Annealing temperature was 55° and $MgCl_2$ concentration 1.5 mM.

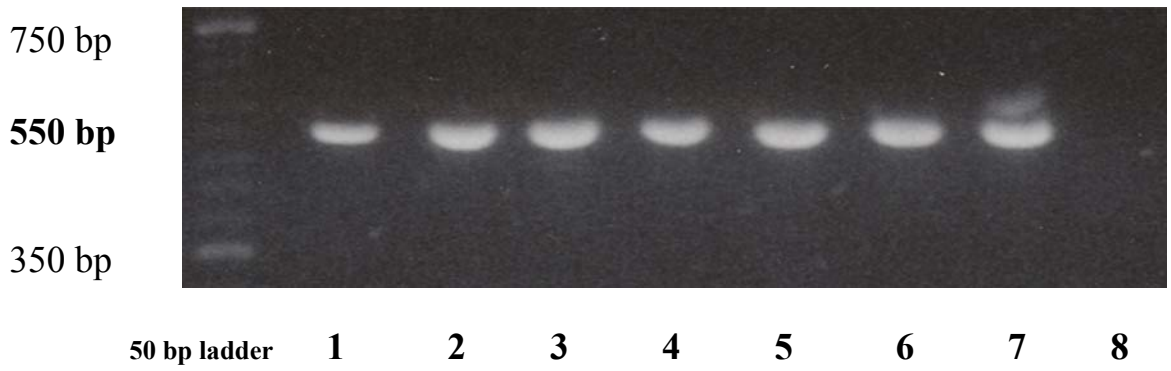

Figure 3.4: p63 mRNA expression in seven (n=7) different primary thyrocyte cultures (lanes 1-7). Lane 8 shows a negative no-template control.

AFP

Positive AFP signal could be detected in only one of the 32 primary thyroid cultures tested (3,125 %). In this one case, AFP was transiently expressed during the first passage but was no longer detectable after the second passage. In all other primary cultures no AFP signal was detectable, although several annealing temperatures, cycle numbers and $MgCl_2$ concentrations were tried.

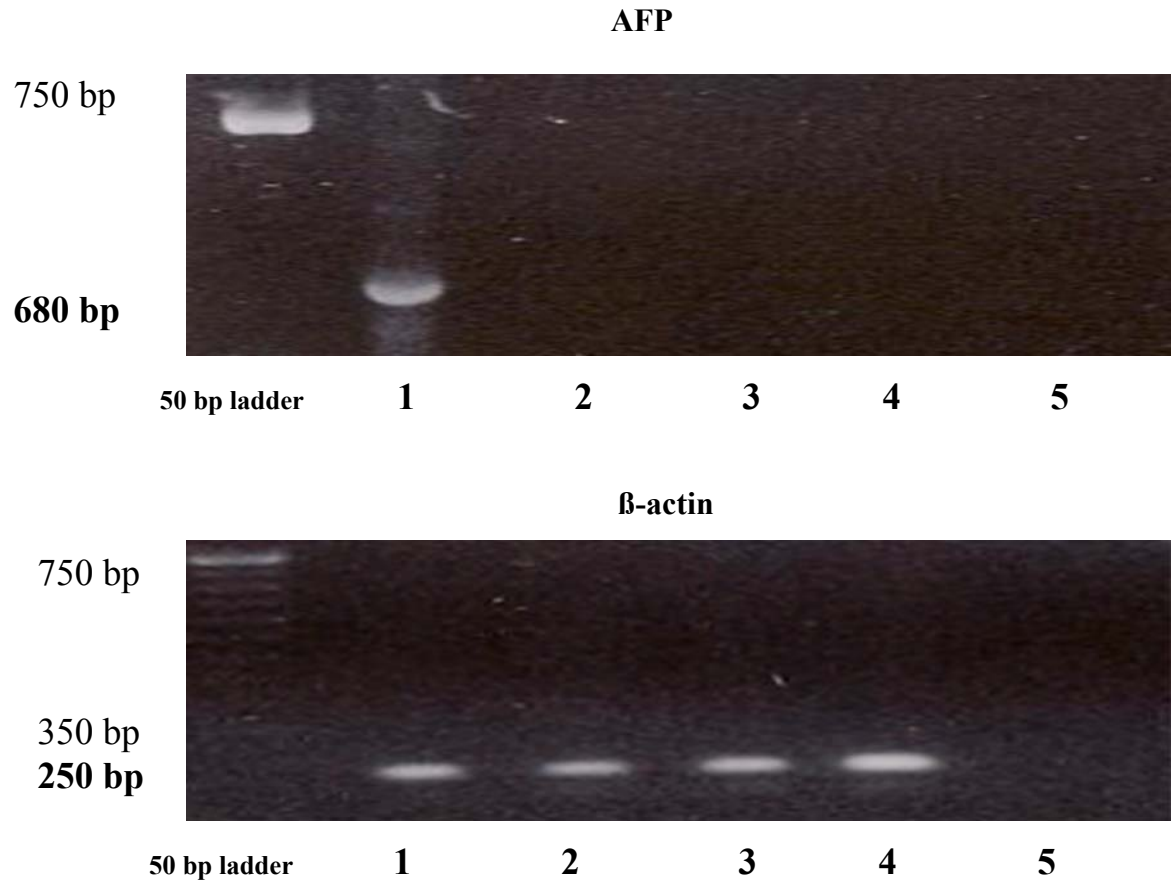

Figure 3.5: AFP mRNA expression in one primary thyrocyte culture. Expression is only observed in the first passage (lane 1) but is not detectable in all subsequent subcultures (lanes 2-4). Lane 5 represents negative no-template controls. Results are shown in comparison to ß-actin controls.

Thyroglobulin

Thyroglobulin was used as a marker of terminally differentiated thyroid cells. As expected, all primary thyroid cell cultures tested positive (for results see next page). The annealing temperature was 55°C and the $MgCl_2$ concentration 1.5 mM. This marker was tested to ensure that the cultures obtained were indeed primary thyrocyte cultures, since thyroglobulin is expressed exclusively by thyroid follicular cells. A representative sample is shown in Figure 3.6:

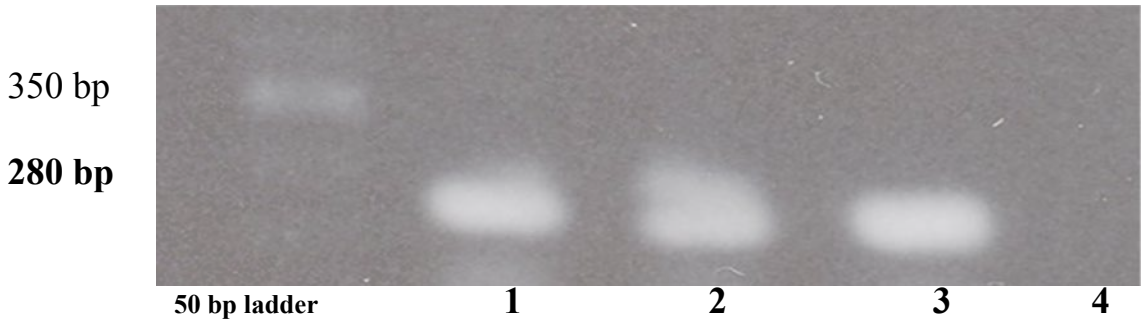

Figure 3.6: Thyroglobulin mRNA expression in three (n=3) primary thyrocyte cultures (lanes 1-3). Lane 4 shows a negative no-template control.

3.3 DNA Restriction

To validate PCR products, restriction endonuclease experiments were performed. To enable electrophoretic visualization, enzymes cleaving at asymmetric sites were picked so that, after restriction, uneven DNA pieces would result. The choice of enzymes was based on analysis of the mRNA sequences for restriction enzyme sites using the Biology Workbench program (Vers. 3.2). DNA sequences, restriction enzymes, cut sites and expected products are shown in Table 3.2:

Marker	Restriction enzyme	Buffer	Cut site	cDNA size before cleavage	Expected product size
Oct4	Alu I	Buffer A	166: AG'CT	215 bp	166 bp - 49 bp
GATA-4	Nco I	Buffer H	182: C'CATG_G	575 bp	398 bp - 182 bp
HNF4α	Xba I	Buffer H	380: T'CTAG_A	501 bp	380 bp - 121 bp
AFP	Pst I	Buffer H	412: C_TGCA'G	680 bp	264 bp - 412 bp
P63	Hpa II	Buffer L	451: C'CG_G	550 bp	451 bp - 99 bp

Table 3.2: Restriction enzymes, cut sites and DNA fragment sizes before and after cleavage.

As shown in figure 3.7, Oct4, GATA-4, HNF4α and p63 were successfully restricted to products of the expected size. The AFP sequence, which was transiently expressed in one of the cultures, was not restricted although two different endonucleases were used. The correctness of the AFP sequence could therefore not be validated.

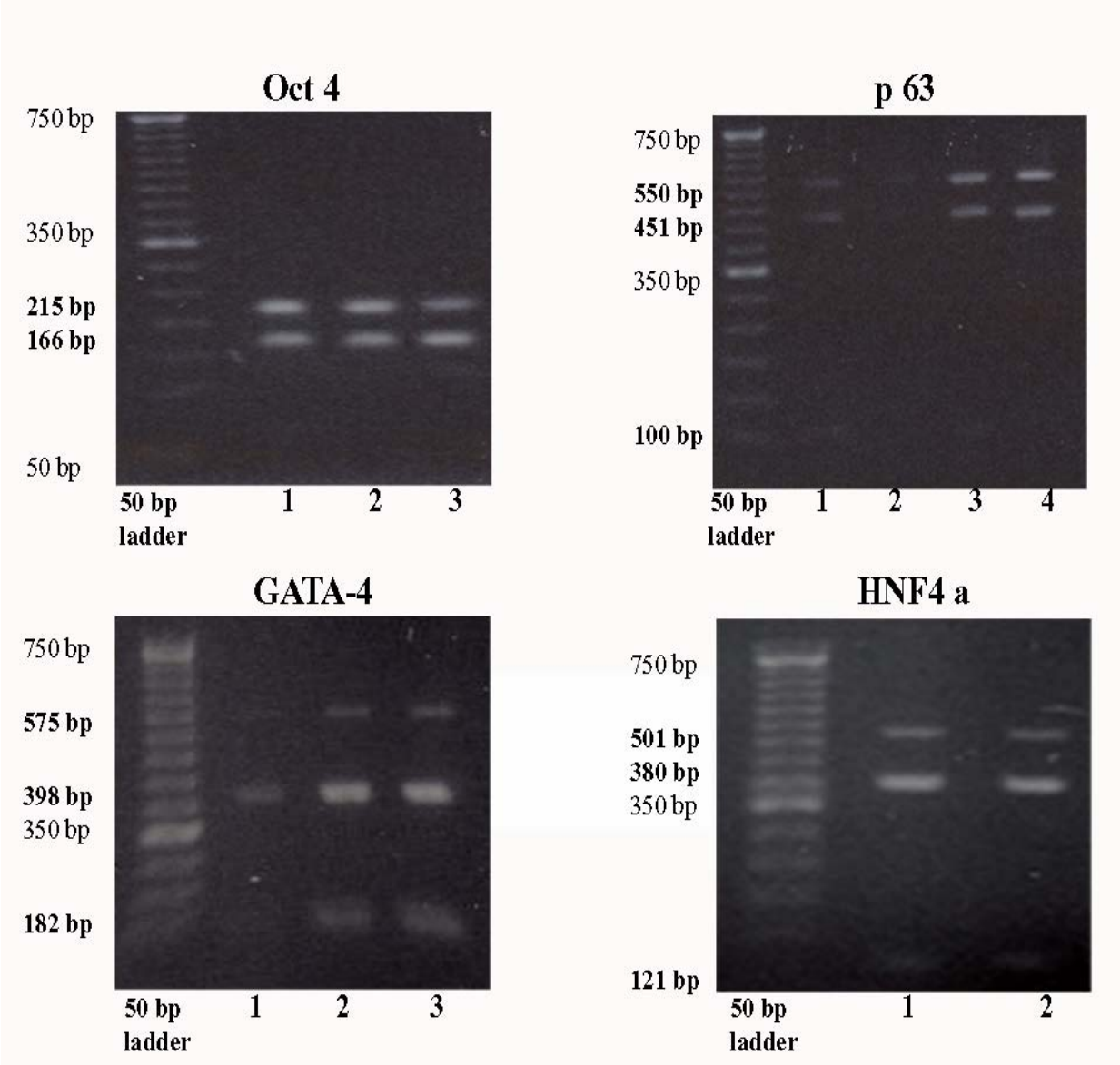

Figure 3.7: Cleavage of the Oct4, GATA-4, p63 and HNF4α cDNA sequences using restriction endonucleases. Numbers represent different thyroid tissues. In the case of Oct4, only the 215 bp unrestricted DNA band and the 166 bp restricted band are shown, the 49 bp band is not visible because of its very small size.

3.4 Stem cell marker expression after a different number of passages

To investigate the timely distribution of stem cell marker signal, cell cultures were passaged several times. When cells had reached the desired confluence, one or two dishes were used for mRNA isolation while all the others were passaged further. Under optimal culture conditions, some of the cells could be subcultured as much as fourteen (n=14) times within a period of 3-4 months. By means of semi-quantitative RT-PCR, it was shown that Oct4, GATA-4, HNF4α and p63 remained readily expressed, even after an increased number of passages (figure 3.8). Although slight differences were occasionally observed between passages, no specific tendency (neither increase, nor decrease) in the level of stem cell marker mRNA expression was observed (Appendix, Table 7.1). Experiments were performed with at least six different primary thyrocyte cultures and similar results were obtained in all cases. A representative sample is shown in figure 3.8:

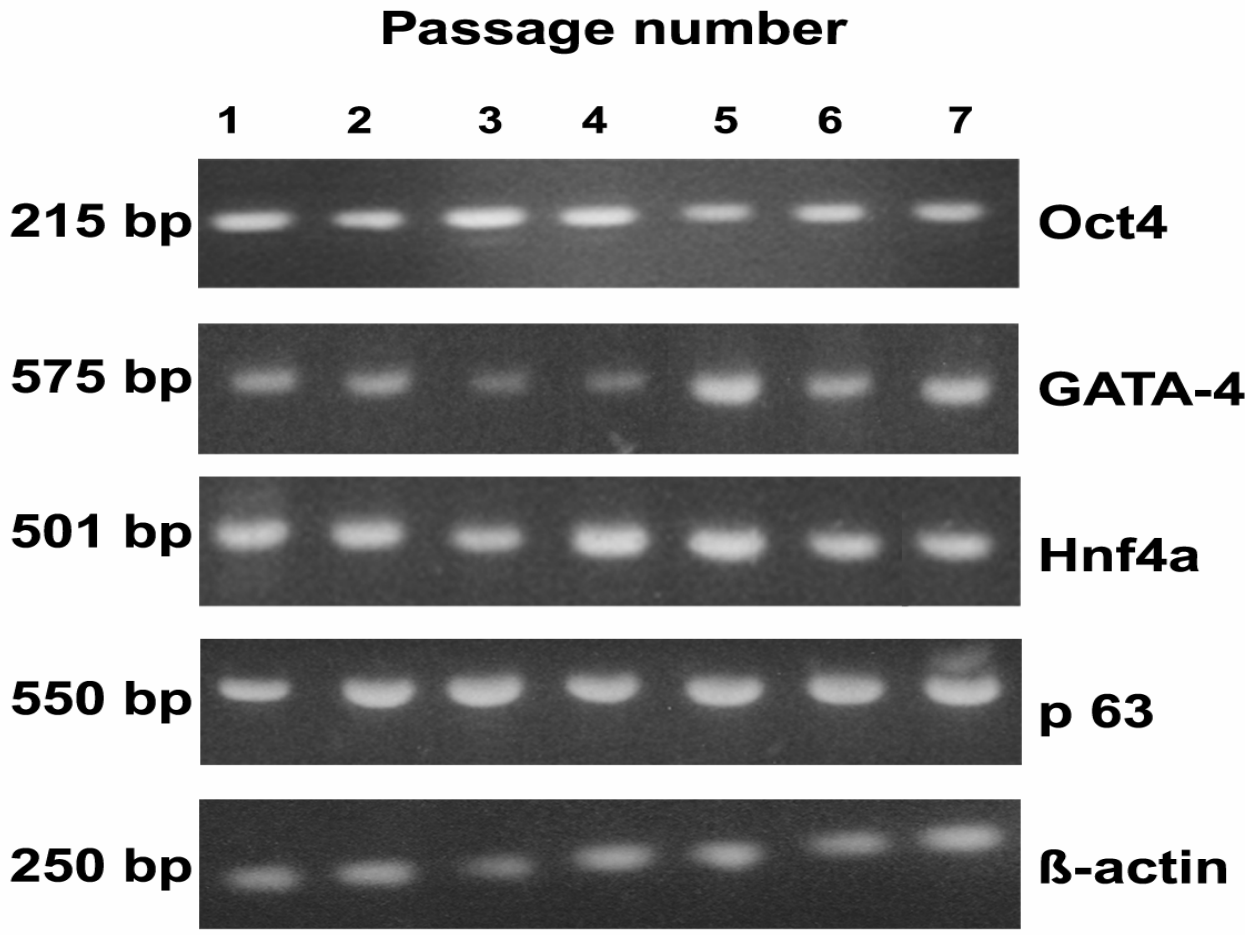

Figure 3.8: Kinetics of stem cell marker expression in primary cultures of cells derived from goiters. No significant difference in Oct4, GATA-4 and HNF4α signal between different passages. Results presented here originate from the same culture. Similar results were obtained for all of the cultures tested (n=6)

3.5 Comparison of stem cell marker expression between nodular and paranodular thyroid regions

For the first part of these experiments, thyroid tissue was classified as nodular (tissue coming direct from a thyroid nodule) or paranodular (tissue from an area adjacent to a thyroid nodule), according to intraoperative surgical assessment. Tissue samples were subsequently cultured separately and mRNA was isolated. In all cases, no significant difference in stem cell marker expression between the two different tissue types was observed (figure 3.9 and Appendix, table 7.2).

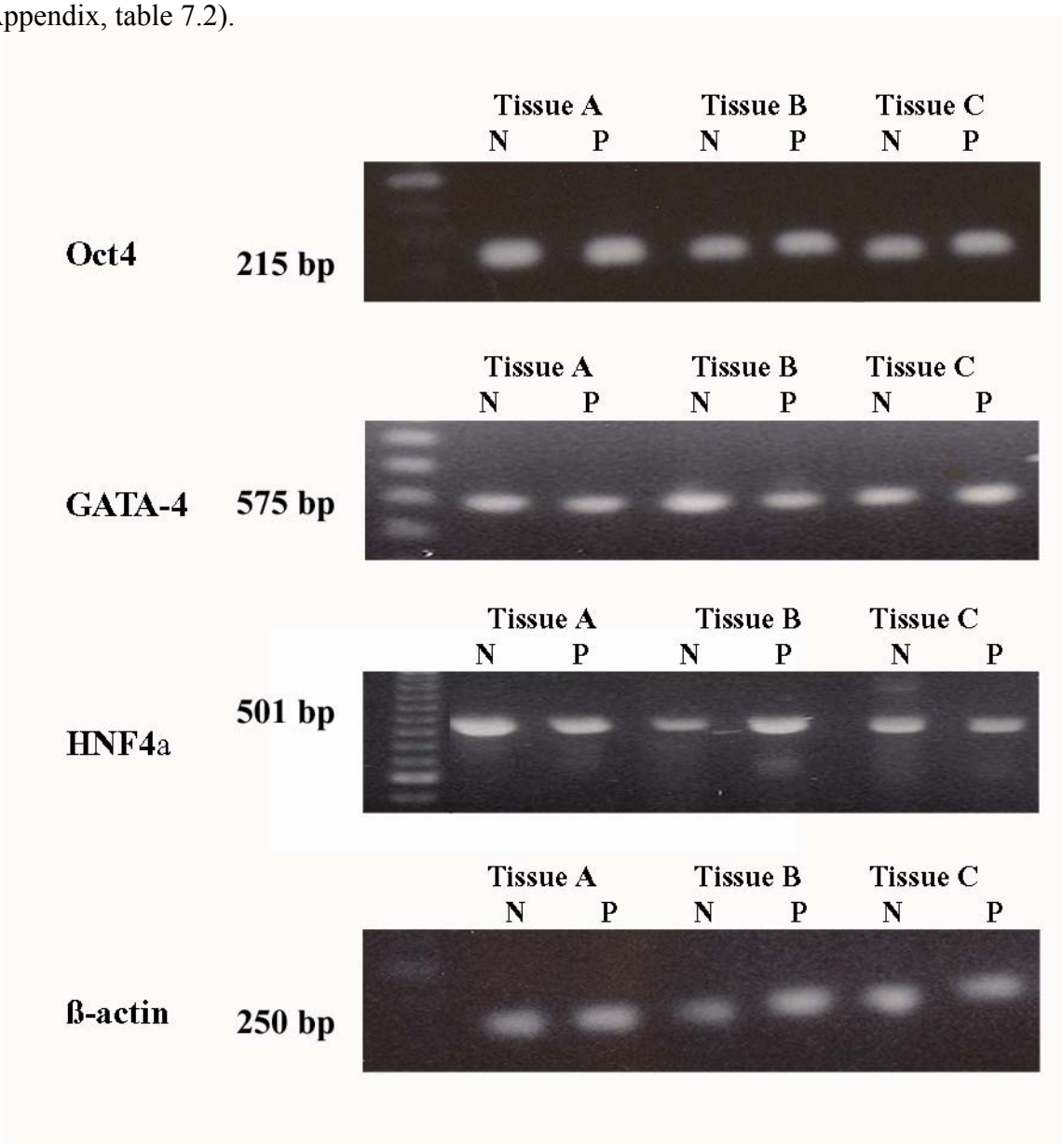

Figure 3.9: Comparison of stem cell marker mRNA expression in nodular (N) and paranodular (P) thyroid regions of three (n=3) different nodular goiters (Tissues A, B and C). Cells obtained from nodular and paranodular regions of the same tissue were isolated and cultured separately.

3.6 Comparison of stem cell marker expression in males and females

Stem cell marker mRNA for Oct4, HNF4α, GATA-4 and p63 was detected in all thyroid cultures coming from male as well as female patients. As shown in table 3.1, there was no difference in marker expression between males or females.

3.7 Expression of stem cell markers in the HTh74, HTC, HeLa and FRTL5 cell lines

All cell lines were stored in liquid nitrogen. For culturing, they were defrosted and grown in suitable culture media as described in Materials and Methods. The results are summarized in figure 3.10. In contrast to the primary thyrocyte cultures, both thyroid carcinoma lines (HTh74 and HTC) displayed no expression of any of the stem cell markers. Similar results were obtained for the FRTL5 cell line. Because of the origin of this cell line from rat species, the human and rat Oct4 genes were compared and Oct4 primers complementary to the rat sequence were designed and used. Rat and human Oct4 mRNAs display 85% similarity with only three base pairs different (all data from analysis with Biology Workbench Vers. 3.2, comparison of nucleotide sequences NM203289 and 001009178 with BLAST). Because of the great similarity between the two nucleotide sequences, the human Oct4 primers were also tested. In both cases, no Oct4 expression was observed. Similar negative results were obtained for GATA-4 and HNF4α (figure 3.11).

HeLa cells share some characteristics common to embryonic stem cells and embryonic carcinoma lines and they have been reported to display nuclear Oct4 expression (43). In the present work, they were used as a positive control for Oct4 expression. The cells were also found to express the HNF4α marker at a lower level, but tested negative for GATA-4 and thyroglobulin (figure 3.11).

All cell lines derived from thyroid tissue (primary cultures, FRTL5, HTh74, HTC) tested positive for thyroglobulin, a marker of differentiated thyroid cells. The HTh74 cells are considered highly undifferentiated, however it has already been reported that they express thyroglobulin at low levels (100)

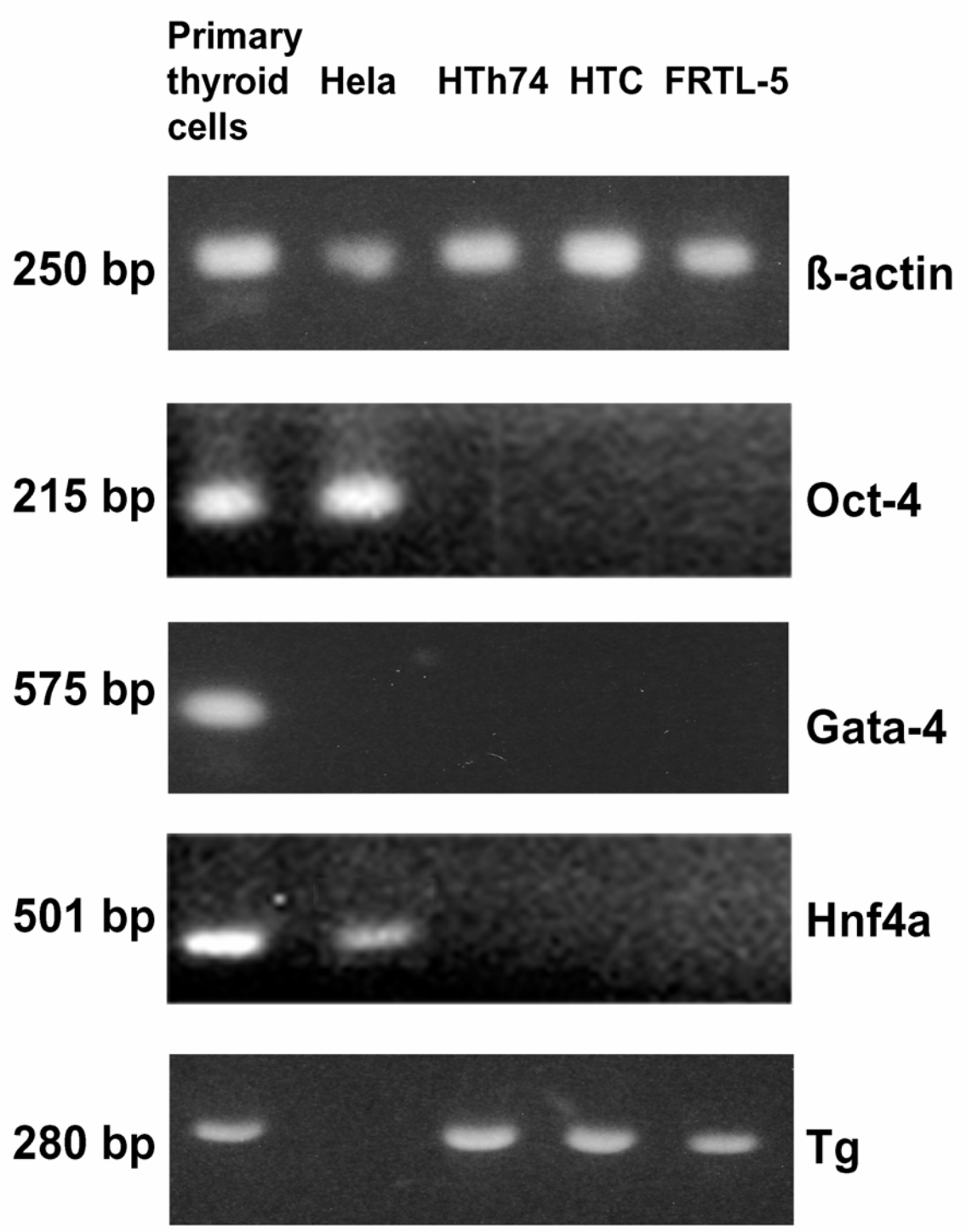

Figure 3.10: Expression of stem cell marker and thyroglobulin mRNA in primary thyrocyte cultures, in the Hela, HTh74 and HTC carcinoma cell lines and in FRTL5 cells

3.8 Xanthosine stimulation

The basic principles of the SACK theory were described in Introduction (paragraph 1.4). In an attempt to apply it to the primary thyrocyte cultures, two different approaches were adopted. Firstly, cultures originating from the same patients and being at the same passage number were selected and randomized in three groups. The first group was treated with 200 μM of xanthosine added to the normal H5+TSH medium, the second with 400μM of xanthosine added to the normal H5+TSH medium and the third, which served as controls, only with H5+TSH medium. Cells were cultured further for a period of one month with mRNA isolations after 48h, 7, 14 and 30 days. Subsequently, RT-PCR for Oct4, GATA-4 and HNF4α cDNA expression was performed. The above mentioned xanthosine concentrations and stimulation times were adopted from the publication of Lee et al, where the successful application of the SACK theory in rat hepatic cell lines led to the isolation of two adult stem cell lines (97) As shown in figure 3.11 (see also corresponding Table 7.3 in Appendix), there was no difference in Oct4, GATA-4 and HNF4α expression after 48 hours, 7 days, 14 days or 30 days of xanthosine treatment (either 200 or 400 μM) in comparison to controls. Moreover, there was no difference between the 200μM and the 400μM treatment groups. Xanthosine therefore did not seem to have the desired effect of selective propagation of stem cell marker positive cells (which would have been detected as an increase of stem cell marker mRNA expression). It should be noted that in two of the cultures treated with 400μM of xanthosine, cell toxicity was observed.

Secondly, in an attempt to reproduce the method used by Lee et al in detail and to achieve a pure stem cell culture, limiting dilution experiments were performed. The number of viable cells obtained directly after thyroid tissue digestion was firstly determined by trypan blue exclusion. Cells were then diluted down to very low viable cell concentrations and plated in 96-well culture plates, so that an expected average of two viable cells per well would result. In this way, the probability of some of the wells containing only stem cells was increased. All wells were then filled with culture medium containing either 200μM or 400μM of xanthosine and cells were grown under normal culture conditions with a medium change every 3-4 days. Wells were observed daily by phase contrast microscopy over a period of one month, however no cell growth was observed in any of the cultures tested.

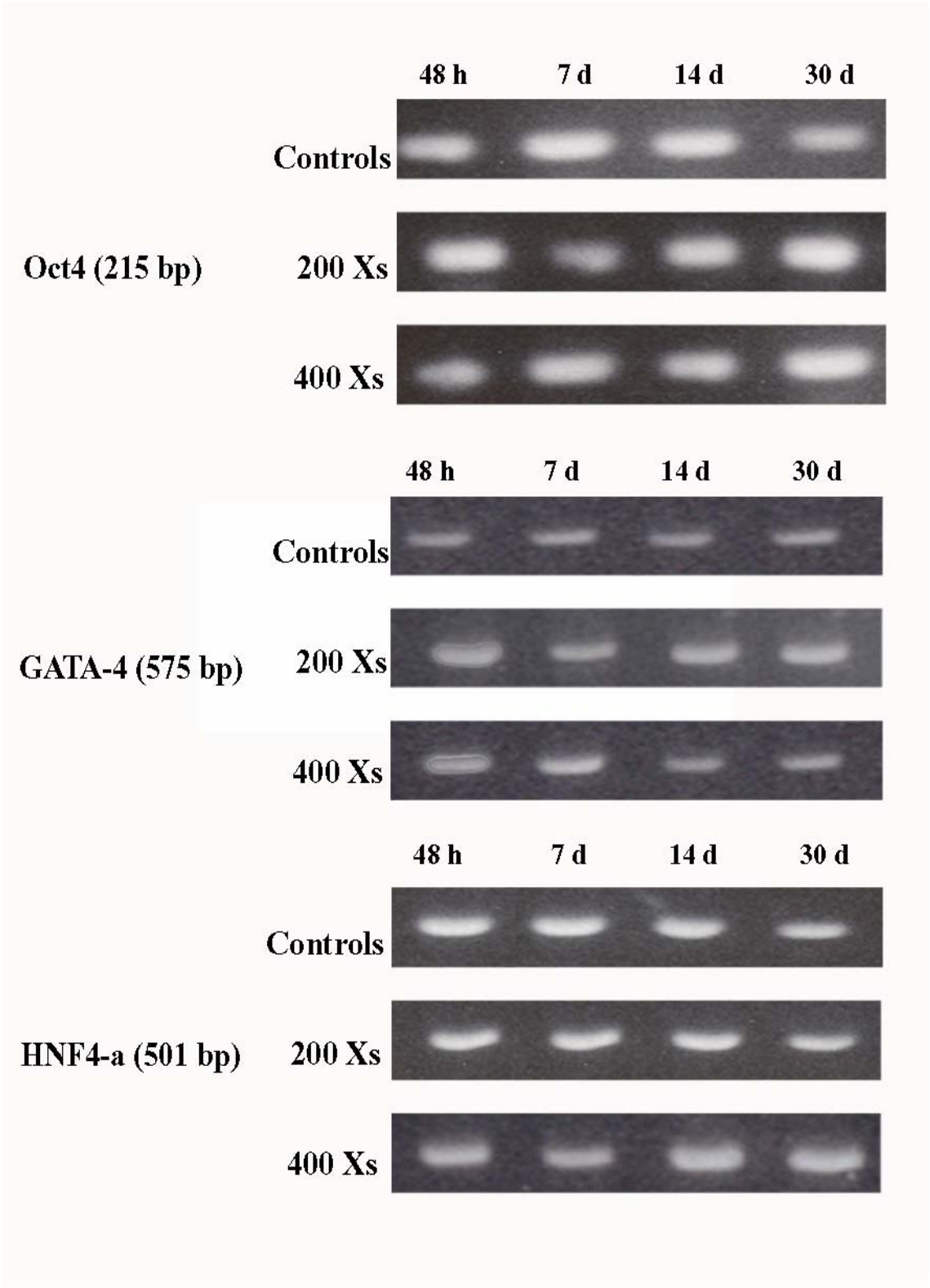

Figure 3.11: Expression of stem cell marker mRNA in primary thyrocyte cultures after stimulation with 200 or 400 μM of xanthosine (Xs) for 48 hours and 7, 14 or 30 days.

3.9 TSH stimulation

To investigate whether TSH has an influence on the growth of stem cell marker-positive cells, TSH stimulation experiments were performed. Cells obtained from the same patient were grown to 30-40% confluency using normal H5 medium with 5 mU/ml TSH. Cells were then randomized in two groups. At time 0, Group A was treated with 200 mU/ml of TSH while group B was treated further with 5 mU/ml TSH (necessary for the preservation of primary thyrocyte cultures) and served as controls. Cells were observed over a period of two weeks with mRNA isolation after 8 and 15 days. RT-PCR for Oct4, GATA-4 and HNF4α expression was performed.

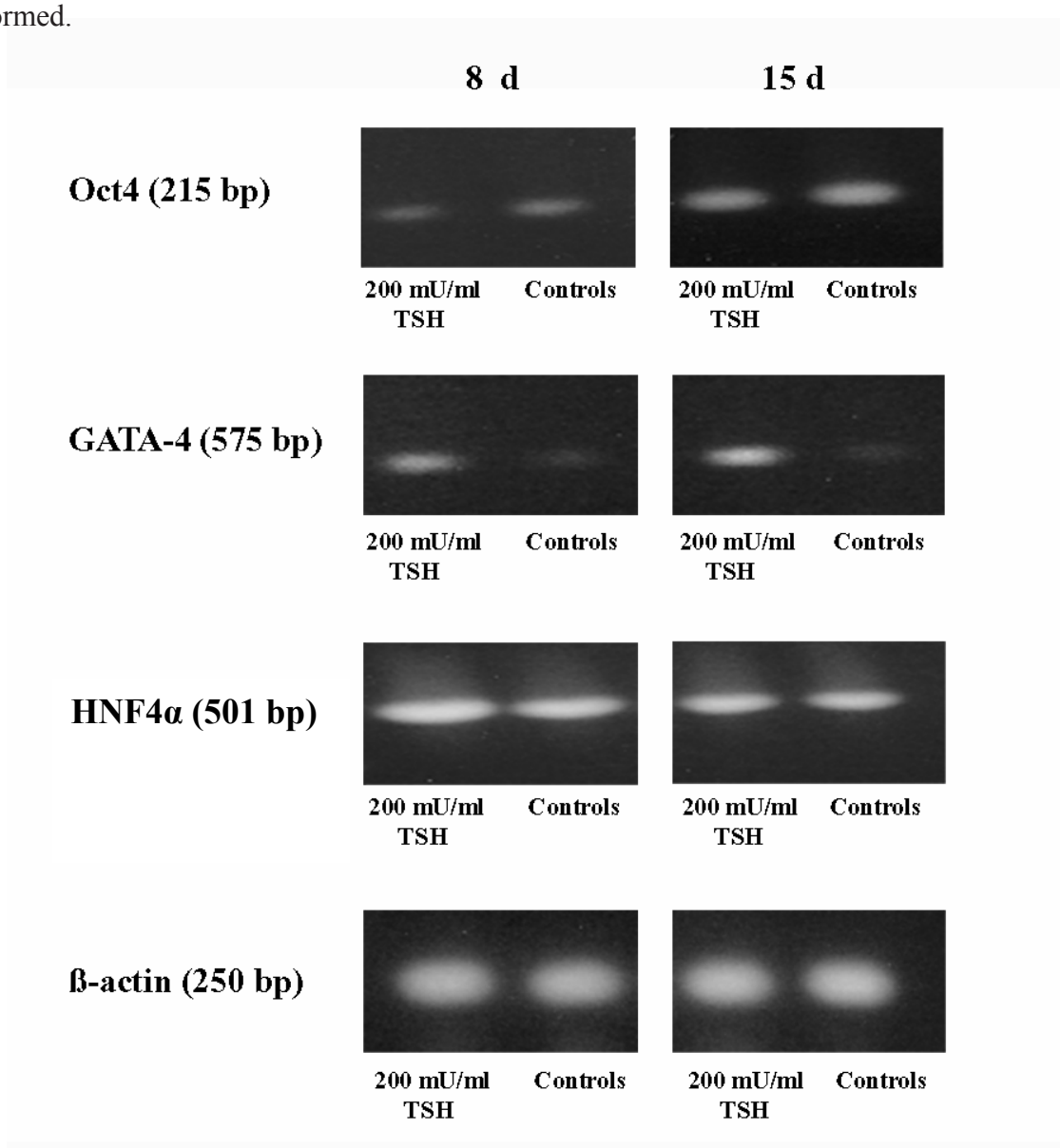

Figure 3.12: Expression of stem cell marker mRNA and ß-actin in primary thyrocyte cultures after treatment with 200 mU/ml of TSH for 8 or 15 days. Primary thyrocyte cultures treated with normal H5 medium (5 mU/ml TSH) were used as controls.

In cells treated with a high TSH concentration, an increase in GATA-4 expression was observed. The effect was detectable after 8 days of high-dose TSH treatment (relative increase 387.6%) and after 15 days of treatment (relative increase 511.9%). Results were normalized to ß-actin controls. (Appendix, table 7.4). These data were obtained after densitometric analysis of the RT-PCR gels (shown in figure 3.12) with ImageJ image analysis software and they clearly highlight a positive effect of a high TSH concentration on the expression of the GATA-4 factor. However, given that the detection method in this case is semi-quantitative, no precise quantitative estimations are possible. For this purpose, an established quantitative method (e.g. Real-Time PCR) would have to be used.

TSH treatment did not affect the expression of Oct4 and HNF4α; the two markers were expressed to the same level after 8 or 15 days of 200 mU/ml of TSH treatment as compared to controls (Figure 3.12 and Appendix, Table 7.4).

3.10 Immunocytochemistry

Primary thyrocyte cultures and established cell lines (HeLa, FRTL5, HTh74, HTC) were grown in polysterene chamber slides as monolayers and immunostained for the expression of Oct4, GATA-4 and HNF4α. Staining was performed using a colorimetric (enzyme-mediated) detection method (see Materials and Methods). For the detection of stem-cell marker positive cells, only nuclear immunoreactivity was considered specific, as all the antigens under detection were transcription factors localized in the nucleus.

Oct4

For the detection of Oct4 positive cells in primary thyrocyte cultures, a human Oct4-specific primary antibody was used at dilutions ranging from 1:100 to 1:400. In all of the seven (n=7) different cultures tested, single cells displaying positive (red) nuclear staining could be observed. Positive cells were very rare and were always found as single cells in the vicinity of Oct4 negative (blue) thyrocytes (figures 3.14 and 3.15).

As a positive control for nuclear Oct4 staining, the HeLa cell line was used. HeLa cells have been reported to display punctate nuclear staining for Oct4 protein when grown in “normal” medium (i.e. not containing any differentiation factors) (43). Using the same staining method as

described above for primary cultures, positive nuclear staining of the majority of cells could be observed (figure 3.16)

GATA-4

Using GATA-4 specific primary antibodies at a concentration of 1:100, GATA-4 protein was detected in the nuclei of single cells in all of the four (n=4) different primary thyrocyte cultures tested. Positive cells were very rare, similar in size to their surrounding thyrocytes and always found isolated amidst big numbers of GATA-4 negative cells (figure 3.17)

HNF4α

Nuclear staining for HNF4α protein could be detected in two of the four primary thyrocyte cultures tested. The pattern of expression was similar to the one for Oct4 and GATA-4: very few single cells displaying nuclear staining could be observed, always surrounded by HNF4α-negative thyrocytes (figure 3.18).

p63

P63-positive cells were detected in all of primary thyrocyte cultures tested (n=4). Their distribution was similar to all other cell markers: rare single cells always in the vicinity of p63-negative thyrocytes (3.19).

Thyroid carcinoma cell lines HTh74 and HTC

The HTh74 anaplastic thyroid carcinoma cell line and the HTC thyroid follicular carcinoma cell line displayed no expression of any of the stem cell markers, a representative picture is shown in figure 3.20.

FRTL5 cell line

As expected according to the RT-PCR results, the FRTL5 cells did not display expression of the stem cell markers. A representative sample of a negative Oct4 staining is shown in figure 3.21), the results were identical after staining for GATA-4, HNF4α and p63.

Negative controls

As a negative control, staining of several primary thyrocyte cultures was performed but with omission of the primary antibody (replacement with TBS+0.5% BSA). No positive cells were detected and no unspecific background staining was observed (figure 3.13)

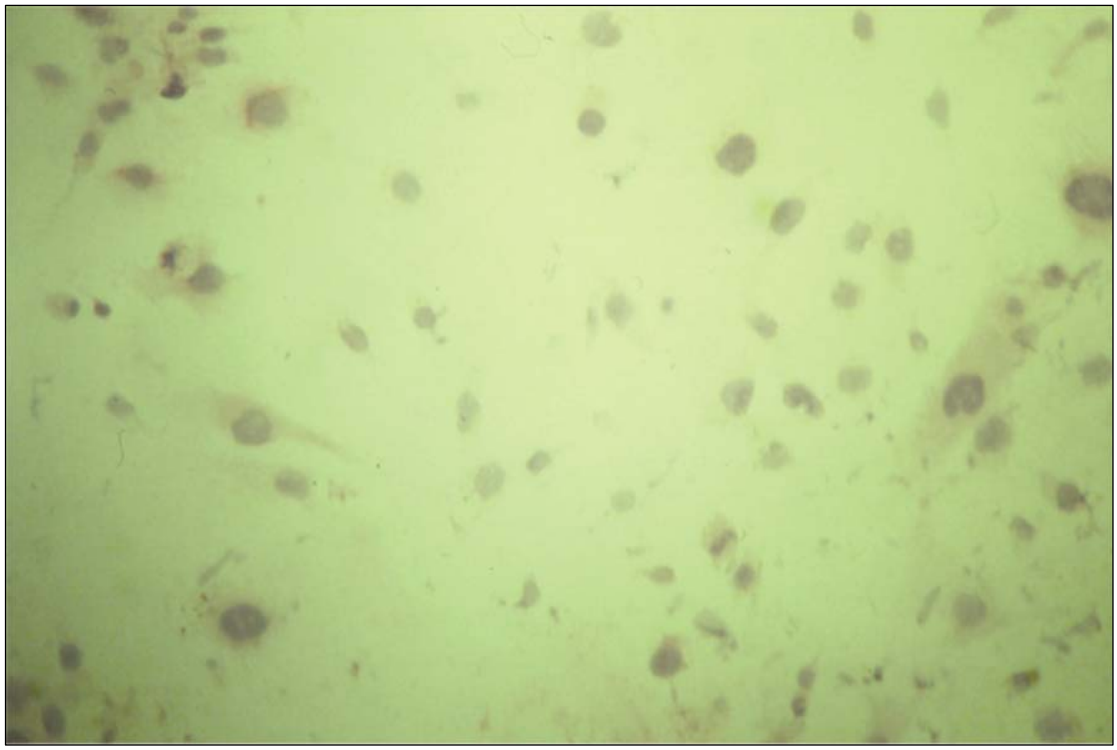

Figure 3.13: Negative staining control of a primary thyrocyte culture after omission of the primary antibody (replacement by TBS+0.5% BSA). No positive cells observed. 100X magnification.

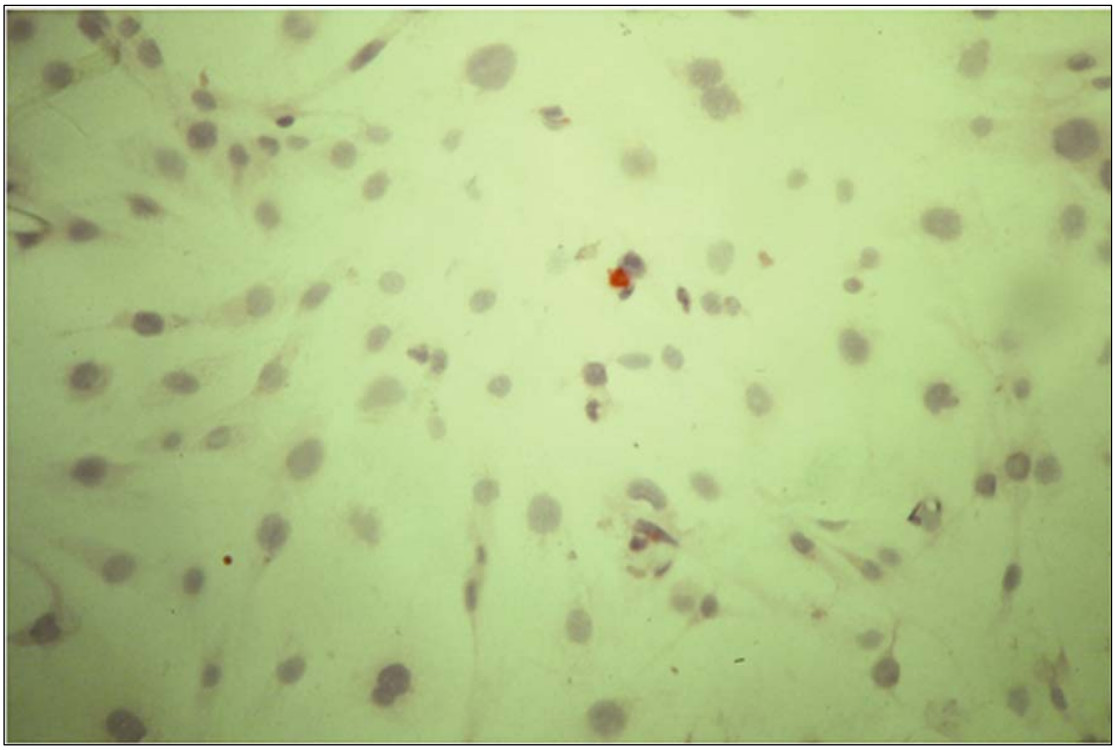

Figure 3.14: Immunocytochemical staining of a primary thyrocyte culture for Oct4, 100X magnification. A single positive cell nucleus is shown, surrounded by Oct4 negative thyrocytes.

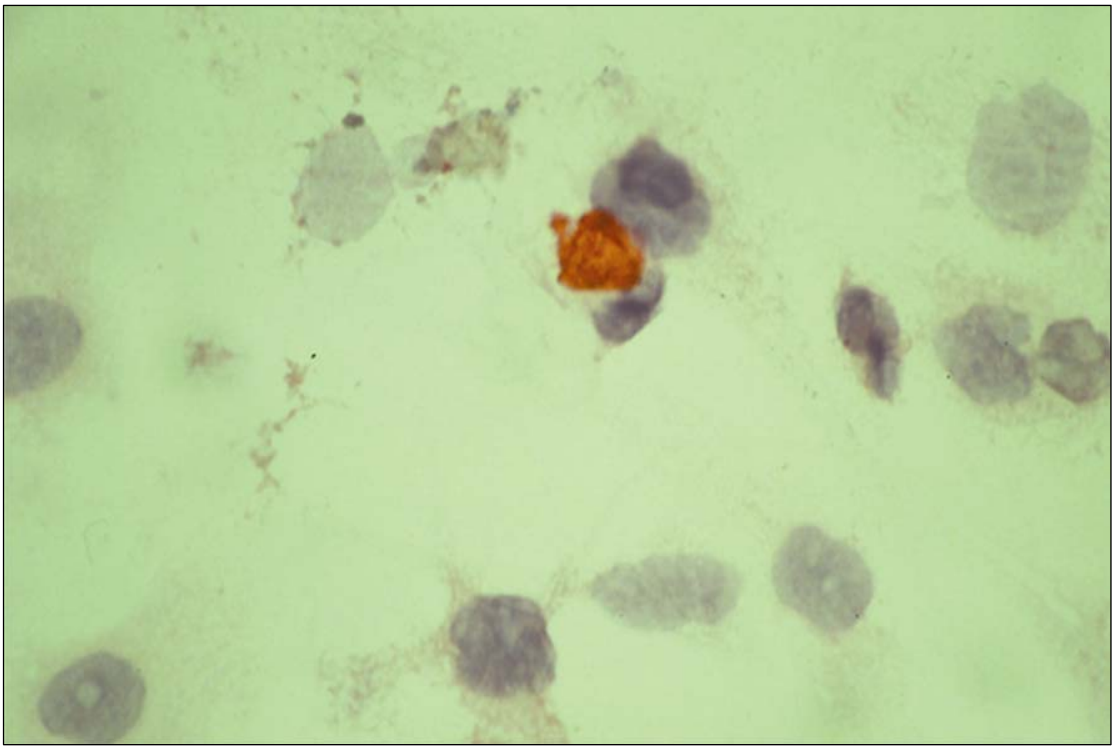

Figure 3.15: 400X magnification of the Oct4 positive cell nucleus shown in figure 3.14

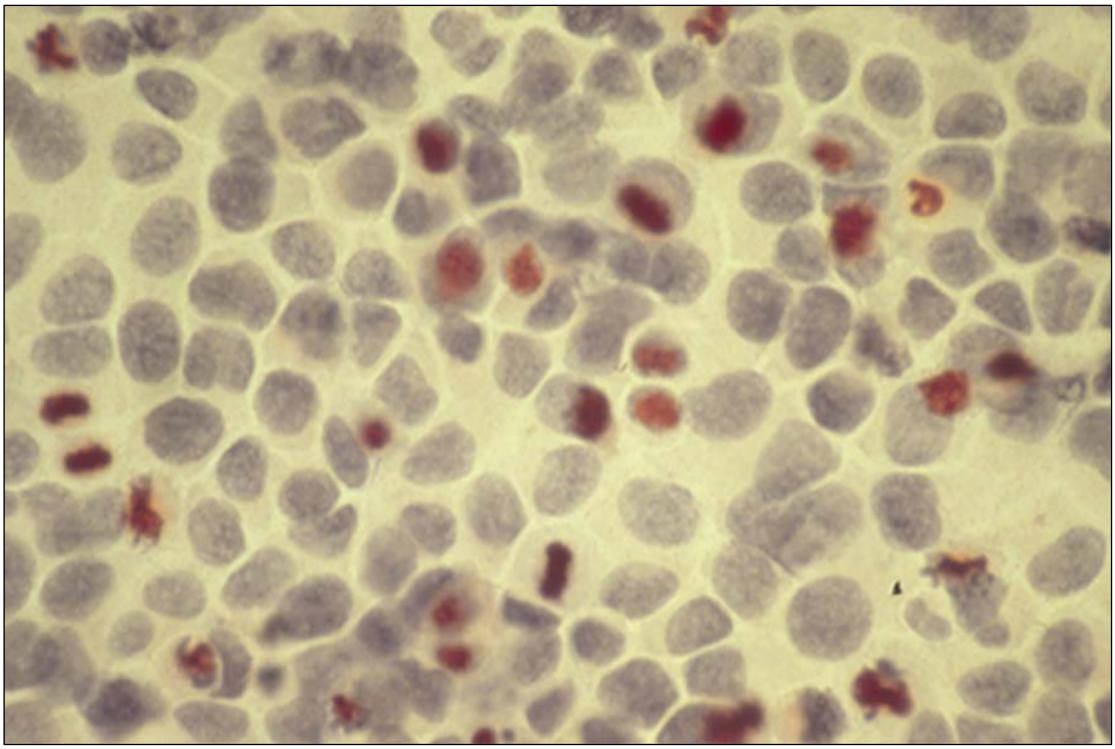

Figure 3.16: Immunocytochemical staining of HeLa cells for Oct4. Nuclear staining of the majority of cells. 200X magnification.

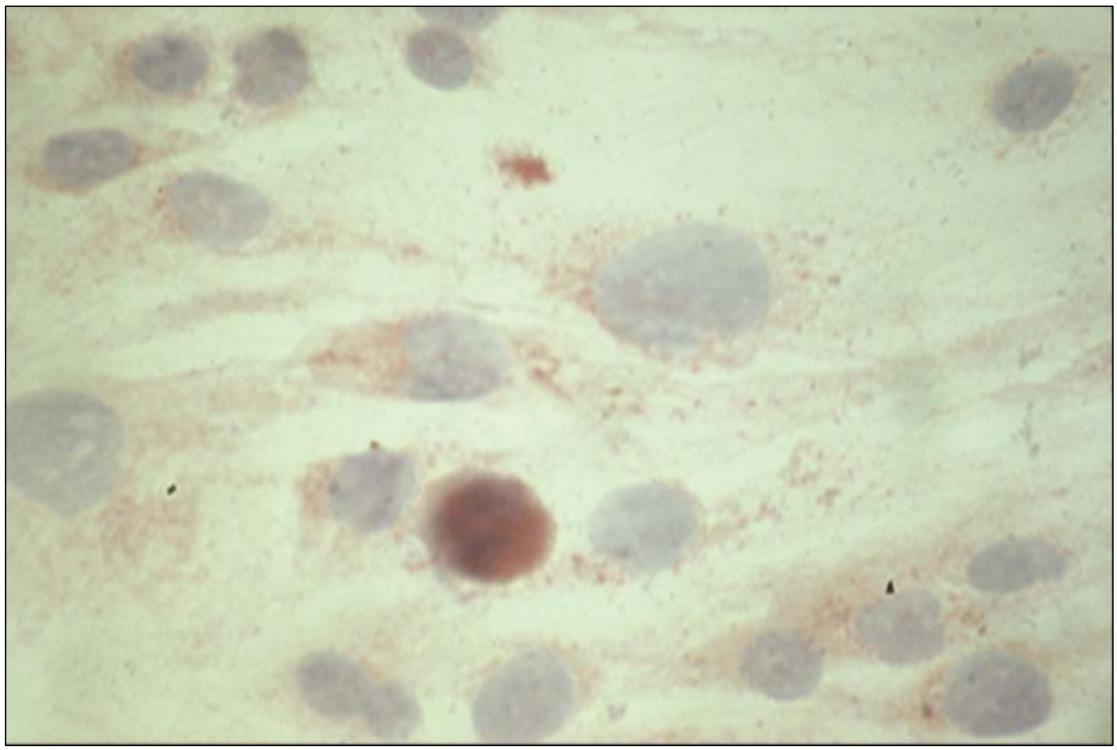

Figure 3.17: Immunocytochemical staining of a primary thyrocyte culture for GATA-4. A single, positive cell nucleus is shown, surrounded by GATA-4 negative thyrocytes. 400X magnification.

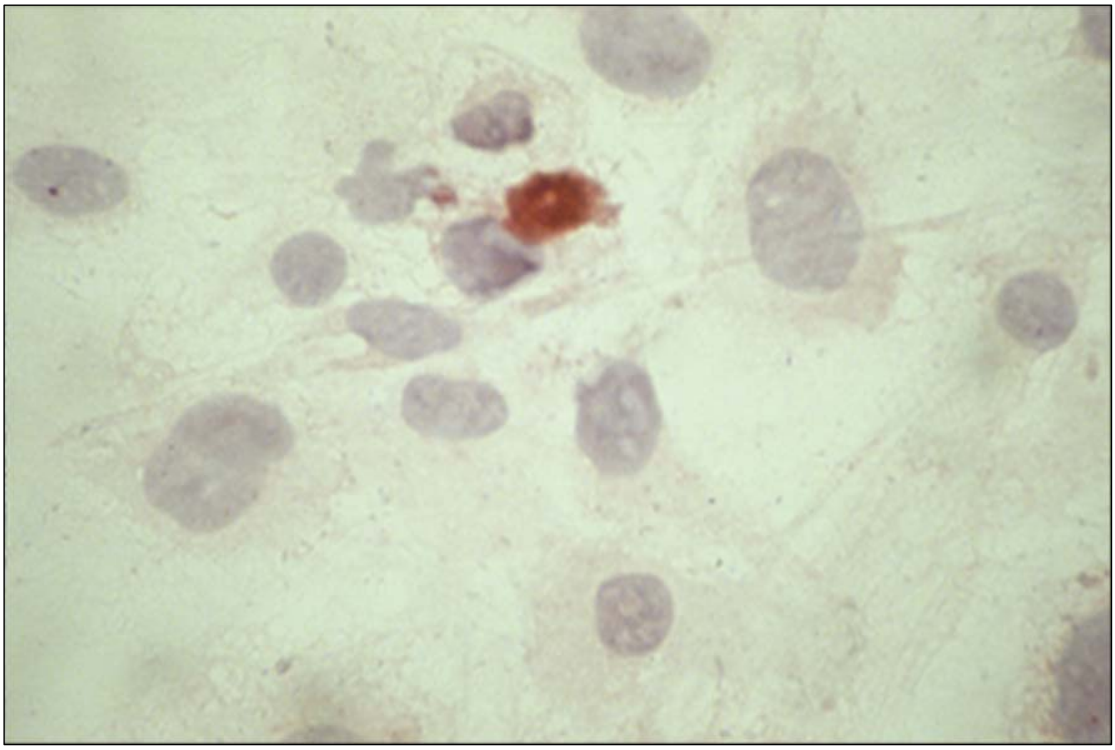

Figure 3.18: Immunocytochemical staining of a primary thyrocyte culture for HNF4α. A single, positive cell nucleus is shown, surrounded by HNF4α negative thyrocytes. 400X magnification.

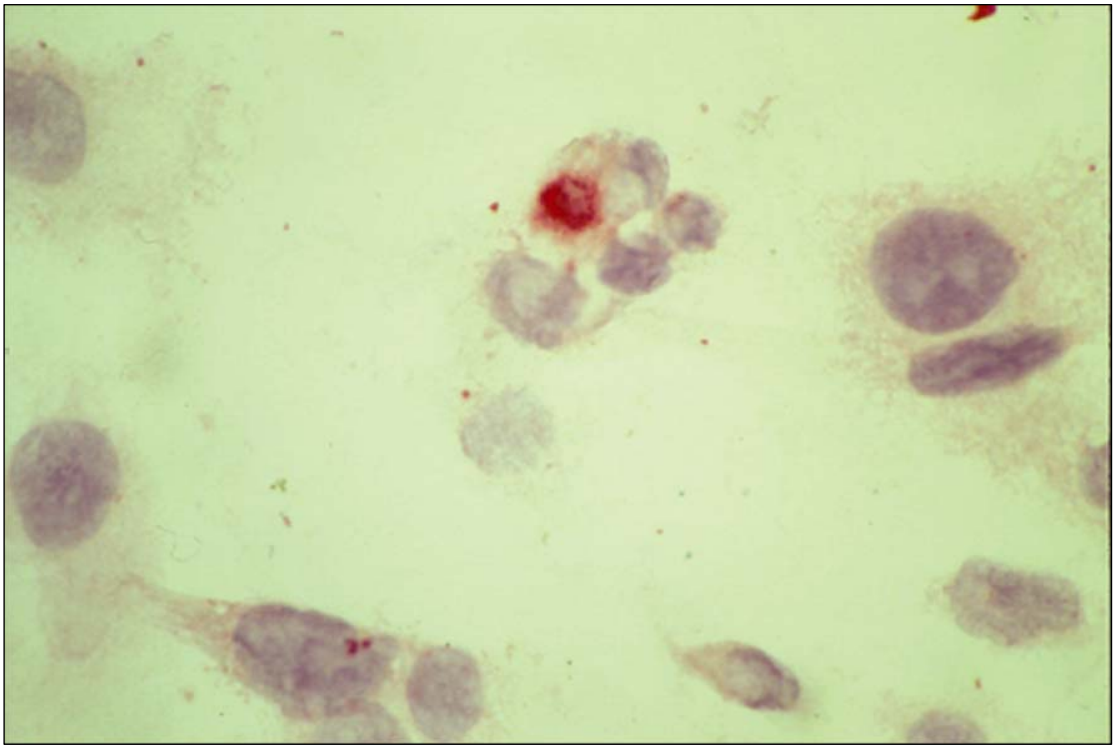

Figure 3.19: Immunocytochemical staining of a primary thyrocyte culture for p63. A single, positive cell nucleus is shown, surrounded by a group of four negative cells. 400X magnification.

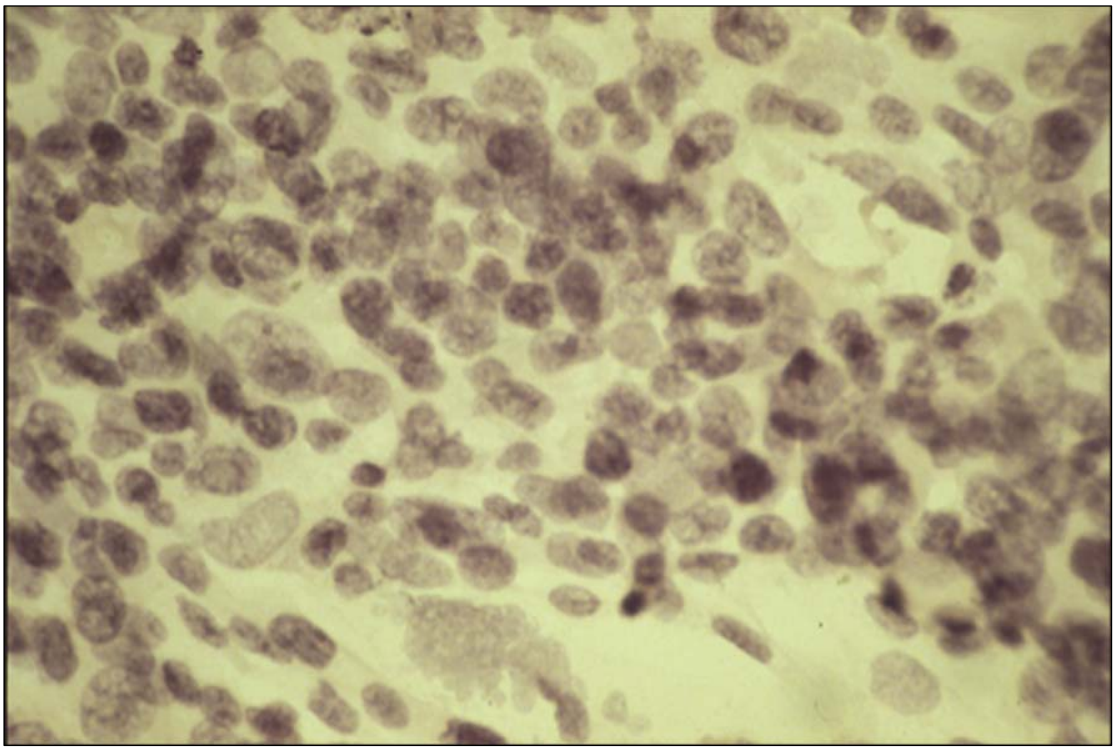

Figure 3.20: Immunocytochemical staining of HTh74 thyroid anaplastic carcinoma cells for Oct4. No positive cells detected. 200X magnification.

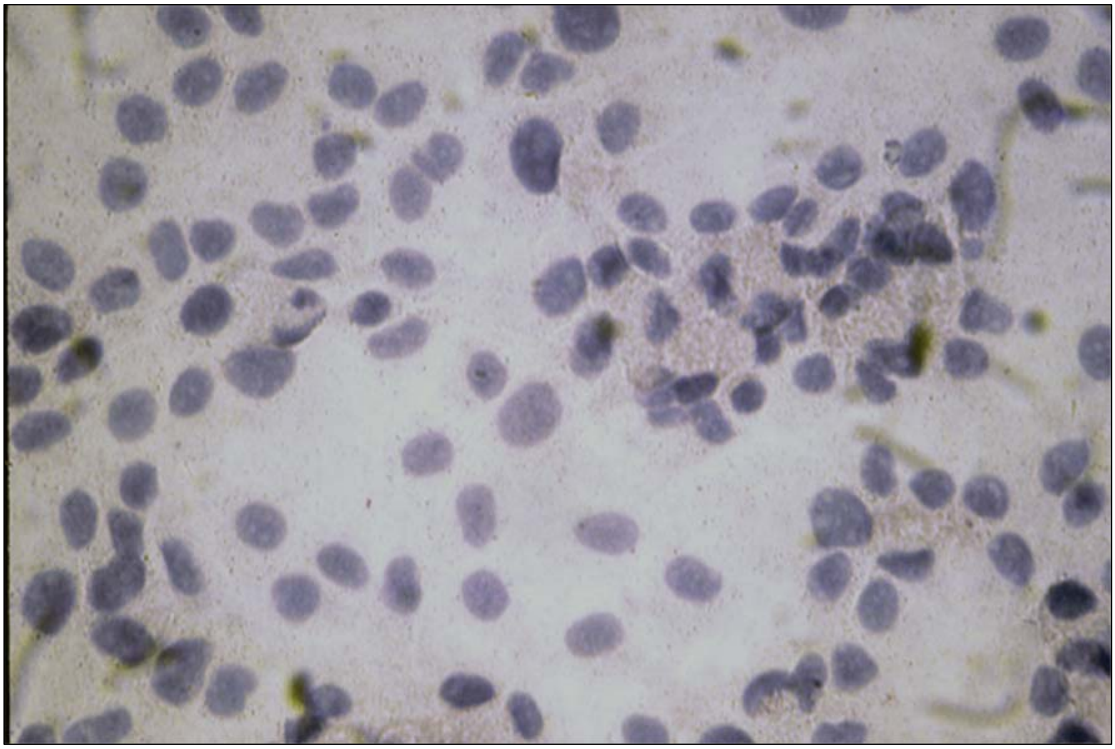

Fig.ure 3.21: Immunocytochemical staining of FRTL5 cells for Oct4. 400X magnification. No positive cells detected. 200X magnification.

11. Immunohistochemistry

To analyze in situ expression of stem cell marker protein, serial sections of paraffin-embedded tissues from 6 nodular goiters were stained for the expression of Oct4,GATA-4 and HNF4α. Pathological diagnosis was established at the Institute of Pathology at Helius Klinikum Berlin (Klinikum Buch). One of the tissue samples was derived from a hyperthyroid nodular goiter (hot nodule in the left thyroid lobe), all others from euthyroid nodular goiters.

Because of insufficient material, the hyperthyroid nodular goiter sections could only be tested for Oct4 protein expression. Although different antibody titrations (1:100 to 1:400) and different Fast Red Napthol exposure times (5 to 9 minutes) were used, no positive cells could be found.

In all of the euthyroid goiter sections examined, very small numbers of single, Oct4 positive cells sparsely scattered amongst Oct4 negative human thyrocytes were detected. Again, only nuclear immunoreactivity was considered specific and only cells displaying a red/pink nuclear staining were scored as positive. Positive cell concentration was markedly lower than in monolayer cultures: in each slide only two to five positive cells could be detected. In most of the cases, positive cells could be found adjacent to normal thyrocytes, as part of the cuboidal epithelium lining the follicles. In one case however, a group of three Oct4 positive nuclei could be found in the centre of a larger Oct4 negative cell group not belonging to a follicle formation (figure 3.22)

In two sections of the tissues (n=2) very few GATA-4 positive cells were detected (figure 3.23) and small numbers of isolated HNF4α positive cells were observed in one of the examined sections (figure 3.24). In all cases, positive cells were detected as part of the normal follicle-forming cuboidal epithelium.

As a positive control for the staining method, ERK-1 was used as the primary antibody. ERK-1 is an extracellular-signal-related kinase whose expression is evident in thyroid cells (99). As can be seen in figure (3.24)., the majority of the cells displayed positive staining.

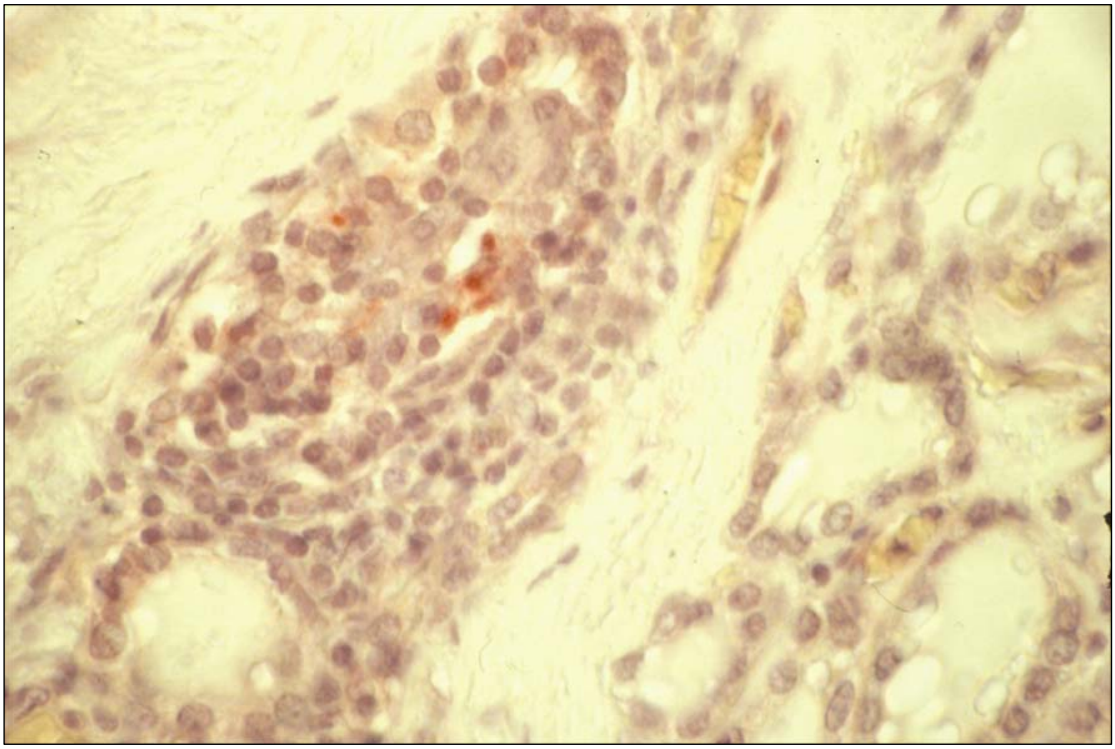

Figure 3.22: Immunohistochemical staining of a human goiter tissue section for Oct4, 400X magnification. Three (n=3) positive cell nuclei are detected, surrounded by a group of negative thyrocytes.

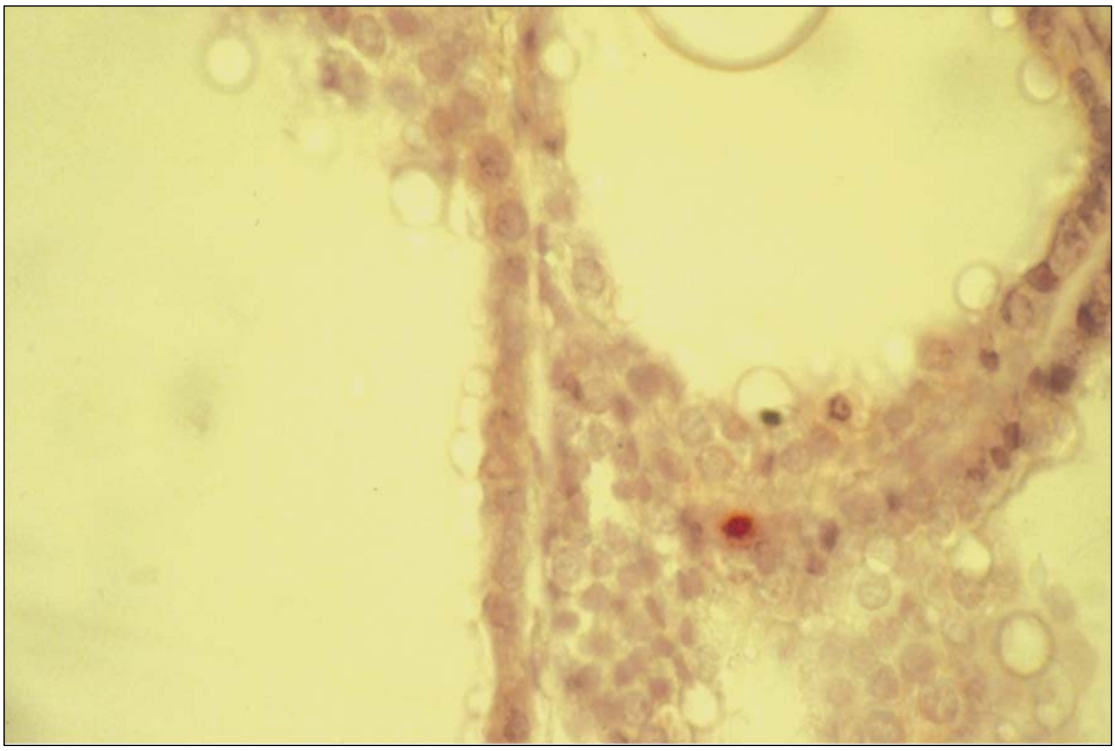

Figure 3.23: Immunohistochemical staining of a human goiter tissue section for GATA-4, 400X magnification. Positive nuclear staining of one cell for GATA-4, all cells in the vicinity are GATA-4 negative,

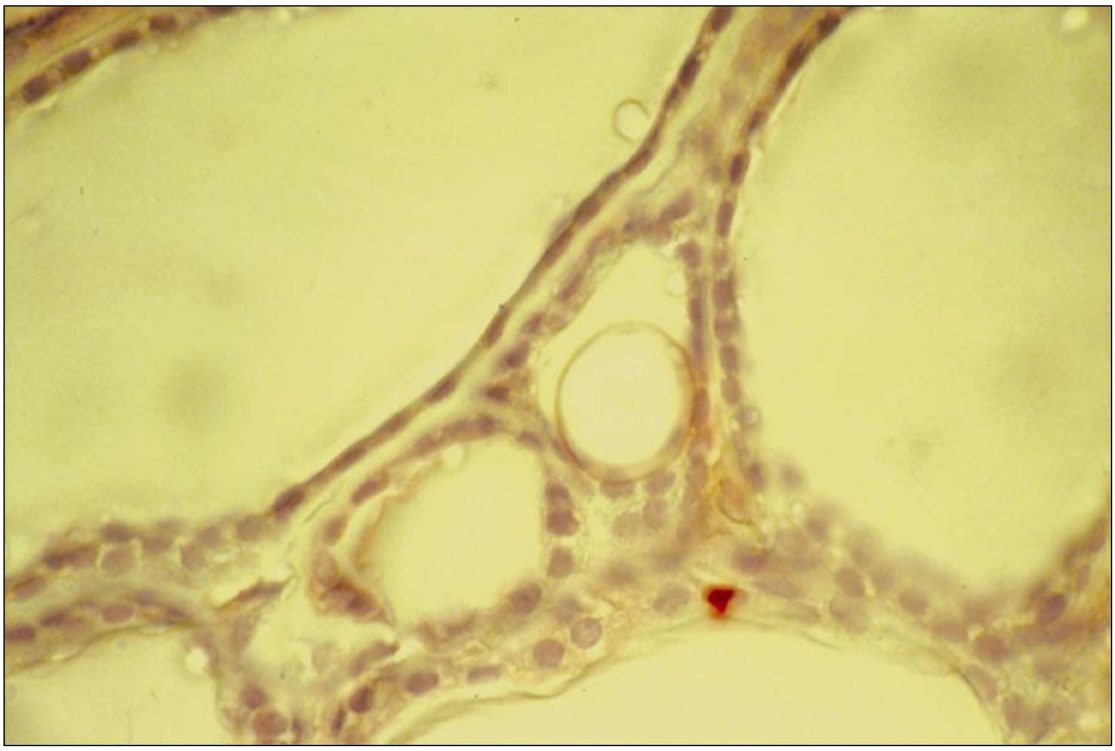

Figure 3.24: Immunohistochemical staining of a human goiter tissue section for HNF4α, 400X magnification. Nuclear staining for HNF4α of a single cell adjacent to a thyroid follicle.

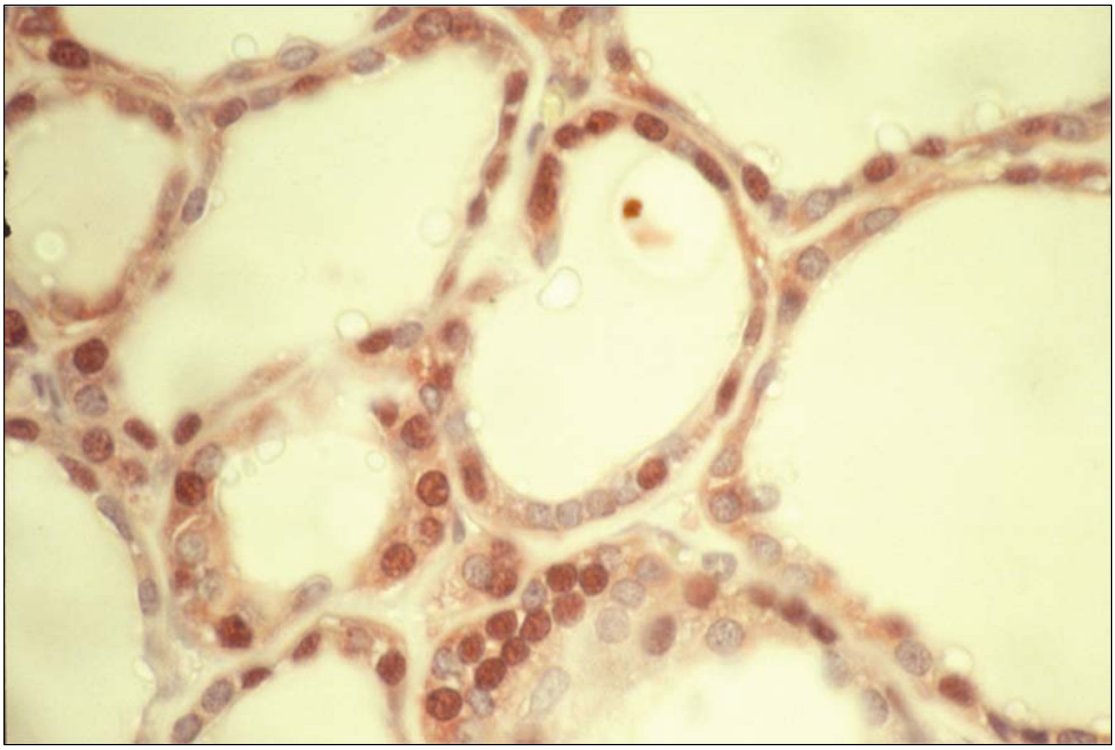

Figure 3.25: Immunohistochemical staining of a human goiter tissue section for ERK-1 (used as a positive control for the staining method). Positive nuclear staining of the majority of cells, 400X magnification.

12. FACS Analysis

To further characterize cells with specific stem cell markers on the protein level, indirect immunofluorescent staining was performed. After incubation with a stem cell marker-specific primary antibody, lysed cells were incubated with a fluorochrome-conjugated secondary antibody (indirect immunofluorescent labelling). Cells were then gated on forward and side scatter and dead cells were sorted out by means of propidium iodide exclusion. In the remaining cell population, cells bearing stem cell marker protein expression were counted and their concentration in respect to the overall cell population was calculated

As can be seen in figure 3.26, small numbers of Oct4, GATA-4 and HNF4α positive cells could be detected. The number of marker-positive cells did not exceed 1.5% of the overall cell population for any of the markers tested.

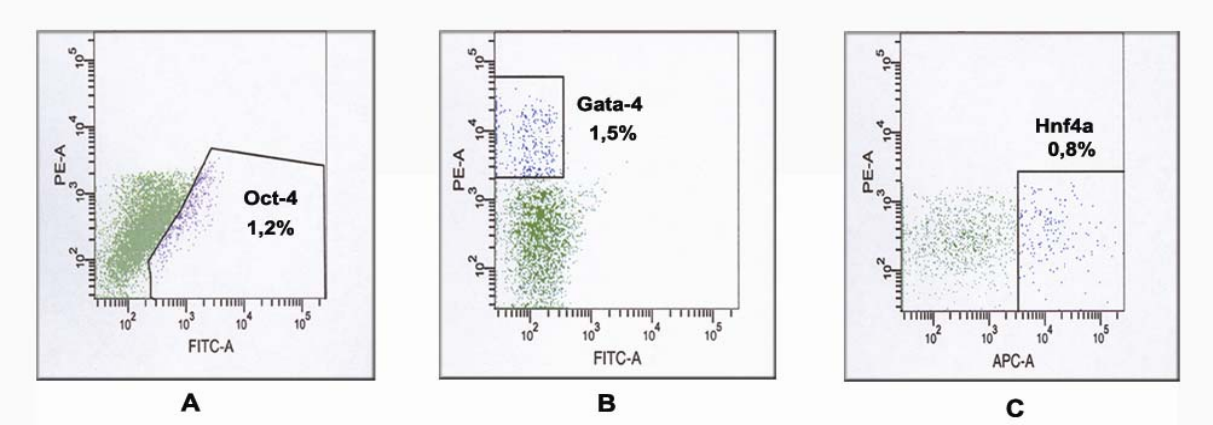

Figure 3.26: Flow cytometric analysis of stem cell marker expression in human goiter cells. Numbers represent the percentage of positive cells in relation to the whole cell population. **A:** Oct4 positive cells (conjugated to FITC fluorochrome). **B:** GATA-4 positive cells (conjugated to FITC fluorochrome). **C:** HNF4α positive cells (conjugated to APC fluorochrome)

Using fluorescence-activated cell sorting (FACS) very small numbers of stem cell marker positive cells (ranging from 150 to 500 cells pro probe) were isolated. Cells were plated in 96-well plates filled with H5 medium with or without 5mU/ml of TSH and observed for a period of two weeks. No cell growth was observed for any of the different cell groups (positive either for Oct4, GATA-4 or HNF4α).