4. DISCUSSION

4.1 Cell elements of primary thyrocyte cultures and their embryonic origin

Adult stem cells have been proven to exist in several human tissues such as the brain , the dermis (9), the gut (18), the breast (115) and the liver (21). In the present work, the hypothesis was tested as to whether they also exist in the human thyroid gland.

To investigate this, thyroid tissue was obtained from patients with nodular goiters directly after thyroidectomy. Malignancy was cytologically and/or histologically excluded in all cases. Tissues were characterized either as nodular or paranodular according to intraoperative macroscopic assessment by the surgeon. Using a modified version of the Rappaport protocol (108), tissues were digested and cells were isolated and grown as monolayers in culture dishes.

All monolayer cultures in the present experiments were obtained after direct cell isolation from surgical preparations. As described in materials and methods (paragraph 2.1), special care was taken during as well as after the operation, to remove all non-thyroidal tissue. Nevertheless, contamination of the culture with several cell elements such as cells of the blood lineages, fibroblasts, neurons or cells of the vascular epithelium could not be ruled out with certainty. A review of the embryological origin of all possible contaminating cells revealed, however, that none of them could be of endodermal origin, as shown in the table below:

Tissue	Cell Type	Germ Layer	Literature
Vessels	Endothelial cells	Mesoderm	(116)
	Mesothelium- Smooth muscle cells		
	Epithelial cells		
Nerves	Neural Cells	(Neuro) ectoderm	(117)
Blood	Red blood cells	Mesoderm	(118)
	White blood cells		
	Platelets		
Connective tissue	Fibroblasts Stromal Cells	Mesoderm	(119)

Table 4.1: Cells that could contaminate a primary thyroid cell culture and their embryological origin.

It can therefore be purported that the only cells in the primary cultures obtained, which are of endodermal origin, can be the thyroid follicular cells or their putative, endodermally derived precursors.

4.2 Detection of mRNA transcripts by RT-PCR

The expression of stem cell marker mRNA in primary thyroid cultures was analysed using reverse transcription PCR. By this very sensitive method (106), even very small amounts of genetic material can be amplified and detected. In the present experimental setting, the method was performed in a semi-quantitative way: In each probe tested, the signal of each marker was quantified by gel densitometry and compared to the expression of the housekeeping gene ß-actin.

By using primers specific for cDNA amplification, evidence for the expression of stem cell marker mRNA in thyroid cell cultures was provided (figures 3.1-3.4). All primers were intron-spanning to avoid amplification of contaminating genomic DNA sequences. All amplified sequences were verified by restriction endonuclease experiments. The detection of stem cell marker protein that followed, using flow cytometry and immunocytochemistry (figure 3.26), provided further evidence for the translation of the detected mRNA sequences.

In contrast to the primary thyroid cultures, the FRTL5 cell line, a homogenous cell line widely accepted as a model for differentiated thyrocytes (120), did not display expression of stem cell marker mRNA. To bypass the problem of potential incompatibility between the human primers and the rat DNA of the FRTL5 cells (although the human and rat Oct4 gene sequences differ in only one base pair) primers showing a 100% match to the rat sequence were designed and used. The fact that these cells were negative for Oct4, GATA-4 and HNF4α expression but positive for markers of differentiated thyrocytes such as thyroglobulin (figure 3.11), provides evidence that it is not differentiated thyroid cells that express these markers but rather a different, hitherto undescribed cell population residing in the human thyroid gland.

In a further approach, two established thyroid carcinoma cell lines, the HTh74 thyroid anaplastic carcinoma cell line and the HTC follicular carcinoma cell line, were screened for stem cell marker mRNA expression. Both these cell lines originate from human thyroid cells and partially retain some of their characteristics, e.g. thyroglobulin expression (100). Both of them displayed no expression of the markers (figure 3.11). This finding supports previously published

reports that expression of Oct4 and other pluripotency-associated genes is restricted to germ-cell cancers and some poorly or entirely undifferentiated cell lines (43). One of them is the HeLa human cervix carcinoma cell line, which was also used in these experiments as a positive control for Oct4 expression. Interestingly, besides Oct4, the HeLa cell line also displayed positivity for HNF4α but for none of the other stem cell markers tested.

4.3 Immunocytochemistry – Immunohistochemistry

Using the RT-PCR method, a strong signal for GATA-4, OCT4, HNF4α and p63 could be detected in all cases of primary cultures. However, given the fact that RT-PCR is extremely sensitive, it could not be ascertained whether the signal originated from all the cells or only a fraction of the cell population. To enable a relative quantification of stem cell marker-positive cells in relation to the overall cell population, immunocytochemical and immunohistochemical staining was employed.

The immunostaining pattern observed for Oct4 in cultured cells (figures 3.14 and 3.15) as well as in tissue sections (figure 3.22) was similar to the one observed by Tai et al when they immunostained normal human skin and dog mast tumor tissue sections for adult stem cells (43). For GATA-4, similar expression patterns as in the present work (figures 3.17 and 3.23) have been observed in hepatocyte-like adult stem cells (121) and for HNF4α (figures 3.18 and 3.24) in oval cells of the liver, which are thought to be the progeny of a liver stem cell compartment (122).

4.4 Flow cytometry

Flow cytometry was used as an alternative method for detecting cells expressing stem cell marker protein, after incubation with fluorochrome-conjugated secondary antibodies. Because the antigens under detection were transcription factors localized in the cell nucleus, cell membranes were permeabilized with alcohol prior to antibody incubation.

The finding that only very small numbers of positive cells could be detected is in accordance with the immunocytochemical results. The density of the stem cell marker-positive cells did not exceed 1.5% of the overall viable cell population in any of the cultures tested. These

cell numbers are very low, they are however within the sensitivity range of flow cytometric detection. Flow cytometry is an established method for isolating cell populations, even at very low numbers, based on their antigen expression profiles. In the field of adult stem cell research, it has been mainly used for isolating hematopoietic progenitors and it has been reported to have a sensitivity of 0.01-0.05% (123, 124).

4.5 Thyroid stem cells: Is there enough experimental evidence?

Definite proof that a certain cell is a stem cell requires experimental demonstration of three basic stem cell properties (3):

1) Undifferentiated state
2) Capacity of self-renewal
3) Ability to give rise to differentiated progeny

The state of differentiation can be assessed according to the set of differentiation (or pluripotency) markers a cell expresses. As described in the introduction, Oct4 has only been detected in pluripotent, undifferentiated cells, either stem cells or cancer cell lines (43), while there are numerous reports of Oct4 being down-regulated when these cells are experimentally forced to differentiate: Embryonic stem cells stop expressing Oct4 when they are induced to become thyrocytes (125) or insulin-producing pancreatic beta-cells (126). Accordingly, Oct4-positive cancer cell lines, such as the HeLa cell line (43) and the F9 embryonic carcinoma cell line (127) stop displaying Oct4 expression when differentiation is experimentally induced. Similarly, GATA-4 and HNF4α are expressed early during endoderm formation in embryogenesis. However, when cells proceed toward differentiation, their expression is lost (50, 59). In brief, the set of markers used for the present experiments is unique for pluripotent (Oct4) or endodermally commited (GATA-4, HNF4α) undifferentiated cells, while terminally differentiated thyrocytes, such as the majority of the cells in the primary cultures or the FRTL5 cell line, do not express them.

Regarding the second condition (proof of self-renewal capacity), the fact that these cells could be detected in the cultures even after an increased number of passages clearly displays their self propagation potential. What is more, no alteration (neither increase nor decrease) in their density was observed, from subculture to subculture. This finding points to the fact that their propagation rate and their ratio to the differentiated cells remain stable, at least in co-culture

with thyrocytes, as in the present experimental setting. A possible explanation of this finding is that the proliferation rate of these cells may be very low. Alternatively, one may speculate that some stem cells differentiate into normal thyrocytes whereas only a constantly low number remain in the state of stem cells. The ability to regulate the relative balance between self-renewal and differentiation is another typical stem cell property (128).

Experimental demonstration of the third condition, namely proving that these cells can produce differentiated progeny, requires their isolation, purification and subsequent cultivation (8). In the present work, all attempts to culture cells isolated after FACS sorting were unsuccessful. The limited number of reports on successful isolation and cultivation of adult stem cells from differentiated human tissues shows that this is a very daunting task: Tissues from which adult stem cells have been successfully isolated and cultured include the placenta (129), the liver (21), the epidermis (17) and human scalp (130), the dental follicle (14), skeletal muscles (16), the retina (20), the bone marrow(13), the mammary glands (115) and the olfactory bulb (12). Most of these tissues in vivo display either a high cell-turnover or an increased self-repair potential after injury, which is not the case for the thyroid. What is more, only in two reports could the adult stem cells be kept in an undifferentiated state for longer periods of time (9, 131).

A possible explanation for the lack of growth of the sorted cells could be the damage inflicted upon them during the preparation and conduction of the sorting procedure (trypsinization, treatment with propidium bromide, permeabilization, injury by the nozzle of the cell sorter etc). Especially permeabilization was unavoidable, since all the stem cell markers detected in the present work are transcription factors localized in the nucleus (see also immunocytochemical results). To our knowledge, to date no surface marker has been described that is exclusively characteristic of adult stem cells or endodermal precursors. The discovery of such a surface antigen would allow cell sorting without permeabilization, a condition which might enable the isolation of viable thyroid stem cells in the future.

Another possible reason could be that the cell concentrations yielded by this method are too low to allow intracellular signaling and subsequent cell growth. The potential role of growth factors should also be taken into consideration: To date, numerous growth factors have been described which are necessary for the growth and differentiation of adult stem cells of different origin (89), however no set of growth factors suitable for the expansion of thyroid stem cells has been reported. The main stimulant of thyrocyte growth is the pituitary hormone TSH (132) which was included in the culture media in all cases, however, the desired results could not be obtained.

Finally, an attractive hypothesis is that the growth of stem cells in vivo and their survival and propagation in primary cultures may be dependent on cellular signaling from neighboring cell populations, a condition which is no longer fulfilled when the cells are purified and cultured separately. The complex mechanisms of intracellular signaling that regulate stem cell growth and activity, which include activation of the canonical Wnt pathway [reviewed in Reya et al (133)], have not been fully elucidated to date.

Although the third criterion could not be demonstrated, these cells displayed another typical adult stem cell property: they were detectable as rare, single cells scattered amongst big numbers of differentiated thyrocytes. This immunocytochemical and immunohistochemical expression pattern is similar to others described in the literature for adult stem cells in vivo (43, 134).

4.6 SACK method: an alternative approach for obtaining a purified stem cell culture.

After the unsuccessful attempts to culture stem cells obtained following FACS sorting, an alternative method was sought to selectively increase their numbers within the primary cultures. In this respect, the theory of Suppression of Asymmetric Cell Kinetics (SACK) was adopted.

The basic principle of the SACK theory is that by experimental suppression of the asymmetric kinetics of stem cells, their numbers in culture can be increased and, by using proper dilution and selection techniques, a pure adult stem cell culture can possibly be derived. Xanthosine is a nucleoside analogue which influences the cell cycle by intervening in p53-depended regulation of guanine nucleotide biosynthesis. It acts by circumventing IMPDH downregulation by promoting guanine ribonucleotide formation via salvage pathways (93). The ability of xanthosine to convert asymmetric cell kinetics to symmetric has been documented in a number of reports (95, 96). Using this compound, Lee et al managed to put this theory to praxis by producing adult liver stem cell lines from male Fischer rats (97). In the present work, the protocols employed by Lee et al were applied to the primary thyrocyte cultures, with the aim to induce the selective propagation of adult thyroid stem cell populations.

The first approach was to explore the effect of xanthosine on the expression of the stem cell markers. After treatment with varying xanthosine concentrations (200 and 400 μM), mRNA

was isolated at designated timepoints and semi-quantitative PCR was performed. No alteration in stem cell marker signal in any of the test groups was observed.

In a second approach, 70-80% confluent primary cultures were trypsinized and cell suspensions were obtained. The number of viable cells was determined by trypan blue exclusion and the cells were diluted down to very low viable cell concentrations and plated in 96-well plates. The principle of these “limiting dilution” experiments is to plate the cells at an expected concentration of 2 cells per well, thus increasing the probability of some of the wells containing only stem cells. Consequent treatment with xanthosine would then lead to the symmetrical propagation of stem cells and thus to a purified adult stem cell culture. However, although several different tissue probes were used and many (at least 8) 96-well plates were prepared, no cell growth was observed.

Several reasons could have attributed to the lack of success of these experiments:

1) The concentration of xanthosine might not have been the appropriate for thyroid cells (the concentrations used were adopted from the publication of Lee et al for liver stem cells).

2) Another possible explanation is that the effect of xanthosine might be tissue-specific: Xanthosine has consistently worked for liver cells but not for other tissues in general (personal communication Prof. Sherley).

3) The toxic effect of xanthosine (in the 400µM concentration) on certain thyrocyte cultures should not be underestimated.

4) Among the reasons that limiting dilution cloning may fail, even with SACK agents present, is that the actual stem cell fraction for thyroid tissue may be prohibitively low. It may be that as many as >10,000 limiting dilution cloning wells have to be observed to have a chance of success. The method has worked for rat liver tissue because the stem cell fraction is estimated to be relatively high and primary rodent cells have a very good plating efficiency in general. In human studies, the method hasn’t produced analogous results, to the present day (personal communication with Prof. Sherley, unpublished data).

4.7 Existing literature on stem cells and the thyroid gland: comparison with the present work

Although the existence of adult stem cells in the human thyroid gland has not been experimentally proven up to date, there is a limited number of reports concentrating on the issue

of stem cells and the thyroid.

4.7.1 p63 as a marker for thyroid stem cells

In a recent publication, Reis-Filho et al characterized a cell population in the thyroid as stem cells based on the immunohistochemical detection of p63 in paraffin-embedded thyroid tissue (26). p63 is a marker for epithelial basal/stem cells (64). p63 expression was detected in the main cells of thyroid cell nests, which are embryonic remnants of unknown biological origin that can be found in most thyroid glands if the search is meticulous (70) (see also introduction). All other thyroid cell populations, such as follicular and parafollicular cells, did not display p63 positivity. In a second publication, members of the same research group further supported this stem cell hypothesis by providing evidence that the p63-positive cells also display increased telomerase expression (a marker of self-renewal potential) and increased proliferation rates in comparison to the rest of the thyroid cells (27). In a similar approach, Burstein et al formulated a stem cell hypothesis of papillary carcinoma oncogenesis based on the immunohistochemical detection of p63 in the main cells of thyroid cell nests as well as in papillary thyroid carcinomas (135)

The present work differs from these studies in three basic aspects. Firstly, the present markers were selected to identify pluripotent (Oct4) or endodermally committed, multipotent (GATA-4, HNF4α) stem cells, based on the knowledge that follicular cells, which constitute the main body of the thyroid cell population, are of endodermal origin. p63 on the other hand is a marker of stem cells of ectodermal origin, such as keratinocyte and myoepithelial stem cells (65, 136). Secondly, the above mentioned studies were confined to the immunohistochemical detection of p63 in serial sections of frozen thyroid tissue, whereas in the present work, not only histological slides but also thyroid cell cultures were examined. The proof of self-renewal potential of the cells in question was therefore not based on the detection of the immunohistochemical expression of proliferation markers (e.g. telomerase catalytic subunit, bcl-2, MIB-1 and MCM2) but rather on the observation of their division kinetics in culture. Thirdly, in the present work, stem-cell marker-positive cells were quantified by flow cytometry and isolated using FACS analysis.

Expression of p63 mRNA was also detected in the present work by means of RT-PCR and immunocytochemistry, thus partially verifying the findings mentioned above.

4.7.2 Fetal cell microchimerism

According to the theory of fetal cell microchimerism, during pregnancy fetal cells of different types transcend through the placenta to reach the maternal circulation and infiltrate a variety of tissues (137). In certain cases these cells persist for many years (138), coexisting with the cells of the host organism, a phenomenon called chimerism. In women with scleroderma who have given birth to male children, male cells have been detected in a variety of tissues, such as the peripheral blood, the skin and the spleen (139-141). In a limited number of reports, male cells have been detected in the thyroids of women with Graves disease (142, 143), Hashimoto disease (143, 144), post-partum thyroiditis (145) and other thyroid diseases (137), suggesting a possible link between the pathogenesis of these diseases and the existence of intrathyroidal fetal microchimerism. In one of these reports (146), the authors postulated that these transplacentally acquired cells were fetal stem cells.

In all of the reports mentioned above, fetal cells were spotted by means of Y chromosome detection, (a characteristic discerning them from the rest of the XX cells of the female hosts) either by PCR (142) or FISH (146, 147). No pluripotency or self-renewal potential was demonstrated, thus the hypothesis that these cells are indeed stem cells of fetal origin is not supported by sufficient experimental data. In any case, the possibility that the cells detected in the present work are not actually adult stem cells but rather stem cells of fetal origin is very remote, given the fact that stem cell marker-positive cells were detected with equal frequency both in male and female specimens (cf. Results, paragraph 3.6).

4.7.3 Thyrocytes from embryonic stem cells

In a rather different but very interesting approach, Lin et al (125) commited embryonic stem cells to differentiate into thyrocyte-like cells in vitro. For these experiments, embryonic stem cells were cultured on feeder cells in DMEM medium containing 15% FCS, Leukemia Inhibitory Factor (LIF) and monothioglycerol. For differentiation, the medium was changed to modified DMEM supplemented with ascorbic acid and monothioglycerol, with or without FCS. In some experiments, cells were treated with varying concentrations of TSH (0,1-10 mU/mL). As in the present work, Oct4 was used as a marker of pluripotency. Thyroglobulin (Tg), TPO, TSH-Receptor, PAX8 and NIS were picked as markers of differentiated thyrocytes.

After 6-8 days of the treatment described above, expression of all the differentiation markers, accompanied by loss of Oct4 expression, was observed. However, the effect of TSH on the differentiation process remained unclear: Although, after 11 days of TSH treatment, a slight increase in intracellular cAMP content was observed (indicating the presence of a functional

TSH receptor), the difference between treatment groups was not statistically significant. Based on these data, the authors concluded that TSH was necessary to maintain the expression level of PAX8 and TSHr during embryoid body differentiation; however they could not identify a specific role for TSH during the differentiation process.

In the present work, cells were treated with different concentrations of TSH in order to evaluate its role on possible differentiation of stem-cell-marker positive cells. Apart from the 5mU/mL TSH concentration, which is necessary for the maintenance of primary thyrocyte cultures, a concentration of 200 mU/ml was also used. TSH did not affect the expression level of Oct4 and therefore failed to induce differentiation of the pluripotent Oct4 cells, which would have been detected as a decrease or a disappearance of Oct4 signal. This might indicate that the Oct4-positive cells do not express the TSH receptor or that its role regarding their development and differentiation is not significant. In this respect, experiments in mouse knockout cell lines are of interest that demonstrated that growth in the fetal thyroid is unaffected by TSH/ TSH receptor signaling (148).

Interestingly, TSH treatment led to an increase in GATA-4 mRNA expression, after both 8 and 15 days of treatment (figure 3.12). Taking into consideration that GATA-4 expression increases as stem cells differentiate into endoderm (126), it could be postulated that TSH may not affect the differentiation process at the very early stage of pluripotent stem cells but it might play a role at a later stage of thyroid embryogenesis, when stem cells have already been endodermally committed. The aforementioned data published by Lin et al on the role of TSH during embryoid body differentiation (125) seem to fit well in this hypothesis. These observations, however, have to be interpreted with great caution, since:

1) The concentration of TSH used for the present experiments (200 mU/ml) is much higher than is the case in vivo.

2) Only the expression of GATA-4 but not of HNF4α (the other endoderm stem cell marker) was influenced by the treatment. This renders the exact effect of TSH unclear. A possible explanation for this finding could be that cells expressing these markers might represent distinguishable cell populations being at a different stage of the differentiation process and perhaps displaying different endogenous properties (e.g. TSH sensitivity or TSH-receptor expression). Further experiments are needed to clarify the exact effect of TSH on the differentiation process of thyroid cell embryogenesis and differentiation.

4.8 A possible link between stem cells and thyroid oncogenesis.

Stem cell biology found its first therapeutic application in the form of bone marrow transplantation (7). In recent years, stem cell research has made great headway, particularly in the field of tissue regeneration. There is an increasing number of reports of stem cells, mainly embryonic ones, being experimentally driven to differentiate into various cell and tissue types. This opens up exciting new therapy prospects, such as regeneration of myocardium after infarction (149), neural tissue repair in demyelinating diseases (150) or after injury (151) using neural stem cells (151), treatment of diabetes by in-vitro obtained insulin-producing beta cells (152), skin, hair (153) and liver tissue regeneration (154) and many others [for a review see (7)].

The thyroid is an organ where tissue regeneration is of limited clinical importance. However, the finding that pluripotent cells of increased proliferation potential reside within the gland could provide new insights into the mechanisms of thyroid oncogenesis.

In recent years there is increasing scientific interest in the intersection of the fields of stem cell biology and cancer. The similarities between stem cells and tumorigenic cells [reviewed in Reya et al, (128)] have led scientists to believe that certain tumours arise from stem cells or progenitors which undergo a process of malignant transformation. Examples of this are certain types of leukemia, which arise from hematopoietic stem cells after accumulation of mutations (155, 156). A theory of fetal stem cell carcinogenesis in the thyroid has recently been described by Takano et al (157). According to these authors, undifferentiated thyroid tumours such as anaplastic thyroid carcinomas originate from fetal stem cells. This theory, although attractive, hasn't been supported by experimental evidence. In the present work, none of the thyroid tumours tested (follicular and anaplastic) displayed stem cell marker expression, however this does not rule out the possibility that the process of malignant transformation might include the down-regulation of the pluripotency markers.

In a very interesting recent publication, Hochedlinger et al reported that Oct4 positive cells may be targets for dysplasia and malignant transformation due to their undifferentiated state (158). It is tempting to assume that benign or malignant thyroid tumours originate from stem cells or thyrocyte progenitors which reside within the gland. Further experiments are needed to test the validity of this hypothesis and to clarify other potential factors (genetic or environmental) that might contribute to the process of such a malignant transformation.