4. Discussion

In recent years, stem cells have become the subject of intense study. They have been analyzed in the context of normal physiology and also associated with the development and propagation of solid tumors (111, 112). Preliminary results have demonstrated their possible links with some benign and malignant tumor formation (113).

Thyroid nodules are very common and nodular transformation with age is a hallmark of the human thyroid gland (3). A marked increase with age of functional nodular autonomy was also observed (4). Depending on the diagnostic approach, i.e., histological examination or molecular analysis, thyroid nodules are described as hyperplastic lesions, adenomatous nodules, adenomas, and clonal or polyclonal nodules (5). In most cases the mechanism underlying autonomous growth of thyroid lesions is the spontaneous proliferation of a benign neoplasia (3). But what is the reason for the spontaneous neoplasia growth in human thyroids, especially in the old? The molecular alterations that cause the formation of these benign neoplasias are largely unknown (6). Although many pathogenetic factors are known as iodine deficiency, mutagenesis, overexpression of growth factors and their related receptors and a genetic predisposition, a comprehensive concept for the pathogenesis of thyroid nodules and nodular goiters is still missing (5, 6).

Recently, stem cells have been hypothesized to be a source of malignant tumors including thyroid carcinomas (20, 21). In the case of colon tumors, the concept of stem cell transformation has also been extended to adenomas (113). The same concept may also apply to benign thyroid tumors. Therefore, the stem cell origin of some nodular formations is hypothesized in our current research, which may provide some evidence to elucidate the underlying molecular mechanisms of the neoplastic transformation that is a hallmark of the ageing thyroid.

Our previous study first demonstrated the existence of stem cells in human thyroid nodule tissues (34). These adult stem cells as well as progenitor cells are detectable as single cells or groups of 2 or 3 cells dispersed throughout the thyroid gland (34). These long-life cells, present in goiter of 50-80 year old patients, express Oct4, a characteristic marker for both adult and embryonic stem cells (13, 34, 36, 37, 104), or endodermal progenitor cell markers such as GATA4 or HNF4 (44, 114-116). When maintained in co-culture with thyroid cells, the number of thyroid stem and

progenitor cells remained constant over 4-6 passages. However, a major obstacle for isolation and further characterization of thyroid stem cells encompasses the absence of a surface antigen that allows the separation by FACS. Based on the recent finding that stem cells express the ABCG2 membrane transporter that provokes the efflux of the Hoechst 33342 DNA binding dye (47, 53), thyroid stem cells were isolated as a side population in our present study to overcome this problem.

Taking advantage of the fact that both embryonic and adult stem cells express ABCG2 transporters (47, 53), Goodell *et al.* first reported a method for the isolation of hematopoietic stem cells (HSCs) based on their ability to efflux a fluorescent dye (54). In 1996, while using Hoechst 33342 dye staining to study the cell cycle distribution of whole bone marrow cells, Goodell *et al*. discovered that the display of Hoechst fluorescence simultaneously at two emission wavelengths (red 675 nm and blue 450 nm) localizes a distinct, small, non-stained cell population (0.1 % of all cells) that express stem cells markers (Sca1+lin$^{neg/low}$). Later, it was established that when various kinds of tissue cells are also subjected to Hoechst 33342 dye staining and FACS analysis, those that actively efflux the Hoechst dye then, likewise, appear as a distinct population of cells -- the “side population”, which is enriched in primitive and undifferentiated cells (65, 66) and closely correlated to ABC transporters--ABCG2. Actually, the Hoechst efflux phenomenon has proven to be a highly useful primary purification strategy for isolating potential stem cells from various tissues in the absence of specific cell-surface markers. Cells with an SP phenotype and typical stemness properties have now been described in many solid tissues, such as the skeletal muscle, lung, liver, heart, testis, kidney, skin, brain, and mammary gland in both human and animal models (52, 65, 67-69, 71-76, 78-80).

But why does a stem cell efflux vital dyes at a greater rate than other cells? One hypothesis to explain this phenomenon is that the overexpression of the membrane transporters responsible for the dye efflux mechanism in SP cells provides a mechanism for long-term survival of stem cell populations via an enhanced ability to pump cytotoxic compounds out of the cell (117). A recent study showed that heme molecules (porphyrins) are detrimental to bone marrow progenitor cells from ABCG2 knock-out (KO) mice under hypoxia (118). ABCG2 specifically binds heme, and cells lacking this molecule accumulate porphyrins (118). Therefore, it seems that stem cells can use ABCG2 to reduce intracellular heme/porphyrin accumulation and overexpression of this membrane transporter confers a strong survival advantage under hypoxic conditions. This stem cell protection mechanism could also be interpreted as an indication that ABCG2 causes the

efflux of a substance important for differentiation (117). Overexpressed ABCG2 may have exported a substance important for the growth or survival of committed lineages. The degree of efflux activity seems to correlate with the maturation state, so that cells exhibiting the highest efflux activity are the most primitive or least restricted in terms of differentiation potential (119). Since the SP phenotype has been correlated with ABCG2 expression and this expression is strongly down-regulated during progenitor commitment (120), the sub-populations found in SP reflect fractions with the lowest degrees of cellular differentiation. Thus, in stem cells, ABCG2 may function as a general protectant against endogenous and exogenous substances of toxicity or differentiation, and have a functional role in stem cell survival and undifferentiation maintenance.

In the present work we first demonstrated that stem cells derived from nodular goiters express ABCG2 transporter mRNA and protein (Fig. 3.1 A and B). As revealed by flow cytometry analysis, only 0.1-0.2 % of total cells expressed the transporter. In comparison to other tissues, this is a considerably lower percentage. *E.g.* about 1.3 % of gastrointestinal cells express the transporter (121). However, the low number of ABCG2 transporter expressing cells from thyroid tissues corresponds to our previous immunohistological studies that showed a very low number of putative stem cells in situ (34). Sorting of cells as a side population confirmed the expression of functional transporters that show an efflux of the Hoechst 33342 dye (Fig. 3.2).

Analyses of expression profiles demonstrated 3 different types of cells within thyroid cell cultures: stem cells, endodermal progenitor cells and differentiated thyroid cells. The SP fractions of stem cells were characterized by expression of stem cell markers ABCG2 and Oct4 and the lack of differentiated markers. Oct4 is a transcription factor not only essential for embryonic stem cell self-renewal, but also very important in some adult stem cells, such as liver, pancreas, breast, kidney (13), skin (36), pituitary (37), and thyroid (34). However, in differentiated cells Oct4 expression is absent (34).

Interestingly, endodermal progenitor cells, detected by expression of GATA4 and HNF4 markers, were not part of the SP fraction but of the major population of thyroid cells (Fig. 3.3). GATA4 is expressed during the process of differentiation into the endoderm and as the transcription factor HNF4 expressed early in the endoderm formation (33, 44). Both markers are absent in differentiated thyroid cells (34). Indeed, in SP sorting (see Fig. 1.4), the SP tail could be divided in different regions according to their dye efflux abilities. It has been demonstrated that cells in

the tip of SP with highest Hoechst efflux activity showed the highest stem cell activity as compared to the distal portion (122-124). The level of Hoechst exclusion may be a direct indicator of cellular differentiation. Since the SP phenotype has been correlated with ABCG2 expression and this expression changes during progenitor commitment (120), the isolation of cells with the highest dye efflux activity within SP could lead to a highly enriched primitive stem cell population with the lowest degree of cellular differentiation. In order to select a specific homogeneous SP from thyroid cells, the SP gate that was selected for cell collection included only verapamil-sensitive cells with the lowest Hoechst incorporation. Therefore, the endodermal progenitor cells from thyroid tissues that may have already lost the expression of ABCG2 transporter were not part of the side population cells.

Moreover, the cytological analysis of cytospun SP and non-SP cells confirmed the distinct isolation of thyroid stem cells. In comparison to differentiated cells, SP cells showed a round shape of the nucleus and were on average smaller in size. Most remarkable was the higher nucleus/cytoplasm ratio of SP cells (Fig. 3.7). Both hallmarks are typical for quiescent adult stem cells (77, 125). Benchaouir *et al*. (126) have reported that SP cells are smaller than non-SP cells. This observation correlates well with the findings of de Paiva *et al*. (125) who proposed that small cell size is a stem cell characteristic. Moreover, SP cells are present in a quiescent state and possess an adhesive endoplasmic reticulum with a few ribosomes suggestive of low metabolic activity, elements common to stem cells (125). Gene expression profiles of the various SP in microarray assays using an in-house platform (65) or Affymetrix 430 2.0 array chips (79) have shown that the cell cycle repressive genes are up-regulated (65). This suggests that SP cells may arrest at a particular phase of the cell cycle. Indeed, G0/G1 cell cycle arrest has been reported within the SP (79), which supports the relationship between SP and stem cells, which are both believed to be slow cycling cells (100) that reside in a quiescent state (127). Taken together, our findings demonstrate that SP isolated from thyroid nodules preserve a primitive stem cell population.

As shown in this study, neither in regular culture conditions for thyroid cells nor in Matrigel-precoated dishes, which offer anchors for attachment and cell-to-cell contact, did isolated SP cells attach and proliferate as monolayer. Matrigel matrix is a continuous sheet of specialized extracellular matrix that has been shown to play a crucial role in thyroid cell attachment, cell-to-cell communication and differentiation (128). Nevertheless, even under intense growth stimulation with EGF and bFGF, the cell counting yielded similar numbers at

plating and after 2 weeks in culture, indicating the absence of proliferation. Similar results were reported on stem cells from breast (129) and kidney (130). This observation that was also made with some other stem cells, may be explained by the absence of interaction between stem cells and microenvironmental cells (niche cells) that nurture stem cells and regulate the balance between cell quiescence and activity (105). The niches are composed of microenvironmental cells and various factors, such as secreted cytokines, extracellular matrix interactions and intercellular adhesion that provide a sheltering environment for preventing stem cells from uncontrolled differentiation and apoptotic stimuli and on the other hand also safeguard against excessive stem cell production that could lead to cancer (105). The lack of these regulatory cells may, in part, explain why adult stem cells isolated as single cells do not proliferate despite intense growth stimulation. As expected, when co-cultured with non-side population cells (differentiated cells) after FACS, the thyroid SP stem cells are capable of self-renewal and differentiation. As shown in the co-culture experiment (Fig. 3.6), in non-SP cell culture, Oct4-positive stem cells were not detectable at any time point and GATA4-expressed endodermal progenitor cells, which were involved in the lineage commitment, gradually disappeared in culture. In co-cultures of SP and non-SP cells, both Oct4 and GATA4-positive cells remained relatively stable, as already shown in our previous study (34), which demonstrated that the number of thyroid stem and progenitor cells remains constant over 4-6 passages via self-renewal. These findings may, again, indicate the vital role of niche cells in the self-renewal and differentiation of stem cells.

The side population is well-documented as an enriched source of stem cells, and it remains a valid and promising tool for identification and isolation of stem cells, especially in the absence of specific surface marker expression. However, given that SP cannot be expanded in our experiment when separated from their niches *in vitro*, additional approaches are required to enrich this source of stem cells for their full characterization and further differentiation.

Therefore, in this study, we adopted a suspension culture, which has been shown to be an extremely useful tool in neural stem cell biology as a strategy for the *in vitro* enrichment and propagation of human thyroid stem cells. In the suspension culture conditions, neural stem cells clonally proliferate to form floating cell colonies called neurospheres. Based on the model of neurospheres, we hypothesized that a small population of thyroid cells with stem cell properties would be able to survive and proliferate in the absence of attachment to an exogenous substratum, whereas the vast majority of cells would undergo “anoikis”. This term (from the ancient Greek

word for homelessness) was first proposed by Frish and Francis in 1994 (131). Anoikis is defined as a process of programmed cell death induced by the loss of cell/matrix interactions and specifically applies to the apoptosis of nontransformed cells which occurs in the absence of anchorage to a substratum. We hypothesized that anoikis was a property of differentiated cells (putative niche cells), but that stem cells could survive anchorage independent conditions. The concept of a stem cell niche was first proposed by Schofield in 1978 (132), as a specific microenvironment, in which adult stem cells reside in their tissue of origin. Within the niche, stem cells are able to maintain their relative quiescent state, with slow cycling of self-renewal. In general, the niche is thought to consist of a highly-organized microenvironment in which various factors, such as secreted cytokines, extracellular matrix (ECM) interactions, intercellular adhesion, and differentiated cells (putative niche cells) are thought to work cooperatively in order to maintain the undifferentiated stem cell phenotype (133, 134).

In the present study, we developed a culture system in which human thyroid cells, isolated from nodules, were cultured on a non-adhesive substratum in serum-free medium in the presence of the strong mitogens EGF and bFGF. Consistent with the hypothesis, we found that a small number of freshly isolated cells was able to survive, proliferate, and form non-adherent, three-dimensional spheres (Fig. 3.8 A and B) after being plated at low density in the serum-free medium containing EGF and bFGF. We termed these spheroids “thyro-spheres” by virtue of their resemblance to neurospheres cultured from primary neural cells. This technique has also recently been proven to be effective in the case of mammary (90), skin (91), cardiac (92), inner ear (93), retina (94), and pancreas tissues (95), that generated the same type of floating spheres in suspension cultures, that were composed of cells with stem and progenitor functional properties.

Analyses of gene expression profiles and secondary FACS of thyro-spheres exhibited the typical cell compositions: enriched primitive stem cells and endodermal progenitor cells. As expected, thyro-sphere cells were characterized by preferential expression of stem cell markers Oct4 and ABCG2, as well as endodermal markers GATA4 and HNF4, and by the lack of differentiation markers (Fig. 3.14). The sphere-derived side population comprised about 5.0 % of stem cells, with the rest being progenitor cells, which were found within the higher fluorescence (non-side population) fraction. This is consistent with observations in neurospheres that a small proportion of cells composing individual neurospheres from a normal brain were stem cells with the ability to self-renew and generate all neural lineages (85, 86), whereas the remaining majority consisted of progenitor cells with more restricted self-renewal capacity and lineage potential.

Taken together, these findings indicate that thyroid stem cells are a small population of cells that are anchorage independent, relatively resistant to apoptosis *in vitro* and highly proliferative in suspension culture in response to intense growth stimulation.

The role of growth factors in the formation of thyro-spheres bears resemblance to the pathogenetic importance of these factors for the growth of thyroid nodules (135, 136). In fact, chronic stimulation of the thyroid gland by growth factors, highly accelerated under the conditions of iodide deficiency (137), resembles in some ways the acute intense stimulation of quiescent stem cells derived from nodule tissues in our experiment. Outgrowth of stem cells as spheres is provoked by the same growth factors that are also involved in the nodular transformation of the thyroid gland (136, 138, 139).

Also interestingly, the isolation of thyroid stem cells from nodule tissues was only possible in a serum-free medium without TSH. When either TSH or serum was added formation of spheres was not observed. Why do stem cells escape the growth control of putative niche cells only in the absence of serum? In serum-free medium differentiated cells may undergo apoptosis since they lose anchorage-dependent growth (90). In 1994 and 1995, Dremier and di Jeso *et al*. reported that serum withdrawal in endothelial cells as well as in canine thyroid primary cultures and transformed rat thyroid cells also induced programmed cell death (140, 141). Serum can be considered for many cell types a complex mixture of growth factors that regulate several cellular functions, including, in certain cell types, the synthesis of ECM, and have important effects on the cellular environment. Follicular cells in the thyroid are surrounded by a continuous rim of matrix proteins. Thyrocytes-ECM interactions can have major effects on the phenotypic features of thyroid cells controlling growth and differentiation (142). Therefore, in the absence of serum, niche cells undergo apoptosis, whereas the stem cells are free from the strictly regulated growth control and highly proliferate in suspension. In addition, TSH has been proved to exert an anti-apoptotic effect for thyroid cells (140). Dog thyrocytes undergo apoptosis after deprivation of TSH (140), suggesting that TSH is involved in the control of growth of the thyroid gland. Kawakami *et al*. reported that Fas antigen is functionally expressed on the surface of thyrocytes, and TSH inhibits Fas antigen-mediated apoptosis of thyrocytes through the inhibitory effect of Fas antigen expression, resulting in the promotion of growth of the thyroid cells (143), which may explain the preserved function of putative niches in response to TSH-containing but serum-free medium.

It has been shown that adhesion is a key factor for the differentiation of stem cells. In particular, ECM is considered a factor of survival and differentiation for many adherent cells. Adhesion generates cell tensional integrity (tensegrity) and repression of apoptotic signals, whereas detachment has the opposite effect. Moreover, lineage-specific differentiation initiated by growth factors has been reported by different groups (114, 144). Several soluble factors have been shown to direct differentiation of mouse ES cells, *e.g.* IL-3, IL-6, retinoic acid, TGF-β 1, bovine fibroblast growth factor, IGF-1 (114), and TSH (144). In a recent study, an ES cell line carrying a fusion gene comprised of an enhanced green fluorescent protein-neomycin-resistant (GFP-Neor) cassette was generated such that the expression of the fusion gene was under the control of the TSHR promoter (145). This ES cell line was subsequently used to generate a TSHR null mouse, which showed severe congenital hypothyroidism, thyroid hypoplasia and died at four weeks. In contrast, heterozygous (TSHR+/-) mice were unaffected (145). Of note is that thyroid follicles in TSHR null mice were poorly developed with fewer follicular cells and more non-follicle-associated interstitial cells (145), implying that follicular growth and differentiation are at least in part dependent on the TSHR. In addition, it has been also demonstrated in previous studies that TSH stimulation of the TSHR activates a cAMP-dependent pathway that leads to the transcriptional regulation of genes necessary for thyroid hormone synthesis and secretion (146). A recent study has reported that TSH was necessary to maintain the expression of the PAX8 and TSHR genes during embryoid body (EB) differentiation. However, some other previous data from cultured human adult thyroid cells (both normal and pathological) have indicated limited or no TSH-induced thyroid cell growth *in vitro* (147-149). TSH is necessary to maintain the thyrocyte architecture in FRTL-5 cells. However, this has not been observed with normal cells because TSHR knockout mice continued to form thyroid follicles (145, 150). The mechanism by which TSH achieves thyroid-specific gene expression is unclear but appears to be intimately associated with its influence on a variety of mitogenic control. Some previous studies have, however, observed that the TSHR was positively regulated by TSH (147). A TSH-dependent differentiation of stem cells has been described for the development of the mice thyroid (151). A directed differentiation of mouse embryonic stem cells into thyroid follicular cells required the involvement of TSH.

In our present study, in serum-enriched medium, thyroid stem cells regained the ability of adhesion and interaction with ECM at the first 3 d of differentiation initiation. Consecutively they underwent the first steps of differentiation including induction of TSHR gene expression. It

is also very interesting to note that the low expression of TSHR inducted by serum can be upregulated and maintained with TSH stimulation, suggesting the dependence of the TSH receptor on TSH itself. Upon differentiation induction with both serum and TSH over time, thyroid sphere-derived stem cells grew as monolayer and differentiated into thyrocytes that showed typical gene expression of differentiation markers (Fig. 3.14). When embedded in collagen for 3-4 d, these cells formed thyroid follicle-like clusters (Fig. 3.16) and displayed specific radioiodide uptake activity, one of the most important characteristics of thyroid follicular cells (Fig. 3.17). Therefore, in our study, both serum and TSH play a pivotal role in stem cell differentiation into thyroid follicular cells.

Taken together, as in the case of neurospheres, we demonstrated that thyro-spheres are highly enriched in undifferentiated cells. In addition, it was shown that single cells isolated from thyro-spheres were able to generate thyroid follicular cells when cultured in the presence of serum and TSH over time.

More than 20 years ago transplantation of nodular goiter tissues on nude mice demonstrated autonomous growth of some thyroid cells with a constitutively higher growth potential (152). In addition, transplantation of autonomously growing embryonic human thyroids suggested that "the autonomously replicating cells that initiate nodule formation in human multinodular goiters reflect the persistence in the adult gland of cells with fetal growth potential (153). It is most likely that these cells correspond to the adult stem cells which are scattered throughout the human thyroid gland.

Therefore, from the present findings we may postulate a role of stem cells in the pathogenesis of thyroid nodules and nodular goiters. This hypothesis is based on epidemiological data, experimental results and the general concept of stem cells as a source of benign and malignant tumors (113). Population studies have demonstrated that nodular transformation increases with age whereas the goiter size decreases (4, 154, 155). Throughout the aging thyroid gland adult stem cells are detectable that maintain the capacity of proliferation and differentiation (34). Normally, stem cells are quiescent or slowly cycling cells maintained in an undifferentiated state until their participation is required in normal function of the organism. Thus, under various environmental signals, i.e. cell destruction, they will divide and give rise to transiently amplified cells (progenitor cells) that will undergo multiple divisions before proceeding to terminal differentiation (33). The proliferation of these quiescent stem cells is controlled by signals from

putative niche cells. In vitro malnutrition (serum deprivation) of cell cultures can limit or even overcome the normal niche control which results in an outgrowth of stem cells. Histological and immunohistochemical studies demonstrated hypofunction, destruction and necrosis of normal thyroid tissue in goiter tissues (5), similar conditions that may affect the control of niches on thyroid cell growth *in vivo*. In addition, there is some experimental evidence that apoptosis of thyrocytes is a main factor of cell loss during goiter formation (156). Apoptosis of thyrocytes is, however, a prerequisite for sphere formation *in vitro*. Moreover, some experimental studies revealed that growth factors, their related receptors and growth-related signaling peptides are highly expressed or even overexpressed in nodular goiters (5). Some of the growth factors are potent stimulators of thyroid stem cell growth as shown in the present work. Thus, the short but intense stimulation of stem cells by these growth factors *in vitro* may correspond to processes of nodular transformation *in vivo* that last for years or even decades.

Further work is necessary to analyze how stem cell growth may potentially contribute to neoplastic thyroid growth that arises in nodular goiters and it also has to focus on the molecular and cellular aberrations that may occur on the long way from adult stem cells to differentiated thyroid cells *in vivo* and may be at the very beginning of thyroid tumorigenesis.