1. Introduction

Thyroid nodules are very common. With the use of ultrasonography for evaluating thyroid and non-thyroid neck disease, the incidental finding of unsuspected thyroid nodules has dramatically increased, with an estimated prevalence that ranges from 4 % by palpation (1) to up to 67 % by ultrasonography (2). The underlying growth mechanism is spontaneous proliferation of benign neoplasias (3). Nodular transformation with age is a hallmark of the human thyroid gland (3). In a previous survey of an iodine deficient community, thyroid nodularity was 0.5 % in children and progressively increased with age in the 56-65-year age group. Besides, a marked increase with age of goiter prevalence, and functional autonomy was also observed (4). Although many pathogenetic factors are known such as iodine deficiency, mutagenesis, overexpression of growth factors and their related receptors and a genetic predisposition, a comprehensive concept for the pathogenesis of thyroid neoplasia (tumor) is still missing (3, 5, 6).

At first glance, the high frequency of nodule and nodular goiter formation in the thyroid gland is surprising, since, compared to highly proliferating tissue such as the colon, breast, skin or prostate, the growth rate of human thyroid cells is considerably low. In vitro labeling of human thyroid slices demonstrated a labeling index of $13.4 \times 10^{(-5)}$ for follicular cells; assuming an S phase of 10 h, this corresponds to a turnover time of the order of 8.5 years for the follicular cells. These results show that human thyroid cells divide only about five times during adulthood and therefore that the steady state level of thyroid cell mass results from a balance between cell division and cell loss. A shorter turnover time was found as expected in the thyroid of an adolescent and in follicular colloid nodules (7). In the context of mutagenesis, proliferation is very important. DNA replication during cell division leads to a fixation of spontaneous mutations into the genome, causing a certain mutation load for dividing cells. Hence, tissue with high cell turnover, such as colon, endometrium, skin, prostate, or breast are more sensitive to mutagenesis and other molecular mechanisms that initiate tumor formation, whereas in resting tissues as thyroid such mechanisms should be less operative (8). To explain this discrepancy, it has been suggested that free radicals resulting from reactive oxygen species in the thyroid generate mutations more frequently (8).

Recently, as an alternative source of benign and malignant tumor formation, adult stem cells have been suggested (9). So what is a stem cell? A stem cell is a special kind of cell that has a

unique capacity to renew itself and to give rise to specialized cell types. In contrast to most cells of the body, such as heart cells or skin cells, that are committed to conduct a specific function, a stem cell is uncommitted and remains undifferentiated, until it receives a signal to develop into a specialized cell.

Stem cells are classified into two principal types: embryonic stem cells and adult stem cells. An embryonic stem cell is derived from inner cell mass, which is part of the early (d 4 to 5) embryo called the blastocyst. An adult stem cell is an undifferentiated cell that occurs in a differentiated tissue. It is a long-lived, generally quiescent cell that generates new stem cells, and thereby maintains the stem cell pool, as well as more committed progeny, which populate the organ through proliferation (9, 10). Adult stem cells are capable of making identical copies of themselves throughout the organism's lifetime. This property is referred to as "self-renewal". Adult stem cells usually divide to generate intermediate cell types before they achieve their fully differentiated state. The intermediate cell is called a precursor or progenitor cell. Progenitor or precursor cells in fetal or adult tissues are partly differentiated cells that divide and give rise to differentiated cells. Because the proliferation of progenitors amplifies the number of differentiated cells that are eventually produced, the progenitors are often called transit amplifying cells (11).

Adult stem cells have been identified in both high and low proliferation tissues such as colon, skin, liver, pancreas, and brain (12-16). For years researchers have looked for ways to use stem cells for replacement of cells and tissues that are damaged or diseased. Recently, stem cells have received much attention because of their putative role in carcinogenesis. Adult stem cells are able to self-renew and differentiate for the lifetime of organism. Due to their pluripotency and undifferentiated state they are widely believed to be involved in the pathogenesis of tumors (17, 18). There is increasing evidence that cancer initiation results from accumulative oncogenic mutations (intrinsic loss of control) in long-lived stem cells or their immediate progenitor, followed by modification of the surrounding microenvironment (loss of extrinsic control) (19). Recently, a role of stem cells has also been hypothesized for the pathogenesis of thyroid carcinomas by two groups (20, 21).

The thyroid gland produces thyroid hormones and calcitonin and consists of several cell types that are derived from all three embryonic germ layers. Among them, two diverse cell types, C cells and thyroid follicular cells, are responsible for the dual endocrine function of the gland, and

originate from two different embryological structures: the thyroid anlage is the site of origin of thyroid follicular cells whereas the ultimobranchial bodies are the source of C cells. C cells are responsible for the production of calcitonin, a hormone which is involved in calcium and phosphate homeostasis, and originate from the (neuro)ectodermal germ layer (22). Thyroid follicular cells represent the most abundant cellular population in the gland and form the thyroid follicles, structures serving as storage for controlled release of thyroid hormones. The thyroid anlage of follicular cell origin is an area enclosing a small group of endodermal cells. It is located on the midline of the embryonic mouth cavity in its posterior part (22). Thyroid follicular cells are, therefore, of endodermal embryological origin. The thyroid gland is the only tissue in the body that can absorb iodine and convert it into thyroid hormones T3 and T4 (23). This gland is of central importance in metabolic homeostasis, growth, and development. Thyroid hormones derive from the degradation of a large precursor, thyroglobulin (Tg), which is iodinated in tyrosine residues by a thyroid-specific enzyme, thyroperoxidase (TPO) (24). Three transcription factors, TTF1 (thyroid transcription factor 1), TTF2 (thyroid transcription factor 2), and PAX8 (paired box gene 8), have been implicated in the control of transcription of Tg and TPO genes (25-28). And four markers of thyroid differentiation, including Na^-/I^- symporter (NIS), TPO, Tg, and TSH receptor (TSHR), dictate the complex machinery of thyroid hormone synthesis (29-32) and can be considered the differentiation markers of thyroid follicular cells.

Previously, it was demonstrated that some specific stem/progenitor cell markers exist, either on the surface (surface marker) or behaving as transcription factor in the nucleus (genetic marker), which makes a stem/progenitor cell distinguishable from the differentiated progeny (33). Several genetic markers of stem/progenitor cells have been identified in thyroid nodule tissues in our previous studies which characterized a small population of stem/progenitor cells in the thyroid gland (34). Some of them are described in the following paragraphs.

Oct-4

Oct4 (also referred to as Oct3/4 or Pou5f1) is a member of the POU (Pit-Oct-Unc) family of transcription factors. POU transcription factors can activate the expression of their target genes through binding of an octameric sequence called the octamer motif (ATGCAAAT) (35). Oct4 has been proposed as a marker for totipotent cells of the developing embryo, embryonic stem (ES) and embryonic germ (EG) cells. Differentiation of totipotent cells to somatic lineages occurs at the blastocyst stage and during gastrulation, simultaneously with Oct4 down-regulation.

Stem cell lines derived from the inner cell mass and the epiblast of the mouse embryo express Oct4 only if undifferentiated. When embryonic stem cells are triggered to differentiate, Oct4 is down-regulated (35). Very recently Tai *et al*. (13) reported Oct4 expression in several human adult stem cells (breast, liver, pancreas, kidney, mesenchyme and gastric stem cells), and in the cancer cell lines Hela. Meanwhile, Oct4 gene expression has been also identified in skin (36) and pituitary stem cells (37).

GATA4

GATA4 belongs to a family of zinc finger transcription factors termed the "GATA-binding proteins", which regulate gene expression, differentiation and cell proliferation in a variety of tissues by binding to the consensus DNA sequence (A/T)GATA(A/G). GATA4 expression has been reported during the early stages of endoderm formation. Using Northern analysis Arceci *et al.* (38) found that GATA4 mRNA was not detectable in undifferentiated F9 embryonal carcinoma cells but was induced during differentiation into parietal or visceral endoderm. Mice null for GATA4 die between embryonic day 8 and 9 because of defects in heart morphogenesis and ventral closure of the foregut. Specifically, GATA4 null mice present with cardia bifida because of ineffective ventral fusion of the lateral aspects of the embryo and the subsequent formation of the foregut. It is likely that aberrant heart formation in GATA4 null mice is a secondary effect associated with an intrinsic defect in the definitive endoderm that underlies the splanchnic mesoderm containing the cardiac field. This interpretation is further supported by the observation that GATA4 null embryonic stem cells can generate cardiac myocytes but are partially defective in their ability to generate visceral endoderm and definitive endoderm of the foregut (39). These data suggest an important role for GATA4 during early endoderm formation and morphogenesis. GATA4 mRNA and protein could be detected in the fetal mouse and human adrenal cortex from embryonic d 14 and gestational wk 9, respectively. In the postnatal adrenal, GATA4 expression is down-regulated (40). GATA4 protein was evident in 12-week-old human fetal testes and was continuously expressed in the Sertoli and Leydig cells through adulthood (41). GATA4 expression was found in a majority of germ cells before puberty but its expression was not detected in germ cells after puberty (42). Immunohistochemical analysis and *in situ* hybridization of adult mouse ovaries showed abundant GATA4 expression in granulosa cells of primary and antral follicles of mature animals. GATA4 signal persisted during follicular maturation but no GATA4 signal was detectable in the corpus luteum after ovulation. Similarly, GATA4 was down regulated during follicular apoptosis, as granulosa cells within large and

small apoptotic follicles lacked GATA4 message (43). It can therefore be deduced that GATA4 is expressed in endodermally derived fetal tissue and potentially mitotic cells in adult tissue (granulosa cells, prepubertal germ cells, Sertoli cells) but not in terminally differentiated (i.e. luteal) or apoptotic cells. All these data taken together suggest that GATA4 is a marker of early endoderm and is expressed early during differentiation of embryonic stem cells to endodermal progenitor cells. In adult endocrine tissues such as the adrenals, the testis and the ovary, GATA4 characterises cells which are not terminally differentiated and maintain an increased proliferation potential.

HNF4

Hepatocyte nuclear factor 4 (HNF4) belongs to the hepatocyte nuclear factor group of transcription factors and it is a member of the nuclear receptor superfamily. Based on *in situ* hybridization analysis in post-implantation mouse embryos, Duncan *et al.* were able to detect HNF4 expression in the extraembryonic endoderm, gut and nephrogenic tissue of the developing mouse embryo and suggested therefore that HNF4 is a marker for primary endoderm in the implanting blastocyst (44). Similarly, HNF3-induced endodermal differentiation of embryonic stem cells led to an increase in HNF4 signal in the resulting endodermal progenitors (45). All these data suggest that HNF4 plays an important role in the earliest stages of endoderm formation and is a marker for progenitor cells of endodermal origin. Therefore, both GATA4 and HNF4 were recommended as markers to identify progenitor cells of endoderm origin (33).

ABCG2

ABCG2, a half-transporter also called Bcrp1, is a member of the ATP-binding cassette (ABC) family of cell-surface transporter proteins, with the capacity to bind ATP as an energy source to transport endogenous or exogenous molecules across the cellular membrane. Unlike other ABC half-transporters localized to intracellular membranes, ABCG2 is expressed exclusively in the plasma membrane (46) and is considered a universal marker for stem cells (47, 48). Analysis of the ABCG2 peptide sequence (49) reveals a single ATP/GTP-binding region and ABC signature motif within a relatively hydrophilic amino-terminal domain (amino acids 1-400), and a relatively hydrophobic carboxyl-terminal domain (amino acids 401-663) containing six putative trans-membrane domains (Fig. 1.1) and four potential N-glycosylation sites (50). Recently, ABCG2 was found in the primitive stem cells of several different tissues (51, 52), which

supports the idea that it plays a significant role in maintaining stem cells in an undifferentiated state (53). Moreover, ABCG2 is highly expressed in a specific population of cells, i.e., the so-called SP (side population) subset, which can be isolated by their ability to efflux Hoechst 33342 dye (53-55). Therefore, in addition to its important use as a determinant of the SP phenotype, the ABCG2 gene is an attractive candidate marker for the use in isolating various stem cells.

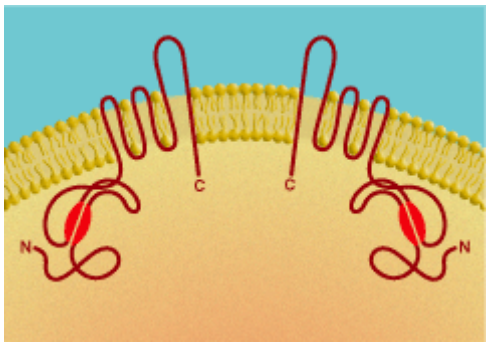

Figure 1.1 Physical structure of ABCG2

PAX8, TTF1, and TTF2

In the embryonic life of thyroid development, three transcription factors are involved at early stages of thyroid morphogenesis and play an important role in the organogenesis of the thyroid gland. These include PAX8, TTF1 and TTF2, whose expression starts early (earlier than other thyroid specific gene expressions such as Tg, NIS, TPO and TSHR) in the formation of thyroid gland. For the rest of its life, a thyroid cell will be hallmarked by the simultaneous presence of PAX8, TTF1 and TTF2 (28).

PAX8 belongs to the mammalian PAX protein family (56) and has been implicated in the control of transcription of Tg, TPO, and NIS genes (23, 27, 57, 58). During embryonic development, PAX8 is expressed in thyroid, kidney, and neural tissue. In the developing thyroid gland, PAX8 first appears in the area of endodermal proliferation from primitive pharynx at day 8.5, coincident with TTF1 expression (28). PAX8 mRNA is present as the primitive thyroid buds off from the floor of the pharynx and migrates caudally. Expression of PAX8 is maintained in thyroid follicular cells during all stages of development and in adulthood (56). In mice lacking the PAX8 locus, the thyroid is small, with no follicular cells (27, 59, 60). In addition to the

important role in morphogenesis of the thyroid follicular cell component of the thyroid gland, it has been shown in cell culture systems that PAX8 is a master gene for the regulation of the thyroid-differentiated phenotype (61). Therefore, PAX8 not only is required for the survival of the thyroid precursor cells but it also holds a specific upper role in the genetic regulatory cascade, which controls thyroid development and functional differentiation.

In our previous study, the expression of stem cell marker Oct4 and the early endodermal progenitor cell markers GATA4 and HNF4 was detected in histological slides and cultured cells derived from goiters using immunohistochemical and flow cytometry analysis, respectively. All these data provided strong evidence for the presence of adult stem and progenitor cells of endodermal origin in the human thyroid gland (34).

Usually, signaling molecules that selectively adhere to the receptors on the surface of the cell are used as a tool to identify stem cells. Many years ago, a technique was developed to attach to the signaling molecule another molecule (or the tag) that has the ability to emit light energy when activated by an energy source such as an ultraviolet light or laser beam (Fig. 1.2). There are multiple fluorescent tags with emitted light that differ in color and intensity.

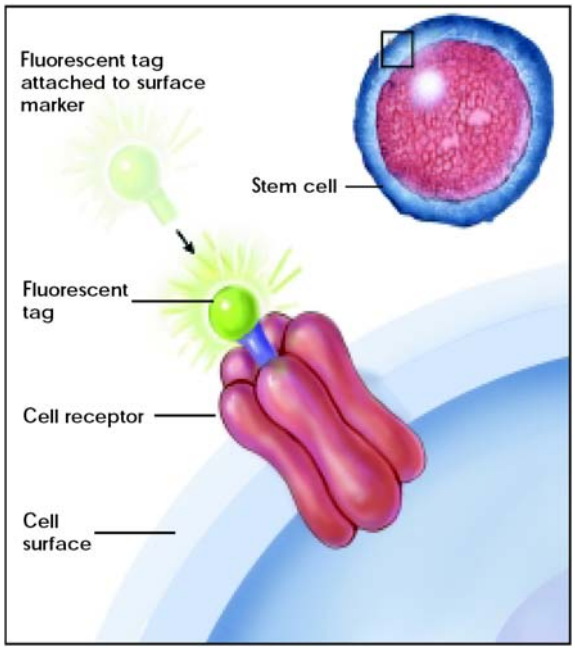

Figure 1.2 Identifying cell surface marker using fluorescence tag
[taken from: Stem Cells: Scientific Progress and Future Research Directions, National Institutes of Health. (http://stemcells.nih.gov/info/scireport/2001report.htm)]

One approach uses the combination of the properties of fluorescence and unique receptor patterns on cell surfaces to identify specific populations of stem cells, known as fluorescence-activated cell sorting (FACS) (Fig. 1.3) (62-64). This technique can be used to sort out the rare stem cells from the millions of other cells. With this technique, a suspension of tagged cells is sent under pressure through a very narrow nozzle — so narrow that cells must pass through it one at a time. Upon exiting the nozzle, cells then pass, one-by-one, through a light source, usually a laser, and then through an electric field. The fluorescent cells become negatively charged, while non-fluorescent cells become positively charged. The charge difference allows stem cells to be separated from other cells.

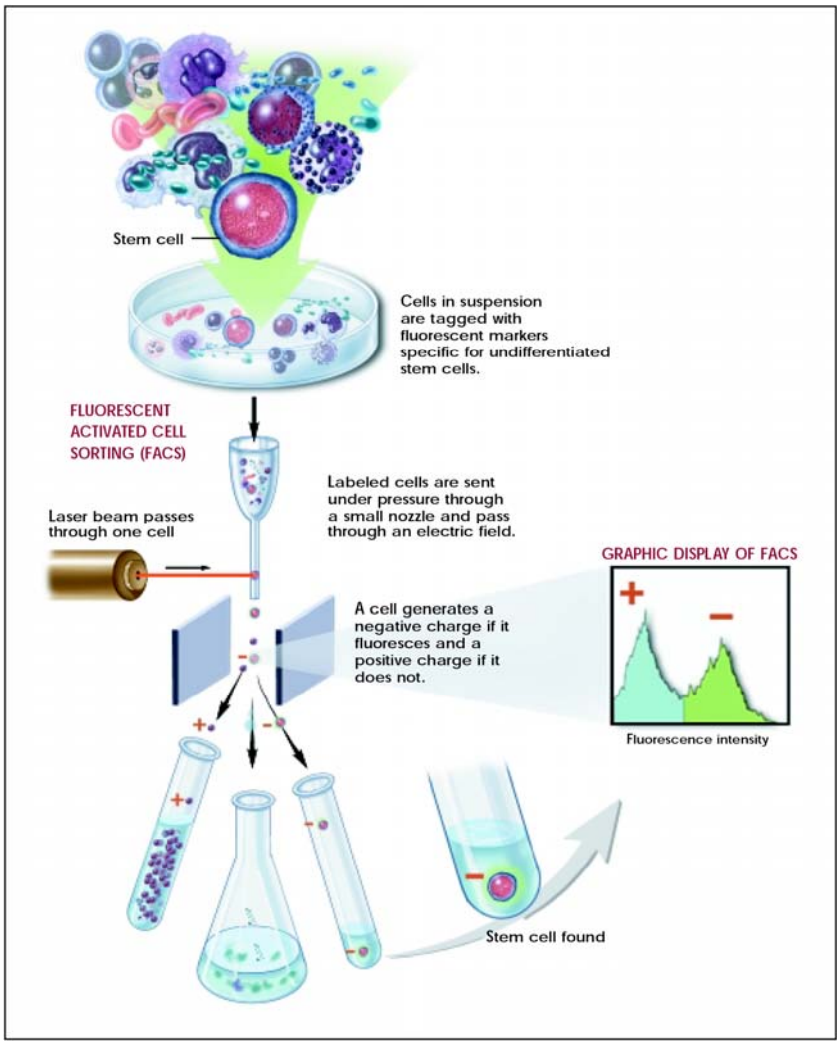

Figure 1.3 Stem cell isolation by FACS [taken from: Stem Cells: Scientific Progress and Future Research Directions, National Institutes of Health. (http://stemcells.nih.gov/info/scireport/2001report.htm)]

In our previous study, the presence of thyroid adult stem cells was identified in human nodule tissue by the detection of some transcription factors indicative of stem cells. However, the ability to purify stem cells always relies largely on the expression of appropriate cell-surface antigens by the cells, so that they can be immunostained with fluorescent conjugated antibodies and isolated by FACS. Therefore, we intend to find a new surface marker or an alternative method to isolate and characterize the stem cells that reside in the thyroid nodule tissues.

As we described above, ABCG2 has been considered a universal marker for stem cells in recent years (47, 48). It was found in the primitive stem cells of several different tissues (51, 52), and played a significant role in maintaining stem cells in an undifferentiated state (53). Furthermore, it has been demonstrated that ABCG2 is highly expressed in a specific population of cells, termed "side population", which can be isolated by their ability to efflux Hoechst 33342 dye (53-55). Therefore, ABCG2 gene is very important as a determinant of the Hoechst 33342 SP phenotype in cells from diverse sources.

The Hoechst efflux phenomenon has already been proven to be a highly useful primary purification strategy for isolating potential stem cells as a side population from various tissues in the absence of specific cell-surface markers. This purification method for stem cells exploits their special spectral emission pattern following staining with the DNA dye Hoechst 33342. In the free state the dye exhibits a fluorescence emission peak at 450 nm (Hoechst blue). In the DNA-bound state in live cells, electronic interactions between the densely packed Hoechst molecules cause a bathochromic shift leading to a secondary emission at 675 nm (Hoechst red). The side population is identified by a characteristic pattern of fluorescence after staining with Hoechst detected in both far red (above 675 nm) and blue (450 nm) emission channels. It has been identified as a particular cell population enriched in primitive and undifferentiated cells (65, 66). Cells with an SP phenotype have now been described in many solid tissues, such as skeletal muscle (67), lung (68, 69), liver (70, 71), heart (52), testis (72, 73), kidney (74), skin (65, 75), brain (76, 77), mammary gland (78-80), limbal epithelium (81), and prostate (82), in both human and animal models.

Goodell *et al*. first reported the method for isolation of human stem cells based on their ability to efflux fluorescent dye Hoechst 33342 (54). If cells are subjected to Hoechst 33342 dye staining and FACS analysis, those that actively efflux the Hoechst dye appear as a distinct small population of cells, the "side population" (Fig. 1.4), in the left lower quadrant of a FACS profile.

These cells were demonstrated to be highly proliferative and relatively resistant to apoptosis *in vitro*. Microarray analysis indicated that several genes implicated in 'stemness', *e.g.* Oct4 and Wnt pathway genes, were substantially up-regulated in the SP cells in comparison to non-SP cells (83).

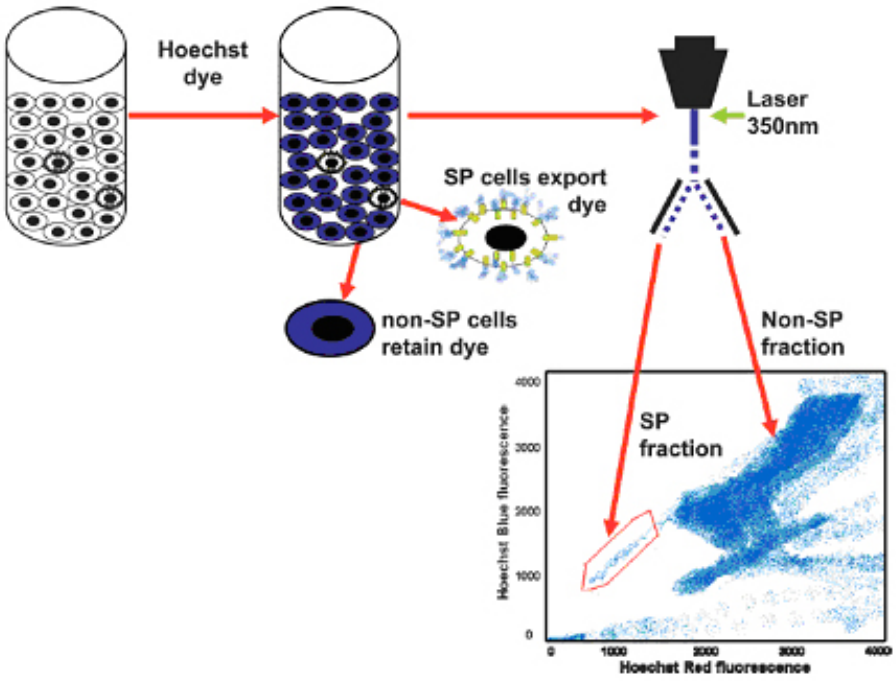

Figure 1.4 The schematic experimental protocol for SP sorting: The cells are stained by the Hoechst 33342 dye and then separated by FACS; those small proportion of cells that are able to efflux the dye have a characteristic fluorescent emission profile in dual wavelength analysis, with a both low red and low blue fluorescence — the SP fraction. [modified from: Forbes SJ, Hepatology, 2006, 44: 23-26]

As described above, most stem cell enrichment/purification protocols rely on FACS and use sets of antibodies against cell surface proteins. As an alternative method, a special culture system, based on previous work in neural stem cell biology, was utilized for isolating adult stem/progenitor cells from other tissues. In 1992, Reynolds and Weis published a study in which they were the first to show that neural cells isolated from the subventricular zone of rat embryos can proliferate in suspension culture, clonally generating spherical colonies, which they termed neurospheres (84). A clonal analysis of neurospheres demonstrated that 20 % of these cells, proliferating *in vitro* in response to epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) stimulation, were capable of both self-renewal and differentiation along multiple lineages, the defining characteristics of stem cells (85). Two years later the same group showed

that neural stem/progenitor cells from adult animals have the same ability to propagate *in vitro* as neurospheres (86). Since then, numerous studies utilizing cells derived from either the central or peripheral nervous system from embryonic and adult tissue showed that the ability to generate neurospheres *in vitro* correlates with the number of stem cells in the tissue of origin (87-89). Therefore, the identification and characterization of neural stem cells has gained fundamental impetus by the creation of a floating sphere culture technique. Meanwhile, this technique has been confirmed in the case of mammary (90), skin (91), cardiac (92), inner ear (93), retina (94), and pancreas stem cells (95).

Based on the observations that some adult stem cells have the ability to survive and proliferate in an anchorage independent manner, we speculated that suspension culture might be utilized as a method for isolating adult stem cells from thyroid nodule tissues.

Objective of this study

The aim of the present work was to further identify cells bearing stem-cell characteristics that exist in the fully differentiated human thyroid, and to isolate and propagate them with new technologies as FACS and sphere culture methods. The second aim was to establish a model of thyroid stem cells that allows further investigation of the relationship between stem cells and nodule transformation, or even thyroid carcinogenesis.