3. Results

3.1 ABCG2 expression and side population

3.1.1 Expression of ABCG2 transporter in primary thyroid cell cultures

In all cell cultures derived from thyroid nodules of different patients, expression of ABCG2 mRNA was detected by RT-PCR with human specific primers (Fig. 3.1). The amplified sequences displayed the expected size of 379 bp, whereas FRTL-5, a cell line with many hallmarks of thyroid differentiation including iodide uptake, TSH-dependent growth and thyroglobulin synthesis, did not display ABCG2 mRNA expression by using rat specific primers.

Meanwhile, to further characterize cells with ABCG2 expression on the protein level, indirect immunofluorescent staining and flow cytometry analysis were performed. Cells were gated on forward and side scatter and dead cells were excluded from further analysis by PI staining. As revealed by flow cytometry analysis, about 0.1-0.2 % of cultured thyroid cells were positive for ABCG2 transporter expression. A representative experiment is shown in Fig. 3.1.

These results confirmed the expression of ABCG2 transporters in human primary thyroid cell cultures both on mRNA and protein levels. Since ABCG2 is a very important determinant of side populations in a wide range of tissues and organs, this finding provided a prerequisite for our further experiment about the isolation of a side population from thyroid tissue cells.

3.1.2 A small side population isolated from human thyroid cells by FACS

To identify cells with functional ABCG2 efflux transporters, thyroid nodule tissue cells derived from patients were incubated with Hoechst 33342 for 120 min and sorted by FACS. The representative dotplots of FACS profile are depicted in Fig. 3.2, in which cells with an efflux of Hoechst were separated as a “side population” from the large majority of cells that accumulate the dye. SP-gated cells accounted for about 0.1-0.2 % of total cells, consistent with that of ABCG2 positive cells demonstrated in flow cytometry analysis (Fig. 3.1). Incubation with verapamil which inhibits the efflux, almost completely abolished SP profile, illustrating the specificity of the staining (Fig. 3.2 B).

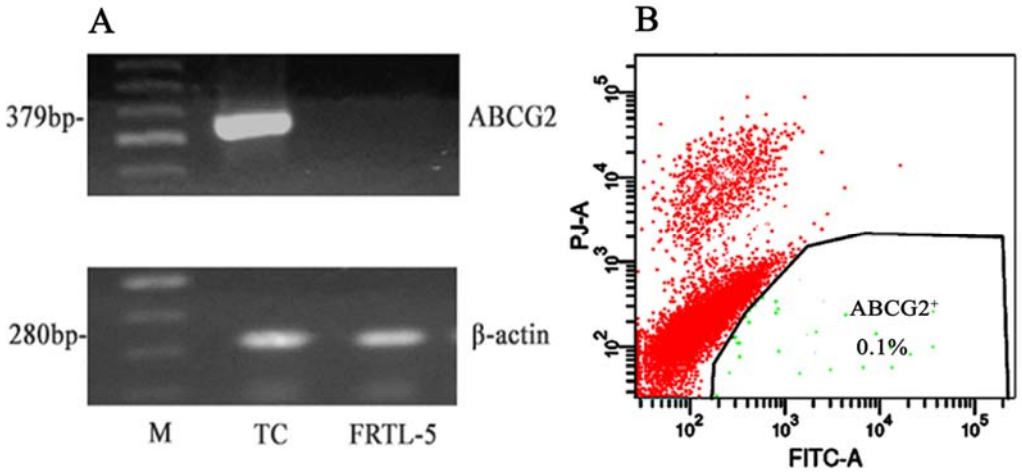

Figure 3.1 Expression of ABCG2 transporter in primary thyroid cell cultures. (A) RT-PCR showed expression of ABCG2 mRNAs in primary cultured thyrocytes. FRTL-5, a differentiated thyroid cell line, was negative for ABCG2 expression. (B) Flow cytometry analysis of cultured human thyrocytes showing a distinctive small population of ABCG2^{+} cells (about 0.1 %). Abbreviations: M, marker; TC, thyroid cells.

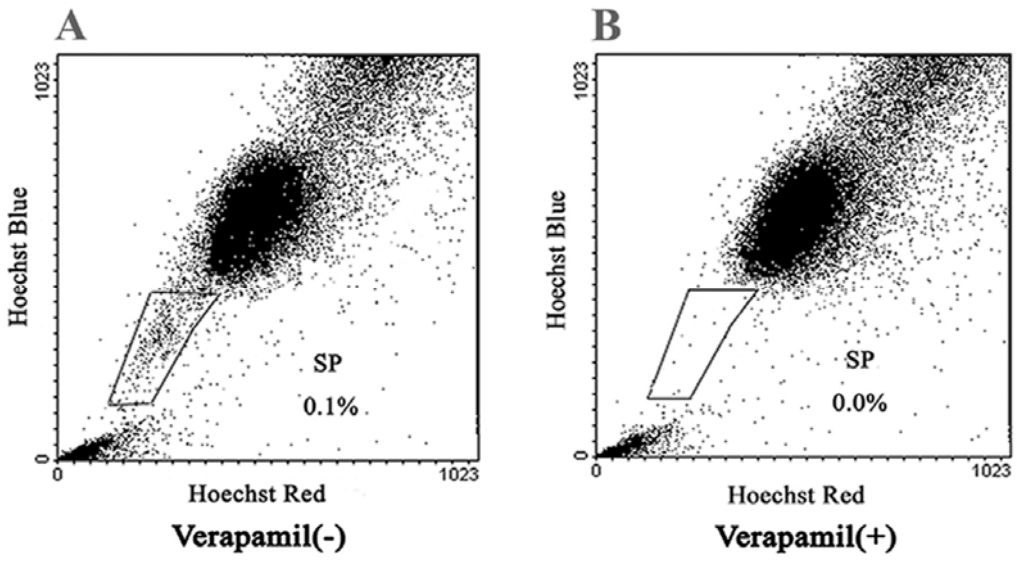

Figure 3.2 A small side population (SP) isolated by FACS. (A) Cells with an efflux of Hoechst 33342 were separated as an SP fraction. The SP-gated cells accounted for 0.1 % of the total cells analyzed. (B) Incubation with 50 µM verapamil which inhibits the efflux, almost completely abolished SP profile, illustrating the specificity of the staining.

3.1.3 Gene expression profiles of human thyroid SP cells

Gene expression profiles of cells isolated as SP fraction and of non-SP cells were further analyzed by RT-PCR (Fig. 3.3). SP fraction of cells strongly expressed ABCG2 and Oct4, a major transcription factor for both embryonic and adult stem cells (13, 34, 36, 37, 104). However, ABCG2 and Oct4 were not detectable in non-SP cells. In contrast, SP cells were negative for a set of thyroid specific genes (Fig. 3.3), i.e. the thyroid transcription factor, PAX8, which is involved at early stages of thyroid morphogenesis and plays an important role in the organogenesis of the thyroid gland; the Na^+ / I^- symporter (NIS), which transports iodide into the thyroid cells; thyroglobulin (Tg); thyroperoxidase (TPO), the enzyme responsible for Tg iodination, and the receptor for TSH (TSHR). NIS, Tg, TPO and TSHR dictate the complex machinery of thyroid hormone synthesis (29-32) and can be considered the differentiation markers of thyroid follicular cells. As expected, the non-SP fraction of thyroid cells expressed thyroid transcription factor PAX8 and the typical differentiation markers (TSHR, TPO, NIS and Tg, Fig. 3.3). Interestingly, the non-SP cells also expressed markers of endodermal progenitor cells, the GATA4 and HNF4 transcription factors that are not expressed in differentiated thyroid cells (34).

This result indicates that the SP fraction of cells may consist of a very small percentage of most undifferentiated stem cells whereas the major fraction of cells contains a heterogeneous cell population of differentiated thyrocytes and of endodermal progenitor cells that have already lost expression of ABCG2 transporter and undergone early differentiation.

3.1.4 Human thyroid SP cells in different culture conditions

Comparable numbers of SP and non-SP cells were recovered in Ham's F12 medium supplemented with penicillin-streptomycin and amphotericin B after FACS, and then plated in 4 different culture conditions as described in Materials and Methods.

1) In normal culture conditions with 10 % FCS and 5 mU/ml TSH, SP cells remained bright and round, but did not attach, as shown in Fig. 3.4 A. Cell counting yielded similar numbers of viable cells at plating and after 2 weeks in culture. As expected, non-SP cells adhered and proliferated as monolayer (Fig. 3.4 B).

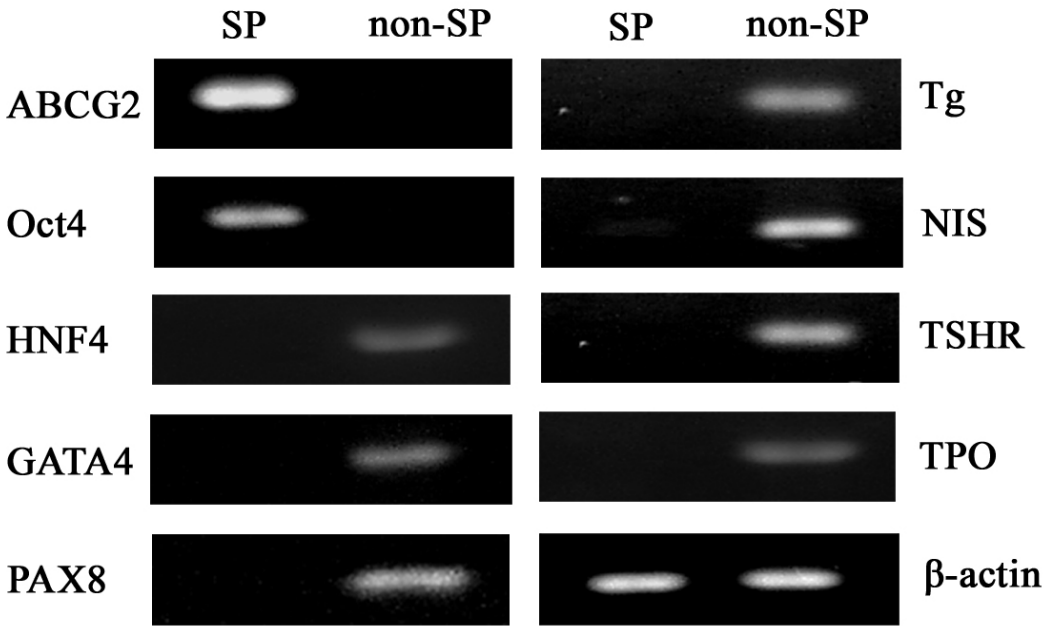

Figure 3.3 Gene expression profiles of FACS-sorted SP and non-SP cells.

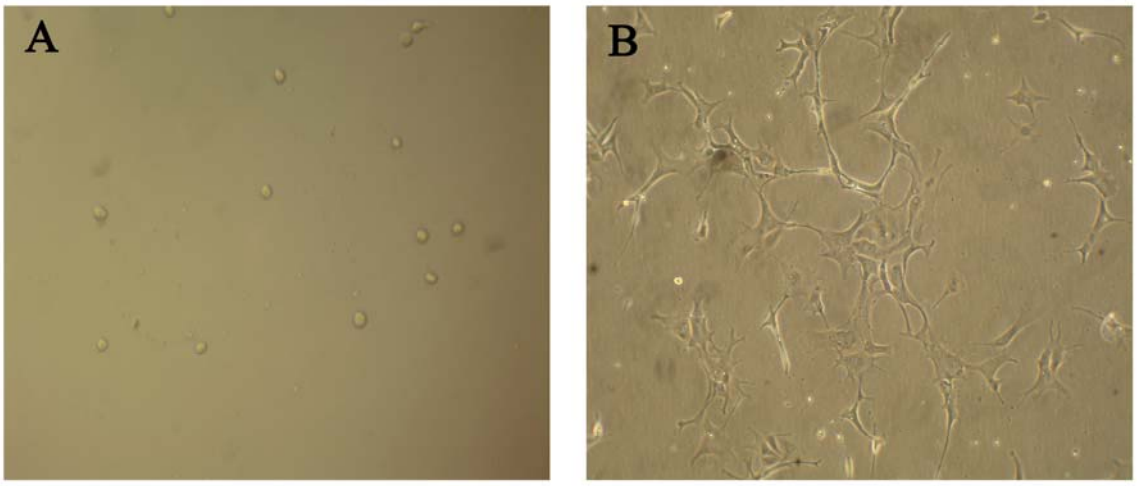

Figure 3.4 SP and non-SP cells in normal culture conditions. (A) SP cells remained bright and round, but did not attach. (B) Non-SP cells adhered and proliferated as monolayer.

2) In the cell culture dishes coated with Matrigel, SP cells were also maintained for 14 d without any attachment and proliferation, as shown in Fig. 3.5 A, whereas non-SP cells attached and expanded as monolayer (Fig. 3.5 B).

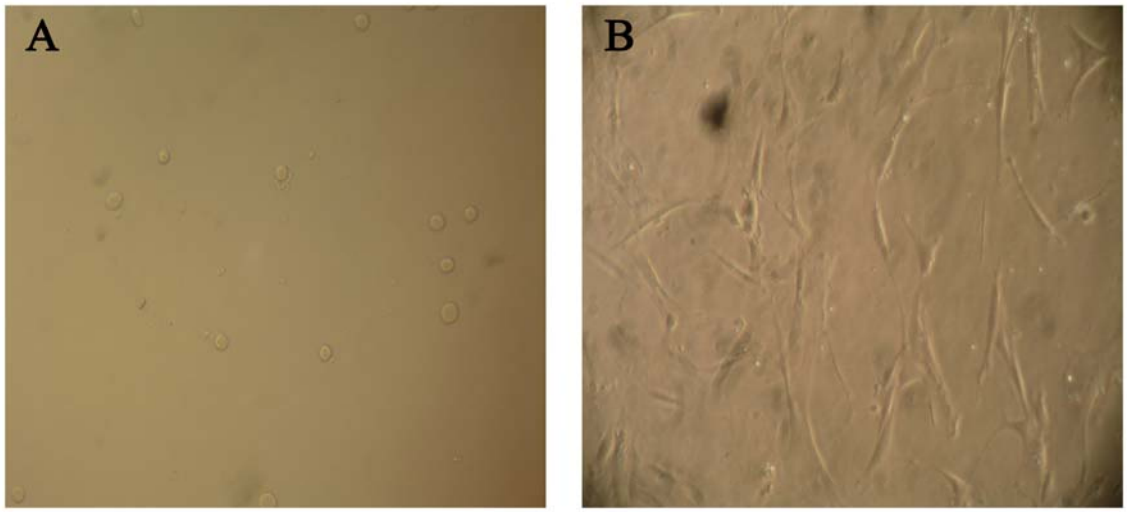

Figure 3.5 SP and non-SP cells cultured in Matrigel-precoated dishes. (A) SP cells, (B) Non-SP cells.

3) Since intense growth stimulation with EGF and bFGF resulted in sphere outgrowth from primary thyroid cells (as described in 3.2.1), SP cells were plated in the same culture condition. However, no division or proliferation was observed up to 14 d either.

Therefore, neither in regular culture conditions nor in Matrigel was cell attachment or growth observed. 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 cell proliferation.

4) Since thyroid SP cells did not show any proliferation in the culture conditions described above, co-cultures of SP and non-SP cells, as well as non-SP cell culture were also performed for 3 weeks. As expected, both groups of cells grew very well as monolayers in regular culture conditions. As shown by RT-PCR, in the non-SP cell culture, expression of stem cell marker Oct4 was not detectable at any time point. Endodermal marker GATA4 was gradually decreased until it became undetectable after 3 weeks of culture (Fig. 3.6). In contrast, in co-cultures, expression of both Oct4 and GATA4 remained at the same levels at 0, 1, 2 and 3 weeks. As demonstrated in our previous study (34), the number of thyroid stem and progenitor cells remained constant over 4-6 passages. This result indicated that, when co-cultured with differentiated non-SP cells, Oct4-positive SP cells (stem cells) sustained the capacity of self-renewal and differentiation. These stem cells could remain constant over passages via

self-renewal and generation of progenitor cells (GATA4-positive cells), which then developed into more specialized cells through differentiation. In contrast, in the singly cultured non-SP cells, GATA4-positive progenitor cells continuously divided, differentiated into specialized cells, and gradually lost the expression of GATA4 after 3 weeks of culture, since no source of Oct4-positive stem cells was present.

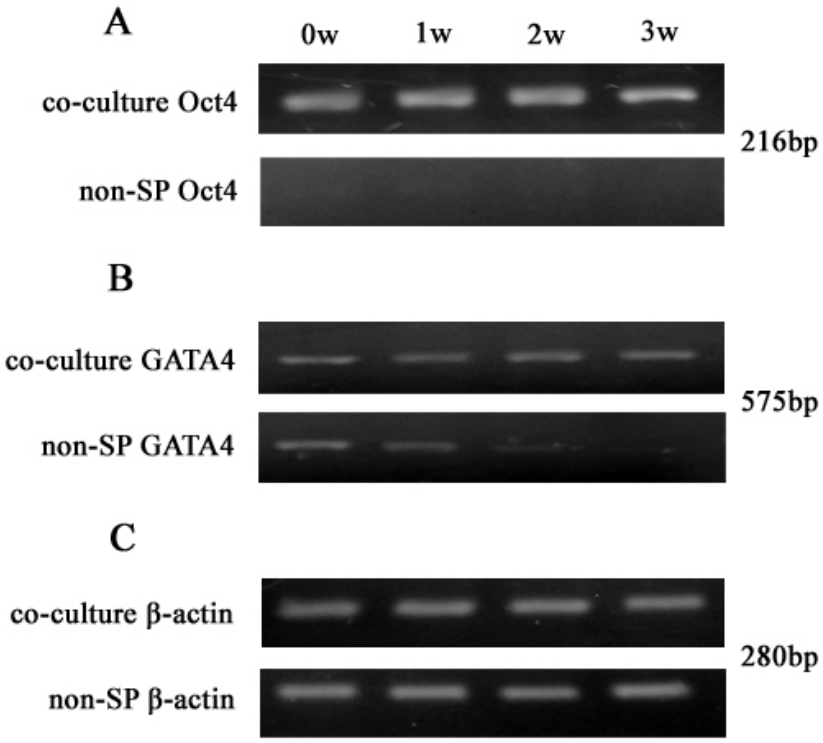

Figure 3.6 Time course of gene expression in SP/non-SP co-cultures and non-SP cells. (A) Oct4 expression, (B) GATA4 expression, (C) β-actin expresssion.

3.1.5 Morphology characteristics of thyroid SP cells

After FACS, both SP and non-SP cells were collected by cytospin, stained with Giemsa and examined under light microscope. We observed that the thyroid SP cells showed an obvious different morphology in comparison to non-SP cells. As depicted in Figs. 3.7 A and C, SP cells were uniformly small with a thin strip of cytoplasm around their nuclei, whereas non-SP cells (Fig. 3.7 B and D) were larger, with increased cytoplasmic area. Importantly, quantitative analysis using morphometry software corroborated these visual observations, confirming that the

SP cells were significantly smaller in cell diameter, in contrast to non-SP (6.6 ± 0.5 μm *vs.* 20.1 ± 1.8 μm, $n = 20$). A small cell size and a high nuclear-to-cytoplasmic ratio are characteristics of quiescent stem cell populations, such as embryonic stem cells, and all other adult stem cell populations, *e.g.* satellite cells in adult skeletal muscle (106), neural stem cells (107), and limbal stem cells (108). Notably, thyroid SP exhibited the typical smaller size and higher nucleus/cytoplasm ratio than that of non-SP, suggesting that they are, indeed, enriched for quiescent cells. All these findings demonstrate that the SP represents the most primitive subset of thyroid cells.

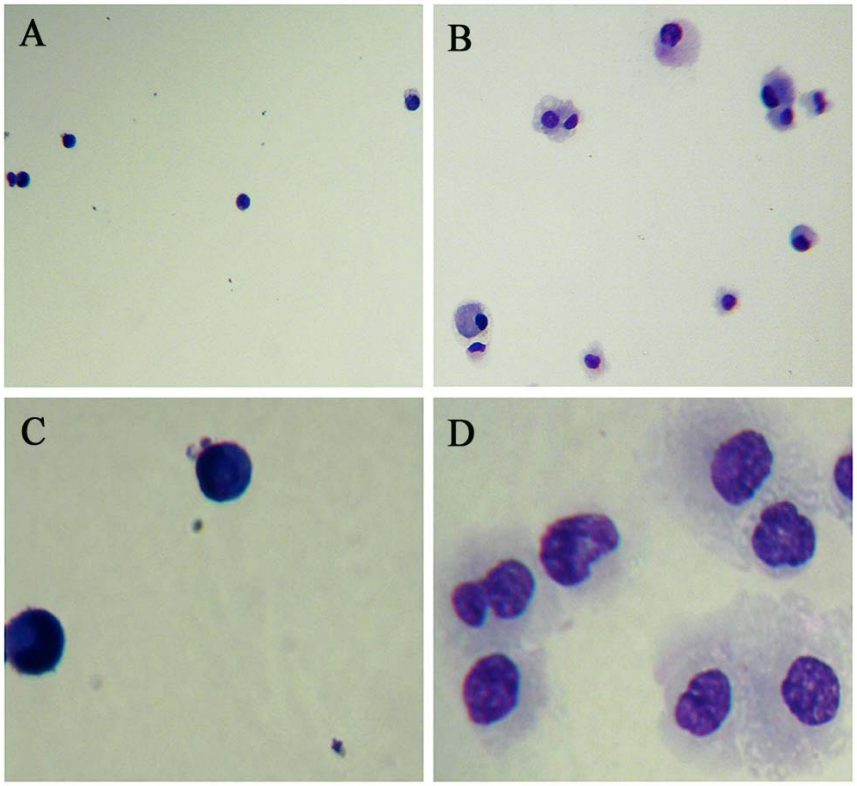

Figure 3.7 Representative micrographs of thyroid SP and non-SP cells collected by cytospin. Thyroid SP cells are conspicuously smaller in size and have a higher nucleus/cytoplasm ratio, compared with non-SP cells. (A) Micrograph of SP cells, 100 ×, (B) non-SP cells, 100 ×, (C) SP cells, 400 ×, (D) non-SP cells, 400 ×. All 4 panels (A-D) show Giemsa staining.

3.2 Stem cell proliferation under intense growth stimulation

3.2.1 Non-adherent spheres develop in thyroid cell cultures

In a complementary approach, single cell suspensions of human thyroid cells derived from nodular goiters were grown in a special medium enriched for growth factors EGF and bFGF, that does not allow adherence to a substratum, as described in Materials and Methods. Following seeding into serum-free growth medium, most primary thyroid cells did not grow under these conditions, but a small number of cells grew out as floating spherical colonies, which were termed “thyro-spheres”, after 1-2 d of culture. During the next few days, more thyro-spheres were progressively developed with increasing cell number and sphere size, in response to the mitogens EGF and bFGF. These spheres were bright, smooth-edged, and compact and were clearly different from irregular clumps of cells (Fig. 3.8 A and B). At first 3-4 d of culture, the spheres expanded in size as well as in number. On d 3 of culture, spheres contained 80 ± 11 cells. After 5-8 d in culture, 29-38 spheres containing 99 ± 15 cells each were observed per 100,000 thyroid cells plated. From d 8-10, both sphere numbers and cell counts in each sphere reached a plateau, and no spheres increased observably in size upon extension of the culture period as shown in Figs. 3.9 A and B. The morphology of the suspended spherical clones under the phase microscope resembled neurospheres, which consist of different numbers of round cells tightly compact together (109, 110).

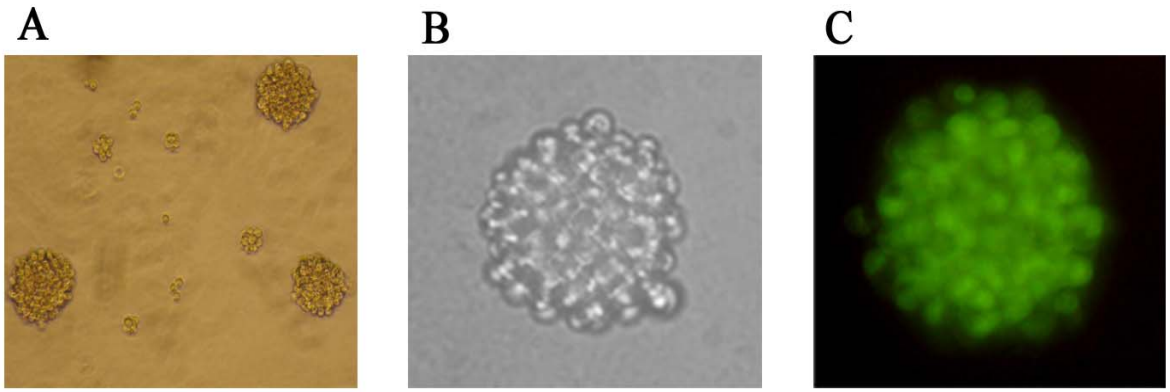

Figure 3.8 Thyro-spheres developed under intense growth stimulation. (A) Floating spheres in culture, 100 ×, (B) Representative graph of a sphere, 400 ×, (C) BrdU incorporation showed most cells in d 5 spheres were labeled with BrdU.

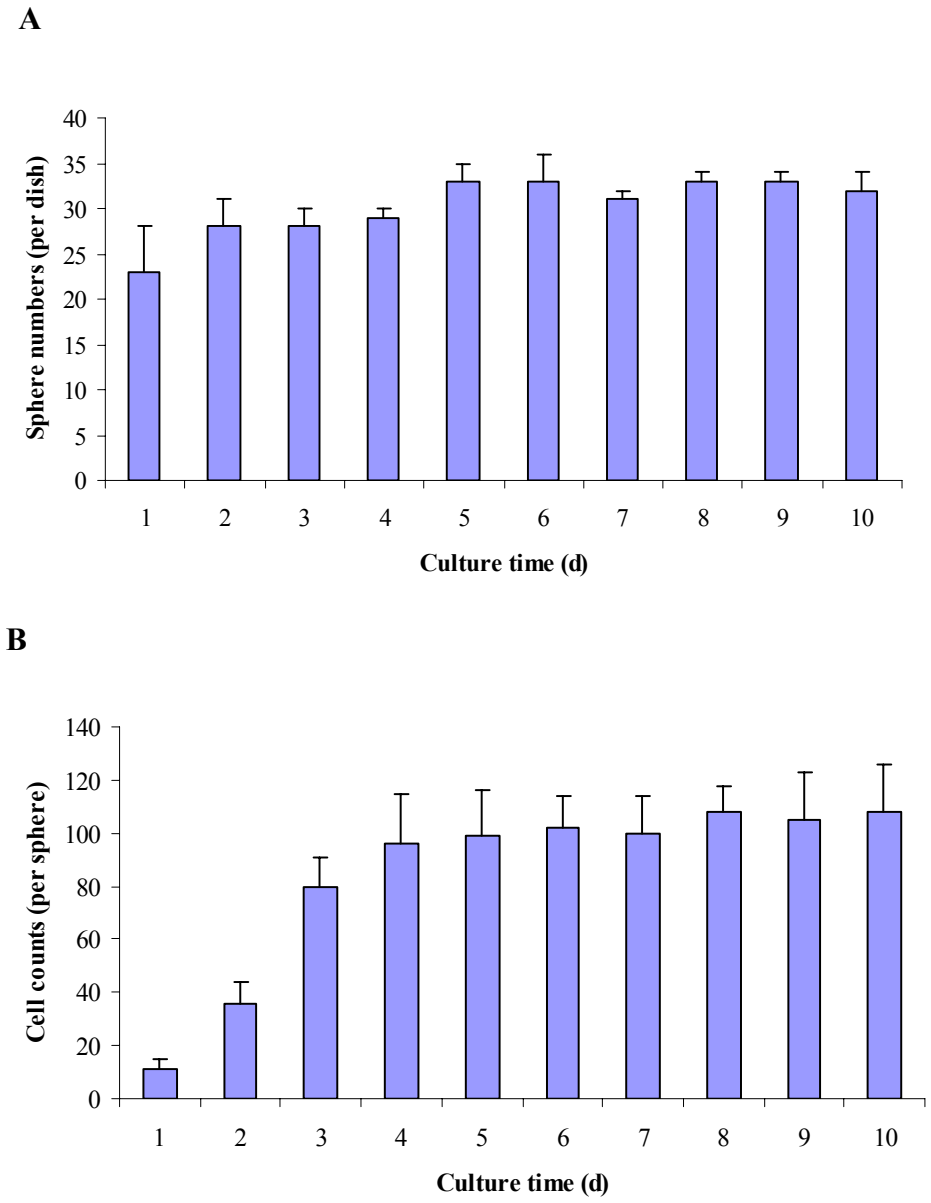

Figure 3.9 Thyro-sphere numbers and sizes during sphere formation. (A) Numbers of spheres in every dish, (B) The average cell counts in each sphere.

In order to investigate the optimal condition for sphere formation, trials with other media were performed to stimulate growth of thyro-spheres. B27 supplement provided essential components, which could not be taken out. EGF and bFGF were found to be indispensable. Interestingly, instead of sphere formation, in the presence of either TSH (Fig. 3.10 A) or serum (Fig. 3.10 B) most cells attached and expanded very slowly.

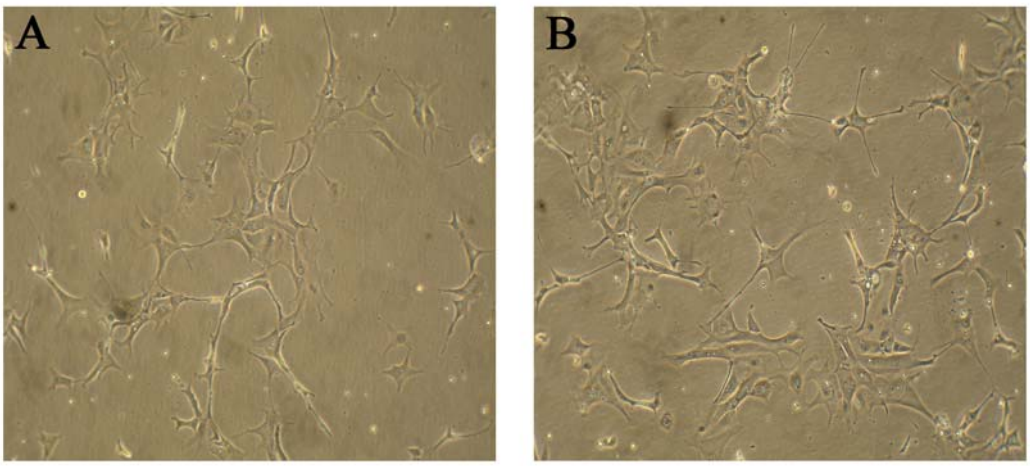

Figure 3.10 No sphere formation in sphere culture medium in the presence of TSH (5 mU/ml) or serum (10 %). (A) in TSH, (B) in serum.

BrdU incorporation experiments were done to confirm that the thyro-spheres were arising from individual cells and not by multicellular aggregation. It was shown that most cells in d 5 spheres were labeled with BrdU (Fig. 3.8 C), indicating that thyro-spheres did not arise by aggregation when thyroid cells were plated at density: 10 cells/ul or lower, but rather developed and grew in size by cell division.

3.2.2 Characteristic expression profiles of thyro-spheres

To determine the stem cell identity of thyro-sphere cells, gene expression profiles of floating sphere cells were detected by RT-PCR. As shown in Fig. 3.14, the important stem cell transcription factor Oct-4, and the universal stem cell marker ABCG2, as well as endodermal progenitor cell markers GATA4 and HNF4 were highly expressed in the floating sphere cells; whereas no expression of thyroid specific genes PAX8, Tg, NIS, TSHR or TPO was detected in these sphere cells. These observations supported the idea that sphere-forming cells were enriched in thyroid stem and progenitor cells.

3.2.3 Thyro-spheres are enriched in SP cells

In view of the largely overlapping expression pattern of SP and thyro-sphere cells, as shown in Figs. 3.3 and 3.14, and in order to determine if the capacity of cells to form spheres correlates

with enrichment in side population cells, we analyzed the thyro-sphere cells by FACS for the Hoechst dye exclusion. As shown above, the SP fraction of stem cells represented only about 0.1 % of the total population (Fig. 3.2 A and B). In contrast, growth factor-stimulated spheres comprised of about 5 % stem cells, as revealed by FACS separation of SP cells (Fig. 3.11). This demonstrates a 50-fold enrichment of stem cells in thyro-spheres. However, the majority of proliferating sphere cells consisted of endodermal progenitor cells that are characterized by their specific markers.

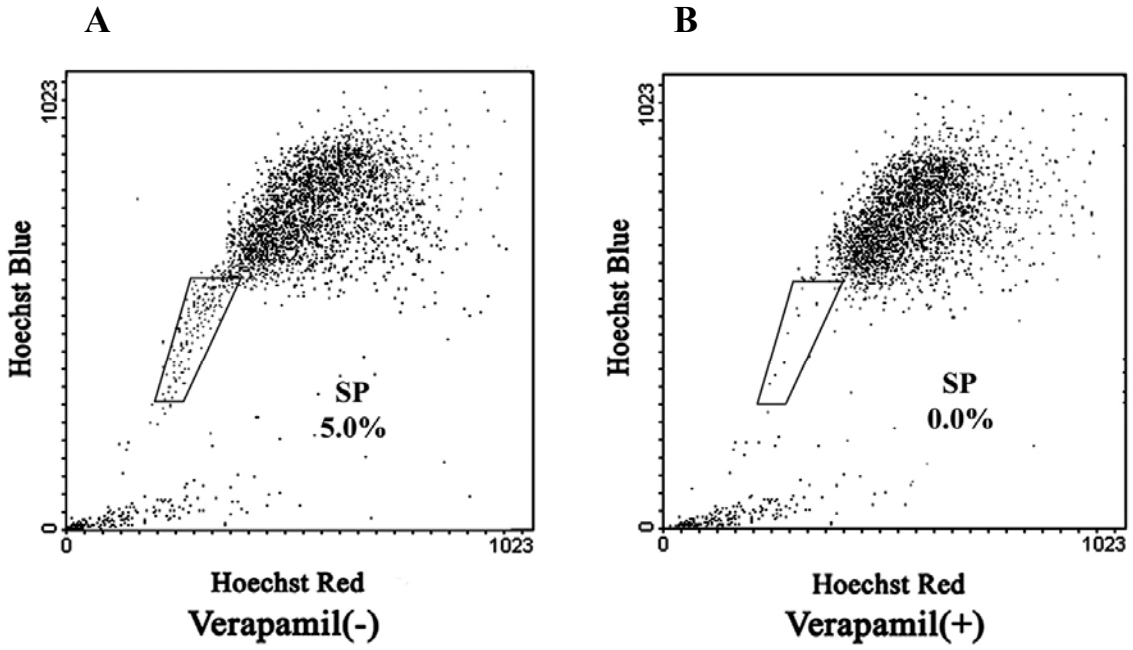

Figure 3.11 Sphere-derived cells were analyzed by FACS for Hoechst dye exclusion. (A) Growth factor-stimulated spheres comprised of about 5 % side population cells, (B) Incubation with 50 µM verapamil which inhibits the efflux, almost completely abolished SP profile, illustrating the specificity of the staining.

We further investigated the gene expression of SP and non-SP sorted from sphere cells. As expected, SP cells of spheres only expressed stem cell markers of Oct4 and ABCG2; whereas the non-SP cells expressed the endodermal progenitor cell markers GATA4 and HNF4 (Fig. 3.12).

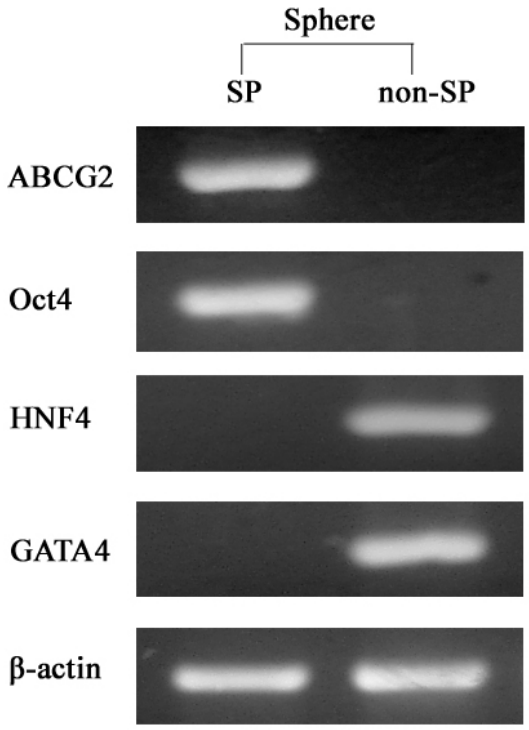

Figure 3.12 Gene expression of SP and non-SP cells sorted from thyro-sphere cells.

3.3 Stem cell differentiation into thyroid cells

3.3.1 TSHR induction by serum and TSH

To determine whether thyro-sphere cells would generate thyroid progeny, we removed thyro-spheres from mitogen-containing medium by gentle centrifugation, dissociated and replated them in a monolayer culture condition in serum-enriched medium, but without growth factors. Within 24 h after plating, almost all cells attached. Three days later, 5 mU/ml TSH was added to the culture medium. The cells were then maintained as monolayer in culture upon differentiation by exposure to both TSH and serum for 18 additional days. RT-PCR analysis demonstrated that, on the first day in culture (d 0), TSHR transcripts were not detectable in sphere cells (Fig. 3.13). Following 3 d of differentiation initiation with serum (d 3), sphere-derived cells began to express TSHR gene at very low level. Notably, TSHR expression gradually increased from d 3-21 of culture in the presence of both TSH and serum, as depicted in Fig. 3.13. These data demonstrated that TSH can stimulate TSHR expression in thyro-sphere cells in the presence of serum.

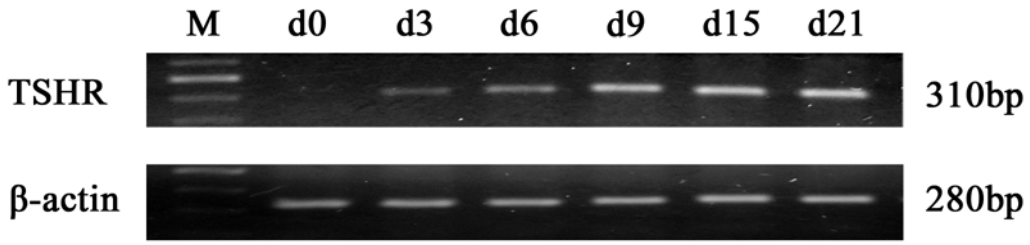

Figure 3.13 Time course of TSHR expression in response to serum and TSH.

3.3.2 Gene expression profiles of differentiated sphere-derived cells

Under the influence of TSH in serum-enriched medium, sphere-derived cells grew as monolayer and differentiated into thyrocytes, as characterized by their typical gene expression profile (Fig. 3.14). All the thyroid differentiation markers (TPO, Tg, TSHR and NIS mRNA) and thyroid transcription factor PAX8 were expressed in these cells, but both the expressions of stem cell markers (Oct4 and ABCG2), and of endodermal progenitor cell markers (GATA4 and HNF4) were not detectable after 21 d of differentiation.

3.3.3 Growth rate of sphere cells

In order to compare the proliferation of sphere cells that underwent intense growth stimulation and differentiation, BrdU incorporation was detected each day. As shown in Fig. 3.15, in response to intense growth stimulation, stem cells proliferated rapidly during the first 3-4 d of sphere formation in response to intense growth stimulation, and then the proliferation slowed down on d 5, which was consistent with the observation about sphere development indicated in Fig. 3.9; upon differentiating stimulation with TSH in serum-enriched medium, sphere-derived cells divided slowly and stably in the same manner as primary cultured thyroid cells.

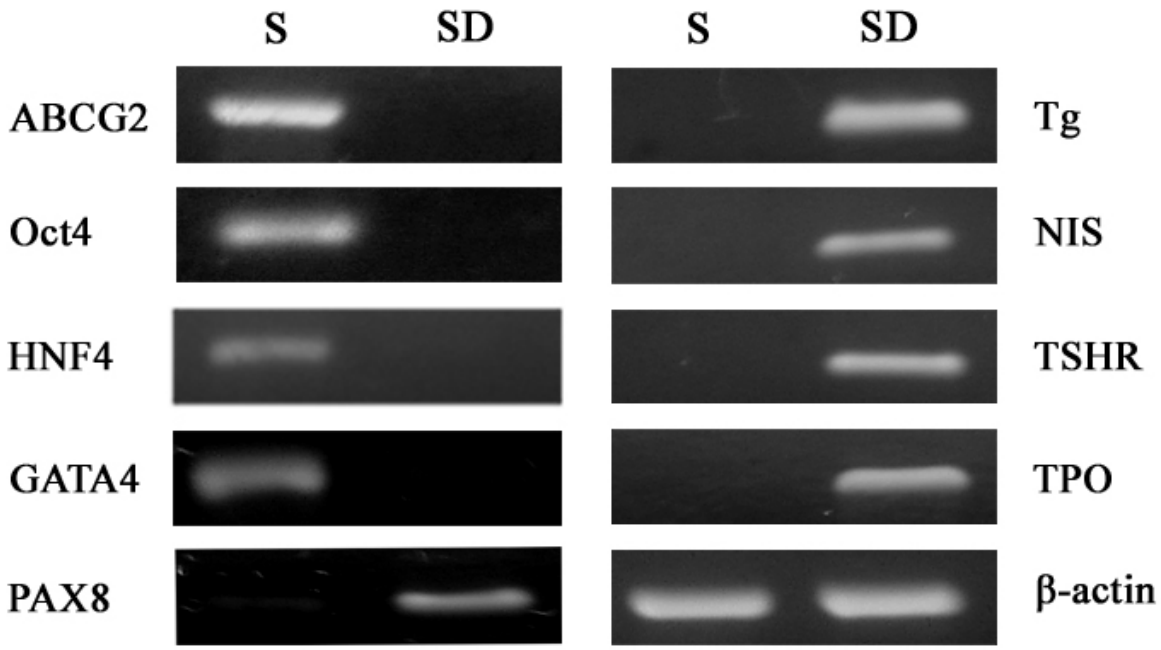

Figure 3.14 Comparison of expression profiles between original thyro-spheres and sphere-derived cells that grew as monolayer under the differentiating conditions for 21 d. Abbreviations: S, original sphere; SD, sphere-derived cells under the differentiating conditions for 21 d.

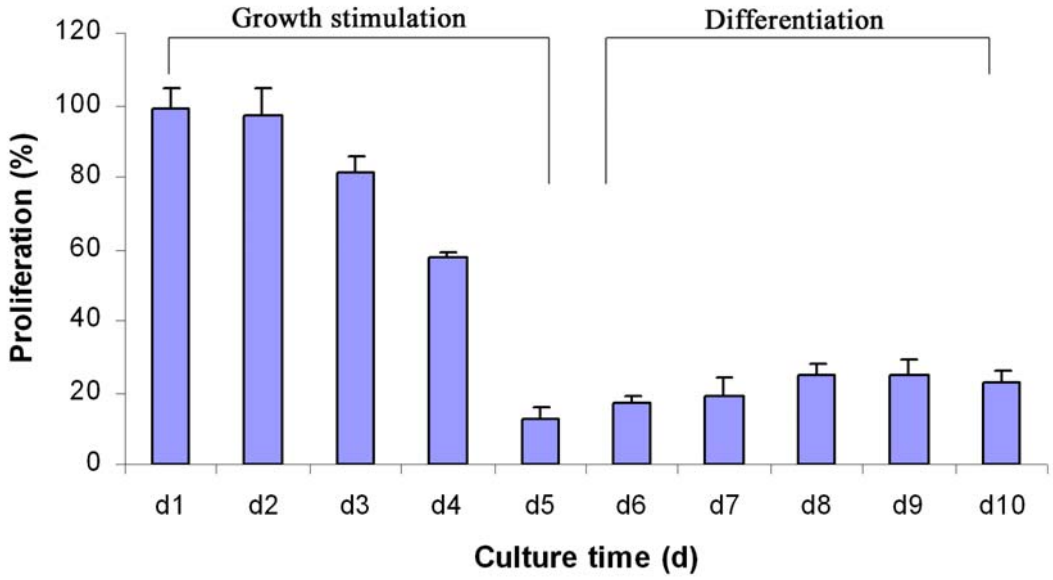

Figure 3.15 Proliferation of sphere cells in response to growth stimulation and differentiation. Growth rates were estimated by counting the percentage of BrdU-labeled cells.

3.3.4 Iodide uptake activity

In addition to the evidence of typical gene expression profile, the commitment of mature thyroid lineage in the differentiated sphere-derived cell cultures was confirmed further by specific iodide uptake activity.

With the support of collagen for 3-4 d, differentiated thyroid cells formed thyroid follicle-like structures with a distinct lumen, as demonstrated in Fig. 3.16. Importantly, in contrast to thyro-sphere cells that maintained in TSH/serum-free medium, these follicles displayed ^{125}iodide uptake activity, one of the most important hallmarks of differentiated thyroid cells, as the primary cultured thyrocytes from the same patients did (Fig. 3.17). These observations proved that thyroid stem cells could differentiate into thyroid cells with functional NIS expression, which displayed TSH-dependent ^{125}iodide uptake.

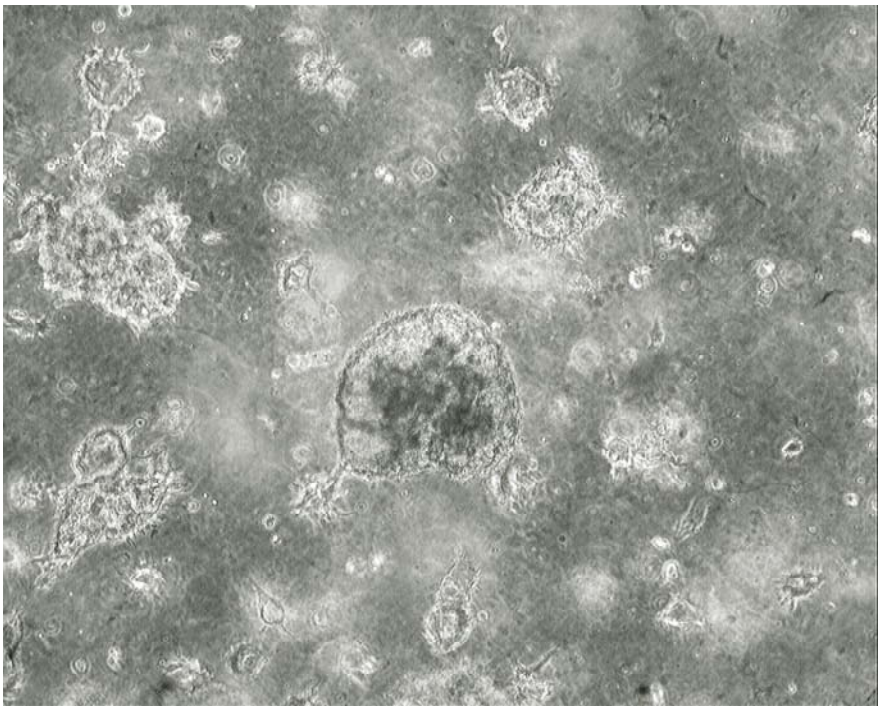

Figure 3.16 Differentiated sphere-derived cells formed thyroid follicle-like structures in collagen

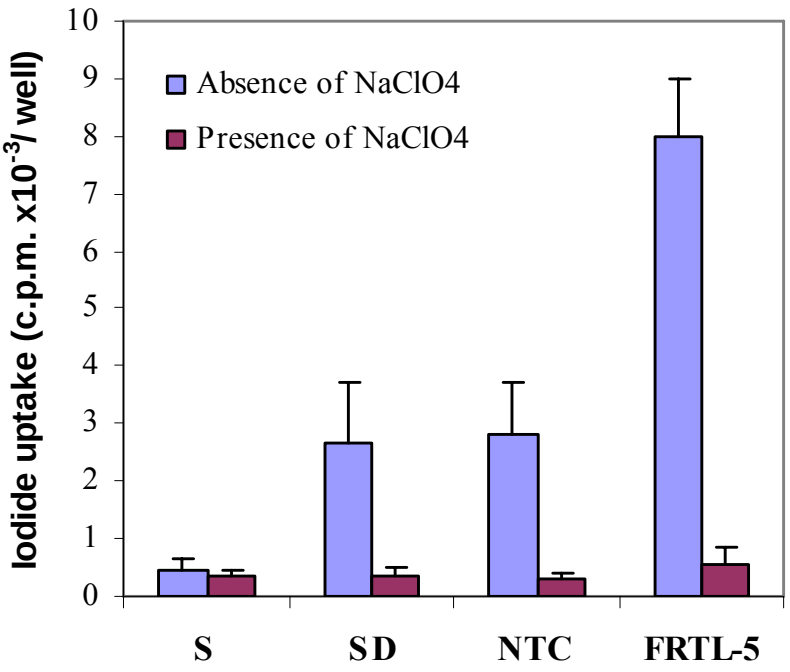

Figure 3.17 Differentiated sphere-derived cells display a specific radioiodide uptake activity. Abbreviation: S, sphere; SD, differentiated sphere-derived cells; NTC, normal cultured thyrocytes from the same patients.