Aus der Medizinischen Klinik des St. Hedwig Krankenhauses
Akademisches Lehrkrankenhaus der Charité –
Universitätsmedizin Berlin

DISSERTATION

Isolation and characterization of a side population in thyroid cancer cell lines: the evidence for the presence of thyroid cancer stem cells

zur Erlangung des akademischen Grades
Doctor medicinae (Dr. med.)

vorgelegt der Medizinischen Fakultät
Charité – Universitätsmedizin Berlin

von

Dai Cui

aus Yancheng, China

Gutachter: 1. PD Dr. med. Heide Siggelkow

2. Prof. Dr. Heiner Mönig

3. Prof. Dr. Harald Klein

Datum der Promotion: 26-Nov-2007

To my parents

CONTENTS

ABBREVIATIONS

Abbreviations

ABCG2	ATP-binding cassette transporter G2
AML	acute myelogenous leukaemia
APC	adenomatous polyposis coli
BCRP	breast cancer resistance protein
bFGF	basic fibroblast growth factor
BrdU	5-bromo-2´-deoxy-uridine
°C	degree Celsius
cDNA	complementary deoxyribonucleic acid
CNS	central nervous system
CSCs	cancer stem cells
DMEM	Dulbecco's modified eagle medium
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotide triphosphates
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EMT	epithelial-mesenchymal transition
ES	embryonic stem cell
FACS	fluorescence-activated cell sorting
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
×g	G-force (unit of measurement of rotation speed of a centrifuge)
HBSS	hank's balanced salt solution
HSCs	hematopoietic stem cells
LAMC2	isolated γ2 chain of laminin
L1CAM	L1 cell adhesion molecule
MDR1	multi-drug resistance gene 1
MEM	non-essential amino acids
$MgCl_2$	magnesium chloride
MMP2	matrix metalloproteinase 2
µM	micromol per liter solution (unit of concentration)

M-MLV-RT	Moloney murine leukemia virus reverse transcriptase
ml	milliliter
mRNA	messenger ribonucleic acid
mU/ml	milliunits per milliliter
µg	microgram
PBS	phosphate buffered saline
PET	polyethylene terephthalate
RT	reverse transcription
PCR	polymerase chain reaction
PH	potentia hydrogenii (negative decimal logarithm of hydrogen-ion concentration)
PI	propidium iodide
rpm	revolutions per minute
SP	side population
TBE	Tris-borate EDTA
TP53	tumour protein p53
TRITC	tetramethylrhodamine isothiocyanate
uPA	urokinase-type plasminogen activator
uPAR	urokinase-type plasminogen activator receptor
UV	ultraviolet

1 Introduction

1.1 The classical theory of thyroid carcinogenesis and its paradox

Thyroid cancer is the most prevalent endocrine neoplasia and accounts for approximately 1 % of all carcinomas [1]. The annual incidence of thyroid cancer per 100,000 individuals in different registries ranges from 1.2 to 2.6 in men and from 2.0 to 4.4 in women, but significantly increasing incidences of this neoplasm have been reported in certain countries [2,3]. Treatment typically involves radioactive iodide, surgical resection, and lifelong levothyroxine medication. For well-differentiated tumours, surgical resection and radioactive iodide could be an effective treatment. However, poorer prognosis is still associated with less differentiated histological types, such as the tall-cell and the Huerthle variants. The even more undifferentiated anaplastic type carries an ominous prognosis (median overall survival of < 6 months). The anaplastic thyroid carcinoma is considered to be one of the most rapidly lethal neoplasms in humans [4]. It can rapidly and extensively spread in the neck, often involving the carotid vessels, larynx, trachea and esophagus, as well as frequently metastases to adrenals, bone, brain, heart and lung. Treatment is mainly palliative, and major advances in the therapy of these neoplasms may benefit from better knowledge of thyroid carcinogenesis.

According to the classical theory of tumorigenesis, cancer is generally regarded as a genetic disease derived from some normal differentiated cells via damage to their genome. Based on this concept, thyroid cancers are believed to originate from normal thyroid follicular cells (thyrocytes) by multi-step gene mutations. For example, follicular carcinomas are believed to originate from follicular adenomas, papillary carcinomas may be derived from some unknown cells that are generated from normal thyrocytes [5] while anaplastic carcinomas are generated from both follicular and papillary carcinomas by genomic changes, such as mutations in tumour protein p53 (*TP53*).

However, some of the recent molecular findings in thyroid carcinoma have raised questions regarding this complicated but widely accepted classic model of thyroid carcinogenesis. For example, mutations in *TP53,* which are most often observed in anaplastic carcinomas, have been recognized to be closely related to the aggressive features of these carcinomas, but recent studies have revealed that no mutation in *TP53* was observed in a considerable percentage of anaplastic

carcinomas. Furthermore, these mutations are also observed in other types of tumours, even follicular adenomas [6,7]. Thus, it seems to be clear that mutations in the *TP53* gene are not necessarily responsible for the aggressive features of anaplastic carcinomas. Furthermore, according to the classical model of thyroid carcinogenesis, the existence of common genomic changes between differentiated carcinomas and anaplastic carcinomas may offer direct proof of the multistep carcinogenesis hypothesis. Mutations in *RAS* and *BRAF* are frequently observed in thyroid follicular and papillary carcinomas, respectively [8,9]. However, in anaplastic carcinomas, increased frequencies of mutations in these genes are not observed and in some studies, decreased frequencies have been reported [10-12]. Tallini *et al*. [13] examined the rearrangement of the *RET* gene in both anaplastic carcinomas and differentiated carcinomas, and found that rearrangement of the *RET* gene is limited to papillary carcinomas and never observed in anaplastic carcinomas. Furthermore, recent studies showed that the *PAX8-PPARγ* rearranged gene is detected only in follicular tumours but not in anaplastic carcinomas [14-16]. In addition, regarding the heterogeneity of thyroid cancer, our understanding of the cancer cellular biology has lagged. The histological appearance and biological behaviors of thyroid carcinoma are extraordinarily diverse. Structures of sheets, nests, trabeculae, follicles or papillae with various differentiations can coexist [17,18]. So far, there is no direct evidence to prove the succession of genomic changes from normal differentiated cells to malignant carcinomas, which raises a question regarding the hypothesis that these aggressive carcinomas are derived from thyrocytes by the accumulation of genetic changes in their genome.

In fact, it has been proven in many other tumours that they do not act as autonomous proliferation machines, but are very heterogeneous, both in their morphological and functional aspects. An individual tumour shows distinct sub-areas of proliferation and cell-cycle arrest, epithelial differentiation and epithelial-mesenchymal transition (EMT), cell adhesion and dissemination. Multi-step carcinogenesis cannot thoroughly explain this heterogeneity.

1.2 Cancer stem cell hypothesis

Recently, there is a growing body of evidence indicating that cancer may not be derived from the normal terminal differentiated cells, instead, they may originate from long-lived adult stem cells which accumulate stepwise genetic mutations, and develop as ‘tumour-initiating cells’ or ‘cancer stem cells’.

That cancer may be a stem cell disease is not a new concept. The notion that tumours begin from stem cells was first formulated in 1875 when Cohnheim proposed the hypothesis that stem cells 'misplaced' during embryonic development where the source of tumours that formed later in life. Later studies involving tumours derived from ascites fluid in rats and teratocarcinomas and leukemias in mice showed that a single tumour cell can give rise to a new tumour and generate heterogeneous progeny [19-22], providing strong evidence for the clonal origin of tumours. After that, several long-term observations reveals that most tumours are heterogeneous, composed of cells showing different degrees of differentiation and transformation, making it difficult to understand how a well-differentiated target cell gives rise to multiple types of less-differentiated cells, even if one considers the possibility that the process of transformation induces some degree of dedifferentiation. In addition, although hundreds of cancer cell lines exist, the majority of such cell lines form tumours in experimental models only after the introduction of a large number of cells [23,24]. If each cancer cell harbors a set of mutations that suffice to program the transformation state, one would expect that every cell or a small number of these cells should be able to initiate new tumours. Indeed, the overall efficiency of obtaining cancer cell lines or even tumour xenografts from patient tumours is low [24], supporting the notion that most tumour-derived cells lack the ability to recreate a tumour, at least in currently used experimental systems. Besides these findings, concerning the dilemma of cancer treatment, a multitude of unresolved issues remains outstanding regarding the molecular basis of carcinogenesis. For example, what makes some tumours difficult to eradicate, why are some tumours more resistant to therapy than others, and why are some tumours highly aggressive?

To resolve these pivotal issues, in recent years another theory of carcinogenesis, which is called "cancer stem cell" theory has been proposed (as shown in Fig. 1.1). Stem cells are undifferentiated and unspecialized cells that can renew themselves and also give rise to one or more specialized cell types with specific functions. Stem cells have three distinctive properties: self-renewal (*i.e.*, at cell division, one or both daughter cells retain the same biologic properties as the parent cell), the capability to develop into multiple lineages, and the potential to proliferate extensively. The combination of these three properties makes stem cells unique. When we sift the properties of stem cells, the attribute of self-renewal is especially notable, because its subversion is highly relevant to oncogenesis and malignancy. Aberrantly increased self-renewal, in combination with the intrinsic growth potential of stem cells, may account for much of what is considered a malignant phenotype.

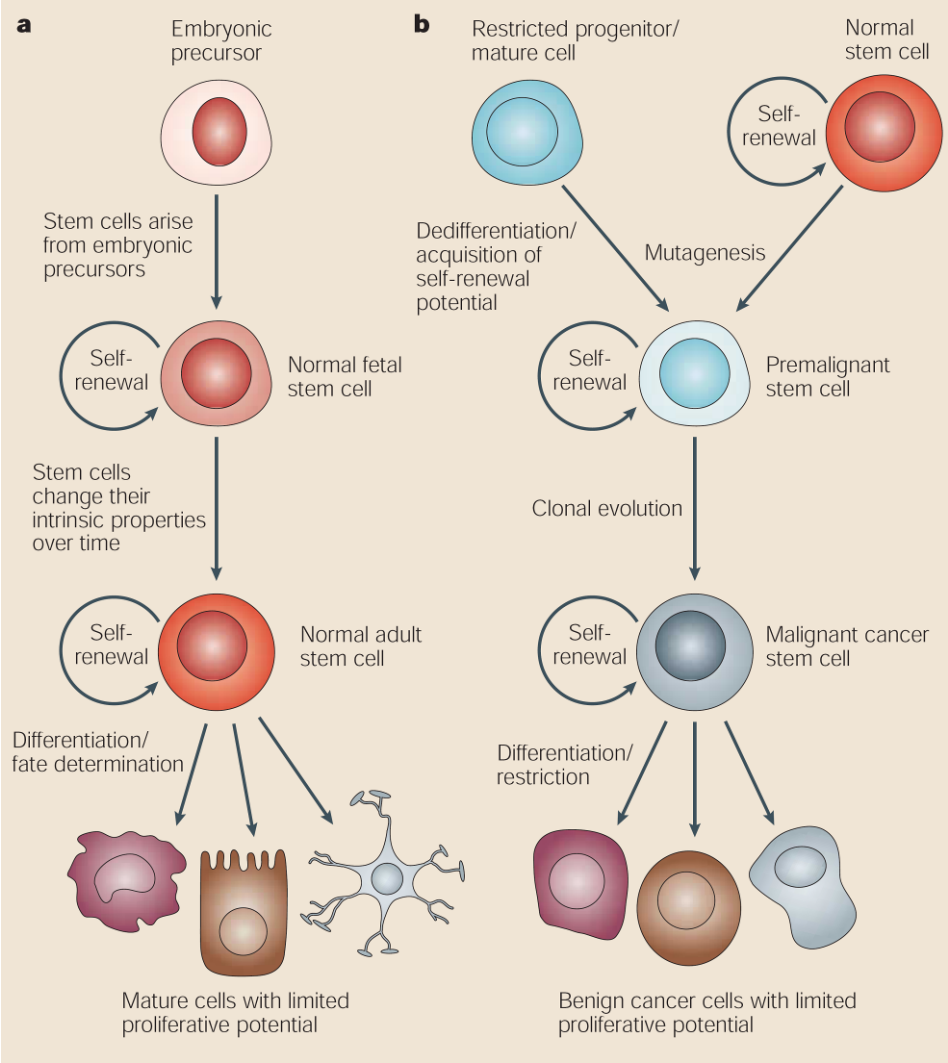

Figure 1.1 (a) Normal adult stem cells arise from embryonic precursors during fetal development. The adult stem cells often continue to self-renew and undergo multilineage differentiation to maintain the adult tissues. (b) In some cases, cancer stem cells can arise from the mutational transformation of normal stem cells, whereas in other cases mutations might cause restricted progenitors or differentiated cells to acquire properties of cancer stem cells, such as self-renewal potential. These pre-malignant stem cells would be distinguished from other cancer cells by their tumorigenic potential, their ability to generate additional cancer stem cells (self-renewal) and their ability to generate phenotypically diverse non-tumorigenic cancer cells (with more limited proliferative potential). In some cancers, like teratocarcinoma, undifferentiated and differentiated cancer cells can clearly be identified histologically. In other cancers, undifferentiated and differentiated cancer cells often cannot be distinguished by histology, although studies have shown that only a subset of cancer cells can form tumours following transplantation into immunocompromised mice. Thus, the growth and progression of many cancers can be driven by a minority population of cancer stem cells, just as the growth of most normal tissues is driven by small populations of adult stem cells in those tissues. [Taken from: Pardal R, *et al*, "Applying the principles of stem-cell biology to cancer", 2003, Nature Reviews, 3: 895-902.]

The basic arguments supporting the idea that tissue adult stem cells may be primary targets for transformation can be summarised as follows:

(1) Stem cells are long-lived, slowly dividing cells that persist in tissues long enough to accumulate multiple genetic alterations required for neoplastic transformation, while somatic cells are constantly replaced through periodic cell turnover; moreover, long-lived cells are exposed to genotoxic insults much longer than are short-lived ones.

(2) Molecular pathways, which play a critical role in governing stem cell self-renewal (*e.g.* Wnt, Notch, Sonic Hedgehog, PTEN) [25] are often deregulated in a number of tumours. It has been revealed that there are lots of mechanistic similarities between the self-renewal of normal stem cells and the proliferation of cancer cells. Stem cells will be renewed in a regulated manner and give source to cell differentiation, providing the amount of cells necessary for a given tissue structure. Tumour cells also exhibit self-renewal capacity; the infinite self-renewal ability (immortalization) is assured by several, so far only partly known, mechanisms. This indicates that cancer can be considered a disease of unregulated self-renewal in which mutations convert normal stem-cell self-renewal pathways into engines for neoplastic proliferation.

(3) Normal stem cells and tumour cells share a number of phenotypic features, such as: a relatively undifferentiated state, the ability to self-renew, extensive proliferative potential, the capacity to give rise to new (normal or abnormal) tissues, the activation of cytoprotective mechanisms (*e.g.* telomerase activity, overexpression of anti-apoptotic proteins, increased trans-membrane molecule efflux capability) as well as a remarkable competence for migration. A brief summary of the similarities between normal stem cells and cancer cells is shown in Fig. 1.2.

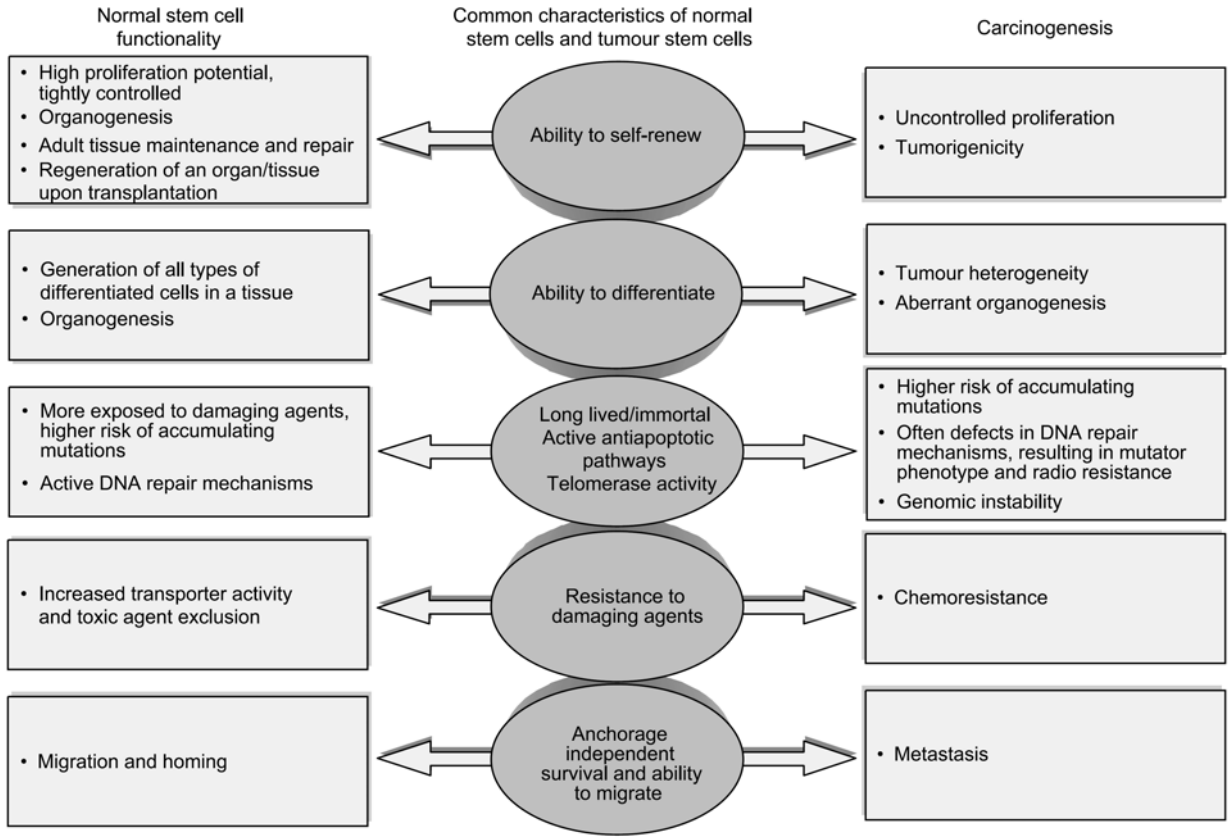

Figure 1.2 Similarities between normal stem cells and cancer cells and their impact on stem-cell functionality and carcinogenesis. [Taken from: Dontu G *et al*, "Stem cells in normal breast development and breast cancer", 2003, Cell Prolif; 36: (Suppl. 1), 59–72.]

According to the cancer stem cell hypothesis, tumour initiation and tumour growth are driven by a small population of cancer stem cells (CSCs), which have an indefinite proliferative and differentiation potential [25,26]. The limited number of such cells in the tumour and their specific phenotypes have been made responsible for tumour formation and relapse [25,27,28]. Thus, for cancer therapy to be curative, it probably must eliminate these cells, which is why it is important to identify and study cancer stem cells.

1.3 The evidence for the existence of cancer stem cells

In addition to providing an elegant model for carcinogenesis, the CSC hypothesis raises several important experimental and clinical implications. First, if a population of biologically unique CSCs exists, then tumour cells lacking stem cell properties will not be able to initiate

self-propagating tumours, regardless of their differentiation status or proliferative capacity. Furthermore, the CSC raises the clinical implication that curative therapy will require complete elimination of the CSC population. Patients who show an initial response to treatment may ultimately relapse if even a small number of CSCs survive. On the other hand, targeted therapies that eliminate the CSC population offer the potential for cure. Given this promise, it is not surprising that the CSC hypothesis has attracted so much attention in recent years.

The existence of cancer stem cells was first clearly documented in the context of leukaemia. Early studies had shown that only a few percent of leukaemia cells proliferated extensively *in vitro* or *in vivo* [20]. Later, John Dick and colleagues showed that only a small subset of cells in human acute myelogenous leukaemia (AML), which could transfer AML when transplanted into immunodeficient mice, was phenotypically similar to normal haematopoietic stem cells. Other AML cells were unable to induce leukaemia [29]. This indicates that AML cells are intrinsically heterogeneous in their proliferative potential, and that AML stem cells give rise to a much larger population of leukaemia cells that lack the ability to proliferate extensively.

Recent experiments have extended this model to include epithelial cancers [30]. It has also been found in breast cancer that only a small population of the tumour cells was able to induce tumour formation in nude mice. These cells were found to express CD44, but little or no CD24. These tumorigenic cells behaved like cancer stem cells in that they not only gave rise to additional $CD44^{+}CD24^{-/low}$ cells, which could be serially passaged from one mouse to another, but they also gave rise to diverse populations of non-tumorigenic breast cancer cells with other phenotypes. These findings indicate that, like AML cells, breast cancer cells intrinsically differ in their tumorigenic potential and only a small fraction among breast cancer cells has the capacity to form new toumors which provided the evidence for the existence of breast cancer stem cells.

Similar results have been observed for cancers of the central nervous system (CNS). Three groups have successfully cultured cells with characteristics of CNS stem cells from various human brain tumours [31-33]. Peter Dirks and colleagues showed that a small subset of cells that express the human neural-stem-cell marker CD133 accounted for almost all *in vitro* proliferative activity. In culture, these $CD133^{+}$ cells gave rise to cells that expressed neuronal and/or glial markers in proportions that mirrored the phenotypes of cells within the original tumours.

So far, other than the definitive evidence found for the existence of cancer stem cells in leukemia [29,34], brain tumour and breast cancer, the putative cancer stem cells/ cancer initiating cells have also been identified in many other tumours, such as lung cancer, melanoma, retinoblastoma, hepatoma, prostate cancer and even some cancer cell lines that persisted in culture over many years [30,31,35-37].

In thyroid carcinomas, it has been well proven that cells within a thyroid carcinoma generally have been derived from a single transformed cell of monoclonal origin [38]. The tumorigenic initiating cancer cells in the thyroid must be able to undergo processes that are similar to the self-renewal and differentiation of normal stem cells to give rise to phenotypically diverse progenies. Based on this hypothesis, it is not surprising that thyroid carcinoma usually contains cancer cells with heterogeneous phenotypes, reflecting abnormal differentiation of cancer stem cells. To date little information about tumour-initiating or cancer stem cells in thyroid cancer has been available, and some has contradicted the existence of cancer stem cells in thyroid carcinoma [39].

1.4 A novel method of identification of cancer stem cells based on the expression of an ATP-binding cassette transporter ABCG2

At present, two general approaches could be utilized to identify and characterize cancer stem cells. First, some specific molecular markers, such as CD44, CD24, CD29, Lin, CD133 and Sca-1 [30-32,37,40], have been proposed for the isolation of cancer stem cells (*e.g.* $CD44^+/CD24^-$ for breast cancer stem cells, $CD34^+/CD38^-$ for leukemia stem cells and $CD133^+$ for brain cancer stem cells).

Another useful approach to the identification and purification of cancer stem cells, specifically in the absence of surface marker expression, has been to utilize the phenomenon that cancer stem cells, just as their normal counterparts, have the ability to efflux lipophilic, fluorescent dyes such as Hoechst 33342 and thus can be isolated as a Hoechst low/negative side population (SP) by Fluorescence-Activated Cell Sorting (FACS). The efflux of the Hoechst 33342 dye has been correlated with ABC transporters, in particular ABCG2 [41], and can be inhibited by the Ca++-channel blocker verapamil.

ABCG2, an ATP-binding cassette transporter, belongs to the ABC transporter family which includes over 50 members [42]. These trans-membrane transporters mediate the transfer of a diverse array of substrates across cellular membranes. The ABC transporter family is characterized by a high level of sequence homology between family members, and also a high level of conservation among species. Several ABC transporters have been associated with antineoplastic drug efflux from tumour cells. The first such transporter was termed P-glycoprotein (P-gp), encoded by the gene MDR1 (multi-drug resistance gene 1), and belonging to the ABCB subfamily (see Fig. 1.3a). P-gp upregulation has been associated with clinical resistance to antineoplastic agents and worsened outcomes in a number of human malignancies [43].

More recently, another transporter, ABCG2, also called breast cancer resistance protein (BCRP), was described in breast and colon carcinoma cell lines selected for high-level resistance to the antineoplastic drug, mitoxantrone [44]. ABCG2 differs from the other transporters of ABC transporter family in that it functions as a homodimer composed of two identical subunits, also referred to as a half-transporter structure (see Fig. 1.3b) [45]. The ABCG2 gene is located on chromosomal locus 4q22 and has extensive homology with the Drosophila White gene. Consistent with other ABC half-transporters, the ABCG2 protein contains a single transmembrane domain (TMD) involved in drug or dye binding and efflux and a single cytosolic nucleotide-binding domain (NBD) which is involved in ATP binding and hydrolysis.

ABCG2 expression is regarded to be associated with side population (SP) phenotype. Stem cells and cancer stem cells share the ability to express ABCG2 membrane transporters, which endow them with the capacity to exclude the Hoechst dye. Using these characteristics, normal or cancer stem cells can be isolated as a Hoechst low/negative side population by FACS after Hoechst staining. As shown in Fig. 1.4, SP is a distinct, small cell population composed of unstained cells in the left lower quadrant of a FACS profile.

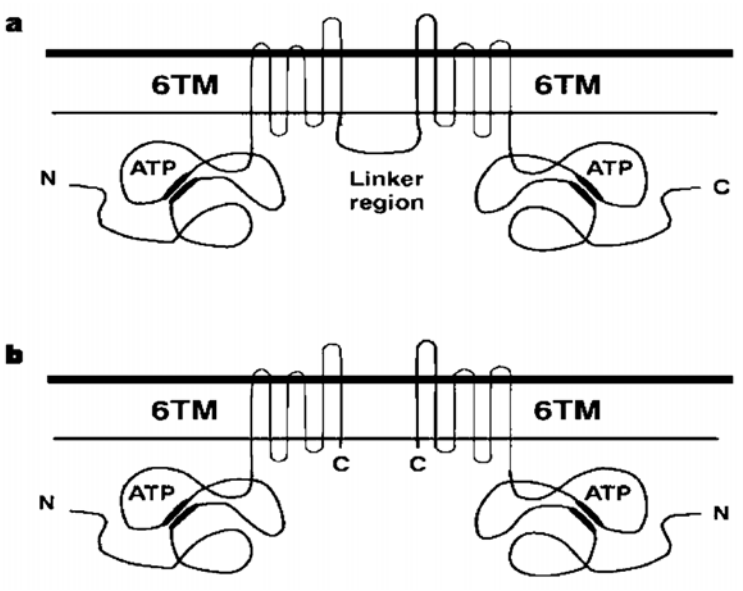

Figure 1.3 Schematic representations of ABC transporters. (a) P-glycoprotein (encode by MDR1) is a full transporter, containing two ATP-binding domains and two transmembrane domains, connected by a linker region. (b) ABCG2 is a half-transporter, requiring homodimerization for functionality. Each molecule contains a single ATP-binding domain and transmembrane domain. [Taken from: Abbott BL, "ABCG2 (BCRP) expression in normal and malignant hematopoietic cells", 2003, Hematol Oncol; 21: 115–130.]

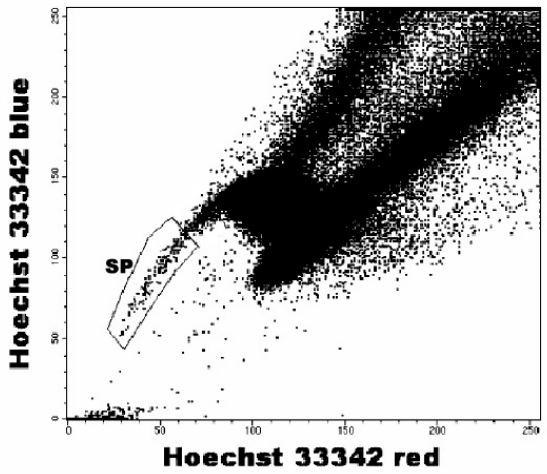

Figure 1.4 Side population (SP) pattern of Hoechst 33342 efflux. The boxed regions contain Hoechst-low staining side population.

Based on this method established by Goodell *et al*, the side population technique has been

adopted to identify putative stem cells and progenitors in multiple tissues/organs including umbilical cord blood [46], skeletal muscle [47-49], mammary glands [50,51], lung [52-54], liver [55] epidermis [56,57], forebrain [58], testis [59,60], heart [61], kidney [62], limbal epithelium [63], and prostate [64]. The strongest evidence linking ABCG2 and the side population phenotype comes from the nearly complete loss of the bone marrow side population phenotype in ABCG2$^{-/-}$ mice [65]. Other supporting evidence is that side population cells preferentially express ABCG2 [52,55,57,59,61,63,66,67] and that ABCG2 expression is detected in known stem/progenitor cells such as hematopoietic stem cells (HSCs) [41], nestin-positive islet-derived progenitors [66], hepatic oval cells [18], limbal basal cells [19], and neural stem cells [68].

Most recently, the side population technique was also applied to identify a cancer stem-like cell enriched SP compartment in various human and rat carcinomas, such as human leukemias, breast, brain, lung, prostate, liver and ovary cancers [67,69-71], which suggest that the side population approach represents a valid method to identify cancer stem cells.

1.5 Wnt signalling

The canonical Wnt cascade has emerged as a critical regulator of stem cells. The discovery of the common origin of the Drosophila segment polarity gene Wingless and the murine proto-oncogene Int-1 laid the keystone of a signalling pathway now commonly referred to as the canonical Wnt cascade (as shown in Fig. 1.5). Wnt proteins are secreted molecules that regulate proliferation and patterning during development. Signalling is initiated when Wnt proteins engage their cognate receptor complex, consisting of a serpentine receptor of the Frizzled family and a member of the LDL receptor family, LRP5/6. The central player is a cytoplasmic protein termed β-catenin whose stability is regulated by the destruction complex. When Wnt receptors are not engaged, two scaffolding proteins in the destruction complex—the tumour suppressors adenomatous polyposis coli (APC) and axin—bind newly synthesized β-catenin. CKI and GSK3, two kinases residing in the destruction complex, then sequentially phosphorylate a set of conserved Ser and Thr residues in the amino terminus of β-catenin. The resulting phosphorylated footprint recruits E3 ubiquitin ligase, which targets β-catenin for proteasomal degradation. Receptor occupancy inhibits the kinase activity of the destruction complex. As a consequence, β-catenin accumulates and travels into the nucleus where it engages the N terminus of DNA-binding proteins of the TCF/LEF family. In the absence of a Wnt signal, TCF/LEF

proteins repress target genes through a direct association with co-repressors. The interaction with β-catenin transiently converts TCF/LEF factors into transcriptional activators which then activate the transcription of genes that promote proliferation.

In many tissues, activation of Wnt signalling has also been associated with cancer. Mutations that activate the Wnt pathway have been implicated in a wide variety of cancers, including those of the colon, prostate and ovary [72]. It has been identified that the Wnt signal pathway also exists and is functionally active in thyroid cells. The finding of mutational activation of the Wnt/β-catenin pathway was recently reported in thyroid cancer. Wnt/β-catenin signalling and thyroid cancer can be linked in several ways: (1) the gene encoding β-catenin, CTNNB1, is frequently mutated in undifferentiated thyroid cancers, (2) there is a high incidence of thyroid neoplasms in patients with germline APC mutations, (3) AKT activation of β-catenin signalling via GSK3β inhibition, and (4) nuclear receptors regulating β-catenin are well documented [73-75].

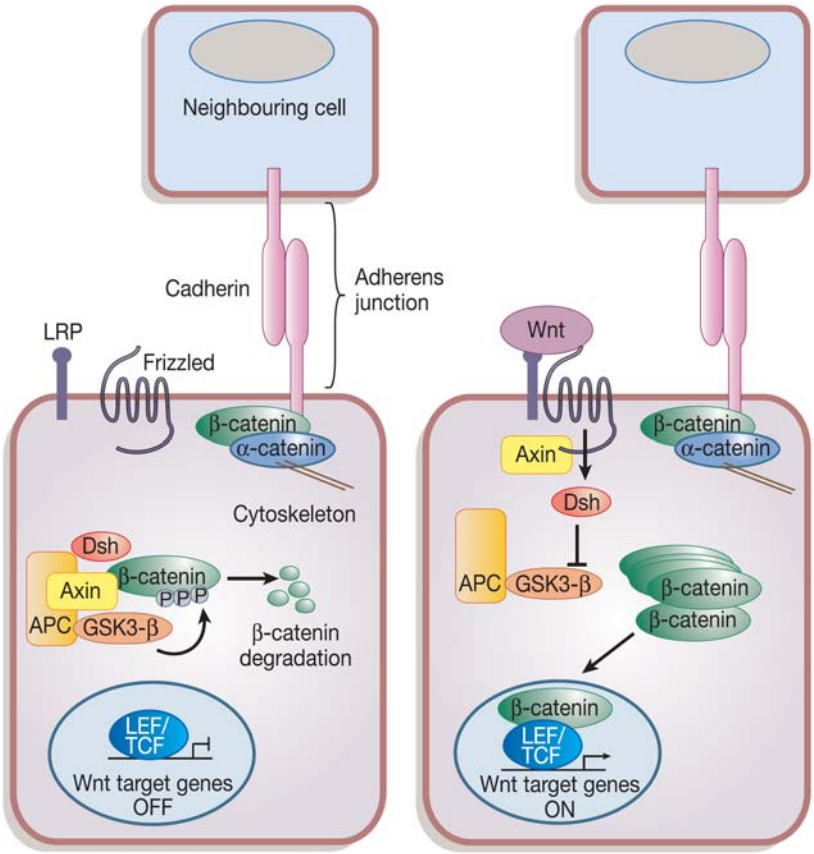

Figure 1.5 The canonical Wnt signalling pathway. In the absence of Wnt signaling (left panel), β-catenin is in a complex with axin, APC and GSK3-β, and gets phosphorylated and targeted for degradation. β-Catenin also

exists in a cadherin-bound form and regulates cell–cell adhesion. In the presence of Wnt signalling (right panel), β-catenin is uncoupled from the degradation complex and translocates to the nucleus, where its binds LEF/TCF transcription factors, thus activating target genes. [Taken from: Reya T, *et al*, "Wnt signaling in stem cells and cancer", 2005, Nature; 434: 843–850.]

1.6 Epithelial-Mesenchymal Transition (EMT) and tumour metastases

Metastasis is defined as the spread of cancer from its primary site to other places in the body. Approximately 90 % of all cancer patients die from metastases [76,77]. The understanding of molecular mechanisms which lead to metastasis is not only a great challenge in experimental cancer research, but it may also reveal key targets against which therapeutic strategies should be directed.

Very recently a new finding of the possible mechanism about tumour metastasis has been shown. The breakdown of epithelial cell homeostasis leading to aggressive cancer progression has been correlated with the loss of epithelial characteristics and the acquisition of a migratory phenotype [78]. This phenomenon is referred to as epithelial-mesenchymal transition (EMT). EMT and the reverse transition from a mesenchymal to an epithelial phenotype [42] are fundamental processes of embryonic development. During embryogenesis, cells that dissociate from primitive embryonic epithelia during tissue patterning often convert to a migrating, mesenchymal cell type. This phenotypic change takes place, for example, in gastrulation, neural crest cell migration, and heart formation [79]. Wnt signalling is involved in the induction of EMT in these physiological processes. EMT can be distinguished on the basis of morphological criteria including the loss of epithelial polarization and the acquisition of a flattened and elongated cell shape [80]. Crucial to the EMT process is the down-regulation of cell-cell contacts, most notably E-cadherin-based adhesion [79].

The hallmarks of malignant tumour are the capabilities of invasion and metastasis. In order for these processes to proceed, tumour cells must be able to detach from the primary tumour, migrate, gain access to blood or lymphatic vessels and disseminate in the body [81]. Finally, disseminated tumour cells have to proliferate again for building up metastases. Recently, EMT is also considered as a crucial event in tumour invasion and metastasis. Accumulating evidence points to a critical role of EMT-like events during tumour progression and malignant transformation, which endow the cancer cell with the invasive and metastatic properties. Several

oncogenic pathways (peptide growth factors, Wnt/β-catenin and Notch, *et al*) induce EMT and a critical molecular event is the downregulation of the cell adhesion molecule E-cadherin.

Recently, there is some evidence suggesting that cancer stem cells may not only initiate the primary tumour formation, but also contribute to cancer metastasis. It has been found that the aberrant activation of Wnt induces EMT in many tumours. Among them, the best understood is the relationship between EMT and the metastases of colorectal cancer. Almost all colorectal cancers show aberrant activation of the Wnt pathway, mostly due to loss-of-function mutations in the APC tumour-suppressor gene [82]. This mutation can inhibit the binding to the cytoplasmic complex and subsequently degradation of β-catenin, which will lead to the nuclear accumulation of β-catenin, the loss of membranous E-cadherin in adherens junctions, and induce EMT of tumour cells. Based on these observations of colorectal cancer described above, Brabletz *et al.* [28] suggested an extended, integrated model that covers all aspects of human tumour progression — the 'migrating cancer stem (MCS)-cell' concept. According to their opinion, although a stepwise, irreversible accumulation of genetic alterations is traditionally considered to be the driving force responsible for tumour dissemination, this traditional model cannot explain the sum of the cellular changes apparent in human cancer progression. For example, the important steps that enable metastasis are reversible, because most metastases re-express E-cadherin, have reduced expression of nuclear β-catenin, are able to recapitulate the differentiated phenotype of their primary tumours, and therefore cannot be explained solely by irreversible genetic alterations [28]. They suggest that the EMT in disseminating tumour cells is only transient [28,83] and such disseminating tumour cells could be regarded as "migrating cancer stem cells" which may be responsible for tumour metastasis.

1.7 Aim of the present study

The aim of the present work was to investigate (1) whether established human anaplastic thyroid cancer cell lines contain side population cells which possess some stemness properties and serve as "cancer stem cells", (2) whether these cancer SP cells have the ability to self-renew and generate different progenies, (3) whether these cancer SP cells have higher tumorigenic and invasive competence in comparison with non-side population cells, and (4) in which culture conditions these cancer SP cells could be enriched.

2 Materials and Methods

2.1 Cell culture

2.1.1 Cell lines

All thyroid cancer cell lines were stored in liquid nitrogen at -196°C**.** For defrosting, cells were placed in 37°C water bath and shaken gently for 1 min and then immediately suspended in pre-warmed culture media.

The HTh74 human anaplastic thyroid carcinoma cell line was isolated from thyroid carcinoma tissue of a 73-year-old woman [84]. HTh74 cells were shown to express some thyroid specific genes like functional TSH receptor protein [84], and mRNA for thyroglobulin, albeit both at very low levels [85].

The cell line C643 was established from a fine-needle biopsy of an anaplastic thyroid carcinoma of a 76-year-old man [86]. The patient died within 5 months after diagnosis. Demonstration of thyroglobulin mRNA ascertained a thyroid epithelial origin of the cell line [87].

Another human anaplastic thyroid cancer cell line SW1736 was originally developed by Leibowitz and McCombs III at the Scott and White Memorial Hospital (Temple, TX) in 1977. Their epithelial origin was confirmed by demonstration of cytokeratin expression [85]. All these three cell lines were kindly provided by Prof. Nils-Erik Heldin (Uppsala University, Uppsala, Sweden).

2.1.2 Culture conditions

HTh74 cells were grown in Ham's F-12 medium (Gibco, Karlsruhe, Germany) with L-glutamine, supplemented with 10 % fetal calf serum (FCS, v/v) (Gibco, Karlsruhe, Germany), 1 % non-essential amino acids (MEM, v/v) (Gibco, Karlsruhe, Germany), 100 U/ml penicillin, 100 µg/ml streptomycin (Roche, Mannheim, Germany), and 2.5 µg/ml amphotericin B (Bristol-Meyer Squibb, Germany).

C643 and SW1736 were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco,

Karlsruhe, Germany), supplemented with 10 % FCS (v/v), 1 % MEM (v/v), 100 U/ml penicillin, 100 µg/ml streptomycin, and 2.5 µg/ml amphotericin B.

For most experiments, all cells were grown as monolayers in 100 mm plastic culture dishes and kept in a humidified incubator at 37°C under 5 % CO_2, with a media change each 3-4 d. Upon reaching the confluency of 70-80 %, cells were passaged. For passaging, the medium was aspirated and the cells were washed briefly with HBSS and incubated in trypsin (0.05 %, w/v)/EDTA-4Na (0.53 mM) solution in the incubator at 37ºC for a time varying from 1~5 min, depending on cell type. Trypsin was then inactivated by adding an equal volume of appropriate cell culture medium, cells were collected by centrifugation at 310 ×g for 5 min, and reseeded at a splitting ratio of 1:5~6. Frozen stocks were prepared in respective cell culture medium containing 10 % (v/v) DMSO and stored in liquid nitrogen.

2.1.3 Cell counting

Following trypsinization and neutralization protocol for cell cultures, a uniform cell suspension was obtained and placed in a centrifuge tube. A 1:2 diluted cell suspension in trypan blue (Sigma-Aldrich, Steinheim, Germany) was loaded on both counting chambers of the hemacytometer and the coverslip was placed over them. The cells were counted under a microscope at 100 × magnification. Cell numbers (total and viable: Trypan blue-unlabeled) overlaying four × 1 mm^2 areas of the counting chamber were determined. And then the total (or viable) cell number of the original cell suspension can be calculated as follows:

Total (or viable) cells recovered =
Cells/ mm^2 divided by dilution (1/2), and multiplied by 10^4 and total volume of cell suspension

2.2 Fluorescent activated cell sorting of side population after incubation of Hoechst 33342

We adopted the original SP identification method of Goodell *et al*. [88] for optimal isolation of thyroid cancer SP cells. The ability of FACS to discriminate Hoechst SP cells is based on the differential efflux of Hoechst 33342 by a transporter of ABC family, ABCG2 transporter. This is

an active biological process, and optimal resolution of the profile is obtained with great attention being paid to the staining conditions. The cell concentration, Hoechst dye concentration, staining time, and staining temperature are all critical. And it is also very important to keep the sample protected from light throughout the staining procedure and during analysis.

Before Hoechst staining, the water bath was kept at precisely 37° C (checked with a thermometer), and DMEM+ medium (see below) was pre-warmed. All the cancer cell lines were cultured in respective culture medium described above. When the cells reached 70~80 % confluence, they were trypsinized from culture dishes, washed, counted accurately, and suspended at 10^6 cells per ml in pre-warmed DMEM+. Cells were mixed well and pre-incubated in a 1.5-ml Eppendorf tube at 37°C for 10 min. Then the cells were labeled in DMEM+ medium with 5 µg/ml (a 200X dilution of the stock) Hoechst 33342 dye at 37°C for 120 min exactly, either alone or in combination with 50 µM verapamil (Sigma, USA), which is a potent inhibitor of ABC membrane transporters, and traditionally used as a guiding parameter to determine where to set the boundary between SP and non-SP cells [88]. During the incubation, the top level of the cell suspension was totally submerged under water in the bath to ensure that the temperature of the cells was maintained at 37^oC. Tubes were gently inverted every 30 min to discourage cell settling and clumping. After 120-min incubation, the cells were spun down at 310 ×g for 5 min at 4°C (in a precooled rotor) and re-suspended in 200 µl cold HBSS+.

When the staining process was over, the cells were kept at 4°C until sorting in order to prohibit further dye efflux. In order to remove cellular aggregates, cells were filtered through a 30 µm porous polyamide mesh prior to analysis. Cells were then counterstained with 2 µg/ml propidium iodide (PI) for dead cell discrimination immediately before sorting. This was not required to see the SP cells, but will help to exclude the dead cells from the profile.

A 350-nm UV laser was used to excite Hoechst 33342 and PI. Analysis was performed on a FACS Calibur Equipment (Becton-Dickinson Biosciences, Heidelberg, Germany) by using a dual-wavelength analysis (blue, 424-444 nm; red, 675 nm) in the facilities of the German Rheumatology Research Center, Berlin. Dead and dying cells (<15 %) were excluded from the analysis on the basis of PI uptake (fluorescence at 564-606 nm), and only data for viable cells were analyzed for Hoechst labeling using WinMDI software (DeNovo Software, Toronto, Canada).

All of the parameters were collected using linear amplification in list mode and displayed in a Hoechst blue versus Hoechst red dotplot to visualize the side population. The side population was identified and selected by gating on the characteristic fluorescence emission profile. In order for a most specific and homogeneous SP selection to be made from thyroid cancer cells, the SP gate for cell collection included only verapamil-sensitive cells with the lowest Hoechst incorporation. A comparable quantity of non-SP cells was also collected. During all staining and sorting procedures, the cells were kept in the dark and on ice. Sorted SP and non-SP cell populations were recovered in culture medium for further experiments or pelleted for RNA isolation. In some experiments, after FACS sorted SP and non-SP cells were maintained in culture separately and re-sorted into SP and non-SP fractions every 2 weeks.

Reagent solutions:

Hoechst 33342: Obtained from Sigma (called Bis-Benzimide) as a powder. Re-suspended at 1 mg/ml (stock concentration) in water, filter sterilized, and frozen in small aliquots.

HBSS+: Hanks Balanced Salt Solution (Gibco, Karlsruhe, Germany) with 2 % FCS and 10 mM HEPES buffer.

DMEM+: DMEM with 2 % FCS and 10 mM HEPES buffer.

Propidium Iodide: Obtained from Sigma. Frozen stock is 10 mg/ml in water. Working stock (covered with aluminum foil and kept in the fridge) is at 200 μg/ml in PBS. Final concentration of PI in samples is 2 μg/ml.

2.3 Reverse transcription and polymerase chain reaction (RT-PCR)

2.3.1 Total RNA isolation of FACS sorted cells

Total RNA was extracted using the RNeasy Micro Kit (Qiagen, Hilden, Germany) according to the manufacturer's specifications. In brief, after cell sorting, the SP cells and the equal number of the non-SP cells were separately collected into the sterile Falcon tube containing 75 μl buffer RLT supplemented with β-ME (add 10 μl β-ME per 1 ml buffer RLT) and vetexed for lysis. Then 5 μl of carrier RNA (4 ng/μl) was added to each tube. Cell lysates were pipetted onto

QIAShredder spin column and centrifuged for 2 min at maximum speed at room temperature for homogenization. 75 µl of 70 % ethanol was added to the homogenized lysate and mixed by pipetting. Samples were then applied to RNeasy MinElute spin columns placed in 2 ml collection tubes and centrifuged for 15 sec at 8000 ×g. Flow-through was discarded and 80 µl of DNase I incubation mix was directly added to the RNeasy MinElute Silica-gel membrane. The tubes were placed on the benchtop at room temperature for 15 min, 350 µl of RW1 buffer was then added and centrifuged for 15 sec at 8000 ×g to wash the columns. Flow-through and collection tubes were discarded and the RNeasy MinElute spin columns were transferred into new 2 ml collection tubes. 500 µl of buffer RPE was pipetted onto the RNeasy columns which were then centrifuged again for 15 sec at 8000 ×g to wash. Flow-through was discarded and 500 µl of 80 % ethanol was added to the RNeasy columns. Tubes were then centrifuged again for 2 min at 8000 ×g to dry the silica-gel membrane. The RNeasy columns were then transferred into a new 2 ml collection tube and centrifuged for 5 min at 8000 ×g with the caps of the spin columns opened. Finally, for elution, the RNeasy columns were transferred to new 1.5 ml collection tubes and 14 µl of RNase-free water was pipetted directly onto the RNeasy MinElute silica-gel membrane. Tubes were closed gently and centrifuged for 1 min at 8000 ×g. The resulting RNA yield was either stored at -20°C or used directly for spectrophotometry and RT-reaction (see below).

For spectrophotometry, 2 µl of RNA was diluted 1:500 in RNase free water and absorption was calculated at OD260. For each probe, the mean value of at least two independent readings was adopted as the result.

carrier RNA stock solution:

carrier RNA	310 µg
RNase-free water	1 ml
Stored at -20 ºC	

carrier RNA working solution (Solution B):

Solution A	carrier RNA stock solution	5 µl
	buffer RLT supplemented with β-ME	34 µl
Solution B	solution A	6 µl
	buffer RLT supplemented with β-ME	54 µl

DNase I working solution:

DNase I stock solution	10 µl
buffer RDD	70 µl

2.3.2 Reverse transcription reaction

For reverse transcription, 2 µl of Oligo-p(dT)15 primer (30 pmol)(Roche) was added to the volume of mRNA solution containing 1 µg of mRNA (as calculated by spectrophotometry). RNase-free water was added to a final volume of 16.75 µl and the mix was heated to 70°C for 5 min. Probes were placed on ice and the following reagents were added consecutively: 5 µl of M-MLV 5× Reaction buffer, 1.25 µl of dNTP nucleotides (10 mM, Roche, Mannheim, Germany), 1 µl (25 Units) of RNAGuard RNase inhibitor (Amersham, Aylesbury, UK) and 1 µl (200 Units) of M-MLV Reverse Transcriptase (Promega, Mannheim, Germany). The mixture was warmed to 42°C for 60 min, 95°C for 5 min and the reaction was terminated at 0°C. cDNA samples were stored at -20°C.

2.3.3 Primer preparation

All primers were obtained in powder form from Invitrogen Inc. Upon delivery, primers were diluted in 1 ml of RNAse-free water and concentration was calculated with spectrophotometry as described above. Primers were then diluted with RNase-free water to a concentration of 4 pmol/µl, aliquoted and stored at -20°C.

2.3.4 Polymerase chain reaction

For PCR amplification the Hot Start method was performed. In brief, 4 µl of complementary DNA was added to a 45.5 µl master mix containing 5 µl 10× reaction buffer, 1.5 mM $MgCl_2$, 1 µl dNTPs (10 mM) and 30 pmol of sense and antisense primers. Negative controls without template cDNA were included in all cases to exclude carry-over contamination with genomic DNA. 50 µl of mineral oil was added to each reaction tube, the lids were closed and the mixture was preheated to 95 ºC for 10 min before adding Taq polymerase (Invitrogen, Karlsruhe, Germany) to reduce non-specific annealing and primer elongation events. Probes were then cooled to 80 ºC and 0.5 µl of Taq DNA polymerase (5 U/µl) was added to each reaction tube. Using a thermocycler, cycling conditions were carried out at 95°C for 30 sec (initial denaturation),

52-63°C for 30 sec (primer specific, annealing) and 72°C for 1 min (extension), followed by a final extension at 72°C for 10 min and termination at 4°C. The number of cycles used was determined to be in the log-linear phase of the amplification reaction. In all PCR analyses, β-actin served as an internal control. Primer pair sequences, product lengths and annealing temperatures were as follows:

Table 2.1 Primer sequences, annealing temperatures, cycles and product sizes for semi-quantitative RT-PCR

Target gene	Primer sequences[#]	Annealing Temp. (^{o}C)	Cycles	Expected size (bp)
ABCG2	S: 5'-AGTTCCATGGCACTGGCCATA-3'	53	30	379
	AS: 5'-TCAGGTAGGCAATTGTGAGG-3'			
Oct4	S: 5'-GACAACAATGAGAACCTTCAGGAG -3'	55	30	216
	AS:5'-CTGGCGCCGGTTACAGAACCA-3'			
MDR1	S: 5'-GCCTGGCAGCTGGAAGACAAATAC-3'	59	30	253
	AS: 5'-ATGGCCAAAATCACAAGGGTTAGC-3			
E-cadherin	S: 5-CTG AAG TGA CTC GTA ACG AC-3	55	30	300
	AS: 5-CAT GTC AGC CAG CTT CTT GAA G-3			
β-catenin	S: 5'- TCTTGGCTATTACGACAG -3'	58	32	459
	AS: 5'- CCTCTATACCACCCACTT -3'			
Snail	S: 5'- CGGGATCCACTATGCCGCGCTCTTT -3'	60	30	779
	AS: 5'- ATCGCGGGGACATCCTGAGCA -3'			
Slug	S: 5'- AGCGAACTGGACACACATAC -3'	58	30	411
	AS: 5'- TCTAGACTGGGCATCGCAG -3'			
uPA	S: 5'- ACTACTACGGCTCTGAAGTCACCA -3'	56	28	200
	AS: 5'- GAAGTGTGAGACTCTCGTGTAGAC-3'			
uPAR	S: 5'- CTGGAGCTTGAAAATCTGCCG -3'	56	28	137
	AS: 5'- CTGGAGCTTGAAAATCTGCCG -3'			
MMP2	S: 5'- AACCCTCAGAGCCACCCCTA- 3'	52	29	287
	AS: 5'- GTGCATACAAAGCAAACTGC -3'			
L1CAM	S: 5'- ACGGGCAACAACAGCAAC -3'	57	35	429
	AS: 5'- CGGCTTCCTGTCAATCATG -3'			
LAMC2	S: 5'- TGGAGAACGCTGTGATAGGTGTCG -3'	63	35	463
	AS: 5'- TGTGTAAGTCTTGGTGAGCCCAC -3'			
β-actin	S: 5'-CCCAGGACCAGGGC GTGAT -3'	59	25	280
	AS: 5'-TCAAACATGATCTGGGTCAT-3'			

[#] S: sense primer; AS: antisense primer

2.3.5 Agarose gel electrophoresis

PCR products were separated on 1.5 % or 2 % (w/v) agarose gels (2 % for expected fragment sizes of 250 bp or lower) containing 0.5 µg/ml ethidium bromide. Gels were run at a voltage of 90V in TBE running buffer for 60 min. Bands were visualized on a UV-transilluminator at 312 nm, and images were documented and analyzed using Image J software.

TBE Buffer (20X):

1.8 M Tris-base

1.8 M boric acid

25 mM EDTA-$Na_2.2H_2O$

PH was adjusted to 8.3

2.4 Immunofluorescent staining

HTh74 cells were double labeled in suspension with Hoechst 33342 and ABCG2 antibody. Following dissociation from culture dishes by trypsin, cells ($2x10^6$) were centrifuged at 200 ×g for 5 min. The supernatant was decanted and cells were resuspended in 1 ml of DMEM+. Hoechst 33342 dye was added at 5 µg/ml, and cells were incubated at 37°C in water bath for 120 min as described in chapter 2.2 above. Cells were then washed twice with PBS, resuspended in 200 µl PBS and incubated with 1:100 diluted ABCG2 antibody (clone BXP21, Santa Cruz, Heidelberg, Germany) on ice for 30 min. After that, cells were washed twice and resuspended in 100 µl PBS. This step was followed by incubation with TRITC conjugated secondary antibody (1:100 dilution, Santa Cruz, Heidelberg, Germany) for 30 min in the dark on ice. After incubation, cells were washed two times with PBS and resuspended in residual supernatant after decanting, then pipetted onto a slide and coverslipped for microscopic viewing. Fluorescent cells were visualized and photographed under a microscope with epifluorescent filters for Hoechst and TRITC.

2.5 In vitro clonal analysis

To evaluate the self-renewal potential of HTh74 SP and non-SP cells *in vitro*, clonal formation assay was performed. HTh74 cells were labeled with Hoechst dye and analyzed by FACS. Two

hundred of side population, non-side population and total HTh74 cells were harvested separately, recovered in 3 ml Ham's F-12 medium supplemented with 10 % FCS and seeded at clonal density (200 cells per 60-mm dish). These cells were then maintained in culture in the incubator. Cell colonies that formed in every dish were photographed and counted at d 5 and d 10 under phase contrast microscope at 100 × magnification. The percentage of cells that initiated a clone was presented as cloning efficiency, which indicated the clonogenecity of cancer cells. Triplicate samples were run for the experiments.

2.6 Cell invasion assay on a Matrigel-coated membrane in a transwell

Cell invasion potential was measured using a transwell migration apparatus (Becton Dickinson, Heidelberg, Germany) according to the manufacturer's instructions. Before invasion assay, the BD Falcon cell culture inserts with 8 μm-pores in their PET (Polyethylene terephthalate) track-etched membranes were coated by Matrigel (Sigma-Aldrich, Saint Louis, USA, 50 μg/well, details see below) and placed in 24-well culture plates. Cells were harvested, washed once, and resuspended in DMEM medium. To start the assay, 1×10^4 of SP or non-SP cells were individually suspended in 300 μl F12 medium containing 10 % FCS and seeded into the upper chamber of the inserts. Additional 700 μl of the same medium was loaded into the lower chamber. The transwell apparatus was incubated at 37°C for 24h, 48h and 72h, respectively. At the end of the incubation, the cells on the top surface of the filters were wiped off with cotton swabs and those on the lower surface of the insert were fixed and stained with Haematoxylin (DAKO, Carpinteria, USA). The number of infiltrating cells was determined by counting in six random visual fields under a light microscope for each membrane. The results were expressed as cells/field, and the values represented the mean ± SD of three independent experiments performed in triplicate.

Matrigel Coating Method:

The Matrigel was diluted according to the manufacturer's instructions. In brief, it was thawed overnight at 4°C before use, and then diluted 1:10 (final concentration, 0.8~1.0 mg/ml) with cold (4°C) DMEM. The applicable number of BD Falcon cell culture inserts (PET membrane, 8 μm pore size, translucent, effective growth area of membrane 0.3 cm^2, Cat No. 353097) were placed aseptically into the wells of a 24- well cell culture plate. By using a sterile pipet syringe, 50 μl of Matrigel solution was added to each of the inserts. Then the cell culture plate was shaken gently

until the Matrigel solution evenly coated the inserts and air-dried overnight in a laminar flow hood. Finally, the cells were seeded in cell culture inserts with appropriate cell density.

2.7 HTh74 cells cultured in the presence of EGF and bFGF

The existence of a cancer stem cell phenotype in brain and breast tumors has been suggested by different groups that utilized *in vitro* cultivation of tumor cells in suspension with EGF and bFGF as neurospheres and mammospheres [31,33,89]. To investigate whether thyroid cancer SP cells could proliferate in the same culture condition, HTh74 cells were cultured in serum-free DMEM/ Ham's F-12 (1:1) medium containing B-27 (1:50, Invitrogen, Karlsruhe, Germany) and EGF (20 ng/ml, Invitrogen, Karlsruhe, Germany), bFGF (20 ng/ml, Invitrogen, Karlsruhe, Germany) or both. Every 2-3 d, B27, bFGF and EGF were added. After 14 d, the cells were photographed and the growth patterns were compared. When cultured in serum-free medium with both EGF and bFGF, some cells formed intact floating spheres. Then the cells were stained with Hoechst 33342 and re-sorted by FACS to detect the SP percentage in each culture condition.

After FACS, the SP fraction of HTh74 cells was cultured in the serum-free medium supplemented with EGF (20 ng/ml) and bFGF (20 ng/ml) to detect whether the cells could reform sphere or not.

2.8 BrdU incorporation assay using immunofluorescent staining

To test the proliferative potential of HTh74 SP cells which were grown as spheres in serum-free medium with EGF and bFGF, BrdU incorporation was performed with 5-bromo-2´-deoxy-uridine Labeling and Detection Kit I (Roche, Mannheim, Germany).

Briefly, the thymidine analog BrdU labeling medium (final concentration: 10 μm) was added to the cell culture medium of d 10 spheres. After incubation for 12 h at 37 ºC, the SP cell derived-spheres were washed and spun down (500 ×g, 10 min) onto a clean glass slide with a centrifuge. Then spheres were fixed with ethanol fixative (ethanol 70 % and 50 mM Glycine 30 %) for 20 min at room temperature and washed once with washing buffer. The peripheral zone of the stained area was carefully dried and the spheres were covered with a sufficient amount of anti-BrdU working solution (1:10 diluted from the supplied stock), and incubated for 30 min at

37°C in a humid atmosphere. Then glass slides were washed 3 times and the peripheral zone of the area to be stained was carefully dried. This step was followed by incubation with a sufficient amount of anti-mouse-FITC working solution (1:10 diluted from the supplied stock) for 30 min at 37°C in a humid atmosphere in the dark. Then the slides were washed 3 times again and mounted in Vectashield (Vector) after being air-dried. For evaluation by fluorescence microscopy, an excitation wavelength in the range of 450–500 nm (e.g., 488 nm) and detection in the range of 515–565 nm were used.

Ethanol fixative:

100 % ethanol 140 ml
Glycine 0.75 g
Distilled water 60 ml
PH was adjusted to 2.0, stored at 4 º C.

2.9 Experimental equipment

2.9.1 Apparatus

FACS Calibur equipment	Becton-Dickinson Biosciences
Laminar flow cabinet	Heraeus, Laminair HB 2448
Cell culture incubator	Heraeus
Phase contrast microscope	Nikon, TMS
Biological microscope	JNOEC, XS-402
Fluorescence microscope	JNOEC
Digital camera	Sony DSC-W7
Water bath	Kotterman Labortechnik
UV Illuminator	Bachofer Laboratoriumsgeräte
UV camera	Polaroid MP4 Land Camera with Polaroid 545 4X5 film holder
Scanner	CanoScan 5000
Thermocycler	Biometra, Trio-Thermoblock
Spectrophotometer	Pharmacia, Ultrospec II
Autoclave	H+P Varioklav

Shake incubator	Infors HT
Precision scale	Advenurer OHAUS
Voltage generators	Consort, Electrophoresis power supply, E455
Homogenisator	Eppendorf Thermomixer 5436
Centrifuges	Hettich Mikro 200R
	Beckmann Microfuge E
	Hettich Rotina 46R
Heating oven	Memmert
Pipets and tips	Eppendorf
Plastic centrifuge tubes (1.5, 15 and 50 ml)	Sarstedt
Falcon tubes	Becton Dickinson
Plastic culture dishes and plates	Sarstedt
Polysterene slide flasks	Nunc (Wiesbaden, Germany)

2.9.2 Software

Biology Workbench version 3.2	DNA and mRNA sequence analysis
Cellquest	Flow cytometry analysis
WinMDI version 2.8	FACS analysis
Adobe photoshop version 7.0	Figure preparation
Image J version 1.34s	Gel Densitometry analysis
	(freely available at http://rsb.info.nih.gov/ij/)

3 Results

3.1 ABCG2 expression and existence of side population cells in human anaplastic thyroid cancer cell lines

3.1.1 Expression of ABCG2 transporter gene in human anaplastic thyroid cancer cell lines

Expression of ABCG2 mRNA could be detected in human anaplastic thyroid cancer cell lines including C643, HTh74 and SW1736 by RT-PCR with human specific primers (Fig. 3.1). The amplified sequences displayed the expected size of 379 bp in all cases.

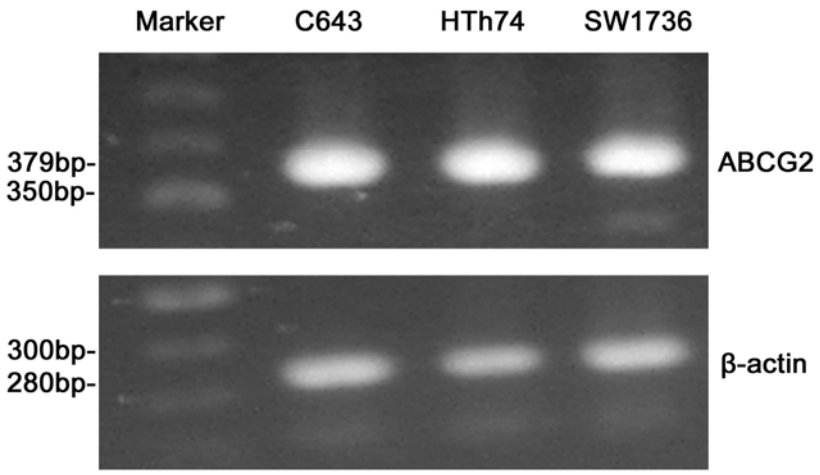

Figure 3.1 Expression of ABCG2 transporter gene in different human anaplastic thyroid cancer cell lines. ABCG2 mRNA was detectable in all of these three different cell lines. β-actin was also detected as a control in all samples.

3.1.2 Existence of side population cells in human anaplastic thyroid cancer cell lines

To determine whether established human anaplastic thyroid cancer cell lines contain side populations, three different cell lines were dissociated from culture dishes by trypsin, incubated with Hoechst 33342 for 120 min and detected by FACS. Representative density dot-plots of FACS profile are shown in Fig. 3.2-3.4, in which cells with an efflux of Hoechst were separated as a “side population” from the large majority of cells that accumulate the dye. The percentage of SP cells in C643, HTh74 and SW1736 was 0.52 %, 0.83 % and 0.41 %, respectively. In each

case, addition of verapamil, which is an inhibitor of Hoechst 33342 dye transporter, completely abolished the SP profile, illustrating the specificity of the staining. Hence, these anaplastic thyroid cancer cell lines do contain a small fraction of side population, despite having been maintained in culture for many years. Further experiments were focused on HTh74 cells.

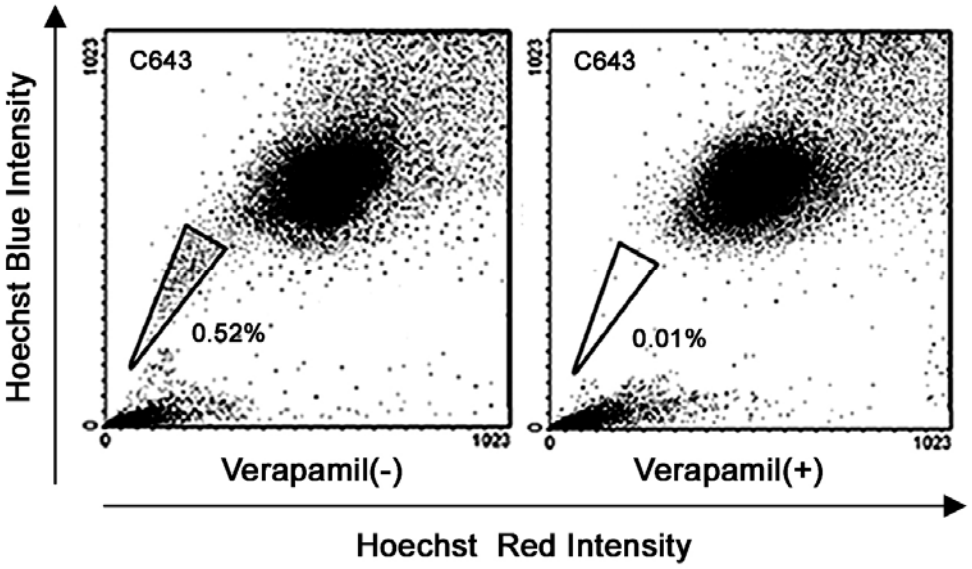

Figure 3.2 Identification of side population (SP) cells in C643 cell line. Left panel: Cells with an efflux of Hoechst 33342 were separated as a SP fraction. The SP-gated cells accounted for 0.52 % of the total cells analyzed. Right panel: Incubation with 50 µM verapamil, which inhibits the efflux, almost completely abolished the SP profile, indicating the specificity of the staining.

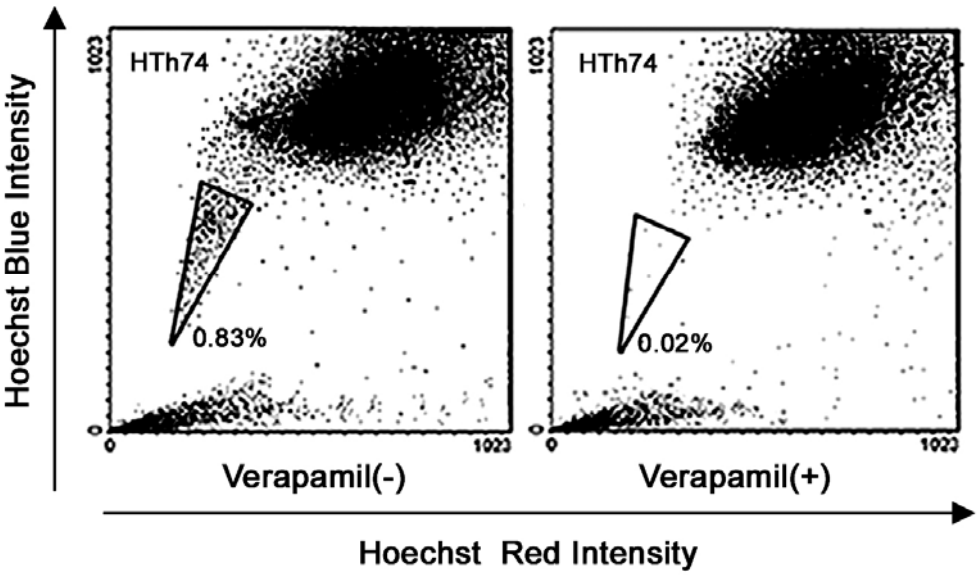

Figure 3.3 Identification of SP cells in HTh74 cell line. Left panel: Cells with an efflux of Hoechst 33342 were separated as a SP fraction (0.83 %). Right panel: Incubation with 50 µM verapamil almost completely abolished the SP profile.

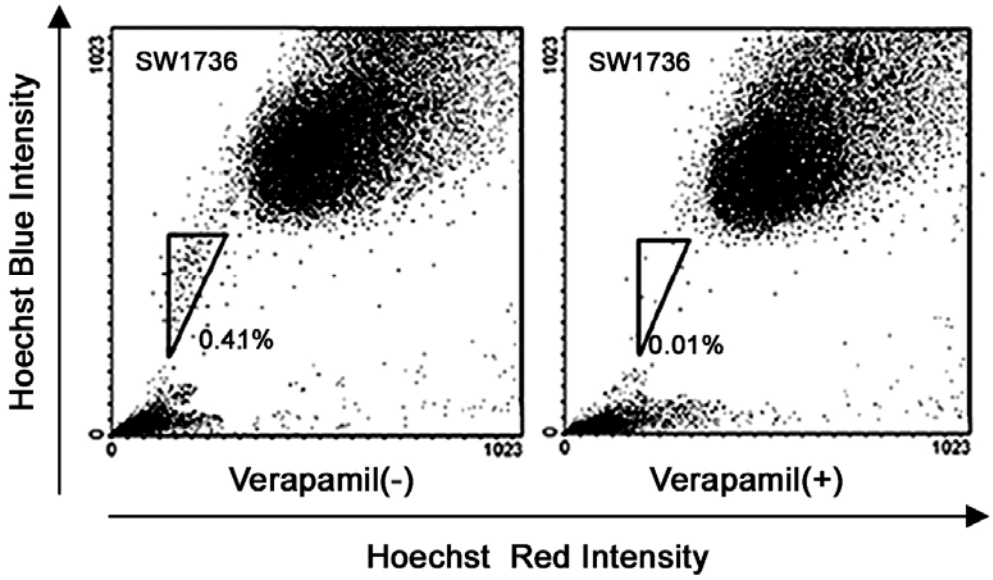

Figure 3.4 Identification of SP cells in SW1736 cell line. Left panel: Cells with an efflux of Hoechst 33342 were separated as a SP fraction (0.41 %). Right panel: Incubation with 50 μM verapamil almost completely abolished the SP profile.

3.2 ABCG2 staining co-localizes with Hoechst-low cells in HTh74 cell culture

The presence of side population in HTh74 cell cultures, as shown above, led us to examine the co-localization of ABCG2 immunoreactivity with Hoechst low-staining cells in HTh74 cell culture. If, in fact, ABCG2 confers on some cells the ability to exclude Hoechst dye, one would predict that cells immunoreactive for ABCG2 would necessarily be Hoechst low-staining. This result is shown in Fig. 3.5. Cells that were Hoechst low-staining (A), were ABCG2-positive (B), as seen definitively in the merged image (C). Brightfield image was captured (D) to ensure that the labeled cells appeared healthy and intact. It was important to determine cell morphology, as less intact cells occasionally took up the TRITC-conjugated secondary antibody in a non-specific manner. Co-localization of Hoechst low-staining and ABCG2 immunoreactivity in HTh74 cells confirmed the presence of SP cells.

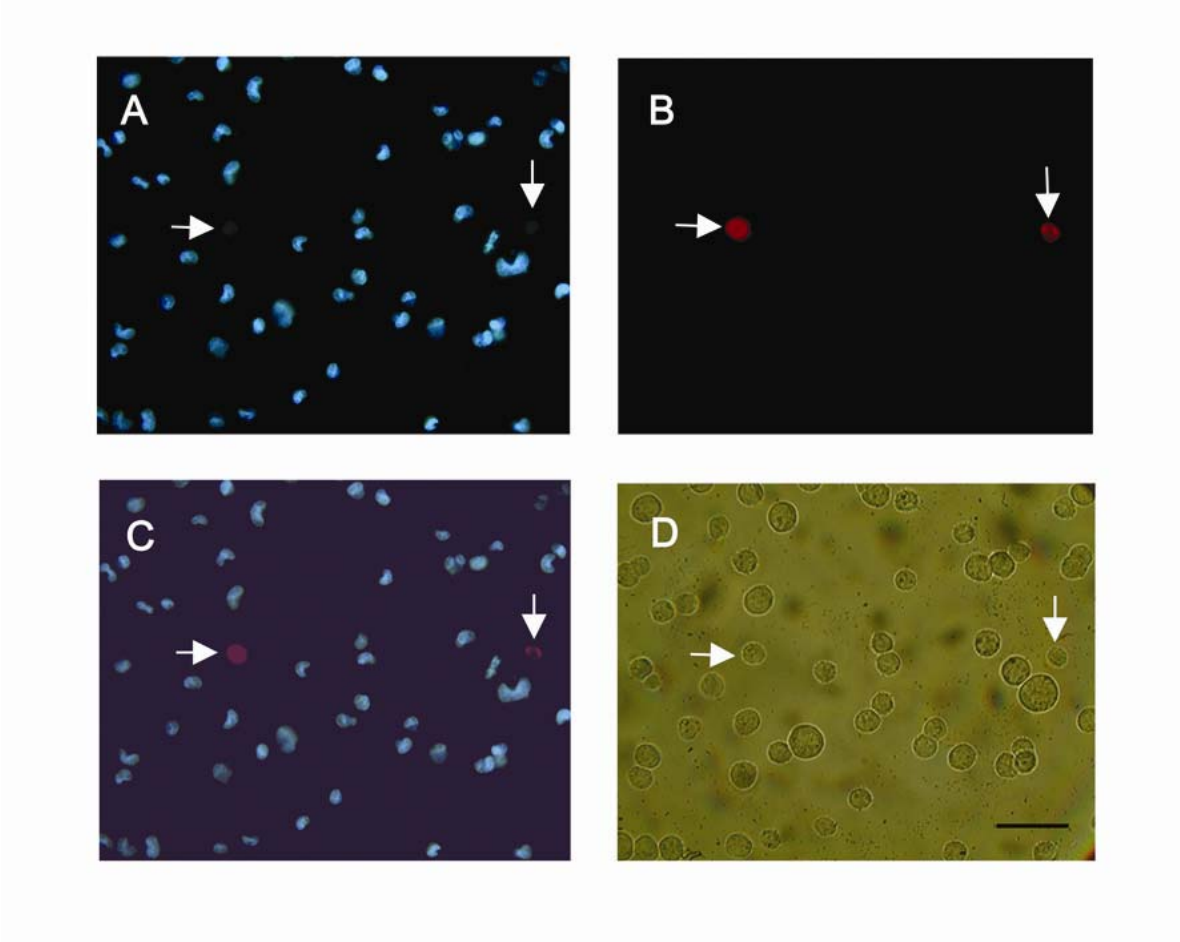

Figure 3.5 Co-localization of ABCG2 positive with Hoechst low-staining cells in HTh74 cells. All four panels (A-D) illustrate the same field. Hoechst low-staining cells (A; arrows) show ABCG2 immunoreactivity (B; arrows). Hoechst low-staining cells co-localize with ABCG2- immunoreactive cells in a merged image (C; arrows). (D) Brightfield images of these cells. Scale bar = 20 μm.

3.3 Identification of ABC transporter genes and stem cell marker in HTh74 side population cells

For characterization of SP cells in HTh74, gene profiles of SP and non-SP cells were analyzed by semi-quantitative RT-PCR. It has already been proven that the side population phenotype was correlated with ABC transporters, in particular ABCG2, and the SP cells isolated from various normal and cancer tissues express high levels of ABCG2 [52,55,57,59,61,63,66,67]. We, therefore, examined the gene expression of ABCG2 in SP and non-SP cells. Meanwhile, multi-drug resistance gene 1 (MDR1), which encodes another ABC transporter and plays a critical role in multiple drug resistance during cancer treatment, was also detected in HTh74 SP

and non-SP cells. As depicted in Fig. 3.6, SP cell populations displayed much higher levels of ABCG2 and MDR1 mRNA expression in contrast to non-SP cells. Furthermore, Oct4, a major transcription factor for embryonic and some adult stem cells was also strongly expressed in SP cells whereas it was almost absent in non-SP cells. As a control, β-actin mRNA levels were equal in all samples.

These results demonstrate that the thyroid cancer SP cells possess high levels of ABC transporter genes and stem cell marker Oct4, which is similar to normal stem cells [41,55,63,66,68].

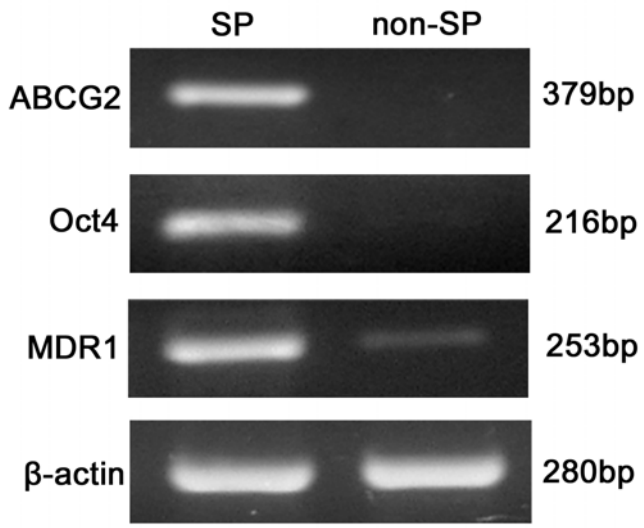

Figure 3.6 Identification of ABC transporter genes and stem cell marker Oct4 in HTh74 side population cells. SP cell fractions showed distinctly higher levels of ABC transporter genes— ABCG2 and MDR1, in contrast to non-SP cells. Moreover, Oct4, a major transcription factor for embryonic and some adult stem cells was also strongly expressed in SP cells.

3.4 HTh74 SP cells display a capacity for self-renewal and can repopulate both SP and non-SP cells

To examine whether cancer SP and non-SP cells have the capacity to self-renew and give rise to diverse progenies, we maintained them individually in culture for 2 weeks, then stained them with Hoechst 33342, and resorted them into SP and non-SP fractions. We found that the cultures initiated with SP cells expanded and contained both SP and non-SP cells (Fig. 3.7 A, C), whereas no SP cells were detected in the cell cultures initiated from non-SP cells (Fig. 3.7 B, D). The results revealed a side population of 0.95 % in the second sorting, which was similar to the

percentage of SP cells in the first-sorted HTh74 cells (0.83 %, Fig. 3.3).

In addition, in order to investigate whether the SP cells could be sustained via serial sorting, the SP cells were cultured and resorted every 2 weeks. As shown in Fig. 3.8, serial sorting and reanalysis showed the SP cells represented 0.83 % for the first sort, 0.95 % for the second sort, 0.78 % for the third sort and 1.02 % for the fourth sort, which demonstrated the constant maintenance of SP fractions over time.

In summary, these observations in HTh74 cells suggest that a tumour hierarchy exists in which only SP cells could divide asymmetrically, display a capacity of self-renewal and give rise to both SP and non-SP cells when recovered and serially sorted in culture.

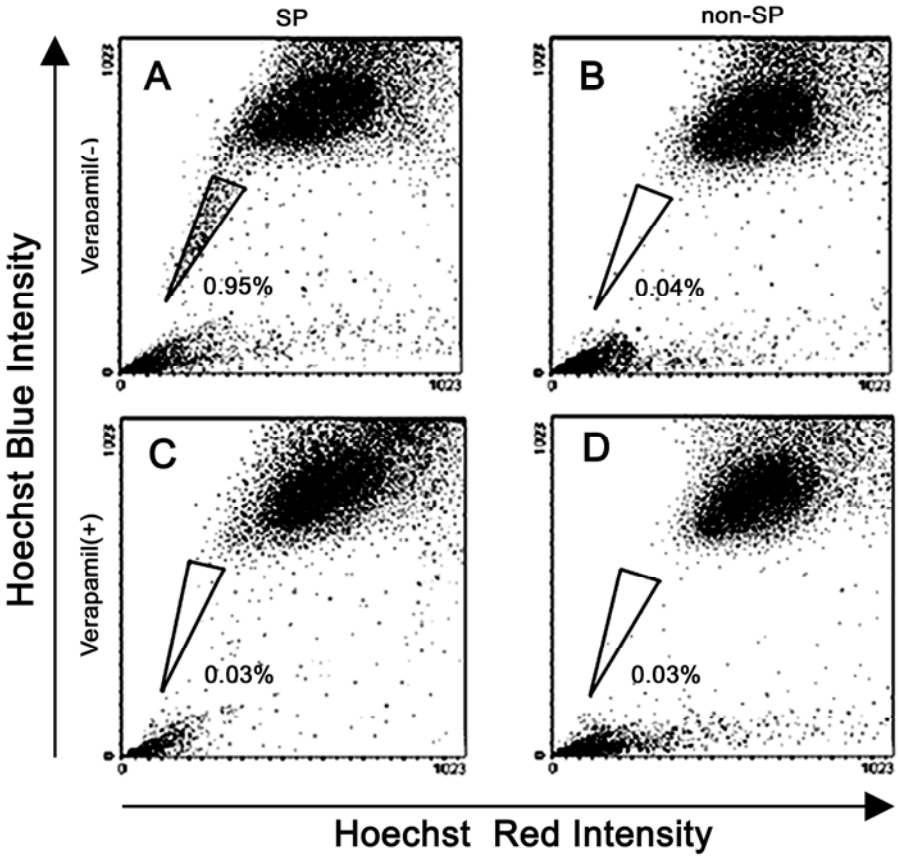

Figure 3.7 HTh74 SP cells display a capacity for self-renewal and can repopulate both SP and non-SP cells. FACS sorted SP and non-SP fraction of cells were maintained in culture individually for 2 weeks and then reanalyzed by FACS. The SP cells expanded in culture and repopulated both SP and non-SP cells (A, C), whereas no SP cells were found in the fraction initiated from non-SP cells (B, D).

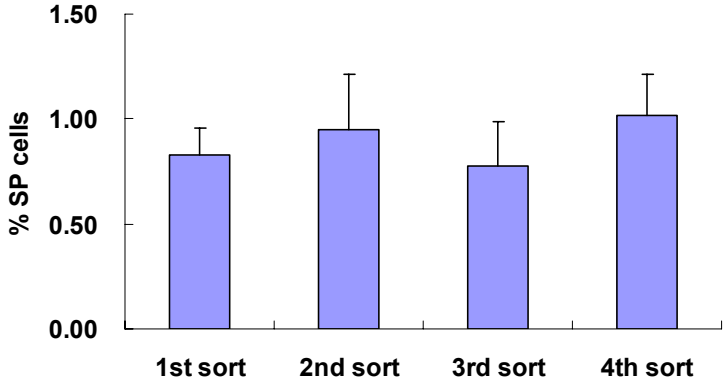

Figure 3.8 HTh74 SP cells were cultured and resorted each 2 weeks. Serial sorting and reanalysis showed the constant maintenance of SP fraction over time.

3.5 Side populations of HTh74 cells are more clonogenic than non-SP cells

To further investigate whether the side population cells might be associated with some of the intrinsic stem cell properties, we used the purified side population and non-side population HTh74 cells in a clonal formation assay, which partially measures the self-renewal capacity of the cells. As shown in Fig. 3.9, whereas 16.5 %-22.5 % of the HTh74 SP cells could sustain a clonal growth and form characteristic compact circular colonies with a cobblestone appearance (Fig. 3.10A), the majority of the non-SP cells were not clonogenic. They scattered and failed to proliferate as clones (Fig. 3.10B). As a control, 3.17 %-3.83 % of total HTh74 cells could grow as colonies.

These differences are not likely to be a consequence of longer retention of potentially toxic Hoechst dye by non-SP because the viability of all cells was identical after sorting (70 %-85 %) and throughout the whole study, confirmed by trypan blue staining (as shown in Fig. 3.11). This finding indicates that side population of cancer cells has some intrinsic properties of stem cells similar to observations in various normal stem cell populations [63,90-92].

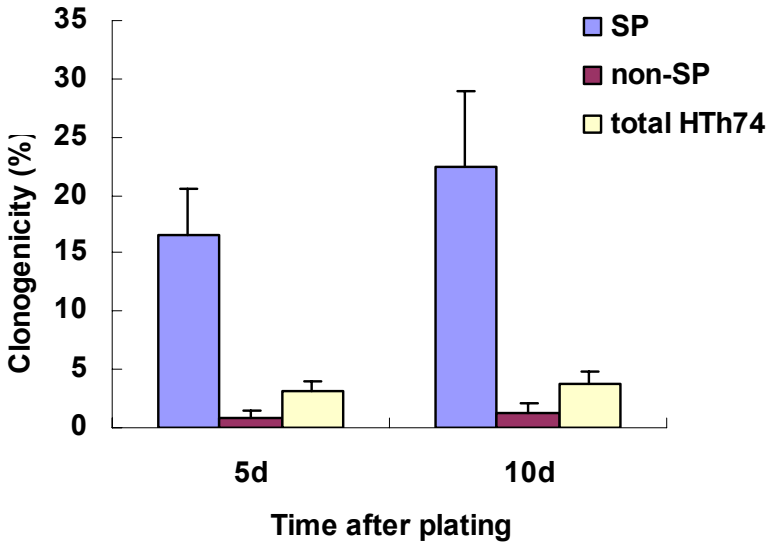

Figure 3.9 The cloning efficiency of SP, non-SP and total HTh74 cells. Each bar indicates the mean and standard deviation of six dishes. The data are representative of three independent experiments.

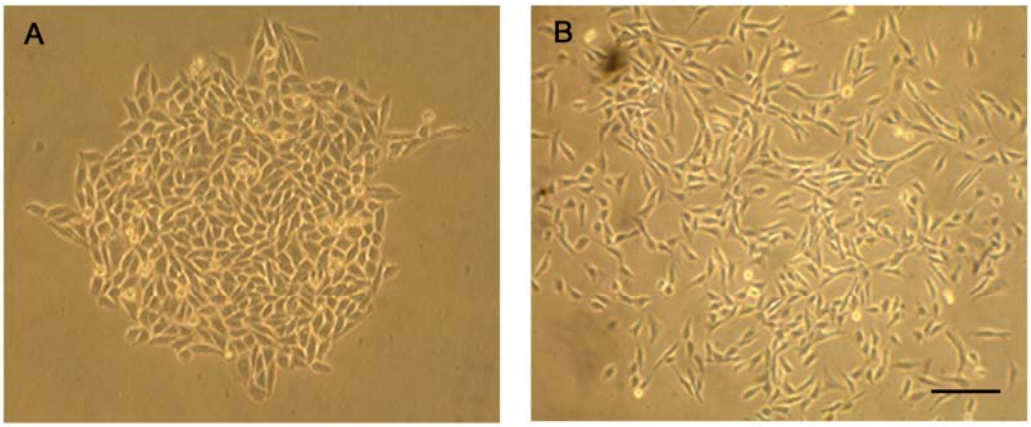

Figure 3.10 Side populations of HTh74 cells are more clonogenic than the non-SP cells. (A) HTh74 SP cells could sustain a clonal growth, and formed characteristic compact circular colonies. (B) The non-SP cells scattered and failed to proliferate as clones. Scale bar = 20 μm.

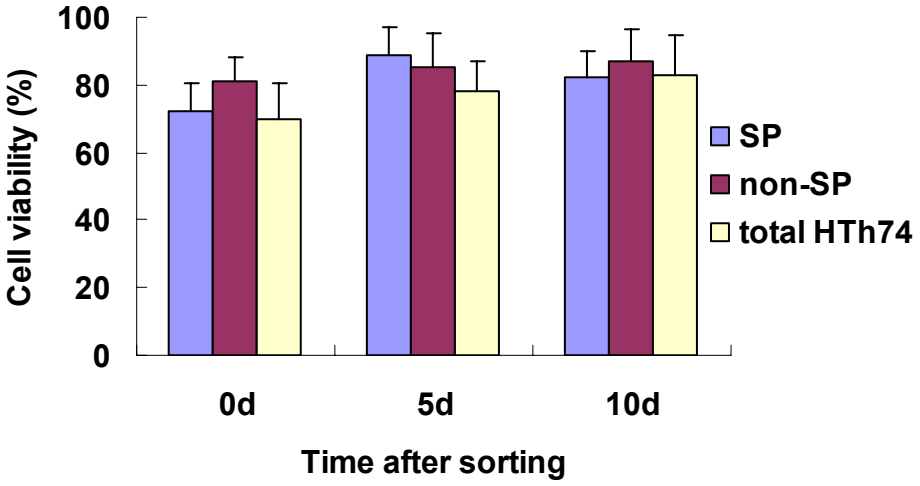

Figure 3.11 The viability of SP, non-SP and total HTh74 cells was identical at d 0, d 5 and d 10 after sorting, respectively.

3.6 HTh74 SP cells show high invasive potential and preferential expression of metastasis-associated genes

Recently, it has been proposed that cancer stem cells may not only initiate the primary tumour formation, but also contribute to cancer invasion and metastasis. The attachment to and penetration of basement membranes by tumour cells is required to complete the metastatic cascade which culminates in the establishment of secondary tumour foci. Therefore, basement membranes are critical barriers to the passage of disseminating tumour cells. Hence, in order to evaluate the invasive potential of HTh74 SP and non-SP cells, an in vitro Matrigel invasion assay was used on reconstituted basement membranes in transwell chambers. The numbers of cells that digested Matrigel and migrated through the pores were counted after 24 h, 48 h and 72 h of incubation. Representative fields with cells that migrated under the membrane were photographed at 24 h (Fig. 3.12 A, B). As depicted in Fig. 3.13, the migrated cells of both SP and non-SP increased in a time-dependent pattern, however, the SP cells exhibited greater invasive abilities than non-SP cells at any time point as assessed by the number of cells that have migrated through the Matrigel-coated filter. These findings suggest that cancer SP cells have higher invasive potential in comparison with non-SP cells, which should be particularly paid attention to in cancer treatment.

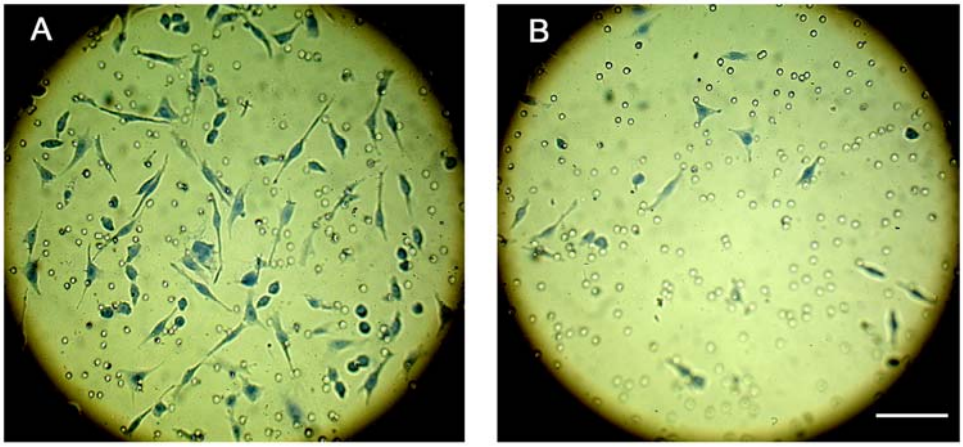

Figure 3.12 Representative fields of SP (A) and non-SP (B) cells that migrated through the Matrigel-coated filter (stained with hematoxylin) were observed under microscope and photographed at 24 h. Scale bar = 20 μm.

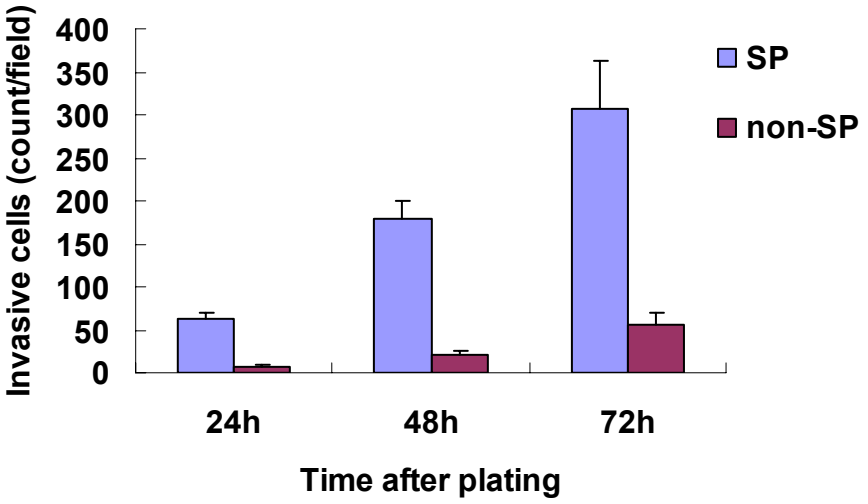

Figure 3.13 The migrated cells of both SP and non-SP increased in a time-dependent manner, and SP cells exhibited higher invasive potential than non-SP cells. Each bar indicates the mean and standard deviation of six transwells.

In recent years it has become increasingly clear that epithelial-mesenchymal transition (EMT), already established as a critical developmental process, plays a major role in the progression of cancer invasion and metastasis. This transition is characterized by the combined loss of epithelial cell junction proteins such as E-cadherin and the gain of mesenchymal markers. The transition to a mesenchymal phenotype, mediated via transcriptional reprogramming by factors such as Snail

[93,94] and Slug, increases the migratory potential of cancer cells and leads to metastasis.
In order to investigate whether the high invasive potential of cancer SP cells is associated with EMT activation, using semi-quantitative RT-PCR, some EMT associated genes were detected. As shown in Fig. 3.14, HTh74 SP cells showed decreased mRNA expression of E-cadherin**,** which is a vital cell junction protein and an important inhibitor for cell motility. Meanwhile, these SP cells exhibited much higher transcriptional levels of β-catenin, Snail and Slug (Fig. 3.14), which inhibit E-cadherin gene transcription. In addition, the cancer SP cells displayed increased gene expression of isolated γ2 chain of laminin (LAMC2) and L1 cell adhesion molecule (L1CAM), both of which are strong inducers for epithelia migration. These findings suggest that thyroid cancer SP cells may possess activated EMT which leads to high invasive competence.

It is well-known that multiple proteinases, particularly the matrix metalloproteinases (MMPs) and members of the urokinase plasminogen activator system, are involved in extracellular matrix (ECM) degradation for tumour invasion and metastasis. In the present study, we found cancer SP cells exhibiting elevated gene expression of matrix metalloproteinases 2 (MMP-2), urokinase-type plasminogen activator (uPA) and uPA receptor (uPAR) (see Fig. 3.14). This may also be a possible explanation for the more invasiveness of SP cells.

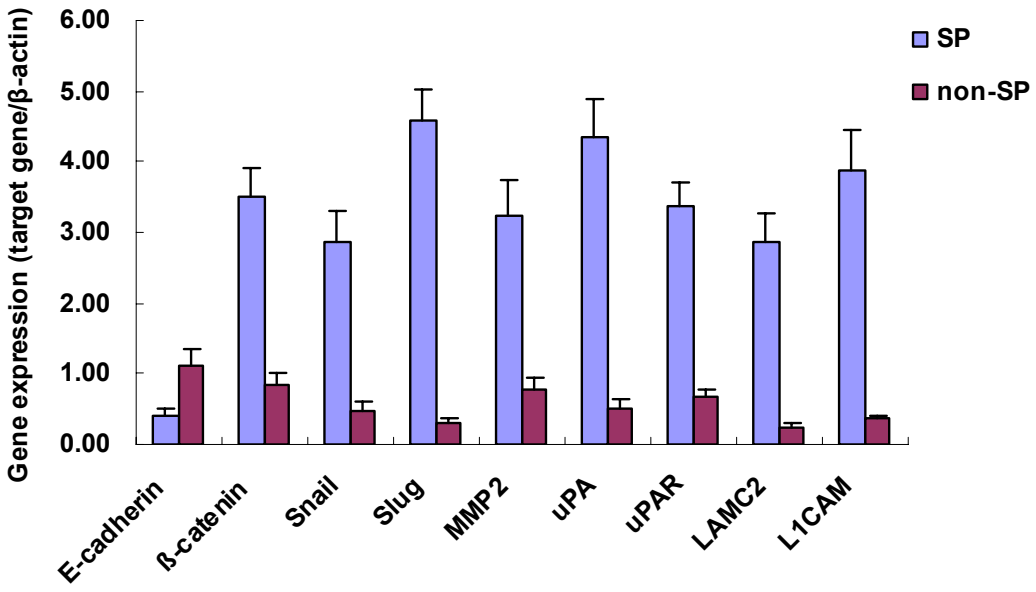

Figure 3.14 HTh74 SP cells show preferential expression of some EMT markers and tumour

metastasis-associated proteinase genes in contrast to non-SP cells. Semi-quantitative RT-PCR analysis showed that in HTh74 SP cells, the gene transcription of E-cadherin was down-regulated, whereas EMT marker β-catenin, Snail, Slug, LAMC2, L1CAM, and metastasis-associated proteinase gene MMP2, uPA, uPAR were preferentially expressed. Image J software was used for gene band intensity analysis. The data are presented as fold increase of each target gene expression *vs.* β-actin and the values represent the mean ± SD of three independent experiments.

3.7 HTh74 SP cells can be expanded in EGF plus bFGF

To further examine the cellular characteristics of HTh74 SP cells, we investigate in which culture condition SP cells can be enriched. The existence of a cancer stem cell phenotype in brain and breast tumours has been suggested by different groups that utilized *in vitro* cultivation of tumour cells in suspension with EGF and bFGF as neurospheres and mammospheres [31,33,89]. We also investigated if EGF and bFGF were suitable candidates for the proliferation of HTh74 SP cells. HTh74 cells were plated in DMEM/F12 (1:1) medium with 10 % FCS, or serum-free DMEM/F12 medium containing EGF (20 ng/ml), bFGF (20 ng/ml) or both, as indicated in Materials and Methods. Interestingly, as shown in Fig. 3.15, we found the morphology of the cells was completely different in these 4 culture conditions. In FCS, after incubation for 24 h, all the cells attached well, had a flat form and proliferated (Fig. 3.15 A). In bFGF, cells half-attached and could propagate (Fig. 3.15 B). However, in the presence of EGF, cells proliferated slowly in a suspension state and developed small cell aggregates (Fig. 3.15 C). When cultured in serum-free medium with both EGF and bFGF, the cells formed intact floating spheres after 10-14 d (Fig. 3.15 D).

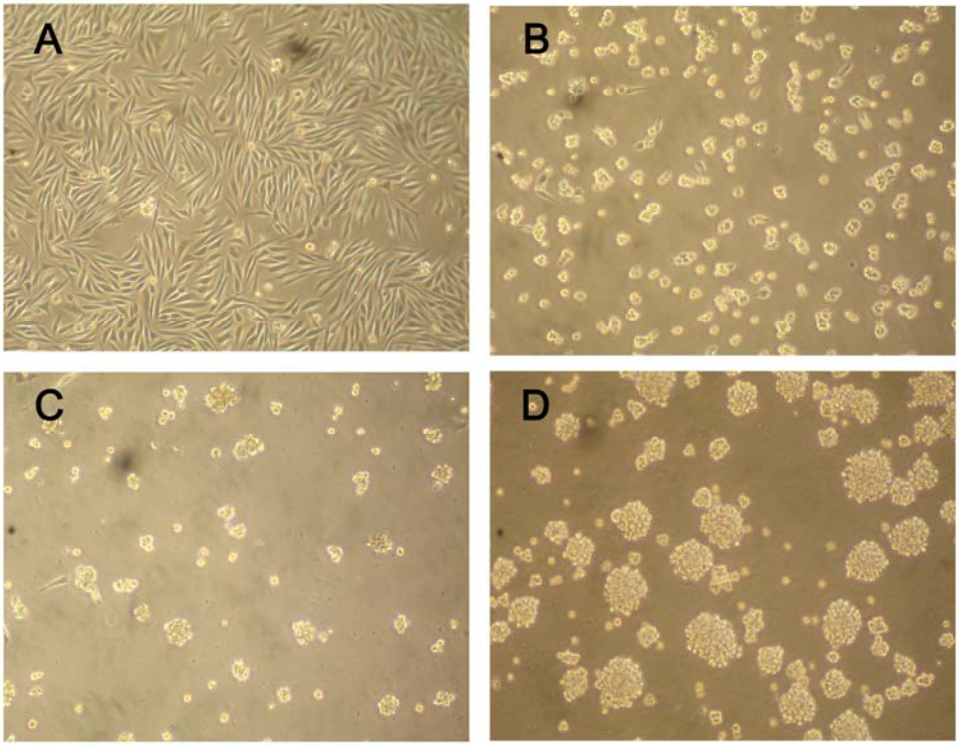

Figure 3.15 HTh74 cells display different morphologies in 4 culture conditions. HTh74 cells were grown in various culture conditions for 2 weeks and then photographed under an inverted phase-contrast microscope.(A) in 10 % FCS, (B) in serum-free medium containing bFGF, (C) in serum-free medium containing EGF, (D) in serum-free medium containing bFGF plus EGF.

To further examine whether cancer SP cells can be enriched in bFGF plus EGF, HTh74 cells were harvested after being cultured in the different conditions for 2 weeks. An equal number of cells (10^6/sample) was stained with Hoechst 33342 and analyzed by FACS. When cultured with bFGF or EGF alone, the percentage of SP was only slightly elevated in comparison with those cultured in FCS (as shown in Fig.3.16). However, when cultured in serum-free medium with EGF plus bFGF, the proportion of SP cells was greatly increased (see Fig. 3.16). The viability of the cells cultured in different conditions was identical, which could confirm the SP sorting results (see Fig. 3.17).

Based on the results above, after FACS we cultured SP fraction of HTh74 cells in the serum-free medium supplemented with EGF and bFGF for expansion. As expected, they also grew as spheres after 10-14 d. BrdU incorporation displayed that most cells in d 10 spheres were labeled with BrdU (see Fig. 3.18), indicating that spheres developed and grew in size by cell division.

Taken together, these results indicate that EGF combined with bFGF may promote the proliferation of HTh74 SP cells.

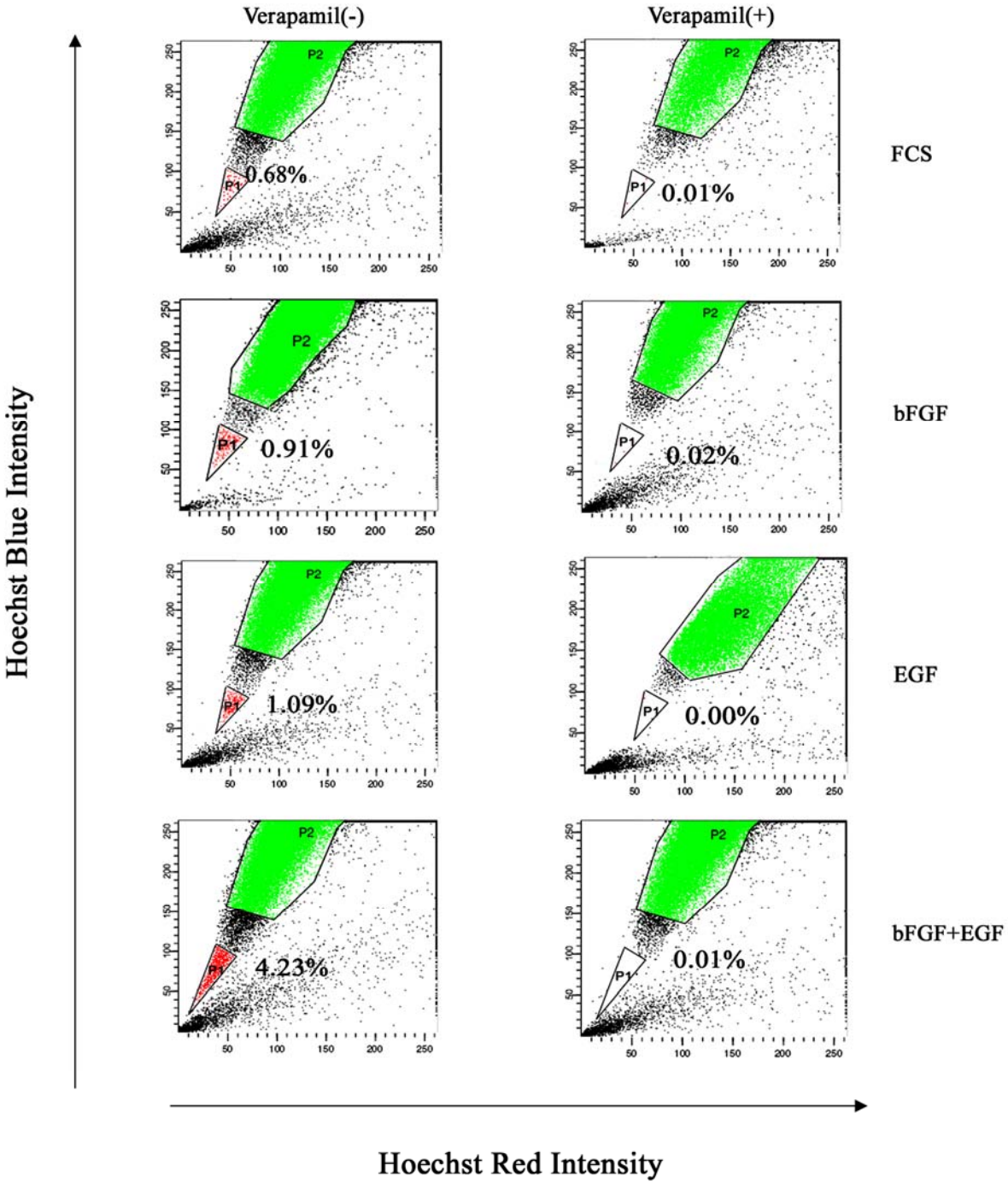

Figure 3.16 HTh74 SP can be enriched in EGF plus bFGF. HTh74 cells were cultured in FCS, bFGF, EGF, or bFGF plus EGF for 2 weeks, and then analyzed for SP fraction by FACS. Only when cultured in bFGF plus EGF, the SP percentage of HTh74 cells was greatly increased.

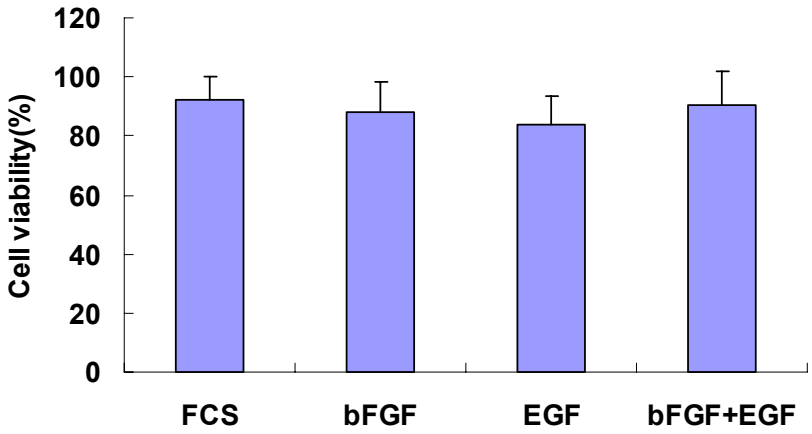

Figure 3.17 The cell viability of HTh74 cells cultured in different conditions was identical before FACS analysis.

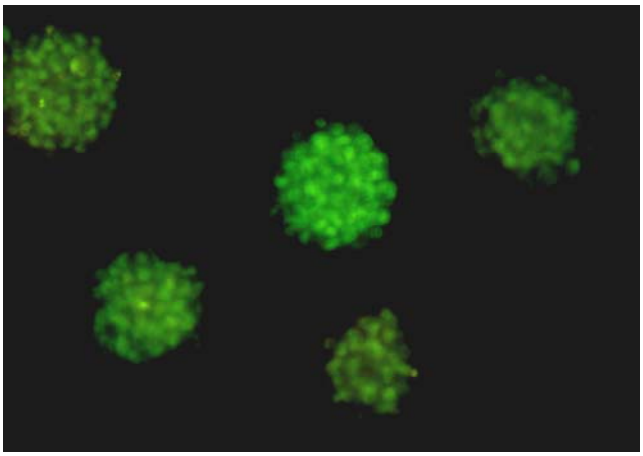

Figure 3.18 BrdU incorporation of d 10 spheres derived from sorted SP cells cultured in the presence of bFGF and EGF showed that most cells in d 10 spheres were labeled with BrdU, indicating that spheres developed and grew in size by cell division.

4 Discussion

An important goal of cancer research is the complete understanding of the mechanisms of cancer initiation and progression for the development of more effective treatments. To succeed in this endeavor a successful bridging between the cellular biology and the molecular biology of cancer must take place. Recently the identification of cancer stem cells (CSCs) in leukemia, as well as in brain and breast cancer has renewed the interest in the hypothesis that cancer may arise from adult stem/progenitor cells. Based on this hypothesis, the failure to eradicate most cancers may be as fundamental as a misidentification of the target. Our current therapies succeed at eliminating bulky disease and rapidly proliferating cells but often miss cancer stem cells that may be the source of disease recurrence and metastasis. Recent advances in the understanding of normal tissue development and repair provide a basis for revisiting the process of oncogenesis, tumour heterogeneity, and drug resistance.

So far two general approaches can be applied to identify CSCs. Some of CSCs were identified using specific markers that identify putative normal stem cells. For example, in the case of breast cancers, a cell surface marker profile $CD44^{+}CD24^{-/Low}Lin^{-}$ was reported [30], and it has been shown that the cancer stem cells isolated from central nervous system tumours express cell surface antigen CD133 [35], which is known as a marker of hematopoietic stem cells. However, distinct regulated molecules characterizing CSCs are largely unknown. In some cases, especially in the absence of specific surface marker expression, cancer stem cells were identified as a Hoechst low/negative side population by FACS due to their properties to express ABCG2 transporter and capacities to efflux Hoechst 33342 dye. Recently our group has demonstrated that adult stem cells also occur in human thyroid glands [95]**.** Due to their undifferentiated state and indefinite proliferative potential they have been hypothesized to be involved in the thyroid carcinogenesis [96,97]. The primary aim of this work was to identify and characterize stem-like side population cells in human anaplastic thyroid cancer cell lines.

The isolation of SP is based on the technique first described by Goodell *et al*. [88] in 1996. While using Hoechst 33342 dye staining to study the cell cycle distribution of whole bone marrow cells, these authors discovered that the simultaneous display of Hoechst fluorescence at two emission wavelengths (red 675 nm and blue 45 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

determined that SP is a particular cell population enriched in primitive and undifferentiated cells [98,99] and the SP phenotype is closely correlated to ABC transporters, in particular ABCG2. At present, SP cells which possess typical stemness properties have been identified in many normal tissues, such as mammary glands [100-102], lung [47,52], muscle [61], heart [61], liver [55,103], brain [58,63], and skin [98,104] in both human and animal models.

In addition to the application of side population in normal cells, they have also been identified in various tumours and cancer cell lines. It has been demonstrated that tumours are composed of heterogeneous cells and that only a small cell population of each tumour bears the capacity to reconstitute a tumour when injected in mice and thus functions as “cancer stem cells”. SP cell isolation from tumours has proven to be an attractive approach to investigate cancer stem cells. Up to now, cancer stem-like SP cells have been identified in diverse malignancies, including retinoblastoma [105], bone marrow from patients with acute myeloid leukemia (AML) [67], melanoma [106] and neuroblastoma [67].

As primary tumours, cancer cell lines also represent a heterogeneous population. It has been demonstrated that the injection of approximately one million human breast cancer cell MCF-7 in SCID mice is required to give rise to a tumour, but 100,000 cells with a specific phenotype, i.e. $CD44^{+}/CD24^{-}$ [107], are sufficient to induce tumour formation. Moreover, when multiple human cancer cell lines, which have been in culture under different conditions for years or even decades, are assessed for their clonal growth and clonogenic potentials, only a small percentage of cells possesses such potentials [70]. These observations led to the speculation that cell lines are composed of cells that are heterogeneous in terms of their tumorigenicity. By the Hoechst exclusion assay, SP and non-SP populations have been identified in a variety of cancer cell lines, such as C6 rat glioma cell line [69], SK-N-SH, IMR-32, and JF human neuroblastoma cell lines [67], human retinoblastoma WERI-Rb27 [105], and various human gastrointestinal cancer cell lines [71].

In the present study, three different anaplastic cancer cell lines C643, HTH74 and SW1736 were stained with Hoechst 33342 and then detected for side population by FACS. We found that all these three cancer cell lines contained a small percentage of distinct side population (0.52 %, 0.83 % and 0.41 %, respectively). The percentages of SP were similar to the side population in multiple normal stem cell or progenitor cell populations [i.e., 0.01-5 %; [57,62,108]]. The immunofluorescent staining showed the co-localization of ABCG2 positive with Hoechst

low-staining cells in HTh74 which confirmed the presence of side population. These findings demonstrate that thyroid anaplastic cancer cell lines do contain a small fraction of side population in spite of being maintained in culture for a long time. Cancer cell lines are a useful tool for analyzing molecular cell markers and cellular behavior under controlled experimental conditions because certain parallels exist between cancer cell lines and malignant tumours. Thus, SP derived from thyroid cancer cell lines may be able to serve as an experimental model to study the tumorigenic population in malignant primary thyroid cancers.

Gene profile analysis demonstrated that cancer SP cells in HTh74 display strong expression of some stemness genes in comparison with non-SP cells. They showed preferential expression of stem cell marker Oct4. Oct4 (also known as Oct-3) , which belongs to the POU (Pit-Oct-Unc) transcription factor family [109], is a pivotal transcription factor for embryonic stem cells to maintain them in an undifferentiation state. The POU family of transcription factors can activate the expression of their target genes by binding an octameric sequence motif of an AGTCAAAT consensus sequence [110,111]. The expression of Oct4 was found in ovulated oocytes, mouse pre-implantation embryos, ectoderm of the gastrula (but not in other germ layers) and primordial germ cells, as well as in embryonic stem cells but not in their differentiated daughters [112]. At maturity, Oct4 expression becomes confined exclusively to the developing germ cells [113,114]. More recently, Oct4 has also been shown in many putative adult stem cells such as liver, pancreas, breast, pituitary, skin and kidney [115-117], as well as in thyroid stem cells [95]. It was suggested that Oct4 might be a specific gene marker for totipotency or a gene required for totipotency [111,118,119]. In addition, the Oct4 gene has been shown to be expressed in some human tumour cells (breast, lung, ovary, colon, prostate cancer cells, pancreatic cancer cell line capan-2, liver cancer cell line, breast cancer cell line MCF7, Hela cell line) but not in normal differentiated cells [114,120,121]. In our study, we found the Oct4 expression was mainly restricted to cancer SP cells, whereas it was almost absent in non-SP cells. These findings support the stem cell theory of carcinogenesis. There is some evidence that Oct4 is not “re-expressed” during the carcinogenic process as declared before [120], but the carcinogenic process may prevent the down-regulation of Oct4, which normally starts the terminal differentiation process in the adult stem cell.

In the present study, in contrast to non-SP cells, the cancer SP cells exhibited higher levels of both ABC transporter genes ABCG2 and MDR1. It has been proven that the side population phenotype is mediated by the ABC family of transporter proteins. One of the major mediators is

ABCG2. It has been shown that the ABCG2 gene knockout mice exhibit almost complete loss of the bone marrow side population. Side population cells derived from many different tissues were demonstrated to express high levels of ABCG2. So far ABCG2 expression has been detected in stem cells such as HSCs, nestin-positive islet-derived progenitors, hepatic oval cells, limbal basal cells, and neural stem cell. Hence, ABCG2 can be regarded as a "universal stem cell marker" [41,122]. The cancer stem cells, just like their normal counterparts, express ABC transporter and have the capacity to pump out diverse toxins and drugs. In this way they can be protected from differentiation and toxicity of chemotherapeutic agents. Resistance to chemotherapy was recognized as an impediment to efficacious cancer treatment. Previous studies have revealed that MDR1 and ABCG2, described in the SP context, exhibit specific exclusion of chemotherapeutic agents, which may increase the risk of early tumour relapse. For example, MDR1 expels vinblastine [47] and paclitaxel [123] while ABCG2 expels imatinib mesylate [124], topotecan [125], and methotrexate [126]. Independent of whether SP are truly a tumour ''stem cell'' population, their high expression of drug efflux transporter genes and their associated high capacity to efflux lipophilic drugs may have a significant influence on treatment outcome. Hirschmann-Jac C. *et al.* found a distinct side population in neuroblastoma cells from patients which expressed high levels of ABC transporter genes and had a greater capacity to expel cytotoxic drugs, resulting in high resistance to cancer treatment [67]. On the other hand, Merry and co-workers also found that the intracellular concentrations of Adriamycin, vincristine, and etoposide were significantly increased in 7 human non-small-cell lung cancer cell lines cocultured in the presence of verapamil, which is an effective inhibitor for ABC transporters [127]. This evidence suggest that cancer SP cells, owing to ABC transporter expression, hold the inherently high resistance to anti-neoplastic drugs and can probably escape from the chemotherapy. Therefore these cancer SP cells should be precisely identified and targeted during tumour treatment. In conclusion, knowledge of the central role that ABC transporters play in protecting normal stem cells has allowed us to further define cancer stem cells and add new insights that may prove relevant to explaining treatment failure, late recurrence and metastasis. Such knowledge may also guide us to design rational therapies that take into account similarities and differences between cancer and normal stem cells. Given the central role of ABC transporters in protecting normal and neoplastic cells, the cancer stem cell theory provides a unified explanation for the effectiveness and failures of cytotoxic antineoplastic therapy (detailed in Fig. 4.1).

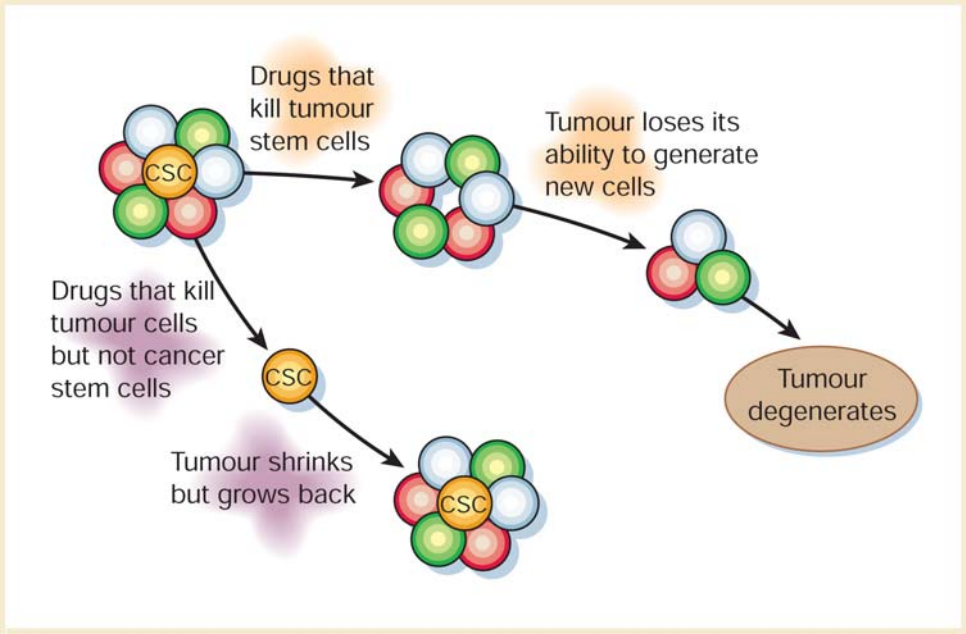

Figure 4.1 Conventional therapies may shrink tumours by killing mainly non-tumorigenic cells with limited proliferative potential. Cancer stem cells are less sensitive to these therapies due to their properties to express ABC membrane transporters, hence they will remain viable after therapy and re-establish the tumour. By contrast, if therapies can be targeted against cancer stem cells, then they might more effectively kill the cancer stem cells, rendering the tumours unable to maintain themselves or grow. [Taken from: Reya T, *et al*, "Stem cells, cancer, and cancer stem cells", 2001, Nature, 414: 105-111.]

Normal tissue homeostasis involves a careful balance between cell loss and cell renewal. In order to maintain this balance, tissues must possess cells capable of self-renewal as well as differentiation. Stem and progenitor cells perform these biologic processes as the functional units of regeneration during both tissue homeostasis and repair. Like normal adult stem cells, CSCs possess stem cell-like properties. They can divide indefinitely, giving rise to both more CSCs and progeny that ultimately differentiate into the different cell types in a tumour. If we view a tumour as an abnormal organ, then the principles of normal stem cell biology [25,128] can be applied to understand better how tumours develop. In fact, many observations suggest analogies between normal stem cells and tumorigenic cells (cancer stem cells). Both normal stem cells and tumorigenic cells have extensive proliferative potential and the ability to give rise to new (normal or abnormal) tissues. Both tumours and normal tissues are composed of heterogeneous combinations of cells, with different phenotypic characteristics and different proliferative potentials [129-132]. Because most tumours have a clonal origin [133,134], tumorigenic cancer cells must give rise to phenotypically diverse progeny, including cancer cells with indefinite

proliferative potential, as well as cancer cells with limited or no proliferative potential. This suggests that tumorigenic cancer cells undergo an aberrant and poorly regulated process of organogenesis that is analogous to the self-renewal and differentiation of normal stem cells and can be thought of as cancer stem cells.

In the present study, we found that the thyroid cancer SP cells can generate both SP and heterologous descendents of non-SP cells in culture, whereas the non-SP cells only produced other non-SP progenies. Moreover, these cancer SP cells can be maintained constantly after serial sorting. These findings indicate that in HTh74 cells, a tumour hierarchy exists in which only SP cells could divide asymmetrically, display a capacity of self-renewal and give rise to both SP and non-SP cells. In fact, there are many different groups which reported that only cancer SP cells could generate both SP and non-SP cells *in vitro*, whereas the non-SP cells can not [67,69-71]. In contrast, some other researchers demonstrated that both SP and non-SP were able to generate SP and non-SP [135]. This discrepancy may be mainly due to the different setting of FACS machine and selection of SP gate. Indeed, the SP tail has been 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 show the highest progenitor activity as compared to the distal portion [62,136,137]. In some reports, side population is identified as a continuous tail of the non-side population. Therefore, the discrimination of the side population from the non-side population is, to a certain extent, arbitrary and can vary significantly from experiment to experiment. In contrast, in our experiment, based on a more stringent staining protocol and specific SP gating setting, we have identified the SP as a distinct ‘‘side’’ population separated from the main non-side population. This gave us relatively pure side population cells which may be more representative of putative CSCs. Thus, isolation of cells with the highest dye efflux activity could lead to a highly enriched stem cell population.

Clonogenic formation assay is a useful method to partially estimate the self-renewal ability *in vitro*. It has long been known that only a small fraction of the cells directly isolated from human tumours is clonogenic under *in vivo* or *in vitro* conditions [138]. The various clonal formation ability is also a typical property of normal and malignant cell lines [25,139]. When normal epithelial cells are plated at low densities *in vitro* they have different abilities to generate colonies [140]. In the present study, we found that thyroid cancer SP cells display remarkable higher clonogenic potential than that of non-SP cells. Whereas 16.5 %-22.5 % of the HTh74 SP cells sustained a clonal growth and formed characteristic compact circular colonies with a

cobblestone appearance, the majority of the non-SP cells were not clonogenic. They scattered and failed to proliferate as clones. However, we cannot formally exclude the possibility that the apparent difference in proliferative and differentiation capacity between SP and non-SP cells is a consequence of damage induced by the Hoechst dye, which is retained longer in the non-SP (Hoechst high-staining) subpopulation. Arguing against this interpretation is our observation that the viability of the two populations remained identical after sorting (70 %-85 %) and throughout the whole study. These findings are in agreement with some previous observations showing that cancer SP cells are more tumorigenic than non-SP cells [69-71,90].

Metastasis is a notable obstacle for tumour treatment. Approximately 90 % of all cancer patients die from metastases [76,77]. Anaplastic thyroid cancer has a strong invasive ability and often invades the abutting tissues, as well as metastasizes to distant organs. Recently, there is growing evidence suggesting that cancer stem cells may not only initiate the primary tumour formation, but also contribute to cancer invasion and metastasis. As we know, invasion is certainly a prerequisite for metastasis, *i.e.* without invasion no metastasis [76-78,141,142]. There are many steps in the cascade from the initiation of cancer to metastasis: initiation → growth → angiogenesis → progression → selection → detachment → adhesion at the basal membranes → destruction of the basal membranes → motility → adhesion at the basal membranes of vessels → migration through the vessel wall → survival in the vessel and embolization → destruction of the vessel membranes in metastatic organs → local factors → invasion and growth of metastasis. Many of these steps require interaction between tumour cells and the extracellular matrix (ECM). Degradation and penetration of the ECM is a hallmark of tumour invasion and metastasis. In our study, to compare the invasive potential between cancer SP and non-SP cells, we performed Matrigel invasion assay using transwell chambers. Our results showed that thyroid cancer SP cells exhibit more invasiveness than non-SP cells. But the underlying mechanisms causing this difference in invasive competence remained unclear.

Recently, epithelial-mesenchymal transition (EMT) as a new putative mechanism for tumour metastasis has been postulated. Epithelial and mesenchymal cells have been identified on the basis of their unique visual appearance and the morphology of the multicellular structures they create (as shown in Fig. 4.2A) [143]. A typical epithelium is a sheet of cells, often only one cell thick, with individual epithelial cells abutting each other in a uniform array. Regularly spaced cell-cell junctions and adhesions between neighboring epithelial cells hold them tightly together and inhibit the movement of individual cells away from the epithelial monolayer. Internal

adhesiveness allows an epithelial sheet to enclose a three-dimensional space and provide it with structural definition and mechanical rigidity. The epithelial sheet itself is polarized, meaning that the apical and basal surfaces are likely to be visually different, adhere to different substrates, or have different functions. Mesenchymal cells, on the other hand, generally exhibit neither regimented structure nor tight intracellular adhesion. Mesenchymal cells form structures that are irregular in shape and not uniform in composition or density. Adhesions between mesenchymal cells are less strong than in their epithelial counterparts, allowing for increased migratory capacity. Mesenchymal cells also have a more extended and elongated shape, relative to epithelial cells. Moreover, mesenchymal migration is mechanistically different from epithelial movement. Epithelial cells move as a sheet en block, whereas mesenchymal migration is considerably more dynamic. Mesenchymal cells move individually and can leave part of the trailing region behind.

The epithelial-mesenchymal transition, where cells undergo a developmental switch from a polarized, epithelial phenotype to a highly motile fibroblastoid or mesenchymal phenotype, has emerged as a central process during normal development. Several developmental milestones, including gastrulation, neural crest formation and heart morphogenesis, rely on the plastic transition between epithelium and mesenchyme.

In recent years, there has been an increasing body of evidence pointing to a critical role of EMT-like events, which endow the cancer cell with invasive and metastatic properties during tumour progression and malignant transformation [144-152]. After undergoing EMT, cells will lose their epithelial properties and acquire a mesenchymal phenotype and increased migratory ability. In tumour development, when some cancer cells possess the active EMT, these metastable cancer cells will have elevated motilities and the abilities for local invasion or metastasis to distance (as shown in Fig. 4.2B).

Turning an epithelial cell into a mesenchymal cell requires alterations in morphology, cellular architecture, adhesion, and migration capacity. A critical molecular feature of EMT is the downregulation of E-cadherin, a cell adhesion molecule present in the plasma membrane of most normal epithelial cells [153]. E-cadherin acts de facto as a tumour suppressor inhibiting invasion and metastasis, and it is frequently repressed or degraded during transformation [154]. Other commonly used molecular markers for EMT include increased expression of N-cadherin and vimentin [155], nuclear localization of β-catenin, and increased production of the transcription

factors such as Snail, Slug and Twist, that inhibit E-cadherin production [156-163].

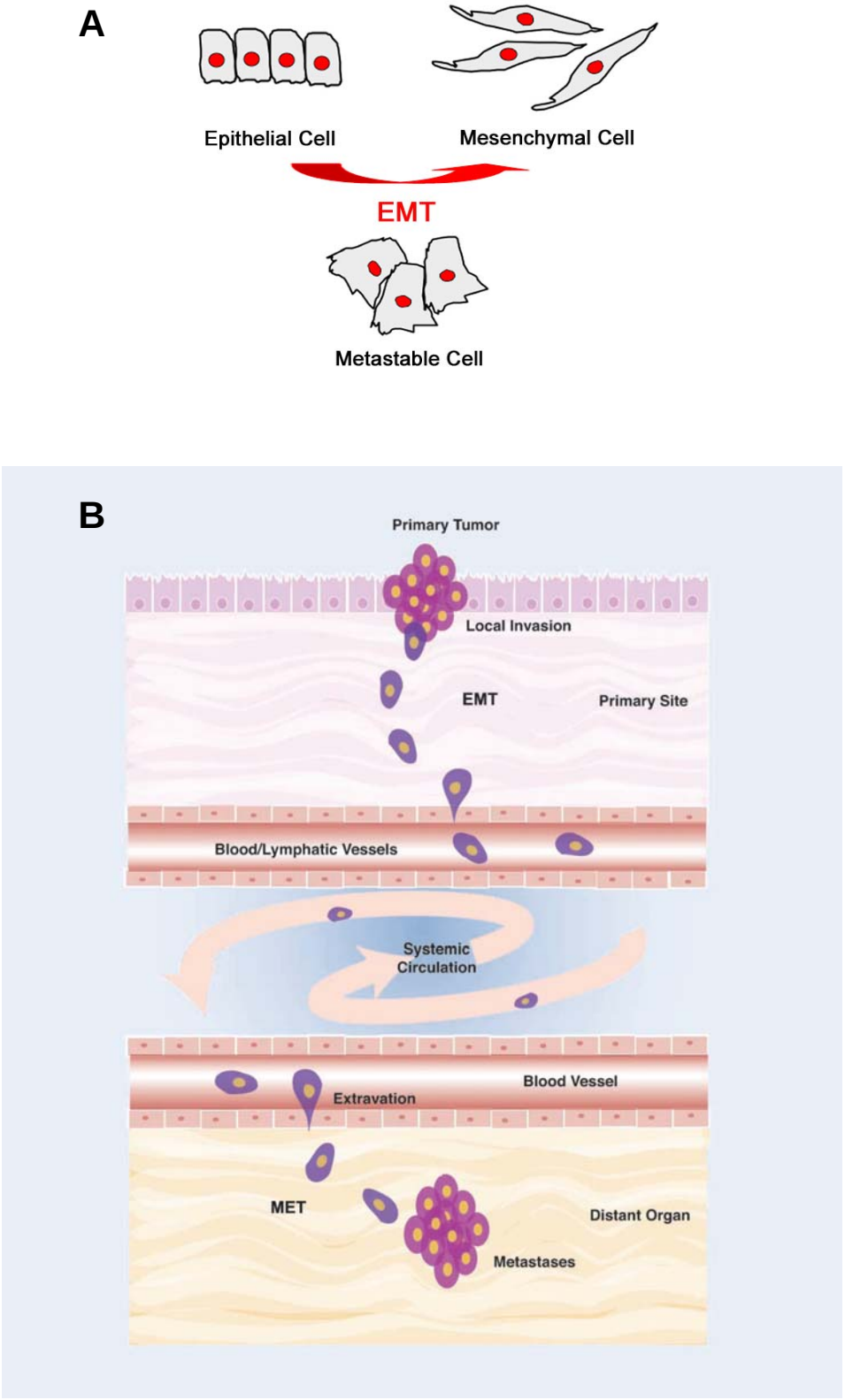

Figure 4.2 (A) The schematic graph of morphology change after EMT in tumour. After undergoing EMT, cells will lose the epithelial properties and acquire a mesenchymal phenotype and increased migratory ability.

(B) A reversible EMT model in tumour metastasis. In tumour development, the metastable cancer cells activate the EMT program to achieve local invasion and dissemination into the systemic circulation. Once distant organs are reached, these mesenchymal tumour cells reverse to an epithelial identity via mesenchymal-epithelial transition (MET) [42] to regain the ability to proliferate. [Taken from: Yang J, *et al*, "Exploring a new twist in tumor metastasis", 2006, Cancer Res; 66: 4549–4552.]

Our results show that thyroid cancer SP cells have elevated expression of β-catenin, Snail, Slug, but decreased expression of E-cadherin which suggested that they have gained active EMT. In addition, these cancer SP cells also exhibited elevated mRNA expression of LAMC2 and L1CAM. LAMC2 and L1CAM are two strong inducers of epithelial-cell migration and are transiently upregulated mainly in dissociated tumour cells. It has been found in colorectal cancers [28] that tumour cells exhibiting high levels of LAMC2 and L1CAM, not only display the EMT activation, but also maintain the expression of some stem cell markers. Such tumour cells can be regarded as putative 'colorectal migrating cancer stem cells' and thus have important consequences in tumour formation and metastasis.

It has been well-documented that the tumour invasion process is accomplished by the concerted action of multiple proteinases, particularly the matrix metalloproteinases (MMPs) and members of the urokinase plasminogen activator system. We found that thyroid cancer SP cells display high mRNA levels of MMP-2, uPA and uPAR, which participate in the process of extracellular matrix degradation for tumour invasion and metastases. MMP-2, also called gelatinase A, belongs to matrix metalloproteinases family and preferentially degrades denatured collagen and basement membrane components such as triple helical regions of type IV collagen and laminins. uPA is one of the best characterized "tumour-associated proteases" and can be activated via bonding to the urokinase-receptor (u-PAR). It is an important component in the fibrinolytic system converting plasminogen to an active enzyme, plasmin. Enhanced activity of plasmin promotes the degradation of extracellular matrix components including fibrin, fibronectin, proteoglycans and, as the main molecules in basement membranes, laminin and collagen IV. Thus, these findings demonstrate that thyroid cancer SP cells may possess activated EMT, and show elevated gene expression of some proteinases involved in ECM degradation and higher invasive potential in contrast to non-SP cells. This indicates that these cancer SP cells may have more opportunities to detach from the primary tumour, migrate, disseminate in the body and then serve as 'cancer stem cells' to form new tumours in distance.

Interestingly, we found that in the presence of EGF and bFGF, HTh74 cells can propagate as spheres which possess increased percentage of SP cells. Furthermore, after sorting, the SP cells could proliferate and re-form three-dimensional spheres in the presence of these two growth factors, whereas the non-SP cells could not. It has been shown that normal neural stem cells can be expanded when grown in suspension culture with EGF and bFGF as neurospheres and this method has been extended to the culture of brain cancer stem cells isolated from brain tumours [31,33]. Suspension methods also allow the growth of malignant epithelial populations from breast tumours [89]. These findings suggest that cells from epithelial malignancies can form expanding populations of tumour spheres in suspension culture and it thus appears that malignant stem cells can be maintained, identified and isolated using a range of *in vitro* methods. EGF and bFGF have already been proven as important growth factors for neural stem cell growth, as well as strong mitogens for normal thyrocytes and thyroid cancer cell proliferation, thus both may play a critical role in thyroid nodular and cancer transformation [164-168]. However, the underlying mechanisms of why cancer SP cells can be expanded in the presence of EGF and bFGF remain unclear and need to be further investigated.

Taken together, our research identifies small fractions of stem-like cells in anaplastic thyroid cancer cell lines. Such cancer stem-like cells are enriched in a side population, which possesses typical stemness characteristics and displays high invasive potential. The stable and readily quantifiable side populations present in thyroid cancer cell lines may provide simple *in vitro* models for investigating biological and molecular features of cancer stem cells in thyroid carcinomas. Further work is necessary to better define and characterize cancer stem-like cells in primary thyroid tumours, which may contribute to elucidating the mechanisms of thyroid carcinogenesis, and finally improve the effectiveness of thyroid cancer therapy.

5 Summary

Recently, several human cancers including leukemia, breast cancer and brain tumors were found to contain stem-like cancer cells called cancer stem cells (CSCs). Most of these CSCs were identified using specific markers that identify putative normal stem cells. In some cases, especially in the absence of specific surface marker expression, stem-like cancer cells were identified by using the side population sorting technique due to their properties to express ABCG2 membrane transporter and their capacities to efflux Hoechst 33342 dye. In this study, using the Hoechst DNA binding dye-based method and Fluorescence-activated Cell Sorting (FACS), we found that the established human anaplastic thyroid cancer cell lines C643, HTh74 and SW1736 contained a small percentage of side populations. Further experiments we focused on HTh74 cells. The immunofluorescent staining showed the co-localization of ABCG2 staining with Hoechst-low cells in HTh74, which confirmed the presence of a side population. These cancer side population cells possess some intrinsic stem cell properties. They display high gene expression of stem cell marker Oct4, as well as ABC transporter genes ABCG2 and MDR1, which endow SP cells with the ability to pump out diverse dyes and drugs, and therefore probably let such SP cells be highly resistant to chemotherapeutic agents and to escape cancer therapy. In vitro, these cancer SP cells exhibited the capacity to self-renew and generate heterologous descendents of non-SP cells, whereas the non-side population (non-SP) cells only produced other non-SP progenies. In clonal formation assay, thyroid cancer SP cells displayed markedly higher clonogenic potential than non-SP cells.

Metastasis is a notable obstacle for tumor treatment. Recent years have seen a growing body of evidence suggesting that cancer stem cells may not only initiate the primary tumor formation, but also contribute to cancer metastasis. In this study, Matrigel invasion assay revealed that the cancer SP cells had a higher invasive potential than non-SP cells. So far, accumulating evidence points to a critical role of the epithelial-mesenchymal transition (EMT) - like events during tumor progression and malignant transformation, endowing the cancer cell with invasive and metastatic properties. Our study demonstrated that cancer SP cells show preferential expression of β-catenin, Snail, Slug, LAMC2 and L1CAM, but decreased expression of E-cadherin, which indicates the activation of EMT in these cells. Furthermore, the SP cells also displayed high levels of metastasis-associated proteinase genes such as MMP-2, uPA and uPAR, which participate in the process of extracellular matrix degradation for tumor invasion and metastases.

Interestingly, we found that in the presence of EGF and bFGF, HTh74 cells can propagate as spheres, which are enriched in SP cells. Furthermore, after sorting, only SP cells could proliferate and re-form three-dimensional spheres in the presence of these two growth factors, which is in agreement with previous findings in brain and breast cancer stem cells.

Taken together, our study identifies a small fraction of cancer stem-like cells, which is termed "side population", in anaplastic thyroid cancer cell lines. These cancer stem-like cells, which may be closely related to tumor recurrence and metastasis, exhibited some typical stemness phenotypes, including preferential expression of stem cell marker and ABC transporter genes, the capacity to generate both SP and non-SP cells, and high invasive potential. Further work is needed to better define and characterize cancer stem-like cells in primary thyroid tumors, which may contribute to elucidating the mechanisms of thyroid carcinogenesis, and to improving the effectiveness of thyroid cancer therapy.

Zusammenfassung in deutscher Sprache

In den letzten Jahren konnten stammzell-ähnliche Tumorzellen bei verschiedenen Malignomen, u.a. bei Leukämien, bei Mammacarcinomen und Hirntumoren nachgewiesen werden. Diese Zellen werden als Carcinom-Stammzellen (Cancer Stem Cells – CSC) bezeichnet. Die meisten dieser CSCs wurden durch stammzell-spezifischen Marker identifiziert. In einigen Fällen jedoch, besonders bei fehlender Expression von spezifischen Membranmarkern, wurden stammzell-ähnliche Zellen mittels Seitenpopulations- Sortierung („side-population sorting“) identifiziert. Diese Technik basiert auf der Eigenschaft von Stammzellen, ABCG2 Membrantransporter zu exprimieren, die den Farbstoff Hoechst 33342 aus der Zelle transportieren.

Durch das auf der Hoechst 33342- Efflux-Methode basierendem FACS Sorting konnte in der vorliegenden Studie gezeigt werden, dass die etablierten menschlichen anaplastischen Schilddrüsenkrebs-Zelllinien C643, HTh74 und SW1736 einen niedrigen Prozentanteil an Seitenpopulation Zellen (SP-Zellen) enthalten. Bei den weiteren Experimenten konzentrierten wir uns vorwiegend auf die HTh74 Carcinom-Zellen. Die Immunfluoreszenz Anfärbung zeigte eine Co-Lokalisierung von ABCG2 Expression und Zellen mit niedrigem Hoechst 33342-Gehalt in HTh74-Zellen als Nachweis für eine Seitenpopulation. Diese SP-Tunorzellen verfügen über einige inhärente Stammzelleigenschaften: sie weisen eine vermehrte Expression des Stammzellmarker Oct4 sowie der ABC – Transportergene ABCG2 und MDR1 auf. Diese Transporter ermöglichen es den SP-Zellen, diverse Farbstoffe, Toxine aber auch Pharmaka aus der Zelle auszuschleusen. Dies verleiht solchen Zellen die Fähigkeit, Resistenzen gegenüber Chemotherapeutika zu entwickeln.

In vitro haben diese Tumor-Stammzellen ein erhöhtes Eigenvermehrungspotenzial sowie die Fähigkeit, heterologe Tumorzellen ohne Stammzelleigenschaften in Kultur zu erzeugen, wie in der vorliegenden Arbeit gezeigt werden konnte. Weitere Experimente wiesen nach, dass Non-SP- Zellen, d.h. Carcinomzellen ohne Stammzelleigenschaften, nur andere non-SP- Zellen nicht aber Tumorstammzellen hervorbringen können. In Klon-Formations-Assays zeigten Schilddrüsencarcinom- Stammzellen ein erheblich stärkeres klonogenes Potenzial als non-SP Zellen.

Eine Metastasierung beeinträchtigt den Erfolg einer Tumorbehandlung wesentlich. In den letzten Jahren konnte gezeigt werden, dass Carcinom-Stammzellen nicht nur die ursprüngliche Tumorbildung initiieren sondern auch zur Metastasierung beitragen. In der vorliegenden Studie konnte mittels in vitro- Matrigel- Invasionsassay gezeigt werden, dass Schilddrüsencarcinom-Stammzellen ein höheres Invasionspotenzial als non-SP Zellen aufweisen.

Für das invasive Wachstum und die Metastasierung von Carcinomen ist die sogen. epithelial-mesenchymale Transition (EMT) von großer Relevanz, da sie eine Voraussetzung für eine invasive und metastatische Tumorausbreitung ist. In der vorliegenden Arbeit wurde gezeigt, dass Schilddrüsencarcinom-Stammzellen vermehrt β-Catenin, Snail, Slug, LAMC2 und L1CAM aber vermindert E-Cadherin exprimieren, was auf eine Aktivierung von EMT in diesen Zellen hinweist. Darüber hinaus zeigten Schilddrüsencarcinom-Stammzellen ein erhöhtes Expressionsniveau von Metastasierungs-assoziierten Proteinase-Genen wie MMP-2, uPA und uPAR. Solche Gene haben eine wichtige Funktion bei der extrazellulären Matrixdegradation während Tumorinvasion und Metastasierung.

Analog zu früheren Experimenten der Arbeitsgruppe mit adulten Stammzellen aus normalem Schilddrüsengewebe konnte auch in dieser Arbeit an Schilddrüsencarcinomzellen gezeigt werden, dass eine intensive Stimulation mit Wachstumsfaktoren in einem speziellen Medium zur Formation dreidimensionaler Sphären führt, die reich an Schilddrüsencarcinom- Stammzellen sind. Dieser Befund entspricht jüngsten Forschungsergebnissen bei Hirn- und Mammacarcinom-Stammzellen.

Zusammenfassend konnte in der vorliegenden Arbeit ein kleiner Anteil Stammzell-ähnlicher Carcinomszellen in anaplastischen Schilddrüsenkrebs-Zelllinien identifiziert werden, die aufgrund ihrer Separation in der FACS-Analyse als Seitenpopulation bezeichnet wurden. Diese stammzell-ähnlichen Carcinomzellen, die in vivo bei Tumorrezidiven und bei der Metastasierung von zentraler Relevanz sind, weisen einige typische Stammzelleigenschaften auf. Dazu gehören das erhöhte invasive Potenzial, die vorrangige Expression von Stammzellmarker- und ABC-Transportergenen sowie die Fähigkeit sowohl SP als auch non-SP Zellen hervorzubringen. Weitere Studien sind erforderlich, um stammzell-ähnliche Carcinomzellen besser zu charakterisieren. Dies könnte zu neuen Erkenntnissen über Patho- genese der prognostisch sehr ungünstigen anaplastischen Schilddrüsencarcinome und perspektivisch zu neuen

Therapieansätzen führen.

6 References

1 Hundahl SA, Fleming ID, Fremgen AM, et al. A National Cancer Data Base report on 53,856 cases of thyroid carcinoma treated in the U.S., 1985-1995 [see commetns]. Cancer 1998;83:2638-48.

2 Gomez Segovia I, Gallowitsch HJ, Kresnik E, et al. Descriptive epidemiology of thyroid carcinoma in Carinthia, Austria: 1984-2001. Histopathologic features and tumor classification of 734 cases under elevated general iodination of table salt since 1990: population-based age-stratified analysis on thyroid carcinoma incidence. Thyroid 2004;14:277-86.

3 Murbeth S, Rousarova M, Scherb H, et al. Thyroid cancer has increased in the adult populations of countries moderately affected by Chernobyl fallout. Med Sci Monit 2004;10:CR300-6.

4 Carcangiu ML, Steeper T, Zampi G, et al. Anaplastic thyroid carcinoma. A study of 70 cases. Am J Clin Pathol 1985;83:135-58.

5 Farid NR, Shi Y, Zou M. Molecular basis of thyroid cancer. Endocr Rev 1994;15:202-32.

6 Fagin JA, Matsuo K, Karmakar A, et al. High prevalence of mutations of the p53 gene in poorly differentiated human thyroid carcinomas. J Clin Invest 1993;91:179-84.

7 Zou M, Shi Y, Farid NR. p53 mutations in all stages of thyroid carcinomas. J Clin Endocrinol Metab 1993;77:1054-8.

8 Namba H, Rubin SA, Fagin JA. Point mutations of ras oncogenes are an early event in thyroid tumorigenesis. Mol Endocrinol 1990;4:1474-9.

9 Kimura ET, Nikiforova MN, Zhu Z, et al. High prevalence of BRAF mutations in thyroid cancer: genetic evidence for constitutive activation of the RET/PTC-RAS-BRAF signaling pathway in papillary thyroid carcinoma. Cancer Res 2003;63:1454-7.

10 Oyama T, Suzuki T, Hara F, et al. N-ras mutation of thyroid tumor with special reference to the follicular type. Pathol Int 1995;45:45-50.

11 Bouras M, Parvaz P, Berger N, et al. Ha-ras oncogene (codon 12) mutation in thyroid carcinogenesis: analysis of 60 benign and malignant thyroid tumors. Ann Biol Clin (Paris) 1995;53:549-55.

12 Nikiforova MN, Kimura ET, Gandhi M, et al. BRAF mutations in thyroid tumors are restricted to papillary carcinomas and anaplastic or poorly differentiated carcinomas arising from papillary carcinomas. J Clin Endocrinol Metab 2003;88:5399-404.

13 Tallini G, Santoro M, Helie M, et al. RET/PTC oncogene activation defines a subset of papillary thyroid carcinomas lacking evidence of progression to poorly differentiated or undifferentiated tumor phenotypes. Clin Cancer Res 1998;4:287-94.

14 Kroll TG, Sarraf P, Pecciarini L, et al. PAX8-PPARgamma1 fusion oncogene in human thyroid carcinoma [corrected]. Science 2000;289:1357-60.

15 Nikiforova MN, Biddinger PW, Caudill CM, et al. PAX8-PPARgamma rearrangement in thyroid tumors: RT-PCR and immunohistochemical analyses. Am J Surg Pathol 2002;26:1016-23.

16 Dwight T, Thoppe SR, Foukakis T, et al. Involvement of the PAX8/peroxisome proliferator-activated receptor gamma rearrangement in follicular thyroid tumors. J Clin Endocrinol Metab 2003;88:4440-5.

17 Kakudo K, Miyauchi A, Katayama S, et al. Ultrastructural study of poorly differentiated medullary carcinoma of the thyroid. Virchows Arch A Pathol Anat Histopathol 1987;410:455-60.

18 Kakudo K, Carney JA, Sizemore GW. Medullary carcinoma of thyroid. Biologic behavior of the sporadic and familial neoplasm. Cancer 1985;55:2818-21.

19 Makino S. Further evidence favoring the concept of the stem cell in ascites tumors of rats. Ann N Y Acad Sci 1956;63:818-30.

20 Bruce WR, Van Der Gaag H. A Quantitative Assay for the Number of Murine Lymphoma Cells Capable of Proliferation in Vivo. Nature 1963;199:79-80.

21 Kleinsmith LJ, Pierce GB, Jr. Multipotentiality of Single Embryonal Carcinoma Cells. Cancer Res 1964;24:1544-51.

22 Park CH, Bergsagel DE, McCulloch EA. Mouse myeloma tumor stem cells: a primary cell culture assay. J Natl Cancer Inst 1971;46:411-22.

23 Al-Hajj M, Clarke MF. Self-renewal and solid tumor stem cells. Oncogene 2004;23:7274-82.

24 Masters JR. Human cancer cell lines: fact and fantasy. Nat Rev Mol Cell Biol 2000;1:233-6.

25 Reya T, Morrison SJ, Clarke MF, et al. Stem cells, cancer, and cancer stem cells. Nature 2001;414:105-11.

26 Bjerkvig R, Tysnes BB, Aboody KS, et al. Opinion: the origin of the cancer stem cell: current controversies and new insights. Nat Rev Cancer 2005;5:899-904.

27 Pardal R, Clarke MF, Morrison SJ. Applying the principles of stem-cell biology to cancer. Nat Rev Cancer 2003;3:895-902.

28 Brabletz T, Jung A, Spaderna S, et al. Opinion: migrating cancer stem cells - an integrated concept of malignant tumour progression. Nat Rev Cancer 2005;5:744-9.

29 Bonnet D, Dick JE. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. Nat Med 1997;3:730-7.

30 Al-Hajj M, Wicha MS, Benito-Hernandez A, et al. Prospective identification of tumorigenic breast cancer cells. Proc Natl Acad Sci U S A 2003;100:3983-8.

31 Singh SK, Clarke ID, Terasaki M, et al. Identification of a cancer stem cell in human brain tumors. Cancer Res 2003;63:5821-8.

32 Hemmati HD, Nakano I, Lazareff JA, et al. Cancerous stem cells can arise from pediatric brain tumors. Proc Natl Acad Sci U S A 2003;100:15178-83.

33 Ignatova TN, Kukekov VG, Laywell ED, et al. Human cortical glial tumors contain neural stem-like cells expressing astroglial and neuronal markers in vitro. Glia 2002;39:193-206.

34 Lapidot T, Sirard C, Vormoor J, et al. A cell initiating human acute myeloid leukaemia after transplantation into SCID mice. Nature 1994;367:645-8.

35 Singh SK, Hawkins C, Clarke ID, et al. Identification of human brain tumour initiating cells. Nature 2004;432:396-401.

36 Kim CF, Jackson EL, Woolfenden AE, et al. Identification of bronchioalveolar stem cells in normal lung and lung cancer. Cell 2005;121:823-35.

37 Xin L, Lawson DA, Witte ON. The Sca-1 cell surface marker enriches for a prostate-regenerating cell subpopulation that can initiate prostate tumorigenesis. Proc Natl Acad Sci U S A 2005;102:6942-7.

38 Moniz S, Catarino AL, Marques AR, et al. Clonal origin of non-medullary thyroid tumours assessed by non-random X-chromosome inactivation. Eur J Endocrinol 2002;146:27-33.

39 Williams ED. Thyroid tumorigenesis. Horm Res 1994;42:31-4.

40 Shackleton M, Vaillant F, Simpson KJ, et al. Generation of a functional mammary gland from a single stem cell. Nature 2006;439:84-8.

41 Zhou S, Schuetz JD, Bunting KD, et al. The ABC transporter Bcrp1/ABCG2 is expressed in a wide variety of stem cells and is a molecular determinant of the side-population phenotype. Nat Med 2001;7:1028-34.

42 Dean M, Allikmets R. Complete characterization of the human ABC gene family. J Bioenerg Biomembr 2001;33:475-9.

43 Kvackajova-Kisucka J, Barancik M, Breier A. Drug transporters and their role in

multidrug resistance of neoplastic cells. Gen Physiol Biophys 2001;20:215-37.

44 Doyle LA, Yang W, Abruzzo LV, et al. A multidrug resistance transporter from human MCF-7 breast cancer cells. Proc Natl Acad Sci U S A 1998;95:15665-70.

45 Rocchi E, Khodjakov A, Volk EL, et al. The product of the ABC half-transporter gene ABCG2 (BCRP/MXR/ABCP) is expressed in the plasma membrane. Biochem Biophys Res Commun 2000;271:42-6.

46 Storms RW, Goodell MA, Fisher A, et al. Hoechst dye efflux reveals a novel CD7(+)CD34(-) lymphoid progenitor in human umbilical cord blood. Blood 2000;96:2125-33.

47 Jackson KA, Majka SM, Wang H, et al. Regeneration of ischemic cardiac muscle and vascular endothelium by adult stem cells. J Clin Invest 2001;107:1395-402.

48 Asakura A, Seale P, Girgis-Gabardo A, et al. Myogenic specification of side population cells in skeletal muscle. J Cell Biol 2002;159:123-34.

49 Montanaro F, Liadaki K, Schienda J, et al. Demystifying SP cell purification: viability, yield, and phenotype are defined by isolation parameters. Exp Cell Res 2004;298:144-54.

50 Welm BE, Tepera SB, Venezia T, et al. Sca-1(pos) cells in the mouse mammary gland represent an enriched progenitor cell population. Dev Biol 2002;245:42-56.

51 Clayton H, Titley I, Vivanco M. Growth and differentiation of progenitor/stem cells derived from the human mammary gland. Exp Cell Res 2004;297:444-60.

52 Summer R, Kotton DN, Sun X, et al. Side population cells and Bcrp1 expression in lung. Am J Physiol Lung Cell Mol Physiol 2003;285:L97-104.

53 Giangreco A, Shen H, Reynolds SD, et al. Molecular phenotype of airway side population cells. Am J Physiol Lung Cell Mol Physiol 2004;286:L624-30.

54 Summer R, Kotton DN, Sun X, et al. Translational physiology: origin and phenotype of lung side population cells. Am J Physiol Lung Cell Mol Physiol 2004;287:L477-83.

55 Shimano K, Satake M, Okaya A, et al. Hepatic oval cells have the side population phenotype defined by expression of ATP-binding cassette transporter ABCG2/BCRP1. Am J Pathol 2003;163:3-9.

56 Terunuma A, Jackson KL, Kapoor V, et al. Side population keratinocytes resembling bone marrow side population stem cells are distinct from label-retaining keratinocyte stem cells. J Invest Dermatol 2003;121:1095-103.

57 Triel C, Vestergaard ME, Bolund L, et al. Side population cells in human and mouse epidermis lack stem cell characteristics. Exp Cell Res 2004;295:79-90.

58 Kim M, Morshead CM. Distinct populations of forebrain neural stem and progenitor cells can be isolated using side-population analysis. J Neurosci 2003;23:10703-9.

59 Lassalle B, Bastos H, Louis JP, et al. 'Side Population' cells in adult mouse testis express Bcrp1 gene and are enriched in spermatogonia and germinal stem cells. Development 2004;131:479-87.

60 Falciatori I, Borsellino G, Haliassos N, et al. Identification and enrichment of spermatogonial stem cells displaying side-population phenotype in immature mouse testis. Faseb J 2004;18:376-8.

61 Martin CM, Meeson AP, Robertson SM, et al. Persistent expression of the ATP-binding cassette transporter, Abcg2, identifies cardiac SP cells in the developing and adult heart. Dev Biol 2004;265:262-75.

62 Iwatani H, Ito T, Imai E, et al. Hematopoietic and nonhematopoietic potentials of Hoechst(low)/side population cells isolated from adult rat kidney. Kidney Int 2004;65:1604-14.

63 Watanabe K, Nishida K, Yamato M, et al. Human limbal epithelium contains side population cells expressing the ATP-binding cassette transporter ABCG2. FEBS Lett 2004;565:6-10.

64 Bhatt RI, Brown MD, Hart CA, et al. Novel method for the isolation and characterisation of the putative prostatic stem cell. Cytometry A 2003;54:89-99.

65 Zhou S, Morris JJ, Barnes Y, et al. Bcrp1 gene expression is required for normal numbers of side population stem cells in mice, and confers relative protection to mitoxantrone in hematopoietic cells in vivo. Proc Natl Acad Sci U S A 2002;99:12339-44.

66 Lechner A, Leech CA, Abraham EJ, et al. Nestin-positive progenitor cells derived from adult human pancreatic islets of Langerhans contain side population (SP) cells defined by expression of the ABCG2 (BCRP1) ATP-binding cassette transporter. Biochem Biophys Res Commun 2002;293:670-4.

67 Hirschmann-Jax C, Foster AE, Wulf GG, et al. A distinct "side population" of cells with high drug efflux capacity in human tumor cells. Proc Natl Acad Sci U S A 2004;101:14228-33.

68 Cai J, Cheng A, Luo Y, et al. Membrane properties of rat embryonic multipotent neural stem cells. J Neurochem 2004;88:212-26.

69 Kondo T, Setoguchi T, Taga T. Persistence of a small subpopulation of cancer stem-like cells in the C6 glioma cell line. Proc Natl Acad Sci U S A 2004;101:781-6.

70 Patrawala L, Calhoun T, Schneider-Broussard R, et al. Side population is enriched in tumorigenic, stem-like cancer cells, whereas ABCG2+ and ABCG2- cancer cells are similarly tumorigenic. Cancer Res 2005;65:6207-19.

71 Haraguchi N, Utsunomiya T, Inoue H, et al. Characterization of a side population of cancer cells from human gastrointestinal system. Stem Cells 2006;24:506-13.

72 Polakis P. The oncogenic activation of beta-catenin. Curr Opin Genet Dev 1999;9:15-21.

73 Jagelman DG. Extracolonic manifestations of familial polyposis coli. Cancer Genet Cytogenet 1987;27:319-25.

74 Plail RO, Bussey HJ, Glazer G, et al. Adenomatous polyposis: an association with carcinoma of the thyroid. Br J Surg 1987;74:377-80.

75 Schneider NR, Cubilla AL, Chaganti RS. Association of endocrine neoplasia with multiple polyposis of the colon. Cancer 1983;51:1171-5.

76 Fidler IJ. The organ microenvironment and cancer metastasis. Differentiation 2002;70:498-505.

77 Fidler IJ. Critical determinants of metastasis. Semin Cancer Biol 2002;12:89-96.

78 Gotzmann J, Mikula M, Eger A, et al. Molecular aspects of epithelial cell plasticity: implications for local tumor invasion and metastasis. Mutat Res 2004;566:9-20.

79 Savagner P. Leaving the neighborhood: molecular mechanisms involved during epithelial-mesenchymal transition. Bioessays 2001;23:912-23.

80 Hay ED. An overview of epithelio-mesenchymal transformation. Acta Anat (Basel) 1995;154:8-20.

81 Woodhouse EC, Chuaqui RF, Liotta LA. General mechanisms of metastasis. Cancer 1997;80:1529-37.

82 Morin PJ, Sparks AB, Korinek V, et al. Activation of beta-catenin-Tcf signaling in colon cancer by mutations in beta-catenin or APC. Science 1997;275:1787-90.

83 Barker N, Clevers H. Tumor environment: a potent driving force in colorectal cancer? Trends Mol Med 2001;7:535-7.

84 Heldin NE, Cvejic D, Smeds S, et al. Coexpression of functionally active receptors for thyrotropin and platelet-derived growth factor in human thyroid carcinoma cells. Endocrinology 1991;129:2187-93.

85 Heldin NE, Westermark B. Epidermal growth factor, but not thyrotropin, stimulates the expression of c-fos and c-myc messenger ribonucleic acid in porcine thyroid follicle cells in primary culture. Endocrinology 1988;122:1042-6.

86 Mark J, Ekedahl C, Dahlenfors R, et al. Cytogenetical observations in five human anaplastic thyroid carcinomas. Hereditas 1987;107:163-74.

87 Heldin NE, Gustavsson B, Claesson-Welsh L, et al. Aberrant expression of receptors for

platelet-derived growth factor in an anaplastic thyroid carcinoma cell line. Proc Natl Acad Sci U S A 1988;85:9302-6.

88 Goodell MA, Brose K, Paradis G, et al. Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. J Exp Med 1996;183:1797-806.

89 Dontu G, Wicha MS. Survival of mammary stem cells in suspension culture: implications for stem cell biology and neoplasia. J Mammary Gland Biol Neoplasia 2005;10:75-86.

90 Szotek PP, Pieretti-Vanmarcke R, Masiakos PT, et al. Ovarian cancer side population defines cells with stem cell-like characteristics and Mullerian Inhibiting Substance responsiveness. Proc Natl Acad Sci U S A 2006;103:11154-9.

91 de Paiva CS, Chen Z, Corrales RM, et al. ABCG2 transporter identifies a population of clonogenic human limbal epithelial cells. Stem Cells 2005;23:63-73.

92 Budak MT, Alpdogan OS, Zhou M, et al. Ocular surface epithelia contain ABCG2-dependent side population cells exhibiting features associated with stem cells. J Cell Sci 2005;118:1715-24.

93 Cano A, Perez-Moreno MA, Rodrigo I, et al. The transcription factor snail controls epithelial-mesenchymal transitions by repressing E-cadherin expression. Nat Cell Biol 2000;2:76-83.

94 Batlle E, Sancho E, Franci C, et al. The transcription factor snail is a repressor of E-cadherin gene expression in epithelial tumour cells. Nat Cell Biol 2000;2:84-9.

95 Thomas T, Nowka K, Lan L, et al. Expression of endoderm stem cell markers: evidence for the presence of adult stem cells in human thyroid glands. Thyroid 2006;16:537-44.

96 Zhang P, Zuo H, Ozaki T, et al. Cancer stem cell hypothesis in thyroid cancer. Pathol Int 2006;56:485-9.

97 Takano T, Amino N. Fetal cell carcinogenesis: a new hypothesis for better

understanding of thyroid carcinoma. Thyroid 2005;15:432-8.

98 Larderet G, Fortunel NO, Vaigot P, et al. Human side population keratinocytes exhibit long-term proliferative potential and a specific gene expression profile and can form a pluristratified epidermis. Stem Cells 2006;24:965-74.

99 Decraene C, Benchaouir R, Dillies MA, et al. Global transcriptional characterization of SP and MP cells from the myogenic C2C12 cell line: effect of FGF6. Physiol Genomics 2005;23:132-49.

100 Dontu G, Abdallah WM, Foley JM, et al. In vitro propagation and transcriptional profiling of human mammary stem/progenitor cells. Genes Dev 2003;17:1253-70.

101 Behbod F, Xian W, Shaw CA, et al. Transcriptional profiling of mammary gland side population cells. Stem Cells 2006;24:1065-74.

102 Alvi AJ, Clayton H, Joshi C, et al. Functional and molecular characterisation of mammary side population cells. Breast Cancer Res 2003;5:R1-8.

103 Hussain SZ, Strom SC, Kirby MR, et al. Side population cells derived from adult human liver generate hepatocyte-like cells in vitro. Dig Dis Sci 2005;50:1755-63.

104 Yano S, Ito Y, Fujimoto M, et al. Characterization and localization of side population cells in mouse skin. Stem Cells 2005;23:834-41.

105 Seigel GM, Campbell LM, Narayan M, et al. Cancer stem cell characteristics in retinoblastoma. Mol Vis 2005;11:729-37.

106 Grichnik JM, Burch JA, Schulteis RD, et al. Melanoma, a tumor based on a mutant stem cell? J Invest Dermatol 2006;126:142-53.

107 Ponti D, Costa A, Zaffaroni N, et al. Isolation and in vitro propagation of tumorigenic breast cancer cells with stem/progenitor cell properties. Cancer Res 2005;65:5506-11.

108 Tamaki T, Akatsuka A, Okada Y, et al. Growth and differentiation potential of main- and

side-population cells derived from murine skeletal muscle. Exp Cell Res 2003;291:83-90.

109 Scholer HR, Ruppert S, Suzuki N, et al. New type of POU domain in germ line-specific protein Oct-4. Nature 1990;344:435-9.

110 Scholer HR. Octamania: the POU factors in murine development. Trends Genet 1991;7:323-9.

111 Pesce M, Scholer HR. Oct-4: gatekeeper in the beginnings of mammalian development. Stem Cells 2001;19:271-8.

112 Solter D. Mammalian cloning: advances and limitations. Nat Rev Genet 2000;1:199-207.

113 Boiani M, Eckardt S, Scholer HR, et al. Oct4 distribution and level in mouse clones: consequences for pluripotency. Genes Dev 2002;16:1209-19.

114 Gidekel S, Pizov G, Bergman Y, et al. Oct-3/4 is a dose-dependent oncogenic fate determinant. Cancer Cell 2003;4:361-70.

115 Tai MH, Chang CC, Kiupel M, et al. Oct4 expression in adult human stem cells: evidence in support of the stem cell theory of carcinogenesis. Carcinogenesis 2005;26:495-502.

116 Dyce PW, Zhu H, Craig J, et al. Stem cells with multilineage potential derived from porcine skin. Biochem Biophys Res Commun 2004;316:651-8.

117 Chen J, Hersmus N, Van Duppen V, et al. The adult pituitary contains a cell population displaying stem/progenitor cell and early embryonic characteristics. Endocrinology 2005;146:3985-98.

118 Pesce M, Gross MK, Scholer HR. In line with our ancestors: Oct-4 and the mammalian germ. Bioessays 1998;20:722-32.

119 Deev IE, Polianovskii OL. [oct-genes and oct-proteins]. Mol Biol (Mosk) 2004;38:48-55.

120 Monk M, Holding C. Human embryonic genes re-expressed in cancer cells. Oncogene 2001;20:8085-91.

121 Abate-Shen C. Homeobox genes and cancer: new OCTaves for an old tune. Cancer Cell 2003;4:329-30.

122 Alison MR. Tissue-based stem cells: ABC transporter proteins take centre stage. J Pathol 2003;200:547-50.

123 Green H, Soderkvist P, Rosenberg P, et al. mdr-1 single nucleotide polymorphisms in ovarian cancer tissue: G2677T/A correlates with response to paclitaxel chemotherapy. Clin Cancer Res 2006;12:854-9.

124 Burger H, van Tol H, Boersma AW, et al. Imatinib mesylate (STI571) is a substrate for the breast cancer resistance protein (BCRP)/ABCG2 drug pump. Blood 2004;104:2940-2.

125 Sparreboom A, Loos WJ, Burger H, et al. Effect of ABCG2 genotype on the oral bioavailability of topotecan. Cancer Biol Ther 2005;4:650-8.

126 Chen ZS, Robey RW, Belinsky MG, et al. Transport of methotrexate, methotrexate polyglutamates, and 17beta-estradiol 17-(beta-D-glucuronide) by ABCG2: effects of acquired mutations at R482 on methotrexate transport. Cancer Res 2003;63:4048-54.

127 Merry S, Courtney ER, Fetherston CA, et al. Circumvention of drug resistance in human non-small cell lung cancer in vitro by verapamil. Br J Cancer 1987;56:401-5.

128 Morrison SJ, Shah NM, Anderson DJ. Regulatory mechanisms in stem cell biology. Cell 1997;88:287-98.

129 Fidler IJ, Kripke ML. Metastasis results from preexisting variant cells within a malignant tumor. Science 1977;197:893-5.

130Fidler IJ, Hart IR. Biological diversity in metastatic neoplasms: origins and implications. Science 1982;217:998-1003.

131 Heppner GH. Tumor heterogeneity. Cancer Res 1984;44:2259-65.

132 Nowell PC. Mechanisms of tumor progression. Cancer Res 1986;46:2203-7.

133 Fialkow PJ. Clonal origin of human tumors. Biochim Biophys Acta 1976;458:283-321.

134 Fearon ER, Hamilton SR, Vogelstein B. Clonal analysis of human colorectal tumors. Science 1987;238:193-7.

135 Benchaouir R, Rameau P, Decraene C, et al. Evidence for a resident subset of cells with SP phenotype in the C2C12 myogenic line: a tool to explore muscle stem cell biology. Exp Cell Res 2004;294:254-68.

136 Parmar K, Sauk-Schubert C, Burdick D, et al. Sca+CD34- murine side population cells are highly enriched for primitive stem cells. Exp Hematol 2003;31:244-50.

137 Camargo FD, Chambers SM, Drew E, et al. Hematopoietic stem cells do not engraft with absolute efficiencies. Blood 2006;107:501-7.

138 Hamburger AW, Salmon SE. Primary bioassay of human tumor stem cells. Science 1977;197:461-3.

139 Resnicoff M, Medrano EE, Podhajcer OL, et al. Subpopulations of MCF7 cells separated by Percoll gradient centrifugation: a model to analyze the heterogeneity of human breast cancer. Proc Natl Acad Sci U S A 1987;84:7295-9.

140 Barrandon Y, Green H. Three clonal types of keratinocyte with different capacities for multiplication. Proc Natl Acad Sci U S A 1987;84:2302-6.

141 Aznavoorian S, Murphy AN, Stetler-Stevenson WG, et al. Molecular aspects of tumor cell invasion and metastasis. Cancer 1993;71:1368-83.

142 Chambers AF, Naumov GN, Vantyghem SA, et al. Molecular biology of breast cancer metastasis. Clinical implications of experimental studies on metastatic inefficiency. Breast Cancer Res 2000;2:400-7.

143 Shook D, Keller R. Mechanisms, mechanics and function of epithelial-mesenchymal transitions in early development. Mech Dev 2003;120:1351-83.

144 Brabletz T, Hlubek F, Spaderna S, et al. Invasion and metastasis in colorectal cancer: epithelial-mesenchymal transition, mesenchymal-epithelial transition, stem cells and beta-catenin. Cells Tissues Organs 2005;179:56-65.

145 Christiansen JJ, Rajasekaran AK. Reassessing epithelial to mesenchymal transition as a prerequisite for carcinoma invasion and metastasis. Cancer Res 2006;66:8319-26.

146 Khew-Goodall Y, Wadham C. A perspective on regulation of cell-cell adhesion and epithelial-mesenchymal transition: known and novel. Cells Tissues Organs 2005;179:81-6.

147 Larue L, Bellacosa A. Epithelial-mesenchymal transition in development and cancer: role of phosphatidylinositol 3' kinase/AKT pathways. Oncogene 2005;24:7443-54.

148 Lee JM, Dedhar S, Kalluri R, et al. The epithelial-mesenchymal transition: new insights in signaling, development, and disease. J Cell Biol 2006;172:973-81.

149 Tarin D, Thompson EW, Newgreen DF. The fallacy of epithelial mesenchymal transition in neoplasia. Cancer Res 2005;65:5996-6000; discussion -1.

150 Thompson EW, Newgreen DF, Tarin D. Carcinoma invasion and metastasis: a role for epithelial-mesenchymal transition? Cancer Res 2005;65:5991-5; discussion 5.

151 Vasko V, Espinosa AV, Scouten W, et al. Gene expression and functional evidence of epithelial-to-mesenchymal transition in papillary thyroid carcinoma invasion. Proc Natl Acad Sci U S A 2007;104:2803-8.

152 Vasko VV, Saji M. Molecular mechanisms involved in differentiated thyroid cancer invasion and metastasis. Curr Opin Oncol 2007;19:11-7.

153 Huber MA, Kraut N, Beug H. Molecular requirements for epithelial-mesenchymal transition during tumor progression. Curr Opin Cell Biol 2005;17:548-58.

154 Lu Z, Ghosh S, Wang Z, et al. Downregulation of caveolin-1 function by EGF leads to the loss of E-cadherin, increased transcriptional activity of beta-catenin, and enhanced tumor cell invasion. Cancer Cell 2003;4:499-515.

155 Nakajima S, Doi R, Toyoda E, et al. N-cadherin expression and epithelial-mesenchymal transition in pancreatic carcinoma. Clin Cancer Res 2004;10:4125-33.

156 Castro Alves C, Rosivatz E, Schott C, et al. Slug is overexpressed in gastric carcinomas and may act synergistically with SIP1 and Snail in the down-regulation of E-cadherin. J Pathol 2007;211:507-15.

157 Kurrey NK, K A, Bapat SA. Snail and Slug are major determinants of ovarian cancer invasiveness at the transcription level. Gynecol Oncol 2005;97:155-65.

158 Shioiri M, Shida T, Koda K, et al. Slug expression is an independent prognostic parameter for poor survival in colorectal carcinoma patients. Br J Cancer 2006;94:1816-22.

159 Toyama T, Zhang Z, Iwase H, et al. Low expression of the snail gene is a good prognostic factor in node-negative invasive ductal carcinomas. Jpn J Clin Oncol 2006;36:357-63.

160 Yang J, Mani SA, Donaher JL, et al. Twist, a master regulator of morphogenesis, plays an essential role in tumor metastasis. Cell 2004;117:927-39.

161 Yang J, Mani SA, Weinberg RA. Exploring a new twist on tumor metastasis. Cancer Res 2006;66:4549-52.

162 Yuen HF, Chua CW, Chan YP, et al. Significance of TWIST and E-cadherin expression in the metastatic progression of prostatic cancer. Histopathology 2007;50:648-58.

163 Zhou BP, Hung MC. Wnt, hedgehog and snail: sister pathways that control by GSK-3beta and beta-Trcp in the regulation of metastasis. Cell Cycle 2005;4:772-6.

164 Bidey SP, Hill DJ, Eggo MC. Growth factors and goitrogenesis. J Endocrinol 1999;160:321-32.

165 Eszlinger M, Krohn K, Kratzsch J, et al. Growth factor expression in cold and hot thyroid nodules. Thyroid 2001;11:125-35.

166 Ollis CA, Davies R, Munro DS, et al. Relationship between growth and function of human thyroid cells in culture. J Endocrinol 1986;108:393-8.

167 Yeh MW, Rougier JP, Park JW, et al. Differentiated thyroid cancer cell invasion is regulated through epidermal growth factor receptor-dependent activation of matrix metalloproteinase (MMP)-2/gelatinase A. Endocr Relat Cancer 2006;13:1173-83.

168 Younes MN, Yigitbasi OG, Park YW, et al. Antivascular therapy of human follicular thyroid cancer experimental bone metastasis by blockade of epidermal growth factor receptor and vascular growth factor receptor phosphorylation. Cancer Res 2005;65:4716-27.

Acknowledgments

This work was planned, performed and completed in the laboratory of Experimental Endocrinology of St. Hedwig Hospital in Berlin. I would like to express my sincere gratitude to the following people for their invaluable help:

... Prof. Dr. Karl-Michael Derwahl, ‘Doctorvater’ and supervisor of this research project. It is thanks to his drive, his ingenious ideas and his constant motivation that this work came to be. Without his encouragement and support I would never have managed to overcome all the difficulties of being a foreigner working and living in Germany and for this I am truly indebted to him.

... Diana, Tini, Theodore and Kathrin, for their excellent technical support, for showing me all the lab methods and protocols, for always being there for me and, most importantly, for the great time we had together!

... Ling, for her great support, kindness and friendly cooperation, for the wonderful two years she accompanies me. I wish her every success with her work and all the best in life!

... Prof. Köhrle and his great team in Charité, for their expert advice and discussions.

... All the members of GK1208 (Graduate College), for their excellent organization of each seminar and the good time we had together.

... Katharina and Toralf, for their technical support of Fluorescence-activated cell sorting.

... Prof. Dr Heldin (Uppsala) for the gifts of the HTh74, C643 and SW1736 cell lines.

... My parents, my husband, my son and all my family members, for they are my life.

Erklärung

„Ich, Dai Cui, erkläre, dass ich die vorgelegte Dissertationsschrift mit dem Thema: „Isolation and characterization of a side population in thyroid cancer cell lines: the evidence for the presence of thyroid cancer stem cells“ selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt, ohne die (unzulässige) Hilfe Dritter verfasst und auch in Teilen keine Kopien anderer Arbeiten dargestellt habe.“

Datum 22-Nov-2007 Unterschrift Dai Cui

Mein Lebenslauf wird aus Datenschutzgründen in der elektronischen Version meiner Arbeit nicht mit veröffentlicht.