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REVIEW

Normal vs cancer thyroid stem cells: the road to transformation

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Recent investigations in thyroid carcinogenesis have led to the isolation and characterisation of a subpopulation of stem-like cells, responsible for tumour initiation, progression and metastasis. Nevertheless, the cellular origin of thyroid cancer stem cells (SCs) remains unknown and it is still necessary to define the process and the target population that sustain malignant transformation of tissue-resident SCs or the reprogramming of a more differentiated cell. Here, we will critically discuss new insights into thyroid SCs as a potential source of cancer formation in light of the available information on the oncogenic role of genetic modifications that occur during thyroid cancer development. Understanding the fine mechanisms that regulate tumour transformation may provide new ground for clinical intervention in terms of prevention, diagnosis and therapy.

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INTRODUCTION

Thyroid cancer (TC) accounts for 96% of endocrine malignancies with 62 980 new cases expected to be diagnosed in the US in 2014, where it represents the second most common cancer among adolescents ages 15–19 (www.cancer.org). Despite of a global increase in incidence over the past three decades, the mortality rate remains low. This is a consequence of a favourable prognosis for the more frequent well-differentiated forms, subdivided into papillary (PTC) and follicular TC (FTC).[1] By retaining the differentiated features of normal thyrocytes, including the ability to concentrate iodine, in most cases these tumours can be treated successfully by surgical resection, followed by radioactive-iodine administration.[2] In contrast, the rare undifferentiated anaplastic TCs (ATCs), have a very-poor prognosis because of their invasiveness and metastatic behaviour (Figure 1) as well as their insensitivity to radioactive-iodine treatment for lack of an iodine symporter.[3]

Alterations in key signalling pathways are proposed for distinct forms of thyroid transformation. Gain-of-function mutations in the thyrotropin receptor (TSH-R) or Gsα encoding genes, result in increased cAMP accumulation and TSH-independent proliferation, which in turn account for hyperfunctional adenomas, benign lesions without propensity towards malignant progression. Constitutive activation of the MAPK pathway seems to be the hallmark of different forms of TC.[2] Genomic alterations of the proto-oncogene tyrosine-protein kinase receptor Ret, the neurotrophic tyrosine kinase receptor, as well as the intracellular signal transducer Ras and the serine/threonine-protein kinase B-Raf, have clearly been implicated in the pathogenesis of PTCs.[4] Similarly, the chromosomal translocation t(2;3)(q13;p25), which fuses the transcription factor paired box protein Pax-8 (Pax-8) and peroxisome proliferator-activated receptor gamma (PPAR-γ) encoding genes, has been identified in significant proportions in FTCs.[5] In addition to *RAS* mutations, another common event of these tumours is the PI3K pathway aberrant activation through mutation of the catalytic subunit p110 (*PI3KCA*) and loss of PTEN (Figure 2).[6]

The multistep carcinogenesis model suggests that ATCs arise by way of a dedifferentiation process from pre-existing FTC or PTC (Figure 3).[7] The additional genetic events involved in the progression towards tumour dedifferentiation are (i) the inactivating point mutation in the *TP53* gene[8–10] and (ii) the activating mutation in the *β*-catenin encoding gene *CTNNB1*.[11,12] Evidence in favour of this multistep carcinogenesis model includes, the presence of well-differentiated TC within ATC specimens and the coexistence of *BRAF* gene and *TP53* gene mutations in both undifferentiated and differentiated carcinomas.[13,14] However, this model is not in accordance with the rare occurrence of *RET/PTC* and *PAX8/PPARG* rearrangements in ATC[15] and the low turnover rate of thyroid follicular cells (about five renewals per lifetime) that reduces the possibility of accumulating the mutations needed for transformation.[8,16–18]

The existence of several differentiation degrees has led to the assumption that TC cells are derived from remnants of fetal thyroid cells, such as stem cells (SCs) or precursors, rather than mature follicular cells.[9,19,20] According to this fetal cell carcinogenesis model supported by gene expression profiling data, ATC arises from fetal thyroid SCs, marked by the onco-fetal fibronectin expression and lack of differentiation markers. Thyroblasts are hypothesised to be at the origin of PTC and are characterised by the concomitant expression of onco-fetal fibronectin, and the more differentiated marker thyroglobulin (Tg). Remnants of prothyrocytes, which represent a more differentiated cell type not expressing onco-fetal fibronectin, would result in FTC (Figure 3).[7,20] Genomic alterations, including mutations in *TP53* and *BRAF* genes, as well as *RET/PTC* and *PAX8/PPARG* rearrangements, have an oncogenic role by conferring proliferative advantages and preventing fetal thyroid cells from differentiating.

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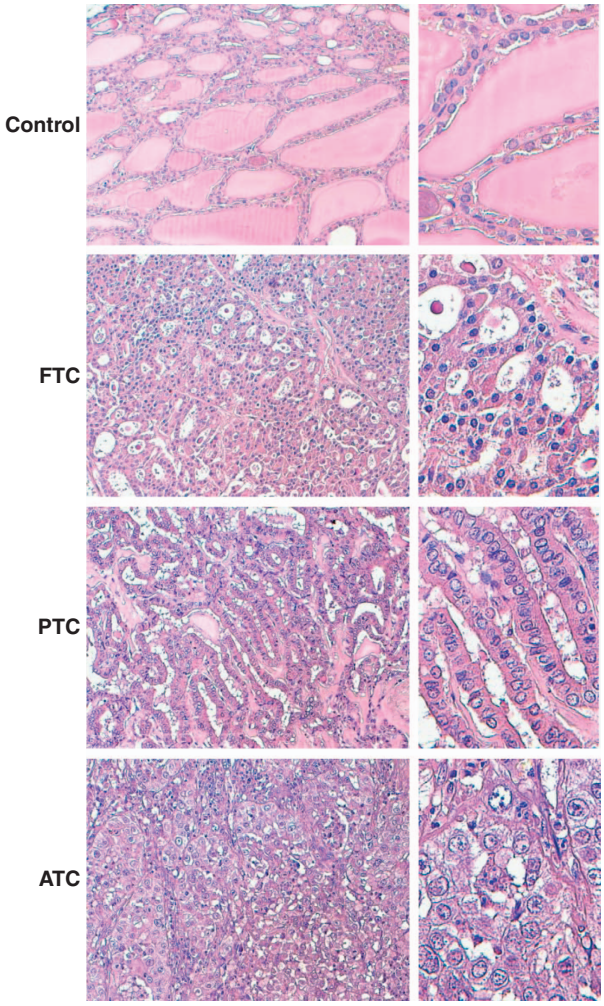

Figure 1. Epithelial thyroid cancer histotypes. Histochemical analysis on paraffin-embedded sections of human thyroid gland control (Control), FTC, PTC and ATC.

This model is sustained by several lines of evidence. Exposure to the radioactive-iodine released after the Chernobyl nuclear accident resulted in a higher incidence of PTCs among infants and young children rather than adults.[21] Furthermore, thyroid-targeted transgenic *RET/PTC1* mice developed PTC and hypothyroidism following the inhibition of thyroid cell differentiation.[7,22]

The third model concerns the so-called cancer SCs (CSCs), a minute population at the apex of a hierarchical pyramid including increasing numbers of progenitors and more differentiated cells. This CSC model is based on the assumption that only a small subset of cells possesses the ability to initiate and spread the tumour. These cells can arise after genetic and epigenetic alterations occurring either in normal SCs or in progenitor/precursor cells that could acquire self-renewal potential in a sort of reprogramming process.[7,23]

Here, we describe the potential role of SCs in thyroid pathogenesis in detail and we address the hypothesis of their possible involvement in metastasis development.

NORMAL THYROID STEM CELLS

SCs are undifferentiated cells characterised by the extraordinary ability to self-renew and give rise to many types of specialised cells.[24] They mostly divide asymmetrically to generate two different daughter cells, one identical to the mother and one more differentiated that will eventually give rise to specialised cells. This mechanism allows to maintain control over the SC pool preserving tissue homeostasis.[25] The protective microenvironment created by the SC niche provides the necessary stimulatory and inhibitory signals for the maintenance of the stemness state.[26] Under particular circumstances requiring the expansion of the SC pool, SCs undergo symmetric division, producing two identical daughter cells able to self-renew and generate a differentiated progeny.[27]

SCs are usually classified into three major groups according to their development potential: embryonic SCs (ESCs), adult SCs (ASCs) and fetal SCs (FSCs).[24] The fate of SCs is influenced by specific signals coming from the microenvironment niche and can be manipulated *in vitro* to form differentiated lineages. ESCs represent a potential source of derived thyroid cells that have a superior predilection to mutate and hence initiate thyroid diseases. During the early stages of embryonic development, visceral endoderm gives rise to extra-embryonic endoderm. Whereas, anterior definitive endoderm migrates to form the primitive gut (foregut, midgut and hindgut). Follicular cells develop from the anterior foregut endoderm and are clearly different from C cells of neuroectodermal origin. Therefore, thyroid cell lineages formed by ESC-derived endoderm could be considered an *in vitro* experimental model adopted for the study of thyroid development.[28–30] Thyroid growth requires a complex network of transcription factors, including Pax-8, thyroid transcription factors 1 (TTF-1, also known as homeobox protein Nkx-2.1) and 2 (TTF-2, also known as forkhead box protein E1, Foxe1), as well as hematopoietically expressed homeobox protein HHEX (Table 1). Each of these factors exhibits distinct roles within different embryonic tissues, but their specific cooperation in progenitor cells is fundamental to uniquely drive thyroid organogenesis.[31] Specifically, TTF-1 and Pax-8 are needed for precursor survival, while TTF-2 promotes their migration. Meanwhile, HHEX works to maintain the expression of these three factors and prevent differentiation.[30] In addition, the surrounding mesenchyme, contributes to expand the pool of thyroid cell progenitors through the release of pro-epidermal growth factor and basic fibroblast growth factor (bFGF, or FGF-2).[31,32] As discussed below, the co-expression of TTF-1 and Pax-8, is an essential event for cell commitment towards follicular cell fate.[33] While, FGF and bone morphogenetic protein (BMP) signalling pathways act in the early stages of thyroid development[34] before TSH/TSH-R and IGF/insulin signalling that promote the full differentiation of follicular cells (Figure 4 and Table 1).[35]

In order to identify early and late markers of thyroid development, several studies are being carried out using mouse ESCs as a source of *in vitro* differentiating thyrocytes. Lin *et al*.[36] demonstrated that mouse ESCs formed embryoid bodies that upon differentiation, express a set of thyroid-specific markers, such as Pax-8, thyroid peroxidase (TPO), Tg, sodium/iodide cotransporter (NIS) and TSH-R. In absence of serum, these differentiated cells did not express Tg. TSH treatment was necessary to maintain Pax-8 and TSH-R expression during embryoid body differentiation, but it was not sufficient to express Tg. This suggests that other factors, besides TSH, are required for long-term maintenance and the maturation of thyrocytes.[36] Later, TSH and activin A, a transforming growth factor β (TGF-β) family member, were proposed as important factors in endoderm specification and thyrocyte differentiation, while insulin and IGF-I promoted the long-term maturation of thyrocytes expressing Tg, NIS and TSH-R.[37] According to these data, TTF-1, TTF-2, and Pax-8 can be considered specific markers of thyroid SC/progenitors, TSH-R a marker of precursor cells, Tg, TPO and NIS markers of fully differentiated thyroid follicles (Figure 4 and Table 1).

Recently, Antonica's research group demonstrated the differentiation of murine ESCs into the thyrocytic lineage, with the

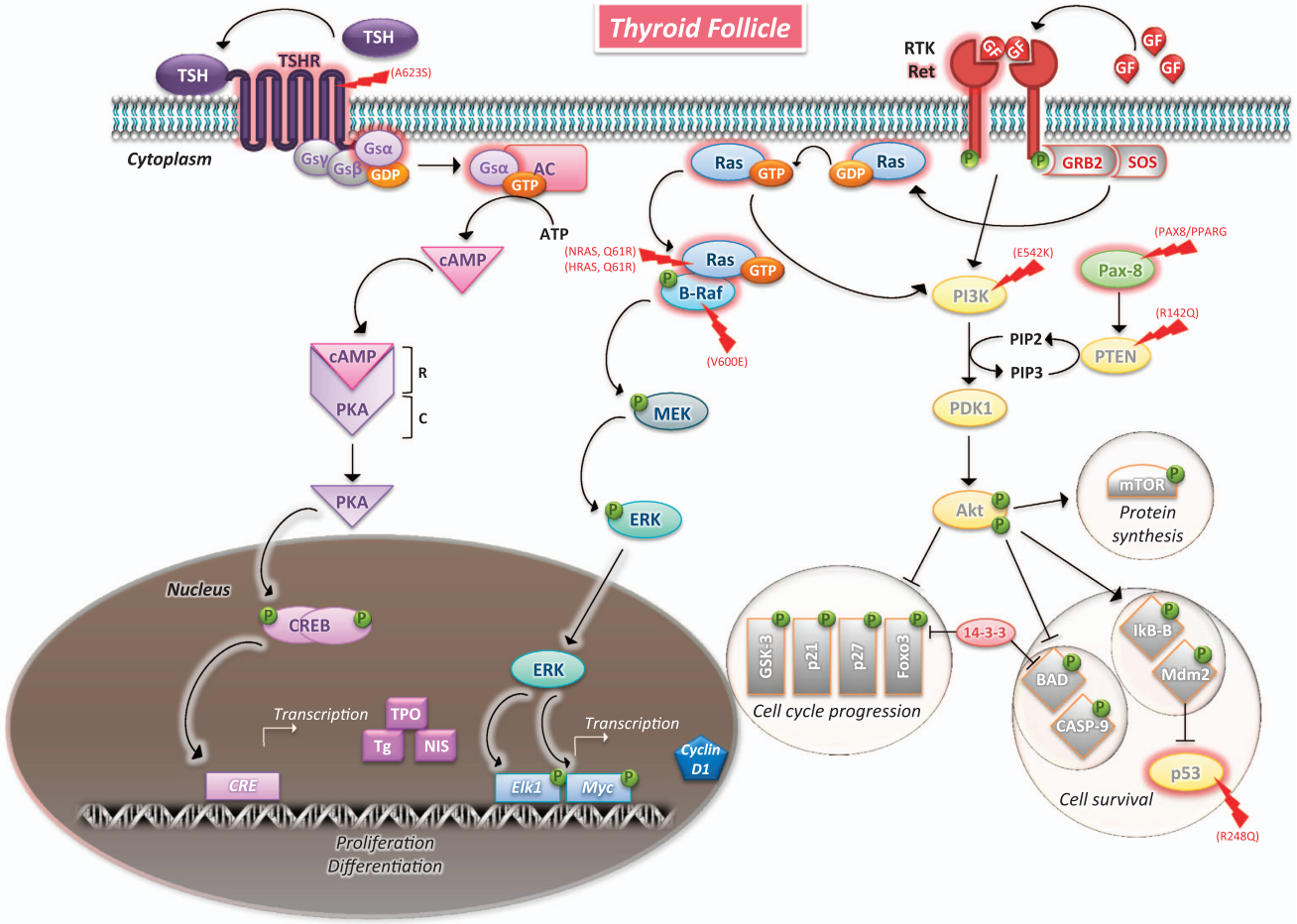

Figure 2. Thyroid follicular cell. The main regulators of thyroid function are TSH signalling through cAMP-dependent pathway, and growth GFs, acting via MAPK and PI3K/Akt signalling pathways. Alterations in key factors of these pathways (indicated in red) could lead to thyroid transformation: gain-of-function, mutations in TSH-R and Gsα encoding genes could result in benign lesions such as hyperfunctional adenomas. *RAS* and *BRAF* mutations in MAPK pathway are frequently observed in PTC. Genetic alterations in genes associated with the PI3K/Akt pathway, i.e., *PAX8* (40%) and *TP53*, are involved in FTC and ATC, respectively. The frequency of BRAFV600E in thyroid carcinomas is 50%. Thyroid tumours have been found to have mutations in *NRAS* (4.6%) and *HRAS* (1.2%) codon 61. Mutations in *TP53* occur in 6% of all thyroid tumours, whereas the frequency of *PTEN* and *PI3K* mutations is 3%. Genetic alterations and mutation frequency reported are referred to http://www.mycancergenome.org and http://cancer.sanger.ac.uk.

formation of thyroid follicles, able to induce iodide organification.[33] In particular, the simultaneous ectopic expression of TTF-1 and Pax-8 induced the *in vitro* differentiation of mouse ESCs into follicular cells. After treatment with TSH, these cells showed molecular, morphological and functional characteristics of thyroid follicles and were able to fulfil thyroid hormone deficits when transplanted orthotopically into an immunodeficient mouse.[33] Pax-8 and TTF-1 co-expression is therefore required for assembling the follicular cells into becoming a follicle-like structure, as recently confirmed.[38]

Longmire *et al.* demonstrated that the presence of activin A led ESCs to differentiate into definitive endoderm, expressing forkhead box protein A2 (Foxa2$^+$). At this stage, if exposed to BMP and activin/TGF-β signalling inhibitors (Noggin and SB431542, respectively) for a specific amount of time, definitive endoderm was shown to be directed towards the lung/thyroid competent definitive endoderm, negative for forkhead box protein A3 (Foxa2$^+$/Foxa3$^-$). Hence, induction of BMP and FGF signalling by exposing cells to high doses of FGF-2 combined with specific factors (that is, BMP-4, Wnt3a, FGF-10, FGF-7, epidermal growth factor and heparin), may promote the initial lineage specification of endodermal TTF-1$^+$ thyroid primordial progenitors. The efficient induction of TTF1 into endodermal progenitors, is restricted by a stage-specific and time-dependent inhibition of BMP and activin/TGF-β signalling, followed by BMP reactivation and combined with FGF signalling.[34,39–41] D'Amour *et al.*[42] reported similar results using human cells, observing that in the presence of FGF-2 high-dose activin A induced a massive differentiation of ESCs into transcription factor SOX-17$^+$/Foxa2$^+$ definitive endodermal cells (Figure 4 and Table 1). Interestingly, Onyshchenko *et al.*[43] used two methods in order to differentiate human ESCs in thyroid follicular cells: (i) an one-step protocol that aims at a direct differentiation through TSH stimulation by avoiding the intermediate endoderm formation and (ii) a two-step protocol with an intermediate passage in endodermal cells, which foresees the TSH stimulation in combination with activin A and FGF-2. In both cases, we were unable to obtain an efficient generation of differentiated cells that express specific thyroid markers. This suggests that, molecular mechanisms involved in mouse and human differentiation are different, and TSH by itself is not sufficient to induce thyrocyte-like cell differentiation *in vitro*.

The thyroid is a low proliferating gland, known for its self-renewal ability.[8,16] It has been suggested that follicle regeneration is maintained by a pool of SCs that reside in the adult gland. ASCs, estimated to be ~0.1% of all thyroid cells, are undifferentiated in a quiescent or slow-cycling state and could replicate themselves to

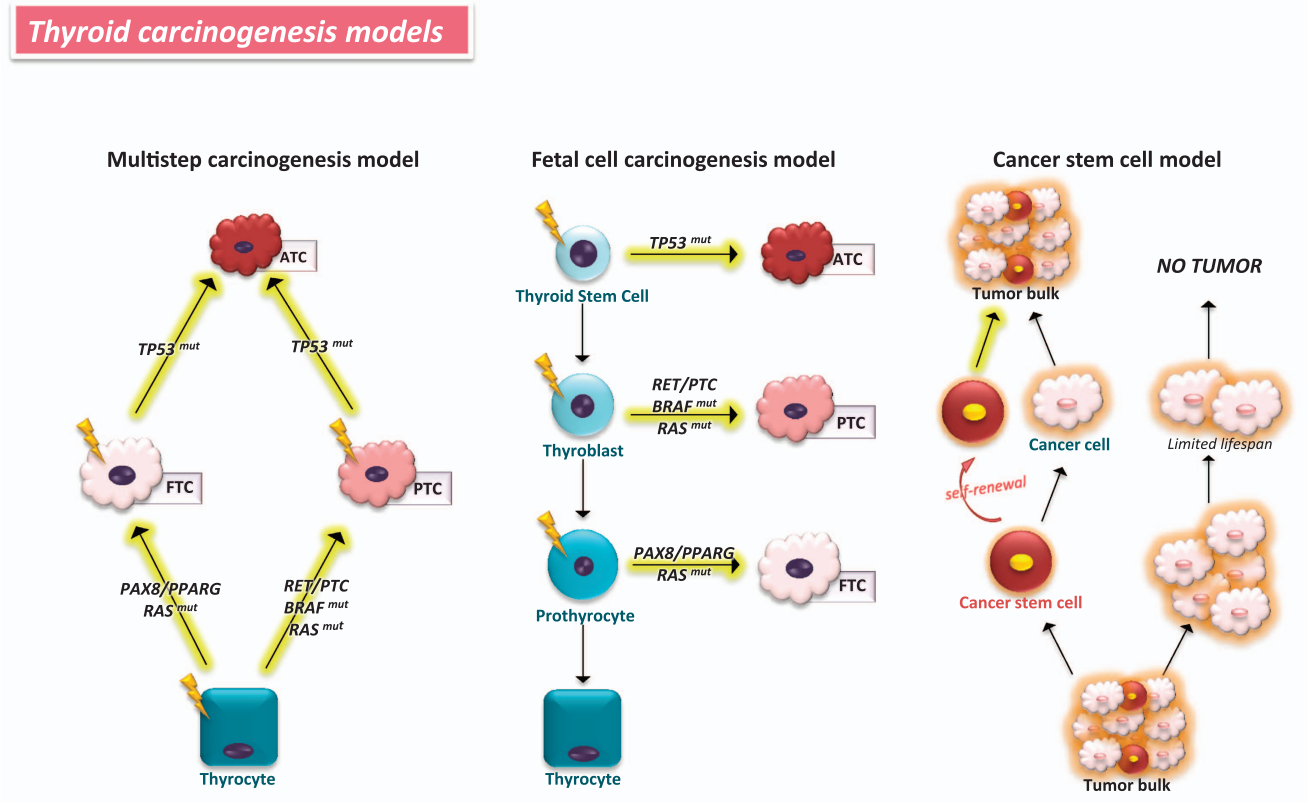

Figure 3. Thyroid carcinogenesis model. The multistep carcinogenesis model suggests that undifferentiated thyroid carcinomas derive from well-differentiated tumours via the sequential accumulation of genetic mutations and dedifferentiation process. According to the fetal cell carcinogenesis model, thyroid cancer cells originate from the transformation of three types of fetal thyroid cells—thyroid stem cells, thyroblasts and pro-thyrocytes, which would result in ATC, PTC and FTC. The CSC model assumes that only a small subset of cells possesses the ability to trigger, reconstitute and sustain tumour growth.

Table 1. Cell markers of thyroid development

Cell specialisation	*Marker*	*Function*	*References*
Thyroid competent definitive endoderm	Transcription factor SOX-17	Transcription factor required for normal development of definitive gut endoderm	32,37,39–43
	Forkhead box protein A2 (Foxa2)	Transcription factor involved in endoderm-derived organ development	32,34,37,39–43,52
Thyroid primordial progenitor	Paired box protein Pax-8 (Pax-8)	Transcription factors that have a role in the regulation of thyroid-specific genes (i.e., *TG*, *TPO*, *TSHR*), needed for the maintenance of thyroid differentiation phenotype. Their co-expression is essential for the survival and the specification towards the thyroid cell type	18,29–34,36,38,41,55
	TTF-1 or Homeobox protein Nkx-2.1		18,29–34,38,40,45,49–51
	TTF-2 or Forkhead box protein E1	Transcription factor involved in thyroid gland organogenesis, promoting cell migration	29–33
	Hematopoietically expressed homeobox protein HHEX	Transcription repressor involved in thyroid development; it maintains gene expression of *PAX8*, *TTF1* and *TTF2* in the thyroid primordium	30–32,34,40,41
Thyroid precursor	TSH-R	Central player in controlling thyroid cell metabolism through the cAMP cascade	18,29,32–34,36–38,43,54,55
Thyrocyte	Tg	Precursor of the iodinated thyroid hormones T3 and T4	29,33,34,36–38,49,50,52–55
	Sodium/iodide cotransporter (NIS)	Symporter that regulates iodide uptake	18,29,33,36–38,43,54,55
	TPO	Enzyme acting in iodination and coupling of the tyrosine residues in Tg to produce T3 and T4	18,29,36–38,43,51,54,55
Solid cell nest	Tumour protein 63 (p63)	Transcription factor implicated in epithelial morphogenesis regulation; the ratio between ΔN/TA-type isoforms may regulate the maintenance of the epithelial stem cell compartment	18,44–50,53

Abbreviations: Tg, thyroglobulin; TPO, thyroid peroxidase; TSH-R, thyrotropin receptor; thyroid transcription factor 1, TTF-1; thyroid transcription factor 2, TTF-2.

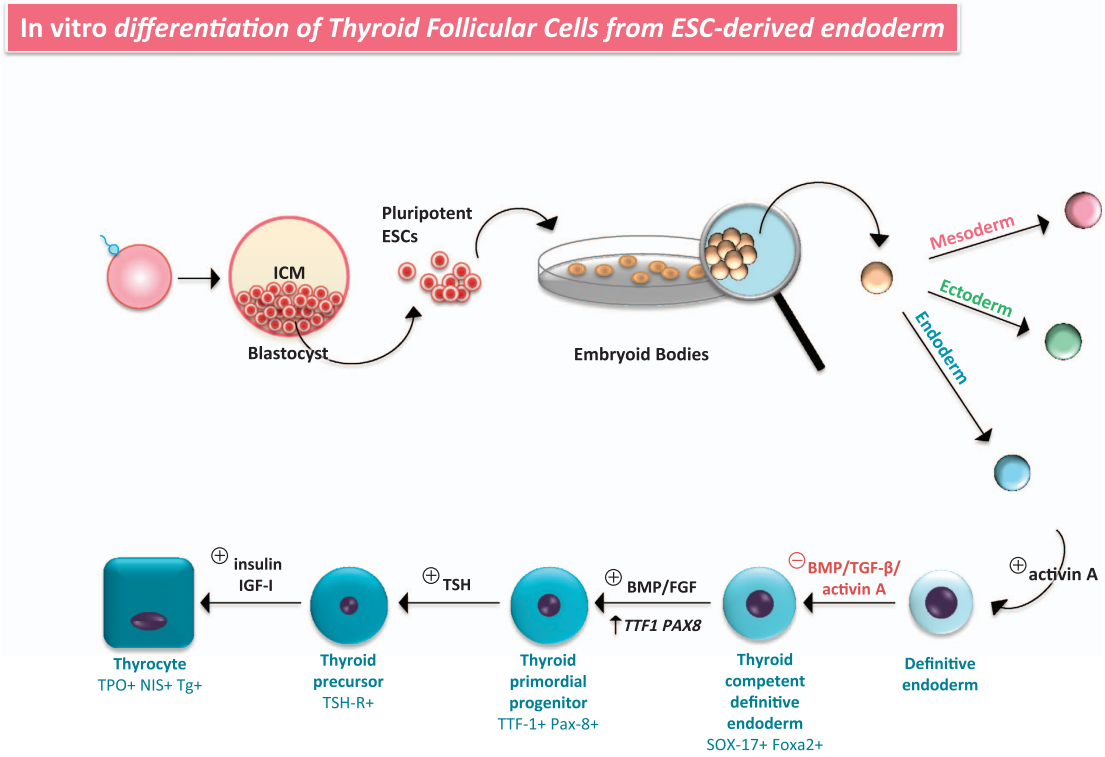

Figure 4. *In vitro* differentiation of thyroid follicular cells from ESC-derived endoderm. Blastocyst-derived ESCs form *in vitro* embryoid bodies able to generate derivatives of the three germ layers. The embryoid bodies differentiate into definitive endoderm in presence of activin A, and into thyroid competent definitive endoderm after exposition to inhibitors of BMP and activin A/TGF-β signalling. At the later stage, the concomitant induction of BMP and FGF signalling, and TTF-1 and Pax-8 co-expression, results in the generation of thyroid primordial progenitors. Thyroid precursors, derived from progenitor cells after TSH treatment, differentiate into Tg-expressing thyrocytes in presence of insulin and IGF-I.

preserve tissue turnover after damage.[17] Embryonic remnants have been identified in adult thyroid tissue as small clusters, the solid cell nests (SCNs), which could represent a potential niche of thyroid SCs defined by the expression of tumour protein 63 (p63, Table 1).[30] SNC is composed of centrally located undifferentiated $p63^-$ cells, and surrounded by $p63^+$ cells with a basal/SC phenotype clustered or structured within a single layer.[44–46] Indeed, some of its stemness features have been demonstrated: the self-renewal capability conferred via telomerase activity and the differentiation potential in more cell types, due to high p63 and Bcl-2 expression.[47,48] Reis-Filho *et al.*[49] identified the SCNs as structures composed of numerous main cells and rare C cells. Main cells are notably characterised with basal/SC marker p63, basal cytokeratins (excluding cytokeratin 20), and the carcinoembryonic antigen. In contrast, C cells lack p63 and are immunoreactive to differentiation markers, such as calcitonin and TTF-1.[49] Hence, it has been proposed that SCN main cells can be considered a pool of multipotent SCs, involved in histogenesis and self-renewal of both follicular and C cells.[47] The potential stem role of SCN main cells was supported by immunohistochemical profile studies.[44,50] Recently, Okamoto *et al.*[51] supported the involvement of SCN in thyroid regeneration, having observed a similar morphology in irregular shaped follicles generated after a partial thyroidectomy in mice. These cells were characterised as SC antigen 1 $(SCA1)^+$/$BrdU^+$/β-gal/$TTF\text{-}1^-$, and temporarily co-expressed cytokeratin 14.[52] They evolve into functional follicles, which express TPO after 120 days, indicating that they consist of newly formed follicular cells, but do not originate from differentiated thyrocytes.[51,52]

Considering the SCN as a source for both follicular and C cells, the presence of three cell types has been hypothesised: (i) progenitors of follicular cells, arisen from the base of the foregut (endodermal origin); (ii) progenitors of C cells, which originate from ultimobranchial bodies (neural crest origin); and (iii) and the follicular and C bipotential progenitor cells.[8]

In 1992, Dumont *et al.*[17] first hypothesised the existence of ASCs within the mature thyroid gland. Thomas *et al.* isolated a population of ASCs from the goitre, characterised by the co-expression of the octamer-binding protein 4 (Oct-4) stem pluripotent marker, the transcription factor GATA-4 and hepatocyte nuclear factor 4-α (HNF-4-α) endodermal markers and Pax-8. These SCs, negative for Tg, were not influenced by TSH treatment.[53] Fierabracci *et al.* obtained a $CD34^+/CD45^-$ subpopulation of cells with self-replicative potential from different human thyroid specimens, characterised by their expression of the pluripotent Oct-4 markers and homeobox transcription factor Nanog (hNanog). Under appropriate culture conditions, these cells were able to generate follicles with thyroid hormonal production.[54]

Hoshi *et al.* identified a side population (SP) able to efflux the vital dye Hoechst 33342 in a thyroid murine. It ranged from 0.3 to 1.4% of the total population and was highly enriched with stem/progenitor cell activity. According to the expression of hematopoietic markers used to define SC populations, SP cells were separated into two cellular subsets, SP1 ($CD45^-$/$c\text{-}kit^-$/$SCA\text{-}1^+$) and SP2 ($CD45^-$/$c\text{-}kit^-$/$SCA\text{-}1^-$). Both SP cells were characterised by high expression levels of ATP-binding cassette sub-family G member 2 and Oct-4, and by a low expression level of thyroid differentiation markers. In particular, SP2 cells seem to have progenitor characteristics because of their low TTF-1 and TSH-R expression that is in line with their involvement in thyroid lineage commitment. Furthermore, the putative SC marker nucleostemin was reported to be downregulated in

undifferentiated thyroid cells.[18] The SP selection method has also been used to isolate ASCs in human thyroid. Lan *et al.* showed that thyroid ASCs isolated from human goitres were able to differentiate into thyroid cells and grow either in a monolayer or embedded in collagen. Under the influence of TSH in a serum-enriched medium, isolated ASCs differentiated into thyrocytes expressing Pax-8, Tg, NIS and TPO. Moreover, when embedded in collagen, they were able to uptake iodine in response to TSH.[55] These results reinforce the reported observations regarding the ability of goitre-derived ASCs, to differentiate into thyroid cells.

THYROID CANCER STEM CELLS

It is becoming increasingly evident that tumours are organised hierarchically similarly to normal tissues, where a small subpopulation named CSCs is responsible for cancer initiation and progression. Assuming the involvement of SCs in thyroid carcinogenesis, it is necessary to clarify the SCs' dual role in the initiation and propagation of a tumour. In cancer initiation, the 'cell-of-origin' concept explains how a normal cell acquires the first mutation able to kick-start the tumour (tumour initiating cells, TICs). TICs constitute a tumour cell subset that sustains tumour growth, but does not necessarily originate from the transformation of normal SCs.[56] However, SCs are able to live for a long time, undergo self-renewal and possess the key features required for the acquisition of genetic or epigenetic changes leading to cancer development.[57] To recognise the cell-of-origin of neoplastic transformation, it is helpful to first enucleate the cellular hierarchy present within the tumour tissue. Putative cells-of-origin are committed progenitor or precursor cells, able to reacquire a SC-like phenotype and functions upon genetic or epigenetic reprogramming.[58] Early progenitors, also known as transit amplifying cells, are facilitated in the acquisition of modifications that drive reprogramming, due to their elevated proliferative capacity and undifferentiated state. Among the TIC population, transit amplifying cells have a predominant role in primary tumour formation, but only a small fraction of them shows detectable self-renewal and metastasis-forming potential. However, initiation and maintenance of the tumour seem to be dynamic processes, characterised by the transition between the self-renewing and transient amplifying phenotypes (Figure 5).[59,60] CSCs are identified due to their ability to self-renew and are represented by: (i) long-term TICs (LT-TICs) able to maintain tumour formation after serial xeno-transplantations and involved in metastasis formation; (ii) delayed contributing TICs (DC-TICs), active only in secondary or tertiary tumour xenografts (Figure 5).[59] The peculiar feature of CSCs concerns the pronounced tendency to undergo symmetric division as compared with normal SCs. This drift is driven by major genetic and epigenetic events conferring unlimited lifespan and to CSCs, which are ultimately responsible for tumour growth and progression.[61] In studying CSCs in the thyroid, several research groups have developed specific methods to isolate TICs from this gland (Table 2). In this overview, we do not take into consideration those studies or single results obtained using ARO, NPA and KAT-4 cell lines, being that they were found not to be of thyroid origin.[62]

Injecting putative CSCs into immunocompromised mouse models[63–65] and following the tumour development constitutes an univocal way of testing their effective tumourigenicity.[66] It has been observed that serial transplantation led to an *in vivo* selection of cells able to generate more aggressive tumours.[67] In particular, transplantations of cells, isolated from secondary and tertiary xenografts, permit to define their long-term tumourigenic potential, as well as their self-renewing ability (Figure 5).

High level of aldehyde dehydrogenase (ALDH) activity is present in stem and progenitor cells, thus it has been used as a functional marker for CSCs isolation in tumours.[30,68] Todaro *et al.* showed that PTC, FTC and ATC contain a small population of tumourigenic cells with high ALDH activity and unlimited replication potential. Expanded indefinitely *in vitro* as tumour spheres, these ALDH^{high} cells contain 25–60% of the clonogenic cells able to generate serial tumour xenografts. Interestingly, the highest percentage (60%) of clonogenic cells present in ATC, defined the cellular subset endowed with a boosted self-renewal activity and a tumourigenic and metastatic potential. This suggests that ATC cells undergo multiple rounds of symmetric division.

In line with this, the injection of ALDH^{high} cells derived from FTC led to a moderately invasive tumour without distant metastasis. Whereas, ALDH^{high} cells derived from ATC, are able to invade adjacent tissues and produce lymph nodes and lung metastasis because of a strong constitutive activation of c-Met/Akt pathways.[63] Elevated ALDH activity was also identified in ATC cell lines: 17–38% of ATC-8505C[30] and 8–13% of SW1736[69] were ALDH^{high}.

hNanog, Oct-4 and transcription factor SOX-2 (encoded by *NANOG*, *POU5F1* and *SOX2* genes, respectively) are transcription factors that establish SC features through a pluripotent regulatory network.[70] Detection of high levels of these transcription factors, together with low or absent markers of thyroid differentiation, can help identify putative thyroid CSCs (Figure 6).[30,63,64,71–73] Such identification can be supported by the analysis of biomarker expression belonging to self-renewing control pathways, such as Wnt/ β-catenin, Sonic hedgehog protein and Notch1 (classified also as epithelial–mesenchymal transition (EMT)-inducing signalling pathways).[74] Malaguarnera *et al.*[75] reported that the self-renewal capacity of thyrospheres is also sustained by the over-expression of the insulin receptor and IGF signalling pathway genes (IGF-I receptor, *IGF-I, IGF-II*) promoting their volume, growth and survival.

Recently, Ahn *et al.* identified a small percentage of CD44^{high}/CD24^{low} cells with tumourigenic capability in the papillary TPC1 cell line, in six human primary PTCs and in four metastatic lymph node specimens, as well as in two PTC-derived ATC samples. Notably, CD44^{high}/CD24^{low} cells showed a higher Oct-4 expression and lower differentiation marker expression than CD44^{high}/CD24^{high} cells, together with the ability to form thyrospheroids. However, no tumour was detected after the inoculation of CD44^{high}/CD24^{low} human specimens into athymic Ncr-*nu/nu* mice.[65]

Different research groups reported controversial results regarding CD133 (also known as Prominin-1) in TC. In anaplastic KAT18 and FRO cell lines, Zito *et al.*[76] did not find CD133^{+} cells; Friedman *et al.*[71] identified CD133^{+} in 6.32% FRO cells, but not in the papillary TPC cell line. Recently, Ke *et al.*[77] described $< 5\%$ CD133^{+} cells in papillary CG3 and follicular WRO and CGTH cell lines. By using primary tumours, Malaguarnera *et al.*[75] and Tseng *et al.*[73] identified a CD133^{+} subpopulation in PTC samples, while it was absent in thyroid spheres derived from PTC, FTC and ATC samples analysed by Todaro's research group.[63] Immunohistochemical examinations reported an elevated expression of CD133 in ATC paraffin-embedded tissue sections,[71,78] but only a low variable expression in FTC and PTC adjacent to ATC samples.[78] Besides the inconsistencies in the reported results, another limit of this marker is that its expression is greatly influenced by the cell cycle phase.[79]

SCs are enriched in the SP compartment. However, dye efflux is not a characteristic common to all SCs, and this feature is not limited only to the SC phenotype. This method seems to be associated with a toxic effect on cells.[80] Mitsutake *et al.*[74] found a very small portion of the SP in follicular WRO (0.02%) and anaplastic FRO (0.1%) human cell lines, but not in papillary TPC1. After sorting, these cells showed a higher clonogenic ability than those referred as main population (MP), and were tumourigenic after being injected into nude mice. The two subpopulations showed a different gene expression profile. Microarray experiments revealed an upregulation of ABCG2, Wnt (*MYC*, *JUN*, *FZD5*) and Notch1 pathway genes (*HES1* and *JAG1*) in SP cells. Following

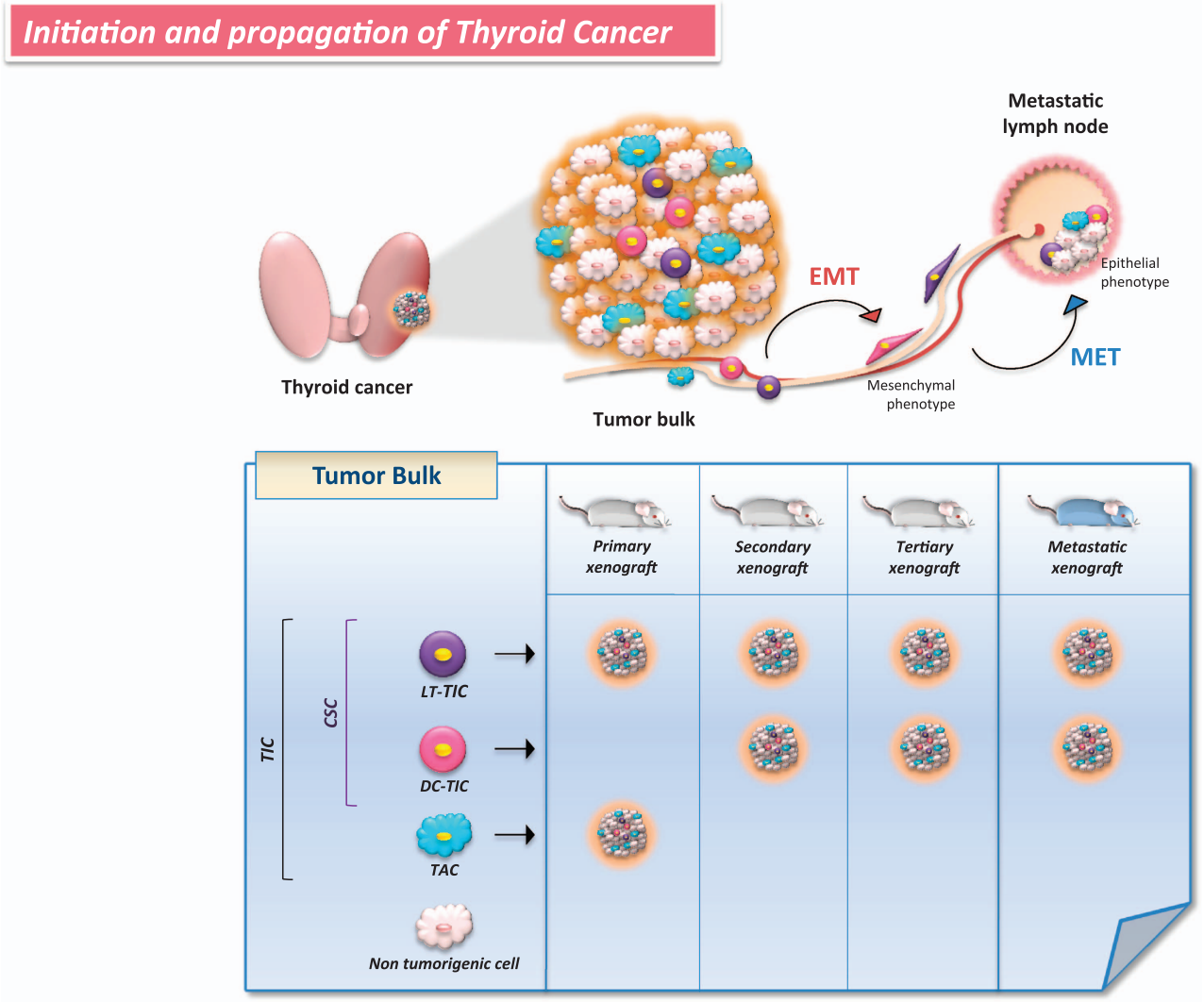

Figure 5. Initiation and propagation of thyroid cancer. Tumour bulk consists of tumourigenic and non-tumourigenic cells. Tumourigenic cells include TICs able to start the tumour; among these, long-term (LT-TIC) and DC-TIC maintain tumour formation after xenograft-transplantation and have characteristics of cancer stem-like cells. Although transit amplifying cells massively contribute to tumour formation, they lack in self-renewal and metastasis-forming features. Some TICs, such as LT-TICs and DC-TICs, are also responsible for tumour dissemination, a mechanism regulated by EMT, whereas the inverse MET drives metastatic colonisation.

10 days *in vitro* expansion, the SP cells reconstituted the full cell population (both SP and MP). MP also generated a small SP, and was tumourigenic in nude mice. This suggests that the SP subset constitutes only a part of the entire CSC component. CSCs are more numerous in the SP, but the two subsets are not identical.[74] Zheng *et al.* identified 0.41–0.83% of the SP in ATC cell lines (SW1736, 0.41%; C643, 0.52%; HTh74, 0.83%) expressing ATP-binding cassette sub-family G member 2 and multidrug resistance protein 1 transporters, which survived a doxorubicin treatment. These cells showed a 10-fold higher clonality and higher invasive features in comparison to the MP. In a 6 months treatment, doxorubicin gradually killed the MP, yet the SP, enriched with Oct-4$^+$ CSCs, constituted 70% of resistant cells. The authors found a composition of thyrospheres in 5% of the SP and $>95\%$ of the MP by FACS analysis.[72] The SP identified in TCs is then characterised by an over-expression of stemness markers and higher clonogenic ability, supported by thyrospheres formation and reconstitution of the MP. Moreover, it displayed chemo-resistance and tumourigenic potential when injected into immunocompromised mice.

THYROID CSCs AND METASTASIS

As observed in the experimental xenograft assay described above, CSCs possess the capacity to seed new tumours when implanted in appropriate animal hosts. This is theoretically analogous to tumour initiation by disseminating tumour cells (DTCs), whose success depends on their ability to spawn an unlimited number of daughter cells (Figure 4). In light of their motility, invasiveness and resistance to apoptosis, CSCs are central players in tumour recurrence and metastasis formation.[81–83] This model is supported by the expression of EMT markers in CSCs and by the activation of SC markers in EMT-induced cells.[84,85]

DTCs are considered the major cause of metastatic disease, chemo-resistance and recurrence, and are characterised by the capacity to migrate from primary tumours to secondary sites. Therefore, DTCs can exist for long time in a quiescent state called dormancy, corresponding to the latent period between primary tumour detection, treatment, recurrence and metastatic spread.[86] The molecular mechanism, responsible for the transition of DTCs from a dormant to a proliferative state, involves a cross-talk between DTCs and the extracellular matrix. Matrix

Table 2. Biological methods for thyroid CSCs isolation and characterisation

Isolation method	*Description*	*Integrative assay*	*References*
Sphere-forming assay	In serum-free non-adherent condition, it allows the purification of CSC population from differentiated thyrocytes and fibroblast, through a negative selection	Clonogenic assay; limiting-diluting assay; colony-forming assay; proliferation and division assay	55,63–65,69,72,73,75
Xenografts assay	The injection of CSCs in immunodeficient mice (NOD/SCID, NSG, nude) led to a tumour formation. Serial transplantations permit the selection of CSCs able to generate more aggressive tumours	Lineage tracing; limiting-dilution injections	63–65
ALDH activity (ALDEFLUOR)	Isolate CSCs based on their elevated ALDH activity: $ALDH^{high}$ cells metabolise their substrate in a fluorescent dye that permits a positive FACS selection		30,63,69
Stemness biomarkers	FACS positive selection based on: ✔*SC transcription factors*: hNanog, Oct-4, transcriptor factor SOX-2 protein ✔*EMT-inducing pathways*: Wnt, Notch1, Sonic hedgehog protein ✔$CD44^{+}/CD24^{-}$ *phenotype* ✔*CD133*	FACS negative selection for differentiation markers: TPO, Tg, NIS, TSH-R	30,63–65,69,71–75,77,78
Side population	Positive selection for drug-resistance based on ABCG2 and ABCB1 activity; these transporters pump out the drug rendering the dye efflux sensitive to verapamil (a Hoechst 33342 inhibitor)		18,72,74

Abbreviations: ALDH, aldehyde dehydrogenase; CSCs, cancer SCs; EMT, epithelial–mesenchymal transition; FACS, fluorescence activated cell sorting; NOD/SCID, non-obese diabetic/severe combined immunodeficiency; NSG, NOD scid gamma; SC, stem cells; Tg, thyroglobulin; TPO, thyroid peroxidase; TSH-R, thyrotropin receptor.

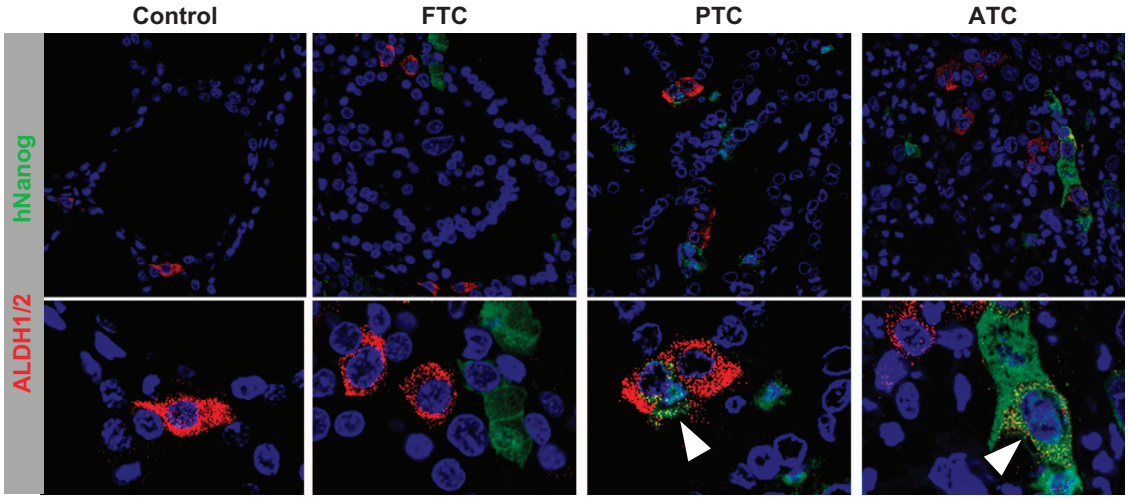

Figure 6. Thyroid CSCs markers. Immunofluorescence analysis of ALDH1/2 and hNanog in control, FTC, PTC and ATC. Arrow heads indicate cells co-expressing ALDH1/2 and hNanog.

metalloproteinases, secreted by stromal cells, induce the switch from tumour dormancy to metastatic growth and vice versa, in some cases through a permissive niche generation. Moreover, integrin signalling regulation directs this mechanism through the extracellular signal-regulated kinase ½ (ERK-1/2) and p38α/β pathways; in particular, high ERK-1/2:p38 signalling ratio promotes primary tumour proliferation and metastatic disease, whereas the opposite determines cellular dormancy.[86,87]

It has become evident that aberrant activation of β-catenin and c-Met is involved in TC progression.[88] Data reported by Todaro *et al.* indicated that Akt, c-Met and β-catenin activation correlates with invasive behaviour of ATC SCs, together with the complete loss of E-cadherin expression. Interestingly, targeted silencing of Akt and c-Met expression, reduced Twist and Snail expression, and abrogated thyroid CSC invasiveness and metastatic capacity.[63]

Other studies identified EMT regulators in TC, with different expression among histotypes. Hardy *et al.* showed decreased E-cadherin expression in differentiated TCs when compared to normal tissues. While *SNAI1* and *SNAI2* were aberrantly transcribed and expressed in follicular (FTC-133), papillary (BCPAP, K1), and anaplastic (CAL-62, 8305C) cell lines, as well as in human cancer samples, in line with the lack of close cellular contact.[89] Vasko *et al.*[90] observed an over-expression of the mesenchymal marker vimentin in PTC human samples, associated with invasion and lymph node metastasis. Moreover, Riesco-Eizaguirre *et al.*[91] reported an over-expression of TGF-β at the invasive front of PTC, suggesting that PTC cells need to undergo EMT and subsequently mesenchymal–epithelial transition (MET), to produce metastasis. In another study, Liu *et al.* found an intense expression of nestin, CD133 and CD44 and an absence of E-cadherin expression in ATC.

Contrarily, PTC and FTC samples showed variability in CD133 and CD44 detection, and were negative for nestin, but positive for E-cadherin.[78]

By tissue microarray analysis, Buehler *et al.* observed that only ATC samples showed a high expression of Snail and Twist associated to a lack of E-cadherin. Otherwise, PTC, FTC and normal specimens were negative for Snail and Twist and, with strong diffused immune reactivity to E-cadherin.[92] Lan *et al.* observed that HIF-1α induced invasive and metastatic properties in follicular cells (FTC-133) through EMT, as confirmed by the downregulation of E-cadherin and upregulation of vimentin. Moreover, it was observed that cells with an over-expression of HIF1α shared stem-like cell features highlighting that EMT induction was directly associated with increased CSC populations.[84]

Several lines of evidence also supported the cross-talk between Twist and ID proteins. Kebebew *et al.*[93] observed over-expression of the DNA-binding protein inhibitor ID-1 in ATC tissues, reporting that the inhibition of ID1 mRNA expression results in decreased growth and reduction of Tg and NIS expression. Moreover, Ciarrocchi *et al.*[94] disclosed that *in vivo* ID-1 expression is associated with aggressiveness and metastatic potential in non-anaplastic tumours, whereas another member of the ID proteins, ID-3 is downregulated in PTC and may be related to the TSH-induced differentiating process.[95]

CONCLUSIONS AND FUTURE PERSPECTIVES

Evidence shows that many pathways underlying the properties of SCs are involved in cancer initiation and progression. A defect in DNA stability is the early event that occurs in tumour transformation followed by a loss of tumour suppressor gene or oncogene activation. Herewith, long-lived SCs rather than short-lived differentiated cells could be the cell compartment in which genetic alterations promote an advantage in clonal propagation. Due to genetic alterations in thyroid cells bearing embryogenesis-associated pathways, transformed SCs represent the undisputed protagonists in the development of TC. In this scenario, further insights into thyroid SC biology could bring to light the molecular mechanisms driving transformation of normal vs cancer SCs and may help design effective anti-cancer strategies.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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