Thyroid cancer is one of the most rapidly increasing malignancies. The reasons for this increase is not completely known, but increases in the diagnosis of papillary thyroid microcarcinomas and follicular variant of papillary thyroid carcinomas along with the enhanced detection of well-differentiated thyroid carcinomas are probably all contributing factors. Although most cases of well-differentiated thyroid carcinomas are associated with an excellent prognosis, a small percentage of patients with well-differentiated thyroid carcinomas as well as most patients with poorly differentiated and anaplastic thyroid carcinomas have recurrent and/or metastatic disease that is often fatal. The cancer stem-like cell (CSC) model suggests that a small number of cells within a cancer, known as CSCs, are responsible for resistance to chemotherapy and radiation therapy, as well as for recurrent and metastatic disease. This review discusses current studies about thyroid CSCs, the processes of epithelial-to-mesenchymal transition (EMT), and mesenchymal-to-epithelial transition that provide plasticity to CSC growth, in addition to the role of microRNAs in CSC development and regulation. Understanding the biology of CSCs, EMT and the metastatic cascade should lead to the design of more rational targeted therapies for highly aggressive and fatal thyroid cancers.

Key Words
- thyroid cancer
- cancer stem cells
- EMT
- signal pathway
- microRNA
- microenvironment

Introduction

Thyroid cancer is one of the most rapidly increasing malignancies in the USA (Siegel et al. 2013). Although most well-differentiated thyroid cancers have a good prognosis, a small percentage of these tumors are associated with recurrence and metastatic disease. Multidisciplinary treatment strategies are used for thyroid cancer, including surgical resection, targeted or cytotoxic chemotherapy, radiation therapy, or a combination of different treatments (Schneider & Chen 2013). A small percentage of patients with well-differentiated thyroid cancers develop rapidly progressive disease, which is resistant to treatment. While patients with anaplastic thyroid carcinomas (ATC) usually succumb to their disease within a year after diagnosis regardless of the treatments used.

One model that may explain the aggressive behavior of some thyroid cancers, such as anaplastic cancer, the development of recurrent disease and metastases after surgery, and the resistance of some thyroid cancers to chemotherapy and radiation therapy involves the existence of cancer stem-like cells (CSCs). The CSC model (hierarchical model) postulates that a small population of
cells in the cancer is responsible for tumor initiation, growth, and recurrence (Reya et al. 2001, Hardin et al. 2013). Such cells are thought to exist in tumors as a distinct subpopulation and cause relapse and metastasis by giving rise to new tumors. Therefore, development of specific therapies targeting CSCs should improve survival and the quality of life of cancer patients with aggressive thyroid cancer (Fig. 1). This model agrees with the fetal cell carcinogenesis of the thyroid, theory of Takano, which suggests that stem cells give rise directly to well-differentiated and undifferentiated thyroid carcinomas (Takano 2007).

**Figure 1**
Cancer stem-like cell (CSC) model in which normal stem cells could undergo transformation to CSCs. CSCs give rise to other CSCs and non-CSCs and a clinically evident cancer is present in the thyroid. The mechanism of transformation is still unknown. Thyroid cancer such as an anaplastic thyroid carcinoma, consisting of CSCs and non-CSCs, can be treated by conventional therapy including surgery, chemotherapy and radioactive iodine. However, the CSCs in the tumor are resistant to conventional therapy such as surgery and radioactive iodine treatment (Sipos & Mazzaferri 2010) and the patient usually has a relapse with re-growth of the cancer, including the CSCs. CSC-specific therapy such as drugs targeting the Notch pathway (Takebe et al. 2014) or the evaluation of MEK and JNK pathway inhibitors as therapeutic agents (Balko et al. 2013) needs to be developed, which would lead to the elimination of the CSCs. If this therapy is combined with conventional therapy it may be possible to eradicate even highly lethal cancers such as anaplastic thyroid carcinomas.
Thyroid stem cells and CSCs identification

The precise tissue microenvironment of thyroid CSCs remains unknown. However, possible precursor cells for thyroid CSCs include solid cell nests (SCNs) in the thyroid, which arise from the ultimobranchial bodies during development. SCNs have been proposed as putative stem cells in the normal thyroid. SCNs in the human thyroid gland are single or multiple clusters of two cell types, referred to as main cells and C cells (Harach et al. 1993, Cameselle-Teijeiro et al. 1995). SCNs harbor the minimal properties of a stem cell phenotype which have capacity for both self-renewal conferred by telomerase activity and differentiation to one or more specialized thyroid cells. Immunohistochemical features of SCNs include high expression of p63 and BCL2, negative immunostaining for thyroglobulin and calcitonin, and variable expression of TTF1. SCNs may represent a pool of stem cells in the adult thyroid gland (Reis-Filho et al. 2003, Preto et al. 2004, Asioli et al. 2009, Rios Moreno et al. 2011). In fact, in mixed follicles, the continuity between main cells and follicular cells was considered to indicate that the latter are produced through a maturation process of the main cells of SCNs (Cameselle-Teijeiro et al. 2005). A histogenetic relationship between SCNs and some thyroid carcinomas has been widely debated (Ozaki et al. 1994, Burstein et al. 2004, Reimann et al. 2006). Burstein et al. proposed that p63-positive embryonal remnants rather than mature follicular cells are the cells of origin for a subset of papillary carcinomas. They considered these p63-positive cells to be pluripotent and remained undifferentiated or underwent benign squamoid or glandular maturation. It was also suggested that they may undergo thyroid follicular epithelial differentiation and subsequent oncogenic changes leading to papillary carcinoma. A large number of histopathological observations have suggested that ATC arises by dedifferentiation from well-differentiated thyroid carcinomas, including papillary thyroid carcinoma (PTC) and follicular thyroid carcinoma (FTC), because of the existence of well-differentiated carcinomas and ATC in the same sections of tumors (Aratake et al. 2006, Albores-Saavedra et al. 2007). When PTC and ATC occur together, they consistently share the same BRAF profile, supporting the notion that many ATC actually represent tumor progression from a pre-existing well-differentiated thyroid carcinoma (Begum et al. 2004). One problem with the SCN hypothesis is that there are no in vitro studies that have demonstrated that SCN can actually differentiate toward specific cell types under the influence of growth factors, such as transforming growth factor β (TGFβ) or epidermal growth factor (EGF), that are highly expressed in the thyroid gland. Another challenge is the identification of a thyroid CSC niche or microenvironment that would be the milieu in which thyroid stem cells or thyroid CSCs normally reside in the thyroid gland.

Thyroid CSC biomarkers

In vitro studies with specific biomarkers have been used to isolate and characterize CSCs derived from thyroid cancers. These include the Hoechst 33342 stain to characterize side populations (SP; Hoshi et al. 2007), the ALDEFLUOR assay to identify and isolate individual CSCs, CD133 cell surface immunoreactivity, and sphere-forming assays. The Hoechst SP was originally developed during cell cycle analysis of hematopoietic cells using the cell-permeable DNA-binding dye Hoechst 33342. When these SP cells were sorted and transferred into irradiated immune-deficient mice, 100 or fewer of these cells could reconstitute all hematopoietic lineages-expressed cell surface markers of stem cells and progenitors, including SCA1 (ATXN1) and cKIT, and were negative for mature lineage markers. Collectively, this evidence suggested that the SP cell population represented stem cells or progenitor cells (Goodell et al. 1996). However, functional studies using Hoechst staining are limited because of the toxicity of this agent. Consequently, if Hoechst-positive cells do not grow in vivo or in vitro, the reason could be a direct toxic effect of the dye, raising doubts on the reliability of the experiments (Pearce & Bonnet 2007). Thus SP technique is no longer the preferred approach for stem cell studies.

The ALDEFLUOR assay is a fluorescent reagent system, which uses the activity of the detoxifying enzyme aldehyde dehydrogenase (ALDH) to identify and isolate ALDH-expressing cells. It was originally used for the isolation of hematopoietic stem cells (Cheung et al. 2007) and is now commonly used for the isolation of CSCs from many cancers, including lung, liver, bone, colon, pancreatic, prostate, head and neck, bladder, thyroid, brain, melanoma, and cervical (Visus et al. 2007, Matsui et al. 2008, Charafe-Jauffret et al. 2010). A growing body of evidence suggests that ALDH activity is a common CSC marker. However, the cause of ALDH activity may differ. Importantly, identification of specific ALDH isoforms prevalent in certain cancers may have critical prognostic applicability. The ALDH enzymes are a family of evolutionarily conserved enzymes comprising 19 isoforms that are localized in the cytoplasm, mitochondria,
or nucleus. ALDHs are responsible for oxidizing aldehydes into carboxylic acids (Marchitti et al. 2008). A few of the isoforms (ALDH1A1, ALDH1A2, ALDH1A3, and ALDH8A1) function in retinoic acid (RA) cell signaling via RA production by oxidation of all-trans-retinal and 9-cis-retinal. This function has been linked to the ‘stemness’ characteristics of CSCs (Sophos & Vasiliou 2003, Ginestier et al. 2009). High ALDH1 activity is associated with several types of murine and human hematopoietic and neural stem and progenitor cells. ALDEFLUOR labeling has been recently used to isolate stem and progenitor cells from thyroid tissues. Thyroid cells with high ALDEFLUOR activity possess the ability to self-renew and reinitiate serial transplantable tumors that recapitulate the phenotype and metastatic behavior of parental tumors (Todaro et al. 2010). However, in a recent report epithelial-to-mesenchymal transition (EMT) and CSC-like properties were induced by SNAIL in ALDEFLUOR-negative thyroid cancer cells (Yasui et al. 2013). These investigators used the ACT-1 anaplastic thyroid cell line and transfection with SNAIL to induce EMT. The ACT–SNAIL cells showed enhanced tumor formation ability in an in vitro sphere assay, but not in vivo subcutaneous tumor growth assay. The cells also showed comparable chemosensitivity as the parental ACT-1 cells. The authors noted that although in vitro sphere formation in the ALDEFLUOR-positive cells was almost unchanged after SNAIL induction, overexpression of SNAIL induced more spheres in ALDEFLUOR-negative cells, suggesting that ALDEFLUOR was no longer a CSC marker in ACT–SNAIL cells.

A few recent studies provide evidence that the ALDH activity as measured by ALDEFLUOR assay is not necessarily due to ALDH1A1 alone (Levi et al. 2009, Rovira et al. 2010). ALDH1A1 deficiency did not reduce ALDEFLUOR activity of hematopoietic stem cells. One or more of the ALDH isoforms may be responsible for the ALDEFLUOR activity and this activity likely varies depending on cancer type and the tissue/cell of origin. For example, ALDH1A1 is predominantly expressed in the epithelium of testis, brain, eye, liver, kidney, and neural and hematopoietic stem cells. Whereas ALDH1A3 is reported to be expressed in the kidneys, salivary glands, stomach, fetal nasal mucosa, and breast stem cells (Marchitti et al. 2008). Intracellular ALDH activity is emerging as an important and reliable universal CSC marker applicable for most cancers. However, while measuring ALDH activity may be an accepted method for the separation of CSC and non-CSC populations for many cancers, at the protein level, the ALDH isoform(s) responsible for ALDH activity is likely different and cancer specific.

The cell surface marker CD133, a glycoprotein also known as Prominin 1 (PROM1), is a member of pentaspan transmembrane glycoproteins which specifically localizes to cellular protrusions. CD133 is expressed in hematopoietic stem cells, endothelial progenitor cells, and many brain CSCs. CD133+ cell population, thought to be a CSC population, undergoes self-renewal, and can propagate tumors when injected into immune-compromised mice. The CD133(+) ATC cells exhibited higher radioresistance and higher expression of OCT4 (POUSF1), NANOG, SOX2, LIN28 (LIN28A), and GLUT1 (SLC2A1) in cell lines or primarily cultured PTC cells, along with lower expression of various thyroid-specific genes (Ke et al. 2013). The first report of CD133 expression in ATC (Zito et al. 2008) found that ARO/CD133(pos) cells had higher proliferation, self-renewal, and colony-forming ability in comparison with ARO/CD133(neg) cells. The KAT-4 ATC cell line also expressed CD133. Subsequent studies demonstrated that the ARO cell line and KAT-4 cell line were not authentic thyroid cell lines because they were contaminated by colon cancer cell line HT-29 (Schweppe et al. 2008, Zhao et al. 2011). In the medullary thyroid carcinoma cell line TT, CD133+ tumor-initiating subpopulation derived from drug-exposed FTTiv cells is significantly more resistant to 5-FU and retains the chemoresistant properties upon FTTiv culture propagation (Kucerova et al. 2013). The CD133(+) cells could be expanded by sphere formation assay, passaged multiple times, and expressed the neural progenitor markers β-tubulin 3 and glial fibrillary acidic protein (Zhu et al. 2010). However, some investigators have not detected CD133 expression in thyroid PTC, FTC, and ATC cells and tissues (Todaro et al. 2010). Other specific biomarkers have been used to characterize thyroid CSCs including OCT4, SOX2, NANOG, SSEA4, and CD144, which detected CSCs in fresh frozen tissues and/or formalin-fixed paraffin-embedded thyroid cancer specimens (TCS; Carina et al. 2013).

Sphere-forming assays

Although multipotent stem cells from the adult thyroid gland have not been isolated to date, cells with stem-like properties have been reported in normal thyroid, in multinodular goiters, and in thyroid cancers. These cells represent a very small percentage of the thyroid follicular cell population (usually <5%) and they can be isolated by culturing them as non-adherent spheres in specialized media (Lan et al. 2007). This method has been used to establish long-term cultures with stem cells and...
progenitor cells in various organs (Pastrana et al. 2011). This approach is based on the properties of stem and progenitor cells to survive in serum-free media and to form spheres that are capable of self-renewal and expressing embryonic stem cell biomarkers (Reynolds & Weiss 1992). In the sphere-forming assay, cells are cultured in ultra-low attachment plates or with extracellular matrix (ECM) in the presence of bFGF and/or EGF in the absence of serum (which can cause differentiation of CSCs). Human thyroid stem and progenitor cells were able to survive in suspension and produce spherical colonies (thyrospheres) composed of both stem and progenitor cells. Thyrospheres from normal tissue could be induced to differentiate by insulin and insulin-like growth factor (Malaguarnera et al. 2011). Thyrosphere cells expressed the stem cell markers NANOG and OCT4 and possessed the ability to self-renew. Injection of these thyrospheres into the thyroids of NOD/SCID Il2rg−/− mice resulted in the formation of metastatic tumors that recapitulated the clinical features of human ATC (Li et al. 2013). Todato et al. (2010) separated primary thyroid cancers using ALDEFLUOR and cell sorting followed by the thyrosphere assay to generate thyrospheres that were used for tumor formation in NOD/SCID mice. They showed that injection of 100 cells into the thyroids of mice resulted in the rapid development of thyroid cancers in 4 weeks and the mice developed metastases to the lung and other sites.

**Animal models used in CSC studies**

Animal models of human thyroid cancers have been used to study CSCs in vivo. These have included subcutaneous xenografts, or orthotopic injections, and transgenic or genetically engineered mice (Teicher 2006). Subcutaneous implantation of thyroid cancer cells into nude mice or NOD/SCID mice has been most commonly used to study CSCs. Subcutaneous xenograft models are technically simple and it is easy to palpate and measure the tumor growth. Subcutaneous transplantation usually leads to the growth of CSCs and serial passage of tumor cancer cells can be done relatively easily. However, these models differ in the tumor microenvironment of the skin implantation site which is different from the thyroid gland (Killion et al. 1999). In xenografted tumors, naturally occurring inhibitors of angiogenesis present in the skin theoretically can limit not only the growth of the primary tumor but also patterns of metastases (Kubota 1994). These differences in the tumor microenvironment may be particularly important in studying tumor invasion and metastasis, because critical aspects of aggressive tumor behavior may depend not only on the tumor cells themselves but also on the stromal, endothelial, and lymphatic elements at the implantation site. Orthotopic transplantation simulates the microenvironment and metastatic patterns of human cancer much better than subcutaneous transplantation. Nucera et al. (2009) describe a novel orthotopic thyroid cancer model using distinctive B505c ATC cells that demonstrated the features of aggressive tumors. Another approach is to use tail vein injection which often leads to lung metastases. Pulmonary metastasis is a common finding in patients with ATCs. This model simulates the metastatic behavior of patients with ATCs. Recently, a novel model of metastatic human thyroid carcinoma combining human adipose tissue-derived stromal/stem cells with the PTC cell lineK1 has been used to obtain metastatic thyroid carcinoma (Kandil et al. 2013).

The mechanism in this model is similar to recent research in melanoma. In melanoma tumor cells, tumor-derived exosomes could act as factors that promote metastatic niche formation by ‘educating’ bone marrow-derived cells toward a pro-vasculogenic and pro-metastatic phenotype via upregulation of c-Met. A specific expression pattern of Ras-related (Rab) proteins was associated with exosome production in melanoma (Peinado et al. 2012, Somasundaram & Herlyn 2012).

**Signaling pathways in stem cells and in CSCs**

The design of new drugs for the treatment of CSCs requires an understanding of the cellular mechanisms that regulate cell proliferation. The first advances in this area were made with hematopoietic stem cells and their transformed counterparts in leukemia. It is now becoming increasingly clear that the Notch, Wnt/β-catenin, and hedgehog (Hh) pathways are involved in thyroid CSC signaling mechanisms as well as in embryonic stem cell signaling. These pathways are critical in the regulation of self-renewal and survival of embryonic stem cells as well as CSCs.

**Notch pathway**

Notch signaling has been reported to promote self-renewal of CSCs in several malignancies and to participate in the interaction of tumors with the stroma and the endothelium in CSC niches in primary and metastatic tumors (Pannuti et al. 2010, Gu et al. 2012). The mammalian cells express four transmembrane Notch receptors: NOTCH1, NOTCH2, NOTCH3, and NOTCH4 (Blaumueller et al. 1997). There are five canonical transmembrane ligands...
Thymic carcinoma stem-like cells

Thyroid cancer stem-like cells

Wnt/β-catenin

Delta-like 1 (DLL1), DLL 3, DLL 4, Jagged-1, and Jagged-2 (Lindsell et al. 1995, Yan & Plowman 2007). The intra-cellular portion of Notch (NIC) is cleaved by the γ-secretase complex (Saxena et al. 2001). This cleaved product translocates to the nucleus and binds to the CBFI-suppressor of hairless/Lag1 (CSL) proteins. The Notch–CSL–MAML complex in turn recruits multiple transcriptional regulators forming the ‘Notch transcriptional complex’ (Hsieh et al., 1999, Wu et al. 2000). Notch activates many genes associated with differentiation and/or survival, including the hairy/ enhancer of split (HES) family, HES related with YRPW motif-like protein (Hey) family of basic helix-loop-helix (bHLH) transcription factors (Maier & Gessler 2000), cyclin D1 (Ronchini & Capobianco 2001), and c-Myc (Weng et al. 2006). Notch signaling plays an important role in endocrine development, through its target gene HES1. HES1, a bHLH transcriptional repressor, was shown to be expressed in the thyroid and regulates the expression of the sodium iodide symporter (NIS). HES1 has a dual role during thyroid development: first, control of the number of both thyrocyte and C-cell progenitors via a p57-independent mechanism; and second, adequate differentiation and endocrine function of thyrocytes and C-cells (Carre et al. 2011).

Notch receptors are expressed during the development of murine thyrocytes and their expression levels parallel those of thyroid differentiation markers. Notch signaling is present in normal adult thyrocytes and is regulated by thyroid-stimulating hormone (TSH). Notch pathway components are variably expressed in human normal thyroid tissue and thyroid tumors, but expression levels are clearly reduced in undifferentiated tumors. Overexpression of NOTCH1 in thyroid cancer cells restores differentiation, reduces cancer cell growth rates, and stimulates NIS expression via a direct action on the NIS promoter (Ferretti et al. 2008). In medullary thyroid cancer (MTC), Notch may lead to altered expression of achaete-scute complex-like 1 (ASCL1). Induction of Notch signaling in MTC cells suppressed ASCL1 expression, cell growth, and hormone secretion. Pharmacological activation of the Notch pathway also successfully suppressed tumor growth in an animal model (Cook et al. 2010). Active Notch1 slowed tumor growth. Furthermore, this activation led to a significant reduction in the levels of ASCL1 and chromogranin A (Jaskula-Sztul et al. 2011). The immunohistochemical expression of Notch1, Notch4, and DLL4 was highly variable in thyrocytes from normal thyroids and Graves’ disease. The staining in tumors was homogeneous and often intense. However, only capillary endothelial cells from Graves’ disease samples were positive for DLL4, the expression being restricted to large vessels in carcinomas and normal thyroids (Geets et al. 2011). Expression of the Notch1 receptor was significantly associated with poor prognostic markers, including large tumor size, nodal metastasis, capsular invasion, and extrathyroidal extension. Notch1 receptor expression showed a significant relationship with lymph node (LN) metastasis. Notch1 receptor may be a predictor of LN metastasis and may be related to poor prognostic markers in patients with PTC (Park et al. 2012). Yamashita et al. (2013) reported that the Notch pathway was activated by MAPK signaling and influenced PTC proliferation. In ATC cells, NOTCH1 knockdown reduced miR-19. SMAD4 was validated as a miR-19 target by luciferase assay, which revealed reduced luminescence associated with miR-19–Smad4 3’-UTR interaction. Moreover, this effect was mimicked in PTC cells treated with anti-miR-19 through Notch pathway inhibition and there was restoration of responsiveness to TGFβ signaling (Fuziwara & Kimura 2014). Notch and TGFβ/Smad3 pathways are involved in the interaction between cancer cells and cancer-associated fibroblasts (CAF) in PTC (Zhang et al. 2014). Recent studies have shown that the novel Notch1 activator, chrysin, inhibits tumor growth in ATC both in vitro and in vivo (Yu et al. 2013a). Resveratrol inhibits cell growth and enhances redifferentiation in ATC cells, dependent upon the activation of Notch1 signaling (Yu et al. 2013b).

It has recently been shown that Notch signaling is activated in endothelial cells, promoting angiogenesis after interaction with cancer cells in head and neck squamous cell carcinomas. These studies indicate that reciprocal interactions of tumor and untransformed stromal cells in the microenvironment play critical roles in the activation of Notch signaling (Zhu et al. 2011, Lee et al. 2013).

Wnt/β-catenin

Wnt/β-catenin or canonical Wnt pathway leads to the accumulation of β-catenin in the cell cytoplasm, which is then translocated into the nucleus to act as a transcriptional coactivator of the TCF/LEF family of transcription factors. When Wnt signaling ligands bind to the Frizzled and LRPS/6 receptors, β-catenin accumulates in the cytoplasm because a complex degrading function becomes disrupted. This complex includes axin, adenomatosis polyposis coli (APC), protein phosphatase 2A, glycogen synthase kinase 3, and casein kinase 1α. This disruption
localizes β-catenin to the nucleus and subsequently induces a cellular response via gene transduction alongside the TCF/LEF transcription factors (Wend et al. 2010). It is well accepted that the Wnt/β-catenin pathway is also necessary for the maintenance of CSCs. Canonical Wnt2/2b and β-catenin signalings are necessary and sufficient to specify lung progenitors in the foregut (Goss et al. 2009). In the colon, the inappropriate activation of this pathway by APC or β-catenin gene mutations promotes the growth of tumor cells exhibiting a stem cell-like expression profile (Vermeulen et al. 2010). In breast cancer, the Wnt pathway is upregulated in CSCs by Wnt ligands secreted by the tumor microenvironment (Malanchi et al. 2012). Increasing evidence supports the existence of CSCs and the role of Wnt pathway in the thyroid gland. Recent data from the studies of Todaro et al. (2010) suggests that CSCs from various types of thyroid carcinomas have different properties. CSCs isolated from ATC are the most aggressive and tumorigenic, followed by CSCs from PTC and FTC. Interestingly, constitutive activation of cMet, Akt, and β-catenin, together with downregulation of E-cadherin, in CSCs derived from the most undifferentiated thyroid tumors correlated with a higher migration capacity and metastatic rate (Todaro et al. 2010). Although the above results are very promising, further studies are needed to evaluate the activation of the Wnt pathway and its role in CSC stemness or maintenance.

**Hedgehog**

The Hh pathway is involved in the maintenance of CSCs in many tumors including gliomas, multiple myeloma, myeloid leukemias (Zhao et al. 2009), colorectal cancers, and gastric cancers (Merchant & Matsui 2010). Several studies have shown that Hh signaling activation is associated with chemotherapy or radiotherapy resistance (Sims-Mourtada et al. 2006), supporting that Hh signaling activation has a role in CSC functions. Hh signaling inhibitors enhanced the delivery of chemotherapy in a mouse model of pancreatic cancer (Olive et al. 2009). Sonic hedgehog (SHH) binding to a 12-pass transmembrane receptor and Patched (PTCH) leads to freeing of transmembrane protein Smoothened (SMO) and subsequent activation of GLI transcription factors. Recently, Bohinc et al. (2013) have reported increased expression of Hh signaling factors in human MTC compared with normal thyroid tissues. In *vitro*, activation of the Hh pathway resulted in increased expression of the key Hh signaling components SMO and GLI2. Conversely, inhibition of the Hh pathway decreased the expression of these genes, leading to significantly reduced cellular growth and increased apoptosis. Another research group reported that SHH, SMO, the receptor PTCH and the target gene GLI1 were clearly expressed in mRNA and protein levels in two ATC cell lines (Hth 74, C643) and in primary tumor samples (41% SHH, 65% SMO, 65% PTCH, and 65% GLI). Treatment with cyclopamine showed a time- and dose-dependent inhibition of cell numbers with IC50 values between 1 and 4 µM in both cell lines (Hinterseher et al. 2014). SHH signaling pathway was also found to be activated in benign tumors such as follicular adenomas (FAs; Nelson et al. 2010, Xu et al. 2012). It is possible that aberrant activation of the Hh pathway might be involved in the biology of thyroid cancer and further evaluation regarding a possible clinical impact of pathway inhibition is warranted. More experiments are needed to determine whether Hh signaling is important for CSCs in thyroid cancer.

**EMT and CSCs**

Epithelial tumor cells gain invasiveness and migratory abilities in the process of EMT, which is essential for successful metastatic spread (Thiery 2002, Potenta et al. 2008). During EMT, epithelial cells lose their polarized organization and cell–cell junctions, undergo changes in shape to a mesenchymal or fibroblast-like cell morphology, and show increased cell migration and invasion (Fig. 2). The process of EMT is likely responsible for decreases in drug efficiency and failure of some anticancer therapies. EMT is a multi-step process involving molecular and cellular changes in epithelial cells and in the adjacent stroma. Restrained and immobile epithelial cells gain a mesenchymal phenotype, characterized by enhanced motility and ability to degrade ECM (Kalluri & Weinberg 2009). This leads to decreased cell–cell adhesion due to the downregulation of epithelial proteins, mainly E-cadherin, but also claudins, occludins and cytokeratins (Christofori 2006). The cells that have undergone EMT show changes in apicobasal polarity, contributing to the spindle-shaped morphology. The characteristic features of a mesenchymal phenotype are high expression of N-cadherin as well as fibronectin (FN), vimentin, tenascin C, collagen VI–α, and laminin-β1. These changes observed in the EMT process are governed by transcription factors such as Twist1, Snail, Slug, ZEB1, and ZEB2 (Yang & Weinberg 2008). To date a number of different EMT inducers such as Wnt, Hh, EGF, and TGFβ (Massague 2008) have been described and some of the molecular pathways have been delineated.
Studies with primary PTCs using gene expression analyses showed that the leading front of PTCs expressed EMT biomarkers. Vimentin overexpression was associated with invasion and nodal metastases (Vasko et al. 2007, Phay & Ringel 2013). Hardy et al. (2007) examined the Snail family of transcription factors and reported that these were associated with thyroid carcinogenesis.

Buehler et al. (2013) reported that well-differentiated thyroid carcinomas and normal thyroid tissues expressed high levels of E-cadherin, but did not commonly express Slug and Twist1 while ATCs frequently expressed Slug and Twist1, but few of these carcinomas expressed E-cadherin (Lloyd et al. 2013). The binding of TGFβ to proteins expressed on the surface of breast cancer cells activates the expression of Snail and Slug which suppress E-cadherin expression (Massague 2008). It appears that stem-like cells increase in numbers during EMT (Mani et al. 2008). EMT induces CSC generation and tumor progression in human thyroid cancer cells in vitro. ATCs show evidence of EMT, including decreased expression of E-cadherin and increased expression of ZEB1 and SMAD7 compared with well-differentiated thyroid carcinomas. The increased expression of SMAD7 may be associated with thyroid tumor progression (Montemayor-Garcia et al. 2013). In a recent study, the FTC133 FTC cell line has been induced to undergo EMT by overexpression of HIF1α. Overexpression of HIF1α which increased the stem-like side population in this cell line. These results suggest that EMT induction promotes CSCs in thyroid tumors (Lan et al. 2013).

In addition to genetic regulation of EMT, a growing body of evidence indicates that epigenetic regulatory mechanisms including changes in the modification of chromatin-associated histones is important to achieve the widespread changes in gene expression observed during EMT (Tam & Weinberg 2013). Although E-cadherin repression is important during EMT, the exact mechanism regulating the E-cadherin (CDH1) gene is not well understood. Recent studies have revealed that the epigenetic silencing of E-cadherin is orchestrated by a variety of histone-modifying enzymes that cooperate to repress the CDH1 promoter (Tam & Weinberg 2013). Further understanding of the role of EMT and CSCs in cancer progression may reveal new therapeutic targets thyroid cancers (Lan et al. 2013).

Mesenchymal-to-epithelial transition and the metastatic cascade

Metastatic disease is usually associated with incurable cancers in most malignancies (Ocaña et al. 2012, Scheel & Weinberg 2012). Metastatic disease remains the least understood step in tumor progression. Ocaña et al. (2012) recently described a new EMT regulator, PRRX1, a paired homeobox factor 1 transcription factor that regulates migratory and invasive properties during EMT. The cells that undergo EMT and disseminate usually go through a mesenchymal-to-epithelial transition (MET) to successfully colonize the metastatic site (Thiery 2002, Gunasinghe et al. 2012). The MET concept is becoming a critical one in the metastatic cascade. Ocaña et al. (2012). used models of breast carcinoma to demonstrate that PRRX1 was increased during EMT and decreased during MET, which facilitated colonization and secondary tumor formation. PRRX1 had a role in invasion and migration of nodes. The cancer cells are dormant or quiescent for variable periods of time and then undergo mesenchymal-to-epithelial transition (MET) with increased proliferation. There is decreased expression of Twist and PRRX1 and increased expression of other biomarkers such as EGFR and cMET. MicroRNAs have regulatory roles during EMT and during the reversal of MET to EMT. With subsequent colonization a secondary tumor becomes apparent clinically as a macrometastasis in the lung.
breast cancer cells undergoing metastasis. They showed that forced expression of PRRX1 blocked the capacity of metastasis-competent cells to produce metastatic tumors and that PRRX1 suppression was needed for MET to proceed. In a related study using squamous cell carcinoma model, Tsai et al. (2012) reported that TWIST1 was also altered during MET and decreased levels of TWIST1 had a role in the metastatic process. In this model, Twist1 was sufficient to promote squamous cell carcinoma to undergo EMT and to disseminate via the circulation. At distant metastatic sites, Twist1 was turned off and allowed reversion of EMT to MET, which was important for the disseminated tumor cells to proliferate and to form metastases. Although such studies have not been performed with thyroid tumors, similar mechanisms may operate in thyroid tumors with metastatic disease to the lung and to other sites. Recent preliminary studies reported by Montemayor-Garcia et al. (2014) in thyroid PTC cell lines have shown that PRRX1 was important for EMT in thyroid cancers (Fig. 2). Their findings agree with earlier studies reported by Ocaña et al. (2012) and of Reichert et al. (2013). The plasticity of CSCs and the EMT and MET processes has major implications for the therapeutic targeting of CSCs. As a great deal of research on EMT is to inhibit cancer cells in the mesenchymal state, these approaches may have stimulatory effects on established metastases or even active dormant cancer cells. The concept of metastatic dormancy during the metastatic cascade is an important feature of metastatic disease (Phay & Ringel 2013). Metastatic dormancy may include physical barriers provided by extracellular matrices, as well as expression of proteins encoded by metastatic suppressor genes (Phay & Ringel 2013). Thus, understanding the biology of EMT and MET should lead to the design of better drugs to target cancer cells including CSCs more effectively (Van Denderen & Thompson 2013).

MicroRNAs in EMT and MET

MicroRNAs are small 18–24 nucleotide molecules that regulate mRNA at post-translational level by binding to an eight-base seed sequence at the 3′-UTR of mRNAs. MicroRNAs play critical roles during development and is linked to various human diseases including cancer (Mendell & Olson 2012). Several profiling studies have also determined the potential implications of high percentage microRNAs in cancer due to their close proximity to cancer-associated genomic regions and fragile sites, chromosomal breakpoints, and dysregulated expression levels in many malignancies (Garg 2012). Dysregulation of CSCs by acquired epigenetic abnormalities may include the aberrant expression of microRNAs (Tam & Weinberg 2013). Over the past few years, research in the area of cancer biology has shown that microRNAs may function as oncogenes and/or tumor suppressor genes in thyroid cancer progression (de la Chapelle & Jazdzewski 2011).

Analyses of microRNAs have provided a great deal of insights into the biology of thyroid tumors, although studies of microRNAs in thyroid CSCs are in their infancy. Several microRNAs regulate EMT and MET by targeting genes that control epithelial or mesenchymal characteristics.

Downregulation of specific microRNAs

Recent studies have shown that downregulated miR-200 and miR-30 families distinguish ATCs from PTCS, FTCs, and normal thyroid tissues. The miR-200 family regulates the EMT induced by EGF/EGFR (Zhang et al. 2012) or by targeting TGFβR1 (Braun et al. 2010) in ATC. The miR-200 family, which includes miR-200a, miR-200b, miR-200c, miR-141, and miR-429, was also shown to target ZEB1 and ZEB2 (Gregory et al. 2008). Expression of these two microRNAs families in mesenchymal ATC-derived cells reduced their invasive potential and induced MET by regulating the expression of MET marker proteins. Downregulation of miR-34b and miR-130b was observed in aggressive PTC. c-MET, a proto-oncogene that encodes hepatocyte growth factor (HGF) receptor, was identified as a potential target gene for miR-34b and miR-1, and significantly higher level of c-MET expression was observed in aggressive PTCs (Yip et al. 2011). Downregulation of miR-138 was associated with overexpression of human telomerase reverse transcriptase protein in human ATC cell lines (Mitomo et al. 2008). Using a squamous cell carcinoma model, it was reported that miR-138 regulated EMT by targeting multiple components of the EMT pathways, such as ZEB2 and the epigenetic regulator EZH2. Both are repressors of E-cadherin expression (Liu et al. 2012a,b). miR-191 was downregulated in FAs, FTC, and follicular variant of PTCS by targeting CDK6, a serine-threonine kinase involved in the control of cell cycle progression (Colaiano et al. 2011). Let-7a downregulation plays a role in follicular-derived thyroid neoplasms by affecting cell adhesion and migration through its ability to target the FXYD5 (Dysadherin) gene (Colaiano et al. 2012). Let-7f effects on thyroid growth and differentiation were reported to attenuate the neoplastic process of RET/PTC papillary thyroid oncogenesis through impairment of MAPK.
signaling pathway activation (Ricarte-Filho et al. 2009). miR-199b-5p and miR-144 were essentially lost in FTCs compared with FAs (Rossing et al. 2012). miR-199b-5p targets HER2 in breast cancer cells (Fang et al. 2013) and is involved in the Notch signaling pathway in osteosarcoma (Won et al. 2013).

Upregulation of specific microRNAs

miR-221/-222 are upregulated in human ATC cell lines (Mitomo et al. 2008), PTCs (Pallante et al. 2006), minimally invasive FTC with distant metastasis, and widely invasive FTC (Jikuzono et al. 2013). miR-221/-222-upregulated carcinomas show dramatic loss of KIT transcript and KIT protein (He et al. 2005). miR-146b-5p is overexpressed in PTCs and has been found to be a potential diagnostic marker for some thyroid tumors because the levels of this microRNA in normal thyroid are very low. Specific inhibition of miR-146b-5p with a locked nucleic acid-modified anti-miR-146b oligonucleotide significantly increased SMAD4 levels in the human PTC cell lines. Suppression of miR-146b-5p increased the cellular response to the TGFβ anti-proliferative signal, significantly decreasing the proliferation rate (Geraldo et al. 2013). PTC tumors with higher expression of miR-146b-5p had significantly poorer disease-free survival rates (Guo et al. 2014). MicroRNA-222 and microRNA-146b are tissue and circulating biomarkers of recurrent PTC (Lee et al. 2013). miR-146a was also shown to be upregulated in ATCs. The inhibition of miR-146a expression in the FRO ATC cell line decreased their oncogenic potential and increased the susceptibility to chemotherapeutic drug-induced apoptosis (Pacífico et al. 2010). miR-146a was identified as a common target of Krüppel-like factor 8 (KLF8) and TGFβ, both of which are known EMT-inducers in breast cancer cell line (Wang et al. 2013). miR-21 was upregulated in human ATC cell lines (Mitomo et al. 2008) by oncogenic Ras (Frezzetti et al. 2011). PTCs with high expression of miR-21 had significantly poorer disease-free survival rates and higher LN metastasis by in situ hybridization (Guo et al. 2014). miR-197 and miR-346 are overexpressed in FC compared with FA and in vitro studies suggest that both miRNAs could have a significant impact on tumour cell proliferation (Weber et al. 2006). miR-10b and miR-92a were significantly upregulated in invasive FTC with distant metastasis (Jikuzono et al. 2013). miR-885-5p was strongly upregulated (> 40-fold) in oncocyty FTCs (Dettmer et al. 2013).

MicroRNAs may also regulate gene expression epigenetically. miR-22 acted as a crucial epigenetic modifier and promoter of EMT and breast cancer stemness toward metastasis by silencing anti-metastatic miR-200. This was accomplished through direct targeting of the ten–eleven translocation family that modifies DNA by hydroxylating 5-methylcytosine (Song et al. 2013). MicroRNAs have been recognized as critical regulators of EMT and CSCs. Understanding the functional role of microRNAs in thyroid cancer may lead to targeting of these molecules in CSCs in order to modify their expression levels.

Role of the microenvironment

Accumulated evidence indicates that the interaction between tumor cells and the local microenvironment at the secondary or metastatic site leads to the development of premetastatic niche(s) or compartment(s) within the microenvironment, which allows for the proliferation of the metastatic cells during colonization. Tumor cells can produce factors leading to the establishment of premetastatic niches (Kaplan et al. 2005). However, the mechanisms allowing tumor cells to influence the behavior of cells in the microenvironment is poorly understood. The tumor stroma is the compartment that serves as a tissue framework, consisting of the vasculature (endothelial, smooth muscle cells, and pericytes), inflammatory and immune cells (macrophages, lymphocytes, and dendritic cells), CAFs, and ECM noncellular components. Dynamic and reciprocal interactions involving cell adhesion molecules (e.g. integrins and CD44), ECM noncellular components (i.e. thrombospondin 1 (TSP1) and FN), and soluble cytokines occur between tumor epithelial cells and tumor microenvironment stromal cells (Hynes 2009). Efficient TGFβ-induced EMT depends on hyaluronan synthase 2 (Porsch et al. 2013). Mesenchymal stem cells (MSCs) are recruited from the bone marrow to the stroma, a process that is mediated mainly by inflammatory factors present in the tumor microenvironment (Hogan et al. 2012). Once recruited to the tumor, MSCs can act as precursors for CAFs, which represents a distinct cell type characterized by heterogeneous expression of alpha-smooth muscle actin, fibroblast activation protein, and fibroblast-specific protein 1. CAFs secrete a variety of inflammatory cytokines, growth factors, and proteinases, all of which contribute to tumor progression. These factors include interleukins, chemokine C-X-C motif ligand cytokines, vascular endothelial growth factor, HGF, and matrix metalloproteinases that affect the surrounding components of the tumor microenvironment, including cancer cells and cells of vasculature and immunity. In addition, factors secreted by CAFs...
modify the ECM, releasing collagens (COL), FN, and periostatin (POSTN) that further act on the cancer cells (Rasanen & Vaheri 2010). In a report by Hoffmann et al. (2005), ten differentiated thyroid carcinoma cell lines (FTC133, 236, 238, HTC, HTC TSHr, XTC, PTC4.0/4.2, TPC1, Kat5) and two ATC cell lines (C643, Hth74), as well as primary cultures of normal thyroid tissue (Thy1, 3) and TCS, displayed profoundly different patterns of integrin receptor molecule expression that appeared to correlate with tumor aggressiveness. The BRAFV600E pathway plays an important role in the progression of PTC through proteins crucial for the ECM remodeling processes, including tumor cell adhesion, migration, invasion, and metastasis. Many of these altered gene sets are involved in the composition and remodeling of ECM such as TSP1 (THBS1), TGFβ1, integrin-α3, -α6, -β1, FN, CD44, cathepsin-B (CTSB), and cathepsin-S (CTSS). These genes appear to be either targeted or affected by the BRAFV600E mutation in PTCs (Nucera et al. 2010). A recent study of PTCs analyzed for BRAF mutation showed that BRAFV600E mutation was associated with hyperactivation of NFkB and upregulation of both TIMP1 and its receptor CD63. These studies provide insights on how BRAFV600E mutant determines cancer initiation, progression, and invasiveness in PTCs and may provide new therapeutic targets for the treatment of aggressive PTCs (Bommarito et al. 2011). CD3−CD16−CD56bright immunoregulatory NK cells are increased in the tumor microenvironment and were found to be inversely correlated with advanced stages in patients with PTC (Gogali et al. 2013). In a recent analysis of four new ATC cell lines, Marlow et al. (2010) have reported that suppressed RHOB was implicated as a molecular target for the treatment of ATCs, because drugs like romidepsin, a histone deacetylase inhibitor, was able to inhibit cell proliferation and upregulate RHOB in these new ATC cell lines.

Summary

The CSC model, EMT, and MET in thyroid cancers are rapidly evolving concepts that are contributing to our understanding of thyroid cancer biology and the pathogenesis and treatment of thyroid cancer. These concepts should contribute to the therapeutic targeting of CSCs in tumors because the CSCs may be responsible for tumor growth, recurrence, and drug resistance. Moreover, emerging research data indicate that the existence of CSCs may be related to a high risk for recurrence and poor prognosis for many tumor types. Further investigations into thyroid CSC biology will require additional technological advances for the visualization, isolation, and characterization of thyroid CSCs, with well-validated biomarkers, and elucidation of the signaling pathways that are altered in these tumor cells. A better understanding of the interaction between thyroid CSCs and their microenvironment should shed light on the existing mechanisms of thyroid carcinogenesis and lead to the development of novel therapeutic strategies targeting thyroid CSCs.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review.

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