PATHOBIOLOGY IN FOCUS

The evolving concept of cancer stem-like cells in thyroid cancer and other solid tumors

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The cancer stem-like cell (CSC) hypothesis postulates that a small population of cells in a cancer has self-renewal and clonal tumor initiation properties. These cells are responsible for tumor initiation, growth, recurrence and for resistance to chemotherapy and radiation therapy. CSCs can be characterized using markers such as SSEA-1, SSEA-4, CD44, CD24, ALDEFLUOR and others. CSCs form spheres when they are cultured in serum-free condition in low attachment plates and can generate tumors when injected into immune-deficient mice. During epithelial to mesenchymal transition (EMT), cells lose cellular adhesion and polarity and acquire an invasive phenotype. Recent studies have established a relationship between EMT and increased numbers of CSCs in some solid malignancies. Non-coding RNAs such as microRNAs and long non-coding RNAs (lncRNAs) have been shown to have important roles during EMT and some of these molecules also have regulatory roles in the proliferation of CSCs. Specific lncRNAs enhanced cell migration and invasion in breast carcinomas, which was associated with the generation of stem cell properties. The tumor microenvironment of CSCs also has an important role in tumor progression. Recent studies have shown that the interaction between tumor cells and the local microenvironment at the metastatic site leads to the development of premetastatic niche(s) and allows for the proliferation of the metastatic cells during colonization. The role of exosomes in the microenvironment during the EMT program is currently a major area of research. This review examines CSCs and the relationship between EMT and CSCs in solid tumors with emphasis on thyroid CSCs. The role of non-coding RNAs and of the microenvironment in EMT and in tumor progression are also examined. This review also highlights the growing number of studies that show the close association of EMT and CSCs and the role of exosomes and other elements of the tissue microenvironment in CSC metastasis. A better understanding of these mechanisms will lead to more effective targeting of primary and metastatic malignancies.

Laboratory Investigation (2017) **97**, 1142–1151; doi:10.1038/labinvest.2017.41; published online 10 April 2017

The cancer stem-like cell (CSC) hypothesis was first proposed for human leukemia based on the observation that a small fraction of cells could generate leukemia in severe combined immunodeficient mice, while the majority of tumor cells failed to engraft and cause disease.¹ A few years later Bonnet *et al*² isolated leukemia cells expressing CD34 but lacking CD38. When injected into immunodeficient mice they were able to reproduce leukemic transformation. Subsequently, isolation of CSCs from human brain gliomas, which were shown to be clonogenic with sphere-forming abilities by Ignatova *et al*³ and characterization of CSCs from breast cancer tissues by Al-Hajj *et al*⁴ who showed that breast carcinoma CSCs had a CD44+/CD24– phenotype and formed tumors in immunodeficient mice demonstrated that solid tumors in addition to hematologic malignancies also

contained CSCs (Figure 1). Various methods permitting isolation and characterization of CSCs have emerged (Table 1). It appears that a small population of cells in cancer that is capable of self-renewal is responsible for tumor initiation, growth and recurrence in a variety of cancers.^{5–12}

Numerous biomarkers to characterize CSCs have been reported. However, the specificity of many of these markers in terms of their ability to differentiate CSCs vs other cancer cells has not been completely demonstrated^{7–11} (Table 1). Non-neoplastic stem cells, both embryonic and somatic, have the ability for self-renewal by continuous division and can be induced to become tissue/organ-specific cells with specialized functions. Non-neoplastic stem cells include embryonic stem cells characterized by pluripotency and can give rise to the three traditional germ layers—ectoderm, endoderm and

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Received 11 January 2017; revised 21 February 2017; accepted 24 February 2017

mesoderm, and somatic or adult stem cells, which are tissue-specific cells with a limited life-span that give rise to cells in a specific lineage. The role of embryonic stem cells and adult stem cells in the development of CSCs remains controversial.^{6,13–15} Recent studies have suggested that the stem cell programs operating in tumor-initiating cells (TICs) and non-neoplastic stem cells of the corresponding normal tissues are likely to differ significantly.⁶

Solid tumors are usually heterogeneous at the histological, as well as the molecular level. Tumor heterogeneity can be explained by two possible models: the stochastic model and the hierarchical model. The stochastic model assumes that cancer results from random mutations within oncogenes and tumor-suppressor genes leading to excessive proliferation.^{16–18} In contrast, the hierarchical model suggests that tumor cells originate from a small population of cells (CSCs) that have similar characteristics to adult stem cells. In this model, tumor development occurs when an adult stem cell bypasses regulation or terminally differentiated cells undergo dedifferentiation. Although some investigators have proposed only one model to explain CSCs in the development of thyroid cancers,^{19,20} it is very likely that both models are operational and contribute to cancer development.

The CSC hypothesis has been challenged by some investigators.^{17,21,22} Kern and Shibata²¹ critiqued the conflicting evidence regarding the presence of CSCs by re-evaluating the published CSC studies and stem cell-related claims in US-awarded patents from a mathematical perspective. They argued that the phenomenon of treatment response and relapse is difficult to explain mathematically when assuming a consistent small population (CSCs) as the only source of tumor regeneration and resistance. Rather, tumorigenic property is more likely to be a common function that most tumor cells are able to assume under certain conditions. Antoniou *et al*²² suggested that there was no convincing evidence to support the CSC concept and that the phenotypic properties ascribed to CSCs represented the result of independent, linked or congruent genetic, epigenetic or other signaling programs.²² However, in spite of these and other objections, the continued accumulation of experimental data provides increasing support for the CSC hypothesis.^{17,23–28}

EPITHELIAL TO MESENCHYMAL TRANSITION

During epithelial to mesenchymal transition (EMT), cells lose cellular adhesion and polarity and acquire an invasive phenotype.^{29,30} EMT is associated with loss of E-cadherin and increased expression of other biomarkers including Snail, Slug, ZEB1, ZEB2 and N-cadherin (Figures 1 and 2; Table 2). EMT is first observed during embryogenesis in the developing fetus, but is also present during wound healing, tumor progression and tumor invasion.^{29–33} In thyroid tumors, EMT has been linked to papillary thyroid carcinoma (PTC) progression with overexpression of vimentin and has been associated with invasion and lymph node metastasis.³⁴ EMT

has been associated with dedifferentiated cancers such as anaplastic thyroid carcinomas (ATCs) with decreased expression of E-cadherin and membranous β -catenin and increased expression of Slug and Twist1.^{29–33} Using a breast cancer model, Mani *et al*²³ showed that acquisition of mesenchymal traits was linked to the acquisition of stem cell markers. They further showed that transformed mammary epithelial cells that had undergone EMT formed spheres, soft agar colonies and formed *in vivo* tumors more efficiently suggesting that the EMT program that enabled cancer cells to spread from a primary tumor may also promote their self-renewal properties.²³ The studies of the Weinberg laboratory with a breast cancer model and primary human breast cancers demonstrated that during the EMT program there was an increase in the CSC population in breast cancers.²⁵ Similar findings have been shown in the thyroid.^{34–37} Hardin *et al*^{36,37} used a model of cultured thyroid cancer cells treated with TGF β 1, which led to the development of EMT. These findings supported the earlier report of Buehler *et al*³⁵ showing that primary thyroid carcinoma specimens underwent EMT during thyroid cancer progression from PTCs to ATCs. The studies of Hardin *et al*^{36,37} showed that when thyroid tumor cell lines, including well-differentiated PTC cell lines, as well as ATC cell lines, undergo EMT, there is also an increase in the number of stem cells in the tumor population. Similar findings were reported in colorectal cancer under the influence of Notch-1 expression.³⁸

Mesenchymal to epithelial transition (MET) is the reverse process of EMT and also has a central role in embryogenesis and tumor progression.^{29–31} The studies of Tsai *et al*³⁹ provided some insight into the mechanism by which EMT and MET were implicated in converting epithelial tumor cells into motile mesenchymal cells during metastases. They used a mouse model to show that Twist1 expression was sufficient to promote squamous cell carcinoma to undergo EMT and disseminate into the circulation. Then, at distant sites Twist1 was turned off and allowed reversion of EMT to MET, which was important for the disseminated tumor cells to proliferate and form metastases.³⁹ Ocaña *et al*⁴⁰ recently described a new EMT regulator, paired homeobox factor 1 transcription factor (PRRX1), which regulates migratory and invasive properties during EMT. Cells that successfully colonized after metastasis usually go through MET to colonize the metastatic site.⁴⁰ This group showed that PRRX1 was increased during EMT and subsequently decreased during MET, which facilitated colonization and secondary tumor formation, therefore, PRRX1 appears to have a role in invasion and migration of cancer cells as they metastasize.⁴⁰ This observation was supported by using forced expression of PRRX1, which blocked the capacity of metastasis-competent cells to produce metastatic tumors and supported the concept that PRRX1 suppression was needed for MET.⁴⁰ Recent studies by Hardin *et al*³⁶ showed that PRRX1 was important for EMT in thyroid cancers. Using a TGF β 1 model of EMT induction in PTC cell lines, it was shown that PRRX1 was overexpressed along with

Figure 1 Diagrammatic illustration of epithelial to mesenchymal transition (EMT) and interaction of cancer stem cells (CSCs) with the tumor microenvironment. EMT is influenced by multiple factors and TGFβ-induced EMT is a useful experimental model. EMT is associated with increases in CSCs as shown in breast cancer²³ and thyroid cancer.³⁶ The mechanism of this increase in CSCs has not yet been elucidated. The figure also highlights CSCs interactions with the microenvironment during tumor progression and metastatic disease. CSCs undergo self-renewal producing other CSCs and non-CSCs. Both CSCs and other cancer cells are surrounded by the tumor microenvironment components, which includes the extracellular matrix (fibronectin, collagen, laminin, integrins, etc.), fibroblasts, cancer-associated fibroblasts (CAF) and blood vessels with endothelial cells associated with angiogenesis, and immune cells. Exosomes released from the CSCs and from other cancer cells have a critical role in communication at both the primary site and at metastatic tumor sites.^{103,106} Exosomes, which contain proteins, lipids, RNA and DNA, influence tumor cell colonization and metastasis.

Table 1 Methods used to isolate and characterize CSCs

1. Flow cytometric cell sorting and immunochemical staining with ALDEFLUOR, ALDH1, Hoeschst dye 33342, CD133, CD44, CD24, SSEA-1 (CD15), SSEA-3, SSEA-4 and Lgr5.
2. Cell culture in non-adherent ultra-low attachment plates or extracellular matrix with serum-free conditions in the presence of FGF and/or EGF.
3. *In vitro* cytotoxicity assays for resistance to chemotherapeutic agents.
4. Xenotransplantation into immunodeficient mice such as nude mice and SCID mice.

References.^{4,5,12,18,23,25,28,31,36,37,39,40,108–110}

Slug and Twist during tumor progression. Analysis of low-grade and highly aggressive human thyroid cancers showed that only the highly aggressive cancers overexpressed PRRX1.³⁶

The plasticity of CSCs and the EMT and MET programs suggest that the therapeutic targeting of CSCs may be very challenging. A great deal of research on EMT and MET is directed at inhibiting cancer cells in their mesenchymal state to prevent metastatic disease. However, such approaches may have stimulatory effects on existing metastatic foci or may even activate dormant cancer cells.^{41,42} The concept of metastatic dormancy during the metastatic cascade is an important feature of metastatic disease.⁴¹ Metastatic dormancy may include physical barriers provided by extracellular matrices or protein expression encoded by suppressor genes. The goals of understanding the biology of EMT and MET should include designing better drugs to target cancer cells including CSCs more effectively.^{41,42}

SIGNALING PATHWAYS IN CSCs

Specific signaling pathways have been described in embryonic stem cells and more recently in CSCs. These pathways are critical in the regulation of self-renewal and survival for both stem cells and CSCs. These pathways include, among others, the Notch, Wnt/β-catenin and hedgehog (HH) pathways. They have been analyzed in various types of cancers including those in the thyroid.^{7,43–50}

NOTCH PATHWAY

Mammalian cells express four transmembrane Notch receptors including NOTCH-1, NOTCH-2, NOTCH-3 and NOTCH-4.^{43–50} Notch signaling has been implicated in self-renewal in CSCs in a variety of cancers. Notch receptors are expressed during the development of thyrocytes in experimental systems and the expression levels of these receptors are aligned with thyroid differentiation markers. Studies have shown that overexpression of NOTCH-1 in thyroid cancer cells restores differentiation, reduces cancer cell growth and stimulates differentiation markers such as sodium iodide transporter (NIS).⁴⁸ In contrast, other studies using clinicopathologic correlation have suggested that Notch-1 receptor may be a predictor of lymph node metastasis and may be a poor prognostic marker in patients with PTCs.⁵⁰ A recent

Figure 2 Epithelial to mesenchymal transition in primary thyroid tissues and tumors. (a) Normal thyroid showing expression of E-cadherin as membranous staining by immunohistochemistry. (Hematoxylin nuclear stain and diaminobenzidine as chromogen; scale bar, 100 μ m). (b) Papillary thyroid carcinoma showing diffuse membranous staining for E-cadherin. (Hematoxylin nuclear stain and diaminobenzidine as chromogen; scale bar, 100 μ m). (c) Anaplastic thyroid carcinoma showing loss of E-cadherin in the tumor cells. (Hematoxylin nuclear stain and diaminobenzidine as chromogen; scale bar, 100 μ m). (d) Anaplastic thyroid carcinoma showing Slug (Snail 2) expression in the tumor cell nuclei. The normal thyroid and the PTC cells were negative for Slug.³⁵ (Hematoxylin nuclear stain and diaminobenzidine as chromogen; scale bar, 100 μ m).

study by Yu *et al*⁴⁷ using primary thyroid cancers showed that Notch-1 expression levels were downregulated in differentiated thyroid cancer tissues compared with benign thyroid tissues. Decreased Notch-1 was more common in more aggressive tumors with extrathyroidal invasion. Restoration of Notch-1 intracellular domain in a stable doxycycline-inducible metastatic differentiated thyroid carcinoma cell line led to a reduction in cell growth and tumor cell migration.⁴⁷

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 and acts as a transcriptional co-activator of the TCF/LEF family of transcription factors. Experimental evidence has shown that Wnt2/2b and β -catenin signaling are necessary and sufficient to specify lung progenitors in the foregut.⁵¹ The role of the Wnt pathway in thyroid cancer and CSCs has been studied using

Table 2 Biomarkers for EMT

Epithelial–mesenchymal biomarkers	References
E-Cadherin (CDH1)	4,6,7,32,35,36
Vimentin	7,32,36
Slug	29–33,36
Twist1	29–33,36
ZEB1	7,30–32,34
ZEB2	23,29–32,34
PRRX1	36,40

primary thyroid cancer cells.⁷ CSCs isolated from ATCs were most aggressive and tumorigenic compared with CSCs isolated from well differentiated papillary and follicular cancers. Constitutive activation of β -catenin, together with

downstream regulation of E-cadherin in CSCs derived from ATCs, correlated with higher migration and metastatic rates compared with well-differentiated thyroid cancers.⁷

HEDGEHOG

The HH pathway is important for the maintenance of CSCs in a variety of tumors including gliomas, colorectal and gastric cancers.^{52–54} *In vitro* activation of the HH pathway has been associated with increased expression of the key HH signaling components such as SMO and GLI1.⁵² Inhibition of the HH pathway has been associated with decreased expression of genes associated with cellular growth and increased apoptosis. Sonic HH (SHH) signaling pathway was activated in benign thyroid tumors such as follicular adenomas.⁵⁴ Heiden *et al*⁵³ reported that SHH signaling maintained the CSC self-renewal of ATCs by inducing Snail expression. When SHH inhibitors were used to knockdown Gli1 in an ATC cell line, there was decreased CSC self-renewal and increased radiation sensitivity. Subsequent studies showed that SHH signaling pathway stimulated ATC cell motility and invasiveness by activating Akt and c-Met.⁵²

EPIGENETIC REGULATION IN CSCs

Epigenetic regulation has been proposed to explain the phenotypic plasticity that has been reported in CSCs.^{13,32,55} Phenotypic plasticity may allow non-CSCs to acquire CSC traits and replenish the CSC pool as has been reported in malignancies such as malignant melanomas^{56,57} and prostate cancers.⁵⁸ Roesch *et al*⁵⁷ used the H3K4 demethylase JARID1B as a biomarker and characterized a small sub-population of slow-cycling melanoma cells that cycled with doubling times of > 4 weeks within the rapidly proliferating main population. Isolated JARID1B-positive melanoma cells gave rise to highly proliferative progeny. Knockdown of JARID1B led to an initial acceleration of tumor growth followed by exhaustion, which suggested that JARID1B-positive sub-population was essential for continuous growth. Lee *et al*⁵⁸ reported that reduced expression of DNA methyltransferase 1 (DNMT1) had an important role in the induction of EMT and CSC phenotype in prostate cancer cells. After reducing DNMT1 by 5-azacytidine, there was promotion of EMT induction, as well as CSC sphere formation. Reduction of DNMT1 was associated with suppression of H3K9me3 and H3K27 on the Zeb2 and KLF4 promoter region of the prostate cancer cells. In other cancers, such as colorectal cancer, aberrant methylation was reported to have important roles in accumulation of damages to cell function and led to malignant transformation.⁵⁹ Similarly, DNA methylation and histone acetylation have been reported to have important roles in the epigenetic regulation in heterogeneity of lung cancer.⁶⁰ Thus, epigenetic regulation appears to have a major role in explaining the plasticity observed in CSCs and non-CSCs *in vitro*.

NON-CODING RNAs AND EMT MicroRNAs and EMT

MicroRNAs are small 18–24 nucleotide molecules that regulate mRNA at a posttranslational level by binding to an eight-base seed sequence at the 3'-UTR of mRNAs. They have a critical role during development and are linked to various diseases including cancers.^{61–65} MicroRNAs have been shown to have a role in tumor development and progression, as they may function as oncogenes and/or tumor-suppressor genes in thyroid and other cancers.⁶⁴ MicroRNAs are increasingly recognized as important regulators of EMT and of CSCs.^{65–70} Recent studies have shown that the miR-200 family has a major regulatory role in EMT.⁶⁵ The miR-200 family has been shown to regulate EMT induced by EGF/EGFR⁶⁶ and also by targeting TGF β R1.⁶⁵ Braun *et al*⁶⁵ used ATC cell lines to show that overexpression of miR-200 and miR-30 in mesenchymal ATC-derived cells reduced their invasive potential and induced MET. Expression of SMAD2 and TGF β R1, which were upregulated in ATC cell lines, was controlled by members of the miR-30 and/or miR-200 families. These findings suggested that microRNA signatures are potent markers for ATCs that leads to EMT and invasion of these cancers. Zhang *et al*⁶⁶ used ATC cell lines to examine the relationship between EGF/EGFR, miR-200 and the induction of EMT or MET. They reported that EGFR silencing in ATC cell lines led to upregulation of E-cadherin and down-regulation of vimentin indicating transition toward MET. Hebrant *et al*⁶⁷ found that specific miRNAs in ATCs had a regulatory role in EMT. They reported that the *LOX* gene, which is a principal factor in EMT, was an upregulated mRNA target in ATCs associated with downregulation of miR-29a.

MiRNAs also have regulatory roles in well-differentiated thyroid cancers during EMT.^{68–70} MiR-146b-5p has been shown to regulate PTC cells during EMT.⁶⁹ Hardin *et al*³⁶ used a TGF β -induced model of EMT to show that miR-146b-5p was initially upregulated during the first fourteen days of TGF β treatment, which was followed by downregulation of this microRNA with subsequent treatment for two weeks. Deng *et al*⁶⁹ later showed that miR-146b-5p targeted ZNRF3, increased the cell surface Wnt receptors Frizzled-6 and LRP6 and decreased Wnt/ β -catenin signaling, as an inhibitor of Wnt/ β -catenin suppressed the effect of miR-146b-5p on migration, invasiveness and EMT induction in PTC cell lines. Other microRNAs are also important in the regulation of thyroid cancer. A recent study by Wang *et al*⁷⁰ examined the role of miR-663 in PTCs. This miRNA has been detected in a large variety of tumor types and has been shown to have both an oncogenic and a tumor-suppressor role in different tumors. In this study, miR-663 expression was inhibited in PTC tissue samples and PTC cell lines as assessed by qPCR. Upregulation of miR-663 inhibited cell invasion and migration in PTC cell lines.⁷⁰ The authors suggested that by targeting TGF β 1 miR-663 regulated EMT, as well as matrix metalloproteinases.⁷⁰

Long Non-Coding RNAs and EMT

Long non-coding RNAs (lncRNAs) are transcripts 200 nucleotides in length or larger that do not contain a protein-coding sequence. lncRNAs are very abundant with estimates of >30 000 lncRNAs transcripts in the human genome.^{71–77} One group of investigators estimated that there were close to 60 000 lncRNAs including 8000 oncologic lncRNAs.⁷⁴ A growing number of these lncRNAs are being isolated and characterized from thyroid and other cancers.⁷⁵ Some lncRNAs are upregulated, whereas others are down-regulated in thyroid cancers, especially in PTCs, which has been the most frequent type of thyroid cancers used to study the role of lncRNAs in thyroid tumors to date. An interesting finding was that some of these lncRNAs have important roles in EMT and in stem cells, as well as in development.^{75–77} Examples of three lncRNAs associated with tumor progression, EMT and CSCs are summarized below.

METASTASIS-ASSOCIATED LUNG ADENOCARCINOMA TRANSCRIPT 1 (MALAT1)

MALAT1 is a widely expressed lncRNA that is highly conserved. MALAT1 has been found to be highly over-expressed in several human malignancies and has been associated with tumor invasion and metastasis in various cancers.^{78–84}

Jiao *et al*⁷⁸ reported that MALAT1 could increase the proportion of pancreatic CSCs, maintain self-renewal capacity, decrease chemosensitivity to anticancer drugs and promote angiogenesis *in vitro* in pancreatic cancer cell lines. Downregulation of MALAT1 reduced self-renewal-associated factors, such as SOX2, Bmi and Nanog. Treatment with siRNA led to less tumor growth in xenografted pancreatic cancer tumor cells.

Recent studies by Zhang *et al*⁷⁹ using a papillary thyroid cancer cell line (TPC-1) showed that MALAT1 expression was markedly increased *in vitro* when the tumor cells were treated with TGF β 1, which induced EMT and led to an increase in expression of EMT markers. As analogous studies by Hardin *et al*³⁶ with these same cell lines had previously showed that EMT is associated with an increase in thyroid CSCs, it is possible that there was also an increase in thyroid CSCs in experiments by Zhang *et al*.⁷⁹

HOX TRANSCRIPT ANTISENSE INTERGENIC RNA (HOTAIR)

HOTAIR is a well-studied lncRNA that binds the polycomb repressive complex 2 and directs it to target genes, and promotes gene silencing by histone H3 lysine 27 trimethylation.^{85–89} HOTAIR expression supports metastasis in breast cancer and has been linked to poor outcome in several malignancies.⁸⁵ Padua Alves *et al*⁸⁶ examined breast and colon cancer cell lines in studies of HOTAIR regulation and found that this lncRNA was increased after TGF β 1 treatment in both breast and colon cancers. This led to an increase in the EMT program, as well as colony-forming capacity of the colon and breast cancer cells. These findings

support a role for HOTAIR in EMT and in stem cell formation. siRNA experiments prevented the EMT program stimulated by TGF β 1 and the colony-forming capacity in the two cell lines.⁸⁶ HOTAIR has also been implicated in the regulation of CSCs in cervical cancer and endometrial cancer.^{87–89}

LINC-ROR

Linc-ROR was first discovered in induced pluripotent stem cells (iPSCs) where it was regulated by the key pluripotency factors Oct4, Sox2 and Nanog.⁹⁰ Linc-ROR has a principal role in maintenance of iPSCs and embryonic stem cells by preventing the activation of cellular stress pathways including the p53 response.⁹⁰ Our laboratory has localized linc-ROR in ATC cell lines and in a CSC clone by *in situ* hybridization (Figures 3c and d) and by RT-PCR (data now shown). The levels of linc-ROR in the CSC clone was noted to be higher than in the parental ATC cell line (Figures 3c and d) from which the CSC clone was derived. Linc-ROR has a regulatory role in multiple cancer types.^{91–96} Recent studies have shown that linc-ROR may function as a molecular sponge for miR-145 targets, such as OCT4, SOX2 and Nanog.⁹² Hou *et al*⁹¹ examined linc-ROR in breast tissue specimens and breast cancer cell lines. They found that linc-ROR enhanced cell migration and invasion, which was associated with the generation of stem cell properties. Linc-ROR was also found to function as a competing endogenous RNA to miR-205 and prevented the degradation of miR-205 target genes including the EMT inducer. In nude mice, silencing of linc-ROR expression inhibited breast cancer tumor growth and lung metastasis.⁹⁰

ROLE OF THE MICROENVIRONMENT AND EXOSOMES IN EMT AND CSCs

A growing body of evidence indicates that the interaction between tumor cells and the local microenvironment at the metastatic site leads to the development of the premetastatic niche(s), which allows for the proliferation of metastatic cells during colonization.⁹⁷ Tumor cells can produce various factors leading to the establishment of the premetastatic niche(s). The microenvironment consist of the tumor stroma, the vasculature including endothelium, smooth muscle cells and pericytes, inflammatory and immune cells such as macrophages, lymphocytes and dendritic cells, cancer-associated fibroblasts (CAFs) and the extracellular matrix non-cellular components. Interactions with adhesion molecules, such as integrins and CD44, proteins, such as thrombospondin and fibronectin, and soluble cytokines all comprise the tumor microenvironment.⁹⁸

Exosomes, which are an important part of the micro-environment, range in size from 40 to 150 nm and represent a subset of multi-vesicular bodies (MVB).^{97–107} They are formed by inward budding of the limiting membrane of MVB and are released from the cell into the extracellular environment upon fusion of the MVB and the plasma

Figure 3 (a) Clonogenic spheres derived from a single anaplastic thyroid carcinoma cell as previously reported.³⁷ The cells were separated into ALDEFLUOR-positive and -negative cells by flow cytometry and cell sorting. The ALDEFLUOR-positive cells formed spheres in serum-free media supplemented with EGF and FGF,³⁶ but the ALDEFLUOR-negative cells did not (scale bar, 50 μ m). (b) Hematoxylin and eosin-stained section of a CSC cloned tumor grown in a nude mouse. Mice were injected with 1×10^4 cells and the tumors were harvested after 8 weeks.³⁷ The tumor cells show aggressive phenotype with large nuclei, prominent mitotic activity (arrow) and focal areas of necrosis (data not shown). (Hematoxylin and eosin; scale bar, 100 μ m). (c) *In situ* hybridization with RNAScope technique using tissues from a tumor grown in nude mice demonstrating ROR-lncRNA in a human anaplastic thyroid carcinoma (THJ-16 T cell line from reference Hardin et al³⁷). The tumor cells show weak (1+) nuclear staining (red arrow), whereas the neutrophilic inflammatory cells in the background (white arrow) show stronger staining (3+). (Hematoxylin nuclear stain and diaminobenzidine as chromogen; scale bar, 100 μ m). (d) *In situ* hybridization with RNAScope technique using tissues from a tumor grown in nude mice demonstrating ROR-lncRNA in a CSC clone derived from parental ATC cell line THJ-16 T as previously reported.³⁷ The CSC clonal cells expressed higher levels of ROR-lncRNA as indicated by stronger (2+) staining in the tumor cell clones (red arrow), whereas the neutrophilic inflammatory cells in the background (white arrow) show stronger staining (3+) (Hematoxylin nuclear stain and diaminobenzidine as chromogen; scale bar, 100 μ m).

membrane. Exosomes contain a concentrated group of functional molecules including proteins, lipids, mRNAs, non-coding RNAs and DNA molecules. Exosomes provide protection for the transported molecules and also serve as intercellular communications locally and systemically.⁹⁸ Exosomes interact with the microenvironment in different ways: (a) they can activate target cells through the transfer of ligands such as different growth factors and (b) they can transfer receptors from one cell to another by fusion with the

plasma membrane of recipient cells. Exosomes get to their targets by endocytosis and transfer the cargo directly into the cytosol of recipient cells. Thus, exosomes are central mediators of cell-cell communication (Figure 1).^{98,103} Prokaryotic and eukaryotic cells release exosomes including cancer cells from a variety of malignancies.

Vesicle-based cell-cell communication by exosomes has been reported during EMT and cancer progression.^{99–101} These molecules can be internalized directly by stromal and

cancer cells and can induce functional changes in the recipient cells. Positive feedback mechanisms can lead to changes in exosome production during tumorigenesis. Deregulation of exosome secretion pathways in tumor cells could lead to an altered microenvironment and modulation of tumor progression.¹⁰⁷ Interactions between cancer cells and components of the tumor stroma have been shown to facilitate EMT induction and drive tumor progression. It has been shown that CAFs facilitate aggressive phenotype of breast cancer cell lines by induction of EMT by TGF β in a paracrine manner.⁹⁹ Other studies have shown that metastatic lung cells were regulated by miR-200 expression and were responsive to TGF β .^{100,101} Metastatic lung cancer cells alternated between epithelial and mesenchymal states and formed polarized epithelial spheres in 3D culture in the presence of TGF β .¹⁰¹ In these experiments, forced expression of miR-200 inhibited the tumor cells from undergoing EMT-associated invasion and metastasis.¹⁰⁰ The regulatory role of exosomes in thyroid cancer has recently been examined. Lee *et al*¹⁰² studied exosomes from the PTC cell line TPC-1 and compared these with a non-neoplastic thyroid cell line NTHY-ori 3-1 (NTHY). Exosomes derived from TPC-1 contained miR-146b-5p and miR-222 as cargo. Both miRNAs showed significantly higher expression in TPC-1 cells compared with NTHY control cells. They found similar miRNA composition within exosomes and in cells suggesting that these miRNAs were actively and selectively packaged into exosomes for secretion.¹⁰²

Other studies have shown that exosomes derived from cancer cells have an important role in premetastatic niche formation (Figure 1).^{103–107} Peinado *et al*¹⁰³ showed in a series of elegant experiments that melanoma exosomes could educate bone marrow progenitor cells into a prometastatic phenotype through MET. The accumulating data would suggest that exosomes mediate communication at both the primary and metastatic tumor sites and that the content of the exosomes can induce a pro-tumorigenic niche and support cell transformation.¹⁰⁴ However, the mode of cargo selection and regulation remain major challenges in understanding the effects of exosomes on CSCs and on the microenvironment.

SUMMARY

The CSC hypothesis has been supported by a great deal of experimental evidence over the past decade. Many biomarkers that recognized CSCs have facilitated their isolation and characterization *in vitro*, but much work is needed to demonstrate the specificity of these biomarkers for CSCs. The close relationship between EMT and CSCs has provided further insights into tumor progression and contribution of CSCs to tumor metastasis and colonization. Recent studies have also established roles for microRNAs and lncRNAs in EMT and in the regulation of CSCs. A growing body of evidence has increased our understanding of the interaction between tumor cells and the local microenvironment, including the role of exosomes, leading to tumor metastasis

and colonization. A better understanding of these mechanisms will lead to more effective targeting of primary and metastatic malignancies.

ACKNOWLEDGMENTS

We thank the Translation Research Initiatives in Pathology Laboratory and the Experimental Pathology Laboratory for technical assistance. The work was supported by the Carbone Cancer Center support grant P30CA014520. Dr R Zhang received a grant from the Department of Pathology and Laboratory Medicine at the University of Wisconsin School of Medicine and Public Health.

DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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