Activation of LINE-1 Retrotransposon Increases the Risk of Epithelial-Mesenchymal Transition and Metastasis in Epithelial Cancer

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Abstract: Epithelial cancers comprise 80-90% of human cancers. During the process of cancer progression, cells lose their epithelial characteristics and acquire stem-like mesenchymal features that are resistant to chemotherapy. This process, termed the epithelial-mesenchymal transition (EMT), plays a critical role in the development of metastases. Because of the unique migratory and invasive properties of cells undergoing the EMT, therapeutic control of the EMT offers great hope and new opportunities for treating cancer. In recent years, a plethora of genes and noncoding RNAs, including miRNAs, have been linked to the EMT and the acquisition of stem cell-like properties. Despite these advances, questions remain unanswered about the molecular processes underlying such a cellular transition. In this article, we discuss how expression of the normally repressed LINE-1 (or L1) retrotransposons activates the process of EMT and the development of metastases. L1 is rarely expressed in differentiated stem cells or adult somatic tissues. However, its expression is widespread in almost all epithelial cancers and in stem cells in their undifferentiated state, suggesting a link between L1 activity and the proliferative and metastatic behaviour of cancer cells. We present an overview of L1 activity in cancer cells including how genes involved in proliferation, invasive and metastasis are modulated by L1 expression. The role of L1 in the differential expression of the *let-7* family of miRNAs (that regulate genes involved in the EMT and metastasis) is also discussed. We also summarize recent novel insights into the role of the L1-encoded *reverse transcriptase* enzyme in epithelial cell plasticity that suggest it might be a potential therapeutic target that could reverse the EMT and the metastasis-associated stem cell-like properties of cancer cells.

INTRODUCTION

The epithelial-mesenchymal transition (EMT) and the reverse process, the mesenchymal-epithelial transition (MET), are key pathways with roles regulating cellular processes such as embryogenesis, gastrulation, neural crest cell migration and tissue regeneration [1]. During the process of EMT, differentiated epithelial cells reorganize their actin cytoskeletal organization and intercellular adhesion structures, undergo changes in cell shape and acquire de-differentiated mesenchymal features, such as a stem cell-like phenotype and enhanced migratory and invasive potential [2]. Although our knowledge of the molecular processes underlying the EMT is still limited, there is evidence to indicate that aberrant EMT activation contributes to the process of metastasis

(spreading of tumor cells from their primary sites to distant sites) of epithelial cancers [3]. For malignant cells to metastasize EMT occurs resulting in loosening of cell-cell adhesions within the primary tumor that permits the initial local invasion and subsequent dissemination. The relevance of the EMT and metastasis process is further supported by the discovery of a direct link between the EMT and the formation of cancer stem cell subpopulations within cancer cells, thereby generating self-renewing metastatic cells, which are capable of initiating the secondary tumor at distant sites [4]. Thus, identifying the factors and genes underlying the EMT is a key step for understanding the complex process of cancer progression and metastasis.

The EMT is a multistage and reversible process mediated by many transcriptional factors and genes operating in the tumor microenvironment. One well-studied EMT-related gene is E-cadherin (*CDH1*), a calcium-dependent cell surface glycoprotein that mediates the formation of tight epithelial cell-cell adhesions and junctions. An early marker of EMT

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induction is the loss of E-cadherin expression, which reinforces the EMT process by inducing the expression of the *Twist1* and *ZEB1* transcription factors in a feed-forward loop [5]. During metastatic progression, as observed in colorectal cancer, expression of E-cadherin is lost in invasive cells [6]. At the same time, there is induced expression of N-cadherin, a form that is normally expressed in mesenchymal and neuronal cells together with the cell surface protein, CD44, a process known as 'cadherin switching' [7]. Strikingly, overexpression of E-cadherin in mesenchymal breast cancer cells induces the MET process [8], suggesting that EMT is a reversible process. During EMT, complex cellular and morphological changes occur in cancer cells that reflect changes in the expression of numerous genes and the cooperation of a large number of signaling pathways and regulators [9]. Based on their function, these can be categorized into three groups: EMT effectors (epithelial junction proteins such as E-cadherin, α-catenin and β-catenin), EMT regulators (transcriptional factors such as *Twist1/2, ZEB1/2, Snail1/2, Slug* and *E12/E47*), and EMT inducers (developmental signaling pathways that include TGF-β, Wnt, Notch, and growth factor receptor signaling pathways such as HGF, EGF, IGF and TNFα) [10]. In addition, many miRNAs directly regulate the expression of EMT-related genes. Notably, the *miR-200* family regulates the EMT by inhibiting the transcription factors, *ZEB1* and *ZEB2* [11], which are involved in repression of E-cadherin. These discoveries emphasize that EMT is a complex process involving many factors and regulators in modulating stemness and cellular plasticity and that the EMT program can be activated in a number of different ways. Indeed, one of the little known factors impinging on the EMT is the aberrant expression of retrotransposons, ubiquitous mobile DNA elements in many eukaryotic organisms that can amplify themselves by utilizing reverse transcriptase and RNA intermediates to relocate within the cellular genome.

Retrotransposons are divided into two subclasses: LTR- (long terminal repeats) and non-LTR retrotransposons. LINE-1 (Long Interspersed Nuclear Element 1 or L1 element) is the most common type of non-LTR retrotransposons in the human genome; with about 500,000 copies, it comprises about 17% of the genome [12]. Unlike exogenous retroviruses, retrotransposons form an integral component of the genome and are normally inactive in somatic tissues due to the presence of repressive cellular mechanisms [13]. Although there is a growing evidence that a small fraction of the active retrotransposons has the capacity to initiate cancer formation due to genomic insertions [14], notably to mutate protein-coding gene expression, almost nothing is known about the impact of retrotransposons on the processes underlying the EMT and development of metastases. In this article we explore the possibility that how unscheduled activation of retrotransposons may increase the risk of EMT and metastasis, through their capacity to reshape gene expression and the associated cellular transformation.

L1 RETROTRANSPOSONS IN CANCER DEVELOPMENT

L1 is capable of generating genetic mutations by inserting copies of itself into genes and affecting gene function (Fig. **1**). While most L1 are defective due to truncations or mutations, L1 belonging to the human-specific Ta1 subfamily are intact, full-length retrotransposons and are potentially active in human cells. At present, at least 100 copies of L1 have been identified as functional elements [14], retaining their ability to move about the genome i.e. they are retrotransposition-competent. An active L1 comprises an internal promoter, two open reading frames and a 3' poly-A tail. The open reading frames encode two proteins: ORF1p with RNA-binding activity and ORF2p containing a reverse transcriptase (RT) and an endonuclease. ORF2p cleaves genomic DNA to form a 3'-end primer from which L1 mRNA is reverse-transcribed into a DNA copy, which is then integrated into a new genomic site, resulting in a newly retrotransposed L1 copy. These L1 insertions are capable of altering the transcriptome by disrupting gene function, altering gene splicing, increasing the frequency of recombination [12], and negatively affecting the stability and integrity of the genome because of their ability to create breaks in genomic DNA during the process of mobilization [15, 16]. In addition, L1 facilitates the mobilization of the *Alu* family of short interspersed nuclear elements (SINE), certain cellular RNAs, and noncoding RNAs to new sites in the genome [17], thereby reshaping cellular function in multiple ways.

L1 is rarely expressed in differentiated stem cells or adult somatic cells. In contrast, the majority of cancer cells and cancer-derived cells are characterized by aberrant expression of L1 [18, 19]. This may be because of changes in the methylation status of L1 promoters [20]. Studies have shown that L1 hypomethylation is associated with activation of L1 expression in various cancers [21]. However, while many studies show a correlation of L1 hypomethylation with cancer formation, some cancers suggest that there is no correlation [22]. In addition, a number of epithelial malignancies and carcinomas from the breast, ovary, lung, liver, colon and rectum, bladder, and prostate have been shown to have a high level of L1 activity [23-26]. When L1 elements become active, they can rapidly increase their copy numbers by a 'copy-and-paste' mechanism. As a consequence, L1 insertions provide a source of genetic mutations by activating oncogenes or inactivating tumor suppressor genes (Fig. **1**). Not unexpectedly, L1 insertions occur in genes that are commonly mutated in epithelial cancers [27]. Several tumor-specific *de novo* L1 insertions have been identified in lung and liver cancer using retrotransposon capture sequencing and whole genome-wide mapping [23]. A recent survey of genome sequencing data, including those reported in the Cancer Genome Atlas (TCGA) project, identified a number of L1 insertions in colon, colorectal, prostatic and ovarian cancers [24]

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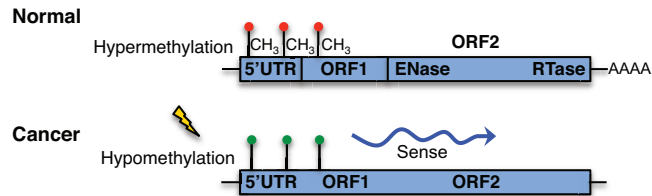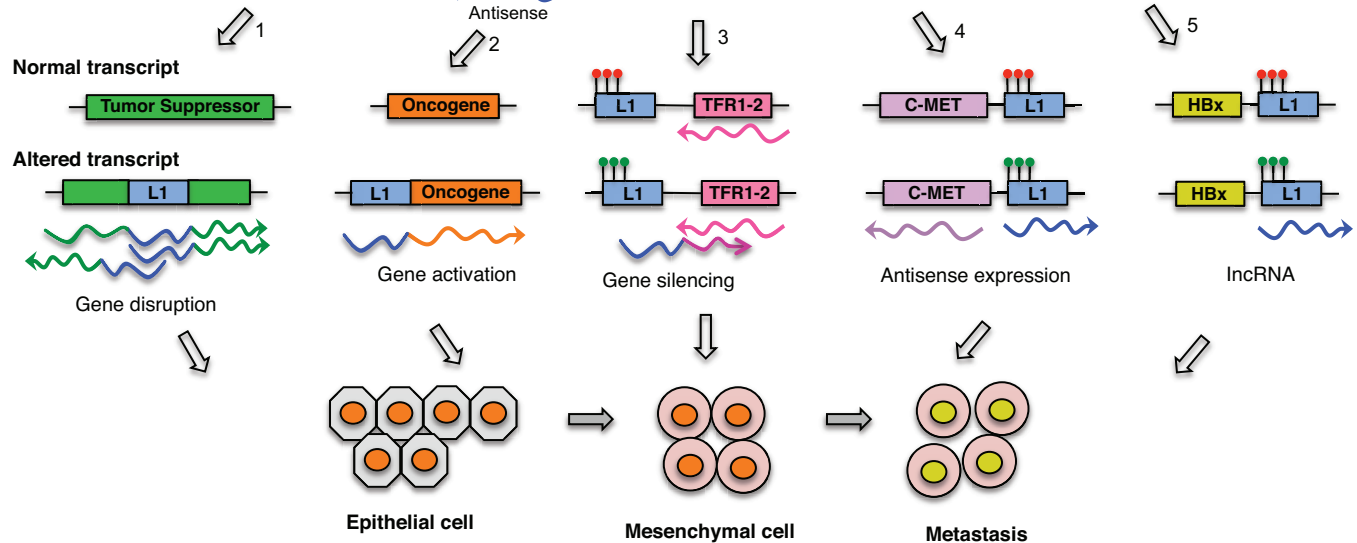
Fig. (1). A schematic picture of L1 retrotransposition. (**A**) Structure of a functional L1 showing the 5'-UTR promoter, ORF1- and ORF2-protein encoding genes, and the poly-A tail. *ENase* endonuclease, *RTase* reverse transcriptase enzyme. L1 expression/activation is mostly undetectable in normal cells due to the presence of DNA hypermethylation. In contrast, the majority of epithelial cancers exhibit high levels of L1 activation. Red and green closed circles on sticks denote hypermethylated and hypomethylated L1 promoters, respectively. Blue arrows indicate bidirectional transcripts from sense and antisense promoters within L1 promoter regions. (**B**) Characteristics of typical L1 activation in cancer cells. **1)** A newly retrotransposed L1 DNA copy (blue coloured box) is commonly inserted into tumor-suppressor genes (e.g., *ST18, SLC21, PHGDH*), resulting in altered expression of the genes or disruption of gene function. **2)** The presence of a retrotransposon in the upstream region of an oncogene can activate its expression and induce tumorigenesis. **3)** The presence of a retrotransposon in the upstream of some tumor-suppressor genes (e.g., *TFP1-2*) may lead to the expression of L1 chimeric sequences (LCT), which suppress gene expression through antisense RNA interference. **4)** A retrotransposon carries sense and antisense promoters that can initiate both upstream and downstream (e.g., *c-MET*) expression, thereby activating normally repressed genes, which can itself eventually lead to metastasis. **5)** L1 retrotransposition often activates the expression of long noncoding RNA-like transcript (lncRNA) by read-through transcription of nearby promoters, which often confer tumor-promoting properties through activation of the ß-catenin/Wnt-signaling pathway. Moreover, changes in the expression of certain types of genes alone may be sufficient to activate the process of EMT and metastasis in multiple ways. (Color images available online).

including one report of 9 somatic L1 insertions across 6 lung cancers [28]. Another recent study [26] of the TCGA project across 290 cancer samples found that L1 insertions are common in the cancer genome, at least one such event in 53% of patients, with colorectal (93% of patients) and lung (73% of patients) cancers being most frequently affected.

Activation of L1 can also lead to the production of chimeric transcripts containing partial L1 sequences as well as flanking genomic sequences that include protein-coding regions, referred to as LCTs (LINE-1 chimeric transcripts). Our understanding of the role of L1 in the epigenetic silencing of tumor suppressor genes has been further enhanced by the discovery that the known metastasis suppressor gene, *TFPI-2*, in breast and colon cancer is silenced by the expression

of an LCT, a large RNA antisense to *TFPI-2* which is located 300 kb away from the LCT site [29]. Moreover, there is evidence that L1 promoter sequences often initiate expression of transcripts in an antisense orientation, resulting in activation of normally repressed genes in a number of malignancies, including chronic myeloid leukemia, and bladder and breast cancers [30]. One such L1-activated proto-oncogene is *c-MET*, a receptor with tyrosine kinase activity, which activates a wide range of cell signaling pathways, including those involved in proliferation, invasion and the development of a cancer stem cell phenotype [31]. L1 activation also contributes to the development of metastases in colorectal and liver cancer by promoting the expression of numerous oncogenes [30] including *RAB3IP* (which modulates actin cytoskeletal organization) and *CHRM3* (which promotes invasion by a COX-2-mediated

cellular mechanism). Collectively, these findings provide direct evidence for L1 expression as a key contributor to cancer development, progression and metastasis.

L1 ACTIVATES THE PROCESS OF EMT AND METASTASES DEVELOPMENT

L1 expression has been reported to be part of cell proliferation in the establishment of the undifferentiated state in embryonic stem cells (ES) and in the proliferative capacity of early embryo development [32]. If siRNAs targeted against active L1 ORF2p are microinjected into male pronuclei after fertilization, development is completely arrested, indicating that L1 is essential to the progression of early embryogenesis [33]. Tissue-regeneration studies in salamander have also shown that L1 is required for the early stages of tissue regeneration [34]. These observations suggest that L1 expression might be involved in the activation of genes that are required for cellular de-differentiation processes in early development. At present however, it is unclear how L1 contributes to the regulation of such genes. One possibility is that L1 might exhibit similar functions to the L1TD1 (LINE-1 type transposase domain containing 1) protein, which is an RNA-binding protein that is involved with pluripotency and the self-renewal of undifferentiated ES cells through interactions with transcriptional factors such as *Nanog, Pou5f1, Sall4, ZNF512b* and *Lin28* [35]. Depletion of *L1TD1* results in downregulation of *Oct4* and *Nanog*, suggesting that L1TD1 plays a crucial role in the regulation of stemness [36]. Importantly, *L1TD1* does not encode a transposase; instead, it is related to the ORF1p of L1, which possesses RNA-binding activity. Several studies have shown that undifferentiated ES cells also support the expression of L1 retrotransposons [37, 38]. Given that L1 are frequently expressed in undifferentiated stem cells and cancer cells, but not normally in differentiated stem cells or in somatic tissues, it is logical to assume that L1 activities are linked to the activation of genes involved in cell proliferation. In this context, recent studies also point to the involvement of the L1-encoded RT enzyme in the regulation of cell proliferation and tumorigenesis [39-42]. This raises questions about the role L1 plays in the process of EMT and metastasis. Thus the link between the EMT and the expression of L1 in cancer cells seems logical.

The transformation of normal epithelial cells to metastatic cancer cells involves multiple genetic and epigenetic changes. Many oncogenes and post-transcriptional regulatory networks, including miRNAs, have already been implicated in EMT and metastasis. During EMT, cells lose their cell-cell junctions, leading to the acquisition of an invasive phenotype. Normal epithelial cells synthesize adhesion proteins and assemble and adhere to extracellular basement membrane proteins such as laminin and fibronectin and a loss of the ability to interact with the basement membrane is one of the early features of carcino-

genesis [43]. Loss of attachment to the basement membrane is also a prerequisite for metastatic dissemination. Having lost their attachments, cells may enter the blood or lymphatic system, invade surrounding tissues and eventually generate metastases by colonizing to distant sites. In recent studies, we have shown that the expression of L1 occurs in almost all high-grade human breast cancers [18] including triple negative breast cancers and estrogen-negative (ER-) tumors, which are characterized by frequent metastases, recurrence, and intrinsic resistance to hormone therapy. L1 expression is associated with high rates of lymph node metastasis and a poor prognosis. In support of these findings, another study has shown that breast carcinoma releases retroviral-like particles that possess high levels of the HERV-encoded RT enzyme [44]. Furthermore, the level of L1-encoded nucleic acid is also high in tumor microvesicles in the plasma of patients with breast cancer, melanoma and lymphoma [45], and after ionizing radiation *in vitro* [46], thus linking L1 activity to metastatic disease development. In conclusion, although the contribution of L1 activity to tumorigenesis has been recognized, little is known about the role of L1 in the EMT and metastasis.

Direct evidence for the role of L1 in the process of EMT comes from a recent immunoprecipitation analysis of L1-encoded proteins in hepatocellular carcinoma cells [47]. In this study, L1-ORF1p has been shown to form a complex with Smad4, which in turn suppresses the nuclear translocation of Smad4 elicited by transforming growth factor β (*TGF-β*), resulting in TGF-β signaling of the EMT and cancer progression. Strikingly, a recent high-throughput transcriptome sequencing study in hepatocellular carcinoma reported that a L1 transcript driven by an HBx promoter, referred to as HBx-LINE-1, was found to be expressed in nearly 25% of hepatocellular carcinoma examined, and correlated with poorer patient survival [48]. Intriguingly, HBx-LINE-1 expression results in formation of a long noncoding RNA (lncRNA), which interacts with and induces the nuclear translocation of β-catenin. Consequently, HBx-LINE-1 activates β-catenin/Wnt signaling and facilitates the process of invasion and cancer colony formation. Although these findings suggest a role for L1 in EMT and hepatocellular carcinoma, it is not clear whether a similar pattern of expression of L1-driven lncRNAs exists in other types of malignancy. Besides interacting with β-catenin, L1 has been reported to activate the c-MET tyrosine kinase receptor in several types of cancers [30]. Activation of the normally silent c-MET leads to its interaction with E-cadherin at the site of cell-cell adhesion complexes [49], resulting in a reduction of E-cadherin and triggering the release of β-catenin and the translocation of β-catenin to the nucleus where it acts as an activator for transcription factors that promote the development of metastases [50]. Together, these studies suggest that L1 can activate the EMT in multiple ways.

INHIBITION OF L1 ACTIVITY REVERSES THE EMT PROCESS

L1 RNA and L1 encoded proteins are abundantly expressed in epithelial cancers and are significantly correlated with poorer patient survival [18, 19]. There have been a number of studies conducted to investigate the potential roles of L1 in the development of malignancy and metastases. Silencing of L1 expression in A-375 melanoma cells, both by transient and stable expression of L1-specific siRNA, leads to reduce levels of L1 expression and induces morphological differentiation, as revealed by changes in cell shape, formation of dendritic-like extensions and increased cell-cell adhesion structures [39, 41]. Significantly, these morphological changes are accompanied by increased expression of *E-cadherin*. At the same time, there is decreased expression of the proliferation genes, *cyclin D1* and *c-Myc*, suggesting that L1 is directly linked to the reprogramming of genes involved in cellular transformation. Importantly, the silencing of L1 expression in melanoma cells strongly reduces their tumorigenicity when the cells are inoculated into nude mice [39]. These findings have opened up the new prospect of developing L1 inhibitors for diagnostic and therapeutic interventions in cancer.

Viruses that use reverse transcription in their life cycles, such as HIV or hepatitis B virus, are a major healthcare problem, and enormous efforts have been focused on developing drugs for these infections. Efavirenz is a non-nucleoside reverse transcriptase inhibitor (NNRTIs) that is widely used to treat HIV infections. It is a broad-spectrum inhibitor, which acts allosterically by binding to a hydrophobic region of the HIV RT enzyme, inducing a conformational change in the enzyme and inhibiting its activity [51]. Recently, efavirenz has also been reported to inhibit the activity of the L1-encoded RT enzyme in a manner similar to that of L1-specific siRNA in A-375 melanoma and PC3 prostate cancer cells [41]. Several *in vivo* mouse studies have validated the cytotoxic effect of efavirenz at inhibiting L1 in H69 lung carcinoma and HT29 colon carcinoma [39]. In addition, we have shown that L1 expression decreases markedly in T47D and MCF7 breast cancer cells after treatment with efavirenz [42]. These cancer cells normally grow in multilayer clumps with indistinct cell borders and shapes due to loss of their cell-cell adhesions and an increased tendency to form aggregates, features that are characteristic of the EMT process. When cancer cells are treated with efavirenz, the cells show significant changes in morphology [39, 42] and regaining epithelial features in a manner that is reminiscent of the reverse process of EMT, i.e. the MET (Fig. **2**).

Cellular differentiation is often characterized by the formation of elongated microtubules, with the cells resuming contact inhibition and developing into monolayer cultures. In contrast, cancer cells lose contact inhibition and proliferate as multilayer clumps with indistinct cell borders and shapes. Using gene expression profiling with microarrays and gene ontology annotation enrichment analysis, we recently identified clusters of genes induced in response to efavirenz treatment [42]. An upregulated cluster contained genes involved in cellular projection and dendritic spine formation including *EXOC4* and *CDC42*. A second upregulated cluster contained genes involved in the formation of cell-cell junction (*PARD3, PDZD3, CX62, UBN1*) and synaptic functions (*ENAH, ITSN1, SYT17*). Furthermore, in this study, we found a number of downregulated genes that are involved in the positive regulation of cell migration and movement and including a number of well-characterized oncogenes, epidermal growth factor receptor (v-erb-b erythroblastic leukemia viral homolog; *EGFR*) and the erythroblastic leukemia viral oncogene homolog (*ERBB4*). Of these, downregulation of *PTP4A1* and *AAMP* is notable and expression of these genes is required for conferring invasive phenotypes in many epithelial cancers [52]. These findings suggest that inhibition of L1-encoded RT by efavirenz, may act in concert with MET reprogramming and contribute to the modulation of key genes, which are functionally associated with cellular plasticity. In fact, it appears that altered expression of these genes alone could be sufficient to reverse the process of EMT and associated differential plasticity.

L1 MODULATES THE EXPRESSION OF THE TUMOR SUPPRESSORS MIRNAs

Activation of L1 expression is kept in control by a variety of genome defence mechanisms, and is mostly undetectable in normal adult cells and tissues. In this context, we recently identified a number of naturally occurring endogenous siRNAs that are differentially expressed in a range of breast cancer cells compared to normal breast cells [18]. Strikingly, forced expression of these siRNAs in breast cancer cells leads to marked silencing of L1 expression through increased DNA methylation of the L1 promoter [13]. This finding suggests that depletion of L1 specific endogenous siRNAs is likely to be one of the causes of the expression of L1 in cancer cells. To investigate whether the silencing of L1 could play any role in the expression of small noncoding RNAs, we carried out a global analysis of small noncoding RNAs using high-throughput deep sequencing analysis [53]. To our surprise, we found that cancer cells in which L1 expression is silenced by overexpression of a specific endogenous siRNA targeting the L1 promoter exhibit greatly increased expression of a number of tumor suppressors miRNAs, and in particular, members of the *let-7* miRNA family [54], which accounted for nearly 40% of the overall increase in global miRNA upregulation, compared to the non-silenced cells in which L1 remains active. All members of the *let-7* family are upregulated in L1 silenced cells and, in some cases, very markedly (e.g. *let-7a, let-7b, let-7c, let-7e,* and *let-7f*). The marked differential expression of *let-7* miRNA family members is an intriguing finding, similar to the differential expression of *let-7* observed in normal and a variety of cancer cells whereby the expression of *let-7* is post-transcriptionally inhibited [55]. In addition, large changes in the expression of other miRNAs including *miR-200c* are also observed in
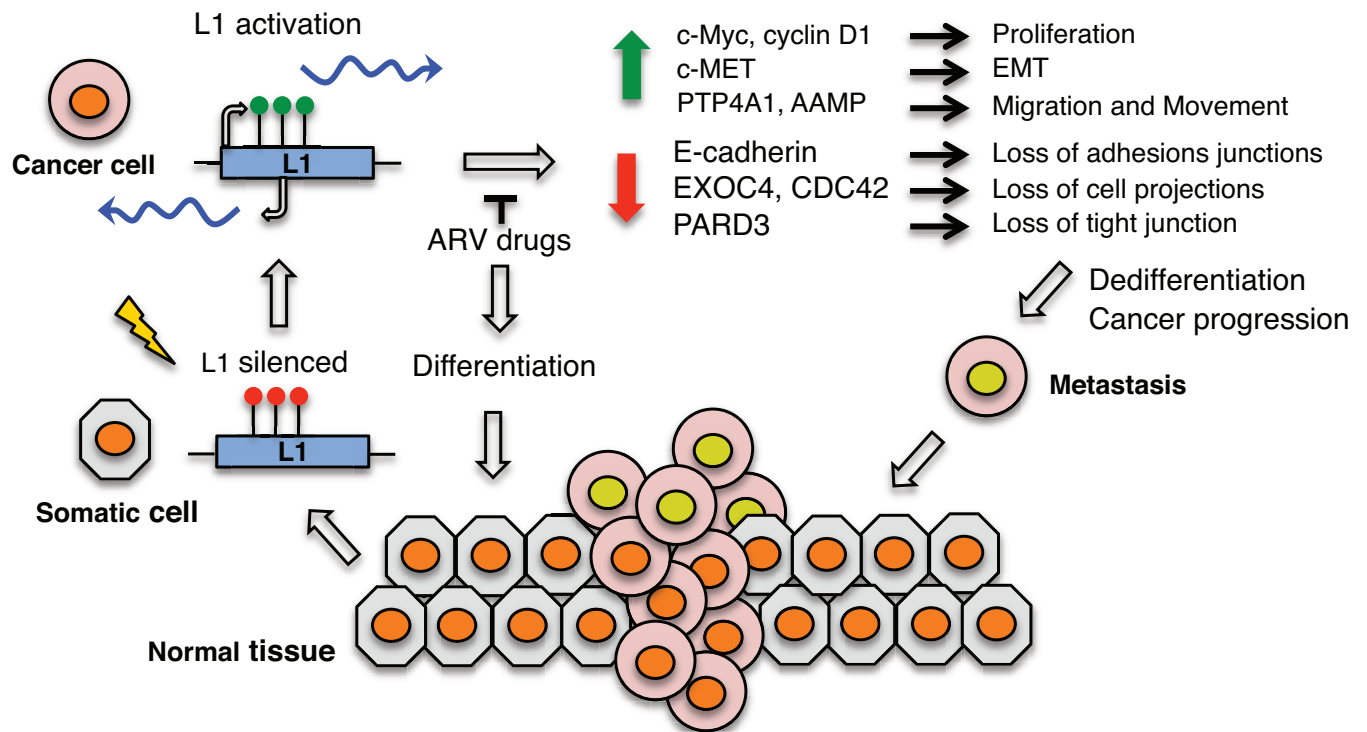
Fig. (2). Expression of L1 occurs at an early stage of cancer formation and is seen all grades and stages of cancer including metastatic cancers. L1 may result in upregulation (green arrow) or downregulation (red arrow) of a number of genes involved in cellular dedifferentiation and cancer progression. Some of the most commonly altered genes and their functions in the process of EMT and metastasis are shown. L1-encoded RT enzyme may be a potential target for anticancer therapy. Inhibition of RT in the early stages of cancer by antiretroviral (ARV) drugs such as efavirenz, nevirapine or combination of RT inhibitors can modulate the expression of genes involved in cell proliferation and migration, thereby promoting cellular differentiation and reducing morbidity and mortality from epithelial cancers. (Color images available online).

the L1-silenced cells [53]. In support of these findings, a recent study in A-375 melanoma cells reported that L1 inhibition with efavirenz downregulates the expression of many oncomiRNAs, miRNAs that are known to activate tumor progression and metastasis. Simultaneously, there is increased expression of several tumor suppressors miRNAs, suggesting that L1 inhibition causes global changes in the transcriptome of cancer cells. Importantly, most efavirenz-modulated miRNAs are found to be typically localized close to or at cancer-associated genomic regions and fragile sites. Although we have only a limited understanding of how the modulation of genes or miRNA expression occurs, the changes observed are reversible when L1 inhibition is stopped. This suggests that L1 could act through reversible epigenetic changes that affect the expression of genes and miRNAs.

Let-7 is one of the most highly conserved miRNAs and is considered to be a vital tumor suppressor targeting cancers and cancer stem cells within tumors. *Let-7* is known to target many oncogenes and "stemness" factors including *c-Myc, Ras, HMGA2, NF-κB* and *Lin28* [56] and studies in lung, breast and prostate cancer reveal that overexpression of *let-7* inhibits cancer cell proliferation, *in vitro* clonal expansion and *in vivo* tumor regeneration by reducing *c-Myc* expression [57]. *c-Myc* is one of the key genes activated by L1 activity in cancer cells [39, 42] and

interestingly, the c-Myc protein also binds to let-7 promoter and decreases *let-7* expression in a feedback loop [58]. During stem cell differentiation, certain members of the *let-7* family of miRNAs are expressed at high levels, playing key roles in differentiation. In contrast, Lin28, an inhibitor of *let-7* miRNAs, is mainly expressed in undifferentiated stem cells and forms part of a pluripotency network regulated by transcriptional factors including Oct4, Sox2 and Nanog [59]. Introduction of *let-7* miRNAs is sufficient to rescue the compromised differentiation phenotype of miRNA-deficient stem cells. A recent study reports that c-Myc protein can also stimulate *Lin28* expression by binding to the Lin28 promoter. Lin28 subsequently represses *let-7* expression post-transcriptionally [60]. We and others have shown that inhibition of L1 activity reduces the expression of *c-Myc*, explaining the ability of L1-silenced cells to activate *let-7* miRNA [39, 42]. Thus it seems that L1 is somehow involved in the *c-Myc/let-7/Lin28* regulatory circuit (Fig. **3**). Whether this involvement is direct or indirect has yet to be proven experimentally. Interestingly, the overexpression of *let-7* miRNA in breast cancer stem cells blocks tumor progression and inhibits the self-renewal capacity of metastatic cells by directly influencing its targeted genes [61]. Moreover, *let-7* also targets several oncogenes that mediate the activation of the mitogen-activated protein kinase (MAPK) and estrogen-receptor

(ER) signaling pathways [62], thereby inhibiting proliferation and suppressing tumor-promoting transcription factors in breast cancer. For these reasons, inhibition of the L1 may be a potential therapeutic strategy to treat cancers and cancer stem cells in clinical applications.
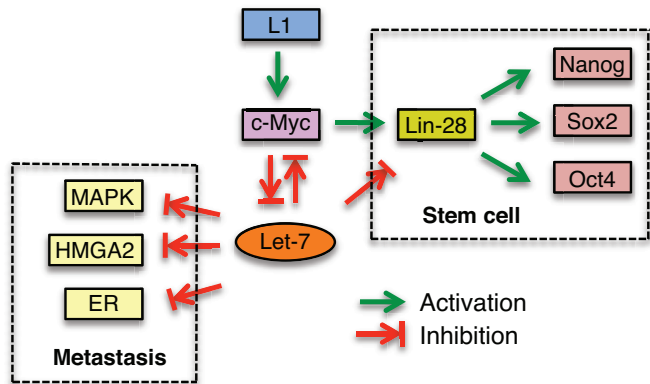
Fig. (3). The interaction of L1 with *c-Myc* and *let-7* and potential mechanisms by which L1 might act to promote processes such as metastasis and cancer stem cells development are shown. In these regulatory circuits, L1 activates c-Myc protein, which in turn decreases *let-7* expression and increases *Lin28* expression *via* multiple feedback loops.

Currently, few studies have addressed the functional role of L1 in the EMT or development of metastases. In addition, it is still unclear whether the effects of L1 activity are direct or indirect process that increases the risk of EMT and cancer growth. Our recent gene expression profiling study combined with deep sequencing of the small RNA transcriptome in L1 silenced and L1 active cells has revealed that activation of L1 substantially affects the transcriptome in a manner that is likely to contribute to the process of epithelial plasticity [53, 54, 63]. Consistent with this, a recent *in silico* analysis proposes that L1 may regulate a network of genes by providing a source of transcriptional regulatory signals not previously known to be present in the promoters of genes [64]. A recent study sequencing the 5'-most nucleotides of RNAs from normal and neoplastic cells supports the idea that highly specific patterns of transcriptional activity occur in these cell types due to the expression of L1 [28]. However, further research is needed to evaluate the precise function of L1 in the activation or repression of genes and miRNAs involved in the EMT or MET processes. Given that L1 encodes an RT enzyme, a strategy in which L1 activity is selectively inhibited by antiretroviral drugs could reduce morbidity and mortality from epithelial cancers and thus provide new therapeutic options for patients with metastatic disease.

PRECLINICAL STUDIES OF L1 BLOCKAGE AND PROSPECTS FOR L1 DRUG INHIBITORS

There is a large body of evidence based on studies in *in vitro* cancer cells and mouse cancer models suggesting that L1 blockade with NNRTIs such as efavirenz or nevirapine is a feasible strategy for treating epithelial cancers. The effects of L1 blockade have been assessed in animal xenograft models of cancer utilizing A-375 melanoma, PC3 prostate carcinoma, H69 small cell lung carcinoma and HT29 colon carcinoma cells inoculated in nude mice [39]. L1 blockade by efavirenz and nevirapine one week after tumor inoculation antagonized the progression of all four tumors, consistent with *in vitro* results of gene signature in cancer cells [41]. Tumor progression quickly resumed on discontinuation of the drug treatment, suggesting that L1 blockade acts through reversible process. An important conclusion emerging from this study was that prolonged L1 inhibition maintained the tumors in a repressed, non-invasive state. Recently, another type of NNRTIs drug belonging to the F2-DABO class (5-alkyl-2-(alkylthio)-6-(1-(2,6-difluorophenyl) propyl)-3,4-dihydropyrimidin-4(3H)-one derivatives or SPV122 compound) has been shown to have a strong anti-proliferative effect and induce differentiation in melanoma cells [65]. This drug also induced apoptosis in a cell-density-dependent manner and antagonized tumor growth in animal models. In this study, cancer cells were inoculated in athymic nude mice which were then subjected to treatment with either 3g or isomer (−)-3g′, endowed with the strongest anti-tumor growth compared to untreated animals. The inhibitory efficacy of the latter compound was comparable to or higher than that of efavirenz. All these effects are similar or even more pronounced than that functional knockout of the L1 RT enzyme by RNAi.

Efavirenz has been shown to be selectively cytotoxic against a range of tumor *in vitro* including breast, colorectal and pancreatic cancer. Despite a lack of understanding of how L1 inhibition regulates cellular processes, Efavirenz is currently undergoing a phase II trial in castration-resistant metastatic prostate cancer [66]. Importantly, this trial has revealed that patients with the highest plasma concentrations of efavirenz (>3000 ng/ml) showed less progression of their cancers compared with patients who received suboptimal concentrations of Efavirenz. This suggests that higher doses of Efavirenz are required for its cytotoxic effects in prostate cancer. Interestingly, this optimal dose for inhibition of metastatic prostate cancer is less than the 1800-mg/day recommended initially by the FDA for the treatment of HIV infections. Because it is an existing drug with a known pharmacokinetic and safety profile and is currently in use against HIV infections, Efavirenz (either alone or in combination with existing chemotherapy) could be rapidly evaluated in clinical trials to evaluate its effect on other types of epithelial cancers such as breast, lung and liver cancer in which L1 activity is known to be present. Knowledge of how L1 inhibition works in cancer could be fundamental importance to understanding NNRTIs as anticancer agents in cancer therapy.

CONCLUSION

In this article, we have discussed how L1 activity impacts on the processes of the EMT and cancer metastasis by changing and fine-tuning gene

expression in cancer cells. The relationship between L1 activity and the EMT was highlighted by showing, firstly, that L1s are expressed in undifferentiated stem cells and cancer cells, but not in differentiated stem cells or adult somatic cells, indicating that L1 activity is linked to cell proliferation and differentiation. Second, L1 expression is closely linked to the growth of cancer cells in multilayers with indistinct cell borders and cell shapes due to a loss of cell-cell junctions and cell-cell adhesion structures. Third, inhibition of L1 activity by the antiretroviral drug, Efavirenz, reduces cell proliferation and induces morphological differentiation of cancer cells, which is accompanied by reduced expression of *cyclin D1* and *c-Myc*. Simultaneously, there is the increased expression of *E-cadherin*, suggesting that L1 is directly or indirectly associated with the reprogramming of genes involved in the EMT. Fourth, L1 inhibition modulates the expression of the tumor suppressors miRNAs that are functionally linked to the control of stemness in human cancer. Together, these studies provide compelling examples confirm the interplay between L1 aberrant expressions and the EMT and metastasis. Although still in its infancy, therapeutic targeting of L1 by antiretroviral drugs offers an unprecedented opportunity to prevent or reverse the process of cancer and cancer-associated stemness. Further studies are required to identify the mechanisms by which L1 affects transcriptome that would help us to understand the role between L1 in the pathogenesis of cancer, which may in turn expedite more effective clinical management of malignant disease.

ABBREVIATIONS

AAMP	=	Angio-associated migratory cell protein
CDC42	=	Cell division cycle 42
CHRM3	=	Cholinergic receptor muscarinic 3
DHX	=	DEAH (Asp-Glu-Ala-His) box helicase
EGF	=	Epidermal growth factor
EMT	=	Epithelial-mesenchymal transition
EXO4	=	Exonuclease 4
HGF	=	Hepatocyte growth factor
IGF	=	Insulin growth factor
L1TD1	=	LINE-1 type transposase domain containing 1
LIN28	=	Lin-28 homolog
LINE-1 or L1	=	Long interspersed nuclear element, type 1
ORF	=	Open reading frame
PTENP1	=	Phosphate and tensin homolog pseudogene 1
PTP4A1	=	Protein tyrosine phosphatase type IVA member 1
RAB3IP	=	RAB3 interacting protein

TFP1	=	Transferrin pseudogene 1
TNF	=	Tumor necrosis factor
Wnt	=	Wingless-type MMTV integration site
ZEB1	=	Zinc finger E-box binding homeobox1

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