Deep Insight Section

LINE-1-encoded Reverse Transcriptase in the genesis and therapy of cancer
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Abstract
Long Interspersed Nuclear Elements (LINE-1) make up a large family of autonomous retrotransposons, accounting for about 17% of the human genome. They constitute the major source of non telomeric Reverse Transcriptase (RT), an essential component of the retrotransposition machinery. Expression of RT-encoding LINE-1 sequences is low in differentiated, non-pathological cells and highly active in early embryos, germ cells and in a broad spectrum of cancers. Growing evidence functionally implicate RT in control of cell growth and differentiation and suggest causative roles in cancer onset. Indeed, inhibition of RT activity reduces proliferation, promotes differentiation and antagonizes cancer progression in animal models. More recently, RT inhibition proved effective in a phase II clinical trial with metastatic prostate cancer patients. Furthermore, the LINE-1-encoded ORF2p product, encompassing the RT-encoding sequence, was found to be already expressed in precancerous lesions, increasing in progressive stages, while being undetectable in normal tissues. RT emerges therefore as a key component of a genome-wide regulatory mechanism that is active in embryogenesis, repressed during cell differentiation, and aberrantly reactivated in cancer cells.

Introduction
A striking, unexpected finding from the Human Genome Project was that protein-coding genes account for a mere 1.2% of the genome, while the vast majority is constituted by a heterogeneous array of non-coding sequences (International Human Genome Sequencing Consortium, 2001; Waterston et al., 2002). That finding challenged the predominant "gene-centric" view in cancer research and represented a historical and scientific turning point: thereafter, the non-coding genome was no longer regarded as a useless genomic burden, but as a new component with potentially relevant informational content, albeit of unclear function(s). The findings that: i) approximately 80% of the genome, essentially constituted by "dark matter" (Clark et al., 2013), is pervasively transcribed (Djebali et al., 2012), and ii) a relevant fraction of these transcripts has roles in regulating genome functions, progressively strengthened the newly emerging view.

Nearly 50% of the “dark matter” is constituted by families of retrotransposable elements. Among those, LINE-1 (long interspersed nuclear elements), HERV (human endogenous retroviruses), Alu and...
SVA (SINE-R-VNTR-Alu; SINE, small interspersed nuclear elements; VNTR, variable number tandem repeats) are most abundant (reviewed by Goodier and Kazazian, 2008). These elements mobilize via a “copy-and-paste” mechanism that uses a reverse transcriptase (RT).

Figure 1. LINE-1 ORF2p product is overexpressed in human cancer. A: Structure of the human LINE-1 retroelement. 5’-UTR and 3’-UTR, untranslated regions; ORF1 and ORF2, open reading frames 1 and 2; the polycistronic ORF2 encompasses regions encoding EN, endonuclease (EN), reverse transcriptase (RT) and cysteine-rich (C) domains. The black box represents the intergenic spacer between the two ORFs. SP, sense promoter; ASP, anti-sense promoter. B: Immunohistochemical staining of ORF2p in human biopitic samples form normal and cancer tissues. Representative tissue sections from: normal colonic mucosa, normal prostatic gland, normal lung epithelium, normal breast (leftmost column) and respective carcinomas (right column).
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enzyme to reverse-transcribe RNA intermediates into cDNA copies, which then integrate in several possible genomic sites. LINE-1s and HERVs encode their own RT and are autonomously replicating elements; in contrast, Alus and SVA do not code for RT and use the RT provided by LINE-1 for their mobilization (Levin and Moran, 2011). The LINE-1 family is the major source of the RT activity required for the overall retrotransposition activity in human cells (Brouha et al., 2003). The human genome contains about 5x10^5 copies of LINE-1 sequences (Fig. 1A), comprising: a 5’ untranslated region (UTR) that functions as an internal promoter, two open reading frames (ORF1 and ORF2), separated by a short intergenic region; ORF1 codes. The RT-containing ORF2p product is expressed in cellular contexts characterized by a high proliferation rate and a low differentiation level, two conditions found in early embryos and cancer cells, both characterized by low levels of DNA methylation (Dean et al., 2003; Gaudet et al., 2003; Miousse and Koturbash, 2015). In contrast, differentiated somatic cells and tissues offer poorly favourable environments for LINE-1 expression (Shi et al., 2007), where LINE-1s are epigenetically suppressed or expressed only at basal level, with the exception of brain tissues that escape this general rule (Coufal et al., 2009). The functional roles of LINE-1 expression in embryogenesis are discussed elsewhere (reviewed by Sciamanna et al., 2011; Spadafora, 2015). Here we focus on roles of LINE-1 retrotransposons in tumorigenesis and their therapeutic and diagnostic implications.

LINE-1 activation in cancer
A growing body of evidence now supports a direct correlation between LINE-1 activation and tumorigenesis. Concomitant with the overall genomic hypomethylation typical of cancer cells and tissues (Gaudet et al., 2003; Miousse and Koturbash, 2015), LINE-1 function is resumed in cancer, and fuels bursts of retrotransposional insertions in the host genome, as typically observed in the progression of a variety of cancers. Indeed, the development of high-throughput technologies has enabled the identification and fine localization of de novo somatic LINE-1 insertions in the genome of many types of cancer, i.e.:

- lung (Iskow et al., 2010),
- colorectal (Lee et al., 2012),
- prostate (Lee et al., 2012),
- multiple myeloma (Lee et al., 2012),
- glioblastoma (Lee et al., 2012),
- hepatoma (Shukla et al., 2013),
- esophagus (Doucet-O’Hare et al., 2015),
- colorectal (Solyom et al., 2012),
- pancreas (Rodic et al., 2015),
- gastric (Ewing et al., 2015),
- ovary (Lee et al., 2012; Tang et al., 2017).

Tumors therefore provide highly permissive environments for retrotransposition. It has long remained unclear, however, whether the new insertions are “driver” mutations that actually promote tumorigenesis, or whether they are accompanying “passenger” mutations (Rodic and Burns, 2013). A causative effect of LINE-1 insertional mutagenesis has been shown only in a limited number of breast (Morse et al., 1988) and colon cancer (Miki et al., 1992). Thus, most retrotransposition events appear to arise in consequence of a global deregulation caused by cancer, rather than being the causes.

Nevertheless, increasing evidence indicate that high LINE-1 activity has roles in cancer, independent on the mutagenic effects of retrotransposition. It is indeed well established that increased expression of LINE-1 is associated with transformed cells and tissues, i.e. mouse embryonal carcinoma cells (Martin, 1991; Martin and Branciforte, 1993) and testicular cancer (Brathhauer and Fanning, 1992). More recently, we have shown that:

i) RT-containing ORF2p is expressed in human breast, lung, prostate and colon cancer tissues (Fig. 1B), and in melanoma (A-375), glioblastoma (U-87), colon (HT-29), small cell lung carcinoma (H-69), pancreas (BxPC-3) and prostate (LuCAP, PC-3, DU145) carcinoma cell lines (De Luca et al., 2016); ii) an abundant RT enzymatic activity (Mangiacasale et al., 2003; Landriscina et al., 2005) is detectable in cancer cell lines, i.e. leukemia (NB4, R4, Kasumi-1, HL60), osteosarcoma (Saos-2), breast (MDA-231, MCF7), glioma (U-343 Mg), colon (HT-29) and thyroid (ARO, FRO); and iii) RT activity increases during breast cancer progression in the transgenic murine model MMTV-PyVT (Gualtieri et al., 2013).

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In parallel, LINE-1 ORF1p expression is a hallmark of colon, renal, hepatocellular, lung, breast, pancreatic, and biliary tract carcinomas, pediatric malignant germ cell and of lymphoma (Rodic et al., 2014; Su et al., 2007). Interestingly, nuclear localization of ORF1p (Harris et al., 2010), or of both ORF1p and ORF2p (Chen et al., 2012a), was associated with poor prognostic outcome breast cancer. Finally, overexpression of LINE-1 endonuclease domain (EN) is associated with gastric cancer and lymph node metastasis (Wang et al., 2013). Together, these findings gradually shifted the focus away from the mutagenic potential of retrotranspositional genomic insertions, rather highlighting the importance of LINE-1 expression in tumorigenesis.

**LINE-1-encoded Reverse Transcriptase as a therapeutic target**

**a) Studies in model cancer cell lines**

A functional link between LINE-1-encoded RT activity and cancer emerged clearly after the finding that RT inhibitors limit cancer cell growth. Our group first discovered that pharmacological inhibition of RT with non-nucleosidic RT inhibitors (NNRTi), nevirapine or efavirenz, reduces the inhibition of RT with non-nucleosidic RT inhibitors has no significant effect on healthy, otherwise fully reversible (Sciamanna et al., 2005). RT inhibitors have no significant effect on healthy, non-expressing RT cells (Sciamanna et al. 2013; Bruning et al. 2017).

**b) Animal models**

Efavirenz shows anticancer therapeutic effectiveness in vivo in nude mouse models xenografted with human cell lines (Sciamanna et al., 2005):

- melanoma A-375,
- prostate PC3,
- colon HT29, and
- non small lung cell carcinoma H-69.

Treatment of the xengrafted animals with efavirenz antagonized tumor progression, though the latter was resumed upon discontinuation of the treatment, consistent with the effects observed in cell lines. These results indicate that an RT-dependent tumor-promoting mechanism plays a key role in the onset and progression of cancer. The mechanism is antagonized by RT inhibitory drugs. Finally, the observation that RT-dependent tumorigenesis can be regarded as a fully reversible process suggests an epigenetic level of control.

Remarkably, transient (Sciamanna et al. 2005) or stably induced (Oricchio et al. 2007) down-regulation of LINE-1 expression via RNA interference (RNAi) in A-375 melanoma cells reduced proliferation and promoted differentiation, reproducing the same effects observed with RT inhibitory drugs. In addition, LINE-1 silencing drastically reduced the tumorigenic potential of A-375 melanoma cells inoculated in nude mice (Oricchio et al. 2007). These findings therefore:
i) suggest a causative role of LINE-1-encoded RT in tumorigenesis,  
ii) strengthen the finding that NNRTIs antagonize cancer by specifically targeting RT,  
iii) point out that RT is a promising target for a novel cancer therapy associated with re-differentiation of the cancer cells.

The anticancer efficacy of RT inhibitors has recently been tested in clinical therapy. A small size phase-II trial on patients with metastatic castration-resistant prostate cancer confirmed the anticancer effectiveness of efavirenz, assessed by PSA non-progression, in a subgroup of patients in which the drug hematic concentration reached an optimal concentration (Houedè et al., 2014). It is worth stressing that heterogeneous fluctuations in the concentration of efavirenz in plasma of treated patients are a known phenomenon (Apostolova et al. 2015, Hecht et al. 2015), and must be taken into account when evaluating the therapeutic efficacy of RT inhibitors. Case reports also confirmed the NNRTIs anticancer potential in HIV-negative patients with thyroid cancer (Landriscina et al., 2006; Modoni et al., 2007), improved the long-term survival of a patient with small cell lung cancer (Kato et al., 2005), and showed that NNRTI-based HAART (highly active antiretroviral therapy) promoted the regression of lymphomas (Amengual et al., 2008; Girard et al., 2005).

**LINE-1-encoded RT as a modulator of transcriptome**

Studies addressing the RT-dependent cancer-promoting mechanism have shown that RT inhibitors induce a global alteration of the transcription profiles, at the level of both coding and non-coding RNA populations: specifically, classes of protein-coding genes involved in proliferation, cell migration, and invasion become repressed (Sciamanna et al. 2013; Patmala et al. 2014), while the expression profile of non-coding sequences, including miRNAs and UCRs (ultraconserved long non-coding RNA) - which often show altered expression in cancer types - is deregulated to various extents. A link between RT activity and miRNAs emerged from the finding that melanoma cells exposed to efavirenz exhibit a reversal in the expression pattern of a sub-group of miRNAs, classified as metastamiRs, with key roles in tumor progression and metastasis (Sciamanna et al. 2013).

Similarly, LINE-1 silencing in breast cancer cells induce differential expression of many miRNA species (in particular, members of the let-7 family), as well as few piRNAs that can potentially regulate gene expression (Ohms et al. 2014). A hypothetical mechanism for the mechanism of miRNA biosynthesis control by LINE-1-encoded RT has been recently proposed (Sciamanna et al. 2013, 2014). In short, LINE-derived RT, overproduced in cancer cells, can “intercept” RNA transcripts and reverse-transcribe them, forming RNA:DNA hybrid molecules. This would be functionally equivalent to “sequestering” RNA strands that are rendered unavailable for double-stranded (ds) RNA formation. Consequently, production of small regulatory RNAs is impaired, ultimately compromising the expression of coding genes. Consistent with this, the biogenesis of LINE-1-derived miRNAs (Lu et al., 2005) and siRNAs (Chen et al., 2012b) is globally reduced in cancer compared to normal cells. Indeed, nucleic acid fractionation experiments show that RT inhibitors prevent RNA:DNA hybrid formation (Sciamanna et al. 2013), restoring the formation of dsRNAs and re-establishing the biogenesis of miRNAs and their control of transcriptome.

**LINE-1-encoded Reverse Transcriptase as a cancer marker and a diagnostic tool**

The finding that LINE-1 is overexpressed in cancer cells, and growing preclinical and clinical data showing cancer responsiveness to RT inhibition, prompted us to undertake a systematic study to evaluate the expression of RT-containing LINE-1-ORF2p in various human cancer tissues. To this end we have developed a highly specific monoclonal antibody raised against a peptide in the ORF2p EN domain, which enabled us to reveal by immunohistochemistry (IHC) LINE-1- ORF2p expression in bioptic tissues from staged carcinomas (De Luca et al., 2016). IHC analysis of prostate, colon, lung and breast bioptic tissues confirmed high ORF2p expression in carcinoma samples, but not in their healthy counterpart tissues (De Luca et al., 2016). This was consistent with a previous study demonstrating high expression of ORF2p in breast cancer using an unrelated antibody (Chen et al., 2012a).
Figure 2. ORF2p in progression of human cancers. A: Immunohistochemical staining of ORF2p in prostate cancer stages. Representative sections from: normal gland; prostatic intraepithelial neoplasia (PIN); adenocarcinoma with Gleason pattern 3 and 4. ORF2p signal intensities (in arbitrary units) were highly significantly different already in PIN compared to normal samples (details in De Luca et al., 2016). B: Immunohistochemical staining of ORF2p in colon cancer tissue sections. Representative sections from: normal colonic mucosa; transitional mucosa; adenoma with medium grade dysplasia and adenocarcinoma. ORF2p signal intensities (in arbitrary units) were highly significantly different in transitional compared to normal mucosa (see De Luca et al., 2016).

Interestingly, high levels of ORF2p expression were observed in very early transformation stages in prostate (Fig. 2A) and colon (Fig. 2B) specimens, before the appearance of typical histological features of carcinoma, and in precancerous lesions; for example, transitional colonic mucosa and prostate intraepithelial neoplasia (PIN) showed significantly increased ORFp signal intensity compared to controls. Interestingly, parallel, independent studies demonstrate that genomic DNA hypomethylation occurs in both colonic mucosa and PIN (Suter et al., 2004; Cho et al., 2009).

In staged samples from both colon and prostate cancer types, ORF2p expression shows a bimodal pattern, with a sharp initial burst in very early stages, followed by a steadier wave in latest stages. It might be speculated that the two waves mark distinct steps in cancer progression: i) the normal-to-precancerous transition, and ii) the evolution from the latter to overt cancer. Aberrant activation of the LINE-1 RT mechanism would induce cell transformation by sequentially converting normal to preneoplastic and eventually to cancer cells through these subsequent steps, implicating the RT enzyme in both phases. The differential activation of LINE-1 RT in different cells might be at the origin of the heterogeneity that characterize tumors (reviewed by Sciamanna et al., 2016).

The finding that L1- ORF2p expression precedes overt tumorigenesis supports the view that high LINE-1 activity could be a trigger to cell transformation, rather than its consequence. Together, these data indicate that early ORF2p expression represents a valuable biomarker for early cancer detection, at least in colon and prostate cancer. It remains to be established whether ORF2p up-regulation is a more widespread phenomenon in other cancers at early onset.

Reverse Transcriptase at the genesis of cancer: a model

The data summarized thus far show that the activation of LINE-1-encoded RT has a cancer-promoting role by impairing the differentiation state of cells, whereas RT repression restores
differentiation in cancer cells with an effective anticancer effect. It is generally accepted that several cancer pathways represent the unscheduled resumption of early embryonic pathways that should have been silenced in adult differentiated cells but erroneously escaped that silencing. In analogy with cancer cells, but in a totally unrelated context, LINE-1-encoded RT is activated at fertilization and highly expressed in very early embryogenesis, then becomes silenced in late preimplantation embryos. The precocious, narrow burst of RT expression is crucial for embryonic development: indeed, both the RT pharmacological inhibition, and the downregulation of LINE-1 expression, result in arrest of embryo development at the 2- or 4-cell stages (reviewed by Sciamanna et al., 2011).

By integrating these lines of evidence, RT activity emerges as a distinctive feature of undifferentiated, or poorly differentiated, cells, regardless of their histological origin. In tumorigenesis, a reverse transition occurs from differentiated back to an embryo-like state, accompanied by altered proliferation rates and morphology reflecting a global reprogramming of the transcriptome.

Available data suggest that, in addition to the known role of retrotransposons in insertional mutagenesis, LINE-1-encoded RT plays a significant epigenetic role in embryogenesis and tumorigenesis by controlling the profile of non-coding regulatory RNAs. As summarized above, LINE-1-encoded RT operates as a master regulator of genome transcription, by regulating the balance between DNA:RNA hybrid and double-stranded RNA formation, and concomitantly acts as a determinant of cell fate. On these grounds, the elevated RT activity observed in virtually every type of cancer can cause the erroneous re-activation of embryonic transcriptional circuits in the wrong environment of adult differentiated cells (reviewed by Spadafora, 2015). Conceptually, therefore, RT-dependent tumorigenesis can be regarded as the specular path to embryogenesis, regressing in the opposite direction and characterized by the re-emergence of embryonic features, including RT reactivation, genome hypomethylation and the erroneous resumption of genome-wide regulatory networks active in embryogenesis (Ma et al., 2010).

LINE-1 expression is highly sensitive to stressing stimuli and readily modulated by them (Hagan and Rudin, 2002; Miousse et al., 2015). Depending on the nature and intensity of endogenous or exogenous stressors, LINE-1 expression can be activated at differential levels in different cells. As schematically represented in Fig.3, differentially intense (or prolonged) bursts of RT activity likely cause different degrees of transformation, generating the heterogeneity typical of cancer cells (reviewed by Sciamanna et al., 2016). A broad spectrum of transformed cells, from mildly de-differentiated primary cancer cells, to highly aggressive metastatic cells, can thus be simultaneously generated upon the burst of RT activity. The invasive potential of metastatic cells favours their spreading and make them the predominant population in the course of time. This model was inspired by the proposed “Big Bang” hypothesis for the genesis of human cancer, in which a single ancestral event is thought to originate the heterogeneity of cancer cell populations (Sciamanna et al., 2015). The model predicts that cell transformation is largely due to the reactivation of embryonic regulatory circuits, mostly at the epigenetic level. DNA mutations, except for characterized oncogenes, have lower weight in the genesis of cancer and would rather accumulate in consequence of checkpoint failure during cancer progression.

Future perspectives

In a critical article, Hanahan (Hanahan, 2014) has pointed out that the war on cancer, if not lost, is certainly not won yet, and has suggested that therapeutic strategies should stop pursuing highly diversified, narrow paths targeting many single proteins, each of which is highly selective for a specific cancer. Rather, effective therapeutic “bullets” should hit fewer targets shared by a large spectrum of cancers. The proposed strategy in Hanahan’s recommendation is strikingly close to that inherent RT inhibitors. Indeed, based on the experience of our and other groups, the RT-dependent cancer-promoting mechanism is shared by a variety of cancers (various carcinoma types, sarcoma, melanoma, and haematological malignancies), suggesting that a common mechanism is at work in the genesis of a broad spectrum of histologically diverse cancers. The LINE-1-encoded RT, with its ability to remodel the profile of regulatory RNAs, would fulfill the criteria predicted by Hanahan, representing both the driving component of a newly emerging cancer-promoting mechanism and a worth-pursuing therapeutic target. In future work it remains to be seen whether RT inhibitors might represent “universal bullets” in a novel differentiation therapy, effective on an ample spectrum of human cancers.

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Figure 3. A model for RT-dependent induction of cancer cell heterogeneity. The onset of deregulated expression of LINE-1 elements in somatic cells (green), often induced by stressing stimuli (red flash), causes a burst of RT activity that deregulates the transcriptome of individual cells at various levels (represented by different color shades): this originates heterogeneous cancer cell populations (rounded shapes). Cancer cell heterogeneity would thus set in following the early burst of RT activity, differentially expressed in individual cells. Cancer would then progress with the expansion of various cell populations (right).

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