Review

PARTICLE - the RNA podium for genomic silencers

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Abstract:

Radiation exposure can evoke cellular stress responses. Emerging recognition that long non-coding RNAs (lncRNAs) act as regulators of gene expression has broadened the spectra of molecules controlling the genomic landscape upon alterations in environmental conditions. Knowledge of the mechanisms responding to low dose irradiation (LDR) exposure is very limited yet most likely involve subtle ancillary molecular pathways other than those protecting the cell from direct cellular damage. The discovery that transcription of the lncRNA *PARTICLE* (promoter of *MAT2A*- antisense radiation- induced circulating lncRNA; *PARTICL*) becomes dramatically instigated within a day after LDR exposure introduced a new gene regulator onto the biological landscape. *PARTICLE* affords an RNA binding platform for genomic silencers such as DNA methyltransferase 1 and histone tri-methyltransferases to reign in expression of tumour suppressors such as its neighbouring *MAT2A* in *cis* as well as *WWOX* in *trans*. *In silico* evidence offers scope to speculate that *PARTICLE* exploits the abundance of Hoogsten bonds that exist throughout mammalian genomes for triplex formation, presumably a vital feature within this RNA silencer. *PARTICLE* may provide a buffering riboswitch platform for S-adenosylmethionine. The correlation of *PARTICLE* triplex formation sites within tumour suppressor genes and their abundance throughout the genome at cancer related hotspots, offers an insight into potential avenues worth exploring in future therapeutic endeavours.

Introduction

Alternative biological responses are induced by low dose irradiation compared to those evoked by medium- or high-dose exposure with limited knowledge existing on the molecular mechanisms under influence (Mullenders, Atkinson, Paretzke, Sabatier, & Bouffler, 2009; Waldren, 2004). Direct cellular damage from ionizing irradiation have been superseded of late with the focus shifting to subtle non-targeted ancillary stresses instigated predominantly after low dose radiation (LDR) i.e., milligray range ionisation exposure typically encountered in the workplace, during medical imaging, and from natural sources (Morgan & Sowa, 2015; Pluder et al., 2011). The carcinogenic effects of LDR stem from retrospective epidemiological studies (Morgan & Sowa, 2015; Pluder et al., 2011; Shvarts, Sevo, Tasic, Shani, & Sadetzki, 2010). Seeking a biomarker sensitive to LDR exposure, an endeavour known as the Dark.Risk project ultimately sought to collect biological samples from people irradiated in childhood against *Tinea capitas* for exploration of individual oncogenic susceptibility variances. Recently, a robust comprehensive review identified several such potential biomarkers of priority with the view to addressing outstanding biological issues related to the impact of LDR (J. Hall et al., 2017). Despite the fact that radiation specific messenger RNA (mRNA) profiles were deemed the sole biomarker at the final stages of development in this regard (J. Hall et al., 2017), the recent accent of long non-coding RNA (lncRNA) should not go unheeded as it quickly gains traction in the search for such a naturally occurring molecule by which radiation exposure and radiotherapeutic outcome can be determined on an individual sensitivity basis. As biological complexity increases so too does the diversity of synthesized cellular transcripts as seen from the larger repertoire of lncRNA versus protein-coding RNAs emanating from the human genome (Taft, Pheasant, & Mattick, 2007). Categorised as RNA transcripts ranging in size from 200 base pairs (bps) up to 100 kbps with limited coding potential, lncRNAs have a lower

expression pattern yet superior tissue-specificity compared to mRNAs, suggestive of their putative regulatory function (Mondal et al., 2015).

LncRNA *PARTICLE*

In the quest for the identification of LDR regulated lncRNA candidates, human lncRNA microarray platform analysis was initially undertaken by the DARK.RISK project team using total RNA extracted from endothelial, breast cancer and osteosarcoma cell lines exposed 4hr previously to medium dose exposure. The lncRNA *PARTICLE* (HUGO gene nomenclature *PARTICL*, promoter of *MAT2A*- antisense radiation- induced circulating lncRNA; NCBI reference sequence NR_038942.1) was amongst the preliminary targets selected for verification and validation. Out of those lncRNAs tested further, it became apparent that the *PARTICLE* transcript was outstanding for its significantly upregulated expression (almost 30 fold) 24 hours after LDR exposure. Indeed, others have identified radiation sensitive upregulated lncRNAs such as *lincRNA-p21* (long intergenic noncoding RNA-p21), GAS5 (growth arrest-specific 5), *PANDA* (p21 associated ncRNA DNA damage activated; *PANDAR*) and *ANRIL* (antisense non-coding RNA in the INK4 locus; *CDKN2B-AS1*) but as part of a DNA damage response to high dose exposure levels (J. R. Hall et al., 2015; Hung et al., 2011; Ozgur et al., 2013). Subsequently, *PARTICLE* expression was confirmed as a ubiquitous tissue responder to LDR but with its higher levels of expression in malignant tissue compared to healthy control counterparts (O'Leary, Maugg, et al., 2017; O'Leary et al., 2015).

PARTICLE silencing in *Cis* - and *Trans*

Efforts of late to assign lncRNAs on the basis of whether they regulate local gene expression *in cis* or leave the site of transcription and perform effects *in trans* assumes an either/or scenario for functional classification purposes (Kopp & Mendell, 2018). In regards to *PARTICLE*,

evidence has been gathered providing proof of its capability for local as well as distant regulatory effects potentially genome wide in human as well as in mouse (O'Leary, Smida, et al., 2017). *PARTICLE* is transcribed in an antisense direction as a 1432bp transcript within the *MAT2A* gene promoter on chromosome 2p11.2. Importantly, *MAT2A* encodes the catalytic subunit of methionine adenosyltransferase (MAT), the enzyme responsible for the production of s-adenosylmethionine (SAM), the principal cellular methyl donor (Mato, Alvarez, Ortiz, & Pajares, 1997). Given that *MAT2A* upregulated expression is instigated by LDR within hours of exposure only to decrease dramatically by 24 hours, the time point at which *PARTICLE* expression peaks, offered the first indication towards a potential silencing mechanism of *MAT2A* transcription by nuclear *PARTICLE*. Almost complete ablation of *PARTICLE* via lentiviral knockdown interference coincided with a concomitant three - fold increase in *MAT2A* transcript expression (O'Leary et al., 2015). Conversely, overexpressing *PARTICLE* via *in vitro* transfection significantly downregulated *MAT2A* in comparison to negative controls (O'Leary et al., 2015). This ability to influence the regulation of its genomic neighbouring gene signified a direct *in cis* relationship between *PARTICLE* and *MAT2A*. In principal, *PARTICLE* might control excess availability of methyl groups required for increased DNA damage repair activity following radiation exposure.

Intriguingly, transiently elevated *PARTICLE* was found to accompany diminished transcript levels and promoter activity in a distantly located tumour suppressor gene known as *WW Domain Containing Oxidoreductase* (*WWOX*) located on chromosome 16 supporting the ability of this lncRNA to also provide regulatory control *in trans* (O'Leary, Smida, et al., 2017). Increased *PARTICLE* levels were found to reduce *WWOX* promoter activity (O'Leary, Maugg, et al., 2017). Such an effect was evident in the osteosarcoma cell line U2OS yet absent from other bone cancer cell lines harbouring FRA16D breakage within this large gene. Intriguingly, *PARTICLE* influenced the *WWOX* tumour suppressor and in the absence of *WWOX FRA16D*

breakage, it was associated with osteosarcoma metastasis free survival (O'Leary, Maugg, et al., 2017). To date, there is conflicting evidence of the correlation between WWOX protein loss and cancer prognosis (Donati et al., 2007; Yang et al., 2010). Studies have been unable to link the occurrence of genetic modifications in *WWOX* with cancer development, leading to suggestions of a non-mutational regulatory influence (Pluciennik et al., 2006). *PARTICLE* association within the *WWOX* gene may forestall *FRA16D* breakage through fork remodelling/scaffolding yet hindering replication and transcription in tumour cells that lead to metastasis (Georgakilas et al., 2014). Chromosomal fragile sites such as FRA16D may act as functional stress sensors cooperating with trans-acting long non-coding elements such as *PARTICLE* recruited to harness osteosarcoma progression in patients.

PARTICLE **triplex formation**

Replication pausing (Hile & Eckert, 2004; Krasilnikova & Mirkin, 2004) and DNA instability in human cancer (Lu et al., 2015; Wang, Carbajal, Vijg, DiGiovanni, & Vasquez, 2008) has been associated with the formation of alternative genomic conformations. DNA strands were initially recognised almost seventy years ago as being capable of engaging in hydrogen bond interactions that form alternative conformations (non B-forms) which deviate from the canonical (Watson-Crick, B form) right-handed double helix (Rich & Watson, 1954). Over-represented in mammalian genomes, non-B DNA forming motifs offer an opportunity for triplex formation (DNA:DNA:RNA) - requiring a duplex (DNA:DNA) and a single-stranded nucleotide sequence acting as the third strand eg. RNA (Frank-Kamenetskii & Mirkin, 1995). The process of predicting putative triplexes in nucleic acid sequence data has been greatly supported of late by sophisticated software development. Triplexator and Triplex Domain Finder (TDF) offer highly efficient computational frameworks worth accessing for *in silico* nucleotide sequence determination of triplex-formation. Computational modelling using

Triplexator indicated that a triplex was highly probable between *PARTICLE* and a site within the *MAT2A* promoter (chromosome 2: 85765239–85765251) (Buske, Bauer, Mattick, & Bailey, 2012). This triplex was subsequently confirmed using surface plasmon resonance (SPR) diffraction. Given such *cis* interaction, the quest began to determine as to whether other potential genomic sites existed for *PARTICLE* triplex formation. Curiously multiple sites (14 in total) were found for *PARTICLE* triplex formation clustered predominantly within the human and mouse *WWOX* gene (O'Leary, Smida, et al., 2017). Surface plasmon resonance diffraction and electrophoretic mobility shift assays with high resolution imaging offered proof consistent with *PARTICLE* triplex formation within human *WWOX*. TDF was deployed to determine if *PARTICLE* could form triplexes extensively throughout the human genome (GRCh37/hg19). Over 1600 human genomic locations were predicted from *in silico* analysis where *PARTICLE* triplex sites might occur. It is tempting to venture that *PARTICLE* may cast its triplex net wide across every human chromosome. It can be speculated that the relevance of such extensive widespread triplex formation may be a feature of the *PARTICLE* scaffold necessary for interaction with protein partners.

A triplex regulatory docking platform linking silencing methylation mechanisms

DNA methyltransferase interaction with PARTICLE: The first indication that *PARTICLE* triplex formation serves as a platform for gene repressors such as DNA methyltransferase came from observations of increased DNA methylation in the ‘shore’ region (456 bp upstream) of a *MAT2A* promoter CpG island 108368 on chromosome 2: 85765695–85766983 (NCBI Homo sapiens build number 37 version 2; GRCh37/hg19 assembly). The transcription initiation site for *MAT2A* resides within this region at position chromosome 2: 85766100 orientated in a forward direction (NCBI refseq NM_005911). The sequence for *PARTICLE* also overlaps this CpG island from position chromosome 2:85765818 for 123 bp, orientated in the antisense

complementary direction. *PARTICLE* was subsequently found to similarly influence the methylation status of a *WWOX* promoter CpG island 105476 on chromosome 16. The transcription initiation site for *WWOX* likewise resides within this region at position chromosome 16: 78133327 orientated in a forward direction. *PARTICLE* interacts with G9a (Euchromatic histone-lysine N-methyltransferase 2 (EHMT2)), predicted to maintain a cooperative partnership with DNA methyltransferase 1 (DNMT1) for chromatin binding activity (O'Leary et al., 2015). Currently, the mechanism by which DNA methyltransferase (DNMT) enzymes are directed to CpG island sites that they are meant to silence is not well understood. Our findings established direct interaction between *PARTICLE* and the maintenance DNA methyltransferase DNMT1 coinciding with increased enzyme activity, a global shift in the methylome and an upsurge in *MAT2A* and *WWOX* CpG island methylation. The *PARTICLE* triplex may govern CpG island methylation to instigate DNMT transcriptional suppression of *MAT2A* and *WWOX*.

Histone methyltransferase interaction with PARTICLE: Chromatin immunoprecipitation and RNA pulldown proved that this lncRNA binds to the Polycomb Repressive Complex 2 (PRC2) subunit Suppressor of Zeste 12 (SUZ12) (O'Leary et al., 2015). It has emerged that SUZ12 is key for locating the PRC2 catalytic subunit responsible for trimethylation (me3) of histone 3 at lysine 27 (H3K27) during heterochromatin formation (Cao & Zhang, 2004). Intriguingly, PRC2 lacks the ability to target genomic regions by itself, relying instead on lncRNAs such as *PARTICLE* for guidance to active chromatin sites. In support of the recognized role of lncRNAs in genomic architectural regulation (Joh, Palmieri, Hill, & Motamedi, 2014) and given the interaction between *PARTICLE* and SUZ12 (O'Leary et al., 2015), it can be envisioned that *PARTICLE* may provide an epigenetic modifying platform to control chromatin structure on chromosome 2 and chromosome 16 at the *MAT2A* and *WWOX* loci respectively and potentially other genomic locations that remain to be analysed. *PARTICLE* and LDR act together to

enhance the H3K27me3 modification. ChIP-seq analysis revealed 24,946 genomic regions with significantly increased H3K27me3 modification in a breast cancer cell line over-expressing *PARTICLE*. ChIP-seq tracking revealed an enhancement of the H3K27me3 modification within all autosomal chromosomes and X-chromosome upon *PARTICLE* overexpression. Of note, there appeared to be significant H3K27me3 enrichment along a 1.1 Mb stretch spanning the *WWOX* locus on chromosome 16 in *PARTICLE* overexpressing (OE) cells versus controls. ChIP-seq tracking revealed a shift in the position and intensity of the H3K27me3 signal upstream of the *MAT2A* promoter CpG island (the established *PARTICLE* triplex region). Enriched H3K27me3 clustering domains from OE samples were merged with TDF data for predicted *PARTICLE* triplex sites within the human genome. This revealed that the *PARTICLE* 627–646 bp domain had significantly higher potential to bind the target H3K27me3 modified domains than randomly chosen similarly sized regions within the human genome ($p = 0.00001$). This would suggest that H3K27me3 modifying enzymes might be guided to specific *PARTICLE* triplex sites to exert their function. *PARTICLE* is capable of enhancing the histone repressive modification mark across the human genome and specifically within *MAT2A* and *WWOX* tumour suppressor genes presumably via its provision of a triplex binding platform (O'Leary, Smida, et al., 2017). Combining the analytical integration power of INGENUITY using ChIP-seq H3K27me3 data and TDF evidence revealed the significance of the *PARTICLE* triplex podium and genes associated with malignancy.

The influence of *PARTICLE* on the central methyl donor S-adenosylmethionine – SAM and the riboswitch.

Riboswitches are RNA elements that change conformation upon binding to a metabolite and modulate gene expression *in cis*. In bacteria, it has been shown that the SAM-II riboswitch forms a triple helix that creates a highly specific binding pocket for S-adenosylmethionine

(Conrad, 2014). *PARTICLE* suppression of *MAT2A* influences SAM availability. Knockdown *PARTICLE* and intracellular SAM levels escalate dramatically with additional effects observed post LDR exposure (O'Leary et al., 2015). Methyl groups are transferred from s-adenosylmethionine to cytosine by DNMTs with the quantity of methylated DNA proportional to enzyme activity. While DNMT activity did not differ with *PARTICLE* knockdown, a significant increase in DNMT activity was noted when *PARTICLE* was over-expressed perhaps in attempts to boost methyl group clearance/transfer. The SAM-II riboswitch in proteobacteria is located upstream of genes involved in methionine and SAM biosynthesis and its structural responsiveness to SAM binding has been demonstrated. *PARTICLE* via triplex formation with *MAT2A* may generate a platform of sorts for SAM to buffer against overproduction in response to radiation. Whether *PARTICLE* is the *bono fide* riboswitch of eukaryotes awaits experimental validation and as such remains the subject of speculation.

PARTICLE **intracellular distribution and extracellular transport via exosomes**

Unlike the majority of lncRNAs which show defined intracellular expression patterns across cell types, *PARTICLE* displays varying distribution patterns between malignant tissues as shown by *in situ* hybridisation (O'Leary, Maugg, et al., 2017; O'Leary et al., 2015). For example, in the metastatic breast cancer MDA-MB-361, higher levels of *PARTICLE* are found in the cytosol compared to the nucleus in sham irradiated cells. Following exposure 24 hr previously to LDR, a substantially increased signal intensity for *PARTICLE* was noted in these cellular compartments yet cytosolic predominance remained. In contrast, the prevalence of this lncRNA within the nucleus of U2OS osteosarcoma cells was striking especially following LDR. Intriguing, differential *PARTICLE* expression was revealed across osteosarcoma cell lines in response to radiation associated with a *WWOX* FRA16D breakage background.

Through the use of bromouridine tracing and north western detection, the discovery was made that *PARTICLE* transports out of the irradiated cell (O'Leary et al., 2015). Exosome isolation from culture media and radiotherapy patient plasma revealed the upregulated expression of *PARTICLE* in exosomes post radiation exposure. *In silico* analysis brought to light an 'exosomal signalling code', be it truncated than previously reported (O'Leary et al., 2015), but none the less evident throughout the length of this lncRNA. *PARTICLE* is transported via exosomes into the bloodstream post radiotherapy presumably for uptake by both neighbouring and distant tissue. The implications of this have yet to be deciphered, with *in vitro* evidence pointing towards its influence on cellular radiation sensitization (O'Leary et al., 2015).

***PARTICLE* activates the silencer- EZH2**

PRC2 is one of the two classes of polycomb-group proteins which play a crucial role in regulating chromatin structure and gene expression. It contributes to chromatin compaction and catalyses the methylation of H3K27. Composed of Suppressor of Zeste 12 (SUZ12), Eed, Ezh2 and Jarid2, this complex is associated with transcriptional repression via the assistance of lncRNAs which act as the genomic 'global positioning system (GPS)', navigating PRC2 to the correct chromatin landing location. *PARTICLE* binds to SUZ12, the protein unit key for locating the PRC2 catalytic subunit responsible for trimethylation of H3K27 during heterochromatin formation. ChIP-seq evidence revealed the double pronged approach of *PARTICLE* for supporting transcriptional repression. Integrated genomics viewer screenshots for ChIP-seq data of *PARTICLE* overexpression compared to wild type radiated breast cancer cells, revealed the absence of H3K27me3 marks along the promoter regions of all members of the PRC2 complex including EZH2 (O'Leary, Hain, et al., 2017). This demonstrated the ability of *PARTICLE* to influence the expression of the transcriptional repressor and as such identifies

this lncRNA as a key upstream regulator of the principal cellular silencer post exposure to LDR.

Why all this silence?

To survive radiation-induced damage, cells mount complex responses that rely on alterations in baseline levels of gene expression. Such transcription signatures are recognised as being related to measurements of individual radiation sensitivity (Amundson et al., 2008). While the up-regulation of the p53 pathway has been shown consistently across studies, the down-regulated expression of genes involved in mitosis represents a pathway of radiation response broadly conserved among cancer cell lines. Such coordinated under expression across an entire NCI-60 panel of genes has been previously shown to be the most conspicuous signature response to low - medium dose ionizing radiation exposure (Amundson et al., 2008). Recently, antisense lncRNAs including *PARTICLE* have been implicated in the silencing of tumour suppressor genes through epigenetic remodelling events (Figure 1). Characterization of lncRNAs involved in the development or maintenance of oncogenic states (Table 1) may define them as potential early biomarkers for the emergence of cancer or indicators of patients' sensitivity to radiation assisting the improvement of radiotherapeutic outcome.

Concluding remarks

It remains to be elucidated as to whether lncRNAs such as *PARTICLE* represent an indicator triggered as part of the cellular stress response to radiation exposure or a molecule assisting in stronger adaptation to environmental strain. Given the identification of thousands of yet to be characterised lncRNAs, it is with confidence that *PARTICLE* will serve to offer an insight into other such non-coding RNAs as a prototype mechanism defining LDR instigation of the dark matter of the genome.

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Conflict of interest

The authors confirm that there is no conflict of interest to declare.

Data sharing statement

Data sharing is not applicable to this article as no new data were created or analysed in this study.

Authors contribution

VBOL wrote the manuscript, designed and conducted the research; SVO designed the graphics; JS undertook bioinformatic analysis and corrected the manuscript; MJA directed the research.

Figure legend:

Figure 1: Schematic overview of the lncRNA *PARTICLE* which has the ability to form triplexes (centre) with various sites within the human genome. *PARTICLE* binds to DNA methyltransferase 1 and histone H3K27 tri-methyltransferases for gene silencing via transcriptional regulation. *PARTICLE* exists in exosomes and undergoes extracellular transport with involvement in cell-cell communication. *PARTICLE* influences the levels of S-adenosylmethionine (SAM) in the cell and may act as a metabolic eukaryotic riboswitch.

Table legend:

Table 1: Summary of the biological or developmental impact of *PARTICLE* on target genes, proteins or cofactors as is currently known. Refer to main text for abbreviation definitions.

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