



Mini review

Non-coding antisense transcripts: fine regulation of gene expression in cancer



Francisco Santos¹, Ana Maria Capela¹, Francisca Mateus¹, Sandrina Nóbrega-Pereira, Bruno Bernardes de Jesus*

Department of Medical Sciences and Institute of Biomedicine – iBiMED, University of Aveiro, 3810-193 Aveiro, Portugal

ARTICLE INFO

Article history:

Received 18 August 2022
Received in revised form 3 October 2022
Accepted 4 October 2022
Available online 10 October 2022

Keywords:

Long non-coding RNAs
lncRNAs
Antisense transcripts
ceRNAs
Cancer

ABSTRACT

Natural antisense transcripts (NATs) are coding or non-coding RNA sequences transcribed on the opposite direction from the same genomic locus. NATs are widely distributed throughout the human genome and seem to play crucial roles in physiological and pathological processes, through newly described and targeted mechanisms. NATs represent the intricate complexity of the genome organization and constitute another layer of potential targets in disease. Here, we focus on the interesting and unique role of non-coding NATs in cancer, paying particular attention to those acting as miRNA sponges.

© 2022 The Authors. Published by Elsevier B.V. on behalf of Research Network of Computational and Structural Biotechnology. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

Contents

| | |
|--|------|
| 1. Introduction | 5652 |
| 2. General overview of antisense lncRNAs | 5653 |
| 2.1. Challenges in the detection of ncNATs and how it impacts on their understanding | 5656 |
| 3. Selected cancer-related ncNATs | 5656 |
| 3.1. Therapeutic and prognostic value of ncNATs in cancer? | 5657 |
| 4. Conclusion | 5657 |
| Funding | 5658 |
| Declaration of Competing Interest | 5658 |
| Acknowledgments | 5658 |
| References | 5658 |

1. Introduction

Over the years, several studies pointed out to the presence of RNA molecules that were transcribed but did not encode proteins, making up the so-called non-coding genome. In fact, comparative analyses carried out between mammalian genomes and transcriptomes led to the important discovery that around three-quarters of

genomic DNA were transcribed [1–3], greatly contrasting with the 2 % that is translated into proteins [1].

Extensive annotation has revealed that about 80 % of the human genome is functional. Indeed, several biochemical functions have been assigned to the non-coding portion of the genome, including transcription, association of transcription factors, chromatin structure and histone modification. This number overwhelmingly exceeds the percentage of the genome assigned to coding proteins, making it clear that the non-coding portion of the genome is not randomly transcribed and is involved in several important biological processes [4].

* Corresponding author at: Department of Medical Sciences and Institute of Biomedicine – iBiMED, University of Aveiro, 3810-193 Aveiro, Portugal.

E-mail address: brunob.jesus@ua.pt (B. Bernardes de Jesus).

¹ These authors equally contributed to this work.

Non-coding RNAs can be divided according to their length into small and long non-coding RNAs (lncRNAs). The latter constitute a class of RNA molecules of more than 200 nucleotides, and account for the largest, yet less described, class of non-coding RNAs [5–6]. Many efforts have been applied to distinguish lncRNA from coding RNA transcripts [5,7–9]. In some cases, the absence of a detectable ORF is the only biochemical dissimilarity between lncRNAs and messenger RNA (mRNA).

lncRNAs are often classified according to their location relative to neighboring genes [10]. As such, these transcripts can be divided into sense, antisense, intronic, intergenic and bidirectional according to their positioning to proximal protein coding genes [11]. Here, we will focus only on the particular characteristics of antisense lncRNAs. These transcripts overlap at least one exon of another gene, and originate from the antisense strand [11]. Antisense lncRNAs usually overlap protein-coding genes, but some may overlap other lncRNAs [8,12–13]. Our main goal is to exemplify antisense transcripts in cancer, with particular emphasis on those acting as microRNA (miRNA) sponges and their role in cancer aggressiveness, metabolism, response to chemotherapy and the epithelial-mesenchymal transition (EMT). miRNA sponges are lncRNAs shown to regulate gene expression through direct binding of specific miRNAs [14–18]. These lncRNAs have been termed competing endogenous RNAs (ceRNAs), and have been implicated in several biological processes and diseases, including cancer [19–22].

2. General overview of antisense lncRNAs

Natural antisense transcripts (NATs) have been considered to be extremely common throughout the mouse and human genomes [23]; in fact, around 70 % of mammalian genes are known to produce NATs [24]. Although these RNA molecules may include coding or non-coding sequences that are complementary to either coding or non-coding transcripts [25–26], here we will specifically describe the biological roles of non-coding NATs (hereafter referred to as ncNATs) complementary to either coding or non-coding sequences.

ncNATs can arise from a variety of promoters, such as independent, bidirectional or cryptic. Bidirectional promoters are shown to generate large numbers of ncRNAs, including in humans. Several factors have been shown to influence promoter bidirectionality, such as chromatin organization and polyadenylation signals that surround the promoter [27–28]. In fact, bidirectional transcription can also be originated from double strand breaks, demonstrating the interactions between transcription and DNA damage [29]. ncNATs can also be generated from cryptic promoters that are positioned either within the transcribed or from the termination regions of the sense gene [30]. Regarding regulation at the RNA level, several ncNATs, in yeast, for example, are considered cryptic unstable transcripts and are targeted for early degradation by nuclear exosomes. Other ncNATs are degraded by cytoplasmic RNA exonuclease [31] or regulated by 5' decapping activity [32].

Depending on the location of their target genes, ncNATs can be classified as: *cis*-NATs, if transcribed from the opposite strand at the same genomic *locus* (and exhibiting perfect sequence complementarity to their targets); or *trans*-NATs, if transcribed from different genomic *loci* (and usually displaying imperfect sequence complementarity) [33–34]. Stalled RNA polymerases, R-loops and triples helices have been shown to help retain NATs at their site of transcription, allowing *cis*-acting NATs to exert their function. On the other hand, the three-dimensional organization of chromatin can lead to the interaction of regions of antisense transcription with other *loci*, thus mediating *trans* effects (reviewed in [35]). *cis*-NATs are further classified according to how they overlap with their targets, and are categorized as: head-to-head, whose 5' end overlaps with the 5' of the target transcript; tail-to-tail, whose 3' overlaps with the 3' of the target gene; and internal, which entirely bind to their target. *cis*-NATs can also be classified as nearby NATs, which are located very close to their targets, but do not, in fact, overlap [24,36] (Fig. 1).

ncNATs, just like other lncRNAs, exhibit tissue-specific expression, which suggests that there are many evolutionarily conserved functional roles associated with these transcripts [37]. Regarding the effect in relation to the cognate sequence, their expression

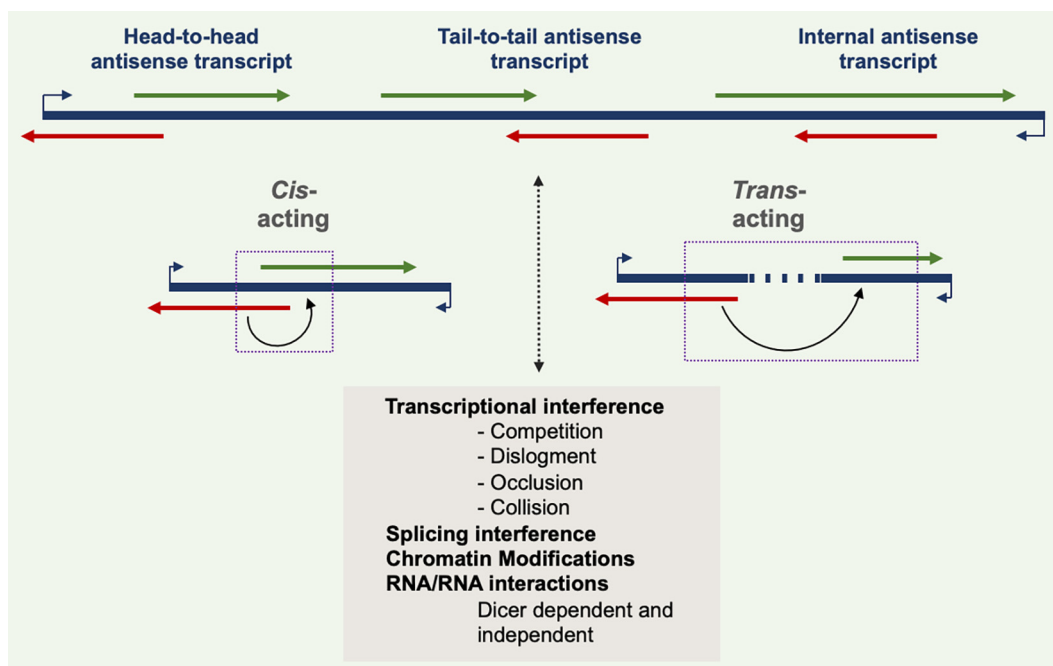


Fig. 1. NAT classification and mechanisms of regulation [35]. Sense and antisense pairs can be head-to-head, tail-to-tail or fully overlapped [26]. NATs can regulate the genes in the vicinity (*cis*-NATs) or at large distance in the genome (*trans*-NATs) [82]. A proposed mechanism of action may involve a direct impact on the transcription of the cognate genes [83], through interference with the process of splicing, affecting the DNA or chromatin epigenetic signature or affect mature RNAs by RNA/RNA interactions, through DICER-dependent or -independent mechanisms [84,85].

Table 1

A variety of techniques can be used to detect novel NATs [86]. Most of the methods include sequencing, being RNA-seq the most basic and versatile application of this principle, followed by microarrays. Depending on the length or other specificities of the target RNAs, enrichment or size selection can be done to optimize the screening. A strand-specific protocol should be preferred for the analysis of the antisense strand and its characteristics. It is also possible to analyze specifically the expression of nascent transcripts, i.e., GRO-seq. CAGE and SAGE are examples of methods that sequence the ends of the transcripts. Techniques such as ChIP-seq or ChIRP analyze the indirect effects of their transcription in chromatin signatures, but this might prove a limitation when NATs overlap coding genes. Polyadenylation is also uncommon in NATs so poly(A)-dependent methods, such as 3P-seq, might not detect all the antisense transcripts present in a sample [87–88]. When NAT structure is already known and annotated, its expression can be further evaluated using RT-qPCR or *in situ* imaging techniques. There has also been a quick evolution with single-cell adaptations, and it is now already possible to apply several omics at once. Finally, only a handful of the techniques listed have been put to practice in NAT identification, so further advances in this field are to be expected, with the consideration that the best analyses may come from a combined application of several techniques.

| Method | Principle | Advantages | Limitations | Ref Original |
|---|---|---|---|--|
| 4Su-seq | Metabolic labelling of nascent RNA using 4sU-tagging | Changes in RNA kinetics are more visible than with total RNA; more sensitive to transient RNA than RNA-seq | More input required; library preparation may introduce some bias for large transcripts; only a short amount of 3' regions are labelled during the short 4sU exposure leading to 5' bias | [89] |
| CAGE (Cap Analysis of Gene Expression) | Sequencing method that analyzes 5' end termini of transcripts | High accuracy and single nucleotide resolution in a high-throughput manner | Not suited for the characterization of novel NATs | [90] |
| ChIP-seq (Chromatin immunoprecipitation) | Detection of chromatin signatures through combination of chromatin immunoprecipitation with RNA-seq, allowing evaluation of expression and primary structure of genes encoding transcripts | High-throughput, sensitivity and specificity, and low cost and input requirement | High complexity; inappropriate to ncRNAs overlapping protein-coding genes and short transcripts; detects all RNA polymerase II (Pol II)-RNA complexes, not just those that are actively engaged | [91] |
| ChIRP-seq (Chromatin Isolation by RNA Purification) | Identification of binding sites and interactions between transcripts and chromatin using tiling oligonucleotides retrieval followed by sequencing | Indicative of putative transcription factor activity for a given ncRNA | Noise caused by precipitation of non-specific DNA fragments from off-target hybridization; prior knowledge of NATs sequence needed | [92] |
| CLIP (Cross-linking immunoprecipitation) | Detects transcripts that interact with RNA Binding Proteins (RBP); RIP followed by sequencing, with variations on the technique depending on the purpose i.e., HITS-CLIP, PAR-CLIP, iCLIP, CRAC | High resolution; can detect unstable transient interactions as well; covalent binding allows washing and purification | UV cross-linking may result in partial degradation of RNA samples; PCR amplification needed to compensate low efficiency if crosslinking | [93] |
| GRO-seq (Global Run-On sequencing) | Sequencing method that analyzes nascent transcripts thus differentiating between transcriptionally active and inactive regions | High resolution and specificity | Detects low but significant amount of antisense transcripts; does not necessarily reflect transcription <i>in vivo</i> | [28] |
| Imaging | Several imaging techniques allow for <i>in situ</i> hybridization of transcripts directly on tissues i.e., smFISH, MERFISH, seqFISH, FISSEQ and SPOTs | Highly specific probes that are easy to use and allow for spatial and contextual information | Small probes can lead to false positives, high background, overcrowding, less sensitivity and spatial resolution | SmFISH- [94–96] MERFISH- [97] SeqFISH- [98] Fisseq- [99–100] Spots- [101] [102–105] |
| Microarrays | Detection of transcripts through hybridization with nucleic acids | Well established, affordable, flexible customization and easy analysis; the use of artificial anti-sense sequence (AFAS) probes identifies unannotated NATs undetectable with cDNA; Affymetrix chips allow a sensitive analysis of most protein coding mRNAs in a more affordable and timely manner | Many non-coding transcripts cannot be detected with standard microarrays due to their design | |
| Tiling microarrays | Detection of transcripts through hybridization with nucleic acids | Identifies more unknown transcripts than RNA-Seq | High false-positive rate; inadequate for low expression transcripts | [106] |
| Nanostring | Directly measures expression of transcripts using probe hybridization and imaging | High automation, specificity, and sensitivity; also detects low expression transcripts without amplification and cDNA production | Requires previous knowledge of the transcript sequence | [107] |
| Nascent-seq | Isolation and sequencing of nascent transcripts | Changes in RNA kinetics are more visible than with total RNA | Many NATs are not polyadenylated and cannot be detected through polyadenylation isolation and sequencing | [108–109] |
| Northern Blot | Probe labelling of RNAs transferred to a membrane that were size separated using gel electrophoresis | Allows estimation of RNA size; cheap and simple | No signal amplification; larger probes are more specific but are more prone to create background; labor-intensive; poor reproducibility; sensitivity impacted by RNA degradation; poor quantitative technique | [110] |
| NET-seq (Native elongating transcript sequencing) | Sequencing of the whole nascent transcriptome that is attached to polymerase | Single-nucleotide resolution of RNA polymerase II (Pol II) associated transcripts | Only detects still attached to RNA polymerase II (Pol II); cannot distinguish between Pol II accumulation and co-transcriptional cleavage | [48] |
| PRO-seq (Precision nuclear Run- | Analyzes nascent RNAs from their 3' ends that are | High sensitivity and base-pair resolution | Does not distinguish between Pol I, II and III | [111] |

Table 1 (continued)

| Method | Principle | Advantages | Limitations | Ref Original |
|---|---|--|---|--------------|
| On and sequencing assay) POINT (Polymerase intact nascent transcript) | attached to polymerase Purification and sequencing of nascent transcripts | Intact nascent RNA and fast | transcription Only detects still attached RNA polymerase II (Pol II); cannot distinguish between Pol II accumulation and co-transcriptional cleavage | [49] |
| PolyA-seq | Sequencing of poly(A)-associated regions of transcripts | High sensitivity; quantitative analysis of novel 3' UTRs without low false positives; better than RNA-seq to detect short transcripts; already strand-specific | Many NATs are not polyadenylated | [112] |
| Poly(A)-position profiling by sequencing (3P-Seq) | Sequencing of 3' UTR of polyadenylated transcripts | High-throughput | Many NATs are not polyadenylated | [113] |
| RT-qPCR | PCR amplification of cDNA | Specific and widely used to evaluate expression | Requires previous knowledge of the transcript sequence | [114] |
| RACE (Rapid amplification of cDNA ends) | Sequencing method following PCR amplification | Efficient and precise annotation of single and low abundance transcripts | Less efficiency with larger fragments, in which cases a cDNA library could be done; requires previous knowledge of a short part of the sequence for primer construction | [115] |
| RIP (RNA immunoprecipitation) | Determination of transcript targets of a given RNA-binding protein (RBP) <i>in vivo</i> | Gold standard that also allows to detect nascent NATs | Only captures the relatively stable RNA–protein complexes and may result in under-representation of transient interactions | [116] |
| RNA CaptureSeq | Deep-sequencing method that constructs tiling arrays of regions of interest against which captured cDNAs of transcripts are hybridized | Quantitative analysis with increased sequencing depth, that allows recognition of rare and unannotated NATs | Hybridization might add undesired artifacts | [117] |
| RNA-PET (RNA paired-end ditags) or GIS-PET | Sequencing of 5' and 3' end regions of transcripts | Validates and maps boundaries of polyadenylated transcripts | Requires large amount of sample; short tags render less mapping specificity; many NATs are not polyadenylated | [118–119] |
| RNA-seq | Sequencing method that identifies and characterizes transcripts; adaptations can be made to sequence the wanted targets i.e., Targeted RNA-seq, Strand-specific RNA (ssRNA-seq), Short RNA-seq, Single-cell RNA-seq (scRNA-seq) | Can detect novel transcripts, of various sizes, expression levels and kinds with a high resolution and sequencing depth | Long RNAs need to be fragmented and assembled in libraries, which may lead to unprecise genomic boundaries; needs complementary experimentation to exclude transcriptional noise | [120–126] |
| RT-PCR-seq | Deep-sequencing method combined with highly multiplexed PCR amplification of cDNA of transcripts | Identifies novel exons of known transcripts | Primer design limits the number of known or predicted testable junctions to be validated | [127] |
| SAGE (Serial Analysis of Gene Expression) | Sequencing method that analyzes 3' end termini of transcripts (poly(A)-associated regions/sites) | Quantitative and qualitative analysis; can identify novel transcripts | Many NATs are not polyadenylated | [128] |
| Single-cell multiomics | Simultaneous sequencing of the transcriptome and other omics, such as genomics i.e., DR-seq and G&T-seq; epigenomics i.e., scM&T-seq and scMT-seq; and proteomics i.e., PLAYR and SPARC | Allows a contextual analysis of the cell, useful in cancer studies and diagnosis | Expensive; amplification errors; allelic and locus dropout; challenging single-base resolution | [129–134] |
| TimeLapse-seq | Metabolic labelling of nascent RNA using 4sU-tagging followed by sequencing | Results in temporal information; already has internal normalization; low input required; changes in RNA dynamics are more visible than with total RNA | Reads beyond the poly-A termination signal are rare, and with restricted time, only 3' ends are labelled, which unables detection of many NATs that are not polyadenylated | [135] |
| TT-seq (Transient transcriptome sequencing) | Metabolic labelling of nascent RNA using 4sU-tagging followed by sequencing | Low input; better than SLAM-seq in analyzing transient RNA species since most ncRNAs are quickly degraded; allows tracking of RNA kinetics; overcomes 4su-seq 5' bias with RNA fragmentation before isolation thus mapping transcribed regions uniformly | With restricted time, only 3' end is labelled and many NATs are not polyadenylated | [136] |
| SLAM-seq (Thiol (SH)-Linked Alkylation for the Metabolic sequencing of RNA) | Metabolic labelling of nascent RNA using 4sU-tagging followed by sequencing | Allows tracking of RNA and kinetics; rapid, low input and high throughput | Many NATs are not polyadenylated; the cellular 4sU uptake kinetics, transcriptional activity and library sequencing depth may limit the detection of nascent labelled transcripts; an assessment should be made to know toxic concentrations of 4sU for the cell type | [137] |

may be concordant (associated with an increased expression) [38] or discordant (associated with a decrease in expression levels) [39]. ncNATs may affect negatively or positively the expression of sense transcription, and their expression is closely related with the regulation of their sense gene [40–41]. Indeed, the regulation of promoter specificities is a potential mechanism of ncNATs in cancer. For instance, *HNF4A-AS1L* selectively activates the *HNF4A* P1 promoter via *HNF1A*, which upregulates the expression of tumor suppressor P1-driven isoforms [42]. RNA-seq data has identified several ncNATs whose expression correlates specifically with the activity of one promoter of their sense gene. Silencing of these ncNATs was shown to alter the promoter usage, demonstrating how these transcripts may regulate promoter-specific programs under different contexts [42].

ncNATs are more frequently located in the nucleus, but they can also be found in the cytoplasm [24], and, like other lncRNAs, contain specific motifs that interact with DNA, RNA and proteins [5], meaning that these transcripts can affect gene expression at the transcriptional, post-transcriptional and translational levels. As such, ncNATs can regulate gene expression by DNA methylation (eg. *LUC7L*), histone modification (eg. *XIST/TSIX*, *HOTAIR*), transcriptional interference (eg. *GNG12-AS*), regulation of alternative splicing (*Zeb2-NAT*), and regulation of mRNA stability, either by masking of miRNA binding sites (eg. *BACE1-AS*) or sponging miRNAs (eg. *HOTAIR*, *NR2F1-AS1*) [24,43]. Interestingly, some ncNATs regulate gene expression through DICER-dependent mechanisms (eg. *NAT6531*), giving rise to small RNAs with roles in the epigenetic regulation of gene expression. The regulation of mRNA stability through miRNA sponging is of particular interest, as several recently identified ncNATs function as ceRNAs (discussed below). Furthermore, several ncNATs have been detected in both the nucleus and cytoplasm, highlighting their role in the regulation of gene expression through multiple mechanisms.

2.1. Challenges in the detection of ncNATs and how it impacts on their understanding

The overlap of antisense transcripts with their sense counterparts, their relative low expression levels and limited evolutionary conservation, have limited the use of high-throughput approaches to identify these transcripts (reviewed in [35]). In fact, antisense transcription has been carefully exploited at a large scale even before the advent of massive sequencing [44–47]. Further, detecting active transcription using global run-on sequencing (GRO-seq), native elongating transcript sequencing (NET-seq) or, more recently, POINT-technology, have been shown to be essential to identify antisense transcription and, therefore, antisense transcripts [28,48,49]. It is important, however, to discriminate between antisense transcription and antisense transcripts, because the former not always gives rise to functional RNAs, either coding or non-coding. In fact, antisense transcription is known to impact locally on gene expression programs through a variety of mechanisms not always mediated through ncRNAs. The advent of new technologies has been supporting the understanding of transcription complexity (Table 1). Indirect detection of antisense transcripts through chromatin modification states has also been employed, although this approach is rather limited due to the lack of strand specificity of chromatin modification and the generally higher expression levels of sense transcripts [50] (reviewed in [43]). The genomic arrangement of ncNATs has made it difficult to determine their function by loss-of-function studies without affecting the expression of the corresponding sense transcript [35,51]. To bypass this problem, the analysis of genome-wide gene expression levels at several time points and its comparison to the transcriptional response of cells after modulation of components that are involved in gene regulation by antisense transcription

(such as histone-modifying enzymes) has proven to be a more suitable approach [52–53].

3. Selected cancer-related ncNATs

Several ncNATs have been associated to cancer, and their roles have been elucidated in several reviews (see references [24,43]). For instance, *TALAM1* (*MALAT1* antisense RNA) is a ncNAT that has been linked to the expression of lncRNA *MALAT1* (metastasis-associated lung adenocarcinoma transcript 1). Zong and colleagues revealed that *TALAM1* interacts with *MALAT1* at its site of transcription, promoting its RNase P-mediated 3' end cleavage. Interestingly, *MALAT1* positively regulates the transcription and RNA stability of *TALAM1*. The dynamic between these two lncRNAs has been explored in breast cancer. Both *MALAT1* and *TALAM1* were shown to be upregulated in MCF7 (luminal A) and MDA-MB-231 (triple negative) cell lines. An upregulation of both *MALAT1* and *TALAM1* was also observed in MCF 10A cells (derived from normal breast epithelium) after exposure to TGF- β . Silencing *TALAM1* negatively impacted the ability of breast cancer cells to migrate *in vitro* and to develop lung metastasis in immunocompromised mice. Ultimately, this study suggests that *MALAT1* and *TALAM1* act together to regulate breast cancer aggressiveness and malignancy [54].

FGD5-AS1 (*FGD5* antisense RNA 1) was shown to be overexpressed in pancreatic cancer and bind to miR-577, which targets β -catenin and LRP6 (low density lipoprotein receptor related protein 6) [55]. Downregulation of *FGD5-AS1* led to increased levels of miR-577, resulting in decreased levels of β -catenin and LRP6. This decrease was associated with a reduction in the Wnt signaling pathway and inhibition of cell proliferation, migration and invasion [55]. *CERS6-AS1* (ceramide synthase 6 antisense RNA 1) was also identified as being overexpressed in both pancreatic tissue samples and cell lines [56]. According to study conducted by Gao and colleagues, *CERS6-AS1* acts as a ceRNA by interacting with miR-195-5p, resulting in an increase of WIPI2. Silencing this antisense transcript resulted in an increase of miR-195-5p and a decrease of WIPI2, which was accompanied by a decrease of cell proliferation, evidenced by a reduction in EdU positive cells, and an increase of apoptotic cells. Remarkably, overexpression of WIPI2 reversed the effects of *CERS6-AS1* silencing. Also in pancreatic cancer, Zhang and colleagues observed increased expression levels of *SLCO4A1-AS1* (solute carrier organic anion transporter family member 4A1 antisense RNA 1) in cancer samples. This lncRNA also targeted a miRNA, miR-4673, to derepress KIF21B. Comparable to other antisense transcripts identified in pancreatic cancer, silencing *SLCO4A1-AS1* led to a decrease in cell viability and migration, and an increase of apoptosis [57].

lncRNA *AFAP1-AS1* (actin filament-associated protein 1 antisense RNA 1) was discovered to play an important role in retinoblastoma, which was shown to be overexpressed in tumor samples and cell lines. *AFAP1-AS1* acts as a ceRNA to suppress miR-545-3p, which in turn derepresses its target GNB1. By silencing *AFAP1-AS1*, miR-545-3p is able to inhibit GNB1, and this was associated with decreased cell proliferation and migration [58].

EMT plays a pivotal role in cancer progression [59]. Recently, Bozgeyik and colleagues identified *VIM-AS1* (vimentin antisense RNA 1), which is transcribed opposite of *VIMENTIN* (*VIM*), a well-known EMT marker, and both are upregulated in oral cancer. Accordingly, the expression of E-cadherin exhibited an opposite trend, being downregulated in tumor samples. The higher expression of *VIM-AS1* was also associated with an advanced clinical stage and the presence of lymph node metastasis [60]. Other crucial EMT-related ncNAT is *ZE2-NAT* (*ZEB2* natural antisense transcript), which was shown to be important for the maintenance of 5'-UTR

ZEB2 intron through direct overlapping with the 5' splice site in the intron [61]. *ZEB2* is a transcriptional repressor of E-cadherin and a major activator of EMT, and expression of *ZEB2-NAT* prevents splicing of the first intron of *ZEB2*, increasing the levels of *ZEB2* protein. Thus, *ZEB2-NAT* expression favors EMT, and is therefore associated with cancer progression and cellular reprogramming [38,62]. This ncNAT has been shown to play an important role in the conservation of stemness properties in quiescent cancer stem cells, supporting the existence of a cellular population with chemoresistance potential [63]. In fact, several ncNATs have been shown to be equally important in cancer and stem cells and/or to modulate response of cancer cells to chemotherapeutic agents. One example is a ncNAT occurring in the *SOX9* locus (*SOX9-NAT*). *SOX9-NAT* expression was significantly lower in cancer tissues or human embryonic stem cells, compared with their matched normal tissues, suggesting that slight modifications in *SOX9-NAT* may result in remarkable changes in *SOX9* expression, making this ncNAT a potential therapeutic target in regenerative medicine and cancer treatment [64]. Another example, *TRPM2-AS* (transcript receptor potential cation channel subfamily M member 2 antisense RNA), was shown to confer paclitaxel resistance in prostate cancer. Shi and colleagues showed that both prostate cancer tissues and cell lines exhibited higher levels of *TRPM2-AS*, compared to normal tissues and cells [65]. Moreover, paclitaxel-resistant prostate cancer cell lines demonstrated even higher expression levels of *TRPM2-AS*. Mechanistically, this transcript was shown to bind to miR-497-5p, whose expression was decreased in prostate cancer tissues, when compared to normal tissues. Furthermore, it was revealed that miR-497-5p negatively regulates *FOXK1*, whose expression levels are higher in prostate cancer tissues and cell lines. The authors revealed that silencing *TRPM2-AS* led to increased and decreased levels of miR-497-5p and *FOXK1*, respectively, resulting in decreased proliferation, migration and invasion and increased apoptosis, and even suppressed tumor growth *in vivo* [65].

Another example of how ncNATs are associated with chemoresistance was brought to light by Ling and colleagues [66]. They identified *FOXD3-AS1* (foxhead box D3 antisense RNA 1) as an over-expressed antisense transcript in glioblastoma associated with a worse prognosis. Furthermore, temozolomide-resistant glioblastoma cell lines evidenced higher *FOXD3-AS1* expression levels comparing to those that were sensitive [66]. In fact, overexpression of *FOXD3-AS1* increased tolerance of temozolomide in sensitive cells. Additionally, this lncRNA was revealed to interact with miR-128-3p, which in turn was shown to negatively regulate cell cycle protein *WEE1*. The authors demonstrated that silencing *FOXD3-AS1* resulted in decreased and increased expression levels of miR-128-3p and *WEE1*, respectively. Furthermore, reduced expression of *FOXD3-AS1* sensitized resistant glioblastoma cells to temozolomide, inhibiting cell growth and inducing an increase of cleaved caspase-3, resulting in apoptosis [66].

In osteosarcoma, *ANRIL* (antisense non-coding RNA in the *INK4* locus) was identified as a potential biomarker for chemosensitivity and clinical outcome. In a study carried out by Lee *et al.*, *ANRIL* was shown to be upregulated in osteosarcoma cell lines, which was correlated with resistance to anti-cancer drugs cisplatin and doxorubicin [67]. By silencing the expression of *ANRIL*, cells became more sensitive to these treatments and less proliferative. On the other hand, overexpression of *ANRIL* resulted in an opposite outcome: increased cell proliferation was observed, and cells developed further resistance to cisplatin and doxorubicin [67]. ncNATs have also been shown to identically play fundamental roles through the regulation of cancer metabolism. Li and colleagues identified lncRNA *OIP5-AS1* in cervical cancer, whose expression was shown to be higher in cancer tissues and cell lines, in comparison to their normal counterparts [68]. Furthermore, patients were

stratified, according to *OIP5-AS1* expression, into low and high expression levels, and the latter was associated with larger tumor size, lymph node metastasis and poor 5-year overall survival. Interestingly, under hypoxic conditions, this transcript became upregulated, along with *HIF-1 α* , *GLUT1* and *LDHA*. *OIP5-AS1* was revealed to bind to miR-124-5p, leading to de-repression of *IDH2* [68].

3.1. Therapeutic and prognostic value of ncNATs in cancer?

Although ncNATs have been widely associated to cancer as prognostic and diagnostic markers [43,69–73] they are still far from reaching a therapeutic significance. Several clinical trials are in progress to assess their potential role as cancer biomarkers, in particular when explored in circulating particles, such as exosomes (see, for example, [43]). Examples of ncNATs in clinical trials are lncRNA *HOTAIR* (coexisting in the *HOX* gene) in thyroid cancers and *MF12-AS1* (melanotransferrin antisense RNA) in localized clear-cell cancers of the kidney. *MF12-AS1* expression was associated with the recurrence and survival of patients with clear-cell kidney carcinoma [74]. Interestingly, *MF12-AS1* may act as a sponge of miR-574-5p, a miRNA with potential roles in cancer metastasis [75–76].

Therapeutics that modulate RNAs in general and ncNATs in particular may be envisioned to play fundamental roles in disease. The development of antisense oligonucleotides (ASOs), duplex RNA technologies (RNAi) or genome editing (e.g. CRISPR), has aided the translational approach of ncNATs to the clinics, in particular when loss of function is projected [77–79]. One example is the application of NATs therapeutics in the Angelman syndrome. Here, targeting NATs may be used to derepress the normally-repressed paternal copy of the *UBE3A* gene in patients experiencing absent expression of the *UBE3A* gene-copy of the maternal allele [79–80].

Additionally, using the knowledge acquired from how NATs may interfere with the expression of associated genes, new therapies may evolve. Recently, Taekyu Ha and colleagues explored a gene-targeting method to deplete ephrinB2 using an inducible lentiviral vector. EphrinB2 promotes colorectal cancer and predicts poor patient survival. They demonstrated that integration and expression of the lentiviral construct in the host DNA may drive divergent transcription. Antisense transcription was associated with cell death through activation of a stress response providing evidence that divergent gene transcription from lentiviral vector integration may have an impact on the regulation of gene expression [81].

4. Conclusion

ncNATs are a class of lncRNAs that overlap either protein- or non-coding sequences. Although many of these transcripts can be regarded as junk RNA, several others present a conservation and cellular specificity, guiding them to important roles during normal tissue functions and diseases, including cancer. In particular, ncNATs have been shown to affect either their neighboring genes, and sequences on different genomic loci, thus adding additional layers to the already complex process of gene regulation.

Many recently identified ncNATs have been shown to derepress miRNA targets by functioning as ceRNAs, thus contributing to the regulation of mRNA stability. Interestingly, some of these transcripts also have nuclear functions, making it clear that they can regulate gene expression through several mechanisms.

The advances of gene editing and targeted techniques show great potential for the tissue-specific determination of the role of ncNATs in several biological processes, allowing the characterization of these transcripts as potential therapeutic targets in disease.

Funding

This work was supported by LISBOA-01-0145-FEDER-028534, projeto cofinanciado pelo FEDER através POR Lisboa 2020 - Programa Operacional Regional de Lisboa, do PORTUGAL 2020 e pela Fundação para a Ciência e a Tecnologia (FCT) and Fundação para a Ciência e a Tecnologia (FCT) (ERA-CVD 2018 / 3599-PPCDT - ERA-CVD/0001/2018 - INNOVATION). FS has an individual scholarship from FCT (SFRH/BD/146204/2019), S. N-P. has a 2020.00355.CEE-CIND assistant research contract and AM-C and FM have a Verão com Ciência 2022 research grant.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

We thank other members of the iBiMED, Bruno B. de Jesus and Sandrina N-Pereira labs for insightful discussions and advice.

References

- [1] Djebali S, Davis CA, Merkel A, et al. Landscape of transcription in human cells. *Nature* 2012;489(7414):101–8. <https://doi.org/10.1038/nature11233>.
- [2] Carninci P, Kasukawa T, Katayama S, et al. The transcriptional landscape of the mammalian genome. *Science* (80-) 2005;309(5740):1559–63. [309/5740/1559 \[pii\]10.1126/science.1112014](https://doi.org/10.1126/science.1112014).
- [3] Maeda N, Kasukawa T, Oyama R, et al. Transcript annotation in FANTOM3: Mouse gene catalog based on physical cDNAs. *PLoS Genet* 2006;2(4):498–503. <https://doi.org/10.1371/journal.pgen.0020062>.
- [4] Dunham I, Kundaje A, Aldred SF, et al. An integrated encyclopedia of DNA elements in the human genome. *Nature* 2012;489(7414):57–74. <https://doi.org/10.1038/nature11247>.
- [5] Guttman M, Rinn JL. Modular regulatory principles of large non-coding RNAs. *Nature* 2012;482(7385):339–46. <https://doi.org/10.1038/nature10887>.
- [6] Batista PJ, Chang HY. Long noncoding RNAs: Cellular address codes in development and disease. *Cell* 2013;152(6):1298–307. <https://doi.org/10.1016/j.cell.2013.02.012>.
- [7] Derrien T, Johnson R, Bussotti G, et al. The GENCODE v7 catalog of human long noncoding RNAs: Analysis of their gene structure, evolution, and expression. *Genome Res* 2012;22(9):1775–89. <https://doi.org/10.1101/gr.132159.111>.
- [8] Ma L, Bajic VB, Zhang Z. On the classification of long non-coding RNAs. *RNA Biol* 2013;10(6):924–33. <https://doi.org/10.4161/rna.24604>.
- [9] Cabili M, Trapnell C, Goff L, et al. Integrative annotation of human large intergenic noncoding RNAs reveals global properties and specific subclasses. *Genes Dev* 2011;25(18):1915–27. <https://doi.org/10.1101/gad.17446611>.
- [10] Rinn JL, Chang HY. Genome regulation by long noncoding RNAs. *Annu Rev Biochem* 2012;81(1):145–66. <https://doi.org/10.1146/annurev-biochem-051410-092902>.
- [11] Ponting CP, Oliver PL, Reik W. Evolution and functions of long noncoding RNAs. *Cell* 2009;136(4):629–41. <https://doi.org/10.1016/j.cell.2009.02.006>.
- [12] Lee JT, Davidov LS, Warshawsky D, Tsix, a gene antisense to Xist at the X-inactivation centre. *Nat Genet* 1999;21(4):400–4. <https://doi.org/10.1038/7734>.
- [13] Zong X, Nakagawa S, Freier SM, et al. Natural antisense RNA promotes 3' end processing and maturation of MALAT1 lncRNA. *Nucleic Acids Res* 2016;44(6):2898–908. <https://doi.org/10.1093/nar/gkw047>.
- [14] Ebert MS, Neilson JR, Sharp PA. MicroRNA sponges: competitive inhibitors of small RNAs in mammalian cells. *Nat Methods* 2007;4(9):721–6. <https://doi.org/10.1038/NMETH1079>.
- [15] Ebert MS, Sharp PA. Roles for MicroRNAs in conferring robustness to biological processes. *Cell* 2012;149(3):515–24. <https://doi.org/10.1016/j.cell.2012.04.005>.
- [16] Ebert MS, Sharp PA. Emerging roles for natural microRNA sponges. *Curr Biol* 2010;20(19). <https://doi.org/10.1016/j.cub.2010.08.052>.
- [17] Kartha RV, Subramanian S. Competing endogenous RNAs (ceRNAs): New entrants to the intricacies of gene regulation. *Front Genet* 2014;5(JAN):8. <https://doi.org/10.3389/FGENE.2014.00008/BIBTEX>.
- [18] Karreth FA, Pandolfi PP. CeRNA cross-talk in cancer: when ce-bling rivalries go awry. *Cancer Discov* 2013;3(10):1113–21. <https://doi.org/10.1158/2159-8290.CD-13-0202>.
- [19] Liu Y, Xue M, Du S, et al. Competitive endogenous RNA is an intrinsic component of EMT regulatory circuits and modulates EMT. *Nat Commun* 2019;10(1). <https://doi.org/10.1038/s41467-019-09649-1>.
- [20] Salmena L, Poliseno L, Tay Y, Kats L, Pandolfi PP. A ceRNA hypothesis: the rosetta stone of a hidden RNA language? *Cell* 2011;146(3):353–8. <https://doi.org/10.1016/j.cell.2011.07.014>.
- [21] Ala U, Karreth FA, Borgia C, et al. Integrated transcriptional and competitive endogenous RNA networks are cross-regulated in permissive molecular environments. *Proc Natl Acad Sci U S A* 2013;110(18):7154–9. <https://doi.org/10.1073/PNAS.1222509110>.
- [22] Poliseno L, Salmena L, Zhang J, Carver B, Haveman WJ, Pandolfi PP. A coding-independent function of gene and pseudogene mRNAs regulates tumour biology. *Nature* 2010;465(7301):1033–8. <https://doi.org/10.1038/NATURE09144>.
- [23] Ozsolak F, Kapranov P, Foissac S, et al. Comprehensive polyadenylation site maps in yeast and human reveal pervasive alternative polyadenylation. *Cell* 2010;143(6):1018–29. <https://doi.org/10.1016/j.cell.2010.11.020>.
- [24] Najafi S, Tan SC, Raei P, et al. Gene regulation by antisense transcription: A focus on neurological and cancer diseases. *Biomed Pharmacother* 2022;145:. <https://doi.org/10.1016/j.biopha.2021.112265>.
- [25] Balbin OA, Malik R, Dhanasekaran SM, et al. The landscape of antisense gene expression in human cancers. *Genome Res* 2015;25(7):1068–79. <https://doi.org/10.1101/gr.180596.114>.
- [26] Latgé G, Poulet C, Bours V, Josse C, Jerusalem G. Natural Antisense Transcripts: Molecular Mechanisms and Implications in Breast Cancers. *Int J Mol Sci* 2018;19(1):123. <https://doi.org/10.3390/ijms19010123>.
- [27] Seila AC, Calabrese JM, Levine SS, et al. Divergent transcription from active promoters. *Science* (80-) 2008;322(5909):1849–51. <https://doi.org/10.1126/science.1162253>.
- [28] Core LJ, Waterfall JJ, Lis JT. Nascent RNA Sequencing reveals widespread pausing and divergent initiation at human promoters. *Science* (80-) 2008;322(5909):1845–1848. doi:10.1126/science.11622Nascent.
- [29] Vitor AC, Sridhara SC, Sabino JC, et al. Single-molecule imaging of transcription at damaged chromatin. *Sci Adv* 2019;5(1). <https://doi.org/10.1126/SCIADV.AAU1249>.
- [30] Kaplan CD, Laprade L, Winston F. Transcription elongation factors repress transcription initiation from cryptic sites. *Science* (80-) 2003;301(5636):1096–9. <https://doi.org/10.1126/science.1087374>.
- [31] Van Dijk EL, Chen CL, Daubenton-Carafa Y, et al. XUTs are a class of Xrn1-sensitive antisense regulatory non-coding RNA in yeast. *Nature* 2011;475(7354):114–9. <https://doi.org/10.1038/nature10118>.
- [32] Geisler S, Lojek L, Khalil AM, Baker KE, Collier J. Decapping of long noncoding RNAs regulates inducible genes. *Mol Cell* 2012;45(3):279–91. <https://doi.org/10.1016/j.molcel.2011.11.025>.
- [33] Zhao Y, Hou Y, Zhao C, et al. Cis-natural antisense transcripts are mainly co-expressed with their sense transcripts and primarily related to energy metabolic pathways during muscle development. *Int J Biol Sci* 2016;12(8):1010–21. <https://doi.org/10.7150/ijbs.14825>.
- [34] Zhao S, Zhang X, Chen S, Zhang S. Natural antisense transcripts in the biological hallmarks of cancer: Powerful regulators hidden in the dark. *J Exp Clin Cancer Res* 2020;39(1):1–18. <https://doi.org/10.1186/s13046-020-01700-0>.
- [35] Pelechano V, Steinmetz LM. Gene regulation by antisense transcription. *Nat Rev Genet* 2013;14(12):880–93. nrg3594 [pii]10.1038/nrg3594.
- [36] Zinad HS, Natasya I, Werner A. Natural antisense transcripts at the interface between host genome and mobile genetic elements. *Front Microbiol* 2017;8(NOV):1–9. <https://doi.org/10.3389/fmicb.2017.02292>.
- [37] Ling MHT, Ban Y, Wen H, Wang SM, Ge SX. Conserved expression of natural antisense transcripts in mammals. *BMC Genomics* 2013;14(1):1–9. <https://doi.org/10.1186/1471-2164-14-243>.
- [38] De Jesus BB, Marinho SP, Barros S, et al. Silencing of the lncRNA Zeb2-NAT facilitates reprogramming of aged fibroblasts and safeguards stem cell pluripotency. *Nat Commun* 2018;9:1–11. <https://doi.org/10.1038/s41467-017-01921-6>.
- [39] Modarresi F, Faghihi MA, Lopez-Toledano MA, et al. Inhibition of natural antisense transcripts in vivo results in gene-specific transcriptional upregulation. *Nat Biotechnol* 2012;30(5):453–9. <https://doi.org/10.1038/NBT2158>.
- [40] Lin S, Zhang L, Luo W, Zhang X. Characteristics of antisense transcript promoters and the regulation of their activity. *Int J Mol Sci* 2015;17(1):9. <https://doi.org/10.3390/ijms17010009>.
- [41] Ozsolak F, Goren A, Gymrek M, et al. Digital transcriptome profiling from attomole-level RNA samples. *Genome Res* 2010;20(4):519–25. [gr.102129.109 \[pii\]10.1101/gr.102129.109](https://doi.org/10.1101/gr.102129.109).
- [42] Molias FB, Sim A, Leong KW, et al. Antisense RNAs influence promoter usage of their counterpart sense genes in cancer. *Cancer Res* 2021;81(23):5849–61. <https://doi.org/10.1158/0008-5472.CAN-21-1859/670645/AM/ANTISENSE-RNAs-INFLUENCE-PROMOTER-USAGE-OF-THEIR>.
- [43] Krappinger JC, Bonstingl L, Pansy K, et al. Non-coding natural antisense transcripts: analysis and application. *J Biotechnol* 2021;340:75–101. <https://doi.org/10.1016/j.jbiotec.2021.08.005>.
- [44] Shendure J, Church GM. Computational discovery of sense-antisense transcription in the human and mouse genomes. *Genome Biol* 2002 39. 2002;3(9):1–14. doi:10.1186/GB-2002-3-9-RESEARCH0044.
- [45] Chen J, Sun M, Kent WJ, et al. Over 20% of human transcripts might form sense-antisense pairs. *Nucleic Acids Res* 2004;32(16):4812–20. <https://doi.org/10.1093/NAR/GKH818>.

- [46] Kiyosawa H, Yamanaka I, Osato N, et al. Antisense transcripts with FANTOM2 clone set and their implications for gene regulation. *Genome Res* 2003;13(6B):1324–34. <https://doi.org/10.1101/GR.982903>.
- [47] Katayama S, Tomaru Y, Kasukawa T, et al. Antisense transcription in the mammalian transcriptome. *Science* 2005;309(5740):1564–6. <https://doi.org/10.1126/SCIENCE.1112009>.
- [48] Churchman LS, Weissman JS. Nascent transcript sequencing visualizes transcription at nucleotide resolution. *Nature* 2011;469(7330):368–73. <https://doi.org/10.1038/nature09652>.
- [49] Sousa-Luís R, Dujardin G, Zukher I, et al. POINT technology illuminates the processing of polymerase-associated intact nascent transcripts. *Mol Cell* 2021;81(9):1935. <https://doi.org/10.1016/j.molcel.2021.02.034>.
- [50] Guttman M, Amit I, Garber M, et al. Chromatin signature reveals over a thousand highly conserved large non-coding RNAs in mammals. *Nature* 2009;458(7235):223–7. <https://doi.org/10.1038/nature07672>.
- [51] Xu Z, Wei W, Gagneur J, et al. Antisense expression increases gene expression variability and locus interdependency. *Mol Syst Biol* 2011;7(468). <https://doi.org/10.1038/msb.2011.1>.
- [52] Margaritis T, Oreal V, Brabers N, et al. Two distinct repressive mechanisms for histone 3 lysine 4 methylation through promoting 3'-end antisense transcription. *PLoS Genet* 2012;8(9). <https://doi.org/10.1371/journal.pgen.1002952>.
- [53] Kim T, Xu Z, Clauder-Münster S, Steinmetz LM, Buratowski S. Set3 HDAC mediates effects of overlapping noncoding transcription on gene induction kinetics. *Cell* 2012;150(6):1158–69. <https://doi.org/10.1016/j.cell.2012.08.016>.
- [54] Gomes CP, Nóbrega-Pereira S, Domingues-Silva B, et al. An antisense transcript mediates MALAT1 response in human breast cancer. *BMC Cancer* 2019;19(1):1–11. <https://doi.org/10.1186/s12885-019-5962-0>.
- [55] Zhang W-T, Zhang J-J, Shao Q, et al. FGD5-AS1 is an oncogenic lncRNA in pancreatic cancer and regulates the Wnt/ β -catenin signaling pathway via miR-577. *Oncol Rep* 2022;47(1):1–11. <https://doi.org/10.3892/or.2021.8232>.
- [56] Gao KF, Zhao YF, Liao WJ, Xu GL, Zhang JD. CERS6-AS1 promotes cell proliferation and represses cell apoptosis in pancreatic cancer via miR-195-5p/WIP1 axis. *Kaohsiung J Med Sci* 2022(January):1–12. <https://doi.org/10.1002/kjm2.12522>.
- [57] Zhang J, Shen Y, Ma D, Li Z, Zhang Z, Jin W. SLC04A1-AS1 mediates pancreatic cancer development via miR-4673/KIF21B axis. *Open Med* 2022;17(1):253–65. <https://doi.org/10.1515/med-2022-0418>.
- [58] Tang W, Zhang L, Li J, Guan Y. AFAP1 antisense RNA 1 promotes retinoblastoma progression by sponging microRNA miR-545-3p that targets G protein subunit beta 1. *Bioengineered* 2022;13(3):5638–52. <https://doi.org/10.1080/21655979.2022.2033464>.
- [59] De CB, Bex G. Regulatory networks defining EMT during cancer initiation and progression. *Nat Rev Cancer* 2013;13(2):97–110. <https://doi.org/10.1038/nrc3447>.
- [60] Bozgeyik E, Ege B, Koparal M, Ceylan O. Clinical significance of Vimentin Antisense RNA 1 and its correlation with other epithelial to mesenchymal transition markers in oral cancers. *Pathol - Res Pract* 2021;2022(232):. <https://doi.org/10.1016/j.prp.2022.153807>.
- [61] Beltran M, Puig I, Pena C, et al. A natural antisense transcript regulates *Zeb2/Sip1* gene expression during *Snail1*-induced epithelial-mesenchymal transition. *Genes Dev* 2008;22(6):756–69. 22/6/756 [pii] 10.1101/gad.455708.
- [62] Eroğlu Güneş C, Güçlü E, Vural H, Kurar E. Knockdown of lncRNA ZEB2NAT suppresses epithelial mesenchymal transition, metastasis and proliferation in breast cancer cells. *Gene* 2021;805:.. <https://doi.org/10.1016/j.gene.2021.145904>.
- [63] Francescangeli F, Contavalli P, De Angelis ML, et al. A pre-existing population of ZEB2+ quiescent cells with stemness and mesenchymal features dictate chemoresistance in colorectal cancer. *J Exp Clin Cancer Res* 2020;39(1):2. <https://doi.org/10.1186/s13046-019-1505-4>.
- [64] Eftekhary M, Mohammadi-Yeganeh S, Bolandi Z, et al. A novel natural antisense transcript at human SOX9 locus is down-regulated in cancer and stem cells. *Biotechnol Lett* 2020;42(2):329–39. <https://doi.org/10.1007/S10529-019-02774-3/FIGURES/5>.
- [65] Shi T, Li R, Duan P, et al. TRPM2-AS promotes paclitaxel resistance in prostate cancer by regulating FOXK1 via sponging miR-497-5p. *Drug Dev Res* 2021;2022:1–12. <https://doi.org/10.1002/ddr.21924>.
- [66] Ling Z, Zhang J, Liu Q. Oncogenic Forkhead box D3 antisense RNA 1 promotes cell survival and confers temozolomide resistance in glioblastoma cells through the miR-128-3p/WEE1 G2 checkpoint kinase axis. *Bioengineered* 2022;13(3):6012–23. <https://doi.org/10.1080/21655979.2022.2042133>.
- [67] Lee AM, Ferdjallah A, Moore E, et al. Long non-coding rna anril as a potential biomarker of chemosensitivity and clinical outcomes in osteosarcoma. *Int J Mol Sci* 2021;22(20). <https://doi.org/10.3390/ijms222011168>.
- [68] Li L, Ma Y, Maerkeya K, Reyanguly D, Han L. LncRNA OIP5-AS1 regulates the warburg effect through miR-124-5p/IDH2/HIF-1 α pathway in cervical cancer. *Front Cell Dev Biol* 2021;9(August):1–14. <https://doi.org/10.3389/fcell.2021.655018>.
- [69] Annie D, Serena D, Sandrine C, et al. LADON, a natural antisense transcript of NODAL, promotes an invasive behaviour in melanoma cells. *bioRxiv*. Published online September 30, 2021:2020.04.09.032375. doi:10.1101/2020.04.09.032375.
- [70] Braicu C, Zimta AA, Harangus A, et al. The Function of non-coding RNAs in lung cancer tumorigenesis. *Cancers* 2019, 11, Page 605. 2019;11(5):605. doi:10.3390/CANCERS11050605.
- [71] Shademan M, Salanghuch AN, Zare K, et al. Expression profile analysis of two antisense lncRNAs to improve prognosis prediction of colorectal adenocarcinoma. *Cancer Cell Int* 2019;19(1):1–12. <https://doi.org/10.1186/S12935-019-1000-1/FIGURES/5>.
- [72] Wenric S, Elguendi S, Caberg JH, et al. Transcriptome-wide analysis of natural antisense transcripts shows their potential role in breast cancer. *Sci Reports* 2017 7(1). 2017;7(1):1–12. doi:10.1038/s41598-017-17811-2.
- [73] Ros G, Pegoraro S, De Angelis P, et al. HMGA2 antisense long non-coding RNAs as new players in the regulation of HMGA2 expression and pancreatic cancer promotion. *Front Oncol* 2020;9:1526. <https://doi.org/10.3389/FONC.2019.01526/BIBTEX>.
- [74] Flippot R, Mouawad R, Spano J-P, et al. Expression of long non-coding RNA MF12-AS1 is a strong predictor of recurrence in sporadic localized clear-cell renal cell carcinoma. *Sci Rep* 2017;7(1):8540. <https://doi.org/10.1038/s41598-017-08363-6>.
- [75] Ji S, Ye G, Zhang J, et al. miR-574-5p negatively regulates *Qki67* to impact β -catenin/Wnt signalling and the development of colorectal cancer. *Gut* 2013;62(5):716–26. <https://doi.org/10.1136/gutjnl-2011-301083>.
- [76] Li C, Tan F, Pei Q, et al. Non-coding RNA MF12-AS1 promotes colorectal cancer cell proliferation, migration and invasion through miR-574-5p/MYCBP axis. *Cell Prolif* 2019;52(4). <https://doi.org/10.1111/cpr.12632>.
- [77] Kim J, Hu C, Moufawad El Achkar C, et al. Patient-customized oligonucleotide therapy for a rare genetic disease. *N Engl J Med* 2019;381(17):1644–52. https://doi.org/10.1056/NEJMOA1813279/SUPPL_FILE/NEJMOA1813279_DISCLOSURES.PDF.
- [78] Messina S, Sframeli M. New treatments in spinal muscular atrophy: positive results and new challenges. *J Clin Med* 2020, Vol 9, Page 2222. 2020;9(7):2222. doi:10.3390/JCM9072222.
- [79] Quemener AM, Bachelot L, Forestier A, Donnou-Fournet E, Gilot D, Galibert MD. The powerful world of antisense oligonucleotides: From bench to bedside. *Wiley Interdiscip Rev RNA* 2020;11(5):e1594.
- [80] Meng L, Ward AJ, Chun S, Bennett CF, Beaudet AL, Rigo F. Towards a therapy for Angelman syndrome by targeting a long non-coding RNA. *Nature* 2015;518(7539):409–12. [nature13975 \[pii\] 10.1038/nature13975](https://doi.org/10.1038/nature13975).
- [81] Ha T, Diprima M, Koparde V, et al. Antisense transcription from lentiviral gene targeting linked to an integrated stress response in colorectal cancer cells. Published online 2022. doi:10.1016/j.omtn.2022.05.029.
- [82] Sun Y, Li D, Zhang R, et al. Strategies to identify natural antisense transcripts. *Biochimie* 2017;132:131–51. <https://doi.org/10.1016/j.biochi.2016.11.006>.
- [83] Vance KW, Ponting CP. Transcriptional regulatory functions of nuclear long noncoding RNAs. *Trends Genet* 2014;30(8):348–55. <https://doi.org/10.1016/j.tig.2014.06.001>.
- [84] Faghihi MA, Wahlestedt C. RNA interference is not involved in natural antisense mediated regulation of gene expression in mammals. *Genome Biol* 2006;7(5):R38. <https://doi.org/10.1186/gb-2006-7-5-r38>.
- [85] Pastori C, Peschansky VJ, Barbouth D, Mehta A, Silva JP, Wahlestedt C. Comprehensive analysis of the transcriptional landscape of the human FMR1 gene reveals two new long noncoding RNAs differentially expressed in Fragile X syndrome and Fragile X-associated tremor/ataxia syndrome. *Hum Genet* 2014;133(1):59–67. <https://doi.org/10.1007/s00439-013-1356-6>.
- [86] Levin JZ, Yassour M, Adiconis X, et al. Comprehensive comparative analysis of strand-specific RNA sequencing methods. *Nat Methods* 2010;7(9):709–15. <https://doi.org/10.1038/nmeth.1491>.
- [87] Wanowska E, Kubiak MR, Rosikiewicz W, Makołowska I, Szczeniak MW. Natural antisense transcripts in diseases: From modes of action to targeted therapies. *Wiley Interdiscip Rev RNA* 2018;9(2):1–16. <https://doi.org/10.1002/wrna.1461>.
- [88] Kung JTY, Colognori D, Lee JT. Long noncoding RNAs: Past, present, and future. *Genetics* 2013;193(3):651–69. <https://doi.org/10.1534/genetics.112.146704>.
- [89] Windhager L, Bonfert T, Burger K, et al. Ultrashort and progressive 4sU-tagging reveals key characteristics of RNA processing at nucleotide resolution. *Genome Res* 2012;22(10):2031–42. <https://doi.org/10.1101/gr.131847.111>.
- [90] Shiraki T, Kondo S, Katayama S, et al. Cap analysis gene expression for high-throughput analysis of transcriptional starting point and identification of promoter usage. *Proc Natl Acad Sci U S A* 2003;100(26):15776–81. <https://doi.org/10.1073/pnas.2136655100>.
- [91] Robertson G, Hirst M, Bainbridge M, et al. Genome-wide profiles of STAT1 DNA association using chromatin immunoprecipitation and massively parallel sequencing. *Nat Methods* 2007;4(8):651–7. <https://doi.org/10.1038/nmeth1068>.
- [92] Chu C, Qu K, Zhong FL, Artandi SE, Chang HY. Genomic maps of long noncoding RNA occupancy reveal principles of RNA-chromatin interactions. *Mol Cell* 2011;44(4):667–78. <https://doi.org/10.1016/j.molcel.2011.08.027>.
- [93] Ule J, Jensen KB, Ruggiu M, Mele A, Ule A, Darnell RB. CLIP identifies novel regulated RNA networks in the brain. *Science* (80-) 2003;302(5648):1212–5. <https://doi.org/10.1126/science.1090095>.
- [94] Raj A, Van Oudenaarden A. Stochastic gene expression and its consequences. *Cell* 2008;135(2):216–26. <https://doi.org/10.1016/j.cell.2008.09.050>.
- [95] Zenklusen D, Larson DR, Singer RH. Single-RNA counting reveals alternative modes of gene expression in yeast. *Nat Struct Mol Biol* 2008;15(12):1263–71. <https://doi.org/10.1038/nsmb.1514>.
- [96] Tan RZ, Van Oudenaarden A. Transcript counting in single cells reveals dynamics of rDNA transcription. *Mol Syst Biol* 2010;6(358):1–7. <https://doi.org/10.1038/msb.2010.14>.

- [97] Chen KH, Boettiger AN, Moffitt JR, Wang S, Zhuang X. Spatially resolved, highly multiplexed RNA profiling in single cells. *Science* 2015;348(6233). <https://doi.org/10.1126/SCIENCE.AAA6090>.
- [98] Shah S, Takei Y, Zhou W, et al. Dynamics and spatial genomics of the nascent transcriptome by intron seqFISH. *Cell* 2018;174(2):363–76. <https://doi.org/10.1016/j.cell.2018.05.035>.
- [99] Lee JH, Daugharthy ER, Scheiman J, et al. Highly multiplexed subcellular RNA sequencing in situ. *Science* (80-) 2014;343(6177):1360–3. <https://doi.org/10.1126/science.1250212>.
- [100] Lee JH, Daugharthy ER, Scheiman J, et al. Fluorescent in situ sequencing (FISSEQ) of RNA for gene expression profiling in intact cells and tissues. *Nat Protoc* 2015;10(3):442–58. <https://doi.org/10.1038/nprot.2014.191>.
- [101] Eng CHL, Shah S, Thomassie J, Cai L. Profiling the transcriptome with RNA SPOTs. *Nat Methods* 2017;14(12):1153–5. <https://doi.org/10.1038/nmeth.4500>.
- [102] Schemm M, Shalon D, Davis RW, Brown PO. Quantitative monitoring of gene expression patterns with a complementary DNA microarray. *Science* (80-) 1995;270(5235):467–70. <https://doi.org/10.1126/science.270.5235.467>.
- [103] Numata K, Osada Y, Okada Y, et al. Identification of novel endogenous antisense transcripts by DNA microarray analysis targeting complementary strand of annotated genes. *BMC Genomics* 2009;10:392. <https://doi.org/10.1186/1471-2164-10-392>.
- [104] Oeder S, Mages J, Flicek P, Lang R. Uncovering information on expression of natural antisense transcripts in Affymetrix MOE430 datasets. *BMC Genomics* 2007;8(1):1–8. <https://doi.org/10.1186/1471-2164-8-200/FIGURES/4>.
- [105] Werner A, Schmutzler G, Carlile M, Miles CG, Peters H. Expression profiling of antisense transcripts on DNA arrays. *Physiol Genomics* 2007;28(3):294–300. <https://doi.org/10.1152/PHYSIOLOGENOMICS.00127.2006>.
- [106] Shoemaker DD, Schadt EE, Armour CD, et al. Experimental annotation of the human genome using microarray technology. *Nature* 2001;409(6822):922–7. <https://doi.org/10.1038/35057141>.
- [107] Geiss GK, Bumgarner RE, Birditt B, et al. Direct multiplexed measurement of gene expression with color-coded probe pairs. *Nat Biotechnol* 2008;26(3):317–25. <https://doi.org/10.1038/nbt1385>.
- [108] Khodor YL, Rodriguez J, Abruzzi KC, Tang CHA, Marr MT, Rosbash M. Nascent-seq indicates widespread cotranscriptional pre-mRNA splicing in *Drosophila*. *Genes Dev* 2011;25(23):2502–12. <https://doi.org/10.1101/gad.178962.111>.
- [109] Lin FR, Niparko JK, Ferrucci L. Nascent-SEQ indicates widespread cotranscriptional rna editing in *drosophila*. *Mol Cell* 2012;47(1):27–37. <https://doi.org/10.1016/j.molcel.2012.05.002>.
- [110] Alwine JC, Kemp DJ, Stark GR. Method for detection of specific RNAs in agarose gels by transfer to diazobenzyloxymethyl-paper and hybridization with DNA probes. *Proc Natl Acad Sci U S A* 1977;74(12):5350–4. <https://doi.org/10.1073/pnas.74.12.5350>.
- [111] Kwak H, Fuda NJ, Core LJ, Lis JT. Precise maps of RNA polymerase reveal how promoters direct initiation and pausing. *Science* (80-) 2013;339(6122):950–3. <https://doi.org/10.1126/science.1229386>.
- [112] Derti A, Garrett-Engle P, MacIsaac KD, et al. A quantitative atlas of polyadenylation in five mammals. *Genome Res* 2012;22(6):1173–83. <https://doi.org/10.1101/gr.132563.111>.
- [113] Jan CH, Friedman RC, Ruby JG, Bartel DP. Formation, regulation and evolution of *Caenorhabditis elegans* 3'UTRs. *Nature* 2011;469(7328):97–103. <https://doi.org/10.1038/nature09616>.
- [114] Mullis K, Faloona F, Scharf S, Saiki R, Horn G, Erlich H. Specific enzymatic amplification of DNA in vitro: the polymerase chain reaction. 1986. *Biotechnology*. 1992;24(Table 1):17–27.
- [115] Frohman MA, Dush MK, Martin GR. Rapid production of full-length cDNAs from rare transcripts: amplification using a single gene-specific oligonucleotide primer. *Proc Natl Acad Sci U S A* 1988;85(23):8998–9002. <https://doi.org/10.1073/pnas.85.23.8998>.
- [116] Gilbert C, Kristjhan A, Winkler GS, Svejstrup JQ. Elongator interactions with nascent mRNA revealed by RNA immunoprecipitation. *Mol Cell* 2004;14(4):457–64. [https://doi.org/10.1016/S1097-2765\(04\)00239-4](https://doi.org/10.1016/S1097-2765(04)00239-4).
- [117] Mercer TR, Gerhardt DJ, Dinger ME, et al. Targeted RNA sequencing reveals the deep complexity of the human transcriptome. *Nat Biotechnol* 2012;30(1):99–104. <https://doi.org/10.1038/nbt.2024>.
- [118] Ng P, Wei CL, Sung WK, et al. Gene identification signature (GIS) analysis for transcriptome characterization and genome annotation. *Nat Methods* 2005;2(2):105–11. <https://doi.org/10.1038/nmeth733>.
- [119] Ng P, Wei C-L, Ruan Y. *Paired-end ditagging for transcriptome and genome analysis*; 2007. doi:10.1002/0471142727.mb2112s79.
- [120] Nagalakshmi U, Wang Z, Waern K, et al. The transcriptional landscape of the yeast genome defined by RNA sequencing. *Science* (80-) 2008;320(5881):1344–9. <https://doi.org/10.1126/science.1158441>.
- [121] Wilhelm BT, Marguerat S, Watt S, et al. Dynamic repertoire of a eukaryotic transcriptome surveyed at single-nucleotide resolution. *Nature* 2008;453(7199):1239–43. <https://doi.org/10.1038/nature07002>.
- [122] Mortazavi A, Williams BA, McCue K, Schaeffer L, Wold B. Mapping and quantifying mammalian transcripts by RNA-Seq. *Nat Methods* 2008;5(7):621–8. <https://doi.org/10.1038/nmeth.1226>.
- [123] Lister R, O'Malley RC, Tonti-Filippini J, et al. Highly integrated single-base resolution maps of the epigenome in *Arabidopsis*. *Cell* 2008;133(3):523–36. <https://doi.org/10.1016/j.cell.2008.03.029>.
- [124] Cloonan N, Forrest ARR, Kolle G, et al. Stem cell transcriptome profiling via massive-scale mRNA sequencing. *Nat Methods* 2008;5(7):613–9. <https://doi.org/10.1038/nmeth.1223>.
- [125] Marioni JC, Mason CE, Mane SM, Stephens M, Gilad Y. RNA-seq: An assessment of technical reproducibility and comparison with gene expression arrays. *Genome Res* 2008;18(9):1509–17. <https://doi.org/10.1101/gr.079558.108>.
- [126] Morin RD, Bainbridge M, Fejes A, et al. Profiling the HeLa S3 transcriptome using randomly primed cDNA and massively parallel short-read sequencing. *Biotechniques* 2008;45(1):81–94. <https://doi.org/10.2144/000112900>.
- [127] Howald C, Tanzer A, Chrast J, et al. Combining RT-PCR-seq and RNA-seq to catalog all genetic elements encoded in the human genome. *Genome Res* 2012;22(9):1698–710. <https://doi.org/10.1101/gr.134478.111>.
- [128] Hu M, Polyak K. Serial analysis of gene expression. *Nat Protoc* 2006;1(4):1743–60. <https://doi.org/10.1038/nprot.2006.269>.
- [129] Frei AP, Bava FA, Zunder ER, et al. Highly multiplexed simultaneous detection of RNAs and proteins in single cells. *Nat Methods* 2016;13(3):269–75. <https://doi.org/10.1038/nmeth.3742>.
- [130] Reimegård J, Tarbier M, Danielsson M, et al. A combined approach for single-cell mRNA and intracellular protein expression analysis. *Commun Biol* 2021;4(624):1–11. <https://doi.org/10.1038/s42003-021-02142-w>.
- [131] Macaulay IC, Haerty W, Kumar P, et al. G&T-seq: Parallel sequencing of single-cell genomes and transcriptomes. *Nat Methods* 2015;12(6):519–22. <https://doi.org/10.1038/nmeth.3370>.
- [132] Hu Y, Fan J, Zhang H, Chen X, Dai G. An estimated method of urban PM2.5 concentration distribution for a mobile sensing system. *Pervasive Mob Comput* 2016;25:88–103. <https://doi.org/10.1016/j.pvmci.2015.06.004>.
- [133] Dey SS, Kester L, Spanjaard B, Bienko M, Van Oudenaarden A. Integrated genome and transcriptome sequencing of the same cell. *Nat Biotechnol* 2015;33(3):285–9. <https://doi.org/10.1038/nbt.3129>.
- [134] Angermueller C, Clark SJ, Lee HJ, et al. Parallel single-cell sequencing links transcriptional and epigenetic heterogeneity. *Nat Methods* 2016;13(3):229–32. <https://doi.org/10.1038/nmeth.3728>.
- [135] Schofield JA, Duffy EE, Kiefer L, et al. Sequencing through nucleoside recoding. 2018;15(3):221–225. doi:10.1038/nmeth.4582.TimeLapse-seq.
- [136] Schwalb B, Michel M, Zacher B, et al. TT-seq maps the human transient transcriptome. *Science* (80-) 2016;352(6290):1225–8. <https://doi.org/10.1126/science.aad9841>.
- [137] Herzog VA, Reichholf B, Neumann T, et al. Thiol-linked alkylation of RNA to assess expression dynamics. *Nat Methods* 2017;14(12):1198–204. <https://doi.org/10.1038/nmeth.4435>.