Interplay between long non-coding RNAs and microRNAs in cancer

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Abstract

In the last decade non-coding RNAs (ncRNAs) have been extensively studied in several biological processes and human diseases including cancer. microRNAs (miRNAs) are the best well-known class of ncRNAs. miRNAs are small ncRNAs of around 23 nt and are crucial post-transcriptional regulators of protein coding genes. Recently, new classes of ncRNAs have been discovered, longer than miRNAs such as long intergenic non-coding RNAs (lincRNAs) and circular RNAs (circRNAs). These novel types of ncRNAs opened a very exciting field in biology, leading researchers to discover new relationships between miRNAs and long non-coding RNAs (lncRNAs) to control protein coding gene expression. One of this new discovery led to formulate the competing endogenous RNA (ceRNA) hypothesis, where a lncRNA acts as a sponge for miRNAs reducing their expression and causing the upregulation of miRNA targets. In this chapter we first discuss some recent discoveries in this field showing the mutual regulation of miRNAs, lncRNAs and protein coding genes in cancer, then we show the general approach for the study of ceRNAs and present with more details a recent computational approach that has been shown to be the most precise and promising.

Key words: microRNAs, long non-coding RNAs, competing endogenous RNAs, sponge, cancer, long non-coding RNA-derived microRNAs, host genes.

1. Introduction

1.1 microRNAs in cancer

microRNAs (miRNAs) are intensively studied small non-coding RNA of 20-22 nucleotides long, which have been recognized over the past decade as key controllers of gene expression by targeting messenger RNAs (mRNAs) and hence involved in several biological processes (Bartel 2004), (Bartel 2009), (Filipowicz, Bhattacharyya et al. 2008). The miRNA molecules recognize their targets by base pairing to partially complementary sequences in the 3'-untranslated region (3'UTR), 5'UTR, or in the open reading frames of the targets. The current version of miRBase (http://www.mirbase.org/), the miRNA registry, contains 1881 precursors and 2588 mature human miRNAs. This represents the set of annotated miRNAs and only a subset of them have been extensively characterized at a functional level.

miRNAs are frequently deregulated in cancer. For instance, in leukemia cells miR-15a and miR-16 are deleted (Calin, Dumitru et al. 2002). Several studies have shown that these two miRNAs are tumor suppressors and their deletion or downregulation lead to the upregulation of anti-apoptotic proteins such as BCL2 (Cimmino, Calin et al. 2005, Calin, Cimmino et al. 2008). Conversely, miRNAs can also be amplified as the miR-17-92 cluster (Hayashita, Osada et al. 2005, Mavrakis, Wolfe et al. 2010).

1.2 Long non-coding RNAs in cancer

The recently acknowledged long non-coding RNAs (lncRNAs) are non-protein coding transcripts longer than 200 nucleotides and lacking of extended open reading frames (Qureshi, Mattick et al. 2010), (Nagano and Fraser 2011), (Clark and Mattick 2011), (Wang and Chang 2011), (Gibb, Brown et al. 2011), (Prensner and Chinnaiyan 2011), (Moran, Perera et al. 2012), (Tano and Akimitsu 2012), (Tang, Lee et al. 2013), (Li, Wu et al. 2013), (Fatica and Bozzoni 2014), (Dey, Mueller et al. 2014), (Yang, Lu et al. 2014), (Li, Wu et al. 2014), (Morlando, Ballarino et al. 2014), (Hansji, Leung et al. 2014), (Iden, Fye et al. 2016), (Parasramka, Maji et al. 2016), (Shi and Yang 2016), (Liu, Zhu et al. 2016).

LncRNAs in cancer are deleted or amplified like miRNAs. For instance, extra copies of the chromosomal region 8q24.21 has been shown to be common in many human cancers and is

associated with poor prognosis (Tseng, Moriarity et al. 2014). This region not only contains the well-known oncogene MYC as well as the lncRNA PVT1; it has been shown that the copy number alteration of MYC was correlated to the increase of PVT1 copies (Tseng, Moriarity et al. 2014).

Copy number alterations and mutations can alter the transcriptional regulation of lncRNAs. Furthermore, specific SNPs can be associated with an increased or decreased risk of specific diseases. It has been shown that the genetic variant rs7763881 in the lncRNA HULC may contribute to the decreased risk of HBV-related hepatocellular carcinoma (Liu, Pan et al. 2012).

1.3 Competing endogenous RNAs

Recent findings show that coding genes are not the only targets of miRNAs. In fact, it has been reported that different non-coding/coding RNAs compete for the same miRNA enabling the reduction of the amount of miRNAs available for interaction via the binding of the miRNA recognition/response elements (MREs) (Ebert, Neilson et al. 2007), (Salmena, Poliseno et al. 2011), (Tay, Rinn et al. 2014), (Ergun and Oztuzcu 2015), (Qi, Zhang et al. 2015), (Kagami, Akutsu et al. 2015), (Guo, Song et al. 2015), (Yang, Wu et al. 2016), (Thomson and Dinger 2016).

These RNA transcripts acts as competing endogenous RNAs (ceRNAs), also known as miRNA 'decoy' or miRNA 'sponges' and appear to be involved in many disease conditions, including cancer development and progression (Wang, Liu et al. 2010), (Poliseno and Pandolfi 2015), (Fan, Li et al. 2013), (Qi, Zhang et al. 2015), (Ergun and Oztuzcu 2015), (Yang, Wu et al. 2016).

CeRNAs recruit the miRNAs and thus effectively de-repressing other targets of that miRNA: the more are expressed the ceRNA that sponges a specific miRNA, the more are expressed the RNAs targeted by that miRNA (**Figure 1**). As a consequence, competing RNAs and "canonical" miRNA targeted RNA have highly correlated expression profiles (Poliseno, Salmena et al. 2010).

Such a mechanism of regulation of miRNA activity was firstly discovered in plants and called 'target mimicry' process (Franco-Zorrilla, Valli et al. 2007). So far, researchers all over the word focused on the study of this mechanism as evidenced by the increased number of publications on ceRNAs in the past few years (Wang, Liu et al. 2010), (Poliseno and Pandolfi 2015), (Fan, Li et al. 2013), (Qi, Zhang et al. 2015), (Ergun and Oztuzcu 2015), (Yang, Wu et al. 2016), (Ebert, Neilson et al. 2007), (Salmena, Poliseno et al. 2011), (Tay, Rinn et al. 2014), (Kagami, Akutsu et al. 2015), (Guo, Song et al. 2015), (Thomson and Dinger 2016) and by the emergence of a lot of publically available databases of miRNA sponge interactions, both predicted (Sarver and Subramanian 2012),

(Li, Liu et al. 2013), (Das, Ghosal et al. 2014) and experimentally confirmed (Wang, Zhi et al. 2015).

In this chapter, we show different levels of regulation between lncRNAs and miRNAs. Then, we describe step by step the recent approach proposed by Paci et al. (Paci, Colombo et al. 2014) for the discovery of ceRNA-miRNA interactions.

2. LncRNA-derived miRNAs

An interesting aspect of miRNA and lncRNA research is the evidence that several lncRNAs are host genes of miRNAs, that is many overlapping transcripts exist (**Figure 2**). The lncRNA-derived miRNAs are an example of a complex gene regulatory network (Mangiavacchi, Sorci et al. 2016). A reciprocal regulation of these two types of RNAs can lead to strong cellular effects, in terms of post-transcriptional regulation and protein expression.

Recent works showed that lncRNA-derived miRNAs are involved in development but also in human diseases such as cancer (Matouk, Halle et al. 2015, Mangiavacchi, Sorci et al. 2016).

Mangiavacchi et al. (Mangiavacchi, Sorci et al. 2016) showed that the lncRNA linc-223 had a crucial role in acute myeloid leukemia (AML). The authors discovered that the alternative production of miR-223 and linc-223 is finely regulated during monocytic differentiation. Furthermore, they demonstrated that endogenous linc-223 localizes in the cytoplasm and acts as a competing endogenous RNA for miR-125-5p, an oncogenic microRNA in leukemia. In particular, they showed that linc-223 directly binds to miR-125-5p and its knockdown increases the repressing activity of miR-125-5p resulting in the downregulation of its target interferon regulatory factor 4 (IRF4), which it was previously shown to inhibit the oncogenic activity of miR-125-5p in vivo. Furthermore, data from primary AML samples showed significant downregulation of linc-223 in different AML subtypes. These findings indicate that the newly identified lncRNA linc-223 may have an important role in myeloid differentiation and leukemogenesis by cross-talking with IRF4 mRNA.

For the purpose of this chapter we mapped the chromosomal locations of human and murine miRNAs to show the landscape of lncRNA-derived miRNAs. We retrieved the chromosomal locations of miRNAs from miRBase (http://www.mirbase.org/) and the coordinates of lncRNAs from Gencode (http://www.gencodegenes.org/). We considered all the miRNAs within lncRNAs

taking into consideration the strand-specificity obtaining in total 180 lncRNAs and 256 miRNAs for human and 69 lncRNAs and 113 miRNAs for mouse (data not shown). When we look at the chromosomal distribution of these overlaps, we can see that some chromosomes have a high number of overlaps for both human and mouse and, looking at the specific genomic positions, we find that this high number is related to the presence of miRNA clusters. Some published examples of these miRNA clusters are the imprinted genomic regions in the chromosome 12qF1 in mouse and 14q32 in human. These regions contain a large number of imprinted miRNAs that are conserved in mammals and seem to be involved in development and highly expressed in the placenta and embryo, whereas in the adult the expression is limited to the brain (Seitz, Royo et al. 2004).

Another example of an imprinted genomic region containing miRNAs, as well as the H19 lncRNA, is the H19-miR-675 axis. In mice these genes are located at the chromosomal position 7qF5, while in human they are within 11p15. The imprinted region H19-miR-675 has been reported to be deregulated in pediatric and adult cancer (Matouk, Halle et al. 2015) and it has been shown that ncRNAs in this region can act as tumor suppressor or oncogene. These results underline the importance of the epigenetic control in cancer and at the same time the potential diagnostic impact of these genomic regions.

3. LncRNAs as ceRNAs

Recent studies have shown that lncRNAs may have a key regulatory role linked not only to their secondary structure (Zhang, Rice et al. 2010, Liang, Bloom et al. 2011, Saxena and Carninci 2011, Novikova, Hennelly et al. 2012, Mercer and Mattick 2013, Mortimer, Kidwell et al. 2014, Fiscon, Paci et al. 2015, Somarowthu, Legiewicz et al. 2015, Engreitz, Ollikainen et al. 2016, Fiscon, Iannello et al. 2016), but also to their primary structure (nucleotides sequence). Indeed, increasing experimental evidence supports the hypothesis that lncRNAs may exploit ceRNA activity (Wang, Liu et al. 2010, Karreth, Tay et al. 2011, Tay, Kats et al. 2011, Marques, Tan et al. 2012, Johnsson, Ackley et al. 2013, Liu, Huang et al. 2013, Wang, Guo et al. 2013, Yu, Yao et al. 2014, Huarte 2015, Xie, Guo et al. 2015, Zheng, Li et al. 2015, Zhou, Ye et al. 2015).

The first experimental evidence of lncRNAs acting as ceRNAs in mammalian cells has been found in wide variety of cancers by Poliseno et al. (Poliseno, Salmena et al. 2010), where the authors investigated the functioning of pseudogenes (i.e., degenerate copies of genes that mostly originate from DNA duplication or retrotransposition of cellular RNAs) as miRNA sponges of their ancestral

genes: the pseudogene PTENP1 competes with its homologous gene PTEN for shared miRNAs (i.e., miR-17, miR-19b, miR-20a, miR-21, miR-26 and miR-214 family), controlling the PI3K signalling and cell proliferation in prostate cancer (Poliseno, Salmena et al. 2010). In addition, they found also the pairs FOXO3B/FOXO3 and KRASIP/KRAS of pseudogene/ancestral gene functioning as miR-182 sponge and miR-143/let-7 sponge, respectively (Poliseno and Pandolfi 2015).

Other lncRNAs functioning as ceRNAs can be also observed in human and mouse muscle cells (Cesana, Cacchiarelli et al. 2011), where the long intergenic non-coding RNA (lincRNA) linc-MD1 controls muscle differentiation by targeting miR-133 and miR-135 to regulate the expression of MAML1 and MEF2C. Then, Wang et al. (Wang, Xu et al. 2013) found that the linc-RoR acts as miR-145 sponge for core transcription factors such as NANOG, OCT4, SOX2, preventing them from miRNA-mediated suppression in cell pluripotency and self-renewing of human embryonic stem cells. Moreover, Fan et al. (Fan, Li et al. 2013) observed as the thyroid-specific lncRNA PTCSC3 can act as ceRNA by targeting the miR-574-5p in human thyroid cancer, and Kallen et al. (Kallen, Zhou et al. 2013) demonstrated that the H19 lncRNA modulates the let-7 miRNAs family availability by acting as a molecular sponge and causing precocious muscle differentiation.

4. CircRNAs as ceRNAs

Most recently, also the new-appreciate circular RNAs (circRNAs) appear acting as miRNA sponges (Memczak, Jens et al. 2013, Jeck and Sharpless 2014, Thomson and Dinger 2016). circRNAs are a class of non-coding RNAs derived mostly from a non-canonical form of alternative splicing, whereby the exon ends are joined to form a continuous loop (Memczak, Papavasileiou et al. 2015, Rybak-Wolf, Stottmeister et al. 2015, Zlotorynski 2015).

circRNAs are much more stable than linear transcripts as more resistant to exonuclease. In view of their higher stability with respect to that of linear transcripts, circRNAs enable a more efficient suppression of miRNA activity.

The first circRNA was discovered over two decades ago and it is the testis-specific cirRNA Sry (sex-determining region Y) (Capel, Swain et al. 1993). Recently, circRNAs have gained a great interest as demonstrated by the many works discussing their widespread and abundant expression in eukaryotes (Hansen, Jensen et al. 2013, Memczak, Jens et al. 2013, Jeck and Sharpless 2014, Guo, Song et al. 2015, Rybak-Wolf, Stottmeister et al. 2015).

Although the function of most circRNAs remains unknown, until now three circRNAs have been experimentally shown to act as miRNA sponges in mammals (Thomson and Dinger 2016): the already mentioned testis-specific cirRNA Sry which serves as sponge for miR-138 in mouse when it is overexpressed (Capel, Swain et al. 1993); the circular CDR1as transcript (also known as cIRS‑7) which has been identified as a miR-7 sponge in the central nervous system (Hansen, Kjems et al. 2013); the transcript circITCH that controls the level of itchy E3 ubiquitin protein ligase (ITCH) by sponging miR‑7, miR-17 and miR‑214 in esophageal squamous cell carcinoma (ESCC) (Li, Zhang et al. 2015).

5. Databases of ceRNA-miRNA interactions

In view of the increasing interest in miRNA sponge interactions, several databases collecting these interactions, both experimentally validated and computationally predicted, were developed. In the following, the most common databases of ceRNA-miRNA interactions are reviewed.

The first miRNA sponge interaction database was ceRDB (Sarver and Subramanian 2012) that allows users to predict miRNA sponge for a specific mRNA target by evaluating the co-occurrence of miRNA response elements in the 3'UTR sequence of mRNA. However, the putative interactions may not be very reliable since the database, for the co-occurrence, depends on TargetScan v5.2, which is outdated.

Another database of miRNA sponge interactions is starBAse (Li, Liu et al. 2013) that utilizes large-scale CLIP-Seq data (HITS-CLIP, PAR-CLIP, iCLIP) of 108 datasets from 37 studies and experiment results providing physical biding information between miRNA-mRNA, miRNA-lncRNA, miRNA-circRNA, miRNA-pseudogene, and miRNA-sncRNA. In order to evaluate if a miRNA sponge pair shares significant common mRNAs, starBAse uses a hypergeometric test.

The database lnCeDB (Das, Ghosal et al. 2014) contains human lncRNAs which can potentially act as miRNA sponges. The miRNA-mRNA interactions in lnCeDB are predicted by using TargetScan (Lewis, Burge et al. 2005) while the miRNA-lncRNA interactions are either retrieved from miRcode (Jeggari, Marks et al. 2012), or predicted by lnCeDB's own algorithm. lnCeDB not only allows users to browse for lncRNA-mRNA pairs sharing the same miRNAs, but also compares the expression data of that pair in 22 human tissues.

LncACTdb (lncRNA-associated competing triplets database) is a database containing 5119 functionally active and over 530,000 computationally predicted lncRNA-miRNA-gene interactions, which are obtained by integrating heterogeneous data from many in silico target prediction studies, Argonaute-CLIP experiments, and RNA-seq expression profiles (Wang, Ning et al. 2015).

HumanViCe (Ghosal, Das et al. 2014) is a comprehensive database that contains a vast number of coding and non-coding RNAs acting as potential miRNA sponges in virus infected human cells.

The first experimentally validated ceRNA-miRNA interaction database is miRSponge (Wang, Zhi et al. 2015), which collects 185 unique miRNA sponge interactions in 11 species by manually curating. This database contains different kinds of miRNA sponges (i.e., lncRNAs, pseudogenes, circRNAs, coding RNAs, viral RNAs and artificial engineered sponges) and so it is a useful tool to verify computational predictions.

Finally, a freely accessible repository that greatly assists the miRNA research community is miRWalk2.0, a comprehensive archive of predicted and experimentally verified miRNA-target interactions (Dweep and Gretz 2015). In particular, miRWalk2.0 combines the information of miRNA binding sites within the complete sequence of a gene with the results of existing miRNA-target prediction databases (e.g. DIANA-microT, miRanda, PicTar, PITA, miRDB, RNA22, RNAhybrid, TargetScan) and also provides experimentally verified miRNA-target interactions obtained via an automated text-mining search and data from existing resources (miRTarBase, PhenomiR, miR2Disease and HMDD).

Obviously, on a case-by-case basis, users can choose to consider a single independent database or combine multiple databases to identify candidate miRNA sponge interactions.

6. Computational approaches for identifying ceRNA-miRNA interactions

In order to analyse and predict the behaviours of the ceRNA regulatory mechanism, different computational approaches have been developed.

Such methods can be classified into: pair-wise correlation-based methods, partial association methods, and mathematical modelling approach (Le, Zhang et al. 2016).

Pair-wise correlation-based methods are based on the principle that the expression levels of pairs of RNAs that compete for the same miRNA are positively correlated (Poliseno, Salmena et al. 2010,

Salmena, Poliseno et al. 2011). Such a principle stems from the observation of the titration mechanism, which states that the increasing (decreasing) of the competing RNA concentrations of a miRNA sponge decreases (increases) the available miRNA by interacting with the other miRNA sponges, relieving (overcoming) the miRNA repression on the other competing RNAs. As a result, the expression levels of the two ceRNAs rise or decrease together, showing a positive correlation (**Figure 1**). Methods belonging to this class share the same procedure: firstly they search for all pairs of RNAs that share the same MREs, then they perform an hypergeometric test to calculate the significance of sharing miRNAs, and finally they predict the positively correlated pairs as miRNA sponges (Zhou, Liu et al. 2014, Chiu, Llobet-Navas et al. 2015, Chiu, Hsiao et al. 2015, Shao, Wu et al. 2015, Xu, Li et al. 2015). In particular, Zhou et al. (Zhou, Liu et al. 2014) built the miRNA sponge interaction network in human breast cancer using matched miRNA and gene expression data, and, by performing a survival analysis, they found that the hub nodes are good candidates biomarker in breast cancer. Similarly, Xu et al. (Xu, Li et al. 2015) inferred the miRNA sponge interactions landscape across 20 cancer types, identifying both cancer-specific and pan-cancer interactions. Shao et al. (Shao, Wu et al. 2015) identified dysregulated ceRNA-miRNA interactions in lung adenocarcinoma by integrating ceRNA expression levels and miRNA-target interactions. Finally, Chiu et al. (Chiu, Hsiao et al. 2015) investigated the optimal conditions of the miRNA sponge regulation mechanism in various cancer type (e.g. glioblastoma, ovarian, and lung carcinoma) by combining ceRNA expression profiles and putative miRNA-target interactions.

Partial association methods take into account both miRNAs and ceRNA expression levels computing either the mutual information (Sumazin, Yang et al. 2011, Chiu, Llobet-Navas et al. 2015) or the partial correlation (Paci, Colombo et al. 2014). In particular, Sumazin et al. (Sumazin, Yang et al. 2011) investigated the ceRNAs activity in human glioblastoma, driven by the a priori information on putative/validated pairs of RNAs sharing a statistically significant number of common miRNAs. Specifically, they combined expression data of RNA-RNA pairs sharing a significant overlap of common miRNAs with predicted miRNA-target regulatory interactions and then, they estimated the difference between the mutual information and conditional mutual information to identify the RNA-miRNA-RNA triplets. Chiu et al. (Chiu, Llobet-Navas et al. 2015) proposed a prediction method, validated on breast cancer data, that allows to simultaneously identify both miRNA-target and miRNA-mediated sponge interaction networks. Paci et al. (Paci, Colombo et al. 2014) developed a purely data-driven approach focused on the identification of new putative lncRNAs acting as ceRNAs by using expression data of breast invasive carcinoma

provided by The Cancer Genome Atlas (TCGA) (Network, Weinstein et al. 2013, Tomczak, Czerwinska et al. 2015).

Mathematical model approaches exploit deterministic or stochastic models to analyse and predict the behaviour of ceRNA regulatory networks (Karlebach and Shamir 2008). Deterministic models exploit the network connectivity information and makes use of the kinetic parameters characterizing the biochemical reactions in order to determine how the system changes in time and space under external stimulation. Each biological network is affected by stochastic components, but, when the number of involved molecules of each species is quite large, the law of mass action can be used to accurately calculate the change in concentrations, and little or no stochastic effect is observable. Conversely, when the number of molecules is small, significant stochastic effects may be seen and then it is preferable to choose a stochastic model. Examples of mathematical modelling approaches aiming to quantitative understanding of ceRNA-miRNA interactions network can be found in (Ala, Karreth et al. 2013, Figliuzzi, Marinari et al. 2013), where a mass-action model is used to determine the optimal conditions of miRNA sponge activity in silico, as well as in (Bosia, Pagnani et al. 2013), where the authors propose a stochastic model to analyse the equilibrium and out-of-equilibrium properties of a network of *M* miRNAs interacting with *N* mRNA targets in terms of a titration mechanism. More recently, Yuan et al. (Yuan, Liu et al. 2015) performed a model-quantitative analysis for miRNA sponge interaction system and validated their computational results by using synthetic gene circuits in human embryonic kidney 293 cells.

7. Case study: algorithm for identifying ceRNA-miRNA interactions in breast cancer data

A recent review (Le, Zhang et al. 2016) reported a comparison study of the widespread computational methods for identifying ceRNA-miRNA interactions. Among these methods the algorithm proposed by Paci et al. (Paci, Colombo et al. 2014) resulted as the best one in terms of the percentage of discovered miRNA sponge interactions associated with breast invasive carcinoma (brca). For this reason, here we present in detail such a computational analysis that aims to identify putative lncRNAs acting as miRNAs sponge in breast cancer.

In this study, the authors used normalized level 3 RNA- and miRNA- sequencing expression data of brca from IlluminaHiSeq platform that were retrieved from TCGA (Network, Weinstein et al. 2013, Tomczak, Czerwinska et al. 2015).

The study concerned 72 samples for which the complete sets of tumor and matched normal profiles (for both RNA-seq and miRNA-seq data) were available. Entries with more than the 10% of missing values were filtered out. Coding versus non-coding RNAs based on entrez gene identifiers and human annotation obtained from NCBI were separated.

The analysis was restricted to those mRNAs with an available 3'UTR sequence at least equal to 500 nt in the curated UTRdb database (Grillo, Turi et al. 2010). Altogether, a total of 10492 mRNAs, 311 miRNAs, and 833 lncRNAs were analysed in (Paci, Colombo et al. 2014).

The computational model developed to analyse these data is based on three hypotheses:

1 *The RNAs competing for the same miRNA are marked by a highly positive correlation.*

The top-correlated mRNA/lncRNA pairs in normal and cancer data sets were selected by setting in both cases the correlation threshold to the 99th percentile of the corresponding overall correlation distribution (**Figure 3A right**).

2 *The interaction between the RNAs competing for the same miRNA is indirect, i.e. mediated by miRNA.*

To investigate the scenario in which specific miRNAs may mediate the interactions of the top-correlated mRNA/lncRNA pairs, the authors applied a well-established tool of multivariate analysis (i.e., the partial correlation) to each selected mRNA/lncRNA pair with respect to each miRNA in their dataset. In general, the partial correlation measures the extent to which an observed correlation between two variables X and Y (here, the expression profiles of a mRNA and a lncRNA) relies on the presence of a third controlling variable Z (here, the expression profile of a miRNA) and it is computed as:

$$\rho_{XY|Z} = \frac{\rho_{XY} - \rho_{XZ}\rho_{ZY}}{\sqrt{1-\rho_{XZ}^2}\sqrt{1-\rho_{ZY}^2}}$$

where ρ_{XY} is the Pearson's correlation. Then, the *sensitivity correlation S* was defined as:

$$S = \rho_{XY} - \rho_{XY|Z}$$

The *XYZ* triplets with $S > 0.3$, corresponding to a drop of about the 30% in the correlation between *XY* when *Z* is removed, were selected. The sensitivity distribution of the top-

correlated mRNA/lncRNA pairs (*XY*) is plotted removing one miRNA (*Z*) molecule at time (**Figure 3A left**).

3 The RNAs competing for the same miRNA harbour one or more MREs for the miRNA that they sponge.

A seed match analysis was performed in order to select only these triplets mRNA/lncRNA/miRNA that are enriched in binding sites of the shared miRNA (hypergeometric test p-value < 0.01).

The minimal pairing requirement to predict a miRNA target recognition is a perfect match to positions 2 to 7 (6-mer miRNA seed) at the 5'-end of the mature miRNA sequence (Lewis, Burge et al. 2005). The miRNA seed sequences were obtained by mapping TCGA miRNA identifiers to miRBase (Kozomara and Griffiths-Jones 2014). Complementary DNA (cDNA) sequences (i.e., without introns) for lncRNAs were obtained querying the Ensembl data portal through its R/Bio-conductor \cite interface provided by the package biomaRt and by using Entrez gene identifiers. For each 3'UTR sequence included in the dataset analysed in (Paci, Colombo et al. 2014), all the occurrences matching the reverse-complement of the 6-mer seed for the miRNAs analysed were recorded. Similarly, for each lncRNA included the dataset analysed in (Paci, Colombo et al. 2014) all the occurrences of short sites matching the reverse-complement of a miRNA seed in the entire transcript sequence were stored. The lists of coding and non-coding RNA identifiers used to retrieve corresponding sequences were built based on gene annotations obtained from the NCBI.

Integrating the results of multivariate statistical analysis and seed match analysis, the so-called miRNA-mediated interactions network (MMI-network) was built both in normal and cancer tissues (**Figure 3C**). Nodes in the networks represent mRNAs and lncRNAs with highly correlated expression profiles while edges represent miRNAs mediating their interactions. Concretely, linked nodes are required to meet three conditions: (i) matching high values of the Pearson correlation between their expression profiles ($\rho > 0.7$); (ii) matching high values of the sensitivity correlation ($S > 0.3$); (iii) sharing binding sites for miRNAs (6-mer miRNA seed match).

The study in (Paci, Colombo et al. 2014) revealed the existence of a complex regulatory network in normal samples that appears to be missing in tumor samples (and vice-versa), highlighting a marked rewiring in the ceRNA program between normal and pathological breast tissues (**Figure 3B**). This

mutually exclusive activation confers an interesting character to ceRNAs as potential oncosuppressive, or oncogenic, protagonists in human cancer. At the heart of this phenomenon is the recently and widely studied oncogene PVT1 (Graham, Adams et al. 1984, Lemay and Jolicoeur 1984, Graham and Adams 1986, Villeneuve, Rassart et al. 1986, Huppi, Siwarski et al. 1990, Huppi and Siwarski 1994, Hodgson, Hager et al. 2001, Gerstein, Bruce et al. 2007, Guan, Kuo et al. 2007, Huppi, Volfovsky et al. 2008, Meyer, Maia et al. 2011, Chapman, Tidswell et al. 2012, Brooksbank, Bergman et al. 2014, Tseng, Moriarity et al. 2014, Wang, Yuan et al. 2014, Colombo, Farina et al. 2015, Zhuang, Li et al. 2015, Cui, Yu et al. 2016, Iden, Fye et al. 2016, Zhou, Chen et al. 2016) that switches from being the first of the hubs in the normal MMI-network to fall outside the list of nodes of the cancer network. In normal network, PVT1 revealed a net binding preference towards the miR-200 family (**Figure 3C**)., which it antagonizes to regulate the expression of hundreds of mRNAs that are known to be related to the cancer development and progression (e.g. GATA3, CDH1, TP53, TP63, TP73, RUNX1, and RUNX3).

The rationale behind this rewiring and the specific conditions required for a ceRNA network to occur are still unknown, but the following hypotheses can be formulated: (i) an exon skipping mechanism, i.e. the presence of alternative transcription start sites causes the skipping of exons where reside the MREs, that could lead to a preferential expression in tumor tissue of some isoform lacking the binding site required for a given miRNA sponge; (ii) a mechanism of titration, i.e. large variations in the ceRNAs expression levels can overcome, or relive, the repression of miRNA on its competitors, or similar over-expression of miRNA can abolish the competition between the two transcripts. Recently, several studies (Salmena, Poliseno et al. 2011) have stressed the importance of the relative concentration of RNA molecules that participate in the sponge mechanism.

Figures

Figure 1. CeRNAs mechanism.

X and Y are two RNA transcripts that compete for binding the same microRNA(s). In the steady state (middle), the microRNA molecules and their targets X and Y are in equilibrium and the microRNA will be equally distributed between its targets. In a downregulation condition of the RNA X (left), the availability of microRNA molecules to bind the RNA Y increased determining the decrease of RNA Y expression. On the contrary, in a overexpression condition of the RNA X

(right), less microRNA molecules are free to bind the RNA Y, and thus the RNA Y abundance increases. Legend. Red dots: microRNA molecules; light red boxes: RNA X; green boxes: RNA Y.

Figure 2. Radar plot of chromosome distribution.

Chromosome distribution of miRNAs within lncRNAs.

Figure 3. Results of computational model proposed by Paci et al. (Paci, Colombo et al. 2014)

A) Heat-map representing the sensitivity correlation *S*, computed using the normal expression data for the top-correlated mRNA/lncRNA pairs (N = 87398) in the normal dataset. Light vertical stripes point to a small pool of miRNA molecules responsible for the high correlation between all top-correlated mRNA/lncRNA pairs (i.e., Pearson correlation values exceeding the 99th percentile of the overall correlation distribution with $\rho > 0.7$). B) Heat-map representing the sensitivity correlation *S*, computed by using the cancer expression data for the top-correlated mRNA/lncRNA pairs in the normal dataset. Legend for panel A) and B). Rows: top-correlated mRNA/lncRNA pairs in normal dataset; columns: miRNAs. Color key: red to blue scale corresponds to low to high *S*. C) Normal MMI-network built from expression data of normal breast tissues. Nodes in this network represent both mRNAs and lncRNAs; edges represent miRNAs. From left to right, the two main components of the normal MMI-network and the lncRNA PVT1 sub-network are highlighted. Color key: one color to each miRNA according to the legend in the panel C (e.g. red color corresponds to the miR-200 family).

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