An Integrated Approach for Experimental Target Identification of Hypoxia-induced miR-210

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miR-210 is a key player of cell response to hypoxia, modulating cell survival, VEGF-driven endothelial cell migration, and the ability of endothelial cells to form capillary-like structures. A crucial step in understanding microRNA (miRNA) function is the identification of their targets. However, only few miR-210 targets have been identified to date. Here, we describe an integrated strategy for large-scale identification of new miR-210 targets by combining transcriptomics and proteomics with bioinformatic approaches. To experimentally validate candidate targets, the RNA-induced silencing complex (RISC) loaded with miR-210 was purified by immunoprecipitation along with its mRNA targets. The complex was significantly enriched in mRNAs of 31 candidate targets, such as BDNF, GPD1L, ISCU, NCAM, and the non-coding RNA Xist. A subset of the newly identified targets was further confirmed by 3′-untranslated region (UTR) reporter assays, and hypoxia induced down-modulation of their expression was rescued blocking miR-210, providing support for the approach validity.

In the case of 9 targets, such as PTPN1 and P4HB, miR-210 seed-pairing sequences localized in the coding sequence or in the 5′-UTR, in line with recent data extending miRNA targeting beyond the “classic” 3′-UTR recognition. Finally, Gene Ontology analysis of the targets highlights known miR-210 impact on cell cycle regulation and differentiation, and predicts a new role of this miRNA in RNA processing, DNA binding, development, membrane trafficking, and amino acid catabolism. Given the complexity of miRNA actions, we view such a multiprong approach as useful to adequately describe the multiple pathways regulated by miR-210 during physiopathological processes.

miRNAs are 21–23-nucleotide non-protein coding RNA molecules that regulate the stability and/or the translational efficiency of target messenger RNAs (1–3). Mature miRNAs are loaded into the RNA-induced silencing complex (RISC) and mediate the translational inhibition of target mRNA, albeit a few opposing examples have been described as well (4–6). The rules that guide miRNA-mRNA interaction are very complex and still under investigation. However, the current paradigm states that a Watson-Crick pairing between the mRNA and the 5′-region of the miRNA centered on nucleotides 2–7, termed “seed sequence,” is required for miRNA-mediated inhibition (7). RISC-miRNA complexes can move the mRNAs they bind to the P-bodies, which are specialized cytoplasmic compartments where translational repression and mRNA turnover is thought to occur (8). Because P-bodies contain many enzymes involved in mRNA exonucleotilic degradation, miRNAs may also have a secondary quantitative inhibitory effect on mRNAs. A role for miRNAs in mRNA destabilization is also suggested by studies reporting robust correlations between the levels of miRNAs and the message of multiple predicted or validated targets (9–11).

miR-210 is currently regarded as “master miRNA” of hypoxic response, because it was found up-regulated by hypoxia in all the cell types tested to date (12). Previously, we characterized miR-210 regulation and its functional relevance in endothelial cell (EC) response to hypoxia (13). We found that miR-210 increases EC tubulogenesis and migration, whereas miR-210 blockade in the presence of hypoxia inhibits these processes and induces apoptosis. These effects are mediated, at least in part, by the direct inhibition of the receptor-tyrosine kinase ligand Ephrin-A3 (EfnA3). Furthermore, other miR-210 targets have been recently identified: E2F3, NPTX1, RAD52, ACVR1B, MNT, CASP8AP2, FGFR1, and HOXA-1 and -9 (14–20), suggesting multiple roles of this miRNA in the cellular adaptation to low oxygen. Considering that the function of each miRNA is mediated by the modulation of a subset-specific mRNA targets and that each miRNA is thought to regulate hundreds of mRNAs (21), the identification of new miR-210 targets represents an important step in elucidating its function. Several tools for predicting miRNA targets have been developed. However, the overlap between their output is rather limited (7), raising the distinct possibility of false positive predic-

The abbreviations used are: RISC, RNA-induced silencing complex; miRNA, microRNA; EC, endothelial cell; GO, gene ontology; HUVEC, human umbilical vein endothelial cells; LNA, locked nucleic acid; UTR, untranslated region.
the identification of a panel of novel targets. These potential targets were validated based on their expression or miR-210 blockade were further analyzed. For instance, a gene repressed by miR-210 up-regulation was considered for further analysis.

**Experimental Target Identification of miR-210**

To test the effect of miR-210 increase, total RNAs derived from HUVEC overexpressing miR-210 or a control sequence were analyzed by two-dimensional electrophoresis (2-DE). Comparison of the 2-DE maps revealed statistically significant differences between the two groups in the expression of 28 proteins (supplemental Table ST1), which were excised from the 2-DE gels and identified by mass spectrometry (supplemental Table ST1). Results are summarized in Table 1. Whereas the 17 up-regulated proteins were most likely indirect targets, the 11 down-modulated proteins may be inhibited by miR-210 either directly or indirectly.

**Transcriptomic Profile Changes Induced by miR-210 Overexpression or Blockade**—To further investigate the effects of miR-210 expression, we analyzed gene expression modifications induced by miR-210 modulation using Affymetrix technology. To test the effect of miR-210 increase, total RNAs derived from HUVEC overexpressing miR-210 or a scramble sequence under control of a retroviral vector were analyzed. Using a reciprocal approach, to determine the effect of miR-210 inhibition, HUVEC in which miR-210 activity was blocked by a complementary LNA oligonucleotide, that binds with high affinity and specificity to miR-210, were analyzed. A scrambled LNA was used as control. To limit the number of false positive, only transcripts displaying inverse modulation following miR-210 overexpression or miR-210 blockade were further analyzed. For instance, a gene repressed by miR-210 up-regulation was con-
considered only if it was induced upon miR-210 inhibition as well. When the previously modulated datasets (supplemental Tables ST2 and ST3) were intersected, we identified a subset of 63 genes (versus modulated genes, Fig. 1). The principal effect of a miRNA is the inhibition of the translation of the target mRNA. However, it was demonstrated that the repression of many miRNA targets is frequently associated with their destabilization (3). Among the inversely modulated genes, 51 were both down-modulated by miR-210 overexpression and up-regulated by miR-210 blockade, representing potential direct targets.

Over-represented Gene Ontology Categories among miR-210-modulated Genes—We next evaluated the cellular processes affected by miR-210, both directly and indirectly, according to Gene Ontology (GO) (30). We analyzed the 63 inversely modulated genes identified by transcriptomics and the 28 modulated genes identified by proteomics, searching for enriched GO categories. Results in supplemental Table ST4 show that miR-210-modulated genes were involved in RNA processing, in DNA binding, in differentiation and development, in the transport through cytoplasmic membranes and through the nucleus, and in the amino acids catabolism. We also analyzed gene interactions within pathways (supplemental Table ST4), and we found that “Hypoxia-Inducible Factor in the Cardiovascular System” was the pathway that displayed the highest enrichment. Other pathways modulated by miR-210 were: ATM pathway, involved in DNA repair; FAS and TNFR1 apoptotic pathways; integrin and agrin pathways, that both regulate proliferation, motility, and cellular shape, by modulating cytoskeleton organization and actin polymerization.

### Potential Targets Identified by Proteomics and Transcriptomics Are Enriched for miR-210 Seed-pairing Sites—To discriminate between direct and indirect miR-210 targets, we took advantage of the many available target-prediction software. Surprisingly, none of the 62 candidates (51 from transcriptomic and 11 from proteomic analyses) was recognized as a direct target by PicTar, TargetScan, and Diana-microT algorithms. Other prediction softwares recognized few candidates as direct miR-210 targets, but their number remained low (BCAS2, SF3B1, and SMCHD1 by Sanger miRBase; AP1S2, KLF12, and ROD1 by microRNA.org; KLF12 by EIMMo). It is worth noting that these software limit the search of miRNA-mRNA interactions to the 3’-UTR regions of mRNA. Considering that many reports have demonstrated the presence of functional miRNA pairing sites both in the coding sequence and in the 5’-UTR of miRNAs (5, 6, 22–24, 27), we performed a low-stringency miR-210 seed-pairing search in the whole sequence of miR-210 potential targets and, as control, in a group of randomly picked genes that were not modulated after miR-210 overexpression (non-target list).

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### Table 1

| Gene      | Description                  | Spot | MW kDa/pI | Fold increase | S.E. | p    |
|-----------|------------------------------|------|-----------|---------------|------|------|
| Up-regulated |
| CNN2      | Calponin-2                   | 34   | 33.7/6.94 | 2.15          | 0.12 | 0.002|
| EHD1      | EH-domain-containing protein 1 | 62   | 60.6/6.35 | 1.75          | 0.07 | 0.002|
| EIF5A     | Eukaryotic translation initiation factor 5A | 6    | 16.8/5.07 | 3.29          | 0.34 | 0.008|
| GPC1      | RGS19-interacting protein 1   | 75   | 36.0/5.90 | 1.64          | 0.11 | 0.037|
| HNRPL     | Heterogeneous nuclear ribonucleoprotein L | 91   | 64.1/4.86 | 1.52          | 0.07 | 0.035|
| HSPD1     | 60 kDa heat shock protein     | 70   | 61.1/5.70 | 1.69          | 0.15 | 0.030|
| KHSRP     | Far upstream element-binding protein 2 | 15   | 73.1/6.84 | 2.69          | 0.30 | 0.009|
| KHSRP     | Far upstream element-binding protein 2 | 81   | 73.1/6.84 | 1.59          | 0.05 | 0.041|
| LMNA      | Lamin A/C                    | 39   | 74.1/6.57 | 5.14          | 0.60 | 0.007|
| LMNA      | Lamin A/C                    | 39   | 74.1/6.57 | 2.04          | 0.21 | 0.026|
| LOC403161 | Nicotinamide phosphoribosyltransferase-like protein | 58   | 57.5/5.51 | 1.80          | 0.12 | 0.007|
| MCCC2     | Methylcrotonyl-CoA carboxylase β-chain | 53   | 61.3/5.81 | 1.85          | 0.24 | 0.046|
| MTHFD1    | C-1-tetrahydrofolate synthase | 21   | 101.6/4.91| 2.59          | 0.27 | 0.011|
| NIP3      | Nucleoprotein 34 kDa         | 87   | 54.6/5.33 | 1.54          | 0.10 | 0.030|
| PCBP1     | Poly(rC)-binding protein 1   | 92   | 37.5/6.66 | 1.51          | 0.03 | 0.002|
| PDHA1     | Pyruvate dehydrogenase E1 component α-subunit | 100  | 43.8/3.35 | 1.43          | 0.11 | 0.036|
| SARS      | Seryl-tRNA synthetase        | 68   | 58.2/5.38 | 1.71          | 0.14 | 0.020|
| TUBA3     | Tubulin α-3 chain            | 87   | 49.9/4.96 | 1.54          | 0.10 | 0.030|
| UCHL1     | Ubiquitin carboxyl-terminal hydrolase isozyme L1 | 79   | 24.8/5.33 | 1.61          | 0.16 | 0.043|

| Down-regulated |
| BCAS2      | Breast carcinoma amplified sequence 2 | 41   | 26.1/5.48 | 0.50          | 0.06 | 0.023|
| CBX1       | Chromobox protein homolog 1     | 28   | 21.4/5.45 | 0.44          | 0.06 | 0.020|
| CTSB       | Cathepsin B precursor           | 60   | 37.8/5.88 | 0.57          | 0.07 | 0.029|
| CTNSB      | Cathepsin B precursor           | 69   | 37.8/5.88 | 0.59          | 0.05 | 0.024|
| EIF1AY     | Eukaryotic translation initiation factor 1A. Y-chromosomal | 97   | 16.5/5.07 | 0.68          | 0.05 | 0.031|
| NDUFV2     | NADH-ubiquinone oxidoreductase 24 kDa subunit | 80   | 27.4/8.22 | 0.62          | 0.05 | 0.040|
| NES        | Nestin                        | 36   | 177.4/4.35| 0.47          | 0.06 | 0.028|
| P4HB       | Protein disulfide-isomerase precursor | 17   | 57.1/4.76 | 0.38          | 0.07 | 0.030|
| PTEN       | Protein phosphatase non-receptor type 1 | 72   | 49.9/5.88 | 0.60          | 0.06 | 0.035|
| SEC13L1    | SEC13-related protein          | 29   | 35.5/5.22 | 0.45          | 0.07 | 0.038|
| TCEB2      | Transcription elongation factor B polypeptide 2 | 78   | 13.3/4.73 | 0.62          | 0.04 | 0.033|
| VIM        | Vimentin                      | 10   | 53.6/5.06 | 0.33          | 0.07 | 0.029|
| VIM        | Vimentin                      | 2    | 53.6/5.06 | 0.21          | 0.01 | 0.000|
| VIM        | Vimentin                      | 12   | 53.6/5.06 | 0.37          | 0.06 | 0.045|

* kDa/isoelectric point.

** Ratio of normalized spot density from cells overexpressing miR-210 versus control cells.
Experimental Target Identification of miR-210

| seed type | miR-210 sequence 5’-3’ |
|-----------|------------------------|
| 8 mer     | CUGUGCGUGUGACACGGCCUGA |
| 7 mer_1   | CUGUGCGUGUGACACGGCCUGA |
| 7 mer_2   | CUGUGCGUGUGACACGGCCUGA |
| 6 mer     | CUGUGCGUGUGACACGGCCUGA |
| 6-1 mer   | CUGUGCGUGUGACACGGCCUGA |

Confirmation of Potential Targets by Immunoprecipitation of miR-210-enriched RISC—To validate experimentally the potential targets identified with the low-stringency miR-210 seed-pairing search, we set up a biochemical assay based on the immunoprecipitation of RISC complexes enriched for miR-210 and its targets. To this aim, we used a c-Myc-tagged allele of Ago2, a core component of the RISC complex, associating both with miRNAs and their mRNA targets (31, 32). Easily transfectable HEK-293 cells were co-transfected with expression vectors for miR-210 and c-Myc-Ago2, yielding cells enriched of miR-210/c-Myc-Ago2-containing RISC complexes. Consequently, we expected miR-210 endogenous targets to be over-represented in the RISC of transfected cells. Then, we immunoprecipitated c-Myc-Ago2 and evaluated the levels of co-immunoprecipitated mRNAs by qPCR. Background controls were represented by c-Myc immunoprecipitates derived from cells transfected with miR-210 but not c-Myc-Ago2. To validate this approach, five well established miR-210 target genes were assayed (13–17). Fig. 3 shows that EFNA3, E2F3, NPTX1, RAD52, and ACVR1B were significantly enriched in immunoprecipitates of miR-210-overexpressing cells compared with cells transfected with a scramble sequence. Conversely, B2M, GAPDH, GUSB, and RPL13, that according to transcriptomic and target-prediction analysis were not miR-210 targets, did not display any enrichment, and thus were used for normalization (Fig. 3F). We next performed the same assay evaluating both the 42 seed-pairing potential targets and the whole list of predicted miR-210 target genes according to Pictar and TargetScan. A selection of cardiovascular-related genes identified as predicted targets by microRNA.org was assayed as well (see supplemental Table ST6 for the complete list of tested genes). Only genes enriched significantly ($p < 0.05$) more than 2-fold were considered as positives. Furthermore, to minimize the number of false positives, each gene was re-assayed with an

FIGURE 2. miR-210 seed-pairing. The figure shows highlighted with a gray background the different types of miR-210 seed sequences that were used for a low-stringency search of miR-210 seed-pairing among the differentially expressed genes identified by transcriptomics and proteomics.

FIGURE 1. Genes modulated by miR-210. Heat map representing mRNAs modulated by miR-210 overexpression (pSUPER-miR-210) that displayed an inverse modulation following miR-210 inhibition (anti-miR-210) ($n = 3$). Gene: official gene symbol. Green, down-modulation; Red, up-regulation. All differences are statistically significant ($p < 0.005$).
independent primer couple. It is worth noting that, with few exceptions such as NCAM and EIF1AY, all the tested genes were easily detectable and similarly expressed in both HEK-293 and HUVEC (median Delta Ct 0.4 ±0.3, supplemental Table ST7) and that the background control immunoprecipitations displayed low-to-undetectable signals for all the assayed genes (data not shown). As expected, target prediction algorithms displayed a 50% validation rate (46% for Pictar and 53% for TargetScan). MiR-210 seed-pairing potential targets identified by transcriptomics and proteomics displayed a lower, but robust validation rate (16/42, 38%). Intriguingly, for most of them (68%), miR-210 seed-pairing sites were in the coding sequence, and CBX1 and TNPO1 even contained miR-210 seed-pairing sites in the 5'UTR. Table 2 shows only genes enriched in miR-210-loaded RISCs, while the complete dataset is shown in supplemental Table ST6. miR-210 seed-pairing sites of the validated targets displayed a poor degree of conservation: whereas all but 2 were conserved in chimp, only 4 were conserved also in mouse and/or rat (supplemental Fig. SF3). The GO analysis of the cellular processes affected by the 31 newly identified targets, along with 5 identified previously, showed a significant enrichment of cell cycle-related GO classes, further demonstrating a role for miR-210 in growth control (supplemental Table ST8).

Further Validation of the Identified miR-210 Targets—To further validate our results, protein levels of a selection of identified targets were evaluated after miR-210 modulation. As shown in Fig. 4A, BDNF, PTPN1, and P4HB were down-modulated following miR-210 overexpression and induced when miR-210 was inhibited (see supplemental Fig. SF4 for Western blotting quantification). A slightly different experimental approach was used to validate the GPD1L target. We were unable to generate an antibody that discriminated GPD1L from GPD1. However, we found that miR-210 overexpression down-modulated and miR-210 blockade increased GPD1L mRNA (Fig. 4B, see supplemental Fig. SF4 for quantification). These regulations

![Graphs and images](image-url)
were even more dramatic when hypoxic cells were analyzed: GPD1L mRNA levels were down-modulated by hypoxia per se, miR-210 overexpression further down-modulated GPD1L; in contrast, miR-210 inhibition completely prevented GPD1L mRNA decrease. MiR-210 ability to modulate RNA levels was further demonstrated when Xist was measured (Fig. 4C). We also confirmed that miR-210 directly regulated the expression of a selection of identified targets. To this aim, miR-210 seed-pairing sites and the immediately surrounding sequences contained in BDNF, CPEB2, DDAH1, NCAM1, PTPN1, and XIST (supplemental Fig. SF2) were cloned downstream of a luciferase open reading frame. The luciferase activity of these constructs was evaluated following the overexpression of either miR-210 or a scramble sequence. Fig. 4D shows that miR-210 inhibited the reporter constructs containing an intact miR-210 binding site (pLUC-210-seed), whereas this effect was prevented by the deletion of the seed complementary nucleotides (pLUC-210-seed del). These data confirm that miR-210 directly inhibits the tested targets. It is worth noting that Xist contains two potential miR-210 seed-pairing sites, and only the one in position 11332–11337 was not functional in the tested targets. It is worth noting that Xist contains two potential miR-210 seed-pairing sites, and only the one in position 6197–6202 (XIST_1) was modulated by miR-210 (Fig. 4D), whereas the one in position 11332–11337 was not functional in the assayed conditions (not shown). GPD1L was confirmed to be a direct target as well. GPD1L-3’-UTR was cloned downstream of a luciferase open reading frame, and the luciferase activity was evaluated in cells overexpressing or down-modulating miR-210. A significant negative effect was observed following miR-210 overexpression (Fig. 4E), whereas miR-210 inhibition increased the luciferase activity, indicating that GPD1L-3’-UTR was also inhibited by basal levels of miR-210.

**DISCUSSION**

In this study, we took a molecular approach encompassing proteomics, transcriptomics, bioinformatics, and biochemistry to investigate miR-210 function. Specifically, we studied how perturbations of miR-210 levels or activity influenced gene expression, both directly and indirectly, affecting specific molecular pathways and cellular functions. Furthermore, we identified, among the modulated genes, 31 new direct miR-210
Our approach to study gene expression modulation by miR-210 was 2-fold, proteomic and transcriptomic. Whereas proteomics is the technique of choice to analyze post-transcriptional regulation, it is also limited by its low sensitivity. Thus, we also measured gene expression modifications induced by both miR-210 overexpression and blockade. Transcriptomic experiments are highly sensitive; however, often yield extensive lists of modulated genes, and it is challenging to evaluate the directly affected genes, or the genes of relevance for a given process. To address this issue, we limited our analysis to those genes that exhibited inverse patterns when miR-210 was overexpressed or down-regulated. A similar approach was recently used to study miR-140 function and targets (33) and to demonstrate that miR-210 modulates the c-Myc antagonist MNT (18).

GO analysis confirmed that miR-210 activity is involved in differentiation and cell cycle regulation (13, 14, 17, 18, 34, 35) and highlighted new functions in RNA processing, DNA binding, membrane trafficking, amino acid catabolism, and development. Of note, one of the most enriched GO classes, “heart development,” was consistent with miR-210 up-regulation observed in cardiac hypertrophy and heart failure (36, 37), events characterized by the re-expression of fetal genes. Furthermore, in keeping with HIF1α-dependent regulation of miR-210 in hypoxic conditions (12), the HIF1α pathway was found as the most enriched miR-210-modulated pathway, suggesting a potential bi-directional relationship between miR-210 and the HIF pathway. Other enriched pathways, such as ATM, FAS, and TNFR1, correlated with the anti-apoptotic role of miR-210 (13, 16, 38). Likewise, integrin and agrin pathway modulations may be instrumental for miR-210-dependent regulation of EC migration and in vitro differentiation previously described by our group (13).

Potential miR-210 direct targets were then assessed for direct association with miR-210 within RISC complexes. To this end, we immunoprecipitated RISC complexes targets, allowing to better delineate the molecular pathways underpinning miR-210 action.
of non-crosslinked RNA-protein complexes, including Ago-miRNAs, may co-purify mRNAs that are not specific targets (39, 40). To minimize this potential artifact source, miR-210-containing RISC complexes were compared with RISC complexes derived from cells transfected with a scramble sequence. Thus, most artifactual targets were presumably excluded by the background subtraction. Results show that 31 of 76 tested genes were targeted by miR-210 directly. However, we cannot exclude that, among non-enriched genes, bona fide miR-210 targets exist. Indeed, our approach was not intended to provide a comprehensive compilation of miR-210 targets, but it was aimed at the identification of a sizable number of targets, allowing to highlight certain nodal elements of the molecular pathways underpinning miR-210 function.

To this aim, very stringent selection parameters were adopted to minimize the number of false positives. Furthermore, it is worth noting that the transcriptomic and the proteomic experiments were performed in HUVEC cells, whereas the IP-RISC experiments were, for technical reasons, performed in the highly transfectable HEK-293 cells. Whereas tested genes were expressed in both cell types, one should consider that alternative splicing and polyadenylation may eliminate miRNA target sites, as demonstrated for highly proliferating cells that express shortened 3′-UTRs (7, 41, 42). Results obtained were not limited to the HEK-293 cell system. Indeed, miR-210 was necessary for the down-modulation of the identified targets in hypoxic HUVEC.

The validity of the RISC immunoprecipitation approach was also confirmed by a recent study that identified a number of miR-210 targets with an experimental approach similar to the one we adopted (20). It is worth noting that the targets identified by Huang et al. display very little overlap with the ones we demonstrated. Indeed, relevant differences in the experimental procedures and in the cell system are present, and none of the studies is saturating, leaving avenues to further investigations in the field.

In our low-stringency research of miR-210 seed-pairing among the differentially expressed genes identified by transcriptomics and proteomics, we kept limitations to a minimum. Thus, our search was not confined to the gene 3′-UTR, but included the coding sequence and the 5′-UTR, both recently demonstrated as involved in miRNA-mRNA binding (5, 6, 22–24, 27). It was found that miR-210 seed-pairing sequences were more frequent among the putative targets compared with controls. Furthermore, we did not impose any conservation bias. Indeed, whereas many miRNAs are evolutionarily conserved, others are species-specific, including human miRNAs not conserved in chimpanzee (43), consistent with miRNA roles ranging from generating cellular to organismal diversity. Thus, it is not surprising that a certain gene may be targeted by miR-210 in humans but not in other vertebrates.

Fig. 5 summarizes the identified cellular processes affected by miR-210, both directly and indirectly, and how some of the new validated targets could be involved in the direct regulation of these events. CLASP2, MDGA1, NCAM1, and P4HB are involved in cellular migration and adhesion and could, at least in part, mediate miR-210 regulation of tubulogenesis and migration observed in hypoxic ECs (13). Furthermore, it has been shown that, following hindlimb ischemia, PTPN1 negatively regulates VEGFR2 and modulates cell-cell adhesions (44). Moreover, in keeping with our findings, previous studies found that CPEB2 and the ternary complex factor ELK-3 proteins are modulated by hypoxia (45, 46). Many identified targets, such as the tumor suppressor APC, ACVR1B, CDK10, SERTAD2, as much the previously identified E2F3 and MNT (14, 18), are involved in cell cycle regulation. These results are in keeping with miR-210 overexpression observed in breast cancer, where miR-210 levels correlate with aggressiveness of lymph node-negative, estrogen receptor-positive human breast cancer (18, 47, 48). Indeed, it has been shown that CDK10 expression is reduced in breast cancer (49), suggesting a role for miR-210 in this regulation. Furthermore, miR-210 is overexpressed in many other solid cancer types (50), it was detected in serum of patients affected by diffuse large B-cell lymphoma (51) and by breast, colon, lung, and ovarian cancer (52). It was also found that elevated expression of miR-210 in melanoma and in pancreatic tumors associates with poorer survival (18, 53). Another novel target is GPD1L, a gene recently linked to sudden infant cardiac death and the Brugada syndrome, a disease that induces syncpe, ventricular arrhythmias, and sudden death (54–56).
Interestingly, acute myocardial ischemia involving the right-ventricular outflow tract is known to induce Brugada-like electrophysiological and electrocardiogram alterations (57–59). Whereas miR-210 and GPD1L levels were not measured in these pathological conditions, it is worth noting that hypoxia is both a crucial component of ischemia and a potent miR-210 inducer (12). To the best of our knowledge, to date a single report shows that miRNAs could target non-coding RNAs (27). Thus, an unexpected target is Xist (60), a long non-protein coding RNA involved in mammalian X-chromosome inactivation during early female embryogenesis. Although Xist is confined to inactivated X-chromosomes (57), recent data demonstrate that miRNAs can be imported to the nucleus (61). In keeping with our data, Xist is down-modulated by ischemia (62), and recently was demonstrated an inappropriate Xist expression/localization on active X-chromosomes in breast cancer (63). Given high miR-210 levels observed in breast cancer (47, 48), one can hypothesize that miR-210 may play a role in this deregulation. Furthermore, miR-210 is overexpressed in breast cancer (47, 48), one can hypothesize that miR-210 may also serve as a model that can be applied for the functional dissection of additional miRNAs.

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