Highly expressed miR-375 is not an intracellular oncogene in Merkel cell polyomavirus-associated Merkel cell carcinoma

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Abstract

Purpose. miR-375 is a highly abundant miRNAs in Merkel cell carcinoma. miR-375 may act as a tumor suppressor or oncogene depending on the cell context. While miR-375 is present as circulating free in the serum of patients with advanced MCC and thus serves as surrogate marker for tumor burden, its function within MCC has not been established.

Methods. miR-375 knockdown was performed using miR-375 antagomiRs via lipofectamine transfection or nucleofection in classical MCC cell lines WaGa and PeTa. Viability and both changes in growth characteristics as well as morphology were determined. Genes targeted by miR-375 were predicted using ENCORI; based on these miR-375 regulated signaling pathways were determined by genes ontology (GO) and gene set enrichment analysis (GSEA). Expression of these genes was analyzed by multiplexed RT-qPCR to check the effect of miR-375 knockdown on these signaling pathways.

Results. Complete knockdown of miR-375 expression by antagomiRs was only achieved by using nucleofection. This knockdown did not affect cell growth pattern, morphology, or proliferative capacity. miR-375 predicted target genes GO analysis revealed that Hippo signaling and focal adhesion-related genes were likely to be regulated by miR-375. GSEA of gene expression data of MCC cell lines further strengthened the regulation of Focal adhesion-related genes by miR-375. However, gene expression analysis revealed that miR-375 knockdown had only a limited effect on expression of these pathways related genes.

Conclusions. Complete miR-375 knockdown did neither change cell viability, morphology, nor oncogenic signaling pathways; these observations render miR-375 unlikely as intracellular oncogene in MCC cells.

Keywords: miR-375, antagomir, Merkel cell carcinoma, Hippo signaling, focal adhesion

Introduction

Merkel cell carcinoma (MCC) is an aggressive skin cancer. Risk factors for MCC include advanced age, ultraviolet (UV) light exposure and immune suppression [1]. Its pathogenesis is associated genomic integration of the Merkel cell polyomavirus (MCPyV) or UV-exposure caused genetic mutations. However, the specific molecular alterations caused by either MCPyV or UV-mutations are just starting to emerge [2,3].

microRNAs (miRNAs) are small, ~21nt single stranded RNAs, which post-transcriptionally regulate the stability and translation of genes mainly by binding to the 3' UTR of mRNAs [4,5]. Each miRNA can bind a specific set of genes, which are referred to as its target genes. Dysregulation of miRNAs has been reported in almost all types of human cancer [4,6]. miRNA expression profiling in MCCs revealed miR-375 as one of most abundant miRNAs in classical MCC cell lines and tumor tissues [7-10]. Physiologically, miR-375 acts as a pancreatic-islet miRNA essential for β-cell formation and regulation of insulin secretion [11,12]. Divergent miR-375 expression was described for multiple cancer types, *e.g.*, reduced expression in gastric [13,14], pancreatic [15], colon [16,17] and liver cancer [18]; high expression in medullary thyroid carcinoma [19], prostate cancer [20] and MCC [7-10]. Therefore, miR-375 was assumed as an oncogenic miRNA in the latter group.

However, when miR-375 function has been studied in MCC by different groups, results were inconsistent. Abraham *et al*. reported that miR-375 was involved in neuroendocrine differentiation and miR-375 knockdown in classical MCC cell lines (MKL-1 and MS-1) did not alter their growth properties [7]. Whereas, Kumar *et al*. reported that miR-375 inhibition in WaGa and MKL-1 cells reduced cell growth and induced apoptosis by targeting lactate dehydrogenase b (LDHB) [21]. To better understand this the functional role of miR-375 in MCC, we established a highly efficient way for miR-375 knockdown in classical MCC cell line and scrutinized the effect with respect to intracellular signaling, phenotype and growth characteristics.

Results

Effective knockdown of miR-375 by nuclear transfection

To explore the function of miRNAs it is essential to achieve largely complete knockdown. To achieve a highly effective knockdown in classical MCC cell lines is not trivial. Thus, tested different transfection conditions, i.e., lipofectamine and nucleofection, in the two classical MCC cell lines WaGa and PeTa using miR-375 antagomiRs.

Transfection miR-375 antagomiRs by lipofectamine reduced miR-375 expression in a dose dependent manner, but was not sufficient for a complete knockdown of the highly expressed miRNA-375 (**Fig. 1A, B**). Next, we performed nucleofection and optimized the transfection conditions. Program D23 with 25nM miR375 antagomiRs was determined as the optimal protocol for knockdown, which rendered miR-375 expression not detectable in both WaGa and PeTa cells (**Fig. 1C, D**). All further experiments were carried out using these conditions.

miR-375 knockdown does neither impact morphology nor proliferative capacity of MCC cells

We scrutinized the phenotypic alteration in WaGa and PeTa cells after complete miR-375 knockdown by nucleofection. Notably, the harsh nucleofection conditions required for complete miR-375 knockdown killed half of the cells which were removed before subsequent analyses (**Suppl. Figure S1**). Even the complete miR-375 knockdown did not alter cell morphology as cells still showed a neuroendocrine growth pattern as loose spheroids or single cell suspension, which was identical to the growth pattern in cells transfected with unspecific control antagomiRs (**Fig. 2A, C**). Similarly, the proliferative capacity was also not affected by miR-375 knockdown (**Fig. 2B, D**).

miR-375 target genes are involved in Hippo and EMT signaling pathways

To further investigate the role of miR-375 in MCCs, we predicted target genes of this miRNA using the miRNA target prediction tool ENCORI. This tool has the advantage that the results can be filtered for experimentally validated target genes. Nevertheless, more than 3000 target genes were predicted, thus, top ranking 500 genes was selected for further analysis (**Suppl. Table 1**). Gene Ontology (GO) analysis shows that miR-375 target genes contribute to several signaling pathways, including Golgi transport, cell junction assembly, Hippo signaling and neuron differentiation (**Fig. 3A**). To test the relevance of these predictions in MCC, we reanalyzed previously published transcriptome microarray data of MCC cell lines [22]. Of this data set, four MCC cell lines were selected according to their miR-375 expression level: WaGa and MKL-1 with high, MCC13 and MCC26 with low miR-375 expression [10]. Gene Set Enrichment Analysis (GSEA) evidenced that particularly genes related in focal adhesion signaling pathway were lower expressed in cell lines with high miR-375 expression (**Fig. 3B)**. Moreover, focal adhesion signaling pathways included most of the experimentally confirmed miR-375 target genes; this notion applies also for miR-375 target genes related to the Hippo signaling pathway. Both pathways regulate epithelial to mesenchymal transition (EMT) [23] (**Fig. 3C)**.

Hippo and EMT signaling pathways related genes are only marginally altered by miR-375 knockdown

Our results suggest that miR-375 may regulate Hippo and EMT-related signaling pathways in MCC cells, which we further scrutinized in the miR-375 knockdown experiments. For this, qRT-PCR based gene expression arrays in MCC cells with or without miR-375 knockdown were performed. miR-375 knockdown resulted in an upregulated expression of eleven genes (11/84, 13.1%) and a downregulation of four genes (4/84, 4.8%) related to the Hippo signaling pathway; for EMT-related pathway related genes , eleven (11/84, 13.1%) were

upregulated and three (3/84, 3.5%) downregulated (**Fig. 4**). However, the changes in gene expression did not reach statistically relevance as the less than +/- two-fold changes in expression. (**Fig. 4, Suppl. Table 2**).

Discussion

Despite the fact that miR-375 is highly expressed in classical MCC cell lines and MCC tumors, its function in MCC is not clear. To study its function in detail, we performed a series of knockdown experiments using specific antagomiRs. Surprisingly, even complete knockdown of miR-375 expression beyond detection level did affect neither proliferation, growth pattern nor cell morphology. Similarly, the impact of miR-375 knockdown on expression of Hippo and EMT signaling pathway related genes, i.e., pathways predicted to be regulated by miR-375, was only marginal. Taking these results together with our previous observations that miR-375 is present in MCC cell line conditioned medium, in sera of preclinical xenotransplantation animal models and in sera of MCC patients [10] suggest, miR-375 may rather serve intercellular than intracellular signaling in MCC. Indeed, miR-375 was recently characterized as an exosomal shuttle miRNA [24,25].

In previous reports, miR-375 knockdown or inhibition in MCC cell lines resulted in different consequences. miR-375 knockdown using antagomiRs did not alter growth properties [7], whereas inhibition of miR-375 using a miRNA sponge suppressed cell growth and induced cell death via downregulation of *LDHB* gene expression [21]. Notably, ectopic expression of miR-375 in variant MCC cell lines decreased their cell viability and migratory potential [7,21]. These conflicting results of may be due to differences in specificity or effectivity of the used methods.

Knockdown of abundant miRNAs can be challenging [26]. In our study, we introduced the respective antagomiRs with two different transfection conditions, which revealed that nuclear transfection was much more efficient and only this method succeeded in a complete

knockdown. Besides miRNA antagomiRs, miRNA sponge is another powerful tool to inhibit miRNA function. Notably, a miRNA sponge was used by Kumar *et al.* to inhibit miR-375 function in MCC cells [21].

Testing for expression of Hippo and EMT related signaling related genes after of miR-375 knockdown, we observed expression changes of only a few genes, however, none of them was bigger than two-fold changes. The gap between the predicted and functional observed effects of miR-375 knockdown are not entirely unexpected. Several reports provide a possible explanation: long non-coding RNAs, such as TINCR, HNGA1 and CircFAT1 act as a miR-375 sponge [27-29]. Thus, the highly expressed miR-375 may be not functionally active in MCC cells.

In summary, we demonstrate that even complete knockdown of the highly abundant miR-375 in classical MCC cells lines, has no relevant impact on cell viability, morphology or oncogenic signaling pathways; these observations renders miR-375 unlikely to function as intracellular oncogene in MCC cells.

Materials and Methods

Cell culture

The classical, MCPyV positive MCC cell lines WaGa and PeTa were maintained in RPMI-1640 (PAN Biotech, Aidenbach, Germany) supplemented with 10% fetal bovine serum (Sigma-Aldrich, Munich, Germany) and 1% penicillin/streptomycin (Biochrome, Berlin, Germany) as previously described [30].

miR-375 knockdown

For miR-375 knockdown specific miR-375 inhibitors (Assay ID: MH10327, Catalog: 4464084, Thermo Fisher Scientific, Frankfurt, Germany) or respective controls (Catalog: 4464076, Thermo Fisher Scientific) were used.

For transfection of MCC cells two methods were compared. Lipofectamine 3000 reagent (Thermo Fisher Scientific) was used according to the instructions of manufacturer, i.e., $2x10^6$ cells were seeded into 6-well-plate 24 hours before transfection with 100nM or 250nM of antagomiRs. Alternatively, the Nucleofector™ 2b Device (Lonza, Basel, Switzerland) with the Cell Line Nucleofector® Kit V (Lonza) were used. D-23 was established as the optional program to transfect MCC cells (https://bioscience.lonza.com/lonza_bs/CH/en/nucleofector-technology). 100µL buffer V were mixed with 10µL miRNA antagomiRs (25nM) and $2x10^6$ MCC cells before being transferred into electroporation cuvette. After the pulse, cells were immediately transferred into 6-well-plates containing pre-warmed culture media.

Real-time reverse transcription-PCR for miRNA

Applied Biosystems TaqMan MicroRNA Assays (Thermo Fisher Scientific) were performed according to the manufacturer's instructions. Pre-designed TaqMan microRNA assays for miR-375 (ID000564) were used. The quantification cycle threshold (Cq) values of miR-375 were normalized to the small nucleolar RNA RNU6B (ID001093) and relative expression to the respective comparator was calculated using the $2^{-\Delta\Delta Cq}$ method.

Prediction of miR-375 targets genes, Gene Ontology (GO) analysis and Gene Set Enrichment Analysis (GSEA)

The Encyclopedia of RNA Interactomes (ENCORI, http://starbase.sysu.edu.cn/index.php) provides miRNA-target gene interactions, which are based on miRNA target prediction programs, *i.e.*, TargetScan, miRanda, microT, PITA, miRmap, and PicTar. All miRNA target predictions are supported by published Argonaute-crosslinking and immunoprecipitation (AGO-CLIP) data [31]. Predicted target genes are ranked based on the predicted efficacy of targeting as calculated using cumulative weighted context++ scores of the sites and related

AGO-CLIP score (clipExpNum, Suppl. Table 2) [31,32]. The top 500 highest ranking predicted target genes were selected for following analysis.

Metascape (http://metascape.org) was applied for GO analysis [33]. Metascape incorporates a core set of default ontologies including GO processes, KEGG pathways, Reactome gene sets, canonical pathways, and CORUM complexes for enrichment analysis.

GSEA the desktop application from the MSigDB of Broad Institute (Cambridge, Massachusetts, United States.) was used (http://software.broadinstitute.org/gsea/msigdb/index.jsp)[34].

Pathway finder gene expression arrays

The RT2 Profiler PCR arrays (SABioscience via Qiagen, Hilden, Germany) for epithelial to mesenchymal transition (EMT) (PAHS-090Z) and Hippo signaling (PAHS-172Z) were performed according to manufacturer's instructions. Total RNA was isolated from MCC cell lines three days after nucleofection with miR-375 inhibitors or the respective control. 200ng of total RNA was transcribed into cDNA using RT2 first strand kit (Qiagen). Relative gene expression was determined using the RT^2 Profiler PCR Array software from Qiagen (https://dataanalysis.qiagen.com/pcr/arrayanalysis.php).

Statistical Analysis

Statistical analyses were performed using GraphPad Prism 8.0 Software (GraphPad Software Inc., San Diego, CA, USA). Experiments containing more than two groups were analyzed using Kruskal–Wallis test, an unpaired nonparametric ANOVA. R studio (version 3.6.0) was applied in statistical analysis as indicated: ggpubr R package (version 3.2.0) for dot plot of gene expression in EMT and Hippo signaling. A p-value smaller than 0.05 was considered significant; the respective p-values are indicated in the figures as follows: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

Conclusions

Complete knockdown of highly expressed miR-375 achieved by miR-375 antagomirs with nucleofection did not cause obvious effects on cell viability, morphology or oncogenic Hippo and EMT signaling pathways; these observations render miR-375 unlikely to function as intracellular oncogene in MCC cells.

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Author Contributions

Conceptualization, J.C.B.; Methodology, F.K., D.S. and A.Z. ; Software, K.F.; Validation, K.F. and K.H.; Formal Analysis, K.F. and K.H.; Investigation, K.F. and A.Z.; Resources, A.Z. and J.C.B.; Data Curation, K.F. and K.H.; Writing, K.F., K.H., and J.C.B.; Visualization, K.F.; Supervision, J.C.B. and D.S.; Project Administration, J.C.B.; Funding Acquisition, J.C.B.

Conflicts of interest

J.C. Becker is receiving speaker's bureau honoraria from Amgen, Pfizer, MerckSerono and Sanofi, and is a paid consultant/advisory board member for eTheRNA, MerckSerono, Pfizer, 4SC and Sanofi. His group receives research grants from Bristol-Myers Squibb, Merck Serono, and Alcedis. None of the activities is related to the submitted work. None of other authors indicated any potential conflicts of interest.

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Figure legends

Figure 1: miR-375 is knockdown in classical MCC cell lines

Relative miR-375 expression was determined in triplicates by RT-qPCR in WaGa (**A, C**) and PeTa (**B, D**) cells transfected with miR-375 antagomiRs or negative control using lipofectamine (**A, B**) or nucleofection (**C, D**). Ct values were normalized to U6 and calibrated to the untreated WaGa cells. All experiments were independently repeated three times. Error bars represent SD, *** indicates $p<0.001$.

Figure 2: miR-375 knockdown does not alter cell morphology and viability of MCC cells

Morphology of WaGa (**A**) and PeTa (**B**) cells 72 hours after nucleofection. Relative cell numbers of WaGa (**B**) and PeTa (**D**) cells seeded at 10^5 cells per well 48 hours after nucleofection with miR-375 antagomiRs or negative control. Scale bar represents 50µm. All experiments were independently repeated three times, error bars represent SD.

Figure 3: miR-375 target genes are involved in Hippo and EMT signaling pathways

A: Gene ontology analysis was performed in Metascape using the top 500 predicted miR-375 target genes. **B**: Gene set enrichment analysis was performed using previously published transcriptome microarray data of MCC cell lines with high (WaGa, MKL1) and low (MCC13, MCC26) miR-375 expression. Enrichment plot of kegg_focal_adhesion signaling pathway is depicted. **C**: miR-375 target genes involved in Hippo and focal adhesion signaling pathway-

Figure 4: Hippo and EMT signaling pathway related genes altered by miR-375 knockdown

Expression of genes related to Hippo (**A**) and EMT (**B**) signaling pathways were determined by multiplexed RT-qPCR expression array in WaGa cells transfected with anta-375 or anta-NC, normalized to the average of Ct values of housekeeping genes (GAPDH, HPRT and RPLP0) and calculated to the ΔCt of WaGa cells transfected with anta-NC.

Suppl. Figure S1: Nucleofection is associated with cell death in MCC cells.

Viable cells of WaGa (**A**)and PeTa (**B**) after nucleofection were determined via Trypan blue staining, percentages of viable cells were depicted.

Figure 1

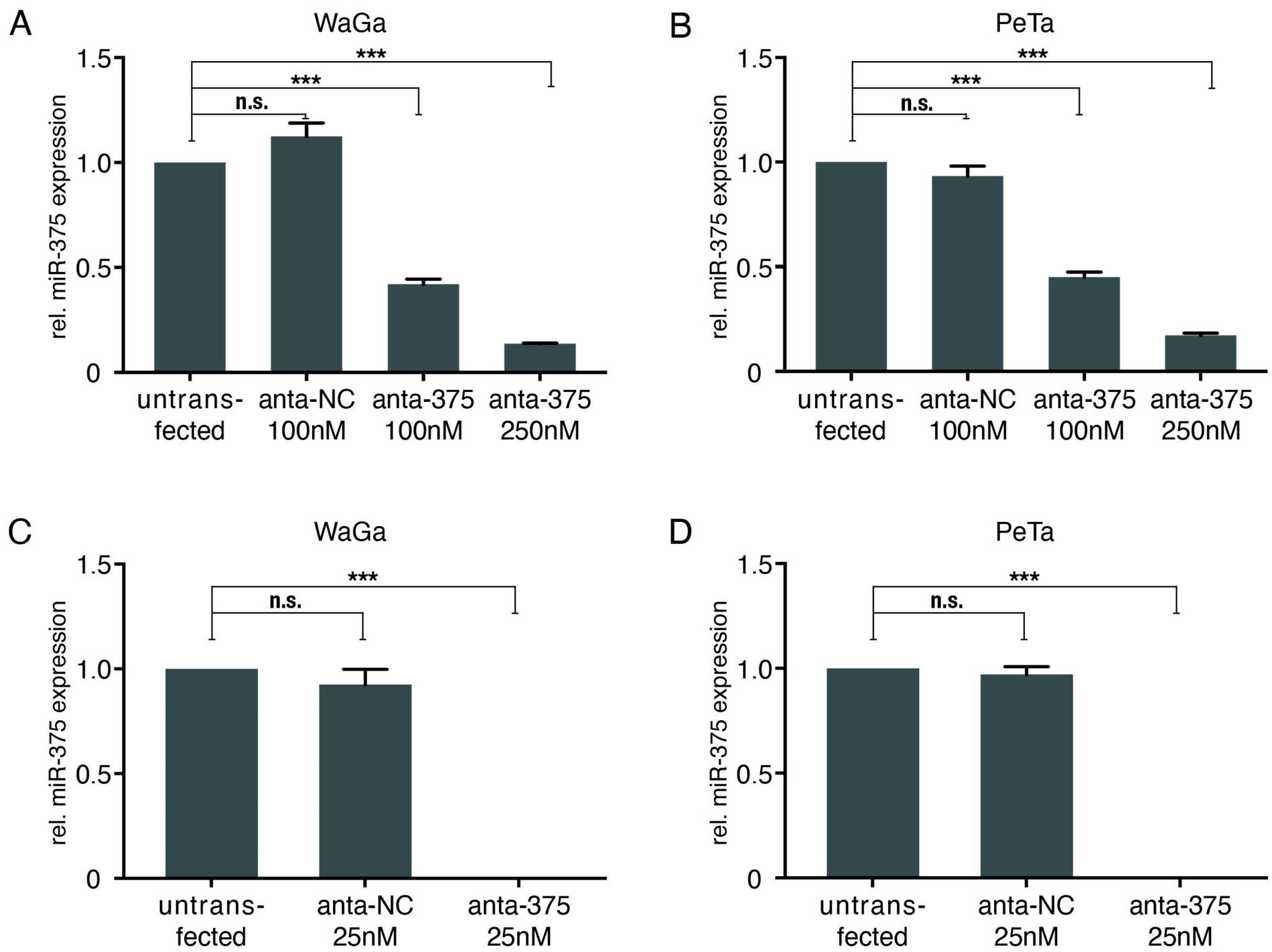
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Figure 2

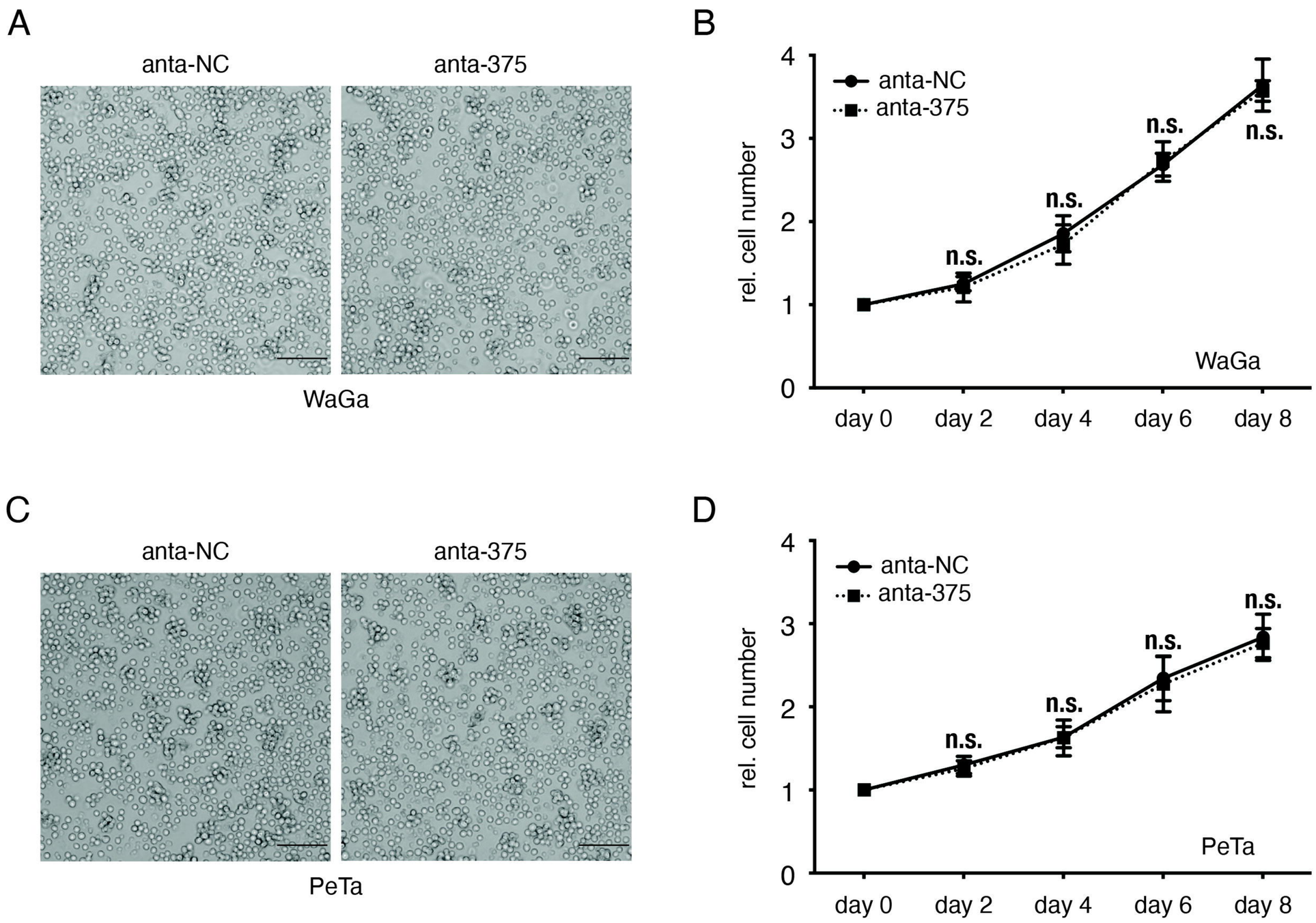
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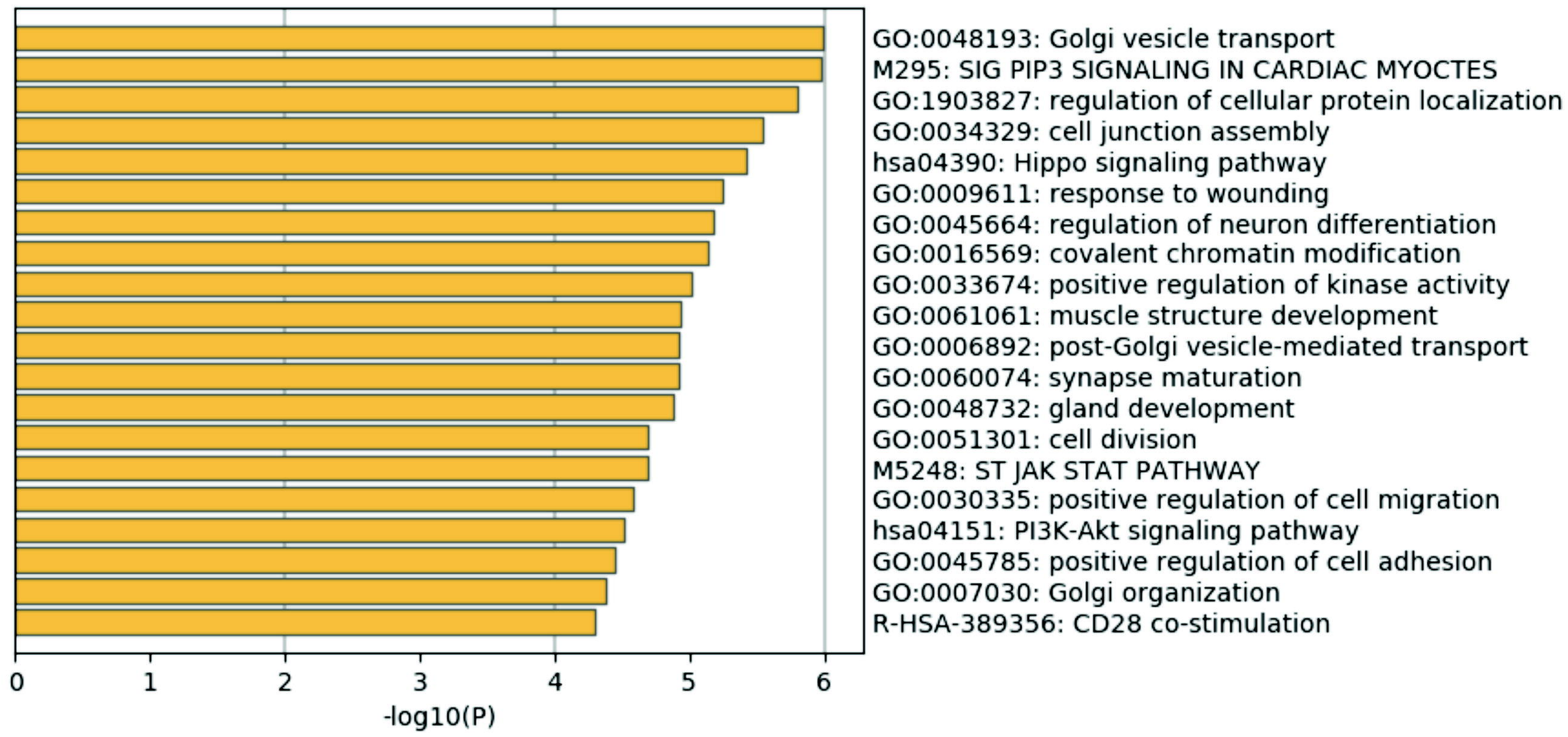
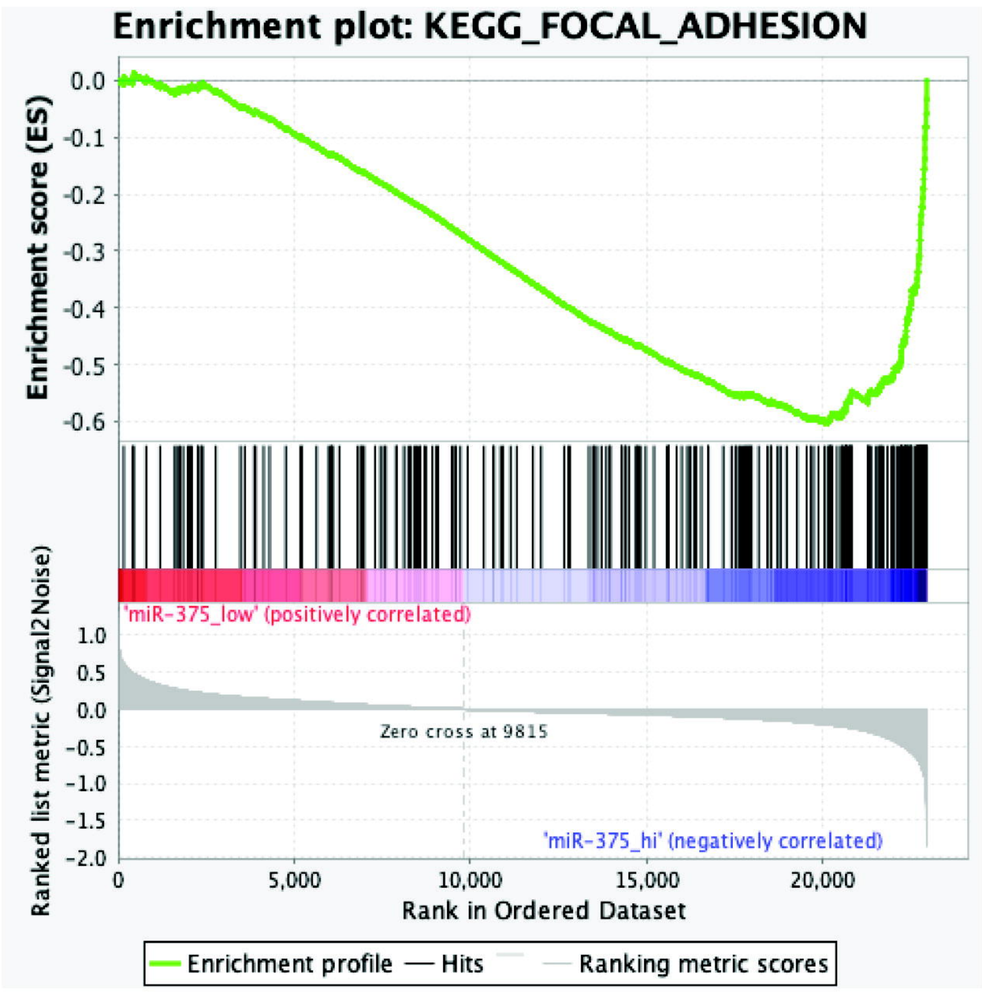
Figure 3

A


GO:0048193: Golgi vesicle transport
M295: SIG PIP3 SIGNALING IN CARDIAC MYOCTES
GO:1903827: regulation of cellular protein localization
GO:0034329: cell junction assembly
hsa04390: Hippo signaling pathway
GO:0009611: response to wounding
GO:0045664: regulation of neuron differentiation
GO:0016569: covalent chromatin modification
GO:0033674: positive regulation of kinase activity
GO:0061061: muscle structure development
GO:0006892: post-Golgi vesicle-mediated transport
GO:0060074: synapse maturation
GO:0048732: gland development
GO:0051301: cell division
M5248: ST JAK STAT PATHWAY
GO:0030335: positive regulation of cell migration
hsa04151: PI3K-Akt signaling pathway
GO:0045785: positive regulation of cell adhesion
GO:0007030: Golgi organization
R-HSA-389356: CD28 co-stimulation
0
1
2
3
4
5
6
-log10(P)

B


Enrichment plot: KEGG_FOCAL_ADHESION
Enrichment score (ES)
0.0
-0.1
-0.2
-0.3
-0.4
-0.5
-0.6
'miR-375_low' (positively correlated)
Ranked list metric (Signal2Noise)
1.0
0.5
0.0
-0.5
-1.0
-1.5
-2.0
Zero cross at 9815
'miR-375_hi' (negatively correlated)
0
5,000
10,000
15,000
20,000
Rank in Ordered Dataset
Enrichment profile
Hits
Ranking metric scores

C

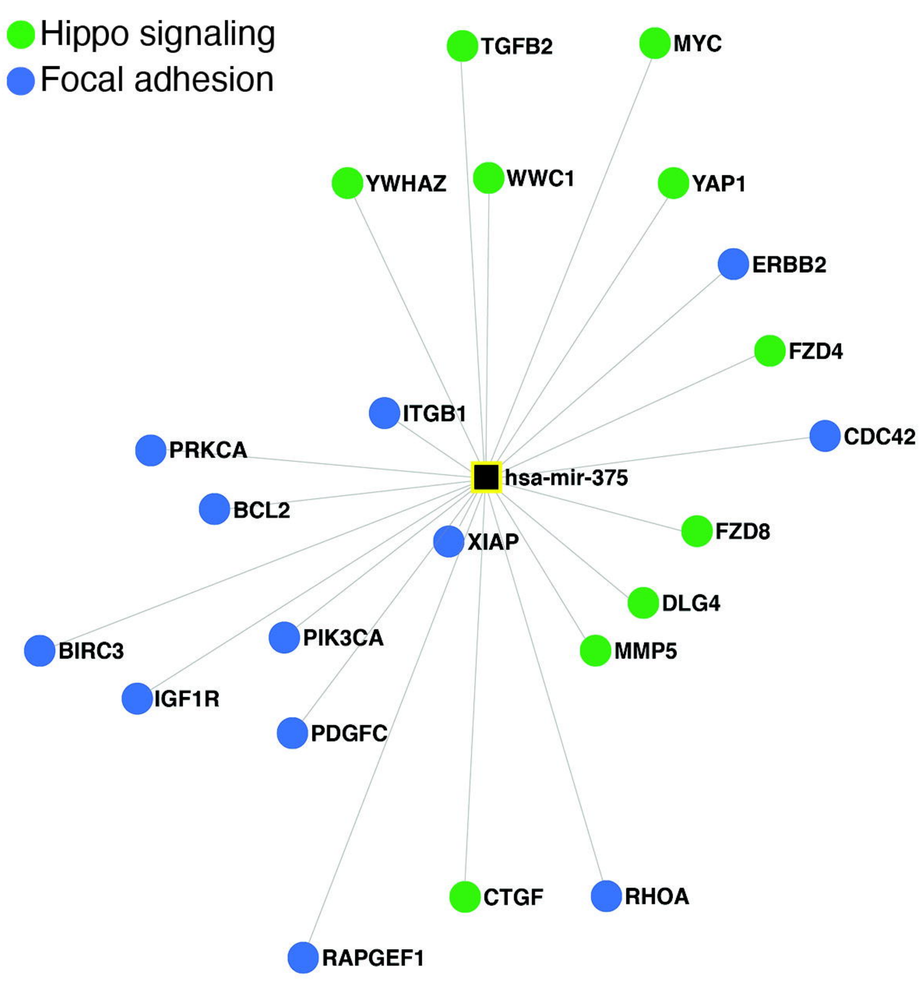
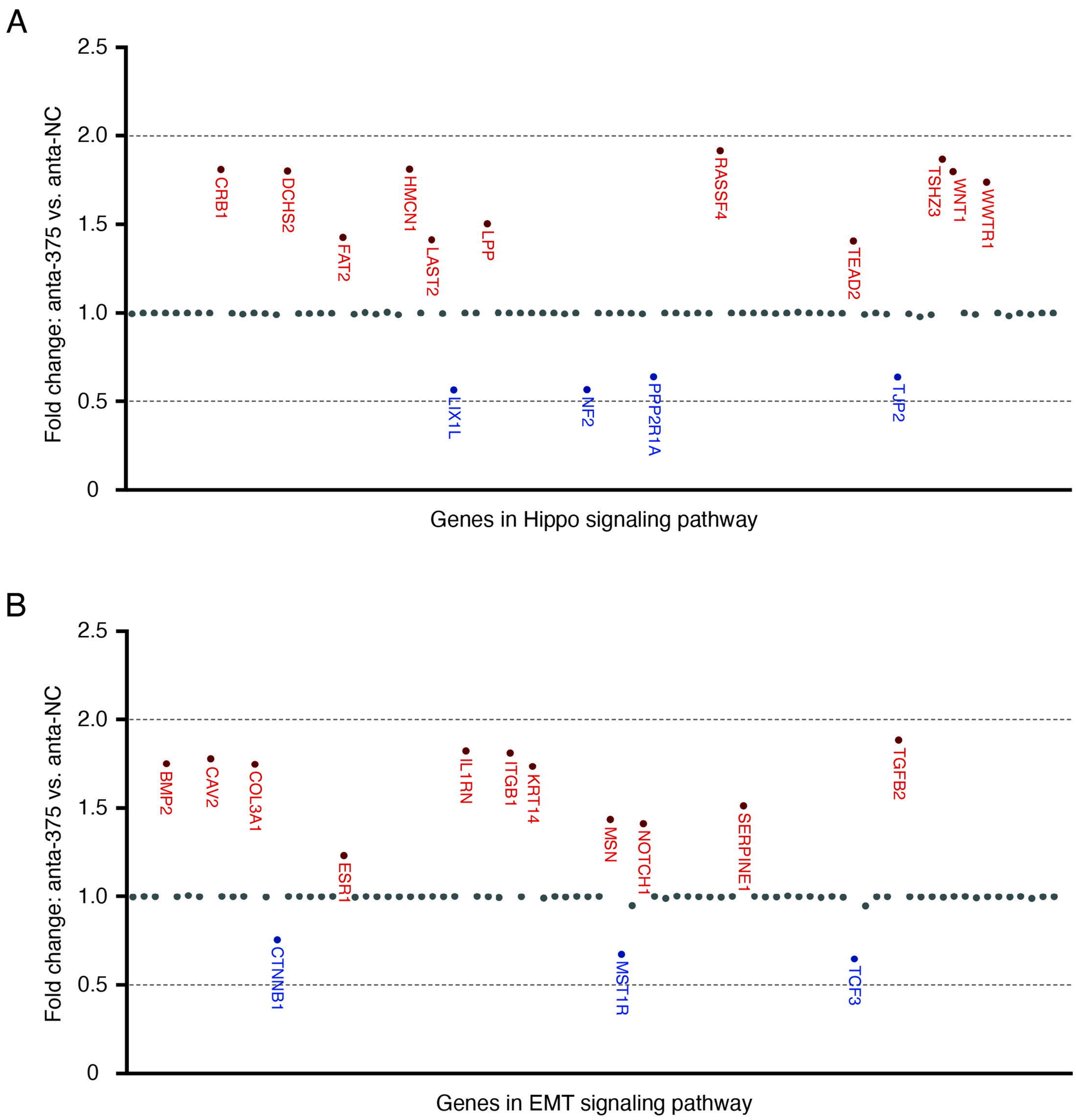
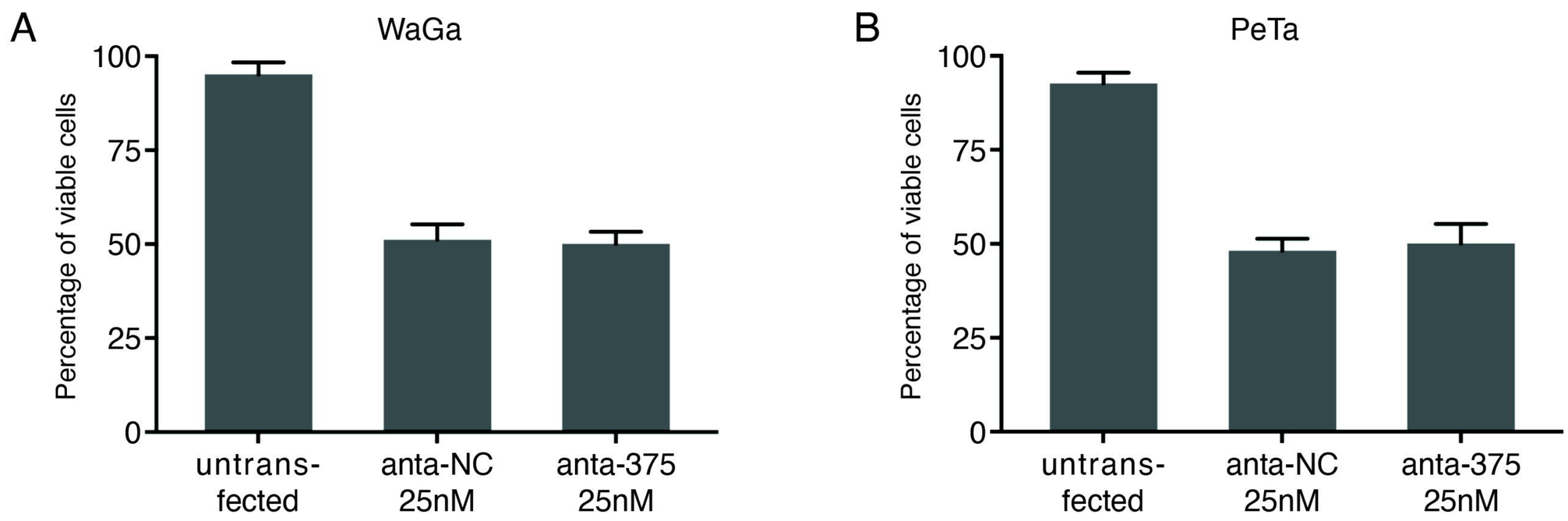

Hippo signaling
Focal adhesion
TGFB2
MYC
YWHAZ
WWC1
YAP1
ERBB2
FZD4
ITGB1
CDC42
PRKCA
hsa-mir-375
BCL2
FZD8
XIAP
DLG4
BIRC3
PIK3CA
MMP5
IGF1R
PDGFC
CTGF
RHOA
RAPGEF1

Figure 4


A
2.5
2.0
1.5
1.0
0.5
0
Fold change: anta-375 vs. anta-NC
CRB1
DCHS2
FAT2
HMCN1
LAST2
LPP
RASSF4
TEAD2
TSHZ3
WNT1
WWTR1
LIX1L
NF2
PPP2R1A
TJP2
Genes in Hippo signaling pathway
B
2.5
2.0
1.5
1.0
0.5
0
Fold change: anta-375 vs. anta-NC
BMP2
CAV2
COL3A1
ESR1
IL1RN
ITGB1
KRT14
MSN
NOTCH1
SERPINE1
TGFB2
CTNNB1
MST1R
TCF3
Genes in EMT signaling pathway

Suppl. Figure S1