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Identification of miRNAs and associated pathways regulated by Leukemia Inhibitory Factor in trophoblastic cell lines

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Abstract

Introduction: Leukemia Inhibitory Factor (LIF) regulates behavior of trophoblast cells and their interaction with immune and endothelial cells. *In vitro*, trophoblast cell response to LIF may vary depending on the cell model. Reported differences in the miRNA profile of trophoblastic cells may be responsible for these observations. Therefore, miRNA expression was investigated in four trophoblastic cell lines under LIF stimulation followed by *in silico* analysis of altered miRNAs and their associated pathways.

Methods: Low density TaqMan miRNA assays were used to quantify levels of 762 mature miRNAs under LIF stimulation in three choriocarcinoma-derived (JEG3, ACH-3P and AC1-M59) and a trophoblast immortalized (HTR8/SVneo) cell lines. Targets and associated pathways of the differentially expressed miRNAs were inferred from the miRTarBase followed by a KEGG Pathway Enrichment Analysis. Expression of miR-21-3p was confirmed by qPCR.

Results: A similar number of miRNAs changed in each tested cell line upon LIF stimulation but low coincidence of individual miRNA species was observed and occurred more often between choriocarcinoma-derived cells. Altered miRNAs were categorized into pathways involved in human diseases, cellular processes and signal transduction. Six cascades were identified as significantly enriched, including JAK/STAT and TGFB-SMAD. miR-21-3p was upregulated in all cell lines but in HTR8/SVneo cells not significantly. STAT3 and SMAD7 were identified as potential miR-21 targets.

Conclusion: Dissimilar miRNA responses may be involved indifferences of LIF effects on trophoblastic cell lines.

Introduction

Leukemia inhibitory factor (LIF) is a pleiotropic cytokine known to be indispensable for human reproduction. LIF controls uterine receptivity and influences trophoblast behavior by promoting proliferation, invasion and differentiation, and its dysregulation is associated with infertility and poor pregnancy outcome (Reviewed in [1, 2]).

To investigate the molecular mechanisms involved in trophoblast response, several cell lines have been employed as models for primary cells [3-7]. Unlike primary cells, these models have the advantage to proliferate easily in culture while maintaining characteristics of desired trophoblast phenotypes. Placental researchers have employed these models for decades, but it is still discussed how their limitations may influence translation of results to the *in vivo* situation and which markers are required to consider them as suitable trophoblast models [8]. Secreted proteins (e.g. IGF2, CSH1), trophoblast lineage markers (e.g. KRT7, VE-Cadherin, HLA-G) and expression of homeobox and imprinting genes (e.g. H19, IGF2, CDKN1C) are among the markers used to characterize trophoblast phenotypes [8, 9]. Recently, specific mRNA and microRNA (miRNA) expression profiles have been added to that list [10-12].

Cumulating evidence demonstrates that physiological characteristics of trophoblast biology (immunity, invasion, proliferation among others) are tightly regulated by miRNAs [13-15]. We have previously reported differences in the miRNA expression profiles of four cell lines widely used as trophoblast models [10]: JEG-3, a human choriocarcinoma cell line preserving several trophoblast-like capacities; two hybrids of a JEG-3 mutant with human first and third trimester trophoblast cells respectively ACH-3P and AC1-M59 cells [3-5]; and the immortalized human first-trimester trophoblast cell line HTR-8/SV40 [6]. Unlike primary trophoblast cells and choriocarcinoma cell lines, HTR8/SV neo cells do not express the placenta-related

Chromosome 19 MicroRNA Cluster (C19MC), but retain expression of C14MC [10]. Likewise, these cell lines have differences in proliferation and invasion rates and respond dissimilarly under stimulation with LIF and other IL-6 family cytokines [16, 17].

In order to gain more insights into the trophoblast response to LIF, the four cell models were investigated to identify canonical and no-canonical miRNAs, their associated pathways and functions.

Materials and Methods

The Placenta Lab strictly applies quality management and is certified after DIN EN ISO 9001.

Cell lines

JEG-3 cell line was purchased from DSMZ (Braunschweig, Germany). ACH-3P and AC1-M59 cells were a kind gift from Prof. G Desoye (Graz, Austria). The immortalized cell line HTR-8/SV40 was kindly provided by Dr. Charles H. Graham, (Queen' University, Kingston, Canada).

Cell culture

Cells cultures were maintained under standard conditions (37ºC, 5% CO_2, humid atmosphere) in Ham's F-12 Nutrient Mixture with L-glutamine (GIBCO) or RPMI Medium (GIBCO; HTR-8 cells) supplemented with 10 % heat-inactivated fetal calf serum (FCS; GIBCO) and 1 % penicillin/streptomycin antibiotic solution (GIBCO). Cells were checked regularly for mycoplasma contamination and their identity was confirmed by Short Tandem Repeat (STR) genotyping compared to the databanks of the American Type Culture Collection (ATCC) and the Leibniz Institute DSMZ - German Collection of Microorganisms and Cell Cultures (DMSZ).

RNA isolation and miRNA TaqMan low density arrays

Cells were cultivated in 12-well plates and allowed to attach overnight. Subsequently, they were serum deprived for at least 2 h and then challenged 4 h with 10 ng/ml LIF (Millipore). Total RNA was isolated using the mirVana isolation kit (Life Technologies), according to the manufacturer's protocol. RNA purity was assessed by the ratio of spectrophotometric absorbance at 260 and 280 nm (A260/280nm)

using NanoDrop ND-1000 (NanoDrop Inc). Total RNA (100 ng) was reverse transcribed using the specific Megaplex RT kit (Life Technologies) followed by a pre-amplification step. Expression of 754 miRNAs was profiled by using the TaqMan® Array Human MicroRNA A+B Cards Set v3.0 (Life Technologies) on the 7900HT Sequence detection system. Experimental data were analyzed using the DataAssist v3.0 Software (Life Technologies) using the formula $2^{-\Delta\Delta Ct}$ with RNU48 and RNU44 as endogenous controls.

Prediction of targets and pathway analyses

Target genes of the deregulated miRNAs (|FC| ≥ 1.5) were obtained from the miRTarBase [18] (release 7.0) to get non-redundant lists of target proteins for each of the different conditions. In the next step, all 299 available regulatory human pathways of the Kyoto encyclopedia of genes and genomes (KEGG) database [19] were ranked individually for the investigated conditions, according to the amount of targeted proteins within each pathway.

To evaluate whether a given pathway was overrepresented in the list of differentially affected miRNA targets, the hypergeometric test [20] was used to calculate corresponding p-values for each pathway:

$$p - Value = \frac{R!n!(N-R)!(N-n)!}{N!} \sum_{i=r}^{\min(n,R)} \frac{1}{i!(R-i)!(n-i)!(N-R-n+i)i},$$

where *N* is the total number of miRNAs that have targets in any of the given pathways, *R* the number of miRNAs of interest (here deregulated miRNAs with an absolute fold change ≥ 1.5), *n* the total number of miRNAs with targets in the currently observed pathway and *r* the number of miRNAs of interest that have targets in the currently observed pathway.

After Benjamini-Hochberg false discovery rate (FDR) adjustment [21], only pathways with an enrichment p-value of ≤ 0.05 were considered statistically significant. Analysis of sub-pathways was performed by manually identifying key altered regions within the pathways reported as significant in the previous step.

Real-time quantitative RT-PCR

Expression levels of miR-21-3p were confirmed by individual TaqMan miRNA Assays according to the supplier's protocol. Reverse transcription was performed with miRNA specific stem-loop RT primer (Assay-ID 002438) using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems). qRT-PCR was done using the specific TaqMan Assay and TaqMan Universal PCR Master Mix no UNG. All reactions were run in duplicates including no-template controls in 96-well plates on a 7300 Real Time PCR System (Applied Biosystems). Fold changes were calculated using the formula $2^{-\Delta\Delta Ct}$ relative to non-stimulated cells with RNU48 as endogenous control.

Results

miRNA profile after LIF stimulation changes in a cell-specific manner

After 4 h of LIF treatment (10 ng/ml), 754 miRNAs were profiled and miRNAs with Ct ≤ 35 displaying more than 1.5-fold change were considered altered. In HTR8/SVneo cells, expression of 110 miRNAs wasinfluenced (39 up- and 71 down-regulated). In the choriocarcinoma-derived JEG3 cell line, 91 affected miRNAs were identified: 45 down- and 36 up-regulated. In the hybrid cell lines AC1-M59 and ACH-3P cells, a total of 88 (45 down- and 43 up-regulated) and 130 (45 down- and 85 up-regulated) were identified, respectively (Figure 1).

Upon LIF stimulation, no miRNA expression was altered in all four tested cell lines. ACH-3P and AC1-M59 cells had the most similar response with 25 altered miRNAs in common (14 up- and 11- down regulated), followed by pairs of JEG-3:ACH-3P and JEG-3:AC1-M59 cells, each with 17 shared altered miRNAs (Figure 1).

Gene oncology and KEGG pathway analysis of the affected miRNAs

Targets of the differentially expressed miRNAs were inferred from the miRTarBase release 7.0. All 299 available regulatory human pathways of the KEGG database were ranked individually according to the amount of targeted proteins after LIF stimulation within each pathway.

Only two pathways were identified in JEG3 as most likely to be affected after LIF treatment: Epithelial cell signaling in Helicobacter pylori infection hsa05120 and Adherens junction hsa04520. In ACH-3P, AC1-M59 and HTR8/SVneo cells,

respectively 17, 41 and 31 statistically significantly enriched pathways were identified (Figure 2A). Among them, a group of 13 pathways were found significantly enriched in at least two cell lines (Figure 2B).

Analysis of subpathway regions

To further establish conserved pathways activated by LIF in trophoblastic cell lines, local areas of high concentration of target genes were identified within high ranked pathways and designated as sub-pathways. Six sub-pathways were identified as influenced by LIF affected miRNAs: a) NFkB, b) TGFB-Smad, c) RAS-RAF-MEK-ERK, d) PI3K-Akt, e) NWASP-ARP2/3-(F-) actin and f) JAK/STAT (Figure 2A). For instance, in “Estrogen Signaling” pathway (hsa04915) identified in HTR8/SVneo cells, an enrichment of the sub-pathway RAS-RAF-MEK-ERK was evident (Figure 2C). Sixty percent of significantly enriched pathways contain at least one of these six sub-pathways (Figure 2A).

Conserved miR-21-3p response to LIF

HTR8/SVneo cells have significantly higher miR-21-3p basal expression levels compared to the other tested cell lines. After 4 h treatment with 10ng/ml LIF, miR-21-3p was up-regulated in all tested cell lines. This increase was higher in JEG3 cells (5.88-fold) than in the other cell lines: ACH-3P (1.79-fold), AC1-M59 (2.40-fold) and HTR8/SVneo (2.13-fold). Only in HTR8/SVneo cells, this increase was not significant (Figure 3A).

miRTar analysis predicted STAT3 in the JAK/STAT and SMAD7 in the TGFB-Smad subpathways as affected by the LIF-induced increase of miR21-3p (Figure 3B).

Discussion

Leukemia Inhibitory Factor (LIF) is one of the most important factors within the uterine environment. It is involved in fundamental processes for human reproduction including endometrium receptivity and blastocyst implantation [2]. LIF and its receptor LIFR are localized in the chorionic villi, decidua and extravillous trophoblast cells (EVTs) of first trimester and term placenta [22]. There, LIF exerts an important role in the regulation of EVT invasion and the communication of trophoblast with decidua and immune cells, which is required for an adequate spiral artery remodeling [23].

To circumvent the disadvantages of working with primary cells and still allowing the study of intracellular signals, several cell lines have been established to investigate functions on trophoblast cells. Some of the most widely used models have been derived from choriocarcinoma (e.g. JEG-3, ACH-3P and AC1-M59) or from primary cells immortalized with the gene encoding for simian virus 40 large T antigen (HTR8/SVneo) [3-6]. Due to the differences in their origin, these models exhibit unique expression patterns of genes, proteins [12, 24] and miRNAs [10]. These differences are reflected in their ability to proliferate, migrate and invade, thus, constituting an important aspect when selected as suitable models for trophoblast research.

To investigate canonical miRNAs altered by LIF in trophoblast cells, expression of 754 miRNAs was investigated in four cell lines. Comparing with each other, a similar number of altered miRNAs were found amongst the four cell lines. Remarkably, overlapping of specific miRNAs was low and occurred mostly between choriocarcinoma cell lines. In fact, ACH-3P and AC1-M59 had the most similar

response on LIF with 25 overlapping miRNA species, followed by JEG3/ACH-3P and JEG3/AC1-M59 each with 17 intersecting miRNAs. This may rely on the shared origin of JEG3, AC1-M59 and ACH-3P cells, but it also may be the result of miRNA redundancy in HTR8/SVneo cells to compensate lack of some placenta-related miRNAs (e.g. C19MC miRNAs). However, further investigation should confirm this assumption.

Since the number of samples was limited (n=3), identification of affected miRNAs was performed under a strict threshold of |fold change|≥1.5 and an expression of Ct ≤ 35. We consider that these strict selection criteria may be causes for the low coincidence of results among cell lines. However, we selected these conditions to identify the most relevant changes and to avoid false positive results.

For the biological interpretation of the array results, an *in silico* analysis was carried out. This strategy has been employed in other studies to assign functional meaning to regulation at mRNA level in trophoblast cells [11, 25]. In our study, upon LIF stimulation, targets or the differentially expressed miRNAs were firstly predicted by miRTarBase [18] and then mapped to KEGG pathways. These pathways were then ranked based on the hit numbers and using the hypergeometric test to identify significantly altered pathways . Once more, the resulting pathways diverge among cell lines. Despite the similarities observed in single miRNA species, analysis of ACH-3P and AC1-M59 cell lines resulted in dissimilar enriched pathways. Likewise, while in JEG3 only two pathways were significantly altered upon LIF stimulation, in AC1-M59 41 affected pathways and HTR8/SVneo cells 31 pathways were identified.

Some of the enriched pathways belong to the KEGG DISEASE and KEGG DRUG databases [19] and thus, they may not necessarily have been described in the context of trophoblast behavior or reproduction. For instance, “Estrogen signaling pathway has04915” and “JAK-STAT signaling hsa04630” pathways significantly

enriched in HTR8/SVneo cells have been respectively identified in endometrium during the implantation period [26] and already recognized as a pivotal cascade for signaling of IL-6 cytokines in trophoblast cells [27]. On the other hand, “Alcoholism hsa05034” with the lowest p-value has never been related to trophoblast behavior, which makes it difficult to extrapolate results.

Recently, analysis of local regions or sub-pathways has been proposed to improve interpretation of biological phenomena associated to enriched KEGG pathways [28]. Using a similar method, mapped regions of high enrichment were identified manually in the pathways of the previous step. E.g. in the “Estrogen signaling pathway has04915” reported in LIF treated HTR8/SVneo cells, the sub-pathway “RAS-RAF-MEK-ERK1/2” was enriched. Using the same method, six sub-pathways were identified: NFkB, TGFB-SMAD, RAS-RAF-MEK-ERK1/2, PI3K-Akt, NWASP-ARP2/3-(f)-actin and JAK-STAT. Because most of the enriched pathways can be organized within these sub-pathways, it can be suggested that they may be major cascades involved in the LIF response of trophoblast cells.

Likewise, miR-21-3p was identified as one of the main important miRNAs in response to LIF. After being identified on the low-density array data, miR-21-3p overexpression was confirmed by qRT-PCR in all but significantly only in the choriocarcinoma-derived cell lines. Previously, we have reported upregulation of the miR-21 guide strand (miR-21-5p) in JEG-3 cells stimulated with LIF [29]. miR-21-5p correlates positively with the ability of trophoblastic cells to invade and proliferate, but again targets and associated pathways differ between JEG-3 and HTR8/SVneo cells [13]. Although the specific function of the miR-21-3p strand remains unclear, the common response in the analyzed cell lines highlights the importance of miR-21 in response to LIF and in controlling major trophoblast functions.

STAT3 in the “JAK-STAT” and SMAD7 in the “TGFB-SMAD” subpathway were identified as putative targets of miR-21-3p. Both cascades are pivotal for regulation of trophoblast behavior. While STAT3 is recognized as an inductor of cell growth and invasion [30], SMAD7 abrogates cell proliferation [31]. HTR8/SVneo and choriocarcinoma cell lines like JEG-3 also differ in the expression of several proteins of the JAK/STAT cascade, including STAT3 isoforms, alpha and beta [32], STAT5 [32] and PIAS1/3 [33]. Likewise, lower levels of SMAD2 and SMAD4, as well as lack of SMAD3 expression has been reported in JEG-3 compared to HTR8/SVneo cells [34].

Previously, we demonstrated that also cellular behavior upon LIF treatment differs among trophoblastic cell lines. For instance, invasion is induced by LIF in JEG-3 and ACH-3P but not in AC1-M59 or HTR8/SVneo cells [17, 35]. Now, we have demonstrated that these dissimilar LIF responses of trophoblastic cells may be influenced by the cell-specific set of altered miRNAs. However, these alterations converged in six major conserved pathways demonstrating the redundancy of miRNA species and highlighting the importance of further studying these pathways. Finally, increased miR-21 expression was identified as a conserved LIF response in different trophoblastic cells, whose predicted targets are relevant for the establishment and maintenance of pregnancy.

This study demonstrates that LIF induces responses in miRNA expression in trophoblastic cells and reveals further differences in the behavior of the most frequently used trophoblast model cell lines.

Acknowledgement

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Legends to Table and Figures

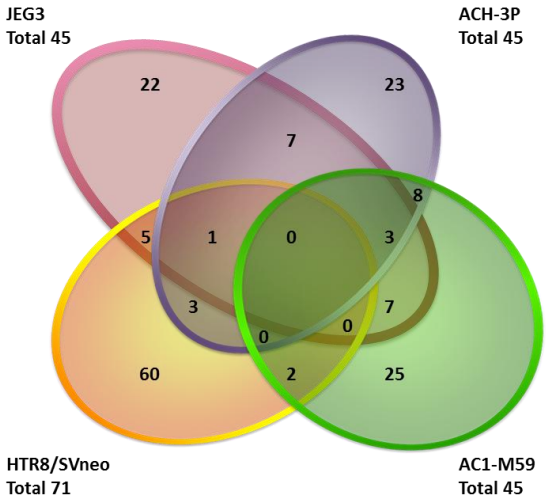
Figure 1. miRNAs in trophoblastic cells regulated upon LIF treatment. Venn diagrams summarizing altered miRNAs (mean fold-change ≥ 1.5; n=3) in cells treated with LIF (10 ng/ml; 4h) compared to controls. miRNAs in boxes were altered in at least two cell lines. miRNA species are reported as hsa-miR-Number-AssayID.

Figure 2. Signal pathway analyses of the target genes of LIF-altered miRNAs in trophoblastic cell lines. Gene targets of miRNAs changed after LIF treatment were inferred by the MirTarBase and classified into Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. A) Pathways were considered as significantly altered when FDR adjusted $p < 0.05$ (vertical dotted line). Letters represent key sub-pathway regions a: NK-kB, b: TGFß-SMAD, c: RAS-RAF-MEK-ERK, d: PI3K-Akt, e: NWASP-ARP/2/3-(F)-actin, f: JAK-STAT. B) Visualized example of sub-pathway identification. Proteins are colored if identified in the regulated set of genes.

Figure 3. Expression and targets of miR-21-3p. A) qRT-PCR confirming elevated miR-21-3p after LIF stimulation (*$p < 0.05$ **$p<0.005$ to non-stimulated control cells). B) miRNA/mRNA alignment of predictive targets for miR-21-3p.

Fig 1.

DOWN



JEG3/HTR8 SVneo
hsa-miR-1276-002843
hsa-miR-1291-002838
hsa-miR-144#-002148
hsa-miR-145#-002149
hsa-miR-573-001615

JEG3/ACH-3P
hsa-let-7a-4373169
hsa-miR-1262-002852
hsa-miR-200b-4395362
hsa-miR-222#-002097
hsa-miR-450b-5p-4395318
hsa-miR-605-001568
hsa-miR-941-002183

JEG3/AC1-M59
hsa-miR-10a-4373153
hsa-miR-204-4373094
hsa-miR-449b-4381011
hsa-miR-494-4395476
hsa-miR-503-4373228
hsa-miR-641-001585
hsa-miR-99b#-002196

HTR8 SVneo/ACH-3P
hsa-miR-1255B-002801
hsa-miR-331-5p-4395344
hsa-miR-516-3p-001149

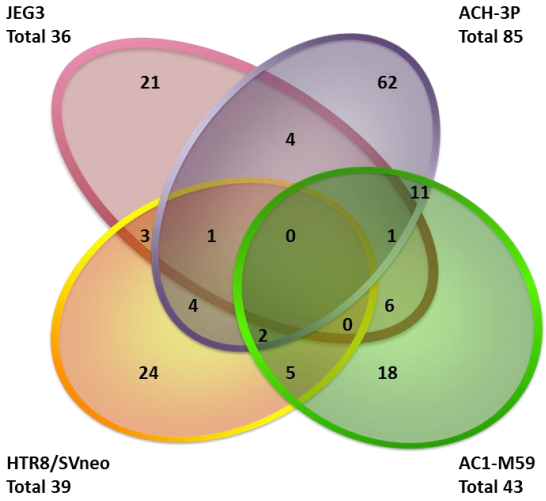
HTR8 SVneo/AC1-M59
hsa-miR-1208-002880
hsa-miR-708-4395452

ACH-3P/AC1-M59
hsa-miR-1300-002902
hsa-miR-148a#-002134
hsa-miR-184-4373113
hsa-miR-23b#-002126
hsa-miR-378-002243
hsa-miR-500-001046
hsa-miR-589-4395520
hsa-miR-93#-002139

JEG3/ACH-3P/AC1-M59
hsa-miR-15b#-002173
hsa-miR-25#-002442
hsa-miR-550-002410

JEG3/HTR8 SVneo/ACH-3P
hsa-miR-885-5p-4395407

UP



JEG3/HTR8 SVneo
hsa-miR-181c-4373115
hsa-miR-184-4373113
hsa-miR-548d-3p-4381008

JEG3/ACH-3P
hsa-miR-1248-002870
hsa-miR-1282-002803
hsa-miR-34b-000427
hsa-miR-96-4373372

JEG3/AC1-M59
hsa-miR-15a-4373123

hsa-miR-190b-002263
hsa-miR-206-000510
hsa-miR-374b#-002391
hsa-miR-489-4395469
hsa-miR-875-5p-002203

HTR8 SVneo/ACH-3P
hsa-miR-150-4373127
hsa-miR-374a#-002125
hsa-miR-628-5p-4395544
hsa-miR-664-002897

ACH-3P/AC1-M59
hsa-miR-1227-002769
hsa-miR-1233-002768
hsa-miR-1260-002896
hsa-miR-142-3p-4373136
hsa-miR-23b-4373073
hsa-miR-26a-2#-002115
hsa-miR-29b-1#-002165

hsa-miR-450a-4395414
hsa-miR-519e#-001166
hsa-miR-520d-5p-4395504
hsa-miR-638-001582

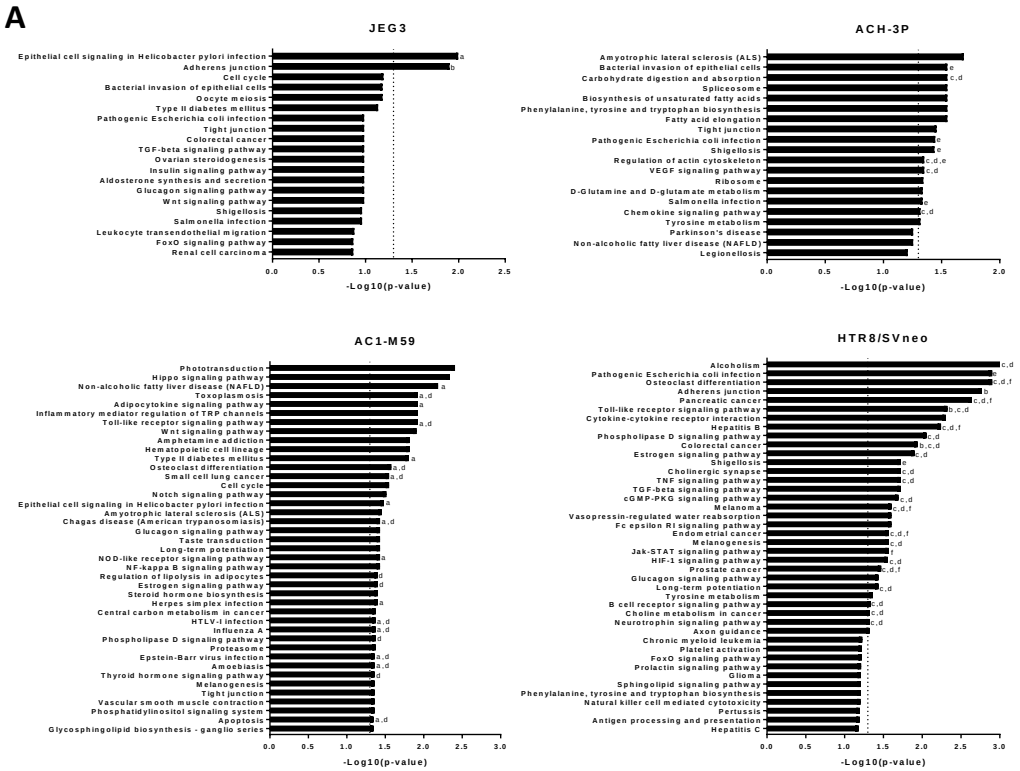
HTR8 SVneo/AC1-M59
hsa-let-7e#-002407
hsa-miR-100-4373160
hsa-miR-338-5P-002658
hsa-miR-545#-002266
hsa-miR-618-4380996

JEG3/HTR8 SVneo/ACH-3P
hsa-miR-202-4395474

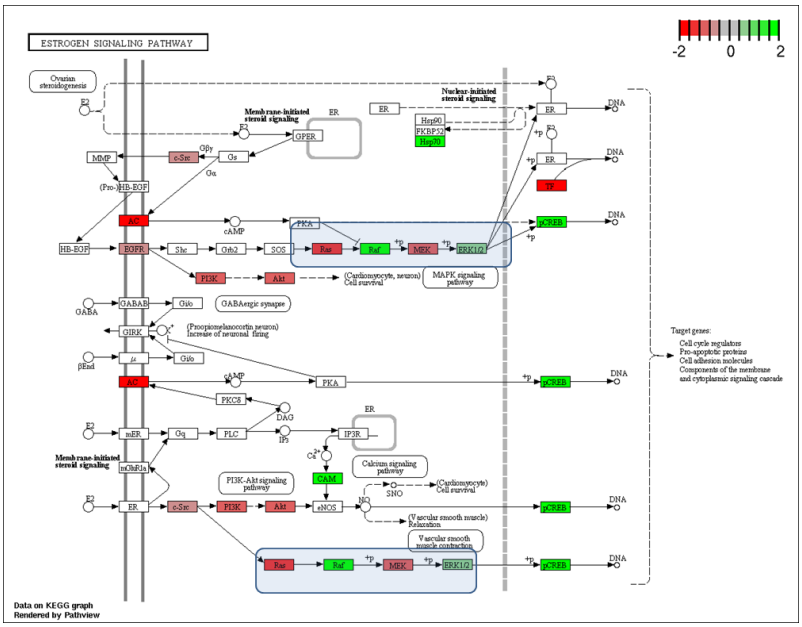
JEG3/ACH-3P/AC1-M59
hsa-miR-21#-002438

HTR8 SVneo/ACH-3P/
AC1-M59
hsa-miR-1303-002792
hsa-miR-29a#-002447

Fig 2.


A
JEG3
Epithelial cell signaling in Helicobacter pylori infection
Adherens junction
Cell cycle
Bacterial invasion of epithelial cells
Oocyte meiosis
Type II diabetes mellitus
Pathogenic Escherichia coli infection
Tight junction
Colorectal cancer
TGF-beta signaling pathway
Ovarian steroidogenesis
Insulin signaling pathway
Aldosterone synthesis and secretion
Glucagon signaling pathway
Wnt signaling pathway
Shigellosis
Salmonella infection
Leukocyte transendothelial migration
FoxO signaling pathway
Renal cell carcinoma
-Log10(p-value)
ACH-3P
Amyotrophic lateral sclerosis (ALS)
Bacterial invasion of epithelial cells
Carbohydrate digestion and absorption
Spliceosome
Biosynthesis of unsaturated fatty acids
Phenylalanine, tyrosine and tryptophan biosynthesis
Fatty acid elongation
Tight junction
Pathogenic Escherichia coli infection
Shigellosis
Regulation of actin cytoskeleton
VEGF signaling pathway
Ribosome
D-Glutamine and D-glutamate metabolism
Salmonella infection
Chemokine signaling pathway
Tyrosine metabolism
Parkinson's disease
Non-alcoholic fatty liver disease (NAFLD)
Legionellosis
-Log10(p-value)
AC1-M59
Phototransduction
Hippo signaling pathway
Non-alcoholic fatty liver disease (NAFLD)
Toxoplasmosis
Adipocytokine signaling pathway
Inflammatory mediator regulation of TRP channels
Toll-like receptor signaling pathway
Wnt signaling pathway
Amphetamine addiction
Hematopoietic cell lineage
Type II diabetes mellitus
Osteoclast differentiation
Small cell lung cancer
Cell cycle
Notch signaling pathway
Epithelial cell signaling in Helicobacter pylori infection
Amyotrophic lateral sclerosis (ALS)
Chagas disease (American trypanosomiasis)
Glucagon signaling pathway
Taste transduction
Long-term potentiation
NOD-like receptor signaling pathway
NF-kappa B signaling pathway
Regulation of lipolysis in adipocytes
Estrogen signaling pathway
Steroid hormone biosynthesis
Herpes simplex infection
Central carbon metabolism in cancer
HTLV-I infection
Influenza A
Phospholipase D signaling pathway
Proteasome
Epstein-Barr virus infection
Amoebiasis
Thyroid hormone signaling pathway
Melanogenesis
Tight junction
Vascular smooth muscle contraction
Phosphatidylinositol signaling system
Apoptosis
Glycosphingolipid biosynthesis - ganglio series
-Log10(p-value)
HTR8/SVneo
Alcoholism
Pathogenic Escherichia coli infection
Osteoclast differentiation
Adherens junction
Pancreatic cancer
Toll-like receptor signaling pathway
Cytokine-cytokine receptor interaction
Hepatitis B
Phospholipase D signaling pathway
Colorectal cancer
Estrogen signaling pathway
Shigellosis
Cholinergic synapse
TNF signaling pathway
TGF-beta signaling pathway
cGMP-PKG signaling pathway
Melanoma
Vasopressin-regulated water reabsorption
Fc epsilon RI signaling pathway
Endometrial cancer
Melanogenesis
Jak-STAT signaling pathway
HIF-1 signaling pathway
Prostate cancer
Glucagon signaling pathway
Long-term potentiation
Tyrosine metabolism
B cell receptor signaling pathway
Choline metabolism in cancer
Neurotrophin signaling pathway
Axon guidance
Chronic myeloid leukemia
Platelet activation
FoxO signaling pathway
Prolactin signaling pathway
Glioma
Sphingolipid signaling pathway
Phenylalanine, tyrosine and tryptophan biosynthesis
Natural killer cell mediated cytotoxicity
Pertussis
Antigen processing and presentation
Hepatitis C
-Log10(p-value)

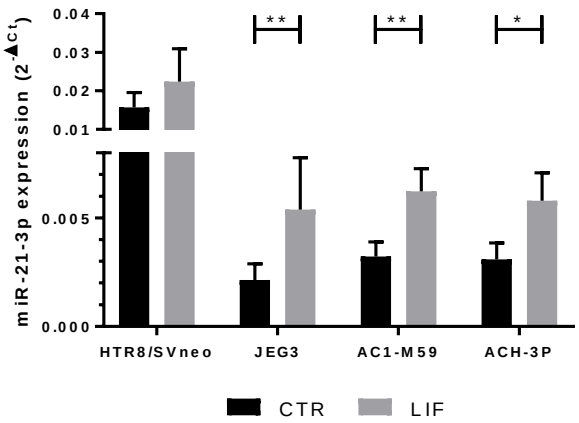
B


ESTROGEN SIGNALING PATHWAY
-2
0
2
Ovarian steroidogenesis
Membrane-initiated steroid signaling
Nuclear-initiated steroid signaling
GPER
ER
Hsp90
FKBP52
Hsp70
MMP
c-Src
Gs
(Pro-)HB-EGF
AC
cAMP
PKA
HB-EGF
EGFR
Shc
Grb2
SOS
Ras
Raf
MEK
ERK1/2
PI3K
Akt
(Cardiomyocyte, neuron) Cell survival
MAPK signaling pathway
GABA
GABAB
Gi/o
GABAergic synapse
GIRK
(Proopiomelanocortin neuron) Increase of neuronal firing
βEnd
μ
PKC δ
DAG
mER
Gq
PLC
IP3
IP3R
Membrane-initiated steroid signaling
mGluRIa
PI3K-Akt signaling pathway
CAM
Calcium signaling pathway
eNOS
SNO
(Cardiomyocyte) Cell survival
(Vascular smooth muscle) Relaxation
Vascular smooth muscle contraction
pCREB
DNA
TF
Target genes:
Cell cycle regulators
Pro-apoptotic proteins
Cell adhesion molecules
Components of the membrane and cytoplasmic signaling cascade
Data on KEGG graph
Rendered by Pathview

Ras
Raf
MEK
ERK1/2

Fig 3

A



B

```
    3' aguuGUAGUCA-G-ACUAUUCGAu 5' hsa-miR-21
          | ||||| | | |||||||
1930:5' cacuCUUCAGUACAUAAUAAGCUu 3' STAT3
```

```
    3' agUUGUAGUCAGACUAUUCGAu 5' hsa-miR-21
         || :| :||:| |||||||
2247:5' auAAGGU-GGUUU-AUAAGCUg 3' STAT3
```

```
    3' aguuguagucagacUAUUCGAu 5' hsa-miR-21
                     |||||||
1108:5' guuuagacuuuaacAUAAGCUa 3' SMAD7
```

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