# Global changes of miRNA expression indicates an increased reprogramming efficiency of induced mammary epithelial cells by repression of miR-222-3p in fibroblasts

**Mingxing Liu** [Equal first author, 1], **Siyi Liu** [Equal first author, 1], **Liangshan Qin** [1, 2], **Danwei Lv** [1], **Guodong Wang** [1], **Quanhui Liu** [1], **Ben Huang** [Corresp., 1, 2], **Dandan Zhang** [Corresp. 2]

[1] School of Animal Science and Technology, Guangxi University, Nanning, Guangxi, China

[2] Guangxi Key Laboratory of Eye Health, Guangxi Academy of Medical Sciences, The People's Hospital of Guangxi Zhuang Autonomous Region, Nanning, Guangxi, Nanning, Guangxi, China

Corresponding Authors: Ben Huang, Dandan Zhang
Email address: bhuang@gxams.org.cn, ddzhang@gxams.org.cn

**Background.** Our previous studies have successfully reported the reprogramming of fibroblasts into induced mammary epithelial cells (iMECs). However, the regulatory relationships and functional roles of MicroRNAs (miRNAs) in the progression of fibroblasts achieving the cell fate of iMECs are insufficiently understood.

**Methods.** First, we performed pre-and post-induction miRNAs sequencing analysis by using high-throughput sequencing. Following that, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment studies were used to determine the primary roles of the significantly distinct miRNAs and targeted genes. Finally, the effect of miR-222-3p on iMECs fate reprogramming in vitro by transfecting.

**Results.** As a result,goat ear fibroblasts (GEFs) reprogramming into iMECs activates a regulatory program, involving 71 differentially expressed miRNAs. Besides, the programming process involved changes in multiple signaling pathways such as GnRH secretion, prolactin signaling pathway, regulation of lipolysis in adipocytes, etc. In contrast, it was discovered that the expression of miR-222-3p downregulation by miR-222-3p inhibitor significantly increase the reprogramming efficiency and promoted lipid accumulation of iMECs.

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[1] Guangxi Key Laboratory of Eye Health, Guangxi Academy of Medical Sciences, The People's Hospital of Guangxi Zhuang Autonomous Region, Nanning,530021, China.
[2] School of Animal Science and Technology, Guangxi University, Nanning, Guangxi, 530004, China.

Corresponding Author:
BenHuang[1,2],Dandan Zhang[1]
Guangxi Key Laboratory of Eye Health, Guangxi Academy of Medical Sciences, The People's Hospital of Guangxi Zhuang Autonomous Region, Nanning,530021, China.
Email address: bhuang@gxams.org.cn, benhuang@gxu.edu.cn (B.Huang), ddzhang@gxams.org.cn (D.Zhang)

## Abstract

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## Introduction

Downstream developmental lineages of cells derived from changes in the initiating cell type are regulated by transcription factors.[1] Therefore, establishing a new balance of the transcriptional

network is essential for a cell to move toward a new fate. Previous research has established that somatic cells can be directly reprogrammed into hepatocytes,[2] neurons,[3] and cardiomyocyte lineages by lineage-specific transcription factors.[4] A growing body of literature has shown that small molecule compounds promote transcription factor-based reprogramming by modulating signaling pathways.[5]

As small noncoding RNA, miRNAs can target many mRNAs and play a role in posttranscriptional gene regulation through protein interactions in the RNA-induced silencing complex.[6] It has been demonstrated that miRNAs are essential to somatic cell reprogramming, allowing mature double-stranded miRNAs to be directly transfected into mouse and human cells to reprogramme them to become pluripotent.[7] It is now well established from a variety of studies that miRNAs are key regulators of mammary epithelial cells (MECs) at different developmental stages.[8] MiRNAs can affect mammary development by affecting hormone level expression.[9] In conclusion, miRNAs are essential for reprogramming and mammary gland development.

MECs have been the only target cell type needed for recombinant protein production in mammary bioreactors due to their unique ability to lactate.[10] MECs can be obtained from the mammary epithelium by collagenase digestion or directly from the breast milk of different species.[11-12] Such approaches, however, have failed to address MECs obtained had too low for transgenic protein expression in milk-producing mammary bioreactors.

We successfully reprogrammed GEFs into iMECs using five small-molecule compounds.[13] Previous studies of the mechanism of this reprogramming process have not addressed the miRNA levels. In the present study, we analyzed small RNA sequencing before and after iMEC induction obtained from a combination of five small molecule compounds. Furthermore, we validated the effect of miR-222-3p on the transition of goat ear fibroblasts to a mammary epithelial cell fate identity.

## Materials & Methods

2.1 Sample Collection

Based on the former research results, goat ear fibroblasts were seeded into a 60-mm cell culture dish at a density of 5 × 105 cells per dish. Following 24 hours, the medium was changed to "VTFBR" (500μg/mL VPA, 10 μM Tranylcypromine, 10 μM Forskolin, 1 μM TTNPB, 10 μM RepSox), a cocktail induction medium based on N2B27 and small molecule chemicals.13 The induction culture was maintained for 8 days with medium changes every two days.

2.2 Creating Small RNA Libraries

Total RNA was isolated from reprogrammed iMECs cells using TRIzol reagent at the predetermined time periods (the initiating GEFs (0 day) and the day 8 post-induction iMECs), applying three replicates for each time point, and then pooled together for sequencing in a total of six libraries. 1% gel electrophoresis was employed to detect total RNA whether degradation. Using 8% polyacrylamide gel electrophoresis, whole RNA was separated into fragments between 15 and 35 nt, and then the fragments were linked with proprietary adapters. Reverse transcription

was used to create complementary DNA (cDNA) in order to create the libraries for sequencing. Using Illumina HiSeq to perform RNA sequencing with the SE50 sequencing strategy.[14]

2.3 Data Analysis

Clean Reads for subsequent analysis were obtained from Raw Bases corresponding to the Sequenced Reads by removing joints, removing low quality, and selecting fragments.[15] Clean Reads in the genome were matched with miRNAs localization information (100% overlap).[16] Using miRDeep2 predicted novel miRNAs whose sequences have been discovered. Normalization was performed for each sample to obtain Reads Per Million (RPM). DESeq was used to identify differentially expressed miRNAs. When |Fold Change|>1 and padj<0.05, the miRNAs were considered as differentially expressed.[17]

2.4 MiRNAs Enrichment Analysis

GO and KEGG analyses of differentially expressed miRNAs were performed using the miEAA (https://www.ccb.uni-saarland.de/mieaa2) database.[18]

2.5 Cell transfection and induction

Goat ear fibroblasts were seeded into a 60-mm cell culture dish at a density of 5×105 cells per dish. Afterwards, Lipofectamine TMRNAiMAX and each group of miRNA fragments were transfected into cells according to the miRNA Transfection Reagents instructions, and three biological replicates of each group were performed. After 12 h, the medium was replaced with N2B27 and small molecule compounds cocktail induction medium “VTFBR” (500μg/mL VPA, 10 μM Tranylcypromine, 10 μM Forskolin, 1 μM TTNPB, 10 μM RepSox), the medium was changed every two days, and the induction culture was continued for 4 days.

2.6 Oil Red O Staining and Quantification

After induction the cells were fixed with 4% paraformaldehyde for 10 min and then stained with oil red O staining solution for 15 min. The cells were then rinsed with 60% isopropanol for 15-30 seconds, washed three more times with PBS, and observed under a microscope. Finally for quantitative analysis of lipid accumulation, lipids were washed off using 100% isopropanol and then absorbance at 510 nm was measured using a spectrophotometer.

2.7 Immunofluorescence staining

For transfection of well induced cells, immunofluorescence was performed as described previously.[13]

2.8. Statistical analysis

All values were presented as the mean ± SEM. And the statistical significance was accepted at $p < 0.05$ using the two-tailed t-test with equal variance.

## Results

3.1 Introduction to Sequencing Data

Six small RNA-seq libraries were constructed for high-throughput sequencing, with three biological repetitions at each time-point (GEFs and iMECs). For the six small RNA libraries, the averages of 13,372,156.33 raw data were obtained, and the averages of 11,180,154.50 clean reads were obtained (Table. S1). Lengths were ranged from 18 to 30 nt, and the peak appeared at 22nt which conformed to the law of animal small RNA (Figure 1.A). The Q20 values of the

miRNA libraries were all higher than 97%. The length distribution of small RNAs in GEFs and iMECs follows the typical read distribution of animal miRNAs, mostly in the range of 22nt. The clean reads were contrasted to the goat reference sequence (Table. S2), and the distribution of sequences on the alignment refers to the density distribution of the best matching position of reads on the genome in each chromosome (with positive and negative chains) (Figure 1.B). Principal component analysis (PCA) (Figure 1.C) was run to cluster the samples based on the expression values and showed a significant difference before and after induction between GEFs and iMECs, the main difference lay in pc1. We also showed the density distribution to visually demonstrate the expression level of all the genes that were detected in the two samples (Figure 1.D). In conclusion, the miRNA libraries used in the current study had high enough data quality to be used for subsequent statistical calculations.

### 3.2 Differentially expressed miRNA and GO and KEGG enrichment analysis

All six miRNA libraries detected a total of 457 miRNAs, including 116 novel miRNAs and 341 known miRNAs.The miRNAs showed were shown differential expression patterns (Figure 2.A). Compared with the GEF libraries, 34 known miRNAs were upregulated in the iMECs, and 37 known miRNAs were downregulated with $|\log2FC| > 1$, $-\log10(Pvalue) < 0.05$ (Figure 2.B, Figure 2.C, Table S3). From the graph above we can see that, chi-miR-34c-3p, chi-miR-34b-3p, chi-miR-34b-5p, chi-miR-34c-5p, and chi-miR-141 were the most obviously upregulated, but chi-miR-100-3p, chi-miR-214-5p, chi-miR-145-5p, chi-miR-214-3p, and chi-miR-296-3p were the most significantly downregulated. This suggested that these miRNAs may play a key role during mammary gland lineage formation. Interestingly, the expression of all 34 family members was observed to be significantly elevated in iMECs.

Both GO and KEGG analyses were carried out to mine cellular biological processes and signaling pathways, respectively, in order to highlight the relevance of differentially expressed miRNAs in the reprogramming of GEFs to iMECs. The KEGG pathway analysis results indicated the involvement of these DE-miRNAs mostly in adherens junction, GnRH secretion, the IL-17 signaling pathway, the prolactin signaling pathway, etc (Figure 2.D). GO enrichment analysis showed that the top ten terms were correlated with the regulation of membrane potential, ribosome processing, multicellular organism development, etc (Figure 2.D). Interestingly, downregulated miRNAs were associated with some hormone pathways(Table. S4) while upregulated miRNAs were associated with some EMT pathways(Table. S5). An explanation for this might be that upregulated miRNAs maintained epithelial cell characteristics by suppressing EMT, and downregulated miRNAs gave hormonal signals that allowed them to become a mammary gland spectrum.

### 3.3 Downregulation of miR-221-3p was promoted induction of reprogramming efficiency in mammary epithelial cells

To verify whether miRNAs improve the efficiency of inducing reprogramming in mammary epithelial cells, we selected miR-221-3p as the target of the study. Mimic/inhibitor of miR-221-3p were transfected into goat ear fibroblasts prior to induction by five small molecule compounds(VTFBR). The results showed that fibroblasts could form more independent and

compact epithelial cell-like colonies in the cultures treated with the inhibitor of miR-221-3p, whereas fewer colonies were found in the miR-221-3p mimic cultures than in the controls at 4 days (Figure 3.A). These observations suggested that miR-221-3p upregulation severely affected small-molecule compound-induced colony formation and decreased the reprogramming efficiency to 0.97% from 3.51% (Figure 3.B). With these findings, we hypothesized that the high expression of miR-221-3p may play a hindering role in the transformation of fibroblasts into iMECs.

3.4 MiR-221-3p affects the biological properties of induced mammary epithelial cells

Next, to better understand the effect of miR-222-3p on the function of iMECs, we evaluated the expression of proteins related to milk fat formation and mammary epithelial cells in iMECs after manipulation of miR-222-3p expression. Secretion of milk lipids is one of the important features of iMECs, and we found that iMECs treated with an inhibitor of miR-221-3p secreted larger lipid droplets around the cytoplasm by saturated oil red O staining. In contrast, miR-222-3P mimic group-treated iMECs significantly reduced periplasmic lipid droplet secretion(Figure 4A, B). In addition, immunofluorescence staining showed higher expression of the mammary epithelial cell-specific antigens CDH1, CK8 and CK14 in iMECs treated with inhibitor of miR-221-3p(Figure 4C, D).

## Discussion

Cell reprogramming techniques allow cells to escape downstream developmental lineages where the initial cell type changes. As we showed in our previous report, iMECs can be chemically reprogrammed from fibroblasts by inhibiting the TGF β R1-Smad3 regulatory site. MiRNAs often operat in feedback loops with transcription factors have been reported in the literature.[25] Posttranscriptional gene regulation by miRNAs might contribute strong and redundant controls to this process. It is worth emphasizing that our reprogramming brings about a reset that is not limited to mRNAs but also includes the reset of other biological process-related miRNAs. However, our previous studies did not focus on changes in miRNA levels. We hope to determine how miRNAs maintain the fate of mammary epithelial cells through this study.

In this study, we profiled miRNA expression and screened miRNAs with differential significant expression. We found that the expression of chi-miR-34b-5p, chi-miR-34b-3p, chi-miR-34c-3p, and chi-miR-34c-5p, which are members of the microRNA-34 family, increased most significantly in the differentially expressed miRNAs. This finding was consistent with that of Bonetti (2000), who discovered that the miR-34 family was essential in the fate commitment and differentiation of mammary epithelial cells.[26] Surprisingly, significantly downregulated mir145, a key target of the tgfB pathway, plays a significant role in the activation of hematopoietic stem/progenitor cells (HSPCs) and fibroblast differentiation.[27-28] By KEGG analysis, we found that downregulated miRNAs were involved in four hormone-related pathways (GnRH secretion, prolactin signaling pathway, thyroid hormone signaling pathway, VEGF signaling pathway), and upregulated miRNAs were significantly enriched in some EMT-related pathways (focal adhesion, leukocyte transendothelial migration, adherens junction). According to these data, we could infer that miRNAs maintain the fate transformation of MEC lineage cells by

activating hormonal signaling and inhibiting EMT signaling. It is worth noting that GSEA of the predicted target genes obtained the same conclusion.

MiRNAs, as important regulators, are involved in almost every step of cellular processes, and although there is a large body of evidence supporting the involvement of miRNAs in the process of cellular reprogramming, little is known about what role miRNAs play in the reprogramming of fibroblasts into mammary epithelial cells. The inhibition of miR-222-3p expression levels was demonstrated by our experiments to be more conducive to fibroblast-to-mammary epithelial cell identity. There is evidence that miR-222-3p affects cancer cell migration by promoting the process of EMT in the development of multiple cancers.[29-30] In our past studies it was thought that fibroblasts were required to undergo MET to achieve the transition to a mammary epithelial cell fate. We therefore speculate that lower expression levels of miR-222-3p may be more helpful for the advancement of the MET process, thereby improving reprogramming efficiency. Next, we experimentally found that miR222-3p inhibitor transfected cells induced to become imec possessed stronger milk fat secretion ability and expression of mammary epithelial signature proteins. This is consistent with the findings of Pere Bibiloni et al. that miR-222 expression is downregulated in 3T3-L1 cells during adipogenesis.[31] High expression of CK14, CK8, and E-cadherin proteins demonstrates better maintenance of epithelial cell identity after inhibitor miR222-3P-induced cellular antigens. These evidences may indicate that miR222-3p is one of the barriers for fibroblasts to achieve mammary epithelial cell fate transition, and inhibition of miR222-3p expression facilitates faster and better mammary epithelial cell fate transition.

## Conclusions

In conclusion, a global view of changes in miRNA levels in GEFs and IMECs before and after reprogramming was obtained by high-throughput sequencing. We found that upregulation of miRNAs may perturb the EMT process to maintain epithelial identity, and downregulation of the gene miRNAs may allow reprogrammed cells to enter the mammary developmental lineage through the response to hormonal signaling. More importantly, we verified that miR-222-3p facilitates goat mammary epithelial cell fate transition. These findings help us to better refine the reprogramming system and accelerate reprogramming efficiency. However, further search for miR-222-3p downstream target genes and further elucidation of the regulatory mechanism for iMECs still require experimental validation.

## Acknowledgements

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'The statistical power of this experimental design, calculated in RNASeqPower is 0.7256308
'

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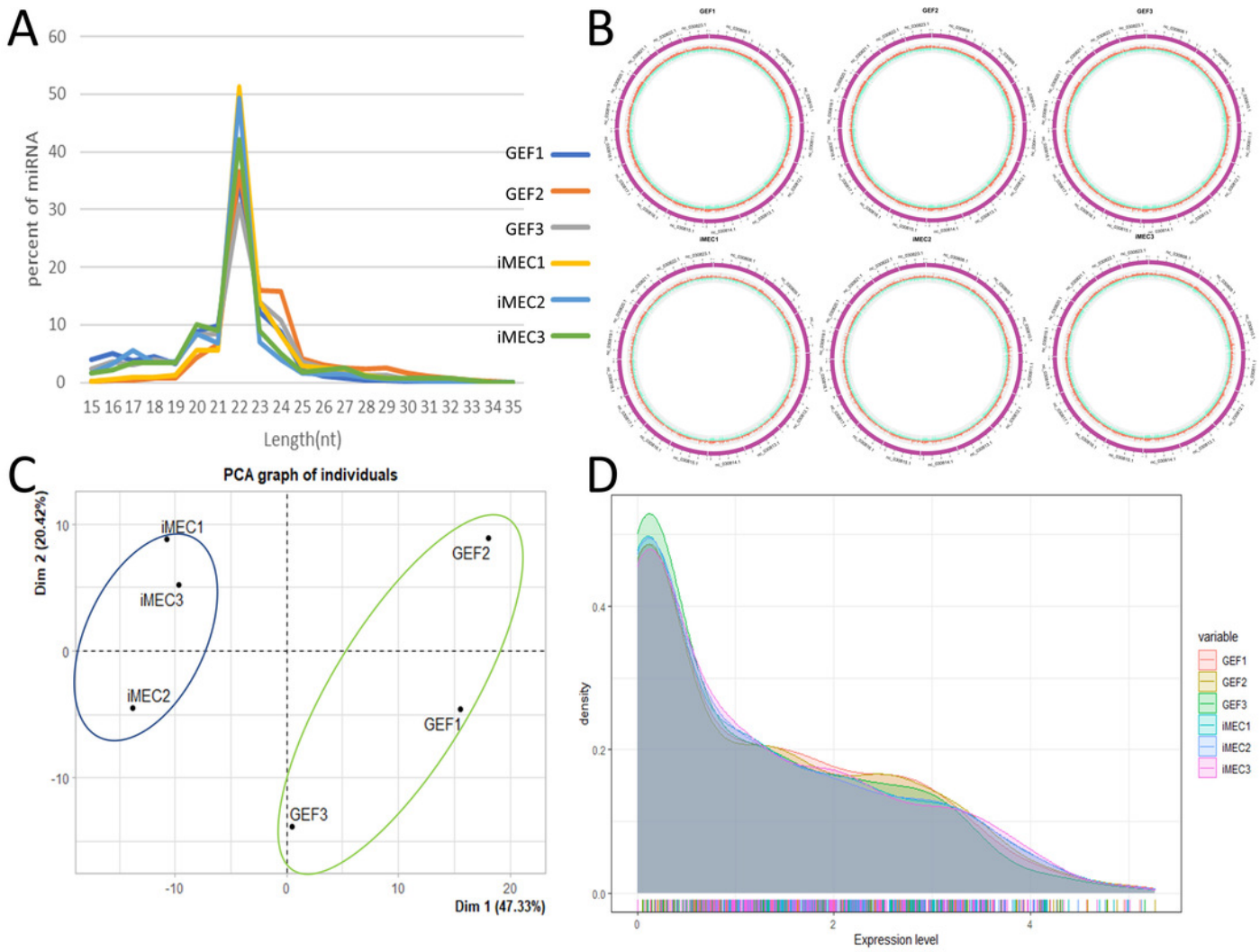
32.

# Figure 1

Fig 1. Quality control.

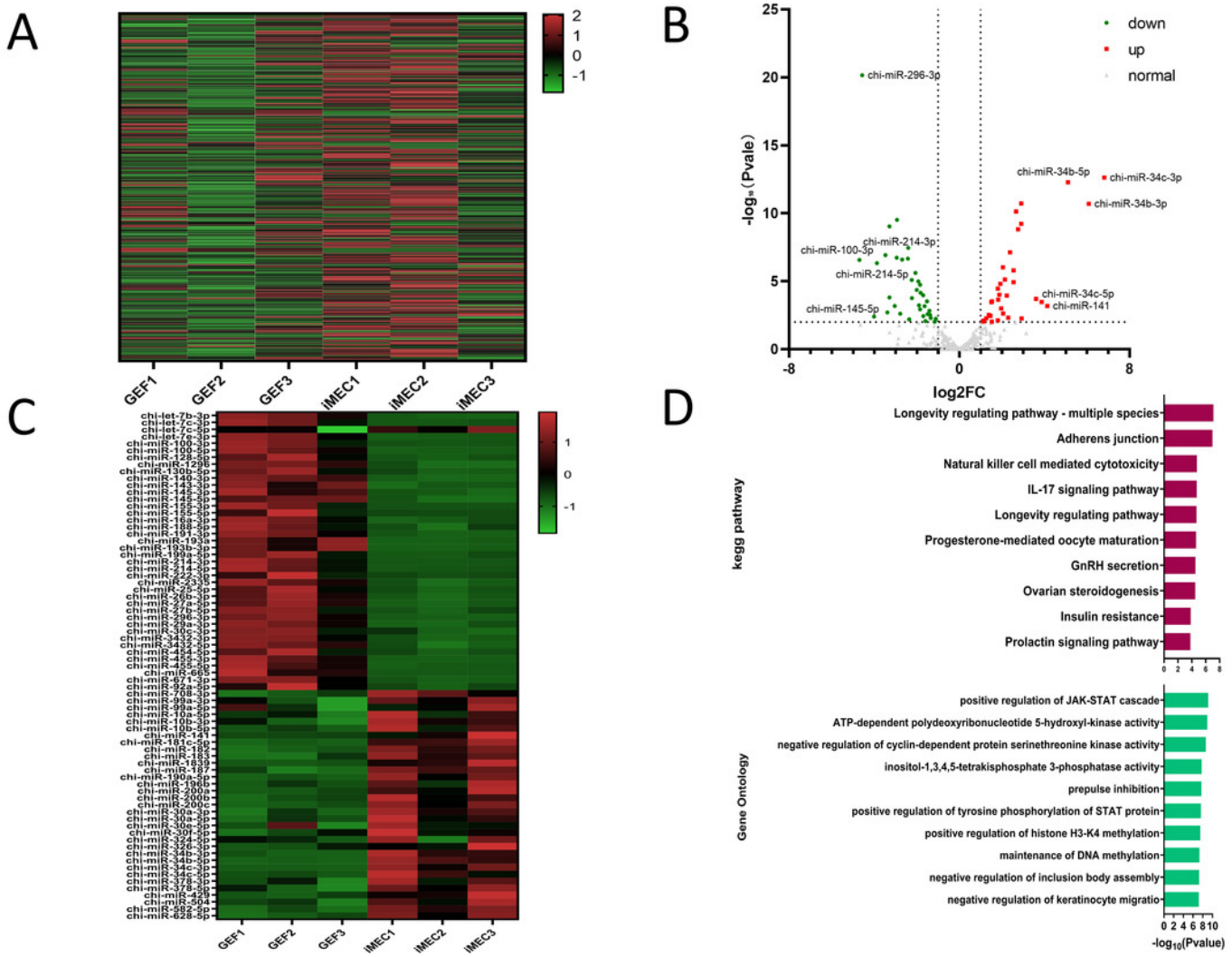
(A) The size distribution of the small RNAs found in the mammary gland between GEFs groups and iMECs groups. (B) The best matching position of Reads on the genome is distributed in the density of each chromosome (with positive and negative chains). (C) Principal component analysis between GEFs groups and iMECs groups. (D) The expression level GEFs groups and iMECs groups.



# Figure 2

Fig 2. Differentially Expressed miRNA And GO and KEGG Enrichment Analysis.

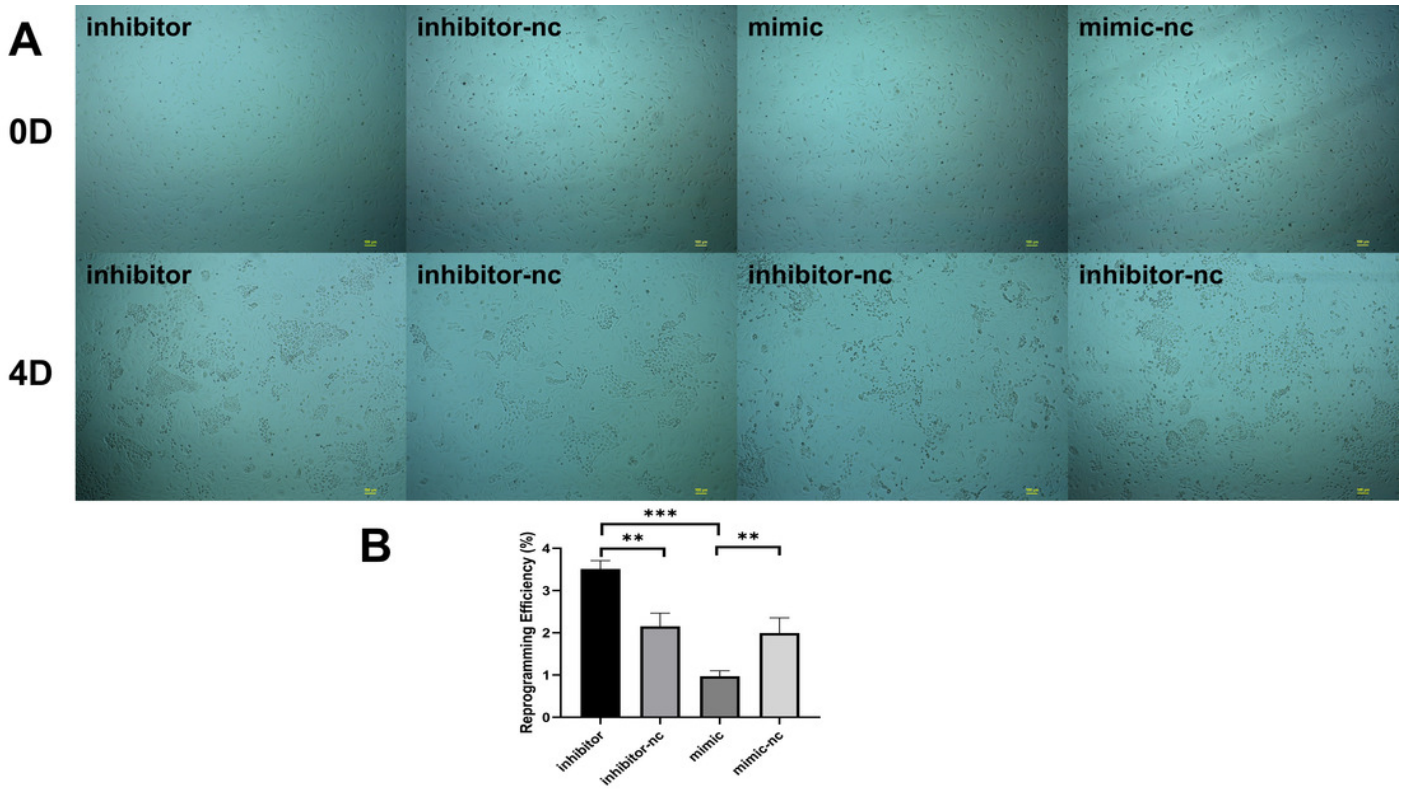
<!--[if !supportLists]--> (A)A heatmap plots 457 miRNAs that were detected in all groups. (B) A volcano plot of miRNAs differentially expressed between GEF groups and iMEC groups (top 5 up-regulated and down-regulated miRNAs were labeled). (C) A heatmap plots 71 differentially expressed miRNAs. (D) KEGG and GO annotation maps of the differentially expressed miRNAs. <!--[endif]-->



# Figure 3

Fig 3. Effect ofmiR-221-3p on reprogramming efficiency of goat induced mammary epithelial cells.

<!--[if !supportLists]-->(A)<!--[endif]-->The ability of cells to form independent clones within 4days under transfection miR-221-3p inhibitor, miR-221-3p inhibitor-NC, miR-221-3p mimic, miR-221-3p mimic-NC. Scale bar, 100 μm. (B) Reprogramming efficiency of goat induced mammary epithelial cells under transfection. All data are presented as mean±SD, and all of these experiments were performed in triplicate. ***p<0.001; *p<0.05, compared to control groups.



# Figure 4

Fig 4. Biological characterization of iMECs under transfection.

Saturated oil red O staining was performed to identify iMECs under transfection miR-221-3p inhibitor, miR-221-3p inhibitor-NC, miR-221-3p mimic, miR-221-3p mimic-NC. Scale bar, 100μm. (B) Histogram showing the quantitation of Oil Red O staining by spectrophotometry.(C) Immunofluorescence staining was performed to detect the expression of iMECs under transfection miR-221-3p inhibitor, miR-221-3p inhibitor-NC, miR-221-3p mimic, miR-221-3p mimic-NC. Scale bar, 100μm. (D) Quantitative analysis of colonies by immunofluorescence. All data are presented as mean±SD, and all of these experiments were performed in triplicate. ***p<0.001; *p<0.05, compared to control groups.