1 ***Microarray screening of differentially expressed genes after up-regulating miR-205 or***
2 ***down-regulating miR-141 in cervical cancer cells***

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23 **Abstract**

24 Cervical cancer is one of the most common gynecological malignancies. However, studies
25 on the expression and molecular mechanism of miR-205 and miR-141 in CC are insufficient
26 recently. Expression profile microarray with 21329 Oligo DNA were used to detect the
27 expression of mRNAs in miR-205 up-regulated or miR-141 down-regulated HeLa and SiHa
28 cells and mRNAs in normal HeLa and SiHa cells. Gene Ontology (GO), Kyoto Encyclopedia
29 of Genes and Genomes (KEGG) pathway were performed to assess the potential pathways
30 of miR-205 in SiHa cell. Compared with normal HeLa cell, there were 38 differentially
31 expressed genes (DEGs) in miR-205 up-regulated HeLa cell. Nine were up-regulation genes
32 and 29 were down-regulation genes. There were 23 DEGs in miR-141 down-regulated HeLa
33 cell. One was up-regulated and 22 were down-regulated. Compared with normal SiHa cell,
34 there were 128 DEGs in miR-205 up-regulated SiHa cell. One hundred and three were up-
35 regulation genes and 25 were down-regulation genes. There were 80 DEGs in miR-141
36 down-regulated SiHa cell. Forty two were up-regulation genes and 28 were down-regulation
37 genes. For miR-205 up-regulated SiHa cell, GO outcome showed that “ubiquitin-protein
38 ligase activity”, “MAP kinase phosphatase activity”, were the most enriched terms ($P < 0.05$).
39 And in KEGG analysis, “Cell cycle” was notably enriched, and Smad4 in this pathway was
40 up-regulated ($P < 0.05$). Expression profile microarray technology can effectively screen out
41 DEGs in cervical cancer cells after up-regulating miR-205 or down-regulating miR-141.
42 Which may enable us to understand the pathogenesis and lay an important foundation for
43 the prevention and treatment of cervical cancer.

44 Keywords: Gene expression profiling, cervical cancer, miRNA.

45 **Introduction**

46 Cervical cancer is one of the most common gynecological malignancies and the fourth
 47 leading cause of cancer-related death worldwide. There were 527,600 new cases of cervical
 48 cancer and 265,700 deaths worldwide in 2012.(1) It is the most common cancer of women in
 49 many low-income countries.(2) Persistent infection of human papillomavirus (HPV) type 16
 50 and 18 are the major risk factors for cervical cancer. In HPV high-risk cervical cancer cases,
 51 HPV16 and 18 phenotypes account for 70%.(3) After HPV DNA integrated into the host DNA,
 52 *E6* and *E7* genes are expressed. *E6* induces the degradation of p53, stimulates telomerase
 53 activity, and induces centrosome distortion and instability of the chromosome.(4) The binding
 54 of *E7* protein with human pRb and E2F transcription factor mediates cell transformation,
 55 leading to the separation of pRb and E2F transcription factor and making cells enter S phase
 56 of cell cycle early.(5) The occurrence of cervical cancer is a multi-factor, multi-stage and
 57 multi-gene process, involving a large number of structural changes and abnormal expression
 58 of tumor-related genes. It is particularly important to study the molecular mechanism of the
 59 oncogenesis and development of cervical cancer and to select effective therapeutic targets.

60 MicroRNAs (miRNAs), usually have 18-25 nucleotides, are a kind of small highly
 61 conserved non-coding RNA and are endogenous expression in many species. Most mature
 62 miRNAs inhibit gene expression by cutting target messenger RNA(mRNAs) or inhibiting
 63 mRNAs' translation by binding to the 3'-UTRs of mRNAs.(6) More and more reports have
 64 shown that miRNA plays an important role in the proliferation, apoptosis, invasion and
 65 migration of tumor cells. Abnormal expression of miRNA can be used for tumor classification,
 66 diagnosis and prognosis.(7) The carcinogenic or cancerocidal effects of miRNAs in the
 67 tumorigenesis and development of cervical cancer depend on the biological effects of their
 68 target genes.(8)

69 Microarray-based gene expression profiling technology has been applied to analyze the
 70 regulatory changes and expression differences of genes in tissues and cells, considerably
 71 facilitating the exploration of molecular mechanisms of tumor development. However,

reports on the expression and molecular mechanism of miR-205 and miR-141 in CC are insufficient recently. In this study, microarray-based gene expression profiling technology was used to detect DEGs after up-regulation of miR-205 or down-regulation of miR-141 in SiHa and HeLa cells. Then screening out the gene groups related to the occurrence and development of cervical cancer, studying their functions, exploring the relationship between DEGs and the tumorigenesis and progression of cervical cancer in order to provide evidence for the selection of therapeutic targets for cervical cancer.

Materials and methods

Cell culture

The *human* cervical cancer cell lines SiHa and HeLa were purchased from American Type Culture Collection (ATCC, Manassas, VA) and cultured at 37°C in a humidified 5% CO₂ atmosphere in DMEM medium (Gibco; Thermo Fisher Scientific, Inc.) with 10% fetal calf serum (Gibco; Thermo Fisher Scientific, Inc.), 100 IU/ml penicillin G, and 100 mg/ml streptomycin sulfate (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany).

SiRNA transfection

Synthetic small interfering RNA (siRNA) used to knockdown miR-141 or overexpress miR-205 were purchased from GenePharma (Suzhou, China). siRNAs were transfected into cells cultured in 10cm culture dish using Lipofectamine 3000 (Invitrogen) according to the manufacturer's instructions. Total RNA was extracted using the Trizol reagent (Invitrogen, Carsbad, CA, USA) according to the manufacturer's protocol 48h after transfection.

The main experimental instruments

The 22K *Human* Genome Array is provided by the CapitalBio Microarray service (CapitalBio, Beijing, China), according to the manufacturer's instructions, and 21329 Oligo DNA of this genome array were from Human Genome Oligo Set Version 2.1, Operon company. The other 193 Oligo DNA were synthesized by CapitalBio company. The scanner

is LuxScan 10KA dual-channel laser scanner (CapitalBio, Beijing, China). The analysis image software is LuxScan 3.0 (CapitalBio, Beijing, China).

Preparation method of chip

For each RNA array experiment, we absorbed 1ul RNA to test the concentration and purity by spectrophotometer at 260nm/180nm, then electrophoresed RNA on gel containing formaldehyde for quality control [Fig1].

Fig1: RNA formaldehyde modified gel electrophoresis. From left to right are SiHa-1, HeLa-1, SiHa-2a, HeLa-2, SiHa-4, HeLa-4, SiHa-2b. The bands are clear, and the brightness of 28S is greater than that of 18S.

For each array experiment, we extracted 4ul of total RNA, added to 50ul reverse transcription reaction system. The samples were fluorescently labeled with cRNA, purified and dried, and then used for chip hybridization. mRNA of HeLa-1, HeLa-2, SiHa-1, SiHa-2 was labeled with cy5-2 dUTP. mRNA of HeLa-4, SiHa-4 was labeled with cy3-2 dUTP. Then we dissolved samples respectively in 20ul hybridization buffer containing 5x sodium chloride-sodium citrate Buffer (SSC), 0.2% sodium dodecyl sulfate (SDS). Hybridization was performed at 42°C overnight. Gene chip and hybridization probes were respectively degenerated 5 min in 95 °c water bath, then added a probe immediately on the chip. We used cover glass sealing piece, placed pieces inside the 60 °c capsule and hybridized 16 h. The mRNA array was washed with 2xSSC and 0.2%SDS solution, 0.1%SSC and 0.2%SDS solution, 0.1%SSC solution respectively at 42°C for 10 min, then dried at room temperature. The array was scanned by a Dual channel laser scanner (LuxScanTM 10K/A, CapitalBio). LuxScan3.0 image analysis software (CapitalBio) was used to analyze the chip image and convert the image signal into digital signal. The Lowess method is used to normalize the data on the chip.

Gene expression analysis, gene ontology, pathway analysis

mRNAs with a IV signal value ≥ 1500 , and at least a 2-fold increase or a 0.5-fold decrease

in mRNA expression were considered to have significant changes, including up-regulation and down-regulation. We selected DEGs between SiHa-1 and SiHa-4 to conduct functional enrichment analysis using Gene Ontology (GO) based on the MAS (molecule annotation system), and perform pathway analysis using Kyoto Encyclopedia of Genes and Genomes (KEGG) database. (<http://www.kegg.jp/>)

Statistical analysis

We use Wilcoxon signed-rank test to determine the statistical difference between two groups. And the hypergeometric distribution test was used to perform GO and pathway enrichment analysis. *P* value of less than 0.05 was considered statistically significant in these texts. Statistical analysis was executed using SPSS software version 20.

Results

The total RNA extracted from all samples ranged from 16.35 to 18.34 ug, and the absorbance of 260nm/280nm ranged from 2.09 to 2.10. The concentration and purity of all RNA samples were good. RNA electrophoresis band [Fig1] showed the quality is up to standard and the quantity is enough.

In all chip images, the signal of positive controls (HEX), external markers (eight intergenic sequences of yeast) were normal, and the signal of negative controls (12 artificially synthesized 70 mer Oligo DNA with no homology with human genes and 50%DMSO) were negative. The repeatability of house-keeping gene was good and the Ratio CV was no more than 0.3, the leakage rate did not exceed 3%.

According to the statistical selection criteria, our results showed that there were 38 DEGs between HeLa-1 and HeLa-4 [Table1]. Nine were up-regulation genes and 29 were down-regulation genes. There were 23 DEGs between HeLa-2 and HeLa-4 [Table2]. One was up-regulated and 22 were down-regulated. There were 128 DEGs between SiHa-1 and SiHa-4 [Table3]. Of these, 103 were up-regulation genes and 25 were down-regulation genes. There were 80 DEGs between SiHa-2 and SiHa-4 [Table4]. Fifty-two were up-regulation genes and

149 28 were down-regulation genes. The outcome of the hybridization scatter plot were the same
150 [Fig2].

151 **Table 1: Part of the differentially expressed genes in miR-205 up-regulated HeLa cell**

Gene name of the Chip ^a	Gene name ^b	Up-regulation rate ^c	Down-regulation rate ^c
DOCK3	-	18.8572	
KIAA1233	ADAMTSL3	7.1471	
ODC1	ODC1	2.3646	
HT007	-	2.1446	
COL4A3BP	COL4A3BP	2.0514	
PPID	PPID	2.0482	
FLJ21802	-	2.0426	
GRWD	GRWD1	2.0381	
CASP1	CASP1		0.3754
IGFBP1	IGFBP1		0.3854
CHRD	CHRD		0.4046
FLJ23058	Q9P281		0.407
MMD	MMD2		0.4102
PROS1	PROS1		0.4129
WFDC1	WFDC1		0.4148
DUSP5	DUSP5		0.4265
SERPINE1	SERPINE1		0.4311
BHLHB2	BHLHB2		0.4341
BF	BF		0.4363
ATIP1	MTUS1		0.4463

IL11RA	IL11RA		0.4464
KIAA0603	TBC1D4		0.4544

152 a The gene name defined by chip

153 b Official gene name

154 c The ratio of Cy5/Cy3. Cy5 is the signal value of the gene detected in HeLa-1. Cy3 is the
155 signal value of the gene detected in HeLa-4

156 **Table2:Part of the differentially expressed genes in miR-141 down-regulated HeLa cell**

Gene name of the Chip a	Gene name ^b	Up-regulation rate ^c	Down-regulation rate ^c
IGFBP3	IGFBP3		0.2691
CREM	-		0.3295
COL15A1	-		0.398
BF	BF		0.4159
GCLC	-		0.4249
UBD	UBD		0.4394
IL6	IL6		0.4556
JDP1	DNAJC12		0.4556
ZNF148	-		0.4593
ETV4	ETV4		0.4601
LOC58489	Q9NPM1		0.4637
CASP8AP2	CASP8AP2		0.4716
HBP17	FGFBP1		0.4746
ATP6C	ATP6V1C1		0.4748

157 a The gene name defined by chip

158 b Official gene name

159 c The ratio of Cy5/Cy3.Cy5 is the signal value of the gene detected in HeLa-2.Cy3 is the
160 signal value of the gene detected in HeLa-4

161 **Table3:Part of the differentially expressed genes in miR-205 up-regulated SiHa cell**

Gene name of the Chip ^a	Gene name ^b	Up-regulation rate ^c	Down-regulation rate ^c
RNP24	P24_HUMAN	4.5834	
PSAP	PSAP	4.1103	
PON2	PON2	3.3356	
COP9	COPS8	3.0867	
FLJ11240	C6orf209	2.7622	
MADH4	SMAD4	2.0531	
PRKAR1A	PRKAR1B	2.1551	
NCK1	NCK1	2.4193	
MADH4	SMAD4	2.0531	
GAS1	-	2.3125	
UMPH1	NT5C3	2.0728	
DUSP5	DUSP5		0.3674
HRY	HES1		0.4964
KIAA1233	ADAMTSL3		0.23
DOCK3	-		0.2523
KRTHA4	KRTHA4		0.2878
DUSP5	DUSP5		0.3674
EGR1	EGR1		0.374
NR4A2	NR4A2		0.3862
KLF2	KLF2		0.4017

CTNND1	CTNND1		0.425
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162 a The gene name defined by chip

163 b Official gene name

164 cThe ratio of Cy5/Cy3.Cy5 is the signal value of the gene detected in SiHa-1. Cy3 is the
165 signal value of the gene detected in SiHa-4

166 **Table4:Part of the differentially expressed genes in miR-141 down-regulated SiHa cell**

Gene name of the Chip ^a	Gene name ^b	Up-regulation rate ^c	Down-regulation rate ^c
FLJ20234	NP_060190	4.3016	
PSAP	PSAP	3.9486	
LOC51714	NP_057359	3.2285	
DPM1	DPM1	3.0777	
SPTLC1	SPTLC1	3.0437	
EIF2S3	EIF2S3	3.0259	
CENPH	CENPH	3.0023	
TLE1	TLE1	2.9681	
SC4MOL	SC4MOL	2.8008	
PON2	PON2	2.7329	
PAFAH1B3	PAFAH1B3		0.3557
DUSP5	DUSP5		0.3891
PPM1B	PPM1B		0.3909
KIAA1233	ADAMTSL3		0.3912
PIGA	PIGA		0.3981
LOC51646	YPEL5		0.4327
DCTN4	DCTN4		0.4409

EGR1	EGR1		0.4445
BACH1	BACH1		0.4471

167 a The gene name defined by chip

168 b Official gene name

169 c The ratio of Cy5/Cy3.Cy5 is the signal value of the gene detected in SiHa-2. Cy3 is the
170 signal value of the gene detected in SiHa-4

171 **Fig2:(A) Hybridization scatter plot of HeLa-1 and HeLa-4.(B) Hybridization scatter**
172 **plot of HeLa-2 and HeLa-4.(C) Hybridization scatter plot of SiHa-1 and SiHa-4.(D)**
173 **Hybridization scatter plot of SiHa-2 and SiHa-4.** The X axis and Y
174 axis coordinates respectively represented the fluorescence signal strength value of two
175 samples, each point in the graph represent one gene. Red and green points respectively
176 represented the ratio of $Y/X \geq 2$ or ≤ 0.5 , black points represented the ratio of Y/X
177 were between 0.5 and 2.0.

178 The function classification of DEGs that was identified between SiHa-1 and SiHa-4 was
179 conducted with GO enrichment analysis. These genes were cataloged according to biological
180 processes (BPs), molecular functions (MFs), and cellular components (CCs) based on GO
181 database, and we showed the most likely (with the lowest P value) relevant 10 Go terms
182 [Fig3].We found that for molecular function, the DEGs mainly involved in the ubiquitin-protein
183 ligase activity, MAP kinase phosphatase activity, transcription factor activity. For biological
184 processes, the DEGs were mostly enriched in ubiquitin cycle, regulation of transcription,
185 DNA-dependent, cell cycle arrest. For cellular Component, the DEGs were distributed mainly
186 in nucleus and mitochondrion.

187 **Fig3: GO enrichment analysis of miR-205 up-regulated SiHa cell, in terms of (A)**
188 **biological process, (B) cellular component and (C) molecular function**

189 The concentration trend of the DEGs between SiHa-1 and SiHa-4 were further defined
190 based on KEGG pathway analysis. Eight pathways with $P < 0.05$ were listed in Table5. The

concentration trend was inversely proportional to the *P* value. The most likely involved pathways were cell cycle, sphingolipid metabolism, ubiquitin-mediated proteolysis, and pyrimidine metabolism. And the DEGs in these signaling pathways were shown in Fig 4.

Table5: The pathways of differentially expressed genes in miR-205 up-regulated

SiHa cell

Pathway Name ^a	Gene name ^b	<i>P</i> value
Cell cycle	SMAD4,MAD2L1,YWHAG	0.004407
Sphingolipid metabolism	DEGS1,SPTLC1	0.007082
Nicotinate and nicotinamide metabolism	NT5C1B,NT5C3	0.007082
Ubiquitin mediated proteolysis	UBE2D3,UBE2D2	0.007735
Valine, leucine and isoleucine degradation	NAT5,ACADM	0.010991
Pyrimidine metabolism	NT5C1B、 NT5C3	0.030525
Bisphenol A degradation	PON2	0.042995
Ethylbenzene degradation	NAT5	0.048587

^a The pathway defined based on KEGG pathway analysis

^b The gene enriched in the signal pathways

Fig4: The differentially expressed genes and KEGG pathways of miR-205 up-regulated SiHa cell were predicted by KEGG.

Discussion

Tumorigenesis is a complex process in which multiple genes are involved. In addition to the expression changes of gene related to cell proliferation, apoptosis and transcription, the changes connected with some biological processes in cells also play a part.

Recent years, miRNAs have been considered as key regulatory molecules of multiple signaling pathways including tumor development, differentiation, proliferation, apoptosis and invasion.(9-10) The increasing maturity of gene expression profile microarray technology provides the foundation for finding out the mechanism of tumorigenesis on the whole. In our research, we up-regulated miR-205 in SiHa cell, discovered that the DEGs covered multiple biological processes, mainly including ubiquitin cycle, DNA-dependent transcriptional regulation, cell cycle arrest and transcription. These genes also involved in multiple signaling pathways such as cell cycle and tumor formation. Therefore, we believe that *miR-205*, a target for the regulation of the occurrence and development of cervical cancer, can provide better support and help for the treatment of cervical cancer by regulating its expression.

MiR-205 is located in chromosome 1q32.2. Low expression of miR-205 have been found in liver cancer, prostate cancer, breast cancer, renal cell cancer and so on.(11-13) While in ovarian cancer, miR-205 is highly expressed.(14) Therefore, we supposed that miR-205 exerts different effects on diverse cancers. Pang, h. and x. Yue's et al. found that the ectopic expression of miR-205 inhibited the proliferation of CC cells and promoted apoptosis. Suggesting that miR-205 may be an anti-oncogene for cervical cancer.(15)

Our study found that after up-regulating miR-205, the expression of Smad4 in SiHa cells increased. Smad proteins are found in the cytoplasm and are signaling transduction elements of the transforming growth factor- β (TGF- β) superfamily.(16) Transforming growth factor- β (TGF- β) family members exert their function via specific type I and type II serine/threonine kinase receptors and intracellular Smad transcription factors.(17) The SMAD family can be divided into three categories: receptor-regulated Smad (R-Smad); Co-mediator Smad (Co-Smad); and the inhibitory Smad (I-Smad).Smad4 is the only Co-Smad. R-smads (Smad1, 2, 3, 5, 8) are directly phosphorylated and activated by type I receptor kinase and undergo homotrimerization and formation of heteromeric complexes with *Smad4*. Then, activated Smad compound are transferred to the nucleus, and regulate

the transcription of target gene together with other nuclear coenzyme factors.(18) The absence of Smad4 plays an important role in skin and upper gastrointestinal squamous cell carcinoma and gastrointestinal adenocarcinoma.(17) Baldus, S.E et al. analyzed the expression of Smad4 in 13 cervical cancer cell lines, 40 precancerous lesions and 41 primary squamous cell carcinoma specimens, found that the loss of Smad4 may play an significant part in the occurrence of cervical cancer.(19)

More and more evidence indicates that microRNAs (MiRNAs) exert an enormous function on TGF- β /smad pathway-induced tumor suppression.(20-22) Members of the TGF- β family regulate a variety of biological processes, including morphogenesis, embryonic development, adult stem cell differentiation, immune regulation, wound healing, inflammation, and cancer. In the early stage of tumorigenesis, TGF- β can inhibit the proliferation but promote apoptosis of epithelial cells, endothelial cells and hematopoietic cells. In advanced tumor, TGF- β can promote the migration and invasion of cancer cells, facilitate angiogenesis and induce immunosuppression, the increase of TGF- β is related to the increased aggression of tumor and poor prognosis of patients.(23)

Smad4 protein significantly affects tumor angiogenesis, tumor proliferation, invasion, metastasis and other biological behavior mainly through TGF- β signal pathway. In pancreatic cancer, TGF- β inhibits cell growth and proliferation by blocking mitogenic growth signals through Smad4. It also induces programmed cell death (PCD) or apoptosis of pancreatic cells by regulating the TIEG, a zinc-finger gene transducted by TGF- β /Smad4 signaling, and takes part in tumor inhibition.(24) Smad proteins regulate the TGF- β signaling pathway, and the absence of the tumor suppressor. Smad4 is one of the molecular mechanisms leading to TGF- β resistance. Zhu, H et al. summarized the expression levels of TGF- β in cervical intraepithelial neoplasia (CIN), cervical cancer and normal tissues, concluded that TGF- β 1 functions as a tumor inhibitor in precancerous lesions of cervical cancer and early cervical cancer, while as a tumor promotor in advanced tumors.(25)

256 The expression proteins of HPV16 push forward an immense influence on the regulation of
 257 TGF- β pathway through Smads protein. Among them, HPV-16 E7 protein is mainly related to
 258 immune stress and the promotion of tumor cell proliferation.(26) Lee, D.K. et al.
 259 demonstrated that E7 protein can bind to Smad2, Smad3 and Smad4, and block
 260 the interaction of Smad complex with the Smad DNA binding element, CAGA. Suggesting
 261 that E7 oncoprotein inhibits the signaling transduction of TGF- β by inhibiting Smad DNA
 262 binding activity.(27) The expression of HPV 16 E5 can attenuate the TGF- β /Smad signaling,
 263 and the loss of signal transduction can lead to the imbalance of epithelial homeostasis in the
 264 early stage of viral infection, which may be an crucial mechanism to promote the occurrence
 265 of HPV mediated cervical cancer.(28) Peralta-zaragoza et al. found that E6 and E7
 266 oncoproteins can regulate the expression of TGF- β 1 through increasing the activity of TGF-
 267 β 1 promoter. Which reveals the contribution of HPV-16 to TGF- β 1 gene expression in
 268 cervical cancer.(29)

269 TGF- β -induced growth responses differ between SiHa and HeLa cells. In SiHa cells, TGF-
 270 β -mediated growth inhibition depends on the nuclear accumulation of activated Smads. In
 271 HeLa cells, the cell cycle progression induced by TGF- β is presumably independent of Smad
 272 activation.(30) Our sequencing results showed that after up-regulating miR-205, the ratio
 273 value of Smad4 gene in HeLa cells was 1.3294, which was not statistically significant, while
 274 the expression of Smad4 gene in Siha cells was up-regulated. HeLa cells integrate with
 275 HPV18 genome. SiHa cells integrated with the HPV16 genome. Therefore, we believe that
 276 Smad4 regulates TGF- signaling pathway to exert carcinogenesis and whether it is related to
 277 HPV phenotype remains to be explored.

278 The results of gene microarray in our study reflected the gene expression changes of
 279 cervical cancer cells HeLa and SiHa after up-regulating miR-205 or down-regulating *miR-*
 280 *141*. Besides, we analysed the signal pathways in which the DEGs of miR-205 up-regulated
 281 SiHa cell participated, but functional experiments were needed to further verify the molecular

mechanism of these gene changes. From the comprehensive analysis of microarray, the expression of genes after up-regulating miR-205 changed towards the direction of inhibiting the malignant phenotype of cervical cancer. A group of genes were found, which provided further research direction for illuminating the molecular mechanism of the carcinogenesis of miR-205.

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Conflicts of interest

There are no conflicts of interest.

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359

Figure1

A

B

C

D

Figure2

A

B

C

Figure3

CELL CYCLE

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Figure4