Acta Biochim Biophys Sin 2022, 54(8): 1090–1099
https://doi.org/10.3724/abbs.2022101
Advance Access Publication Date: xx xx 20xx
Original Article

Original Article

Multiple circRNAs regulated by QKI5 conjointly sponge miR-214-3p to antagonize bisphenol A-induced spermatocyte toxicity

Huimin Li[1,2], Yunhan Zhao[1], Qiuzi Shen[1], and Honggang Li[1,3,*]

[1]Institute of Reproductive Health/Center of Reproductive Medicine, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China, [2]Guilin Medical University, Guilin 541000, China, and [3]Wuhan Tongji Reproductive Medicine Hospital, Wuhan 430030, China

*Correspondence address. Tel: +86-27-83692651; E-mail: lhgyx@hotmail.com

Received 28 September 2021 Accepted 07 January 2022

Abstract

Although circular RNAs (circRNAs) are found to play important roles in many pathophysiological processes, the canonical theory that they act as microRNA sponges is now more and more challenged, given that most circRNAs only have few binding sites in a particular microRNA. Our previous study revealed that some up-regulated circRNAs play protective roles in bisphenol A (BPA)-induced toxicity in GC-2 germ cells. Here by CCK-8 assay, apoptosis assay, qRT-PCR and western blot analysis, we further discover that circRNAs (represented by circDcbld2, circMapk1 and circTbcld20) can cooperatively sponge miR-214-3p and then up-regulate AKT1 in ameliorating BPA-induced reproductive toxicity. They share binding sites with miR-214-3p and collectively reinforce the sponging effects. In addition, the upstream regulation mechanism, proven by bioinformatics analysis and *in vitro* gain- and loss-of-function study, shows that down-regulation of RNA binding protein QKI5 after BPA exposure can increase the expressions of these protective circRNAs, and thus activate the cell protective process. The QKI5-circDcbld2/circMapk1/circTblcd20-miR-214-3p-AKT1 axis ameliorates the toxic effect of BPA on GC-2 cells. Many other circRNAs up-regulated upon BPA treatment and QKI5 down-regulation also show binding sites with miR-214-3p. Thus the above axis may also be extrapolated to other circRNAs. Our results enrich the context of circRNA sponge mode and may provide new ideas in future multiple nucleic acid therapy.

Key words circular RNA, BPA, QKI5, miR-214-3p, AKT1

Introduction

Circular RNAs (circRNAs) are covalently closed RNA loops that were discovered more than 40 years ago [1]. With the widespread application of high-throughput sequencing, researchers came to realize their wide and abundant existence across various species [2,3]. Since the discovery of circRNA CDR1as as a miR-7 sponge in 2013 [4], interest in the research of circRNAs has been increasing [5]. The biogenesis of circRNAs is mainly driven by "exon skipping" or "direct back splicing" in which 3′ and 5′ terminus of circRNAs are covalently joined together to form single-stranded continuous loop [6]. circRNAs can work as microRNA (miRNA) sponges, transcription regulators, protein scaffolds and peptide translators [5,7]. With prominent features like high stability, abundance, evolutionary conservativeness and tissue/development specificity [5], circRNAs hold great promise to be potent regulators of gene expression. Meanwhile, circRNAs have also been found to take active part in many pathophysiological processes [5], like cancer, cardiovascular diseases, neuropathies, osteoarthritis, reproductive disorders, toxic disease, *etc.*

However, with the in-depth research in circRNAs, a number of bewildering issues emerged [5]. One of the most noticeable questions is their roles in serving as miRNA sponges. Most circRNAs revealed by high-throughput sequencing are at the median length of approximately 300–500 nt [8]. At such short length, circRNAs are very limited in providing enough space for sponging miRNAs, especially for a particular miRNA. Many of the subsequently reported miRNA-sponging circRNAs only have one binding site or very few binding sites for a specific miRNA [8]. This inevitably

made researchers doubt their actual sponging potency. The only two circRNAs that are widely recognized as classical circRNA sponges are CDR1as [4] and circSry [9]. They each have 73 and 16 binding sites for one particular miRNA respectively. Many subsequent studies revealed that circRNAs have very limited binding sites for one particular miRNA [5]. Apart from the limited binding sites in circRNAs for one particular miRNA, the abundance of most circRNAs is also generally low [10]. These facts obviously challenge the sponge theories.

However, if we see the fact the other way around, *i.e.*, multiple circRNAs may cooperatively sponge one miRNA rather than the one-to-one circRNA/miRNA mode, it will be much more reasonable. In fact, under real *in vivo* circumstances, the ways circRNAs interact with miRNAs might be manifold. One circRNA may interact with many miRNAs and many different circRNAs may sponge one miRNA. This assumption has been preliminarily confirmed by one recent research concerning the function of circRNAs in pig testis [11]. By in-depth bioinformatics analysis, the researchers concluded that collective role of different circRNAs should exist, as opposed to, or in addition to functions of individual circRNA. Experimentally, a group of other researchers also found that many circRNAs are activated and play protective roles in benzoapyrene-induced carcinogenicity by binding to miR-181-1-3p [12]. These circRNAs that sponge miR-181-1-3p seemed to function in a cooperative way. However, this is only a bioinformatic prediction that was not experimentally confirmed in their study. The above results from one aspect support the presumption that cooperative effects of circRNAs may exist, or even, be the major way of most circRNAs.

Our previous study also suggested the cooperative effects of circRNAs. We used next-generation sequencing method to unveil the RNA (circRNAs, miRNAs and mRNAs) landscape in bisphenol A (BPA)-exposed and unexposed GC-2 cells, a mouse spermatocyte-like cell line [13]. BPA is a synthetic organic compound that has been widely used in industrial materials, food packaging and personal hygiene products [14]. BPA has weak estrogenic, antiandrogenic and antithyroid activities and it is considered as one of the most important and representative endocrine disrupter [14]. A number of studies have explored the mechanism of spermatocyte cell death caused by BPA. BPA may trigger germ cell apoptosis by activating the mitochondria-mediated pathway [15], the estrogen receptors and GPR30 pathway [16], the reactive oxygen species (ROS)-regulated PERK/EIF2α/chop pathway [17], *etc.* In our previous study, we surprisingly found that a number of circRNAs were up-regulated after BPA exposure and showed protective roles in BPA-induced cell toxicity [13]. Notably, these circRNAs show synergistic effects in ameliorating cell death. However, how these circRNAs act together into the conjoint downstream mechanism, and how these protective circRNAs are generated under BPA exposure still need to be further investigated.

In this study, we found that circRNAs (represented by circDcbld2, circMapk1 and circTbcld20) can cooperatively sponge miR-214-3p and then up-regulate AKT1 in ameliorating BPA-induced reproductive toxicity. The down-regulated RNA binding protein QKI5 after BPA exposure can increase the expressions of these protective circRNAs, and thus activate the cell protective process. Our results enrich the context of circRNA sponge mode and may provide new ideas in future nucleic acid therapy for better efficacy.

Materials and Methods

Cell culture and treatment

GC-2 spd(ts) (#CRL-2196) cells were purchased from ATCC (Manassas, USA) and cultured in DMEM high glucose medium (Hyclone, Logan, USA), with 10% fetal bovine serum (Sijiqing, Hangzhou, China) and 1% penicillin-streptomycin (Hyclone) at the temperature of 37°C and 5% CO_2. After synchronizing in DMEM without FBS for 12 h, the cells were treated with various concentrations of BPA (Sigma-Aldrich, St Louis, USA) for 48 h in 6-well plates (Corning, New York, USA). BPA was dissolved in DMSO and diluted with complete medium to desired concentration before use. Cells treated with complete medium with 0.1% (v/v) DMSO were used as the control. Our dose-inhibition curve showed that the IC_{50} of BPA for GC-2 cells is approximately between 120 μM and 160 μM (results shown in our previous study [13]).Though in human exposure studies, BPA was generally set at lower levels, higher levels of BPA were usually used in *in vitro* studies [18]. Our previous study also examined the expression of several circRNAs under exposure to different concentration of BPA (0 μM, 40 μM, 80 μM, 120 μM, 160 μM). They all showed consistently elevated trend along with the increase of the BPA concentration [13]. Therefore, in the present study we chose 120 μM as the representative dose.

Cell transfection

miRNA mimics and siRNAs were synthesized by GenePharma (Suzhou, China). siRNA for QKI was designed at the mutual sequences of all mouse QKI isoforms. The sequences used are listed in Table 1. Transfection was performed using Lipofectamine 2000 (Life Technologies, Carlsbad, USA) according to the manufacturer's instructions.

circRNA overexpression vectors were constructed with the pLCDH-ciR vector (Geenseed Biotech, Guangzhou, China) as previously described [13]. A mock vector contains a nonsense stuffer was used as the control for circRNA overexpression. As to the amount of plasmid, 10 μg of circRNA overexpression plasmid and mock vectors were transfected into the cells. In the co-transfection experiment, 3.33 μg of circMapk1, circDcbld2 and circTblcd20 were added together to the total amount of 10 μg. The primers used for vector construction were listed in Supplementary Table S1. For regular gene/protein overexpression, overexpression vectors were constructed using pVAX1 vector with restriction-enzyme digestion and ligation methods. An empty pVAX1 plasmid was used as the control. Primers used are listed in Supplementary Table S2. The constructed transcripts were shown in Supplementary Figure S1. All constructs were verified by Sanger sequencing. Plasmids were delivered into the GC-2 cells (a mouse spermatocyte-like cell line)

Table 1. Sequences of miRNA mimics

Name	Sense sequence (5′→3′)	Antisense sequence (5′→3′)
NC	UUCUCCGAACGUGUCACGU	ACGUGACACGUUCGGAGAA
miR-214-3p	ACAGCAGGCACAGACAGGCAGU	UGCCUGUCUGUGCCUGCUGUUU
si-QKI	CGCCAACAUUAAAUCACCAGCCCUU	AAGGGCUGGUGAUUUAAUGUUGGCG
si-ADAR1	CCAGUACUGUGUAGCAGUA	UACUGCUACACAGUACUGG

using the GenJet™ Plus DNA In Vitro Transfection Reagent (SignaGen Laboratories, Shanghai, China) according to the manufacturer's protocol.

Cell viability assay

CCK-8 assay was done to assess cell proliferation rate of the cells. Briefly, GC-2 cells were seeded in a 96-well plate at a density of 5000 cells per well and cultured overnight. And 36 or 48 h after transfection, 10 μL of CCK-8 reagent (Dojindo, Kumamoto, Japan) was added to each well and incubated for 1–4 h at 37°C and 5% CO_2. The absorbance of each well was assessed at 450 nm with a microplate reader (Bio-Tek, Winooski, USA).

Detection of cell apoptosis by flow cytometry

The apoptosis assay kit (Sungenebiotech, Beijing, China) was used to examine apoptotic cells 48 h after transfection and BPA treatment according to the manufacturer's instructions. In brief, 5×10^5 cells were collected and resuspended in 100 μL 1× binding buffer (Sungenebiotech). Five microliters Annexin V-APC and 5 μL 7-AAD staining solution (Sungenebiotech) were added, followed by incubation at room temperature for 10 min in the dark. Then 400 μL 1× binding buffer was added, and cells were analyzed by flow cytometry on a BD flow cytometer (BD Biosciences, Franklin Lakes, USA) within 1 h.

Dual-luciferase reporter assay

pmirGLO dual luciferase reporter vector (Promega, Beijing, China) was used for dual-luciferase reporter assay. The wild-type sequences of circMapk1, circDcbld2 and circTbcld20 were respectively cloned into the vector. Primers used for vector construction were listed in Supplementary Table S3. And so were the mutated sequences of the above circRNAs with corresponding mutated miRNA binding sites. Wide-type circRNA vector/mutated vector and miRNA mimics/negative control were co-transfected into cells in 96-well plates using Lipofectamine 2000 according to the manufacturer's instruction. Lysates were harvested and processed 36 h after transfection as the instructions of Luc-Pair Duo-Luciferase HS Assay Kit (GeneCopoeia, Rockville, USA). The luciferase activity was measured with a dual luciferase reporter assay system (Bio-Tek). For comparison, the FL (Firefly luciferase) activity was normalized with RL (*Renilla* luciferase) activity.

PCR and quantitative real-time PCR

PrimeScript RT reagent kit DRR047 (Takara, Dalian, China) was used for the cDNA synthesis of circRNAs and mRNAs. For miRNA cDNA synthesis, specific stem-loop RT primers for *U6* and miRNA were used instead of the RT primer Mix in the kit. qPCR analysis was performed using SYBR Premix Ex Taq II (Takara). *β-Actin* (*Actb*) was used as the internal control for circRNAs and mRNAs, and *U6* for miRNAs. The sequences of primers are listed in Supplementary Tables S4, S5 and S6.

Western blot analysis

Cells were lysed with RIPA buffer (Beyotime, Shanghai, China). The protein concentration was quantified using BCA protein assay kit (Beyotime). The following steps were done as mentioned in previous study [24]. Immunoreactive bands were revealed using BeyoECL Plus kit (Beyotime). The images were acquired by using Bio-Rad ChemiDoc™ XRS + System (Bio-Rad, Hercules, USA). Antibodies against the following proteins were used: QKI5 (13169-1-AP, 1:1000 dilution; Proteintech, Rosemont, USA), AKT1 (55230-1-AP, 1:1000 dilution; Sanying, Wuhan, China), β-actin (60008-1-Ig, 1:1000 dilution; Sanying), α-tubulin (ab7291, 1:1000 dilution; Abcam, Cambridge, UK). HRP-conjugated goat anti-rabbit IgG (AS1107, 1:1000 dilution; ASPEN, Wuhan, China) or goat anti-mouse IgG (AS1106, 1:1000 dilution; ASPEN) was used as the secondary antibody.

Bioinformatics analysis

circRNA/miRNA interaction were predicted by circPrimer2.0 (https://www.bio-inf.cn) and RegRNA 2.0 (http://regrna2.mbc.nctu.edu.tw) with default parameters. miRNA/mRNA interactions were predicted by miRanda with Top Energy ≤ −10. The circRNA-miRNA-mRNA interaction network was constructed and visualized by Cytoscape(3.9.1) based on the same target miRNAs of circRNAs and mRNAs. DIANA miRPath v.3.0 (http://www.microrna.gr/miRPathv3) was applied for miRNA pathway analysis with the criterion of $P < 0.05$. The interaction between RCMs and RBPs was predicted with the website RNA-Protein Interaction (RPISeq) (http://pridb.gdcb.iastate.edu/RPISeq).

Statistical analysis

Data are expressed as the mean ± SEM. All experiments were performed at least three times. Western blot analysis and FCM results are representative of three independent experiments. The unpaired *t* test was used for statistical analyses. Results were analyzed by Student's *t*-test or one-way ANOVA using Prism version 5 (Graphpad, La Jolla, USA). Post hoc comparisons were done with Dunnett's *t* test. Statistic tests were all two tailed and $P < 0.05$ was considered as significant. *P* value was adjusted in the post hoc comparison according to the multiple comparison times.

Results

circDcbld2, circMapk1 and circTbcld20 serve as miR-214-3p sponges

Our previous study demonstrated that most up-regulated circRNAs have protective effects in antagonizing BPA toxicity in GC-2 cells [13], and synergistic effects of multiple circRNAs were observed. Though BPA exerts toxic effects on these cells, somehow a self-protective mechanism was induced. A number of circRNAs were up-regulated under BPA treatment, including circCnot2, circCnot6l, circDcbld2, circMapk1, circMpp6, circPphln1, circPpm1b, circTbcld2, circUsp3, and circZranb1 [13]. Meanwhile, some of these circRNAs like circDcbld2, circMapk1, circMpp6 and circTbcld20 showed protective effects on the cells. Notably, co-transfection with circDcbld2, circMapk1 and circTbcld20 showed better effect than single circRNA transfection with the same total amount (Figure 1A). These results prompt us to present the hypothesis that these circRNAs may act synergistically through sponging the same miRNA. We predicted that many of the up-regulated circRNAs showed binding sites with one miRNA, *i.e.*, miR-214-3p (Figure 2). Among these circRNAs, the overexpression plasmids of circDcbld2, circMapk1 and circTbcld20 were of relatively high efficiency [13]. Since the effects of circRNAs with low expression levels are difficult to observe, we choose these three circRNAs as representatives for the possible cooperative effects.

Subsequent confirmation experiments using apoptosis assay after circRNAs transfection demonstrated that these three circRNAs can

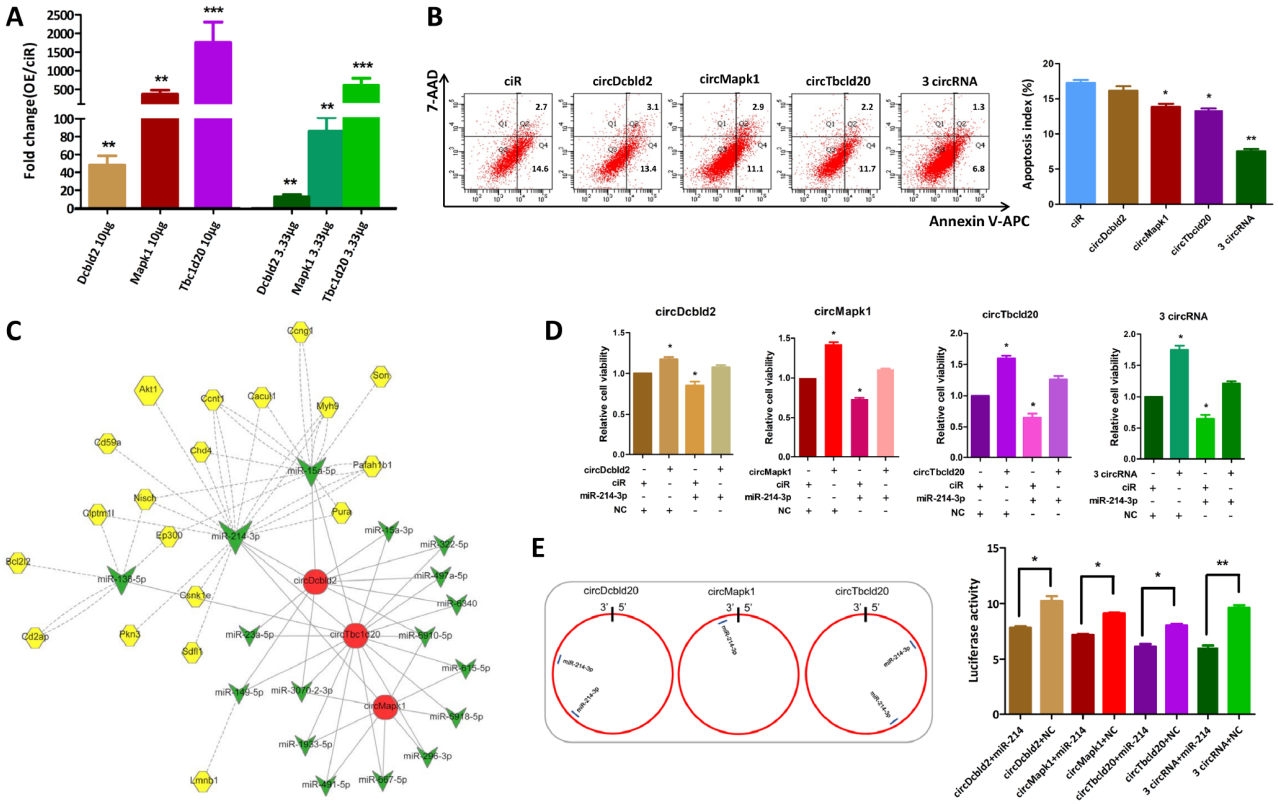

Figure 1. The cooperative effect of circDcbld2, circMapk1, circTbcld20 by sponging miR-214-3p in GC-2 cells (A) The expression level of circRNA after overexpression plasmid was transfected individually and jointly. OE: overexpression. circRNAs were transfected in the amount of 10 μg individually. (B) The cooperative effect of circDcbld2, circMapk1, circTbcld20 by sponging miR-214-3p in GC-2 cells. Left panel: apoptosis assay revealed that circDcbld2, circMapk1 and circTbcld20 can all alleviate BPA toxicity respectively and collectively. ciR: empty vector serves as circRNA control. Right panel: quantitative apoptosis assay data. GC-2 cells were treated with 120 μM BPA in all groups. Apoptosis was determined by flow cytometry using Annexin V-APC/7-AAD staining. (C) The ceRNA network of circDcbld2, circMapk1, circTbcld20 and their downstream miRNAs and target genes. Red circles: circRNAs; Green arrows: miRNAs; Yellow hexagon: mRNAs. miRNAs (miR-15a-5p, miR-138-5p, and miR-214-3p) that are involved in cell viability and death processes are presented as larger arrows. (D) CCK-8 assay revealed that co-transfection of these three circRNAs respectively and synergistically relieved the cell viability-inhibiting role of miR-214-3p. NC: random sequence serves as miRNA control. The result of circMapk1 can also be seen in our previous work [13]. (E) The binding sites of circRNAs with miR-214-3p. Luciferase reporter assay confirmed the interaction of miR-214-3p with the three circRNAs separately and collectively. *$P < 0.05$, **$P < 0.01$.

Figure 2. The putative binding sites of site of circRNAs and with miR-214-3p (A) circCnot2. (B) circDcbld20. (C) circMapk1. (D) circMpp6. (E) circPphln1. (F) circTbcld20-miR-214-3p. (G) circZranb1-miR-214-3p.

achieve the best protective effects in GC-2 cells when transfected together (Figure 1B). We found that the group of circDcbld2, circMapk1 and circTbcld20 achieved the best protective effect among single circRNA transfection, though the total amount of circRNAs were equal in all the groups (BPA = 120 μM in all groups). Then ceRNA network was delineated to explore the possible downstream mechanism of these three circRNAs (Figure 1C). Among these downstream miRNAs, three of them (miR-15a-5p, miR-138-5p and miR-214-3p) are significantly involved in processes like cell division, cell cycle and cell death in miRPath GO analysis. Meanwhile, miR-214-3p was the mutual downstream miRNA of these three circRNAs, whereas the other two miRNAs, miR-15a-5p and miR-138-5p, were only predicted to be sponged by two circRNAs. Subsequent miR-214-3p transfection experiment showed that it can markedly decrease cell viability (Figure 1D). Co-transfection with circRNAs and miR-214-3p showed that these circRNAs can remarkably alleviate the viability-inhibiting role of miR-214-3p (Figure 1D). The binding sites of miR-214-3p were predicted by RegRNA 2.0 and shown in Figure 1E. Dual-luciferase assay also confirmed the interaction of miR-214-3p with circDcbld2, circMapk1 and circTbcld20 (Figure 1E). They each can interact with miR-214-3p and the co-transfection of the reporter of the three circRNAs showed the best effect. These results suggest that circDbcld2, circMapk1 and circTbcld20 can all sponge miR-214-3p and alleviate its toxic effect on GC-2 cells.

miR-214-3p inhibits cell viability by targeting Akt1

To find out the downstream target genes of miR-214-3p, miRTarBase (https://mirtarbase.cuhk.edu.cn) was searched for the already validated target genes of miR-214-3p reported by other researchers. Twelve possible function-related genes, including *Akt1*, *Ccl9*, *Cd59a*, *Csnk1e*, *Ctgf*, *Ezh2*, *Golga2*, *Itgav*, *Msrb3*, *Pkn3*, *Pon2* and *Sdf2l1*, were searched out (References for the twelve genes were listed in Supplementary Table S7), and subject to further experimental screening. After ectopic expression of miR-214-3p in GC-2 cells, five genes, including *Akt1*, *Cd59a*, *Csnk1e*, *Pkn3* and *Sdf2l1*, were significantly down-regulated (Figure 3A), and they were tentatively supposed as the target genes of miR-214-3p in GC-2 cells. We then overexpressed circDcbld2, circMapk1 and circTbcld20 respectively in GC-2 cells to explore which genes were up-regulated by these circRNAs. Those genes that are down-regulated by miR-214-3p and up-regulated by circRNAs should be their mutual targets. After intersecting the genes that were down-regulated by miR-214-3p and up-regulated by circRNA overexpression, three potential mutual downstream genes, i.e., *Akt1*, *Ctgf* and *Pkn3*, were preliminarily screened out. Further gain-of-function study (overexpression efficiency shown in Figure 3B) showed that only overexpression of *Akt1* can observably increase the cell viability of GC-2 cells (Figure 3C). AKT1 is one of three closely related serine/threonine-protein kinases (AKT1, ATK2 and AKT3) and generally has anti-apoptosis effects on cells. *Akt1* may be the actual common target gene of circRNAs and miR-214-3p [19]. The alignments of miR-214-3p and the 3′UTR region of *Akt1* were revealed by miRTarBase and illustrated in Figure 4. Subsequent dual luciferase assay (Figure 3D) and western blotting (Figure 3E) further confirmed that miR-214-3p can bind to the 3′UTR region of *Akt1* and inhibit its

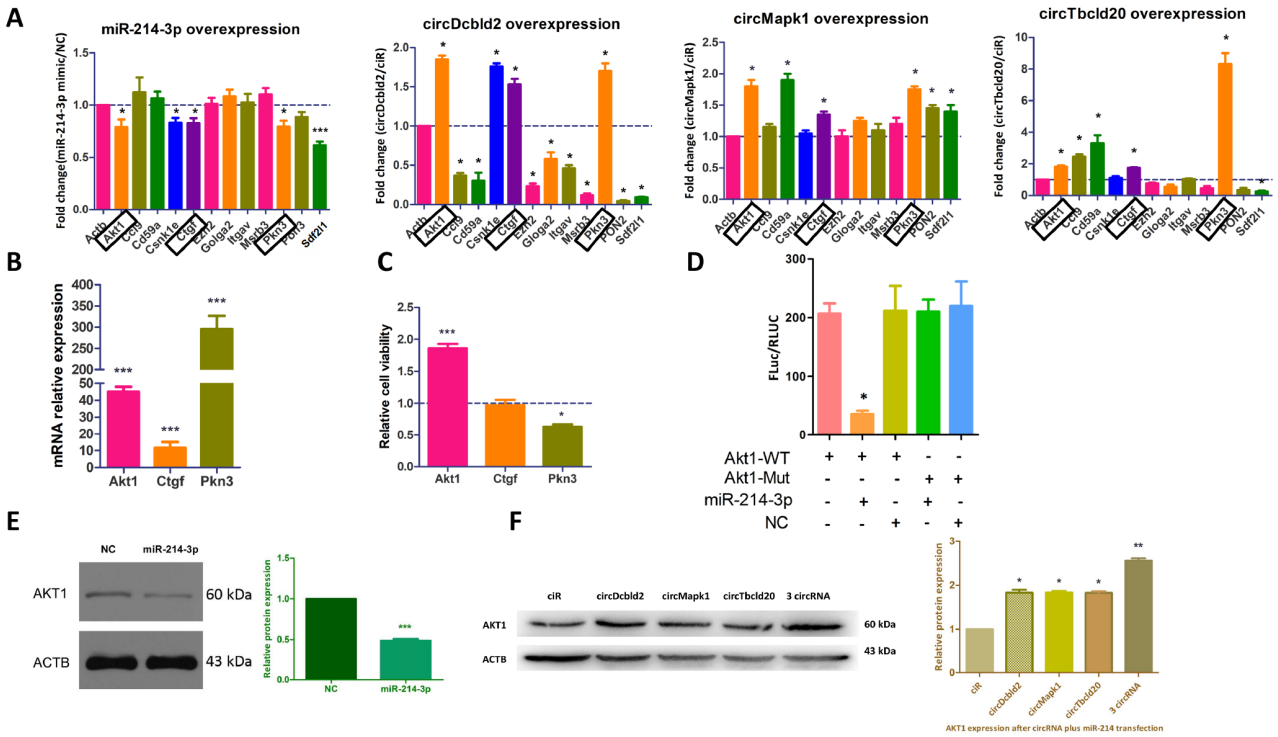

Figure 3. *Akt1* is the mutual target gene of these three circRNAs by sponging miR-214-3p (A) The expressions of predicted target genes of miR-214-3p after miR-214-3p transfection in GC-2 cells. The expressions of the genes after circDcbld2, circMapk1 and circTbcld20 overexpression. The rectangles mark the mutual downstream target genes of circDcbld2, circMapk1 and circTbcld20 via sponging miR-214-3p. (B) The overexpression efficiency of Akt1, Ctgf, and Pkn3 overexpression plasmid. (C) Cell viability of GC-2 cells after overexpression of three potential mutual target genes. Only AKT1 overexpression showed protecting effect. (D) Luciferase reporter assay confirmed the interaction of miR-214-3p with the 3′ UTR region of Akt1. (E) Overexpression of miR-214-3p down-regulated the expression of AKT1. (F) Western blot analysis showed that these three circRNAs can all upregulate the expression of AKT1, with co-transfection showing the best effect. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

expression.

circDcbld2, circMapk1 and circTbcld20 protect cells against BPA toxicity by upregulating AKT1

We then explore whether circDcbld2, circMapk1 and circTbcld20 can regulate the expression of *Akt1*. Overexpression of circDcbld2, circMapk1 and circTbcld20 can up-regulate the protein level of *Akt1* both individually and synergistically, with synergistic transfection showed the most prominent effect (Figure 3F). All these results collectively suggest that circDcbld2, circMapk1 and circTbcld20 can promote the cell viability by sponging miR-214-3p and upregulating AKT1. The enhanced cooperative effects of them may be resulted from the increased binding site of the same miRNA. Though limited binding sites of miRNA are found on one single circRNA, the mutual effects of multiple circRNAs provide sufficient sites for the sponging effects. circDcbld2, circMapk1 and circTbcbld20 altogether sponge miR-214-3p, restrain its effect, and upregulate the expression of *Akt1*. The up-regulation of AKT1 can enhance the viability of GC-2 cells. Thus these three circRNAs were found to protect cells from BPA toxicity in a cooperative way.

ID	Duplex structure	Position	Score	MFE
1	miRNA 3' ugaCGGACA--GACAC--GG-ACGACa 5' Target 5' ccaGTTTGTTGCTGTGTCCCATGCTGt 3'	545 - 571	135.00	-20.10
2	miRNA 3' ugaCGGAC-----AGACACGGACGACa 5' Target 5' cccGCATGGATCATCTGAACCTGATGt 3'	57 - 83	123.00	-11.90
3	miRNA 3' ugACGGACA--GACACGGACGACa 5' Target 5' gaTGCTT-TCCCTCT-CCCGCTGt 3'	712 - 733	120.00	-11.00

Figure 4. The binding sites of miR-214-3p with the 3′UTR region of *Akt1* The three binding sites were all mutated in luciferase reporter assay.

High expression of ESR2 has no effect on circRNA biogenesis

The above results demonstrated that these three circRNAs can sponge the same miRNA to act cooperatively. We then asked the question whether a common or a main mechanism may exist in the up-regulation of circRNA expression after BPA exposure. We first tried to seek if there are some transcription factors that may influence the expression of circRNAs. Potential promoter regions (2000 bp upstream region from TSS) of these three circRNAs were retrieved from UCSC (https://www.ucsc.edu) and were searched in JASPAR (https://jaspar.genereg.net) for possible binding transcription factors. After taking intersections and focusing on those transcription factors that are involved in estrogen-related pathways, we selected *Esr2* for further analysis. Motif of *Esr2* is shown in Figure 5A. *Esr2* mRNA was upregulated after BPA exposure (Figure 5B) and was predicted to bind to the promoter regions of circDcbld2, circMapk1 and circTbcld20 (Supplementary Figure S2). However, in the gain-of-function study of ESR2 (overexpression efficiency shown in Figure 5C), expressions of these three circRNAs were not elevated after overexpression of this transcription factor, with or without BPA treatment (Figure 5D,E). Additionally, the corresponding mRNA levels of these three circRNAs were not changed (Figure 5D,E). These results suggest that ESR2 may not be involved in the regulation of BPA-altered circRNA expression.

BPA down-regulates QKI5 and promotes circRNA biogenesis

Next we sought to explore if these circRNAs are influenced by RNA

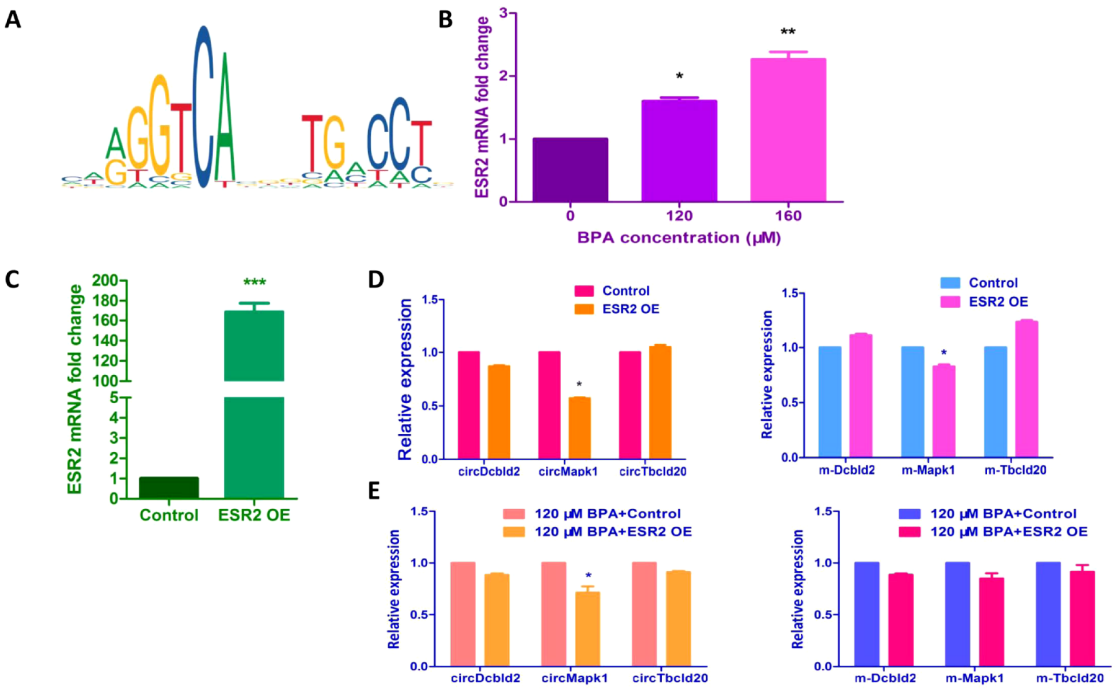

Figure 5. ESR2 does not regulate the expressions of circDcbld2, circMapk1 or circTbcld20 in GC-2 cells (A) ESR2 motif logo presented by JASPAR database. (B) ESR2 mRNA expression levels were elevated after BPA exposure, especially under higher BPA dose. (C) pVAX1-based ESR2 overexpression vector realized high ESR2 overexpression efficiency. (D) Overexpression of ESR2 did not promote neither circDcbld2, circMapk1, circTbcld20 (left panel) nor the mRNAs of the three corresponding genes (right panel). (E) Under BPA exposure, ESR2 overexpression did not upregulate the expressions of these circRNAs and mRNAs. $*P<0.05$, $**P<0.01$, $***P<0.001$.

binding proteins (RBPs) like QKI [20], ADAR1 [21], FUS [22], DHX9 [23], *etc.* RBPs are generally considered to bind to the reverse complementary matches (RCMs) flanking circRNAs and promote or inhibit the biogenesis of circRNAs. After retrieving and analyzing the intron sequences flanking the previously-studied circRNAs [13], we found that half of the circRNAs, which were up-regulated after BPA exposure, including circCkap5, circMapk1, circMpp6, circPpm1b, circTbcld20 and circUsp3, possess RCMs (Supplementary Table S8), and the length and the matching extent of these RCMs are rather considerable (Supplementary Table S9 and Supplementary Figures S3–S6). These provide solid basis for the effect of RBPs. In the prediction of interaction between different RBPs and these RCMs, we found the highest scores of possible interaction between QKI5/ADAR1 and RCMs of these circRNAs (Supplementary Tables S10 and S11). So we chose these two RBPs for experiment validation. siRNAs for these two RBPs were synthesized according to the literatures [24,25] and high knockdown efficiencies of these two proteins were achieved (Figure 6A,B), and circRNAs were significantly upregulated upon *QKI5* knockdown, but not altered after *ADAR1* knockdown (Figure 6C). In the gain-of-function study, QKI5 overexpression (efficiency shown in Figure 6D) inhibited the expressions of most circRNAs (Figure 6E).

In addition, BPA exposure can significantly suppress the level of QKI5, and the trend was pronounced along with the increase of BPA concentration (Figure 6F). On the other hand, knockdown of *QKI5* in GC-2 cells led to increased cell viability, while overexpression of QKI5 inhibited the viability of GC-2 cells (Figure 6G), consistent with the results of other researchers who found that QKI5 can promote apoptosis in germ cells [26]. All these data suggest the promoting effect of QKI5 on these anti-apoptotic circRNAs. So here we summarize the regulating axis in Figure 7. BPA exposure restrains the expression of QKI5, and then the lower level of QKI5 promotes the biogenesis of circRNAs. These protective circRNAs function as a group in serving as pro-apoptotic miRNA sponges in the anti-toxic mechanism of cells. Multiple circRNAs regulated by QKI5 conjointly sponge miR-214-3p to antagonize BPA-induced spermatocyte toxicity.

Discussion

With the help of the next-generation sequencing method, those circRNAs that were used to be sporadically reported in the past are now growing at a tremendous speed across various species [13]. Their functions and action mechanisms have been much concerned by researchers. In the present study, we found that besides the one-to-one circRNA/miRNA sponge mode, a number of circRNAs can synergistically sponge one miRNA and then work on one target gene to protect GC-2 cells from BPA-caused toxicity. Our work enriches the context of circRNA regulation mode and may enlighten future circRNA research and treatment therapy. Moreover, we found that these circRNAs were regulated by the altered level of RNA binding protein QKI5 after BPA exposure, possibly through the interaction of QKI5 with the reverse complementary elements flanking these circRNAs.

As a new member of the RNA family, circRNAs are widely reported to participate in various pathophysiological processes [13]. Many studies have explored their roles in toxicology. circRNAs were found to play important roles in atrazine-induced testicular degeneration of developing male *X. laevis* [27]. Another study revealed

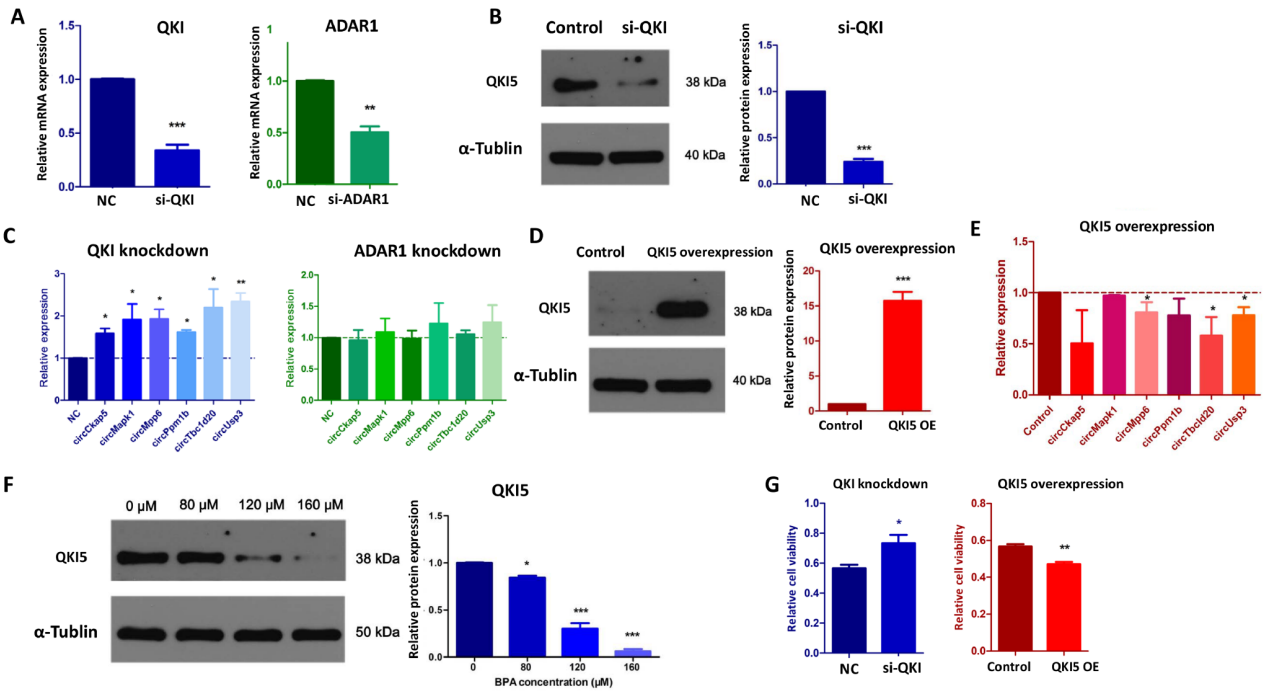

Figure 6. The effects of RNA binding protein QKI5 on the biogenesis of circRNAs (A) The effects of knockdown of QKI and ADAR1 siRNAs on their mRNA expressions. (B) QKI siRNA decreased its protein level confirmed by western blot analysis. (C) QKI knockdown significantly promoted the expressions of all determined circRNAs, while ADAR1 showed no obvious effect. (D) The overexpression efficiency of pVAX1-based QKI5 OE vector. (E) Overexpression of QKI5 showed down-regulating role for circRNAs. (F) BPA exposure observably inhibited the expression of QKI5 protein, especially under higher doses. (G) CCK-8 assay revealed the cell viability upon QKI5 knockdown or overexpression. OE: overexpression. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

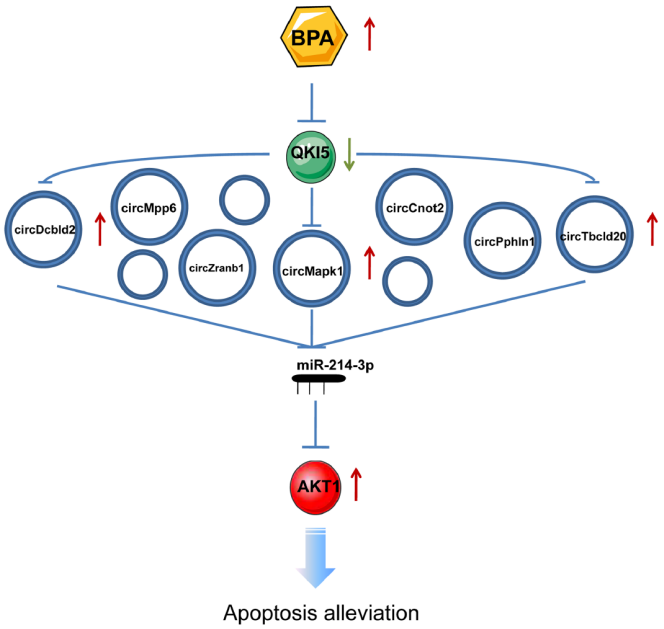

Figure 7. Schematic illustration of the biogenesis and function mechanism of circRNAs Under normal condition, relatively high level of QKI5 inhibits the production of circRNAs, so the self-protection mechanism of cells is at rest. After BPA exposure, the down-regulation of QKI5 liberates the production of many protective circRNAs. These circRNAs sponge miR-214-3p and release the viability promoting AKT1, thus relieve the toxic effect of BPA to some extent.

that circRar1 participates in lead-induced neuronal apoptosis by sponging miR-671 [28]. In our study, we proposed a model in which multiple circRNAs act on one target gene by sponging one miRNA. The overexpression of circDcbld2, circMapk1 and circTbcld20 altogether resulted in the up-regulation of AKT1 through sponging miR-214-3p, and in turn alleviated the BPA-induced germ cell toxicity. *Akt1* is one of the key genes in the PI3K-mTOR pathway [29]. BPA was previously reported to activate the expression of *Akt1*, the subsequent mTOR activation and the induction of cell growth [29,30]. However, the involved mechanisms have not been clearly elucidated. The circRNAs presented here may be an important part in this process. For the feasibility of the experiments, we only explored the roles of three representative circRNAs. A number of other circRNAs were also up-regulated in our previous experiment [13]. They all showed predicted binding sites with miR-214-3p (Figure 2). These circRNAs may act together to sponge miR-214-3p to protect GC-2 cells from BPA toxicity.

Cooperative effects of non-coding RNAs apart from circRNAs have already been observed previously by other researchers. For example, miR-524-3p and miR-524-5p are both suppressed in glioblastoma and they can significantly inhibit the expressions of *Smad2*, *Tead1* and *Hes1*, which eventually results in an elevation of c-Myc [27]. circ-ITCH was found to sponge miR-7, miR-17 and miR-214, and in turn conjointly up-regulated the expression of *ITCH* gene itself. piRNA clusters are also known to repress transposable elements in germ cell development in a cooperating way [31]. The cooperative effects of non-coding RNAs are reasonable, since their effects are generally moderate compared with protein-coding genes. The cooperative effects of non-coding RNAs may reinforce their roles in pathophysiological processes. In a recent published article [11], after in-depth analysis of massive high-throughput sequence datasets, the authors predicted that collective functions of different circRNAs may exist in addition to functions of individual circRNAs. Experimentally, our study is the first to confirm that the cooperative effects of circRNAs actually exist.

The current research not only enriches the way circRNAs act on miRNAs, but also provides new insights into the way circRNAs act in pathological and physiological processes. Generally, most up-regulated circRNAs in disease group seem to promote the relevant disease progression and down-regulated circRNAs do the opposite. To our best knowledge, only two studies reported that up-regulated circRNAs in disease group play protective roles in the progress of diseases [32, 33]. As a rising star in epigenetic regulation, more and more circRNAs will be found to play multi-facet roles in pathological and physiological processes.

The upstream regulation mechanism of circRNAs is also in dispute [5]. Some studies found that they are also regulated by transcription factors like mRNAs do. However, increasing evidence indicated that they seemed to be regulated by RNA binding proteins. For example, many RNA binding proteins [13] like ADAR1, DHX9, FUS and QKI can bind to the complementary intronic elements flanking circRNAs and modulate the biogenesis of circRNAs. In our study, we initially tried to figure out if these dys-regulated circRNAs are regulated by transcription factors. Estrogen receptors are widely expressed in various cells in the testis [33], and play important roles in BPA-induced reproductive disorders. These receptors are also competent transcription factors which initiate the expressions of a wide variety of genes after BPA exposure. Notably, ESR2 was found to be expressed in pachytene spermatocytes [34] and also showed upward trend under BPA treatment in our experiment. However, gain-and-loss of function study showed that these dysregulated circRNAs seemed not to be affected by ESR2.

We further found that down-regulation of RNA binding protein QKI5 can facilitate the biogenesis of these circRNAs, and reverse complementary elements were found in the intronic flanking intronic sequences of these circRNAs. QKI may interact with these elements and inhibit the expressions of these protective circRNAs. However, it is worth noting that QKI was generally considered as a kind of RNA binding protein that promotes circRNA biogenesis [35]. A number of circRNAs were found to be inhibited by QKI overexpression in EMT process [35] and doxorubicin-mediated cardiotoxicity [36] by other researchers. This is understandable, since the intronic flanking elements vary in different circRNAs. They may interact with different motifs of the same RNA binding proteins and the effects are diversified and individualized. Similarly, RNA binding protein FUS also has bidirectional regulating effects on the formation of different circRNAs in embryonic stem cell differentiation [37]. In addition, the downward trend of QKI5 expression after BPA treatment and cell viability inhibiting roles of QKI5 also to some extent supported the following regulatory pathway we proposed: under BPA exposure, the expression of *QKI5* is lowered, which promotes the biogenesis of many protective circRNAs. These circRNAs may cooperatively work on some important anti-apoptosis genes like *Akt1* by sponging the same miRNA, and in turn protects germ cells from BPA-induced toxicity.

In summary, we found the QKI5-circDcbld2/circMapk1/circTbcld20-miR-214-3p-AKT1 axis in BPA-induced germ cell apoptosis, which may provide new targets in BPA reproductive toxicity intervention. We also predicted that many other circRNAs

up-regulated upon BPA treatment show binding sites with miR-214-3p. And these circRNAs can also be regulated by QKI5. Thus the above axis should also be extrapolated to other circRNAs. All these up-regulated circRNAs may altogether alleviate the toxic effects of BPA through the same mechanism. This study proved the co-operative effects of multiple circRNAs through sponging one miRNA. Future nucleic acid therapy may consider this multi-targets regimen. The inhibitory effects of QKI5 on circRNAs also enrich the context of individualized circRNA regulation. Nevertheless, this study still has some limitations. For example, we only chose three representative circRNAs to study their functions. There are many other circRNAs whose expression is elevated in response to BPA stimulation and with protective effects [13]. Future studies are needed to find out whether these circRNAs can act together through sponging miR-214-3p or other miRNAs to protect GC-2 cells from BPA toxicity.

Supplementary Data

Supplementary Data is available at *Acta Biochimica et Biphysica Sinica* online.

Funding

This work was supported by the grants from the National Key Research and Development Program of China (No. 2017YFC1002001), the China National Natural Science Foundation (No. 81801435), and the China National Natural Science Foundation Youth Project (No. 82001621).

Conflict of Interest

The authors declare that they have no conflict of interest.

References

1. Hsu MT, Coca-Prados M. Electron microscopic evidence for the circular form of RNA in the cytoplasm of eukaryotic cells. *Nature* 1979, 280: 339–340
2. Salzman J, Chen RE, Olsen MN, Wang PL, Brown PO. Cell-type specific features of circular RNA expression. *PLoS Genet* 2013, 9: e1003777
3. Ebbesen KK, Kjems J, Hansen TB. Circular RNAs: identification, biogenesis and function. *Biochim Biophys Acta* 2016, 1859: 163–168
4. Memczak S, Jens M, Elefsinioti A, Torti F, Krueger J, Rybak A, Maier L, *et al.* Circular RNAs are a large class of animal RNAs with regulatory potency. *Nature* 2013, 495: 333–338
5. Li HM, Ma XL, Li HG. Intriguing circles: conflicts and controversies in circular RNA research. *WIREs RNA* 2019, 10:
6. Jeck WR, Sharpless NE. Detecting and characterizing circular RNAs. *Nat Biotechnol* 2014, 32: 453–461
7. Zhang Y, Zhang X, Xing Z, Tang S, Chen H, Zhang Z, Li J, *et al.* circStrn3 is involved in bone cancer pain regulation in a rat model. *Acta Biochim Biophys Sin* 2020, 52: 495–505
8. Zheng Q, Bao C, Guo W, Li S, Chen J, Chen B, Luo Y, *et al.* Circular RNA profiling reveals an abundant circHIPK3 that regulates cell growth by sponging multiple miRNAs. *Nat Commun* 2016, 7: 11215
9. Hansen TB, Jensen TI, Clausen BH, Bramsen JB, Finsen B, Damgaard CK, Kjems J. Natural RNA circles function as efficient microRNA sponges. *Nature* 2013, 495: 384–388
10. Patop IL, Wüst S, Kadener S. Past, present, and future of circRNAs. *EMBO J* 2019, 38: e100836
11. Robic A, Faraut T, Djebali S, Weikard R, Feve K, Maman S, Kuehn C. Analysis of pig transcriptomes suggests a global regulation mechanism enabling temporary bursts of circular RNAs. *RNA Biol* 2019, 16: 1190–1204
12. Caiment F, Gaj S, Claessen S, Kleinjans J. High-throughput data integration of RNA–miRNA–circRNA reveals novel insights into mechanisms of benzo[a]pyrene-induced carcinogenicity. *Nucleic Acids Res* 2015, 43: 2525–2534
13. Li HM, Dai YW, Yu JY, Duan P, Ma XL, Dong WW, Li N, *et al.* Comprehensive circRNA/miRNA/mRNA analysis reveals circRNAs protect against toxicity induced by BPA in GC-2 cells. *Epigenomics* 2019, 11: 935–949
14. Ma Y, Liu H, Wu J, Yuan L, Wang Y, Du X, Wang R, *et al.* The adverse health effects of bisphenol A and related toxicity mechanisms. *Environ Res* 2019, 176: 108575
15. Sidorkiewicz I, Czerniecki J, Jarząbek K, Zbucka-Krętowska M, Wołczyński S. Cellular, transcriptomic and methylome effects of individual and combined exposure to BPA, BPF, BPS on mouse spermatocyte GC-2 cell line. *Toxicol Appl Pharmacol* 2018, 359: 1–11
16. Wang C, Zhang J, Li Q, Zhang T, Deng Z, Lian J, Jia D, *et al.* Low concentration of BPA induces mice spermatocytes apoptosis via GPR30. *Oncotarget* 2017, 8: 49005–49015
17. Yin L, Dai Y, Cui Z, Jiang X, Liu W, Han F, Lin A, *et al.* The regulation of cellular apoptosis by the ROS-triggered PERK/EIF2α/chop pathway plays a vital role in bisphenol A-induced male reproductive toxicity. *Toxicol Appl Pharmacol* 2017, 314: 98–108
18. Siracusa JS, Yin L, Measel E, Liang S, Yu X. Effects of bisphenol A and its analogs on reproductive health: a mini review. *Reproductive Toxicol* 2018, 79: 96–123
19. Cai J, Chen Z, Wang J, Wang J, Chen X, Liang L, Huang M, *et al.* circHECTD1 facilitates glutaminolysis to promote gastric cancer progression by targeting miR-1256 and activating β-catenin/c-Myc signaling. *Cell Death Dis* 2019, 10: 576
20. Conn SJ, Pillman KA, Toubia J, Conn VM, Salmanidis M, Phillips CA, Roslan S, *et al.* The RNA binding protein quaking regulates formation of circRNAs. *Cell* 2015, 160: 1125–1134
21. Rybak-Wolf A, Stottmeister C, Glažar P, Jens M, Pino N, Giusti S, Hanan M, *et al.* Circular RNAs in the mammalian brain are highly abundant, conserved, and dynamically expressed. *Mol Cell* 2015, 58: 870–885
22. Enuka Y, Lauriola M, Feldman ME, Sas-Chen A, Ulitsky I, Yarden Y. Circular RNAs are long-lived and display only minimal early alterations in response to a growth factor. *Nucleic Acids Res* 2016, 44: 1370–1383
23. Kristensen LS, Okholm TLH, Venø MT, Kjems J. Circular RNAs are abundantly expressed and upregulated during human epidermal stem cell differentiation. *RNA Biol* 2018, 15: 280–291
24. Guo W, Shi X, Liu A, Yang G, Yu F, Zheng Q, Wang Z, *et al.* RNA binding protein QKI inhibits the ischemia/reperfusion-induced apoptosis in neonatal cardiomyocytes. *Cell Physiol Biochem* 2011, 28: 593–602
25. Liu Z, Zhou Y, Liang G, Ling Y, Tan W, Tan L, Andrews R, *et al.* Circular RNA hsa_circ_001783 regulates breast cancer progression via sponging miR-200c-3p. *Cell Death Dis* 2019, 10: 55
26. Li K, Zhong S, Luo Y, Zou D, Li M, Li Y, Lu Y, *et al.* A long noncoding RNA binding to QKI-5 regulates germ cell apoptosis via p38 MAPK signaling pathway. *Cell Death Dis* 2019, 10: 699
27. Zhao K, Wang Q, Wang Y, Huang K, Yang C, Li Y, Yi K, *et al.* EGFR/c-myc axis regulates TGFβ/Hippo/Notch pathway via epigenetic silencing miR-524 in gliomas. *Cancer Lett* 2017, 406: 12–21
28. Nan A, Chen L, Zhang N, Liu Z, Yang T, Wang Z, Yang C, *et al.* A novel regulatory network among lncRpa, circRar1, miR-671 and apoptotic genes promotes lead-induced neuronal cell apoptosis. *Arch Toxicol* 2017, 91: 1671–1684
29. Goodson Iii WH, Luciani MG, Sayeed SA, Jaffee IM, Moore Ii DH, Dairkee SH. Activation of the mTOR pathway by low levels of xenoestrogens in breast epithelial cells from high-risk women. *Carcinogenesis* 2011, 32: 1724–1733

30. Kang NH, Hwang KA, Lee HR, Choi DW, Choi KC. Resveratrol regulates the cell viability promoted by 17β-estradiol or bisphenol A via down-regulation of the cross-talk between estrogen receptor α and insulin growth factor-1 receptor in BG-1 ovarian cancer cells. *Food Chem Toxicol* 2013, 59: 373–379
31. Li F, Zhang L, Li W, Deng J, Zheng J, An M, Lu J, *et al.* Circular RNA ITCH has inhibitory effect on ESCC by suppressing the Wnt/β-catenin pathway. *Oncotarget* 2015, 6: 6001–6013
32. Cheng X, Zhang L, Zhang K, Zhang G, Hu Y, Sun X, Zhao C, *et al.* Circular RNA VMA21 protects against intervertebral disc degeneration through targeting miR-200c and X linked inhibitor-of-apoptosis protein. *Ann Rheum Dis* 2018, 77: 770–779
33. Matuszczak E, Komarowska MD, Debek W, Hermanowicz A. The impact of bisphenol a on fertility, reproductive system, and development: a review of the literature. *Int J Endocrinol* 2019, 2019: 1–8
34. Wersinger SR, Haisenleder DJ, Lubahn DB, Rissman EF. Steroid feedback on gonadotropin release and pituitary gonadotropin subunit mrna in mice lacking a functional estrogen receptor α. *Endocrine* 1999, 11: 137–144
35. Han J, Meng J, Chen S, Wang X, Yin S, Zhang Q, Liu H, *et al.* YY1 complex promotes quaking expression via super-enhancer binding during emt of hepatocellular carcinoma. *Cancer Res* 2019, 79: 1451–1464
36. Gupta SK, Garg A, Bär C, Chatterjee S, Foinquinos A, Milting H, Streckfuß-Bömeke K, *et al.* Quaking inhibits doxorubicin-mediated cardiotoxicity through regulation of cardiac circular rna expression. *Circ Res* 2018, 122: 246–254
37. Errichelli L, Dini Modigliani S, Laneve P, Colantoni A, Legnini I, Capauto D, Rosa A, *et al.* FUS affects circular RNA expression in murine embryonic stem cell-derived motor neurons. *Nat Commun* 2017, 8: 14741