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Research paper

Full-length nanopore sequencing of circular RNA landscape in peripheral blood cells following sequential BNT162b2 mRNA vaccination

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ABSTRACT

Circular RNAs (circRNA) lack 5' or 3' ends; their unique covalently closed structures prevent RNA degradation by exonucleases. These characteristics provide circRNAs with high pharmaceutical stability and biostability relative to current standard-of-care linear mRNAs. CircRNA levels are reportedly associated with certain human diseases, making them novel disease biomarkers and a noncanonical class of therapeutic targets. In this study, the endogenous circRNAs underlying the response to BNT162b2 mRNA vaccination were evaluated. To this end, peripheral blood samples were subjected to full-length sequencing of circRNAs via nanopore sequencing and transcriptome sequencing. Fifteen samples, comprising pre-, first, and second vaccination cohorts, were obtained from five healthcare workers with no history of SARS-CoV-2 infection or previous vaccination. A total of 4706 circRNAs were detected; following full-length sequencing, 4217 novel circRNAs were identified as being specifically expressed during vaccination. These circRNAs were enriched in the binding motifs of stress granule assemblies and SARS-CoV-2 RNA binding proteins, namely poly(A) binding protein cytoplasmic 1 (PABPC1), pumilio RNA binding family member 1 (PUM1), and Y box binding protein 1 (YBX1). Moreover, 489 circRNAs were identified as previously reported miRNA sponges. The differentially expressed circRNAs putatively originated from plasma B cells compared to circRNAs reported in human blood single-cell RNA sequencing datasets. The pre- and post-vaccination differences observed in the circRNA expression landscape in response to the SARS-CoV-2 BNT162b2 mRNA vaccine.

1. Introduction

According to the World Health Organization (WHO) Coronavirus disease (COVID-19) dashboard, over 773 million people had been infected with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) as of December 2023, with over 7 million confirmed deaths

(Geneva: World Health Organization, 2020). Meanwhile, more than 5.4 billion people, approximately 68.7 % of the world's population, have been vaccinated against SARS-CoV-2 (Mathieu et al., 2021); the rapid pace of vaccination has contributed to the development of mRNA-based vaccines, such as the BNT162b2 (Pfizer-BioNTech) and mRNA-1273 vaccines (Moderna). These vaccines were designed using mRNA

Abbreviations: BSJs, 3' and 5' back-splice junctions; circRNAs, Circular RNAs; DEGs, differentially expressed genes; GO, gene ontology; BP, biological process; IFN, interferon; ISG, interferon stimulated gene; PCC, Pearson's correlation coefficient; PKR, protein kinase R; PBMC, peripheral blood mononuclear cell; RBPs, RNA binding proteins; WHO, World Health Organization; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; scRNA-seq, single cell RNASeq; SG, stress granules.

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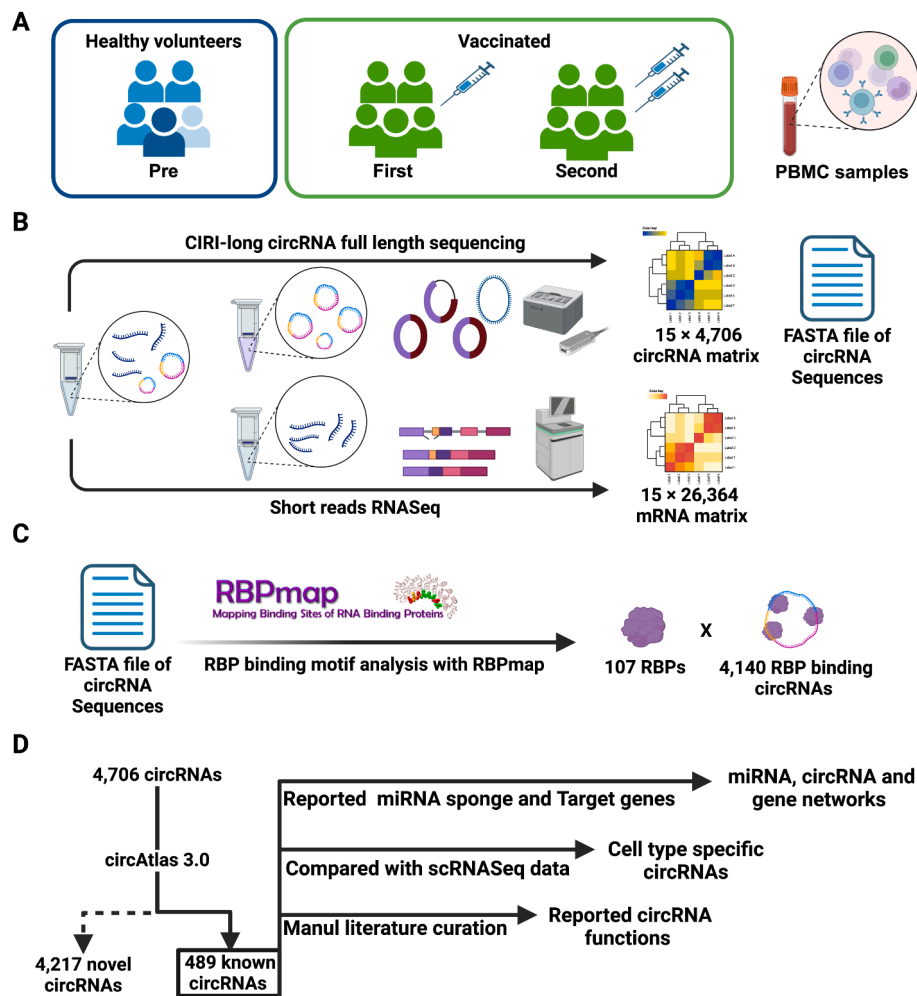


Fig. 1. Summary of the experiment and data analysis (A) Overview of the experimental design. (B) Experiment procedures. (C) RNA binding protein motif detection on circRNA sequences. (D) Data analysis steps taken. (Pre: Pre-vaccination. First: Samples collected immediately after first vaccination. Second: samples collected immediately after second vaccination dose).

encoded by the SARS-CoV-2 spike protein and were among the first to demonstrate effective induction of SARS-CoV-2 antigen-specific antibodies and T-cell responses (Huang et al., 2021). Comparison of blood samples collected from medical workers without neither COVID19 infection history, prior to the introduction of vaccines in early onset of the pandemic, to the later induction of multiple vaccination provides valuable insights.

In our previous study, we found that consecutive BNT162b2 mRNA vaccination induces short-term epigenetic memory in innate immune cells. Adaptive immune memory after vaccination can be assessed by monitoring memory B- and T-cell persistence following the administration of BNT162b2 vaccine boosters. An increase in the frequency of CD14⁺CD16⁺ monocyte clusters and enhanced interferon (IFN)-stimulated gene (ISG) responses have been observed in single-cell (sc) RNA-seq studies following the second vaccination (first booster), confirming the development of an innate immune response (Yamaguchi et al., 2022).

Circular RNAs (circRNAs) are generally referred to as RNAs that lack 3' and 5' back-splice junctions (BSJs) (Yu and Kuo, 2019). Functional endogenous circRNAs are conserved in mammals, ubiquitously expressed (Salzman et al., 2012), and may originate from intronic and exonic regions. Additionally, they have myriad roles in mammalian cellular physiology (He et al., 2021). However, among the millions of categorized human endogenous circRNAs, only a few hundred have been identified as having specific functional roles. With enriched

miRNA-targeting site sequences, circRNAs can serve as natural miRNA sponges for post-transcriptional regulation. Similar sponging effects have been reported for RNA binding proteins (RBPs); that is, the binding of RBPs to mRNA decreases following upregulation of RBP binding site-enriched circRNAs (Zang et al., 2020). Most importantly, circRNAs reportedly play pivotal roles in immune responses by competitively binding double-stranded protein kinase R (PKR), regulating innate immunity (Li and Chen, 2021). Meanwhile, viral infections activate RNase L, which degrades circRNAs, leading to the release of PKR, which binds to double-stranded viral RNAs and inhibits infection (Liu et al., 2019).

Considering the regulatory role of endogenous circRNAs in the immune response, it is reasonable to hypothesize that vaccination induces a potential shift in circRNA expression profiles. However, well-established and reliable circRNA detection experiments are required to verify the underlying changes in circRNAs following BNT162b2 mRNA vaccination. Conventionally, high-throughput detection of circRNAs has been conducted via computational detection of BSJ sites in transcriptome sequencing data (Zheng et al., 2020). However, these methods only provide head-tail genome loci and strand information on the detected circRNAs. Meanwhile, assembly of full-length circRNA sequences from short reads is based on the presumption that circRNAs are transcribed from genetic regions and share exonic or intronic sequences. Hence, with advances in high-throughput long-read sequencing technology, several protocols for full-length sequencing of circRNAs have been developed (Hou et al., 2023; Liu et al., 2021; Xin et al., 2021).

This study employs a well-known protocol, i.e., circRNA identifier using long-read sequencing data (CIRI-long) (Hou et al., 2023), to conduct nanopore sequencing experiments on peripheral blood mononuclear cell (PBMC) samples collected from healthcare workers with no history of SARS-CoV-2 infection or previous vaccination. The primary objectives of this study are to (1) establish accurate expression profiles of the mRNA and full-length sequenced circRNAs following BNT162B2 mRNA vaccination; (2) perform a comprehensive analysis of the correlations between sequence-based circRNAs and mRNA expression profiles; and (3) provide insights into the functional roles of circRNAs in response to BNT162B2 mRNA vaccination.

2. Material and methods

Full-length nanopore sequencing of PBMCs was conducted on samples from five healthcare workers, before and after vaccination during the SARS-CoV-2 pandemic (Fig. 1). The ethical approval was obtained and informed consent was provided by all participants. CircRNAs within the samples were enriched with RNase R following the addition of poly (A) tails. Reverse-transcribed cDNA was PCR-amplified and sequenced with a Nanopore Sequencer MinION per the methods described by Lu et al. (Lu et al., 2016). The resulting libraries were processed using the CIRI-long software (Hou et al., 2023; Zhang et al., 2021). All sequencing data were deposited in the Sequence Read Archive (SRA) of the National Center for Biotechnology Information (NCBI) under the BioProject ID PRJNA939269.

2.1. PBMC RNA isolation and library construction

The PBMCs from healthy volunteers were isolated before and after vaccination via density gradient separation using a Lymphoprep (STEMCELL Technologies, Vancouver, Canada). Total RNA was isolated using the PAXgene™ Blood RNA System (BD Biosciences, Franklin Lakes, NJ, USA). Eluted RNA was dissolved in RNase-free water. RNA quality and quantity were evaluated using a Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, CA, USA). Double-stranded cDNAs were synthesized from RNA, and libraries were prepared using the SMART-seq HT kit (Takara, Shiga, Japan) according to the manufacturer's protocol. The libraries were quantified using an Illumina Library Quantification Kit (Kapa Biosystems, Wilmington, MA, USA), and the fragment size distribution was determined using a Bioanalyzer 2100 system (Agilent Technologies).

Following the Regulations on Human Genome Research at Osaka University, the Osaka University Research Ethics Committee has approved this study. Samples were collected at Osaka University Hospital and collaborative institutes (Approval number 885, HP; <http://www.osaka-u-tacc.com/occonomix/index.html>).

2.2. Nanopore sequencing of circRNA

Poly (A) tailing treatment of linear RNAs was applied using *E. coli* poly (A) polymerase (NEB) according to the manufacturer's protocol. RNAs were purified using Agencourt RNAClean XP (Beckman Coulter, Inc., IN, USA), and linear RNAs were digested with RNase R (Epicenter). RNase R-treated RNAs were purified using Agencourt RNAClean XP and reverse transcribed with the SMARTer cDNA synthesis kit (Takara Bio); the 3' SMART CDS Primer II A provided with the kit was replaced with a random hexamer (5'-AAGCAGTGGTATCAACGCAGAGTACNNNNNN-3') to reverse transcribe circular RNAs without poly (A) sequences. Amplification of reverse-transcribed cDNAs was performed using LongAmp™ Taq 2X Master Mix (NEB) with SMARTer primers containing a SMARTer cDNA synthesis kit. The amplified products were washed with Agencourt AMPure XP magnetic beads (Beckman Coulter, Inc.) and sequenced via Oxford Nanopore MinION (Lu et al., 2016).

2.3. Transcriptome sequencing and differential expression profiling

Transcriptome sequencing was performed using an MGISEQ 2000 system (MGI Tech Co., Ltd., Shenzhen, China) with 100-bp paired-end reads converted into FASTQ files. The alignments were read with Tophat2 v2.1.1 (Kim et al., 2013) using the human reference genome (hg19). The BAM files were converted to raw count files using FeatureCount v2.0.3 (Liao et al., 2014). Raw counts were analyzed using iDEGES/edgeR in the TCC package (version 1.36.0) (Sun et al., 2013). Enrichment analysis was conducted via DAVID (Sherman et al., 2022) using z-score results of condition-wise *t*-tests for the top 200 differentially expressed genes (DEGs) under the different vaccination statuses.

2.4. Data analysis of the full-length sequenced circRNAs

Nanopore sequencing data were processed using the CIRI-long analysis pipeline (Zhang et al., 2021). CircRNA information and expression profiles were collected from the collapsed folders and formatted into an expression-profile matrix. Normalization, differential expression analysis, comparison with database data, and mRNA expression profiling, along with the sample metadata, were formatted into annotated data format (Virshup et al., 2021). Differential expression analysis of circRNAs and mRNAs was conducted with the integrated *t*-test using Scanpy (Wolf et al., 2018) and SciPy (Virtanen et al., 2020). All graphs were created by Seaborn (Waskom, 2021) using Bio-Render.com.

2.5. Functional annotation and manual curation of sequenced circRNAs

The BSJ sites of the identified circRNAs were compared with those in public databases, namely, circAtlas 3.0 (Wu et al., 2023) and CircSC (Wu et al., 2022). Manual curation of functional roles was performed on the top 25 differentially expressed circRNAs ranked by Z-Score of T-test results. Expression of circRNAs in samples within each condition were compared against rest of the samples through T-test integrated in the Scanpy (Wolf et al., 2018) function `scanpy.tl.rank_genes_groups()` to find condition wise specific expressed circRNAs. The application integrates the `scipy.stats.ttest_ind()` function of SciPy (Virtanen et al., 2020) to calculate the T-test of the mean expression values of circRNAs within the samples of the specific vaccination condition, against all other samples. The resulting P values and the corresponding z score, which indicate the underlying number of standard deviations of certain circRNA away from the average, was ranked. Hence the top ranked circRNAs with the highest z-score represent the most specific to the vaccination condition circRNA sequenced. The same approach was also conducted into the analysis of transcriptome short read sequencing to acquire the conditional specific differential expressed genes. Through manual curation with the circRNAs named with circBase (Glazar et al., 2014) identifiers, reported functions of the condition specific circRNAs were summarized.

2.6. Compare with circRNAs found in vaccination short-read sequenced samples

To assess whether the full-length sequenced circRNAs could be verified in publicly available vaccination short-read sequenced samples, the circRNAs were searched in 115 public PBMC RNA-seq samples from an octogenarian cohort-based study (NCBI GSE190747) (Lee et al., 2022) were collected and processed with CIRI-full (Zheng et al., 2019). The resulting BSJs were compared with the results of CIRI-long full-length sequenced results.

2.7. Target genes of the miRNAs sponged by circRNAs are differentially expressed upon vaccination

To identify the potential post-transcriptional regulatory effects of the identified circRNAs on miRNA, verified miRNA target genes were

Table 1
Summary of collected PBMC samples.

Volunteer	Age	Sex	Days	Condition
P1	55	Male	0	Pre-Vaccination
			14	First Vaccination
			28	Second Vaccination
P2	46	Male	0	Pre-Vaccination
			18	First Vaccination
			30	Second Vaccination
P3	44	Male	0	Pre-Vaccination
			14	First Vaccination
			37	Second Vaccination
P4	43	Male	0	Pre-Vaccination
			19	First Vaccination
			29	Second Vaccination
P5	24	Female	0	Pre-Vaccination
			17	First Vaccination
			30	Second Vaccination

screened from the MiRTarBase (Huang et al., 2020). The mRNA expression profiles of the 15 collected samples were compared, and the regulatory network of circRNAs, miRNAs, and target genes, potentially corresponding to the identified differentially expressed circRNAs, was summarized. The network was constructed based on the reported correlation between circRNA and miRNA, as well as the experimentally verified miRNA target genes recorded.

2.8. CircRNA sequence-based RNA binding motif analysis

To explore the potential functional roles played by the sequenced circRNAs in their interactions with RBPs, a motif-based analysis on the sequenced full-length circRNA FASTA file (Supplemental File 2) using RBPmap was conducted (Paz et al., 2014). RBPs interact with SARS-CoV-2 viral RNA were identified through comparing the results with ScoV2-proteome-atlas (Schmidt et al., 2021). Enrichment analysis via DAVID (Sherman et al., 2022) was also applied on the RBP gene symbols.

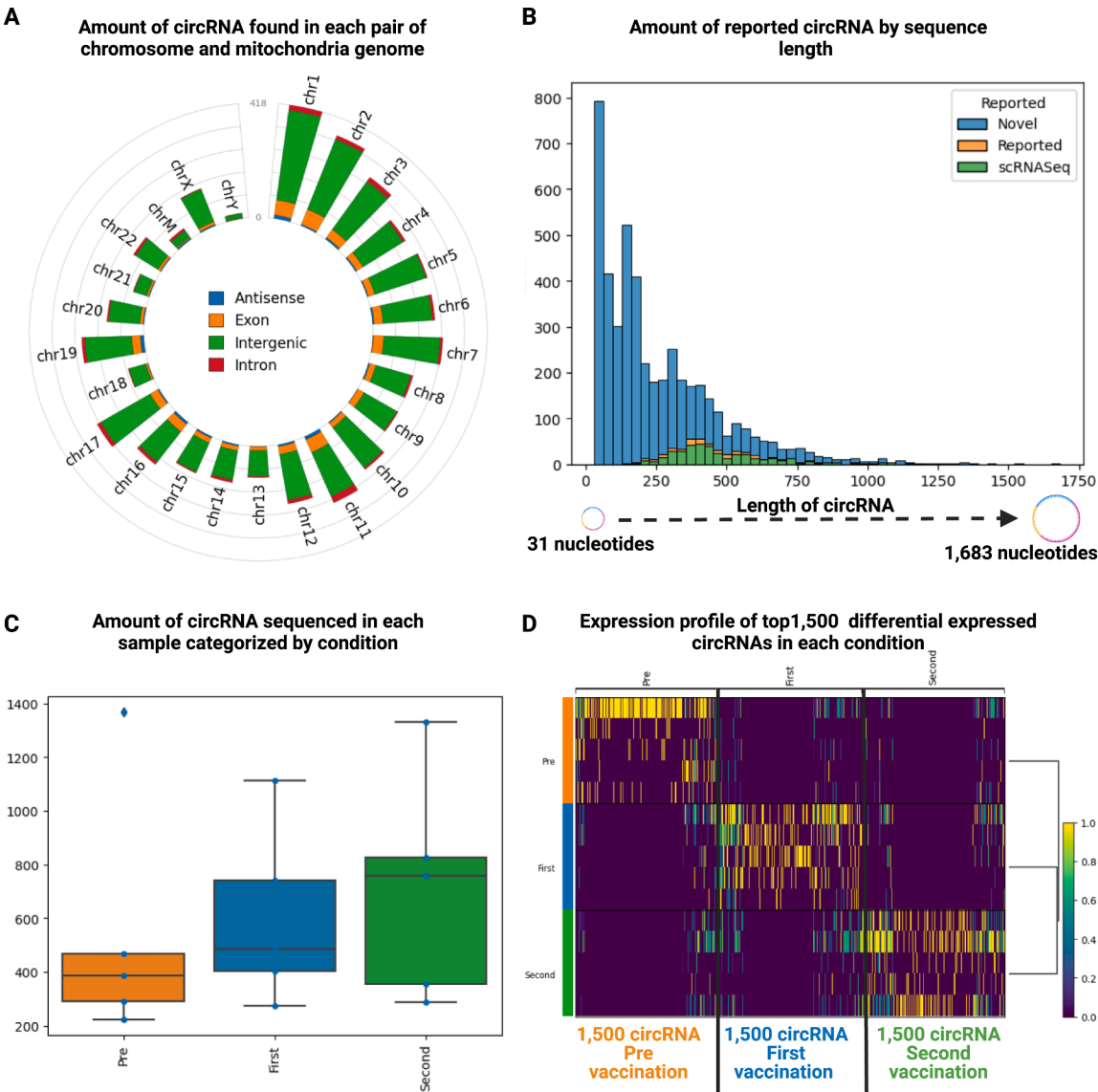


Fig. 2. Summary of the circRNA full-length sequencing results. (A) Amount of circRNAs sequenced from human genomes, categorized by four types: antisense, exonic, intronic, and intergenic. (B) Distribution of circRNAs by the transcript length. Putative circRNAs ranges from 31 nucleotide to 1683 nucleotide long. (C) Amount of circRNAs sequenced from each sample categorized by vaccination status. (D) General expression profile heatmap of the top 4,500 sequenced circRNA.

Table 2
Reported functional roles of top differential expressed circRNA.

Condition	circRNA	Reported functional role	Reference
Pre-Vaccination	hsa_circ_0044177	Breast Cancer-Related	(Wang et al., 2019)
	hsa_circ_0007723	Placenta of preeclampsia expressed	(Zhou et al., 2018)
	hsa_circ_0007059	Sponges miR-421	(Hui et al., 2022; Su et al., 2019)
	hsa_circ_0006856	Silicosis and idiopathic pulmonary fibrosis associated	(Cheng et al., 2022; Lv et al., 2018)
	hsa_circ_0004313	Human Breast Cancer associated	(Sheng et al., 2021)
	hsa_circ_0001467	Glioma associated	(Geng et al., 2021)
	hsa_circ_0001346	Sponge miRNA-1224-5p	(Zhang et al., 2020)
	hsa_circ_0000233	Vogt-Koyanagi-Harada Disease associated	(Shu et al., 2021)
	hsa_circ_0000942	Serum and serum exosome circRNAs	(Kang et al., 2022a)
	hsa_circ_0085323	Crohn's disease associated	(Hu et al., 2021)
	hsa_circ_0001178	Sponge miR-382,miR-587 and miR-616	(Ren et al., 2020)
	hsa_circ_0008732	Sponge miR-223-3p	(Liu et al., 2022)
	hsa_circ_0028319	Expressed in placentas of women with gestational diabetes mellitus	(Wang et al., 2019)
	hsa_circ_0006508	Warburg effect in colorectal cancer cells	(Xu et al., 2023)
	hsa_circ_0001073	Sponge miR-626 in lung cancer	(Liu et al., 2021)
	hsa_circ_0004417	Potential involved in the inflammation progress of pulpitis.	(Lei et al., 2022)
	hsa_circ_0001258	Sponge hsa-miR-744-3p	(Zhu et al., 2019)
First Vaccination	hsa_circ_0000681	Plasma circRNA	(Huang et al., 2018)
	hsa_circ_0000894	Cytomegalovirus infection associated	(Huo et al., 2021; Lou et al., 2019)
	hsa_circ_0001492	Serum and serum exosome circRNAs	(Kang et al., 2022a)
	hsa_circ_0001522	Up-regulates miR-181b	(Li et al., 2022)
	hsa_circ_0001535	Sponges miR-433-3p	(Mao et al., 2022)
	hsa_circ_0004587	Plasma circRNA	(Hu et al., 2020)
	hsa_circ_0005965	Potential miRNA sponge, Exosome circRNA	(Chen et al., 2022; Mao et al., 2021)
	hsa_circ_0051680	Serum exosome circRNA	(Li et al., 2022)
	hsa_circ_0057742	Skin aging-related	(Wang et al., 2021)
	hsa_circ_0065301	Multiple sclerosis associated	(Cardamone et al., 2023)
	hsa_circ_0025705	Differential expressed in prostate cancer	(Luo et al., 2021)
	hsa_circ_0002316	Sponging miR-1294	(Shi et al., 2022)
	hsa_circ_0015454	Differentially expressed in pheochromocytomas and paragangliomas.	(Yu et al., 2020)
Second Vaccination	hsa_circ_0006127	Down regulated in infrapatellar fat pad/synovium unit	(Jiang et al., 2021)
	hsa_circ_0000643	Interact with miR-647 in breast cancer	(Huang et al., 2023)
	hsa_circ_0005927	Plasma circular RNA	(Li et al., 2020)
	hsa_circ_0037515	Biomarkers for non-small cell lung cancer	(Zhao et al., 2020)
	hsa_circ_0004771	Sponges miR-399-5p and miR-653	(Huang et al., 2020; Qiao et al., 2023)
	hsa_circ_0001801	Sponges miR-224-5p	(Zheng et al., 2019)
	hsa_circ_0002904	Sponges miR-338-3p	(Xiong et al., 2019)
	hsa_circ_0004872	Sponges miR-224	(Ma et al., 2020)
	hsa_circ_0005576	Sponges miR-512-5p	(Liu et al., 2022)
	hsa_circ_0007052	Dengue virus serotype 3 infection correlated	(Long et al., 2021)
	hsa_circ_0002374	Potential association with Triple-negative breast cancer	(Li et al., 2022)
	hsa_circ_0000110	Biomarker for gastric cancer	(Ma et al., 2021)
	hsa_circ_0007099	Co-expressed in diffuse large B-cell lymphoma with PIP4K2A	(Wang et al., 2023)
	hsa_circ_0008450	Sponges miR-431	(Du et al., 2020)
	hsa_circ_0030720	Potential association with Non-Small-Cell Lung Cancer	(Huang et al., 2020)
	hsa_circ_0002500	Associated with severity of coronary atherosclerosis in human coronary artery	(Hou et al., 2020)
	hsa_circ_0074368	Potential miRNA sponge	(Chen et al., 2022)
	hsa_circ_0088300	Exosome circRNA,miR-1305 sponge	(Shi et al., 2021)

2.9. Estimation of correlation between mRNA and circRNA expression

To explore the potential correlations between the genetic loci of circRNAs and the corresponding genes within the PBMC samples, correlations between circRNAs and genes originating from overlapping genomic loci were calculated using Pearson's correlation coefficient (PCC). The expression profiles of antisense, exonic, and intronic circRNAs were normalized and compared with the short-read RNASeq expression profiles. The expression profiles of RBPs with RNA binding motifs on the circRNA sequences were extracted from the whole transcriptome sequencing results. By comparing the available full-length expression profiles of circRNAs and transcriptome sequencing of the same 15 samples, the correlations between circRNAs and RBPs were estimated. PCCs were calculated based on the expression profiles of the circRNAs and the mRNAs of the corresponding RBPs within the 15 samples. PCC was calculated through the function `scipy.stats.pearsonr()` of Python package SciPy (Virtanen et al., 2020). The normalized mRNA expression profile of specific gene within 15 collected samples from short read transcriptome sequencing was applied to the function as input x vector, and the corresponding normalized circRNA expression

profile from full length sequencing within the collected 15 samples was applied as input y vector.

3. Results

3.1. Nanopore sequencing and identification of reported circRNAs

The cohort comprised four males aged 43–55 years and one female aged 24 years (Table 1). The expression profiles and full-length sequences of 4,706 circRNAs were acquired (Supplemental Files 1 and 2). Given the fact that circRNA expression profiling and detection in this study was purely based on the CIRC-long protocol (Wang et al., 2019) without further experiment verification, functional discussion will only focus on circRNAs reported with functional roles in other studies.

Most of the identified circRNAs (3713/4706; 78.9 %) originated from intergenomic loci (Fig. 2A). A total of 48 mitochondrial circRNAs were identified through default criteria provided in the CIRC-long analysis pipeline (Zhang et al., 2021) The lengths of the sequenced circRNAs ranged from 31 nucleotides long putative circRNA located in chr1:156704810–156704840 to 1683 nucleotides long mitochondria

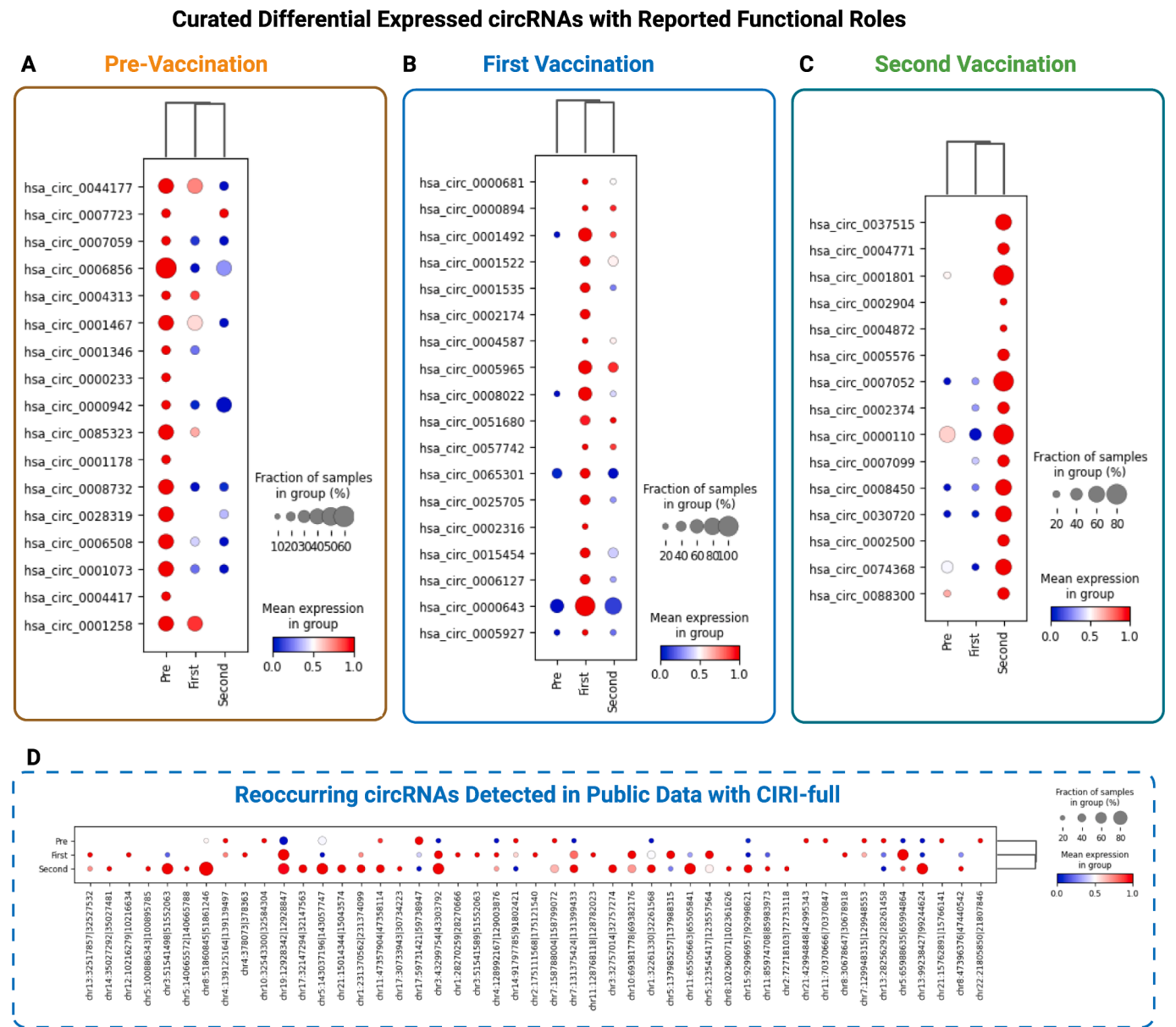


Fig. 3. Curated circRNAs categorized by condition (A) Pre-vaccination specific circRNAs with functional roles identified; (B) Specifically expressed circRNAs after first vaccination; (C) Specifically expressed circRNAs after second vaccination; (D) Sequenced circRNAs detected in public vaccinated RNA-seq PBMC samples.

putative circRNA located in chrM:3322–8422. Comparison of the discovered BSJ sites with circAtlas 3.0 (Wu et al., 2023) and CircSC (Wu et al., 2022) is summarized in Fig. 2B. A total of 489 (10.4 %) circRNAs were reported in the circAtlas, and 386 (8.2 %) were identified in the public single-cell RNA-seq data collected from CircSC(Wu et al., 2022). Information on these circRNAs is provided in Supplemental File 3. Through manual curation of the top differentially expressed circRNAs with identifiers in the circBase (Glažar et al., 2014) format, the circRNAs were reported to have specific functional roles (Table 2).

Gender and age information of the five participants is summarized in this table. The column Days refer to the exact days pass from the first introduction of the vaccine. Samples were categorized into three conditions: Pre-Vaccination, First Vaccination and Second Vaccination.

3.2. Fundamental shift in the circRNA landscape from pre- to post-vaccination

The number of circRNAs sequenced from the 15 samples ranged from

223 to 1369 (Fig. 2C), categorized by vaccination period Pre-Vaccination, First Vaccination and Second Vaccination. Most of the expressed circRNAs were specific to the vaccination period, as summarized in the expression profiling heatmap of 4,500 sequenced circRNA (Fig. 2D). Manual curation of functional roles was performed on the top differentially expressed circRNAs (Fig. 3A–C). The size of the circles within the figures reflects the fraction of the specific circRNA found within the vaccination period samples, while the mean expression of the circRNA was normalized and scaled into 1 to 0, illustrated in red and blue color bar. Differential expressed circular RNAs reported with specific functions, along with corresponding references were summarized in Table 2.

Sequenced circRNAs differential expressed and reported with functional roles are summarized in this table. The referred name of the circRNAs is based on the recorded serial numbers of circBase, same as the references.

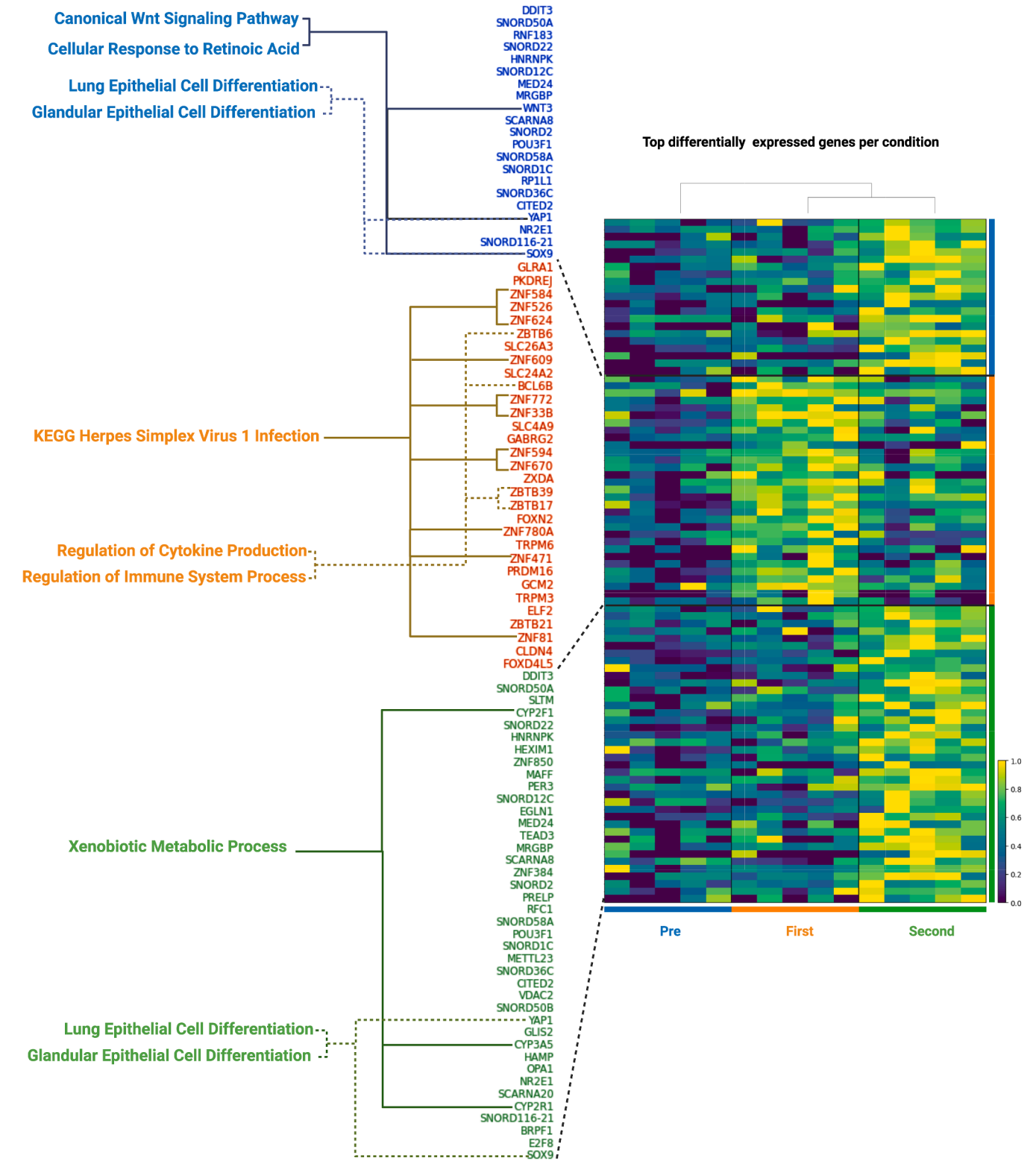


Fig. 4. Top differentially expressed genes and enrichment results from transcriptome sequencing Condition-wise differentially expressed genes and corresponding GO: Biological Process or KEGG pathway. Genes and enriched terms corresponding to Pre-Vaccination samples were labeled in blue color, First Vaccination in orange and Second Vaccination in green.

3.3. Detection of circRNAs from octogenarians receiving mRNA vaccination

Within the collected 115 public PBMC RNA-seq samples from an octogenarian cohort-based study (NCBI GSE190747) (Lee et al., 2022),

circRNAs identified in this study were also detected in the octogenarian study samples. Functionally differentially expressed circRNAs such as hsa_circ_0001746, hsa_circ_0000896, hsa_circ_0006508, hsa_circ_0001535, hsa_circ_0001522, and hsa_circ_0000233 were identified (Fig. 3D).

Table 3
Source Genes Correlated circRNAs.

BSJ	Type	Gene	circBase	PCC (r)
chr11:66509288 66509456	antisense	DPP3		0.709123
chr15:90638446 90641199	exonic	CRTC3	hsa_circ_0003010	0.699144
chr7:27851086 28049705	antisense	JAZF1		0.690393
chr19:56580596 56580658	exonic	ZNF470		0.660849
chr1:15717893 15721388	exonic	PLEKHM2	hsa_circ_0000021	0.62513
chr15:48331800 48332199	intronic	DUT		0.620069
chr1:167440173 167518477	intronic	CD247		0.61738
chr19:57861047 57861916	exonic	ZNF587		-0.63106
chr3:50065031 50066502	exonic	RBM6	hsa_circ_0006531	-0.6462
chr10:121790169 121841209	exonic	ATE1		-0.69692
chr3:47675475 47678311	exonic	SMARCC1	hsa_circ_0008639	-0.71757

3.4. Differentially expressed circRNAs in cell-free serum exosomes and plasma

CircRNAs hsa_circ_0004587, hsa_circ_0000681, and hsa_circ_0005927 were detected in blood plasma (Hu et al., 2020; Huang et al., 2018; Li et al., 2020). In contrast, hsa_circ_0001492, hsa_circ_0000942, hsa_circ_0088300, and hsa_circ_0051680 were found in serum exosomes (Kang et al., 2022a; Kang et al., 2022b; Li et al., 2022; Mao et al., 2021).

3.5. Genes associated with viral infections are differentially expressed within the PBMC samples

Within the gene expression profiles through transcriptome sequencing, enrichment analysis was conducted. The associated processes and pathways were assessed via gene ontology (GO) (Blake et al., 2015). Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa et al., 2021) terms revealed the DEGs correlated with the zinc-finger proteins ZNF584, ZNF670, ZNF81, ZNF624, ZNF780A, ZNF772, and ZNF33B associated with the hsa05168 herpes simplex virus 1 infection pathway. These genes were upregulated in the PBMC samples collected following the first vaccination, comparing to the Pre-vaccination and Second-vaccination samples. The enriched genes ($P < 0.05$) are summarized in Fig. 4. The color bar of the heatmap represents the expression level of the mRNA scaled into 0 to 1, acquired from short read transcriptome sequencing experiments.

Table 4
Reported miRNA regulatory roles of circRNAs.

Condition	circRNA	miRNA	Target genes	Reference
Pre-Vaccination	hsa_circ_0001178	miR-382	YBX1, PCBP1, RFX1, RABGAP1L, TLN1, C11orf74, STAT2	(Ren et al., 2020)
		miR-587		
		miR-616		
First Vaccination	hsa_circ_0001346	miRNA-1224-5p	NDUFA2, SOCS7	(Zhang et al., 2020)
	hsa_circ_0001258	miR-744-3p	EMC3	(Zhu et al., 2019)
	hsa_circ_0007059	miR-421	PPP2CB	(Hui et al., 2022; Su et al., 2019)
	hsa_circ_0001522	miR-181b(up)	QKI, ZNF503, TAF5L	(Li et al., 2022)
	hsa_circ_0001535	miR-433-3p	POTEG	(Mao et al., 2022)
Second Vaccination	hsa_circ_0000643	miR-647	ZNF609	(Huang et al., 2023)
	hsa_circ_0004771	miR-653	ZNF813	(Huang et al., 2020; Qiao et al., 2023)
	hsa_circ_0001801	miR-224	EGLN1, SMKR1, ZNF850, PHAX	(Zheng et al., 2019)
	hsa_circ_0004872			(Ma et al., 2020)
	hsa_circ_0005576	miR-512-5p	PHAX	(Liu et al., 2022)
	hsa_circ_0088300	miR-1305	USP48, LRRC8A	(Shi et al., 2021)

3.6. Correlation between circRNAs and the potential source genes in expression profiles

Regarding the circRNAs sequenced and found to originated from the exon locus of specific genes, the correlation between the expression profile of mRNA acquired from transcriptome short read sequencing and the circRNA expression profile acquired from nanopore long read sequencing were calculated as described in the Method section 2.9. CircRNAs exhibiting strong correlations with genes (Pearson Correlation Coefficient $r > 0.6$ or < -0.6) were selected and summarized in Table 3.

CircRNAs exhibiting strong correlations with genes ($r > 0.6$ or < -0.6) were summarized in this table. The BSJ is shortened for Back Splice Junction locus and PCC is shortened for Pearson Correlation Coefficient.

3.7. Target genes of the miRNAs sponged by circRNAs are differentially expressed upon vaccination

The mRNA expression profiles of the 15 collected samples were compared, and the regulatory network of circRNAs, miRNAs, and target genes, potentially corresponding to the identified differentially expressed circRNAs, was summarized (Table 4 and Fig. 5). Pre-vaccination sample-specific circRNA hsa_circ_0001178 reportedly sponges multiple miRNAs, including miR-382, miR-587, and miR-616 (Ren et al., 2020). The target genes of the three miRNAs, YBX1, PCBP1, RFX1, RABGAP1L, TLN1, C11orf74, and STAT2, were upregulated in the pre-vaccination samples. In contrast, hsa_circ_0001522 reportedly upregulates miR-181b expression (Li et al., 2022); its target genes QKI, ZNF503, and TAF5L were downregulated after the first vaccination. Of the circRNAs specifically expressed in the five PBMC samples collected after the second vaccination, hsa_circ_0001801 and hsa_circ_0004872 were previously reported to sponge the same miRNA miR-224 (Ma et al., 2020; Zheng et al., 2019). The target genes of miR-224, EGLN1, SMKR1, ZNF850, and PHAX were upregulated following the second vaccination (Table 4 and Fig. 5). The expression profile of the listed circRNAs categorized by the condition is summarized in the dot plot of the upper right of Fig. 5, where the size of the dot represents the fraction of the samples in the condition the circRNAs were sequenced. Average expression level of circRNA were scaled from 0 to 1 and illustrated in the red and blue color bar. The reported regulation roles (Table 4) of the circRNA and miRNA were labeled in the color lines of blue in Pre-Vaccination samples, orange in First Vaccination and green in Second Vaccination samples. Target genes with differential expression pattern corresponding to the circRNA were summarized in the matrix plot with expression level scaled into 0 to 1 as well.

The circRNA with reported miRNA targeting functional roles. Target genes of miRNA recorded in MiRTarBase demonstrating expression patterns summarized in Fig. 5 are listed in this table.

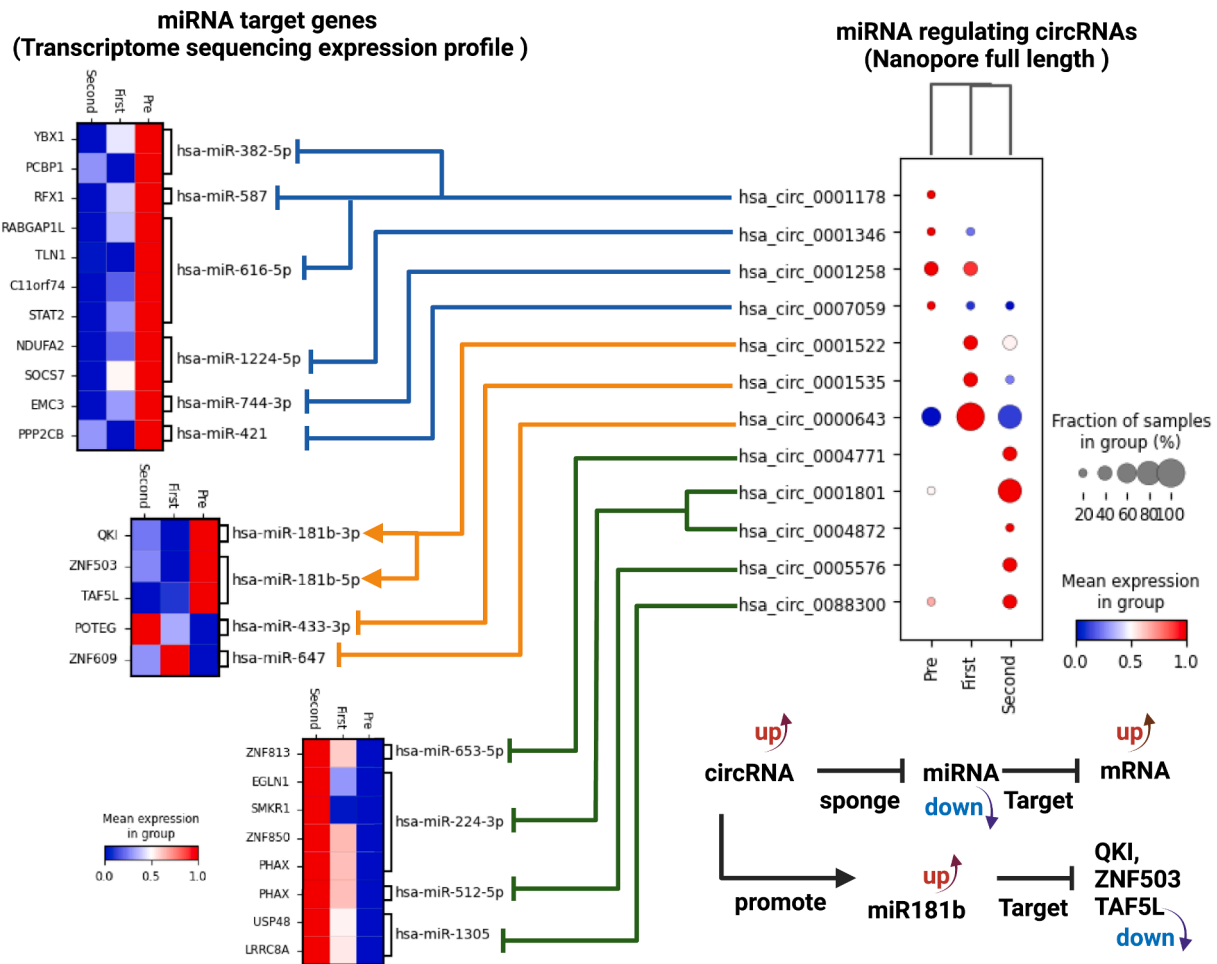


Fig. 5. Competing endogenous RNA activities Expression profiles of the miRNAs regulating circRNAs and the curated target genes of the miRNAs. Target genes, categorized by the miRNAs with corresponding expression pattern were summarized in the matrix plot. The network lines corresponding to the regulation roles summarized in Table 4 were labeled in blue for Pre-Vaccination samples, orange in First Vaccination and green in Second Vaccination samples.

3.8. Differentially expressed circRNAs are within the peripheral blood plasma cells of scRNA-seq samples

To investigate the potential cellular origin of the differentially expressed circRNAs in PBMCs, cell-type scRNA-seq data was collected from the public circRNA single-cell portal CircSCs (Wu et al., 2022). A total of 386 circRNAs were identified across 5,452 human scRNA-seq samples originating from 55 different tissue types (Wu et al., 2022). Considering that the samples examined in the current study were all PBMCs, the comparison of cellular origin was based on the 230 samples in the databank that also originated from blood, peripheral blood, and T-cells. Within the selected 75 circRNAs containing the top 25 differentially expressed circRNAs in public blood or T-cell scRNA-seq samples corresponding to the three conditions (pre-, first-, and second-vaccination), 23 circRNAs were identified within the 230 samples. The cell types associated with these 23 circRNAs are summarized in Table 5 and Fig. 6.

The number of public scRNA-seq samples found with differential expressed circRNAs is summarized in this table.

3.9. CircRNA sequence-based RNA binding motif analysis

A motif-based analysis on the sequenced full-length circRNA FASTA file (Supplemental File 2) using RBPmap was conducted (Paz et al., 2014). A complete list of identified motifs is provided in Supplemental File 4. The results revealed binding sites for 107 human and 24 mouse

RBP motifs. These results were compared with the list of RBPs that were reported to interact with SARS-CoV-2 viral RNA in the SCoV2-proteome-atlas (Schmidt et al., 2021). The results yielded 26 RBPs: A1CF, FUBP1, FUBP3, G3BP2, IGF2BP1, IGF2BP2, IGF2BP3, KHSRP, PABPC1, PABPC4, PCBP1, PCBP2, PTBP3, PUM1, PUM, RBM3, RBM4, RBM47, RBMS1, RBMS2, SRSF1, SRSF2, SRSF5, SRSF7, SRSF9, and YBX1. Enrichment of the associated RBP genes was performed using the DAVID software (Sherman et al., 2022) (Supplemental File 5). Among the 107 RBPs associated with the identified circRNAs, 21 were enriched in cellular component: cytoplasmic stress granules (SGs; GO:0010494): CELF1, FMR1, G3BP2, IGF2BP1, IGF2BP2, IGF2BP3, KHSRP, LIN28A, PABPC1, PABPC3, PABPC4, PABPC5, PUM1, PUM2, RBFOX1, RBM4, RC3H1, TARDBP, TIA1, YBX1, and ZFP36; nuclear SGs (GO:0097165): TIA1 and RBFOX1. Among them, TIA1, G3BP2, and PUM2 were associated with the BP GO term GO:0034063 and SG assembly, whereas TIA1 and YBX1 were associated with GO:1903608 and protein localization to cytoplasmic SGs. Interestingly, 11 of the 26 reported SARS-CoV-2 viral RBPs (i.e., IGF2BP2, IGF2BP3, RBM4, G3BP2, IGF2BP1, PABPC1, YBX1, PUM2, KHSRP, PABPC4, and PUM1) were associated with SGs.

3.10. Correlations between RBPs and circRNAs in expression profiles

The resulting 168,760 correlations between 107 RBP genes and 4140 unique circRNA, with r values ranging from 0.85 (between chr14:22551180|22574416 and DAZ) to -0.84 (between

Table 5

Detected circRNAs within blood scRNA-seq samples.

Condition	circRNA	Annotated cell type	Sample
Pre-vaccination	hsa_circ_0000233	B cell	1
		CD4-positive CD25-positive alpha-beta regulatory T cell	1
		Central memory CD4-positive, alpha-beta T cell	1
		Effector memory CD4-positive, alpha-beta T cell	1
		Plasma cells from peripheral blood	6
		Plasma cells from peripheral blood	1
	hsa_circ_0000722	Plasma cells from peripheral blood	1
	hsa_circ_0001073	Plasma cells from peripheral blood	2
	hsa_circ_0001178	Plasma cells from peripheral blood	1
	hsa_circ_0006508	Plasma cells from peripheral blood	1
	hsa_circ_0006530	Effector memory CD4-positive, alpha-beta T cell	1
		Plasma cells from peripheral blood	1
	hsa_circ_0008351	Plasma cells from peripheral blood	1
First vaccination	hsa_circ_0000643	Plasma cells from peripheral blood	1
	hsa_circ_0001436	Plasma cells from peripheral blood	1
		B cell	9
	hsa_circ_0001522	CD8-positive alpha-beta T cell	4
		Central memory CD4-positive, alpha-beta T cell	1
		plasma cells from peripheral blood	17
		B cell	1
	hsa_circ_0001679	Plasma cells from peripheral blood	3
	hsa_circ_0002174	Plasma cells from peripheral blood	1
	hsa_circ_0007163	Plasma cells from peripheral blood	3
	hsa_circ_0008967	Plasma cells from peripheral blood	1
Second vaccination	hsa_circ_0001306	Plasma cells from peripheral blood	3
	hsa_circ_0003959	Plasma cells from peripheral blood	1
	hsa_circ_0007099	Plasma cells from peripheral blood	1
	hsa_circ_0017636	Plasma cells from peripheral blood	1
	hsa_circ_0030720	B cell	1
	hsa_circ_0030720	In vivo single T cell	1
	hsa_circ_0069338	Ex vivo single T cell	1

chr4:147633765|147634581 and *PABPC1*) were calculated (Fig. 7). The top 200 positive correlations ($r > 0.74$) were nested between the 161 unique circRNAs and 16 RBPs. The top RBPs (RBM41, TRA2A, RBM24, NOVA1, and PABPC5) with the most positively correlated circRNAs are illustrated in Fig. 7C. The expression profiles of the 106 circRNAs positively correlated with the expression profile of RBM41. The distribution of the correlations between circRNAs and RBP appeared to have no observable bias with respect to the sequence length of the circRNAs (Fig. 7D). In contrast, the strongest 200 negative correlations ($r < -0.68$) comprised nested correlations between 147 unique circRNAs and 30 RBPs. Poly(A) binding protein cytoplasmic 1 (*PABPC1*) negatively correlated with 46 circRNAs (Fig. 7E). Additionally, pumilio RNA binding family member 1 (*PUM1*) was negatively correlated with the expression profile of 28 circRNAs with RNA-binding motifs detected using the RBPmap. Among the most negatively correlated RBPs, *PABPC1*, *PUM1*, and Y box binding protein 1 (*YBX1*) are associated with SARS-CoV-2 and SGs (Matsuki et al., 2013; Schmidt et al., 2021).

4. Discussion

In this study, PBMCs were collected before vaccination and after the first and second vaccinations (primary and first booster). To compare the correlation between expressed circRNAs and mRNAs, short-read RNA-seq was conducted. Among the 15 collected samples, 4706 circRNAs were identified, 484 of which were previously reported in public databases; 386 can be found in public scRNA-seq datasets. These results suggest the consistency and reliability of the experimental protocol (Zhang et al., 2021). The expression profiles of the sequenced circRNAs demonstrated extremely high specificity during vaccination. The substantial difference observed between the first and second vaccinations suggests that circRNA expression levels are potentially correlated with the innate immune response to vaccination. Among these, 50 circRNAs (recorded in CircBase (Glazar et al., 2014)) had functional roles. Potentially differentially expressed mRNAs due to competing endogenous RNA (ceRNA) activity were identified for 12 circRNAs.

Several circRNAs have been identified in blood plasma and serum exosomes. Comparison with public blood scRNA-seq data (Wu et al., 2022) suggested that most of the differentially expressed circRNAs likely originated from cells with molecular expression properties similar to those of plasma B cells.

Finally, RBP-binding motifs on the full-length sequenced circRNAs were detected using the RBPmap tool (Paz et al., 2014). Compared with SARS-CoV-2-associated RBPs in the SCoV2-proteome-atlas (Schmidt et al., 2021). Enrichment analysis of the 107 RBP gene symbols revealed that (Sherman et al., 2022) differentially expressed circRNAs enriched with SG binding motifs were correlated with TIA1, G3BP2, PUM2, and YBX1, as well as the viral RNA genome replication regulators PCBP1 and PCBP2. The binding motifs that correlated with SFPQ and ANKHD1 were also identified. Further examination revealed that PABPC1, PUM1, and YBX1 were strongly correlated with the corresponding circRNAs. The RBPs with binding motifs in the differentially expressed circRNAs were also associated with SGs or SARS-CoV-2 viral activity. The shift in the circRNA expression landscape within PBMCs before and after vaccination suggests that the sequenced circRNAs contribute to the immune response to the BNT162B2 mRNA vaccine. The speculation made here is based on the motif sequences found in the sequenced circRNAs. Further application with the orthogonal method to prove the functional interactions between circRNAs and the listed RBP is necessary for the future study.

SGs are dense complexes comprising proteins and RNA that appear when cells are subjected to various stressors (Wheeler et al., 2016). GTPase-activating protein-binding proteins, including Ras GTPase-activating protein-binding protein (G3BP1 and G3BP2, are core components of SG assemblies (Matsuki et al., 2013; Sidibé et al., 2021). The SARS-CoV-2 nucleocapsid (N) protein impairs SG formation by inactivating PKR autophosphorylation and forming a complex with G3BP1 or G3BP2 (Zheng et al., 2021). As part of the innate immune response, GTPase-activating protein-binding SG assembly can be triggered by the auto-phosphorylation of viral double-strand RNA binding PKR (Reineke and Lloyd, 2015). In addition, the association between SGs, RBPs, and circRNAs may contribute to the observed circRNA landscape shift. Various circRNAs have been identified in SGs, including G3BP2-binding circRNA and other SG assembly-related RBP-binding circRNAs (Chen et al., 2022). Specifically, circRNA-CREIT suppresses SG assembly by forming a complex with PKR and HECT domain and ankyrin repeat-containing E3 ubiquitin protein ligase 1 (HACE1) in triple-negative breast cancer (Wang et al., 2022). In addition to G3BP2, TIA1, PUM2, YBX1, and 17 other SG-associated RBP-binding motifs were enriched in the sequences of the discovered circRNAs. PUM2 was also identified in a recent study on host circRNA expression profiles in human lung epithelial cells infected with SARS-CoV-2 (Yang et al., 2021). Meanwhile, YBX1 is reportedly associated with multiple circRNAs in chemotherapeutic procedures (Xu et al., 2020) and promotes oncogenesis in hepatocellular carcinoma (Razpotnik et al., 2022). According to

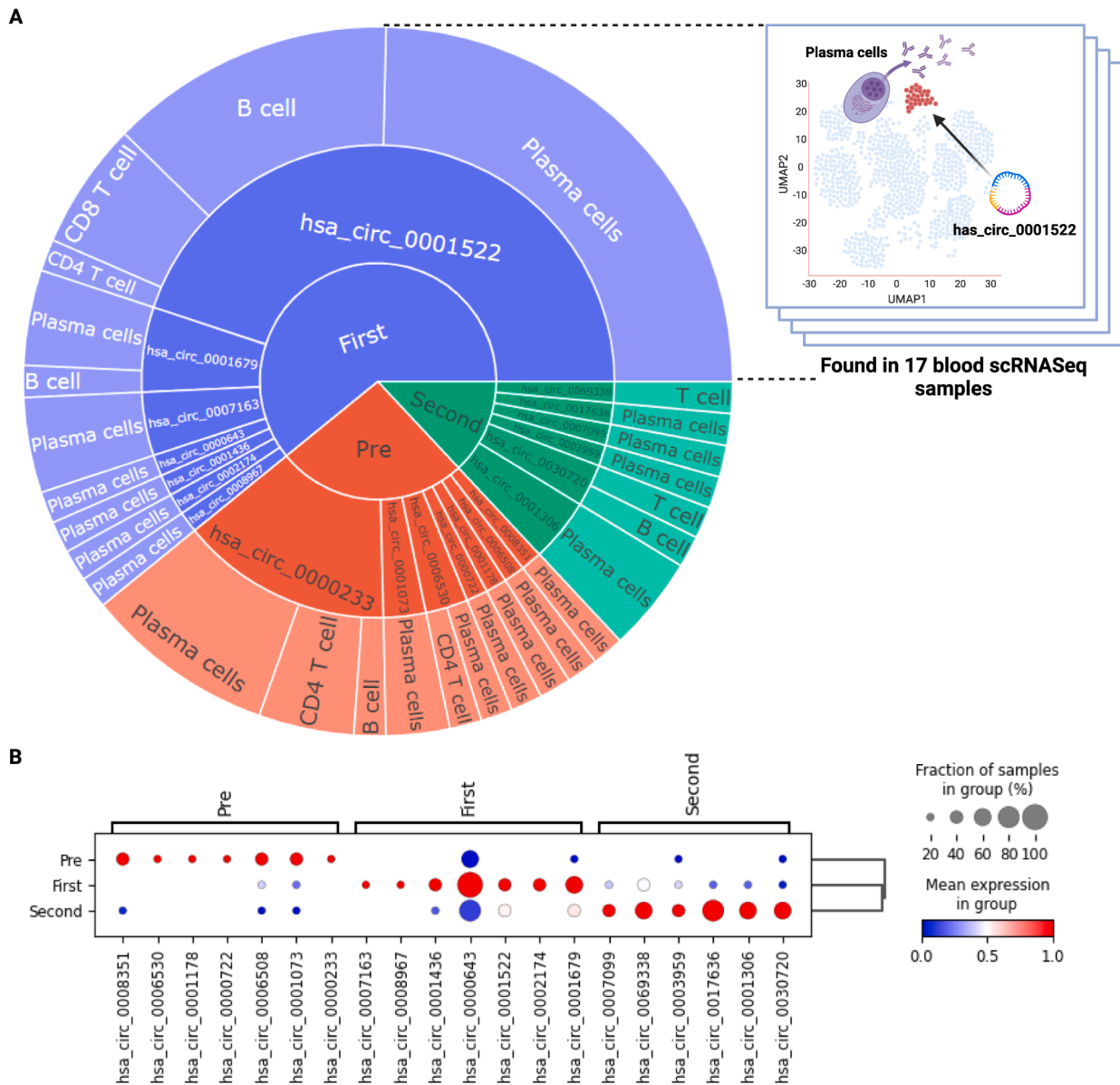


Fig. 6. Differentially expressed circRNAs in blood scRNA-seq samples. (A) scRNA-seq samples with corresponding cell types the circRNAs as listed in Table 5. (B) Differential expression profiles of the listed circRNAs.

multiple glioblastoma studies, SRSF family proteins are sponged by certain circRNAs, such as circSMARCA5 (Barbagallo et al., 2019; Barbagallo et al., 2018). Interestingly, 11 of these SG-correlated RBPs reportedly interact with SARS-CoV-2 viral RNA (Schmidt et al., 2021).

The correlation between circRNAs and the listed RBPs was further examined by computing the correlations between the expression of circRNAs and RBP mRNAs in 15 samples. These results suggest a strong correlation between the appearance of RBP-binding motifs and differential circRNA expression. In addition, the motifs of the viral RNA genome replication regulators PCBP1 and PCBP2 were enriched in differentially expressed circRNAs. The binding motifs of innate immunity GO terms associated with RBP, SFPQ, and ANKHD1 were also identified. Albeit the estimation appears to be promising, the corresponding coefficients were calculated based on the expression profile acquired from the sequencing experiments. Change of RNA expression profile is not direct evidence of the interaction between RBP and circRNA in this study.

Although only 10.3 % of the sequenced circRNAs in this study were detected in the major circRNA databases (Glažar et al., 2014; Wu et al.,

2020), various studies on human diseases other than COVID-19 have described the functional roles played by many of the top differentially expressed circRNAs. In particular, the functional roles of circulating circRNAs in cell proliferation, tumor metastasis, and drug resistance have been widely reported (Wang et al., 2019). However, whether differentially expressed circRNAs induced by vaccination appear in cell-free exosomes requires further investigation.

Most circRNAs identified through full-length nanopore sequencing in this study were intergenic. Prior to the establishment of recently developed full-length circRNA sequences (Liu et al., 2021; Rahimi et al., 2021; Xin et al., 2021; Zhang et al., 2021), circRNAs were assumed to be functional isoforms of mRNAs primarily within exonic regions. These newly discovered intergenic circRNAs may have various functional roles. Similar to the findings of the original CIR-long protocol (Zhang et al., 2021), mitochondrial circRNAs were also identified in this study. Steatohepatitis-associated circRNA ATP5B regulator (SCAR), a functional mitochondrial circRNA that inhibits mitochondrial ROS (mROS) output and fibroblast activation in the liver (Zhao et al., 2020). Moreover, mROS is associated with the activation of T- and B-cells in the

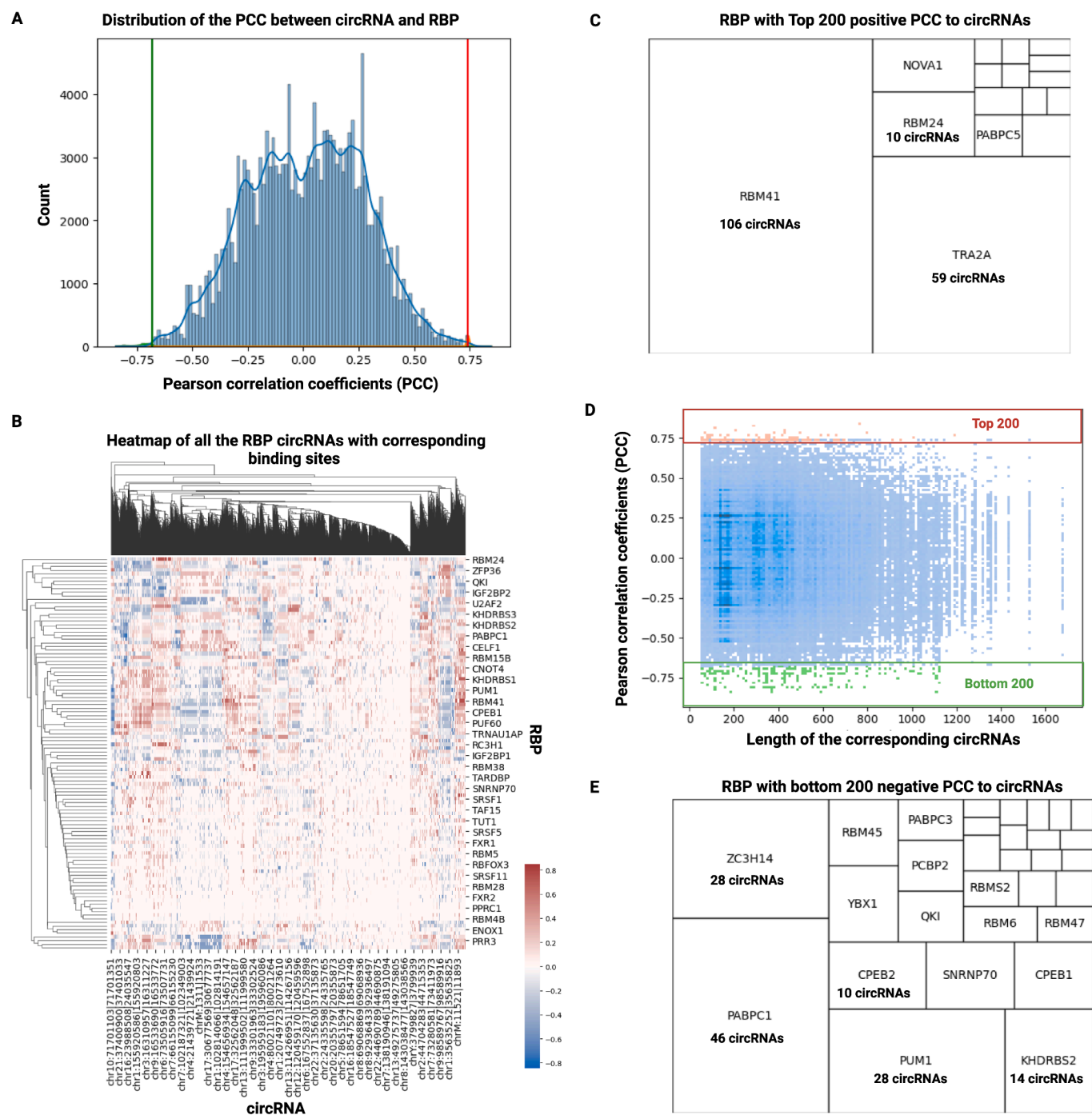


Fig. 7. Correlations between circRNA and RBP expression profiles. (A) Distribution of all circRNA RBP Pearson Correlation Coefficients (PCCs/ r) and the top 200 positive and negative correlations. (B) Summary clustering heatmap of all discovered circRNA RBP pairs. (C) Treemap summary of the most positively correlative RBPs; square size represents the number of unique circRNAs detected with binding sites. (D) Distribution of all circRNA RBP PCCs across the sequence lengths of the circRNAs. (E) Treemap summary of the most negatively correlative RBPs.

presence of antigens (Capasso et al., 2010; Sena et al., 2013). Hence, the potential association of the mitochondrial circRNAs detected within the PBMCs in this study and vaccination warrants further exploration. A comparison between the results of this study and public blood scRNA-seq data (Wu et al., 2022) indicated that the most differentially expressed circRNAs were detected within plasma B-cells, suggesting a potential correlation between blood circRNAs and adaptive immunization.

This study has certain limitations. First, despite the superiority of the reported nanopore long-read sequencing protocols over conventional

circRNA detection techniques, there are no empirical benchmarks for the robustness of the reported circRNA full-length sequence protocols. Second, the RBP motif-based binding site analysis in this study was computationally based and requires further experimental verification. Third, the circRNA expression profile of the blood cell samples did not reflect an immediate response to vaccine injection. To provide solid evidence of the existence of novel circRNA, qPCR with inverse direction primers designed for specific BSJ should be applied. Full length Sanger sequencing should be conducted to prudently verify the full-length sequences of selected circRNAs. Interaction between circRNA and RBP

should be verified through RNA immunoprecipitation (Zang et al., 2020).

Further exploration of the functional roles of the identified circRNAs can pave the way toward novel strategies for enhancing vaccine efficacy and immune system modulation.

Authors' contributions

This research project was conceptualized by Y. C. Liu under the supervision of D. Okuzaki. Resource management was performed by Sakakibara, Sugihara, Matsumoto, and Hirata under the supervision of Ogura. Experiments including library preparation and sequencing were performed as described by Naito et al. The circRNA enrichment and amplification experiments were conducted by M. Ishikawa. Generation and processing of sequence data were carried out under the supervision of D. Motooka. The data and deposits were recorded by Al-Kadi. Analysis and interpretation of the results were conducted by Liu. All authors were involved in the writing and editing of the manuscript.

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CRediT authorship contribution statement

Yu-Chen Liu: Writing – review & editing, Writing – original draft, Visualization, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Masakazu Ishikawa:** Resources, Methodology, Investigation, Conceptualization. **Shuhei Sakakibara:** Investigation. **Mohamad Al Kadi:** Resources. **Daisuke Motooka:** Supervision, Software, Resources, Methodology, Investigation, Data curation. **Yoko Naito:** Resources, Methodology. **Shingo Ito:** Resources, Methodology. **Yuko Imamura:** Resources, Methodology. **Hisatake Matsumoto:** Supervision, Investigation, Conceptualization. **Fuminori Sugihara:** Resources, Data curation. **Haruhiko Hirata:** Supervision. **Hiroshi Ogura:** Supervision, Methodology. **Daisuke Okuzaki:** Supervision, Software, Resources, Methodology, Investigation, Data curation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.gene.2024.148971>.

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