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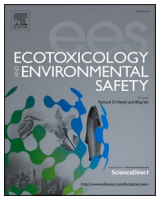

Transcriptional analyses provide novel insights into the transgenerational effects of Poly (I:C) on chickens

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ABSTRACT

Pathogenic microorganisms that are ubiquitous in the environment threaten human health and food safety. Polyinosinic:polycytidylic acid (Poly (I:C)) is a macromolecule with a double-stranded RNA structure, which is often used to simulate viruses. Our previous study found that Poly (I:C) maternal stimulation could affect the reproduction of laying hens and their offspring, but the underlying mechanism needed to be explored. In the present study, splenic transcriptomes were sequenced and analyzed from two groups (Poly (I:C) treatment as the challenged group and saline treatment as the control) and in three generations (maternal stimulated F0 hens, unchallenged F1 and F2 generations). The results showed that Poly (I:C) maternal stimulation affected gene expression patterns in laying hens and their offspring. A total of 27 differentially expressed genes (DEGs) with the same regulating trend were discovered in the F0 and F1 generations, indicating an influence of the intergenerational transmission effect. Functional enrichment analysis of Gene Ontology (GO) showed that lymphocyte differentiation, positive regulation of leukocyte differentiation, positive regulation of MAPK cascade, and T cell differentiation were the common biological processes between F0 and F1 generations, revealing Poly (I:C) could affect the immunity of the treated F0 hens and the unchallenged subsequent generations. Further study showed that pathways associated with growth, development, biosynthesis, and metabolism of F2 chicks were also affected by Poly (I:C) maternal stimulation. Correlation analysis between DEGs and reproductive traits revealed that *PHLDA2 (pleckstrin homology-like domain family A member 2)* and *PODN (podocan)* with inheritable effect were highly correlated with egg-laying rate and egg weight in F1 hens, suggesting their potential long-term role in regulating reproductive traits. *ARHGAP40*, *FGB*, *HRH4*, *PHLDA2*, *PODN*, *NTSR1,* and *NMU* were supposed to play important roles in regulating chickens' immunity and reproductive traits. This study reveals the far-reaching effect on transcriptome induced by Poly (I:C), reflecting the influence of the mother's living environment on the offspring. It is an important reference for future research into the multi-generational transmission of maternal stimulation and harmful environmental factors.

Abbreviations: Poly I:C, Polyinosinic:polycytidylic acid; P, Group P, treated with Poly (I:C); C, Group C, control, treated with saline; DEGs, Differentially expressed genes; FPKM, Fragments Per Kilobase of transcript per Million mapped reads; FC, Fold change; GO, Gene ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; PPI, Protein-protein interaction; RT-qPCR, Reverse transcription quantitative real-time PCR; coDEGs, Common DEGs; EW, Egg weight; LR, Egg-laying rate.

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1. Introduction

Public environmental health has been paid more and more attention by people. However, the widespread pathogenic microorganisms in the environment still pose a non-negligible threat to the health of humans and animals (Baghani et al., 2022). For example, COVID has swept the world in recent years and has had a large impact on people's lives and health (Bittmann, 2022). Nevertheless, the effects of COVID on the offspring of patients are rarely reported. Maternal exposure to various external stimuli factors, such as pathogens and toxins, have been reported to profoundly impact their offspring (Gong et al., 2021; Liu et al., 2020). Yet, similar studies are still insufficient on domestic animals.

Healthy animals and their offspring are particularly important for animal husbandry production, food safety and human health. Although people are paying more and more attention to the breeding environment of livestock and poultry, due to factors such as the closed nature of intensive breeding and the inability to clean up manure in time, there are still many pathogenic microorganisms in the living environment of livestock and poultry (Hu et al., 2017; Kumar et al., 2013). The influence of these pathogenic microorganisms on the growth and production performance of domestic animals and the underlying molecular mechanism needs to be further elucidated. As laying hens can produce a huge offspring population (Belousov et al., 2021), they are often taken as a model to study the influence of maternal stimulation on their offspring.

Polyinosinic:polycytidylic acid (Poly (I:C)) is a virus mimic whose structure is similar to double-stranded RNA. It can simulate the pathogenic mechanism of RNA virus infecting animals (Ai et al., 2021), and stimulate them to produce antiviral immune responses and inflammatory responses (Albarracin et al., 2017). Our previous studies have already found that Poly (I:C) maternal stimulus could cause the intergenerational transmission effect of alterations in the growth and reproductive performance of laying hens (Liu et al., 2019, 2018), but the molecular mechanism underlying still remains a lack. Radford et al. have shown that the changes in offspring traits caused by maternal stimulation may be regulated by the transcriptome (Radford et al., 2014). Considering that a better understanding of the mechanism of transmission between generations would be beneficial for disease prevention and reducing the economic loss for the livestock and poultry industry, the objective of this study is to investigate the regulation of the multi-generational transcriptome in the effects of Poly (I:C) maternal stimulation on the offspring.

Based on the influence of Poly (I:C) on the immune system of the organism (Chen et al., 2022), the current study detected the changes in the transcriptome of the spleen in the F0, F1, and F2 generations after Poly (I:C) treatment to the F0 hens, to study the long-term effect of Poly (I:C) on transcriptome and its regulation of immunity and reproduction of laying hens. Moreover, Poly (I:C) maternal stimulation also affected pathways associated with growth, development, biosynthesis, and metabolism of F2 chicks, revealing the durability of its effect. The current study reveals the significant impact of the mother's living environment on future generations, which provides an important reference value for animal breeding and human health, gaining evidence on the application of Poly (I:C) as drugs and vaccine adjuvants in humans and farmed animals.

2. Material and methods

2.1. Experimental animals and sample preparation

The experimental design has been described in detail in our previous research (Liu et al., 2018). In short, a total of 64 Rhode Island White hens at 53 weeks of age, raised under the same conditions, were randomly selected and equally divided into two groups, one being challenged with a dose injection of Poly (I:C) (1 mg/kg, MilliporeSigma, group P) and the other with equivalent sterile saline (group C) under the wing vein. Artificial insemination avoiding half-sib mating was performed 12 h before the challenge with 16 cocks and hatching eggs were collected till 56 weeks of age. Then, 528 F1 hens (group P, n = 270; group C, n = 258) were generated, out of which 64 F1 hens (34 from group P and 30 from group C) were randomly selected and inseminated intra-grouply to generate 587 F2 chicks (group P, n = 307; group C, n = 280). Totally, 18 individuals were randomly selected and sampled from the F0, F1 and F2 generations (six hens (group P, n = 3; group C, n = 3) per generation). Spleen tissues were harvested at 53 weeks of age for F0 and F1 generations. For some difficulties in the farm, F2 chickens were not reared to 53 weeks and spleens were collected at one day of age to explore the far-reaching effect of Poly (I:C) on the grandchildren. All tissues were snap-frozen in liquid nitrogen and then stored at − 80 ℃ until RNA extraction. All animals used in the current study were approved by the Animal Welfare Committee of China Agricultural University, Beijing, China (permit number: DK996).

2.2. RNA extraction and quality control

Total RNA was extracted from spleen tissues using Trizol (Thermo Fisher Scientific, Waltham, MA, USA) according to the protocol. The concentration and purity of total RNA were measured with the NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA), and the integrity was estimated by the Bioanalyzer 2100 System (Agilent Technologies, Santa Clara, CA, USA). RNA samples with high quality (RIN > 6.8, OD260/280 > 2, Supplementary Table S1) were used to construct the sequencing libraries.

2.3. Library construction and transcriptome sequencing

NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (NEB, Ipswich, MA, USA) was used for constructing cDNA libraries with 2 μg of total RNA for each sample. TruSeq Rapid PE Cluster Kit (Illumina, USA) was used to cluster the cDNA libraries. Finally, Illumina HiSeq 2500 platform (Illumina, USA) was used to sequence the libraries and 125 paired-end reads were obtained.

2.4. RNA-seq data analysis

The raw reads (fastq format) were primarily processed through FastQC v.0.11.5 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Trimmomatic software version 0.38 was used to filter out the adapter sequence and low-quality bases/reads with the default parameters (Bolger et al., 2014). Further quality assessment of the sequence reads was then undertaken using FastQC v.0.11.5. After these QC procedures were completed, the sequence reads were aligned to the reference genome GRCg6a (ftp://ftp.ensembl.org/pub/release-99/) through HISAT2 using basic options. Then, differential analyses were performed between group P and group C in the F0, F1, and F2 generations separately through Cuffdiff v.2.2.1 (Trapnell et al., 2010).

2.5. Functional enrichment and protein interaction network analysis

The DEGs ($P < 0.05$ and |fold change| (|FC|) > 1.5) in each generation were used to perform the following functional enrichment analysis. Gene Ontology (GO) terms of biological process and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway enrichment were conducted using the online software Metascape (Zhou et al., 2019). To further study the relationships among the GO terms, process enrichment analysis was performed. The biological processes with $P < 0.05$, count > 3, and enrichment factor > 1.5 (enrichment factor is the ratio of counts observed divided by the counts expected by chance) were retained and clustered based on the similarity of their membership. Word cloud analysis was performed with the GO terms used by the online software Free World Cloud Generator (https://www.freewordcloudgenerator.com/generatewordcloud). Finally, the Protein-Protein Interaction (PPI) network was performed through the database STRING v.11.0

(Szklarczyk et al., 2019).

2.6. Reverse transcription quantitative real-time PCR (RT-qPCR) validation

To quantitatively assess the reliability and accuracy of the RNA-seq data, we randomly selected 11 genes in the F0 generation for RT-qPCR, of which four genes were also verified in the F1 generation, with the same samples used for RNA-seq. The primers were shown in Supplementary Table S2. PrimeScript™ RT reagent kit was used to reverse transcribe the RNA samples into cDNA following the instructions (Takara Bio, Dalian, China). Real-time detection of specific PCR products was performed with the Sybr Green I Master Mix Kit (Roche Diagnostics, Mannheim, Germany) on the LightCycler 480 II (Roche Diagnostics Ltd., Basel, Switzerland) according to the manufacturer's protocol. The thermocycler program was as follows: one cycle of pre-incubation at 95 °C for 10 min and 45 cycles of amplification (95 °C for 10 s, 60 °C for 10 s, and 72 °C for 10 s). Duplicate RT-qPCR assays were performed on each cDNA sample and relative expression values were calculated using the $2^{-\Delta\Delta CT}$ method with the gene *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) as the internal reference. The $\log_2|FC=$ values were calculated from RT-qPCR data, and the results were compared with RNA-seq results.

2.7. Statistical analysis

For RT-qPCR data, the linear model fitting function was used to perform regression analysis with the $\log_2|FC=$ results of RT-qPCR and

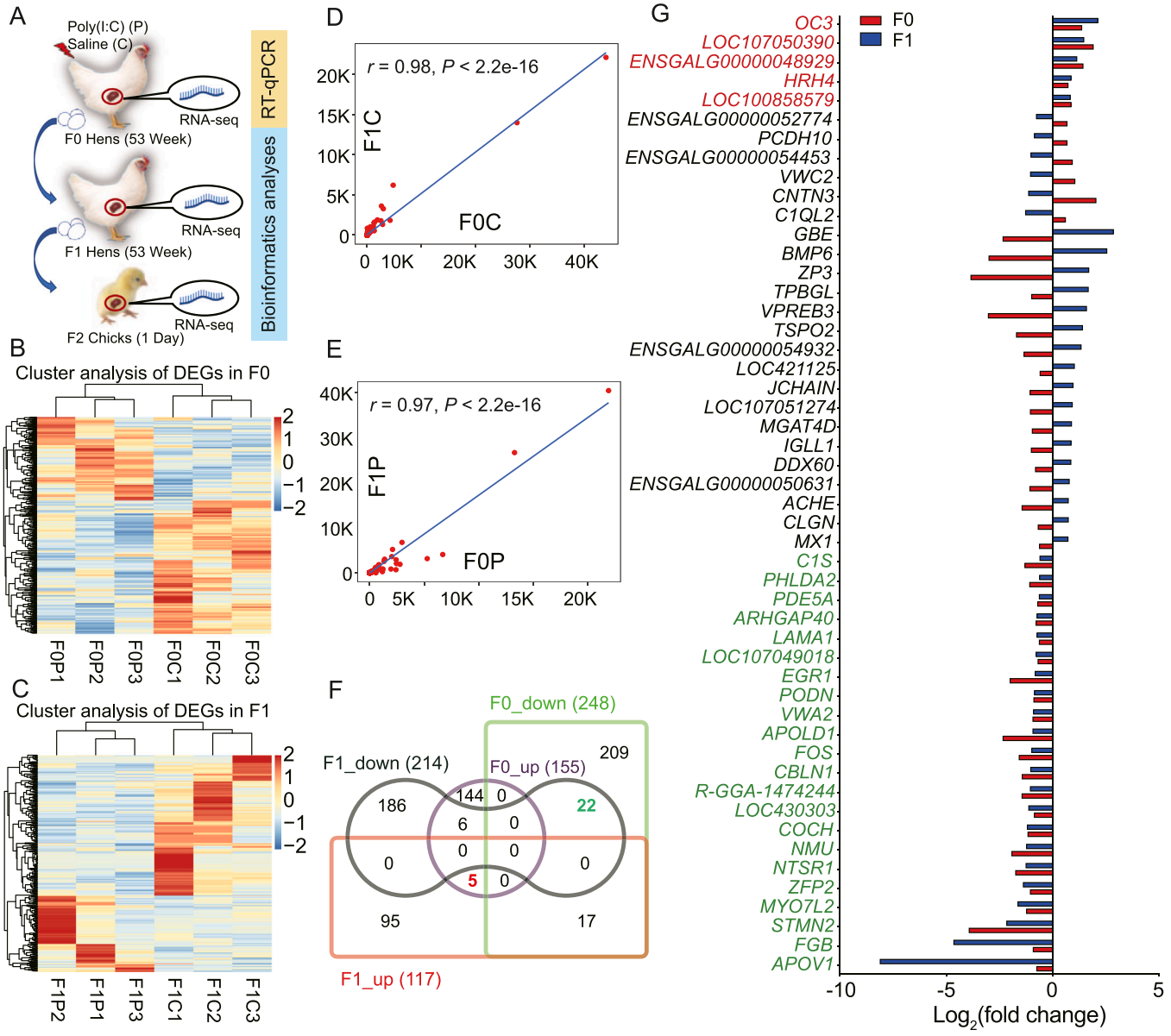

Fig. 1. Intergenerational effect of the transcriptome between the Poly (I:C) treated F0 and F1 hens. (A) The experimental design of the study. (B, C) Cluster heatmap of differentially expressed genes (DEGs) in the 12 individuals from the two generations. (D, E) Pearson correlation analysis of the gene expression between F0 and F1 generation in the control group (D) and the Poly (I:C) group (E). *r*: correlation coefficient. The X and Y axes indicate the FPKM value of DEGs in the F0 and F1 generations, respectively. 1 K = 1000. (F) Venn diagrams of the up-regulated and down-regulated DEGs of the F0 and F1 generations. (G) DEGs shared between F0 and F1 generations. The X-axis represents $\log_2$(fold change). Positive and negative values indicate the gene was up-regulated and down-regulated in the Poly (I:C) group, respectively. DEGs with the same trend in the two generations are marked separately on the Y-axis, with up-regulated DEGs in red and down-regulated ones in green.

RNA-seq in R software (v4.0.2). Moreover, Pearson correlation analysis was also performed between the key differentially expressed genes (DEGs) and reproductive traits (egg weight, EW, and egg-laying rate, LR). The "stat_cor" function in R software was used, and "Pearson" was selected for the method. The correlation coefficient and *P* value were calculated with default parameters. In addition to this, the fitted curve was also added using the "stat_smooth" function with the "lm" method. EW and LR data were from our previous study (Liu et al., 2018). The difference was reported to be statistically significant when $P < 0.05$.

3. Results

3.1. Poly (I:C) stimulation induces intergenerational transmission in gene expression between F0 and F1 hens

After Poly (I:C) stimulation in the F0 hens, spleen tissues were harvested at the same period (53-week-old) in F0 and F1 generations, and transcriptome alterations were studied (Fig. 1A). The results showed that Poly (I:C) maternal stimulation changed the splenic transcriptome both in F0 and F1 generation hens (Fig. 1B, C). A total of 403 and 331 DEGs were separately detected in the F0 and F1 generation based on $P < 0.05$, |fold change| (|FC|) > 1.5. And the down-regulated DEGs in the Poly (I:C) group of F0 and F1 separately accounted for 61.54 % and 64.65 %, implying that Poly (I:C) may have an inhibitory effect on the gene expression of the spleen.

Further, DEGs in the F0 and F1 generations were compared and a total of 684 DEGs were found to be involved, out of which 50 were common DEGs (coDEGs) that were shared in both generations. Pearson correlation analysis of the 684 DEGs with the expression level (FPKM) between the two generations was carried out. The results showed highly positive correlations both in group C and group P (Fig. 1D, E, $P = 2.2e\text{-}16$), indicating the transmission effect of the transcriptome caused by the Poly (I:C) stimulation in parental females. Moreover, among the 50 common DEGs, 27 coDEGs expressed with the same trend (5 up-regulated and 22 down-regulated) between the two generations (Fig. 1F), for example, *APOLD1, ARHGAP40, EGR1, FOS, FGB, HRH4, PHLDA2,* and *PODN* (Fig. 1G), and these genes are considered to be intergenerational heritable and could be used as potential marker genes for further study.

3.2. Poly (I:C) affects the immunity of the treated F0 and unchallenged F1 hens

Online software Metascape was used to separately perform functional enrichment analysis with DEGs in the F0 and F1 generations. A total of 340 and 274 significantly enriched pathways were identified in the F0 and F1, respectively (Supplementary Table S3 and S4, $P < 0.05$), of which 70 terms were shared by F0 and F1 generations (Supplementary Table S5). These GO items are mainly related to immunity and cell differentiation, hormone secretion and regulation, female pregnancy, skeletal and tissue development and cellular component morphogenesis, *etc*. (Supplementary Table S5). Focusing on the immune-related biological processes, we found five GO items shared in F0 and F1 generations, including regulation of MAPK cascade, lymphocyte differentiation, positive regulation of leukocyte differentiation, positive regulation of MAPK cascade, and T cell differentiation (Table 1). In the five shared pathways, 32 and 22 DEGs were separately involved in F0 and F1, out of which three genes, *EGR1*, *FGB,* and *FOS*, were overlapped. Interestingly, these three genes were regulated by the same trend in the challenged group (Fig. 1F). These findings revealed that Poly (I:C) challenging could impact the immune system and other biological processes of the treated F0 hens, such as the tissue development and female pregnancy. And the regulation influence on biological processes could be remembered and be translated to the unchallenged subsequent offspring.

Further, DEGs from F0 and F1 were merged and 684 genes were taken into Metascape to perform the functional enrichment analysis. Biological process enrichment analysis showed that the biological processes mainly clustered into chemotaxis, cell differentiation, proliferation, and system development, based on the similarity of their membership (Fig. 2A). And among them, chemotaxis was the most enriched and clustered term (Fig. 2B), indicating the pivotal role of immune response to Poly (I:C) challenging in F0 hens and their offspring. Furthermore, the leukocytes and lymphocytes were activated, further inducing the occurrence of immune response (Fig. 2C). As expected, in most terms the F0 generation had more genes than those of the F1 generation. However, we also noticed some terms possessed more dysregulated genes in the F1 generation than F0, such as regulation of system process, regulation of secretion, regulation of response to wounding, behavior, and response to alcohol (Fig. 2D), revealing the potential importance of these biological processes in F1 hens, which might be attributed to the Poly (I:C) stimulation on their parental dams.

3.3. Poly (I:C) affects biosynthesis and metabolism pathways in F2 generational chicks

Additionally, splenic transcriptome data from the F2 chicks were also explored and a total of 1742 DEGs were detected, including 849 down-

Table 1
Overlapped biological processes in F0 and F1 generations.

	Biological processes	*P* value	Gene symbols
F0 generation	regulation of MAPK cascade	9.64E-05	*ADRA2C, ATF3, CDH2, CCN2, DUSP1, FGB, FN1, CCN1, NPPA, ROR1, RGS4, SEMA7A, FGF18, GPR55, SPRY1, DIRAS2, PDGFC, ALKAL2*
	lymphocyte differentiation	1.68E-03	*ANXA1, ATP7A, PRDM1, EGR1, FUT7, IFNG, IRF4, POU2AF1, SLAMF8, AICDA*
	positive regulation of leukocyte differentiation	3.05E-03	*ANXA1, CA2, FOS, IFNG, JUN, SLC9B2*
	T cell differentiation	7.35E-03	*ANXA1, ATP7A, PRDM1, EGR1, FUT7, IFNG, IRF4*
	positive regulation of MAPK cascade	9.00E-03	*ADRA2C, CDH2, CCN2, FGB, ROR1, SEMA7A, FGF18, GPR55, DIRAS2, PDGFC, ALKAL2*
F1 generation	regulation of MAPK cascade	7.14E-04	*ADORA2B, AMBP, AR, DRD4, EDN3, FGA, FGB, RAP2A, TGFBR3, XDH, FZD7, SEMA3A, EDAR, SPRY4, IQGAP3*
	positive regulation of MAPK cascade	3.86E-03	*ADORA2B, AR, DRD4, EDN3, FGA, FGB, XDH, FZD7, SEMA3A, EDAR, IQGAP3*
	positive regulation of leukocyte differentiation	8.80E-03	*FOS, IL7, LGALS1, MYB, ZBTB16*
	lymphocyte differentiation	2.66E-02	*EGR1, IL7, LGALS1, MYB, ZBTB16, FZD7, HDAC4*
	T cell differentiation	4.96E-02	*EGR1, IL7, MYB, ZBTB16, FZD7*

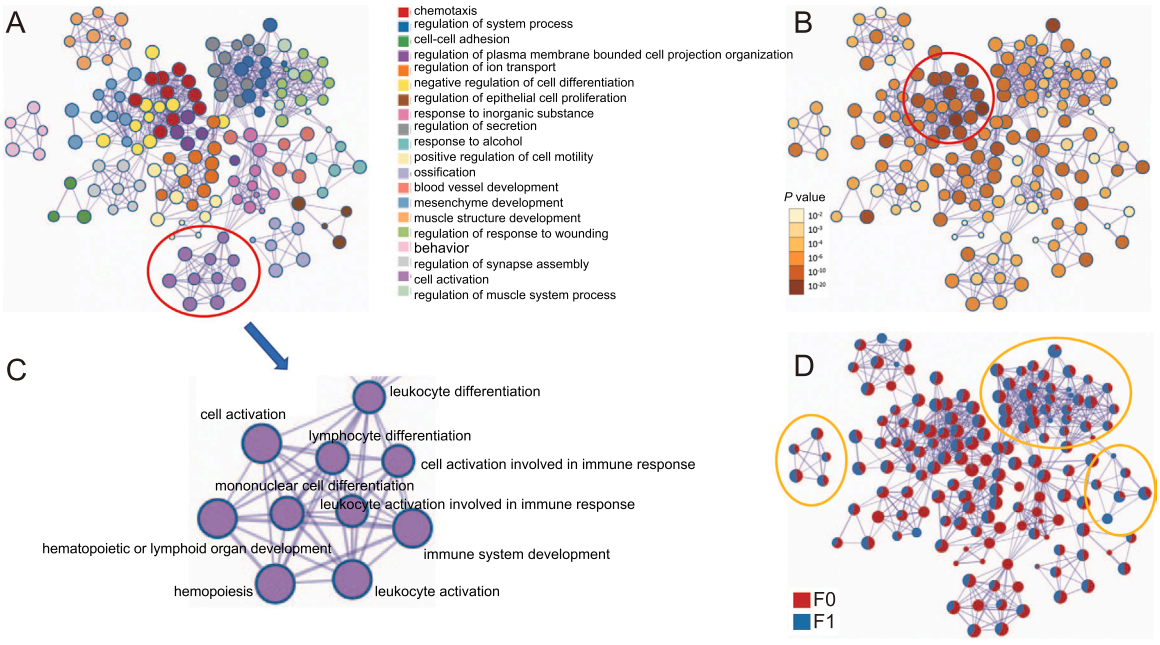

Fig. 2. Process enrichment analysis of biological processes in F0 and F1 generations. (A) The biological processes colored by cluster ID. The nodes with similar membership are typically close to each other. For each cluster, only the most significant terms are displayed. The size of the node represents the difference in gene numbers. (B) The biological processes colored by the *P* value, with the red circle showing the chemotaxis. The terms containing more genes tend to have a more significant *P* value. (C) The biological processes contained in the cluster of cell activation. All of the terms were immune-related biological processes. (D) Network of enriched terms represented as pie charts. The pies are color-coded based on the identities of the gene lists. The area of the pie represents the proportion of the number of genes. The orange circles show the biological processes with more genes in F1 than in F0.

regulated and 893 up-regulated DEGs. Although the splenic transcriptome data of F2 was obtained from 1-day-old chickens, the study still has reference value for discussing the effect of maternal Poly (I:C) stimulation on the generation of grandchildren, especially at the early developing stage.

Firstly, we performed biological processes of GO terms and KEGG pathway enrichment analysis on F2 generation DEGs. A total of 268 significantly enriched GO terms were found ($P < 0.05$). Word cloud analysis revealed that the development, morphogenesis, and growth of the unchallenged F2 chickens were mainly affected by the Poly (I:C) maternal stimulation (Fig. 3A). The interaction of these terms was shown in Fig. S1. Top 10 biological processes also included the development, morphogenesis, and growth-related terms, namely anatomical structure formation involved in morphogenesis, circulatory system development, and growth (Fig. 3B).

Moreover, there were 22 KEGG pathways predicted with the F2 DEGs, including histidine metabolism, mucin-type O-glycan biosynthesis, inositol phosphate metabolism, various types of N-glycan biosynthesis, drug metabolism - cytochrome P450, and so on (Fig. 3C). It was worth noting that all of the DEGs located in one carbon pool by folate (Fig. 3D), primary bile acid biosynthesis (Fig. S2A) and melanogenesis pathways (Fig. S2B) were up-regulated, indicating that these pathways in F2 generation chicks were activated by Poly (I:C) maternal stimulation. Also, we found that DEGs in drug metabolism - cytochrome P450 pathway, codeine & morphine were activated, while DEGs in tamoxifen, cyclophosphamide & ifosfamide, and citalopram were inhibited (Fig. S2C). Besides, N-Glycan biosynthesis in F2 generation chicks was also activated (Fig. S2D). Taken together, we speculated that some pathways relating to biosynthesis and metabolism were evidently influenced in F2 generational chicks of the challenged group.

3.4. Poly (I:C) affects transgenerational gene expression in the unchallenged F2 generation

Additionally, we compared the DEGs detected in spleens from F0 and F1 layers as well as F2 chicks, and eight DEGs (*PODN, PHLDA2, OC3, LOC430303, JCHAIN, FGB, ENSGALG00000050631*, and *ARHGAP40*) were found to be shared by three generations. Among them, three genes, *LOC430303*, *PHLDA2,* and *PODN*, were expressed in the same direction (Fig. 4A, B) and could be regarded as transgenerational inheritable DEGs. These results indicated that Poly (I:C) stimulation might induce a long-term effect on splenic transcriptome throughout at least three generations. Furthermore, the results of the PPI network analysis showed that the proteins FGB and ARHGAP40 were connected with TLR3 (Fig. 4C, D), which was reported to be the receptor of the Poly (I:C) (Matsumoto and Seya, 2008), further proving that the enduring effect was caused by the Poly (I:C) maternal stimulation. The gene *FGB* was enriched in six of the ten terms and its fold change between group P and the control was 41.21(P = 5E-05, Fig. 3B), indicating its critical role and the effect may be induced by the Poly (I:C) maternal stimulation.

In order to verify the accuracy of the RNA-seq data in this study, we randomly selected eleven genes for verification with RT-qPCR in the F0 generation and four were also tested in F1. The results showed that the expression trends of all the detected genes were indeed the same in RNA-seq and RT-qPCR (Fig. 4E), and the correlation between the two techniques was as high as 0.88 (Fig. 4F, $P < 0.01$), confirming the accuracy and reliability of the RNA-seq results in this study.

3.5. Correlation of the inheritable DEGs with hen's reproductive traits in the F1 generation

As our previous study showed, Poly (I:C) stimulation could significantly affect the reproduction traits (egg weight and laying rate) and had an intergenerational effect on chicken. Considering the close relationship between animal reproductive performance and immunity (Chao and Lee, 2001; Zhuo et al., 2020), the association analysis of the 27 intergenerational inheritable DEGs with chicken reproduction traits was performed in the F1 generation. The expression level (FPKM) of the above 27 coDEGs with the same expression trend were extracted in the six F1 hens and their correlation with the average egg weight (EW) and

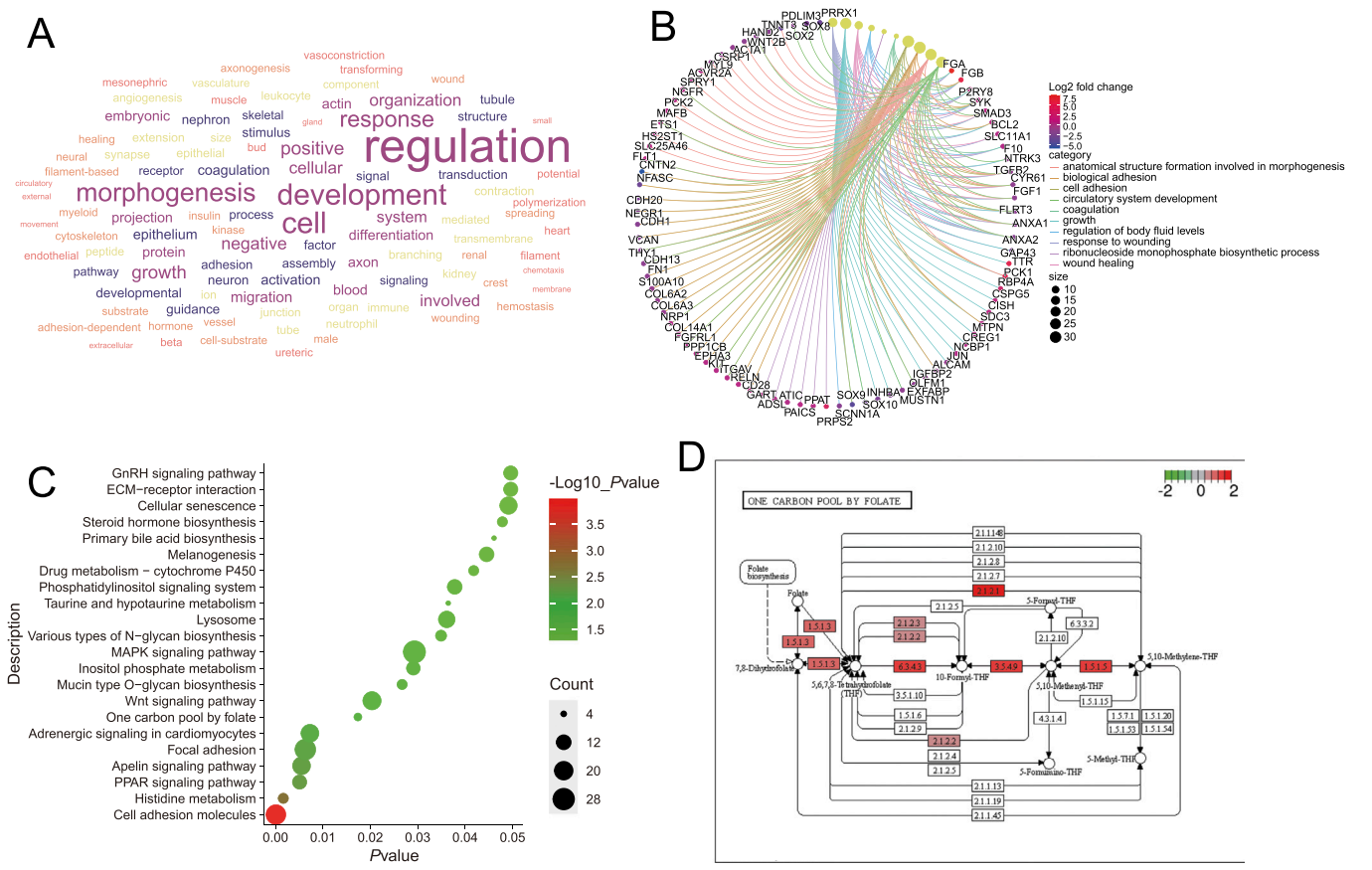

Fig. 3. Functional enrichment analysis in the F2 generation. (A) Word cloud of the biological processes GO terms predicted with DEGs in the F2 generation. The larger the font size of the word, the more frequently it appears in the terms. (B) Cnetplot of top 10 biological processes. The yellow dots represent GO terms, and the larger the dots, the more genes. The color of the gene point represents the value of $\log_2$(fold change), with red showing up-regulated and blue showing down-regulated. (C) The significant KEGG pathways of F2 generation with the criterion of $P < 0.05$. (D) The pathway of one carbon pool by folate. The red box indicates that the gene is up-regulated. All genes involved are up-regulated, indicating the pathway is activated.

egg-laying rate (LR) at 53 weeks old were analyzed (Fig. 5A). The results disclosed that egg weight had a highly negative correlation with the egg-laying rate, with a correlation coefficient of 0.75 (Fig. 5B, $P = 8.1e\text{-}06$). Regarding the EW trait, the correlation coefficient of 14 (51.58 %) DEGs was higher than 0.5, of which 78.57 % (11/14) were positively correlated with egg weight. For the LR trait, the correlation coefficient of 9 (33.33 %) DEGs were higher than 0.5, of which 77.78 % (7/9) were negatively correlated with the egg-laying rate (Fig. 5A). It showed that the intergenerational transmission of the changes in reproductive traits might be related to these genes.

Among these coDEGs, three were detected to be significant with reproductive traits. *NTSR1* and *HRH4* had a significantly positive and negative correlation with the EW trait ($P < 0.01$), respectively, while *NMU* was significantly and negatively correlated with LR ($P < 0.05$, Fig. 5C, D, E). The gene expression results showed that *NTSR1* and *NMU* were significantly down-regulated and *HRH4* was significantly up-regulated in the Poly (I:C) group of both F0 and F1 generations (Fig. 5F, G, H).

As aforementioned, the expression of the *LOC430303*, *PHLDA2*, and *PODN* genes had a transgenerational inheritable effect. We found that all three genes were down-regulated in Poly (I:C) group across all three generations (Fig. 6A, B, C). Likewise, we extracted the FPKM in the F1 hens of these three DEGs and performed a correlation analysis with the reproductive traits. The correlation coefficient of *PHLDA2* and *PODN* with both LR and EW were greater than 0.5 (Fig. 6D, E), suggesting a moderate or high correlation in the F1 generation. All three genes were positively correlated with EW but negatively correlated with LR (Fig. 6D, E).

4. Discussion

Maternal exposure to infectious agents or maternal stimuli increases the risk of disease in offspring (Rousset et al., 2013). Maternal stimulation may inhibit the reproductive performance of animals, and the negative impacts can be passed on to the next generation (Bale, 2015; Liu et al., 2022). Our previous studies have shown that maternal injection with a single dose of 1 mg/kg of Poly (I:C) causes an increase in egg-laying rate and a decrease in egg weight both in the challenged laying hens and the subsequent unchallenged generation (Liu et al., 2019, 2018). In this study, we investigated the effects of Poly (I:C) stimulation on the spleen transcriptome of the treated laying hens and the next two generations to reveal the underlying mechanisms of enduring effect on the transcriptome level, and further explored the potential impact of Poly (I:C)-mediated and changed genes on the reproductive performances of laying hens.

Based on whether the object is directly affected by the stimulus, the multi-generational transmission can be divided into intergenerational and transgenerational transmission (Perez and Lehner, 2019). With the chicken model, the intergenerational and transgenerational transmission can be separately observed in F1 and F2 generations (Liu et al., 2018). In the current study, a total of 27 coDEGs having a consistent expression trend and 70 shared significantly enriched biological process GO terms ($P < 0.05$) were found between F0 and F1 hens, indicating that Poly (I:C) could induce the intergenerational transmission of the transcriptome of layer spleen. It is reported that MAPK signaling proteins could be upregulated by Poly (I:C) (Farrelly et al., 2015). In this study, GO terms of positive regulation of MAPK cascade were found in

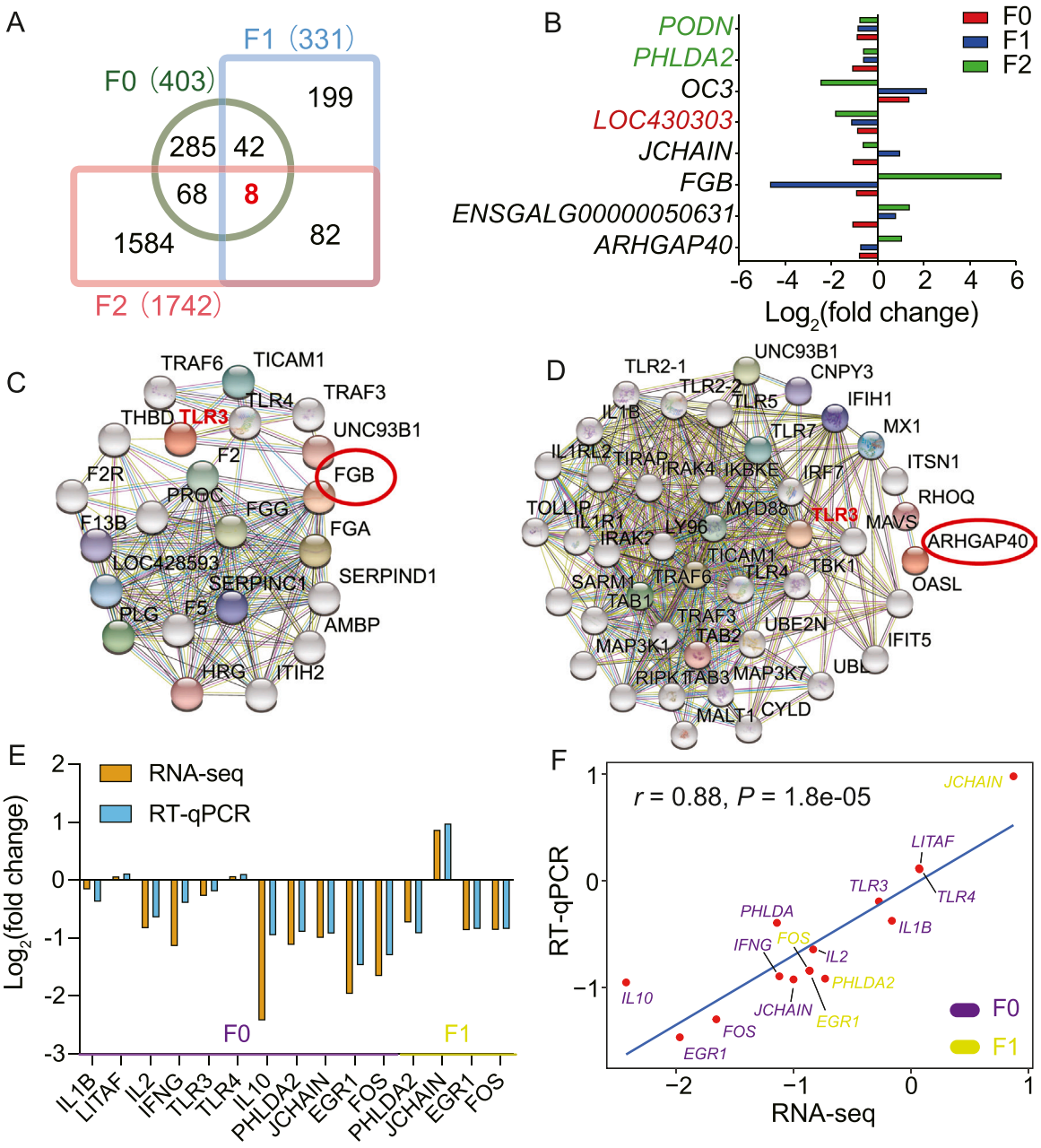

Fig. 4. The study of shared differentially expressed genes (DEGs) across the three generations induced by Poly (I:C). (A) Venn diagram of DEG numbers in F0, F1, and F2 generations. (B) The shared DEGs across the three generations. The X-axis represents $\log_2$(fold change). Positive and negative values indicate the gene was up-regulated and down-regulated in Poly (I:C) group, respectively. The up-regulated and down-regulated DEGs with the same expression direction in the three generations were marked in red and green, respectively, on the Y-axis. (C, D) The PPI network analysis of FGB (C) and ARHGAP40 (D). (E) RT-qPCR confirmation results for the eleven and four randomly selected genes tested in F0 and F1 generations, respectively. The *GAPDH* gene was used as an internal reference control gene in RT-qPCR. (F) Regression analysis between RNA-seq and RT-qPCR validation. The values on X-axis and Y-axis represent $\log_2$(fold change). *r*: correlation coefficient.

both F0 and F1 generations. Moreover, GO terms of lymphocyte differentiation, positive regulation of leukocyte differentiation and T cell differentiation were also found in the two generations, indicating that Poly (I:C) maternal stimulus could result in the alteration of immunity in the offspring (Arsenault et al., 2014) which might be affected by the differentiation of immune cells in laying hens and their offspring.

Poly (I:C) prenatal immune activation can induce the transgenerational transcriptional transmission in the brain of mouse (Weber--Stadlbauer et al., 2017). Here, the first time we found that Poly (I:C) maternal stimulation affected the splenic transcriptional patterns of the unchallenged F2 generation chicks. A recent study has shown that Poly (I:C) could delay the growth and development of mice (Arsenault et al., 2014). In the present study, the DEGs enrichment analysis also revealed that Poly (I:C) maternal stimulation might affect the growth and development of F2 chicks. Coincidentally, a decrease in the birth weight of the offspring induced by Poly (I:C) maternal stimulation was observed in our previous study (Liu et al., 2018). The alteration in biosynthesis and metabolism pathways may be the potential mechanism of the changed growth and development of the F2 chicks. In addition, the results of PPI showed that the proteins FGB and ARHGAP40 were related to TLR3, which was the receptor of the Poly (I:C) (Matsumoto and Seya, 2008), proving that the multi-generational transcriptional alteration might be caused by the Poly (I:C) maternal stimulation. However, further study is needed to explain why the expression trend of these two genes changed in the F2 generation.

The intergenerational transmission of phenotypic alterations may be

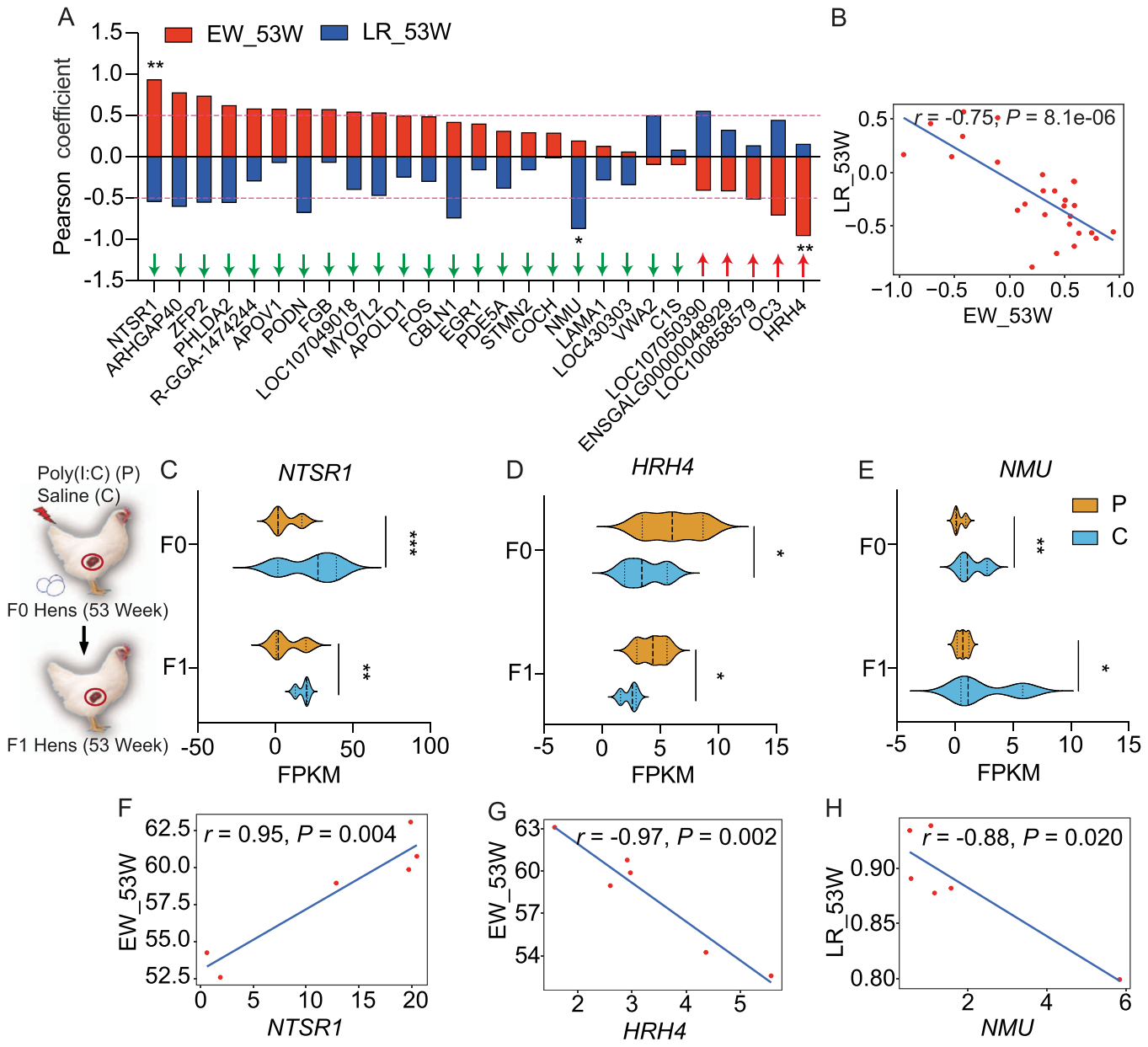

Fig. 5. Correlation analysis of intergenerational heritable DEGs with reproductive traits in the F1 generation. (A) Correlation analysis between reproductive traits and gene expression of 27 common DEGs (coDEGs) with the same regulation trend in F0 and F1 generations. The reproductive traits include egg-laying rate (LR, blue bars) and egg weight (EW, red bars) at 53 weeks of age in the F1 generation. The green-down and red-up arrows separately indicate the down-regulated and up-regulated genes. (B) Regression analysis between egg weight and egg-laying rate. (C, D, E) Intergenerational heritable coDEGs of *NTSR1*, *HRH4,* and *NMU*, expressed with the same trend in F0 and F1 generations. (F, G) Correlation analysis of gene expression of *NTSR1* (F) and *HRH4* (G) with egg weight. (H) Correlation analysis of gene expression of *NMU* with egg-laying rate. * : $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$. *r*: correlation coefficient.

affected by the intergenerational transmission of the transcriptome (Arsenault et al., 2014; Liu et al., 2022). Our previous studies have shown that Poly (I:C) maternal stimulation can cause an increase in egg-laying rate and a decrease in egg weight in F0 and F1 generations (Liu et al., 2019, 2018). In this study, we found that all of the up-regulated DEGs shared by the F0 and F1 generations were positively correlated with egg-laying rate and negatively correlated with egg weight, and 90.91 % (20/22) of the down-regulated genes were negatively correlated with egg-laying rate, and positively correlated with egg weight. From the transcriptional level, the above results further prove that Poly (I:C) can increase the egg-laying rate and decrease the egg weight of hens, indicating that the intergenerational transmission of phenotypic changes may be regulated by the transcriptome. It is reported that Poly (I:C) can improve the body's immune resistance (Apostolico et al., 2019), which might further increase the egg-laying rate of hens in the current study. And based on the conservation of energy, the increase in egg-laying rate leads to a decrease in egg weight. In terms of *NTSR1, HRH4,* and *NMU* genes, their expression levels were first discovered to be highly correlated with egg-laying traits in this study. The regulatory mechanism needs to be further revealed in future research.

It is noteworthy that after challenging, three genes, *LOC430303*, *PHLDA2,* and *PODN* were all significantly down-regulated in F0, F1, and F2 generations, suggesting that the Poly (I:C) effect may have a transgenerational transmission effect. *PHLDA2* is reported to play a vital role in regulating placenta growth (Angiolini et al., 2021; John, 2017). As a maternally expressed imprinted gene, its expression level is linked to fetal birth weight and growth restriction in humans (Xing et al., 2019).

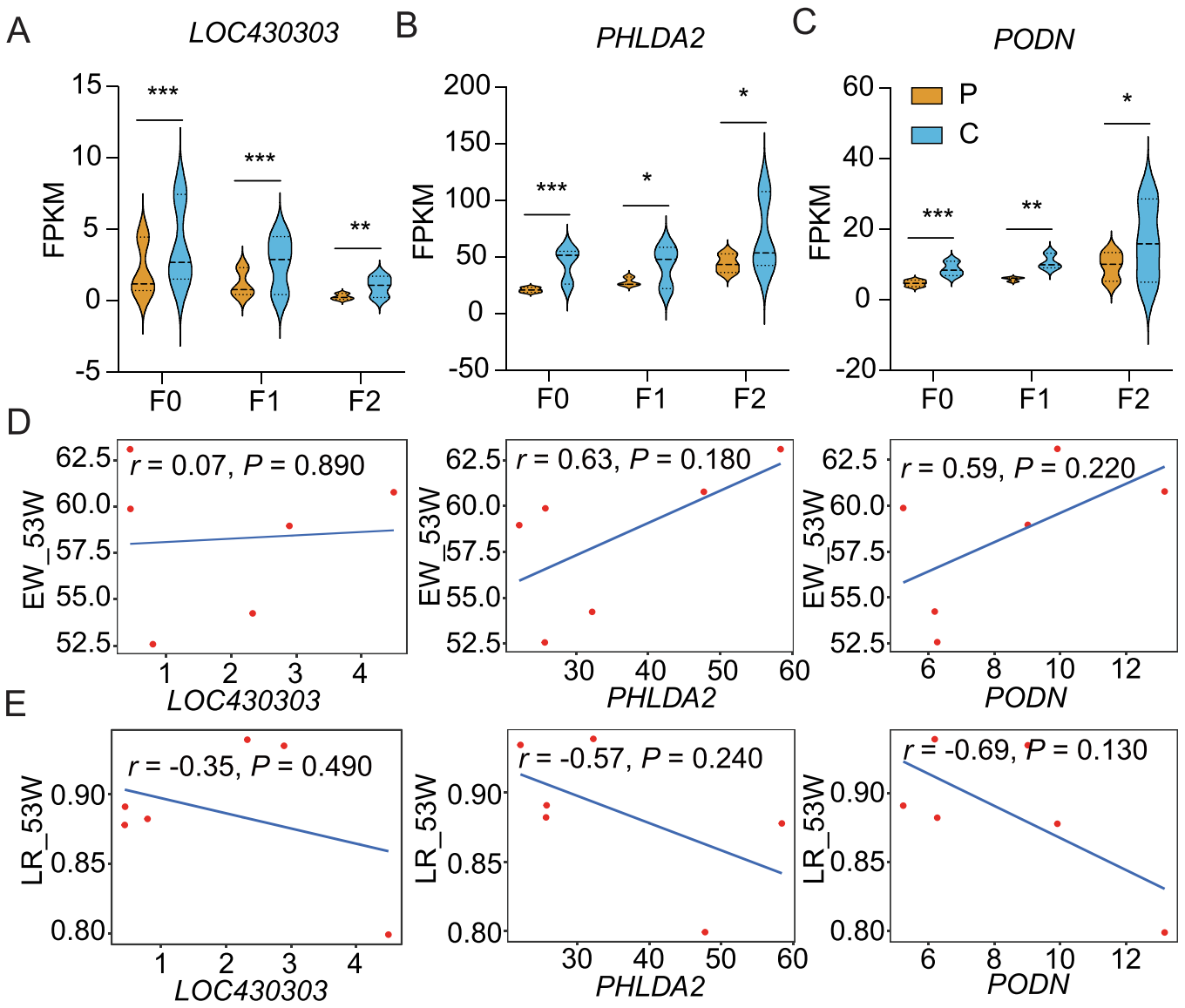

Fig. 6. The transgenerational heritable DEGs and their relationship with reproductive traits. (A, B, C) The gene expression of *LOC430303* (A), *PHLDA2* (B), and *PODN* (C) across the three generations in the Poly (I:C) group and the control. (D) Correlation analysis of *LOC430303*, *PHLDA2,* and *PODN* with egg weight at 53 weeks of age (EW_53W) in the F1 generation. (E) Correlation analysis of the three DEGs and egg-laying rate at 53 weeks of age (LR_53W) in the F1 generation. * : $P < 0.05$, **: $P < 0.01$, *** : $P < 0.001$, *r*: correlation coefficient.

PODN had a profound clinical significance in clinical diagnosis (Yao et al., 2021). It negatively regulates cell proliferation and cell migration (Shimizu-Hirota et al., 2004) and the silencing of *PODN* reduced by miR-3180–5p could lead to a significant promotion of cell proliferation in human bladder smooth muscle cells (Sun et al., 2016). For the first time, our study suggested that the *PHLDA2* and *PODN* gene expression levels were related to reproduction performances in chickens, with a negative relationship with the egg-laying rate but a positive relationship with egg weight. The long-lasting impact of chickens posted by the Poly (I:C) treatment process indicated that the three genes could be used as marker genes in chicken breeding and deserved further research.

In the present study, F0 generation hens were treated only once, and the offspring were not directly stimulated. However, a large number of DEGs were induced in the F2 generation at birth, which means that environmental stimulating factors have a substantial impact on the hens and their unexposed offspring. It was reported that when the somatic cells of the offspring are affected by the maternal exposure factor, the next generation will also be affected (Hao et al., 2017). This effect can be passed on to the subsequent generations only when the germ cells of the fetus are affected by the exposure factor (Gapp et al., 2014). From the transcriptional level, this study revealed the mechanism of maternal stimulus, which could result in changes in the reproductive performance of the offspring. The transmission of information between generations needs to be carried out through germ cells. The alteration of the spleen transcriptome indicated that the gene expression information in somatic cells might be transmitted to germ cells directly (O'Brien et al., 2020) or through miRNA and other mediators (Sharma, 2015; Silva et al., 2021), which in turn leads to the inheritance of phenotypic traits in untreated offspring.

5. Conclusions

This study uses a layer model to reveal that Poly (I:C) stimulation in females can cause a long-lasting effect at the transcriptional level, which in turn affects the immunity and production performances of the laying hens and their offspring. This research has important reference significance not only for human environmental health research but also for livestock and poultry production management and breeding. Full

consideration of intergenerational transmission effects in conventional breeding will speed up the breeding process, but how to apply the phenotypic results and molecular mechanisms obtained from multi-generational transmission studies in breeding practice is a long way to go.

CRediT authorship contribution statement

Lei Liu: Conceptualization, Methodology, Resources, Formal analysis, Writing – Original Draft. **Di Wang**: Resources, Formal analysis, Writing - Original Draft. **Yang Fu**: Formal analysis, Writing – review & editing. **Zhongyi Duan**: Resources, Writing – review & editing. **Adeyinka Abiola Adetula and Huagui Liu**: Writing - review & editing. **Ying Yu and Qin Chu**: Conceptualization, Writing – review & editing, Supervision.

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

The dataset supporting the conclusions of this article is available in the National Center for Biotechnology Information database under the accession number PRJNA763763.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2022.114216.

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