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# Knockdown of gga-miR-142-3p in developing chicken embryo: expression profile of XPO1

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**ABSTRACT**

Differential expression of gga-miR-142-3p microRNA of haemopoeitic origin during immune organ development and functional stages in chicken embryos creates new opportunities for understanding its pivotal role during embryonic developmental stages. To decipher the role of gga-miR-142-3p *in-ovo* knockdown was carried out with LNA modified anti-miR-gga-miR-142-3p *via* intravenous route at developmental and functional stages of the immune organs and other organs. Bioinformatic analysis of the genes targeted by gga-miRNA-142-3p revealed that the predicted gene XPO1 conserved binding sites at 3'UTR. The target gene XPO1 was evaluated as the validated target of gga-miR-142-3p by employing qPCR SYBR green based technology, which was evidenced by its increased expression in the tissues of gga-miR-142-3p knockdown chicken embryos. Histopathological alterations in the immune organs and visceral organs indicated that the systemic knockdown of gga-miR-124-3p led to over expression of the XPO1 gene during the embryonic stages, and changed the environment of the immune organs related to structural integrity, immune response, signal transduction and migration of B and T cells during the embryonic developmental stage in the chicken embryos. The results clearly indicated that these changes could alter the postnatal development and functions of these immune organs, and may lead to development of immuno-compromised chickens.

**Key words:** gga-miR-142-3p knockdown, chicken embryo, XPO1 expression

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## Introduction

MicroRNAs are small non-coding endogenous RNA (21 to 23 nt in length) which are generated from endogenous hairpin-shaped transcripts. MicroRNAs are one of the largest

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gene families, constituting approximately 1 to 3% of the genome, and are predicted to regulate 30% of human genes (LEWIS et al., 2005; SANDHU et al., 2011). Micro RNAs also act as delicate regulatory switches and fine tuners of gene expression, by binding mainly to 3' UTR sites, as well as to the ORF and 5' UTR sites occasionally, in a sequence specific manner to regulate stability and translation of the target mRNAs. Micro RNAs interact with mRNAs and either block protein translation or lead to protein degradation (BARTEL, 2009). In addition, miRNAs also play a major role in normal biological pathways, such as cell cycle, survival, differentiation, proliferation and migration of cells.

gga-miR-142-3p was highly expressed in all the cDNA libraries of day 15 and day 20 in the immune organs. In the spleen and bursa on day 20 it accounts for the 5.9% and 9.7%, respectively of the total reads (HICKS et al., 2008; HICKS et al., 2009). As miR-142-3p is highly expressed in the developmental stages of immune organs near to hatching, it has a major role in modulating the expression of genes involved in immune response, signal transduction, and maturation and migration of T and B cells. Knockdown of this miRNA at developmental and functional stages of these immune organs and or other organs will elucidate the functional role of the gga-miR-142-3p in respect to genes regulating the target organs involved in signal transduction and the immune response of B and T cells during embryonic developmental stages.

XPO1 gene encoding Exportin 1, also known as CRM1, an export protein, facilitates the nuclearcytosolic transport of large macromolecules, including RNA and protein, across the nuclear membrane to the cytoplasm. In addition to nuclear-cytosolic transport, CRM1 also plays a role in centrosome duplication and spindle assembly, especially in response to DNA damage during cell division and cell biogenesis. XPO1 regulates various cellular signalling pathways and shuttling of proteins: Nucleophosmin (NPM) involved in centrosome duplication, NF-κB during cell growth (ZHANG et al., 2012), (cellular FLIP) cFLIP in apoptosis signalling pathway and modulation of Wnt signalling (KATAYAMA et al., 2010), and regulates the avian geminin protein involved in targeting the embryonic patterning control genes of the HOX family in chickens (LUO et al., 2007).

Hence the present study was conducted using bioinformatic analysis in predicting the target binding site of gga-miR-142-3p on the 3' UTR of the XPO 1 gene, and also to analyse the effect of gga- miR-142-3p knock down on the expression pattern of the XPO1 in immune and visceral organs during the embryonic developmental stage of chickens.

## Materials and methods

*Bioinformatics analysis.* All the different genes targeted by gga-miR-142-3p involved in signal transduction and immune response were analysed using different miRNA databases *viz*., miRBase 19, miRDB, Target scan 6.2, PicTar, and analysed by RNA hybrid, DIANA-microT-CDS (V 5.0), etc. From all the analysed genes involved in signal transduction and immune response, XPO1 was selected for the proposed study.

*Experimental design for in-ovo knockdown of gga-miR-142-3p***.** *In-ovo* knock down of microRNA gga-miR-142-3p was done by E14 embryonated eggs (n = 6) by injecting the inhibitor i.e LNA antimiR gga-miR-142-3p (5'AAGTAGGAAACACTAC3') via. i/v @ of 7.5 mg/kg b.m. (100 μL). Similarly, a Scramble Control group of embryonated eggs (n=6) were inoculated with scramble control oligo/probes (5'TACGTCTATACGCCCA3'). Further, the miRNA inhibitor group and the Scramble control group embryonated eggs were incubated at 38 $^0$C with suitable relative humidity in a BOD incubator until the embryonic age of 20 days. Inoculated embryonated eggs were candled every day twice (i.e. morning and evening) to check the viability and movement of the embryo, and provided with 45$^0$ egg rotation every 2 hours during incubation period.

*Harvesting of target organs and histological studies.* Immune organs (Bursa, Spleen and Thymus) and other organs (Heart, Lung, Kidney, Liver, Bone, etc.) were harvested from the chicken embryo inoculated with LNA-antimiR gga-miR-142-3p and scramble control oligonucleotides aseptically on day 20 of incubation. Harvested organs from different embryos in the same group were pooled tissue wise and divided into two halves. One half was stored at -70 $^0$C until used for RNA isolation, and the other half was fixed in 10 per cent neutral buffered formalin for 48 hours and processed by routine paraffin technique, as per the method of DRURY and WALLINGTON (1980). Sections were cut at four microns (4 μm) thickness and stained by the Haematoxylin and Eosin staining method, as per DRURY and WALLINGTON (1980) for histopathological studies.

*Optimization of PCR.* Target organs/tissues were homogenized in TRIzol (Life Technologies, Invitrogen), and total cellular RNA was isolated and purified following the manufacturer's instructions. Concentration was calculated using a NANODROP spectrophotometer (Thermo scientific, USA) instrument. For mRNA analysis, 500 ng of total RNA was converted into cDNA, using the SuperScript First-Strand Synthesis System (Invitrogen). The primers targeting the XPO1 gene were designed using primer designing the bioinformtics software tool Primer Express (XM_001231647) For-XPO (5'GAG GAG TGT TGC TCA TTT GTG3') and Rev-XPO (5'GTT CTT TAA GCA CTA CAG CAT GG3'). Amplification reaction was performed in 25 μL reaction volume containing 1 μL (50 ng) of template cDNA, 2.5 μL of 10X Taq DNA polymerase buffer, 1 μL of dNTP mixture containing10 mM of each dNTP, 1.5 μL of 50mM $MgCl_2$, 1 μL (5pM) each of forward and reverse primer for target genes, and 0.3 μL of TaqDNA polymerase (5U/ μL). The PCR programme included initial denaturation at 94 $^0$C for 2 min, followed by 35 cycles (94 $^0$C for 30 sec.), annealing (57 $^0$C for 1min.) and extension (72 $^0$C for 45 sec.). Final extension was carried out at 72 $^0$C for 10 min. The product was subjected to electrophoresis on 2.0% agarose gel and visualized under a UV Transilluminator.

*Optimization of qPCR.* The quantitative real time PCR (qPCR) was carried out using a Fast SYBR® Green (Applied Biosystems, USA) and was performed in 10 μL reaction

volume containing 1 μL (50 ng) of template cDNA, 5 μL of SYBR® Green Master mix (2x), 1 μL (5 pM) each of forward and reverse primer for target genes, with the same cycle conditions mentioned above for 40 cycles, along with a default melting step at the end of the reaction for melt-curve analysis. A housekeeping gene 18S of *Gallus gallus* (BAUDET et al., 2003) used as the endogenous control, was also optimised at annealing temperature of 57 $^{0}$C.

*Designing of qPCR assay & Relative quantification of target gene*. Real-time quantitative PCR was performed using Fast SYBR® Green with the StepOne™Real time PCR (Applied Biosystems, USA) to amplify samples in triplicate replicates along with negative controls. Relative gene product amounts were reported for each gene, compared with 18S ribosomal RNA using Applied Biosystems StepOne™ Real time PCR software v2.0 by ΔΔCT method keeping scramble control tissue as a reference sample. The assay also included the melt-curve analysis. The flags were set as default, and the primer efficiency was set at 95% confidence.

**Results and discussion**

Bioinformatic analysis of the genes based on the available literature pertaining to the expression profile of gga-miRNA-142-3p and targeted genes TGFβ2, XPO1, CD200, Cathelicidins B, Fowlicidins 1 to 3, Nr13, Ephirins, Bmp2, GH, Myc, SDF-1, Shh, Mx involved in structural integrity, immune response, signal transduction and migration of B and T cells during the embryonic developmental stage in chicken embryos revealed that XPO1 had sites for binding of miR-142-3p on 3’ UTR, indicating the predicted targets of gga-miR-142-3p (Fig. 1). This indicates that XPO1 plays a vital role during embryonic developmental stages, and maintains the homeostasis of the transport mechanism (CALLANAN et al., 2000). Results of bioinformatic analysis revealed a conserved site for binding of miR-142-3p and hence over-expression of XPO1 in different tissues could be a possible target of miR-142-3p.

*qPCR Expressional analysis of XPO1in gga- miR-142-3p knockdown tissues.* Intravenous injection of LNA antimiR gga-miR-142-3p during late embryonic stage revealed early amplification of the XPO1 gene in the miR-142-3p knockdown tissue, compared with the control tissue by qPCR, indicating the increased expression of the XPO1 gene. The amplification mean Ct values of XPO1 and 18S for miRNA inhibitor & scramble groups, ΔΔCT analysis and RQ values for tissues are summarized in Table 1. The aim of this study was to validate XPO1 as the target of miR-142-3p as it facilitates the nuclearcytosolic transport of large macromolecules, including RNA and protein, across the nuclear membrane to the cytoplasm. Further work is being carried out at the proteomic level to identify the function of XPO1’s role during the aberrant expression of miR-142-3p.

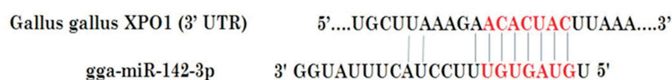


Fig. 1. Conserved binding site for gga-miR-142-3p in the 3' UTR region of XPO1 gene

Table 1. Expressional analysis data of targeted gene XPO1 in of miRNA knockdown organs compared with Scramble control group

| Tissue | Sample | CT Mean | ΔCT Mean | ΔCT SD | ΔΔCT | RQ | RQ Min | RQ Max |
|---|---|---|---|---|---|---|---|---|
| Bursa | SC | 27.87 | 15.436 | 0.102 | 0 | 1 | 0.821 | 1.218 |
| | mi-Ih | 22.52 | 13.646 | 0.066 | -1.79 | 3.458 | 2.986 | 4.004 |
| Thymus | SC | 36.868 | 21.898 | 0.079 | 0 | 1 | 0.791 | 1.265 |
| | mi-Ih | 33.53 | 18.69 | 0.198 | -3.208 | 9.243 | 6.319 | 13.52 |
| Spleen | SC | 33.668 | 18.912 | 0.186 | 0 | 1 | 0.7 | 1.429 |
| | mi-Ih | 29.904 | 10.798 | 0.239 | -8.115 | 277.116 | 174.987 | 439.01 |
| Heart | SC | 35.56 | 23.05 | 0.32 | 0 | 1 | 0.54 | 1.852 |
| | mi-Ih | 32.59 | 20.105 | 0.121 | -2.945 | 7.699 | 6.103 | 9.711 |
| Lung | SC | 35.769 | 23.035 | 0.045 | 0 | 1 | 0.905 | 1.105 |
| | mi-Ih | 36.92 | 23.165 | 0.198 | 0.13 | 0.914 | 0.59 | 1.415 |
| Kidney | SC | 28.438 | 13.745 | 0.147 | 0 | 1 | 0.754 | 1.327 |
| | mi-Ih | 27.815 | 12.439 | 0.134 | -1.307 | 2.474 | 1.911 | 3.201 |

SC- Scramble control group, mi-Ih- miRNA knockdown group

Relative quantification analysis, keeping 18S as an endogenous control for normalisation, revealed that in the immune organs XPO1 gene was upregulated in the bursa up to 3.458, 9.243 fold in the thymus, and 277.166 fold in the spleen (Fig. 2a). Similarly in the visceral organs XPO1 was upregulated in the heart and kidney up to 7.699 and 2.474 times respectively, but in lungs it was slightly down regulated - 0.914 times (Fig. 2b). Similarly, it has been reported that gga-miR-142-3p expression was high on day 15 and day 20 in the immune organs (HICKS et al., 2008; HICKS et al., 2009). Knockdown of miR-142-3p led to the increased expression of the target gene XPO1 in the immune organs and visceral organs compared with the miR-142-3p scramble oligo/probe inoculated group, clearly indicating the inverse relationship between the expression of miR-142-3p and XPO1 gene expression.

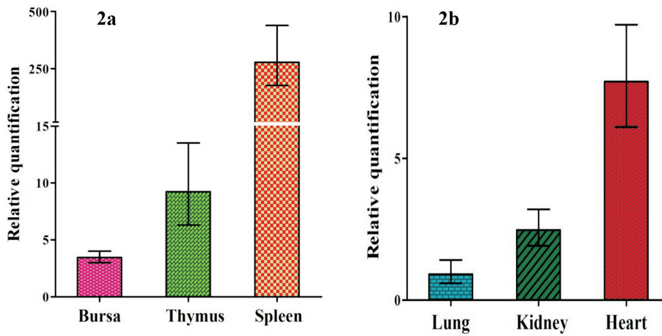


Fig. 2. Relative expression (RQ) analysis of XPO 1 gene in gga-miR-142-3p knockdown tissues; (2a): RQ of XPO 1 gene in immune organs, (2b): RQ of XPO 1 gene in visceral organs

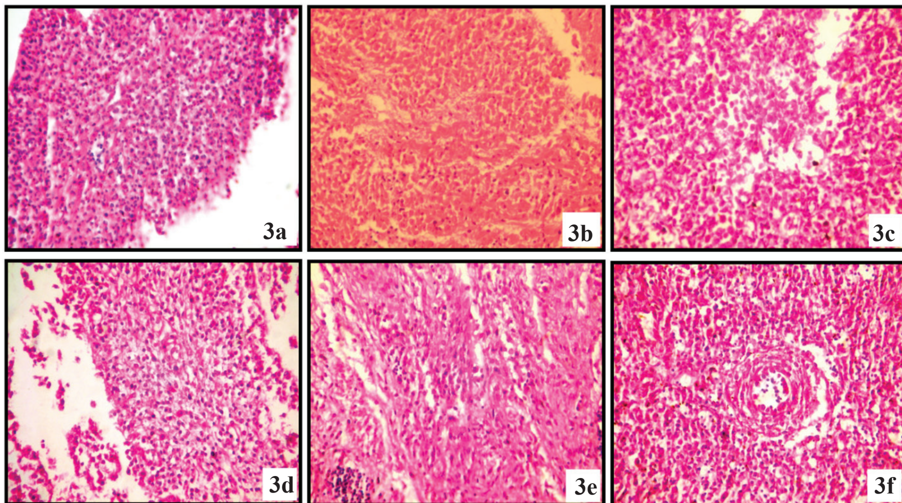


Fig. 3. Histopathological changes detected by HE staining in the immune organs of gga-miR-142-3p knockdown developing chicken embryo. (3a): Densely populated lymphocytes and absence of follicular arrangement in the bursa, (3b): Vascular degenerative changes with undifferentiated lobes of thymus, (3c): Distortion of spleenic parenchyma & depopulation of lymphocytes with proliferative reticular tissue in the spleen. (3d), (3e) and (3f): Scramble control bursa, thymus and spleen respectively. ×400.

*Histopathology of immune and visceral organs*. Follicular arrangement was completely absent, with a dense population of bursal lymphocytes with reticular tissue, revealing multiple gaps and multiple vascular fatty cells, indicating aplasia of the bursal

parenchyma (Fig. 3a). In the thymus, the thymic lobes were not well differentiated, with depopulation of lymphocytes, and there was a round mass of reticular tissue with plenty of vascular spaces, indicating vascular degenerative changes and aplasia of the thymus (Fig. 3b). However, in the spleen the architecture of the splenic parenchyma was completely absent and showed proliferation only of reticular tissue, which was evident from the multiple vascular spaces, and with depopulation of lymphocytes, indicating developmental disturbances in the splenic tissue (Fig. 3c). Several authors have reported that XPO1 is involved in T-cell activation (SHAPIRO et al., 2005), and regulates IP3KB involved in development of immune cells (NALASKOWSKI et al., 2011), regulating IFN-α1 mRNA transport involved in host innate immunity (KIMURA et al., 2010). These changes could be attributed to the fact that XPO1 regulates shuttling of proteins, such as nucleoplasmin, involved in centrosome duplication (WANG et al., 2005), NF-κB during cell growth (ZHANG et al., 2012), and cellular FLIP in apoptosis signalling (KATAYAMA et al., 2010), and regulates avian geminin protein involved in targeting the embryonic patterning genes of the HOX family (LUO et al., 2007).

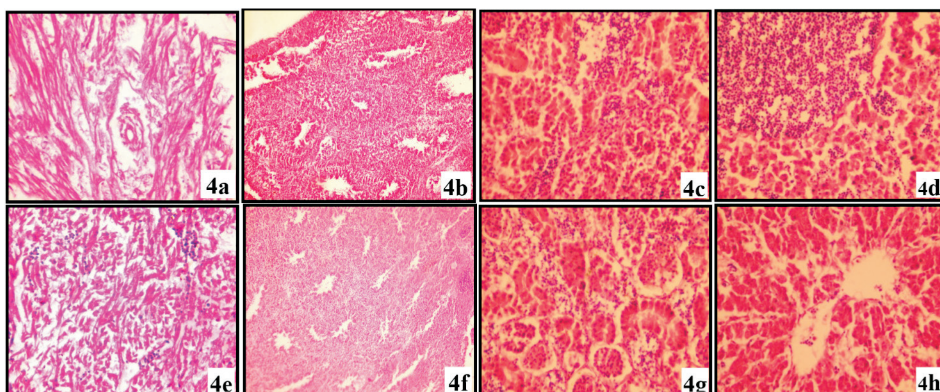


Fig. 4. Histopathological changes detected by HE staining in the visceral organs of gga-miR-142-3p knockdown developing chicken embryo. (4a): heart with separated cardiac fibres with multiple vacuolar gaps in the myocardium, (4b): absence of parabronchial arrangement in the lungs, (4c): Vascular degenerative changes, with infiltration of lymphocytes in the kidney, (4d): Extramedullary follicular lymphopoiesis, along with congestion of blood vessels and sinusoidal in Liver. (4e), (4f), (6g) and (4h): Scramble control heart, lungs, kidney and liver respectively. ×400.

The heart showed separated cardiac fibres, and revealed multiple vacuolar gaps in the myocardium, there were very prominent changes around the blood vessels with infiltration of lymphocytes, indicating the myocardial muscles were not fully developed (Fig. 4a). The lung tissue appeared to be a consolidated mass of tissue, which was infiltrated with

lymphocytes with non-uniform bronchial and para-bronchial spaces indicating distortion of the lungs (Fig. 4b). Similarly, in the kidney the glomeruli were not formed, there was arrested tubular development and infiltration of RBCs, with possibly extramedullary lymphopoiesis observed (Fig. 4c). In the liver the well-developed hepatic portal triad was observed, but evidence of extramedullary follicular lymphopoiesis was prominent, along with congestion of blood vessels and sinusoids (Fig. 4d). Considering the role of XPO1 in the shuttling of biomolecules during the embryonic developmental stages, the gene expression analysis of XPO1 during the later stages of embryonic development in miRNA inhibitor embryos revealed that XPO1 was highly up-regulated in the immune organs viz., the spleen, bursa, thymus and even in organs such as the heart and kidney, whereas it was slightly down-regulated in the lungs.

## Conclusion

XPO1's major role in regulating signal transduction involved in the signalling pathway during immune response, elucidates the functional role of the XPO1 gene in the immune organs during embryonic developmental stages. The knock down of miR-142-3p during embryonic stages resulted in over-expression of the XPO1 gene and the histopathological evidence indicates the crucial role of XPO1 in the development and function of immune organs and other organs during the embryonic developmental stage. Hence, it may be concluded that gga-miR-142-3p plays a vital role in the embryonic developmental stages of the chicken, regulating the genes involved in signal transduction and immune response in the immune organs and other organs. As this miRNA is found in different species, it may be evaluated as a future therapeutic target for immunity related pathological conditions.

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## References

BARTEL, D. P. (2009): MicroRNAs: Target recognition and regulatory functions. Cell 36, 215-233.

BAUDET, M. L., E. J. SANDERS, S. HARVEY (2003): Retinal growth hormone in the chick embryo. Endocrinology 144, 5459-5468.

CALLANAN, M., N. KUDO, S. GOUT, M. BROCARD, M. YOSHIDA, S. DIMITROV, S. KHOCHBIN (2000): Developmentally regulated activity of CRM1/XPO1 during early Xenopus embryogenesis. J. Cell Sci. 113, 451-459.

DRURY, A. A., E. A. WALLINGTON (1980): Carleton's Histological Technique-5th ed., Oxford University press, New York, Toronto.

HICKS, J., P. A. TEMBHURNE, H. C. LIU (2008): MicroRNAs expression in chicken embryos. Poultry Sci. 87, 2335-2343.

HICKS, J., P. A. TEMBHURNE, H. C. LIU (2009): Identification of microRNA in the developing chick immune organ. Immunogentics 61, 231-240.

KATAYAMA, R., T. ISHIOKA, S. TAKADA, R. TAKADA, N. FUJITA, T. TSURUO, M. NAITO (2010): Modulation of Wnt signaling by the nuclear localization of cellular FLIP-L. J. Cell Sci. 123, 23-28.

KIMURA, T., I. HASHIMOTO, M. NISHIZAWA, S. ITO, H. YAMADA (2010): Novel cisactive structures in the coding region mediate CRM1-dependent nuclear export of IFN-α 1 mRNA. Med. Mol. Morphol. 43, 145-157.

LEWIS, B. P., C. B. BURGE, D. P. BARTEL (2005): Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell 120, 15-20.

LUO, L., Y. UERLINGS, N. HAPPEL, N. S. ASLI, H. KNOETGEN, M. KESSEL (2007): Regulation of geminin functions by cell cycle-dependent nuclear-cytoplasmic Shuttling. Mol. Cell Biol. 27, 4737-4744.

NALASKOWSKI, M. M., R. FLIEGERT,O. ERNST, M. A. BREHM, W. FANICK, S. WINDHORST, H. LIN, S. GIEHLER, J. HEIN, Y. N. LIN, G. W. MAYR (2011): Human inositol 1,4,5-Trisphosphate 3-Kinase isoform B (IP3KB) is a nucleocytoplasmic shuttling protein specifically enriched at cortical actin filaments and at invaginations of the nuclear envelope. J. Biol. Chem. 286, 4500-4510.

SANDHU, S. K., C. M. CROCE, R. GARZON (2011): Micro-RNA expression and function in lLymphomas. Adv. Hematol. 2011, 1-12.

SHAPIRO, M. J., Y. Y. CHEN, V. S. SHAPIRO (2005): The carboxyl-terminal segment of the adaptor protein ALX directs its nuclear export during T cell activation. J. Biol. Chem. 280, 38242-38246.

WANG, X. W., J. ZHANG, F. LI, J. GU, T. HE, X. G. ZHANG, Y. D. LI (2005): MicroRNA identification based on sequence and structure alignment. Bioinformatics 21, 3610-3614.

ZHANG, M., B. HU, T. LI, Y. PENG, J. GUAN, S. LAI, X. ZHENG (2012): A CRM1- dependent nuclear export signal controls nucleocytoplasmic translocation of HSCARG, which regulates NF-κB activity. Traffic 13, 790-799.

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**MANESH KUMAR, P., P. A. TEMBHURNE, P. RAJA, V. C. INGLE, D. R. KALOREY: Utišani gga-miR-142-3p u pilećem embriju: ekspresija profila XPO1. Vet. arhiv 87, 67-76, 2017.**

**SAŽETAK**

Različita ekspresija gga-miR-142-3p mikroRNA hemopoetskog podrijetla daje nove mogućnosti razumijevanja njezine ključne uloge u embrionalnom razvoju limfnih organa i funkcionalnih zbivanja u pilećem zametku. Za otkrivanje uloge gga-miR-142-3p *in ovo* provedeno je utišavanje zaključanom nukleinskom kiselinom što preko anti-miR-gga-miR-142-3p preinačuje razvojne i funkcionalne sposobnosti limfnih i drugih organa. Bioinformatička analiza ciljnih gena za gga-miRNA-142-3p otkriva da gen XPO1 ima konzervirana mjesta vezanja na 3'UTR. Gen XPO1 bio je potvrđen kao cilj za gga-miR-142-3p uporabom tehnologije temeljene na qPCR SYBR zelenilu, što je bilo dokazano njegovom povećanom ekspresijom u tkivima pilećih zametaka s utišanim gga-miR-142-3p. Patohistološke promjene u imunosnim i unutarnjim organima pokazuju da sustavno utišavanje gga-miR-124-3p vodi do prevelike ekspresije gena XPO1 tijekom embrionalnog razvoja. To mijenja zadaću imunosnih organa s obzirom na strukturni integritet, imunosni odgovor, prijenos poruka te migraciju B i T limfocita tijekom razvoja pilećih zametaka. Rezultati jasno naznačuju da te promjene mogu preinačiti postnatalni razvoj i funkcije imunosnih organa te mogu dovesti do razvoja imunološki oslabljenih pilića.

**Ključne riječi:** utišana gga-miR-142-3p, pileći zametak, ekspresija gena XPO1