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Differential Expression Profile of TGFβ2 in Immune and Visceral Organs of gga-miR-142-3p Knockdown Chicken Embryo

Pannerselvam Manesh Kumar[1*], Paramasivam Raja[1], Prabhakar Abhimanji Tembhurne[2], Sanjay Gohain[2] and Vijay Chokharam Ingle[2]

[1]Department of Animal Biotechnology, Madras Veterinary College, TANUVAS, Chennai-7, INDIA
[2]Department of Veterinary Microbiology and Animal Biotechnology Teaching & Research Cell, Nagpur Veterinary College, MAFSU, Nagpur, Maharashtra, INDIA

**Corresponding author: PM Kumar; Email: manesh.balu@gmail.com*

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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 opens the new avenue towards understanding its pivotal role during embryonic developmental stages. To decipher the role of gga-miR-142-3p, *in-ovo* knockdown was carried out with locked nucleic acid (LNA) modified anti-miR-gga-miR-142-3p *via*, intravenous route at developmental and functional stages of these immune organ and visceral organs. Bioinformatic analysis revealed that predicted gene TGFβ2 have conserved binding sites at 3'UTR for gga-miR-142-3p which is involved in immune response and signal transduction during the embryonic developmental stage in the chicken embryos. TGFβ2 was evaluated as validate targets of gga-miR-142-3p by employing qPCR SYBR green based technology which was evidenced by aberrant expression of targeted gene, which could alter the postnatal development and functions of these immune organs and may lead to immune compromised chickens.

Keywords: gga-miR-142-3p, Knockdown, Chicken embryo, TGFβ2 Expression

The development of a new life is a spectacular process and scientists have been proved that different genes determine the early development of the individual during their embryonic stage. The genes are expressed at defined stages of development and determine the body plan pattern of the growing embryo. Over the past decade, it has become clear that, these genes are controlled by a large class of small non-coding RNAs, which are functioning as important regulators of a wide range of cellular processes by modulating gene expression. Three classes of small RNAs have been identified to play major role in biological system, including microRNAs (miRNAs), siRNAs and Piwi-interacting RNAs (piRNAs) (Farazi *et al.,* 2008). The small RNA sequence is able to regulate the expression of multiple genes because it can bind to target genes as either an imperfect or perfect complement (Ha *et al.,* 2008).

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 gene families, constitutes 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 act as the 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 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). miRNA found to play a major role in normal biological pathways such as cell cycle, survival, differentiation, proliferation and migration of cells.

Micro RNA profiling in chicken embryo at day 20 revealed higher expression of gga-miR-142-3p in immune organs spleen (5.9%) and bursa (9.7%) compared with 15 day old embryo (Hicks *et al.,* 2009). As miR-142-

3p is highly expressed in the late developmental stages of immune organs approach the hatching where T and B cells maturation, migration, differentiation playing a vital role modulating the expression of genes involved in immune response, signal transduction, structural integrity, maturation and migration of these cells. Hence, it is important to find out the why these miRNA are highly expressed at this stages, whether these miRNA needed for maintaining the functional activity of these immune organ by regulating the gene expression in these organs. Therefore, we carried out *in-ovo* knockdown gga-miR-142-3p at developmental and functional stages of these immune organ and visceral organs with LNA modified anti-miR-gga-miR-142-3p to elucidate the functional role of the gga-miR-142-3p in respect to TGFβ2 gene regulating the target organs involved in signal transduction, immune response during embryonic developmental stages.

MATERIALS AND METHODS

Animal model

Eleven day old fertilized embryonated eggs were procured from Regional Government hatchery, Nagpur. All the embryonated eggs were incubated at 39ºC provided with required humidity and used as model for the current study.

In-ovo knockdown of gga-miR-142-3p

Fourteen day old embryonated eggs (N=6) were injected with LNA antimiR gga-miRNA-142-3p (5'AAGTAGGAAACACTAC3') via. i/v @ of 7.5 mg / kg body weight (100µl). Similarly, scramble control group of embryonated eggs (N=6) were inoculated with scramble control oligo/probes (5'TACGTCTATACGCCCA3'). Further, miRNA inhibitor group and scramble control group embryonated eggs were incubated at 39ºC with suitable relative humidity in the BOD incubator till embryonic age of 20 days. Inoculated embryonated eggs were candled every day to check the viability and movement of the embryo.

Harvesting of target organs

Immune organs (Bursa, Spleen and Thymus) and visceral organs (Heart, Lung and Kidney) from the chicken embryo inoculated with LNA-antimiR gga-miR-142-3p and scramble control oligonucleotides were harvested aseptically on day 20 of the incubation. Harvested organs from the different embryos in the same group were pooled tissue wise and were stored at -70ºC until use.

RNA extraction and RT-PCR

Target organs were homogenized in TRIzol® reagent (Life Technologies, Invitrogen), and total cellular RNA was isolated and purified following the manufacturer's instructions and concentration was calculated using NANO DROP spectrophotometer (Thermo scientific, USA) instrument. For mRNA analysis, 500ng of total RNA was converted into cDNA using the SuperScript First-Strand Synthesis System (Invitrogen). Conventional RT-PCR & qPCR for gga-miR-142-3p targeted gene TGF β2 was standardised at 60ºC using the designed primers For-TGF (5'GGG CTC TTT TTG ATC ACT CAG A3'), Rev-TGF (5'AAA TGG AGT CAG GTA GCT TAT GTC A3') with 5 pmol of primer concentration. Housekeeping gene 18S rRNA of *Gallus gallus* (Baudet *et al.*, 2003) used as endogenous control.

Designing of qPCR assay & Relative quantification of the target genes

Real-time quantitative PCR was performed using Fast SYBR® Green with the StepOne™ Real time PCR (Applied Biosystems, USA) to amplify samples in triplicate along with negative controls. Relative gene product amounts were reported for each gene compared with 18S ribosomal RNA as housekeeping gene using Applied Biosystems StepOne™ Real time PCR software v2.0 by ΔΔCT method (Schmittgen and Livak, 2008) keeping scramble control tissue as reference sample or calibrator. The assay also included the melt-curve analysis, the flags were set as default, as well as the primer efficiency was set at 95% confidence.

RESULTS

Bioinformatic analysis of miR-142-3p targets

Bioinformatic analysis of the target gene TGFβ2 revealed the presence of binding site for miR-142-3p on 3' UTR

indicating the predicted targets of gga-miR-142-3p (Fig. 1). Presence of conserved site for binding of miR-142-3p and over expression of TGFβ2 in the different tissues could be a possible target of miR-142-3p.

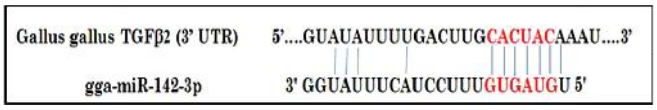

Fig. 1: Conserved binding site for gga-miR-142-3p in the 3' UTR region of TGFβ2 gene

RT-PCR for TGFβ2

The results of RT-PCR showed that the target gene TGFβ2 and the endogenous control 18S rRNA genes were amplified and analysis of the PCR products on the agar gel electrophoresis revealed the size of the amplified products of TGFβ2 and 18S rRNA gene were 164 bp and 154 bp respectively (Fig. 2) without any non-specific amplification. It indicated that the primers designed were highly specific for the amplification of target genes.

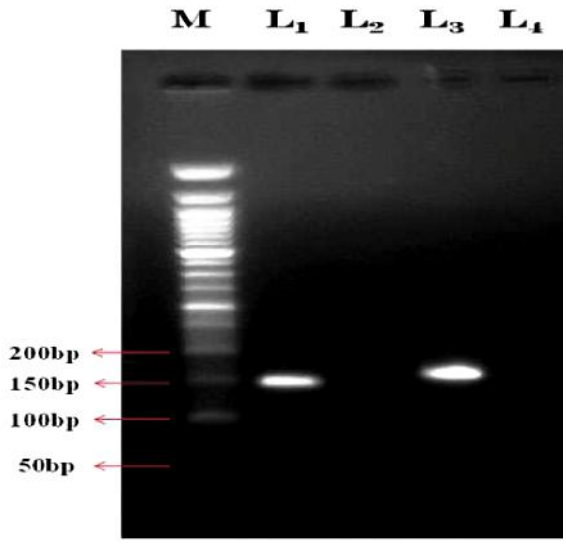

Fig. 2: Agarose gel electrophoresis of 18S rRNA and TGFβ2 genes

M- DNA Marker (50bp)

Lane: 1 – 18S rRNA endogenous control gene (152bp)

Lane: 2 – 18S rRNA Negative control

Lane: 3 – TGFβ2 gene (164bp)

Lane: 4 – TGFβ2 gene Negative control

Differential expressional of TGFβ2 in gga- miR-142-3p knockdown tissues

Expression profile of the target gene TGFβ2 was analysed in immune organs and visceral organs of gga-miR-142-3p knockdown chicken embryo. The amplification mean Ct values of TGFβ2 and 18S rRNA for miRNA inhibitor & scramble groups, ΔΔCT analysis and RQ values for tissues are summarized in the Table 1.

The relative quantification analysis keeping 18S as endogenous control for normalisation revealed that in immune organs TGFβ2 gene was upregulated in thymus and spleen up to 2.2 folds and 60.143 folds but it is slightly downregulated in bursa to 0.966 folds (Fig. 3a). Similarly in visceral organs TGFβ2 was upregulated in heart and kidney up to 4.579 folds and 30.564 folds respectively but in lungs was slightly downregulated 0.82 folds (Fig. 3b).

DISCUSSION

TGFβ2 is a multifunctional gene that regulates the proliferation, differentiation, migration and other functions in many cell types as well as plays a pivotal role in the embryonic developmental stages of mice and chicken embryo. Several workers even reported the normal expression of the TGFβ2 at different levels in the different organs of the developing chicken embryo indicating the vital role of TGFβ2 during somitogenesis (Aramaki *et al.*, 2005) and myogenesis (Saxena *et al.*, 2007). Increased expression was noticed in spleen of newly hatched chicks compared to embryo and adult (Jakowlew *et al.*, 1997), heart, lung, brain, muscle however lower levels of expression was noticed in kidney and liver (Jakowlew *et al.*, 1994). To decipher the role of gga-miR-142-3p and its effect on TGFβ2 during the late embryonic developmental stages, knocking down of gga-miR-142-3p during late embryonic stages of chicken embryo revealed the increased expression of TGFβ2 in thymus, spleen, heart and kidney, where as in bursa and lungs it is slightly down-regulated.

The differential expression of TGFβ2 in the miRNA inhibitor tissue compared to scramble control indicates that the TGFβ2 is a valid target of miR-142-3p could play a major role in disturbing the normal immune organ functions and development during the embryonic stages. Even Lei *et al.* (2012) reported that the KSHV virus miR-K10 shares the same seed sequence of miR-142-

Table 1: Expressional analysis data of targeted gene TGFβ2 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	26.865	13.593	0.057	0	1	0.961	1.041
	mi-Ih	23.792	13.643	0.277	0.05	**0.966**	0.797	1.17
Thymus	SC	29.874	17.036	0.069	0	1	0.876	1.141
	mi-Ih	28.763	15.898	0.085	-1.138	**2.2**	1.869	2.59
Spleen	SC	26.553	14.04	0.05	0	1	0.908	1.101
	mi-Ih	24.943	8.13	0.056	-5.91	**60.143**	54.007	66.975
Heart	SC	28.889	17.327	0.191	0	1	0.693	1.444
	mi-Ih	26.906	15.126	0.085	-2.201	**4.597**	3.9	5.418
Lung	SC	28.388	17.704	0.184	0	1	0.702	1.424
	mi-Ih	29.866	17.99	0.086	0.286	**0.82**	0.695	0.968
Kidney	SC	24.694	10.956	0.26	0	1	0.607	1.648
	mi-Ih	23.413	9.122	0.06	-1.834	**3.564**	3.124	4.067

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

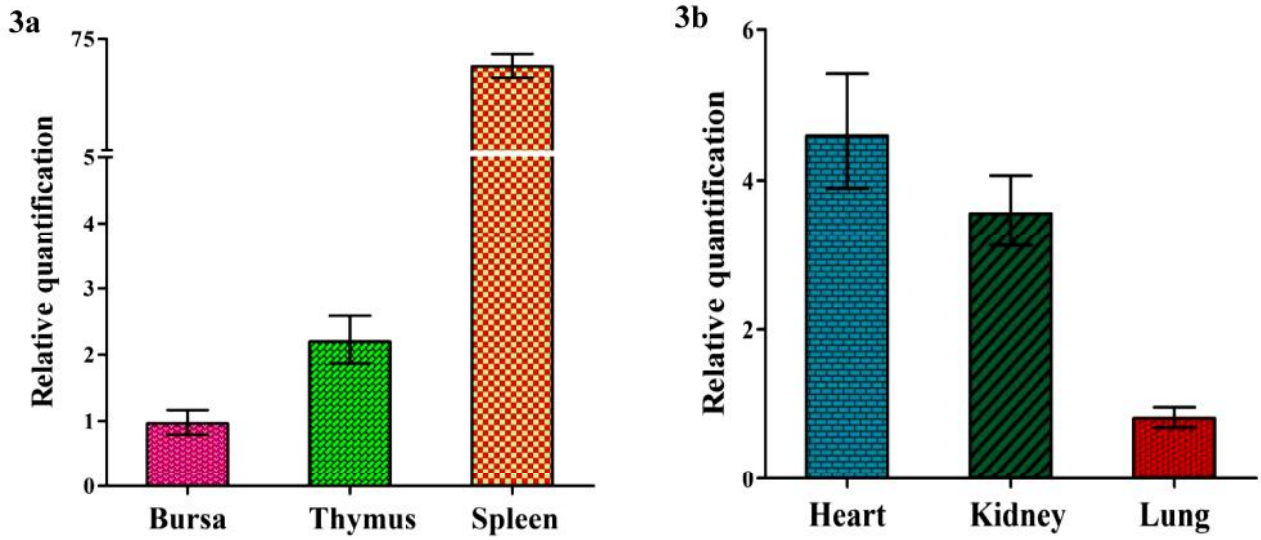

Fig. 3: Relative expression (RQ) analysis of TGFβ2 gene in gga-miR-142-3p knockdown tissues; (a): RQ of TGFβ2 gene in immune organs, (b): RQ of TGFβ2 gene in visceral organs

3p targeting TGFβ pathway promoting viral latency and malignancy in cellular transformation.

Higher expression of the TGFβ2 in the immune organs especially in spleen and thymus revealed the crucial role of TGFβ2 during embryonic stages which was also in support with the micrometry analysis indicating the densely populated B-cells in bursa of Fabricius however the immature lymphocytes were noticed in the spleen and thymus (Raja *et al.*, 2015). As the TGFβ2 mainly involved in regulation of the immune response and the transduction of signals involved in the immune signalling pathway by inhibiting NF-κB pathway and T-cell function by blocking both proliferation and differentiation (Rautava *et al.*, 2012; Gorelik *et al.*, 2002), inhibits the cell division and apoptosis by inducing Foxp3 expression in CD4+ and CD25- precursors cells (Fu *et al.*, 2004). Other reports also suggest that TGFβ2 as an antifibrotic cytokine regulating the fibrotic alterations and cardiac remodelling and

regulation of development of other vital organs in during embryonic stages (Prelog *et al.,* 2005; Zheng *et al.,* 2008). gga-miR-142-3p knockdown histopathological sections of organs heart revealed the disturbance in the architecture of the cardiac fibre arrangement, in lungs with un-uniform para bronchial arrangement and kidney with lymphopoiesis (Raja *et al.,* 2015) indicating that the aberrant expression of TGFβ2 as a result of knockdown of gga-miR-142-3p evidenced by disturbance in the development of heart, lung and kidney at embryonic stage. Our results from the present study pertaining to TGFβ2 expression under miR-142-3p regime along with bioinformatic analysis revealed the binding of gga-miR-142-3p to 3' UTR sequence of TGFβ2 indicate that gga-miR-142-3p expression in the developing embryo is utmost important to regulate the TGFβ2 expression.

CONCLUSION

The miR-142-3p is also found conserved in different species, considering the chicken as the model organism to study the miRNA effects, can employ for understanding the development of immune organs in different animals and can be evaluated as future therapeutic target for immunity related pathological conditions and developmental defects pertaining to immune organs.

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