

# MOLECULAR AND CELLULAR BIOLOGY

## Changes in renal gene expression associated with induced ochratoxicosis in chickens: activation and deactivation of transcripts after varying durations of exposure

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**ABSTRACT** Exposure to ochratoxin A (OTA) can lead to changes in global gene expression. This study investigated the individual expression of genes turned on and off in renal cells of chicks after different durations of exposure to dietary OTA. One hundred and eighty day-old male broiler chicks (Ross 308) were randomly assigned to a 3 × 3 factorial arrangement of treatments (3 levels of OTA: 0, 1 and 2 mg OTA/kg diet and 3 time periods: 7, 14 and 21 d). Birds were allocated to 36 pens (4 replicate pens of 5 birds each per treatment). For RNA-Sequencing analysis (RNA-Seq), kidney samples were collected weekly from 3 controls and 3 chicks fed 1 mg OTA/kg. Birds fed 2 mg OTA/kg diet were not chosen for analysis because their reduced feed intake could affect gene expression. The libraries were constructed by Illumina's TruSeq RNA protocol. NextGENe software was used for alignment and transcript quantification. Reads per kilobase of target per

million tiled reads (RPKM) were used in the Binary test analysis ( $P < 0.05$ ). The highest RPKM values were used as criterion for the selection of the genes described. A total of 27,638,976 50-bp RNA-Seq reads were produced over the 3 time periods. Transcripts (40,782) were assembled de novo and annotated by homology to either *G. gallus* or *H. sapiens*. The genes activated at 7 d were *IL9* and *TULP1*, at 14 d was *GHSR* and at 21 d were *GRK6* and *GAPDH*. Unlike all other genes, *LOC396365* was activated during all time periods. In contrast, the genes deactivated at 7 d were *SPAG4* and *LOC100857131*, at 14 d were *LOC771469*, *NKX2-1*, *NKX2-8*, *FOXO1*, *MyHC* and *CLDN18* and at 21 d was *XPC*. The *B-G* gene was turned off at 7 and 21 d. All of these genes were involved in kidney toxicity. Therefore, the turning on and off responses of these genes may contribute to carcinogenic and tumorigenic effects of OTA in birds.

**Key words:** broiler, mRNA abundance, nephrocarcinogenicity, ochratoxin A, tumor

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## INTRODUCTION

The poultry industry plays a significant role in the worldwide economy, as evidenced by the global record in broiler meat production when compared to beef and pork production. This industry is important for the economy of the United States, which leads the world in broiler meat production (18.0 million metric tons), followed by Brazil and China (13.1 and 13.0 million metric tons, respectively). Brazil leads exports with 3.7 million metric tons, followed by the United States with 3.0 million metric tons (USDA, 2015).

Large losses in the poultry industry may occur if feedstuffs contaminated with mycotoxins, in particular

ochratoxins, are provided to the birds. Ochratoxin A (OTA), which is the most toxic member of this family, is a secondary metabolite produced by *Penicillium verrucosum* and several related *Aspergillus* species (*A. ochraceus*, *A. carbonarius*, and a small percentage of *A. niger*; JECFA, 2008). Due to the presence of these fungi in different geographical regions, contamination by the mycotoxin occurs in many cereal grains used in animal feed, such as corn, wheat, maize, rye, barley, and oats (Al-Anati and Petzinger, 2006).

Ochratoxin A is a moderately heat-stable molecule that can survive most industrial food processing, making it one of the most abundant food-contaminating mycotoxins (Bullerman and Bianchini, 2007). Due to its long half-life, the carry-over effect of OTA from feed to animals can occur, whereby products of animal origin may contribute to the contamination of human food supply (Duarte et al., 2010). Ochratoxin A is po-

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tently nephrotoxic, strongly carcinogenic (IARC group 2B), as well as genotoxic, hepatotoxic, teratogenic, embryotoxic, mutagenic, neurotoxic, and immunosuppressive (JECFA, 2008), but its multiple mechanisms of toxicity are not yet fully elucidated, and sometimes results are even controversial (Ringot et al., 2006).

Although problems associated with OTA have been known for decades and a great amount of research on their effects in animals has already been performed, studies based on the effects of OTA at the cellular level (genes) are relatively recent. There is much less known about chicken gene responses in comparison to mammalian species. For comprehension of structure and function relationships, chickens represent a good biomedical model for understanding disease mechanisms because of their defined genetics (Kaufman et al., 1999).

In the present study, we investigated the changes in gene expression associated with an induced experimental ochratoxicosis in broiler chicks fed 1 mg OTA/kg feed at 7, 14 and 21 d. Using the technique of RNA sequencing (RNA-Seq), we isolated the genes transcriptionally activated and deactivated in renal cells. In particular, carcinogenic and tumorigenic effects were investigated.

## MATERIALS AND METHODS

### *Animal Procedures and Experimental Conditions*

Bird handling and experimental protocols were approved by the University of Missouri, Columbia, MO, Animal Care and Use Committee. One hundred and eighty 1-day-old male broiler chicks (Ross 308) were obtained from a commercial hatchery (Hoover's Hatchery Inc., Rudd, IA) and were randomly assigned to a  $3 \times 3$  factorial arrangement of treatments to receive 3 levels of OTA (0, 1 and 2 mg OTA/kg diet) for 3 time periods (7, 14 and 21 d). The birds were weighed, sorted, wing-banded, and allocated to 36 pens (4 replicate pens of 5 birds each per treatment) for 21 d. The average BW of the birds at the beginning of the experiment (d 1) was 37.4 g across all treatments ( $P > 0.05$ ).

The basal diet was a commercial type maize-soybean meal diet (23% CP and 3,200 kcal ME/kg), formulated to meet or exceed the nutritional requirements of growing chicks (1 to 21 d) according to the NRC (1994). The ingredient composition and the calculated nutrient composition of the basal diet were previously described by Zeferino et al. (2016). For OTA supplemented diets, OTA was produced by *Aspergillus ochraceus* NRRL 3174 grown on shelled corn (Ciegler, 1972), at the Veterinary Medical Diagnostic Laboratory (College of Veterinary Medicine, University of Missouri, Columbia, MO). The culture material contained 1,100 mg OTA/kg. Dietary OTA concentrations of supplemented diets were determined (Zeferino et al., 2016).

Over the entire experimental period (1 to 21 d), birds were allowed ad libitum access to feed and water and

maintained on a 24 h continuous light schedule. They were inspected daily for any health problems and mortality was recorded. The room temperature was adjusted daily to ensure the thermal comfort of the birds. Average temperatures of 31.4°C, 30°C and 28.5°C were recorded for wk 1, 2, and 3, respectively.

### *Renal Gene Expression Profiling*

According to the performance results on d 21 (Zeferino et al., 2016), chicks fed 1 mg OTA/kg diet had a numerical decrease in feed intake of 5.4% ( $P > 0.05$ ), whereas chicks fed 2 mg OTA/kg diet had a significant decrease in feed intake of 26.5% ( $P < 0.05$ ). No effect of OTA ( $P > 0.05$ ) was detected on mortality across all treatments, with a mortality of 2/20, 1/20, and 4/20 observed in birds fed 0, 1 mg/kg, or 2 mg/kg OTA, respectively.

In order to remove the possible effects of the reduced feed intake on gene expression, birds fed 2 mg OTA/kg diet were not chosen for analysis, thus, kidney samples were collected weekly from 3 chicks fed the basal diet (0 mg OTA/kg) and 3 chicks fed the basal diet supplemented with 1 mg OTA/kg diet.

Each bird, apparently healthy with uniform weight, was taken randomly from a different replicate pen and euthanized with carbon dioxide. Kidney cortex samples (approximately 2.0 g) were aseptically collected from the cranial portion of the right kidney, immediately snap-frozen in liquid nitrogen, and stored at  $-80^{\circ}\text{C}$ . Total RNA was extracted by the RNeasy Mini Kit (Qiagen Inc., Valencia, CA), according to the manufacturer's instructions. The quality and integrity of all purified RNA was confirmed by agarose gel electrophoresis. The concentration was measured by absorbance at 260 nm and the purity was monitored by inspection of the 260/280 nm ratio using a spectrophotometer (ND-1000, NanoDrop Technologies Inc., Wilmington, DE). Total RNA samples were submitted for analysis at a concentration of 100 ng/ $\mu\text{L}$  in nuclease free water in a minimum volume of 25  $\mu\text{L}$  (2.5  $\mu\text{g}$  total).

High-throughput sequencing was performed at the DNA Core Facility (University of Missouri, Columbia, MO). RNA quality analysis on each sample was performed before library preparation using the Eukaryote Total RNA StdSens Assay (Bio-Rad Experion System, Hercules, CA). The libraries were constructed following the manufacturer's protocol with reagents supplied in Illumina's TruSeq RNA sample preparation kit (RS-930-2001, Illumina Inc., San Diego, CA). The final construct of each purified library was evaluated using the BioAnalyzer 2100 (Agilent Technologies, Palo Alto, CA) automated electrophoresis system. The libraries were also quantified with the Qubit fluorometer (Invitrogen, Carlsbad, CA) using the Quant-iT dsDNA HS reagent kit (Invitrogen), and diluted according to Illumina's standard sequencing protocol for sequencing on the HiSeq 2000 (Illumina). For generation of sequences, 18 stranded RNA-Seq libraries were pre-

pared, multiplexed into 3 pools (6-plex) and each pool sequenced in a single lane of a  $1 \times 50$  HiSeq run, totaling 3 lanes.

NextGENe-NG Release v. 2.17 (beta) software (Soft-Genetics, State College, PA) was used for read alignment and transcript quantification. To build a more complete reference, reads were filtered to remove data for transcripts that can be found in public data. The remaining reads were assembled de novo. These transcripts were further assembled using CAP3 (Huang and Madan, 1999) to remove redundancy and extend sequence. Lastly, the public data were combined with the RNAs from this study and further consolidated with CAP3. The resulting data includes *Gallus gallus* (*Gga*) RefSEQ (<http://www.ncbi.nlm.nih.gov/refseq/>) and data from this study and represents both full-length transcripts as well as non-overlapping fragments of transcripts. For this study, each constructed RNA sequence was referenced as a transcript. However, mRNAs may be represented in the data more than once when only non-overlapping fragments were assembled. Transcripts were assembled de novo and first annotated by homology to *Gga*. Transcripts that had no counterpart in the public *Gga* data were then compared to the mRNA reference for *Homo sapiens* (*Hsa*) because the functional definitions of chicken genes are not as complete as those in mammals and it is expected that conserved genes have the same function in different species.

## Statistical Analysis

Reads per kilobase of target per million tiled reads (RPKM) values of the transcripts were used in the analysis as the measurement of mRNA abundance. The results of RNA-Seq were obtained by the arithmetic mean of 3 replicates per treatment (control and treated with 1 mg OTA/kg) for each time period (at 7, 14, and 21 d). Through the Binary test (Microsoft Excel, Microsoft Corporation) it was possible to select the transcripts that were activated (i.e., transcripts with zero RPKM mean for control and RPKM mean greater than zero in treated group) and deactivated (i.e., transcripts with RPKM mean greater than zero in control group and zero RPKM mean for treated group). A *P*-value of less than 0.05 was considered to be statistically significant. An individual gene approach was chosen for this study, this way the highest RPKM values were used as a criterion for the selection of the genes described.

## RESULTS

The results of dietary OTA concentrations analysis confirmed the expected levels in the control diet (below the limits of detection) and the treated diets with 1 and 2 mg OTA/kg (1.130 and 2.080 mg OTA/kg, respectively). Analysis of the basal diet indicated that it did not contain detectable amounts

of OTA, and other mycotoxins such as aflatoxin B1 (**AFB1**), zearalenone (**ZEAN**), fumonisin B1 (**FB1**), or deoxynivalenol (**DON**).

All samples used for RNA-Seq exceeded minimum quality requirements based on Eukaryote Total RNA StdSens results. The results of RNA quality indicator were higher than 9.3 on a scale of 1.0 (fully degraded) to 10.0 (intact) which demonstrated the high quality of the isolated RNA.

A total of 27,638,976 50-bp RNA-Seq reads were produced over the 3 time periods. These reads were tiled onto a custom reference that included 40,782 mRNA transcripts and transcript fragments. For the purpose of this study, both full-length transcripts and transcripts fragments will be referenced as "transcripts". Our findings on gene expression response by duration of exposure to dietary OTA are based on mRNA levels only.

Considering the activation of transcripts, on d 7, there were 668 mRNA transcripts that were undetectable in the control animals but became detectable when OTA was fed. However, only 131 of the 668 transcripts (19.6%) were statistically different ( $P < 0.05$ ) when compared to controls. This group included 33 transcripts that could be identified (Table 1), 7 uncharacterized transcripts (i.e., transcripts that are present but unannotated in the chicken genome build; Table 1), and 91 transcripts that had no counterpart in the public *Gga* and *Hsa* data (i.e., transcripts that could not be identified by homology to known chicken nor human transcripts). On d 14, 625 mRNA transcripts became detectable, but only 89 transcripts (14.2%) were statistically different from controls ( $P < 0.05$ ). This group included 19 identified and 4 uncharacterized transcripts (Table 2), and 66 transcripts that had no counterpart in the public *Gga* and *Hsa* data. On d 21, 632 mRNA transcripts became detectable, but only 117 (18.5%) were statistically different from controls ( $P < 0.05$ ). This group included 30 identified and 9 unannotated transcripts (Table 3), and 78 transcripts that could not be identified by homology to known chicken nor human transcripts.

Concerning the deactivation of transcripts, on d 7, there were 320 mRNA transcripts that were present in control animals but became undetectable with the inclusion of OTA in the diet. However, only 18 of the 320 transcripts (5.6%) were statistically different ( $P < 0.05$ ) when compared to controls. This group included 9 identified and 2 uncharacterized transcripts (Table 4), and 7 transcripts that had no counterpart in the public *Gga* and *Hsa* data. On d 14, 355 mRNA transcripts became undetectable, but only 21 transcripts (5.9%) were statistically different from controls ( $P < 0.05$ ). This group included 16 identified and 2 uncharacterized transcripts (Table 4), and 3 transcripts that could not be identified by homology to known chicken nor human transcripts. On d 21, 396 mRNA transcripts became undetectable, but only 27 (6.8%) were statistically different from controls ( $P < 0.05$ ). This group included 12 identified and

**Table 1.** Genes differentially expressed (turned on) from RNA-Seq results in chickens fed ochratoxin A (1 mg OTA/kg diet) for 7 d.<sup>1</sup>

Gene identification	Official symbol	Gene name <sup>2</sup>	RPKM value <sup>3</sup>
64085120	<i>TSHR</i>	<i>Hsa</i> thyroid stimulating hormone receptor (TSHR), transcript variant 1, mRNA	0.014
238624128	<i>KCNMA1</i>	<i>Hsa</i> potassium large conductance calcium-activated channel, subfamily M, alpha member 1 (KCNMA1), transcript variant 1, mRNA	0.014
148806931	<i>KY</i>	<i>Hsa</i> kyphoscoliosis peptidase (KY), mRNA	0.015
289547604	<i>CELF3</i>	<i>Hsa</i> CUGBP, Elav-like family member 3 (CELF3), transcript variant 3, mRNA	0.027
363733921	<i>LRIT3</i>	Predicted: <i>Gga</i> leucine-rich repeat, immunoglobulin-like and transmembrane domains 3 (LRIT3), mRNA	0.028
122937280	<i>SLC38A8</i>	<i>Hsa</i> solute carrier family 38, member 8 (SLC38A8), mRNA	0.028
118095198	<i>LOC424944</i>	Predicted: <i>Gga</i> cytochrome P450 2J2-like (LOC424944), mRNA	0.034
363730082	<i>LOC100859294</i>	Predicted: <i>Gga</i> uncharacterized LOC100859294 (LOC100859294), miscRNA	0.037
45383573	<i>ID4</i>	<i>Gga</i> inhibitor of DNA binding 4, dominant negative helix-loop-helix protein (ID4), mRNA	0.037
363743493	<i>LOC100858293</i>	Predicted: <i>Gga</i> retinol dehydrogenase 8-like (LOC100858293), mRNA	0.038
47551304	<i>LGALS3</i>	<i>Gga</i> lectin, galactoside-binding, soluble, 3 (LGALS3), mRNA	0.039
289177194	<i>CNGB3</i>	<i>Hsa</i> cyclic nucleotide gated channel beta 3 (CNGB3), mRNA	0.050
288869473	<i>HSF4</i>	<i>Gga</i> heat shock transcription factor 4 (HSF4), mRNA	0.051
363737360	<i>LOC424943</i>	Predicted: <i>Gga</i> cytochrome P450 2J6-like (LOC424943), mRNA	0.052
363727552	<i>LOC417741</i>	Predicted: <i>Gga</i> secreted frizzled-related protein 2-like (LOC417741), mRNA	0.071
210147587	<i>HAPLN4</i>	<i>Hsa</i> hyaluronan and proteoglycan link protein 4 (HAPLN4), mRNA	0.074
363745857	<i>LOC100857580</i>	Predicted: <i>Gga</i> abhydrolase domain-containing protein 8-like (LOC100857580), mRNA	0.085
363747217	<i>LOC100857951</i>	Predicted: <i>Gga</i> T-cell-interacting, activating receptor on myeloid cells protein 1-like (LOC100857951), mRNA	0.088
311771629	<i>SRC</i>	<i>Gga</i> v-src sarcoma viral oncogene (SRC), mRNA	0.091
363735798	<i>LOC769520</i>	Predicted: <i>Gga</i> uncharacterized LOC769520 (LOC769520), mRNA	0.091
363735237	<i>LOC423793</i>	Predicted: <i>Gga</i> kinesin-like protein KIF20B-like (LOC423793), miscRNA	0.094
363741760	<i>LOC419182</i>	Predicted: <i>Gga</i> neuritin-like (LOC419182), mRNA	0.100
363746664	<i>LOC100858545</i>	Predicted: <i>Gga</i> leukocyte immunoglobulin-like receptor subfamily A member 2-like (LOC100858545), mRNA	0.102
116534899	<i>DBH</i>	<i>Hsa</i> dopamine beta-hydroxylase (dopamine beta-monoxygenase) (DBH), mRNA	0.103
221554495	<i>CPLX4</i>	<i>Hsa</i> complexin 4 (CPLX4), mRNA	0.109
224458363	<i>PLA2G1B</i>	<i>Gga</i> phospholipase A2, group IB (pancreas) (PLA2G1B), mRNA	0.126
317108133	<i>MSMP</i>	<i>Hsa</i> microseminoprotein, prostate associated (MSMP), mRNA	0.127
118090753	<i>LOC771108</i>	Predicted: <i>Gga</i> uncharacterized LOC771108 (LOC771108), mRNA	0.134
363735726	<i>LOC100857643</i>	Predicted: <i>Gga</i> uncharacterized LOC100857643 (LOC100857643), mRNA	0.149
313850960	<i>IL22</i>	<i>Gga</i> interleukin 22 (IL22), mRNA	0.179
363740143	<i>VPREB3</i>	Predicted: <i>Gga</i> pre-B lymphocyte 3 (VPREB3), mRNA	0.182
45383254	<i>SS2</i>	<i>Gga</i> somatostatin II (SS2), mRNA	0.374
363737262	<i>LOC100858812</i>	Predicted: <i>Gga</i> uncharacterized LOC100858812 (LOC100858812), miscRNA	0.629
363745644	<i>LOC100858522</i>	Predicted: <i>Gga</i> interleukin-28B-like (LOC100858522), mRNA	0.772
83721989	<i>IL9</i>	<i>Gga</i> interleukin 9 (IL9), mRNA	3,757
301171470	<i>MIR1630</i>	<i>Gga</i> microRNA mir-1630 (MIR1630), microRNA	3,832
363732552	<i>LOC422008</i>	Predicted: <i>Gga</i> uncharacterized LOC422008 (LOC422008), mRNA	4,303
363747017	<i>LOC776798</i>	Predicted: <i>Gga</i> uncharacterized LOC776798 (LOC776798), partial mRNA	10,088
45382320	<i>LOC396365</i>	<i>Gga</i> preprogastrin (LOC396365), mRNA	17,414
45382922	<i>TULP1</i>	<i>Gga</i> tubby like protein 1 (TULP1), mRNA	56,240

<sup>1</sup>The table comprises identified transcripts and uncharacterized (i.e., transcripts that are present but unannotated in the chicken genome build).

<sup>2</sup>Transcripts that were annotated by homology to *Gallus gallus* mRNA are identified by *Gga* and transcripts that were compared to the mRNA reference for *Homo sapiens* are identified by *Hsa*.

<sup>3</sup>The RPKM (reads per kilobase of target per million tiled reads) value describes the magnitude of activation ( $P < 0.05$ , Binary test) between OTA and control treatments (numbers were compared to zero). The comparison among genes is not valid.

1 unannotated transcript, and 14 transcripts that could not be identified by homology to known chicken nor human transcripts.

According to the individual gene response for activation, the *Gga* interleukin 9 (**IL9**) and the *Gga* tubby

like protein 1 (**TULP1**) genes were transcribed at high levels at 7 d (Table 1), the *Hsa* growth hormone secretagogue receptor (**GHSR**), transcript variant 1a gene was transcribed at high levels at 14 d (Table 2), and finally, the *Gga* G protein-coupled receptor kinase 6 (**GRK6**)

**Table 2.** Genes differentially expressed (turned on) from RNA-Seq results in chickens fed ochratoxin A (1 mg OTA/kg diet) for 14 d.<sup>1</sup>

Gene identification	Official symbol	Gene name <sup>2</sup>	RPKM value <sup>3</sup>
162139832	<i>PCDHA1</i>	<i>Gga</i> protocadherin alpha 1 (PCDHA1), mRNA	0.013
162287058	<i>PCDHA2</i>	<i>Gga</i> protocadherin alpha 2 (PCDHA2), mRNA	0.013
363738999	<i>NMUR2</i>	Predicted: <i>Gga</i> neuromedin U receptor 2 (NMUR2), mRNA	0.014
319655688	<i>ADCYAP1</i>	<i>Gga</i> adenylate cyclase activating polypeptide 1 (pituitary) (ADCYAP1), mRNA	0.019
319803039	<i>NKX3-2</i>	<i>Gga</i> NK3 homeobox 2 (NKX3-2), mRNA	0.023
118100636	<i>LOC771136</i>	Predicted: <i>Gga</i> protein-glutamine gamma-glutamyltransferase 6-like (LOC771136), mRNA	0.023
61743913	<i>CHRNA7</i>	<i>Hsa</i> cholinergic receptor, nicotinic, gamma (muscle) (CHRNA7), mRNA	0.031
363746653	<i>LOC100858294</i>	Predicted: <i>Gga</i> free fatty acid receptor 2-like (LOC100858294), partial mRNA	0.032
363728111	<i>C1H12ORF35</i>	Predicted: <i>Gga</i> chromosome 1 open reading frame, human C12ORF35 (C1H12ORF35), mRNA	0.051
363747527	<i>LOC100859293</i>	Predicted: <i>Gga</i> olfactory receptor 14A16-like (LOC100859293), partial mRNA	0.062
45383340	<i>TNFSF8</i>	<i>Gga</i> tumor necrosis factor (ligand) superfamily, member 8 (TNFSF8), mRNA	0.069
363747549	<i>LOC100859820</i>	Predicted: <i>Gga</i> uncharacterized LOC100859820 (LOC100859820), mRNA	0.099
363740079	<i>LOC100859504</i>	Predicted: <i>Gga</i> uncharacterized LOC100859504 (LOC100859504), mRNA	0.122
363734320	<i>MTL5</i>	Predicted: <i>Gga</i> metallothionein-like 5, testis-specific (tesmin) (MTL5), mRNA	0.142
363738979	<i>SPINK7</i>	Predicted: <i>Gga</i> serine peptidase inhibitor, Kazal type 7 (putative) (SPINK7), miscRNA	0.152
363744294	<i>CZH9ORF24</i>	Predicted: <i>Gga</i> chromosome Z open reading frame, human C9ORF24 (CZH9ORF24), mRNA	0.183
122891863	<i>IKZF4</i>	<i>Hsa</i> IKAROS family zinc finger 4 (Eos) (IKZF4), mRNA	0.242
363745462	<i>LOC100857300</i>	Predicted: <i>Gga</i> uncharacterized LOC100857300 (LOC100857300), partial mRNA	0.256
363727450	<i>LOC100859445</i>	Predicted: <i>Gga</i> sucrase-isomaltase, intestinal-like (LOC100859445), mRNA	0.277
363744385	<i>LOC100858520</i>	Predicted: <i>Gga</i> uncharacterized LOC100858520 (LOC100858520), mRNA	0.743
301172701	<i>MIR1329</i>	<i>Gga</i> microRNA mir-1329 (MIR1329), microRNA	3,706
295424119	<i>GHSR</i>	<i>Hsa</i> growth hormone secretagogue receptor (GHSR), transcript variant 1a, mRNA	16,121
45382320	<i>LOC396365</i>	<i>Gga</i> preprogastrin (LOC396365), mRNA	19,463

<sup>1</sup>The table comprises identified transcripts and uncharacterized (i.e., transcripts that are present but unannotated in the chicken genome build).

<sup>2</sup>Transcripts that were annotated by homology to *Gallus gallus* mRNA are identified by *Gga* and transcripts that were compared to the mRNA reference for *Homo sapiens* are identified by *Hsa*.

<sup>3</sup>The RPKM (reads per kilobase of target per million tiled reads) value describes the magnitude of activation ( $P < 0.05$ , Binary test) between OTA and control treatments (numbers were compared to zero). The comparison among genes is not valid.

and the *Hsa* glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), transcript variant 2 genes were transcribed at high levels at 21 d (Table 3). After the peaks, transcription of each gene declined rapidly. Unlike all other genes, the *Gga* preprogastrin (**LOC396365**) was transcribed at high levels during all time periods (Tables 1, 2 and 3).

For deactivation, the *Gga* sperm associated antigen 4 (**SPAG4**), *Gga* sperm associated antigen 4 protein-like (**LOC100857131**) and the *Gga* V-region-like B-G antigen, and *Gga* MHC class IV antigen (*B-G*) genes were transcriptionally turned off at 7 d (Table 4). The *Gga* zinc finger B-box domain-containing protein 1-like (**LOC771469**), the *Hsa* NK2 homeobox 1, transcript variant 2, and *Hsa* NK2 homeobox 8 (**NKX2-1** and **NKX2-8**, respectively), the *Gga* forkhead box O1 (**FOXO1**), the myosin heavy chain (**MyHC**) and the *Hsa* claudin 18 (**CLDN18**), transcript variant 2 genes were transcriptionally turned off at 14 d. Finally, the *Gga* V-region-like B-G antigen (*B-G*) and the *Gga* xeroderma pigmentosum, complementation group C (**XPC**) genes were transcriptionally turned off at 21 d.

## DISCUSSION

In order to support and help clarify our gene expression results, a histopathology exam was conducted on kidney samples from control birds and birds fed 1 mg OTA/kg diet but no significant microscopic changes were detected. We speculate that the level of 1 mg OTA/kg diet was not high enough or was not fed for a long enough period for the emergence of kidney lesions.

Ochratoxin A is unlikely to act through a single, well-defined mechanism of action (Marin-Kuan et al., 2008), therefore we can suggest that there would be damage in different tissues, which resulted in individual gene responses. Although OTA studies on most of the genes described below are limited to humans and the effects have been reported in organs other than the kidneys, our data on turned on and off genes provide a description of ochratoxicosis in broilers.

### Activated Genes

**IL9** Dysregulated interleukin 9 (**IL-9**) response in vitro can lead to cell growth and malignant

**Table 3.** Genes differentially expressed (turned on) from RNA-Seq results in chickens fed ochratoxin A (1 mg OTA/kg diet) for 21 d.<sup>1</sup>

Gene identification	Official symbol	Gene name <sup>2</sup>	RPKM value <sup>3</sup>
345091031	<i>CACNA1B</i>	<i>Hsa</i> calcium channel, voltage-dependent, N type, alpha 1B subunit (CACNA1B), transcript variant 1, mRNA	0.007
363727786	<i>SEPT3</i>	Predicted: <i>Gga</i> septin 3 (SEPT3), mRNA	0.015
188497622	<i>NMUR1</i>	<i>Hsa</i> neuromedin U receptor 1 (NMUR1), mRNA	0.017
222136646	<i>FBXO47</i>	<i>Hsa</i> F-box protein 47 (FBXO47), mRNA	0.023
289177194	<i>CNGB3</i>	<i>Hsa</i> cyclic nucleotide gated channel beta 3 (CNGB3), mRNA	0.024
363735977	<i>PCDP1</i>	Predicted: <i>Gga</i> primary ciliary dyskinesia protein 1 (PCDP1), mRNA	0.025
363733514	<i>LOC770479</i>	Predicted: <i>Gga</i> gamma-aminobutyric acid receptor subunit beta-1-like (LOC770479), mRNA	0.026
363743630	<i>RAX</i>	Predicted: <i>Gga</i> retina and anterior neural fold homeobox (RAX), miscRNA	0.027
307078132	<i>SYT10</i>	<i>Hsa</i> synaptotagmin X (SYT10), mRNA	0.032
118100502	<i>LOC769590</i>	Predicted: <i>Gga</i> uncharacterized LOC769590 (LOC769590), mRNA	0.034
236456829	<i>CELA1</i>	<i>Hsa</i> chymotrypsin-like elastase family, member 1 (CELA1), mRNA	0.035
363742708	<i>LOC771882</i>	Predicted: <i>Gga</i> uncharacterized LOC771882 (LOC771882), mRNA	0.053
295424119	<i>GHSR</i>	<i>Hsa</i> growth hormone secretagogue receptor (GHSR), transcript variant 1a, mRNA	0.063
15451899	<i>FGF3</i>	<i>Hsa</i> fibroblast growth factor 3 (FGF3), mRNA	0.063
45383749	<i>HCRT</i>	<i>Gga</i> hypocretin (orexin) neuropeptide precursor (HCRT), mRNA	0.064
45382904	<i>CAPZA1</i>	<i>Gga</i> capping protein (actin filament) muscle Z-line, alpha 1 (CAPZA1), mRNA	0.064
190194402	<i>AGBL4</i>	<i>Hsa</i> ATP/GTP binding protein-like 4 (AGBL4), mRNA	0.064
363741957	<i>LOC100857778</i>	Predicted: <i>Gga</i> uncharacterized LOC100857778 (LOC100857778), mRNA	0.065
363746521	<i>LOC100857978</i>	Predicted: <i>Gga</i> uncharacterized LOC100857978 (LOC100857978), mRNA	0.072
223633970	<i>GPR83</i>	<i>Hsa</i> G protein-coupled receptor 83 (GPR83), mRNA	0.072
118102562	<i>LOC428272</i>	Predicted: <i>Gga</i> gastricsin-like (LOC428272), mRNA	0.073
363734651	<i>LOC100858994</i>	Predicted: <i>Gga</i> uncharacterized LOC100858994 (LOC100858994), mRNA	0.073
363730116	<i>LOC100857519</i>	Predicted: <i>Gga</i> uncharacterized LOC100857519 (LOC100857519), miscRNA	0.090
363746514	<i>LOC100857852</i>	Predicted: <i>Gga</i> zinc finger protein 519-like (LOC100857852), partial mRNA	0.091
363732882	<i>AKAP14</i>	Predicted: <i>Gga</i> A kinase (PRKA) anchor protein 14 (AKAP14), mRNA	0.094
363738776	<i>LOC427594</i>	Predicted: <i>Gga</i> 2-epi-5-epi-valiolone synthase-like (LOC427594), mRNA	0.099
363740245	<i>LOC100858749</i>	Predicted: <i>Gga</i> zinc finger protein RFP-like (LOC100858749), mRNA	0.113
48255876	<i>TNNT2</i>	<i>Hsa</i> troponin T type 2 (cardiac) (TNNT2), transcript variant 1, mRNA	0.125
363730725	<i>LOC100858887</i>	Predicted: <i>Gga</i> uncharacterized LOC100858887 (LOC100858887), mRNA	0.127
363734320	<i>MTL5</i>	Predicted: <i>Gga</i> metallothionein-like 5, testis-specific (tesmin) (MTL5), mRNA	0.149
363746525	<i>LOC100858082</i>	Predicted: <i>Gga</i> uncharacterized LOC100858082 (LOC100858082), mRNA	0.165
50807014	<i>SLC5A12</i>	Predicted: <i>Gga</i> solute carrier family 5 (sodium/glucose cotransporter), member 12 (SLC5A12), mRNA	0.269
301172712	<i>MIR1764</i>	<i>Gga</i> microRNA mir-1764 (MIR1764), microRNA	0.308
301172618	<i>MIR1754</i>	<i>Gga</i> microRNA mir-1754 (MIR1754), microRNA	0.782
363738963	<i>GRK6</i>	Predicted: <i>Gga</i> G protein-coupled receptor kinase 6 (GRK6), mRNA	3,693
378404907	<i>GAPDH</i>	<i>Hsa</i> glyceraldehyde-3-phosphate dehydrogenase (GAPDH), transcript variant 2, mRNA	3,995
45382320	<i>LOC396365</i>	<i>Gga</i> preprogastrin (LOC396365), mRNA	5,676
301171152	<i>MIR2188</i>	<i>Gga</i> microRNA mir-2188 (MIR2188), microRNA	6,835
363747017	<i>LOC776798</i>	Predicted: <i>Gga</i> uncharacterized LOC776798 (LOC776798), partial mRNA	7,106

<sup>1</sup>The table comprises identified transcripts and uncharacterized (i.e., transcripts that are present but unannotated in the chicken genome build).

<sup>2</sup>Transcripts that were annotated by homology to *Gallus gallus* mRNA are identified by *Gga* and transcripts that were compared to the mRNA reference for *Homo sapiens* are identified by *Hsa*.

<sup>3</sup>The RPKM (reads per kilobase of target per million tiled reads) value describes the magnitude of activation ( $P < 0.05$ , Binary test) between OTA and control treatments (numbers were compared to zero). The comparison among genes is not valid.

transformation of lymphoid cells associated with constitutive activation of the Janus kinase-signal transducer and activator of transcription (**JAK-STAT**) pathway (Demoulin et al., 1996; Li and Rostami, 2010). The activation of signal transducer and activator of transcription 3 (**STAT3**), can promote lung cancer cell prolif-

eration and migration, and facilitate the intercellular adhesion of cancer cells (Ye et al., 2012). In contrast, neutralized IL-9 can inhibit tumor growth in the murine lymphoma and this process is associated with down-regulation of the cells involved in immune suppression: regulatory T cells and mast cells (Feng et al., 2011). In

**Table 4.** Genes differentially expressed (turned off) from RNA-Seq results in chickens fed ochratoxin A (1 mg OTA/kg diet) for 7, 14 and 21 d.<sup>1</sup>

Gene identification	Official symbol	Gene name <sup>2</sup>	RPKM value <sup>3</sup>
<i>7 Days</i>			
363732468	<i>LOC100858959</i>	Predicted: <i>Gga</i> uncharacterized LOC100858959 (LOC100858959), mRNA	0.045
217330575	<i>NXPH3</i>	<i>Hsa</i> neurexophilin 3 (NXPH3), mRNA	0.063
363742772	<i>LOC100859756</i>	Predicted: <i>Gga</i> scale keratin-like (LOC100859756), mRNA	0.078
239937510	<i>CHIR-AB1</i>	<i>Gga</i> immunoglobulin-like receptor CHIR-AB1 (CHIR-AB1), mRNA	0.080
363729257	<i>LOC100857654</i>	Predicted: <i>Gga</i> uncharacterized LOC100857654 (LOC100857654), mRNA	0.182
363733676	<i>ATRN</i>	Predicted: <i>Gga</i> attractin (ATRN), mRNA	0.439
301173186	<i>MIR1571</i>	<i>Gga</i> microRNA mir-1571 (MIR1571), microRNA	5,466
363742649	<i>SPAG4</i>	Predicted: <i>Gga</i> sperm associated antigen 4 (SPAG4), mRNA	34,725
363742651	<i>LOC100857131</i>	Predicted: <i>Gga</i> sperm associated antigen 4 protein-like (LOC100857131), partial mRNA	36,670
71897284	<i>B-G</i>	<i>Gga</i> V-region-like B-G antigen (B-G), mRNA	92,344
150247076	<i>B-G</i>	<i>Gga</i> MHC class IV antigen (B-G), mRNA	203,996
<i>14 Days</i>			
223671937	<i>PCDH9</i>	<i>Hsa</i> protocadherin 9 (PCDH9), transcript variant 2, mRNA	0.010
118092394	<i>LOC423605</i>	Predicted: <i>Gga</i> protein NDNF-like (LOC423605), mRNA	0.014
212549565	<i>CAMK2A</i>	<i>Hsa</i> calcium/calmodulin-dependent protein kinase II alpha (CAMK2A), transcript variant 2, mRNA	0.022
380036025	<i>PAX1</i>	<i>Hsa</i> paired box 1 (PAX1), transcript variant 2, mRNA	0.031
197245365	<i>KCNK3</i>	<i>Hsa</i> potassium channel, subfamily K, member 3 (KCNK3), mRNA	0.033
126165215	<i>F-KER</i>	<i>Gga</i> feather keratin I (F-KER), mRNA	0.035
363739339	<i>GRIFIN</i>	Predicted: <i>Gga</i> galectin-related inter-fiber protein (GRIFIN), mRNA	0.043
363746389	<i>LOC431128</i>	Predicted: <i>Gga</i> platelet glycoprotein VI-like (LOC431128), partial miscRNA	0.077
134288905	<i>TRIM67</i>	<i>Hsa</i> tripartite motif containing 67 (TRIM67), mRNA	0.084
363739353	<i>LOC100858949</i>	Predicted: <i>Gga</i> uncharacterized LOC100858949 (LOC100858949), mRNA	0.154
363744058	<i>LOC100859308</i>	Predicted: <i>Gga</i> uncharacterized LOC100859308 (LOC100859308), mRNA	3,655
363737408	<i>LOC771469</i>	Predicted: <i>Gga</i> zinc finger B-box domain-containing protein 1-like (LOC771469), mRNA	3,800
31377776	<i>NKX2-8</i>	<i>Hsa</i> NK2 homeobox 8 (NKX2-8), mRNA	4,407
45383501	<i>FOXO1</i>	<i>Gga</i> forkhead box O1 (FOXO1), mRNA	5,859
386220	<i>MyHC</i>	myosin heavy chain (chickens, gizzard, smooth muscle, mRNA Partial, 51 nt)	6,522
269847375	<i>MIR32</i>	<i>Gga</i> microRNA mir-32 (MIR32), microRNA	7,128
60115825	<i>CLDN18</i>	<i>Hsa</i> claudin 18 (CLDN18), transcript variant 2, mRNA	11,941
31881814	<i>NKX2-1</i>	<i>Hsa</i> NK2 homeobox 1 (NKX2-1), transcript variant 2, mRNA	21,690
<i>21 Days</i>			
363733404	<i>PPM1K</i>	Predicted: <i>Gga</i> protein phosphatase, Mg <sup>2+</sup> /Mn <sup>2+</sup> dependent, 1 K (PPM1K), mRNA	0.010
207113121	<i>DYNC1I1</i>	<i>Hsa</i> dynein, cytoplasmic 1, intermediate chain 1 (DYNC1I1), transcript variant 2, mRNA	0.018
118089498	<i>LOC422276</i>	Predicted: <i>Gga</i> sodium/hydrogen exchanger 2-like (LOC422276), mRNA	0.020
384871686	<i>SAMD3</i>	<i>Hsa</i> sterile alpha motif domain containing 3 (SAMD3), transcript variant 1, mRNA	0.034
363740242	<i>IL4I1</i>	Predicted: <i>Gga</i> interleukin 4 induced 1 (IL4I1), miscRNA	0.034
118101196	<i>LOC429936</i>	Predicted: <i>Gga</i> arylacetamide deacetylase-like 4-like (LOC429936), mRNA	0.035
30795197	<i>LHX5</i>	<i>Hsa</i> LIM homeobox 5 (LHX5), mRNA	0.040
363728680	<i>LOC100857528</i>	Predicted: <i>Gga</i> uncharacterized LOC100857528 (LOC100857528), mRNA	0.056
301172119	<i>MIR1704</i>	<i>Gga</i> microRNA mir-1704 (MIR1704), microRNA	4,094
53139476	<i>TAP2</i>	<i>Gga</i> TAP2 mRNA, 5'UTR, clone 1.5B	10,764
71897284	<i>B-G</i>	<i>Gga</i> V-region-like B-G antigen (B-G), mRNA	67,529
363738710	<i>XPC</i>	Predicted: <i>Gga</i> xeroderma pigmentosum, complementation group C (XPC), mRNA	78,396
71897284	<i>B-G</i>	<i>Gga</i> V-region-like B-G antigen (B-G), mRNA	81,027

<sup>1</sup>The table comprises identified transcripts and uncharacterized (i.e., transcripts that are present but unannotated in the chicken genome build).

<sup>2</sup>Transcripts that were annotated by homology to *Gallus gallus* mRNA are identified by *Gga* and transcripts that were compared to the mRNA reference for *Homo sapiens* are identified by *Hsa*.

<sup>3</sup>The RPKM (reads per kilobase of target per million tiled reads) value describes the magnitude of deactivation ( $P < 0.05$ , Binary test) between OTA and control treatments (numbers were compared to zero). The comparison among genes is not valid.

our study, the *IL9* gene was transcriptionally turned on in chicks fed 1 mg OTA/kg diet, at d 7. Based on the literature, we can infer that IL-9 activated regulatory T cells and mast cells to mediate immune suppression, which can lead to tumor growth in the renal cells of chickens fed OTA.

**TULP1** Tubby-like protein 1 (*TULP1*) was recently identified as a Mer Tyrosine Kinase (**MeRTK**) ligand. It means that *TULP1* interacts with MeRTK, one of the members of the Tyro3, Axl, and MerTK (**TAM**) receptor, to facilitate phagocytosis (Caberoy et al., 2010). Overexpression of MeRTK ligands has been shown in many cancers and plays a role in oncogenic processes (Newlaczyk and Yu, 2011) but the roles for *TULP1* in those processes have not yet been studied (Cummings et al., 2013). According to our results, the *TULP1* gene was transcriptionally activated in birds fed 1 mg OTA/kg diet compared with control birds, at 7 d. Interestingly, it was shown that *TULP1* had response similar to the other known ligands in the MeRTK signaling for cancer processes, but at this time its function is still not clear. More studies on *TULP1* as a ligand in MeRTK signaling are necessary for the elucidation of its possible role in oncogenic processes.

**GHSR** Growth hormone secretagogue receptor (**GHSR**) gene encodes a member of the G-protein coupled receptor family. The transcript variant 1a (**GHSR1a**) encodes the receptor for the ghrelin ligand and, therefore, defines a neuroendocrine pathway for growth hormone release (NCBI, 2016) among other functions (Gahete et al., 2014). It is known that dysregulation in some of the components of the ghrelin system can lead to the development and progression of several types of cancers (Gahete et al., 2014) but their role is still unclear. Some studies support the idea of cell proliferation (Rossi et al., 2008) mediated through the activation of key signaling pathways such as the mitogen activated protein kinase (**MAPK**) pathway, namely MAPK p44/p42 (ERK 1/2) or the phosphoinositide 3-kinase (**PI3K**)-AKT pathway (Nanzer et al., 2004; Rossi et al., 2008). In our study, we observed that the *GHSR* gene was turned on in chicks fed 1 mg OTA/kg diet compared with controls, at d 14. This finding suggests that the high levels of *GHSR* induced by OTA resulted in dysregulation of the ghrelin system and may potentially play a role in cancer development.

**GRK6** G-protein-coupled receptor kinase 6 (**GRK6**) is a member of the versatile G protein-coupled receptor kinase (**GRK**) superfamily (Nakaya et al., 2013). This gene can regulate the activity of chemokine receptor by phosphorylation (Vroon et al., 2004), and it is related to the adhesion and movement of many cancer cell lines (Li, 2013). According to Li (2013), the GRK6 may participate in the regulation for migration and invasion of hepatocellular carcinoma cell, but the molecular mechanism is still unclear. We report that the *GRK6* gene is transcriptionally activated in birds fed OTA compared with controls at 21 d. Our results indicate that, similar

to Li (2013), changes in *GRK6* expression may be a sign of renal tumor response to the inclusion of OTA in the diet.

**GAPDH** The levels of glyceraldehyde-3-phosphate dehydrogenase (**GAPDH**) are elevated under stressed conditions, such as exposure to DNA-damaging agents (Tarze et al., 2007). As a key intermediate component of glycolysis, GAPDH could serve an important role in cancer cell development and tumor progression, because the hypoxic environment in which tumor cells reside leads to an increase in glycolytic metabolism (Wang et al., 2013a). However, when cells are exposed to a stressor, the binding of GAPDH to Siah (a protein E3 ligase that aids in the translocation of *GAPDH* to the nucleus) may regulate gene expression, which results in cellular dysfunction and death (Hara et al., 2006; Tristan et al., 2011). In our study, the *GAPDH* gene was activated in birds fed 1 mg OTA/kg diet, at 21 d. This result is consistent with the literature and could suggest that the high levels of GAPDH increased the glycolytic metabolism, leading to cancer cell development and tumor progression. We also could speculate that a post-translational modification of *GAPDH* by OTA in the GAPDH/Siah1 cascade was initiated, leading to disorders and cell death. Therefore, altered expression of *GAPDH* in OTA-treated chickens might have a number of implications. This issue has yet to be clearly understood.

**LOC396365** This is represented by preprogastrin. Preprogastrins are peptides derived from the gastrin precursor (Dockray et al., 2001). The growth of carcinoma and tumor cells is stimulated by autocrine secretion of gastrin (Rehfeld, 1998), thus it seems that gastrin acts as a co-carcinogen, in other words, it does not initiate tumorigenesis, just expands the pool of transformed cells (Dockray et al., 2001). In the present study, the preprogastrin was transcriptionally turned on in birds fed 1 mg OTA/kg diet compared with controls over the entire experimental period (at 7, 14 and 21 d). We propose that the elevated levels of preprogastrin mRNA contributed to the synthesis of gastrins in the stomach of the birds. These high levels of secretion of gastrin could be a sign of cancer in the stomach and also other organs, such as kidneys, but further studies are necessary for substantiation.

## Deactivated Genes

**SPAG4 and LOC100857131** We report that the sperm associated antigen 4 (*SPAG4*) and sperm associated antigen 4 protein-like (*LOC100857131*) mRNA were transcriptionally deactivated in kidneys of birds fed 1 mg OTA/kg diet at 7 d. Similar to our results, Ryu et al. (2007) demonstrated lower *SPAG4* mRNA levels in testes of mice chronically exposed to the di(*n*-butyl)phthalate, considered as a carcinogenic substance. We believe that the silenced expression of *SPAG4* may be a sign of renal tumor response to the

inclusion of OTA in the diet, however further studies are necessary for a better understanding of the relationship between this gene and renal cells.

**LOC771469** Zinc finger proteins containing the Krüppel-associated box (**KRAB-ZFP**) are the largest family of transcriptional repressors in mammals (Huntley et al., 2006) and it is suggested that loss of the KRAB domain in KRAB-ZFPs due to alternative splicing might contribute to carcinogenesis (Wang et al., 2013b). In our experiment, the zinc finger B-box domain-containing protein 1-like (*LOC771469*) mRNA was switched off in birds fed OTA compared with control birds at 14 d. According to Huntley et al. (2006) and Wang et al. (2013b), it suggests that repression in zinc finger B-box domain-containing protein 1-like expression could be responsible for a transcriptional derepression which consequently could lead to cancer development, but more investigation is necessary for an elucidation of the gene function and the possible role in carcinogenic and tumorigenic processes.

**NKX2-1 and NKX2-8** The NK-2 family encodes sequence-specific DNA-binding transcriptional activators. The NK2 homeobox 1 (*NKX2-1*) gene is overexpressed in many pulmonary adenocarcinomas (Anagnostou et al., 2009). Lin et al. (2013) proposed that NK2 homeobox 8 (*NKX2-8*) plays a role in the regulation of nuclear factor kappa-light-chain-enhancer of activated B cells (**NF-B**) transcriptional activity: *NKX2-8* represses NF- $\kappa$ B by restraining nuclear localization of NF- $\kappa$ B p65 via downregulation of A-kinase-interacting protein 1 (**AKIP1**), a NF- $\kappa$ B p65 binding partner, and also by directly targeting the AKIP1 promoter. Some studies demonstrated that *NKX2-8* and *NKX2-1* have opposite effects on transcription in lung cancer cells (Harris et al., 2011). Our findings indicated that *NKX2-1* and *NKX2-8* were both turned off in birds fed OTA compared with control birds at 14 d. Based on different tissue results, we hypothesized that deactivation of these genes might be associated with the potential development of renal cancer cells. However, we still do not know if these genes are acting individually or are acting in concert on transcription.

**FOXO1** The mechanisms of dysregulation of the forkhead box (**FOX**) family are inactivation via an increased phosphoinositide 3-kinase (**PI3K**)-AKT signaling, gene deletion, involvement in oncogenic protein fusions and leukaemia. The consequences are unscheduled cell proliferation, apoptosis resistance, independence of growth factor signaling, androgen independence of prostate cancer (Myatt and Lam, 2007; Paik et al., 2007; Greer and Brunet, 2008). The specific function of the forkhead box O1A (*FOXO1A*) gene has not yet been determined, but it has been suggested that this gene may play cellular and developmental roles by mediating insulin signaling, suppressing proliferation, or inducing apoptosis (Tuteja and Kaestner, 2007). In our study, *FOXO1* gene was deactivated in chicks fed 1 mg OTA/kg diet compared with control birds at 14 d. We suggest that the dysregulation of this renal gene

indicates an induced cell proliferation with potential cancer formation.

**MyHC** Our results showed that the myosin heavy chain (*MyHC*) mRNA was turned off in chicks fed 1 mg OTA/kg diet at 14 d. Several studies associated alteration in *MyHC* expression with cardiovascular diseases (Morkin, 2000) or even gastrointestinal tumor (Sakurai et al., 1999), but in fact, their mechanisms are still not well defined. In the present study, we hypothesized that the silenced expression of *MyHC* occurred as an adaptive response to inclusion of OTA in the diet. The possibility of a role for this gene in the nephrocarcinogenicity of OTA requires further development and demonstration.

**CLDN18** The tight junction-based permeability barrier is modified in tumorigenesis (Arabzadeh et al., 2007). In epithelial cancer cells, changes in claudins at junctions are associated with defective cell-to-cell contacts owing to faulty E-cadherin function and the loss of adherence junctions and tight junctions is characteristic (Quinonez and Simon, 1988). The loss of polarity in epithelia is associated with increased cellular proliferation, while disruptions in tight junction complexes alter cell-cell interactions allowing invasion and metastasis (Shin et al., 2006). In our study, the claudin 18 (*CLDN18*) gene was switched off in chicks fed 1 mg OTA/kg diet at 14 d. We inferred that the loss of *CLDN18* in renal epithelial cells modified the tight junction permeability barrier, promoting the progression of tumorigenesis and the invasion of cancer cells.

**B-G** Chicken major histocompatibility B complex (*MHC-B*) haplotypes have conflicting associations with disease (Taylor, 2004). The role of the B-G (also known as class IV MHC; Zekarias et al., 2002) molecules in antibody response is speculated to be through enhancing the antigen recognition by B and T cells and in the selection of B cells. The B-G haplotypes have divergent antibody responses that in effect can be linked with differences in resistance to infectious pathogens attributed to differences in antibody production (Kaufman and Salomonsen, 1992; Zekarias et al., 2002). In the present study, the *B-G* (V-region-like B-G antigen, and MHC class IV antigen) gene was turned off in chicks fed OTA at 7 d and *B-G* (V-region-like B-G antigen) gene was turned off in chicks fed OTA at 21 d. Based on our previous work (Zeferino et al., 2016), we propose that the loss of *B-G* caused by OTA may have different effects at 7 and 21 d. At 7 d, there was a direct effect of the toxin on the kidneys (the deactivated expression cancelled the immune protection, thus not conferring resistance to diseases). Thereafter, the *B-G* gene was turned on due to an adaptive response (the body was combating the OTA damage by increased immune activity). At 21 d the *B-G* gene was switched off because the kidney and other related organs had been repaired or the damage had been contained.

**XPC** The xeroderma pigmentosum (XP) disorder is caused by a defect in the nucleotide excision repair (NER) pathway (Schäfer et al., 2013). In this

disorder, cells are defective in DNA damage repair or DNA replication induced by chemical agents or UV radiation and it seems that these deficiencies involve most of the genome (damage to the whole genome) (Cleaver, 2005; Schäfer et al., 2013). In the current study, the xeroderma pigmentosum complementary group C (*XPC*) gene was turned off in chicks fed OTA at 21 d. It appears that OTA induced an XP disease in the kidneys with potential development of cancer. According to the literature, studies on this gene are limited to humans and the effects have been reported in organs other than the kidneys. Our finding might be interesting, but more studies are necessary for substantiation.

## CONCLUSIONS

This study is the first demonstration of genes individually activated and deactivated in renal cells of chickens as a result of different durations of exposure to dietary OTA. Although these changes in gene expression were not associated with gross or histopathological changes it does indicate that these concentrations of OTA can affect kidney gene expression in birds as early as d 7. All of these genes were involved in kidney toxicity, which is consistent with the reported carcinogenic and tumorigenic effects of OTA in birds. Therefore, we conclude that the turning on and off responses may contribute to carcinogenic and tumorigenic effects of OTA in birds. Further investigation into the molecular mechanisms regulating the observed changes in gene expression is required for a fuller understanding of carcinogenesis and tumorigenesis effects.

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