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Transcriptional profiling of gallinacins antimicrobial peptides in the chicken reproductive tract and embryos

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Gallinacins (Gals) are antimicrobial peptides that play significant roles in the innate immune system in chickens. These genes, which are clustered on chromosome 3 of the chicken genome, encode a group of 14 cationic antimicrobial peptides characteristic of β-defensins. Although many studies have reported the expression of Gals in various chicken organs, little is known about the function of these genes in the chicken reproductive tract and embryos. Therefore the aim of this study was to identify the types of gallinacin genes expressed in the chicken male and female reproductive tract and in the chicken embryos during embryonic development. Total RNA was extracted from ovary, oviduct, testis and epididymis from six month old male and female birds and from embryos from day three to day ten of chicken embryogenesis. The expression pattern of the 14 gallinacin genes was analyzed using reverse transcription-polymerase chain reaction (RT-PCR). Expression analysis revealed that most of the gallinacin genes were expressed in the chicken reproductive tract and embryos, suggesting a role of these antimicrobial peptides in the protection of the reproductive tract and embryos from microbial assaults.

Key words: gallinacins, antimicrobial peptides, reproductive tract, chicken embryos.

INTRODUCTION

Antimicrobial peptides (AMPs) play a significant role in the innate immune system of many species, providing the first line of defence against potential pathogens (Lehrer & Ganz, 2002; Sugiarto & Yu, 2004; Higgs *et al*., 2005). During the last years, hundreds of AMPs have been identified in vertebrates, invertebrates, plants and fungi (Lehrer & Ganz, 2002; Schutte & McCray, 2002; Thomma *et al*., 2002; Froy & Guverevitz, 2003; Ganz, 2003). One of the most important peptides and the prime targets for boosting of innate immunity is the family of antimicrobial peptides, defensins (Zasloff, 2002). Defensins constitute a large family of small cationic antimicrobial peptides, characterized by the presence of a conserved 6 cysteine defensin motif. These peptides are capable of killing a broad spectrum of pathogens, including bacteria, fungi, and specific enveloped viruses (Harmon, 1998; Lehrer & Ganz, 2002; Thomma *et al*., 2002; Ganz, 2003) and thus play a critical role in defence and disease resistance by protecting the hosts against infections. These peptides have been identified in various species including plants, insects, animals and humans and are divided into five groups, namely plant, invertebrate, α-, β-, and θ- defensins (Lehrer & Ganz, 2002; Thomma *et al*., 2002; Ganz, 2003). In the chicken genome only the family of β-befensin exists, where it is known as avian β-defensins or gallinacins (Gals) (Zhao *et al*., 2001; Xiao *et al*., 2004). Gallinacins attack a wide range of microorganisms including Gram-positive and Gram-negative bacteria, fungi, and yeast (Evans *et al*., 1995; Harmon, 1998; Sugiarto & Yu, 2004). A total of 14 avian β-defensin genes (Gals-1 to -14) have been recently identified through *in silico* studies (Lynn *et al*., 2004; Xiao *et al*., 2004; van Dijk *et al*., 2008).

It is well known that salmonellosis is a major cause of food-poisoning worldwide with outbreaks usually associated with *Salmonella enteriditis* and connected to the consumption of either contaminated poultry meat and/or derived products including eggs

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(Guard, 2001). During the last years the EU ban on the use of growth promoting antibiotics in animal feeds, including those provided for laying hens, may result, unintentionally, to an increase in contaminated poultry flocks and poultry products. Within commercial flocks *Salmonella* can be transmitted horizontally by faecal shedding, but transmission can also occur vertically through the trans-ovarian route, and this latter route is often the cause of *Salmonella* contaminated eggs (Guard, 2001). In addition, contamination of the male reproductive organs such as testis and epididymis may present a threat to the successful fertilization and also have as a result in microbial pathogens being incorporated into the newly forming eggs (Palladino *et al*., 2003). Thus, the knowledge of the endogenous innate immune mechanisms operating in birds (functioning naturally to protect birds from the colonisation, infection and transmission of microbial pathogens) is essential.

Although there are many reports on the expression of gallinacins in various chicken organs and the interactions between *Salmonella* and the chicken gastrointestinal tract (Hancock & Diamond, 2000; Methner *et al*., 2004; Ledeboer *et al*., 2006) little is known about the roles of gallinacins in protecting the avian reproductive system from microbial assault. Recent studies have reported that the cessation in egg-laying associated with feed-withdrawal and oviduct regression is correlated with the reduced expression of gallinacins 1, 2 and 3 (Yoshimura *et al*., 2006). Furthermore, in chicken vaginal cells cultured *in vitro* gallinacin expression has been shown to increase in response to challenge with *S. enteriditis* (Yoshimura *et al*., 2006). In addition, the expression of many gallinacin genes have been reported in the theca and granulosa layers of chicken ovarian follicles (Subedi *et al*., 2007). These findings provide evidence to suggest that the gallinacins host defence peptides function *in vivo* to protect the avian reproductive tract and the newly forming eggs from microbial infection.

The aim of this study was therefore to examine the expression pattern of the 14 gallinacin genes in the chicken (*Gallus domesticus*) reproductive tract *in vivo* and specifically in the chicken ovary, oviduct, testis and epididymis, as well as in embryos during chicken embryonic development.

MATERIALS AND METHODS

Collection of tissues and embryos

Six-month old egg laying female (n = 4) and sexual mature male (n = 4) birds (Rhode Island Reds) provisioned by a commercial supplier were sacrificed by cervical dislocation. Chicken ovary, oviduct (uterus), testis and epididymis were dissected, snap frozen in liquid nitrogen and stored at –80˚C. Large yolky preovulatory follicles from the chicken ovaries were removed before storage.

Freshly-laid chicken eggs (Rhode Island Reds) were obtained from a commercial supplier and incubated at 38˚C and 65-75% relative humidity in a forced air incubator. Because after day 10 the chicken embryo becomes too large for total embryo expression (Meade *et al*., 2009), the focus of this study was on differential gene expression during early embryogenesis (up to and including day 10 of embryonic development). Therefore embryos were removed from eggs at day 3 to day 10 of incubation (n = 4 at each day). The allantoic fluid from the eggs was removed, the embryos were sacrificed, minced, snap frozen in liquid nitrogen and stored at –80˚C until analysed.

Salmonella test

Experimental birds were tested for *Salmonella* species using Salmonella/Shigella (SS) agar (Sigma). Each bird was tested for *Salmonella* by plating faecal swabs on SS selective agar plates. Briefly, 63 g of SS agar were suspended in 1000 ml of distilled water. The solution was boiled with frequent agitation to dissolve the medium completely and was mixed and poured into sterile Petri plates. When cooled, different dilutions of faeces, resuspended in 1xPBS (Ambion), were poured into the plates. Using this medium, growth of the *Salmonella* species is uninhibited and appears as a colourless colony with a black center.

DNA isolation, PCR amplification, cloning and sequencing

Chicken genomic DNA was isolated using the NucleoSpin Tissue kit (Macherey Nagel, Germany) according to the instructions provided by the manufacturer. The integrity of the DNA samples was examined by electrophoresis through a 1.5% Tris Borate EDTA (TBE) agarose gel. For amplification of the 14 gallinacin genes specific primers were designed based on the nucleotide sequences reported in the GenBank database. The sequences of these primers are presented in Table 1. PCR amplification was performed using 0.1 μg genomic DNA as template, 200 nM of each primer, 1 mM Deoxynucleotide Triphosphates (dNTPs) and 1 U of Taq DNA Polymerase Recombinant (Invitrogen) in 50 μl total volume reac-

TABLE 1. Nomenclature, nucleotide primer sequences and exon location, length of PCR products in cDNA and DNA and accession numbers of primers used in the expression experiments using RT-PCR

Gene	Forward Primer	Reverse Primer	Exons	cDNA (bp)	DNA (kb)	GenBank acces. numbers
Gal-1	GAAACCATTGTCAGCCCTGT	TCAGCCCCATATTCTTTTGC	1-4	187	0.7	AF033335
Gal-2	GCATAAACACTTCATGAGTCCATC	GAAGAAAGGCAGTGCAGAAGATA	1-4	166	1	AF033336
Gal-3	CCTTCTTCCTCTTGTTTCTCCAG	ATCAACCTCATATGCTCTTCCAC	1-4	158	2.5	NM_204650
Gal-4	GATCCTTTACCTGCTGCTGTCT	TCCTCACACAGCAAGATTTTAGTC	2-4	185	0.9	NM_001001610
Gal-5	GATCCTTTACCTGCTGCTGTCT	AGCAAGAGCCTATTCCATTGTTAC	1-4	176	1.1	NM_001001608
Gal-6	AGGATTTCACATCCCAGCCGTG	CAGGAGAAGCCAGTGAGTCATC	1-4	249	1.2	NM_001001193
Gal-7	CTGCTGTCTGTCCTCTTTGTGG	CATTTGGTAGATGCAGGAAGGA	2-4	230	0.6	NM_001001194
Gal-8	CTGTTCTCCTCTTCCTCTTCCAG	AATCTTGGCACAGCAGTTTAACA	2-4	170	0.9	NM_001001781
Gal-9	GCAAAGGCTATTCCACAGCAG	AGCATTTCAGCTTCCCACCAC	1-4	211	1.8	NM_001001611
Gal-10	GCTCAGCAGACCCACTTTTC	GTTGCTGGTACAAGGGCAAT	3-4	189	1.9	NM_001001609
Gal-11	CAGAATTGCAGAAAGCCACA	TTCTACGTGTGCGTGTGTGA	3-4	240	0.8	NM_001001779
Gal-12	CCCAGCAGGACCAAAGCAATG	GTGAATCCACAGCCAATGAGAG	2-4	335	0.7	NM_001001607
Gal-13	ACAGCTGCGAGTTCAAGGAG	GCCCGGTAGAGGTTGTATCC	3-4	181	0.4	NM_001001780
Gal-14	TGGGCATATTCCTCCTGTTT	TTGCCAGTCCATTGTAGCAG	2-4	156	0.8	AM402954

tion. PCR conditions were 94˚C for 3 min, 35 cycles of 94˚C for 30 sec, 55˚C (for Gals-2, -4, -5, -6, -12) or 57˚C (for Gals-1, -3, -9, -14) or 60˚C (for Gals-7, -8, -10, -11) for 30 sec, 72˚C for 2 min and a final extension period at 72˚C for 10 min.

The PCR products were cloned and sequenced in order to confirm that these products were amplicons of the gallinacins genes and were not PCR artifacts or amplification of another gene. PCR products were separated on a 1% TBE agarose gel, cloned into the pCR 2.1-TOPO vector using the TOPO TA cloning Kit (Invitrogen) according to the manufacturer's protocol and sequenced. Sequencing was performed by Macrogen (Korea).

RNA Isolation and cDNA Synthesis

Total RNA was isolated from chicken tissues and embryos stored at –80˚C. Initially, the tissues and embryos were ground to a fine powder with sterile pestles and mortars and the RNA was extracted using the Total RNA Isolation (TRI) Reagent (Ambion) according to the instructions provided by the manufacturer. To reduce degradation, RNase inhibitor (Invitrogen) was added to each sample (1 U μg^{-1} of RNA) before storage at –80˚C. All samples were pretreated, before reverse transcription (RT), with DNAse (Fermentas) at a concentration of 1 U μg^{-1} of RNA.

One μg of total RNA was reverse transcribed to cDNA using the SuperScript II Reverse Transcriptase kit (Invitrogen) according to the manufacturer instructions.

Two μl of the RT products were subjected to PCR amplification. For the expression analysis experiments all primer pairs were designed to cross intron sequences and resulted in amplification of larger sequence if the samples were contaminated with genomic DNA (Table 1).

RT-PCR products were resolved by electrophoresis using 1% TBE agarose gels, visualised with ethidium bromide and imaged under UV illumination.

Amplification of chicken β-actin was performed as a control to check the quality of the synthesized cDNAs, using the primers: 5′-CTCCCTGATGGTCAGGTCAT-3′ and 5′-ATGCCAGGGTACATTGTGGT-3′, which amplify a region of 203 bp of the chicken β-actin mRNA and an annealing temperature of 56˚C.

In negative control samples, reverse transcriptase was omitted to demonstrate that PCR amplification was not due to contamination with genomic DNA.

The cDNA products were identified by cloning the PCR products into the pCR 2.1-TOPO vector and DNA sequencing as described above.

Protein sequence comparisons

The amino acid sequences of the chicken gallinacin genes were aligned using the multiple sequence alignment program T-COFFEE ver. 5.72 (Notredame *et al*., 2000). The GenBank accession numbers of the sequences were: Gal-1: AF033335 (Brockus *et al*., 1998), Gal-2: AF033336 (Brockus *et al*., 1998), Gal-3: NM_204650 (Yoshimura *et al*., 2006), Gal-4: NM_00100

1610 (Lynn *et al*., 2004), Gal-5: NM_001001608 (Lynn *et al*., 2004), Gal-6: NM_001001193 (Lynn *et al*., 2004), Gal-7: NM_001001194 (Lynn *et al*., 2004), Gal-8: NM_001001781 (Xiao *et al*., 2004), Gal-9: NM_ 0010 01611 (Xiao *et al*., 2004), Gal-10: NM_001001609 (Xiao *et al*., 2004), Gal-11: NM_001001779 (Xiao *et al*., 2004), Gal-12: NM_001001607 (Xiao *et al*., 2004), Gal-13: NM_001001780 (Xiao *et al*., 2004), Gal-14: AM402954 (direct submission).

RESULTS

Amino acid alignment

In silico amino acid sequences alignment of the gallinacin antimicrobial peptides revealed a conservation of the signal sequence at the N-terminus domain. In addition, the characteristic six-cysteine defensin motif was also identified at the C-terminus domain of all these peptides (Fig. 1).

Expression of gallinacins in the chicken reproductive tract

In order to determine the expression pattern of gallinacins mRNA, the initial experiments were focused on identifying the optimal conditions for the PCR amplification of these genes. As illustrated in Fig. 2, this was successful for all Gals, apart from Gal-13. We were unable to confirm Gal-13 amplification in the chicken genome despite using a number of different PCR primers, annealing temperatures and genomic DNA extracted from different chicken tissues.

As many studies have reported that synthesis of antimicrobial peptides increases in response to challenge with *Salmonella* serovars, one of the objectives of this study was to include in the expression experiments only *Salmonella* free birds. Using the SS selection method described in materials and methods, we isolated *Salmonella* free birds. These birds were fur-

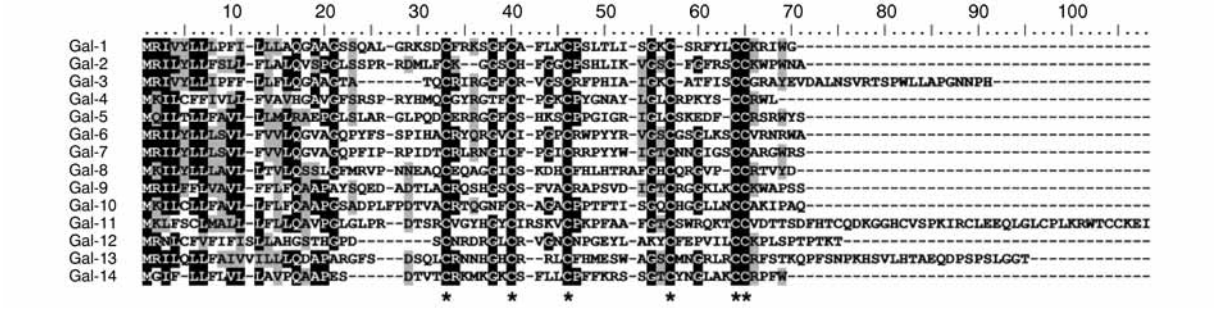

FIG. 1. Alignment of the chicken gallinacin antimicrobial peptides. Identical amino acids in all proteins are in white letters highlighted by black background, while similar amino acids of the sequences are highlighted by grey background. Dashes indicate gaps to maximize alignment. Asterisks indicate the 6 conserved cysteines.

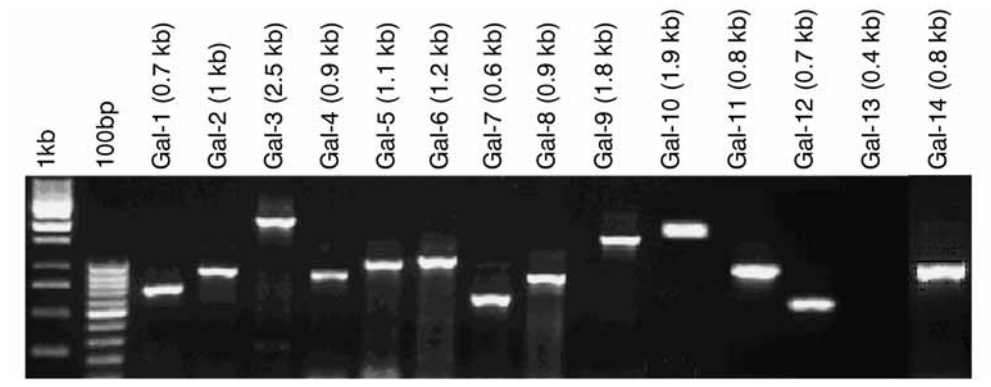

FIG. 2. PCR amplification of chicken gallinacin (Gals) genes in chicken genomic DNA.

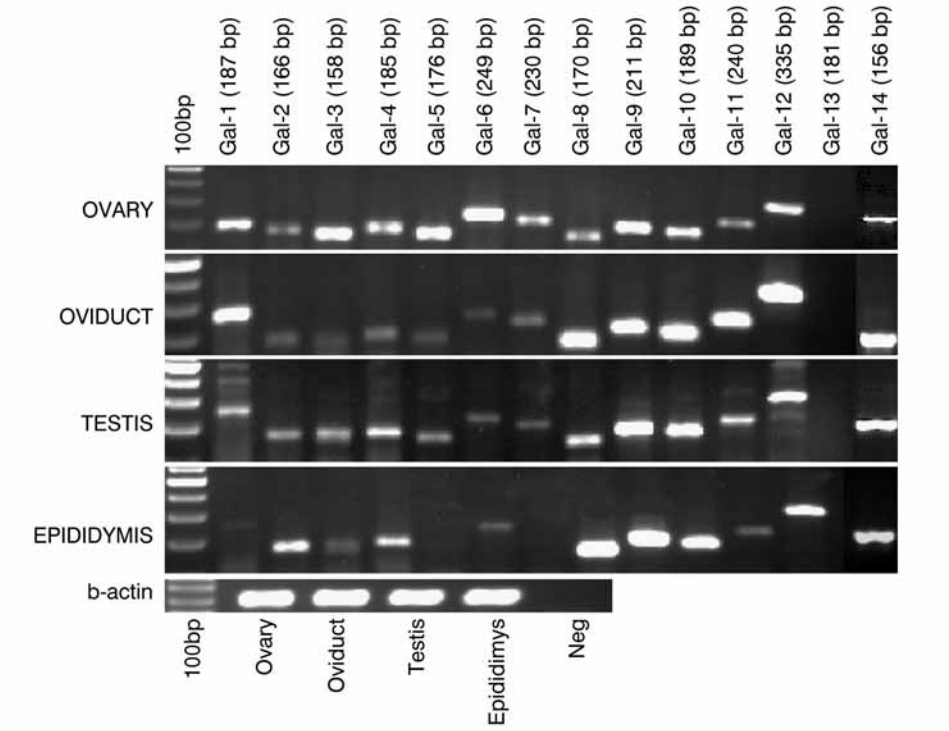

FIG. 3. Expression of chicken gallinacin (Gals) genes in chicken ovary, oviduct, testis and epididymis.

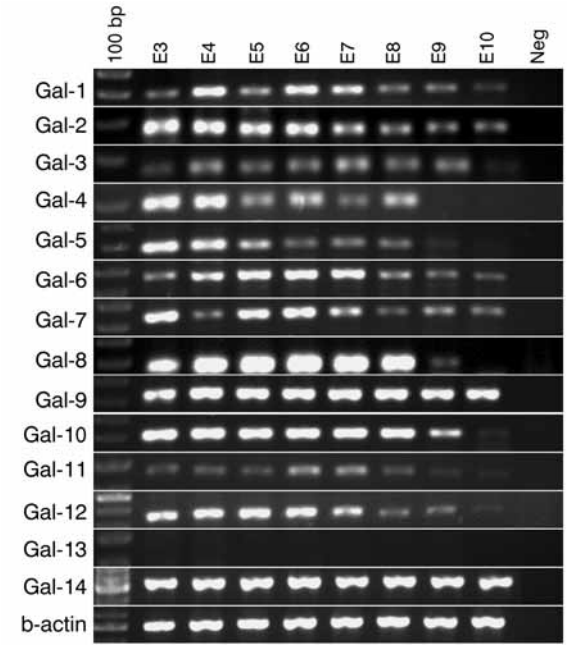

FIG. 4. Expression analysis of gallinacin (Gals) genes in chicken embryos from day three (E3) to day ten (E10) of chicken embryonic development. Neg: Negative control.

ther investigated for the expression of antimicrobial peptides. Birds that were found to be infected by *Salmonella* species were excluded from the expression analysis experiments. As illustrated in Fig. 3, RT-PCR analysis revealed that most of the gallinacin genes were expressed in the chicken reproductive tract. In the female reproductive tract all Gals were expressed in the chicken ovary and oviduct. In addition, in the male reproductive tract mRNA levels were detected for all Gals, whereas in the epididymis all Gals were expressed apart from Gal-5 and -7, for which no mRNA transcripts were detected.

Expression of gallinacins during chicken embryogenesis

Expression analysis using RT-PCR was performed in chicken embryos in order to investigate the expression pattern of gallinacin genes during chicken embryogenesis. Expression analysis was performed in embryos between day 3 (E3) and day 10 (E10) of chicken embryonic development. As illustrated in Fig. 4, whole embryo gene expression profiling revealed that all gallinacin genes, apart from Gal-13, were expressed at some stages of early chicken embryonic development. Interestingly, no mRNA transcripts were detected for Gal-3, -4, -5 and -8 at day 10 of embryonic development.

DISCUSSION

In the present study we report the types of gallinacins antimicrobial peptides expressed in the chicken reproductive tract *in vivo*, as well as in embryos *de novo*, during embryonic development.

In silico analysis studies revealed that similar to other families of antimicrobial peptides, the gallinacin proteins constitute a family of small, cysteine-rich cationic peptides, which vary from 59 to 104 amino acids in length. The signal sequences of all chicken gallinacin proteins are rich in leucines, consistent with has been observed for other vertebrate β-defensins (Xiao *et al*., 2004). Furthermore all Gal proteins are characterized by the presence of surplus arginines and lysines in the C-terminal region of these sequences.

Expression analysis data revealed that all of the members of the family of gallinacin genes were expressed in the chicken gonads, as well, as in the oviduct, while only Gal-5 and -7 mRNA transcripts were absent from epididymis. These data suggests that gallinacins antimicrobial peptides function *in vivo* in order to protect the chicken reproductive tract from infections. Recent studies have reported that Gal-1 to -7 are predominantly expressed in bone marrow and respiratory tract, whereas Gal-8 to -13 are restricted to the liver and urogenital tract (Xiao *et al*., 2004). Expression of Gal-3 was observed in the ovary of immature chicken (Zhao *et al*., 2001) and more recently expression of Gal-4, -7 and -9 were observed in the ovary of 38-day-old chicken (Milona *et al*., 2007). In addition, expression of Gal-1, -2, and -3 was observed in the chicken vaginal mucosa (Yoshimura *et al*., 2006), while the expression of Gal-1, -2, -7, -8, -10, and -12 in the theca layer and Gal-1, -8, -10, and -12 in the granulosa layer was identified in white and yellow chicken follicles (Subedi *et al*., 2007). Gal-12 protein has also been localized in the theca interna, granulosa and perivitelline layers of chicken ovarian follicles and its amount was increased with follicular growth from a white follicle to a yellow follicle, whereas the protein may be released from the cells by LPS stimulation (Subedi *et al*., 2008). Furthermore, expression of most gallinacin genes was recently reported in chicken oviduct epithelial cells cultured *in vitro*, as well as changes in their expression as a result of *Salmonella enterica* infection (Ebers *et al*., 2009). Our expression analysis data are in consistent with previous reports which indicated mRNA transcripts of Gal-3, -4, -7 and -9 in the chicken ovary (Zhao *et al*., 2001; Milona *et al*., 2007) and expression of Gal-1, -2 and -3 in the chicken oviduct (Yoshimura *et al*., 2006). However, we extended the expression pattern of 13 avian β-defensins in the reproductive tract in both male and female birds.

In this study, we report the expression of gallinacin antimicrobial peptides in the chicken embryos *de novo* during embryonic development. Although the expression of antimicrobial peptides during embryonic development has been investigated for other species, little is known about their expression during chicken embryogenesis. Human amnion epithelial cells have been found to express β-defensin 3, which was induced in response to lipopolysaccharide and peptidoglycan (Buhimschi *et al*., 2004), suggesting a role for antimicrobial peptides in defence during embryonic development. In addition, cathelicidin (CRAMP) gene expression was detected in mouse embryos at day 12 of embryonic development (Gallo *et al*., 1997). Our RT-PCR analysis data revealed mRNA transcripts from all gallinacin genes, apart from Gal-13, during various stages of chicken embryogenesis. These data were in consistent with a recently published study, which reported expression of gallinacins at day 3 of chicken embryonic development (Meade *et al*.,

2009). The expression of gallinacin antimicrobial peptides during chicken embryonic development provides evidence to suggest a mechanism of protection of the developing embryo and the newly hatched birds from pathogenic colonization.

Collectively, the data provided in this study reveal that gallinacins antimicrobial peptides were expressed in the chicken reproductive tract and embryos during embryonic development. These findings provide evidence to suggest that gallinacins host defence peptides play a significant role in the chicken reproductive tract. These antimicrobial peptides probably function *in vivo* to protect the female reproductive tract for successful follicular development, successful fertilization and also in preventing microbial pathogens from being incorporated into the newly forming eggs. It is well established that egg formation is completed during the passage of the yolk through the oviduct. The expression of gallinacins in the chicken oviduct suggests that these peptides may probably play an essential role in protection of the oviductal tissues from infections and production of pathogen-free eggs. In addition the novel findings of the expression of Gals in the male reproductive organs such as testis and especially the epididymis suggest that these peptides function to prevent male infection that may affect temporary or permanent fertility and have a significant role in avian sperm maturation and capacitation.

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