Characterization of chicken TNFR superfamily decoy receptors, DcR3 and osteoprotegerin☆

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Abstract

Tumor necrosis factor (TNF) family ligands bind to death domain-containing TNF receptors (death receptors), which can subsequently activate intracellular signaling pathways to initiate caspase activity and apoptotic cell death. Decoy receptors, without intracellular death domains, have been reported to prevent cytotoxic effects by binding to and sequestering such ligands, or by interfering with death receptor trimerization. The chicken death receptors, Fas, TNFR1, DR6, and TVB, are constitutively expressed in a relatively wide variety of hen tissues. In this study, two chicken receptors with sequence homology to the mammalian decoys, DcR3 and osteoprotegerin, were identified and their pattern of expression was characterized. Unlike the previously identified chicken death receptors, the newly characterized decoy receptors show comparatively limited expression among tissues, suggesting a tissue-specific function. Finally, characterization of these chicken receptors further contributes to understanding the evolutionary divergence of TNFR superfamily members among vertebrate species.
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Keywords: Tumor necrosis factor receptor superfamily; TNF ligands; Death receptor; Decoy receptor; Ovary; Chicken; Apoptosis

Death receptors (DRs) are characterized by the presence of a variable number of extracellular cysteine-rich motifs that comprise the ligand binding domain, a single-pass (type I) transmembrane domain and, in particular, a cytoplasmic death-domain. When bound to their cognate ligands, DRs typically form homotrimers, a process that modifies the conformation of the cytoplasmic domain and initiates cell signaling. DRs are capable of initiating apoptotic pathways in a cell type-dependent fashion by signaling through binding of their death-domains with death domains of cytoplasmic adaptor proteins. The adaptor proteins (e.g., Fas associated via death domain, FADD), in turn, interact with initiator caspases (e.g., caspase-8) via a death effector domain (DED). These interactions ultimately promote a cascade of caspase enzyme activation leading to cell death [1].

Alternatively, many of the death receptors are capable of signaling through intracellular pathways that actually promote cell survival. For example, TNFR1 is known to activate nuclear factor κB (NFκB) resulting in increased transcription of cell survival signals such as X-linked inhibitor of apoptosis protein (XIAP) [2], Bcl-X_{Long} [3], and FLICE-like inhibitory protein (FLIP) [4].

The ability of TNF family ligands to activate DR signaling is proposed to be modulated by the presence of decoy receptors (DcRs) that can bind to and sequester ligands from the death inducing receptors and/or interfere with receptor trimerization. Similar to DRs, DcRs contain cysteine-rich ligand binding domains, but do not possess intracellular death domains and may, or may not, contain a transmembrane domain. Mammalian DcRs are known to exist for TNF-related apoptosis inducing ligand (TRAIL) and Fas ligand (FasL). Additionally, splice variants encoding soluble forms of Fas, which are predicted to function as decoys, have been identified in the human [5] and chicken [6], and soluble Fas variants are capable of inhibiting apoptosis induced

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by the FasL in human T (HUT) lymphoma cells [5]. It has been proposed that the relative ratio of DR to DcR may determine susceptibility of cells to DR-mediated cell signaling [7].

More specifically, in the mammalian ovary there is limited immunohistochemical evidence to suggest that DcR-1 (a TRAIL DcR) is expressed in porcine granulosa cells and that decreased levels of DcR-1 are associated with follicle death via atresia [8]. This finding is of potential significance, given the importance of granulosa cell survival to the viability of differentiating ovarian follicles [9]. Brook trout (*Salvelinus fontinalis*) granulosa cells also express a decoy receptor (TDcR) that becomes upregulated just after the completion of ovulation [10], presumably at the time of ovarian regression.

In ongoing studies, we utilize the hen ovary as a model system to study DR activation and function in relation to ovarian follicle viability and development (e.g., [6,11,12]). The potential importance of signaling through death receptors as one pathway to initiate ovarian follicle atresia has been proposed for several vertebrate species (bovine, [13]; rat, [14]; and chicken, [6]). Significantly, four recently identified chicken death receptors (Fas, TNFR1, DR6, and TVB) are expressed at higher levels in atretic follicles as compared to healthy follicles [6,11,12]. These studies also demonstrate that levels of receptor expression are regulated by growth factors and gonadotropins that promote granulosa cell survival. The presence of multiple death receptors in this single cell type, each controlled by a unique set of intracellular signaling pathways, suggests a precise and intricate regulation of DR activation and signaling, including the potential for modulation by DcRs. Accordingly, in the present report, we provide evidence for two new chicken DcRs, DcR3 and osteoprotegerin (OPG), and characterize their selective expression in ovarian and extra-ovarian tissues.

Materials and methods

Isolation and characterization of chicken (ch) decoy receptors. The chDcR3 sequence was initially identified in the University of Manchester Institute of Science and Technology (UMIST) expressed sequence tagged (EST) database (http://www.chick.umist.ac.uk/; [15]) as an assembled and tentatively identified sequence (No. 048474.1). This nucleic acid sequence includes an open reading frame corresponding to the entire coding region when compared to the human DcR3 gene. Homologous primers were designed based upon this sequence to amplify a 558 bp product representing the region from amino acid 17 to 186 of the human 300 amino acid DcR3 protein (including the cysteine-rich TNF ligand binding domain). Primers were: forward 5′-ATG CTG TTC TGT CCC GAC CCG-3′ and reverse 5′-TTG GTT TCC TGG CAC ATT GG-3′. Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed with Applied Biosystems MuLV reverse transcriptase (Foster City, CA) using chicken spleen total RNA as template and TaKaRa ExTaq enzyme (Fisher Scientific, Chicago, IL) with the amplification conditions of: 94 °C for 30 s, 62 °C for 1 min, and 72 °C for 1 min, for 30 cycles.

Two chicken EST sequences with similarity to human OPG were identified in the University of Delaware Chicken EST database (pituitary gland, hypothalamus, and pineal gland library, clone No. BI393190, and breast muscle, leg muscle, and epiphyseal growth plate library, clone No. BM488261; http://www.chickest.udel.edu/). These sequences encompass a partial open reading frame with homology to the cysteine-rich domain of mammalian OPGs, but lacking the C-terminus. Homologous primers based on the two EST sequences were used to amplify a 454 bp fragment (forward primer 5′-TTC TTC CAC CAA CCA CAA GA AC-3′ and reverse primer 5′-GGG CAA CTT TTA CAA ACA GTG TCA C-3′) using an ovarian stromal tissue template with the identical amplification conditions described above.

Each PCR product was cloned into TA cloning vector (Invitrogen, Carlsbad, CA), transformed into INVαF′cells (Invitrogen), and subsequently sequenced using the dideoxychain-termination method with Sequenase version 2.0 (USB Corporation, Cleveland, OH). Final sequences for full-length clones were obtained through Canadian Molecular Research Service (Ottawa, Ont., Canada). Sequence analysis and alignments were performed using MacVector 6.5 software (Oxford Molecular, Genetics Computer Group, Madison, WI).

Animals and tissue collection. Single-comb white Leghorn hens (H&H Poultry, Portland IN), 25–35 weeks of age and laying regular sequences of at least 5–6 eggs, were used in all studies described. Birds were housed individually in laying batteries, provided with free access to feed (Purina Layeena Mash, Purina Mills, St. Louis, MO) and water, and were exposed to a photoperiod of 15 h light, 9 h darkness, with lights on at midnight. Individual laying cycles were monitored by the daily timing of oviposition. Hens were killed approximately 16–18 h prior to a midsequence ovulation by cervical dislocation. All procedures described herein were reviewed and approved by the University of Notre Dame Institutional Animal Care and Use Committee, and were performed in accordance with the Guiding Principles for the Care and Use of Laboratory Animals.

Tissues were collected from the chicken ovarian stroma, post-ovulatory follicle, brain, spleen, bone marrow, oviduct, kidney, and heart for isolation of total RNA. Ovarian follicle tissues included granulosa and theca from 3 to 5 and 6 to 8 mm diameter prehierarchal (undifferentiated) follicles, and the largest (F1) and second largest (F2) preovulatory follicles. Morphologically normal and atretic follicles (3–8 mm) were processed without separating the granulosa and theca layers, as previously described [16]. Samples were collected and processed immediately or frozen at −70 °C.

Northern blot analysis. EcoRI restriction digests were performed to isolate the chDcR3 558 bp and chOPG 454 bp inserts from the clones described above for use as cDNA probe templates. Templates were random-prime labeled with the Megaprime DNA Labeling System (Amersham, Piscataway, NJ) and [^{32}P]dCTP (3000 Ci/mmol; Amersham). Prehybridization was carried out for at least 30 min at 60 °C, at which time probes were added and hybridization was allowed to proceed overnight. Stringent washes were conducted by rinsing with 2× SSC (single strength SSC = 150 mM sodium chloride and 15 mM sodium citrate, pH 7.0) at room temperature, two 15 min washes in 2× SSC and 1% SDS at 60 °C, and one 15 min wash with 0.1× SSC and 0.1% SDS at 60 °C, followed by exposure to phosphorimaging plates. Images were captured using the Storm 840 PhosphorImager system equipped with the ImageQuant data reduction system (Molecular Dynamics, Sunnyvale, CA). Where appropriate, blots were subsequently reprobed with chicken 18S ribosomal RNA to standardize for loading of RNA [6].

Results and discussion

Two additional decoy receptors belonging to the TNFR superfamily, chDcR3 and chOPG, have been characterized for the first time in an avian species,

Table 1
Currently identified human, mouse, avian, and piscine decoy receptors from the tumor necrosis factor superfamily, together with representative GenBank accession numbers for either nucleic acid or amino acid sequences

Decoy receptors	Mammalian		Avian	Piscine
	Human	Mouse		
DcR3	AAD03056		AAP03889	
OPG	AAB53709	AAB53708	AAP03890	
Soluble Fas	I37383		AAG02243	
TRAIL-DcR1	AAB67104			
TRAIL-DcR2	AAD03477			
TNFRSF22		NP_076169		
TNFRSF23		NP_077252	AY251409	
TNF decoy receptor				AAK91758
TDcR				AAD56428

bringing the total number of identified chDcRs to four (Table 1). Each of the newly identified receptors displays a characteristic and limited pattern of tissue distribution compared to previously reported chDRs [6,11,12]. Accordingly, it is proposed that each chDcR plays a role in regulating DR function in a tissue-specific fashion.

Nucleic acid and deduced amino acid sequences for chDcR3 and chOPG

The predicted full length amino acid sequence of the chDcR3 is based upon the cloned nucleic acid sequence presented herein (GenBank Accession No. AY251406), combined with additional sequence obtained from a tentatively identified assembled EST from the UMIST database (No. 048474.1). The full-length protein is 51% identical (68% similar) to human DcR3 (GenBank Accession No. AAD03056; [17]. The partial nucleic acid sequence of chOPG (GenBank Accession No. AY251407) includes a predicted coding region that was determined to be 68% identical (80% similar) to amino acids 1–146 of the 401 amino acid human OPG (GenBank Accession No. AAB53709) (Fig. 1).

Both the chDcR3 and chOPG receptors contain the highest sequence identity to known TNFR decoy sequences within the cysteine-rich regions (CRDs), including 19 aligned cysteine residues (Fig. 1). The N-linked glycosylation site is also conserved in both DcR3 and OPG between human and chicken predicted amino acid sequences (Fig. 1). The trout decoy receptor (TDcR, Accession No. AAD56428; [10]) is reported to contain the four CRDs in common with both DcR3 and OPG of mammalian species. These four cysteine-rich domains are also conserved in both chDcR3 and chOPG sequences that were obtained in this study (Fig. 1).

DcR3

The human DcR3 is a receptor that, like soluble Fas, binds with FasL, as well as LIGHT, and TL1A. Recombinant DcR3 protein added to human Jurkat T leukaemia cells was shown to specifically inhibit FasL-mediated apoptosis [17]. A broad range of human tumors, including lung, colon, and breast cancers are known to frequently express high levels of DcR3. Accordingly, over-expression of this decoy has been proposed to incur a growth or survival advantage to the tumor and/or to inhibit the anti-tumor effects of FasL, LIGHT or TL1A [18]. To date, no other mammalian orthologs to human DcR3 have been reported, and notably, there has yet to be a gene encoding DcR3 identified in mice [18]. Therefore, it has not been possible to conduct mouse knockout experiments to firmly establish a physiological role for this decoy.

Interestingly, a rainbow trout (*Onchorynchus mykiss*) TNF receptor homolog has recently been reported [19] that shows some similarity to the brook trout (*Salvelinus fontinalis*) TDcR (35% identity), but more similarity to chDcR3 (41% identity) and human DcR3 (40% identity). The identification of two apparently unique DcRs in related genera of trout indicates an early divergence of DcRs during vertebrate evolution similar to that shown for DRs [20].

Northern blot analysis of various chicken tissues revealed two transcripts of approximately 1.2 and 0.8 kb in size that are predicted to encode chDcR3 mRNA. These are expressed in hen oviduct, in the postovulatory follicle (POF), and to a lesser extent in ovarian stroma (Fig. 2). Although chDcR3 is not constitutively expressed at detectable levels in either granulosa or theca cells of preovulatory follicles (Fig. 3), it is expressed in POF (Fig. 2) and is upregulated by 3.5-fold in atretic follicles (Fig. 4A). It has yet to be determined whether this increase reflects a cause or an effect of atresia, however, the potential significance of this DcR being upregulated under circumstances associated with cell death indicates that Fas receptor mediated events are induced during avian follicle atresia. The correlative findings that human DcR3 binds FasL [17] and that both FasL and its receptor Fas are upregulated in atretic bovine [13] and hen [6] ovarian follicles further implicate these factors in determining the fate of developing follicles.

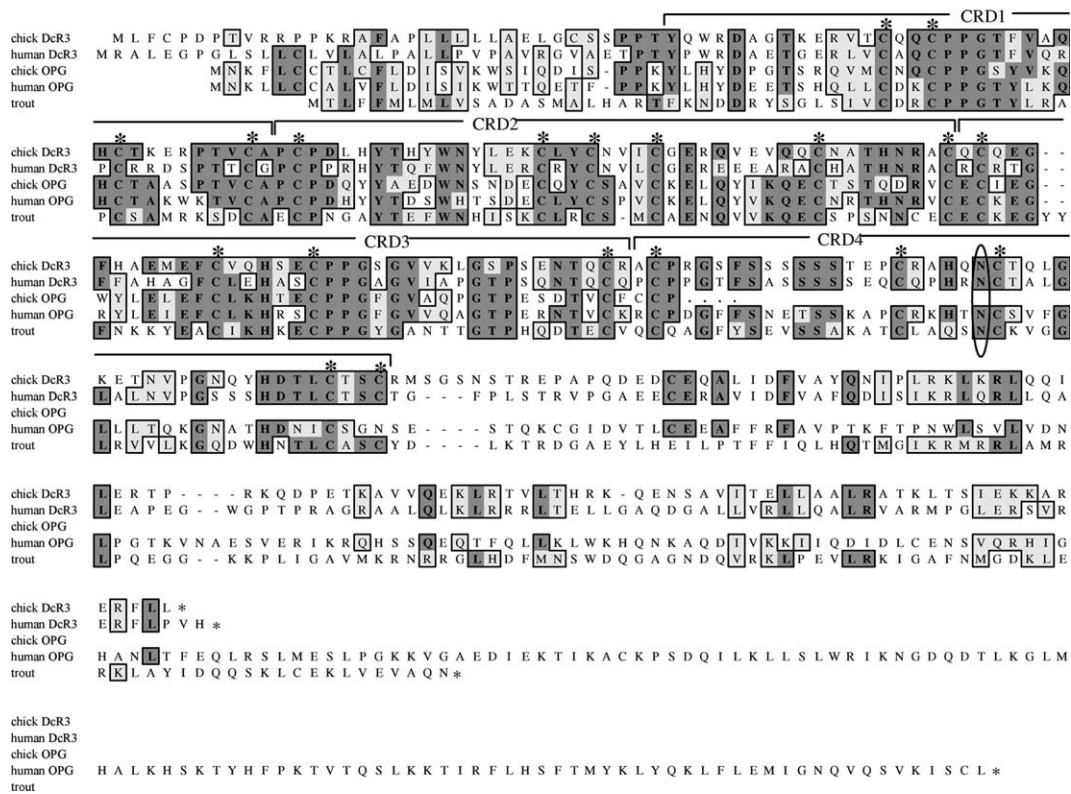## Decoy Receptor Amino Acid Alignment

Fig. 1. Chicken DcR3 (Accession No. AY251406) and partial OPG (Accession No. AY251407) deduced amino acid sequences aligned with homologous sequences of human DcR3 (Accession No. AAD03056) and OPG (No. AAB53709), and trout TDcR (*Salvelinus fontinalis*; No. AAD54628). Dark boxes indicate identical amino acids; lightly shaded boxes represent conserved substitutions. The four cysteine-rich domains (CRD1-4), conserved cysteine residues (asterisk), and N-linked glycosylation site (circled) are noted [17]. The putative N-terminal signal peptide cleavage site for human DcR3 resides at LLP-VP, for chicken DcR3 is at GSC-SP, and chicken OPG is at QWS-IQ, as predicted using the program available from the Center for Biological Sequence Analysis at http://www.cbs.dtu.dk/services/SignalP/.
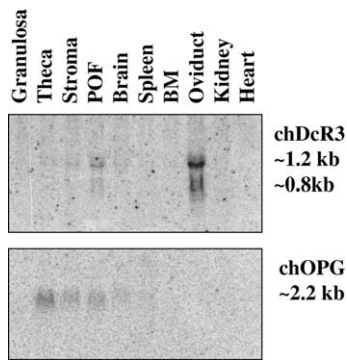

Fig. 2. Northern blot analysis of chicken (ch) DcR3 (top panel) and chOPG (bottom panel) mRNA expression in several hen tissues. POF, postovulatory follicle; BM, bone marrow. Each blot was replicated once with similar results.
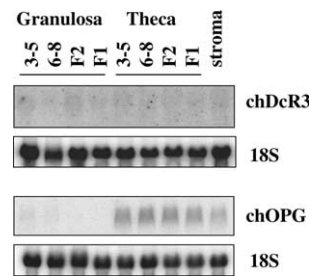

Fig. 3. Northern blot analysis of chDcR3 (top panel) and chOPG (bottom panel) mRNA expression in granulosa and theca cells at different stages of development. F1 and F2 represent the largest and second largest preovulatory follicles, respectively; 3–5 and 6–8 refer to diameters of undifferentiated follicles (mm).

Osteoprotegerin

Mammalian OPGs bind with two TNF family ligands, TRAIL and receptor activator of nuclear factor (NF-κB) ligand (RANKL). Although the physiological significance of OPG binding with TRAIL has yet to be characterized, this receptor plays an important role in regulation of bone metabolism. RANKL activates its receptor RANK and signals osteoclast formation, fusion, activation, and survival, thus resulting in bone resorption and bone loss. Conversely, when RANKL is bound by OPG this process is blocked. OPG knock-out

A

Normal　　Atretic

← 1.2 kb chDcR3

18S

DcR3
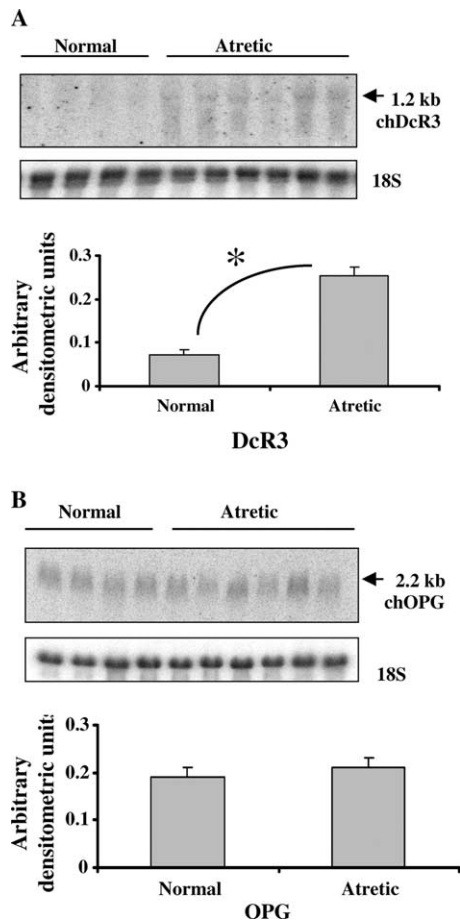
B

Normal　　Atretic

← 2.2 kb chOPG

18S

OPG

Fig. 4. (A) Northern blot analysis of chDcR3 (only 1.2 kb transcript measurable) and (B) chOPG mRNA isolated from healthy (normal) versus atretic prehierarchal follicles. Summarized data represent means ± SEM, standardized to levels of 18S rRNA. *$P < 0.05$ by unpaired t test.

mice display severe osteoporosis, whereas over-expression of OPG in transgenic mice causes osteopetrosis [21,22]. Additionally, the RANKL/OPG/RANK system is an important regulator of mammary gland development and has been implicated in severe bone metabolic disorders, as well as abnormal vascular calcification [21].

Northern blots depicting tissue expression of OPG mRNA (Fig. 2) indicate that a single chOPG transcript (~2.2 kb) is present in ovarian tissues, including theca, stroma, and POF, but not in the granulosa cell layer. Highest levels of chOPG mRNA are found in the theca layer, however, the level of chOPG expression does not change with follicle development (Fig. 3) or atresia (Fig. 4B).

The partial sequence thus far identified for chOPG predicts high homology to human OPG (80% similar) through 3 of the 4 cysteine-rich domains, suggesting both evolutionary conservation of amino acid sequence and conservation of function. It is worth noting, however, that the C-terminal sequence of chOPG is not yet known, and thus identity to the remaining motifs pres-

ent in mammalian OPG sequences is uncertain. For instance, human OPG is known to lack a transmembrane domain and to contain two separate death domain like regions in the C-terminal portion of the protein [23]. The death domain regions apparently do not promote intracellular signaling, since OPG is a secreted protein and is known to bind to and prevent signaling via RANKL and TRAIL [24,25]. In the hen ovary, highest levels of chOPG are detected within the theca layer, a finding which suggests that this receptor can act in a paracrine-like fashion, being secreted from the theca layer to potentially protect granulosa cells from signaling via their own membrane DRs.

Similar to both chDcR3 and chOPG, trout TDcR is expressed predominantly in reproductive tissues [10]. TDcR mRNA is positively regulated, in vitro, by an activator of protein kinase C signaling (the phorbol ester, PMA), and TDcR expression is upregulated, in vivo, at the completion of ovulation. In situ hybridization studies of trout postovulatory follicles demonstrated that TDcR is expressed most highly in the granulosa cell layer [10]. By comparison, in the hen ovary chDcR3 is upregulated in follicles undergoing atresia (Fig. 4A), whereas chOPG mRNA was expressed in the theca layer and was not differentially expressed during atresia (Figs. 3 and 4B).

Expression of both chDcR3 and chOPG in the hen is somewhat novel, as thus far humans represent the only other vertebrate species known to express both genes (Table 1). Interestingly, even within the same vertebrate class, humans and mice express distinctly different decoy receptors that bind with TRAIL [26]. As more comparative sequence information from non-mammalian species becomes available, the evolutionary relationships and potential functional specialization of DcRs can be addressed, as has been recently reviewed for vertebrate DRs [20].

In summary, chDcR3 and chOPG represent chicken orthologs to known mammalian DcRs that have previously been shown to bind to and inhibit signaling through FasL and RankL, and TRAIL, respectively. The chDcRs are differentially expressed among various tissues from the hen, as well as expressed in unique patterns within the ovary. Accordingly, chDcR3 and chOPG likely represent extracellular factors that can modulate the apoptosis inducing effects of DRs in the hen. Finally, given a recent report that TRAIL can actively promote the survival and proliferation of cultured human endothelial cells [27], future studies should address the possibility that DcRs, such as chOPG, also function to modulate non-death-related effects.

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