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Mutant oocytic low density lipoprotein receptor gene family member causes atherosclerosis and female sterility

(germ cells/differential splicing/familial hypercholesterolemia)

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ABSTRACT **The so-called very low density lipoprotein receptors (VLDLRs) are related to the LDLR gene family. So far, naturally occurring mutations have only been described for the prototype LDLR; in humans, they cause familial hypercholesterolemia. Here we describe a naturally occurring mutation in a VLDLR that causes a dramatic abnormal phenotype. Hens of the mutant restricted-ovulator chicken strain carry a single mutation, lack functional oocyte receptors, are sterile, and display severe hyperlipidemia with associated premature atherosclerosis. The mutation converts a cysteine residue into a serine, resulting in an unpaired cysteine and greatly reduced expression of the mutant avian VLDLR on the oocyte surface. Extraoocytic cells in the mutant produce higher than normal amounts of a differentially spliced form of the receptor that is characteristic for somatic cells but absent from germ cells.**

Receptors belonging to the low density lipoprotein receptor (LDLR) supergene family play important biological roles in addition to mediating lipoprotein metabolism (1, 2). A strain of mutant chickens termed restricted ovulator (R/O), characterized by female sterility via failure to lay eggs associated with severe hyperlipidemia (3, 4), constitutes a prime example in support of this notion. At an average age of 18 months, R/O hens show striking aortic atherosclerotic lesions; in parallel, oocytes do not reach full size, become necrotic, and fail to ovulate. The hens' levels of serum triglycerides are elevated 4- to 5-fold, total cholesterol is elevated 6-fold, and phospholipids are elevated 3-fold compared to normal; roosters of the mutant strain show no apparent abnormality. Breeding studies demonstrated that the sterile hyperlipidemic phenotype was due to a single gene defect at a locus (*ro*) on the Z chromosome (5–7). We have shown (8, 9) that the lack of oocyte growth (vitellogenesis) in R/O hens is due to the absence of a functional 95-kDa member of the LDLR family [termed oocyte vitellogenesis receptor (OVR)] that normally mediates massive uptake of yolk precursors from the serum. Molecular cloning of wild-type OVR demonstrated that it is the avian homologue of the recently discovered mammalian receptors termed very low density lipoprotein receptors (VLDLRs), whose exact physiological function(s) have not been clarified yet (7, 10–17). VLDLRs possess eight (and in contrast, LDLRs possess seven) tandemly arranged so-called binding repeats, complement-type cysteine-rich domains displaying a negatively charged surface for ligand binding. One unusual feature of OVR is the absence of a region that in LDLRs contains clustered O-linked sugar chains (7). Chicken oocyte OVR is able to bind apparently unrelated serum-borne yolk precursor molecules such as vitellogenin, VLDL, riboflavin-binding protein, and α_2-macroglobulin (18–20). Identification of the ro^- mutation was thus expected to shed light on the molecular basis for the dramatic dual effects of functional disruption of this receptor.

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MATERIALS AND METHODS

Northern Blot and Reverse Transcriptase (RT)–PCR Analysis. For Northern blot analysis, poly(A)$^+$ RNA (2.5 μg) from the indicated tissues of adult (>6 months old) normal and R/O hens was denatured by using 0.8 M glyoxal/45% (vol/vol) dimethyl sulfoxide, separated by electrophoresis on 1.0% agarose gels, and blotted onto Hybond-C Extra membrane (Amersham) by using standard methods (21). Hybridization and washings were performed as described (7), using as probes cDNA fragments covering the ligand binding and epidermal growth factor (EGF)-precursor homology regions of OVR or a 90-nt probe corresponding to an alternatively spliced exon, respectively (see *Results and Discussion*). For preparation of the 90-nt probe, we synthesized two 50-mer oligonucleotides corresponding to its 5′ side (sense) and 3′ side (antisense), respectively, annealed them, and labeled the DNA by using the Klenow fragment of DNA polymerase (21). Membranes were exposed for 2 days to Fuji RX film with intensifying screens. After stripping the probe, the membrane was used for hybridization with a β-actin probe.

For RT–PCR with the OVR mRNA from R/O hens, poly(A)$^+$ RNA was isolated from the ovary (7). Single-stranded cDNA was synthesized by using SuperScript RT (GIBCO/BRL) and random primers. The synthesized cDNA was used for subsequent PCR amplifications. The following oligonucleotides, P1–7, were used for PCR primers: P1, 5′-CGACGGGATATCAGGAAGATTGGCC-3′; P2, 5′-AGGAAGAACAGCCCAAGCTCCTGCT-3′; P3, 5′-ACCCTAGTAAACAACCTCAATGATG-3′; P4, 5′-TGGAGGAAGTCTTTCAGCCACAAGC-3′; P5, 5′-AATAGAAGCTGGCTCTCTCAGCTCA-3′; P6, 5′-GTCAAGAAGACATGTGCTGAATCTG-3′; and P7, 5′-TTGACTGAGGACCACAGCTGATCTC-3′. The nucleotide sequences of primers corresponded to nt 1396–1420 (sense), nt 2358–2382 (antisense), nt 2098–2122 (sense), and nt 2656–2680 (antisense), nt 1719–1743 (antisense), nt 256–280 (sense), and nt 403–427 (antisense) of the chicken OVR (7), respectively. PCR amplifications of cDNA were performed with the three pairs of primers, P1/P2, P3/P4, or P5/P6, respectively, and the GeneAmp PCR kit (Perkin–Elmer) on a Perkin–Elmer ther-

Abbreviations: LDLR, low density lipoprotein receptor; VLDLR, very LDLR; OVR, oocyte vitellogenesis receptor; R/O, restricted ovulator; RT, reverse transcriptase; EGF, epidermal growth factor; FH, familial hypercholesterolemia.

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mal cycler model 480. PCR parameters were 94°C for 1 min, 60°C for 1 min, and 72°C for 2 min for 30 cycles. To amplify the sequence of the 5′ end of the receptor mRNA, 5′ rapid amplification of cDNA ends was carried out with primer P7 and the 5′ rapid amplification of cDNA ends system (GIBCO/BRL). Amplified products were separated by agarose gel electrophoresis, and the resulting amplified fragments were subcloned into the pGEM-T vector (Promega). Several clones from each fragment were isolated and sequenced on both strands by using Sequenase (United States Biochemical).

Immunoblot Analysis. Membrane fractions of ovaries from mature (>6 months old) and immature (2 months old) normal and adult R/O chickens, respectively, were prepared and detergent extracts were applied without heating or adding dithiothreitol to a 4.5–18% gradient polyacrylamide/SDS gel. Electrophoresis, transfer to nitrocellulose membrane, and immunodetection were performed as described (7). The polyclonal rabbit IgGs used for the analysis are directed against the purified protein and a synthetic peptide corresponding to the last 14 amino acids of chicken OVR, respectively (7).

Cell Surface Binding. Plasma LDL from normal hens was prepared and labeled with ^{125}I by the iodine monochloride method as described (22). Skin fibroblasts from normal and R/O hens were obtained and maintained as described (22). Lipoprotein binding to cultured fibroblasts was determined as outlined (22). Briefly, after incubation for 24 h in lipoprotein-deficient serum, the monolayers were incubated with the indicated concentrations of ^{125}I-labeled LDL (316 cpm/ng of protein) in the absence (total binding) or presence (nonspecific binding) of unlabeled LDL (500 μg/ml) for 5 h at 37°C. After washing, the cells were incubated with dextran sulfate buffer [50 mM NaCl/10 mM Hepes/dextran sulfate (M_r, 500,000) (4 mg/ml), pH 7.4], and radioactivity in the collected buffer was measured to determine the amount of ^{125}I-labeled LDL released from the cell surface.

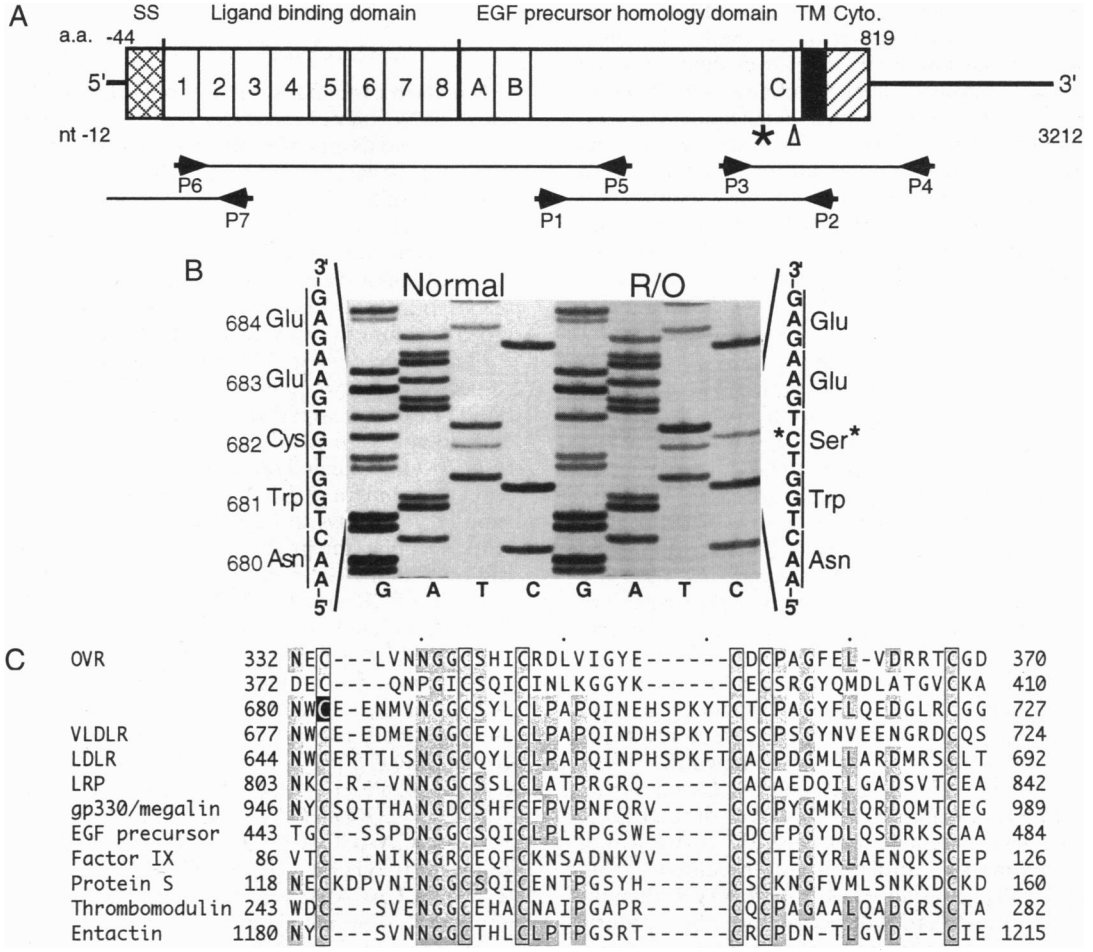

FIG. 1. Nucleotide sequence analysis of OVR in R/O hens. (*A*) Schematic representation and strategy for RT–PCR amplification of OVR in R/O hens. The signal sequence (SS) and the following four functional domains in OVR are shown (7): eight binding repeats in the ligand binding domain, boxes 1–8; the cysteine-rich repeats A, B, and C in the EGF-precursor homology domain, boxes A–C; the transmembrane domain, TM; the cytoplasmic domain, Cyto. Amino acid and nucleotide numbers are shown above and below the schematic receptor, respectively (7). The asterisk indicates the mutated site in the R/O gene, and the open arrowhead indicates the site (between nt 2311 and 2312 of the cDNA) of insertion of the 90 nt via differential splicing. Primers P1–P7 used for PCR amplification are indicated by short arrows. (*B*) Nucleotide sequence of a part of the OVR cDNA from R/O hens. Normal and mutated sequences are compared. The mutated nucleotide (G → C) at position 2177 (7) and the resulting altered amino acid (Cys → Ser) at residue 682 (7) are shown by asterisks. (*C*) Amino acid sequence alignment of EGF-precursor homology domains in OVR with those in various proteins of the LDLR supergene family. All sequences correspond to EGF-precursor homology domains, type B (23), and are aligned to match the six cysteine residues. Gaps introduced to optimize alignments are shown as dashes, and the mutated cysteine in the R/O OVR is indicated with white type on a black background. All conserved cysteine residues are boxed, and residues conserved in more than 50% of the sequences are shaded. Parts of sequences of chicken OVR (7), human VLDL receptor (12), human LDL receptor (24), chicken LDLR-related protein (LRP) (25), rat gp330/megalin (26), mouse EGF precursor (27), human factor IX (28), human protein S (29), human thrombomodulin (30), and mouse entactin (31) are shown. Amino acid numbers at the beginning and the end of each aligned sequence indicate the position of that amino acid in the respective protein.

RESULTS AND DISCUSSION

Sequence analysis of the complete coding region of the abnormal receptor mRNA by RT–PCR revealed a single nucleotide substitution (G → C), which results in the Cys-682 → Ser replacement (indicated by an asterisk in Fig. 1 *A* and *B*). The mutation was also observed in genomic DNA of R/O hens (data not shown). The missense mutation affects the first of six cysteines in a characteristic extracellular domain of LDLR family members, the so-called EGF-precursor homology repeat C (Fig. 1*A*). As a result, there is an unpaired cysteine residue in this structural element located close to the plasma membrane-spanning domain of the receptor. EGF-precursor homology repeats with six cysteines are found in a large number of proteins including members of the LDLR family, proteins of the blood coagulation system, and the basement membrane protein entactin (Fig. 1*C*) (23).

Transcriptional activity of the gene containing the point mutation appeared normal, as determined by blot analysis of poly(A)$^+$ RNA isolated from ovarian follicles (Fig. 2). However, we consistently observed that the size of the hybridizing signal(s) obtained with full-length OVR cDNA as a probe was somewhat larger in the mutant than in the normal hens (e.g., Fig. 2). Detailed PCR analysis of several regions of the normal and mutant mRNAs revealed the surprising reason for the apparent size difference; the key observations are summarized in Fig. 3. As stated above, the chicken OVR lacks the O-linked sugar domain (7), which in LDLRs is located between the EGF-precursor homology repeat C and the membrane-spanning domain (Fig. 1*A*). However, when we used oligonucleotides corresponding to the amino acid residues flanking this domain in a RT–PCR of ovarian RNA, we obtained two products (Fig. 3*A*). One was a fragment of the size expected based on the published sequence of OVR (7), and the other minor fragment was 90 nt larger. These 90 nt, 5′-G[T^{1}T(Val)-TCA(Ser)GGT(Gly)ACT(Thr)GGA(Gly)ACA(Thr)-ACT(Thr)GTG(Val)GCT(Ala)TAC(Tyr)ACT(Thr)GAG-(Glu)GCT(Ala)AAA(Lys)GAT(Asp)ACC(Thr)AGC(Ser)-ACA(Thr)ACT(Thr)GAA(Glu)AAA(Lys)TCT(Ser)CCA-(Pro)ACT(Thr)GTT(Val)GGA(Gly)CTA(Leu)GTT(Val)-CCT(Pro)GGA(Gly)G^{90}]-G-3′, encode a typical 30-residue O-linked sugar domain located at the consensus site in LDLR family members and arise by differential splicing of the primary OVR transcript (H.B., Ken A. Lindstedt, M.H., L. M. Dalmau, J.N., and W.J.S., unpublished data). The larger amplified fragment was clearly detectable in R/O ovaries (Fig. 3*A*, lane 1) but required prolonged exposure for visualization from normal organs (Fig. 3*A*, lane 2). However, the longer transcript was directly identified by Northern blot analysis of mRNA prepared from the heart of normal animals (Fig. 3*B*, lanes 2 and 3). This experiment furthermore clearly demonstrates, by using a probe corresponding exactly to the 90 nt (Fig. 3*B Lower*), the dramatic differences in expression levels of the two transcripts in chicken tissues.

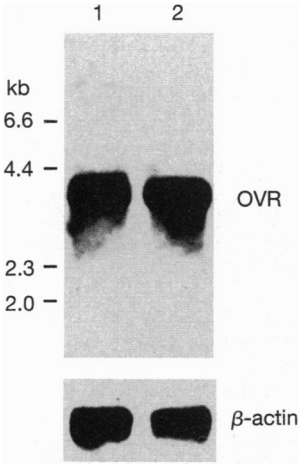

FIG. 2. Expression of OVR mRNA in ovaries. Autoradiograms of blot hybridization analysis of ovarian poly(A)$^+$ RNA from R/O (lane 1) and normal (lane 2) hens using cDNA probes for OVR (*Upper*) or β-actin (*Lower*). The size markers used were the *Hin*dIII cleavage products of phage λ DNA.

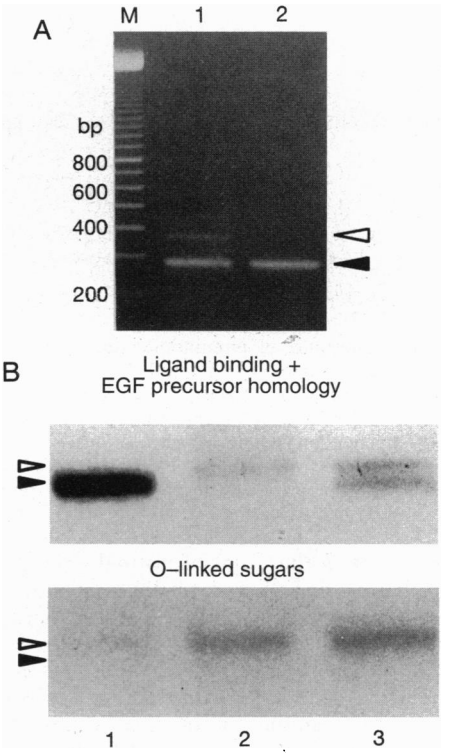

FIG. 3. Expression of the OVR variants in R/O and normal tissues. (*A*) RT–PCR-amplified fragments from 0.2 μg of ovarian poly(A)$^+$ RNA by using specific primers encompassing the alternatively spliced O-linked sugar domain (P2/P3; see Fig. 1*A*) are shown. One-tenth of the amplified products was used for gel electrophoresis. Lanes: 1, R/O; 2, wild type. Lane M contains as size markers the 100-bp ladder (Pharmacia). (*B*) Northern blot analysis of OVR transcripts in tissues of normal hens by using two different probes. Denatured poly(A)$^+$ RNAs from undissected ovary (2 μg; lane 1), heart (5 μg; lane 2), and a mixture of both (0.1 μg from ovary and 15 μg from heart; lane 3) were separated by electrophoresis on a 2.0% agarose gel. Northern blot analysis was performed with probes corresponding to the common region (ligand binding and EGF precursor homology) (*Upper*) or with the 90-bp probe specific for the O-linked sugar domain (*Lower*). Exposure time was 1 day (*Upper*) and 7 days (*Lower*), respectively. Open and solid arrowheads indicate the amplified fragments (*A*) and transcripts (*B*) corresponding to the receptor forms with or without the 90-nt O-linked sugar domain, respectively (see text).

Figs. 2 and 3*A* suggest that R/O ovaries express higher than normal levels of the longer transcript and possibly its translated product. Thus, we next tested whether the mutant ovaries that lack OVR function (8, 9) show a relative increase in the production of the receptor translated from the differentially spliced transcripts containing the 90-nt insert. IgG directed against purified OVR (Fig. 4, lanes 1–3) and against the common C terminus of both forms of the receptor (Fig. 4, lanes 4–6) detected OVR in only 2 μg of protein of ovarian membrane extract from normal laying hens (Fig. 4, lanes 1 and 4) and immature hens (lanes 3 and 6); under these and other conditions (data not shown), only a single 95-kDa band was visualized. In contrast, both IgGs recognized a protein pair at a similar position to the normal OVR in ovarian membranes of R/O hens (Fig. 4, lanes 2 and 5), but only if a greater amount

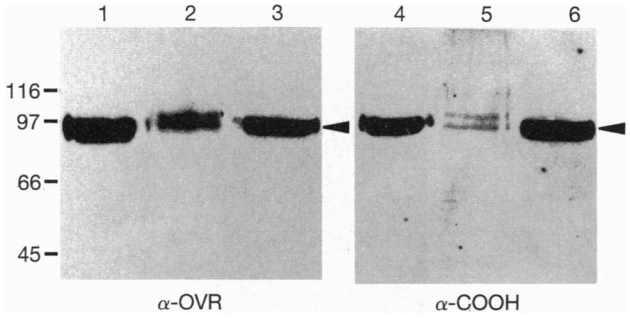

FIG. 4. Autoradiograms of immunoblot analysis of OVR from normal and R/O hens. Lanes: 1 and 4, follicles from a normal laying hen (2 μg of protein per lane); 2 and 5, follicles from a R/O hen (40 μg per lane); 3 and 6, previtellogenic follicles from an immature normal hen (2 μg per lane). The membranes were incubated with anti-OVR IgG (2 μg/ml) (lanes 1–3) or anti-C-terminal IgG (20 μg/ml) (lanes 4–6). The position of migration of the normal OVR (95 kDa) is indicated by the arrowhead.

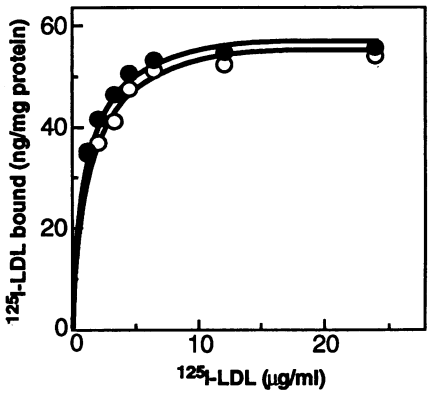

FIG. 5. Binding activities of cultured skin fibroblasts from normal and R/O hens. Surface binding of ^{125}I-labeled LDL as a function of ^{125}I-labeled LDL (^{125}I-LDL) concentration in normal (open circles) or R/O (solid circles) cells. Specific binding was calculated by subtraction of nonspecific from total binding. Each data point represents the average of duplicate incubations.

of membrane protein than that of normal animals was analyzed. Fig. 4, lanes 3 and 6, confirms that immature (previtellogenic) oocytes in the ovaries of sexually immature normal hens also express high levels of OVR transcripts and protein (7, 32). An important aspect of ovarian biology is the crosstalk between the oocyte and neighboring somatic cells that probably harbor the small amount of the larger transcript detected in the ovary (Fig. 3*B*, lane 1, *Lower*). The relative increase in production of the larger form of OVR in R/O hens (Fig. 3*A*) could be the result of a futile effort by somatic cells of the ovary to overcome the oocytes' deficiency. Furthermore, such redistribution of transcripts among the different cell types is consistent with the observed larger size of hybridizing signals on Northern blots of ovarian mRNA from mutant animals (Fig. 2).

Human familial hypercholesterolemia (FH) is an autosomal dominant disorder leading to premature atherosclerosis due to alterations in the LDLR gene (23, 33). One of the mutations, described as a French Canadian FH founder mutation, occurs exactly at the position (Cys-646; ref. 34) corresponding to the mutation in OVR causing the R/O phenotype. The intracellular processing of this mutant LDLR is blocked and degradation is accelerated, leading to lack of expression on the cell surface (34). We show that the oocyte plasma membrane fraction of R/O hens contains much less mutant OVR than the amount of wild-type OVR found in normal membranes (Fig. 4). Thus, the current and our previous results (8, 9) support the notion that defective intraoocytic transport of the mutant receptor causes atherosclerosis and sterility in R/O hens.

In addition to the OVR described here, chicken somatic cells express a classical LDLR gene, the product of which was originally identified as a sterol-regulated 130-kDa membrane protein in cultured fibroblasts from normal hens (22). The R/O strain allowed us to test whether fibroblasts from hens carrying the mutation are indeed indistinguishable from normal fibroblasts in terms of LDLR activity and protein. The results of Fig. 5 confirm that expression of the 130-kDa protein, as determined by cell-surface binding of ^{125}I-labeled LDL, in mutant fibroblasts is identical to that in normal animals. From a physiological point of view, this finding lends further support to the causative connection between the mutation in OVR and the R/O phenotype.

The different mutations in the human LDLR lead to a wide range of disease severities for FH. The R/O mutation in OVR, to our knowledge the first naturally occurring mutation described for VLDLRs, has become accessible because of the particularly dramatic dual phenotypic expression. In analogy to FH, yet unidentified milder forms of oocyte-specific receptor gene defects may exist; since human ovaries have been reported to express VLDLR(s) as well (14), human health and reproduction could also be affected by such defects. In chickens and other birds, OVR mutations might result in less pronounced yet significant hyperlipidemia and altered body composition, reduced egg production, and abnormal yolk composition, parameters of considerable commercial impact.

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