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The Laying Hen: An Animal Model for Human Ovarian Cancer

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ABSTRACT

Ovarian cancer is the most lethal world-wide gynecological disease among women due to the lack of molecular biomarkers to diagnose the disease at an early stage. In addition, there are few well established relevant animal models for research on human ovarian cancer. For instance, rodent models have been established through highly specialized genetic manipulations, but they are not an excellent model for human ovarian cancer because histological features are not comparable to those of women, mice have a low incidence of tumorigenesis, and they experience a protracted period of tumor development. However, the laying hen is a unique and highly relevant animal model for research on human ovarian cancer because they spontaneously develop epithelial cell-derived ovarian cancer (EOC) as occurs in women. Our research group has identified common histological and physiological aspects of ovarian tumors from women and laying hens, and we have provided evidence for several potential biomarkers to detect, monitor and target for treatment of human ovarian cancers based on the use of both genetic and epigenetic factors. Therefore, this review focuses on ovarian cancer of laying hens and relevant regulatory mechanisms, based on genetic and epigenetic aspects of the disease in order to provide new information and to highlight the advantages of the laying hen model for research in ovarian carcinogenesis.

(Key words : Chicken, Ovary, Cancer, Animal model)

INTRODUCTION

Ovarian cancer is a fatal gynecological disease and the fifth leading cause of cancer-related deaths in women (Siegel *et al*., 2011). It is difficult to control with chemotherapeutic and surgical strategies, and ovarian cancer poses significant challenges in making an early stage diagnosis of the disease. It is typically detected at later stages of metastasis as cells are transported to other organs through blood vesicles or lymphatics to colonize those tissues which are linked directly to high rates of mortality. Indeed, about 70 percent of ovarian cancer patients are diagnosed at advanced stages, while only 30% of them survives longer than five years following diagnosis (Bovicelli *et al*., 2011). However, detection of ovarian cancer at early stages increases survival rates to over 70%. In order to allay its fatal effects, novel biomarkers for early stage detection must be identified in appropriate animal models for research on human ovarian cancer. In addition, further genetic and epigenetic insights are needed for a better understanding of regulatory mechanisms responsible for ovarian tumorigenesis.

The laying hen is perhaps the most appropriate animal model for studying human ovarian cancer when compared to other animal models such as primates and rodents. Primates share similar physiological and anatomical characteristics with humans, but biological and ethical limitations, as well as higher costs and non-spontaneous development of ovarian cancer are serious obstacles (Vanderhyden *et al*., 2003). Laboratory rodents have the advantages of easy handling, high efficiency of genetic changes, and low cost, but they are impractical for research on epithelial cell-derived ovarian cancer (EOC) due to a wide variety of histological types, a low incidence of ovarian cancer and the protracted periods required for the appearance of ovarian tumors (Stakleff and Von Gruenigen, 2003). On the other hand, the laying hen is an appropriate experimental animal model for human ovarian cancer because approximately 83% develop EOC after 3 to 4 years of

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continuous laying of eggs (Barua *et al*., 2009). Moreover, several well-developed molecular biomarkers used to detect ovarian cancer in women are also expressed in ovarian tumors in laying hens.

In order to increase understanding of ovarian carcinogenesis, many researchers are investigating and elucidating multiple genetic and epigenetic mechanisms using a variety of molecular and cellular biological techniques. Indeed, various genetic alterations are involved in ovarian carcinogenesis. For example, during menstruation and ovulation, the disruption and repair of the epithelial surface of ovaries leads to genomic damage, DNA mutations, and insertion and deletion of the surface ovarian epithelium, increasing the risk of EOC (Fathalla, 1971). In addition, genetic alterations, abnormal cell cycles and their alteration of regulatory genes such as cyclins, cyclin-dependent kinases and cyclin-dependent kinase inhibitors are common features of various types of cancer. Furthermore, different methylation patterns in normal and cancerous ovaries may provide clues to allow discovery and elucidation of epigenetic mechanisms responsible for ovarian cancer. Indeed, DNA methylation has critical roles in embryogenesis, organogenesis and carcinogenesis including ovarian cancer and it is regulated by several types of DNA methyltransferases. Recently, many researchers have attempted to determine the relationship between epigenetics and microRNAs and determine whether they directly or indirectly regulate epigenetic mechanisms such as methylation and post-transcriptional gene regulation in ovarian cancers. Therefore, this review addresses general and histological characteristics of ovarian cancer, and the application of results from the laying hen for ovarian cancer research into genetic/epigenetic regulatory mechanisms during ovarian carcinogenesis.

Characteristics and Classification of Ovarian Cancer

General Characteristics

Ovarian cancer is the fifth deadliest cancer in female patients in the United States. Approximately 1 in 70 women have a lifetime risk of ovarian cancer, and 1 in 100 women die from it (Siegel *et al*., 2011). The most probable cause of the high risk and high death rate is late diagnosis when the cancer is usually in its advanced stages of acute metastasis status to other organs has occurred. In fact, approximately 70% of ovarian cancer patients are diagnosed at the advanced stages, of which only 30% are expected to survive past five years (Bovicelli *et al*., 2011). More than 50% of ovarian cancers appear after the age of 40, as the incidence rises sharply during peri-menopausal and post-menopausal periods in women; the peak incidence of EOC occurs at age 60. However, mortality rates from ovarian cancer decrease sharply after 65 years of age (Jemal *et al*., 2008; Parazzini *et al*., 1991).

In general, it is well known that approximately 3% of ovarian cancers, such as choriocarcinomas, originate from germ cells, about 7% of ovarian tumors arise from sertoli or granulosa cells which come from sex chord stromal cells, whereas 90% of ovarian cancers are germinal/surface epithelia-derived or originate from epithelium of the oviduct (Auersperg *et al*., 2001; Kurman and Shih Ie, 2008; Kurman *et al*., 2008). EOCs are divided histologically into several sub-types such as endometrioid, serous, clear cell, mucinous, and undifferentiated carcinomas (Auersperg, 2011; Auersperg *et al*., 2001). Indeed, this high rate of EOC likely results from incessant ovulation and menstrual cycles that lead to genomic damage and mutations in genes in the ovarian surface epithelium (Auersperg *et al*., 1998; Murdoch *et al*., 2005). To investigate and elucidate the etiological and pathological aspects of EOC, several rodent models have been developed through biotechnological manipulation, but they have many limitations and obstacle associated with clinical relevance because of the non-spontaneous nature and physiologically distinct differences in their EOC (Barua *et al*., 2009; Stakleff and Von Gruenigen, 2003; Vanderhyden *et al*., 2003). Meanwhile, the laying hen spontaneously develops EOC at a high rate as occurs in women and shows very similar morphological characteristics to that of EOC in women (Ahn *et al*., 2010; Ansenberger *et al*., 2009; Barua *et al*., 2009; Lim *et al*., 2011; Stammer *et al*., 2008). Therefore, the laying hen EOC is most likely to provide positive outcomes in efforts to develop anti-cancer drugs and biomarkers for early diagnosis and therapies to prevent adverse outcomes of EOC in women.

It is believed that the aberrant gene regulation, expression, and mutational damage in the ovarian surface epithelium by repeated ovulation events during menstrual cycles increase the incidence of EOC in women. In addition, DNA damage caused by reactive oxygen species influences various regulatory mechanisms to control hormone synthesis and secretion for a variety of physiological and developmental events in the female reproductive tract. To prevent such genomic damage, most animals maintain several self-protective mechanisms including the production of anti-oxidant enzymes or molecular regulatory systems, but these provide less than complete protection (Cooke *et al*., 2003; Marnett, 2000; Murdoch *et al*., 2005). In addition, numerous genetic and epigenetic alterations such as microRNA mutation, activation and inactivation as well as alterations in DNA methylation or histone acetylation patterns occur during ovarian carcinogenesis.

Classification of EOC

There are five histological subtypes of EOC, of which the serous type comprises approximately 60%, the endometrioid type 10～20%, the clear cell type less than 10%, the mucinous type less than 5%, and undifferentiated carcinomas less than 1% (Bocker, 2002). Of these, serous carcinoma is the most common and lethal subtype of ovarian cancer. This cancer type is characterized by high levels of abnormal alterations in DNA copy number and low levels of point mutations in genes. It exhibits papillary structures with histological features including nuclear atypia and abnormal slit glandular locations (Barua *et al*., 2009). Endometrioid ovarian cancer is the second most common subtype of ovarian cancer. It is influenced by hormones such as estrogen and progesterone and shows distinct differentiation with nuclear pleiomorphism as well as characteristics including less invasion into the myometrium and a low potential for lymph node metastasis. In the histological features of the endometrioid type, complex glandular, microglandular foci and solid growth patterns exist (Barua *et al*., 2009; Sherman, 2000).

Genetic and Epigenetic Mechanisms of Ovarian Carcinogenesis

Genetic Mechanisms

It is well established that development and progression of cancers are results from accumulation of genomic changes like gene amplification, chromosomal translocation, point mutations, promoter insertion and deletions (Holschneider and Berek, 2000. These may contribute to many genetic abnormalities which are associated with ovarian cancer such as oncogenic activation or inactivation, cell signaling transition, epithelial mesenchymal transition, abnormalities of the cell cycle regulatory system and loss of function in tumor suppressor genes. Furthermore, these may involve alterations in gene expression that are major features of ovarian carcinogenesis and their identification is useful for clinical trials of ovarian cancer. Hence, in our previous studies, we mainly focused on several dynamic points of genomic regulation and function, because these modalities occur through multiple simultaneous interactions. In general terms, alterations in tumor suppressor genes and expression of oncogenes leads to carcinogenesis. For instance, BRCA1, BRCA2, and P53 mutations are common features of high-grade serous ovarian cancers. K-Ras over-expression (Enomoto *et al*., 1991), alterations in ERBB2 expression (Lancaster *et al*., 2006), as well as mutations in c-Myc, AKT, PTEN and CTNNB1 have been discovered as critical regulators of ovarian carcinogenesis (Xing and Orsulic, 2005). Furthermore, over-activation and abnormal over-expression via gene amplification (i.e. RAB25, FGF1, PI3R1 and AURKA), and genomic mutations (i.e. CDKN2A, K-Ras, SMAD4 and KIT), deletions, mutations, and loss of heterozygosity (PTNE, BRCA1, BRCA2, TP53 and ARH1) have also been discovered to play a role in ovarian tumor development (Bast *et al*., 2009).

Meanwhile, various aberrations in activation of signaling pathways (such as LPA, NF-KB, PI3K, JAK/STAT, and MAPK pathways) related to regulatory mechanisms in cell metabolism, proliferation, differentiation and apoptosis in ovarian cancerous tissues have been discovered and elucidated. For example, the LPA (lipoprotein) signaling pathway is activated in 90% of ovarian cancers, and it has functions that affect initiation, progression and metastasis of cancers (Bast *et al*., 2009; Song *et al*., 2009). In general, concomitant with activation of this signaling pathway, there is activation of cyclin D1 and matrix metalloproteinases, and cyclooxygenase which is well known to accompany development of ovarian carcinogenesis (Bast *et al*., 2009). The NF-KB signal transduction cascade is activated in one-half of ovarian cancers and it is mainly initiated by epidermal growth factor and pro-inflammatory cytokines such as interleukin 6 and tumor necrosis factors, and it can also be driven by MAPK and PI3K signaling pathways. It mainly prevents apoptosis in various tissues, but it also stimulates cell proliferation, angiogenesis, and inflammation in ovarian cancerous cells/tissues (Annunziata *et al*., 2008; Bast *et al*., 2009; Hernandez *et al*., 2010). In the MAPK signaling cascade, MAPK is initially activated by epidermal growth factor and it generally brings about several oncogenic mutations in ERBB2, BRAF, and KRAS that lead to uncontrolled cell proliferation in cancer (Bast *et al*., 2009; Cho and Shih Ie, 2009). Furthermore, the PI3K pathway is constitutively activated in most of ovarian cancers through several growth factors such as epidermal growth factor, bone morphogenetic proteins, and tumor necrosis factors. It is also stimulated by inactivating PTEN mutations or activating PIK3CA mutations (Bast *et al*., 2009; Kuo *et al*., 2009; Yang *et al*., 2006). As well as, it accelerates cell proliferation by inactivating cell cycle inhibitory genes such as P21 and P27 (also known as CDKN1A and CDKN1B, respectively) (Bast *et al*., 2009). As a matter of fact, these examples of abnormal regulation and expression of cell cycle related gene are among the most common genetic characteristics associated with initiation and development of cancer. The cell cycle in most eukaryotic cells includes a series of highly coordinated events consisting of cell growth, replication of genetic materials, segregation of the duplicated chromosomes and cell division (Vermeulen *et al*., 2003). Specifically, the cell division cycle in mammals

is precisely and harmoniously regulated in a timely manner by different active heterodimeric complexes that include cyclin dependent kinases (CDKs) and their cognate cyclin partners, as well as CDK inhibitors (CDKIs) (D'Andrilli *et al*., 2004). Thus, tumor development frequently occurs when there is deregulation of the cell cycle control system including abnormal regulation of expression of cell cycle genes (Bovicelli *et al*., 2011). In human cancerous tissues including EOC, different families of cell cycle genes and regulators are frequently mutated and dysfunctional (D'Andrilli *et al*., 2004).

The epithelial-mesenchymal transition (EMT) is a necessary process for morphological organogenesis and embryogenesis (Thiery and Sleeman, 2006) and it also correlates with ovarian carcinogenesis (Liliac *et al*., 201). During ovarian tumor invasion and metastasis, EMT is commonly detected and it is involved in various events such as cellular reprogramming, loss of epithelial characteristics and production of the extracellular matrix (Kalluri and Weinberg, 2009).

Epigenetic Mechanisms

Until recently, it is believed that tumorigenesis is caused by the accumulation of genetic changes such as gene mutation, rearrangement, deletion and translocations at the genomic level. However, these classical theories alone were unable to clarify the basis for carcinogenesis because, in contrast to genetic regulation, epigenetic modifications alter gene expression without changes in DNA sequences. Thus, it is now fully understood that epigenetic events involving multiple interactions with DNA methylation, histone modification, and small non-cording RNAs lead directly to ovarian cancer. Indeed, beside genetic changes, alterations in chromatin conformation have been shown to contribute to carcinogenesis (Hatziapostolou and Iliopoulos, 2011). In addition, many research groups have demonstrated that epigenetic mechanisms are closely associated with the development and progression of ovarian cancer, and gradual stimulation is involved in advanced stages of tumorigenesis (Balch *et al*., 2009). Recently, the field of epigenetics has become highly relevant for clinical trials in cancer research and as a result, histone deacetylase and DNA methylation inhibitors have undergone rapid development in the anti-cancer drug industry.

DNA methylation is one of the major epigenetic modifications that have critical roles in embryogenesis, organogenesis and carcinogenesis, including ovarian cancer. It is mainly regulated by several types of DNA methyltransferases (DNMTs). Theoretically, DNA methylation is added to the carbon-5 cytosine ring of CpG islands by DNMTs which have three established forms: DNMT1, DNMT3A and DNMT3B. DNMT1 mainly plays an enzymatic role in the maintenance of DNA methylation, and is a hallmark of endometrioid carcinomas and prostate cancer (Lan *et al*., 2010). It is also responsible for both *de novo* maintenance of methylation of tumor suppressor genes in various human cancer cells (Jair *et al*., 2006). On the other hand, DNMT3A and DNMT3B function in *de novo* methylation and over-expression of either DNMT3A or DNMT3B which is associated with tumorigenesis depending on cancer types in humans (Robertson *et al*., 1999; Socha *et al*., 2009). Recent research has elucidated the relationship between epigenetics and microRNAs, and determined whether they directly or indirectly regulate mechanisms such as methylation and post-transcriptional gene regulation in ovarian cancer. Indeed, we previously reported alternative DNA methylation patterns of several genes in EOC of laying hens that are influenced directly by changes in expression of genes associated with initiation and progression of cancer (Jeong *et al*., 2012; Lee *et al*., 2012; Lim *et al*., 2012). In the same vein, most cancer related genes, especially tumor suppressor genes, are commonly regulated by DNA methylation events. According to previous reports, several cancer associated genes have hypermethylation patterns and oncogenes display hypomethylation patterns during cancer development. It has becoming evident that hypermethylation can lead to gene transcriptional silencing while hypomethylation may increase gene transcription in tumorigenesis including ovarian cancer.

MicroRNAs (miRNA) are small non-coding segments of RNA that regulate post-transcriptional processing by binding to the 3'UTR region of a target gene to trigger down-regulation. MiRNAs have been shown to play crucial roles in a wide range of biological and pathological processes. Previously, we reported the association between chicken ovarian cancer-related genes and several miRNAs (see Table 1 and Fig. 1). In addition, results of many recent studies revealed that miRNAs are down-regulated in various cancer types containing human ovarian cancer (Dahiya *et al*., 2008; Laios *et al*., 2008; Nam *et al*., 2008; Zhang *et al*., 2008). In addition, the term epi-miRNA refers to a complex connection between an epigenetic mechanism and a miRNA molecule. This complex is affected by miRNA expression, which generates an epigenetic feedback mechanism (Valeri *et al*., 2009). Moreover, epigenetic regulation has been shown to mediate several miRNA-related instances of cancer development and disease.

Models for Human Ovarian Cancer Research

Primate Models

Resembling humans in pathologies, anatomy and phy-

Table 1. MicroRNA profiling for chicken ovarian carcinoma related genes

microRNA	Target transcript	References
gga-miR-101 *gga-miR-1668* *gga-miR-1681*	*SERPINB3*	Lim *et al* (2012)
gga-miR-1615	*AvBD-11*	Lim *et al* (2013)
gga-miR-499 *gga-miR-1709*	*PTN*	Lee *et al* (2012)
gga-miR-140	*SPP1*	Lim *et al* (2012)
gga-miR-1798	*CCND1*	Lee *et al* (2012)
gga-miR-1699	*CCNE2*	Lee *et al* (2012)
gga-miR-223	*CDK1*	Lee *et al* (2012)
gga-miR-1744	*CDK3*	Lee *et al* (2012)
gga-miR-1626	*CDKN1A*	Lee *et al* (2012)
gga-miR-222 *gga-miR-1787* *gga-miR-1812*	*CDKN1B*	Lee *et al* (2012)

siological characteristics including menstrual cycles, primate models are crucial for ovarian cancer research. For instance, chemoprevention studies for ovarian cancer have been conducted on monkeys (*cynomolgus macaques*). In those studies, researchers combined and administrated sex steroid hormones (estrogen and progesterone) and oral contraceptives, and found that the sex steroids increased apoptosis levels of ovarian surface epithelium (Rodriguez *et al*., 1998). Similarly, ovarian cancer researches using rhesus monkeys suggested that primate models have a unique potential for comparative research on human ovarian cancer (Brewer *et al*., 2007). However, primate models do have severe biological limitations, including non-spontaneous ovarian cancer development, a deficiency of surrogate biomarkers, and practical challenges due to requirements for scientists and technicians with specialized skills, and high cost of obtaining and maintaining primates (Lu *et al*., 2009).

Rodent Models

Genetically modified mouse models for ovarian cancer research are available and, due to a high level of inbreeding, provide for reproducibility in results when used to investigate functional interactions and specific signaling pathways associated with cancer-related genes during *in vivo* tumor development. Historically, rodent models for ovarian cancer research were first developed by Orsulic and colleagues who systematically researched several introduced oncogenes in mouse ovarian cells using an avian retroviral gene delivery system (Orsulic *et al*., 2002). Later, Flesken-Nikitin generated an EOC mouse model by inactivating Trp53 and Rb in

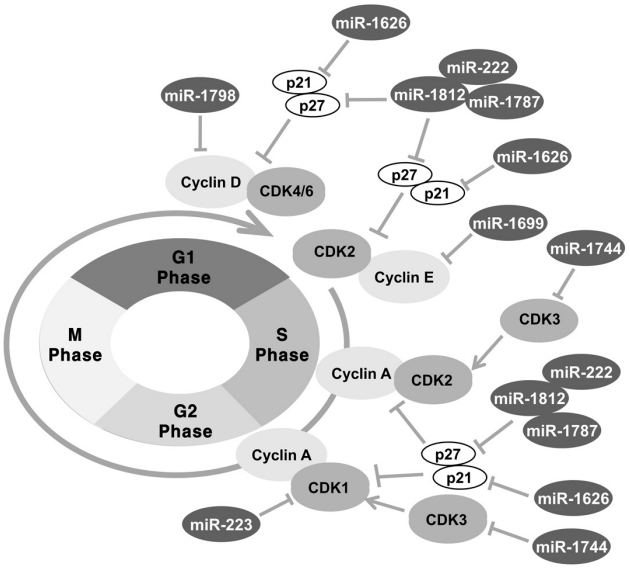

Fig. 1. Schematic diagram of cell cycle regulatory genes and microRNAs on the each phase of cell cycle. Diagram of mechanism of cell cycle regulatory genes as activator or inhibitor functions on the cell cycle like cyclin D, cyclin E, cyclin A, CDK4/6, CDK2 and CDK1. MicroRNA targeted cell cycle regulatory genes as down-regulation of each target genes which are *miR-1798, miR-1626, miR- 1812, miR-222, miR-1787, miR-1699* and *miR-1744*.

List of abbreviation

AKT	Serine/Threonine protein kinase Akt
BRCA1/2	Breast cancer1/2
CDK1	Cyclin-dependent kinase 1
CDK3	Cyclin-dependent kinase 3
CDK4/6	Cyclin-dependent kinase 4/6
CDK5	Cyclin-dependent kinase 5
CDKN1A	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)
CDKN1B	Cyclin-dependent kinase inhibitor 1B (p27, Kip1)
DNMT1	DNA (cytosine-5-)-methyltransferase 1
DNMT3A	DNA (cytosine-5-)-methyltransferase 3 alpha
DNMT3B	DNA (cytosine-5-)-methyltransferase 3 beta
EGFR	Epidermal growth factor receptor
EMT	Epithelial–mesenchymal transition
ERBB2	Erythroblastic leukemia viral oncogene homolog2
miRNA	MicroRNA
PI3K	Phosphoinositide 3 kinase
PTEN	Phosphatase and tensin homolog
PTN	Pleiotrophin
TP53	Tumor protein p53

ovarian surface epithelium (Flesken-Nikitin *et al*., 2003). Certainly, ovarian tumors occasionally develop spontaneously with aging in some abnormal mice, in Sprague-Dawley rats and Wistar rats (Gregson *et al*., 1984; Tillmann *et al*., 2000; Walsh and Poteracki, 1994). However, these models are not feasible for ovarian cancer research due to a wide variety of histological types, low incidence, and long durations needed for disease development.

Avian Models

Fredrickson first developed the laying hen as a model for ovarian cancer in 1987 (Fredrickson, 1987). In fact, avian species, as experimental animal models have many advantageous characteristics for EOC research as compared with other animal models. Approximately 83% of avian species have genital tumors including ovarian cancer, which is of considerable value to researchers as compared to other species. About 45% of laying hens develop reproductive tract tumors at two years of age. Actually, ovarian cancers spontaneously develop age-dependently in laying hens, with 12% at age 3.9 years, 32% at age 4.2 years and more than 50% at age 6.1 years (Fredrickson, 1987). Laying hens start laying eggs at 20 to 22 weeks of ages and reach their peak in egg laying at 30 to 32 weeks of age. Also, their egg production rate is highest during the first laying years and the ovulatory cycle ranges from 24 to 26 hours depending on the age of the laying hen (Barua *et al*., 2009). Likewise with humans, both ovulatory cycles and follicular development are regulated by ovarian steroid hormones and pituitary gonadotrophins (Robinson and Etches, 1986). These similarities in aspects of reproductive physiological functions between humans and chickens, as well as accessibility and high relevance are evidence that laying hens are the most appropriate experimental animal model for elucidation of the etiology human ovarian cancer. Furthermore, diagnostic molecular biomarkers for human ovarian cancer are usually expressed in chicken ovarian cancers. CA125 is an important biomarker for diagnosing human ovarian cancer and it is expressed in spontaneous ovarian adenocarcinomas in chickens (Jackson *et al*., 2007). In addition, expression of several growth factors and their receptors (e.g., TGFA, EGFR), as well as human ovarian cancer signaling pathways (e.g., EGFR/PI3K and AKT/PI3K), and markers of cell proliferation (e.g., cytokeratin AE1/AE3, PCNA) coincide with development of both human and chicken ovarian cancers (Liu *et al*., 2009; Liu *et al*., 2010; Rodriguez-Burford *et al*., 2001). Collectively, the laying hen is likely the best animal model for epithelia-derived ovarian cancer research due to its similar morphological aspects and etiological characteristics when compared with ovarian cancer in women.

CONCLUSION

This present review provides information on unique similarities in the characteristics of human and chicken ovarian cancers, and the potential of the laying hen as the appropriate animal model for human ovarian cancer research. This review also provides new insights into both genetic and epigenetic regulation for initiation, progression and development of human EOC, and discusses highly relevant molecular targets for the development of therapeutic agents and applications for treatment and monitoring of epithelia-derived ovarian cancers in women.

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