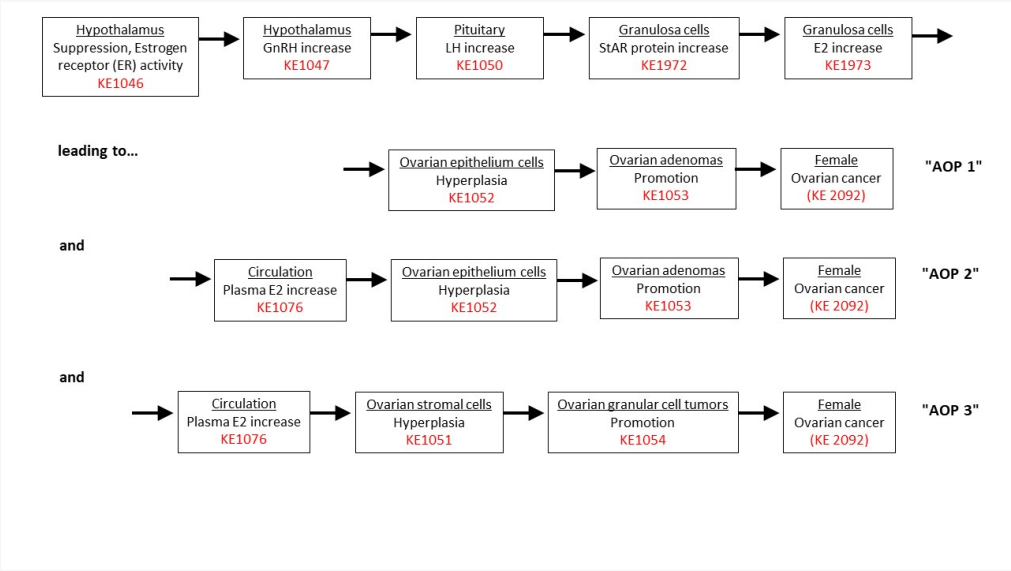
AOP ID and Title:

AOP 440: Hypothalamic estrogen receptors inhibition leading to ovarian cancer
Short Title: Hypothalamic estrogen receptors inhibition leading to ovarian cancer

Graphical Representation



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Status

Author status	OECD status	OECD project	SAAOP status
Under development: Not open for comment. Do not cite	Under Development	1.104	Included in OECD Work Plan

Abstract

Malfunctioning of sex hormones (e.g., estradiol, estrone and progesterone) may result in ovarian cancer (Fooladi et al., 2020; Meehan and Sadar, 2003). Exposure to endocrine-disrupting chemicals (EDCs) in the form of occupational usage of pesticides, fungicides, herbicides, plasticizers, cosmetics, etc. are the causes of ovarian cancer (Samtani et al., 2018). Some stressorsmolecules (e.g., clomiphene citrate, Tamoxifen, Toremifene) act on neuronal cell in the hypothalamus (molecular initiating event, MIE), where they inhibit hypothalamic Estrogen Receptors selectively and these chemicals increase the risk of ovarian cancer (McLemore et al., 2009). These stressors molecules stimulate the releasing of gonadotropin-releasing hormone (GnRH) from hypothalamic region of brain bythe suppression of hypothalamic Estrogen Receptors. Subsequently, secretion of luteinizing hormone (LH) from pituitary becomes high(Cassidenti et al., 1992; Mungenast and Thalhammer, 2014a; Tomao et al., 2014). This hormone regulates the synthesis of sex hormones (e.g., estrogens) at cellular level (Shoemaker et al., 2010a; Tomao et al., 2014). These sex hormones are primarily produced in the gonads through a series of enzyme-mediated reactions from cholesterol (precursor) and control through complex signalling pathway along hypothalamus – pituitary – gonadal (HPG) axis (Shoemaker et al., 2010a; Perkins et al., 2019). High estrogen level increases the risk of ovarian cancer (McLemore et al., 2009; Tomao et al., 2014).

Background

Development and progression of certain types of cancer disease (e.g. ovarian cancer, breast cancer, prostate cancer etc.) is related with the hormonal levels in human. Lack of proper diagnosis at early stage of the disease increase the mortality rate of the cancer. Among many types of cancer ovarian cancer hasthe high mortality rate (~50%) due to the lack of proper diagnosis at early stage of the disease progression. Circulating levels of the steroidal sex hormones in conjunction with the gene expression is related with the progression of this disease. Some important sex hormones which are related with many cancer diseases include oestrogen, progesterone and testosterone. Oestrogen hormone mainly involved in female sex organ development, controlling of menstruation cycle etc. Progesterone also involved in controlling menstrual cycle, maintaining pregnancy and spermatogenesis. Testosterone hormone regulates sexual development, bone mass development, red blood cell production in male. In females sexual

hormone balance protects the ovaries from the tumor development. A number of researchesrevealed that molecular level perturbation leading towards sex hormone imbalance plays important role in the development of the ovarian cancer.

Summary of the AOP

Events

Molecular Initiating Events (MIE), Key Events (KE), Adverse Outcomes (AO)

Sequence	Type	Event ID	Title	Short name
	MIE	1046	Suppression, Estrogen receptor (ER) activity	Suppression, Estrogen receptor (ER) activity
	KE	1047	Increased, secretion of GnRH from hypothalamus	Increased, secretion of GnRH from hypothalamus
	KE	1050	Increased, secretion of LH from anterior pituitary	Increased, secretion of LH from anterior pituitary
	KE	1972	Increased, Steroidogenic acute regulatory protein (StAR)	Increased, Steroidogenic acute regulatory protein (StAR)
	KE	1973	Increased, estrogens	Increased, estrogens
	KE	1076	Increased, circulating estrogen levels	Increased, circulating estrogen levels
	KE	1051	Hyperplasia, ovarian stromal cells	Hyperplasia, ovarian stromal cells
	KE	1052	Hyperplasia, ovarian epithelium	Hyperplasia, ovarian epithelium
	AO	1053	Promotion, ovarian adenomas	Promotion, ovarian adenomas
	AO	1054	Promotion, ovarian granular cell tumors	Promotion, ovarian granular cell tumors
	AO	2092	Promotion, Ovarian Cancer	Promotion, Ovarian Cancer

Key Event Relationships

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Suppression, Estrogen receptor (ER) activity	adjacent	Increased, secretion of GnRH from hypothalamus	High	Not Specified
Increased, secretion of GnRH from hypothalamus	adjacent	Increased, secretion of LH from anterior pituitary	High	Moderate
Increased, secretion of LH from anterior pituitary	adjacent	Increased, Steroidogenic acute regulatory protein (StAR)	High	Moderate
Increased, Steroidogenic acute regulatory protein (StAR)	adjacent	Increased, estrogens	High	Moderate
Increased, estrogens	adjacent	Increased, circulating estrogen levels	High	Moderate
Increased, circulating estrogen levels	non-adjacent	Hyperplasia, ovarian stromal cells	High	Not Specified
Increased, circulating estrogen levels	non-adjacent	Hyperplasia, ovarian epithelium	High	Not Specified
Hyperplasia, ovarian epithelium	non-adjacent	Promotion, ovarian adenomas	High	Not Specified
Hyperplasia, ovarian stromal cells	non-adjacent	Promotion, ovarian granular cell tumors	High	Not Specified
Promotion, ovarian adenomas	non-adjacent	Promotion, Ovarian Cancer	High	Low
Promotion, ovarian granular cell tumors	non-adjacent	Promotion, Ovarian Cancer	High	Low

Stressors

Name	Evidence

Name	Evidence
Tamoxifen	Moderate
Raloxifene	Moderate
Clomiphene citrate (1:1)	High

Overall Assessment of the AOP

Suppression, Estrogen receptor (ER) activity [Evidence- Strong]:There are number of reports available related to suppression of Estrogen receptor activity (ER) (Baez-Jurado et al., 2018; Cosman, 2003; Haskell, 2003; Ng et al., 2009; Kang et al., 2001; Roy et al., 1999; Marques P, 2018; Mungenast and Thalhammer, 2014b; Ghasemnejad-Berenji et al., 2020; J. H. Liu, 2020; Oride et al., 2020; Zhang et al., 2020; John F. Kerin et al., 1985b; The Practice Committee of the American Society for Reproductive Medicine, 2013; Moskovic et al., 2012 ; Bryan J. Herzog, 2020). Stressors act on neuronal cell in the hypothalamus, where it inhibits hypothalamic Estrogen Receptors selectively. A number of compounds or molecules (e.g. Clomiphene citrate, Tamoxifen, Toremifene etc.) are detected which show the modulation activity of estrogen receptor in brain leading to high GnRH pulses (Haskell, 2003; Cosman, 2003).

Increased, secretion of GnRH from hypothalamus[Evidence- Strong]:A number of evidencesare found by the researchesthat the increased secretion of gonadotropin-releasing hormone (GnRH)(Shander and Goldman, 1978; Tsourdi et al., 2009). Studies had shown that of inhibition of Estrogen receptor activity (ER) enhances the secretion of GnRH in human (Adashi et al., 1980; Bussenot et al., 1990; JOHN F KERIN et al., 1985a; Tan et al., 1996), rat and mice (Bharti et al., 2013; Kumar and Pakrasi, 1995; Zoeller and Young, 1988) . Studies on human patient had shown the application of clomiphene is able to promote response of GnRH secretion (Goerzen et al., 1985; Tan et al., 1996).

Increased, secretion of LH from anterior pituitary [Evidence- Strong]:Good evidence may be acquired from different published articles for the increased secretion of LH increases from anterior pituitary (Plouffe and Siddhanti, 2001; Wright et al., 2012; Shoemaker et al., 2010b). It is also reported that increased secretion of the GnRH in hypothalamus leads to high levelofLH in human (John F Kerin et al., 1985a; Adashi et al., 1980; Bussenot et al., 1990), mice/rat.(Bharti et al., 2013; Kumar and Pakrasi, 1995; Botte et al., 1999) and cow (Fields et al., 2009).

Increased, Steroidogenic acute regulatory protein (StAR) [Evidence- Strong]:Steroidogenic acute regulatory protein (StAR) plays critical role in luteal steroidogenesis by controlling the transport of cholesterol from the outer to inner mitochondrial membrane(Wu et al., 2003; Shoemaker et al., 2010b).It had been reported that increase in LH level leads to increase StAR protein concentration in human(Tsang et al., 1980; Johnson and Bridgham, 2001; Murayama et al., 2012; Rekawiecki et al., 2005), rat(T. Liu et al., 2007; Martinat et al., 2005) and mice(Eacker et al., 2008; Tsuchiya et al., 2003).

Increased, estrogens [Evidence- Strong]:Aromatase is a key enzyme for estrogen formation in human tissues. In female, one of the important sites of estrogen enzyme synthesis is ovarian granulose cells(Holesh et al., 2017; Shoemaker et al., 2010b). Although ovarian aromatase enzyme expression in postmenopausal female is very low, high estrogen level is maintained in the blood through aromatase expression in other tissues. A number of researches had shown increased synthesis of StAR Protein increases the estrogen in ovarian granulosa cellsin human (Kiriakidou et al., 1996; Fang et al., 2016; Men et al., 2017), rat (Ronen-Fuhrmann et al., 1998; Nimrod, 1981) and fish (Kusakabe et al., 2002).

Increased, circulating estrogen levels [Evidence- Strong]:Researches had shown increased synthesis of estrogen in ovarian granulosa cells leads to maintain the high circulating estrogen levels in blood (Holesh et al., 2017; Shoemaker et al., 2010b).

Hyperplasia, ovarian stromal cells[Evidence- Strong]: High concentration of circulating estrogen drives the endometrial hyperplasia of the stromal cells in the postmenopausal ovaries. Many scientific evidences are available which supports this event. Number of evidence may be found on the formation of tumors in the ovarian granulosa cells due to the high levels of circulating estrogen in the plasma (Janson et al., 1980; Scirpa et al., 1984; Shoemaker et al., 2010b).

Hyperplasia, ovarian epithelium [Evidence- High]: Ovarian surface is covered by the epithelium cells often called as ovarian mesothelium tissue. High evidence is available which supports that hyperplasia of the stromal cells might lead towards the hyperplasia of the ovarian epithelium tissue(Nyboe Andersen et al., 2008; Kang et al., 2001).

Promotion, ovarian adenomas[Evidence- Moderate]:Ovarian adenoma or cystadenoma is classified as benign tumor in the epithelial tissue. Evidence on the promotion of ovarian adenoma due to the hyperplasia in the ovarian epithelial tissue is available.

Promotion, ovarian granular cell tumors [Evidence- Strong]: Tumors in the granulosa cells is most common type of tumors found in females. High number of evidences is available which shows the association of the ovarian granulosa cell tumors with the hyperplasia of the ovarian epithelium tissue(Nyboe Andersen et al., 2008; Kang et al., 2001).

Promotion, ovarian cancer [Evidence- Strong]: Promotion of ovarian adenomas and promotion of ovarian granular cell tumors leads to the phenotype outcome of ovarian cancer at individual level (Johansson et al., 2022, Christine Stewart et al., 2019).

Domain of Applicability

Life Stage Applicability

Life Stage	Evidence
Adult, reproductively mature	High

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	High	NCBI
rat	Rattus norvegicus	High	NCBI
mice	Mus sp.	High	NCBI

Sex Applicability

Sex	Evidence
Female	High

Sex: This particular AOP is mainly applicable for the females. Sex hormone regulation in female is more complex compare to the male. Development and growth of the ovaries depend on the hormonal balance in the body. This hormonal balance in female changes often observed during the menstrual cycle and pregnancy. Imbalance in the hormonal levels leads to the abnormal function of the ovaries.Predominant form of estrogen (estradiol) hormone also found in male and plays critical role in sexual behavior and spermatogenesis. However, males more likely experiences imbalance in testosterone hormonelevels.

Life stage: This AOP is closer to the adult female. In particular the females (at the age of 45-55) going through the menopause are having greater chance of developing ovarian cancer compared to the young adult female. Young female undergoing through the hormonal therapy (usually estrogen) also having high risk of developing ovarian cancer. Risk factor of ovarian cancer is high in case of adult females who are taking ovulation stimulating drugs to increase fertility.

Taxonomic: For this AOP taxonomic domain is applicable to the different species like mice, rat, guinea pig and human.

Essentiality of the Key Events

In this AOP the essentiality of the proposed events are supported by a number of scientific works.

Kettel et al., had shown the treatment of seventeen females with clomiphene citrate with 150mg/day dose for 5 days enhance the estrogen levels. Analysis of the other hormones (follicle-stimulating hormone, luteinizing hormone, gonadotropin-releasing hormone) levels suggest the clomiphene citrate involved in the modulation in hormonal secretion at the hypothalamic site (Kettel et al., 1993).

Koch et al, had shown female rat injected with the clomiphene citrate (1-100 ng/kg) for 20 days increase the gonadotropin-releasing hormone (GnRH) release in the hypothalamus region (Koch et al., 1971).

Research by Kurosawa et al., on 293T cells (transfectable derivative of human embryonic kidney 293 cells, revealed that effect of clomiphene citrate depend on the concentration of the molecule. Clomiphene citrate at higher concentration (10^{-10} - 10^{-12} M) showed the estrogenic activity. However at higher concentration (10^{-6} - 10^{-12} M) no estrogenic activity was observed. Results of the study also suggest that clomiphene citrate either act as agonist or as an antagonist depends on the presence of 17β-estradiol (E2) receptor(Kurosawa et al., 2010).

Weight of Evidence Summary

Overall assessment of the biological plausibility, empirical support and quantitative understanding of the KEs and KERs associated with this AOP shows that molecular mechanism or signaling pathway of tumor development in the female ovaries due to the suppression of estrogen receptors activities in the hypothalamus is still unclear.

Empirical evidence is available which shows the release of gonadotropin-releasing hormone (GnRH) depends on the concentration of the Selective Estrogen Receptors Modulator (SERM) compound (e.g. clomiphene citrate). However, molecular mechanism for the enhancement of GnRH by suppression of Estrogen receptor activity is poorly known.A number of researches had shown secretion of luteinizing hormone (LH) from anterior pituitary depends on the GnRH concentration or dose. Scientific reports have shown the both stimulatory and inhibitory effects on the GnRH secretion exhibited by the estradiol depending on the concentration of stressor (clomiphene) molecules and presence of types of receptors. The requirement of the GnRH dose for the secretary release of the LH in the different species varies widely.

A number of articles had shown that release of LH from the anterior pituitary regulates the steroidogenic function of cells by controlling the cholesterol transportation to the mitochondria. Biological plausibility of this event is very high as a number of studies have shown the similar results using different biological models (e.g. granulosa cells of adult female, bovine luteal cells, leydig cells of mice and rat etc.) in their study. Estradiol synthesis during menstrual cycle is governed via expression of StAR protein synthesis. Quantitative estimation of the event has been performed through indirect measurement (e.g. Northern blot analysis of mRNA collected from ovarian follicle granulosa cells). Therefore in many studies finding results are inconsistent. Circulating estrogen levels increases due to the increased estradiol

synthesis and concentration controlled by the negative feedback loop of the other steroidal hormone synthesis.Biological evidence of tumor formation in the ovarian granulose cells due to the high circulating estrogen levels in the plasma is pretty high. High circulating estrogen drives the endometrial hyperplasia towards the progression of endometrial cancer.

Quantitative Consideration

Quantitative understanding in many KEs and KERs are available. However, exploitation of different biological models and use of different assay techniques provide incoherent results. Inconsistent results also have been mentioned in many KEs and KERs. A few assay techniques such as radioimmunoassay, radioreceptor assay, estrogen receptor binding assay etc. are sensitive enough to measure the concentration of a molecule at pictogram level. Some other techniques such as quantitative real time PCR (qRT-PCR), northern blot analysis of RNA also have been used for quantitative estimation of molecules at low concentration. Some indirect methods such as immunohistochemistry also have been employed for identification and quantitative estimation of biological molecule.

Considerations for Potential Applications of the AOP (optional)

This AOP provides the valuable informations regarding chemical messengers and different glands of endocrine system that are related for the risk and promotion of ovarian cancer. Linkage of qualitative and quantitative informations of different chemical messengers for the promotion of ovarian cancer would be beneficial for the cancer therapy and cancer drug development. Further, this AOP would be helpful to evaluate the hazardous long-term effects of the endocrine-disrupting chemicals and drugs which may lead towards the development of the ovarian cancer. This AOP would also help to regulate the uses of these stressor molecules which have inhibitory effects on the hypothalamic Estrogen Receptors. Understanding of the molecular events related with this AOP would help to screen these molecules and provide guideline to access the risk associated with these stressors.

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Appendix 1

List of MIEs in this AOP

Event: 1046: Suppression, Estrogen receptor (ER) activity

Short Name: Suppression, Estrogen receptor (ER) activity

Key Event Component

Process	Object	Action
estrogen receptor activity	estrogen receptor	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:165 - Antiestrogen activity leading to ovarian adenomas and granular cell tumors in the mouse	KeyEvent
Aop:440 - Hypothalamic estrogen receptors inhibition leading to ovarian cancer	MolecularInitiatingEvent

Stressors

Name
Clomiphene citrate (1:1)
Tamoxifen
Raloxifene

Biological Context

Level of Biological Organization
Molecular

Cell term

Cell term
neuron

Organ term

Organ term
hypothalamus

Evidence for Perturbation by Stressor

Overview for Molecular Initiating Event

Clomiphene citrate (a stressor) at 10^{-10} - 10^{-12} M concentrations exhibits approximately 30% of the estrogenic activity which is same from 17β-estradiol (at 10^{-10} M) in ERα-expressing cells where as no activity in ERβ cells.

Clomiphene citrate at the concentration of 10^{-10} M reveals weak estrogen agonist activity in the presence of 17 β - estradiol (E2) at the concentration of 10^{-14} M in ERα-expressing cells, and no activity was found in ERβ cells.

Clomiphene citrate at lower doses (10^{-10} - 10^{-12} M), but not higher doses (10^{-6} - 10^{-8} M) showed estrogenic activity via ERα. However, clomiphene citrate at concentrations between 10^{-6} M and 10^{-12} M did not reveal any estrogenic activity via ERβ. In the presence of E2, clomiphene citrate worked as either as an agonist or an antagonist through ERα depending on the concentrations of E2. Clomiphene citrate worked as antagonistic when it is combined with the higher E2 concentrations and worked as agonistic with the lower E2 concentrations. On the other hand, via ER β, clomiphene citrate acted as an estrogen antagonist irrespective of the concentration of E2. (Kurosawa et al., 2010).

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	High	NCBI
rat	Rattus norvegicus	High	NCBI
mice	Mus sp.	High	NCBI

Life Stage Applicability

Life Stage	Evidence
Not Otherwise Specified	High

Sex Applicability

Sex	Evidence
Mixed	High

Neuronal cell in Hypothalamus

Key Event Description

Estrogen receptors are produced in all vertebrates and located in either the cell cytoplasm or nucleus(Bondesson et al., 2015; Eick and Thornton, 2011). Estrogen receptors are localized either in cytoplasm, or on the cell surface.

Site of action: Stressors (e.g., clomiphene) act on neuronal cell in the hypothalamus, where it inhibits hypothalamic Estrogen Receptors selectively.

Responses at the macromolecular level: Stressors activate the Estrogen Receptor α in the presence of lower level of estrogen and partially blocks the same for higher level of estrogen and works as antagonist for the Estrogen Receptor β(Trost and Khera, 2014). Stressors appear to act in the brain's pituitary gland to secrete an increased amount of gonadotropins hormone (GnRH) in hypothalamus leading towards increased GnRH level in blood.

Estrogen Receptor α: ERα (Estrogen Receptor α or NR3A1 or ESR1) - A nuclear receptor and it is activated by the estrogen (sex hormone). Estrogen located at chromosome number 6 (6q25.1)

Estrogen Receptor β: ERβ (Estrogen Receptor β or NR3A2 or ESR2) – This is also nuclear receptor and activated by the sex hormone estrogen which is located at chromosome number 14 (14q23.2). I ERβ has both N-terminal has DNA binding domain and C-terminal has ligand binding domain. This is localized to the nucleus, cytoplasm, and mitochondria. Selective estrogen receptor modulators (SERM) inhibits the ERβ. Drugs used as SERM are clomiphene, tamoxifen, raloxifene etc.

Biological compartments: Estrogen receptors (ER) are present in the plasma membrane. Both ERα and ERβ have diverse functions depending on cells and organs. ERs have also been loacated in cytoplasmic organelles including mitochondria and the endoplasmic reticulum(Levin, 2009).

General role in biology: Estrogen receptors (both estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ) binds the estrogens to promote the the biological functions of estrogens. Depending upon a balance between ERα and ERβ activities in target organs, estrogen signaling is selectively stimulated or inhibited (Welboren et al., 2009). ERβ has a

high degree of sequence homology with the classical estrogen receptor. Interestingly, ERβ is detected in many tissues, including those previously assumed to be estrogen insensitive. In tissues where both ERs are expressed, such as the hypothalamus, uterus, mammary glands, and immune system, ERα promotes proliferation whereas ERβ has pro-apoptotic and pro-differentiating functions(Morani et al., 2008). ERα is present mainly in ovary (thecal cells) where as ERβ is found mainly in ovary (granulosa cells)(Paterni et al., 2014). ERα and ERβ is identical approximately 97% in the DNA-binding domain and approximately 56% in the ligand-binding domain(Dahlman-Wright et al., 2006).

How it is Measured or Detected

Radioreceptor assay/The estrogen receptor binding assay (using Rat Uterine Cytosol): This assay identifies chemicals that have the potential to interact with the estrogen receptor (ER) *in vitro*. Principle of this particular assay is based on the competitive protein-binding methods. A radiolabelled ligand and an unlabelled ligand are presented together to a specific receptor. The radioactivity measurement provides the quantitative estimation of the bound and unbound fraction of the ligand with the receptor. All cytosolic estrogen receptor subtypes that are expressed in the specific tissue, including ERα and ERβ are used for the determination of estrogen receptor binding. This assay is simple and rapid to perform when optimal conditions for binding are determined. Assay determines if a ligand/chemical can interact and displace the endogenous hormone 17β-estradiol (Freyberger et al., 2010).

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List of Key Events in the AOP

Event: 1047: Increased, secretion of GnRH from hypothalamus

Short Name: Increased, secretion of GnRH from hypothalamus

Key Event Component

Process	Object	Action
hormone secretion	Gonadotropin Releasing Hormone	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:165 - Antiestrogen activity leading to ovarian adenomas and granular cell tumors in the mouse	KeyEvent
Aop:440 - Hypothalamic estrogen receptors inhibition leading to ovarian cancer	KeyEvent

Biological Context

Level of Biological Organization
Cellular

Cell term

Cell term
gonadotropin releasing neuron

Event: 1050: Increased, secretion of LH from anterior pituitary

Short Name: Increased, secretion of LH from anterior pituitary

Key Event Component

Process	Object	Action
luteinizing hormone secretion	Luteinizing hormone	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type

AOP ID and Name	Event Type
Aop:165 - Antiestrogen activity leading to granulosa cell tumors and granular cell tumors in the mouse	KeyEvent
Aop:440 - Hypothalamic estrogen receptors inhibition leading to ovarian cancer	KeyEvent

Biological Context

Level of Biological Organization

Cellular

Event: 1972: Increased, Steroidogenic acute regulatory protein (StAR)

Short Name: Increased, Steroidogenic acute regulatory protein (StAR)

Key Event Component

Process	Object	Action
increased luteinizing hormone level	StAR-related lipid transfer protein 3	increased
increased luteinizing hormone level	StAR-related lipid transfer protein 4	increased
increased luteinizing hormone level	StAR-related lipid transfer protein 5	increased
increased luteinizing hormone level	StAR-related lipid transfer protein 6	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:440 - Hypothalamic estrogen receptors inhibition leading to ovarian cancer	KeyEvent

Biological Context

Level of Biological Organization

Cellular

Cell term

Cell term
steroid hormone secreting cell

Organ term

Organ term
reproductive organ

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	High	NCBI
mice	Mus sp.	High	NCBI
rat	Rattus norvegicus	High	NCBI
Monkey	Monkey	Low	NCBI

Life Stage Applicability

Life Stage	Evidence
Adult, reproductively mature	High

Sex Applicability

Sex	Evidence
Female	High
Male	Low

In Granulosa cells

Key Event Description

Biological state: Steroidogenic acute regulatory protein (StAR) plays important role in luteal steroidogenesis(Christenson and Devoto, 2003). Steroidogenic acute regulatory protein (StAR) controls the transport of cholesterol from the outer to inner mitochondrial membrane(Stocco, 2000). There are several pathways involved for the transport of cholesterol from different subcellular pools into the inner mitochondria(Martin et al., 2016).

Biological compartments: Cholesterol is one type of lipid which is crystalline solid with yellow colour. It is biosynthesized by animal cells and is an essential structural component of animal cell membranes (Hanukoglu, 1992). It is the precursor molecule for the synthesis all steroid hormones(Payne and Hales, 2004). Cytochrome P450 enzymes are present in most tissues of the body, and play important roles in hormone synthesis in mitochondria using cholesterol as precursor(Poderoso et al., 2013).

General role in biology: It is been reported that high in cholesterol levels in mitochondrial resulted several diseases like cancer, neurodegenerative diseases, steatohepatitis ischemia, and influence disease (Martin et al., 2016). The alteration in mitochondrial cholesterol import may change the cholesterol concentrations that may lead to proper mitochondrial function along with biophysical properties of mitochondrial membranes. In absence of StAR protein, cholesterol transport into the mitochondria did not occurs leading to no conversion of progesterone from cholesterol precursors doesn't occur(Kiriakidou et al., 1996; Pescador et al., 1996). All Steroidogenic acute regulatory protein (StAR) promoters contain steroidogenic factor 1 binding sites which is responsible for sex hormones regulation(Manna et al., 2002).

One of the important function of the steroid hormones is maintaining reproductive capacity. For this purpose, steroidogenic cells must move large amounts of cholesterol from the outer mitochondrial membrane to the inner membrane. In the granulosa cells, this cholesterol is ultimately converted to progesterone. The initial transport of cholesterol across the mitochondrial membrane requires Steroidogenic Acute Regulatory (StAR) protein. Expression of StAR protein in preovulatory cells of the developing follicle is low. The dramatic upregulation of StAR protein expression within the dominant follicle is found after the luteinizing hormone (LH) surge. This upregulation allows the corpus luteum to produce substantial amounts of progesterone to maintain the reproductive capacity in human/animal (Men et al., 2017; Stocco, 2000).

How it is Measured or Detected

StAR protein is measure by quantitative real time PCR (qRT-PCR):

For qRT-PCR analyses, cDNA is synthesized using reagent kit in a 20-µl reaction containing 0.5 µg of total RNA collected from human ovarian granulosa tumor cell line (KGN cells), mouse Leydig cells. qPCR is performed in a 25-µl reaction containing 0.5 to 1.5 µl of cDNA using fluorescein in real-time PCR detection systems. PCR was performed by initial denaturation at 95°C for 5 minutes, followed by 40 cycles of 30 seconds at 95°C, 30 seconds at 60°C, and 30 seconds at 72°C. The threshold cycle values of each sample are used to calculate mRNA levels. The PCR primers for the indicated human and mouse genes are as follows (Men et al., 2017).

Human H19 forward: 5′-GCACCTTGGACATCTGGAGT

Human H19 reverse: 5′-TTCTTTCCAGCCCTAGCTCA

Human StAR forward: 5′-GGCATCCTTAGCAACCAAGA

Human StAR reverse: 5′-TCTCCTTGACATTGGGGTTC

Mouse StAR forward: 5′-TTGGGCATACTCAACAACCA

Mouse StAR reverse: 5′-GAAACACCTTGCCCACATCT

Indirect immunohistochemistry for the detection of Steroidogenic Acute Regulatory Protein (StAR):

Ovarian or peritoneal tissues from the human patients are collected. Ovarian or peritoneal tissues from the patient are fixed using 10% paraformaldehyde. Tissues are embedded in paraffin. Serial sections of 5 µm are made using microtome. Tissue sections are prepared by microwave heating in 10× citrate buffer, pH 6.0, for 10 min. Tissues are rinsed three times in 20 mM phosphate buffered saline (PBS), pH 7.2, for 10 min each, before incubation with 1:200

dilutions of polyclonal anti-human StAR antibodies at 37°C for 60 min. Tissue sections were washed three times in 20 mM PBS, pH 7.2, for 2 min each, before incubation with a 1:1000 dilution of secondary mouse– anti-rabbit antibody at 37°C for 30 min. Indirect immunohistochemistry kits were used according to the manufacturer's instructions to visualize StAR protein stained tissue under microscope and image collected. A pathological image analysis system is used to measure mean optical density (MOD) analysis under high-magnification (×400) microscopy. The MOD, which reflected the positive staining intensity, and the positive staining ratio (area %) of every positively stained area, are measured. The area % is calculated as ([the area of positive staining]/[total nuclear area in the field of view]) × 100. The MOD and area % are used to calculate the expression index, EI (%) = MOD × area %(Tian et al., 2009).

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Event: 1973: Increased, estrogens

Short Name: Increased, estrogens

Key Event Component

Process	Object	Action
estrogen secretion	Estrogen	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:440 - Hypothalamic estrogen receptors inhibition leading to ovarian cancer	KeyEvent

Biological Context

Level of Biological Organization
Cellular

Cell term

Cell term
steroid hormone secreting cell

Organ term

Organ term
reproductive organ

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	High	NCBI
rat	Rattus norvegicus	High	NCBI
mice	Mus sp.	High	NCBI

Life Stage Applicability

Life Stage	Evidence
Adult, reproductively mature	High

Sex Applicability

Sex	Evidence
Female	High
Male	Moderate

It is applicable in reproduction system, cell growth and cell function

Key Event Description

Biological state: The most predominant form of estrogens is 17β-estradiol (E2) which is sex hormone. In women having premenopausal it is mainly produced in the ovaries. For postmenopausal women, it E2 primarily is sythesized from testosterone by aromatase enzyme in extragonadal tissues(Simpson, 2003). Estradiol stimulates both cell growth and cholesterogenesis in the MCF-7 line (breast cancer cell line) (Cypriani et al., 1988). Cholesterol increases neuronal estradiol release into the medium through synapse formation(Fester et al., 2009).

Biological compartments: Estrogen is considered as the risk of developing cholesterol gallstones by enhancing the hepatic secretion of biliary cholesterol leading to an increase in cholesterol(Wang et al., 2009).

General role in biology: When estrogen levels decline, levels of low-density lipoprotein, the harmful kind of cholesterol increases, and levels of high-density lipoprotein, the positive kind of cholesterol decrease, due to which fat build up in the body and cholesterol in the arteries that causes heart attack and stroke(Fåhraeus, 1988; Wahl et al., 1983). Granulosa cells are the primary cell which provides the support and microenvironment required for the developing oocyte in the ovary(Sen and Hammes, 2010; Sterneck et al., 1997).

How it is Measured or Detected

Radioimmunoassay (RIA) and analytical method based on mass spectroscopic are used for estrogen measurement present in serum (Smy and Straseski, 2018; Giese, 2003).

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Event: 1076: Increased, circulating estrogen levels

Short Name: Increased, circulating estrogen levels

Key Event Component

Process	Object	Action
	estrogen	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:168 - GnRH pulse disruption leading to mammary adenomas and carcinomas in the SD rat.	KeyEvent
Aop:169 - GnRH pulse disruption leading to pituitary adenomas and carcinomas in the SD rat.	KeyEvent
Aop:440 - Hypothalamic estrogen receptors inhibition leading to ovarian cancer	KeyEvent

Biological Context

Level of Biological Organization

Organ

Event: 1051: Hyperplasia, ovarian stromal cells

Short Name: Hyperplasia, ovarian stromal cells

Key Event Component

Process	Object	Action
hyperplasia	stromal cell of ovary	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:165 - Antiestrogen activity leading to ovarian adenomas and granular cell tumors in the mouse	KeyEvent
Aop:440 - Hypothalamic estrogen receptors inhibition leading to ovarian cancer	KeyEvent

Biological Context

Level of Biological Organization

Cellular

Cell term

Cell term
stromal cell of ovary

Event: 1052: Hyperplasia, ovarian epithelium

Short Name: Hyperplasia, ovarian epithelium

Key Event Component

Process	Object	Action
hyperplasia	epithelium of female gonad	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:165 - Antiestrogen activity leading to ovarian adenomas and granular cell tumors in the mouse	KeyEvent
Aop:440 - Hypothalamic estrogen receptors inhibition leading to ovarian cancer	KeyEvent

Biological Context

Level of Biological Organization

Tissue

Organ term

Organ term
epithelium of female gonad

List of Adverse Outcomes in this AOP

Event: 1053: Promotion, ovarian adenomas

Short Name: Promotion, ovarian adenomas

Key Event Component

Process	Object	Action
	Adenoma	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:165 - Antiestrogen activity leading to ovarian adenomas and granular cell tumors in the mouse	AdverseOutcome
Aop:440 - Hypothalamic estrogen receptors inhibition leading to ovarian cancer	AdverseOutcome

Biological Context

Level of Biological Organization
Tissue

Event: 1054: Promotion, ovarian granular cell tumors

Short Name: Promotion, ovarian granular cell tumors

Key Event Component

Process	Object	Action
	Granular Cell Tumor	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:165 - Antiestrogen activity leading to ovarian adenomas and granular cell tumors in the mouse	AdverseOutcome
Aop:440 - Hypothalamic estrogen receptors inhibition leading to ovarian cancer	AdverseOutcome

Biological Context

Level of Biological Organization
Cellular

Event: 2092: Promotion, Ovarian Cancer

Short Name: Promotion, Ovarian Cancer

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:440 - Hypothalamic estrogen receptors inhibition leading to ovarian cancer	AdverseOutcome

Biological Context

Level of Biological Organization
Organ

Appendix 2

List of Key Event Relationships in the AOP

List of Adjacent Key Event Relationships

Relationship: 2580: Suppression, Estrogen receptor (ER) activity leads to Increased, secretion of GnRH from hypothalamus

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Hypothalamic estrogen receptors inhibition leading to ovarian cancer	adjacent	High	Not Specified

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	High	NCBI
rat	Rattus norvegicus	High	NCBI
mice	Mus sp.	High	NCBI

Life Stage Applicability

Life Stage	Evidence
Adult, reproductively mature	High

Sex Applicability

Sex	Evidence
Female	High
Male	Low

Negative feedback action on GnRH secretion had shown in female guinea pig (Kelly et al., 1984).

Reduced firing of GnRH neurone was shown in adult female mice (Chu et al., 2009).

Alterations in the concentrations of oestrogen receptors in the hypothalamus was shown in rat (Adashi et al., 1980).

Negative Feedback of estrogen on GnRH secretion was studied in adult woman (Shaw et al., 2010).

Key Event Relationship Description

Study on female human patient had shown Selective Estrogen Receptors Modulator (Clomiphene) act on the hypothalamic site and increase the hypothalamic GnRH secretion significantly (KERIN et al., 1985). Study on female rat had shown increased gonadotropin hormone secretion upon administration of very low dose (1-100 ng/kg) of clomiphene citrate. However, high dose (1µg/kg -2 mg/kg) of clomiphene citrate in female rat inhibit the gonadotropin

hormone secretion (Koch et al., 1971).

Estradiol i.e. Estrogen receptor beta acts as a potent feedback molecule between the ovary and hypothalamic GnRH neurons, and exerts both positive and negative regulatory actions on GnRH synthesis and secretion (Hu et al., 2008). ESR_2 control the GnRH release through the intracellular calcium ions release (Kenealy et al., 2011). Research had shown that nanomolar concentration of membrane-associated G protein-coupled estrogen receptor alter the patterns of Ca2+ release in GnRH neurone (Komatsuzaki and Kawato, 2007). Studies on mouse have shown several molecules such as, eastradiol, non-peptide neurotransmitters, gasotransmitters can modulate the GnRH neuron activity and GnRH secretion and control the reproductive functions (Spergel, 2019; Temple et al., 2004; Temple and Wray, 2005).

Evidence Supporting this KER

Koch et al., had shown the ~107% increase in GnRH secretion after administration of clomiphene citrate (1-100 ng/kg) in adult female rat (Koch et al., 1971).

Boyer et al., had also shown the increasing GnRH secretion after administration of clomiphene citrate (1.0 mg/kg/day)in immature female rats (Boyar, 1970).

Roy et al., had shown that 17β-estradiol at 1 nm concentration over a 48 h time period down regulate (~55%) the expression of GnRH mRNA in GnRH-secreting, hypothalamic cell line (GT1–7) (Roy et al., 1999).

Chu et al., had shown using whole cell electrophysiology of the brain slice in adult female mice 10 picomolar concentration of estradiol reduce the firing of GnRH neurone (Chu et al., 2009).

Biological Plausibility

Molecular mechanism for the enhancement of GnRH by suppression of Estrogen receptor activity is poorly known.

Empirical Evidence

Compound class	Species	Study type	Dose	KER findings	Reference
Clomiphene citrate	Adult Rat (female)	Quantification of GnRH	(1-100 ng/kg, 21-48 days)	GnRH – 37.0± 3.6 µg/mg (Control) GnRH – 76.7±5.8 µg/mg (Clomiphene treated)	(Koch et al., 1971)
Clomiphene citrate	Immature female rats (female)	Quantification of pituitary concentra-tion of FSH (ovarian weight augmenta-tion method)	1.0 mg/kg/day for 20 days	FSH-RF – 32.0 (22.3- 41.7) µg/mg (Control) FSH-RF – 122.0 (29.2- 215.8) µg/mg (Clomiphene treated)	(Boyar, 1970)
17β-estradiol	Hypothalamic cell line of transgenic mice (GT1–7)	Expression of GnRH mRNA	1 nm	~55% down regulation of the expression of GnRH mRNA.	(Roy et al., 1999)

Uncertainties and Inconsistencies

The release GnRH neurons depends on the concentration of the Selective Estrogen Receptors Modulator compound (Clomiphene). Scientific reports have shown the both stimulatory and inhibitory effects on the GnRH secretion exhibited by the estradiol depending on the concentration of clomiphene molecules and presence of types of receptors (Chu et al., 2009; Micevych and Kelly, 2012; Boyar, 1970).

Quantitative Understanding of the Linkage

Not Specified

Response-response relationship

Not Specified

Time-scale

Neural activity and elevated hormone release are observed for hours in in vivo study (Chu et al., 2009).

Known modulating factors

GnRH secretion from the neurone can be modulated by prostaglandin, glutamate, ATP, carbon monoxide, nitric oxide, neurotransmitters (norepinephrine, epinephrine, GABA, histamine and acetylcholine) (Spergel, 2019).

Known Feedforward/Feedback loops influencing this KER

Not Specified

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Relationship: 1089: Increased, secretion of GnRH from hypothalamus leads to Increased, secretion of LH from anterior pituitary

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Antiestrogen activity leading to ovarian adenomas and granular cell tumors in the mouse	adjacent	High	
Hypothalamic estrogen receptors inhibition leading to ovarian cancer	adjacent	High	Moderate

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	High	NCBI
rat	Rattus norvegicus	High	NCBI
mice	Mus sp.	High	NCBI
cow	Bos taurus	Low	NCBI

Life Stage Applicability

Life Stage	Evidence
Adult, reproductively mature	High

Sex Applicability

Sex	Evidence
Female	High
Male	Low

Adult

Key Event Relationship Description

The release of gonadotrophin-releasing hormone (GnRH) stimulate the secretion of luteinising hormone (LH) (Fields et al., 2009). GnRH causes the pituitary gland to secrete LH. Gonadotropin releasing hormone (GnRH) is the key regulator of the secretion of luteinising hormone (Marques et al., 2018; Bowen et al., 1998; Tsutsumi and Webster, 2009). Metastin or kisspeptin in the control of gonadotropin-releasing hormone (GnRH) release and then it causes for pulsatile release of luteinizing hormone(Ohkura et al., 2009).

Evidence Supporting this KER

- Gonadotropin-releasing hormone (GnRH) is the master hormone for regulating the reproduction. GnRH pulses stimulate the synthesis and secretion of LH from the anterior pituitary(Tsutsumi and Webster, 2009).
- Nicol et al., reported that high GnRH dose enhances the secretion of LH (Nicol et al., 2002)

Biological Plausibility

GnRH was isolated from porcine hypothalamus. It was structurally identified as a decapeptide (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly·NH2)(AV et al., 971). During the childhood, GnRH levels are low but as puberty begins. GnRH levels start to rise and when the testes and ovaries are fully developed. GnRH regulates LH and these hormones to control the production of sex hormones in adult (Marques et al., 2018). GnRH secretion have been described in pulsatile (in minutes) and surge modes. Pulsatile mode refers to episodic release of GnRH while the surge mode of GnRH secretion occurs in females during the pre-ovulatory phase (Maeda et al., 2010). Secretion of LH is also in pulsatile nature (in hrs)(Bolt, 1971).

Empirical Evidence

Compound class	Species	Study type	Dose	KER findings	Reference
GnRH	Cows	In-vivo	GnRH dose of 100 micro gram	GnRH dose increases LH secration	(Fields et al., 2009)
GnRH	Possums	In-vivo		GnRH dose increases LH secration	(Crawford et al., 2009)
GnRH	Bitches	In-vivo	GnRH dose of 06.-2.4 microgram/kg	GnRH dose increases LH secration	(Concannon et al., 2006)
GnRH agonist (triptorelin acetate) dose	Humans	In-vivo	GnRH dose of 3.75 mg / person	GnRH agonist (triptorelin acetate) dose decrease LH seccretion	(Sonntag et al., 2005)

Uncertainties and Inconsistencies

Not Specified

Quantitative Understanding of the Linkage

- Fields et al., studied the dose response of GnRH (100 micro gram) on cows and observed greater release of LH (25 %) aftrer 12-18 hours (Fields et al., 2009)
- Crawford et al., used PCR techniques to study the effect of GnRH on LH in vivo on Possums. They reported the increase of LH quantitavely in absence of pulse of LH(Crawford et al., 2009)
- Guillaume et al., studied the two GnRH antagonist Antarelix and Cetrorelix (0.01 mg/kg) on mare and observed that there is strong suppression of LH (Guillaume et al., 2002)
- Washington et al., developed one mathematical model for the respose of LH under the pulsatile and continuous exposure of GnRH (Washington et al., 2004
- Shoemaker et al., developed a mathematical model on steroidogenesis in the fathead minnow. They quantified the relationship between GnRH and LH(Shoemaker et al., 2010)

Response-response relationship

Not Specified

Time-scale

- Generally time scale is in hours (6-18) between GnRH and LH response (Fields et al., 2009).
- GnRH is degraded by proteolysis within a few minutes(Kenealy et al., 2011).
- It has very low activity during childhood, and is activated at puberty or adolescence and in reproductive years, pulse activity is critical for successful reproductive function(Berger et al., 1983).

Known modulating factors

- Protein kinase C cross-talk with gonadotrope progesterone receptor is involved in GnRH-induced LH secretion (Garrido-Gracia et al., 2006)

Known Feedforward/Feedback loops influencing this KER

Not Specified

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Relationship: 2581: Increased, secretion of LH from anterior pituitary leads to Increased, Steroidogenic acute regulatory protein (StAR)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Hypothalamic estrogen receptors inhibition leading to ovarian cancer	adjacent	High	Moderate

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	High	NCBI
rat	Rattus norvegicus	Moderate	NCBI
mice	Mus sp.	Moderate	NCBI

Life Stage Applicability

Life Stage	Evidence
Adult, reproductively mature	High

Sex Applicability

Sex	Evidence
Female	High
Male	Low

Adult

Key Event Relationship Description

The activity of StAR protein in theca cells is control by LH (Murayama et al., 2012). Subsequently, StAR protein regulates cholesterol transportation to the mitochondria and therefore, the production of steroid hormones is regulated by StAR protein (Clark and Stocco, 1995).

Evidence Supporting this KER

- Murayama et al. studied the in vitro LH pulse dose in Bovine ovaries and reported LH dose enhances the activity of StAR protein (Murayama et al., 2012).
- Johnson and Bridgham performed in vitro studied in granulosa cells from prehierarchal and preovulatory hen follicles to examine the regulation of steroidogenic acute regulatory protein (StAR) by LH. They reported the treatment with LH rapidly increased StAR mRNA and protein (Johnson and Bridgham, 2001).

Biological Plausibility

In mammalian species (e.g., rat,rabbit, human), LH stimulates the StAR protein to increase the cholesterol transport in to the inner mitochondrial membrane. Cholesterol is the precursor of sex hormones. Therefore, LH regulate the steroidogenic function of theca cells (Murayama et al., 2012; Johnson and Bridgham, 2001; Rekawiecki et al., 2005).

Empirical Evidence

Compound class	Species	Study type	Dose	KER findings	Reference
LH	Bovine ovaries, Human	In vitro	5-50 ng/ml LH dose	LH increases StAR protein activity	(Murayama et al., 2012)
LH	granulosa cells,Human	In vitro	LH dose	LH increases StAR protein activity	(Johnson and Bridgham, 2001)
LH	Leydig cells, Rat	In vivo	LH dose	LH-induced StAR protein expression	(Martinat et al., 2005)
LH	Bovine luteal cells, Human	In vitro	LH dose	LH increases StAR protein expression	(Rekawiecki et al., 2005)
LH	Leydig cells, Rat	In vivo	LH dose	LH increases StAR protein expression	(Liu et al., 2007)
LH	Leydig cell, Mice	In vivo	LH dose	LH increases five fold StAR protein expression	(Eacker et al., 2008)

Uncertainties and Inconsistencies

No uncertainties and inconsistencies are observed

Quantitative Understanding of the Linkage

- Rekawieck et al. conducted the in vitro study on Bovine luteal cells to investigate the effect of LH on steroid acute regulatory protein (StAR). They reported the LH enhances the activity of StAR protein (Rekawiecki et al., 2005).
- Liu et al. investigated the effect of LH on StAR protein using rat as model and reported the positive correlation between Lh and StAR protein (Liu et al., 2007)
- Eacker et al. reported that LH up regulates the StAR protein (around five fold) using mice model (Eacker et al., 2008)

Response-response relationship

Not specified

Time-scale

Time scale for the response between LH to StAR protein in hours (3-20 h) (Johnson and Bridgham, 2001; Martinat et al., 2005; Rekawiecki et al., 2005).

Known modulating factors

Not specified

Known Feedforward/Feedback loops influencing this KER

Not specified

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Relationship: 2582: Increased, Steroidogenic acute regulatory protein (StAR) leads to Increased, estrogens

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Hypothalamic estrogen receptors inhibition leading to ovarian cancer	adjacent	High	Moderate

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	High	NCBI
rat	Rattus norvegicus	Low	NCBI
mice	Mus sp.	Low	NCBI
fish	fish	Low	NCBI

Life Stage Applicability

Life Stage	Evidence
Adult, reproductively mature	High

Sex Applicability

Sex	Evidence
Female	High
Male	Low

Adult

Key Event Relationship Description

Steroidogenic acute regulatory (StAR) protein (37-kDa) is synthesized with a mitochondrial leader sequence in response to the cell stimulation to produce steroid and plays a crucial role in steroidogenesis (Hanukoglu, 1992). Research had shown in human ovary StAR protein was produced in response to the Luteinizing Hormone (LH) surge (Kiriakidou et al., 1996). In particular, StAR protein involved in the transportation of the cholesterol (substrate for steroid hormone) from outer to inner mitochondrial membrane. This step is crucial and rate limiting in steroid biosynthesis. In the inner membrane of the mitochondria with the help of cleaved cholesterol pregnenolone is formed, which is the precursor to the different steroid hormones including estrogen (P. R. Manna et al., 2016). Effects of StAR protein on steroidal biosynthesis had been studied by number of researchers (Pulak R Manna et al., 2002; Pescador et al., 1996; Stocco, 2001).

Estradiol synthesis during menstrual cycle is governed via expression of StAR protein synthesis. Presence of StAR protein allows follicular production of androgens which allows the progesterone dominated microenvironment and help in

sexual differentiation, growth, reproduction, development and metabolism. Kusakabe et al., had shown in trout fish (*Salvelinus fontinalis*) model that peak of StAR protein coincide with the menstrual hormone production peak (Kusakabe et al., 2002). Research had shown some toxic chemicals can caused alteration in steroidal regulation and resulted in the agonist effect on estrogen receptors (Lauretta et al., 2019).

Evidence Supporting this KER

Study on immature female rat model had shown rapid changes of the StAR protein level in the ovary during follicular development facilitate the production of estrogen (Ronen-Fuhrmann et al., 1998).

Fang et al., had studied StAR protein expression under the influence of amphiregulin protein in cultured primary human granulosa cells collected from female. Results of the study had shown that human chorionic gonadotropin (hCG) rapidly induces amphiregulin (AREG) expression in the culture cells. Treatment with amphiregulin increase StAR expression and progesterone production in the cells (Fang et al., 2016).

Biological Plausibility

StAR protein catalyzes the movement of cholesterol in the outer mitochondrial membrane to the inner membrane. There, cytochrome P450scc converts cholesterol to the steroid pregnenolone. Studies have shown (in mouse and rat model) some molecules (e.g. 25-hydroxycholesterol) can serve as a substrate for inducing the expression of StAR and influence the steroid production in different tissues. Other oxysterols molecules also capable of increasing STAR expression and pregnenolone synthesis in human endometrial stromal cells (P. R. Manna et al., 2016).

Empirical Evidence

Compound class	Species	Study type	Dose	KER findings	Reference
StAR Protein	Trout fish (*Salvelinus fontinalis*)	*In situ* hybridization, cloning of cDNAs, Northern blot analysis of mRNA	Stressor concentration (0.5 ml/liter 2-phenoxyethanol)	Increased in StAR transcripts in tissues exhibiting enhanced steroid production and increased circulating levels of 17β-estradiol and maturation inducing steroid (17α,20β - dihydroxy-4-pregnen-3-one).	(Kusakabe et al., 2002)
StAR Protein	Hen (Single-comb white Leghorn)	Northern blot analysis of mRNA collected from ovarian follicle granulosa cells	MAP kinase inhibitor (U0126)-50 mM, Follicle stimulating hormone(FSH) - 100 ng/ml, TGFa (50 ng/ml)	Acute increase in progesterone production in response to LH treatment	(Johnson et al., 2002)
StAR Protein	The mouse MA-10 Leydig tumor cell line	Northern blot analysis of total RNA	Fadrozole (100 µM)	StAR Protein inhibition by antifungal drugs econazole and miconazole	(Walsh et al., 2000)

Uncertainties and Inconsistencies

Chang et al., had investigated the effects of antimullerian hormone (AMH) on estradiol production in primary culture of human granulosa-lutein (hGL) cells. In the control cell estradiol concentration was found 43.2–93.7 ng/mL. Whereas,treatment with AMH (10 ng/mL) significantly reduced the estradiol accumulation in the cells (Chang et al., 2013).

Quantitative Understanding of the Linkage

Pescador et al., had studied the StAR mRNA levels in the bovine corpus luteum. Result of the study had shown that expression of StAR mRNA was low in developing corpus luteum. In mid to late luteal phase the concentration increased 9- to 15-fold compared to the expression of StAR mRNA during developing stage. Results confirms that StAR mRNA and protein are tightly coupled in the corpus luteum cells and present at low levels during CL development and present elevated concentrations during the midluteal phase (Pescador et al., 1996).

Response-response relationship

Not specified

Time-scale

Observed for hours

Known modulating factors

Arukwe had shown nonylphenol (15 µg/L) can induce the StAR protein in juvenile Atlantic salmon (Salmo salar) fish (Arukwe, 2005).

Known Feedforward/Feedback loops influencing this KER

Not specified

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Relationship: 2583: Increased, estrogens leads to Increased, circulating estrogen levels

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Hypothalamic estrogen receptors inhibition leading to ovarian cancer	adjacent	High	Moderate

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	High	NCBI
rat	Rattus norvegicus	High	NCBI
mice	Mus sp.	High	NCBI

Life Stage Applicability

Life Stage	Evidence
Adult, reproductively mature	High

Sex Applicability

Sex	Evidence
Female	High
Male	High

Judd et al, had measured the circulating estrogen level in the male and female lizards (*Iguana iguana*) (Judd et al., 1976).

Roberts et al., had estimated the circulating estrogen in the plasma collected from human volunteer (Roberts and Szego, 1946).

Truan et al., had shown the high circulating estrogen levels in the mice model (Truan et al., 2010).

Key Event Relationship Description

Ovaries are the principle source of estrogen hormone in premenopausal women. This estrogen functions as a circulating hormone to act on different tissues. In postmenopausal women, estrogen is produced in a number of extragonadal sites and acts locally at these sites as a paracrine or even intracrine factor. The monthly menstrual cycle in female is controlled through unique co-ordination between secreted hormones by the hypothalamus, the pituitary gland, and the ovary. Estrogen is synthesized from androgen, upon calalysis of aromatse enzyme present in the endoplasmic reticulam of the cells. Presence of aromatase enzyme is found majorly in the ovarian granulosa cells (premenopausal female), in the skin and adipose tissue (postmenopausal woman). Estrogen was synthesized in postmenopausal women due to the aromatization of steroids, found in the adipose and skin tissue.

Aromatase is a key enzyme for estrogen formation in human tissues. In men and postmenopausal women C19 steroids undergoes aromatization in different tissues (e.g. skin, adipose) to generate estrogen. In men, testicular steroidogenesis accounts for 15% of the circulating level of estrogen.

In women, the ovarian granulosa cells are important sites of estrogen formation for local use within the ovary as well as for endocrine signalling to the target tissues (e.g. uterus, skin, breast, brain, bone). In case of postmenopausal female, ovarian aromatase expression is stopped, but estrogen level is maintained in the plasma by the increased aromatase expression in other tissues (adipose and skin). Research had shown elevated circulating estradiol may persist at sufficient levels to cause postmenopausal uterine bleeding, endometrial hyperplasia, and even cancer.

Evidence Supporting this KER

Steger et al., had shown the age related changes in steroid productions in the ovaries of rat model. In this work researchers had shown the elevated serum estrone and estradiol level in the rats (mid–aged) (Steger and Peluso, 1982).

Biological Plausibility

Estrogens in humans are classified as estrone (E1) and estradiol (E2). E2 is synthesized majorly in ovaries and testes by aromatization of testosterone. Small amounts of estrogens are produced in the adrenal glands and some peripheral tissues (e.g. skin, fat tissues). E2 and E1 are interchangeable, and both can be deactivated via hydroxylation. E2 has 1.25 to 5 times higher biological potency of E1. E2 circulates at 1.5 to 4 times more concentration of E1 in premenopausal women. E2 levels in men and postmenopausal women are much lower than in nonpregnant women. E2 levels in premenopausal women fluctuate during the menstrual cycle. An E2 level is lowest during the early follicular phase, then rise gradually until 2 to 3 days before ovulation. In the ovulatory phase E2 levels again declined.

Empirical Evidence

Compound class	Species	Study type	Dose	KER findings	Reference
Circulating estrogen	lizards (*Iguana iguana*)	Assay of plasma protein	-	Estrogens mean concentrations were higher in the females compared to males. Estradiol (258 ± 46 pg/ml), Estrone (205 ± 147 pg/ml) – In Females. Estradiol, 79 ± 42 pglml, Estrone, 37 ± 2 pglml – In Male	(Judd et al., 1976)
Circulating estrogen	Human Plasma	Assay of plasma protein fractions	-	1.5 to 2.0 µg of estriol (1.8 µg – in average) per 100 ml. of original plasma.	(Roberts and Szego, 1946)
Circulating estrogen	Mice	Ovariectomized athymic mice (BALB/c nu/nu, 4–5 wk old)	A 100-µL cell suspension ($4 x10^6$ MCF-7 cells)	High circulating estrogen (E2) concentration (E2 pellet (0.36 mg/ 60-day release) simulating premenopause	(Truan et al., 2010)

Uncertainties and Inconsistencies

Leung et al., had shown estradiol-17β (I mg) administration in the female rat for 3 days decrease the ovarian androgen levels (13 ± 2 pg/mg) compared to the control (34 ± 7 pg/mg). Results of the study suggest estrogen levels controlled by the negative feedback loop of testosterone production (Leung et al., 1978).

Quantitative Understanding of the Linkage

Estrone concentrations in human

Males: 10-60 pg/mL, Females: Premenopausal: 17-200 pg/mL, Postmenopausal: 7-40 pg/mL

Estradiol concentrations in human

Males: 10-40 pg/mL, Females: Premenopausal: 15-350 pg/mL, Postmenopausal: <10 pg/mL (Cummings et al., 1998; Elmlinger et al., 2002)

Response-response relationship

Leung et al., had shown estradiol-17β (I mg) administration in the female rat for 3 days decrease the ovarian androgen levels (13 ± 2 pg/mg) compared to the control (34 ± 7 pg/mg) (Leung et al., 1978).

Time-scale

Elevation of the circulating estrogen can be observed in days

Known modulating factors

Estrogen levels changes due to the following reasons.

- High androgen levels caused by tumors
- Androgen therapy
- Elevations in estrogen due to aromatization
- Obesity with increased tissue production of E1
- Decreased estrogen clearance in liver disease

- Estrogen producing tumors
- Estrogen ingestion

Known Feedforward/Feedback loops influencing this KER

Not specified

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List of Non Adjacent Key Event Relationships

Relationship: 2584: Increased, circulating estrogen levels leads to Hyperplasia, ovarian stromal cells

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Hypothalamic estrogen receptors inhibition leading to ovarian cancer	non-adjacent	High	Not Specified

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	High	NCBI
rat	Rattus norvegicus	High	NCBI
mice	Mus sp.	High	NCBI

Life Stage Applicability

Life Stage	Evidence
Adult, reproductively mature	High

Sex Applicability

Sex	Evidence
Female	High

Increase in circulating estrogen level causing increase in the ovarian stromal cells observed in adult female (human) also in rodents.

Key Event Relationship Description

Ovarian tumor termed as hyperplasia is characterized as enlarged ovary with increased numbers of corpora lutea and tertiary follicles. In some cases cystic/incompletely lutenised corpora lutea may also be observed. Ovarian tumors may contain Leydig cells and originate within the specific ovarian stroma cells (Sternberg and Roth, 1973). Studies on the female rats have shown increased hormonal levels (e.g. estradiol 17-β, progesterone, prolactin) in the plasma are causing the tumor formation in the ovarian granulosa cell (Long et al., 2001).

High levels of circulating estrogen in the plasma can produce tumors in the ovarian granulosa cells. Magnetic resonance (MR) imaging was used for the detection of the ovarian tumors directly (Tanaka et al., 2004). Eriksson et al., had shown the estrogen levels (1 pg/mL ±0.048) in men samples using gas chromatography - mass spectrometry (GC-MS) or liquid chromatography tandem mass spectrometry(Eriksson et al., 2018). In another study the serum estradiol concentration ranges was determined (~20 - 80 pg/mL) in females during the early to mid-follicular phases of the menstrual cycle and before puberty (~ 20 pg/mL) (Carmina et al., 2019). Barr Fritcher et al., had found that the expression of estrogen receptor (ER) is proportional with age and diagnosed with atypical hyperplasia (Barr Fritcher et al., 2011).

Evidence Supporting this KER

Barr Fritcher et al., had studied the expression of estrogen receptors (ER) over 246 women with atypical hyperplasia and found that 87 (35%) had atypical ductal hyperplasia (ADH), 141 (57%) had atypical lobular hyperplasia (ALH), and 18 (7%) had both type of hyperplasia and also found the increasing ER expression in atypical hyperplasia with increasing age(Barr Fritcher et al., 2011).

In a diiferent study Shaaban et al., had shown the positive correlation between ER-α and cellular proliferation causing hyperplasia with an increased risk of subsequent breast cancer development (Shaaban et al., 2002).

Biological Plausibility

Estradiol is the most biologically active estrogen, primarily secreted by ovarian granulosa cells and the conversion of estradiol to estrone occur with the action of 17β-hydroxysteroid dehydrogenase enzyme(Melmed et al., 2015).

Samavat, H. and M.S. Kurzer, found that in postmenopausal women endogenous estrogens are associated with breast cancer. But for premenopausal women this relationship has not been firmly established but it may possible during the menstrual cycle due to the large variations in hormone levels (Samavat and Kurzer, 2015).

Hankinson, S.E. and A.H. Eliassen, found that a positive association has been observed to the women with high levels of estrogen consistently with approximate two fold increases in invasive breast cancer risk(Hankinson and Eliassen, 2007).

Empirical Evidence

Compound class	Species	Study type	Dose	KER findings	Reference
Estradiol level	Human (Female)			20 to 80 pg/mL	(Carmina et al., 2019)
Estrogen and Estrogen metabolite	Rodents (Female)	*In Vivo*	-	estrone (2.65 (1.09–6.45) ng/mL estradiol (2.72 (1.04–7.14) ng/mL	(Wood et al., 2007)
Estradiol	rats and mice	Detection with GC-MS	-	<0.3 pg/mL	(Nilsson et al., 2015)
Estrone	rats and mice	Detection with GC-MS	-	<0.5 pg/mL	(Nilsson et al., 2015)

Uncertainties and Inconsistencies

Zhao et al., had shown serum estrogen concentration decreased to naormal level after three days of the removal of ovarian tumor (Zhiyi Zhao et al., 2019).

Montgomery et al., had reviewed the works on endometrial and mentioned that unopposed estrogen in woman taking the hormone replace therapy increase the risk of endometrial hyperplasia (Montgomery et al., 2004).

Travis et al., had suggested that circulating oestrogens have strong corelation with the increased risk of breast cancer in postmenopausal women (Travis and Key, 2003).

Quantitative Understanding of the Linkage

Not specified

Response-response relationship

Not specified

Time-scale

Observed in months

Known modulating factors

Regulation of gonadotropin secretion, Dysregulation of ovarian function, Insulin-resistant hyperinsulinism, Modulation of androgen action (Rosenfield and Ehrmann, 2016).

Known Feedforward/Feedback loops influencing this KER

Wood, et al., had found that circulating estrogen level positively correlated with uterine width and stromal cell proliferation and negatively correlated with glandular epithelial proliferation and stromal compartments in the rodents (Wood et al., 2007).

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Relationship: 2585: Increased, circulating estrogen levels leads to Hyperplasia, ovarian epithelium

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Hypothalamic estrogen receptors inhibition leading to ovarian cancer	non-adjacent	High	Not Specified

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	High	NCBI
rat	Rattus norvegicus	High	NCBI
mice	Mus sp.	High	NCBI

Life Stage Applicability

Life Stage	Evidence
Adult, reproductively mature	High

Sex Applicability

Sex	Evidence
Female	High

Increase in circulating estrogen level causing increase in the ovarian stromal cells observed in adult female (human) also in rabbit and rodents.

Key Event Relationship Description

Hyperplasia of the ovarian epithelial cells characterized by aggregates of tubular like structures or cleft lines. In the mammalian ovary tissue presence of germ cells surrounded by the somatic cells is known as follicles. During the oestrous cycle early stage follicles either go through atresia or ovaluation to produce mature egg for fertilization. With the age ovaries run out of follicles and female undergo menopause. Repetitive rupture and repair of the epithelium tissue of the ovarian cells causes genetic aberrations causing the abnormal growth of these cells ultimately leads towards hyperplasia (Bajwa et al., 2016).

Yamagata et al., studied that the increased estrogen were reflected in such target tissues proliferation, hyperplasia, atypical hyperplasia of the endometrium were observed in patients with ovarian tumors (Yamagata et al., 1989). Goad et al., had shown that unopposed estrogen drives the endometrial hyperplasia leads towards the progression of endometrial cancer in the uterine epithelium (Goad et al., 2018). During the menstrual cycle, epithelium tissue of the ovary proliferate under the influence of higher estrogenic level, and the increased mitotic activity is likely to enhance the risk of the mutation in the cells (Harvey A. Risch, 1998b).

Evidence Supporting this KER

Nash et al., had shown 50% increase in the growth rate of the epithelial ovarian cancer cell line (PE04) with the treatment of 17β-estradiol in vitro cell culture study (Nash et al., 1989).

Meissner et al., had shown the endometrial hyperplasias and cancers by excessive estrogenic stimulation in the female rabbit (Meissner et al., 1957).

Biological Plausibility

There are many kinds of ovarian tumors that are related with the estrogen or androgen levels. Granulosa cell tumor and thecoma are well-known estrogen-producing tumors. Metastatic ovarian tumors often have androgen-producing stroma and that mucinous cystadenoma produces estrogens. Many other ovarian tumors also can produce sexual hormones in their stroma (Tanaka et al., 2004).

Empirical Evidence

Compound class	Species	Study type	Dose	KER findings	Reference
Estrogen	women	*In vivo*	Increase in concentration of estrogen	Atypical hyperplasia of the endometrium	(Yamagata et al., 1989)
Estrogen	women	*In Vitro*	Increase in concentration of estrogen	Endometrial cancer by endometrial hyperplasia	(Goad et al., 2018)

Uncertainties and Inconsistencies

Ho et al., had shown that steroid hormones, primarily estrogens and progesterone, are implicated in ovarian carcinogenesis and estrogens favor neoplastic transformation of the ovarian surface epithelium (Ho, 2003).

Quantitative Understanding of the Linkage

Not specified

Response-response relationship

Vuong et al., had shown estrogen replacement therapy in the primary culture of the mouse ovarian surface epithelium cells increases the risk of ovarian cancer. Study had demonstrated that exogenous estradiol accelerates the onset of ovarian cancer in mouse models via the ESR1 pathway to result in the down-regulation of a tumour suppressor gene (Vuong et al., 2017).

Time-scale

Observed in months to years

Known modulating factors

Not specified

Known Feedforward/Feedback loops influencing this KER

Not specified

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Relationship: 1093: Hyperplasia, ovarian epithelium leads to Promotion, ovarian adenomas

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Antiestrogen activity leading to ovarian adenomas and granular cell tumors in the mouse	non-adjacent	High	
Hypothalamic estrogen receptors inhibition leading to ovarian cancer	non-adjacent	High	Not Specified

Relationship: 1094: Hyperplasia, ovarian stromal cells leads to Promotion, ovarian granular cell tumors

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Antiestrogen activity leading to ovarian adenomas and granular cell tumors in the mouse	non-adjacent	High	
Hypothalamic estrogen receptors inhibition leading to ovarian cancer	non-adjacent	High	Not Specified

Relationship: 2829: Promotion, ovarian adenomas leads to Promotion, Ovarian Cancer

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Hypothalamic estrogen receptors inhibition leading to ovarian cancer	non-adjacent	High	Low

Relationship: 2830: Promotion, ovarian granular cell tumors leads to Promotion, Ovarian Cancer

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Hypothalamic estrogen receptors inhibition leading to ovarian cancer	non-adjacent	High	Low