【S-8】

Effects of TCDD and DES on the Gene Expression of Reproductive System

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We have identified sensitive genes to endocrine disrupters expressed in mammalian reproductive system. Combination of subtraction cloning (Representational Difference Analysis) and DNA microarray technologies enables identification of several hundreds of genes that were induced or suppressed by endocrine disrupters in reproductive system. The induction or suppression of the genes was confirmed by a quantitative real-time PCR method. We newly identified hundreds of genes that were actually induced or suppressed by certain endocrine disrupting chemicals in the ovary, placenta, or cultured cells originated from reproductive organs. In this symposium, I would like to talk about effects of dioxin (TCDD) and diethylstilbesterol (DES) on the reproductive system based on the data presented by the DNA database.

Effects of TCDD and DES on the gene expression of reproductive system

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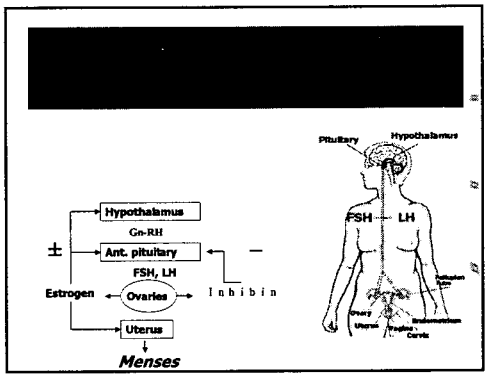
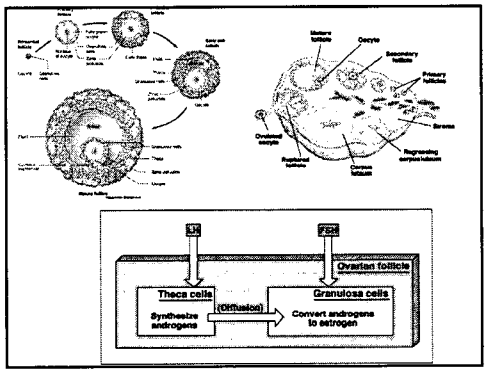
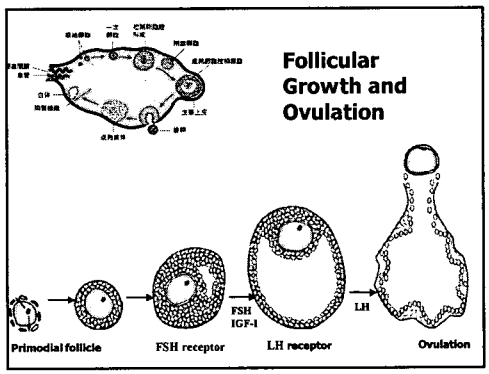
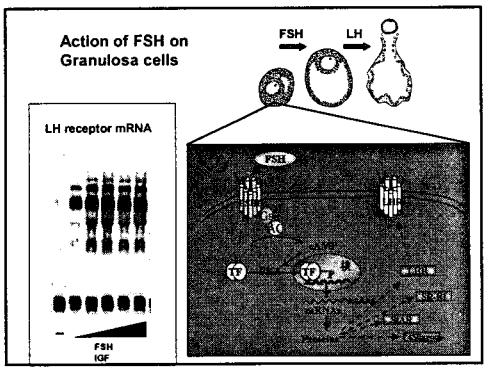








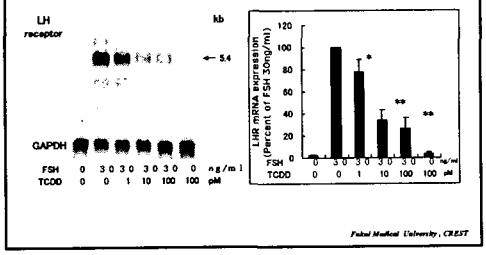
Low Dose Effects of Endocrine Disrupters
(Dioxin: TCDD)
Tolerance for Daily Intake (TDI) ; 4pg/kg/day
Exposure of daily life ; 0.5-3.5pg/kg/day
Intake from mother's milk ; 100pg/kg/day

Effects on Reproductive Systems
Suppression of E2 effects on MCF7 cells ;
300-3000pg/ml (Safe S et al. 1993)

Increase in the incidence of endometriosis in monkey ; 126pg/kg/day (Rier SE et al., 1993)

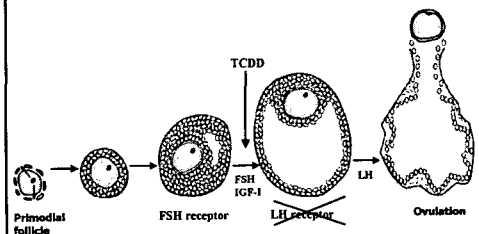
Suppression of LH receptor expression in rat granulosa cells; 0.3-3pg/ml
(Hirakawa T, Miyamoto K, Minegishi T et al., 2000)

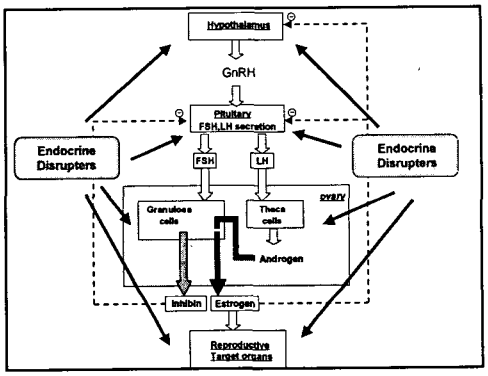
Effects of TCDD on LH receptor expression



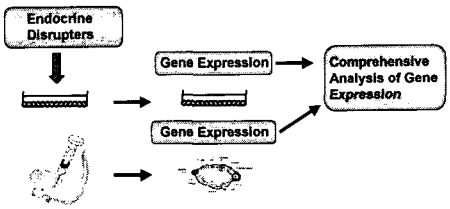
Fukui Medical University, CREST

Follicular Growth and Ovulation





Analysis of Actions of Endocrine Disrupters



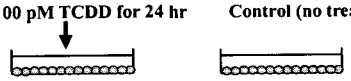
Identification of tissue specific genes

- Northern blotting, quantitative RT-PCR.
- Differential Display
- DNA Microarray.
- SAGE (Serial Analysis of Gene Expression) .
- Subtraction cloning.
  - ①Hydroxyl appatite method
  - ② Directed cDNA library method
  - ③ PCR-subtraction.
    - a) SSH (Suppressive Subtractive Hybridization).
    - b) MOS-SSH (Mirror Orientation Selection-SSH).
    - c) RDA (Representational Difference Analysis).

Identification of genes induced or suppressed by low concentrations of Dioxin in rat granulosa cells

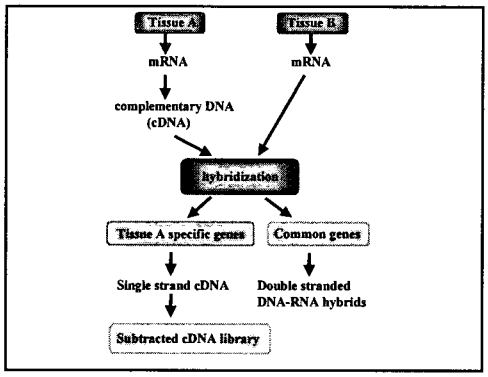
Primary culture of rat ovarian granulosa cells

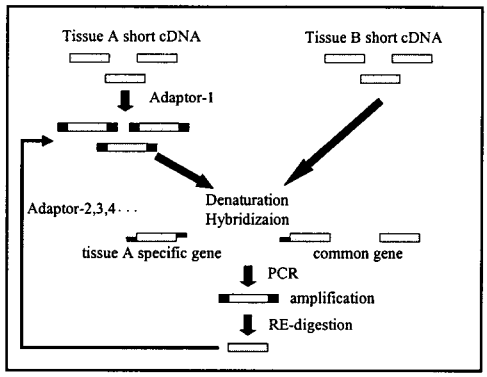
100 pM TCDD for 24 hr | Control (no treatment)

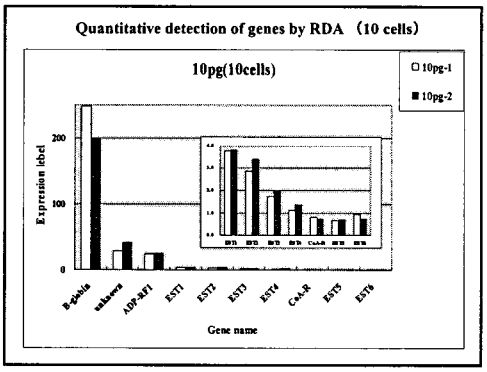


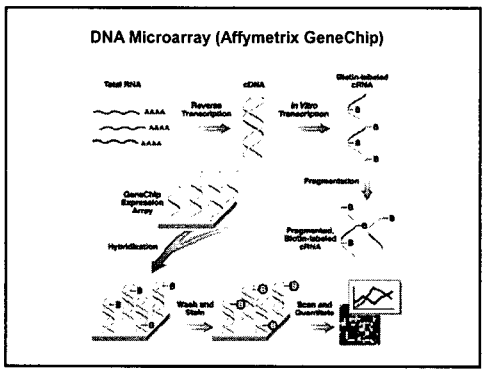
(A) (B)

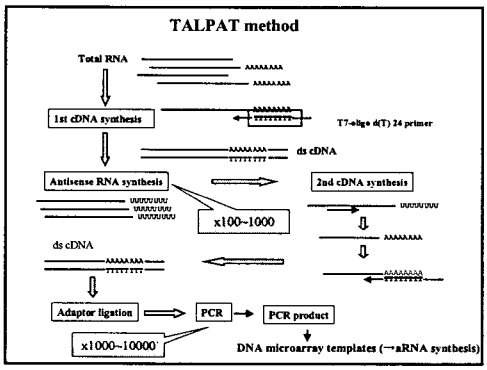
Subtraction cloning
genes(A) minus genes(B) : TCDD inducible genes
genes(B) minus genes(A) : TCDD suppressive genes

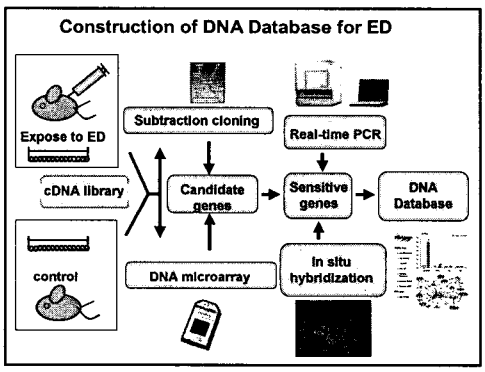

Tissue A
Tissue B
mRNA
mRNA
complementary DNA
(cDNA)
hybridization
Tissue A specific genes
Common genes
Single strand cDNA
Double stranded
DNA-RNA hybrids
Subtracted cDNA library

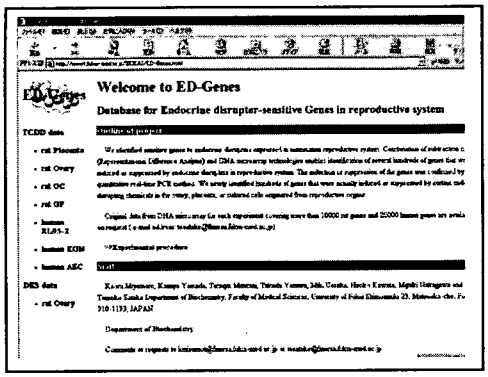

Tissue A short cDNA
Tissue B short cDNA
Adaptor-1
Adaptor-2,3,4···
Denaturation
Hybridizaion
tissue A specific gene
common gene
PCR
amplification
RE-digestion

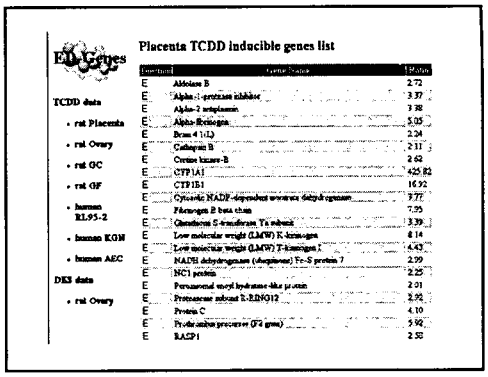

Quantitative detection of genes by RDA (10 cells)
10pg(10cells)
10pg-1
10pg-2
Expression label
200
100
0
Gene name

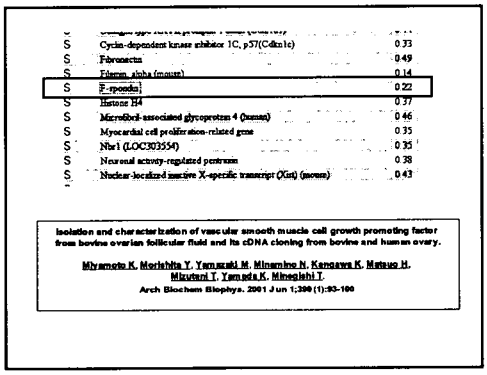

DNA Microarray (Affymetrix GeneChip)

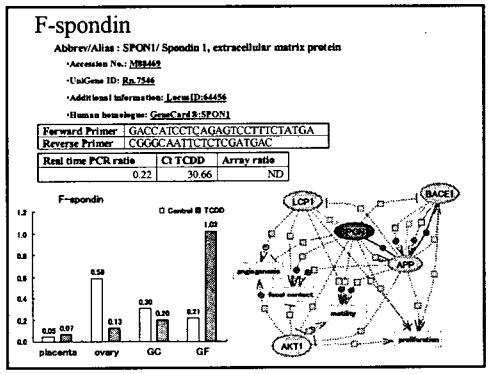

TALPAT method
Total RNA
1st cDNA synthesis
T7-oligo d(T) 24 primer
ds cDNA
Antisense RNA synthesis
2nd cDNA synthesis
x100~1000
ds cDNA
Adaptor ligation
PCR
PCR product
x1000~10000
DNA microarray templates (→aRNA synthesis)

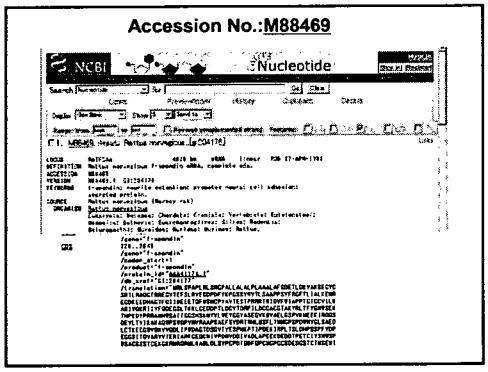

Construction of DNA Database for ED
Expose to ED
Subtraction cloning
Real-time PCR
cDNA library
Candidate genes
Sensitive genes
DNA Database
control
DNA microarray
In situ hybridization

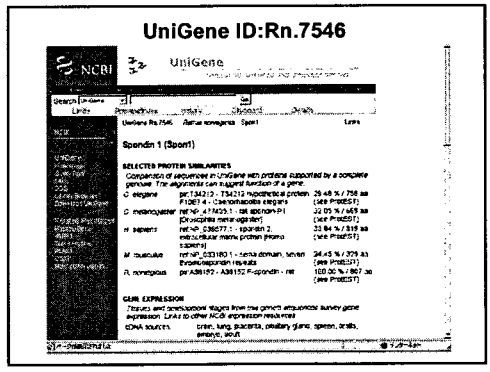

Welcome to ED-Genes
Database for Endocrine disrupter-sensitive Genes in reproductive system
TCDD data
• rat Placenta
• rat Ovary
• rat GC
• rat GF
• human RL95-2
• human EOM
• human AEC
DES data
• rat Ovary

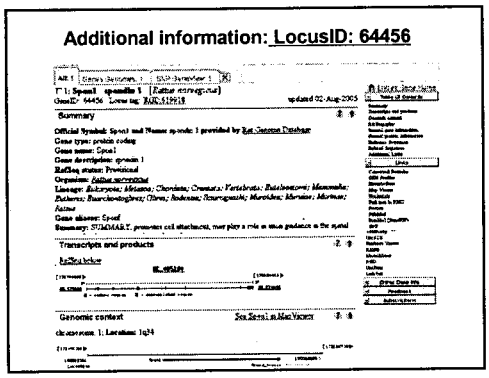

Placenta TCDD inducible genes list
TCDD data
• rat Placenta
• rat Ovary
• rat GC
• rat GF
• human RL95-2
• human EOM
• human AEC
DES data
• rat Ovary

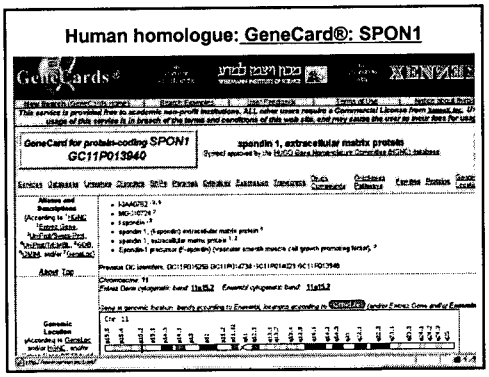

Cyclin-dependent kinase inhibitor 1C, p57(Cdkn1c) 0.33
Fibronectin 0.49
Fibrinogen, alpha (mouse) 0.14
F-spondin 0.22
Histone H4 0.37
Microfibril-associated glycoprotein 4 (human) 0.46
Myocardial cell proliferation-related gene 0.35
Nbr1 (LOC303554) 0.35
Neuronal activity-regulated pentraxin 0.38
Nuclear-localized inactive X-specific transcript (Xist) (mouse) 0.43
Isolation and characterization of vascular smooth muscle cell growth promoting factor from bovine ovarian follicular fluid and its cDNA cloning from bovine and human ovary.
Miyamoto K, Morishita Y, Yamazaki M, Minamino N, Kangawa K, Matsuo H, Mizuno T, Yamada K, Hineglahl T.
Arch Biochem Biophys. 2001 Jun 1;390 (1):93-100

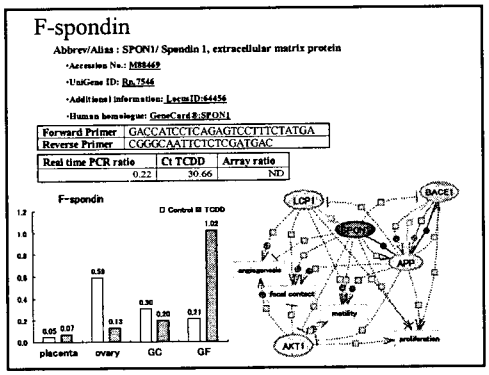

F-spondin
Abbrev/Alias : SPON1/ Spondin 1, extracellular matrix protein
•Accession No.: M88469
•UniGene ID: Rn.7546
•Additional information: LocusID:64456
•Human homologue: GeneCard S:SPON1
Forward Primer GACCATCCTCAGAGTCCTTTCTATGA
Reverse Primer CGGGCAATTCTCTCGATGAC
Real time PCR ratio 0.22
Ct TCDD 30.66
Array ratio ND
F-spondin
placenta ovary GC GF
LCP1
BACE1
APP
AKT1
proliferation

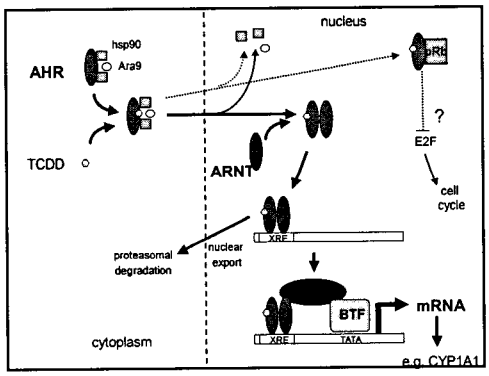

Accession No.:M88469
NCBI
Nucleotide

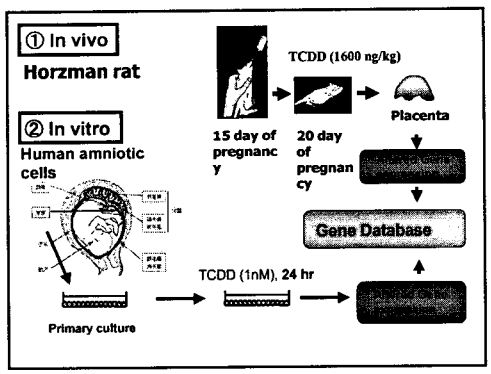

UniGene ID:Rn.7546
NCBI
UniGene
Spondin 1 (Spon1)

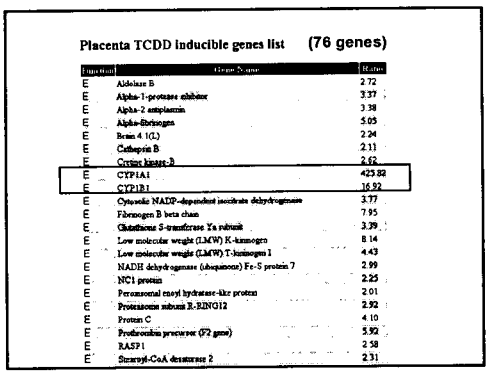

Additional information: LocusID: 64456
Summary
Transcripts and products
Genomic context


Human homologue: GeneCard®: SPON1
GeneCards
GeneCard for protein-coding SPON1
GC11P013940
spondin 1, extracellular matrix protein


F-spondin
Abbrev/Alias : SPON1/ Spondin 1, extracellular matrix protein
Forward Primer GACCATCCTCAGAGTCCTTTCTATGA
Reverse Primer CGGGCAATTCTCTCGATGAC
Real time PCR ratio Ct TCDD Array ratio
0.22 30.66 ND
F-spondin
placenta ovary GC GF
LCP1
BACE1
SPON1
APP
AKT1
motility
proliferation

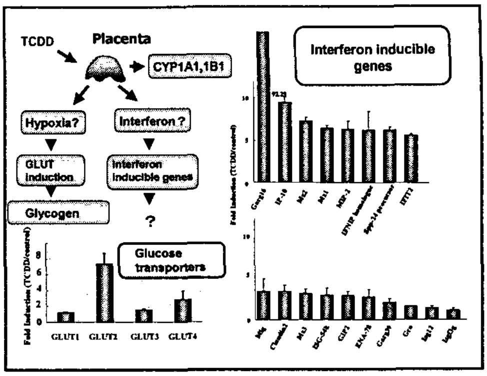

nucleus
hsp90
Ara9
AHR
TCDD
ARNT
pRb
E2F
cell cycle
XRE
proteasomal degradation
nuclear export
BTF
mRNA
TATA
cytoplasm
e.g. CYP1A1


① In vivo
Horzman rat
② In vitro
Human amniotic cells
TCDD (1600 ng/kg)
Placenta
15 day of pregnancy
20 day of pregnancy
Gene Database
TCDD (1nM), 24 hr
Primary culture


Placenta TCDD inducible genes list (76 genes)
Ligand Gene Name Ratio
E Aldolase B 2.72
E Alpha-1-protease inhibitor 3.37
E Alpha-2 antiplasmin 3.38
E Alpha-fibrinogen 5.05
E Brain 4.1(L) 2.24
E Cathepsin B 2.11
E Cytoic kinase-B 2.62
E CYP1A1 423.82
E CYP1B1 16.92
E Cytosolic NADP-dependent isocitrate dehydrogenase 3.77
E Fibrinogen B beta chain 7.95
E Glutathione S-transferase Ya subunit 3.39
E Low molecular weight (LMW) K-kininogen 8.14
E Low molecular weight (LMW) T-kininogen 1 4.43
E NADH dehydrogenase (ubiquinone) Fe-S protein 7 2.99
E NC1 protein 2.25
E Peroxisomal enoyl hydratase-like protein 2.01
E Proteasome subunit R-RING12 2.92
E Protein C 4.10
E Prothrombin precursor (F2 gene) 5.92
E RASP1 2.58
E Stearoyl-CoA desaturase 2 2.31

TCDD inducible genes in rat placenta

Tf	Zinc finger homeobox 1b (mouse)	2.99
St	Beta ig-h3	2.73
St	CXC chemokine LIX	2.60
St	Inhibin alpha-subunit	1.98
St	Interferon beta	5.07
St	Interferon inducible protein 10	9.48
St	Macrophage inflammatory protein-2	6.29
St	Monokine induced by gamma interferon	3.28
St	Prolactin-related protein	1.82
Tr	Alpha-fetoprotein	5.47
Tr	Apolipoprotein A-I	6.26
Tr	Apolipoprotein A-IV	4.24
Tr	Apolipoprotein B	7.07
Tr	Apolipoprotein H	6.28
Tr	Beta-globin	3.71
Tr	GLUT2	7.39
Tr	GLUT4	2.84
Tr	Retinol-binding protein	5.39
Tr	Transferrin	4.12
S	Angiopoietin-related protein 3 (mouse)	8.90
S	Claudin 2 (mouse)	3.27
S	Collagen alpha 1 type XI	2.60
S	Collagen type XXVII proalpha 1 chain (Col27a1)	1.98
S	Decorin	2.07
S	Glucocorticoid-attenuated response gene 16 product	92.29
S	Hypothetical protein RMT-7	2.09



Identification of genes induced or suppressed by low concentrations of Dioxin in rat granulosa cells

Primary culture of rat ovarian granulosa cells

100 pM TCDD for 24 hr　　Control (no treatment)

(A)　　(B)

Subtraction cloning

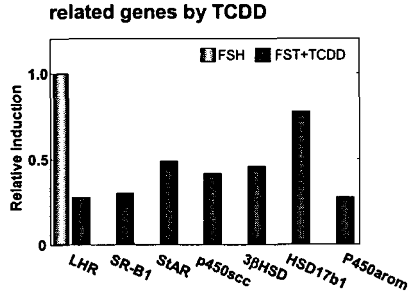
genes(A) minus genes(B) : TCDD inducible genes

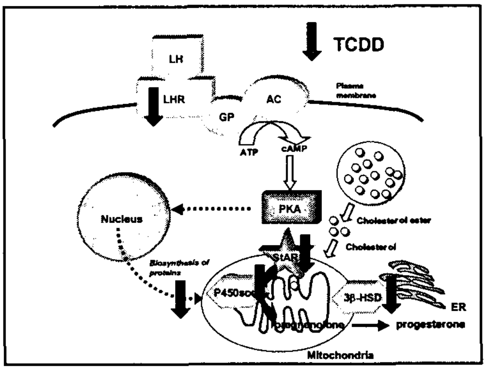
genes(B) minus genes(A) : TCDD suppressive genes

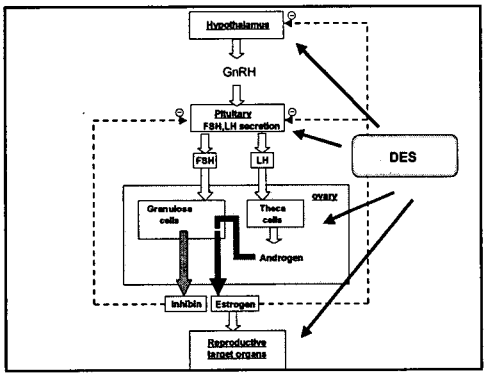
GF TCDD suppressive genes list (24 Genes)

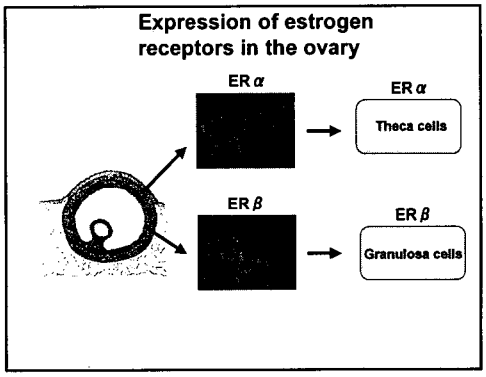
Function	Gene Name	Ratio
E	3Beta-HSD II	0.36
E	*ADP-ribosyltransferase (NAD+,poly(ADP-ribose)polymerase)2	*0.33
E	*Aldehyde dehydrogenase 1, subfamily A1	*0.28
E	*Aromatase	*
E	*C1galt1	*0.25
E	DNA-directed RNA polymerase II largest subunit (LOC363633)	0.49
E	Glutathione S-transferase Ya subunit	0.46
E	*P450SCC	*
E	Peptidyl-glycine alpha-amidating monooxygenase	0.49
E	*Stearoyl-CoA desaturase 2	*0.37
E	*UDP-glucuronate decarboxylase	*0.40
Tf	*Zinc finger homeobox 1b (mouse)	*0.27
St	*Cation-dependent mannose-6-phosphate receptor (M6pr)	*0.32
St	Inhibin alpha-subunit	0.41
St	Lutropin-choriogonadotropic hormone receptor	0.52
Tr	Scavenger receptor class B type I	0.15
Tr	*Solute carrier family 23 (nucleobase transporters),member 2 (mouse)	*0.44
Tr	Steroidogenic acute regulatory protein	0.49
S	*CIIDBP	*0.36
U	*Ekar ras-associated intracisternal-A	*0.08

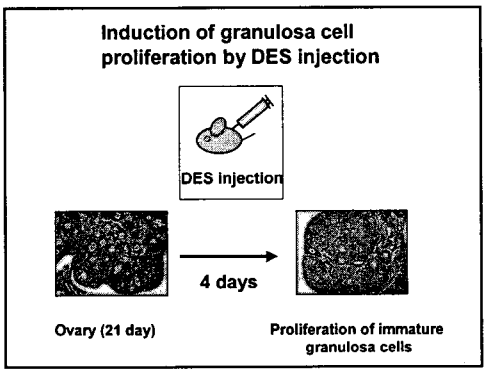
Suppression of steroidogenesis related genes by TCDD





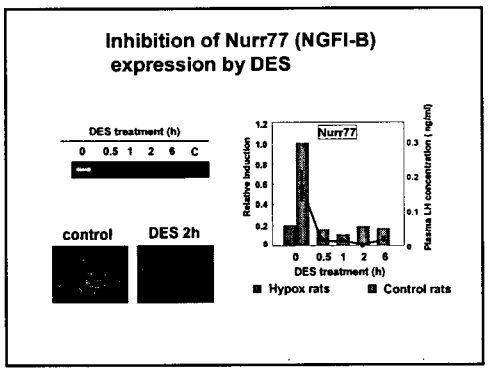


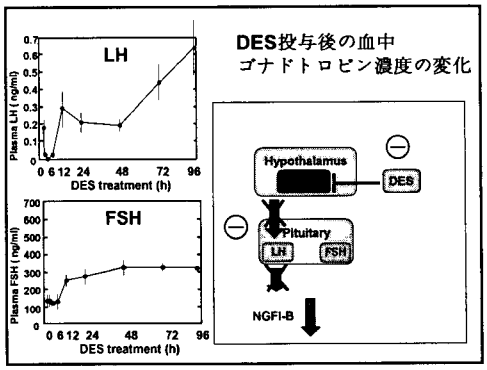




DES suppressive genes (24 Genes)

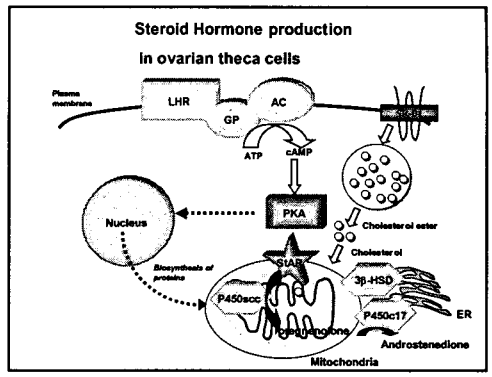
Gene Name	Ratio
Immediate early gene transcripton factor NGFI-B (Nur77)	0.19
DAX-1	*0.06
17-alpha hydroxylase (CYP17)	*0.05
Aromatase cytochrome P450	*0.07
Cholesterol side-chain cleavage enzyme mRNA (P450SCC)	*0.07
Luteinizing hormone/choriogonadotropin receptor (Lhcgr)	*0.40
Scavenger receptor class B type I	*0.03
Steroidogenic acute regulatory protein	*0.09
5-aminolevulinate synthase	0.20
Adrenodoxin	0.43
BM1 or1 MHC class Ib antigen, strain DA	0.39
Clone ROVDV15	0.42
Clone RPCAT02	0.44
C-type natriuretic peptide	0.47
Gonadotropin inducible ovarian transcripton factor 2 beta (GIOT2beta)	0.45
Heat shock protein 70	0.44
Interferon regulatory factor 7 (LOC293624)	0.47
MHC class I RT1 C/E	0.49
N-acetyltransferase CML5 (Cml5)	0.23
Nerve growth factor-induced (NGFI-A)	0.47
Phosphoenolpyruvate carboxykinase (GTP)	0.51

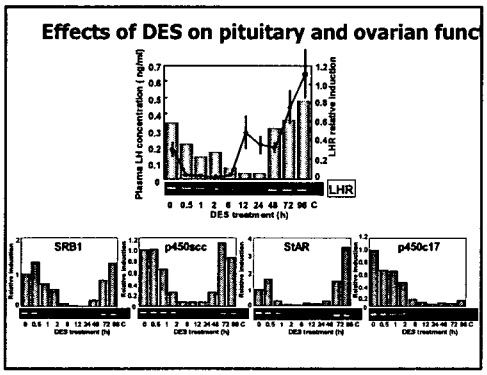


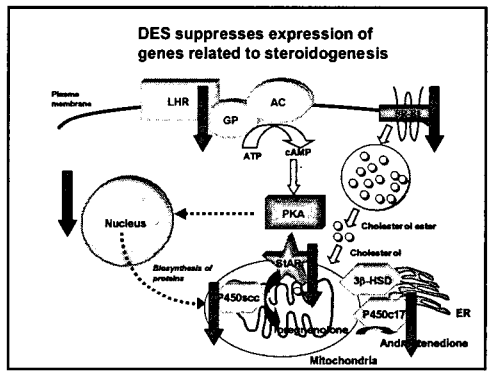


DES suppressive genes (24 Genes)

Gene Name	Ratio
Immediate early gene transcription factor NGFI-B (Nur77)	0.19
DAX-1	*0.06
17-alpha hydroxylase (CYP17)	*0.05
Aromatase cytochrome P450	*0.07
Cholesterol side-chain cleavage enzyme mRNA (P450SCC)	*0.07
Luteinizing hormone/choriogonadotropin receptor (Lhcgr)	*0.40
Scavenger receptor class B type I	*0.03
Steroidogenic acute regulatory protein	*0.09
5-aminolevulinate synthase	0.20
Adrenodoxin	0.43
BM1#1 MHC class Ib antigen, strain DA	0.39
Clone ROVDV15	0.42
Clone EPCAT02	0.44
C-type natriuretic peptide	0.47
Gonadotropin inducible ovarian transcription factor 2 beta (GIOT2beta)	0.45
Heat shock protein 70	0.44
Interferon regulatory factor 7 (LOC293624)	0.47
MHC class I RT1.C/E	0.49
N-acetyltransferase CML5 (Cml5)	0.23
Nerve growth factor-induced (NGFI-A)	0.47
Phosphoenolpyruvate carboxykinase (GTP)	0.51







DES inducible genes

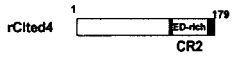
Gene Name	Ratio
3-beta-hydroxysteroid dehydrogenase/delta-5-delta-4 isomerase type II	*1.55
Cyclin D2	*1.68
Follicle stimulating hormone receptor (Fshr)	*1.68
Hydroxysteroid (17-beta) dehydrogenase 1 (Hsd17b1)	1.83
Inhibin alpha-subunit	*1.77
Inhibin beta-A (Activin beta-A)	*1.42
Inhibin beta-B (Activin beta-B)	*3.86
Vascular endothelial growth factor	*1.86
Cited4	3.54
Cytosolic branched chain aminotransferase (Bcatc gene)	3.39

DES suppressive genes

Gene Name	Ratio
Immediate early gene transcription factor NGFI-B (Nur77)	0.19
DAX-1	*0.06
17-alpha hydroxylase (CYP17)	0.06
Aromatase cytochrome P450	0.24

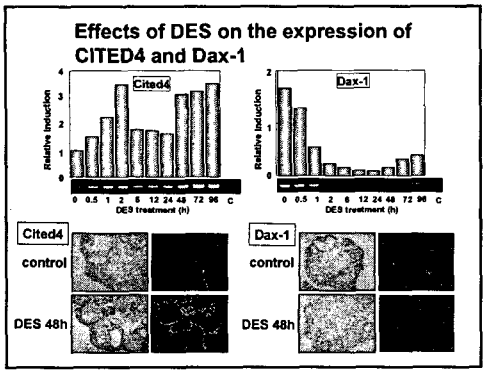
Cited-4

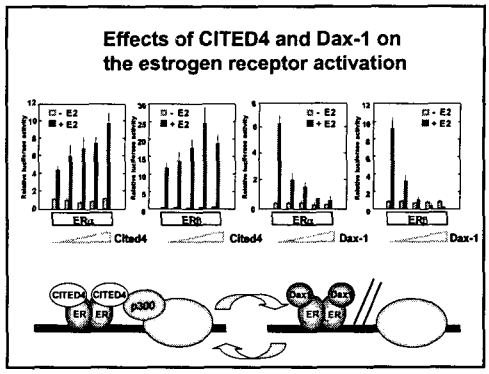
(CBP/p300-interacting transactivator with ED-rich tail (Cited) 4)

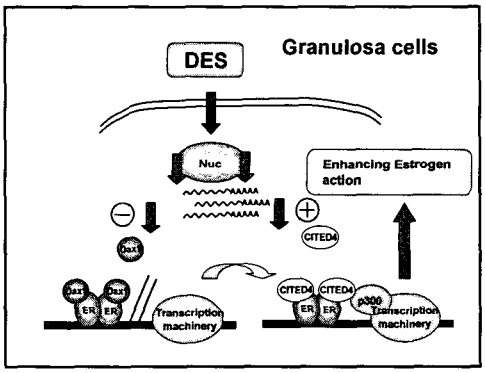


Dax-1

(Dosage-sensitive sex reversal, Adrenal hypoplasia congenita, critical region on X-chromosome, gene-1)







DES inducible genes

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Inhibin beta-B (Activin beta-B)	*3.86
Vascular endothelial growth factor	*1.86
Cited4	3.54
Cytosolic branched chain aminotransferase (Bcatc gene)	3.39
Hypoxia inducible factor 2 alpha (Hif-2a gene)	2.12
Serine threonine kinase (pim-3)	1.92