# 2. 5. エストロゲン受容体の神経幹細胞における発現

身のまわりの化学物質のうち，ホルモン活性が報告されているものにはエストロゲンアゴニスト様作用，あるいはアンドロゲンアンタゴニスト作用を有するものが多いことが知られている．特にエストロゲン受容体系は，ピコモル($10^{-12}$ M)の低い濃度域で作動することから，外因性影響を受ける可能性が高いことが考えられる．われわれは，エストロゲン受容体を重要な標的として位置づけ，まず神経幹細胞での発現を検討した．神経幹細胞が豊富な胎生14.5日のマウス終脳を分離し(図2・3，口絵8参照)，ニューロスフェアを形成させ，RNAを抽出した後，RT-PCRによってエストロゲン受容体α型およびβ型のmRNA発現を調べた．図2・4(口絵9参照)に示すように，どちらの型の受容体もニューロスフェア内で発現していることが確認された．なお，データは示さないが，同じRNAにおけるNestinの発現も検出されている．さらに，α型についてはタンパク質レベルでもニューロスフェア細胞に発現していることが確認されている(図2・4B)．これらのことから，前述のラットでの報告[17]とともに，エストロゲン受容体は胎児神経幹細胞において発現していることが強く示唆される．この事実は，内分泌撹乱化学物質が胎児神経幹細胞に対して何らかの作用を発揮する可能性を示唆するものである．

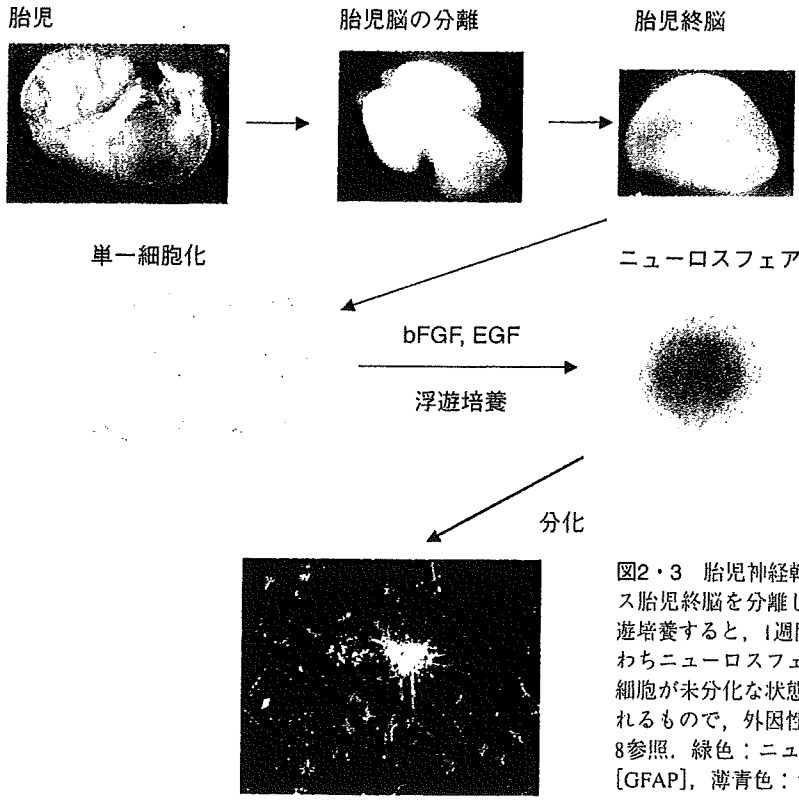


図2・3 胎児神経幹細胞の培養(ニューロスフェア法)．マウス胎児終脳を分離し単一細胞化した後，bFGF, EGF存在下浮遊培養すると，1週間ほどで単クローン性の細胞凝集体，すなわちニューロスフェアが得られる．ニューロスフェアは単一細胞が未分化な状態を保ったまま自己複製することで形成されるもので，外因性刺激により，神経系の3種類の細胞(口絵8参照．緑色：ニューロン[MAP2]，赤色：アストロサイト[GFAP]，薄青色：オリゴデンドロサイト[O4])に分化する．

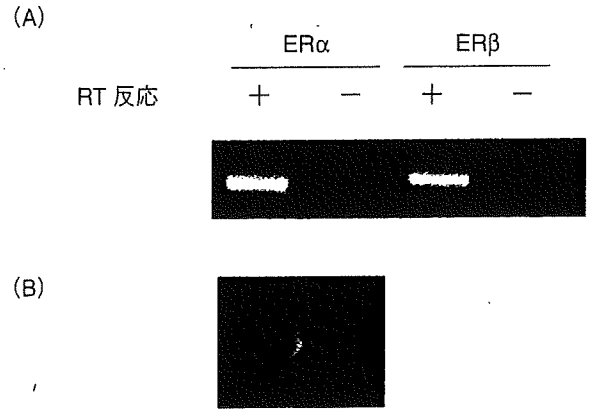


図2・4　神経幹細胞におけるERα，ERβの発現．(A) mRNA検出．ニューロスフェアより抽出した全RNAをオリゴdTで逆転写し，ERα，ERβ特異的なプライマーでPCRした．ERα，ERβともにニューロスフェアに発現していることが示された．(A)タンパク質検出．ニューロスフェアをNestin((口絵9参照：緑色)，ERα(口絵9参照：赤色)に対する抗体で免疫染色した．Nestin陽性のニューロスフェアでERαの発現が検出された．(B)タンパク質検出．ニューロスフェアをNestin(緑色)，ERα(赤色)に対する抗体で免疫染色した．Nestin陽性のニューロスフェアでERαの発現が検出された．

## 2．6．内分泌撹乱化学物質の神経幹細胞分化・増殖に対する影響をモニターする方法
### ――神経系細胞種特異的マーカーとニューロスフェア法――

神経幹細胞に対する影響を調べるためには，脳内または培養下で神経幹細胞を含む神経系の細胞を正確に同定する技術と，神経幹細胞の増殖能・分化能を評価する技術が必要である．

前者の目的には，神経幹細胞および分化した細胞に特異的なマーカータンパク質が利用されている(図2・2参照)．神経幹細胞のマーカーとして汎用されているのはNestinであり，ニューロンはMAP2，アストロサイトはGFAP，オリゴデンドロサイトはO4が用いられている．Nestinは中枢神経系前駆細胞の中間径線維タンパク質の主要な構成成分である．その神経幹細胞における特異的な発現を利用し，岡野らはNestinのプロモーター下流にEGFPを接続したトランスジェニックマウスを用い，神経幹細胞をFACS(fluorescence activated cell sorting)により分離できることを報告している[18]．MAP2は，ニューロンの中間径線維タンパク質に結合し，中間径線維タンパク質の集合を促進することが知られているタンパク質である．GFAPは中枢神経系でおもにアストロサイトに発現が検出される中間径線維タンパク質である．O4はI型およびII型のオリゴデンドロサイトによって形成されるスルファチド(硫脂質)で，ミエリンの構成脂質である．

一方，後者の目的である神経幹細胞の増殖・分化能を評価する方法としては，1)単一細胞から細胞凝集塊(ニューロスフェア)を形成する自己複製能を調べる方法(ニューロスフェア法)や，2)ニューロスフェアの多分化能を調べる方法がある．以下にそれらの方法の概略を示す．

1) 神経幹細胞の機能の1つである自己複製能を反映する指標であるニューロスフェアの大きさの検討：胎児終脳(胎生11.5日から14.5日曝露)からbFGF，EGF存在下，1週間培養することにより形成されるニューロスフェアについて，その径の分布を計測する．溶媒対照群で直径200 μm以上のものが20%程度存在する条件下で，曝露群との比較を行う．
2) 神経幹細胞の分化能に対する胎内曝露の影響の検討：胎内曝露胎児由来(胎生11.5日から14.5

日)のニューロスフェアを20個取り，容器に接着させ血清存在下1週間培養し，MAP2，GFAP，O4の抗体を用いた三重染色によってニューロスフェアの3系統への分化を半定量的に測定する．溶媒対照群では，NAO(Neuron，Astrocyte，Oligodendrocyte)，すなわち，3種類の細胞すべてに分化するニューロスフェアが90%を占める条件下で，検体投与群におけるNAOの割合の変化，2種類の細胞にしか分化しないものや，1種類の細胞にのみ分化するものの割合の変化をもって神経幹細胞分化能への影響とする．

## 2. 7. DESの神経幹細胞自己複製能に対する影響

われわれはDES(ジエチルスチルベストロール)を内分泌撹乱化学物質のモデル化合物に選び(DESは17β - エストラジオールと同等のエストロゲン活性を有するが，血漿タンパク質との結合が比較的弱いことが知られ，そのため胎児影響が強調されることが考えられる)，その胎生期曝露による神経幹細胞への影響を検討した．

神経幹細胞がさかんに増殖する時期であると同時にそれらにエストロゲン受容体や，Hoxをはじめとする分節マーカーが順次発現する胎生11.5日から14.5日の神経管形成中期におけるエストロゲン影響を検討する目的で，母親体重1 kgあたり2 μgのDESを母獣の皮下に3日間反復投与した．胎生15日目に胎児終脳を採取し，前述の方法によりニューロスフェアを得た．

まず，神経幹細胞の機能の1つである自己複製能を反映する指標であるニューロスフェアの大きさについて検討した結果，図2・5に示すように，DESに曝露された胎児終脳から形成されたニューロスフェアはその径が小さく，溶媒対照群でみられるような直径300 μmを超えるサイズのものは形成されないことが明らかとなった．この結果はDESが胎児脳において神経幹細胞の自己複製能に影響する可能性があることを示唆するものであると考えられる．

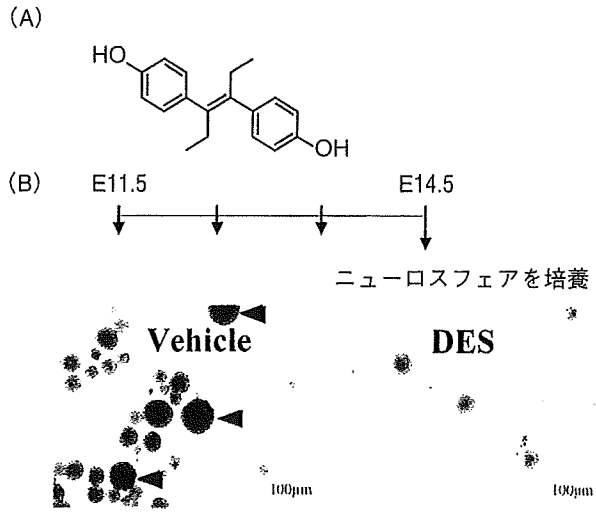


図2・5　胎生期神経幹細胞に対するDESの影響．(A) DESの化学構造，(B)胎生期DES *in utero*曝露による胎児終脳神経幹細胞に対する影響の検討．DESをマウス胎生11.5日から14.5日まで連日2 μg/kg dam body weight/day投与し，胎児終脳からニューロスフェアを得た．DES投与群では溶媒対照投与群にみられるような径の大きいニューロスフェアの形成が阻害されていた．

## 2. 8. DESの神経幹細胞分化に対する影響

次に，DES曝露胎児由来のニューロスフェアの分化能について検討した(図2・6)．DES曝露胎児由来のニューロスフェアを20個取り，容器に接着させ血清存在下1週間培養し，MAP2，GFAP，O4の抗体を用いた三重染色によって分化程度を検討した．Vehicle投与群では，NAO，すなわち，3種類の細胞すべてに分化するニューロスフェアが90%を占めたのに対し，DES 0.2 μg/kg以上投与した群ではNAOの割合は低下し，2種類の細胞にしか分化しないものや，1種類の細胞にのみ分化するものの割合が増えていた．特に，2 μg/kg投与群ではアストロサイトにのみ分化するものが40%以上を占めた．

以上のことから，胎内DES曝露により，胎児の神経幹細胞の自己複製能および分化能の両方に影響が生じることが明らかとなった．殊に，胎児脳から細胞を分離し，ニューロスフェアを得る1週間の培養期間中の培養液中にはDESが存在していないにもかかわらず，これらの影響が観測されたことから，DESの神経幹細胞に対する作用は一過性のものではなく持続する性質のものであることが示唆された．そのメカニズムは現在不明であるが，1週間の培養期間中には，神経幹細胞はおおよそ10回の分裂を繰り返していると計算されることから，われわれはDNAメチル化などのエピジェネティックなメカニズムが関与している可能性を考えている．

## 2. 9. まとめ

以上紹介してきたように，内分泌撹乱化学物質は発生・発達の過程で，内分泌系，免疫系と共に

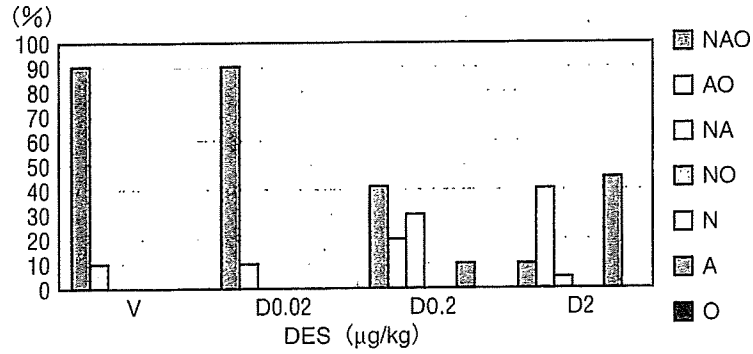


図2・6　DESの神経幹細胞分化に対する影響．図2・5と同様のスケジュールでDESに曝露した胎児終脳から形成されたニューロスフェアの分化能を調べた．bFGF，EGF存在下で未分化状態を保って浮遊培養したニューロスフェアを，コーティングした容器に移し，ウシ胎仔血清1%を加え1週間培養することで分化させた結果をグラフにまとめた．グラフ中の略号は以下を示す．N：ニューロン，A：アストロサイト，O：O4．たとえば，NAOはニューロン(N)，アストロサイト(A)，オリゴデンドロサイト(O)の3種類の細胞に分化したニューロスフェアを示す．Vehicle群(V)およびDES 0.02 μg/kg/day群(D0.02)では，3種類の細胞に分化したニューロスフェア(NAO)が90%を占めたのに対し，DES 0.2 μg/kg/day(D0.2)以上の群では，3種類の細胞に分化するニューロスフェアの割合は減り，アストロサイトにのみ分化するもの(A)の割合が増える傾向があった．

神経系，殊にその基幹となる神経幹細胞の機能にも影響を与える可能性があり，その影響を具体的に検討する研究が徐々に展開して来ている．エストロゲン受容体が神経幹細胞に発現していること，オーファン受容体であるTLXが成体神経幹細胞に発現し，少なくとも成体においてはその未分化性の維持に必須であること，甲状腺ホルモン$T_3$が神経幹細胞からオリゴデンドロサイトへの分化を促進しうることなどが，内分泌撹乱化学物質の神経幹細胞への影響の可能性を具体的に示しつつある例である．しかし，その影響が実際にどのような表現形として，何時どこに現れるのかは現在不明な点が多い．よって，これらの影響の分子メカニズムに踏み込んだ解析から表現型を見定めていく方策と，器質的変化としては捕らえにくい行動異常などの機能障害を詳細に検討する方策との両面からのアプローチが今後重要となろう．分子メカニズムの面からは，一時的な曝露によるエピジェネティックな変化が，何回もの細胞分裂を経た後にまで影響を及ぼす可能性が示唆されており，機能障害の詳細の解明とともにリスク評価の観点からも重要な毒性学的検討課題の1つであると考える．

## 参考文献


1. Reynolds, B.A., W. Tetzlaff, and S. Weiss, A multipotent EGF-responsive striatal embryonic progenitor cell produces neurons and astrocytes. J Neurosci, 1992. **12**(11): p. 4565-74.
2. McKay, R., Stem cells in the central nervous system. Science, 1997. **276**(5309): p. 66-71.
3. Nakashima, K., Yanagisawa, M., Arakawa, H., Kimura, N., Hisatsune, T., Kawabata, M., Miyazono, K., Taga, T., Synergistic signaling in fetal brain by STAT3-Smad1 complex bridged by p300. Science, 1999. **284**(5413): p. 479-2.
4. Suslov, O., Kukekov, V., Ignatova, T., and Steindler, D. (2002). Neural stem cell heterogeneity demonstrated by molecular phenotyping of clonal neurospheres. Proc Natl Acad Sci U S A ***99***, 14506-14511.
5. Hitoshi, S., Tropepe, V., Ekker, M., and van der Kooy, D., Neural stem cell lineages are regionally specified, but not committed, within distinct compartments of the developing brain. Development. 2002 Jan;129(1):233-44, 2002. **129**: p. 233-244.
6. Qian, X., Shen, Q., Goderie, S., He, W., Capela, A., Davis, A., and Temple, S., Timing of CNS cell generation: a programmed sequence of neuron and glial cell production from isolated murine cortical stem cells. Neuron, 2000. **28**: p. 69-80.
7. Reynolds, B.A. and S. Weiss, Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. Science, 1992. **255**(5052): p. 1707-10.
8. Temple, S. and A. Alvarez-Buylla, Stem cells in the adult mammalian central nervous system. Curr Opin Neurobiol., 1999. **9**: p. 135-141.
9. Gage, F., Mammalian neural stem cells. Science. 2000, 2000. **287**: p. 1433-1438.
10. Gould, E., Reeves, A., Fallah, M., Tanapat, P., Gross, C., and Fuchs, E., Hippocampal neurogenesis in adult Old World primates. Proc Natl Acad Sci U S A., 1999. **96**: p. 5263-5267.
11. Eriksson, P., Perfilieva, E., Bjork-Eriksson, T., Alborn, A., Nordborg, C., Peterson, D., and Gage, F., Neurogenesis in the adult human hippocampus. Nat Med., 1998. **4**: p. 1313-1317.
12. Doetsch, F., Caille, I., Lim, D. A., Garcia-Verdugo, J. M., and Alvarez-Buylla, A., Subventricular zone astrocytes are neural stem cells in the adult mammalian brain. Cell, 1999. **97**(6): p. 703-16.
13. Johansson, C. B., Momma, S., Clarke, D. L., Risling, M., Lendahl, U., and Frisen, J., Identification of a neural stem cell in the adult mammalian central nervous system. Cell, 1999. **96**(1): p. 25-34.
14. Shi, Y., Chichung Lie, D., Taupin, P., Nakashima, K., Ray, J., Yu, R. T., Gage, F. H., and Evans, R. M., Expression and function of orphan nuclear receptor TLX in adult neural stem cells. Nature, 2004. **427**(6969): p. 78-83.

15. Ciana, P., Ghisletti, S., Mussi, P., Eberini, I., Vegeto, E., and Maggi, A., Estrogen receptor alpha, a molecular switch converting transforming growth factor-alpha-mediated proliferation into differentiation in neuroblastoma cells. J Biol Chem, 2003. **278**(34): p. 31737-44.

16. Tanapat, P., Hastings, N. B., Reeves, A. J., and Gould, E., Estrogen stimulates a transient increase in the number of new neurons in the dentate gyrus of the adult female rat. J Neurosci, 1999. **19**(14): p. 5792-801.

17. Brannvall, K., L. Korhonen, and D. Lindholm, Estrogen-receptor-dependent regulation of neural stem cell proliferation and differentiation. Mol Cell Neurosci, 2002. **21**(3): p. 512-20.

18. Sawamoto, K., et al., Generation of dopaminergic neurons in the adult brain from mesencephalic precursor cells labeled with a nestin-GFP transgene. J Neurosci., 2001. **21**: p. 3895-3903.

生体統御システムと内分泌撹乱　　定価（本体5,800円＋税）

発　行　2005年5月27日
編　集　井上 達（いのうえ とおる）／井口 泰泉（いぐち たいせん）
発行者　平野 皓正
発行所　シュプリンガー・フェアラーク東京株式会社
〒113-0033 東京都文京区本郷3丁目3番13号
TEL (03) 3812-0757（営業直通）
印刷所　株式会社シナノ


ISBN 4-431-71112-0 C3045　　http://www.springer-tokyo.co.jp

Research Signpost
37/661 (2), Fort P.O., Trivandrum-695 023, Kerala, India


Carcinogenesis and Modification of Carcinogenesis, 2005: 135-151 ISBN: 81-308-0059-4
Editors: Takuji Tanaka and Hiroyuki Tsuda

# 9 Uterine carcinogenesis based on estrogen or metabolite driven pathways in the Donryu rat

**Midori Yoshida[1] and Akihiko Maekawa[2]**
[1]Department of Pathology, [2]Director, Sasaki Institute, 2-2 Kanda-Surugadai
Chiyoda-ku, Tokyo 101-0062, Japan

## Abstract

*Endometrial adenocarcinomas of the uterine corpus have been increasing in incidence in many developed countries and are a leading cause of cancer death. In the Donryu rat strain, cancers of the endometrium develop spontaneously or can be induced which possess many morphological, endocrinological and molecular similarities to those of human lesions, especially of endometrioid type. The goal of this chapter is to propose 3 different pathways of endometrial carcinogenesis based on mechanisms established using our 2-stage model in the Donryu rat: one driven by a relatively high 17-β*

Correspondence/Reprint request: Dr. Midori Yoshida, Department of Pathology, Sasaki Institute, 2-2 Kanda-Surugadai, Chiyoda-ku, Tokyo 101-0062, Japan. E-mail: midoriy@sasaki.or.jp

*estradiol(E2) status; the second by excess estrogens or estrogenic compounds; and the third by estrogen metabolites or catechol estrogens. In addition, examples of agents exhibiting promotion or inhibition effects on uterine carcinogenesis with each pathway are also presented. The data covered in this chapter provide compelling evidence that the Donryu rat model is a powerful tool for elucidation of mechanisms which can be extrapolated to human uterine cancer.*

# Introduction

Endometrial adenocarcinomas in the uterine corpus have been increasing in women of many developed countries and are now a leading cause of cancer death. With sub-classification based on epidemiological, clinico-pathological and molecular findings, endometrial adenocarcinomas are divided into endometrioid adenocarcinomas and serous carcinomas, the former, most common type, being considered to be estrogen driven [1]. In rodents also, estrogens are considered to play a crucial role in uterine carcinogenesis [2-7]. While naturally occurring endometrial adenocarcinomas are generally very rare in rats, Maekawa and co-workers [8,9] have described a high-incidence of spontaneous uterine endometrial adenocarcinomas in aged Donryu rats and established a rat model with particular morphological and endocrinological similarities to the human case, in particular regarding endometriod adenocarcinomas [1]. This chapter focuses on the analysis of uterine endometrial carcinogenesis based on estrogen or estrogen metabolite driven pathways using the Donryu rat model, with discussion of its implications for human cancer prediction and/or prevention.

# Spontaneous occurring endometrial adenocarcinomas in Donryu rats, and their morphological and endocrinological features

Maekawa and co-workers [8] first found a high-occurrence of spontaneous uterine endometrial adenocarcinomas in aged Donryu rats (Table 1), and their subsequent analyses with this strain have established many morphological and endocrinological similarities to the human case, as described in detail below.

In middle-aged Donryu female rats (approximately 10 months of age, and equivalent to the menopausal phase in women) ovarian dysfunction, characterized by anovulation and consequently abnormal estrous cyclicity, starts to increase with time and most rats eventually show a persistent estrus (PE) status on examination of vaginal cytology (Table 2). Simultaneously, focal atypical hyperplasias of the glandular epithelium begin to develop in the uteri which exhibit morphological similarities to endometrial adenocarcinomas

**Table 1.** Incidence data (%) for histological findings in the uterine, ovarian and vaginal epithelium of Donryu and F344 rats [modified from ref. 5].

| | Strain | |
|---|---|---|
| | Donryu (94)# | F344 (146)# |
| ***Uterus*** | | |
| Adenocarcinoma | 35.1 | 4.1 |
| ***Ovary*** | | |
| Cyst | 23.4* | 8.2 |
| Absence of corpus luteum | 57.4* | 7.5 |
| Absence of follicles | 2.1 | 8.2 |
| Atrophy | 24.5 | 15.8 |
| ***Vagina*** | | |
| Cornification | 23.4* | 7.5 |
| Mucification | 27.7* | 79.5 |

# No. of rats *, Significantly different from F344 rats ($P<0.01$, $\chi 2$-test)

**Table 2.** Sequential change in the incidence of persistent estrus in female Donryu and F344 rats [3].

| Strain | Incidence(%) in rats | | | | | | |
|---|---|---|---|---|---|---|---|
| | 4 | 5 | 6 | 8 | 10 | 12 | 15 (months of age) |
| Donryu | 0 | 17* | 32** | 64** | 87** | 88** | 85** |
| F344 | 0 | 0 | 2 | 0 | 6 | 11 | 4 |

*;**, Significantly different from the F344 case ($p<0.05$ and 0.01, respectively)

and increase in both number and size with time, finally giving rise to a 30 to 50% incidence of adenocarcinomas at 24 months of age [8, 9]. Therefore, the atypical hyperplasias are recognized as precursors of endometrial adenocarcinomas and have been classified into 3 stages, slight, moderate and severe (Figure 1). Slight hyperplasias are characterized by localized proliferation of glandular epithelial cells with no or slight cellular atypia in the endometrium, while moderate examples have moderately atypical cells and severe hyperplasias feature irregular groupings of atypical glandular epithelial cells, often morphologically indistinguishable from those in well-differentiated adenocarcinomas. The adenocarcinomas themselves are diagnosed on the basis of invasion of atypical glandular epithelial cells and are characterized by tubular/glandular growth, with twist or cribiform patterns, into the muscularis and/or the serosa. They are subdivided into well, moderately and poorly differentiated types with reference to the degree of morphological atypia and depth of invasion: limited to the uterus, invading into the serosa and/or the

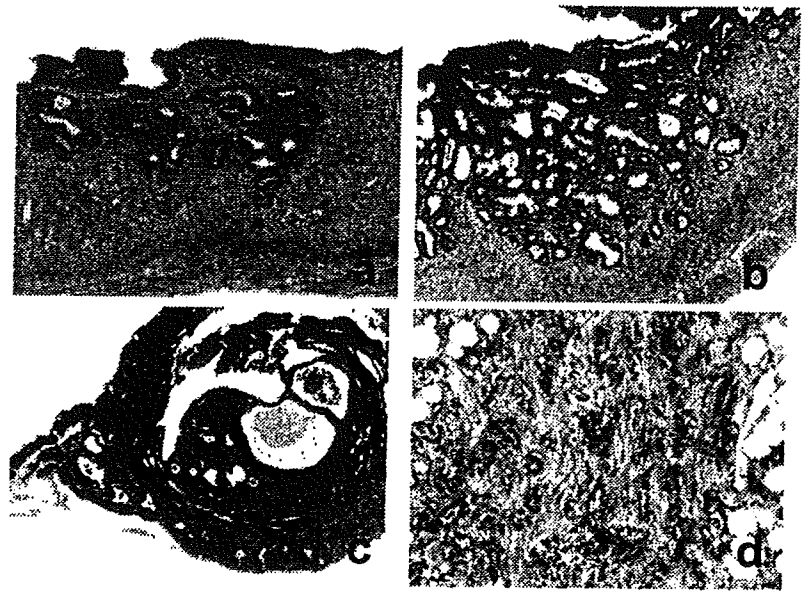


**Figure 1.** Uterine proliferative lesions. a, Atypical hyperplasia, slight. b, Atypical hyperplasia, severe. c, Endometrial adnocarcinoma, well-differentiated. d, Endometrial adenocarcinoma, poorly-differentiated.

surrounding adnexae, and with distant metastasis, in accordance with the simplified FIGO histopathological grades for human uterine cancers [10].

Another characteristic feature related to uterine cancer in this strain is an early occurrence of ovarian hormonal imbalance leading to elevation of serum E2 levels relative to progesterone (P), for example as compared to F344 strain rats [5, 8] (Table 3). This imbalance is morphologically detectable as atrophic ovaries with small polycystic atretic follicles and loss of corpora lutea manifested as PE status in vaginal cytology. PE appears from 4 months of age in Donryu rats and is prevalent in most animals at 11 months of age when other rat strains are still capable of reproduction. Long term exposure to a high E2:P ratio is also known to increase the risk of endometrial adenocarcinoma development in women.

In Donryu rats, ERα is consistently expressed in aged normal epithelia as well as the various degrees of atypical hyperplasia and well- and moderately differentiated adenocarcinomas, and the intensity is similar in all these cases.

**Table 3.** Changes in mean plasma estrogen: progesterone (E2:P) ratios in F344 and Donryu rats [5].

| Strain | No of rats/months | $10^{-3}$xE2/P for rats aged (months) | | | | |
|---|---|---|---|---|---|---|
| | | 1.5 | 6 | 8 | 10 | 12 |
| Donryu | 5-9 | 3.83 | 3.31 | 2.57 | 3.20 | 4.92 |
| F344 | 4-11 | 2.52 | 3.99 | 1.54 | 2.10 | 0.96 |

In addition, expression homogeneous in proliferative lesions. In clear contrast, no antibody binding is detected in any of the poorly-differentiated adenocarcinomas. Thus, the ERα expression in normal endometrial epithelium in the aged uteri, uterine atypical hyperplasias and well-differentiated adenocarcinomas suggests that up-regulation is not necessary for stimulation by estrogens. The loss of ERα in poorly-differentiated adenocarcinomas is linked with estrogen-independent growth of implanted tumors, as has been established for human endometrial adenocarcinomas, with no effects of hormone therapy on advanced malignancies [12, 13].

## Establishment of a uterine endometrial adenocarcinoma model using Donryu rats

Based on the morphological and endocrinological similarities of uterine cancers in Donryu rats to those in women, a 2-stage uterine carcinogenesis model was established by Makeawa and his co-workers to detect promoting or preventive effects of test-chemicals [14]. As the first step of this model (Figure 2), female Donryu rats at 10 or 11 weeks of age are treated with *N-ethyl-N'-nitro-N-nistroguanidine* (ENNG) at the concentration of 20mg/kg dissolved in polyethylene glycol, introduced into a unilateral uterine horn via vagina using a stainless steel catheter for initiation. Then the rats are exposed to test materials for 12 months. At 15 months of age, the animals are sacrificed to determine incidences or multiplicities of uterine neoplastic lesions for comparison with control (ENNG initiation only) data. The intrauterine treatment with a single dose of ENNG results in earlier development and higher yields of endometrial adenocarcinomas, as well as precursor lesions, compared with intact Donryu females not receiving carcinogen (Table 4) [14]. The tumorigenesis is specific to the uterus and no other organs are affected and this 2-stage uterine carcinogenesis model has proven to be useful for detection of promotive or preventive effects of test materials [2, 15-17]. In an attempt to enhance uterine carcinogenesis in this model, ENNG at 10mg/kg body weight

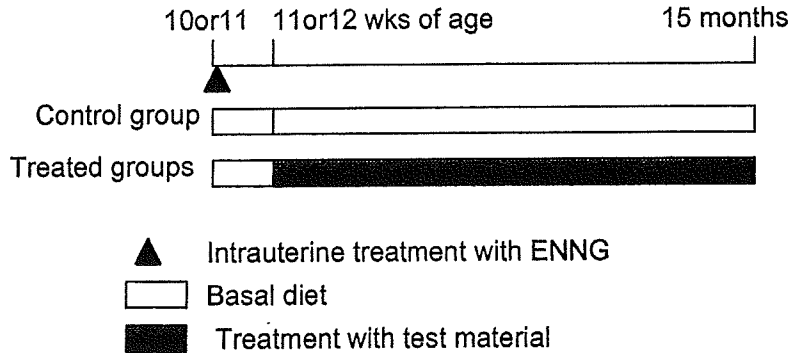


**Figure 2.** Protocol for the 2-stage uterine carcinogenesis model using Donryu rat.

was administered to Donryu rats once a week 4 times. The repeated treatment with ENNG succeeded in increasing the incidence of endometrial adenocarcinomas, but concurrently induced serious bleeding into the lumina of the uteri or abdominal cavity from hematomas or angiosarcomas (Table 5). In addition, associated exfoliation of endometrial epithelium resulted in difficulty of detailed histopathological analysis.

**Table 4.** Sequential changes in the uterine endometrium of Donryu rats after ENNG treatment [Modified ref. 14].

| | 3 | 6 | 9 | 12 | 15(months of age) |
|---|---|---|---|---|---|
| ***Control group*** | | | | | |
| Number of rats examined | 4 | 4 | 4 | 8 | 30 |
| Hyperplasia | | | | | |
| Slight | 0 | 0 | 0 | 1 | 7 |
| Moderate | 0 | 0 | 0 | 0 | 6 |
| Severe | 0 | 0 | 0 | 1 | 0 |
| Adenocarcinoma | 0 | 0 | 0 | 0 | 0 |
| ***ENNG-treated group*** | | | | | |
| Number of rats examined | 6 | 6 | 6 | 8 | 49 |
| Hyperplasia | | | | | |
| Slight | 0 | 1 | 2 | 1 | 7 |
| Moderate | 0 | 0 | 1 | 1 | 7 |
| Severe | 0 | 0 | 1 | 2 | 6 |
| Adenocarcinoma | 0 | 0 | 1 | 4 | 24 |

**Table 5.** Comparison of data for uterine adenocarcinomas and hematomas, and cause of death, with repeated ENNG treatment.

| | Repeated ENNG treatment |
|---|---|
| Number of rats examined | 29 |
| ***Incidence of uterine lesions*** | |
| Uterine hematoma | 9 |
| Endometrial adenocarcinoma | 16 |
| Angiosarcoma | 5 |
| ***Cause of death*** | |
| Uterine hematoma | 8 |
| Endometrial adenocarcinoma | 10 |
| Uterine stromal sarcoma | 3 |
| Uterine angiosarcoma | 4 |
| Uterine choriocarcinoma | 1 |
| Mammary tumor | 1 |
| Leiomyosarcoma in small intestine | 1 |
| Pneumonia | 1 |

## Relatively high E2 status driven uterine carcinogenesis

The simplest pathway for uterine carcinogenesis in the Donryu rat model is relatively high estrogen status (elevated E2:P ratio)-mediated (Figure 3). The early occurrence of ovarian hormonal imbalance leading to elevation of serum E2 levels relative to P as compared to other rat strains, a characteristic of Donryu rats, was described above. This imbalance is morphologically reflected in atrophic ovaries with small polycystic atretic follicles and lack of corpora lutea showing vaginal cornification or a PE status of vaginal cytology. In contrast, other rat strains such as Sprague-Dawley or Fisher 344 rats demonstrate corpus luteum predominant ovaries with increasing age, with vaginal mucification and only low incidences of endometrial adenocarcinomas [5]. This ovarian hormonal imbalance and the associated changes in ovarian morphology with cystic follicles and lack of corpus lutea are crucial for rat uterine carcinogenesis.

One example of promoting effects is provided by concurrent oral administration of ethylenethiourea (ETU) and sodium nitrate, which was found to cause an early occurrence of PE (Table 6) and enhance endometrial adenocarcinoma development at the termination (Table 7) [16]. In this study, the rats were initiated by *N-ethyl-N-nitrosourea* (ENU) and its profile of uterine endometrial adenocarcinoma development was very similar to that initiated with ENNG [16]. As an example of inhibitory effects, long-term dietary treatment with hydroxymatairesinol (HMR), a lignan derived from spruce trees, delayed the occurrence of PE (Table 8) and also exerted an inhibitory effect on uterine carcinogenesis in this rat model (Table 9) [15].

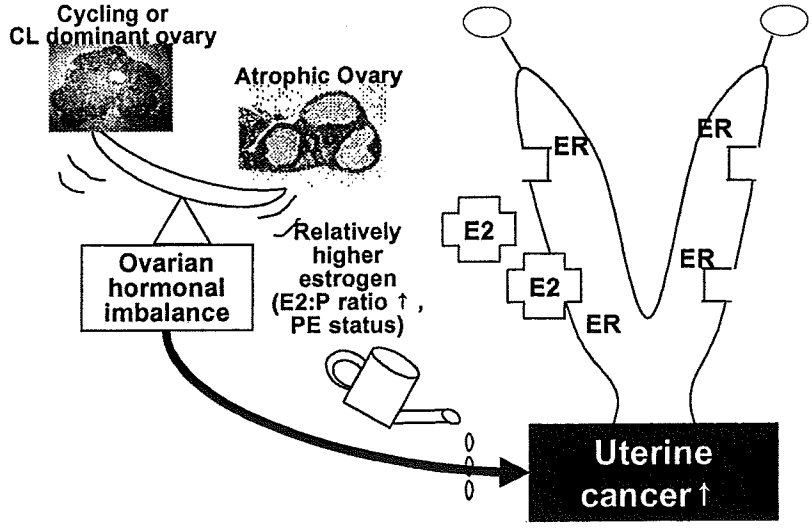


**Figure 3.** Relatively high E2 driven pathway of uterine carcinogenesis using the rat model. CL, Corpus luteum; PE, persistent estrus; E2, 17β-estradiol; P, progesterone; ER, estrogen receptor.

**Table 6.** Sequential changes in the incidence of persistent estrus [Modified ref.16].

| | Incidence (%) of persistent estrus | | | | |
|---|---|---|---|---|---|
| Group | 3 | 4 | 5 | 6 | 7 (months of age) |
| Control | 0 | 0 | 50.0 | 87.5 | 87.5 |
| ETU+sodium nitrate | 0 | 44.4 | 66.6 | 77.8. | 87.5 |
| ETU+sodium nitrate+ENU | 0 | 80.0 | 80.0 | 80.0 | 80.0 |

Control, Oral administration of distilled water once a week by stomach tube without ETU+sodium nitrate, Concurrent oral administration of ETU(80mg/kg) and sodium nitrate (56mg/kg) in distilled water once a week by stomach tube.
ETU+sodium nitrate+ENU, Concurrent oral administration of ETU(80mg/kg) and sodium nitrate (56mg/kg) in distilled water once a week by stomach tube after ENU initiation (15mg/kg) into the uteri instead of ENNG treatment.
Estrous cycles were checked monthly in 8-10 rats in each group by vaginal smear.

**Table 7.** Uterine proliferative lesions at the termination [Modified ref.16].

| Group | Control | ENU | ETU+ Sodium nitrate | ENU+ETU Sodium nitrate |
|---|---|---|---|---|
| No. of rats examined | 21 | 21 | 31 | 37 |
| ***Endometrial hyperplasia*** | | | | |
| Slight | 4 | 1 | 12 | 2 |
| Moderate | 1 | 10* | 6 | 9 |
| Severe | 1 | 1 | 7 | 5 |
| ***Endometrial adenocarcinoma*** | 0 | 6* | 4 | 21** |

Control, Oral administration of distilled water once a week by stomach tube.
ENU, intrauterine treatment with ENU (15mg/kg) instead of ENNG
ETU+sodium nitrate, Concurrent oral administration of ETU(80mg/kg) and sodium nitrate (56mg/kg) in distilled water once a week by stomach tube.
ETU+sodium nitrate+ENU, Concurrent oral administration of ETU(80mg/kg) and sodium nitrate (56mg/kg) in distilled water once a week by stomach tube after ENU initiation (15mg/kg) into the uteri instead of ENNG treatment.
*;**, Significantly different from the control group ($p<0.05$ and $p<0.01$, respectively)

**Table 8.** Delay of persistent estrus by hydroxymatairesinol(HMR) dosing [15].

| Group | Mean week of age of persistent estrus start |
|---|---|
| Control-conventional diet (CRF-1) | 30.3±1.2 |
| Control-basal diet (1324 diet alone) | 32.1±1.3 |
| 200 ppm HMR in basal diet | 35.4±1.6* |
| 600 ppm HMR in basal diet | 35.3±1.6* |

Values are mean±SEM, for n=25, 27, 27, and 26 rats in the groups, respectively. Means of 200 and 600 ppm HMR in basal diet groups are significantly different (*$p<0.05$) from both control values.

**Table 9.** Numbers and incidences of uterine endometrial proliferative lesions at 15 months of age in the four experimental groups [Modified ref. 15].

| | No. of rats examined | Atypical hyperplasia None | Slight | Moderate | Severe | Adenocarcinoma |
|---|---|---|---|---|---|---|
| Control-conventional diet (CRF-1) | 25 | 0 | 2 | 8 | 6 | 9 |
| Control-basal diet (1324 diet alone) | 27 | 2 | 2 | 8 | 7 | 8 |
| 200 ppm HMR in basal diet | 27 | 3 | 5 | 9 | 7 | 3* |
| 600 ppm HMR in basal diet | 26 | 1 | 4 | 12 | 5 | 4* |

Significantly different from both control values (*$p<0.05$).

None, animal bearing no atypical hyperplasia and/or adenocarcinoma

Although precise mechanisms of the promotive or inhibitory effects of these compounds on the ovarian function have yet to be fully determined, these results clearly demonstrate that treatment-related ovarian changes play essential roles. In human beings, anovulatory women with the polycystic ovary syndrome are defined as a high risk group for endometrial cancer [18]. Any factors that might disturb the ovarian hormone balance would be expected to modulate risk of uterine tumorigenesis in women as well as experimental animals.

## Long term exporsure to estrogens or estrogenic compound driven uterine carcinogenesis

A second pathway of uterine carcinogenesis is with long-term with estrogen or estrogenic compound treatment (Figure 4). Enhancement of uterine carcinogenesis by estrogenic compounds involves binding to ERα and consequent shift of E2 dependent tissues into a proliferation mode. Long term treatment with effective doses of E2 is well known to promote endometrial uterine carcinogenesis in rats [4, 7], whereas extremely high dose E2-exposure does not induce uterine cancers, but rather development of squamous metaplasia in the luminal and/or glandular epithelium in the uteri or serious pyometra in rats, suggesting over dose treatment with estrognes can not induce any uterine cancer in rats.

Katsuda et al. [2] reported that long-term subcutaneous injection of high dose *p-tert* octylphenol (100mg/kg/day), a weak environmental xenoestrogen, significantly promotes endometrial adenocarcinoma development in our rat model (ovary intact females) whereas no adenocarcinomas developed in ovarictomized (OVX) rats exposed to *p-tert* octylphenol as the same dose and manner (Table 10). The dose level examined is known to be effective for estrogenic activity in rats [19, 20]. In addition, the *p-tert* octhylphenol treatment disturbed the vaginal cytology with large amounts of epithelial cells

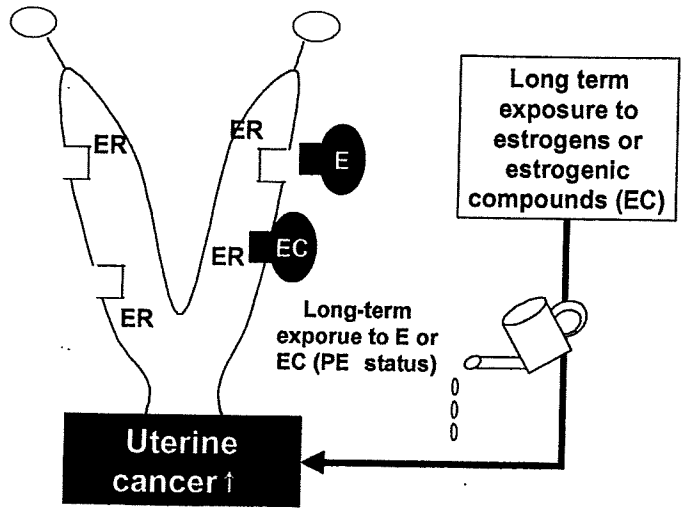


**Figure 4.** Long term exposure to estrogens or estrogenic compound driven pathway of uterine carcinogenesis using the rat model. E, excess estrogens; EC, estrogenic compounds; PE, persistent estrus; ER, estrogen receptor.

**Table 10.** Incidence of uterine proliferative lesions [Modified ref. 2].

| Age (months) | Group | n | | Hyperplasia | | | Adenocarcinoma |
|---|---|---|---|---|---|---|---|
| | | | None | Slight | Moderate | Severe | |
| 9 | Control | 6 | 1 | 5 | 0 | 0 | 0 |
| | OP-treated | 6 | 2 | 4 | 0 | 0 | 0 |
| | Control(OVX) | 3 | 3 | 0 | 0 | 0 | 0 |
| | OP-treated(OVX) | 6 | 6 | 0 | 0 | 0 | 0 |
| 12 | Control | 6 | 1 | 2 | 3 | 0 | 0 |
| | OP-treated | 8 | 0 | 4 | 1 | 0 | 3 |
| | Control(OVX) | 3 | 3 | 0 | 0 | 0 | 0 |
| | OP-treated(OVX) | 7 | 6 | 1 | 0 | 0 | 0 |
| 15 | Control | 23 | 2 | 2 | 8 | 7 | 4 |
| | OP-treated | 26 | 0 | 1 | 8 | 5 | 12* |
| | Control(OVX) | 15 | 15 | 0 | 0 | 0 | 0 |
| | OP-treated(OVX) | 29 | 25 | 3 | 1 | 0 | 0 |

*, Significantly different from the control value. ($p<0.05$)
OP, *p-tert* octylphenol
None, Animal bearing no atypical hyperplasia and/or adenocarcinoma
OVX, ovariectomized

at metestrus and/or diestrus stages in ovary-intact rats and uterotropic effects in OVX rats. On the other hand, dietary treatment with *p-tert* octylphenol at concentrations of 10, 100 and 1000ppm, which are neither uterotrophic nor steroid hormone modulating, did not exhibit any promotive effects on uterine carcinogenesis in the rat model. Therefore, estrogens and/or estrogenic compounds that impact directly upon the uterus by binding to ER can promote uterine carcinogenesis.

## Estrogen metabolite or catechol estrogen driven uterine carcinogenesis

Recently, a hypothesis has been presented that high tissue levels of E2 could promote carcinogenesis via two mechanisms; stimulating proliferation as described above and by producing DNA damage [1]. 4-Hydroxyestradiol (4HE), a hydroxylation metabolite of E2, has been reported to be a stronger carcinogen then the parent E2 due to production of DNA damage [1, 21-23]. Of the cytochrome P450 enzymes related to estrogen metabolism, CYP1B1 which is widely distributed in many human and mammalian tissue has been nominated as important for transformation of E2 into 4-hydroxyestradiol (4HE) [24, 25]. Concerning E2 metabolites, 2-hydroxyl estradiol (2HE), which is a major metabolite by 2-hydroxylation route in the liver, results in little estrogenic activity, and therefore limited carcinogenicity effects in estrogen dependent target organs [26]. On the other hand, 4HE, which is a minor product of estrogen metabolism in the liver, has proven to enhance tumorigenesis via production of DNA adducts, despite this catechol estrogen having weak estrogenic activity compared to parent E2. There is also experimental evidence that 4HE promotes tumor development in the hamster kidney and mouse uteri [27, 28]. Furthermore, in the Donryu rat model long term subcutaneous treatment with 4HE promote uterine carcinogenesis in terms of both incidence and multiplicity (Table 11)[29].

Recently, a number of supplements extracted from vegetables have been produced, some of which are known to induce CYPs related to estrogen metabolism in the liver and estrogen dependent organs [30, 31]. Since these products exert no direct estrogenic activity on target organs, their induction of CYPs and consequent modulation of estrogen metabolism indirectly impacts on estrogen dependent organ carcinogenesis. Indole-3-carbinol (I3C), a cruciferous vegetable, is reported to induce the CYP1 family in the liver [31, 33] and has been shown to suppress or promote carcinogenesis depending on the animal model [33-35]. Regarding its preventive effects, I3C acts as an anti-estrogen and can induce apoptosis [36-38], but precise mechanisms remain to be determined. Nevertheless, I3C does not exert any estrogenic or anti-estrogenic activity to the rat uteri using uterotropic assays in OVX rats or any disturbance of the ovarian hormone balance [29]. When I3C was administered to Donryu rats at dietary concentrations of 500 or 2000ppm for 12 months, incidences of uterine adenocarcinomas and/or multiplicities of uterine neoplastic lesions were increased (Table 11). In addition, I3C treatment caused consistent elevation of estradiol 2- and more especially 4-hydroxylase activities in the liver, but no effects on estradiol 16α-hydroxylase activity (Figure 5). Expression of mRNAs for CYP1A1, 1A2 and 1B1 was increased in the liver by the treatment (Figure 6), with

translation confirmed immunohistochemically. These studies provide new evidence that modulation of estrogen metabolism to increase 4HE through induction of CYP 1family members is a new pathway to promote uterine carcinogenesis in rats (Figure 7).

**Table 11.** Incidences of uterine proliferative lesions and their multiplicities at 15 months of age [Modified ref. 29].

| | None | Atypical hyperplasia | | | Adenocarcinoma | Multiplicity(a) |
|---|---|---|---|---|---|---|
| | | Slight | Moderate | Severe | | |
| Control (n=24) | 4 | 2 | 5 | 7 | 6 | 1.04±0.62 |
| I3C 500ppm (n=30) | 1 | 2 | 3 | 7 | 17* | 1.50± 0.63* |
| Control (n=18) | 2 | 2 | 7 | 3 | 4 | 1.17±0.62 |
| I3C 2000ppm (n=18) | 1 | 2 | 5 | 2 | 8 | 1.78±0.73** |
| E2 1μg/kg (n=16) | 0 | 3 | 2 | 3 | 8 | 1.50±0.52 |
| 4HE 5μg/kg (n=16) | 0 | 0 | 5 | 1 | 10* | 1.69±0.60** |

(a) Multiplicities are average numbers of uterine proliferative lesions per rat, mean±SD. *;**, Significantly different from the relevant control group ($p<0.05$ and 0.01, respectively.

I3C, indole –3- carbinol

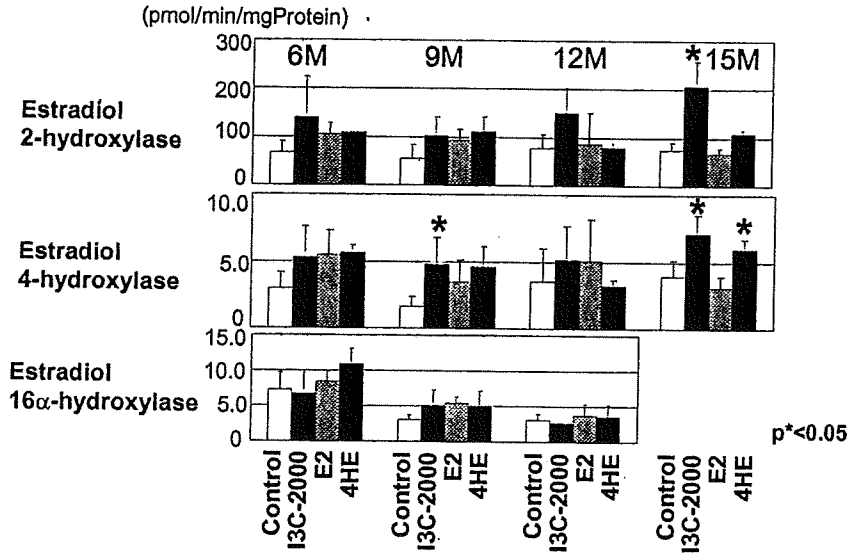


**Figure 5.** Sequential changes of enzyme activity related to estrogen metabolism in the liver. M, months of age; Control, control group given basal diet only; I3C-2000, indole-3-carbinol at 2000ppm in basal diet; E2, subcutaneous injection of 17β-estradiol at 1μg/kg; 4HE, subcutaneous injection of 4-hydroxyestradiol at 5μg/kg.

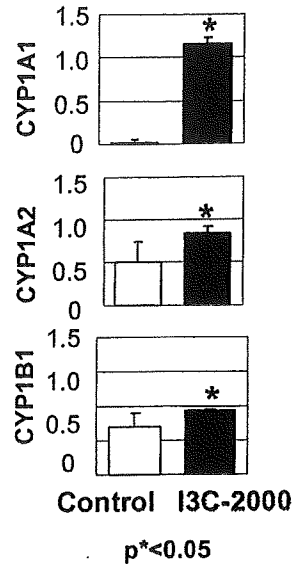


**Figure 6.** Levels of expression of cytochrome P450s 1A1, 1A2 and 1B1 mRNA relative to GAPDH mRNA in the liver (calculated as %). Control, control group given basal diet only; I3C-2000, indole-3-carbinol at 2000ppm in basal diet. [Modified ref. 29]

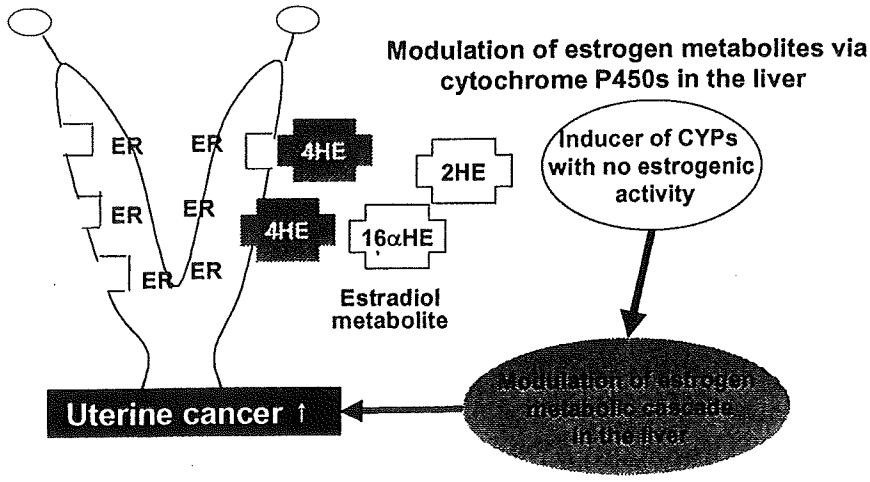


**Figure 7.** Modulation of estrogen metabolite or catechol estrogen driven pathway of uterine carcinogenesis using rat model. CYP, cytochrome P450s; ER, estrogen receptor; 2HE, 2 hydroxyestradiol; 4HE, 4 hydroxyestradiol; 16α-E, 16α-hydroxyestradiol. [Modified ref. 29]

# Molecular evidence for rat uterine carcinogenesis

In women, most molecular studies of endometrial carcinoma have been performed on small series of cases from single institutions [1]. In rodents, there have only been a few molecular based attempts to elucidate endometrial adenocarcinoma development. Nonetheless, the studies have produced several findings common with human cases as described below.