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Morphogenetic action of retinoids and estrogens

BART VAN DER BURG, EDWIN SONNEVELD, JOSEPHINE G. LEMMEN and PAUL T. VAN DER SAAG*

Hubrecht Laboratory, Netherlands Institute for Developmental Biology, Utrecht, the Netherlands

ABSTRACT Retinoids and estrogens are small lipophilic compounds fulfilling important biological roles in vertebrate development, reproduction and homeostasis. Both types of ligands are regulators of gene transcription by binding to (nuclear) proteins acting as ligand-activatable transcription factors, members of the nuclear receptor gene superfamily. Retinoids and their multiple receptors (RARs/RXRs) are particularly well-known for their role in early development and spermatogenesis, while much less is known about the two estrogen receptors (ERα/β) during development. In this article we describe some of our previous and present work in both areas of research.

KEY WORDS: *retinoids, estrogens, cancer, development, CYP26*

Introduction

Members of the superfamily of nuclear hormone receptors are transcription factors that are essential in a large variety of morphogenetic processes. Upon ligand binding, a conformational change occurs allowing such receptors to bind to specific DNA sequences in promoter regions of target genes. Due to their lipophilic nature, receptor ligands readily enter cells and can bind to their cognate receptors intracellularly. The ligand-dependency of the receptor activity allows modulation of activity *in vivo*, and endogenous ligand concentrations are tightly regulated. This superfamily also comprises a variety of so-called orphan receptors for which no ligand is known, several of which are expressed in early embryos, suggesting that they could also be involved in development. While it is well established that retinoid receptors are essential in guiding early developmental processes, sex-steroid receptors are known for their role in the shaping and maintenance of the reproductive system. We are interested in the molecular processes that govern the morphogenetic action of these receptors. We are using a variety of *in vitro* and *in vivo* tools to dissect these signaling routes, among which transgenic reporter mice and fish in which reporter plasmids are introduced that respond to the presence of the liganded receptor. With such animals, the specific sites in the embryo where such receptors are active, and also probe for the existence of the putative ligand gradients involved in nuclear receptor, mediated morphogenesis. Since our main focus has been on estrogens and retinoids in the past years, this overview will mainly describe that part of our work.

Retinoids

The first group of molecules that received our attention comprises derivatives of vitamin A (retinol): the retinoids. Morphogens such as retinoids can be defined as small (lipophilic) molecules which are involved in cellular growth regulation, differentiation and patterning during early development (De Luca *et al.,* 1994; Gudas *et al.,* 1994; Durston *et al.,* 1998). Highly controlled and tissue-specific conversions of retinoids may be crucial in their morphogenetic activity through the generation of gradients of specific ligands, as proposed earlier for all-*trans*-retinoic acid (RA) (Durston *et al.,* 1989). It is our aim to elucidate the role of local metabolism in the morphogenetic action of these molecules. Presently, our focus is on the oxidative metabolism of retinoids, and in particular the metabolism of RA.

Retinoids are important molecules, as they play key roles during development mediated by their cognate RA receptors (RARs) and retinoid X receptors (RXRs) (Mangelsdorf *et al.,* 1994). Different endogenous retinoid ligands in combination with a varying receptor composition, various receptor heterodimer combinations between RXR and RAR subtypes, and different retinoid-responsive elements present in the promoters of target

Abbreviations used in this paper: RA, retinoic acid; RAR, retinoic receptor; RXR, retinoic X receptor; VAD, vitamin A-deficient; CYP, cytochrome P450; E, embryonic day; CNS, central nervous system; ER, estrogen receptor; DES, diethylstilbestrol.

***Address for reprints:** Hubrecht Laboratory, Netherlands Institute for Developmental Biology, Uppsalalaan 8, 3584 CT Utrecht, the Netherlands. FAX: 31-30-2516464. e-mail: paul@niob.knaw.nl

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genes are determinative for the outcome at the level of gene expression during development. An important role for RARs and RXRs during development was already clear from their specific expression patterns, coinciding with most of the target tissues affected in vitamin A-deficient (VAD) mouse embryos (Dollé *et al.,* 1989, 1990). Furthermore, extensive receptor knockouts of members of the RAR/RXR subfamily by the group of Chambon has allowed a genetic dissection of retinoid function *in vivo* and provided extensive support that RARs and RXRs are indeed the mediators of known functions of retinoids during development (Kastner *et al.,* 1995, 1997). In contrast to the retinoid receptors which converge the retinoid signals, relatively little is known about the specific biological functions of a variety of receptor ligands themselves, and about the mechanisms providing the specific retinoid at the right time and place.

Retinoid metabolism

Multiple metabolic pathways responsible for the conversion of retinol to active metabolites including RA are known and besides several enzymes responsible for the synthesis of RA, enzymes for further metabolism of RA exist (Fig. 1). Hydroxylation of RA into 4-hydroxy-RA and 18-hydroxy-RA has been considered to be the main metabolic route to metabolize RA into more polar retinoids, which for long were thought to have no signaling function anymore. In the past, however, Durston c.s. have already shown that 4-oxo-RA can act as a strong (stronger than RA) morphogen in providing anterior/posterior (A/P) axis specification during *Xenopus* development (Pijnappel *et al.,* 1993) and is a potent inducer of *in vivo* proliferation of growth-arrested A spermatogonia in VAD mouse testis (Gaemers *et al.,* 1996). Furthermore, it was shown that 4-oxo-RA binds with the same affinity to RARs as RA, and is equally effective in transactivation (Pijnappel *et al.,* 1993; Sonneveld *et al.,* 1999b). This observation indicates that also other (more) polar metabolites of RA may act as physiologically significant ligands in RAR-mediated signal transduction.

The formation of 4-oxo-RA could be catalyzed enzymatically by a cytochrome P450 (*CYP*), since formation is inhibited by CYP inhibitors such as liarozole (Wouters *et al.,* 1992) and ketoconazole (Van Wauwe *et al.,* 1988). CYP enzymes are active in hydroxylation of endogenous and exogenous (xenobiotic) compounds (Nebert, 1991; Nedelcheva and Gut, 1994) and some members of this gene superfamily, such as CYP1A2, CYP2B4 and CYP2C8, have been reported also to hydroxylate RA (Leo *et al.,* 1989; Roberts *et al.,* 1992). In general, however, the specificity of these CYPs for RA is low. Recently, a novel and specific RA hydroxylase has been cloned from regenerating dorsal zebrafish fin, representing a new *CYP* subfamily designated as *CYP26* (White *et al.,* 1996). Subsequent cloning from mouse (Fujii *et al.,* 1997; Ray *et al.,* 1997; Abu-Abed *et al.,* 1998), *Xenopus* (Hollemann *et al.,* 1998) and humans (White *et al.,* 1997) has shown that *CYP26* is highly conserved across these species. All CYP26 homologs possess specific properties of the CYP superfamily, such as a transmembrane anchoring domain, a proline-rich domain, a steroid- and oxygen binding site, and a conserved heme-binding domain including the highly conserved cysteine residue at amino acid residue 442 (Fig. 2). Amino acid sequence comparison of CYP26 revealed 93% identity between human and mouse, 66% between human and *Xenopus*, and the human and zebrafish sequences were 65% identical (Fig. 2).

We and others have shown that CYP26 is rapidly induced by RA itself in a variety of cell lines (Ray *et al.,* 1997; White *et al.,* 1997; Abu-Abed *et al.,* 1998; Marikar *et al.,* 1998; Sonneveld *et al.,* 1998, 1999a), including F9 and P19 mouse embryonal carcinoma (EC) cells, and CYP26 expression coincides temporally with earlier described RA-inducible RA metabolism (Takatsuka *et al.,* 1996; van der Leede *et al.,* 1997). CYP26 enzyme activity is detectable in microsomal preparations within 60 min of RA addition to the cells, and is indeed responsible for the metabolism of RA (Sonneveld *et al.,* 1998). Induction at the mRNA level by RA is protein synthesis-independent, indicating that RA is acting directly at the transcriptional level, probably through RARs. Induction is only seen in cell lines known to be sensitive for RA. e.g. with respect to growth inhibition. Stable introduction of different RAR subtypes in RA-resistant HCT-116 colon carcinoma cells showed that CYP26 can be restored under these conditions subsequently leading to restored RA metabolism (Sonneveld *et al.,* 1998). HPLC analysis of the products formed by CYP26-containing microsomes in the presence of labeled RA shows a pattern of five peaks, comprising mainly 4-hydroxy-RA, but also 4-oxo-RA, 18-hydroxy-RA and two unknown peaks, still to be identified. Strong selectivity of the enzyme for all-*trans*-RA was found as 13-*cis*- and 9-*cis*-RA were hardly hydroxylated (Sonneveld *et al.,* 1998). It was suggested that a metabolite of RA, rather than the parent compound itself is responsible for the cell growth inhibition of breast cancer cells (Takatsuka *et al.,* 1996), however, we have shown that retinoid products of CYP26 are less active than RA in RA-sensitive T-47D and MCF-7 breast cancer cells, indicating that RA itself is the most active retinoid in these cells, and that metabolism of RA is not required to confer growth inhibition of human cancer cells (van der Leede *et al.,* 1997). This is also confirmed by the fact that overexpression of CYP26 in RA-resistant MDA-MB-231 breast cancer and HCT 116 colon cancer cells does not lead to re-induced growth inhibition by RA (Sonneveld *et al.,* unpublished results). On the other hand, overexpression of RARs in both RA-resistant cell lines resulted in RA-mediated growth inhibition (van der Leede *et al.,* 1997; Sonneveld *et al.,* 1998), suggesting that growth inhibition depends on RAR status and that RA metabolism is a consequence of RAR activity. This also suggests that the RA-induced catabolism of RA by CYP26 protects RA-sensitive cells from continuous exposure to this active retinoid and serves as a negative feedback mechanism.

CYP26 in embryonic development

In situ hybridization experiments during mouse development indicated that *CYP26* is expressed throughout a large time window and the expression pattern is complex. *CYP26* was already expressed at embryonic day (E) 7.5 in the anterior neural fold ectoderm and associated mesoderm of the mouse embryo, while one day later (E8.5) this anterior expression was lost and expression appeared more posterior in the caudal midline ectoderm including primitive streak and newly formed mesoderm (Fig. 3A-C) (Fujii *et al.,* 1997; de Roos *et al.,* 1999). This could indicate that the enzyme has a role in creating and/or maintaining A/P differences in retinoid levels during development. Also in embryos, *CYP26* expression can be induced and enhanced by treating the mother animal with exogenous RA. Later in development, *CYP26* is more restricted to specific structures with high expression in craniofacial mesenchyme, probably including mi-

grating neural crest cells (Fig. 3D), the caudal neural pore (Fig. 3D), the neural retina, limb buds, around differentiating cartilage and bone, and the outer mesenchyme of oesophagus and stomach (Fujii *et al.,* 1997; de Roos *et al.,* 1999; Iulianella *et al.,* 1999). During embryonic development, there is great similarity in temporal and spatial *CYP26* expression patterns of mouse and *Xenopus laevis* homologs. *Xenopus CYP26* transcripts are present in the mesoderm around the future blastopore and within the dorsal animal hemisphere at the onset of gastrulation. Expression is intensified in cells surrounding the blastopore and extended to the dorso-anterior region during gastrulation, and parallels the expression observed in the mesoderm of early-primitive streak mouse embryos (Hollemann *et al.,* 1998; de Roos *et al.,* 1999). In *Xenopus* embryos, C*YP26* expression is also observed in the tip of the tail (caudal neural plate), in presumptive neural crest cells, in somitic mesoderm, the branchial arches, the eye, and the olfactory placode, as in mouse embryos. Despite some differences between mouse and *Xenopus* during early development, many expression sites are the same, suggesting conserved functions for *CYP26* during embryogenesis. Based on its expression pattern, the role of *CYP26* in embryonic development is probably to control RA levels, as suggested earlier: some regions where *CYP26* is expressed, such as neural crest, limb and eye are very sensitive to high RA levels, and in these regions the concentration of RA needs to be tightly regulated.

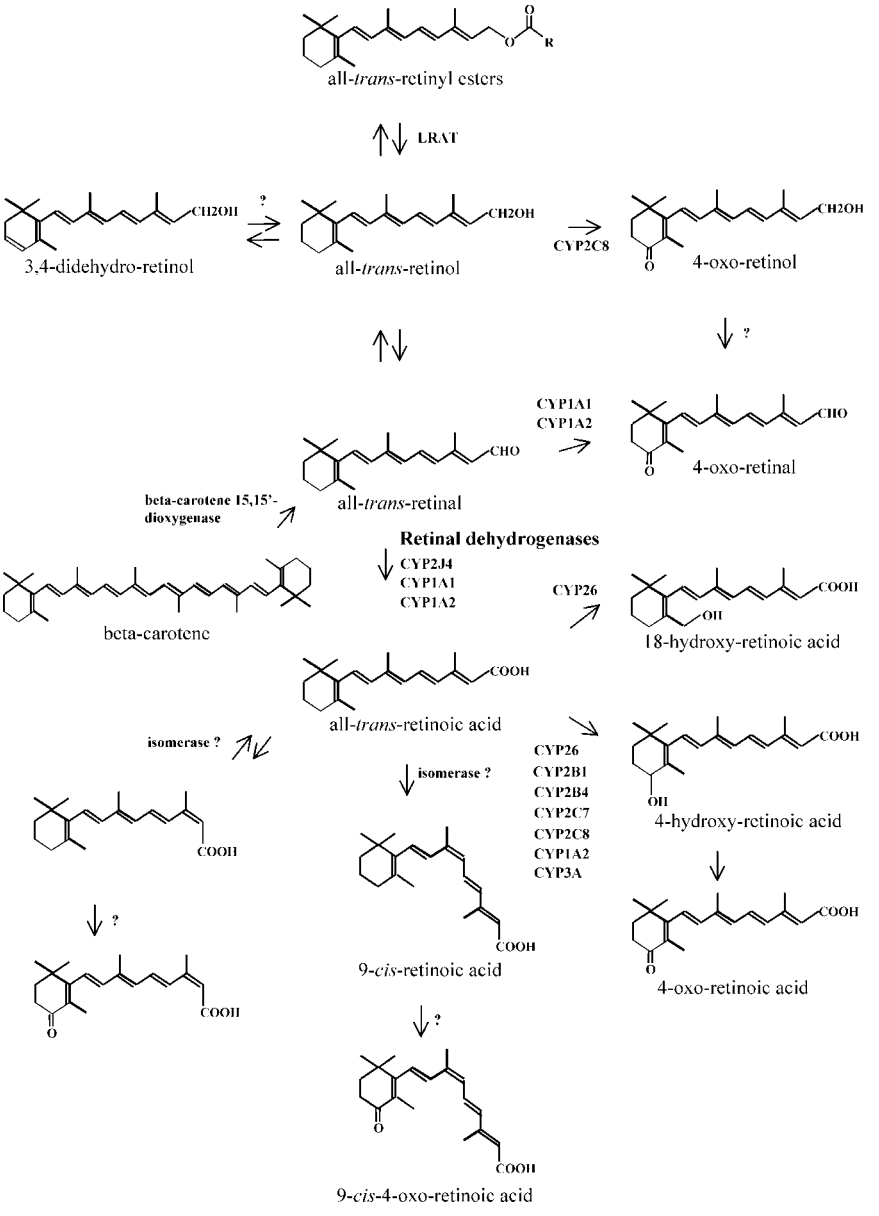

Fig. 1. Chemical structures of naturally occurring retinol derivatives and proposed pathways of retinol metabolism. *Abbreviations: CYP, cytochrome P450; LRAT, lecithin:retinol acyltransferase.*

In addition, we have used the P19 EC cell system as an *in vitro* tool to study the role for *CYP26* in development. P19 EC cell lines stably expressing *CYP26* undergo extensive and rapid neuronal differentiation in monolayer already at low concentrations of RA, while normally P19 EC cells under these conditions differentiate only in endoderm like cells (Sonneveld *et al.,* 1999a). Moreover, it was found that this phenomenon is accompanied by precocious expression of a variety of neural-specific transcription factors which normally are induced by higher RA concentrations at later time points. Based on these observations, we have developed the working hypothesis that the onset of neural differentiation and subsequent expression of neuronal markers is mediated by CYP26 via conversion of RA into hydroxylated products, while the effects on growth and RARβ induction in P19 EC cells are mediated by RA directly. Thus, CYP26 and subsequent RA metabolism might be playing a crucial role during early development of the central nervous system (CNS). Recently, more evidence has been accumulated that retinoids are important in the development of the CNS, specifying positional identity and determination of specific CNS cells (Gould *et al.,* 1998; Maden *et al.,* 1998; Sockanathan and Jessell, 1998; Solomin *et al.,* 1998), but it is unknown whether this is due to the direct action of RA itself, or whether it could be caused by specific RA metabolites. Based on our findings in the P19 EC cell system described above, the importance of the retinoid pathway in the developing CNS, and the high expression of *CYP26* in the CNS during early embryonic development, we propose that *CYP26* might also have an active role in RA-dependent processes, probably by generating specific RA metabolites involved in the onset of neuronal differentiation.

To investigate the *CYP26* function *in vivo*, we have started to overexpress *CYP26* during mouse embryogenesis. This resulted in phenotypes previously reported for the embryonic VAD syndrome, again suggesting a RA-inactivation function for *CYP26* as shown for *Xenopus* embryos, where *CYP26* overexpression resulted in a stepwise anteriorization of the molecular identity of

Fig. 2. Amino acid sequence alignment of CYP26 from human, mouse, *Xenopus,* and zebrafish. *Comparison of different CYP26 homologs indicate that these proteins are highly conserved across species. The alignment, shown with the single-letter amino acid codes, was done using Megalign (Lasergene). Human and mouse sequences are 93% identical, the human and Xenopus sequences 66% and the human and zebrafish sequences are 65% identical. The anchor region, proline rich region, oxygen binding, steroid binding and heme binding sites are indicated. The conserved cysteine is indicated with a box.*

individual rhombomeres (Hollemann *et al.,* 1998) as observed for *Xenopus* embryos with inhibited RAR-mediated signaling (van der Wees *et al.,* 1998). However, in mouse embryos we also found new phenotypes such as exencephaly (Sonneveld *et al.,* unpublished results), a commonly observed phenotype in RA-treated embryos (Sulik *et al.,* 1995). Based upon these observations, it is tempting to speculate that overexpression of *CYP26* in mouse embryos can result in premature differentiation of specific stem cells in the CNS, as seen for P19 cells stably expressing *CYP26*. To test the latter hypothesis, it is important to show the precocious expression of neuronal markers in *CYP26* overexpressing embryos. This work is in progress.

Estrogens

Natural estrogens (estrone, estriol, estradiol) are cholesterol derivatives which are synthesized in males and female vertebrates via a series of highly regulated metabolic conversions. Estrogen metabolism is better understood than retinoid metabolism, and the enzymes of almost all steps have been cloned and sequenced. However, some surprising discoveries have recently been made with respect to the role of aromatization of one of the principle androgens in the male, testosterone, in androgen action. Recent data have suggested that conversion of androgens to estrogens is essential in male reproductive functioning. First of all, male knock-out mice in which the classical estrogen receptor (now designated as ERα) has been inactivated are infertile (Lubahn *et al.,* 1993). In addition, we found high expression levels of the newly discovered ERβ in testicular germ cells and all types of spermatocytes up till the elongation phase (van Pelt *et al.,* 1999). The occurrence of aromatase activity in adult tissue allows local conversion of testosterone to estrogen and suggested us the possible involvement of this receptor in testosterone enhancement of sperm development. Since ERβ can be activated by

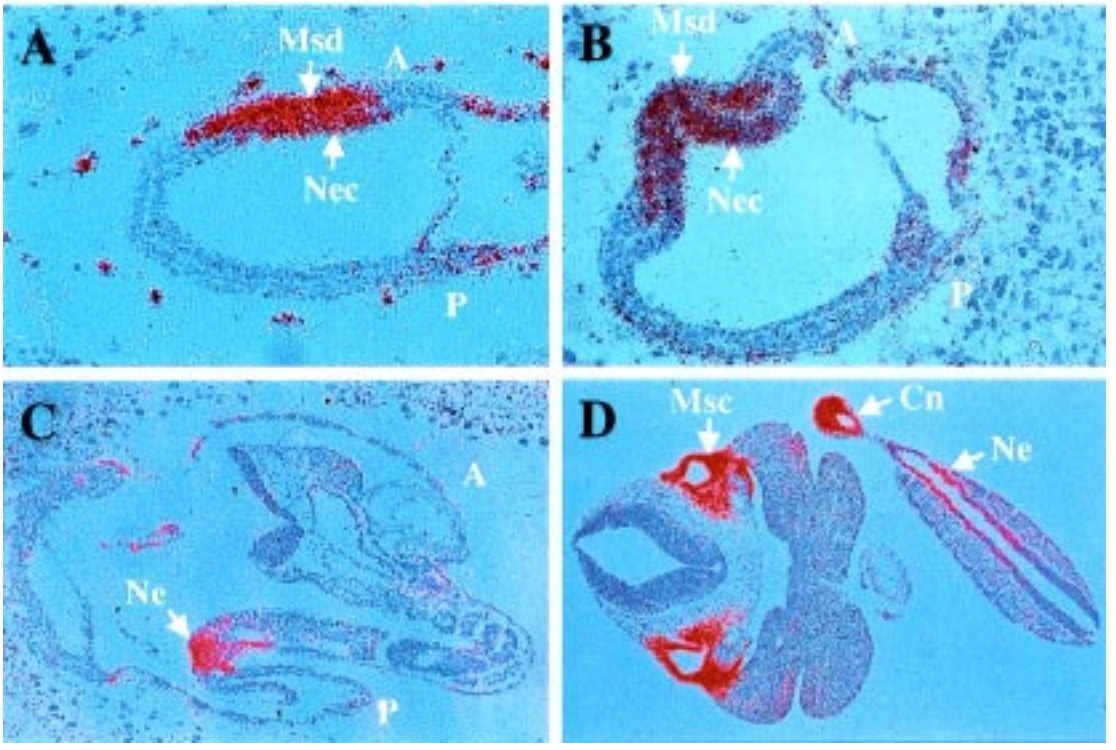

Fig. 3. *CYP26* gene expression in early mouse embryos analyzed by *in situ* hybridization on sections. *The red staining is signal for mCYP26 expression.* **(A)** *E7.5 sagittal section.* **(B)** *E7.5 transversal section.* **(C)** *E8.5 sagittal section.* **(D)** *E10.5 transversal section. Abbreviations: A, anterior; Cn, caudal neural pore; Msc, mesenchyme; Msd, mesoderm; Ne, neural epithelium; Nec, neural ectoderm; P, posterior.*

various hormonal pollutants (Kuiper *et al*., 1998), it may be also involved in hormonal disruption by exogenous estrogens.

It has been the traditional view that estrogens are only involved in female reproduction and the development of secondary sex characteristics. However, it has become clear that estrogens are important for the optimal functioning of a variety of organs/tissues in both males and females, as is illustrated by the variety of disorders associated with the post-menopausal drop of estrogen levels in humans. The number of sites in the body in which ERs have been found has been increasing gradually over the years. Importantly, estrogen excess may lead to excessive stimulation and cancer development in target tissues such as the breast, endometrium, vagina, ovary, prostate and testis.

Compared to the vast amount of data on the role of estrogens and their receptors in postnatal life, very little is known about their role in prenatal development. This is surprising since it has been found that the developing fetus is particularly sensitive to exposure to excess estrogens, which was observed in humans with the estrogen homolog diethylstilbestrol (DES) causing reproductive problems and cancer during adulthood (Herbst *et al.,* 1971). We aim at improving our insight in the possible role of estrogens in causing cancer by exposure of the target tissue during development. Initially, we have focussed on late stages of development but more recently our focus is shifting to the earliest stages of development in which estrogen receptor expression is found.

Estrogens and proliferation of mammary cancer cells

Estrogens are essential for proliferation of the normal mammary gland. A large part of human breast tumors still are hormone-dependent at the time of detection, and regress following a therapy which interferes with estrogen action. The estrogen-dependence often is lost upon progression of the disease, and regrowth occurs of a more aggressive estrogen-independent tumor. Much of our previous work has focused on determining the basic growth regulatory pathways operative in estrogen responsive mammary epithelial cells. Insight in these pathways may provide the targets for novel therapy for this major disease.

Previously we have found in estrogen-dependent breast tumor cells that induction of proliferation is controlled by a synergistic combination of estrogens and insulin (Van der Burg *et al.*, 1988) which is linked to induction of the proto-oncogenes c-fos, c-jun, and the resulting dimeric AP1 complex (Van der Burg *et al.*, 1989, 1990). Formation of this complex is generally thought to be an important event in the mitogenic cascade. Retinoic acid (RA) inhibited proliferation of such estrogen-dependent breast cancer cell lines, but surprisingly, all estrogen-independent cell lines tested were RA-insensitive (Van der Burg *et al.*, 1993). This RA-unresponsiveness of estrogen-independent lines is partly due to low RA receptor expression levels, but may also involve the AP1 complex (Van der Burg *et al.*, 1993, 1995). We found that in the estrogen-dependent and RA-sensitive MCF7 cell line, RA inhibited AP1 activity. In all hormone-independent breast cancer cells AP1 inhibition by RA was insignificant, even in cell lines with relatively high levels of RA receptors. Evidence was found that AP1 overexpression prevented RA inhibition of AP1 activity to occur (Van der Burg *et al.*, 1995).

We found that the cdk-4/cyclin D1/p21 complex has a central role in regulating cell cycle progression in breast cancer cells. Estrogen-induced cyclin D1 expression was found to be an essential part of mitogenic signaling in breast cancer cells (Zwijssen *et al.*, 1996). Enhanced expression of p21 was found to be linked to growth inhibition by the phorbol ester TPA and progesterone

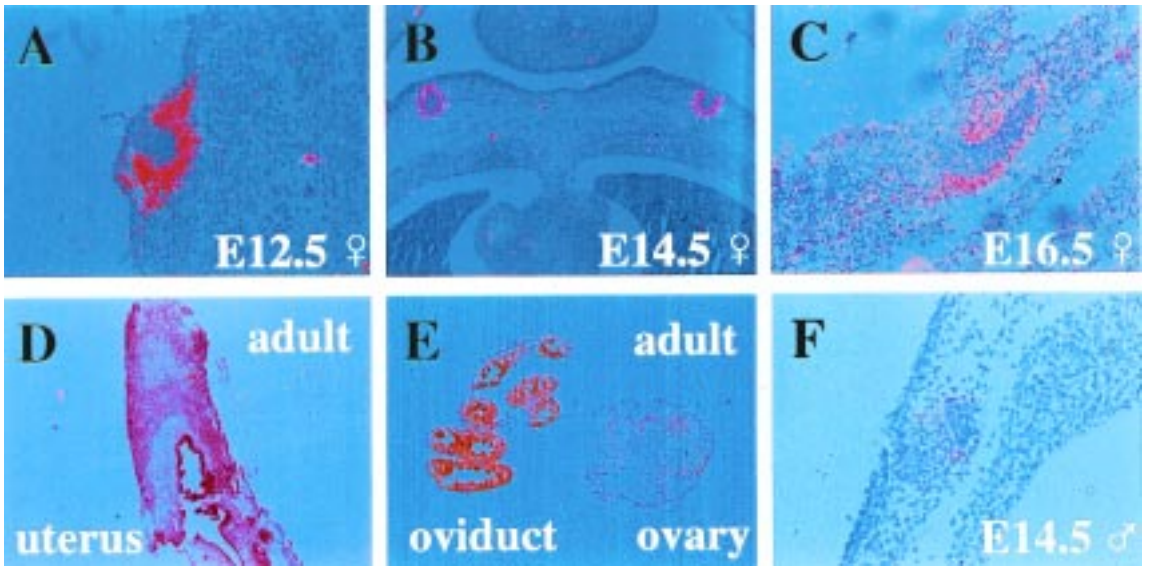

Fig. 4. Expression of ERα mRNA in the developing mammary gland by radioactive *in situ* hybridization. *The red/fuchsia staining is signal for ERα mRNA.* **(A-C)** *Expression of ERα mRNA in the mesenchymal cells of the female mammary bud.* **(D)** *ERα mRNA in the adult uterus.* **(E)** *ERα mRNA in the adult ovary and oviduct.* **(F)** *ERα mRNA in the mesenchymal cells of surrounding the regressing male mammary bud.*

(Alblas *et al*., 1998; Kester *et al.,* in preparation). Surprisingly, growth arrest by TPA was dependent on prolonged activation of Erk-2, a pathway linked to mitogenic signaling in other cell types (Dufourny *et al*., 1997; Alblas *et al*., 1998).

Our studies so far have shown that signal transduction pathways involved in mitogenic signaling in human epithelial breast cancer cells show several characteristics which are distinct from those in rodent fibroblasts, which are often used as models for human cancer. The use of more physiological models for human cancer therefore certainly is of benefit, and could be further refined by employing appropriate culture conditions using matrix- and stromal components.

Estrogens and early development

Classical experiments by Jost (1946) have shown that androgens produced by the fetal gonad are essential for the development of the male phenotype, while the female phenotype is considered to be the default state in mammals. In other vertebrates, like chicken, the male is considered the default phenotype. Clearly, these generalizations are far too simple, and based on rather crude experiments in which embryonal gonads are removed, assuming the removal of all relevant steroids, or the use of enzyme inhibitors. Until now, however, the results of ER knockout studies, generally seem consistent with the model, and single knock-out animals of ERα or ERβ have no striking prenatal phenotype (Lubahn *et al*., 1993; Krege *et al.,* 1998). However, functional redundancy seems possible and double ER knock out mice may lead to much more severe phenotypes.

Although it is not clear what role endogenous estrogens have in embryonic development, it is clear that embryonic exposure to estrogens such as DES can lead to a range of malformations in male and female reproductive organs, bone, and the mammary gland (Raynaud, 1956; Newbold, 1995; Migliaccio *et al.,* 1996). It has been proposed that developmental exposure to environmental estrogens may be involved in the rising cancer incidence in target tissues and decreased fertility in humans and animals (Carlsen *et al.,* 1995; Ekbom, 1998). We have started to study the role of estrogens in normal and disrupted development of reproductive organs.

Since no detailed study has been made so far, we mapped the embryonic sites of expression of the ERα and the recently discovered second estrogen receptor, ERβ (Kuiper *et al.,* 1996; Mosselman *et al.,* 1996), by radioactive *in situ* hybridization (Lemmen *et al.,* 1999). Our data suggest that ERα is the most abundantly expressed receptor type in the developing reproductive tract, and is also expressed in kidney, brain, heart, bone primordia, perichondria and mammary gland. Interestingly, very strong expression was detected in the mesenchymal cells surrounding the developing mammary buds in both female and male mice, as shown in Figure 4 A-C and F. Figure 4D and E shows the ERα expression in adult uterus and oviduct, which served as positive controls. The mesenchyme is known to be essential for mammary gland development and the target of suppressive effects of androgens in males on gland development. Surprisingly, no ER expression has been reported in literature, although it is known that prenatal estrogen exposure may increase breast cancer risk. In addition, early studies by Raynaud and Raynaud (1956) have shown that treatment of a pregnant mouse with estrogens on E12.5 will have profound effects on the developing mammary buds of the offspring. Future studies aim at a more clear understanding of the functioning of estrogens in early mammary gland development.

Environmental estrogens and cancer in target tissues

Fertility in wildlife, and possibly humans, is declining in many areas of the Western world (Colborn *et al*., 1993; Carlsen *et al*., 1995). This seems linked to the environmental accumulation of chemicals that mimic endogenous hormones, thereby disturbing normal endocrine functions. The dramatic drop in wildlife reproduction in the most exposed, often aquatic, environments is giving a warning for the possible impact on human health. Pollution with chemicals that mimic estrogens may explain many of the problems noted. As stated above, it seems that the developing embryo is particularly vulnerable to these hormonal pollutants.

Prediction of estrogenic activity of chemicals will be essential

to prevent further accumulation of these chemicals in the environment. However, this has been proven to be extremely difficult since estrogenic substances often have very different chemical structures, hampering risk assessment on a structural basis. Tests on laboratory animals such as mice are available but are laborious, costly, and require large amounts of animals. As an alternative and more simple *in vitro* screening method we have developed cell lines in which an estrogen-responsive reporter gene is stably introduced (Legler *et al.*, 1999). However, major drawbacks of such cell lines are, compared to *in vivo* measurements in animals, that important aspects of *in vivo* functioning such as metabolic conversion and breakdown can be missed. Moreover, no assessments can be made of the vulnerability of developing embryos to the hormonal disrupting compounds. For this reason, we have started to develop a test system for environmental estrogens using zebrafish in which an estrogen responsive reporter gene is stably introduced. Because of their large and rapidly developing offspring, transgenic reporter zebrafish are expected to combine the advantages of *in vitro* and *in vivo* systems and provide a rapid and simple *in vivo* model to screen for hormonally active xenobiotics.

We now have set up a very efficient procedure to generate transgenic zebrafish. Using this procedure we have generated a transgenic zebrafish line (the first permanent transgenic zebra fish line to be produced in the NIOB), expressing a highly specific estrogen reporter construct (Legler *et al.*, in preparation). It shows high luciferase expression following exposure of juvenile F2 and F3 generation fish to estradiol (up to 100 fold induction relative to wild type). Induced expression was found not to be confined to the liver only, as is the case for the endogenous estradiol-inducible gene vitellogenin, but is also found in the gonad, and presumably some other estrogen target tissues such as the brain. This may greatly extend the use of the model and makes it potentially possible to probe for tissue-specific effects elicited by xenoestrogens. A segment of the zebrafish ERβ-like receptor has been cloned. Using RT-PCR we have found expression in 1-5 day old embryos, after which expression drops, to rise again after 3 weeks, at a time when sexual development starts (Broekhof *et al.*, unpublished results). This pattern of expression is consistent with transgene expression, which is readily measurable at 3 weeks. These transgenic zebrafish will be used as tools to determine the pattern of ER receptor activity during normal and disrupted development.

Estrogens and disrupted development: do prenatal changes in hormonal imprinting affect the biological behavior of target tissues?

So far there is little insight in the molecular processes that underlie the teratogenic changes elicited by estrogen excess. It has long been known that in certain tissues like the avian liver, a secondary estrogen pulse elicits a much faster hormonal response that the primary one (Edinger *et al.*, 1997). This so-called estrogen memory effect may therefore prime tissues for later hormonal responses. To what extent such priming mechanisms operate in the prenatal period is unknown, but if relevant, such an early pulse may enhance the sensitivity to hormones during adulthood. Many different mechanisms may be operative in the permanent changes that can be elicited by a single estrogen pulse during development. Interestingly, it has been found that hormonal treatment during development can change the imprinting of gene expression patterns, and can lead to constitutive expression of otherwise hormone-dependent genes (Gray-Nelson *et al.*, 1994). This may involve permanent changes in the methylation patterns of estrogen responsive promoters. Our future research aims at determination of the mechanisms underlying these hormonal imprinting mechanisms. Since estrogen exposure shows a direct relation to major human diseases, such as mammary cancer, even subtle changes in hormonal responsiveness may have important consequences.

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