ABSTRACT

Porter, Karen Louise. 17β-Estradiol is Abundant in Skin and Regulates the Hair Follicle Cycle and Mirex Tumor Promotion. (Under the direction of Dr. Robert C. Smart)

Skin is a complex, hormone responsive tissue that functions as a barrier against water loss and infection. Estrogens have been shown to influence dermal thickness, vasodilatation and hair growth in skin. Remarkably, cutaneous E2 levels and capacity for E2 synthesis have not been fully assessed. We have determined that cutaneous17β-estradiol (E2) levels average nine times greater than serum E2 levels in female mice and that skin E2 is independent of serum E2. Additionally, we determined that estrogens are a major metabolite of testosterone in mouse skin explants, indicating that skin is a major site of extraglandular estrogen biosynthesis. Earlier studies have shown that castration accelerates hair growth in mice, and we have determined that castration induces a greatly diminished telogen phase, of the hair cycle. Previously our laboratory has shown that E2 blocks telogen to anagen transition of the hair cycle. We observed that only twice weekly 1 nmol E2 treatment reversed the effects of castration while daily treatment with 100 nmol testosterone or 25 nmol DHT was required, indicating that E2 is up to 100 times more potent than androgens. Previous studies have shown that mirex, a non-phorbol ester skin tumor promoter, promotes three times more tumors in female mice than OVX mice. E2 implants were able to restore 80% of the intact female mirex promotion response to OVX mice, indicating that E2 is the primary ovarian hormone that regulates mirex promotion. Since mirex promotes three times more tumors in female mice than in male mice, we conducted a tumor promotion study on intact and castrate mice given

empty or E2 containing implants, and found that intact mice develop three times more tumors than castrated mice and that E2 implants fully restore intact male response to castrate mice, indicating that E2 also regulates mirex tumor promotion sensitivity in male mice. Collectively, these data indicate that skin is an important extraglandular source of E2 and skin E2 influences the hair cycle and chemical carcinogenesis.

17β-ESTRADIOL IS ABUNDANT IN SKIN AND REGULATES THE HAIR FOLLICLE CYCLE AND MIREX TUMOR PROMOTION

by
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A dissertation submitted to the graduate
faculty of North Carolina State University in
partial fulfillment of the requirements for the
Degree of Doctor of Philosophy

MOLECULAR AND CELLULAR TOXICOLOGY

Raleigh

2001

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DEDICATION

I would like to dedicate this work to my father, Woodrow S. Porter, who always supported my interests in science, and accepted the person that I am, not the person I could not be.

BIOGRAPHY

Karen Louise Porter was born on July 23, 1959 in Madisonville, Kentucky, USA. Her family relocated to Henderson, Kentucky in 1971. She graduated from Henderson County High School *Cum Laude* in 1977. She attended Kentucky Wesleyan College in Owensboro, Kentucky and graduated in 1981 with a B. S. in Medical Technology and passed the American Society of Clinical Pathologists registry exam in 1981. She worked as a medical technologist in hospitals in Kentucky, Indiana and North Carolina for 14 years. She attended East Carolina University in the Department of Environmental Health in 1991 for two semesters and transferred to the Department of Toxicology at North Carolina State University in 1992. While working as a medical technologist at Wake Medical Center in Raleigh, North Carolina, she earned a Master of Toxicology degree from North Carolina State University in 1995. She rejoined the Department of Toxicology in 1996 and joined the laboratory of Dr. Robert C. Smart to pursue a Ph.D. in Molecular and Cellular Toxicology. She has accepted a postdoctoral fellowship in the laboratory of Dr. Marvin Meistrich at M.D. Anderson Cancer Center in Houston, Texas.

ACKNOWLEDGEMENTS

I would like to thank my mentors, Dr. Robert C. Smart and Dr. C. Lee Robinette and the members of my committee, Dr. Ronald Baynes and Dr. G. A. LeBlanc, for their support and guidance. Additionally, I would like to thank Dr. Hye-Sun Oh for her help when I first started and my laboratory colleagues, Dr. Hui-Qin Wang, Dr. Sanjay Chanda, Dr. Songyun Zhu, Dr. Jackie Akunda, Dr. Michael Viana, Mr. Minsub Shim, Ms. Kyungsil Yoon, and Ms. Tina Powers for their help and friendship. Finally, I would like to thank my cat, Teddy, for his unfailing emotional support and the laboratory mice, for their ultimate sacrifice, without which this work would not be possible.

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GENERAL INTRODUCTION

Steroid Hormone Biology in Skin

Skin is a complex, cyclic, regenerative tissue capable of periodic remodeling. Skin is the largest organ in the body and functions as a barrier, as a thermoregulation system, in expression of secondary sex characteristics and as a sensory organ. Hormones regulate skin growth, differentiation and homeostasis. Skin expresses the androgen receptor (AR) (20, 68), estrogen receptor-α (ER-α) (90), the progesterone receptor (PR) (120), the prolactin receptor (PRL) (95), the glucocorticoid receptor (GR) (98) and the thyroid hormone receptor β1 (15).

Skin's primary function is to serve as a barrier against transdermal water loss and infection. The outermost layer of skin, the stratum corneum, is a two-compartment system of nucleated corneocyctes embedded in a intercellular matrix containing hydrophobic lipids, packaged in lamellar bodies, which mediate the barrier function. Studies have shown that estrogens accelerate skin barrier maturation in fetal rats by encouraging multilayered stratum corneum development and increasing neutral lipid deposition. Additionally, studies have shown that androgens delay barrier formation (54). Furthermore, castrated mice exhibit enhanced barrier recovery after tape stripping compared to intact male mice (65). However, other studies have shown that testosterone and hydrocortisone increases fatty acid synthase (FAS) activity, the primary enzyme responsible for fatty acid synthesis in skin (118). Other hormonal influences on skin barrier function include glucocorticoids, which accelerate lamellar body formation and

increase total lipid, cholesterol and polar ceramide content (10) and thyroxine, where hypothyroidism delays stratum corneum development *in utero* (123).

Wound healing is also an essential component of barrier function. Estrogens have been shown to influence the inflammation, proliferation and remodeling phases of skin repair (23). Estrogen treatment has been shown to enhance phagocytic activity of neutrophils and limit inflammatory exudate formation by decreasing vascular permeability (23), while impairing neutrophil chemotaxis and elastase activity (9). Studies have shown that topical estrogen enhances wound healing by increasing collagen deposition and hydroxyproline content (9) and that estrogen treatment enhances wound healing in postmenopausal women by increasing wound TGF-β1 activity leading to acceleration of reepithelialization (8). Additionally, aging mice treated with estrogen exhibit decreased dermal collagen solubility (104). Furthermore, estrogen treatment has been shown to increase vascular endothelial proliferation and accelerate endothelial recovery via a VEGF mediated pathway (31, 71).

Skin also plays a role in thermoregulation through vasodilatation. Estrogen and progesterone treatment promotes vasodilatation in response to local warming by increasing endothelial nitric oxide synthase (NOS) activity (27). The estrogen receptor antagonists, ICI 182,780 and tamoxifen, reverse E2 induced NOS activity in endothelial cells indicating that estrogen stimulates NOS via an ER mediated pathway (56). Both endogenous and exogenous estrogens enhance skin vascular perfusion (7), and decrease skin temperature in response to heat, indicating that estrogen is an important factor in regulating body temperature (11).

Skin contains the steroid hormone metabolizing enzymes, aromatase, 17β-hydroxysteroid dehydrogenase (89), 5α-reductase (34) and 3β-hydroxysteroid dehydrogenase (106) and others. Aromatase irreversibly converts testosterone to E2 and androstenedione to estrone and in men and postmenopausal women, aromatization in peripheral tissues is thought to be the primary source of circulating estrogens (89). Increased extraglandular aromatase activity has been associated with familial gynecomastia (13) and with the feminized henny feathering trait in Sebright Bantam roosters (74). Aromatase is expressed in the hair follicle and sebaceous glands of human skin (106), and in follicular keratinocytes and follicular fibroblasts of cultured human hair follicle cells (13, 75). Studies have shown that the apparent K_m of aromatase activity in cultured human foreskin fibroblasts is similar to that of adipose tissue stromal vascular cells (13). Interestingly, studies have shown that dihydrotestosterone (DHT) stimulates aromatase activity in cultured human fibroblasts, but FSH, which stimulates aromatase activity in gonadal tissue, does not (26). Conversely, testosterone inhibits aromatase activity in cultured genital fibroblasts but the presence of estrogen has no effect (12), indicating that androgen levels may regulate estrogen synthesis.

The enzyme, 5α-reductase type 2, which converts testosterone to DHT, is expressed in the sebaceous gland, the hair follicle outer root sheath and the dermal papilla (34). The dermal papilla has the most 5α-reductase activity, and in androgen dependent male beard follicles, the dermal papillae have about three times more 5α-reductase activity than in male or female scalp, and male frontal scalp had more activity than male occipital scalp, indicating that increased 5α-reductase activity is associated with androgen dependent

sites (41). DHT is converted to 5α-androstan-3α,17β-diol, a less potent androgen, in skin, and the rate of skin conversion to 5α-androstan-3α,17β-diol is ten times higher than in liver, muscle and prostate (93). Additionally, testosterone has been shown to be metabolized primarily to androstenedione and 5α-androstanedione in human keratinocytes and sebocytes in culture (48).

The enzyme, 17β-hydroxysteroid dehydrogenase (17β-HSD or 17β-hydroxysteroid oxidoreductase), reversibly converts E1 to E2 and converts testosterone and DHT to less potent androgens and its activity in skin is at roughly twice the rate of adipose tissue and almost the same rate of adrenal glands in mice (85). Additionally, studies have shown that 1,25-$(OH)_2D_3$ (vitamin D) treatment increases to rate of E2 to E1 conversion by 17β-HSD accompanied by decreased proliferation in cultured keratinocytes, indicating that E2 induced proliferation may be regulated by 17β-HSD activity (61). 17β-HSD type 1 and 2 are expressed in the outer root sheath of the hair follicle and type 2 only in the sebaceous gland but neither is expressed in the dermal papilla (34, 114). Additionally, the enzyme 3β-hydroxysteroid dehydrogenase, which converts DHT to 5α-androstan-3β,17β-diol, is expressed exclusively in the sebaceous gland (106).

Percutaneous absorption of steroids in skin has been studied extensively. Studies have shown that when CH3 mouse skin explants are mounted in skin penetration chambers and topically treated with ^{3}H-E2 or ^{14}C-testosterone, approximately 18% of the E2 and 65% of the testosterone is passed through the epidermis and dermis to the media. The media contained 59% of the testosterone as parent compound and 79% of the E2 as parent compound, indicating that there is more retention of E2 than T, and that E2 tends to

remain in its parent form (66). Additionally, studies in human skin confirm that E2 conversion to E1 is minimal (76). Distribution of ^{3}H-E2 after topical application has been studied in rat skin by high-resolution autoradiography and it was found that the stratum corneum is the highest reservoir of ^{3}H-E2 followed by the sebaceous gland and the hair follicle (14). However, percutaneous absorption studies in human skin show that testosterone is primarily held in the dermis (83).

Since E2 is important in skin physiology, we hypothesize that skin contains high levels of E2 and in order to sustain E2 levels, skin has a significant capacity to synthesize estrogens. Remarkably, E2 levels have not been determined in skin nor has the capacity of skin to synthesize estrogens from testosterone. In our current study we determine cutaneous E2 levels in male and female mouse skin and conduct testosterone metabolism studies on mouse skin.

Hormonal Regulation of the Hair Follicle Cycle

The hair follicle is a complex, cyclic epidermal structure capable of periodic remodeling and regeneration. Anatomically, the hair follicle consists of the infundibulum, the upper third of the follicle down to where the sebaceous duct inserts, the isthmus, down to the bulge area or attachment of the arrector pili muscle, and the bulbar region, including the dermal papilla, an area of specialized mesenchymal cells (1). The hair cycle consists of a telogen, resting phase, an anagen active growth phase and a brief transitional catagen phase where the follicle shortens to one-third its anagen length before transitioning to telogen (5, 28). The hair follicle contains a bulge stem cell thought to be governed by an unknown diffusible factor from the dermal papilla and during late telogen, the bulge stem

cell is stimulated to divide into transient amplifying cells which differentiate to form the hair matrix and the inner root sheath (33).

In mice, the initial pelage is completed by 16 days of age, followed by the second synchronous anagen phase beginning at 20 to 30 days of age, then the third synchronous anagen phase at 9 to 13 weeks of age (5). Hair growth in rats and mice proceeds in waves and is synchronous through the third anagen phase, and becomes asynchronous in subsequent hair cycles as the animal ages (40, 62). Studies have shown that in male C57 Black mice with plucked skin, telogen lasts 2 to 4 days, catagen lasts 2 days, anagen lasts 18 days and the hair fiber erupts from the skin at about the eighth day of anagen (28).

Early studies have shown that gonadectomy in male and female mice of various strains from 38 to 252 days old shortens the time to restore normal hair coat after clipping (60), indicating that removal of testicular and ovarian hormones removes a component of hair growth regulation. Previous studies have shown that androgens reverse the effects of castration in rats (63) and mice (60). AR has been located in the dermal papilla of telogen and anagen hair follicle (20, 29), and in higher numbers in balding human scalp skin (58). Androgens, which are implicated in the etiology of male pattern baldness, have been shown to reduce proliferation of dermal papilla cells in culture (68). In our laboratory, we have found that the dermal papilla also expresses the estrogen receptor-α (ER-α) only in telogen and that topical 17β-estradiol (E2) blocks telogen-anagen transition in mice (90). An early study has shown that rats given twice weekly subcutaneous estradiol benzoate injections experienced marked loss of hair, rats given

both testosterone and estradiol benzoate experienced no change in hair growth (59) and dogs treated with topical estrone displayed an inhibition of hair regrowth (124). Additionally, castrate male rats implanted with E2 experience greater time to full hair growth than intact males, and when implanted with testosterone only experience restoration of intact male response, indicating that E2 may be more potent than testosterone in hair growth delay (63).

Studies have shown that DHT was only produced from beard dermal papilla cells incubated in testosterone containing media and not from scalp and pubic dermal papilla cells (101). Also, hair follicles from men with androgenic alopecia express increased amounts of 5α-reductase and decreased aromatase (105). Additionally, studies have shown that conversion of testosterone to the more potent androgen, DHT, increases during telogen, and conversion to less potent androgens increases during anagen (100). Additionally, conversion of E2 to estrone, the less potent estrogen, increases during anagen and decreases in telogen (99), meaning that the most potent estrogen and androgen are present at higher levels in telogen. Moreover, E2 biosynthesis from testosterone by aromatase is stimulated by DHT in human skin fibroblasts (26), but decreased by testosterone and unaffected by E2 (12), adding another layer of metabolic regulation.

While the effects of castration on hair growth have been noted for many years, detailed observations of hair growth patterns in castrated mice have not been recorded. Additionally, although androgens have been implicated in alopecia in humans and E2 has been shown to block hair growth in female mice, comparisons of the ability of androgens

and E2 to reverse the effects of castration have not been made. In our study, we examine the effects of castration on the hair follicle cycle and compare the effects of androgen and E2 treatment on hair growth in castrated mice.

Mirex Tumor Promotion in Mouse Skin

Mirex, an organochlorine pesticide, was used as an industrial fire retardant and to control fire ants in the southeastern United States until its cancellation by the US Environmental Protection Agency in 1977. Additionally, the United Nations Environment Programme has recently identified mirex as one of the twelve most important persistent organic pollutants that threaten global human and wildlife health (44, 96). Mirex was primarily manufactured near Niagara Falls, NY and State College, PA and the Hooker Chemical and Plastics Corporation released mirex at rates up to 200kg/year between 1957 and 1976, accounting for mirex contamination in the Great Lakes area (3). Mirex has been found in Lake Ontario sediment cores at depths corresponding with the mid-1960's (3) and has also been found in the Great Lakes area in bald eagle eggs and nestlings, herring gulls, cormorants, mussels and trout (18, 39, 45, 47, 102, 103). Additionally, mirex has been found in plasma from fish and waterfowl consumers in Ontario in 1999 (67), in human breast milk in New York in 1996 (82), and in human brain, liver and adipose tissues in Greenland in 1999 (38). In 1985, a US EPA national survey of chemicals in adipose tissue estimated that 10.2% of the southern US population has measurable levels of mirex in adipose tissue(73). The US EPA and the New York Department of Health have issued fish consumption advisories for mirex in Great Lakes fish in 2001 indicating that mirex continues to be a concern for human health (2, 57).

The US EPA has classified mirex as a probable human carcinogen in 2000 (42). Additionally, mirex was listed as "reasonably anticipated to be a human carcinogen" in the 2001 Ninth Report on Carcinogens (96). The International Agency for Research on Cancer (IARC) has also classified mirex as a possible human carcinogen (44). Mirex has been observed to be an epigenetic, non-phorbol ester hepatocarcinogen in rats and mice, producing neoplastic nodules, adenomas and hepatocellular carcinomas (117, 119).

Mirex is a potent, nonphorbol ester tumor promoter in 7,12-dimethylbenz[*a*]anthracine (DMBA) initiated mouse skin (87), and like the phorbol ester-type tumor promoter, 12-*O*-tetradecanoylphorbol-13-acetate (TPA) , promotes tumors with a 90% incidence of A →T transversion in the 61st codon of Ha-*ras* (86). Skin carcinogenesis has been characterized as a multihit, multistage process involving initiation, promotion and progression (92). The classical model for chemical carcinogenesis in skin involves initiation with a single dose of a carcinogen, such as 7,12-dimethylbenz[*a*]anthracine (DMBA), followed by multiple applications with a tumor promoter, such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA). About 10 weeks later papillomas would develop and most of them would express mutant Ha-*ras* (97). TPA, other phorbol ester-type and nonphorbol ester-type skin tumor promoters produce a sustained epidermal hyperplasia, whereas mirex promotion induces only one additional layer of epidermal cells, and only a slight increase in PCNA S-positive cells over those in acetone treated skin. Retinoic acid, necessary for normal epidermal differentiation, inhibits ornithine decarboxylase activity and tumor formation by TPA, mezerein and some non-phorbol ester-type promoters.

Fluocinolone acetonide, a synthetic anti-inflammatory glucocorticosteroid, inhibits inflammation, epidermal DNA synthesis and hyperplasia produced by TPA and other phorbol ester and non-phorbol ester tumor promoters. However, mirex tumor promotion is resistant to both retinoic acid and fluocinolone acetonide inhibition, unlike the potent response of TPA to these compounds, pointing to a mechanism of mirex tumor promotion not involving the inflammatory response (70). ODC, the rate limiting enzyme in polyamine biosynthesis, is also associated with PKC activation, and is considered necessary but not sufficient for tumor formation. Studies have shown that mirex does not activate PKC which is activated by ornithine decarboxylase and phorbol esters (127). Additionally, mirex does not induce cornifin-α/SPRR1, the putative precursor protein of the cornified cell envelope associated with advanced terminal differentiation, which is induced by TPA (91). Furthermore, DMBA initiated mice promoted with a submaximal promoting dose of TPA and mirex develop the same number of papillomas as mice promoted with a maximal promoting dose of TPA added to the number of papillomas produced from mice promoted with mirex alone, indicating that mirex has a synergistic interaction with TPA (84). Moreover, when DMBA initiated mice were maximally promoted with mirex until tumor number reached a plateau then maximally promoted with TPA or the reverse, the total tumor yield was additive, indicating that mirex promotes a unique population of initiated keratinocytes (69).

Studies have shown that male mice and ovariectomized female mice exposed to mirex tumor promotion exhibit 70% fewer tumors/mouse, decreased incidence, increased time to first tumor and increased latency, compared to intact female mice. However, TPA does

not exhibit sexual dimorphism (86). The hair follicle bulge stem cell may be the target cell for chemical carcinogenesis (33). As mentioned earlier, the dermal papilla of the telogen hair follicle expresses the estrogen receptor and studies have shown that E2 may be an endogenous regulator of telogen to anagen transition since topical E2 treatment blocks this transition. This effect may occur through the direct effect of estrogen or by estrogen modulating factors that influence proliferation and differentiation of the bulge stem cell and transient amplifying cells (90). The bulge stem cell receives instruction to divide from the dermal papilla during early anagen. It produces transient amplifying cells which proliferate and differentiate into inner root sheath, medulla and cortex cells, and which, with the cuticle cells, terminally differentiate into the hair fiber (33). Early studies have shown that when an initiating carcinogen is topically applied to shaven skin, tumor yield will often depend on the phase of the hair cycle at application (33). Additionally, studies have shown that squamous cell hyperplastic foci in DMBA initiated, 12-*O*-tetradecanoylphorbol-13-acetate (TPA) promoted mouse skin histologically involve the hair follicle (16). Since mirex tumor promotion is influenced by estrogen and the follicle dermal papilla expresses the estrogen receptor, it is possible that initiated follicular bulge stem cells may be the origin of mirex promoted papillomas.

Sexual dimorphism in tumor multiplicity and tumor incidence is not uncommon in chemical carcinogenesis. Chronic feeding studies show that female rats are more sensitive than male rats to the hepatocacinogen 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and that ovarian hormones increase sensitivity to TCCD (78). In BALB/c x A/J hybrid mice, a single i.p. dose of urethane induced 45% more lung tumors in male mice

than in female mice (43). Female Sprague-Dawley rats given atrazine in their diets developed mammary tumors, whereas male rats and OVX female rats developed no mammary tumors (110). E2 stimulates proliferation in the human breast cancer cell lines MCF-7 and T47D, which contain ER at higher levels than nonresponsive breast cancer cell lines (80). Early studies in humans have shown that ER positive human breast tumors regress when treated with ethinyl estradiol and in hypophysectomized or radiocastrated women, and ER negative tumors do not respond to these treatments (81). DMBA induced mammary tumors in rats will regress after ovariectomy and resume growth when the rats are given E2 implants (126). Eker rats treated with E2 develop more hereditary renal tumors than untreated rats and OVX rats developed fewer tumors (125). Additionally, studies in mouse skin initiated with 3-methylcholantrene (MCA) have shown that the number of squamous cell carcinomas are three times higher in castrate male mice treated with E2 and MCA than in castrate mice treated with MCA alone, indicating that E2 is a factor in MCA induced skin carcinogenesis (79). Together, these data indicate that sensitivity to carcinogenesis in ER containing, estrogen responsive tissue can be affected by estrogen.

Mirex has previously been shown to have no affinity for ER-α in an *in vitro* competitive binding assay, unlike the structurally similar kepone (19), which is inactive as a tumor promoter (86). However, mirex may be an endocrine disrupter since it has been shown to inhibit E2 uptake in cultured rat hepatocytes (113) mirex also increases 2-hydroxylation of estradiol in rat liver (22) and mirex decreases glucocorticoid binding sites in rat liver (116). Furthermore, the European Commission lists mirex as having evidence for

endocrine disruption in wildlife and humans in at least one study with intact animals as of high concern and highly persistent by the most stringent criteria (30), and the Illinois State EPA lists mirex as a chemical probable to cause endocrine disruption based on the preponderance of the evidence in both intact animals and bioassays (64).

Our hypothesis is that E2 is the primary hormone that regulates mirex tumor promotion sensitivity in male and female mice. In our study, we conducted mires tumor promotion studies on intact mice, gonadectomized mice and gonadectomized mice with subcutaneous E2 containing implants to determine if E2 implants can restore intact mouse tumor response to gonadectomized mice. Additionally, we conducted mirex tumor promotion studies on intact female mice treated with ICI 182,780, an ER antagonist, to determine whether ER blockade can reproduce the effects of OVX in intact mice. Since mirex may be an endocrine disrupter, we tested mirex estrogenicity in a sensitive mammalian cell ER transactivation assay.

MANUSCRIPT ONE

Skin Contains Significant Levels of17β-Estradiol and has a High Capacity to Synthesize Estrogens from Testosterone

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Abbreviated Title: Skin maintains high levels of 17β-Estradiol

Key Words: skin; 17β-estradiol; testosterone; metabolism; synthesis

ABSTRACT

Recent studies indicate that skin is an estrogen responsive tissue and that17β-estradiol (E2) enhances wound healing, vasodilatation and affects hair follicle cycle regulation. While estrogen receptor-α is expressed in skin, the endogenous level of E2 in skin has not been determined nor has the capacity of skin to form estrogenic metabolites from testosterone been fully assessed. We report here that skin E2 levels in female mice average 9 fold greater than serum E2 levels. Moreover, ovariectomy does not change skin E2 levels, indicating that skin E2 levels are independent of serum E2 levels and skin may have a high capacity for E2 synthesis. Mouse skin explants were incubated with ^{3}H-testosterone to evaluate the skin's capacity to synthesize estrogenic metabolites as well as androgenic metabolites. Estrone, E2 and estriol accounted for 33% of the total metabolites while dihydrotestosterone (DHT), androstanedione, androstenedione, and androstanediol accounted for 44% of the total metabolites, indicating that skin has a high capacity for estrogen synthesis. Of total metabolites, E2 was the second most abundant metabolite, while DHT was the fifth most abundant metabolite. When mouse skin explants were incubated with ^{3}H-E2, there was a time dependent increase in ^{3}H-E2, and estrone and estriol were the major metabolites. Collectively these data indicate that skin E2 levels are significantly higher than serum E2 levels and that skin has a high capacity to synthesize E2 from testosterone, demonstrating that skin may be a significant extraglandular source for estrogens.

INTRODUCTION

Skin, a complex, regenerative tissue, is the largest organ in the body and functions as a barrier, as a sensory organ, in thermoregulation, and in expression of secondary sex characteristics and . Skin is a hormone responsive tissue and has been shown to express the androgen receptor (6, 7, 10, 25) and to contain androgens and androgen metabolizing enzymes (5, 15, 22-24, 27, 32, 39, 41, 42). Although skin has not traditionally been thought of as an estrogen responsive tissue, estrogen receptor-α (ER-α) is expressed in human and mouse skin (26, 30, 45) and topical 17β-estradiol (E2) treatment has been found to block transition from telogen, the resting phase of the hair follicle cycle to anagen, the growth phase of the hair follicle cycle in mice (30). Studies have shown that estrogen accelerates skin barrier maturation in fetal rats (20) and can increase skin thickness in human adults (16). Additionally, topical estrogen enhances wound healing by increasing collagen deposition and reducing elastase activity in human skin (2) and estrogen – progesterone hormone replacement therapy reverses age related reductions in wound healing ability in postmenopausal women (1). Moreover, estrogen treatment enhances skin vascular reactivity in response to heat and cold suggesting a potential role for estrogen in thermoregulation and vasodilatation (3).

Although skin is an estrogen responsive tissue, cutaneous estrogen levels have not been determined nor has the capacity of skin to produce E2 been well characterized. Aromatase activity has been found in skin fibroblasts (4) and androgen metabolism to androgenic metabolites alone has been assessed in whole skin (5, 32, 34, 42) and in cultured dermal papilla cells (33, 35), but the capacity of skin explants to form both

estrogenic and androgenic metabolites from testosterone has not been compared in the same system. Skin contains numerous steroid hormone metabolizing enzymes including aromatase, 17β-hydroxysteroid dehydrogenase (29), and 5α-reductase (11). Aromatase irreversibly converts testosterone to E2 and also converts androstenedione to estrone and is expressed in follicular keratinocytes, sebocytes and fibroblasts in human skin (4, 18, 40). Skin expresses 17β-hydroxysteroid dehydrogenase (17β-HSD) which reversibly converts estrone to E2 and also converts testosterone and DHT to less potent androgens (11, 28, 43) and 16α-hydroxylase which converts E2 to estriol (46). 5α-Reductase irreversibly converts testosterone to DHT, the most potent androgen, and is expressed in male and female human hair follicles, sebaceous glands and sweat glands (15, 22).

Since estrogens play an important role in skin function, we have determined cutaneous E2 levels in male and female mice, measured estrogen synthesis in comparison with androgen metabolism in mouse skin explants and separately measured E2 metabolism in mouse skin explants. Our study provides evidence that skin contains high levels of E2, has a high capacity for estrogen synthesis and may be an important site of extraglandular estrogen biosynthesis.

MATERIALS AND METHODS

Materials

17β-estradiol, dihydrotestosterone, 4-androstene-3,17-dione, 5α-androstane-3β,17β-diol, 5α-androstane-3,17-dione, estrone, estriol, testosterone, androsterone, dextrose, bovine serum albumin (fraction V) and thimersol were purchased from Sigma (St. Louis, MO). Bloom 275 gelatin and norit A charcoal were purchased from Fisher (Fairlawn, NJ). Ultra-resi-analyzed acetonitrile, ultra-resi-analyzed methanol and Bakerbond C18 SPE columns were purchased from JT Baker (Phillipsburg, NJ). HPLC grade, carbonyl free ethyl acetate was purchased from Burdick Jackson (Muskegon, MI). Linear-K silica gel glass TLC plates were purchased from Whatman (Clifton, NJ). Rabbit anti-estradiol antibody was a kind gift from J.H. Britt (U. of TN, Knoxville). [2,4,6,7-^{3}H] 17β-estradiol and [2,4,6,7-^{3}H] testosterone were purchased from New England Nuclear (Boston, MA). Estradiol-6-(O-carboxymethyl)oximino-)2-[^{125}I]iodohistamine tracer (specific activity = 2000 Ci/mmol) was purchased from Amersham (Arlington Heights, IL).

Animals

CD-1 mice were purchased from Charles River Laboratories (Raleigh, NC) and kept in our animal facility for 1 week prior to use. The mice were fed rodent chow *ad libitum,* kept on corncob bedding and placed on a 12-hour light/dark cycle. Female CD-1 mice (7 weeks old) were shaved on their dorsal surface with electric clippers, then two days later the mice were subjected to halothane anesthesia and ovariectomized (OVX) or sham OVX as previously described (9). Thirteen weeks later skin E2 levels were analyzed.

Estradiol Radioimmunoassay (RIA)

Blood was drawn by cardiac puncture from the mice under xylazine anesthesia, allowed to clot, and centrifuged for 10 minutes at 1550 x g. The serum was separated from the cells and stored at -20°C. Dorsal skin was removed, finely minced and homogenized on ice in 1 ml PBS-gel/200 mg tissue (0.01 M PBS, 0.1% gelatin, pH 7.0) using a Polytron tissue homogenizer. The homogenate was filtered through gauze to remove hair, and stored at -20°C until analysis. Skin samples were extracted as follows: 200µl of each skin homogenate containing internal recovery standard (100 µl ^{125}I-E2 tracer diluted to approximately 900 cpm/100 µl) was freeze extracted twice with 2 ml ethyl acetate and evaporated to dryness under nitrogen. To remove lipids, 2 ml cold 70% methanol was added to each sample, the samples were vortexed, and stored at -20° C overnight (17, 36). The next day, samples were centrifuged at 1550 x g for 10 minutes at 4° C, and the supernatants were poured off. The lipid precipitate was rinsed with 1 ml 70% methanol, centrifuged and the supernatants were pooled. The precipitate was discarded and the supernatant evaporated to one-third volume under nitrogen. The samples were added to a Bakerbond C18 SPE column that was preconditioned with 1.5 ml acetonitrile, 2 ml methanol and 3 ml distilled water. Three ml of distilled water were added to the column and eluted water was discarded. E2 was eluted from the column with successive 0.5 ml volumes of 60, 70, 80, 90 and 100% acetonitrile: water and the entire eluate collected and evaporated to one-third volume under nitrogen. The acetonitrile: water mixture was freeze extracted twice with 2 ml ethyl acetate and evaporated to dryness under nitrogen and counted using a gamma counter to determine extraction efficiency, which ranged from 60 to 90 %. The samples were reconstituted in 500µl PBS-gel and 100 µl of sample

were added into each assay tube and analyzed for E2 (9, 12). Serum (100 μl) was freeze extracted with 2 ml ethyl acetate, evaporated at 37°C under nitrogen and reconstituted with PBS-gel. For the RIA, skin or serum samples were incubated overnight with 200 μl anti-estradiol antibody (diluted 1:1,500,000 with PBS-gel). The next day, 100 μl ^{125}I-tracer diluted with PBS-gel (approximately 8000 cpm) were added and incubated at 4°C for 6 hours. Dextran coated charcoal (500 μl, 0.05% dextran, 0.5% charcoal in PBS-gel) was added, vortexed, then incubated for 45 minutes at 4°C and centrifuged at 1550 x g for 15 minutes. The supernatant was decanted and counted. E2 levels were determined from a standard curve. Skin E2 was corrected for extraction efficiency. The RIA was validated for skin and serum using sample dilution linearity studies and by sample added mass studies.

Skin Explant ^{3}H-Testorerone and ^{3}H-E2 Metabolism

Seven week old male CD-1 mice were sacrificed by cervical dislocation, the dorsal skin was harvested, scraped clean of subcutaneous fat, placed on PBS soaked paper towels and cut into 25 mm disks, three per skin. The skin explants, distributed so that each time point had 3 explants from 3 different mice, were floated on 2 ml isolated perfused porcine skin flap (IPPSF) media (3), containing 1.25 μCi ^{3}H-testosterone (specific activity = 95 Ci/mmol) or 0.04 μCi ^{3}H-E2 (specific activity = 72 Ci/mmol) per ml in 6 well tissue culture plates, one explant per well, at 37° C for 0.25, 0.5, 1 and 2 hours. At each time point, the explants were removed, rinsed with PBS, finely minced and homogenized on ice in 2 ml PBS using a Polytron tissue homogenizer. The homogenates and the incubation media were immediately poured into separate glass conical extraction tubes, 8 ml ethyl acetate were added, mixed by inversion and vortexing and centrifuged at 500 x g

for 10 minutes. The organic supernate was taken off and evaporated under nitrogen. A sample of the aqueous layer was taken to determine the amount of aqueous soluble metabolites and for the testosterone metabolism study, to analyze metabolites after acid hydrolysis. The media samples were stored at -20° until ready to analyze. The organic extract from the skin samples was delipidated overnight in cold 70% methanol, then the methanol: water mixture was evaporated to one-third volume under nitrogen, extracted with ethyl acetate and evaporated to dryness under nitrogen. TLC was performed as previously described (37, 38). Skin or media samples were reconstituted in chloroform and spotted onto glass linear-K silica gel TLC plates with cold estrone (E1), E2, estriol (E3), testosterone (T), dihydrotestosterone (DHT), 4-androstene-3,17-dione(androstenedione), 5α-androstane-3β,17β-diol (androstanediol) and 5α-androstane-3,17-dione(androstanedione) standards for the testosterone metabolism study and E1, E2 and E3 standards for the E2 metabolism study. The TLC plates were placed in a TLC chamber containing chloroform: diethyl ether 7:3 mobile phase and a filter paper wick. After migration, the plate was developed in an iodine tank and the spots corresponding to E1, E2, E3, T, DHT, androstenedione, androstanediol, androstanedione and the origin scraped, added to scintillation vials containing scintillation cocktail and counted in a scintillation counter. For the testosterone metabolism study, the aqueous fraction was acid hydrolyzed by adding 200μl 6N HCl to each tube, boiling for one hour, neutralizing with 6N NaOH, then extracting and analyzing by TLC as above. The R_f values for the metabolites were 0.71 for androstanedione, 0.66 for E1, 0.57 for androstenedione, 0.45 for DHT, 0.41 for E2, 0.36 for T, 0.28 for 5α-androstane-3β,17β-diol and 5α-androstane-3α,17β-diol, which co-migrate in this system, and 0.04 for E3. Since the E1 and

androstanedione spots were smeared, the samples were also run using a chloroform: methanol 98:2 mobile phase to verify E1 and androstenedione relative amounts.

RESULTS

Skin 17β-Estradiol Levels

To determine cutaneous E2 levels, we utilized dorsal skin from intact and OVX female mice and male mice, processed the skin and quantitated E2 by RIA as described in the methods section. Serum E2 levels from the same mice were also quantitated by RIA. Female mouse skin E2 levels were on average 9 times greater than serum E2 levels (Figure 1A). Skin E2 levels for individual mice ranged from 5 to 30 times greater than serum values (Figure 1B). Ovariectomy produced a significant decrease in serum E2 levels, however, skin E2 levels did not change significantly (Figures 1A and 1B). At the time skin and serum samples were collected, the intact female mice were actively going through the estrus cycle as determined by examination of vaginal rinse for cornified epithelial cells, uterine wet weight, and serum E2 levels. However, cutaneous E2 levels appear to be independent of circulating serum E2 levels (Figure 1B), phase of the estrus cycle (data not shown) and ovariectomy. Linear regression analysis of all skin and serum E2 values yields a correlation coefficient (R^2) of 0.8856, indicating that there is no correlation between skin and serum E2 levels (Figure 1C). Collectively these data demonstrate that skin has higher skin E2 levels than serum E2 levels, skin E2 levels are independent of serum E2 levels and suggest that skin has a high capacity to synthesize E2. Male mouse skin E2 levels averaged 6 times higher than serum E2 levels and were approximately half that of female mice (Figures 1A and 1B), indicating that there may be sex related differences in mechanisms underlying skin E2 levels.

17β-Estradiol synthesis and ^{3}H-testosterone metabolism in mouse skin explants

To assess the capacity of skin to form estrogenic and androgenic metabolites from testosterone, we incubated mouse skin explants with ^{3}H-testosterone and analyzed metabolites retained in skin and those released into the incubation medium. There was a time dependent increase in organic soluble estrogenic and androgenic metabolites within the skin (Figures 2A and 2B). In terms of estrogenic metabolites, E2 and E1 were the major metabolites formed, while androstanedione and androstenedione were the most abundant androgenic metabolites. In terms of total organic soluble metabolites, 17β-estradiol was the second most abundant metabolite, whereas DHT was a minor metabolite, being only the fifth most abundant metabolite. Aqueous soluble metabolites were subjected to acid hydrolysis to remove conjugates and the products were analyzed. E2 was the most abundant acid hydrolyzed aqueous metabolite, followed by E1 and androstanedione. (Figures 2C and 2D). Overall, estrogens comprised 33% of total metabolites, compared to androgens at 44% of total metabolites, indicating that estrogens are a major product of testosterone metabolism in skin (Figure 2E). Forty percent of all aqueous soluble metabolites were acid hydrolyzible, 60% were resistant to acid hydrolysis and these comprise the remaining 23% of total metabolites (Figure 2E).

We also analyzed the testosterone metabolites released by skin explants into the medium. As shown in Figure 3A, testosterone was rapidly metabolized as both estrogenic and androgenic metabolites that were rapidly excreted into the medium. In terms of estrogenic metabolites, E3 and E2 were the major metabolites formed, while androstanediol and androstenedione were the most abundant androgenic metabolites (Figures 3A and 3B). Aqueous soluble metabolites were subjected to acid hydrolysis to

remove conjugates and the products were analyzed. E3 was the most abundant acid hydrolyzed aqueous metabolite, followed by E2 and androstanediol. (Figures 3C and 3D). Overall at two hours, estrogens comprised 30% of total metabolites, compared to androgens at 35% of total metabolites, indicating that estrogens are a major product of testosterone metabolism in skin (Figure 3E). Twenty percent of all aqueous soluble metabolites were acid hydrolyzible, 80% were resistant to acid hydrolysis and these comprise the remaining 35% of total metabolites (Figure 3E). Collectively, these data indicate that estrogens are a major fraction of testosterone metabolism and E2 synthesis from testosterone may be a major mechanism for maintenance of high skin E2 levels.

^{3}H-17β-Estradiol metabolism in mouse skin explants

In order to characterize E2 metabolism in skin, we incubated male mouse skin explants with ^{3}H-E2 and analyzed metabolites in the skin and excreted into the incubation media. There was a time dependent increase in the accumulation of parent E2 in the skin explant and ^{3}H-E2 was primarily metabolized to aqueous soluble metabolites in the skin (Figure 4A). ^{3}H-E2 was primarily metabolized to aqueous soluble metabolites in the media (Figure 4B). Overall, E1 comprises 2.2% of total metabolites, E3 comprises 2.8% of total metabolites, and aqueous soluble metabolites comprise 95% of total metabolites. Higher rates of ^{3}H-E1 production observed in the ^{3}H-testosterone metabolism study may be due to conversion from androstenedione by aromatase.

DISCUSSION

Our results demonstrate that skin E2 levels are, on average, nine times higher than serum E2 levels in female mice and six times higher in male mice. To our knowledge this is the first report to quantify skin E2 levels. E2 levels have been previously determined in other tissues including human abdominal adipose tissue (170 ± 120 pg/g tissue) (14), human breast tissue (120pg/g tissue) (44), human myometrium (550-1400 pg/tissue) (44), and fetal guinea pig brain (227 ± 97 pg/g) (19). Thus, the E2 levels we observed in skin are within the range of, or higher than other estrogen responsive non-reproductive organ tissues. When one takes into account the total surface area of skin, it is apparent that skin is a major depot for total body estrogens. Collectively, our findings support a role for skin as an important extraglandular source of estrogen.

Previous studies of testosterone metabolism in skin have focused on androgen production (5, 13, 24, 27, 32, 41, 42). Our results demonstrate that skin has a high capacity to produce estrogenic metabolites from testosterone and further suggests that this capacity contributes to the high levels of E2 observed in skin. The production of high levels of E2 from testosterone suggests that aromatase and 17β-HSD are abundantly expressed in skin. Aromatase expression has been localized to the hair follicle and sebaceous glands of human skin (40) and in follicular keratinocytes and follicular fibroblasts of cultured human hair follicle cells (26), indicating that estrogen synthesis may occur in the hair follicle, the sebaceous gland and in fibroblasts. Androgen levels may regulate estrogen synthesis since DHT has been shown to stimulate aromatase activity in cultured human fibroblasts (8). In addition to aromatase, E2 is also produced from E1 by 17β-HSD in

skin, at twice the rate of adipose tissue (28). Adipose tissue is thought to be a major extraglandular source of estrogens (14). Since our study used explants without subcutaneous fat, skin may also be a major source of extraglandular estrogens. In rat skin, the rate of E2 to E1 conversion varies with the phase of the hair cycle(31), indicating that 17β-HSD activity may regulate the effects of E2 on the hair cycle. Aromatase activity combined with 17β-hydroxysteroid dehydrogenase activity may account for the relatively high rate of E2 synthesis seen in our study. Additionally, in mammary tissue, deconjugation is a major source of estrogens, indicating that deconjugation of sulfated or glucuronidated E2 may be an additional source for E2 and a possible mechanism for regulating E2 levels (46).

In general, testosterone is metabolized to estrogenic and androgenic metabolites to approximately the same extent in our study. For estrogenic metabolites, a similar metabolic profile was observed on both the skin explants and the incubation medium. However, for androgenic metabolites, a greater amount of androstanediol is observed in the incubation media and a greater amount of androstanedione was measured in the skin explants. Androstanediol is more water soluble than androstanedione and there may be an active transport process that selectively targets androstanediol for excretion.

In skin explants incubated with ^{3}H-E2, there was a time dependent uptake of parent E2 into the skin. In studies where mouse skin explants were topically treated with ^{3}H-E2 or ^{14}C-testosterone, three times more testosterone than E2 was excreted into the media (23), indicating that E2 is more likely to be retained in skin. Additionally, the partition

coefficient (log K_{ow}) of E2 is 4.01, and the partition coefficient of testosterone is 3.32 (21) indicting that E2 is a more lipophilic compound.

In summary, our study has shown that; i) cutaneous E2 levels in female mice average 9 times higher than serum E2 levels, ii) skin E2 levels are independent of serum E2 levels, iii) skin has a high capacity to synthesize E2 from testosterone. Considering the importance of estrogens in skin function, it is not surprising that skin E2 levels are significant, and that there are mechanisms in place for maintaining them.

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FIGURE LEGENDS

Figure1. Cutaneous E2 levels and serum E2 levels in mice. Dorsal skin from 20 week old mice was homogenized, extracted and analyzed for E2 by RIA. *A*. Skin (□) and serum (▨) E2 levels in intact female (n=3), OVX female (n=4) and male (n=4) mouse skin. * denotes significant difference between male and female E2 levels, analyzed using Student's t test, $p< 0.05$. Data are expressed as mean ± SD. *B*. Skin (□) and serum (▨) E2 levels in individual mice. *C*. Scattergram plot of skin and serum E2 values for linear regression analysis. Slope = -0.0298, y intercept = 25, $R^2 = 0.0886$, and R = 0.2976.

Figure 2. ^{3}H-Testosterone metabolism in mouse skin explants. Dorsal skin explants from male mice(~200mg) were incubated in media containing ^{3}H-testosterone for 0.25, 0.5, 1 and 2 hours, then removed and homogenized, ^{3}H-metabolites in the skin homogenate was extracted and analyzed by TLC. *A*. Estrogenic metabolites. E1 (-O-), E2 (-■-), E3 (-◆-). *B*. Androgenic metabolites. DHT (-□-), androstenedione (-●-), 5α-androstane-3β,17β-diol (-x-), androstanedione (-∗-). *C*. Estrogenic acid hydrolyzible aqueous metabolites. E1 (-O-), E2 (-■-), E3 (-◆-). *D*. Androgenic acid hydrolyzible aqueous metabolites. DHT (-□-), androstenedione (-△-), 5α-androstane-3α,17β-diol and 5α-androstane-3β,17β-diol (-x-), androstanedione (-∗-). *E*. Percent total metabolites in skin N=3 explants for each time point. Data are expressed as mean ± SD.

Figure 3. ^{3}H-Testosterone metabolites in incubation media from mouse skin explants. Dorsal skin explants from male mice were incubated in media containing ^{3}H-

testosterone for 1 and 2 hours and then removed. The media was extracted and analyzed by TLC. *A*. Estrogenic metabolites. E1 (□), E2(▨), E3 (⁝). *B*. Androgenic metabolites as DPM. DHT (□), androstenedione (▩), 5α-androstane-3β,17β-diol (▨), androstanedione (▩) *C*. Estrogenic acid hydrolyzed aqueous metabolites. E1 (□), E2 (▨), E3 (⁝). *D*. Androgenic acid hydrolyzed aqueous metabolites. DHT (□), androstenedione (▩), 5α-androstane-3β,17β-diol (▨), androstanedione (▩). *E*. Percent total metabolites in media, aqueous fraction consists of aqueous soluble, unhydrolyzible metabolites. One hour of incubation (□), two hours of incubation (▩). N=3 plates for each time point. Data are expressed as mean ± SD.

Figure 4. ^{3}H-E2 metabolites in incubation media from male mouse skin explants. Mouse dorsal skin explants were incubated in media containing ^{3}H-E2 for 0.25, 0.5, 1 and 2 hours and then removed. The media was extracted and analyzed by TLC. *A*. Parent E2 and metabolites in explants. E1 (-□-), E2 (-▲-), E3 (-*-), and aqueous soluble metabolites (-o-). *B*. Parent E2 and metabolites in the incubation media. E1 (-□-), E2 (-▲-), E3 (-*-), and aqueous soluble metabolites (-o-). N=3 explants per time point. Data is expressed as mean ± SD.

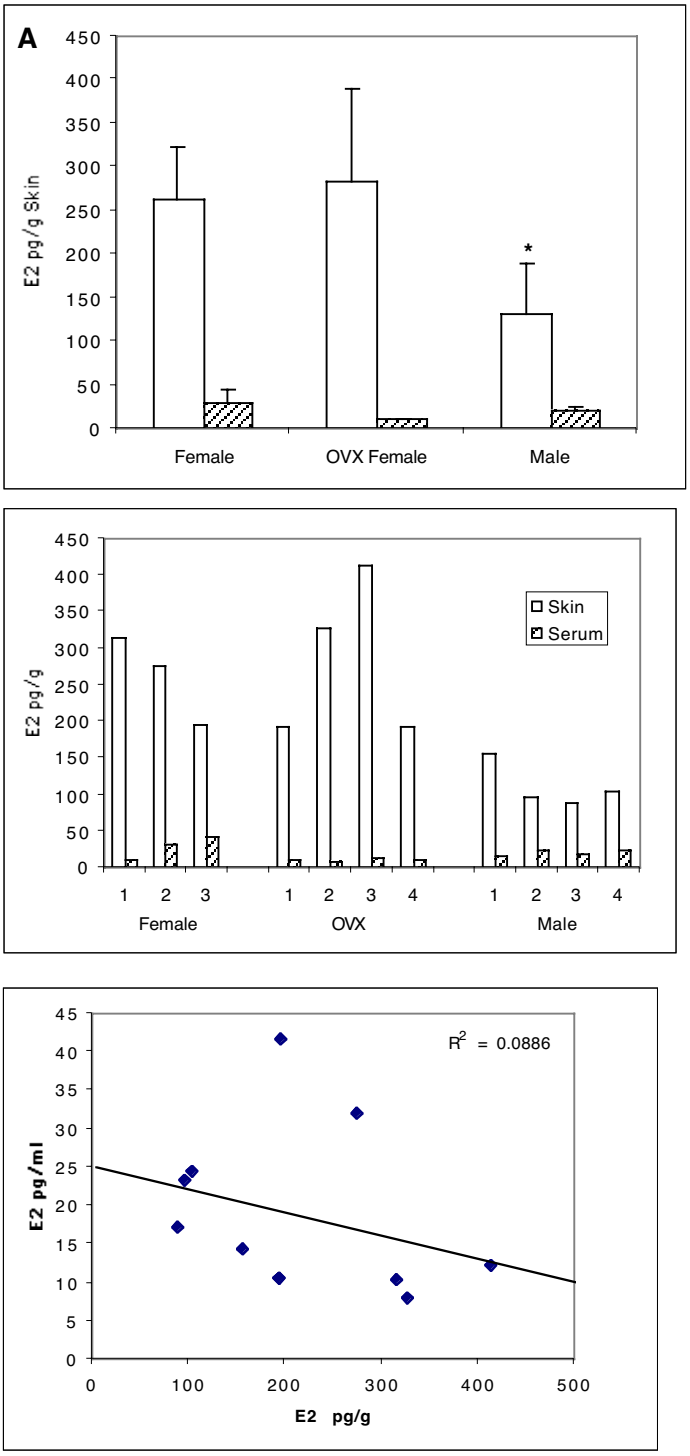

Figure 1.1 Cutaneous E2 levels are higher than serum E2 levels.

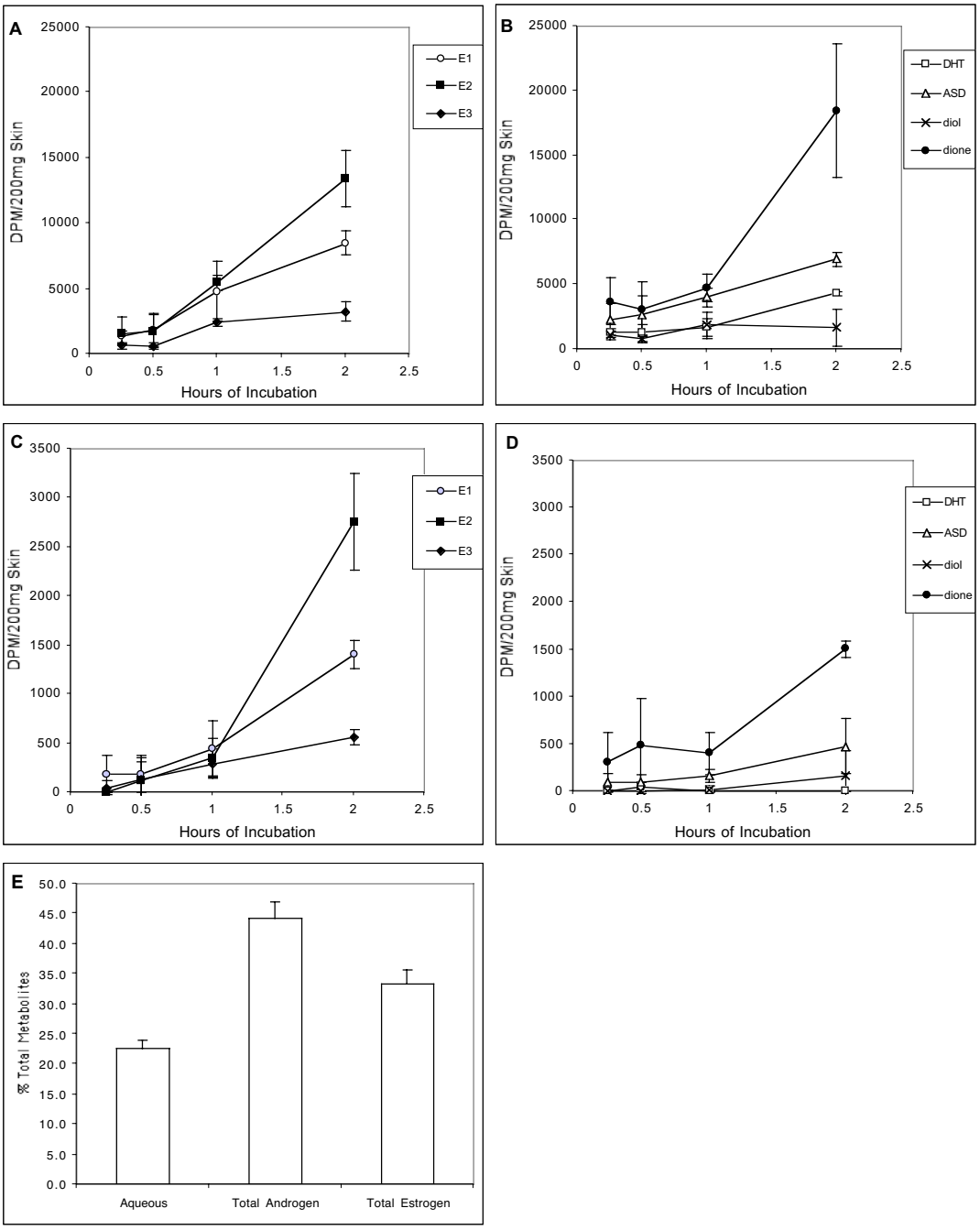

Figure 1.2 ^{3}H-Testosterone metabolism in male mouse skin explants.

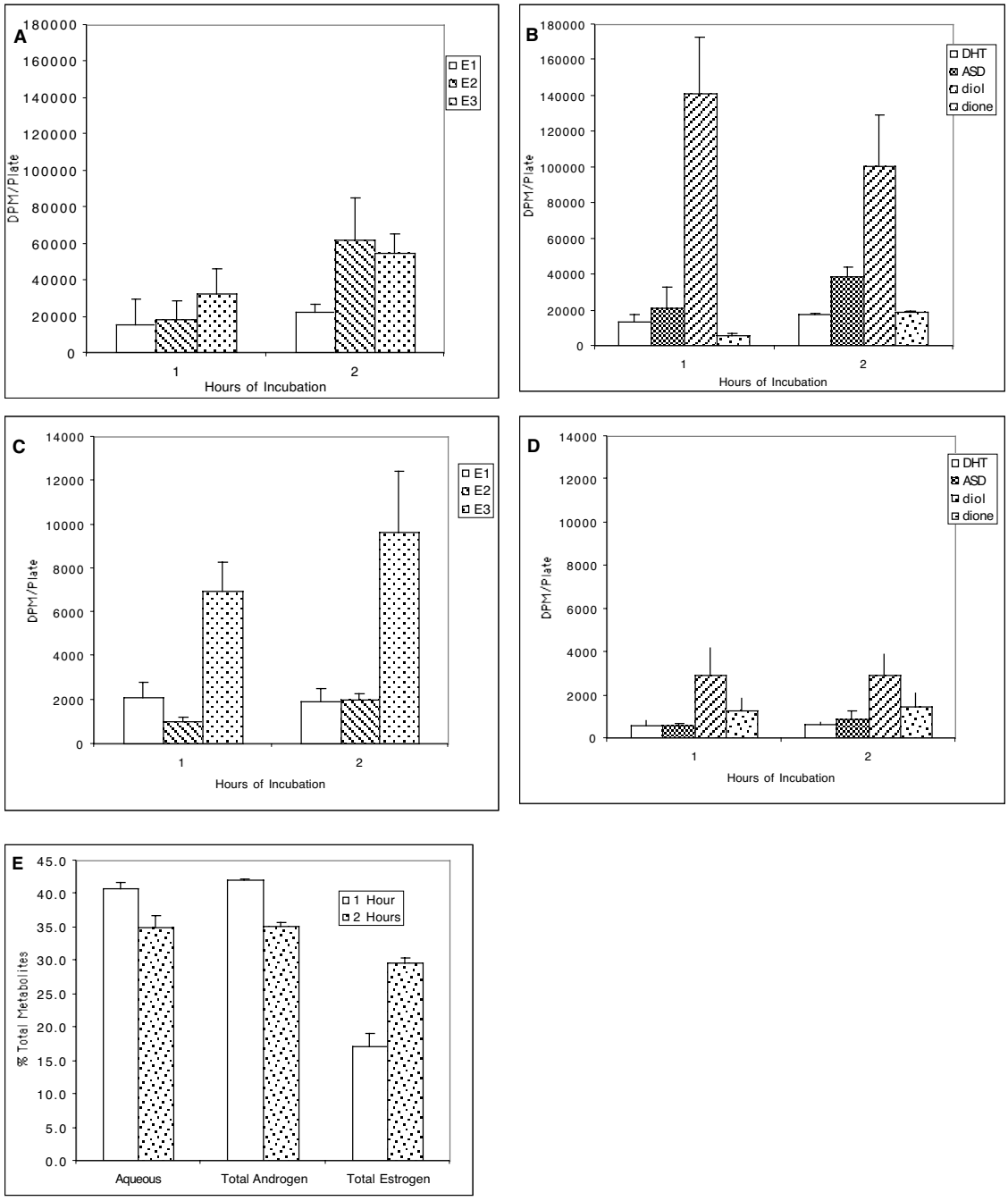

Figure 1.3 ^{3}H-Testosterone metabolites in mouse skin explant incubation media.

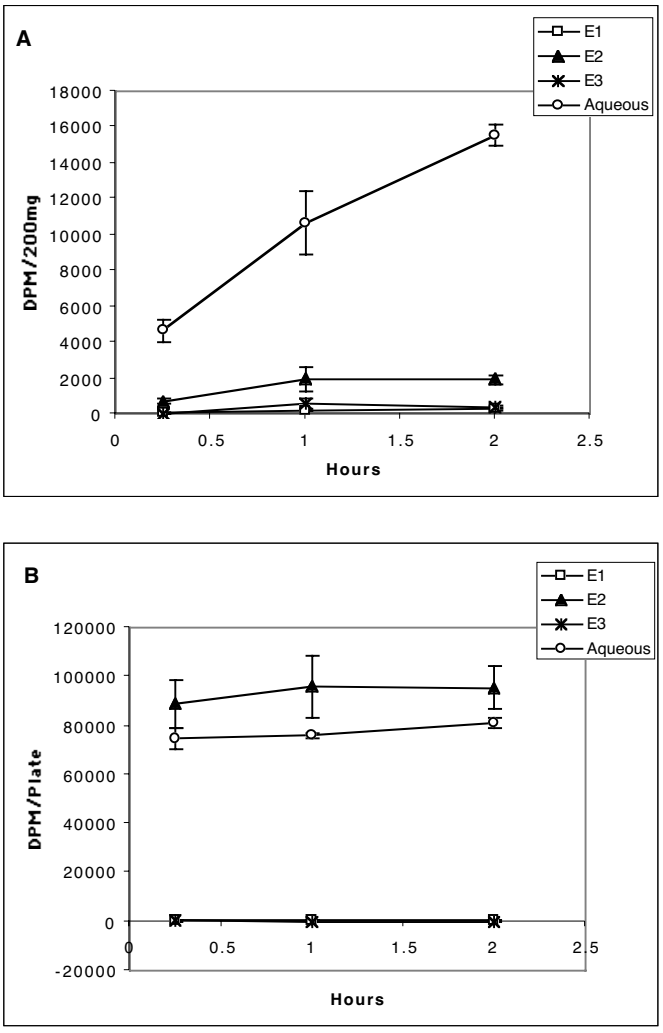

Figure 1.4 ^{3}H-E2 metabolism in mouse skin explants and incubation media.

MANUSCRIPT TWO

17β-Estradiol Treatment Reverses Castration Induced Deregulation of the Hair Follicle Cycle Significantly More Effectively than Androgens

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Abbreviated Title: 17β-Estradiol reverses the effects of castration

Key Words: skin; 17β-estradiol; hair cycle; androgen; regulation

ABSTRACT

The hair follicle is a complex, self-renewing, cyclic epidermal structure that is influenced by both androgens and estrogens. Previous studies have shown that castration of male mice induces hair growth. To test this hypothesis, dorsally shaved male mice (six weeks old) in the telogen phase/resting phase of the hair follicle cycle were castrated or left intact. Intact male mice exhibited full hair regrowth in the shaved region within nine weeks. In contrast, castrated mice rapidly entered the anagen phase/growth phase of the hair follicle cycle and by two weeks all mice exhibited full hair growth in the shaved region, indicating that castration induced a synchronous telogen to anagen transition. Hair follicles of castrated mice exhibited a normal anagen time period, however the follicles continued to display a deregulated cycle in which telogen was absent or greatly diminished for next two to three hair follicle cycles. Surprisingly, 17β-estradiol (1nmol) topically applied twice weekly, but not DHT (25nmol) or testosterone (100nmol), could reverse the effects of castration on the hair follicle cycle. Daily topical applications of DHT (25 nmol) or testosterone (100 nmol) were required to reverse the effects of castration on the hair follicle cycle. These data indicate that castration greatly shortens or eliminates telogen, deregulating the hair cycle and that high dose androgen or low dose estrogen treatment can reverse the effects of castration, indicating that hair growth regulation may depend on both estrogen and androgen influences.

INTRODUCTION

The hair follicle is a complex, cyclic epidermal structure capable of periodic remodeling and regeneration. The hair cycle consists of a telogen, resting phase, an anagen active growth phase and a brief transitional catagen phase where the follicle shortens to one-third its anagen length before transitioning to telogen (1, 9). The hair follicle contains a bulge stem cell thought to be governed by an unknown diffusible factor from the dermal papilla and during anagen, the bulge stem cell is stimulated to divide into transient amplifying cells which proliferate, then terminally differentiate to form the inner and outer root sheath and the hair matrix (11).

In mice, the initial postnatal period of hair growth is completed by 16 days of age, followed by the second synchronous period of hair growth beginning at 20 to 30 days of age, then the third synchronous period of hair growth at 9 to 13 weeks of age (1). Hair growth in mice proceeds in waves and is synchronous through the third anagen phase, and becomes asynchronous in subsequent hair cycles as the animal ages (12). Early studies have shown that gonadectomy in male and female mice of various strains shortens the time to restore normal hair coat after clipping (15). Moreover, high doses of oral testosterone propionate can inhibit hair growth in castrated mice (15).

The androgen receptor (AR) has been located in the dermal papilla of telogen and anagen hair follicles (5, 10), and in higher numbers in balding human scalp skin (14). Androgens, which are implicated in the etiology of male pattern baldness, have been shown to reduce proliferation of dermal papilla cells in culture (16). In our laboratory, we

have found that the dermal papilla also expresses the estrogen receptor-α (ER-α) only in telogen and that topical 17β-estradiol (E2) blocks telogen-anagen transition in mice (17), while the ER antagonist, ICI 182,780, stimulates hair growth (8, 17).

Because hormones can influence the hair follicle cycle, we examined in detail the effects of castration. We observed that castration causes: i) a synchronous telogen to anagen transition, ii) telogen that is absent or greatly diminished in the next two hair cycles, iii) low dose E2 or high dose androgen reversed the effects of castration on the hair cycle.

MATERIALS AND METHODS

Materials

17β-estradiol, dihydrotestosterone, and testosterone were purchased from Sigma (St. Louis, MO). Acetone was purchased from Fisher (Fairlawn, NJ).

Animals

Male CD-1 mice, 5 weeks old, were purchased from Charles River Laboratories (Raleigh, NC) and kept in our animal facility for 1 week prior to use. The mice were fed rodent chow *ad libitum,* kept on corncob bedding and placed on a 12 hour light/dark cycle. At 6 weeks of age, mice were dorsally shaved using electric clippers two days prior to surgery. Mice who exhibited no hair growth were put under halothane anesthesia and castrated or sham castrated. Mice in topical treatment groups were treated with acetone, E2, DHT or testosterone within two hours of castration.

Castration and Hair Growth Study

Male mice in the telogen stage of the hair cycle were castrated or sham castrated at 6 weeks of age, six mice per group. The dorsal region of each mouse was shaved every other day; before shaving, hair growth was scored as determined by the presence or absence of 1mm hair fibers protruding above the skin and full hair growth was recorded at 95% coverage of the shaved area. Cessation of hair growth indicated that the hair follicles had exited anagen. The experiment was terminated at the fourth cycle of hair regrowth when asynchrony was visually achieved.

Effect of Steroid Treatment on Castrate Mouse Hair Growth Study

Male mice in the telogen stage of the hair cycle were castrated or sham castrated at 7 weeks of age. Mice were topically treated with 200ul acetone, 1 or 5 nmol E2, 25 nmol DHT or 100nmol testosterone in 200ul acetone within two hours of castration. The mice were treated with E2 twice weekly or with androgen daily or twice weekly for 12 weeks. Mice were monitored daily for hair growth, recorded at the first appearance of hair above the epidermis, and full hair growth was recorded at 95% coverage of the shaved area. Pilot studies were done to determine the correct dose and frequency of dose for each compound.

RESULTS

Castration causes a synchronous telogen to anagen transition. In order to examine castration induced telogen-anagen transition, we castrated or sham castrated dorsally shaved male mice and observed hair growth. Intact male mice exhibited full hair regrowth by three to nine weeks (Figure 1A-1F). In contrast, castrated mice exhibit synchronous visible hair growth covering the entire shaved area within 12 to 13 days post castration (Figure 1G-1L). Castrated mouse hair follicles underwent a normal period of anagen, as determined by the cessation of hair growth. However, the hair follicles of castrated mice continued to exhibit a deregulated cycle in which telogen was absent or greatly diminished as determined by the time interval between hair growth and no hair growth. This diminished telogen effect was observed for the next two to three hair follicle cycles. Additionally, there was less variation in hair growth patterns between individual castrated mice, compared to intact mice that have great variation in hair growth patterns between individual mice. Furthermore, hair growth erupted simultaneously over the entire shaved area in the castrated mice compared to intact mice who regrew their hair in anterior to posterior waves, indicating that castration eliminates the hair growth pattern usually seen in rodents.

17β-Estradiol reverses the effects of castration, but testosterone or DHT treatment has no effect. In order to compare the effectiveness of E2 treatment with androgen treatment in reversal of the effects of castration, we treated castrate mice with acetone, 1nmol E2, 5nmol E2, 100nmol testosterone or 25 nmol DHT twice weekly for 12 weeks. Castrated, acetone treated mice attained full hair growth 2 weeks after castration and intact mice

attained full hair growth from 5 to 12 weeks post surgery (Figure 3). Twice weekly treatment with1 nmol E2 reversed the effect of castration on the hair cycle and castrated mice treated with 5 nmol E2 never attained full hair growth in the course of the study. Twice-weekly treatment with testosterone or DHT had no effect on castrated mouse rapid telogen to anagen transition.

Daily topical androgen treatment is required to reverse the effects of castration. In order to determine whether daily treatment with testosterone or DHT can reversecastration induced telogen to anagen transition, we treated castrated mice topically with acetone (vehicle), testosterone (100nmol) or DHT (25nmol) daily for 12 weeks. Acetone treated, castrated mice attained full hair growth 2 weeks after castration (Figure 2). Castrate mice treated daily with testosterone or DHT attained full hair growth from 7 to 12 weeks post castration, indicating that daily treatment with DHT or testosterone is required to reverse the effects of castration. DHT, the more potent androgen, is four times more effective than testosterone, indicating that the effectiveness of androgen induced reversal of castration may be related to the potency of the androgen employed.

DISCUSSION

Previous studies have shown that in male mice with plucked skin, the hair fiber erupts from the skin at about the eighth day of anagen and full length hair growth is reached about ten days later (9). In the current study we observed that castrate mice exhibited full hair growth 12 to 13 days after castration, while intact mice exhibited full hair regrowth within three to nine weeks after sham castration. These results demonstrate that castration greatly shortens the telogen phase of hair growth and based on hair fiber eruption through the skin surface it appears castration induces a telogen to anagen transition within two to three days post castration. Castrated male mice have approximately 25 times less plasma testosterone than intact male mice (2) but both intact and castrate mice have statistically the same serum E2 levels (6), indicating that castration induced hair cycle deregulation may be dependent on systemic androgen levels but independent of systemic estrogen levels.

Local temporal control of androgen and estrogen metabolism may be an important component of hair cycle regulation. Both 5α-reductase, which converts testosterone to DHT, and aromatase, which converts testosterone to E2, have been localized to the hair follicle (21, 22). Conversion of testosterone to DHT, increases during telogen, and decreases during anagen (20), and conversion of E2 to estrone increases during anagen and decreases in telogen (19). Taken together these studies indicate that the most potent estrogen, E2, and the most potent androgen, DHT, are present at higher levels in telogen. Moreover, E2 biosynthesis from testosterone by aromatase is stimulated by DHT in human skin fibroblasts (7), but decreased by testosterone (4).

E2 has been shown to be a potent inhibitor of hair growth by blocking telogen to anagen transition (17). The fact that castration induces telogen to anagen transition prompted us to evaluate the effects of E2 on this transition. Surprisingly, E2 was approximately two orders of magnitude more potent than DHT or testosterone in reversing the effects of castration on the hair follicle cycle. These results are consistent with the idea that endogenous levels of E2 are important in regulating that hair follicle cycle.

An additional factor in hair growth regulation may be the growth factor TGF-β1, which inhibits keratinocyte proliferation, induces keratinocyte apoptosis (18), and is expressed *in vivo* in the outer root sheath in the area below the bulge region only during anagen and catagen (13). TGF-β1 regulates entry into and completion of the catagen phase of hair growth, and TGF-β1 treatment induces premature catagen in mice that are in synchronous anagen (13). Since E2 treatment increases TGFβ1 levels in dermal fibroblasts (3), it is possible that E2 and TGFβ interact to produce hair growth blockade.

Collectively, these data indicate that androgen and estrogen hair cycle regulation are intertwined, and suggests that castration induced deregulation of the hair cycle results from loss of androgen mediated telogen arrest. That only a small quantity of exogenous E2 is required to tip the balance toward normal regulatory control suggests that E2, acting in concert with androgens, is the primary hormone responsible for hair cycle regulation.

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FIGURE LEGENDS

Figure 1. Castration deregulates the hair cycle in mice. Castrate and intact male mice were shaved on alternate days and observed for hair growth for 18 weeks post castration. Individual intact mice (A-F), individual castrate mice (G-L), n = 6. Area above the x-axis represents visible hair growth, area below the x-axis represents no visible hair growth.

Figure 2. Twice-weekly topical E2 treatment reverses the effects of castration, but twice-weekly topical testosterone or DHT treatment has no effect. Castrate mice were treated twice-weekly with topical acetone (-♦-), 1 nmol E2 (-△-), or 5 nmol E2 (-*-). Intact male mice were treated daily with acetone (-□-). Hair growth was reported on alternate days for 12 weeks post surgery. N=6 for each group.

Figure 3. Daily topical testosterone or DHT treatment reverses the effect of castration on hair regrowth. Castrate male mice were treated daily with topical acetone (-♦-), 100nmol testosterone (-△-) or 25 nmol DHT (-O-) or twice weekly with 100nmol testosterone (-∇-) or 25nmol DHT (-*-). Intact male mice were treated daily with acetone (-□-). Hair growth was reported on alternate days for 12 weeks post surgery. N=6 for each group.

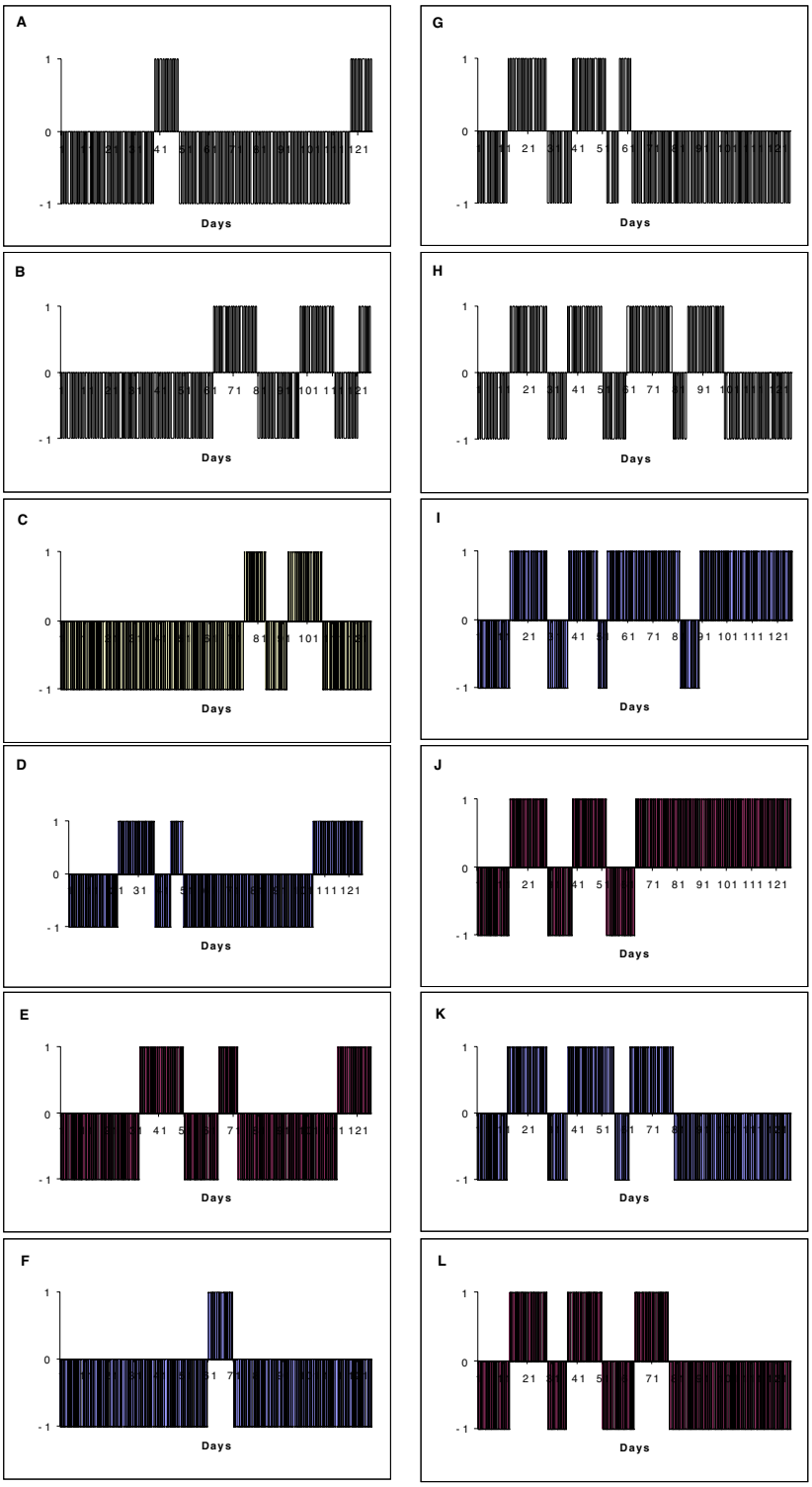

Figure 2.1 Castration deregulates the hair cycle in mice.

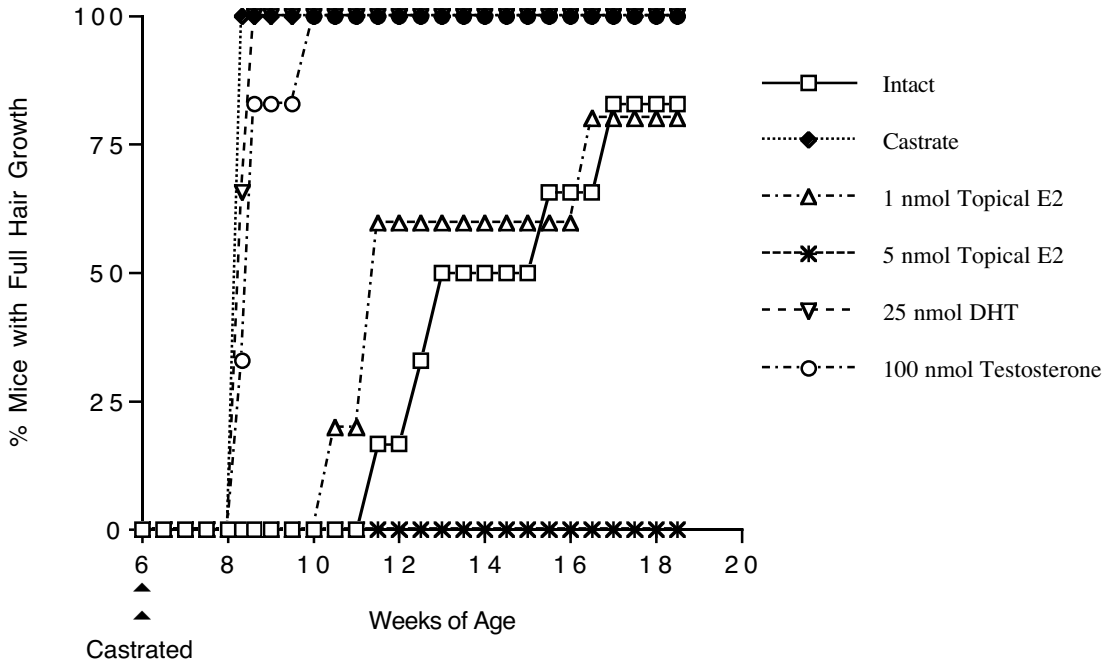

Figure 2.2. Twice-weekly topical E2 treatment reverses the effects of castration, but twice-weekly testosterone or DHT treatment has no effect.

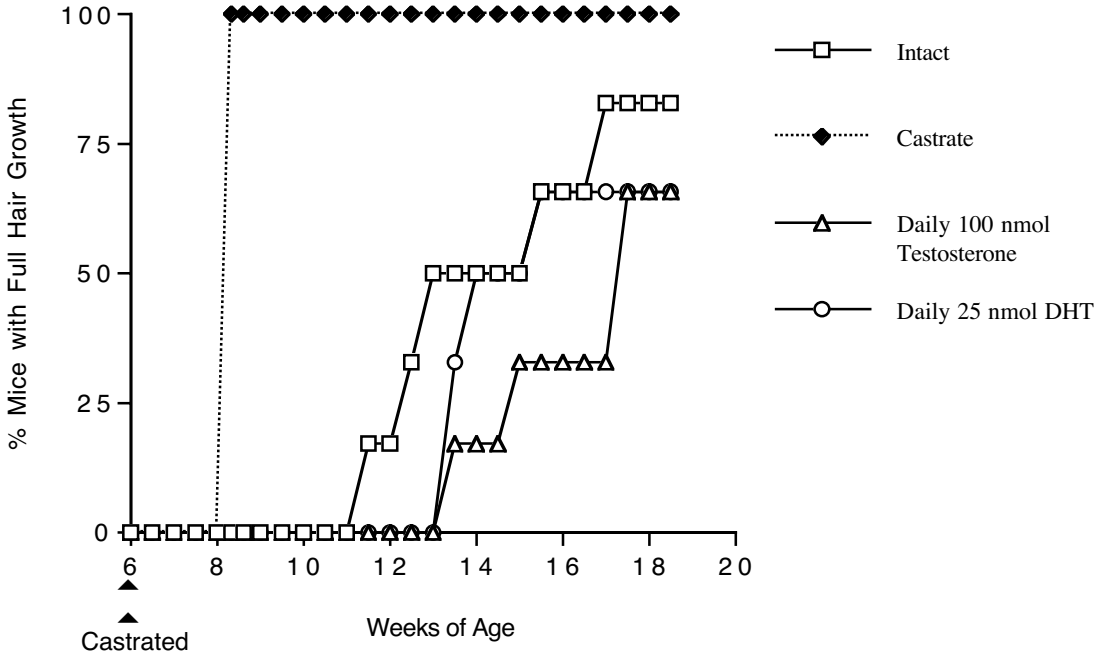

Figure 2.3. Daily topical testosterone or DHT treament is required to reverse the effects of castration.

MANUSCRIPT THREE

17β-Estradiol is the Primary Hormonal Regulator of Mirex Tumor Promotion Sensitivity

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Abbreviated Title: 17β-Estradiol regulates mirex tumor promotion

Key Words: mirex; skin; 17β-estradiol; tumor promotion; endocrine

ABSTRACT

Mirex, an organochlorine pesticide, is a potent non-phorbol ester tumor promoter in mouse skin. Previous studies have shown that female mice are three times more sensitive to mirex tumor promotion than male mice, and that ovariectomized (OVX) female mice are resistant to mirex tumor promotion, suggesting a role for ovarian hormones in mirex tumor promotion. To determine if the ovarian hormone, 17-β estradiol (E2), is responsible for the sensitivity of female mice to mirex, E2 containing implants were placed in ovariectomized (OVX), 7,12 dimethylbenz[*a*]anthracine (DMBA) initiated female mice to replenish serum E2 and a mirex tumor promotion experiment was conducted. Analysis of serum from the E2 implanted mice demonstrated high normal physiologic levels of E2 throughout the tumor promotion experiment. E2 implants restored by 80% the mirex tumor promotion sensitive phenotype to the OVX mice. However we found that mirex does not stimulate ER-α or ER-β transactivation in a sensitive mammalian cell ER reporter gene assay. Futhermore, topical application of the ER antagonist, ICI 182,780, inhibited mirex tumor promotion by 30% in female mice indicating that mirex may act through an indirect ER pathway. We conducted a similar tumor promotion study in male mice in order to determine how E2 implants affect mirex tumor promotion sensitivity in males. While E2 implants in male mice did increase the sensitivity to mirex tumor promotion, the implants could not produce the full female sensitivity to mirex tumor promotion, possibly due to E2-sex specific toxicity. Collectively these studies indicate that E2 is the major ovarian hormone responsible for mirex tumor promotion sensitivity in female mice.

INTRODUCTION

Mirex, an organochlorine pesticide, was used as an industrial fire retardant and to control fire ants in the southeastern United States until its cancellation by the US Environmental Protection Agency in 1977, and continues to be used in South America and South Africa (16, 34). The United Nations Environment Programme has recently identified mirex as one of the twelve most important persistent organic pollutants that threaten global human and wildlife health [1, 2,]. Mirex has been found in Lake Ontario sediment cores at depths corresponding with the mid-1960's (2) and has also been found in the Great Lakes area in bald eagle eggs and nestlings, herring gulls, cormorants, mussels and trout (5, 14, 17, 18, 35, 36). Additionally, mirex has been found in plasma from fish and waterfowl consumers in Ontario in 1999 (25), in human breast milk in New York in 1996 (30), and in human brain, liver and adipose tissues in Greenland in 1999 (13). In 1985, a US EPA national survey of chemicals in adipose tissue estimated that 10.2% of the southern US population has measurable levels of mirex in adipose tissue (27). The US EPA and the New York Department of Health have issued fish consumption advisories for mirex in Great Lakes fish in 2001 indicating that mirex continues to be a concern for human health (1, 23).

Mirex has been observed to be an epigenetic, non-phorbol ester hepatocarcinogen in rats, producing neoplastic nodules and hepatocellular carcinomas (38, 39). The US EPA has classified mirex as a probable human carcinogen in 2000 (15). Additionally, mirex was listed as "reasonably anticipated to be a human carcinogen" in the 2001 Ninth Report on Carcinogens [2]. The International Agency for Research on Cancer (IARC) has also

classified mirex as a possible human carcinogen (16). We have found that mirex, like phorbol esters, is a potent tumor promoter in 7,12 dimethylbenz[*a*]anthracine(DMBA) initiated mouse skin with a 90% incidence of A→T transversion in the 61st codon of Ha-*ras*. However, mirex skin tumor promotion is refractory to the classical phorbol ester and non-phorbol ester skin tumor promoter inhibitors, retinoic acid and the synthetic anti-inflammatory steroid, fluocinolone acetonide (26). Also, unlike phorbol esters, mirex skin tumor promotion is sexually dimorphic, promoting three times more tumors in female mice than in male mice. Additionally, ovariectomized (OVX) female mice promoted with mirex exhibited about one-third the number of papillomas per mouse as intact female mice, indicating that ovarian hormones are factors likely to influence mirex tumor promotion sensitivity (31, 32).

The hair follicle contains a stem cell located in the bulge region of the follicle. Early studies have shown that when an initiating carcinogen is topically applied to shaven skin, tumor yield will often depend on the phase of the hair cycle at application (11). Additionally, studies have shown that squamous cell hyperplastic foci in DMBA initiated, 12-*O*-tetradecanoylphorbol-13-acetate (TPA) promoted mouse skin histologically involve the hair follicle (4). Also, in H-*ras* transgenic TG.AC mouse skin treated with TPA, papillomas arise from hyperplastic foci of the follicular epithelium (22). These data indicate that the hair follicle containing the follicular bulge stem cell may be considered the target for chemical-induced skin carcinogenesis. Recently, we have found that skin expresses estrogen receptor-α (ER-α) in the dermal papilla of the hair follicle and that 17-β estradiol (E2) and ICI 182,780, an E2 antagonist, have

opposing effects on the hair cycle, indicating that skin is an estrogen responsive tissue (9, 33). Studies in mouse skin initiated with 3-methylcholantrene (MCA) have shown that the number of squamous cell carcinomas are three times higher in castrate male mice treated with E2 and MCA than in castrate mice treated with MCA alone, indicating that E2 is a factor in MCA induced skin carcinogenesis (29). Since the bulge stem cell is a putative target for chemical carcinogenesis and the hair follicle is responsive to E2, estrogen may be the ovarian hormone responsible for regulating mirex tumor promotion sensitivity.

The present study was conducted to determine the role of E2 in mirex tumor promotion by examining the ability of E2 implantation and gonadectomy to alter the number of skin papillomas promoted by mirex treatment. Since OVX female mice develop fewer papillomas than intact female mice and male mice develop fewer papillomas than female mice, it was hypothesized that an ovarian hormone, specifically E2, was regulating mirex tumor promotion.

MATERIALS AND METHODS

Materials

Analytical standard (99% purity) mirex (dodecachlorooctahydro-1,3,4-methano-1H-cyclobutal[*c,d*] pentalene) was purchased from Radian Corporation (Dallas, TX). 17β-estradiol (1,3,5[10]-estratriene-3, 17β-diol), kepone and thimersol were purchased from Sigma (St. Louis, MO). Polyethylene tubing was purchased from Becton Dickinson (Sparks, MD) while silastic tubing was obtained from VWR. Teflon beading was purchased from Cole Parmer (Vernon Hills, IL). Bloom 275 gelatin and norit A charcoal were purchased from Fisher (Fairlawn, NJ). Sodium phosphate, monobasic and dibasic, was purchased from JT Baker (Phillipsburg, NJ). HPLC grade, carbonyl free ethyl acetate was purchased from Burdick Jackson (Muskegon, MI). Rabbit anti-estradiol antibody was a kind gift from J.H. Britt (NCSU). Estradiol-6-(O-carboxymethyl)oximino-)2-[^{125}I]iodohistamine tracer was purchased from Amersham (Arlington Heights, IL). ICI 182,780 was a kind gift from Zeneca Pharmaceuticals (Wilmington, DE). Materials used in the HepG2 transcription assay are as previously described (29) except that the transfection reagent used was TransIT-LT1 from Pan Vera (Madison, WI).

Animals

Male and female CD-1 mice (6 weeks old) were purchased from Charles River Laboratories (Raleigh, NC) and kept in our animal facility for 1 week prior to use. The mice were fed rodent chow *ad libitum,* kept on corncob bedding and placed on a 12-hour light/dark cycle. Male or female CD-1 mice (7 weeks old) were shaved on their dorsal

surface with electric clippers. One week later, the mice that did not show hair regrowth were treated with a single topical application of 200 nmol DMBA in 200 μl acetone for males and 50 nmol DMBA in 200 μl acetone for females. Two weeks after initiation, the mice were put under halothane anesthesia and either castrated or ovariectomized (OVX), or sham operated, and given subcutaneous empty or 17β-estradiol (E2) containing silastic implants over the scapula. Beginning two weeks later, the mice were promoted with 200 nmol mirex in 200ul acetone twice weekly for 27 weeks. Control female mice were given E2 implants and either initiated with DMBA and promoted with acetone or initiated with acetone and promoted with mirex. A separate group of mice were DMBA initiated, given silastic E2 implants and promoted with mirex for various periods of time up to 31.5 weeks to provide timed interval determination of serum estradiol levels. Blood was drawn at the start of mirex promotion, which was two weeks post implantation, at 18 weeks of promotion (twenty weeks post implantation) and at 29.5 weeks of promotion (31.5 weeks post implantation) by cardiac puncture under halothane anesthesia prior to sacrifice. The blood was allowed to clot, centrifuged for 10 minutes, serum separated from cells and stored at -20° until analysis. At the end of each tumor promotion study, blood was drawn; serum was separated and stored at -20° until analysis. In the ICI 182,780 study, female mice were initiated with DMBA at 8 weeks of age, then promoted twice weekly with 200 nmol mirex in 200 μl acetone two weeks later for 25 weeks. The mice were treated with 10 nmol ICI 182,780 in 200 μl acetone 30 minutes prior to each mirex treatment.

Implants

Implants for male mice were made from 12mm lengths of polyethylene tubing (1.77mm I.D., 2.80mm O.D.) filled with an 8mm crystalline E2 column, plugged with Teflon beading and heat-sealed. Implants for female mice were made from1 cm lengths of silastic tubing, (1.57mm I.D., 3.12mm O.D), filled with 2.95 mg crystalline E2 (2.5 mm length) and plugged with Teflon. This tube was inserted into a second 1.2 cm length of silastic tubing, (2.64 mm I.D., 4.88 mm O.D.). This outer tube was then sealed at both ends with silicone medical adhesive and cured overnight. Male mice were given 4 polyethylene implants each, and female mice were given 1 silastic implant. All implants were conditioned overnight in carrier mice.

Estradiol Radioimmunoassay (RIA)

Serum estradiol was assayed by a method previously described (9, 12). Briefly, 100 µl of serum was freeze extracted with 2 ml ethyl acetate, evaporated at 37°C under nitrogen, reconstituted with PBS-gel Buffer (0.01 M PBS, 0.1% gelatin, pH 7.0) and incubated overnight with 200 µl antibody (diluted 1:1,500,000 with PBS-gel). The next day, 100 µl tracer diluted with PBS-gel (approximately 8000 cpm) were added and incubated at 4°C for 6 hours. Dextran coated charcoal (500 µl, 0.05% dextran, 0.5% charcoal in PBS-gel) was added, vortexed, then incubated for 45 minutes at 4°C, then centrifuged at 1550 x g for 15 minutes. The supernatant was decanted and counted using a gamma counter (1272 Clinigamma, Wallac Instruments, Gaitherburg, MD). Estradiol levels were determined from a standard curve. Recovery was measured using a pooled serum sample spiked with tracer.

HepG2 Cell Luciferase Assay for Estrogenicity

Briefly, HepG2 cells were grown in complete phenol red-free MEM with stripped fetal bovine serum and transiently transfected with plasmids containing β-galactosidase, ER-α or ER-β, and an ERE-containing luciferase reporter gene as previously described (29) except that TransIT transfection reagent (2μl/μg of plasmid DNA) was used. After transfection, cells were incubated 3 hours, then media was removed and the plates were washed with PBS. Mirex, kepone and E2 were serially diluted in DMSO to yield final concentrations in culture media from 10^{-5} M to 10^{-11} M after adding 1μl of the DMSO solution to l ml of media. Each chemical solution was added to the plates, three wells per chemical per dilution, and incubated for 24 hours.

After incubation, media was aspirated, and the plates were washed with PBS. Cells were lysed for 30 minutes in 65 μl lysis buffer per well (25 mM Tris-phosphate, pH 7.8, 2 mM 1,2 diaminocyclohexane-N,N,N',N'-tetraacetic acid, 10% glycerol, 0.5% Triton X-100, 2 mM dithiothreitol). For the β-galactosidase assay for transfection efficiency, 30 μl of each lysate was put into a 96 well microtiter plate with 170 μl CPRG reagent (20 μl of a 4 mg/ml solution of chlorophenol red-β-D-galactopyranoside added to 150 μl of CPRG buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, l mM $MgSO_4$, 50 mM β-mercaptoethanol, pH 7.8). The plate was read on a spectrophotometric plate reader to a kinetic endpoint at 570nm for 30 minutes at one-minute intervals. For the luciferase assay, 20 μl of each lysate was put into a 96 well luminometer plate with 100 μl Promega luciferase assay reagent and immediately read in a luminometer. Luciferase activity was corrected for β-galactosidase activity.

RESULTS

Effect of Subcutaneous 17β-Estradiol Containing Implants on Mirex Tumor Promotion in Ovariectomized Female Mice

In order to determine whether E2 is the ovarian hormone that is responsible for the sensitivity of female mice to mirex tumor promotion, we placed E2 containing subcutaneous implants in OVX female mice in an attempt to restore sensitivity to mirex tumor promotion. Female mice were initiated with DMBA, then two weeks later groups of mice were OVX or sham OVX and silastic implants with or without E2 were surgically implanted subcutaneously. Two weeks later, the mice were promoted with mirex, twice weekly, for 26 weeks. As shown in Figure 1, intact female mice developed 7.5 papillomas/mouse and 100% of the mice developed papillomas while OVX female mice with sham implants developed only 3 papillomas/mouse and 65% tumor incidence. In contrast, OVX mice with E2 containing implants developed 6 papillomas/mouse and 90% of the mice developed papillomas. A preliminary experiment performed using the same protocol also showed similar results. As shown in Figure 1, control mice with E2 containing implants did not develop any papillomas. These results demonstrate that chronic systemic administration of E2 can increase the sensitivity to mirex tumor promotion in OVX female mice to nearly that observed in intact female mice

Since silastic E2 implants were capable of restoring the sensitivity of OVX female mice to mirex promotion, it was important to determine the serum concentration of E2 in OVX E2 implanted mice and compare these values to the serum E2 concentration of E2 in

intact and OVX mice. As shown in Figure 2, serum E2 levels in intact female mice range between 5.8 and 79.6 pg/ml, while E2 levels in OVX mice were low and ranged between 3.6 and 9.4 pg/ml. The broad range of E2 values measured in the intact female mice is due to the mice being in different stages of the estrous cycle. In E2 implanted OVX mice, E2 levels were measured at various time points after implantation. At two weeks post implantation, which represents the start of tumor promotion, serum E2 ranged from 82 to 125 pg/ml with an average of 106 ±pg/ml, at 20 weeks implantation, which represents 18 weeks of promotion, serum E2 levels range from 40 to 85 pg/ml with an average of 60 ± 13 pg/ml, and at 31.5 weeks post implantation, which represents 29.5 weeks of promotion, serum E2 levels ranged from 50 to 75 pg/ml with an average of 62 ± 9.7 pg/ml. These data indicate that silastic E2 implants can raise OVX female mouse serum E2 levels to high physiological intact female mouse levels and sustain those levels through the end of the tumor promotion study.

Effect of Subcutaneous 17β-Estradiol Containing Implants on Mirex Tumor Promotion in Male Mice

In order to determine if E2 is responsible for sensitivity to mirex tumor promotion in male mice, we placed E2 containing subcutaneous implants in castrate male mice in an attempt to induce intact female mouse mirex tumor promotion sensitivity in male mice. Male mice were initiated with DMBA, and two weeks later, groups of mice were castrated or sham castrated and polyethylene implants with or without E2 were surgically implanted subcutaneously. Two weeks later, the mice were promoted with mirex, twice weekly, for 27 weeks. As shown in Figure 3, intact male mice with sham implants

developed 2.8 papillomas/mouse and 55% of the mice developed papillomas, while castrate mice with sham implants developed ~1.0 papillomas/mouse and 25% of the mice developed papillomas. In contrast, castrate E2 implanted mice developed 3.4 papillomas/mouse and 60% of the mice developed papillomas. These results demonstrate that castration decreases mirex promotion sensitivity and that chronic systemic administration of E2 can increase the sensitivity to mirex tumor promotion in castrate male mice to that observed in intact male mice, but not to that observed in intact female mice.

Since polyethylene implants are capable of restoring the sensitivity of castrate male mice to mirex promotion, we assayed serum E2 levels in intact male mice, castrate male mice and castrate E2 implanted male. Serum E2 levels in intact male mice ranged from 6.5 to 13.7 pg/ml and surprisingly, serum E2 levels in castrate male mice ranged from 6.0 to 12.2 pg/ml. However, serum E2 levels in E2 implanted mice range between 9.8 and 21.2 pg/ml at 29 weeks after implantation, 55% higher and significantly statistically different using Student's t test from E2 levels observed in intact male mice and within the low normal serum E2 range observed in intact female mice. Mortality due to urinary retention and hydronephrosis was observed in three of 25 E2 implanted mice early in the experiment when male mice received implants that released higher levels of estradiol and these animals were not included in the tumor promotion data.

Effect of Topical E2 Antagonist Treatment on Mirex Tumor Promotion in Female Mouse Skin

To determine if an ER-α mediated pathway modulates the sensitivity of female mice to mirex tumor promotion, we initiated intact female mice with DMBA, then two weeks

later, they were promoted topically with mirex and ICI 182,780, an ER antagonist, or mirex alone twice weekly, for 25 weeks. As shown in Figure 4, mice treated with mirex alone develop 11.8 papillomas/mouse, while mice treated with mirex and ICI 182,780 develop 8.2 papillomas/mouse, a 30% decrease in mirex tumor promotion sensitivity. Additionally, there was a two-week delay in tumor formation in the ICI 182,780 treated group. These results demonstrate that although ER-α blockade can reduce tumor yield, it cannot reduce tumor incidence or replicate the effects of ovariectomy at the dose used.

HepG2 Cell Luciferase Reporter Assay for Mirex and Kepone Estrogenicity

To determine whether mirex can induce ER-α or ER-β transactivation and reporter gene transcription in mammalian cells, we transiently transfected HepG2 cells with ER-α or ER-β, β-galactosidase and an estrogen response element (ERE) containing luciferase reporter gene construct. The transfected HepG2 cells were then treated with increasing concentrations of mirex or kepone, an organochlorine pesticide structurally similar to mirex. As shown in Figure 5A, mirex at concentrations up to 1×10^{-5} M did not induce luciferase activity, but kepone at 1.5×10^{-7} M did induce luciferase activity in the ER-α system. However, as shown in Figure 5B, neither mirex nor kepone induced luciferase activity in the ER-β system. We were unable to test concentrations of mirex higher than 10^{-5} M since mirex is a lipophilic compound with low solubility in aqueous solutions. These data show that mirex does not induce ER-α or ER-β transactivation activity in mammalian cells unlike structurally similar, but tumor promotion inactive kepone.

DISCUSSION

In this study we found that subcutaneous E2 implants were able to restore eighty percent of the intact female mouse sensitivity to OVX female mice indicating that E2 is the primary ovarian hormone responsible for regulating mirex tumor promotion sensitivity. The E2 implants induced continuous, chronic serum E2 levels that were within the upper range of intact cycling female mice. However, intact female mice cycle from high E2 levels in proestrus, decreasing E2 levels in estrus and low E2 levels in diestrus during the four to five day estrus cycle [16], which may explain why E2 implant induced mirex tumor promotion sensitivity was only partial. Furthermore, other hormones may also be important since the ovary is the primary source of testosterone in females, and LH, FSH and progesterone levels also rise and fall during the estrus cycle [17]. Additionally, studies have shown that female mice given silastic implants, 6 mg E2 per implant, experience a marked increase in serum prolactin levels [18].

We have found that although castrate male mice develop one-third the tumors that intact male mice develop, intact and castrate male mice had statistically the same levels of serum E2. Furthermore, studies in nude mice confirm that intact and castrate male mice have similar serum E2 levels (40). These data indicate that mirex tumor promotion sensitivity in castrate male mice is independent of endogenous levels of circulating E2, but exogenous E2 at levels above physiological levels for male mice is sufficient to overcome the effects of castration and restore intact male mouse response. We observed a higher mortality rate in E2 implanted male mice from E2 toxicity and no mortality in E2 implanted female mice from E2 toxicity. This observation is consistent with the findings

of earlier studies where male mice develop urinary retention and hydronephrosis resulting in increased mortality after receiving silastic E2 implants (7).

ICI 182,780, a pure estrogen antagonist, was only able to reduce tumor multiplicity by 30% at the dose used, so it is possible that higher doses may yield a greater reduction. However, since mirex does not induce ER-α or ER-β transactivation in a HepG2 mammalian cell reporter assay, ER-α and its classical pathway may reflect only part of the picture. Additionally, mirex has previously been shown to have no affinity for ER-α in an *in vitro* competitive binding assay, unlike the structurally similar kepone (6), which is inactive as a tumor promoter (32). However, mirex may be an endocrine disruptor since it has been shown to inhibit E2 uptake in cultured rat hepatocytes (37) and mirex also increases 2-hydroxylation of estradiol in rat liver (8). Furthermore, the European Commission lists mirex as having evidence for endocrine disruption in wildlife and humans (10), and the Illinois State EPA lists mirex as a chemical probable to cause endocrine disruption (24).

An area of future investigation involves the role of transforming growth factor beta (TGF-β) in mirex skin tumor promotion. TGF-β1, TGF-β2, and TGF-β3 are all expressed in normal epidermis; TGF-β1 in the stratum granulosum and stratum corneum, TGF-β2, and TGF-β3 in the suprabasal layers (21). Recent studies have shown that E2 can activate TGF-β3 via a novel activated Ras dependent pathway (28). Since our laboratory has determined that greater than 90% of mirex promoted tumors express mutant Ha-*ras* (32), it is possible that E2 activation of TGF-β3 can occur in initiated mutant Ha-*ras* containing keratinocytes. Additionally, studies have shown that both

TGF-β3 and TGF-β1 are upregulated in spindle cell carcinoma [39]. Furthermore, studies have shown that low risk papillomas in skin express TGF-β1, and loss of TGF-β is associated with squamous cell carcinomas (19), and that TGF-β1 can act early in tumor progression as a tumor suppressor, but later enhance malignancy [39]. However, when initiated mutant Ha-*ras* containing TGF-β1 null keratinocytes were grafted onto nude mice, they developed multifocal squamous cell carcinomas, whereas normal initiated, mutant Ha-*ras* containing keratinocyte grafts developed into papillomas, indicating that there are opposing effects of autocrine and paracrine TGFβ1 on tumor progression (20). Interestingly, E2 has been demonstrated to increase levels of TGF-β1 in dermal fibroblasts (3), which may indicate an additional role for E2 in skin carcinogenesis.

Collectively, these data indicate that E2 is the primary ovarian hormone responsible for modulating mirex skin tumor promotion sensitivity in female mice, but other hormonal signals may also influence mirex tumor promotion. In male mice, exogenous E2 increases mirex skin tumor promotion sensitivity, but cannot replicate female mouse response. Castration decreases male mouse mirex skin tumor promotion sensitivity that is independent of endogenous E2 levels. It is possible that different mechanisms underlie male and female mirex skin tumor promotion sensitivity. Since E2 antagonist treatment only partially decreases intact female mouse response at the dose used, classical ER-α mediated pathways may be only a part of the overall mechanism of mirex skin tumor promotion. Non-classical or downstream ER-α mediated pathways, local skin hormones

or other ovarian or testicular hormones and TGF-β mediated pathways may also be factors in regulating mirex skin tumor promotion sensitivity.

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FIGURE LEGENDS

Figure. 1. Mirex tumor promotion response of intact (-□-), OVX (-♦-), and OVX with subcutaneous estradiol implants (-O-) female mice. Seven week old female mice were initiated with 50 nmol DMBA, two weeks later were sham OVX and given sham implants, OVX and given sham implants, or OVX and given subcutaneous 17β-estradiol implants, then promoted two weeks later with 200 nmol mirex twice weekly for 26 weeks. N = 25 mice per group. Two control groups of female mice were initiated with 50 nmol DMBA (-Δ-) or acetone (-X-) then given subcutaneous 17β-estradiol implants, then promoted two weeks later with 200 nmol mirex (-X-) or acetone (-Δ-) twice weekly for 26 weeks. N = 15.

Figure. 2. Serum 17β-estradiol ranges for OVX (-□-) n=22, and intact (-Δ-) n=34 female mice, sacrificed at 26 weeks of promotion, and E2 implanted OVX female mice sacrificed at 2 weeks (-O-) n=6, 20 weeks (-●-) n=9, and 31.5 weeks (-X-) n=7, post implantation.

Figure. 3. Mirex tumor promotion response of intact (-□-), castrate (-♦-), and castrate with subcutaneous estradiol implants (-O-) male mice. Seven week old male mice were initiated with 200 nmol DMBA, two weeks later were sham castrated and given empty implants, castrated and given empty implants, or castrated and given subcutaneous 17β-estradiol implants, then promoted two weeks later with 200 nmol mirex twice weekly for 27 weeks. N = 25 mice per group.

Figure 4. Mirex tumor promotion response of acetone treated (-□-) and ICI 182,780 treated (-♦-) intact female mice. Seven week old female mice were initiated with 50 nmol DMBA, then promoted two weeks later with 200 nmol mirex twice weekly and treated topically with acetone or 10 nmol ICI 182,780 twice weekly for 25 weeks. N = 25 mice per group.

Figure. 5. Luciferase activity from HEPG2 cells transiently transfected with ERα or ERβ and luciferase promoted by an ERE containing promoter then exposed to 17β-estradiol (-□-), mirex (-O-), or kepone (-▲-).

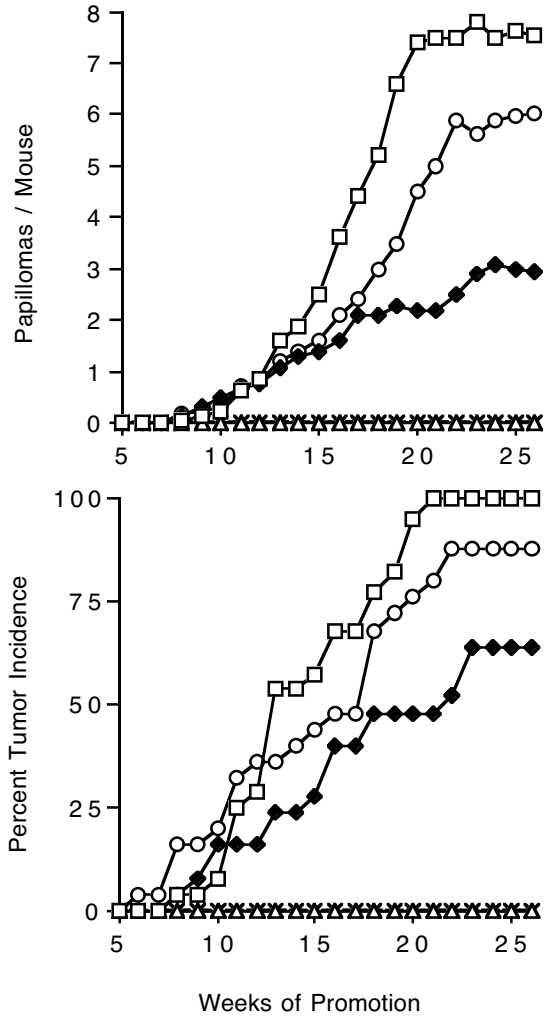

Figure 3.1 Mirex tumor promotion response of intact, OVX, and OVX with subcutaneous estradiol implants female mice.

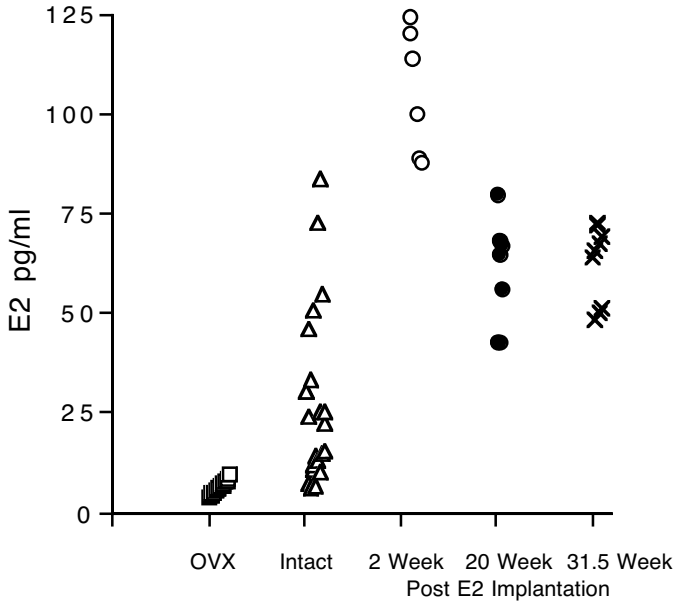

Figure 3. 2. Serum 17β-estradiol ranges for OVX, and intact female mice, sacrificed at 26 weeks of promotion, and E2 implanted OVX female mice sacrificed at 2 weeks, 20 weeks, and 31.5 weeks post implantation.

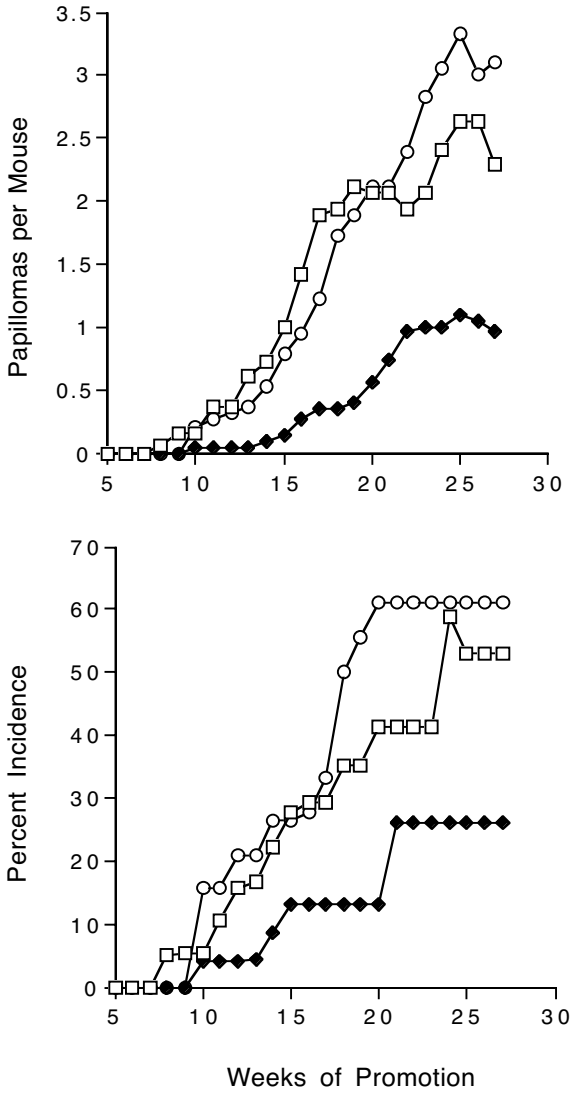

Figure 3.3. Mirex tumor promotion response of intact, castrate, and castrate with subcutaneous E2 implant.

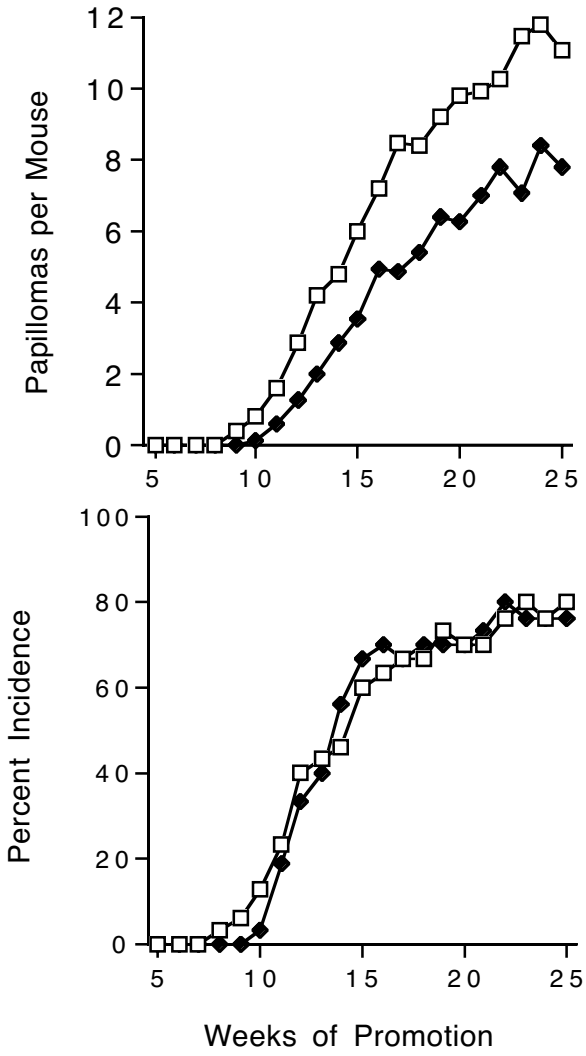

Figure 3.4. Mirex tumor promotion response of acetone treated and ICI 182,780 treated intact female mice.

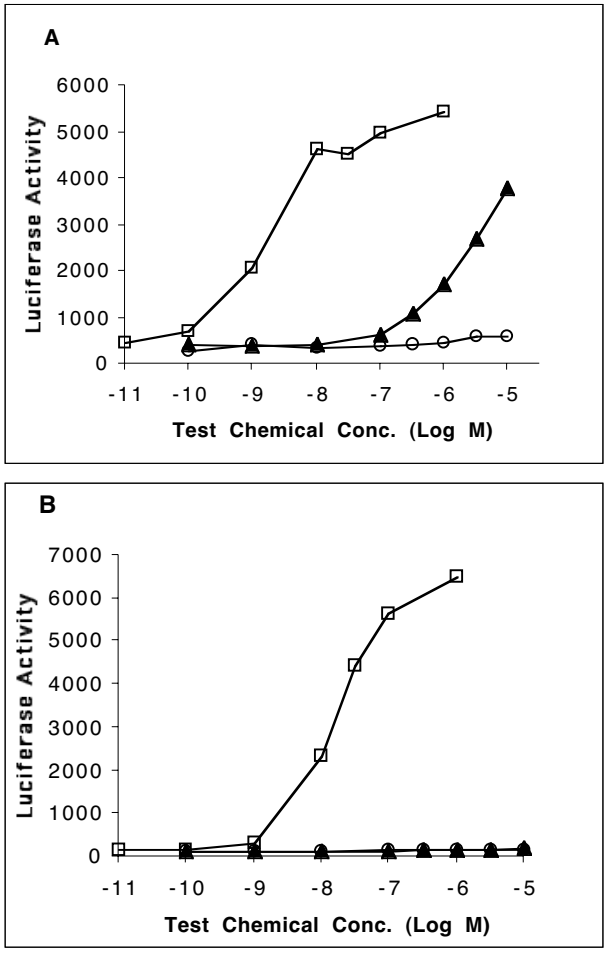

Figure 3.5. Luciferase activity from HEPG2 cells transiently transfected with ERα or ERβ and luciferase promoted by an ERE containing promoter then exposed to 17β-estradiol, mirex, or kepone.

GENERAL DISCUSSION

Manuscript One

Our data have shown that E2 in female mouse skin is abundant and levels are maintained by synthesis. In contrast, testosterone levels in balding human scalp skin, which is androgen sensitive, have been determined to be 3 times lower than serum testosterone levels (28). However, testosterone levels in human lower limb skin are 440 pg/g, 10 times lower than serum testosterone (29). Serum testosterone levels in male mice range from 3.2 pg/ml to 7520 pg/ml (5, 95), 2550 ± 1500 pg/ml in rats (75), and DHT levels in male mice are 340 ± 40 pg/ml (5), compared to E2 levels of 11.4 pg/ml in male mice and 15 pg/ml in rats (75, 95). In order to compare the relationship between skin and serum testosterone and DHT in mice, quantitation of testosterone and DHT levels in male and female mouse skin and serum levels should be done.

Androgen synthesis and metabolism in skin have been studied extensively in the past, but since skin has not been thought of as an estrogen responsive tissue, estrogen synthesis has had less attention. Some have suggested that aromatase activity in adipose tissue is the major source of estrogen in postmenopausal women and elderly men due to the correlation between increased obesity and age and increased estrone levels (108). Additionally, studies have shown that the V_{max} of aromatase activity in cultured human foreskin fibroblasts is similar to the V_{max} of aromatase activity for adipose tissue stromal vascular cells and since adipose has been thought of as major source of E2, this indicates that skin may also be an important site for estrogen synthesis in extraglandular cells (12). Additionally, studies have shown that DHT stimulates aromatase activity in cultured human fibroblasts (21), indicating that androgen levels may regulate estrogen synthesis.

E2 is also produced from E1 by 17β-hydroxysteroid dehydrogenase in skin, at roughly twice the rate of adipose tissue and almost the same rate of adrenal glands in mice (72). It is possible that the activity of estrogens may be influenced by local enzyme activity at or near ER's and by aromatization of androgens and deconjugation of estrogen conjugates (108). The enzyme estrone-3-sulfatase which hydrolyzes estrone-3-sulfate to estrone and is thought to be an important precursor to E2 in postmenopausal women. Estrogen β-glucuronidase converts E2 and E1 glucuronides to their parent compounds and is present in large quantities in rat mammary tissue at puberty (108). Additionally, vitamin D has been shown to regulate 17β-hydroxysteroid dehydrogenase activity in cultured keratinocytes and push the reaction equilibrium in the direction of E2 to E1 conversion, indicating that local17β-hydroxysteroid dehydrogenase activity may tightly regulate the effects of E2 in skin (50). Aromatase activity combined with 17β-hydroxysteroid dehydrogenase activity may account for the relatively high rate of E2 synthesis seen in our study. In order to determine if conjugation and deconjugation enzymes regulate skin E2 levels, additional work could be done to characterize estrogen conjugation by using glucuronidase, sulfatase, or other deconjugation enzymes and measure estrone-3-sulfatase and estrogen β-glucuronidase activity.

In our study of ^{3}H-E2 metabolism in mouse skin explants, we found that a greater percentage of E2 was converted to aqueous soluble metabolites than testosterone. Additionally we found that there was a time dependent increase in the accumulation of ^{3}H-E2 in the skin. We have performed a study of *in vivo* ^{3}H-E2 uptake and retention and found that E2 is retained in skin up to 18 hours post intravenous injection, 3 times longer

than in plasma and fat. In CH3 mouse skin explants topically treated with ^{3}H-E2 or ^{14}C-testosterone, approximately 18% of the E2 and 65% of the testosterone is passed through the epidermis and dermis into the media. The media contained 59% of the testosterone as parent compound and 79% of the E2 as parent compound, indicating that there is more retention of E2 than testosterone, and that E2 tends to remain in its parent form (54). The partition coefficient (log Kow) of E2 is 4.01 compared to 3.32 for testosterone (44), indicating that E2 is more lipophilic than testosterone and thus more likely to be retained in tissue. Additional studies to assess both E2 and testosterone uptake and retention in skin should be performed to verify this hypothesis.

Manuscript Two

In the current study we observed that castrated mice immediately enter another anagen phase after full-length hair growth is attained, indicating that castration had greatly shortened or eliminated the telogen phase of hair growth. Castration induced deregulation of the hair cycle may be more dependent on systemic androgen levels than on systemic endogenous estrogen levels, and only comparatively small quantities of exogenous E2 is required to overcome the effects of castration whereas high doses of androgen are required daily to produce the same effect. We had performed a hair growth study to chemically castrate intact male mice by treating them twice daily with topical flutamide, an androgen receptor antagonist, but the treatment had no effect on hair growth. The study could be repeated using castrate male mice with testosterone

containing implants to avoid the feedback effect, where androgen receptor blockade induces increased androgen production.

Conversion of testosterone to the more potent androgen, DHT, increases during telogen, and conversion to less potent androgens increases during anagen (87). Additionally, conversion of E2 to estrone, the less potent estrogen, increases during anagen and decreases in telogen (86), meaning that the most potent estrogen and androgen are present at higher levels in telogen. Moreover, E2 biosynthesis from testosterone by aromatase is stimulated by DHT in human skin fibroblasts (21), but decreased by testosterone and unaffected by E2 (11), adding another layer of metabolic regulation.

Another consideration is TGFβ expression during the hair cycle. TGFβ1 has been demonstrated to inhibit proliferation in hair follicles *in vitro* (81), and is expressed *in vivo* only during anagen and catagen, and is located in the outer root sheath in the area below the bulge region (38). Additionally, TGFβ1 deficient mice experience a greatly prolonged period of anagen and TGFβ1 injection in wild type mice induces premature catagen and increased apoptosis (38). Since E2 treatment increases TGFβ1 levels in dermal fibroblasts (7), it is possible that E2 and TGFβ interact to produce hair growth blockade. Studies using TGFβ1 and TGFβ receptor antagonists along with E2 and ICI 182,780 treatment would be useful to determine how E2 and TGFβ1 may interact in hair cycle regulation.

Quantitation of estrogen and androgen receptor expression in the hair follicle and dermal papilla in different phases of the hair cycle could be done by laser capture microdissection

followed by RT-PCR or RPA, or in situ RT-PCR to find site specific receptor expression and eliminate the dilution effect of skin thickening in anagen. We have done a preliminary study using ER-α knock out (ERKO) mice that showed surprisingly, topical E2 treatment blocked hair growth in both ERKO and wild type mice, but treatment with ICI 182,780, an ER antagonist, induced anagen in wild type mice, but not in ERKO mice, indicating that hair growth blockade may not be entirely receptor mediated, but telogen to anagen transition may require a receptor mediated pathway. However, the numbers of mice used were not sufficient to make definite conclusions. More extensive studies with ERKO-α and perhaps ARKO mice could help define the role of ER-α and AR in hair cycle regulation.

Manuscript Three

In our mirex tumor promotion study we found that subcutaneous E2 implants were able to restore eighty percent of the intact female mouse sensitivity to OVX female mice indicating that E2 is the primary ovarian hormone responsible for regulating mirex tumor promotion sensitivity. We have found that although castrate male mice develop one-third the tumors that intact male mice develop, intact and castrate male mice had statistically the same levels of serum E2. These data indicate that mirex tumor promotion sensitivity in castrate male mice is independent of endogenous levels of circulating E2, but exogenous E2 treatment is sufficient to overcome the effects of castration and restore intact male mouse response, similar to the response to E2 treatment we saw in the hair growth studies. Since we know from our skin E2 study that there is no correlation

between serum E2 levels and whole skin E2 levels, the influence of E2 levels may be concentrated in specific microenvironments within the tissue and regulated by steroid hormone metabolizing enzymes such as aromatase, 17β-HSD, sulfatases or glucuronidases.

ICI 182,780, a pure estrogen antagonist, was only able to reduce tumor multiplicity by 30% at the dose used, and additional studies using higher doses would be useful to determine if there is a dose-response relationship. However, since mirex does not induce ER-α or ER-β transactivation in a HepG2 mammalian cell reporter assay, ER-α and its classical pathway may reflect only part of the picture. Recently, a non-nuclear membrane bound form of ER-α has been described (20, 61, 94). Studies have shown that membrane bound ER- α binds the regulatory subunit of phosphatidylinositol-3-OH kinase (PI3K) and that E2 stimulation of membrane bound ER- α leads to activation of the Akt anti-apoptotic pathway via PI3K (94). Additionally, membrane bound ER-α can activate PI3K independent of E2 as a possible mechanism of tamoxifen resistance in mammary tumors (20) and membrane bound ER- α activates the extracellular signal-regulated kinase (ERK) pathway and that this activation is resistant to tamoxifen and ICI 182,780 (61). Thus far, membrane bound ER- α has not yet been described in skin and this represents a target for future investigation.

Although this is a minor metabolic pathway, a controversial theory has been proposed that the production of catechol estrogens by 2-hydroxylation or 4-hydroxylase which are then converted to quinones, which produce free radicals that can induce DNA damage,

leading to carcinogenesis (108). However, mirex may be an endocrine disrupter since it has been shown to inhibit E2 uptake in cultured rat hepatocytes (97) and mirex also increases 2-hydroxylation of estradiol in rat liver (18). Since hydroxylated estradiol can be converted to quinones (108), mirex induced endocrine disruption may lead to carcinogenesis along this pathway. Studies to determine the ability of mirex to increase hydroxylated E2 and downstream products and cause DNA damage in keratinocytes treated with E2 and mirex could be done to explore this hypothesis.

An additional area of future investigation involves the possible role of transforming growth factor beta (TGF-β) in mirex skin tumor promotion. Recent studies have shown that E2 can activate TGF-β3 via a novel activated Ras dependent pathway (65). Since our laboratory has determined that greater than 90% of mirex promoted tumors express mutant Ha-*ras* (73), it is possible that E2 activation of TGF-β3 can occur in initiated mutant Ha-*ras* containing keratinocytes. When initiated mutant Ha-*ras* containing TGF-β1 null keratinocytes were grafted onto nude mice, they developed multifocal squamous cell carcinomas, whereas normal initiated, mutant Ha-*ras* containing keratinocyte grafts developed into papillomas, indicating that there are opposing effects of autocrine and paracrine TGFβ1 on tumor progression (41). E2 has been demonstrated to increase levels of TGF-β1 in dermal fibroblasts (7), which may indicate an additional role for E2 in skin carcinogenesis. Studies to localize and quantitate TGFβ1 and TGFβ3 expression in mirex promoted preneoplastic skin, papillomas and carcinomas compared to TPA would be important to determine if a relationship between TGFβ and mirex exists. Since TGFβ1 null mice die within 3 to 4 weeks of birth and TGFβ null mice of other isoforms

die at birth (38), tumor promotion studies using knockout mice would not be possible, however, transgenic mice using a tetracycline inducible system (42) or wild type mice treated with TGFβ or TGFβ antagonists may be used in mirex tumor promotion studies.

Future Directions

Collectively, our studies show that skin is an important extraglandular source and storage depot for estrogens and that estrogens are a potent hormonal regulator of the hair follicle cycle and mirex tumor promotion sensitivity. A global hypothesis for future studies could be stated as: microenvironment estrogen and androgen levels and follicular ER-α, AR and TGFβ expression interact to regulate the hair cycle and mirex tumor promotion sensitivity. Studies using ER, AR and TGFβ receptor agonists and antagonists or using ERKO or ARKO mice in hair growth and tumor promotion studies would be useful to determine the relationship between estrogen, androgen and TGFβ1 regulation of hair growth and carcinogenesis. As stated earlier, determination of testosterone and DHT levels, *in vivo* uptake and retention in mouse skin, along with determination of conjugated metabolites and sulfatase and glucuronidase deconjugation activity would also be important to completely assess the hormonal environment in skin. Additionally, to provide information on the microenvironment of hair follicles and papillomas, studies should be done using immunohistochemistry, laser capture microdissection or *in situ* RT-PCR to localize steroid metabolizing enzymes, receptors and their ligands to specific locations in normal skin during the hair cycle, mirex treated skin, preneoplastic foci and mirex promoted papillomas. We have done studies where mutant Ha-*ras* containing BALB/MK-2 keratinocytes were treated with DMSO, mirex, E2 or mirex and E2 and

were unable to find any significant, reproducible differences between treatments in cell growth, confluence or morphology. If we determine that BALB/MK-2 keratinocytes do not contain ER-α, AR or TGFβ receptors, studies where mutant Ha-*ras* containing BALB/MK-2 keratinocytes are transfected with ER-α, AR or TGFβ receptors and treated with mirex, E2, DHT, TGFβ1 or any combination would be useful to try and construct an *in vitro* model for mirex tumor promotion. Additionally, the same studies could be tried using primary keratinocytes from CD-1 mice and dermal papilla cells transfected with mutant Ha-*ras,* since mirex has been shown to promote a separate population of initiated cells than TPA. These studies could answer important questions regarding cutaneous steroid hormone physiology, hair cycle regulation and chemical carcinogenesis in skin.

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