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Developmental Action of Estrogen Receptor-α Feminizes the Growth Hormone-Stat5b Pathway and Expression of *Cyp2a4* and *Cyp2d9* Genes in Mouse Liver

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ABSTRACT

We have studied the roles of estrogen receptor-α (ERα) and the Stat5b form of STAT (signal transducers and activators of transcription) in sex-specific expression of *Cyp2a4* (steroid 15α-hydroxylase) and *Cyp2d9* (steroid 16α-hydroxylase) genes using ERα-deficient mice. ERα deficiency resulted in the repression of the female-specific *Cyp2a4* and expression of the male-specific *Cyp2d9* genes, respectively in females. In ERα-deficient males, the *Cyp2d9* gene continued to be expressed. Nuclear localization of Stat5b occurs in both sexes of ERα-deficient mice, although it is normally observed in only wild-type males. Nuclear localization of Stat5b correlates with the repression of *Cyp2a4* and expression of *Cyp2d9*, respectively. Because Stat5b was not detectable in liver nuclear extracts prepared from hypophysectomized ERα-deficient females, the regulation by ERα appeared to be mediated through a pituitary hormone (i.e., growth hormone). Thus, ERα appears to play a key role in the mechanism that inhibits nuclear localization of Stat5b in female mice, leading to feminization of a ERα-GH-Stat5b pathway and *Cyp* expression. Defaulting to this ERα-dependent mechanism results in localization of Stat5b to nuclei, which masculinizes the expression of *Cyp* genes in male mice.

Hepatic metabolism of steroids and xenochemicals is sexually dimorphic in rodents and other animals (Negishi et al., 1993). Sex-specific metabolism is catalyzed by cytochrome P-450s (CYPs) that are expressed either in male or female animals. Certain metabolism by sex-specific CYPs may lead to sex-dependent susceptibility for chemical toxicity and carcinogenicity (Waxman and Chang, 1995a and references therein). Various molecular and/or cellular mechanisms that may regulate transcription of sex-specific *CYP* genes have been proposed: DNA methylation (Yokomori et al., 1995), hepatocyte nuclear factor 6 (Lahuna et al., 1997) and the Stat5b form of STAT (signal transducers and activators of transcription) (Subramanian et al., 1995; Udy et al., 1997; Teglund et al., 1998). However, defining the regulatory mechanism of sex-specific *CYP* expression remains to be a major interest in continuous investigations.

Hormonally, growth hormone (GH) is known to play a central role in regulating sex-specific *CYP* genes. Gustafsson and Stenberg (1976) first suggested the existence of a pituitary factor that feminizes steroid metabolism by rat liver microsomes and called it "a feminization factor". Later, this pituitary factor was found to be GH (Kramer and Colby, 1976). In rats, pulsatile GH secretion (in males) is required for activation of the male-specific *CYP2C11* gene, whereas continuous GH secretion (in females) is essential for activating the female-specific *CYP2C12* gene (Mode et al., 1981). CYP2A4 and CYP2D9 are the well-characterized sex-specific mouse steroid hydroxylases; the former is the female-specific steroid 15α-hydroxylase, whereas the latter is the male-specific steroid 16α-hydroxylase (Harada and Negishi, 1984a,b, 1988). Using GH-deficient Little mice, we previously showed that GH activates and represses the male-specific *Cyp2d9* and female-specific *Cyp2a4* gene expression, respectively, dictating the male phenotype of the *Cyp* genes (Noshiro and Negishi, 1986; Aida and Negishi, 1993). On the other hand, the female phenotype (i.e., expression and repression of the *Cyp2a4* and *Cyp2d9* genes, respectively) is not regulated by GH (Noshiro and Negishi, 1986). In mice, the sex-specific expression is hormonally regulated by GH in males, whereas it is constitutive in females. GH, thus, is permissive for female-specific *Cyp* expression in mice. Recently, the cellular mechanism mediating the GH-regulated sex-specific *Cyp* expression has begun to unfold (Udy et al., 1997; Teglund et al., 1998).

STAT represents a group of cellular proteins that can be activated by extracellular peptide signals including GH (Darnell, 1997). In response to GH, for example, tyrosine protein kinases called Janus kinases (JAKs) phosphorylate Stat pro-

ABBREVIATIONS: CYP, cytochrome P-450; ERα, estrogen receptor-α; STATs, signal transducers and activators of transcription; Stat5a and Stat5b, 5a and 5b forms of STATs; GH, growth hormone; PCR, polymerase chain reaction; RT, reverse transcription.

teins that undergo nuclear translocation to activate target genes. Waxman and his associates demonstrated that pulsatile GH secretion (i.e., male pattern) elicits a Stat5b nuclear translocation, suggesting Stat5b as a direct transcription factor regulating the sex-specific *CYP2C11* and *CYP2C12* genes in rats (Waxman et al., 1995b; Gebert et al., 1997). Stat5b-deficient mice have recently provided unequivocal evidence that Stat5b regulates the sex-specific *Cyp2a4* and *Cyp2d9* genes in male mice, whereas the deficiency does not affect these *Cyp* genes in female mice (Udy et al., 1997; Teglund et al., 1998). In conjunction with our previous findings (Noshiro and Negishi, 1986), a GH-Stat5b pathway may be a regulatory mechanism that masculinizes transcription of the *Cyp* genes in murine liver.

Neonatal action of androgen is thought to regulate sex-specific expression of *CYP* genes in adult rats (Waxman and Chang, 1995a and references therein). Neonatal castration alters the sex-specific expression of *Cyp* genes in mice (Wong et al., 1987). To understand the developmental role of estrogen and/or estrogen receptor in the GH-Stat5b regulation of sex-specific *Cyp* genes, we have now examined expression of *Cyp2a4* and *Cyp2d9* genes and nuclear localization of Stat5b in estrogen receptor-α (ERα)-deficient mice. Disruption of the ERα gene has dramatically altered sex-specific *Cyp* expression and Stat5b nuclear localization. These findings implicate a developmental role of ERα in feminizing *Cyp* expression in mouse liver via a GH-Stat5b-CYP pathway.

Experimental Procedures

Animals. ERα-deficient homozygous ERKO mice were produced through gene targeting as previously reported (Lubahn et al., 1993). Hypophysectomy of both male and female mice and ovariectomy were performed by the Comparative Medicine Branch at the National Institute of Environmental Health Sciences according to an approved National Institute of Environmental Health Sciences animal study protocol. Mice were housed following surgery for at least 2 weeks before removal of liver tissues or estradiol treatment. Estradiol was administrated i.p. in saline at a dose of 25 μg/kg b.wt./day for 7 consecutive days.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR). Liver RNA was prepared using Trizol reagent (Life Technologies, Gaithersburg, MD). RT was performed with 2 μg of RNA and 50 ng of random hexamer using the Superscript Preamplification System (Life Technologies). cDNAs were then purified using QIAquick spin columns (Qiagen Inc., Chatsworth, CA) and eluted with 40 μl H_2O. Two micoliters of cDNA solution was used as template for a 50-μl PCR reaction that contained: 1× PCR reaction buffer (Boehringer Mannheim, Indianapolis, IN), 0.4 U *Taq* polymerase and *Taq* antibody (Clontech Inc., Palo Alto, CA), 0.1 μg each of forward and reverse primers, and 200 μM dNTP. Thermal cyclizations were performed at 94°C for 30 s, 55°C for 30 s, and 72°C for 40 s with the Gene Amp System 9600 (Perkin Elmer, Norwalk, CT). RT-PCR amplifications for CYP2A4, CYP2D9, and Stat5 were 28, 28, and 33 cycles, respectively. These amplification reactions were shown to be within linear range. Primers used were as follows: CYP2A4 (Lindberg et al., 1989), 5′-CTACCTTCGACTGGCTTTTC-3′ and 5′-GCATTCGGATGAGGAAGGAG-3′; CYP2D9 (Wong et al., 1989), 5′-CTTTGGGGACATTGTTCCAG-3′ and 5′-AAGAATACCATAGACTCCAG-3′; and Stat5 (Liu et al., 1995), 5′-CAGGTGAAGGCGACCATCAT-3′, and 5′-TGCTGTTGTAGTCCTCGAGG-3′. Amplified cDNAs for CYP2A4 and Stat5 were purified with QIAquick spin columns and subsequently digested with *Hin*dIII and *Nco*I, respectively. Following digestion, CYPs 2A4 and 2A5 or Stat5a and Stat5b DNA fragments were separated on an agarose gel. Fragments derived from CYP2A4 were 503 bp; from CYP2A5 were 299 bp and 204 bp; from Stat5a were 310 bp and 240 bp; and from Stat5b were 550 bp. Fragment size of the amplified CYP2D9 DNA was 347 bp.

Western Blotting of Nuclear Stat5b. Nuclear extracts were prepared from mouse livers as previously reported (Sueyoshi et al., 1995). Thirty micograms of nuclear extract was incubated with 1 μg of anti-Stat5b (SC-835; Santa Cruz Biotechnology, Santa Cruz, CA) for 2 h at 4°C. The antibody-antigen complex was precipitated using protein A Sepharose (Pharmacia, Piscataway, NJ) by incubation for 1 h at 4°C. The complex-bound Sepharose was washed four times with 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1.0% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 100 μM sodium vanadate, and 1 μg/ml leupeptin. One third of the precipitated protein was applied on a 8% SDS-polyacrylamide gel, transferred to a polyvinylidene difluoride membrane and visualized with anti-Stat5b antibody and enhanced chemiluminescence Western blotting detection reagents (Amersham, Arlington Heights, IL). For Western blots of nuclear extracts for Stat5b or retinoid X receptor α (RXRα), 10 μg of liver nuclear extracts were separated on a 8% SDS-polyacrylamide gel, transferred and detected using anti-Stat5b or anti-RXRα antibody (SC-553; Santa Cruz Biotechnology).

Results

Expression of Sex-Specific CYPs in ERα-Deficient Mice. As expected from our previous work (Squires and Negishi, 1988), CYP2A4 mRNA was expressed only in wild-type females (Fig. 1A). This female-specific mRNA was completely repressed in ERα-deficient females, whereas mRNA levels varied significantly in ERα-deficient males, ranging from levels nearly as high as those observed in wild-type females to undetectable (Fig. 1A). The other subfamily-member CYP2A5 mRNA (Squires and Negishi, 1988; Lindberg et al., 1989) was measured simultaneously using a specific digestion method (Negishi et al., 1991). CYP2A5 mRNA was

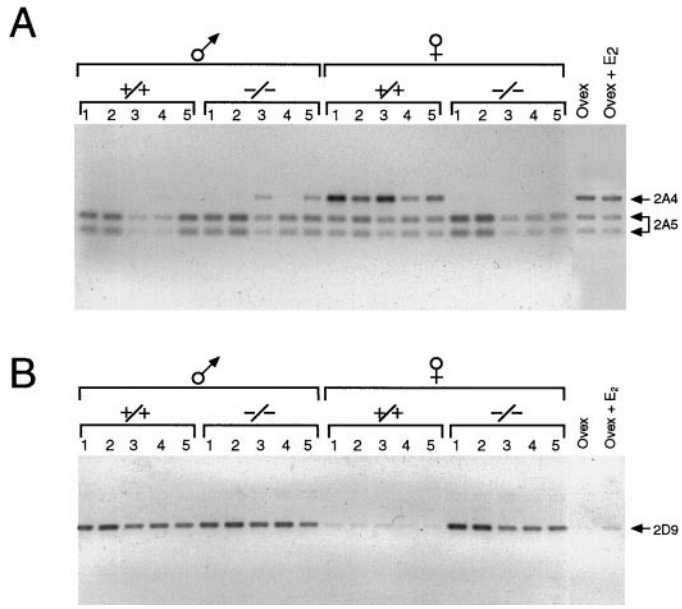

Fig. 1. Hepatic levels of the sex-specific CYP mRNAs in ERα-deficient mice. Amplified cDNAs for CYP2A4 (A) and CYP2D9 (B) from liver RNAs (lanes 1–5) of individual wild-type (+/+) and ERα-deficient (−/−) mice were separated on a 1.5% agarose gel. DNA bands were visualized by ethidium bromide in the gel as well as in the electrode buffer. Wild-type females were ovariectomized and treated with estradiol as described in *Experimental Procedures*, and liver RNAs prepared from pools of the ovariectomized females (Ovex) and ovariectomized/estradiol-treated (Ovex + E_2) were subjected to RT-PCR.

consistently expressed in all male and female mice examined (Fig. 1A), thereby indicating that ERα does not regulate non-sex-specific expression of the *Cyp2a5* gene. These results indicate that the presence of the functional ERα gene is an essential factor required for expression of the *Cyp2a4* gene in female mice. The regulatory mechanism that represses this gene in males however, appears to be more complex, and a factor other than ERα may be involved.

Consistent with our previous findings (Wong et al., 1989), male-specific CYP2D9 mRNA was expressed at high levels only in wild-type males (Fig. 1B). In contrast, both males as well as females of ERα-deficient mice showed high expression of CYP2D9 mRNA (Fig. 1B). CYP2D9, thus, becomes sexually nonspecific in ERα-deficient mice, indicating that the presence of a functional ERα gene is an essential factor required for repression of the *Cyp2d9* gene in female mice. ERα, on the other hand, does not seem to play a role in expression of the *Cyp2d9* gene in male mice.

Neither ovariectomy nor ovariectomy combined with estrogen treatment of wild-type females affected expression of *Cyp2a4* gene (Fig. 1A, last two lanes). Ovariectomy further decreased CYP2D9 mRNA, whereas estrogen treatment slightly increased CYP2D9 mRNA to the levels as observed in wild-type females (Fig. 1B, last two lanes). ERα does not regulate expression of *Cyp2a4* gene in adult females, whereas it may play only a minor role in repressing *Cyp2d9* gene. Thus, the role of ERα in regulation of the expression of the sex-specific *Cyp* genes appears to be developmental.

Nuclear Localization of Stat5b in ERα-Deficient Mice. First, the liver nuclear extracts from two animals from each experimental group were separated on a SDS-polyacrylamide gel, transferred, and immunostained using anti-Stat5b antibody (Fig. 2). Nuclear Stat5b was not detectable in nuclear extracts from both wild-type female mice, whereas it was present in one of two individuals of wild-type male mice and in both males and females of ERα-deficient mice. For further investigation, then we precipitated Stat5b using anti-Stat5b antibody from various liver nuclear extracts and subjected these immunoprecipitates to Western blot analysis (Fig. 3A). Again, Stat5b was present in the extracts from wild-type males but not from wild-type females, suggesting that nuclear localization of Stat5b occurred in only wild-type male livers. In contrast, Stat5b localized to liver nuclei in both males and females of ERα-deficient mice (Fig. 3A). Intriguingly, of the five wild-type males examined, only three males showed nuclear localization of Stat5b and only two of the five ERα-deficient males and three of ERα-deficient females, respectively. Individual differences in nuclear Stat5b levels implied that nuclear localization of Stat5b is episodic in its nature. In contrast, RXRα was always localized in nuclei and was neither sex-specific nor affected by disruption of the ERα gene (Fig. 3B). Stat5b mRNA was expressed in all mice and remained at the same levels regardless of sex difference and ERα deficiency (Fig. 3C). Thus, ERα appears to imprint the mechanism that prevents nuclear localization of Stat5b in female mice.

Effect of Hypophysectomy on CYPs and Stat5b in ERα-Deficient Females. To further examine the role of ERα in the nuclear localization of Stat5b, we measured Stat5b in liver nuclear extracts from hypophysectomized ERα-deficient females. Consistent with the finding with wild-type males and ERα-deficient females (Fig. 3A), nuclear Stat5b was readily detected from three of four sham-operated ERα-deficient females (Fig. 4A). Hypophysectomy of ERα-deficient females decreased nuclear Stat5b to practically undetectable levels (Fig. 4A), which was reminiscent of nuclear Stat5b in wild-type female mice (Fig. 3A). Episodic nuclear localization of Stat5b appeared to be under pituitary control and moreover, correlated with pulsatile (male) GH secretion pattern. In accordance with the decreased Stat5b in nuclei, CYP2A4 mRNA was induced in hypophysectomized females and CYP2D9 mRNA was reduced (Fig. 4B). The period for episodic nuclear localization of Stat5b must be much shorter than the half-life of CYP2D9 mRNA. Thus, ERα appears to

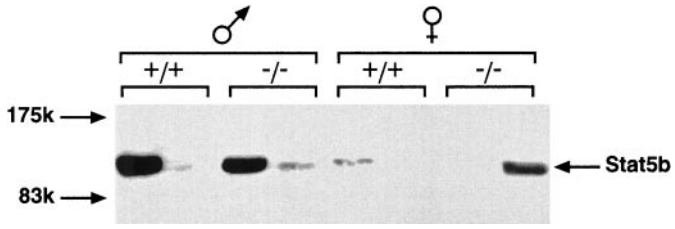

Fig. 2. Western blot for Stat5b in liver nuclear extracts. Hepatic nuclear extracts were prepared from individual wild-type (+/+) and ERα-deficient (−/−) mice. Nuclear extracts were separated and Western blots performed as described in *Experimental Procedures*.

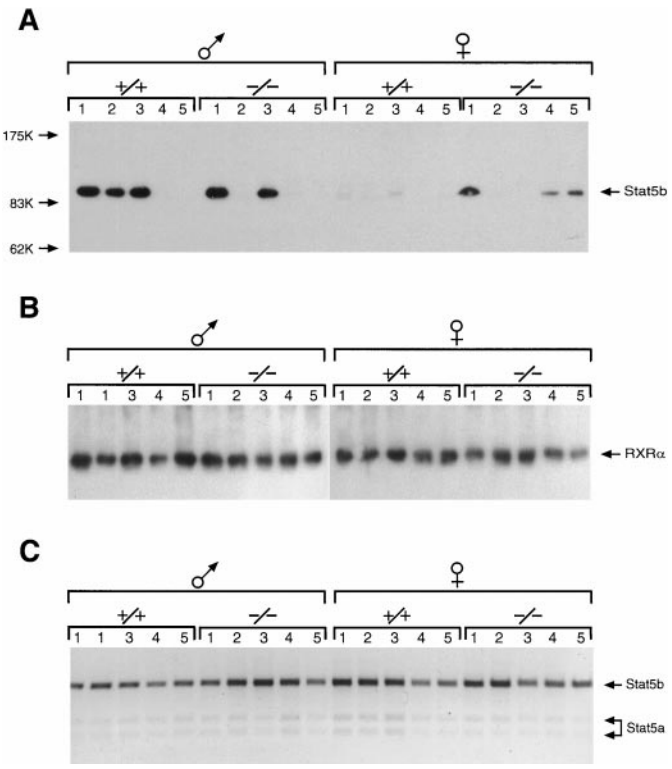

Fig. 3. Expression and localization of Stat5b in ERα-deficient mice. Liver nuclear extracts were prepared from the individual mice that was used to isolate total RNA in Fig. 1. A, nuclear Stat5b was measured using Western blot analysis: Stat5b was immunoprecipitated, transferred to a polyvinylidene difluoride membrane, and stained with anti-Stat5. Molecular size makers (Broad range prestained marker; New England Biolabs, Beverly, MA) are indicated by arrows with molecular weights. B, Western blot analysis of RXRα. Nuclear extracts from individual mice used in Fig. 1 were separated on a SDS polyacrylamide gel, transferred, and immunostained using anti-RXRα antibody. C, RT-PCR of Stat5a and Stat5b mRNAs was performed as described in *Experimental Procedures*, and amplified products were analyzed by electrophoresis on a 1.5% agarose gel.

regulate Stat5b nuclear localization through the pituitary gland, presumably by the secretion pattern of GH.

Discussion

A regulatory role of ERα in sex-specific *Cyp* expression is proposed in Fig. 5. The sex-specific *Cyp2a4* and *Cyp2d9* genes are either constitutively expressed or repressed in female mice because hypophysectomy does not affect their expressions (Yoshioka et al., 1990). Our present studies with ERα-deficient female mice have clearly indicated that this constitutive expression and repression is developmentally imprinted by an action of ERα. To the contrary, GH is an essential factor for masculinizing *Cyp* expression in male mice, as suggested by our previous finding that the male-specific *Cyp2d9* and female-specific *Cyp2a4* genes are repressed and expressed, respectively in GH-deficient Little male mice (Noshiro and Negishi, 1986; Yoshioka et al., 1990). It is paradoxical to see that GH and ERα act as if they were independent and unrelated factors, although both are involved in the regulation of the sex-specific *Cyp* genes. This paradox may be explained by hypothesis that a developmental action of ERα is to imprint GH secretion pattern in female mice, whereas the lack of ERα defaults GH to the male secretion in both ERα-deficient males and females. The GH secretion patterns in ERα-deficient mice remains to be established in the future.

A role of GH in the sex-specific CYP expression is to regulate nuclear translocation of Stat5b in liver. Waxman and his associates (Waxman et al., 1995b; Gebert et al., 1997) have previously shown that nuclear translocation of Stat5b is male-specific in rat liver. The nuclear hepatic Stat5b content of individual male rats varied significantly, implying that Stat5 shuttles between the cytoplasm and nucleus. Moreover, the hepatic nuclear Stat5b contents of individual male rats correlated positively with plasma GH levels. Our present study reveals that the individual variability in a nuclear Stat5b level appears to be pronounced in male mice. The regular periodicity of the GH pulse is shorter in male mice (2.5 h) than male rats (4 h) (Jansson et al., 1985; MacLeod et al., 1991). The shorter periodicity may make the time of nucleocytoplasmic shuttle of Stat5b faster in male mice, reflecting the strong individual differences in nuclear Stat5b level. Despite the variable Stat5b levels, the male-specific *Cyp2d9* gene is expressed in every individual of wild-type males and ERα-deficient males and females examined. This suggests that the half-life of Stat5b in nucleus must be much shorter than that of CYP2D9 mRNA in mouse liver. Nuclear Stat5b represses the female-specific *Cyp2a4* gene, whereas it activates the male-specific *Cyp2d9* gene. Because Stat5b can be translocated only under the male secretion pattern of GH, either a physiological condition of the female GH secretion or the pathological condition of no GH results in no nuclear translocation of Stat5b in liver. The *Cyp* expression thus becomes the male phenotype in both ERα-deficient male and female mice as well as in wild-type males.

In general, neonatal imprinting is considered to occur during sexual differentiation (i.e., feminization versus masculinization) of brain function (MacLusky and Naftolin, 1981; Bardine and Catterall, 1981), including sexual differentiation of GH secretion pattern and sex-specific expression of *CYP* genes (Gustafsson et al., 1983; Jansson et al., 1985). The majority of these studies were performed in rats, concluding

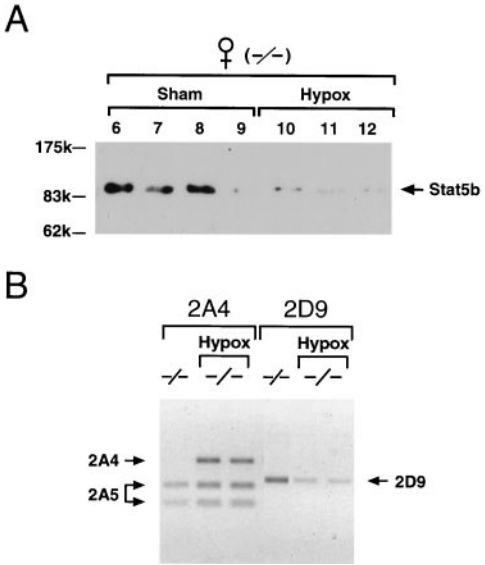

Fig. 4. Hepatic nuclear Stat5b and sex-specific CYP mRNAs in ERα-deficient hypophysectomized females. A, hepatic nuclear extracts were prepared from individual hypophysectomized female mice and subjected to Western analysis as described in *Experimental Procedures*. B, CYP mRNAs were amplified and analyzed using the methods as described in *Experimental Procedures* and also in the legend of Fig. 1. RNAs were pooled from at least three female mice for each experiment. Symbols used are same as those in Fig. 1, except that Hypox denotes hypophysectomy.

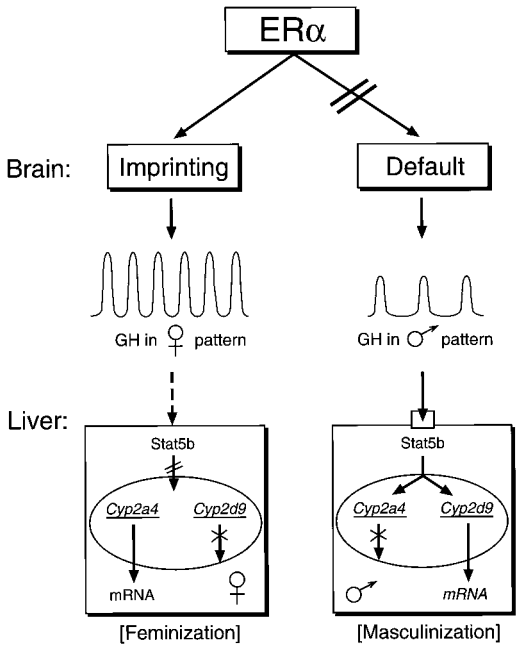

Fig. 5. Proposed mechanism for the feminization of the sex-specific CYPs. GH secretion patterns are depicted from MacLeod et al. (1991). GH in male mice, via GH receptor (open box with arrow), translocates Stat5b into nuclei. Nuclear Stat5b, then activates transcription of *Cyp2d9* gene and represses that of *Cyp2a4* genes. Under the female secretion pattern of GH, however, hepatic nuclear translocation of Stat5b does not occur. The absence of nuclear Stat5b in female mice results in the constitutive activation and repression of *Cyp2a4* and *Cyp2d9* genes, respectively. It should be emphasized that the female pattern of GH secretion is permissive for the female phenotype of *Cyp* expression in mice, meaning that GH plays no regulatory role in females. ERα may imprint the mechanism regulating the GH secretion pattern in female mice, so that Stat5b is not translocated into hepatic nuclei.

that a neonatal action of androgen is a key factor for imprinting. Androgen can be aromatized to estrogen, which imprints masculinization in male rats, and the lack of androgen (i.e., estrogen) results in feminization in female rats. Studies using ERα-deficient mice have provided some evidence that ERα-mediated estrogen may, infact, act as a masculinization factor for sexual differentiation. ERα-deficient females exhibit similar dopaminergic neurons to those observed in males, whereas the female-type dopaminergic neurons are largely retained in ERα-deficient females (Simerly et al., 1997). Given the fact that ERα feminizes the *Cyp* expression in mice, sexual differentiation may be diverse processes that are centered around ERα and/or estrogens. It is increasingly evident that any given biological signal can be transduced in positive as well as negative manners. It may not be so surprising, even if the apparent regulation of GH-Stat5b-CYP pathway observed in ERα-deficient mice is contrary to a general imprinting mechanism previously established in rats. Is ERα-mediated feminization of the GH-Stat5b-CYP pathway regulated by estrogen? It is difficult to speculate this at the present time. There may or may not be sufficient levels of estrogen to activate ERα in neonatal mouse brain. Because steroid receptors including ERα can be activated in the absence of cognate ligands (Cenni and Picard, 1999), even if there is absolutely no estrogen in brain, ERα may be activated by a ligand-independent mechanism, thereby feminizing the GH-Stat5b-CYP pathway. The regulation mechanism of sex-specific *CYP* genes is rather complex and diverse. The complexity and diversity appear to arise from the fact that ERα does not directly regulate the *CYP* genes. There are multiple regulatory steps leading ERα action to *CYP* genes. Whether ERα also acts a factor that feminizes *CYP* expression in other species remains a subject for further investigation. Nevertheless, our present study using ERα-deficient mice unequivocally shows that ERα plays a central role in feminizing *Cyp* expression in mice.

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