

Regulation of Progesterone Levels during Pregnancy and Parturition by Signal Transducer and Activator of Transcription 5 and 20 α -Hydroxysteroid Dehydrogenase

Roland P. Piekorz,* Sébastien Gingras,* Angelika Hoffmeyer,* James N. Ihle, and Yacob Weinstein

Howard Hughes Medical Institute (R.P.P., S.G., A.H., J.N.I.), Department of Biochemistry (R.P.P., S.G., A.H., J.N.I.), St. Jude Children's Research Hospital, Memphis, Tennessee 38105; and Faculty of Health Sciences (Y.W.), Department of Microbiology and Immunology, Ben Gurion University of the Negev, Beer Sheva, Israel 84105

The two highly related signal transducers and activators of transcription (Stats), Stat5a and Stat5b, are major mediators of prolactin signaling in both the mammary gland and in the ovary. Deficiencies in Stat5b, or in both Stat5a and Stat5b, result in loss of pregnancy during midgestation and are correlated with an increase in ovarian 20 α -hydroxysteroid dehydrogenase (20 α -HSD) and a decrease in serum progesterone, which normally declines only immediately before parturition. To determine the relative contribution of 20 α -HSD to progesterone metabolism and Stat5 function during pregnancy and parturition, we created a 20 α -HSD-deficient strain of mice

by gene disruption. Mice deficient for 20 α -HSD sustain high progesterone levels and display a delay in parturition of several days demonstrating that 20 α -HSD regulates parturition downstream of the prostaglandin F_{2 α} receptor in an essential and nonredundant manner. Moreover, 20 α -HSD deficiency partially corrected the abortion of pregnancies associated with Stat5b deficiency, supporting the concept that prolactin activation of Stat5b is important in suppressing 20 α -HSD gene expression and thereby allowing the maintenance of progesterone levels that are required to sustain pregnancy. (*Molecular Endocrinology* 19: 431-440, 2005)

THE CYTOKINE PROLACTIN (PRL) has two major physiological roles based on the phenotype of mice lacking either PRL (1) or the receptor (2, 3). One function is to induce mammary gland differentiation for lactation whereas the second function is to control ovarian function during pregnancy. Both of these physiological functions are mediated by the two highly related signal transducers and activators of transcription (Stat) transcription factors, Stat5a and Stat5b. Mice deficient in Stat5a alone (4) have a severe loss of mammary gland development during the first pregnancies whereas the lack of Stat5b (5) is associated with early abortions that are due to reduced progesterone levels during midgestation. The phenotype of mice lacking both Stat5 proteins (6) is more severe, and abortions occur earlier than in the Stat5b-deficient mice.

The critical targets of Stat5 transcriptional regulation in PRL target tissues are largely unknown. One potential gene target is the progesterone-metabolizing enzyme 20 α -hydroxysteroid dehydrogenase (20 α -HSD) (7). The enzyme was initially detected as a progesterone-metabolizing enzyme in the ovary (8) and was subsequently shown to be also highly expressed in thymocytes (9-11). Based on this observation, its induction in splenocytes was used as an assay for the identification of T cell-derived factors that potentially regulated T cell development (12). This activity was subsequently found to be a function of IL-3 (13). However, the significance or role of the expression of 20 α -HSD by thymocytes and lymphocytes, as well as myeloid lineage cells, is unknown.

In addition to hematopoietic cells, 20 α -HSD is highly expressed in the adrenal gland (14) and in granulosa cells of the corpus luteum (15). The potential functions of 20 α -HSD in the adrenal gland are largely unknown. In contrast, 20 α -HSD expression by granulosa cells is widely accepted to be involved in the regulation of progesterone levels during the estrous cycle and in pregnancy (8, 16). After oocyte release in response to LH, granulosa cells initiate the formation of a corpus luteum and the cells begin to express 20 α -HSD. In rodents, in the absence of coital stimulation, the granulosa cells undergo apoptosis and the corpus luteum regresses (17). With coital stimulation, PRL is produced by the anterior pituitary gland (18), and PRL stimulation of granulosa cells suppresses the expression of 20 α -HSD and

First Published Online October 7, 2004

* R.P.P., S.G., and A.H. contributed equally to this work and should all be considered first authors.

Abbreviations: ES cell, Embryonic stem cell; 20 α -HSD, 20 α -hydroxysteroid dehydrogenase; 20 α -OHP, 20 α -hydroxyprogesterone; P14, d 14 of pregnancy; PGF_{2 α} , prostaglandin F_{2 α} ; PRL, prolactin; StAR, steroidogenic acute regulatory protein; Stat, no. 5, signal transducer and activator of transcription, no. 5.

Molecular Endocrinology is published monthly by The Endocrine Society (<http://www.endo-society.org>), the foremost professional society serving the endocrine community.

apoptosis (7, 19) and allows the development of a secretory corpus luteum producing the progesterone required for the initial stages of implantation and maintenance of pregnancy. At term, the ovarian release of progesterone is terminated, and it has been proposed that 20 α -HSD contributes to the rapid elimination of progesterone (8, 15). To explore the role of 20 α -HSD and its regulation by Stat5 in various cell lineages, we have created a strain of mice lacking the enzyme. The results demonstrate that Stat5 plays a critical role in suppressing 20 α -HSD during pregnancy and that the primary function of 20 α -HSD is to reduce progesterone levels at term of pregnancy.

RESULTS

Regulation of Ovarian 20 α -HSD Expression during Pregnancy and Influence of Stat5a/b

As illustrated in Fig. 1A, high levels of the progesterone-metabolizing enzyme 20 α -HSD are initially

present during early pregnancy in mouse ovaries, and this activity is due to previous cycling of the ovaries. Thereafter, 20 α -HSD protein levels drop dramatically, and only low or no protein is detectable throughout mid- to late gestation. At parturition, however, the protein levels dramatically increase. These results were comparable to previous studies showing a stringent regulation of ovarian 20 α -HSD mRNA expression during rat pregnancy (15). In contrast, the protein levels for 3 β -HSD, an enzyme involved in the last step of progesterone biosynthesis, did not change significantly during the whole course of pregnancy.

A potential role for Stat5 transcription factors in influencing 20 α -HSD expression during pregnancy has been previously proposed (6). In ovarian tissue the predominant Stat5 protein expressed is Stat5b, and deletion of Stat5b alone is associated with spontaneous abortions during midgestation (5). Correlated with the abortions is a precipitous decline in progesterone concentrations, and adminis-

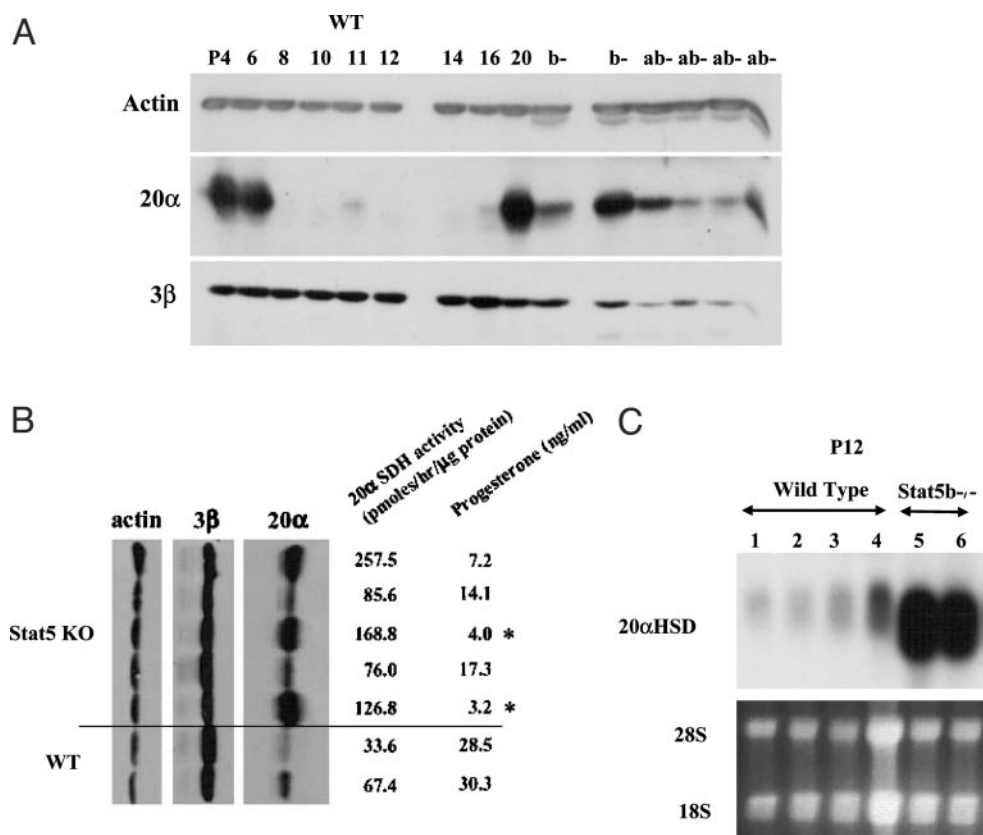


Fig. 1. Expression of 20 α -HSD during Pregnancy in Normal and Stat5-Deficient Mice

A, Expression of 20 α -HSD and 3 β -HSD in the mouse ovary during pregnancy. Total cell lysates were prepared from ovaries of wild-type mice at the indicated stages of pregnancy (P4–P20) or from Stat5a/b-deficient mice (b–) or from Stat5a/b-deficient mice (ab–) at P14–P16, and the expression of 20 α -HSD and 3 β -HSD was determined by Western blot analysis. B, Increased 20 α -HSD protein expression and activity in Stat5a/b-deficient ovaries. Western blot analysis of 20 α -HSD and 3 β -HSD expression using ovarian cell lysates from wild-type and Stat5a/b-deficient females at P10–P12. Indicated are also the specific 20 α -HSD enzymatic activities (picomoles 20 α -OHP formed/h/ μ g) and concomitant serum progesterone levels (ng/ml). Asterisk indicates mice with resorbed embryos. C, Northern blot analysis of 20 α -HSD expression in P12 ovaries from wild-type mice (tracks 1–4) and Stat5b-deficient mice (tracks 5 and 6).

tration of progesterone was shown to allow the maintenance of pregnancy (5). In contrast, Stat5a-deficient mice are fertile and carry the embryos to term (4, 6). The levels of 20 α -HSD protein in ovaries from Stat5b-deficient and Stat5a/b-deficient females at 14–16 d of pregnancy (P14–P16) are also shown in Fig. 1A. As illustrated, 20 α -HSD protein was readily detectable in these tissues at mid-gestation and, although the protein levels varied among individual females, the range of protein levels observed was comparable between Stat5a/b-deficient and Stat5b-deficient mice. Among a group of Stat5a/b-deficient females examined, there was a direct correlation between the levels of protein detected by Western blotting and the amount of enzyme activity as well as the circulating levels of progesterone (Fig. 1B). Lastly, the levels of 20 α -HSD protein are regulated at the transcriptional level because, in the absence of Stat5b, the levels of 20 α -HSD transcripts are dramatically elevated relative to the transcript levels in ovaries of wild-type mice at P12 (Fig. 1C).

The effects of Stat5a/b deficiency on circulating progesterone levels throughout pregnancy are illustrated in Fig. 2. In wild-type mice the circulating concentrations of progesterone increase dramatically from P4–P6 and are maintained at high levels throughout pregnancy (Fig. 2). In contrast, the levels of circulating progesterone show an initial increase in Stat5a/b-deficient mice but drop at P6–P8, although the rates are variable among individual mice. Comparable declines are seen in Stat5b-deficient mice (data not shown) as previously described (5). Together, the results support the hypothesis that 20 α -HSD expression is suppressed during mid- to late gestation, that this is mediated, in part, through a Stat5b-dependent mechanism, and that this regulation is critical for maintaining the high levels of progesterone required for maintenance of the pregnancy.

Generation of Mice in Which the Gene for 20 α -HSD Is Disrupted

To more definitively establish the physiological role of 20 α -HSD, a strain of mice was established in which the gene was disrupted by homologous recombination. The targeting vector (Fig. 3A) was designed to delete exons 2 and 3, encoding essential components of the active site of the enzyme (20), and would be predicted to generate a null mutation. Genotyping of F1 mice by PCR (Fig. 3B) revealed a normal frequency of homozygously null mice. Absence of 20 α -HSD expression in homozygously deleted mice was verified by Western blotting in the adrenal, ovary, and kidney, which normally express high protein amounts (Fig. 3C). In addition, the determination of the enzymatic activity of 20 α -HSD *in vitro*, by conversion of radiolabeled progesterone to the biologically inactive form of 20 α -hydroxyprogesterone (20 α -OHP), demonstrated that enzymatic activity is absent in 20 α -HSD-deficient tissues (Fig. 4). The residual activity in the adrenal tissue is due to the activity of another metabolizing enzyme the product of which migrates at the 20 α -OHP region on the thin layer chromatography plate. In summary, these results demonstrate that the mutated locus creates a 20 α -HSD protein null phenotype, and that the enzyme is nonredundantly required for all 20 α -HSD enzyme activity.

Normal Hematopoiesis in 20 α -HSD-Deficient Mice

High expression of 20 α -HSD has been observed in hematopoietic tissues including the thymus and bone marrow (9, 11), and its expression can be induced by treatment with IL-2 or IL-3 in thymocytes or bone marrow cells, respectively (21, 22). These observations had led to the hypothesis that 20 α -HSD plays a role in hematopoiesis. However, extensive analysis of hematopoietic functions has failed to detect any phe-

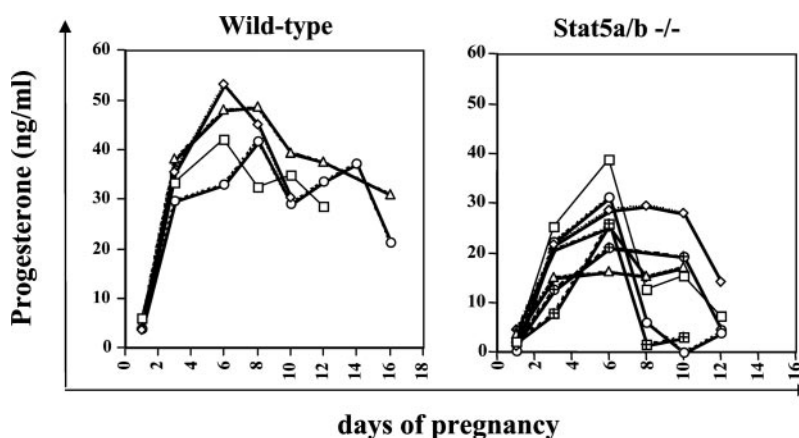


Fig. 2. Failure to Maintain High Progesterone Levels in Pregnant Stat5a/b Females

Wild-type and Stat5a/b-deficient females were bled during the course of pregnancy, and serum progesterone levels were analyzed by RIA.

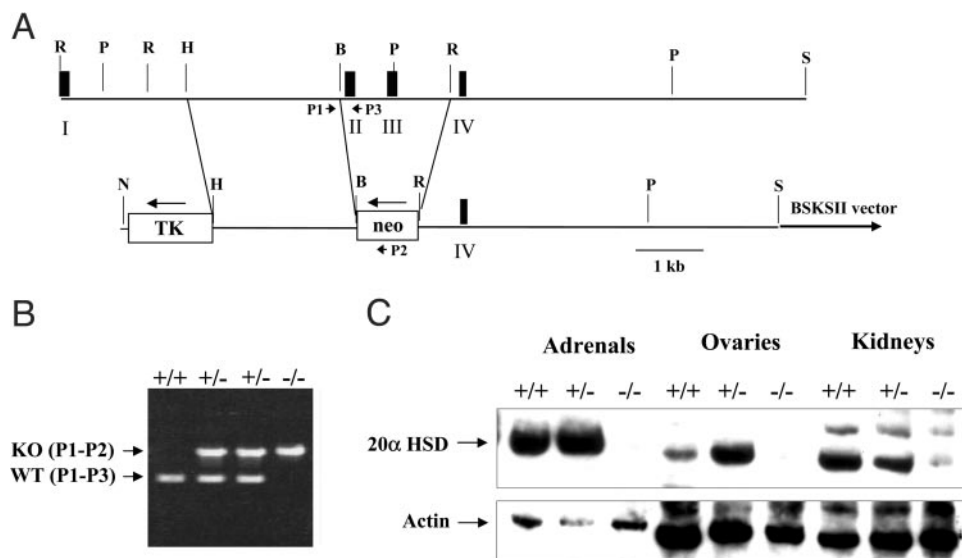


Fig. 3. Targeted Disruption of the 20 α -HSD Gene Generates a Null Mutation

A, Genomic organization of the 20 α -HSD locus and structure of the gene-targeting vector. Black rectangles indicate exons I-IV; the 3'-exons are not shown. Restriction enzyme sites are as follows: R, *EcoRI*; P, *PstI*; H, *HincII*; B, *BamHI*; S, *SalI* (derived from the bacteriophage polylinker). B, Screening PCR/genotyping of F1 mice for the presence of the disrupted allele. C, Western blot analysis for the expression of 20 α -HSD protein in various tissues of 20 α -HSD-deficient, heterozygous, and wild-type mice. Approximately 1 μ g of adrenal extract and 10 μ g of ovary or kidney extracts were loaded on the gel. Antiactin antibodies were used to control protein loading.

notypic alterations. For example, single cell suspensions from bone marrow, spleen, and thymus were subjected to fluorescence-activated cell sorting analysis. Analysis of the various subpopulations of the thymus (CD4, CD8), spleen (Thy1.2, B220), and bone marrow (Mac1, Gr1) using specific markers did not reveal any differences between wild-type and homozygously deficient mice in the cellular composition (data not shown). In addition, colony-forming activities in methylcellulose were comparable between 20 α -HSD-deficient and wild-type bone marrow cells (data not shown). The absence of enzymatic 20 α -HSD activity in the spleen and thymus and in IL-3-stimulated

bone marrow cells was confirmed by enzyme assays (data not shown). In summary, these results indicate that 20 α -HSD is dispensable for the normal development of hematopoietic cells.

Increased Progesterone Levels and Delayed Parturition in 20 α -HSD-Deficient Females

To establish the putative role of 20 α -HSD in reproduction and parturition, the reproductive function of 20 α -HSD-deficient female mice was addressed. Both homozygously deficient males and females showed a normal fertility, and females maintained pregnancy

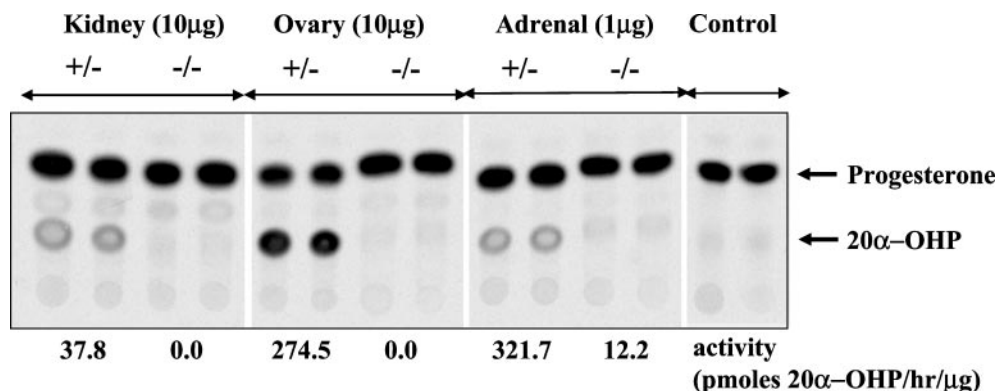


Fig. 4. Enzymatic Activity of 20 α -HSD in Various Tissues of 20 α -HSD-Heterozygous or -Deficient Female Mice

¹⁴C-labeled progesterone was incubated with tissue homogenates. The substrate (progesterone) and its metabolite (20 α -OHP) were separated on thin layer chromatography plates, visualized, and quantified by phosphoimaging. The specific enzymatic activities are indicated.

normally. However, 20 α -HSD-deficient females failed to induce parturition at the term of pregnancy (d 20) as compared with wild-type or heterozygous animals. Parturition was consistently delayed by 2–3 d (Fig. 5A) leading to the death of pups *in utero* (data not shown). This phenotypic alteration was directly associated with a failure to completely down-regulate progesterone levels at the end of pregnancy. In wild-type mice, serum progesterone concentrations fell below 10 ng/ml on d 20 of pregnancy, and parturition occurred (Fig. 5B). In contrast, the levels of progesterone in pregnant 20 α -HSD-deficient females were still at 25–30 ng/ml at P19–P20, and these levels did not decline below 10 ng/ml for the next 2–3 d. These results demonstrate that 20 α -HSD plays a critical role in the down-regulation of progesterone levels at the end of pregnancy, and that this down-regulation is obligate for the induction of parturition.

To determine whether the phenotypic changes seen with 20 α -HSD deficiency were related solely to the changes in progesterone metabolism, RU486 was administered to pregnant females at P19. Administration of this progesterone antagonist to pregnant 20 α -HSD females resulted in normal parturition on the next day, although progesterone blood concentrations remained high (data not shown). The pups were born normal and were nursed normally by their 20 α -HSD-deficient mothers. The results are consistent with the hypothesis that the only role for 20 α -HSD during pregnancy and parturition is to control the levels of circulating progesterone.

20 α -HSD Is Necessary for Prostaglandin F_{2 α} (PGF_{2 α})-Dependent Progesterone Down-Regulation before Parturition

PGF_{2 α} signaling induces the down-regulation of blood progesterone concentrations by inhibition of its biosynthesis (23, 24) and by the up-regulation of 20 α -

HSD expression before parturition (25, 26). Comparable to the 20 α -HSD deficiency, parturition is delayed in females deficient for the PGF_{2 α} receptor because blood progesterone levels remain high at the end of pregnancy (27). To determine whether 20 α -HSD is an essential part in the PGF_{2 α} -activated pathway toward the induction of parturition, wild-type and 20 α -HSD-deficient females were injected on day P16–P17 of pregnancy with either 5 μ g or 10 μ g of cloprostenol, a potent PGF_{2 α} receptor agonist. As shown in Fig. 6A, induction of premature births occurred in approximately half of the wild-type females treated with 10 μ g cloprostenol as well as in heterozygous mice (data not shown). The induction of parturition was correlated with the levels of progesterone 24 and 48 h after cloprostenol injection (Fig. 6B). In particular, induction of parturition occurred in those females in which progesterone levels were reduced to less than 10–15 ng/ml. In contrast to the induction of parturition in wild-type mice, cloprostenol did not induce parturition in any of the 20 α -HSD-deficient females treated under the same conditions. Moreover, 20 α -HSD-deficient animals displayed mean serum progesterone levels of approximately 25–30 ng/ml even 48 h after cloprostenol treatment (Fig. 6B). These findings demonstrate that 20 α -HSD plays a critical role in PGF_{2 α} regulation of parturition.

PGF_{2 α} also affects the levels of progesterone by inhibition of its synthesis, and the decreases that were observed in 20 α -HSD-deficient mice could be speculated to be due to this component of regulation. One important regulatory component of the progesterone synthetic pathway that is affected by PGF_{2 α} is the protein StAR (steroidogenic acute regulatory protein) (28, 29). In the sequence of events leading to the reduction of progesterone levels, the decrease in StAR, a protein required for translocation of intracellular cholesterol in progesterone synthesis, occurs

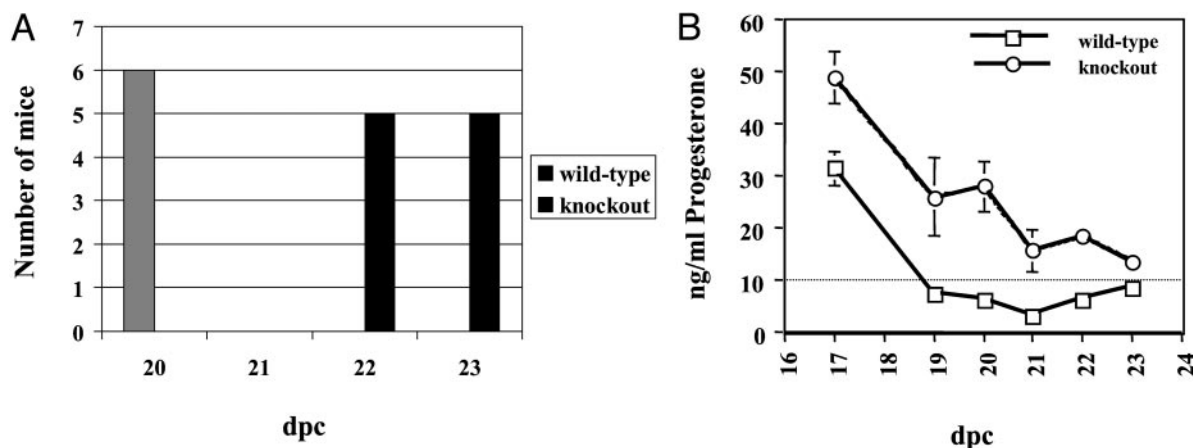


Fig. 5. Increased and Sustained Progesterone Levels and Delayed Parturition in 20 α -HSD-Deficient Females

A, Number of wild-type and knockout females giving birth at the indicated days post coitum. B, Time course of progesterone blood concentrations in pregnant wild-type/heterozygous and 20 α -HSD-deficient mice as analyzed by RIA. Values shown are average \pm SEM ($n > 5$ for all time points of pregnancy). dpc, Days post coitum. When error bars are not shown, the SEM was less than 1.5.

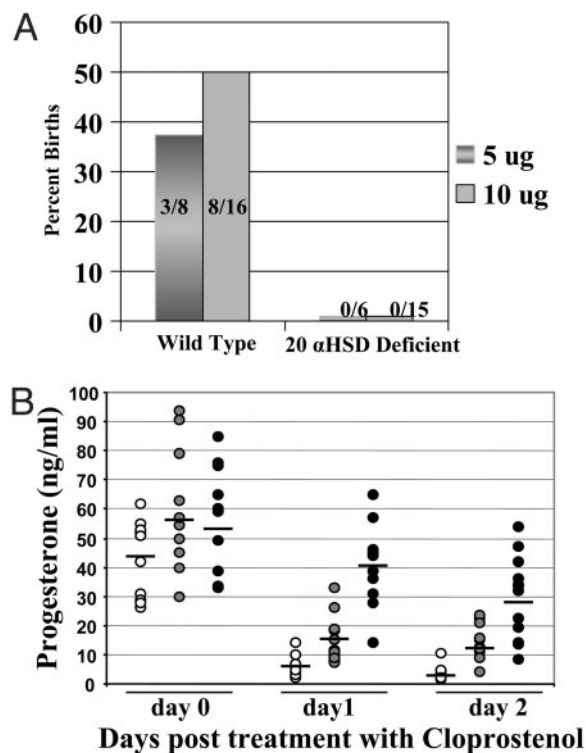


Fig. 6. The PGF_{2 α} Agonist Cloprostenol Induces Parturition through 20 α -HSD

A, Percent premature parturition recorded for wild-type mice and 20 α -HSD-deficient mice 24 h after two injections with a total of 5 μ g or 10 μ g of cloprostenol. B, Progesterone blood levels in pregnant wild-type and 20 α -HSD-deficient mice after treatment with two injections of cloprostenol. Mice were bled before injection (d 0) and on the following 2 d, and progesterone levels were determined by RIA. *Open circles*, wild-type females, birth induced; *gray circles*, wild-type females, no birth induced; *black circles*, knockout females.

rapidly and is followed by the induction of 20 α -HSD enzyme activity. We therefore examined the affects of 20 α -HSD deficiency on the initial reductions in progesterone levels. As illustrated in Fig. 7A, cloprostenol induced a decrease in progesterone levels of approximately 40% by 3 h in wild-type mice when injected at P17–P18. This reduction occurred in the absence of detectable 20 α -HSD protein that became detectable only at 24 h (Fig. 7B). As with wild-type mice, cloprostenol induced a decrease in progesterone levels of approximately 40% in 20 α -HSD-deficient mice. The results are therefore consistent with the hypothesis that PGF_{2 α} induces a loss of progesterone by initially affecting its synthesis, independent of 20 α -HSD, and subsequently inducing the expression of 20 α -HSD.

The Reproductive Defects of Stat5b-Deficient Females Are Rescued by 20 α -HSD Deficiency

The above results suggested that a critical function of Stat5b is the suppression of 20 α -HSD. If correct, it would be predicted that a deficiency of 20 α -HSD

should correct the abortion of embryos during mid-gestation. We therefore crossed the 20 α -HSD deficiency onto the Stat5b deficiency and examined the status of the pregnancies at P18.5–P19.5. The numbers of viable or dead/resorbed embryos among the individual females is illustrated in Fig. 8. Among five females that were Stat5b deficient and wild-type or heterozygous for the loss of 20 α -HSD, only one female had maintained pregnancy as defined by more than 80% of the embryos present remaining viable and among 41 embryos that could be detected, only nine were viable. In contrast, six of seven females that were 20 α -HSD deficient and Stat5b deficient had maintained pregnancy resulting in 62 visible embryos of which 54 were viable. In contrast to the ability to rescue the defects of Stat5b-deficient mice with 20 α -HSD deficiency, the defects associated with Stat5a/b deficiency could not be rescued (data not shown).

DISCUSSION

Our studies were directed at defining the functions of 20 α -HSD *in vivo* and determining whether Stat5 regulation of its expression contributed to the physiological functions associated with this transcription factor. Although 20 α -HSD has been studied in the context of a variety of cell lineages and organs, the consequences of deleting the gene are remarkably specific. In particular, in contrast to the predictions of a variety of studies of hematopoietic lineage cells, a deficiency of 20 α -HSD had no impact on the parameters of differentiation and function that we have examined. The lack of any phenotypic changes is unlikely to be due to the presence of redundant enzyme activities because, as demonstrated, the deletion of the 20 α -HSD gene completely eliminates the ability to convert progesterone to 20 α -hydroxyprogesterone in all the tissues examined. It is possible that 20 α -HSD is important in stress conditions associated with high sustained levels of progesterone. Indeed, it has been shown that progesterone can affect thymic cellularity and various immunological responses. Consistent with this, female mice lacking the progesterone receptor are resistant to estrogen/progesterone-mediated thymic involution that occurs during pregnancy (30). Based on these observations, it will be important to examine very defined physiological and pathophysiological states for potential roles for 20 α -HSD.

In the context of reproductive physiology, it is notable that 20 α -HSD deficiency did not affect the mating behavior of females, the regulation of the estrous cycle, or the onset of pregnancy. Indeed, the primary, nonredundant function of 20 α -HSD established from the gene deficiency is to contribute to the reduction of progesterone levels that must occur at term to allow parturition to take place. It is important to note, however, that significant reduction of progesterone levels does occur in the absence of 20 α -HSD (Fig. 5B), and

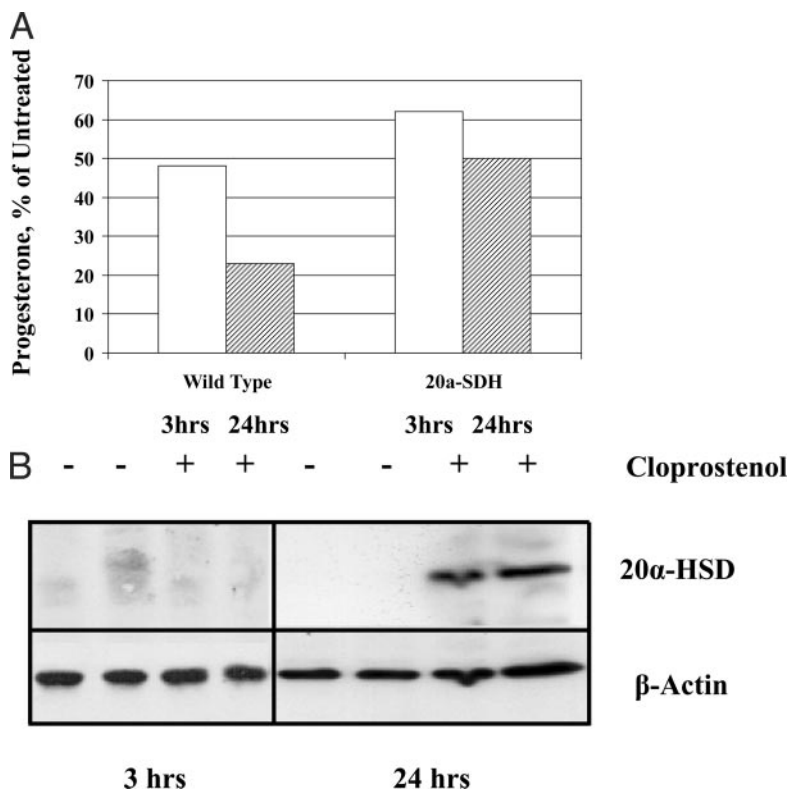


Fig. 7. Early Cloprostenol-Mediated Down-Regulation of Progesterone Is Independent of 20 α -HSD

A, Pregnant wild-type or 20 α -HSD-deficient mice were injected with 10 μ g cloprostenol, and serum progesterone levels were measured 3 h and 24 h thereafter (expressed as percentage of PBS-treated control mice). B, Late induction of ovarian 20 α -HSD protein expression by cloprostenol treatment. Pregnant wild-type mice (P16) were injected with either one injection (3 h time point) or two injections (24 h time point) of 10 μ g of cloprostenol (+) or PBS alone (-). The ovaries were removed after 3 or 24 h for the detection of 20 α -HSD protein by Western blot analysis.

thus the overall process leading to parturition involves multiple components.

The regulation of the expression of 20 α -HSD in granulosa cells involves different factors at different stages of function. Early in the development of the corpus luteum, 20 α -HSD expression is induced, and the factors controlling this expression are unknown. After coital stimulation, the production of PRL and its activation of Stat5 in granulosa cells are responsible for the down-regulation of expression as established by the persistent expression of 20 α -HSD in ovaries of pregnant Stat5-deficient females and consistent with previous studies. Interestingly, PRL has been shown to be responsible for both the induction and repression of genes in ovaries of pregnant rodents. The mechanisms by which Stat5 represses 20 α -HSD gene expression are not known and, in particular, it is not known whether Stat5 represses gene expression directly or whether it induces a transcriptional repressor. It should be noted, however, that the promoter region of the gene does not contain a consensus Stat5-binding site.

The ability of the 20 α -HSD deficiency to rescue the premature abortions that occur in Stat5b-deficient mice demonstrates the importance of the function of Stat5 to suppress enzyme expression. This result is

consistent with the observation that progesterone administration could similarly rescue the pregnancies of Stat5b-deficient mice (5). However, 20 α -HSD deficiency was unable to rescue the pregnancies of Stat5a/b-deficient mice, and again consistent with this is the observation that progesterone administration was not able to rescue the pregnancies (data not shown). These results suggest that Stat5 proteins have additional functions in ovarian activity that are critical for maintenance of pregnancy. Thus removing Stat5b, which is the predominant protein expressed in the ovary, impairs suppression of 20 α -HSD expression sufficient to affect progesterone levels, but the residual Stat5a activity is sufficient for the other critical functions. In particular, we have shown previously that p27 expression in the ovary is dependent upon the presence of both Stat5a and Stat5b (6). The importance of this expression is shown by the loss of ovarian function in p27-deficient mice (31).

The results are consistent with the hypothesis that Stat5a/b play a critical role in the physiological responses to PRL. However, it is important to note that the deficiency of either PRL or the PRL receptor results in lack of implantation at an earlier stage than that seen in Stat5a/b-deficient mice. This would suggest

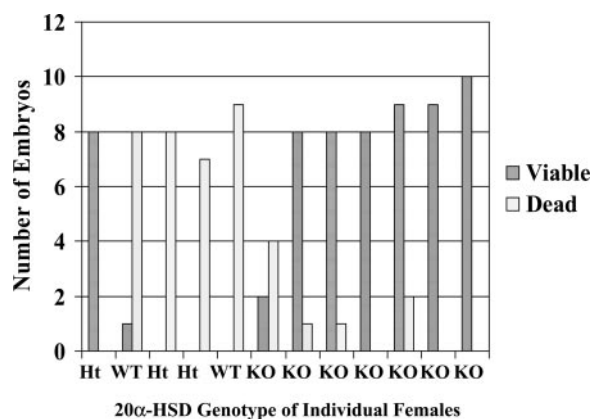


Fig. 8. Rescue of Pregnancies by Combining Deficiencies of 20 α -HSD and Stat5b

Females that were Stat5b deficient and were either wild type (WT), heterozygously (Ht) or homozygously deficient for 20 α -HSD were bred. At P18.5–P19.5 the status of the pregnancies was evaluated. The numbers of viable or dead/resorbed embryos were determined for individual females and are indicated by the bars.

that there are additional, Stat5a/b-independent functions provided by signaling through the PRL receptor.

Immediately before term, 20 α -HSD expression is strongly induced at the transcriptional level. Based on a number of observations, this induction is dependent upon PGF_{2 α} . In particular, failure to reduce progesterone levels at term and to deliver is a phenotype that is also seen in PGF_{2 α} receptor-deficient mice (27). However, the overall reduction of progesterone levels is clearly dependent upon additional regulation beyond degradation. In particular, mice lacking the enzymes required for its synthesis including phospholipase A₂ (32, 33) and cyclooxygenase 1 (34) have a similar phenotype. This is consistent with the observation that PGF_{2 α} induces the rapid decline in the expression of the sterol carrier protein-2 (35) and StAR, the steroidogenic acute regulatory protein (28, 29). The inability of the PGF_{2 α} agonist, cloprostenol, to induce parturition in 20 α -HSD-deficient pregnant females emphasizes the importance of 20 α -HSD induction in the process. Together the studies support a model in which both the synthesis of progesterone is affected at term as well as its degradation and these result in sufficient reductions in progesterone levels to allow parturition.

Mechanistically, PGF_{2 α} may either induce transcription of 20 α -HSD in a manner that overrides the suppression due to Stat5 or may function to remove the Stat5-dependent suppression at various stages of signal transduction. In this regard it is important to note that PGF_{2 α} has been shown to induce SOCS3 (36), which could potentially inhibit PRL signaling. It has also been proposed that PGF_{2 α} may inhibit the expression of the PRL receptor (37). Alternatively it has been proposed that PGF_{2 α} -induced expression of 20 α -HSD is mediated via the transcription factor NUR77 (26). However, the lack of reproductive defects

in NUR77-deficient mice (38) would indicate that there are other, perhaps redundant, pathways. Nevertheless, the studies presented here demonstrate that the regulation of 20 α -HSD expression is an essential function for normal reproductive activity.

MATERIALS AND METHODS

Antibodies, SDS-PAGE, and Western Blotting

Antibodies against 20 α -HSD were raised in rabbits using a C-terminal peptide (amino acids 294–313, KILDGLDRNLRY-FPADMFKA) of murine 20 α -HSD (324 amino acids) coupled to keyhole limpet hemocyanin. Total cell extracts were prepared in lysis buffer containing 0.5% Nonidet P-40 (39). Antibodies against human 3 β -HSD (type I) were a generous gift of Dr. F. Labrie (40). Proteins were separated by SDS-10% PAGE and transferred to nitrocellulose. Membranes were probed by using the 20 α -HSD antiserum (1:1000) or anti- β -actin antibodies (clone C4, 1:5000, ICN Biomedicals, Inc., Aurora, OH) and visualized with the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Arlington Heights, IL).

Northern Blot Analysis

Total RNA from ovaries was isolated using RNAzol (Tel-Test, Friendswood, TX) and separated on 1% formaldehyde agarose gels. A cDNA fragment of murine 20 α -HSD was labeled using [α -³²P]dCTP and a random priming labeling kit (Amersham, Arlington Heights, IL) and used as probe. Membranes were hybridized using a rapid hybridization solution (Amersham) followed by final stringent washes in 0.5 \times saline sodium citrate/0.2% SDS at 65 C.

Construction of 20 α -HSD Targeting Vector and Generation of 20 α -HSD-Deficient Mice

The 20 α -HSD gene was isolated from a 129/SVJ mouse embryonic cell (ES) cell genomic phage library (Stratagene, La Jolla, CA) using a full-length 20 α -HSD cDNA expressed sequence tag clone from a mouse macrophage library (GenBank accession no. AA105098). A 14-kb *Sa*I phage insert, identified by Southern blotting, was subcloned into pBlue-scriptIIISK(+). For the 20 α -HSD targeting vector a 1.9-kb *Bam*HI/*Eco*RI fragment containing exons 2 and 3 was replaced with a neomycin resistance cassette previously described (41). The sizes of the flanking genomic regions were 1.8 and 5 kb, 5' and 3' of the *neo* gene, respectively. A herpes simplex thymidine kinase cassette mediating negative selection was inserted in the 5'-end of the 20 α -HSD-*neo* construct. Twenty micrograms of the *Not*I linearized targeting vector (20 μ g) were electroporated into E14 (129/Ola mouse strain) ES cells, and cells were grown under double selection as described (42). Clones were picked and expanded 7–9 d after electroporation. Correctly targeted ES-clones were identified by genomic PCR (data not shown), and a karyotypically normal clone was injected. Conditions for blastocyst injection of ES cells and breeding to generate mice homozygous for the mutated 20 α -HSD gene were followed as described elsewhere (43).

Genotyping of 20 α -HSD-Deficient Mice by PCR

Genomic DNA was amplified in 50- μ l reactions using 2.5 U of AmpliTaq Gold (Perkin-Elmer Corp.) in PCR buffer with a final concentration of deoxynucleotide triphosphates at 0.2 mM

and MgCl₂ at 2 mM. The PCR primer consisted of P1 primer (5'-TGCAAAATCTGACAACTACTC-3') at 0.6 μ M, P3 primer (5'-TCTGCTTGGAGTAATGGATC-3') at 0.2 μ M, and the neomycin primer P2 (5'-AGTATCCATCATGGCTGATGC-3') at 0.6 μ M. The PCR cycle profile was as follows: 1 cycle at 94 C for 10 min followed by 35 cycles at 94 C for 1 min, 55 C for 1 min, 72 C for 1 min with 5-sec autoextension in every cycle, and finally one cycle at 72 C for 10 min. PCR products were analyzed on 2% agarose gels. A 250-bp fragment indicates the presence of the wild-type allele, whereas a 800-bp fragment is amplified from the mutated allele.

Measurement of 20 α -HSD Enzymatic Activity

The enzymatic activity of 20 α -HSD was determined essentially as described previously (9, 10). Tissues were disrupted in glass Teflon homogenizers in PBS, the homogenate was centrifuged at 5000 rpm for 5 min, and the supernatants were analyzed for 20 α -HSD activity (conversion of progesterone as substrate to 20 α -OHP). The activity assay was performed in glass tubes, containing 1–50 μ g protein in a 0.25-ml reaction mixture consisting of 0.01 M sodium phosphate, pH 7.4; 0.1 M sucrose; 1.0 mM MgCl₂; 10⁻⁵ M progesterone; 0.02 μ Ci ¹⁴C-marked progesterone (PerkinElmer Life and Analytical Sciences, Inc., Boston, MA); and a reduced nicotinamide adenine dinucleotide phosphate regenerating system consisting of 1.0 mM reduced nicotinamide adenine dinucleotide phosphate, 10 mM glucose-6-phosphate, and 1.5 U glucose-6-phosphate dehydrogenase (all reagents from Sigma Chemical Co., St. Louis, MO). After an incubation period of 30 min at 37 C, reactions were stopped by adding 3 ml diethyl ether containing 1 μ g progesterone and 1 μ g 20 α -OHP/ml as carriers. The tubes were vigorously vortexed to extract the steroids into the organic phase that was subsequently separated and evaporated overnight in a chemical hood. The extracted steroids were separated on fluorescent silica gel 60 F 254 plates (Merck, Darmstadt, Germany) in a diethyl ether/chloroform 3:10 (vol/vol) system. Radioactive progesterone and 20 α -OHP were visualized and quantitated by phosphorimaging on a Storm 860 (Molecular Dynamics, Inc., Sunnyvale, CA). Enzymatic activities were calculated as the amount of picomoles 20 α -OHP generated/h/ μ g protein.

Treatment of Pregnant Mice with Cloprostenol (PGF_{2 α} Agonist) or Mifepristone (RU486, Progesterone Receptor Antagonist)

Pregnant mice (P15–P17) were injected sc in the upper back with 5–10 μ g cloprostenol (Sigma) freshly dissolved in 200 μ l PBS (two doses of 100 μ l at 1000 h and 1400 h, or one dose at 1000 h). Control mice received PBS alone. Premature birth was observed usually 24 h after the second injection, but all mice were monitored for another 24 h. Mifepristone/RU486 (Sigma, 0.2 mg in 100 μ l olive oil) was injected sc into pregnant mice at d 19 of gestation. Parturition occurred 24 h later.

Measurement of Serum Progesterone Levels by RIA

Serum progesterone levels were measured using a solid-phase ¹²⁵I RIA kit (Diagnostic Products Corp., Los Angeles, CA). Female mice were anesthetized and bled into tubes containing 20 μ l Heparin (Elkins-Sinn, Inc., Cherry Hill, NJ). Sera were recovered after centrifugation at 3500 rpm for 5 min and frozen at –80 C. Serum samples were either diluted with calibrator serum (containing no progesterone) or analyzed directly in the RIA procedure exactly as described by the manufacturer. Radioactivity counts were determined in a γ -counter (1-min counting time), and the progesterone concentrations were calculated from a standard curve (0–40 ng/ml progesterone) that was run in parallel.

Acknowledgments

We thank Kristen Rothhammer and Margalit Krup for technical assistance.

Received July 27, 2004. Accepted September 27, 2004.

Address all correspondence and requests for reprints to: James N. Ihle, Howard Hughes Medical Institute, Department of Biochemistry, St. Jude Children's Research Hospital, Memphis, Tennessee 38105. E-mail: james.ihle@stjude.org.

This work was supported in part by Grant 1998128 (to J.N.I. and Y.W.) from the United States-Israel Binational Science Foundation (BSF) Israel; a grant from the Israel Cancer Research Fund and a grant from the Israel Science Foundation (ISF) (Grant 435/03) (to Y.W.); by Cancer Center CORE Grant CA21765; and by the American Lebanese Syrian Associated Charities (ALSAC).

Current address for R.P.P.: Institute for Biochemistry and Molecular Biology II, Heinrich-Heine-University, Duesseldorf D40225, Germany.

REFERENCES

- Horseman ND, Zhao W, Montecino-Rodriguez E, Tanaka M, Nakashima K, Engle SJ, Smith F, Markoff E, Dorshkind K 1997 Defective mammapoiesis, but normal hematopoiesis, in mice with a targeted disruption of the prolactin gene. *EMBO J* 16:6926–6935
- Ormandy CJ, Camus A, Barra J, Damotte D, Lucas B, Buteau H, Edery M, Brousse N, Babinet C, Binart N, Kelly PA 1997 Null mutation of the prolactin receptor gene produces multiple reproductive defects in the mouse. *Genes Dev* 11:167–178
- Bole-Feysot C, Goffin V, Edery M, Binart N, Kelly PA 1998 Prolactin (PRL) and its receptor: actions, signal transduction pathways and phenotypes observed in PRL receptor knockout mice. *Endocr Rev* 19:225–268
- Liu X, Robinson GW, Garrett L, Wynshaw-Boris A, Hennighausen L 1997 Stat5a is mandatory for adult mammary gland development and lactogenesis. *Genes Dev* 11:179–186
- Udy GB, Snell RG, Wilkins RJ, Park S-H, Ram PA, Waxman DJ, Davey HW 1997 Requirement of STAT5b for sexual dimorphism of body growth rates and liver gene expression. *Proc Natl Acad Sci USA* 94:7239–7244
- Teglund S, McKay C, Schuetz E, van Deursen J, Stravopodis D, Wang D, Brown M, Bodner S, Grosfeld G, Ihle JN 1998 Stat5a and Stat5b proteins have essential and non-essential, or redundant, roles in cytokine responses. *Cell* 93:841–850
- Albaracin CT, Parmer TG, Duan WR, Nelson SE, Gibori G 1994 Identification of a major prolactin-regulated protein as 20 α -hydroxysteroid dehydrogenase: coordinate regulation of its activity, protein content, and messenger ribonucleic acid expression. *Endocrinology* 134:2453–2460
- Wiest WG 1968 On the function of 20 α -hydroxypregn-4-en-3-one during parturition in the rat. *Endocrinology* 83:1181–1184
- Weinstein Y 1977 20 α -Hydroxysteroid dehydrogenase: a T lymphocyte-associated enzyme. *J Immunol* 119:1223–1229
- Weinstein Y, Lindner HR, Eckstein B 1977 Thymus metabolizes progesterone—possible enzymatic marker for T lymphocytes. *Nature* 266:632–633
- Hirabayashi K, Suzuki M, Takahashi M, Nishihara M 2001 Expression of ovarian 20 α -hydroxysteroid dehydrogenase in rat thymus. *Endocr J* 48:557–563

12. Ihle JN, Peppersack L, Rebar L 1981 Regulation of T cell differentiation: in vitro induction of 20 α hydroxysteroid dehydrogenase in splenic lymphocytes from athymic mice by a unique lymphokine. *J Immunol* 126:2184–2189
13. Ihle JN, Keller J, Oroszlan S, Henderson L, Copeland T, Fitch F, Prystowsky MB, Goldwasser E, Schrader JW, Palaszynski E, Dy M, Lebel B 1983 Biological properties of homogenous interleukin-3. I. Demonstration of WEHI-3 growth factor activity, mast cell growth factor activity, P-cell stimulating factor activity, colony stimulating factor activity and histamine producing cell stimulating factor activity. *J Immunol* 131:282–287
14. Ertel RJ, Ungar F 1968 20- α -Hydroxysteroid dehydrogenase and reductive pathways in mouse adrenal glands *in vitro*. *Endocrinology* 82:527–534
15. Akinola LA, Poutanen M, Vihko R, Vihko P 1997 Expression of 17 β -hydroxysteroid dehydrogenase type 1 and type 2, P450 aromatase, and 20 α -hydroxysteroid dehydrogenase enzymes in immature, mature, and pregnant rats. *Endocrinology* 138:2886–2892
16. Gibori G 1992 The corpus luteum of pregnancy. In: Adashi EY, Leung PCK, eds. *The ovary*. New York: Raven Press; 261–317
17. Amsterdam A, Gold RS, Hosokawa K, Yoshida Y, Sasson R, Jung Y, Kotsuji F 1999 Crosstalk among multiple signaling pathways controlling ovarian cell death. *Trends Endocrinol Metab* 10:255–262
18. Terkel J 1986 Neuroendocrinology of coitally and non-coitally induced pseudopregnancy. *Ann NY Acad Sci* 474:76–94
19. Richards JS, Williams JJ 1976 Luteal cell receptor content for prolactin (PRL) and luteinizing hormone (LH): regulation by LH and PRL. *Endocrinology* 99:1571–1581
20. Jez JM, Bennett MJ, Schlegel BP, Lewis M, Penning TM 1997 Comparative anatomy of the aldo-keto reductase superfamily. *Biochem J* 326:625–636
21. Aflalo E, Ofir R, Apte RN, Weinstein Y 1987 Characterization of mouse thymocyte subpopulations by the enzymatic marker 20- α -hydroxysteroid dehydrogenase: differential responses to IL-1 and IL-2. *Clin Exp Immunol* 70:578–584
22. Keller JR, Weinstein Y, Hursey M, Ihle JN 1985 Interleukins 2 and 3 regulate the in vitro proliferation of two distinguishable populations of 20- α -hydroxysteroid dehydrogenase-positive cells. *J Immunol* 135:1864–1871
23. McCracken JA, Custer EE, Lamsa JC 1999 Luteolysis: a neuroendocrine-mediated event. *Physiol Rev* 79:263–323
24. Niswender GD, Juengel JL, Silva PJ, Rollyson MK, McIntush EW 2000 Mechanisms controlling the function and life span of the corpus luteum. *Physiol Rev* 80:1–29
25. Lamprecht SA, Lindner HR, Strauss III JF 1969 Induction of 20 α -hydroxysteroid dehydrogenase in rat corpora lutea by pharmacological blockade of pituitary prolactin secretion. *Biochim Biophys Acta* 187:133–143
26. Stocco CO, Zhong L, Sugimoto Y, Ichikawa A, Lau LF, Gibori G 2000 Prostaglandin F_{2 α} -induced expression of 20 α -hydroxysteroid dehydrogenase involves the transcription factor NUR77. *J Biol Chem* 275:37202–37211
27. Sugimoto Y, Yamasaki A, Segi E, Tsuboi K, Aze Y, Nishimura T, Oida H, Yoshida N, Tanaka T, Katsuyama M, Hasumoto K, Murata T, Hirata M, Ushikubi F, Negishi M, Ichikawa A, Narumiya S 1997 Failure of parturition in mice lacking the prostaglandin F receptor. *Science* 277:681–683
28. Fiedler EP, Plouffe Jr L, Hales DB, Hales KH, Khan I 1999 Prostaglandin F(2 α) induces a rapid decline in progesterone production and steroidogenic acute regulatory protein expression in isolated rat corpus luteum without altering messenger ribonucleic acid expression. *Biol Reprod* 61:643–650
29. Sandhoff TW, Hales DB, Hales KH, McLean MP 1998 Transcriptional regulation of the rat steroidogenic acute regulatory protein gene by steroidogenic factor 1. *Endocrinology* 139:4820–4831
30. Tibbetts TA, DeMayo F, Rich S, Conneely OM, O'Malley BW 1999 Progesterone receptors in the thymus are required for thymic involution during pregnancy and for normal fertility. *Proc Natl Acad Sci USA* 96:12021–12026
31. Fero ML, Rivkin M, Tasch M, Porter P, Carow CE, Firpo E, Polyak K, Tsai LH, Broudy V, Perlmutter RM, Kaushansky K, Roberts JM 1996 A syndrome of multiorgan hyperplasia with features of gigantism, tumorigenesis, and female sterility in p27(Kip1)-deficient mice. *Cell* 85:733–744
32. Bonventre JV, Huang Z, Taheri MR, O'Leary E, Li E, Moskowitz MA, Sapirstein A 1997 Reduced fertility and postischemic brain injury in mice deficient in cytosolic phospholipase A2. *Nature* 390:622–625
33. Uozumi N, Shimizu T 2002 Roles for cytosolic phospholipase A2 α as revealed by gene-targeted mice. *Prostaglandins Other Lipid Mediat* 68–69:59–69
34. Gross GA, Imamura T, Luedke C, Vogt SK, Olson LM, Nelson DM, Sadovsky Y, Muglia LJ 1998 Opposing actions of prostaglandins and oxytocin determine the onset of murine labor. *Proc Natl Acad Sci USA* 95:11875–11879
35. McLean MP, Billheimer JT, Warden KJ, Irby RB 1995 Prostaglandin F_{2 α} mediates ovarian sterol carrier protein-2 expression during luteolysis. *Endocrinology* 136:4963–4972
36. Curlewis JD, Tam SP, Lau P, Kusters DH, Barclay JL, Anderson ST, Waters MJ 2002 A prostaglandin F_{2 α} analog induces suppressors of cytokine signaling-3 expression in the corpus luteum of the pregnant rat: a potential new mechanism in luteolysis. *Endocrinology* 143:3984–3993
37. Stocco C, Djiane J, Gibori G 2003 Prostaglandin F_{2 α} (PGF_{2 α}) and prolactin signaling: PGF_{2 α} -mediated inhibition of prolactin receptor expression in the corpus luteum. *Endocrinology* 144:3301–3305
38. Lee SL, Wesselschmidt RL, Linette GP, Kanagawa O, Russell JH, Milbrandt J 1995 Unimpaired thymic and peripheral T cell death in mice lacking the nuclear receptor NGFI-B (Nur77). *Science* 269:532–535
39. Wang D, Stravopodis D, Teglund S, Kitazawa J, Ihle JN 1996 Naturally occurring dominant negative variants of Stat5. *Mol Cell Biol* 16:6141–6148
40. Luu T, V, Lachance Y, Labrie C, Leblanc G, Thomas JL, Strickler RC, Labrie F 1989 Full length cDNA structure and deduced amino acid sequence of human 3 β -hydroxy-5-ene steroid dehydrogenase. *Mol Endocrinol* 3:1310–1312
41. van Deursen J, Lovell-Badge R, Oerlemans F, Schepens J, Wieringa B 1991 Modulation of gene activity by consecutive gene targeting of one creatine kinase M allele in mouse embryonic stem cells. *Nucleic Acids Res* 19:2637–2643
42. Parganas E, Wang D, Stravopodis D, Topham DJ, Marine J-C, Teglund S, Vanin EF, Bodner S, Colamonici OR, van Deursen JM, Grosveld G, Ihle JN 1998 Jak2 is essential for signaling through a variety of cytokine receptors. *Cell* 93:385–395
43. van Deursen J, Heerschap A, Oerlemans F, Ruitenbeek W, Jap P, ter Laak H, Wieringa B 1993 Skeletal muscles of mice deficient in muscle creatine kinase lack burst activity. *Cell* 74:621–631

Molecular Endocrinology is published monthly by The Endocrine Society (<http://www.endo-society.org>), the foremost professional society serving the endocrine community.