Reproductive Endocrinology

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The past year has once again seen the boundaries of reproductive 'possibility and promise' extended. Ovarian cryopreservation seems to be an increasing reality in primates as well as in mice, new physiological systems and steroidogenic pathways involved in reproductive development and function are being elucidated, the genetic bases of several fairly common (as well as some extremely rare) endocrine conditions have been determined, and the era of stem cell/germ cell potential is truly upon us. The major challenge ahead will be to facilitate translation of this scientific progress into patient care, in a calculated and ethically considered manner. As always, selecting such a limited number of papers is not easy and many excellent papers could not be included in this collection.

Mechanism of the year: FSH binding becomes crystal clear

Structure of human follicle-stimulating hormone in complex with its receptor

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Background: Follicle-stimulating hormone (FSH) regulates reproduction in mammals through a gonadal G-protein-coupled receptor.

Methods: The structure of a partially deglycosylated complex of human FSH bound to the extracellular hormone-binding domain of its receptor (FSHR(HB)) has been solved to 2.9 Å.

Results: The hormone is bound in a hand-clasp fashion to an elongated, curved receptor. Three important new observations have been made based on this structure. First, the buried surface area of the complex is large (2,600 Å²) and has a high charge density. Secondly, FSH undergoes a conformational change when it binds receptor that affects loops thought to be important to receptor activation. Finally, the FSH-FSHR(HB) complexes form dimers in the crystal and this topology may be important to transmembrane signal transduction (fig. 1).

Conclusions: This study provides a model for the binding of all glycoprotein hormones to their receptors and shows that binding specificity is mediated by key interaction sites involving both the common α - and hormone-specific β -subunits.

The structural basis of reproductively-relevant dimeric protein hormones binding to specific cell surface receptors has been significantly advanced in the past decade with the solution of dimeric activin binding to its receptor, hCG bound to its high affinity receptor and now the structure of FSH with the G-protein-coupled receptor, FSHR. Dimers provide intriguing opportunities in nature, namely to make more specific contacts out of a more limited repertoire of proteins. In the case of the glycoprotein hormones, a common α -subunit is used as a scaffold for a functional β -subunit. Structural data are important for the understanding of the protein interactions themselves as well as providing important insight into potential agonists or antagonists that might be used to develop the ever-elusive orally bioactive FSH-like small molecule or an effective non-steroidal contraceptive. In addition, we know that the superfamily of cytokine and growth hormone receptors require dimerization for signal transduction. The data presented here suggest that dimerization of G-protein receptors may also be important for transmembrane signaling.

Fig. 1. Model for FSH receptor structure (**a**) and activation by FSH (**b**). The extracellular hormone-binding (HB) domain of the receptor is shaded whereas the linker and transmembrane domain of the receptor are shown as a black dotted trace. Crystallization of the FSH/FSHR complex revealed several surprising features, such as interactions between the hormone and transmembrane domain of the receptor as well as dimerization of hormone-receptor complexes, which may be important for signal transduction [reproduced with permission of the authors and Nature Publishing Group, 2005].

New paradigms: early puberty – preventing the breakdown

Early onset of puberty and early ovarian failure in CYP7B1 knockout mice

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Background: CYP7B1 is the enzyme responsible for hydroxylation of the androgen metabolite, 5 α -androstane-3 β ,17 β -diol (3 β Adiol), resulting in termination of its potential estrogenic activity. Consequently, 3 β Adiol is estrogenic in ER α - or ER β -positive cells only if they do not express CYP7B1.

Methods: CYP7B1(–/–) mice were created and phenotyped.

Results: Female CYP7B1(–/–) mice experienced early-onset uterine growth and mammary development, and enter reproductive cyclicity 2 days earlier than wild-type littermates. The mammary glands and uteri of adult animals have phenotypic features consistent with chronic estrogenic stimulation.

Conclusion: By cell-specific regulation of the estrogenicity of 3 β Adiol, CYP7B1 performs two major tasks: (i) it allows 3 β Adiol to have growth inhibitory effects through ER β and (ii) it permits estradiol-specific

Fig. 2. Biosynthesis and excretion of 3βAdiol, a non-aromatized derivative of dihydrotestosterone (DHT) with estrogenic activity. Following targeted deletion of CYP7B1 in the mouse, elevated concentrations of 3βAdiol can act indiscriminately through estrogen receptors, resulting in early uterine maturation and mammary development.

activation of estrogen receptors by protection of certain cells from the estrogenic effects of 3βAdiol. When CYP7B1 is inactivated, 3βAdiol activates estrogen receptors indiscriminately, resulting in prolonged and inappropriate estrogen exposure.

Much of our focus in endocrinology is on the mechanisms regulating biosynthesis of hormones. However, hormone action is also influenced by clearance mechanisms, degradation and/or conversion to bio-inactive derivatives, which may occur in a tissue- or cell-specific manner, and which may be regulated at a paracrine level. The phenotype of the CYP7B1(−/−) mouse is an important example of this concept. CYP7B1 is important for the hydroxylation of 3βAdiol (fig. 2). 3βAdiol is a non-aromatase-dependent metabolite of DHT that is a natural ligand of ERβ. Circulating levels of 3βAdiol from the prepubertal ovary likely have an anti-proliferative effect through ERβ stimulation. However, targeted deletion of CYPB1(−/−) in mice results in early puberty and evidence of excessive and chronic estrogenic stimulation as estrogen receptors (ERβ and ERα) are activated indiscriminately by 3βAdiol in multiple tissues. This study shows that we still have much to learn about steroidogenic pathways, especially at the cellular level, and that ‘alternative pathways’ may have important biological roles [1]. Mechanisms regulating inactivation of biologically active compounds may be as significant as those regulating synthesis in the first place. These results suggest that non-aromatizable androgens can have estrogenic action through 3βAdiol. However, the physiological relevance of this in the absence of CYP7B1 changes and in humans remains unknown.

New hope: (cryo)preserving reproductive potential

Live birth after orthotopic transplantation of cryopreserved ovarian tissue

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Background: The lifesaving treatment experienced by cancer patients leads, in many women, to early menopause and subsequent infertility. Ovarian tissue cryopreservation is a promising option to restore fertility in women who undergo chemotherapy. Biopsy samples of ovarian cortex were taken from a woman with stage IV Hodgkin’s lymphoma (1997) and cryopreserved prior to chemotherapy. Subsequent to treatment, the patient had premature ovarian failure.

Methods: Orthotopic autotransplantation of thawed ovarian cortical tissue was done by laparoscopy in 2003.

Results: Basal body temperature, menstrual cycles, vaginal ultrasonography, and hormone concentrations were observed 5 months after transplant indicated recovery of regular ovulatory cycles. Laparoscopy confirmed the ultrasonographic data and showed the presence of a follicle at the site of reimplantation, clearly situated outside the ovaries, both of which appeared atrophic. From 5 to 9 months, the patient had menstrual bleeding and development of a follicle or corpus luteum with every cycle. Eleven months after reimplantation, human chorionic gonadotrophin concentrations and vaginal echography confirmed a viable intrauterine pregnancy, which resulted in a live birth.

Conclusion: A live birth was obtained following orthotopic autotransplantation of cryopreserved ovarian tissue. These data suggest that cryopreservation of ovarian tissue should be offered to all young women diagnosed with cancer.

The development of fertility preservation methods for women has great public attention. However, the number of 'take-home' babies is still extremely small. This paper by Donnez et al. supports last year's studies of Lee et al. [2] and Oktay et al. [3] and at last raises the real possibility of cryopreservation as a viable option for women likely to suffer premature ovarian failure, such as those undergoing cancer treatment or potentially even Turner girls who enter puberty spontaneously. Although some criticism of the study has come from the *orthotopic* transplantation of tissue into the fallopian area (thereby raising the possibility of spontaneous ovarian recovery resulting in ovulation), the authors provided evidence of follicular development within the transplanted cryopreserved tissue. This report does represent a major advance. However, the opportunity for 'all young women diagnosed with cancer' to cryopreserve ovarian tissue is associated with cost and ethical issues which must be addressed. In the USA, the ability of a young woman, newly diagnosed with cancer to accrue additional costs of a laparoscopy and fertility options may result in a decision of economics rather than fertility management. In public healthcare systems, such options may not yet be readily available. In both systems, healthcare providers should look at the option to preserve fertility as an available procedure to ensure that economic disparities do not arise. Secondly, the definition of 'young' suggests that girls with cancer should also be considered. The ovarian status of the young girl is, in many ways, 'better' from the reproductive standpoint because more follicles are available. However, issues of decision-making for a problem that might arise in future years places additional stresses on the caregiver (usually the parents) in an already stressful time. Social policy, ethics, and economics must dialogue with reproductive scientists and care-givers to ensure that the universal access that is suggested does not create new issues.

New concerns: when fragile ovaries start to fail

Evidence of early ovarian aging in fragile X premutation carriers

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Background: As many as 28% of female fragile X premutation carriers develop premature ovarian failure. The present study examines whether hormone changes characteristic of early ovarian aging occur in fragile X premutation carriers with ovulatory menstrual cycles.

Methods: Eleven regularly cycling fragile X premutation carriers, 24–41 years old (34.5 ± 5.7 years, mean $\pm$ SD), were enrolled and daily blood samples were obtained across one menstrual cycle. LH, FSH, estradiol, progesterone (P4), inhibin A, and inhibin B levels were compared with levels in 22 age-matched, regularly cycling women, at each cycle stage.

Results: Total cycle (26.1 ± 1.0 vs. 28.2 ± 0.4 days; $p < 0.05$) and follicular phase length (12.9 ± 0.8 vs. 14.5 ± 0.4 days; $p < 0.05$) were decreased in fragile X premutation carriers compared with age-matched controls, whereas luteal phase length was similar. FSH was elevated across the follicular (21.9 ± 3.5 vs. 11.2 ± 0.5 IU/l; $p < 0.001$) and luteal phases (14.6 ± 3.9 vs. 7.9 ± 0.5 IU/l; $p < 0.05$)

in fragile X premutation carriers compared with age-matched controls. Inhibin B in the follicular phase, inhibin A and P4 in the luteal phase were decreased in fragile X premutation carriers compared with age-matched controls, whereas there was no difference in estradiol or LH.

Conclusions: Despite regular ovulatory cycles, FSH was increased in fragile X premutation carriers compared with age-matched controls. The increased FSH was accompanied by decreased inhibin B in the follicular phase and inhibin A and P4 in the luteal phase. These hormonal changes, reflecting a decrease in follicle number, suggest that fragile X premutation carriers exhibit early ovarian aging despite regular menstrual cycles.

Fragile X syndrome is one of the most common genetic defects affecting mental development in boys, and typically presents with learning difficulties, autistic-like behavior and attention-deficit/hyperactivity disorder. Physical features include an elongated jaw and enlarged testes – a sign that has puzzled pediatric endocrinologists for years, but that is generally not associated with any endocrine derangements. Fragile X syndrome results from expansion (>200) of an unstable CGG repeat in the 5'-untranslated region of the FMR1 gene, resulting in promoter methylation and severely attenuated transcription of the FMR protein. Female carriers of the premutation (55–200 repeats, which expands on transmission) do not have neurological defects. However, ovarian age greatly exceeds chronological age in the carriers. This study by Welt et al. clearly demonstrates impaired ovarian function in fragile X premutation carriers, and stresses the importance of counselling for the risk of premature menopause in this group. The hormone data are consistent with fewer numbers of follicles present in the premutation carriers. It is interesting to speculate whether the premutation impacts the number of initial follicles that migrate into the gonadal ridge or alters the kinetics of postnatal follicle development. Whilst the study's sample size is understandably small, these data point to the need for more studies on the premutation as it relates to follicle dynamics and to the related questions regarding male fertility. The authors suggest that women with idiopathic infertility and high day 3 FSH undergo genetic testing for the fragile X premutation. In the adolescent endocrinology clinic, this approach might be considered in girls with irregular menses and elevated FSH. However, an increased prevalence of fragile X syndrome in IVF babies has not been documented. Larger studies will be needed to establish the prevalence of fragile X premutation changes in women with impaired fertility in the absence of a history of a son with a behavioural phenotype suggestive of fragile X. However, the ovarian clock could be ticking for those mothers or sisters of boys where the diagnosis has been made.

Concepts revised: SF-1 – out of the orphanage?

Structural analyses reveal phosphatidylinositols as ligands for the NR5 orphan receptors SF-1 and LRH-1

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Background: Vertebrate members of the nuclear receptor NR5A subfamily, which includes steroidogenic factor 1 (SF-1) and liver receptor homolog 1 (LRH-1), regulate many crucial aspects of development, endocrine homeostasis, and metabolism. Mouse LRH-1 is believed to be a ligand-independent transcription factor with a large and empty hydrophobic pocket. However, it remains debated whether a natural ligand exists for human or murine SF-1 or even for human homologs of LRH-1.

Methods: Following crystallization, structural and biochemical data were obtained for these three other NR5A members (hSF-1, m-SF-1, hLRH-1).

Results: Structural analysis reveals that these receptors bind phosphatidylinositol second messengers and that ligand binding is required for maximal activity. Evolutionary analysis of structure-function

relationships across the SF-1/LRH-1 subfamily indicates that ligand binding is the ancestral state of NR5A receptors and was uniquely diminished or altered in the rodent LRH-1 lineage.

Conclusions: Phospholipids regulate gene expression by directly binding to NR5A nuclear receptors.

Similar findings were reported in:

Crystallographic identification and functional characterization of phospholipids as ligands for the orphan nuclear receptor steroidogenic factor-1

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Steroidogenic factor-1 is a nuclear receptor that plays a pivotal role in regulating the development and function of the adrenal gland, gonad and reproductive axis at multiple levels. Point mutations in SF-1 can be associated with adrenal failure gonadal dysgenesis and persistent müllerian structures in humans [4], whereas it is emerging that apparent haploinsufficiency of SF-1 may be a relatively frequent cause of gonadal dysgenesis with preserved adrenal function in humans [5–7]. As SF-1 has such a crucial role in regulating gene transcription in these systems, it is likely that even small changes in SF-1 activity might have quite significant effects on endocrine function. Although oxysterols were once proposed to be ligands for SF-1, it has widely been held that SF-1 belongs to the *orphan* nuclear receptor subfamily of factors that can function in the absence of ligand. This subfamily contrasts with the *classic* liganded nuclear receptors that bind high affinity hormone (e.g. estrogen receptor) or *intermediate* group of nuclear receptors that have low-affinity metabolic ligands (e.g. PPAR γ). Crystallization of the ligand-like binding domain of SF-1 by several groups has revealed that human SF-1 binds a phospholipid ligand that is required for maximal activity. Although the exact significance of this ligand action in regulating SF-1 activity in vivo is not totally clear yet, phospholipid binding represents an important means of ‘fine-tuning’ SF-1 activity by upstream signaling pathways. This interface with signaling pathways may help to confer tissue specificity to the effects of SF-1 (e.g. gonadal vs. adrenal regulation) or influence the effects of SF-1 differently at different stages of development. It is already known that MAP kinase-related serine phosphorylation of SF-1 may upregulate its activity, whereas post-translational modification by SUMOylation of lysine residues may be involved in repressing its basal function. The discovery of phospholipid binding to SF-1 by several independent groups may represent another important mechanism by which endocrine function can be regulated.

Homozygous disruption of P450 side-chain cleavage (CYP11A1) is associated with prematurity, complete 46,XY sex reversal, and severe adrenal failure

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Background: Disruption of the P450 side-chain cleavage cytochrome (P450_{scc}) enzyme due to deleterious mutations of the CYP11A1 gene is thought to be incompatible with fetal survival because of impaired progesterone production by the fetoplacental unit.

Method: CYP11A analysis was performed in a 46,XY patient born prematurely with complete sex reversal and severe adrenal insufficiency. Laboratory data showed diminished or absent steroidogenesis in all pathways.

Results: Molecular genetic analysis of the CYP11A1 gene revealed a homozygous single nucleotide deletion leading to premature termination at codon position 288. This mutation will delete highly conserved regions of the P450_{scc} enzyme and thus is predicted to lead to a non-functional protein. Both healthy parents were heterozygous for this mutation.

Conclusions: Severe disruption of P450scc can be compatible with survival in rare instances. Defects in this enzyme are inherited in an autosomal-recessive fashion, and heterozygote carriers can be healthy and fertile.

Side-chain cleavage by P450scc is the rate-limiting step in the production of pregnenolone from cholesterol, necessary for the biosynthesis of all steroid hormones in the gonad, adrenal and placenta. Progesterone is necessary for the maintenance of pregnancy. In rodents, the maternal corpus luteum is able to produce sufficient progesterone to support pregnancy throughout gestation, and a naturally-occurring rabbit model of P450scc deficiency exists. In higher primates, progesterone production is thought to transfer from the corpus luteum to the placenta in the second trimester ('luteo-placental shift'). As the placenta is fetally derived, complete loss of P450scc activity was thought to be incompatible with survival to term. In this report, Hiort et al. describe a homozygous severely disruptive mutation in the gene encoding P450scc (*CYP11A*) in a patient with complete 46,XY sex reversal and severe adrenal failure, born prematurely at 31 weeks' gestation. This finding shows that in rare occasions, loss of P450scc function can be compatible with survival. The possibility of *CYP11A* mutations in families with a history of late spontaneous miscarriages or preterm labour must be considered, and karyotypic analysis of phenotypically female fetuses might be appropriate in such situations. One would expect that the gonads of such a child would differentiate normally, yet these were not detectable by ultrasound or MRI. It is possible that secondary atrophy occurred due to cholesterol accumulation as observed in lipid adrenal hyperplasia.

Sox9 is sufficient for functional testis development producing fertile male mice in the absence of Sry

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Background: 'Odd sex' (Ods) is a dominant mutant mouse model (XXOds/+) where XX animals develop as phenotypic males due to male-pattern expression of Sox9 in the developing embryonic gonads. However, mice are sterile as they lack Y chromosomal material necessary for spermatogenesis.

Methods: To test whether SOX9 was sufficient to generate a fully fertile male in the absence of Sry, an XY(Sry–)Ods/+ male mouse was constructed in which the male phenotype is controlled autosomally by the Ods mutation.

Results: Mice were initially fertile, but progressively lost fertility until 5–6 months when they were sterile with very few germ cells in the testis. XY(Sry–)Ods/+ males also failed to establish the correct male-specific pattern of vascularization at the time of sex determination, which could be correlated to an inability of XY(Sry–)Ods/+ males to fully down-regulate Wnt4 expression in the embryonic gonad. Increasing the amount of SOX9 by producing homozygous XY(Sry–)Ods/Ods males was able to completely rescue the phenotype and restore correct vascular patterning and long-term fertility.

Conclusions: These data indicate that activation of SOX9 in the gonad is sufficient to trigger all the downstream events needed for the development of a fully fertile male.

Recent evidence from overexpression of Sox9 in the 'Odd-sex' (XX) mouse model [8], as well as in a masculinized 46,XX patient with mosaic duplication of the locus containing SOX9 [9], has suggested that Sox9/SOX9 can function as a 'testis-determining factor' in its own right, downstream of Sry. However, as many genes on the Y chromosome (such as *Daz*) are necessary for spermatogenesis, it has not been known whether SOX9 overexpression is able to form a gonad capable of sustaining fertility, independent of Sry. In this simple and logical experiment, Qin and Bishop overexpressed Sox9 in a mouse with a full Y chromosome complement but lacking the Sry gene. They demonstrate that Sox9 is sufficient to induce full fertility, which is maintained when higher doses of Sox9 are expressed (i.e., homozygous Ods mouse on XY(Sry–) background). This study confirms that Sox9 is a testis-determining factor in its own right, highlights the importance of dosage effects in reproductive development and function, and demonstrates the benefits of crossing various transgenic mouse strains to address simple biological questions.

A single luteinizing hormone determination two hours after depot leuprolide is useful for therapy monitoring of gonadotropin-dependent precocious puberty in girls

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Background: Long-acting GnRH analogs are the standard treatment for gonadotropin-dependent precocious puberty (GDPP). The aim of this study was to determine the hormonal parameters for monitoring the adequacy of depot leuprolide acetate treatment in girls with clinical and hormonal diagnosis of GDPP.

Methods: Eighteen girls with GDPP were treated with 3.75 mg depot leuprolide acetate monthly. The LH peak after a classical GnRH test was compared with a single LH measurement obtained 2 h after depot leuprolide acetate administration, before and during GnRH analog treatment.

Results: Adequate hypothalamic-pituitary-gonadal axis suppression during treatment was achieved in 16 of the 18 girls according to clinical parameters and prepubertal LH levels. In these 16 well-controlled girls, the mean $\pm$ SD LH peak after a classical GnRH test was 18.4 ± 11.2 IU/l before therapy, and it was 22.6 ± 8.3 IU/l 2 h after the first depot leuprolide dose. During therapy, the mean $\pm$ SD of LH peak after classical GnRH test was 1.4 ± 0.6 IU/l (ranging from <0.6 to 2.3 IU/l), and it was 2.7 ± 1.9 IU/l (ranging from 0.7 to 6.6 IU/l) 2 h after depot leuprolide. Peak and 2-hour LH values correlated significantly before and during treatment. In contrast, the 2 girls who were not adequately controlled with 3.75 mg depot had higher peak (4.3 , 5.7 IU/l) and 2 h (7.5 , 11 IU/l) LH concentrations, which were subsequently suppressed when high doses of leuprolide (7.5 mg depot) were used.

Conclusions: LH cut-off values for adequate depot leuprolide therapy are an LH peak <2.3 IU/l after a classical GnRH test or <6.6 IU/l 2 h after depot leuprolide. The latter measurement may replace the classical GnRH test as a reliable and convenient tool for monitoring therapy in female GDPP.

Depot leuprolide (3.75 mg monthly) is usually an effective treatment for central precocious puberty in girls, but some individuals require higher doses of leuprolide (7.5 mg) to prevent pubertal progression. Ideally, this subgroup would be identified before clinical evidence of ongoing estrogenization is seen. GnRH stimulation tests can be useful in monitoring response to treatment, but are invasive and relatively expensive. In this report, Brito et al. propose that a single LH measurement taken 2 h after depot leuprolide administration is as reliable as the classic GnRH test for monitoring therapy. As only 2 of the 18 girls were poorly controlled, some caution is needed in interpreting the exact cut-off value of LH (6.6 IU/l) proposed. However, it seems that a single LH measurement 2 h after depot could be a good screening test to identify those girls who might need closer vigilance for adequacy of treatment and who are at risk of 'breaking' through suppressive therapy by the end of the monthly treatment period.

Activating GNAS1 gene mutations in patients with premature thelarche

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Background: The association of activating GNAS1 gene mutations (R210H) with McCune-Albright syndrome (MAS) or isolated precocious puberty is well established, but the incidence of these changes in other forms of thelarche is less clear.

Methods: GNAS1 gene mutation from genomic (leukocyte) DNA by allele-specific polymerase chain reaction and enzymatic digestion was performed in 23 girls with exaggerated (n = 9) and/or chronic fluctuating (n = 14) thelarche for at least 1-year duration with no other signs of precocious puberty, skeletal dysplasia, or typical skin lesions of MAS.

Results: The activating GNAS1 change (R201H) was identified in 6 girls. Three R201H(+) girls reached their menarche at a mean chronologic age of 10.8 years and 9 of the R201H(−) girls at a mean age of 11 years.

Conclusions: Activating mutations of the GNAS1 gene may be found in some girls with chronic fluctuating and/or exaggerated thelarche, without other classic signs of MAS.

Activating mutations in GNAS1, the Gs protein involved in G-protein-coupled receptor signaling, are associated with gonadotropin-independent precocious puberty (GIPP), polyostotic fibrous dysplasia of the bones, and typical pigmented skin lesions in MAS. This is a mosaic condition, as homozygous activation of GNAS1 in all somatic cells is likely to be lethal. Thus, presenting features can be variable depending on the mosaicism in different tissues, and a significant proportion of girls with activating GNAS1 mutations in the recent European Collaborative Study [10] presented with isolated GIPP alone. This report by Roman et al. shows that activating GNAS1 changes can be found in a proportion of girls with chronic or exaggerated premature thelarche (i.e., at least two self-limiting episodes of breast budding within 1 year with advanced bone age or increased growth velocity). The changes were identified in genomic DNA extracted from leukocytes by allele-specific PCR, and confirmed by enzyme digestion. Whilst this is a selected group that by no means represents girls with 'simple' premature thelarche, the prevalence of activating mutations was surprisingly high and it is possible that an even higher prevalence of changes could be found if ovarian tissue or fluid from ovarian cysts were studied. Long-term follow-up will be necessary to determine whether these girls are at risk of developing other features of MAS (e.g., thyrotoxicosis, fibrous dysplasia) with time.

Hypogonadism in a patient with a mutation in the luteinizing hormone β -subunit gene

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Background: Luteinizing hormone (LH) plays a crucial role in post-natal testicular steroidogenesis but human LH- β subunit mutations are rare.

Method: The LH- β subunit gene was sequenced in a 30-year-old man who presented with delayed puberty, hypogonadism and infertility, and who was found to have an absence of circulating LH.

Results: The patient had a homozygous missense mutation in the gene that encodes the LH- β (Gly36Asp). This mutation disrupts a vital cystine knot motif and abrogated the heterodimerization and secretion of LH. Treatment with human chorionic gonadotropin increased circulating testosterone, promoted virilization, and was associated with the appearance of normal spermatozoa in low concentrations.

Conclusion: This case illustrates the important physiological role that LH plays in male sexual maturation and fertility.

It is now over a decade since the first report of a LH β -subunit gene mutation in a man with delayed puberty, hypogonadism/micropenis and elevated LH concentrations [11]. The homozygous Gln54Arg mutation found in that patient did not affect hormone secretion or heterodimerization, but did prevent receptor binding and activation. Thus, high circulating LH concentrations were found. This second report of a LH β -subunit mutation (Gly36Asp) describes a man with delayed puberty, hypogonadism and absent circulating LH, due to a homozygous Gly36Asp change in the cystine knot motif that affects heterodimerization and secretion. This change is similar to the small number of FSH β -subunit changes reported in women with impaired reproduction and low FSH [12]. These cases show that different mutations in different domains of the β -subunit can present with a similar reproductive phenotypes but variable biochemical profiles. This report raises several important points.

Firstly, LH β -subunit changes might be found in a greater spectrum of patients than originally thought. Secondly, placental hCG is sufficient to virilize the developing human male fetus and for testicular descent, whereas fetal LH is necessary for supporting penile growth in later gestation. Thus, this patient presented with micropenis, but not hypospadias. Finally, the appearance of spermatozoa following hCG treatment alone adds to the growing body of evidence that intratesticular testosterone has an important role in facilitating spermatogenesis in humans and mice.

Clinical trials, new treatments

New testosterone buccal system (Striant®) delivers physiological testosterone levels: pharmacokinetics study in hypogonadal men

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Background: Various modes of testosterone delivery are available, but each method has advantages and disadvantages.

Methods: A new mucoadhesive testosterone buccal system (Striant®), 30 mg testosterone (T), was applied twice daily in 82 hypogonadal men for 3 months. Serum T, free T, and 5 α -dihydrotestosterone (DHT) were measured during this period. T pharmacokinetics were determined during a 24-hour sampling at week 12.

Results: Physiological mean serum T concentrations were steady and consistently maintained. Over a 24-hour period, total serum T concentration was above the lower limit of adult male range 80.1% of the time. During treatment, mean serum 5 α -DHT, free T, and estradiol concentrations paralleled serum T. T pharmacokinetics were not significantly affected by age, BMI, food or beverage, gum abnormalities, or medications known to cause dry mouth. Gum-related adverse events occurred in 16.3% of subjects. Except for 3 subjects, the gum adverse effects occurred early during treatment, did not cause interruption of treatment, and resolved rapidly and completely.

Conclusions: The T buccal system is a novel T formulation that offers a safe, effective, and convenient approach for physiological T replacement therapy in hypogonadal men.

Testosterone replacement for hypogonadal men remains a challenge, both in terms of formulation as well as delivery system. Buccal testosterone delivery systems have been developed recently, and launched by several pharmaceutical companies. This study reports the profiling and pharmacokinetic data, as well as patient adverse events and compliance, for a buccal testosterone delivery system in 82 hypogonadal men over 3 months. To our knowledge, no data have been published yet for the use of buccal testosterone to induce puberty in adolescence. However, this system may represent a more physiological and better tolerated approach than depot injections, patches or oral preparations.

Insulin sensitization for girls with precocious pubarche and with risk for polycystic ovary syndrome: effects of prepubertal initiation and postpubertal discontinuation of metformin treatment

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Background: Low birth weight (LBW) girls with precocious pubarche (PP) (i.e. early pubic hair development) are at risk of developing polycystic ovary syndrome (PCOS) (hyperinsulinemic hyperandro-

genism, dyslipidemia, dysadipocytokinemias) and central fat excess. Recent studies have shown a benefit from metformin treatment (and improved insulin sensitization) in preventing the progression from PP to PCOS in formerly LBW girls with PP who were postmenarcheal at the initiation of therapy.

Methods: This study of formerly LBW girls with PP investigated (1) the effects of early insulin sensitization with metformin before puberty, and (2) the effects of discontinuing metformin in girls who had started metformin treatment after menarche. Prepubertal LBW-PP girls ($n = 33$; mean age 8.0 years; body mass index 18.5 kg/m^2) were randomly assigned to remain untreated or to receive metformin (425 mg/day) for 6 months. Postpubertal LBW-PP girls ($n = 24$; age 12.4 years; body mass index 21.0 kg/m^2) had been randomized (at -12 months) to remain untreated or to receive metformin (850 mg/day) for 12 months, at which time (0 month) a treatment crossover was performed for 6 months. Fasting blood glucose and serum insulin, SHBG, dehydroepiandrosterone sulfate, androstenedione, testosterone, lipid profile, IL-6, and adiponectin were assessed at 0 and 6 months, as was body composition (by dual x-ray absorptiometry).

Results: In the prepubertal study (group A), comparisons of untreated vs. treated girls disclosed normalizing effects of metformin on SHBG, androstenedione, dehydroepiandrosterone sulfate, low- and high-density lipoprotein cholesterol, triglycerides, IL-6, adiponectin, total and abdominal fat mass, and lean body mass. In the postpubertal study (group B), treatment crossover at 0 month was in each subgroup followed by a striking reversal in the course of the endocrine-metabolic state, adipocytokinemias, and body composition; all changes pointed to normalizing effects of metformin treatment.

Conclusion: These studies provide the first evidence that (1) prepubertal metformin therapy has normalizing effects on PCOS features in high-risk girls with a combined history of LBW and PP, and (2) in adolescence, metformin's normalizing effects are reversed as soon as metformin therapy is discontinued.

Girls with a history of low-birth weight and precocious pubarche (pubic hair development) are at risk of central obesity, polycystic ovarian syndrome and dyslipidemia. Recently, Ibanez and colleagues have produced a series of studies showing the benefits of metformin treatment for this group during adolescence, either alone or with an anti-androgen. The report cited here shows that early treatment with metformin is effective in reversing adverse indicators (e.g., androgens, central fat) in prepubertal girls with LBW and PP, but also shows that in treated pubertal girls the normalizing benefits of metformin are reversed once it is discontinued. Metformin has been available for many years, is a relatively inexpensive agent and has relatively well-documented side effects (e.g., nausea, gastrointestinal disturbances and, rarely but importantly, lactic acidosis). However, data on the long-term use of metformin in young children are not extensive. This series of studies is encouraging that metformin may be beneficial for this patient group but, if metformin is to be used for long-term treatment, ongoing vigilance will be needed.

Recombinant human leptin in women with hypothalamic amenorrhea

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Background: Low levels of the adipocyte-secreted hormone leptin may result in hypothalamic amenorrhea. Therefore, it was hypothesized that exogenous recombinant leptin replacement to women with hypothalamic amenorrhea would improve reproductive and neuroendocrine function.

Methods: Eight women with hypothalamic amenorrhea due to strenuous exercise or low weight were studied for 1 month before receiving recombinant human leptin and then while receiving treatment for up to 3 months. Six control subjects with hypothalamic amenorrhea received no treatment and were studied for a mean ($\pm$ SD) of 8.5 ± 8.1 months.

Results: Luteinizing hormone (LH) pulsatility, body weight, ovarian variables, and hormone levels did not change significantly over time in the controls and during a 1-month control period before recombinant leptin therapy in the treated subjects. In contrast, recombinant leptin treatment increased mean LH levels and LH pulse frequency after 2 weeks and increased maximal follicular diameter, the number of dominant follicles, ovarian volume, and estradiol levels over a period of 3 months. Three patients

had an ovulatory menstrual cycle ($p < 0.05$ for the comparison with an expected rate of spontaneous ovulation of 10%); 2 others had preovulatory follicular development and withdrawal bleeding during treatment ($p < 0.05$). Recombinant leptin significantly increased levels of free triiodothyronine, free thyroxine, insulin-like growth factor 1, insulin-like growth factor-binding protein 3, bone alkaline phosphatase, and osteocalcin but not cortisol, corticotropin, or urinary N-telopeptide.

Conclusion: Leptin administration for the relative leptin deficiency in women with hypothalamic amenorrhea appears to improve reproductive, thyroid, and growth hormone axes and markers of bone formation, suggesting that leptin, a peripheral signal reflecting the adequacy of energy stores, is required for normal reproductive and neuroendocrine function.

It is becoming clear that leptin plays an important role in facilitating reproductive development as individuals with leptin deficiency fail to enter puberty, but do so once leptin treatment is initiated (see Yearbook 2004–5). However, the exact role of leptin in regulating the onset of puberty, and sex differences in leptin actions, are still actively debated. This study shows leptin administration improves reproductive function in women with hypothalamic amenorrhea and hypoleptinemia due to low body weight or strenuous exercise. Thus, leptin likely forms the link between the reproductive axis and energy stores, preventing pregnancy when insufficient calorie stores are available. Whilst there may be more cost-effective approaches to addressing the consequences of hypothalamic amenorrhea, this study does provide direct evidence for a role of leptin in facilitating reproductive function and also highlights the effects of leptin on other endocrine systems such as bone.

New mechanisms: puberty – it started with a kiss(peptin)

Kisspeptin directly stimulates gonadotropin-releasing hormone release via G-protein-coupled receptor 54

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Background: Recently, the G-protein-coupled receptor (GPR54) has been identified as a potential molecular ‘gatekeeper’ of the hypothalamic-pituitary-gonadal axis, and its requirement for the onset of pulsatile luteinizing hormone (LH) and follicle-stimulating hormone (FSH) secretion at puberty has been demonstrated.

Method: The possible central mode of action of GPR54 and its ligand, kisspeptin, were investigated.

Results: First, GPR54 transcripts are colocalized with gonadotropin-releasing hormone (GnRH) neurons in the mouse hypothalamus, suggesting that kisspeptin, the GPR54 ligand, may act directly on these neurons. Second, GnRH neurons seem anatomically normal in *gpr54*^{−/−} mice, with projections to the median eminence. These findings suggest that the hypogonadism seen in *gpr54*^{−/−} mice is not due to an abnormal migration of GnRH neurons (as in Kallmann syndrome), but more likely due to a lack of GnRH release or absence of GnRH neuron stimulation. Third, intraperitoneally injected kisspeptin injected, which stimulates robust LH and FSH release in wild-type mice, has no effect in *gpr54*^{−/−} mice, suggesting that kisspeptin acts directly and uniquely through GPR54. Finally, central (intracerebroventricular) administration of kisspeptin in sheep produces a dramatic release of GnRH into the cerebrospinal fluid, with a parallel rise in serum LH.

Conclusions: Kisspeptin/GPR54 act directly on the hypothalamo-pituitary-gonadal axis at the level of GnRH release. The localization of kisspeptin/GPR54 and the GnRH-releasing effects of kisspeptin thus define GPR54 as a major control point in the reproductive axis and suggest that kisspeptin is an important new neurohormonal effector.

See also next page:

Characterization of the potent luteinizing hormone releasing activity of KiSS-1 peptide, the natural ligand of GPR54

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Endocrinology 2005;146:156–163

Advanced vaginal opening and precocious activation of the reproductive axis by KiSS-1 peptide, the endogenous ligand of GPR54

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Increased hypothalamic GPR54 signaling: a potential mechanism for initiation of puberty in primates

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The KiSS-1-derived peptide (kisspeptin)/GPR54 system emerged as a potentially important regulator of puberty and reproductive function last year, following the generation of hypogonadal *Gpr54*(–/–) knockout mice and the description of a limited number of patients with hypogonadotropic hypogonadism due to GPR54 mutations (Yearbook, 2004–5) [13–15]. Several studies in the past year have confirmed the importance of the kisspeptin/GPR54 system in pubertal regulation and hypothalamic-gonadotrope function. Messenger et al. demonstrated co-localization of GPR54 with GnRH neurons, suggesting that the kisspeptin/GPR54 system acts by regulating hypothalamic GnRH release – a potential ‘gatekeeper’ that modulates GnRH activity. Intracerebroventricular injection of kisspeptin in sheep increased GnRH release, and produced a subsequent rise in LH. The ability of kisspeptin to release LH and FSH in the rat was confirmed in reports by Navarro et al. Several studies have also addressed the role of kisspeptin/GPR54 in regulating the timing of puberty. For example, Shahab et al. showed that hypothalamic GPR54 transcript expression increases during the peripubertal period in primates, and Navarro et al. reported advanced vaginal opening and precocious activation of the reproductive axis by administration of kisspeptin to immature female rats. Taken together, these data further support the role of kisspeptin/GPR54 as a ‘gatekeeper of puberty’, and it is possible that alterations in this system might be identified in early puberty disorders in humans, as well as in pubertal failure.

The Gs-linked receptor GPR3 maintains meiotic arrest in mammalian oocytes

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Science 2004;306:1947–1950

Background: Mammalian oocytes are held in prophase arrest by an unknown signal from the surrounding somatic cells. The identification of this factor or its signaling pathway will provide critical clues about the mechanism of oocyte meiotic arrest after development of the follicle to the antral stage.

Methods: The receptor was identified through in silico investigation of expressed sequence tag (EST) database derived from a cDNA library of fully grown prophase-arrested mouse oocytes, looking for proteins predicted to be G-protein-coupled receptors (GPCRs) based on their 7-transmembrane structure. Of the 1,000 mammalian genes that encode 7-transmembrane proteins, 15 were found in the oocyte EST database. One of these proteins, the orphan receptor GPR3, was investigated.

Results: The orphan Gs-linked receptor GPR3, which is localized in the oocyte, maintains prophase arrest. Oocytes from Gpr3 knockout mice resume meiosis within antral follicles, independently of an increase in luteinizing hormone, and this phenotype can be reversed by injection of Gpr3 RNA into the oocytes.

Conclusion: It was concluded that the GPR3 receptor is a link in communication between the somatic cells and oocyte of the ovarian follicle and is crucial for the regulation of meiosis.

Whether the somatic cells or oocyte dictate meiotic arrest had been debated. However, the identification and localization of Gpr3 to the oocyte supports the notion that the egg controls meiotic arrest. Further, it is likely that the agonist is derived from the granulosa cells and the discovery of this ligand will add important new clues to the mechanism that is necessary for control of meiotic progression in the postnatal ovary.

New genes: focusing on female development at last

A WNT4 mutation associated with müllerian-duct regression and virilization in a 46,XX woman

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Background: WNT4, a secreted protein that suppresses male sexual differentiation, is thought to repress the biosynthesis of gonadal androgen in female mammals.

Methods: Mutational analysis of WNT4 was undertaken in an 18-year-old woman with primary amenorrhea, an absence of müllerian-derived structures, unilateral renal agenesis, and clinical signs of androgen excess – a phenotype resembling the Mayer-Rokitansky-Kuster-Hauser syndrome, and similar to that of female Wnt4 knockout mice.

Results: A heterozygous loss-of-function mutation in the WNT4 gene was found.

Conclusions: WNT4 appears to play an important role in müllerian-duct formation and control of ovarian steroidogenesis in humans.

Hypergonadotropic ovarian failure associated with an inherited mutation of human bone morphogenetic protein-15 (BMP15) gene

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Background: Hypergonadotropic ovarian failure is a common cause of female infertility. It is a heterogeneous disorder that, in the most severe forms, is a result of ovarian dysgenesis (OD). Most OD cases are associated with major X-chromosome abnormalities, but the pathogenesis of this disorder is still largely undefined in patients with a normal karyotype. Animal models showed the important role in female reproduction played by the product of a gene located at Xp11.2 in humans (BMP15). BMP15 is an oocyte-specific growth/differentiation factor that stimulates folliculogenesis and granulosa cell (GC) growth.

Methods: The authors report 2 sisters with a normal karyotype who are affected with hypergonadotropic ovarian failure due to OD. The familial presentation suggested a genetic origin, and candidate genes were screened for mutations.

Table 1. Important genetic advances relevant to pediatric reproductive endocrinology (listed in order as featured)

Gene	Protein	Phenotype	Reference
<i>FMRP</i>	FMRP	Premature ovarian failure	Welt et al.
<i>NR5A1</i>	SF-1	46,XY gonadal dysgenesis	5–7
<i>CYP11A</i>	P450scc	46,XY sex-reversal Primary adrenal failure Prematurity	Hiort et al.
<i>GNAS1</i>	Gs subunit	Premature thelarche	Roman et al.
<i>LHB</i>	LH β -subunit	Micropenis/hypogonadism Delayed puberty Infertility	Valdes-Socin et al.
<i>GPR54</i>	GPR54	Hypogonadotropic hypogonadism	15
<i>WNT4</i>	WNT-4	Absent uterus Unilateral renal agenesis Androgen excess	Biason-Lauber et al.
<i>BMP15</i>	BMP15	Ovarian dysgenesis	Di Pasquale et al.
<i>DHH</i>	Desert hedgehog	Testicular dysgenesis	16
<i>TSPYL</i>	SIDDT	Sudden-infant death Testicular dysgenesis	17
<i>CHD7</i>	CHD7	CHARGE syndrome	18

Results: A heterozygous non-conservative substitution in the pro region of BMP15 (Y235C) was identified in both sisters but not in 210 control alleles. This mutation was inherited from the father. Mutant BMP15 appears to be processed abnormally, is associated with reduced GC growth, and antagonizes the stimulatory activity of wild-type protein on GC proliferation.

Conclusions: In conclusion, the first natural mutation in human BMP15 is associated with familial OD, indicating that the action of BMP15 is required for the progression of human folliculogenesis. This condition represents an exceptional example of X-linked human disease exclusively affecting heterozygous females who inherited the genetic alteration from the unaffected father. BMP15 defects are involved in the pathogenesis of hypergonadotropic ovarian failure in humans.

This year has seen the focus of molecular genetics drift slowly from the testis to the ovary. Biason-Lauber et al. extrapolated the phenotype of the Wnt4 knockout mouse to humans, and reported a heterozygous WNT4 change in a woman with ovarian failure, absent uterus and evidence of androgenization. Although not all women with Mayer-Rokitansky-Kuster-Hauser syndrome have hyperandrogenization, this finding does support the current interest in the WNT4 signaling system in gonadal transdifferentiation and ovarian versus testicular development. The story of BMP15 and fecundity has led to the sheep farms of New Zealand, mouse knockouts that suggest independent roles for the ligand in the ruminant and sheep and now to Italian siblings who have hypergonadotropic ovarian failure due to ovarian dysgenesis. BMP is a secreted member of the TGF β superfamily of growth and differentiation factors. It is made exclusively by the oocyte and acts on the somatic cells to regulate FSH receptor expression. BMP15 mutations in the sheep result in superfecundity. In contrast, deletion of the gene encoding BMP15 in mice results in perfectly normal ovarian development, follicle development and fertility, likely due to the fact that biologically active BMP15 is not made in mice due to inappropriate processing. In the patients reported here, the proband and her sister acquired a mutation in the prodomain of the ligand which creates an additional cysteine

and, apparently, additional homo-associations within the protein which renders the ligand (or the molecular milieu in which the ligand is absent) dominant negative. Further studies of both WNT4 and BMP15 are likely to shed light on their relevance to human gonadal development and associated disorders in the near future. Other important reports in the field of human gonadal genetics (table 1) include the description of desert hedgehog (DHH) mutations in three 46,XY males with complete gonadal dysgenesis but no evidence of polyneuropathy by Canto et al. [16]. A single DHH mutation has been published previously in a patient with partial gonadal dysgenesis and neurological features. Also, Puffenberger et al. [17] mapped a gene for 'sudden infant death with dysgenesis of the testes syndrome (SIDDT)' to the long arm of chromosome 6 in several large Amish kindred. Homozygous frameshift mutations in the testis-specific Y-like gene, TSPYL, were identified in affected individuals. This protein has unknown function but has a peptide domain with homology to the nucleosome assembly protein family. The prevalence of this condition in non-Amish populations is unknown, but shows the importance of assessing the karyotype at post-mortem in all phenotypic girls with sudden infant death syndrome. Finally, the genetic basis of CHARGE syndrome was elucidated by Vissers et al. More than 50% of patients with this condition have microdeletions or point mutations in CHD7, a member of the chromodomain gene family [18].

New hormones: relaxin-like about testicular function

Insulin-like factor 3 (INSL3) serum levels in 135 normal men and 85 men with testicular disorders: relationship to the LH-testosterone axis

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Background: Insulin-like factor 3 (INSL3) (relaxin-like factor) is a hormone produced and secreted by Leydig cells in the fetal testis, which plays a key role in stimulating the intra-abdominal phase of testicular descent. It is unclear whether serum INSL3 measurement might be a useful marker of Leydig cell integrity in adulthood.

Method: INSL3 serum levels were measured in 135 andrologically well-characterized normal men and 85 patients with testicular disorders using a newly developed fluorescence immunoassay. The relation between INSL3 serum testosterone (T), LH and semen quality was assessed.

Results: Median (2.5–97.5 percentiles) INSL3 serum levels were as follows: Normal men (n = 135): 0.99 (0.55–1.73) ng/ml; infertile men (n = 23): 1.11 (0.60–2.07) ng/ml; anorchid men (n = 21): non-detectable (ND); patients with 47, XXY, Klinefelter syndrome (n = 21): 0.12 (ND–0.78) ng/ml; men with hypogonadotropic hypogonadism and T substitution (n = 11): ND; and men with hypogonadotropic hypogonadism and hCG (human chorionic gonadotropin) treatment (n = 5): 0.36 (0.13–0.73) ng/ml. Before testicular biopsy, 2 infertile men had blood samples drawn directly from vena spermatica. Here the serum INSL3 levels were 15-fold higher than in serum from peripheral blood samples (13.84 and 14.00 ng/ml, respectively). In 2 unilaterally orchiectomized former testis cancer patients, who underwent hCG stimulation test, INSL3 serum levels were unchanged 72 and 96 h post-hCG stimulation.

Conclusion: This study (1) provides a normal range for INSL3 serum levels in adult men and (2) shows that the majority, if not all, circulating INSL3 derives from the testes. Furthermore, the data strongly indicate that (3) INSL3 secretion is dependent on the differentiating effect of LH on Leydig cells, but independent of the steroidogenic LH-mediated action. Thus, even though T and INSL3 are both dependent on LH, these two Leydig cell hormones are regulated differently.

INSL3 (relaxin-like factor) was originally shown to be a key regulator of the intra-abdominal stage of testicular descent – a non-gonadotropin-dependent phenomenon. The development of an assay for human INSL3 may be a useful adjuvant for assessing for Leydig cell integrity, independent of LH/hCG-stimulated steroidogenic capacity.

One tissue, two fates: molecular genetic events that underlie testis versus ovary development

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An outstanding review of recent advances in our understanding of gonad development in the mouse, with obvious applications to humans.

Food for thought

Oxytocin increases trust in humans

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Background: Trust is indispensable in friendship, love, families and organizations, and plays a key role in economic exchange and politics. In the absence of trust among trading partners, market transactions break down. In the absence of trust in a country's institutions and leaders, political legitimacy breaks down. Little is known, however, about the biological basis of trust among humans.

Methods & Results: Intranasal administration of oxytocin causes a substantial increase in trust among humans, thereby greatly increasing the benefits from social interactions. This effect of oxytocin on trust is not due to a general increase in the readiness to bear risks. On the contrary, oxytocin specifically affects an individual's willingness to accept social risks arising through interpersonal interactions.

Conclusion: These results concur with animal research suggesting an essential role for oxytocin as a biological basis of prosocial approach behavior.

The 'forgotten' reproductive hormone finds a new role in Swiss economics – Donald Trump: 'You're (not) fired!'

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