Opening conference

Human mutations and genetically modified mice unravelling the pathophysiology of pituitary-gonadal function. Ilpo Huhtaniemi (Institute of Reproductive and Developmental Biology, Hammersmith Campus, Imperial College London, Du Cane Road, London W12 0NN, UK, and Department of Physiology, University of Turku, Kiinamyllynkatu 10, 20520 Turku, Finland).

Numerous genetically modified mouse models have recently been developed for the study of the pituitary-gonadal function. The first of such models was the *hpg* mouse which was devoid of gonadotropin releasing hormone (GnRH) and gonadotropin secretion due to a spontaneous deletion mutation in the *GnRH* gene. More recently developed models are knockouts for all gonadotropin subunit genes (common alpha, LH beta and FSH beta) and gonadotropin receptors, as well as transgenic mice overexpressing LH or FSH. The knockouts offer good phenocopies of respective mutations observed in humans, and therefore provide good models for the study of molecular pathogenesis of these conditions. In addition, knockouts for several transcription factors crucial for the development of the hypothalamic-pituitary-gonadal axis have been developed, again offering relevant models for the respective human mutations. These models have to a great extent elucidated the normal and pathological hyper- and hypofunctions of gonadotropins and gonadal hormones, and the various para- and autocrine factors modulating their physiological actions. In addition, they have elucidated the potential tumorigenic role of chronically elevated gonadotropin secretion as well as ectopic gonadotropin function. The purpose of this presentation is to review the key observations on the currently available genetically modified mouse models related to normal and pathological functions of the hypothalamic-pituitary-gonadal axis. In particular, we concentrate on the relevance to human pathophysiology of the hCG overexpressing and LH receptor knockout mice developed recently in our laboratory. The studies reviewed here were supported by grants from The Academy of Finland and Wellcome Trust.

Gametogenesis

Oocyte differentiation and maturation. Pascal Mermillod (INRA, Unité de Physiologie de la Reproduction et des Comportements, UMR INRA/CNRS/Université de Tours, 37380 Nouzilly, France).

During fertilisation, each gamete brings half of the DNA complement of the future embryo. In addition, the oocyte brings most of the zygotic cytoplasm. This cytoplasmic complement appears very important for the success of embryo development since its protein and mRNA content will support all embryo requirements during the genome silencing occurring during the first embryonic cleavages. The quality of the oocyte may be defined as the ability of its cytoplasm to support this critical period of early development. This quality is the cumulated result of the long differentiation process occurring during folliculogenesis and of the profound changes in cytoplasmic structure and content occurring during oocyte maturation, just before ovulation. During folliculogenesis, the differentiating oocyte sequentially first acquires the ability to resume and complete meiosis up to metaphase II in early antral follicles and then the ability to sustain early embryo development at later follicular stages. This differentiation occurs as the oocyte is maintained at the germinal vesicle (prophase I) meiotic stage by inhibitory signals originating from the follicular environment. When oocytes from antral follicles are extracted from this environment, they spontaneously resume meiosis. This phenomenon is the basis of in vitro maturation (IVM) and in vitro embryo production (IVP). However, all these oocytes do not reach the fully differentiated status necessary for the success of early embryo development. As a consequence, most oocytes aspirated from growing ovarian follicles will be able to mature and to be fertilised but will not be able to support embryo development up to the blastocyst stage. The kinetics of oocyte differentiation inside growing follicles is not homogeneous. Some oocytes already acquire full competence in early antral follicles whereas others reach this competence at a later stage or never if the follicle turns into atresia. Several parameters like puberty of the female or its genetic background could affect this differentiation kinetics. The basis of these

successive stages of differentiation are not well understood. Some features of morphological evolution of the oocyte are known. However, it is likely that this differentiation requires specific sequences of gene expression. Knock out experiments in mice have allowed the recent identification of several genes that should be expressed in oocytes to allow early embryos to develop. These genes, called "maternal effect genes" are encoding proteins involved in several cellular functions including transcription regulation, chromatin remodelling and DNA repair. However, most of these genes are already expressed at early follicular stages, far before oocyte competence acquisition. Oocyte differentiation culminates during maturation. In parallel to meiotic resumption and progression to the metaphase II stage, several morphological and molecular changes occur. Adequate oocyte maturation depends on both the oocyte intrinsic quality (differentiation stage) and environment (follicle quality or maturation medium). During maturation, gene expression control mainly relies on postranscriptional regulation, as evidenced by the profound changes in polyadenylation patterns of maternal messengers. In conclusion, the developmental potential of a mature oocyte depends both on the differentiation status reached before maturation and on the maturation conditions. In vitro techniques may be developed to mimic the intra follicular oocyte differentiation and to provide an optimised in vitro maturation environment.

The extracellular matrix: a morphological and functional support of folliculogenesis. Danielle Monniaux, Frédérique Le Bellego, Clotilde Huet, Claudine Pisselet, Stéphane Fabre, Agnès Nicolle, Michel Bosc, Philippe Monget (Physiologie de la Reproduction et des Comportements, UMR 6175 INRA/CNRS/Université de Tours/Haras Nationaux, 37380 Nouzilly, France).

From the primordial to the preovulatory follicular stage, the outer layer of granulosa cells lays on a basal lamina containing extracellular matrix (ECM) components that separates them from theca layers and interstitial ovarian tissue. Laminin, fibronectin and collagens are also present within the walls of follicular cells and are synthesised by both granulosa and theca cells. Growth of antral follicles is associated with an increase in levels of type I collagen and laminin in granulosa cell layers in ovine and bovine species, respectively. Moreover, levels of laminin increase strongly in granulosa cell layers of ovine antral follicles during the follicular and the preovulatory phases of the cycle. Furthermore, granulosa cells express integrins that are known to bind collagens ($\alpha_3\beta_1$) and laminin ($\alpha_6\beta_1$, $\alpha_3\beta_1$, $\alpha_V\beta_3$), suggesting that these changes may play a role in the regulation of granulosa cell functions. In vitro experiments have shown that type I collagen and laminin exert opposite effects on both proliferation and steroidogenesis of ovine granulosa cells. Type I collagen contributes to maintaining a low proliferative and a high estrogenic activity of granulosa cells, whereas it inhibits progesterone secretion. On the contrary, laminin exerts clear proliferative and luteinising effects on granulosa cells. Part of these effects of ECM components are mediated by changes in granulosa cell shape. Cell rounding, obtained by culturing granulosa cells on type I collagen gel, or on poly-hema (a factor impairing cell adhesion) coated wells, or by addition of heparin in culture medium, is associated with low survival and proliferation rate, high estradiol and low progesterone secretion. In contrast, laminin, fibronectin or RGD peptides induce cell spreading, enhance survival and proliferation and reduce estradiol secretion by granulosa cells. The addition of a function-blocking antibody raised against the α_6 integrin subunit (GoH3 antibody) to the medium of granulosa cells cultured on laminin specifically impairs cell spreading, decreases proliferation rate, increases apoptosis rate, enhances estradiol secretion, and inhibits progesterone secretion and steroidogenic enzymes P450scc and 3β-HSD expression. This indicates that most effects of laminin are exerted through $\alpha_6\beta_1$ integrin activation. These changes are mimicked to a large extent by the addition of cytochalasin D (an inhibitor of microtubule polymerisation), suggesting a major role of the cytoskeleton in mediating laminin effects. Interestingly, examples of uncoupling between steroidogenesis and cell shape suggest that other signalling pathways are also involved in the action of ECM components on granulosa cells. Firstly, when cultured on type I collagen coated wells, granulosa cells spread but, unexpectedly, maintain high estradiol and low progesterone secretion. Secondly, when granulosa cells are cultured on wells coated with different laminin concentrations,

cell rounding induced by the addition of the GoH3 antibody is not correlated to estradiol or progesterone secretion. Thirdly, inhibition of ERK phosphorylation by the addition of PD 198059 in culture medium of granulosa cells cultured on laminin has no effect on cell spreading but mimicks to a large extent the effects of GoH3 on steroidogenesis and expression of steroidogenic enzymes, suggesting that ERK activation might be involved in laminin signalling. In conclusion, ECM modulates survival, proliferation and differentiation of granulosa cells by both triggering changes in cell shape and cytoskeletal tension, and by inducing ligand specific and cell shape independent changes. Thereby, ECM components participate functionally to the microenvironment of follicular cells.

In vitro maturation of male germ cells.
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During spermatogenesis, diploid spermatogonia divide mitotically several times to provide a population of spermatocytes that proceed through meiosis to give birth to haploid spermatids; these latter undergo, during spermiogenesis, a morphological differentiation leading to spermatozoa. Spermatogenesis takes place in the seminiferous epithelium where the germ cells are in close association with the Sertoli cells. Multiplication, differentiation and survival/apoptosis of germ cells, are finely regulated by hormones, mainly by FSH, acting on Sertoli cells, and LH, through the production of testosterone by Leydig cells, and a multitude of cell-cell interactions involving membrane bound or secreted factors such as growth factors, cytokines and neurotrophins. It is becoming clearer and clearer, that hormones and intratesticular regulatory factors may compensate, at least in part, for the absence of some of them including FSH and androgen or LH receptors. Thus, it is likely that synergism and/or redundancy between regulatory molecules is a characteristic of the spermatogenic process on which depends species survival. Many teams have studied which regulating factors are produced within the testis. However, for many of these factors, their specific action on germ cell differentiation is unknown. This search has been hampered by the lack of long-term culture systems creating the in vitro conditions necessary for male germ cell development. Indeed, such culture systems should be helpful in establishing the role of paracrine factors, either by adding them to the culture medium, or by preventing their expression or blocking their action in vitro with the use of oligonucleotides or specific antibodies. Such studies should be important to identify some of the causes of male infertility. Moreover, in vitro maturation of male germ cells would allow to preserve the potential of reproduction of children treated with chemotherapy or radiotherapy, and could allow to cure some infertility, due to defects in somatic cells of the testis, by culturing germ cells in a suitable environment for their maturation. Moreover, germ cells (spermatogonia) might be used as cellular vectors for transgenesis in domestic animals. Hence several groups, during the last years, have tried to settle culture systems allowing some steps of multiplication/differentiation of mammalian germ cells to occur in vitro in several species: the rat, mouse, pig, bull, and also in the human. However, several parameters should be evaluated in order to ascertain the functionality of these culture systems and the normality of the in vitro differentiated gametes. The mitotic phase of spermatogenesis is a complex series of events involving likely differentially regulated specific factors. The spermatogonial stem cells undergo self-renewal and simultaneously produce more differentiated stages of spermatogonia. In non-primate mammals, the As (single) spermatogonia are considered to be the stem cells of spermatogenesis. Upon division, they produce daughter cells, which either become new single stem cells or remain connected through an intercellular bridge giving A-paired (Apr) spermatogonia. These latter develop further into chains of A-aligned (Aal) spermatogonia. The Aal spermatogonia differentiate into A1 spermatogonia and, after several mitotic divisions into A2, A3, A4 and A intermediate spermatogonia, give birth to B spermatogonia. The B spermatogonia will give rise to preleptotene spermatocytes at the ultimate mitotic division. Hence, the undifferentiated spermatogonia include As, Apr and Aal spermatogonia, whereas the differentiating spermatogonia include the A1, A2, A3, A4, intermediate and B spermatogonia. Many attempts to settle culture systems allowing proliferation of spermatogonial stem cells and differentiation into meiotic spermatocytes have been reported during the last decade. The earlier studies were

based on light microscopy, identification of germ cells, and labelling S phases with ^{3}H-thymidine or Bromo-2'-deoxyuridine (BrdU). Since morphological differences between undifferentiated spermatogonia and A1-A2 spermatogonia are quite small in the rat, it is very difficult to distinguish the undifferentiated spermatogonia from the A1 and A2 types of differentiating spermatogonia, especially in cultures. Hence, in the second generation of experiments, specific markers for the different types of spermatogonia and for somatic cells were used in immunocytochemical experiments, and were often associated to electron microscopy. Moreover, the evaluation of stem cell activity of cultured spermatogonia has been evaluated by transplantation of germ cells in the testes of infertile or nude mice, in the case of heterospecific transplantations. These latter experiments have allowed the identification of specific markers of spermatogonial stem cells. Meiosis is a unique event, which is restricted to germ cells. It includes the pairing and recombination of chromosomes during prophase of meiosis I, and the segregation of homologous chromosomes during anaphase of meiosis I. After the last mitotic division, diploid spermatogonia develop into preleptotene spermatocytes which become 4C cells after premeiotic DNA replication. The first meiotic division then generates haploid secondary spermatocytes, which possess two copies of each gene (2C cells). After the second meiotic division, the secondary spermatocytes generate haploid spermatids, which will differentiate into spermatozoa. Recently a part then the whole meiotic stage of spermatogenesis was reproduced in vitro in different species. The meiotic process was monitored by four criteria: (i) morphological identifications of newly formed spermatids from seeded spermatocytes, by both light and electron microscopy; (ii) determination of the change in ploidy of the cell population seeded with time in culture; (iii) assessment of the ability of germinal cells to transcribe genes expressed during the post meiotic phase; (iv) monitoring the fate of BrdU-labelled preleptotene spermatocytes over the culture period until the identification of BrdU-labelled round spermatids. A recent study compared some features of the meiotic process, which develops in the testis of pubertal rats, in vivo and in vitro, paying special attention to the time-course of the phenomenon. The differentiation of spermatocytes was assessed in the testes of 20- to 46-day-old rats and in tubule segments of 20- or 28-day-old rats cultured over a 4-week period. Very similar results were obtained in vivo and in vitro, during the first week of culture, when considering the changes in the cell populations of different ploidy, the gene expression of germ cells, the kinetics of differentiation of BrdU-labelled spermatocytes and the levels of apoptosis in the different cell populations. However, during the second week of culture, the decrease in the proportion of the 4C cell population was not associated with an increase in the 1C cell population as large as in vivo. This result could be explained partly by a high proportion of apoptotic 1C cells beyond one week of culture. Concomitantly, the rate of in vitro differentiation of BrdU-labelled spermatocytes slowed down when reaching the stage of middle pachytene spermatocytes and BrdU-labelled round spermatids were observed 6–11 days later than when BrdU-labelled spermatocytes differentiated in vivo. These results therefore identified a bottleneck for the development of the rat meiotic cells in vivo at the transition from middle to late pachytene spermatocytes. These experiments also allowed to make a rough comparison between the efficiency of the meiosis event, which occurs in vivo, and in cultured seminiferous tubules from 20-day-old rats. Indeed, by comparing the number of round spermatids obtained to the number of leptotene spermatocytes seeded, it could be estimated that one leptotene spermatocyte gave birth to about 0.2 round spermatid which is 4–6 fold lower than in vivo at 35 days. It has to be noted that in most instances, however, many abnormalities of in vitro formed round spermatids were observed. The include incomplete or delayed nuclear condensation, cytoplasmic elongation, acrosome formation or development of several flagella. Nevertheless, it has been reported very recently that round spermatids developed in vitro from spermatocytes can produce normal mice when injected into mature oocytes, but with a rather low yield (see below). Currently, there are very few recent data on in vitro spermiogenesis in animal models. In vitro formed round spermatids can generate one to several flagella, all emerging from the same cellular pole. Moreover, in some instances, actively propagating bending waves have been reported. However, it is important to emphasise that the presence of flagellated cells in germ cell Sertoli cell coculture systems does not ascertain that these cells are spermatids, since any cell with a centriole-derived basal body is able to generate an axoneme. The composition of the intratubular fluid in which are released mature

spermatozoa is very different from that of the extracellular medium. Hence, it is likely that performing the whole spermiogenic process in vitro will need a specific culture medium different from those used for germ cell multiplication and meiosis. There is increasing evidence that in vitro culture of preimplantation embryos can be associated with aberrant growth and phenotypic abnormalities during fetal and postnatal development. Among the several hypotheses proposed to explain these results, is that in vitro culture leads to aberrant epigenetic modifications in the genome. Genome imprinting results in non-equivalent expression of the paternal and maternal alleles of certain genes. The nature of the imprint that marks the parental alleles is not fully understood. However, it must be capable of being erased and then reset sometimes during gametogenesis, so that oocytes carry the maternal imprint and spermatozoa carry the paternal imprint. It has been shown that the expression of imprinted genes in mouse embryos obtained by round spermatid injection into oocytes do not differ from controls. This strongly suggests that paternal genes are correctly imprinted by the round spermatid stage. However, as mentioned above, the ability of in vitro formed round spermatids to give birth to live pups is rather low. Thus it appears important now to compare the profile of gene expression and the imprinting status of in vitro and in vivo differentiated gametes. In conclusion, considerable advances for achieving spermatogenesis in vitro have been made during the last decade. An intriguing feature is that it appears that the culture conditions may vary greatly between species and even between different genetic backgrounds in the same species. Hence it appears necessary to identify specific factors which govern the balance between germ cell survival, progression and attrition in the different species to achieve more efficient and normal spermatogenesis in vitro.

The bank vole: a good model to study the role of estrogens in spermatogenesis. Barbara Biliska (Laboratory of Endocrinology and Tissue Culture, Department of Animal Physiology, Institute of Zoology, Jagiellonian University, Kraków, Poland).

In seasonal breeders, terminal differentiation of the gonads can be retarded or even stopped by changes in the photoperiod. Earlier observations have shown that season and length of the light cycle regulate changes in hormonal activity, affecting reproduction effectiveness and fertility parameters of the sperm. Thus, it is suggested that photoperiod is one of the most important factors inducing changes in reproductive activity. Moreover, the reproductive cycle of seasonally breeding rodents, such as the bank vole can be easily mimicked under laboratory conditions by subjecting the animals to different light cycle regimes. The voles behave similarly to wild animals and show different phases of reproduction, active or regressive, that reflect spring or autumn photoperiods. Thus, bank voles can serve as a useful model for studies on male reproduction since the recrudescence of spermatogenesis is physiologically regulated. Based on recent literature postulating the role of estrogens in the male genital tract, efforts have been made to show the cellular site of aromatisation in the bank vole testis by means of several techniques. These include studies on aromatase immunoexpression at the light and electron microscopic levels, the presence of aromatase, and its enzymatic activity. Additionally, to show the necessity of estrogens in germ cell differentiation, the effects of 17β-estradiol and a pure anti-estrogen, ICI 182,780 on immature vole testes were investigated. All experiments were performed on animals that were kept either in long or short light cycles. Irrespectively of the photoperiod in the testis of mature voles aromatase was found in Leydig, Sertoli, and germ cells, mainly in spermatocytes and spermatids, whereas keeping immature voles under long light cycles induced aromatase expression in spermatogonia and spermatocytes. The technique with colloidal gold allowed for the demonstration of the gold particles distributed over the cytoplasm of elongating spermatids and residual bodies. Western blot analysis revealed the presence of a protein about 52 kDa in size within microsomal preparations of the whole testes or isolated seminiferous tubules. The activity of functional P450 aromatase was confirmed by the measurement of tritiated water release after incubation of microsomal preparations with labelled androstendione. All the studied parameters revealed a close correlation with the length of the photoperiod being distinctly higher in bank voles kept under long day conditions when compared with those of short ones. Exposure to the low dose of estradiol caused acceleration of the onset of spermatogenesis in the testes of immature bank voles, whereas treatment with a high dose of estradiol or ICI 182,780

induced defects in testicular structure associated with increased apoptosis of germ cells and impaired reproductive function. Such apparent alterations seen after a 2-week-period of estradiol or ICI treatment could be explained by a great sensitivity of bank voles to overexposure or deprivation of estrogens. In conclusion, in bank voles, not only somatic cells but also germ cells produce estrogens, representing an additional source of estrogens, and also possess estrogen receptors, as described in other rodents. It can also be suggested that induction of spermatogenesis is up-regulated by low doses of estrogens. Therefore, the bank vole testis as an androgen- and estrogen-dependent tissue provides a good experimental system for the study of the involvement of estrogens in the regulation of testicular function and especially the development of spermatogenesis. The author thanks Dr M. Kotula-Balak, B. Frczek, M. Gancarczyk, A. Hejmej, and Dr S. Carreau, S. Bourguiba (UPRES EA 2608 USC-INRA, University-IBFA, Caen, France) who have contributed to the data presented. Supported in part by a Solicited Project PBZ-KBN-084/PO6/2002.

Regulation of reproduction

Regulation of aromatase expression by orphan nuclear receptors in male gonads. Vincenzo Pezzi (Department of Pharmaco-Biology, University of Calabria, Rende, Italy).

Estrogens play a critical role in regulating testicular function and development. Target disruption of the genes encoding the Estrogen Receptor β (ERβ) or the cytochrome P450 aromatase (the enzyme catalysing the synthesis of estrogens from androgens) indicates that estrogen is essential for normal male fertility. On the contrary, estrogen excess stimulates Leydig cell hyperplasia in rodents and has been associated with cryptorchidism, testicular cancer, and impaired spermatogenesis. In rats according to age there is a shift in the aromatase activity which is mainly expressed in Sertoli cells in immature animals then in Leydig cells in adults. Aromatase expression in the testis is driven principally through the use of the promoter II, and requires the presence of a nuclear receptor half-site that binds the orphan receptor SF-1 (NR5A1) to mediate basal transcription and, in part, cAMP-induced transcription. We hypothesised that LRH-1 (NR5A2), a receptor closely related to SF-1, could also play a role in regulating aromatase expression in the testis. We demonstrated the expression of LRH-1 in the adult rat and immature mouse Leydig cells (LHR-1 > SF-1) as well as in pachytene spermatocytes and round spermatids, but not in Sertoli cells, which in contrast express high levels of SF-1. In transient transfection assays using mouse TM3 (Leydig) and TM4 (Sertoli) cell lines, a rat promoter II luciferase reporter construct was stimulated by co-transfection of increasing concentrations of either LRH-1 or SF-1 expression vectors. Transfected into TM3 cells, in the absence of stimulation, LRH-1 dose-dependently increased promoter II activity reaching a maximum of 8-fold over basal at 0.05 µg plasmid. Treatment for 24 h with adenylyl cyclase activator forskolin (FSK) increased basal promoter activity 3-fold; however in the presence of this agent LRH-1 strongly induced PII activity reaching a maximum of 20-fold. Analogous transfection experiments using SF-1 instead of LRH-1 revealed a similar pattern, although the maximum levels of induction were lower. Interestingly, in TM4 cells in basal conditions neither LRH-1 nor SF-1 increased luciferase activity. However, once activated by forskolin, both LRH-1 and SF-1 further increased luciferase activity. Mutation analysis showed that induction by LRH-1 in TM3 and TM4 cells requires an AGGTCA motif at position –90, to which LRH-1 bound in gel shift analysis. Primary Leydig cell nuclear extracts produced a protein/DNA complex of mobility similar to that formed with TM3 cell extracts or in vitro translated LRH-1, whereas primary Sertoli cell nuclear extracts produced a complex of mobility similar to that formed with TM4 extracts. The LRH-1 antibody supershifted the binding activity from both TM3 and primary rat Leydig cell extracts, but not from TM4 or primary rat Sertoli cells. We also investigated the role of two other orphan receptors DAX-1 (NR0B1) and the short heterodimer protein (SHP; NR0B2), lacking DNA binding domains but exhibiting the capacity to bind other nuclear receptors, repressing their transcriptional activities. In this study, we investigated their role in regulating SF-1- and LRH-1- induced aromatase transcription. Co-transfection with SHP inhibited in a dose dependent manner the aromatase promoter II induction by LRH-1 in basal condition as well as after FSK stimulation. In contrast, SHP did not inhibit SF-1 induction

of this promoter. This mechanism is different from DAX-1 which is able to block in a dose dependent manner the induction of promoter activity induced by either SF-1 or LRH-1. This last result could explain the mechanism at the base of the aromatase overexpression observed in testis of DAX-1 knockout mice. Our data suggest that not only SF-1 and DAX-1, but also transcription factors which are considered mainly as regulators of bile acid biosynthesis such as LRH-1 and SHP, may play an important role in the regulating estrogen production in the testis. Moreover, further investigation on the role of these orphan nuclear receptors on aromatase expression could be useful in elucidating the mechanisms involved in the regulation of age-related aromatase expression in testicular cells which may influence testis development, spermatogenesis and testicular carcinogenesis.

Oestrogen receptors in male human and primate reproduction. Jayne E Sierens, Shiela Macpherson, Michael Millar, Julie Wilson, Frances Collins, Philippa TK Saunders (MRC Human Reproductive Sciences Unit, 49 Little France Crescent, Edinburgh, EH16 4SB, UK).

Oestrogens have a major impact on reproductive function in both males and females. In mammals, two subtypes of the oestrogen receptor known as ERα (*NR3A1*) and ERß (*NR3A2*), have been identified and shown to be the products of different genes. These receptors are expressed in the nuclei of target cells where they are reported to exist as homo- or heterodimers. Splice variant isoforms of the ERß gene have been identified in humans (Moore et al., 1998) and rodents and it has been suggested they can influence oestrogen responsiveness (Ogawa et al., 1998). We examined the expression of full length ERs (ERα, ERß1) and the ERß2 variant (Ogawa et al., 1998) in human testicular tissues using immunohistochemistry. We cloned ERß cDNA from primate male tissues in order to determine whether alternatively spliced isoforms exist in these species. We performed transfections using plasmid constructs encoding ERs tagged with fluorescent proteins in order to visualise the formation of homo- and hetero-dimers, and the ability of the receptors to activate an ERE-containing reporter construct. Immunohistochemical analysis revealed that the pattern of expression of ERα and ERß in human and primate testicular tissues was receptor dependent; cells containing one, or both receptors were identified (Saunders et al., 2000; 2001; Critchley et al., 2001). The ERß2 variant protein was expressed in multiple human cell types (Critchley et al., 2002; Saunders et al., 2002; Gaskell et al., 2003). We cloned full length ERß cDNA (ERß1) from stump-tailed macaque (*Macaca artoides*) an Old World primate, and the common marmoset (*Callithrix jacchus jacchus*), a New World primate. We demonstrated for the first time that splice variant mRNA homologous to hERß2 are formed in both species (Sierens et al., 2004); this splice variant isoform is distinct from those found in rodents. In transient transfection assays ERß2-containing constructs were unable to induce transcription of an ERE reporter plasmid in the presence of oestradiol. ERß1 from humans, macaques and marmosets exhibited minor differences in their ability to induce transcription of the ERE reporter when incubated with different natural and synthetic oestrogenic ligands and this may be due to amino acid substitutions within their ligand binding domains. Confocal and FRET analysis confirmed that hERα, hERß1 and hERß2 are able to form heterodimers with each other and the impact of ERß2 on the ability of ERα or ERß1 to induce expression of the ERE reporter gene in the presence of oestradiol was host cell dependent. In conclusion, the impact of endogenous and exogenous oestrogens on reproductive function depends on cell and tissue specific patterns of expression of ERs and ER variant(s). Our data indicate that the marmoset monkey provides a suitable animal model in which to investigate the impact of ERß variant expression on tissue responsiveness to oestrogens.

Rapid responses to estrogens. Michaela Luconi, Elisabetta Baldi, Gianni Forti (Department Clinical Physiopathology, Andrology Unit, University of Florence, Florence, Italy).

Although they have always been regarded as female reproductive hormones, a pivotal role in the regulation of male sexuality and reproduction is now emerging for estrogens (ES). ES have been described to act primarily through classical genomic mechanisms by binding to their specific cytosolic/nuclear receptors (ERα and ERβ)

finally leading to regulation of target gene expression. However, these steroids also exert rapid effects (non genomic) mediated by a membrane mechanism which can involve either the nuclear receptors somehow associated to the plasma membrane or totally different membrane receptors. These actions are too rapid to be ascribed to the activation of gene expression and can also be mimicked by steroidal molecules impermeable to the membrane and do not require protein synthesis. In this scenario, spermatozoa represent a useful model for studying the rapid action of ES as well as for the characterisation of their membrane receptors, since in these cells there are no interfering effects due to the genomic receptors. Indeed, sperm DNA is highly packed and not accessible for transcription and these cells lack the apparatus for protein synthesis. However, the expression of classical ER in mature spermatozoa is still a matter of debate (Makinen et al., 2001; Aquila et al., 2004). Moreover, the presence of aromatase in human spermatozoa (Carreau et al., 2002; Aquila et al., 2002) and the presence of high levels of ES not only in the female but also in the male reproductive tract (Labard et al., 2003), suggest that these cells can respond to ES and actively synthesise them from testosterone during their "journey" to the site of fertilisation. Our group has recently characterised a novel functional ER localised on the human sperm plasma membrane which mediate rapid effects of ES in these cells (Luconi et al., 1999 and 2001). By Western blot analysis using an antibody directed against the conserved ligand binding domain of the genomic ER (H222) and ligand blot technique using peroxidase-conjugated estradiol, we detected in sperm total lysates and in purified membranes a protein of about 29 kDa molecular weight capable of ES binding, whereas nongenomic ER were not observed. On the contrary, a different antibody against the DNA-binding domain of the ER (H226) could not reveal any band in spermatozoa, compared to the classical ER detected in the uterus, suggesting the presence on the human sperm surface of a novel ER of 29 kDa conserving the hormone binding domain only. In human spermatozoa, physiological concentrations of 17βestradiol (17βE_2), exert rapid (seconds up to minutes) non genomic effects, such as the stimulation of tyrosine phosphorylation of sperm proteins including extracellular-signal regulated kinases (ERKs) and a rapid and sustained increase in intracellular calcium levels (Luconi et al., 1999; Baldi et al., 2000). All these rapid events finally result in a significant interference with the well known nongenomic effects exerted by progesterone (P) (Baldi et al., 1998) both on calcium response and acrosome reaction (AR) (Luconi et al., 1999; Baldi et al., 2000). Indeed, priming of spermatozoa with 17βE_2 results in a significant inhibition of the biphasic calcium response induced by the subsequent addition of P, in particular on the plateau phase of the calcium wave which is regulated by tyrosine kinase and is essential for AR. In fact, also P-stimulated AR is blunted following pre-exposure to 17βE_2 whereas 17βE_2 alone has no effect on the spontaneous process. Interestingly, when added together, the two steroids induce a calcium wave even higher than the one stimulated by the single molecules. Similar effects were observed with the impermeant ES analogue E_2-BSA, further indicating the involvement of a membrane ER. Moreover, the inhibitory effect of sperm preincubation with H222, but not H226, antibody on intracellular calcium increase stimulated by 17βE_2 and on its inhibition of P response, strongly suggests that the 29 kDa membrane protein detected by ligand and Western blot analyses mediates rapid effects of ES in human spermatozoa. Altogether, our findings suggest a potential physiological role for ES in epididymal sperm maturation in the male and in restraining P activation of spermatozoa in the female genital tract allowing appropriate timing of capacitation and AR at the site of fertilisation.

Nutritional signals in reproduction. Jacques Bringer (Service des Maladies Endocriniennes, Hôpital Lapeyronie, 34295 Montpellier Cedex 5, France).

The achievement of puberty, ovarian folliculogenesis and the whole reproductive process depend upon energy balance, weight, body composition, fat distribution, food intake and eating attitudes. Many candidates have been involved as links between nutritional status and reproductive disorders: (1) alteration in metabolic fuels, mainly fatty acids and glucose; (2) changes in nutritional signals such as insulin, insulin growth factors (IGF) and binding proteins (IGF-BP), leptin, all being able to modulate gonadotrophic secretion and action; (3) variations in hypothalamic neuropeptides regulated by peripheral signals. What predominant metabolic, hormonal or

peptidic factors interfere with the reproductive axis has not been fully determined. Among them, leptin may be an important player of ovarian physiology and pathophysiology, as clearly shown in the rodent model and, more controversially, in primates and humans. Assertion of leptin's specific role in ovarian function is not validated whereas conflicting results pointed out that variations in leptin levels and effects are intimately tied to insulin, IGF and other metabolic and reproductive effectors. In support of the role of leptin in reproduction is the finding of the location of leptin receptors both in the hypothalamus and in follicles where there is evidence of the expression of leptin nRNA in human ovaries. The levels of food intake and body fat stores can be transmitted via leptin to the GnRH neurons, in part by modulation of neuropeptide Y. The demonstration that a high ratio of leptin to body mass index (BMI) appears to be a better predictor of the failure of ovarian stimulation than BMI or waist-to-hip ratio (being independent of insulin sensitivity) reenforces the potential role of leptin on woman's fertility. In contrast, the observation of a spontaneous ovulatory cycle and pregnancy in insulin-treated woman with lipoatrophic diabetes and the lack of leptin point out a limited permissive role instead of an indispensable one. The real contribution of leptin among other peptidic signals remains controversial notably because insulin and leptin have changes in similar ways in various pathophysiological conditions such as the reduction or increase of food intake and weight. Effective nutritional and drug approaches that act on insulin sensitivity to improve fertility may modify levels and effects of leptin as well. Therefore, in the tight leptin-insulin couple, clarification of a specific and dominant role for one of these two peptides continues to be challenged in humans.

Fetal and neonatal testis development. René Habert (Unité de Gamétogenèse et Génotoxicité, INSERM U566/CEA/Université Paris 7, Fontenay-aux-Roses, France).

The fetal testis originates from a proliferation of the mesonephrons and the coelomic epithelia which is colonised by the primordial germ cells. In the fetal testis, the development and functions of the three main cell types (Leydig, Sertoli and germ cells) do not depend on gonadotrophins. Numerous intra and extratesticular factors are candidates to control its development and functions. Furthermore, physical and chemical environmental perturbators can alter normal development of the testis during fetal or neonatal life. In order to study the potential involvement of these factors and perturbators, we developed an organotypic culture system. In the absence of any growth factors or hormone, this system allowed the development of the 3 main cell types which mimics that which is observed in vivo. The effects of different candidates (GnRH, FSH, TGFß, IGF-I, AMH, retinoic acid, estrogens) were tested in this system. Whether some of the effects observed in vitro have a physiological relevance was evaluated using appropriate transgenic mice. We conclude that the fetal testis cannot be considered as an adult mini-testis since it has a specific physiology which largely differs from that of the immature or adult testis.

In utero exposure to anti-androgens and fetal programming of male infertility. Mohamed Benahmed, Aline Bozec, Asma Omezzine, Anne Florin, Magali Maire, Bénazir Siddek, Claire Friry, Franck Chuzel, Rémi Bars, Claire Mauduit (Unité INSERM U407, Faculté de Médecine Lyon-Sud, BP12, 69921 Oullins Cedex, France).

Reports have indicated a decline in human male reproductive health during the last decades: high and probably increasing prevalence of cryptorchidism and hypospadias, low and probably decreasing semen quality, a rising incidence of testicular cancer and a growing demand for assisted reproduction. It has been suggested that these adverse trends in male reproductive health in humans may be symptoms of an underlying entity, the testicular dysgenesis syndrome (TDS). The rates of the different symptoms are rising synchronously at a speed that strongly suggests that environmental factors are operating. A current hypothesis is that TDS may be caused by fetal exposure to endocrine disruptors (ED, estrogen-like, anti-androgens). However, the molecular and cellular mechanisms involved in the action of the ED remain to be investigated. By using, as an experimental model, rats exposed in utero to an anti-androgen ED (the prototype molecule: flutamide), we studied the molecular mechanisms of action on testicular cells. Adult rats exposed in utero to increasing doses of flutamide (0, 0.4, 2, 10 $mg \cdot kg^{-1} \cdot d^{-1}$) displayed a

chronic apoptosis in the testicular germ cells (particularly pachytene spermatocytes and round spermatids) but not in somatic cells (particularly Sertoli cells). This long-term apoptosis could explain the hypospermatogenesis observed at higher dose (> 25 mg·kg^{-1}·d^{-1}) of the anti-androgen reported by other laboratories. Moreover, we showed that the persistent apoptotic process in testicular germ cells is related to a modification of the equilibrium of the death effector proteins in the testicular germ cells. Indeed, we observed chronic upregulation and activation of the effector caspases-3 and -6 associated with a long-term decrease of the expression of the inhibitor of apoptosis (IAPs) cIAP1 and cIAP2 in the testicular germ cells. The chronic activation of effector caspases is probably related to an activation of the mitochondrial pathway. Indeed, the Bax/Bclw ratio in the testicular germ cells is increased in the testis of adult rats exposed in utero to flutamide. By contrast, the Bak/Bclw ratio was not affected. Since Bak is mainly expressed in Sertoli cells, such observations could explain that adult Sertoli cell number was not affected in the in utero flutamide model. Finally, while flutamide effects are irreversible and chronic if the exposure occurred in utero, they are transient if rats are exposed during adulthood suggesting that the expression of apoptotic genes in the testis is related to an androgeno-dependant fetal programming process. Indeed, in utero endocrine disruption may affect the epigenetic control of the androgen target genes specifically through changes in the methylation pattern of their promoters. In conclusion, the hypospermatogenesis observed in adult male rats exposed in utero to flutamide may be related to a chronic activation of effector caspases in the testicular germ cells. Several genes encoding apoptotic proteins might be, in the testis, under epigenetic control exerted by androgens during the fetal period.

Physiopathology of reproduction

Spermatozoa chromosome abnormalities and hypospermatogenesis. Nathalie Rives (Laboratoire de Biologie de la Reproduction/CECOS/CHU Charles Nicolle, Rouen, France).

Chromosome pairing during male meiosis is a major event for the normal segregation of chromosomes during anaphase I and the normal process of spermatogenesis. Meiotic disturbances induce activation of checkpoints in spermatocytes I responsible for apoptotic death of these germ cells. Meiotic errors are observed at different stages of the meiotic process: (i) abnormal pairing or synapsis, (ii) abnormal recombinations, (iii) disturbances during chromosome migration and orientation on metaphases I and II equatorial plates. Chromosome non disjunctions induced by these meiotic errors are responsible for aneuploid spermatozoa production. Assessment of spermatozoa chromosome aneuploidy is an indirect tool to explore male meiotic non disjunctions. Initially sperm nuclei karyotypes were performed using the human sperm – hamster oocyte fusion assay. This technique was extremely difficult to carry out with a poor productivity and an uneasy interpretation of the karyotypes. More recently, fluorescence in situ hybridisation (FISH) was used to screen a large number of human spermatozoa. The aim of such analysis was to identify the male population at increased risk of aneuploidy in spermatozoa and consequently of major risk of aneuploidy for their progeny. Spermatozoa resulting from an apparently normal meiotic process have low rates of aneuploidy (0.24% per autosome, 0.46% for sex chromosomes). Several factors are susceptible to interfere with the normal meiotic process and to induce an increase of chromosome meiotic non disjunctions (tobacco, professional toxic exposition, anticancer treatment, …). Furthermore, meiotic studies performed several years ago in infertile males with sperm alterations suggested an increase of meiotic pairing abnormalities in this population. Spermatozoa chromosome constitution from oligoasthenoteratozoospermic patients with a normal constitutional karyotype revealed an increased rate of aneuploid spermatozoa. The incidence of spermatozoa chromosome abnormalities is negatively correlated to the sperm count and a low sperm count should be considered as one of the strong predictive factors for spermatozoa aneuploidy. Furthermore, in the situation of non obstructive azoospermia histologically classified hypospermatogenesis, a significant rate of aneuploidy has been observed in spermatids and spermatozoa. Abnormal chromosome pairing as well as excess of spermatocyte I elimination via apotosis have also been reported in this group. A decreased frequency of meiotic chromosome pairing may activate the cell cycle checkpoint responsible for meiotic arrest and/or may also

induce meiotic non disjunctions. Meiotic arrest in some germ cells leads to spermatogenesis impairment, and non disjunctions to post-meiotic cell aneuploidy. Furthermore, teratozoospermia should also be considered as a predictive factor for post meiotic cell aneuploidy especially when teratozoospermia is specific and concerns spermatozoa head and tails. Identification of novel markers predictive for spermatozoa aneuploidy is currently necessary.

The molecular basis of cryptorchidism. Richard Ivell (School of Molecular and Biomedical Science, The University of Adelaide, SA 5005, Australia).

The failure of the testis to descend into the scrotum at birth is the most common congenital ailment affecting newborn boys with an average incidence of between 1 and 4 percent. Normally the testes descend at about the 29th week of pregnancy, going through a two-stage process first involving a transition from a perirenal to a lower abdominal position, and then a second stage through the inguinal canal. The transabdominal phase involves the differentiation and thickening of the gubernacular ligament, which anchors the gonad into the inguinal region. Recent studies using knockout mice have shown that the peptide hormone insulin-like factor 3 (INSL3) and its recently discovered cognate receptor LGR8 are responsible for this essential change in the gubernaculum, at least in rodents. Very little is still known about the role of this hormone system in humans, nor about the relative importance of the developing HPG axis in the aetiology of this phenotype. All evidence to date points to a multifactorial origin of cryptorchidism in humans. Similarly, very little is known about the regulation of this hormonal system and its possible disruption by exogenous factors. The recent development of new assay systems, together with our knowledge of testicular descent in animal models will greatly help our understanding of this very common ailment, which nevertheless can have severe consequences in adult life. Failure of the testes to descend (cryptorchidism), even if corrected, is associated with later infertility and testicular cancer.

Estrogen regulates human seminoma cell proliferation. Adil Bouskine[a], Baharia Mograbi[a], Sophie Lambard[b], Marielle Nebout[a], Cyril Roger[a], Serge Carreau[b], Patrick Fénichel[a] ([a] INSERM EMI 00-09 – IFR 50, Faculté de Médecine, Nice, France; [b] EA 2608-USC INRA, Université de Caen, France).

Is testicular cancer estrogen-dependent? We first verified by RT-PCR, aromatase gene expression, in 5 human seminomas (the most frequent testicular cancer) and in a pure human seminoma cell line, and identified by Western blot the estrogen receptor beta (ERβ) but not ERα. The effects of estrogens (E2, E2-BSA, 10^{-6} M to 10^{-10} M) on seminoman cell proliferation were then studied by cell counting during six days using steroid free culture medium as the control. E2-BSA was able to increase cell proliferation dramatically and rapidly (D1), reaching +40% over control at D3 but normalised at D5. This positive effect was not inhibited but partially reproduced by ICI 182780 alone, a classical ER antagonist. This proliferative effect was associated with a rapid activation (5 to 15 min) of ERK1/ERK2 observed on Western blot and prevented by PD 98059 an inhibitor of the MAP kinase pathway. E2 induced a significant decrease of cell proliferation beginning at D2 and still present at D6 (–25%), which was completely abolished by ICI 182370. Human seminoma cells are able to transform androgens into estrogens and to respond to estrogens through at least two different pathways: (1) a rapid proliferative, non genomic membraneous effect which involves the MAP kinase pathway but not ERβ (not influenced by ICI 182370), and (2) a long lasting inhibitory effect which may involve a genomic effect through ERβ (antagonised by ICI 182370). These two different effects of estrogens on human seminoma cells support the hypothesis on the role of the testicular estrogen/androgen inbalance in male physiological and pathological germ cell proliferation.

Induction of ovulation in women with clomiphene-resistant polycystic ovary syndrome (PCOS). Slawomir Wolczynski (Department of Gynecological Endocrinology, Bialystok, Poland).

PCOS is the most common endocrine disorder affecting 5–8% of women of the reproductive age. According to the 2003 Rotterdam consensus, PCOS is characterised by ovarian dysfunction with clinical or biochemical evidence of hyperandrogenism and polycystic ovary morphology in ultrasonography. Anovulation affects 80% women with PCOS. In all PCOS women, ovulation can be induced by increasing serum concentration of FSH. The first step in management in obese women is reducing body mass index by diet and exercise, but it effects ovulation in some cases. Usually, ovulation is induced by the first line therapy clomiphene citrate. However, 20–40% women are resistant to clomiphene and do not ovulate and in addition, the cumulative pregnancy rate (30–40%) is much lower that the ovulation rate. This discrepancy seems to be related to the antiestrogenic activity of clomiphene which includes long lasting estrogen receptor depletion. Induction of ovulation in women with clomiphene – resistant PCOS presents a therapeutic dilemma. Recently, the insulin sensitisers (metformin, rosiglitasone) have been proposed for inducing ovulation after unsuccessful treatment with clomiphene. In a systematic review, supply of metformin only restores regular menses and induces monoovulation. However, there were no data supporting the increase of pregnancy rate. Metformin is effective as a co- inductor with clomiphene and gonadotrophins. From our own observation three months of treatment with metformin before clomiphene reinduction gives about a 15% pregnancy rate. Oral administration of aromatase inhibitor, letrozole is an interesting option for induction of ovulation especially in the group of patients with strong antiestrogen effects on the endometrium thickness after therapy with clomiphene. Letrozole enables to avoid the antiestrogenic effects on the endometrium. Stimulation of ovulation with gonadotrophins is the next possibility for women with clomiphene- resistant polycystic ovary syndrome. Almost all PCOS patients are highly sensitive to gonadotrophins. The conventional doses of hMG were associated with a multiple pregnancy rate up to 25%, ovarian hyperstimulation syndrome (OHSS) and a high rate of miscarriage. New recommendation for stimulation, with recombinant gonadotrophin-administration at low doses, with careful titration up is safe and highly effective in producing high rates of monofolicular ovulation and a high rate of pregnancy. In our own group of 146 clomiphene-resistant PCOS patients, the ovulation rate was 86%, cumulative pregnancy rate 64% for 4 months, twin rate 12%, triplet rate 0.5% miscarriage rate 9%, one patient had mild OHSS. Starting doses of rFSH were 37.5 or 50 $IU \cdot d^{-1}$. In most patients ovulation was induced by FSH dose maximum 75 $IU \cdot d^{-1}$. Some patients required higher doses of FSH up to 225 $IU \cdot d^{-1}$. The main problem of this method is the prediction of individual FSH thresholds and the limitation by the high cost of the treatment. Laparoscopic ovary cauterisation may also be used in this group of patients. Good results of laparoscopic ovarian surgery could be an argument to recommend this method as the second line treatment for anovulation in PCOS. The potential complications are adhesion formation and premature ovarian failure. The pregnancy rate after laparoscopic procedures is 20–88%. For women who ovulate after gonadotropin treatment, but who do not become pregnant, the IVF-ET programme is the next step for achievement of pregnancy. The long protocol with GnRH analogs and gonadotrophins gives the same pregnancy rate as in other indications for the IVF-ET programme. The high rate of OHSS in the group of PCOS patients in the IVF-ET programme is still a severe problem. The recent advances in human reproduction have become a chance for much better results in the management of pregnancy in women with clomiphene-resistant PCOS.

Physiopathological role of estrogens in man.
Yves Reznik (Endocrinologie, CHU Côte de Nacre and EA 2608-USC INRA, Caen, France).

The precise role of estrogens in man has remained poorly understood until naturally-occurring inactivating mutations of both the estrogen receptor- and aromatase gene were reported in humans. These case reports in man are corroborated by their animal counterparts, i.e. the aromatase gene knock-out (ArKO) and the estrogen-receptor gene knock-out (ERKO) in the mouse. Altogether, these data have highlighted the multiple roles of estrogens on puberty and fetal growth, regulation of the gonadotrope axis, but also on yet unknown targets such as bone development, glucose and lipid metabolism and liver metabolism. Aromatase deficiency in man is caused by mutations in the CYP19 gene within exons V, VI, IX and X,

which leads to a truncated protein and an almost complete loss of enzyme activity. Such aromatase deficiency results in maternal virilism during pregnancy, underlining the protective role of the placenta against androgen deleterious effects. During the developmental period, estrogen deficiency does not have harmful consequences on fetal growth, which remains harmonious. Estrogen deficiency does not have any obvious consequence on male or female sexual identity. A role of estrogens on male sexual behaviour and spermatogenesis is still debated. In contrast, estrogens have a crucial role on bone maturation, as proven by defective epiphyseal closure and major osteoporosis in male and female patients with complete estrogen deficiency. Such patients exhibit a complete absence of pubertal growth peak, and continuing bone growth leading to a tall stature. The metabolic consequences of estrogen deficiency in man are multiple, with an equivalent "metabolic syndrome" which may preclude to premature atherogenesis. Insulin resistance, abnormal lipid profile, mainly HDL-cholesterol and apolipoprotein-AI lowering, as well as endothelial dysfunction are the main features of the metabolic syndrome. Steatohepatitis with the features of the "NASH syndrome", i.e. hepatic enzyme disturbance and morphological liver abnormalities have been described in subjects with estrogen deficiency. Estrogen replacement therapy in patients with aromatase deficiency results in the correction of bone, metabolic and hepatic derangement. These human naturally-occurring or animal experimental models of estrogen deficiency are concordant with the lessons brought from observational and experimental studies in man aimed at analysing the roles of estrogens on their diverse targets.

Aging and reproduction

Place of androgen substitution in the androgen deficiency of ageing males. Jean Marc Kuhn (Service d'endocrinologie et maladies métaboliques, CHU de Rouen, Unité INSERM 413 Mont-Saint-Aignan, France).

The androgen deficiency of ageing males (ADAM) is defined as a decrease in plasma free testosterone level, considered as its active fraction. ADAM occurs in at least 50% of men older than 65 years. The consequences of ADAM look like the symptoms of the hypogonadism of the young adult man. They include physical and psychological asthenia, decreased libido and clinical signs of androgen deficiency. They are frequently associated with gynecomastia, reduced mineral bone density with sometimes osteoporosis, a rise in fat mass and relative sarcopenia, which represent the most characteristic features of ADAM. The androgen deficiency is not isolated but is a part of the endocrine changes observed in ageing including a decrease in GH secretion and a dramatic drop in DHEA production by the adrenal glands. The initiation of an androgen substitution in the ageing male is only considerable in a man complaining of symptoms compatible with the diagnosis of ADAM and in whom either the plasma bioavailable or calculated free level of testosterone is below the set-point considered as the lowest limit of normal range. A pathologic status needing a specific therapy or a treatment known to modify either gonadotroph or Leydig cell secretions should be identified before deciding to initiate androgen supplementation. Furthermore, an absolute contraindication (namely prostatic) should be ruled out before starting such a substitution. The modalities of the treatment will be chosen as a function of both the patient preferences and the medical end-points. After initiating androgen substitution, a control of prostatic status should be performed twice a year for the first year of treatment and then yearly. The occurrence of either a prostatic carcinoma or a pronounced dysuria due to a benign prostatic obstruction should lead to treatment interruption. The appearance of a gynecomastia should be systematically detected during the androgenic supplementation. Either its emergence or its aggravation should lead to modifying the modalities of therapy. Several biological markers should also be steadily measured during supplementation using oral androgens. With these cautions and with an adaptation of the modalities of the treatment to the results obtained, the initiation of a substitution with androgens is indicated in the cases of well-proven ADAM. The beneficial effects of such a treatment on bone and possibly on the cardiovascular system, should lead to prefer the use of an aromatisable androgen for hormonal substitution. Conversely, the treatment of a gynecomastia due to an underlying hypogonadism will lead to the use of a dihydrotestosterone for a few months, and as to whether an etiological therapy is not possible. When it is well-indicated, the

substitution of ADAM, for which the aims are to restore or to maintain an adequate androgen status, does not suffer any limit of age. However, its follow up has to be all the more careful if the treatment is initiated in an older man.

Factors contributing to the estrogenic status in postmenopausal women. Claude Ribot (Unité Ménopause et Maladies Osseuses Métaboliques, Hôpital Paule de Viguier, 330 avenue de Grande-Bretagne TSA 70034, 31059 Toulouse Cedex 9, France).

The postmenopausal period is characterised by a new hormonal state, which is mainly the consequence of the cessation of the reproductive function. Within a short period of time, a local, intracrine production of estrogens, which mainly results from the aromatisation of adrenal steroids replace the systemic endocrine production by the ovaries. After menopause, several factors are likely to modulate such estrogen activity: (i) the quantity of available androgens and the local aromatase enzyme activity; (ii) the steroid activity as a function of their local bioavailability and metabolism; (iii) the tissue responsiveness depending on the number, distribution and structure of the different estrogen receptors as well as other genetic regulators. Estrogens play a key regulatory role in the process of bone mineralisation. Bone tissue and bone-derived cells both express the two isoforms of estrogen receptors (α and ß) and all the necessary enzymes for the local metabolism of estrogens. A quantitative approach of the relationship between bone metabolism and estrogens has been made possible by the development of accurate measurement methods of bone mass as well as biochemical markers of bone remodelling, namely bone resorption and formation. Indeed, bone tissue represents a potential valuate candidate to demonstrate the relationship between changes in estrogen activity and/or synthesis and the local tissue response. Numerous studies have documented the influence of changes in SHBG concentrations and differential regulatory pathways of estrogens on postmenopausal bone loss and the risk of fracture. More recently, changes in the expression of the key enzyme of estrogen biosynthesis, aromatase p450, as well as those of the estrogen receptors α and ß have also been associated to bone mineral density, fracture incidence and the bone response to HRT in postmenopausal women. Aromatase enzyme activity represents undoubtedly one of the major determinants of estrogen activity during the postmenopausal period. Its key role in bone tissue metabolism is supported by the major troubles in skeleton growth and mineralisation, which are observed in male subjects with congenital aromatase deficiency. The mechanisms through which aromatase gene activity controls bone remodelling are only beginning to be better understood. On the contrary, over-expression of aromatase activity has been reported in different uterine, ovarian and breast disorders. Interestingly, several recent epidemiological studies have shown a strong relationship between the risk of breast cancer, uterine diseases and higher bone mineral density, which raises the hypothesis of a higher estrogen activity and/or synthesis in some women after menopause. Differences in estrogen activity and/or synthesis at the individual level can now be better demonstrated, especially after menopause, when the intracrine production of estrogens replace the endocrine production. These differences have been linked to the risk of estrogen-dependent disorders such as osteoporosis, atherosclerosis and gynecological cancers. Bone tissue is likely to reflect the cumulative measure of endogenous estrogen and could allow to easily evaluate the differences in estrogen activity and/or synthesis which are observed in postmenopausal women.

Plenary lecture

New perspectives on ovulation. Stephen G. Hillier, Michael T. Rae, Oliver Gubbay, Deborah Niven, Christopher R. Harlow (University of Edinburgh Centre for Reproductive Biology, The Chancellor's Building, 49 Little Crescent, Edinburgh EH16 4SB, UK).

During the regulation of follicular development and ovulation, gonadotrophin action depends vitally on locally produced steroidal and nonsteroidal factors that mediate and modify the actions of FSH and LH (Hillier, 2001). Once a month during reproductive years, usually a single follicle in the ovaries develops sufficiently to secrete oestrogen and ovulate. In spontaneous menstrual cycles, the intercyclic blood FSH

level rises sufficiently to "recruit" multiple antral follicles into advanced stages of development through altering the expression of genes in granulosa cells promoting proliferation and differentiation. Paracrine factors produced in LH-stimulated thecal cells modulate sensitivity and responsiveness to FSH. FSH-regulated genes include LH receptor, which confers direct granulosa responsiveness to LH and ultimately determines which follicle is 'selected' to ovulate. Induction of monovulation therefore depends on appropriate timing and duration of exposure to adequate amounts of FSH and LH (Loumaye et al., 2003). The mid-cycle LH unleashes a cascade of biochemical changes in follicular cells and macrophages, leading to dissolution of the wall of the preovulatory follicle and shedding of the oocyte. These changes include cessation of oestrogen production, onset of progesterone production, increased formation of pro-inflammatory cytokines, activation of cyco-oxygenase-2 (COX-2) and prostaglandin synthesis, increased formation of free-oxygen radicals and release of histamine, and increased proteolysis mediated by matrix-metaloproteinases (MMP) (Hillier, 2003). Each ovulation is associated with a wave of apoptotic cellular degeneration in the overlying surface epithelium (OSE). Since the OSE undergoes inflammatory damage during ovulation, it follows that compensatory anti-inflammatory mechanisms exist to promote its repair. There is evidence that cytokine induced changes in glucocorticoid metabolism influence the anti-inflammatory milieu at this time (Hillier, 2003). Thus within the preovulatory follicle, a switch occurs in the expression of 11betahydroxysteroid dehydrogenase (11betaHSD) isoforms during granulosa cell luteinisation, which determines the reversible inter-conversion of cortisone and cortisol, and hence the availability of cortisol to bind glucocorticoid receptors (GR) and activate anti-inflammatory gene expression (Tetsuka et al., 1997). Immature (i.e. non-luteinised) granulosa cells express mainly the 11betaHSD2 gene and little or no 11betaHSD1, which dictates low-level conversion of cortisone to cortisol. After exposure to LH or human chorionic gonadotrophin (HCG), the pattern is reversed, with 11betaHSD1 induced and 11betaHSD2 suppressed. This translates functionally into locally elevated follicular fluid concentrations of cortisol (Yong et al., 2000). Pro-inflammatory cytokines are believed to mediate these effects of LH on granulosa cells and IL-1alpha has been shown to induce similar changes in cultured human OSE cells (Yong et al., 2002). With the demonstration that cortisol can dose-dependently suppress inflammatory (e.g. COX-2, MMP-9) gene expression in IL-1alpha-stimulated HOSE cells (Rae et al., 2004), we postulate an anti-inflammatory role for 11betaHSD1 in establishing a steroidal milieu conducive to the resolution of ovarian inflammation. Furthermore, since cortisol itself dose-dependently augments IL-1alpha-stimulated 11betaHSD1expression in HOSE cells, a means is available to sustain the regeneration of this anti-inflammatory signal at the site of ovulation. The discovery of this natural anti-inflammatory process has implications for the diagnosis and treatment of disease states involving chronic or excessive ovarian inflammation, including endometriosis and ovarian cancer. (Supported by MRC Programme Grant 0000066.)

Selected abstracts for oral presentations

Estrogens affect protein expression in fetal gonocytes. Ans M.M. van Pelt, Dirk G. de Rooij (Department of Endocrinology, Faculty of Biology, University of Utrecht, The Netherlands).

It is becoming more and more clear that exposure to endocrine disruptors with estrogenic activity during fetal life result in malformations in the male gonadal tract. In order to investigate the possible direct effects of estrogens on the fetal germ cells, we used our established gonocyte cell line in an in vitro system. Gonocyte cell lines were established by isolation of testicular cells from embryonic day 16 rat testes, and cotransfection with pSV3-neo (containing the SV40 large T-antigen) and LTRp53cGg (containing a temperature sensitive p53 (Hofmann et al., 1994)). Transfected cells were single cell cloned and characterised for germ cell specific markers. One gonocyte cell line was used to investigate the direct effects of increasing concentrations of estrogen on protein expression in the fetal germ cells. At least 3 of the established cell lines express the proteins Hsp90α and oct-4, which in the embryonic testes are specific for gonocytes. These gonocyte cell lines have now been cultured for several years with more than 100 passages without changes in morphology. Normally, gonocytes from this age express ERβ

(Van Pelt et al., 1999). We investigated the expression of ERβ in these cell lines. All three gonocyte cell lines show protein expression of ERβ. This expression was upregulated with increasing concentration of estrogen. ICI, the specific inhibitor for estrogen receptor activity, prevents this upregulation. In gonocytes, estrogens act via a similar pathway as PDGF (Li et al., 1997) and fetal gonocytes express the PDGFRβ (Basciani et al., 2002). At least one of our gonocyte cell lines showed expression of PDGFRβ. The protein expression for this receptor was downregulated after increasing the concentration of estrogen. This decrease was not affected by ICI. In conclusion, a fetal gonocyte cell line was used to investigate the direct effect of estrogen on the protein expression of ERβ and PDGFRβ. Increasing concentrations of estrogen enhances the protein levels of ERβ, while PDGFRβ is downregulated. The upregulation of ERβ is an estrogen receptor mediated process, while the down regulation of PDGFRβ seemed to be independent of estrogen receptor activity. The fact that PDGF increases proliferation of neonatal gonocytes (3) and that PDGFRβ is downregulated in developing germ cells (4), indicate that this protein is important in normal germ cell development. Downregulation of PDGFRβ by estrogenic active endocrine disruptors during fetal life may disturb normal gonocyte development and as a consequence male fertility. This work was supported by the Commission of the European Communities Grant QLK4-2000-00305, specific RTD programs "Quality of Life and Management of Living Resources" titled: "The Impact of Developmental Exposure to Weak (Environmental) Estrogens on the Incidence of Diseases in Target Organs Later in Life".

Expression of p63 in the male mouse gonocytes. Béatrice Petre-Lazar, G. Livera, B. Dutrillaut, René Habert, Hervé Coffigny (Gametogenesis and Genotoxicity Laboratory – CEA/INSERM U566/University Paris 7 and Paris 11, CEA/DSV/DRR/SEGG/LDRG, Fontenay-aux-Roses, France).

In the male mouse testis, the development of the gonocytes has several phases: by 12.5 to 13.5 embryonic days gonocytes proliferate rapidly, from 14.5 dpc (days postcoitum) until birth gonocytes are arrested in G1 phase and after birth gonocytes resume mitosis and give rise to A spermatogonia. p53 is a transcription factor with four important domains: TA (transactivation), DBD (DNA binding sites), OD (oligomerisation) and C terminus regulatory domain. p63 and p73 are regarded as member of p53 family, based on their remarkably structure similarity. Between them there are some difference. p53 has only one promoter which encode for a single protein. P63 and p73, each has two different promoters which produced two opposite group of proteins with different N terminus: one group contains TA domain (TA isoforms) and another group lacks it (ΔN isoforms). Additionally, TA and ΔN isoforms are alternatively spliced at C terminus which produced three different isoforms for each group, designate as p63 alpha, beta and gamma. Since the activities of p63 and p73 depend on their isoforms, we analysed the expression of these isoforms in the male mouse gonocytes throughout foetal and neonatal life. In the gonocytes from 12.5 dpc to 4 dpp staining was observed in the cytoplasm for p53 and p73 and the nucleus for p63 using an antibody which detects all the isoforms. To determine the pattern of p63 isoforms expression at cellular and molecular level, we performed immunohistochemistry and RTPCR analysis specific for each isoform. During development of the testis, the gonocytes have a differential expression of p63 isoforms. At 12.5 dpc, the gonocytes express at weak level the following p63 isoforms: TA, DN, alpha and gamma. From 15.5 dpc until birth gonocytes express strongly TAp63gamma and at small level ΔNp63 alpha. After birth, we detected TA and ΔNp63 alpha. p63 beta was absent all the time. Taken together these results suggest that: p53 and p73, always localised in the cytoplasm, are probably not involved in the gonocytes development. p63 is a specific nuclear marker of the germ cells with a sequential expression during different phases of the gonocytes: TA p63gamma was correlated with arrested phase and p63alpha was correlated with proliferation phase which suggest that these isoforms could be involved in cell cycle regulation of the gonocytes. Transgenic p63 mice will be an useful tool to understand the role of p63 in the development of the gonocytes.

Progesterone receptors in the testis: cellular and molecular characterization. Chirag Shah, Deepak Modi, Geetangali Sachdeva, Sushama Gadkar-Sable, Serena D'Souza, Chander Puri

(Primate Biology Division, National Institute for Research in Reproductive Health, Parel Mumbai, 400012, India).

The steroid hormone progesterone shows remarkable diurnal variation in male circulation, high levels have been reported in the testicular tissue suggesting its role in male physiology. Progesterone acts through high affinity intracellular receptors PR-A and Pr-B by a genomic mode of action; progesterone is also known to act through membrane receptors via a non-genomic mode of action. By RT-PCR and Nothern blotting, transcripts corresponding to PR-B and PR-A isoforms were detected in testis and spermatogenic cell RNA. PR mRNA isoforms were in situ localised in the spermatogonia, spermatocytes and spermatids; except Sertoli cells, other somatic components were generally PR negative. The PR protein was immunolocalised in the nucleus and the cytoplasm of spermatogonia, primary, secondary spermatocytes and round spermatids in a stage specific manner. Intense PR immunoreactivity was evident in stages IV, V and VI of spermatogenesis suggesting the requirement of PR mediated transcriptional events post spermation. Western blot analysis of testicular lysates revealed two bands, 120 kDa and 90 kDa corresponding to the conventional PR. In addition to these, a band of ~ 55 kDa was also observed in the testis that corresponded to a protein of the same size in sperm that is known to have the membrane PR. The presence of membrane bound PR on the spermatogenic cells was confirmed by progesterone bound fluorescein conjugate. By immunogold electron microscopy PR was localised in the nucleus of most of the spermatogenic cells. In addition, integral expression of PR was found in the membrane of round and elongated spermatids particularly in the peri-acrosomal region. These results demonstrate the existence of both the isoforms of the intracellular PR and the membrane bound form. The varying levels of intracellular PR during different stages of spermatogenesis and the presence of the membrane bound PR imply the significance of progesterone in male reproductive events such as the regulation of spermatogenesis.

Nuclear oxysterol receptors, LXRs, are involved in the maintenance of mouse adult caput epididymis structure and functions. Jean-Marie Frenoux[§a], Patrick Vernet[§a], David H. Volle[b], Aurore Britan[a], Fabrice Saez[a], Ahyan Kocer[a], Joelle Henry-Berger[b], David J. Mangelsdorf[c], Jean-Marc A. Lobaccaro[b], Joël R. Drevet[a] ([§] Both authors contributed equally to this work, [a] EMG: Epididyme et Maturation des Gamètes, CNRS UMR 6547, Université Blaise Pascal, Aubière, France; [b] PCEM: Physiologie Comparée et Endocrinologie Moléculaire, CNRS UMR 6547, Université Blaise Pascal, Aubière, France; [c] Department of Pharmacology, University of Texas Southwestern Medical Center, Dallas, Texas 75390-9050, USA).

In this study we looked at the epididymis and spermatozoa of mice knocked-out for LXR receptors. We show that LXR-deficient mice exhibited upon aging a severe disruption of their caput epididymides associated with abnormal accumulation of neutral lipids. The epididymis defaults are correlated with sperm head fragility and infertility. In agreement with the observed caput defect in transgenic animals in which both LXRα and LXRß isoforms were disrupted, we show here that both receptors are expressed in caput and cauda epididymidis regions. LXRß is predominantly expressed throughout the mouse epididymis while the expression of LXRα is weaker. In addition, the expression of selected genes that can be considered as markers of adult epididymis function was monitored via Northern blot in the different single and double LXR-deficient backgrounds. Altogether, the data presented here suggests that LXR receptors are important actors in epididymis function.

Molecular basis of bone morphogenetic protein-4 inhibitory action on progesterone secretion by ovine granulosa cells. Alice Pierre[a], Claudine Pisselet[a], Joëlle Dupont[a], Béatrice Mandon-Pépin[b], Danielle Monniaux[a], Philippe Monget[b], Stéphane Fabre[a] ([a] Physiologie de la Reproduction et des Comportements, UMR 6175 INRA/CNRS/Université de Tours/Haras Nationaux, 37380 Nouzilly, France; [b] Biologie du Développement et de la Reproduction, UMR 1198 INRA-ENVA, 78350 Jouy-en-Josas, France).

From recent accumulating in vivo and in vitro evidence, it appears that members of the Bone Morphogenetic Proteins (BMP) family of cytokines and their receptors are strongly implicated in ovarian function, controlling folliculogenesis and ovulation rate. In sheep, we have

shown that a substitutive "loss-of-function" mutation (Q249R) in the gene encoding BMP Receptor-1B (BMPR1B, also called Alk6), one of the type-1 receptors triggering the BMP intracellular signalling, is responsible for an increase in ovulation rate in the Booroola strain. We have recently reported that Bone Morphogenetic Protein-4 (BMP-4), a BMPR-IB ligand can inhibit progesterone production by ovine granulosa cells (GC). Thus, we investigated the underlying mechanisms of this inhibitory effect in basal and in Follicle-Stimulating Hormone (FSH)-induced conditions. By using ovine GC primary culture, we confirmed that treatment with BMP-4 decreased basal progesterone secretion and totally abolished FSH stimulating action. This inhibitory action was associated with a decrease in expression of cAMP-regulated genes, Steroidogenic Acute Regulatory protein (StAR) and P450 side-chain cleavage (P450scc) at mRNA and protein levels. However, BMP-4 did not alter basal cAMP production by GC. In contrast, BMP-4 decreased by half the FSH-induced cAMP production and strongly inhibited cAMP-induced progesterone production. Thus, the inhibitory effect of BMP-4 was exerted both upstream and downstream of cAMP signalling. We next examined downstream effects focussing on cAMP-dependent transcription factors, SF-1 and CREB, through the BMP factor signalling intermediary, Smad1. As expected, BMP-4 induced phosphorylation and transcriptional activity of Smad1 in ovine GC. BMP-4 activated Smad1 did not affect CREB activity but inhibited the transcriptional activity of SF-1 on canonical SF-1 responsive element. Interestingly, this transcriptional inhibitory mechanism occurred on transfected StAR and P450scc promoter. Based on these results, we propose that SF-1 is a key target in the inhibitory mechanism exerted by BMP-4 on progesterone synthesis by ovine GC in culture. Because SF-1 plays an essential role in the differentiation of GC, our findings could have new implications in understanding the role of BMP family members in the control of ovarian folliculogenesis.

Involvement of CRE-like and SF-1 responsive elements in the regulation of the aromatase promoter II in rabbit granulosa cells. Thomas Andrieu, Annie Benhaïm, Hervé Mittre, Colette Féral (EA2608 USC INRA, Estrogènes et Reproduction, Université de Caen, 14032 Caen Cedex, France).

Androgens are converted into estrogens by aromatase cytochrome P450 encoded by the CYP19 gene. The transcription of the CYP19 gene is controlled by FSH and LH during follicle development mainly through their common second messenger cAMP. The rabbit aromatase gene is driven by three tissue-specific promoters PI.1, PI.r and PII. The proximal promoter PII is the main promoter controlling aromatase expression in ovaries. To characterise the molecular mechanisms by which this regulation occurs in rabbit granulosa cells, we explored on the promoter PII, cyclic adenosine 3',5'-monophosphate (cAMP) response elements that are required for transcriptional activation of aromatase. Rabbit granulosa cells were transiently transfected with deletion mutants of rabbit aromatase promoter II ligated to the luciferase reporter gene and treated or not by dibutyryl cAMP (db cAMP). Directed mutagenesis and co-transfections with vectors coding for nuclear factors were performed to confirm these experiments. Analysis of activities of deleted fragments demonstrated that two regions are required for cAMP activation of the aromatase promoter, the –881/–491 base pairs (bp) region which contains potential enhancers SP-1 and AP-1, and the –273/–121 bp region which contains the cAMP-Responsive-Element-Like (CRE-Like) and Steroidogenic Factor-1 (SF-1) sequences. The 5' deletions and site directed mutagenesis experiments clearly demonstrated a crucial role of CRE-Like in the cAMP induction of the aromatase gene expression, whereas an intriguing result is that the –187/–121 bp region containing one SF-1 binding motif seemed to be unresponsive to cAMP. Preliminary co-transfection experiments indicated that the rabbit SF-1 sequence may bind the factor SF-1 suggesting that the SF-1 site is functional. To assess that the db cAMP activation defect of the –187/–121 bp region is not due to a lack of SF-1, we performed mutagenesis experiments where a consensus SF-1 sequence replaces the rabbit element. Preliminary results showed that this construction restored the db cAMP stimulation, suggesting that the rabbit SF-1 element presents a weak affinity to SF-1. This study underlines the major role of the CRE-Like element and the minor role of the SF-1 element in the transcription of the aromatase gene in rabbit granulosa cells. The combined

action of both sites, by synergic or additive action, should now be investigated by mutagenesis experiments.

The interleukin-1 system in ovarian function.
Nadine Gérard[a], Maud Caillaud[a], Alain Martoriati[b] ([a] Physiologie de la Reproduction et des Comportements INRA-UMR 6073, 37380 Nouzilly, France; [b] Pathologie Infectieuse et Immunologie INRA, 37380 Nouzilly, France).

Interleukins are polypeptide cytokine components of the immune system that were originally defined by their role in leukocyte interactions. Thus interleukins (IL) are known for their involvement in the immune system and their role during inflammation. Interleukin-1 was first described in 1972 by Géry and Waksman. Identified as a Lymphocyte Activating Factor (LAF), it was named interleukin 1 (IL-1) in 1979 at the 2nd international congress on lymphokines. IL-1 is organised as a gene system that includes two bioactive ligands, IL-1 and IL-1, and one natural receptor antagonist (IL-1ra). These three molecules are encoded by separate genes and bind to two types of receptors: type 1 (IL-1R1) and type 2 receptors (IL-1R2). Interleukin-1 is produced by a large variety of cells and acts as a paracrine/autocrine factor on target cells. Mice deficient in components of the IL-1 system are widely studied in order to better understand its implication in various physiological processes (Fantuzzi, 2001). Since then, the interleukin-1 system components (IL-1α, IL-1β, IL-1 receptor antagonist, IL-1 receptors) have been demonstrated as having several synthesis sites in the ovary. Thus ovarian cells could represent sources and targets of interleukin-1. Moreover in the ovary, a growing body of evidence suggests that the ovarian follicle is a site of inflammatory reactions, as hypothesised by Espey in 1980. IL-1, IL-1ra and IL-1 receptors have been localised in the various ovarian cell types, such as the oocyte, granulosa and theca cells, in several mammalian species. IL-1-like bioactivity has been reported in human and porcine follicular fluid at the time of ovulation. The role of IL-1 in local processes are still poorly known, although there is evidence of its involvement in the ovulation process, and in oocyte maturation. More precisely, IL-1 may be involved in several ovulation-associated events such as the synthesis of proteases, regulation of plasminogen activator activity, prostaglandin and nitric oxide production. IL-1 also regulates ovarian steroidogenesis. The different ways in which the IL-1 system plays an important role in female reproduction will be presented.

Early menopause and genetics of FOXL2.
Marc Fellous (INSERM U 0021, Hôpital Cochin, Pavillon Baudelocque, 123 boulevard Port-Royal, 750014 Paris, France).

The genetic analysis of the majority of Premature Ovarian Failure cases (POF) is not frequently conclusive, leading to difficult diagnosis, prognostic and genetic counselling. Nevertheless the presence of POF in a syndrome make genetic analysis easier such as POF, associated with Turner Syndrome, metabolic diseases such as galactosemia or BPES (Blepharophimosis, Ptosis, Epicantus, Syndrome). The isolation of the FOXL2 gene allows us important progress in the knowledge of the BPES syndrome, a genetic disease characterised by eyelid malformations (type II) associated with POF (type I). The analysis of mutations allows us to establish a genotype/phenotype correlation between the phenotype of POF in both types of BPES. Furthermore, the FOXL2 knockout mice permit to clarify the functional role of FOXL2 in the genetic cascade of folliculogenesis in mammals including man.

Closing conference

Domestic mammal abortions and zoonoses.
Daniel Tainturier (Biotechnologies et Pathologie de la Reproduction, École Nationale Vétérinaire, BP 40706, 44307 Nantes Cedex, France).

For many years, brucellosis was identified as the principle cause of abortion, particularly among bovines. A medical and then sanitary prophylaxis has practically enabled its eradication from France. Sporadic cases still occur in some regions and alongside rivers, the wildlife (hares, deers, wild boars, …) being the usual source of contamination. Sea mammals (dolphins) may also be a potential source of contamination. The after-effects of this disease may explain the frequency of rheumatism in the countryside. Cases

of abortions in ewes caused by *Campylobacter foetus* variety *intestinalis* and *Campylobacter jejuni* are observed every year. Chlamydiosis is responsible for at least 25% of ewe abortions, 20% of goat abortions and 5% of cow abortions. Miscarriages have been observed with women who have participated in deliveries of contaminated ewes or goats. Q fever (Query Fever) that is found throughout France and due to *Coxiella burnetti* is responsible for premature deliveries in cows, ewes (3%) and also in domestic carnivores. Contamination occurs by respiratory means. In France, 600 people a year suffer the acute form and 60 the chronic form: bad head, hyperthermia, sweat, lung disease, miscarriage, hepatitis. Human mortality may be as high as 3%. In 2002, 128 human cases were identified in the Chamonix valley with a peak during the 1st week of July, when manure spreading takes place. Salmonellosis (*Salmonella dublin*, *typhimurium*, …) affects bovines throughout France and small ruminants especially in the South of France. (*S. abortus ovis*). Sources of infection are the pasture, contaminated water, liquid manure, waste water, man, small wild mammals and birds. Leptospirosis is responsible for 3% of cow abortions and when affecting a flock of sheep, it can cause the interruption of up to 20% of gestations, especially for females less than 3 years old. In humans, it manifests itself as hépatonéphritis. Veterinarians and sewage workers are most at risk but it is also becoming a leisure disease (i.e. caught while fishing, swimming or hunting). The frequency of listeria has been multiplied by a factor of 40 since bovines are not fed with traditional food but with corn or grass silage contaminated by rodents from silos. The disease causes Méningo-encéphalitis in adults. Women can suffer miscarriages. Other bacteria, more rare, can be the cause of abortion in domestic animals and accidents in human: erysipelasa for instance affects sows and can cause endocarditis in humans. Among parasites, two should be mentioned: toxoplasmosis and néosporosis. Toxoplasmosis is well known to cause miscarriage in women and the birth of hydrocephalic babies. It is also responsible for 10% of abortions in small ruminants. This frequency can be controlled by medical prophylaxis. A new disease has been identified in the last fifteen years: *Neospora caninum*, responsible for 25% of abortions in bovines and a smaller percentage in other domestic animals. The question of human contamination has been raised but has so far not been proven and requires further study. It has, however, been observed that women who have suffered repeated miscarriages and who live surrounded by many cats and dogs can be positive to this disease. As for viruses causing abortions in domestic animals in France, there does not seem to be any inter species contamination. Herpes viruses with genital and pulmonary tropism should, however, be compared on a pathogenic point of view.

Other abstracts

Intracytoplasmic sperm injection (ICSI): results and prognostic factors in the center of Tunisia (Study of 199 cases). Mounir Ajina, Radhouane Neifer, Imed Harrabi, Ghaya Merdassi, Samira Ibala, Meriam Mehdi, Ali Saâd (Service d'Epidémiologie, Service de Cytogénétique et de Biologie de la Reproduction, CHU Farhat Hached, Sousse, Tunisia).

The retrospective study of 199 cycles of ICSI during a 2 year-and-a-half period between September 2001 and February 2004. The procedure of ICSI included several stages: collection and preparation of the semen, stake in culture of oocytes, removing of cumulus cells and microinjection of oocytes, control of fertilisation and embryo transfer respectively 18 to 22 h and 48 h after the microinjection. Our data were computerised on the SPSS version 08 software. Two statistical tests were used with a level of significance of 5%: the "Independent Samples Test" for the comparison between 2 groups and the "ONE WAY ANOVA" for the analysis of variance between several groups. The mean age of the patients was 32.4 years (20–48). The mean duration of infertility was 7 years (0.6–28 years). The mean fertilisation and segmentation rates were respectively 50% and 66%. The mean number of embryos transferred was 2.46 (1–7). We got 41 pregnancies of which 36 were clinical pregnancies (87.8%). The pregnancy rate was 26.1% by transfer and 21% by retrieval. The prognostic factors of ICSI in our study were the women's age and the number of 4 cell embryos transferred. The pregnancy rate was 27% before the age of 35 years, decreases with age and annul itself after 40 years ($P = 0.02$). The pregnancy rate increased significantly with the number of 4 cell embryos transferred: 15% after transfer of only one embryo versus 43% after transfer of

3 embryos or more ($P = 0.032$). The ICSI prognostic was not influenced in a significant way by the origin or the sperm mobility, by the duration of infertility and by the total number of embryos transferred. In conclusion, the ICSI represents currently the treatment of choice of a couple having extreme spermatic changes. The women's age and the number of 4 cell embryos transferred are the main factors predicting the ICSI prognostic.

Hormonal study of the follicular fluid of patients programme for in vitro fertilisation. Samira Ibala, Mounir Ajina, Monia Zaouali-Ajina, Radhouane Neifer, Raouf Abdelali, Meriam Mehdi, Ali Saâd (Laboratory of Cytogenetic and Biology of Reproduction, CHU Farhat-Hached, Sousse, Tunisia).

Our study consisted in comparing the rates of androgens in the follicular fluid of two groups of women undergoing ovarian stimulation for in vitro fertilisation (IVF), who each profited from a different ovulation induction protocol (short protocol vs. long protocol), as well as the study of the E2/androgen ratio of a second population of patients treated by a long protocol. The patients studied had a variable origin of infertility, of 6 years average duration. The first studied population included 58 patients. They profited from 58 stimulated cycles of IVF for which half was treated by a long protocol (group 1) and the other half by a short protocol (group 2). The second studied population included 26 patients, half of whom became pregnant following the IVF. Estradiol (E_2) and androgens (testosterone T, androstenedione A, dehydroepiandrosterone sulphate DHEAS) concentrations in follicular fluid were measured using a commercially available radioimmunoassy kit. The average age of the patients was respectively 36 years and 33 years in groups 1 and 2 with a significant difference ($P = 0.02$). The rate of the DHEAS in the follicular fluid was significantly higher in group 2 than in group 1 ($P = 0.02$). In the second study, all patients were treated by the long protocol, two sub-groups were compared for E_2/androgen ratio. For the first sub-group whose IVF led to pregnancy, the E_2/A ratio was significantly higher than the second sub-group (IVF without pregnancy, $P = 0.05$). In conclusion, the patients treated by a long protocol had lower rates of androgens in the follicular fluid, and thus a higher E_2/androgen ratio. This group of patients was characterised by a rate of oocyte cleavage, oocyte fertilisation and pregnancy better than those treated by the short protocol. We can thus conclude to the harmful effect of the androgens on the follicular growth and oocyte maturation.

Expression of aromatase gene in male rat germ cells. Dorothée Silandre, Sonia Bourguiba, Christelle Delalande, Serge Carreau (Laboratoire de biochimie, IBFA, UPRES-EA 2608-USC INRA, Université de Caen, 14032 Caen Cedex, France).

The mammalian testis has two main functions: biosynthesis of steroids and production of spermatozoa. These functions are under the control of gonadotrophins and numerous factors produced locally including cytokines, androgens and estrogens. The enzymatic complex responsible for the irreversible conversion of androgens into estrogens is called aromatase. It is present in the endoplasmic reticulum and is composed of a specific cytochrome P450 aromatase (P450 arom) and a ubiquitous non specific flavoprotein, the NADPH cytochrome P450 reductase. Human P450 arom is encoded by a unique gene called cyp19. Its expression is regulated by the alternative use of various first exons preceded by tissue specific promoters. Actually, nine non coding first exons have been characterised. The expression of functional aromatase in the male gonad has been known for a long time. Sertoli cells, Leydig cells but also germ cells (pachytene spermatocytes, round spermatids and spermatozoa) of many species including humans, rats, mice, bank voles are able to produce estrogens. In order to continue the characterisation of aromatase expression in rat germ cells, we underlined the presence of P450 arom mRNA transcript in earlier germ cells: spermatogonia and preleptotene spermatocytes. Their levels are lower than those in pachytene spermatocytes and in round spermatids (divided by 3). Aromatase is expressed in a constitutive manner in testicular cells suggesting the importance for estrogen in spermatogenesis and spermiogenesis. Recent data from our laboratory have shown that the expression of aromatase in rat germ cells is regulated by the second messenger: cAMP, by the cytokines: TNF and TGF and by steroids including testosterone

and estradiol. These modifications of the quantity of aromatase transcripts are corroborated with estradiol levels of culture medium. In gonads, the major promoter responsible of the expression of cyp19 is the promoter PII but we can not exclude that another promoter is implicated such as P1.4. Moreover, in mice, it was shown that a promoter testis specific (Ptes) controls the expression of aromatase. The molecular mechanisms implicated in the regulation of expression of aromatase in testicular cells are not yet elucidated notably, the sequences of cis elements on cyp19 promoter and the nature of trans factors.

Differential effects of glyphosate and roundup on human placental cells and aromatase. Sophie Richard, Safa Moslemi, Herbert Sipahutar, Nora Benachour, Gilles-Eric Seralini (EA2608-USC INRA, IBFA, esplanade de la Paix, Université de Caen, 14032 Caen Cedex, France).

Roundup is a glyphosate-based herbicide used worldwide including on most genetically modified plants in which it can be tolerated. Its residues may thus enter the food chain and glyphosate is found as a contaminant in rivers. Some agricultural workers using glyphosate have pregnancy problems, but its mechanism of action in mammals is questioned. Here we show that glyphosate is toxic on human placental cells within 18 hr with concentrations lower than those in agricultural use, and this effect increases with concentration and time, or in the presence of Roundup adjuvants. Surprisingly, Roundup is always more toxic than its active ingredient. We tested with lower non-toxic concentrations its effect on aromatase, the enzyme responsible for estrogen synthesis. It acts as an endocrine disruptor on aromatase activity and mRNA levels. We conclude that endocrine and toxic effects of Roundup and not only glyphosate need to become clearer in mammals. We suggest that the presence of Roundup adjuvants enhance glyphosate bioavailability and/or bioaccumulation.

Roundup alters mammalian aromatases and human embryonic cells more efficiently than glyphosate. Herbert Sipahutar, Safa Moslemi, Céline Nativelle Serpentini, Marie-Josèphe Simon, Gilles-Eric Séralini (EA 2608-USC INRA, Université de Caen, esplanade de la Paix, 14032 Caen Cedex, France).

We report here the effects of both Roundup and glyphosate on mammalian aromatase activities and their cytotoxicity on human embryonic E293 cells. Aromatase is a crucial steroidogenic enzyme responsible for the irreversible synthesis of estrogens from androgens. It is composed of two moieties: a specific cytochrome P450 and a ubiquitous reductase. The experiments were carried out on microsomes from human placenta or equine testis, and on both enzymatic moieties purified from equine testis, as models. Roundup, a glyphosate-based herbicide, is used worldwide and also with genetically modified plants; in most of these it is tolerated at very high levels. Our results indicate that glyphosate and Roundup inhibit the two mammalian aromatases, and to a lesser extent reductase activities also dose-dependently. However, Roundup is considerably more efficient on human aromatase than glyphosate. After pH adjustment, the acidity of the commercial formulation accounted for a maximum of 32% inhibition. Moreover, inhibition, kinetic and spectral studies reveal that the compounds interfere at the P450 aromatase active site level. Cytotoxicity studies showed that both Roundup and glyphosate were toxic on human embryonic kidney (E293) cells, more than 90% of cell mortality was reached with 0.84% Roundup, after only 24 h of culture, whereas dilutions from 1 to 2% are proposed for eliminating normal plants by the supplier. Again Roundup was in the same manner more potent than glyphosate. It is concluded that Roundup could be considered as an endocrine disruptor at the steroidogenic level in mammals, and that its adjuvants considerably enhance glyphosate effects on human embryonic cells.

Male and female hypofertility in mice mutant for a histone deacetylase. Virginie Guiraud[a], Hélène Humbertclaude[a], Patrick Hery[a], Wolfgang Wurst[b], Carine Travert[c], Serge Carreau[c], Edwige Col[d], Saadi Khochbin[d], Roberte Pelletier[d], Sophie Rousseaux[d], Matthieu Gérard[a] ([a] Laboratoire de Transgenèse, DBJC/SBMS, CEA Saclay Bat 142, 91191 Gif-sur-Yvette, France; [b] GSF Center for Environment and Health, Institute of Mammalian Genetics, Gene Trap Project,

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Histone deacetylases (HDAC) belong to a highly conserved protein family. These enzymes target chromatin proteins and play multiple roles in the regulation of transcription in mammals. Nucleosomes, which are the basic unit of chromatin, contain 147 base pairs of DNA wrapped around a histone octamer. Numerous post-translational modifications of histones have been documented and analysed in detail: acetylation or methylation of specific lysine residues, serine phosphorylation, etc. These modifications have important consequences on the activity of our genome. For example, a hyperacetylated chromatin is generally associated with transcriptionally active regions of our genome. Hypoacetylated nucleosomes, however, are a hallmark of silent genomic regions. HDAC, which remove acetyl residues from histones, are involved in transcriptional repression. We are studying the phenotypes of a mouse line carrying a gene trap mutant allele of HDAC2. This enzyme is widely expressed during embryogenesis and adult life, with a high level of expression in the testis. After birth, HDAC2 homozygous mutant mice display an important growth defect and a high rate of lethality. A proportion of these mice survives however to adulthood. At this stage, the growth defect has been compensated and is no longer detectable. Both male and female mutant homozygous are affected by a severe hypofertility. Strikingly, the penetrance of this phenotype is variable in different homozygous individuals. In addition, females are also affected by a defect in the late stages of gestation. We are currently investigating the cellular and molecular alterations that might explain these phenotypes.

Expression of the Insl3/LGR8 hormone-receptor system in the testis. Ravinder Anand-Ivell, Marga Balvers, Ralph Telgmann, Richard Ivell (Institute for Hormone and Fertility Research at the University of Hamburg, 20251 Hamburg, Germany, and School of Molecular and Biomedical Sciences, University of Adelaide, SA 5005, Australia).

The peptide hormone Insl3 (insulin-like factor 3, alternatively known as relaxin-like factor, RLF) is a major secreted product of the Leydig cells in all species of mammals. In rodents it has been shown to be responsible for the transabdominal phase of testicular descent, its absence or down-regulation in the fetus leading to cryptorchidism. Recently, a novel G-protein coupled receptor, LGR8, was identified as the specific receptor for Insl3. In order to characterise possible testicular roles for the Insl3/LGR8 system, we developed and validated novel antibodies against the LGR8 receptor, and used these in immunohistochemistry in both human and rodent tissues to identify cells potentially able to respond to the peptide hormone. Furthermore, we applied RT-PCR analysis for the specific receptor transcripts to testicular cells, cell-lines and tissues, in order to corroborate these findings. Both immunohistochemistry and RT-PCR analysis confirmed the Leydig cells as a major site of LGR8 receptors in the adult testis, supporting an autocrine role for Insl3. In addition LGR8 receptors appear to be present in an intratubular location, most probably the germ cells, since Sertoli cell-lines and germ cell-depleted testes have no or reduced LGR8 mRNA, respectively. This research was funded in part by the Innovation Funds of the City of Hamburg and by the German Research Council (DFG Graduiertenkolleg 336).

Alterations in proteoglycan synthesis selectively decrease FSH-stimulated particulate phosphodiesterase 4 (PDE4) activities in immature rat Sertoli cells. Guénaëlle Balavoine, Jérôme Levallet, Hélène Bouraïma, Magali Demoor-Fossard, Pierre-Jacques Bonnamy (Laboratoire de Biochimie, UPRES EA 2608, USC INRA, Université de Caen, 14032 Caen, France).

Proteoglycans (PG), composed of sulphated glycosaminoglycans (GAG) covalently linked to a core protein, are not only components of the extracellular matrix but are also expressed as integral membrane molecules. Because of their structural diversity and cell distribution, PG are involved in many cellular processes, including

modulation of the PKC signaling pathway. We have previously shown that a decrease in cell PG content, consecutive either to an alteration of GAG synthesis by *para*-nitrophenyl-β-D-xyloside (PNPX) or to inhibition of GAG sulphation by sodium chlorate, induce an increase in follicle-stimulating hormone (FSH)-stimulated estradiol production in cultured testicular Sertoli cells from the immature rat. Part of both chlorate and PNPX effects result from a decrease in cAMP-phosphodiesterase activities as evidenced by the disappearance of the up-regulation of FSH-stimulated steroidogenesis induced by these molecules in the presence of MIX, an unspecific inhibitor of cyclic-nucleotide phosphodiesterases. Since rolipram-sensitive phosphodiesterases (PDE4) are the main cAMP catabolising enzymes in Sertoli cells and the only PDE regulated by FSH, we have studied the effects of both PNPX and chlorate upon PDE4 activities in cultured Sertoli cells from 20-day old rats. Cyclic AMP-PDE activities in subcellular fractions were determined by hydrolysis of [^{3}H]cAMP according to the method of Thompson and Appleman. In cultured Sertoli cells, cAMP-PDE activities were distributed in both cytosolic (40%) and particulate (60%) fractions, the rolipram-sensitive PDE4 accounting for half of total PDE activity in both fractions. The addition of PNPX (1mM) or chlorate (10 mM) abrogated the 5-fold increase in particulate PDE4 activity induced by FSH (100 ng·mL^{-1}) but, in contrast, had no significant effect on the 2-fold increase in cytosolic PDE4 activity induced by the gonadotropin. Moreover, total PDE and PDE4 activities were unaffected by chlorate or PNPX in Sertoli cells cultured in the absence of the gonadotropin. By using a semi-quantitative RT-PCR assay, we have shown that, of the 8 PDE4D isoforms already identified, Sertoli cells expressed mainly 2 short (D1 and D2) and two long isoforms (D4 and D5). Only transcription of the D1 and D2 were up-regulated by FSH. Chlorate and PNPX were without an effect on all PDE4D isoform transcription in Sertoli cells cultured in the absence as well as in the presence of FSH. Immunoblot analysis of the main PDE4D proteins (D1, D2, D4 and D5) in the particulate fraction did not reveal any apparent effect of chlorate or PNPX in both unstimulated and FSH-treated Sertoli cells. Collectively, these data suggest that alteration in cell membrane PG induces abolition of the FSH-stimulated particulate PDE4 activities by a post-translational effect, whose nature remains to be determined. Transmembrane PG, by regulating the cAMP catabolising activities at the close vicinity of adenylate cyclases, could serve to limit intracellular diffusion of cAMP and to prevent overflow of the cyclic nucleotide signalling pathway in FSH-stimulated Sertoli cells.

Effect of an intrafollicular injection of interleukin-1β on equine oocyte cytoplasmic maturation. Maud Caillaud, Guy Duchamp, Nadine Gérard (UMR INRA/CNRS/Université de Tours/Haras Nationaux, PRC, 37380 Nouzilly, France).

It is accepted that the IL-1 system plays a role in the ovulatory process. Under our experimental conditions, ovulation is induced by an intravenous injection of Crude Equine Gonadotropin (CEG). Recently, it has been shown in the mare that interleukin-1β (IL-1β), when intrafollicularly injected in the dominant follicle, induces ovulation in the same way as CEG. It has also been shown that an intrafollicular injection of IL-1β induces oocyte nuclear maturation, an increase in intrafollicular content of progesterone and prostaglandin F2α and a decrease in intrafollicular content of 17β-estradiol. However, pregnancy rate at day 14 was only 25% after an intrafollicular injection of IL-1β. To explain this low level, one hypothesis could be due to the poor quality of oocytes, and the lack of a normal cytoplasmic maturation. In addition, the migration of cortical granules and mitochondria are an important step in cytoplasmic maturation and have been used as criteria of oocyte maturity assessment. At the end of maturation, cortical granules form a monolayer at the border of the cytoplasm and mitochondria aggregate in the cytoplasm. The aim of the present work was to determine the effect of an intrafollicular injection of IL-1β on oocyte cytoplasmic maturation. Twenty-three cyclic Welsh pony mares (3–15 years old) were allocated to 2 experimental groups. These were 1/PBS/CEG group, intrafollicular (i.f.) injection of PBS plus i.v. injection of crude equine gonadotropins (CEG; 15 mg) and 2/IL-1β/saline group, i.f. injection of 2 mL of IL-1β (0.5 μ·mL^{-1} in PBS) plus i.v. injection of saline. Transvaginal ultrasound-guided injections were performed into the dominant follicle when its diameter reached 30–34 mm. Thirty-eight hours after the i.f. injection, transvaginal ultrasound-guided aspirations were performed

to collect the oocyte from each injected dominant follicle. Cortical granules and mitochondria were labelled with FITC- PNA and mitotracker orange respectively. Laser confocal microscopy was used to analyse the labelling. The majority of oocytes in the PBS/CEG group showed complete cortical granule migration at the border of the cytoplasm. On the contrary, most oocytes in the IL-1β/saline group displayed a network of cortical granules throughout the cytoplasm. Moreover, the aggregation of mitochondria was observed in the majority of oocytes in the PBS/CEG group whereas in the IL-1β/saline group the distribution of mitochondria in the oocytes was homogeneous. These results may explain the low pregnancy rate at day 14 observed in our previous study.

Age-related expression of hyaluronidases in peritubular and Sertoli cells from the immature rat. Nadia Coudray, Hélène Bouraïma, Guénaëlle Balavoine, Jérôme Levallet, Magali Demoor-Fossard, Pierre-Jacques Bonnamy (Laboratoire Aromatase et Oestrogènes dans la Reproduction, UPRES-EA 2608, USC INRA, IBFA, Université de Caen, 14032 Caen, France).

Hyaluronan (HA) is a high molecular weight and lineary, polysaccharide widely distributed in the extracellular matrix and composed of thousands of dissacharide units of N-acetyl-D-glucosamine and D-glucuronic acid. Despite its very simple structure, HA is thought to have active tissue-organising functions mediated through specific HA-binding proteins and cell surface receptors such as CD44 and RHAMM. By using alkaline phosphatase-linked hyaluronectin, we have previously shown that HA was abundantly present in intertubular space on testicular slices from 10 day-old rats but decreased progressively on testicular slices from older rats so far as to disappear in peripubertal animals. The age-related changes of testicular HA suggest that HA or oligosaccharides resulting from its degradation by hyaluronidases could exert yet unknown functions in the testis. To delineate the involvement of testicular cells in the age-related decrease in intertubular HA, we studied the expression of hyaluronidases, the HA catabolising enzymes, by RT-PCR in purified cultures of peritubular and Sertoli cells. Amongst the 4 hyaluronidases tested (HYAL-1, -2, -3 and PH-20), only HYAL-1 and -2 were expressed in peritubular and Sertoli cells from 20 day-old rat testis. The regulation of gene expression for both hyaluronidases was cAMP-dependant in Sertoli cells as shown by the 2-fold increase in the amount of HYAL-1 and -2 transcripts in semi-quantitative RT-PCR using actin for normalisation of the data. In contrast, HYAL-1 and -2 expression in peritubular cells appeared independent of the cAMP/PKA pathway. Interestingly, the pattern of HYAL-1 expression level was age-related in both peritubular and Sertoli cells. While differentiation of Sertoli cells was accompanied by a fall in HYAL-1 expression, an increase in HYAL-1 expression occurred in peritubular cells between 10 and 30 days. In contrast, expression of HYAL-2 did not exhibit any significant changes in both cell types during that period. The shift in HYAL-1 expression from Sertoli cells towards peritubular cells from day 10 to day 30 could explain (i) the absence of HA within the seminiferous tubule and (ii) the age-related decrease in intertubular HA within the rat testis. The pattern of hyaluronidase expression is likely of interest in the occurrence of some intratesticular events. Indeed, the two hyaluronidases are thought to act in concert, HYAL-2 cleaving high-molecular-weight hyaluronan polymers to intermediate size fragments of approximately 20 kDa, which are further catabolised to low-molecular-weight oligosaccharides by the action of HYAL-1. Thus, peritubular cells are potentially able to generate significant amounts of angiogenic oligosaccharides which could be involved in the neovascularisation of intertubular space at the onset of spermatogenesis.

Identification of a new expanding family of genes characterised by atypical LRR domains. Localisation of a cluster preferentially expressed in oocytes. Sébastien Dadé[a], Isabelle Callebaut[b], Pascal Mermillod[a], Philippe Monget[a] ([a] Physiologie de la Reproduction et des Comportements, UMR 6073 INRA/CNRS/Université François Rabelais de Tours, 37380 Nouzilly, France; [b] Systèmes Moléculaires et Biologie Structurale, LMCP, CNRS UMR 7590, Universités Paris 6 et Paris 7, case 115, 4 place Jussieu, 75252 Paris Cedex 05, France).

As shown by knock-out experiments in mice, most of oocyte-specific genes play key roles in oogenesis, folliculogenesis or early embryonic development. In particular, GDF-9 and BMP-15

are required during early ovarian folliculogenesis enabling primordial and primary to secondary follicle transition in mice and sheep, respectively. Moreover, maternal factors such as maternal antigen that are embryo required (MATER), Zygotic arrest 1 (Zar1) and nucleoplasmin 2 (Npm2) are necessary for normal embryonic development beyond the 1- or 2-cell stage. Several genes that are specifically expressed in oocytes have recently been identified by mRNA differential display, or using in silico subtraction. In the present work, we used the in silico subtraction methodology to identify novel oocyte-specific genes in mice. By this way, we identified in silico a new family of genes composed of more than eighty members. Sequence analysis showed that these genes belong to the superfamily of leucine-rich repeat (LRR) proteins. However, the LRR of this family display some variability in length and amino-acid composition within the ß-strands region, since more leucine residues are substituted by other hydrophobic amino acids as compared to canonical LRR. Interestingly, for nine of these genes, the EST were represented almost exclusively in mouse egg libraries. Three of them are localised in a cluster on mouse chromosome 4, in the vicinity of another recently discovered oocyte-specific gene called oogenesin, that we also found to belong to the same family. We thus renamed this latter gene "oogenesin-1", and the three genes identified here were named oogenesin-2, -3 and -4. Oogenesin-2, -3 and -4 were selected for experimental study. By RT-PCR, virtual northern and in situ hybridisation, we confirmed their specific expression in the mouse oocyte from primary to preovulatory follicles.

Estrogens inhibit testis development during fetal/neonatal life in rodents – in vitro and in vivo study. Géraldine Delbès[a], Christine Levacher[a], Clotilde Duquenne[a], Andrée Krust[b] René Habert[a] ([a] Unité de Gamétogenèse et Génotoxicité, INSERM U566/CEA/Université Paris 7, Fontenay-aux-Roses, France; [b] GBMC – INSERM U184 CNRS/INSERM/ULP, Collège de France, BP 163, 37404 Illkirch Cedex, France).

Epidemiological, clinical and experimental studies have suggested that excessive exposure to estrogens or estrogen-like endocrine disruptors during fetal/neonatal life can induce a decrease of sperm production in adulthood. However, the action of estrogens on testicular functions during fetal/neonatal life is poorly documented. Therefore we investigated the effects of exogenous and endogenous estrogens on both testicular functions: gametogenesis and steroidogenesis during fetal and neonatal life. We developed in vitro systems allowing the development of rat fetal and neonatal germ cells (gonocytes): organ cultures and dispersed testicular cell cultures. In both systems, additions of high concentrations (10^{-6} M) of estradiol-17ß or diethylstilbestrol decreased the number of gonocytes during the first fetal proliferative period (14.5 to 18.5 days post conception (dpc)) but not during the second neonatal proliferative period. Until 18.5 dpc, estrogens inhibited testosterone production by testes in organ culture or in dispersed testicular cell cultures. In later stages, the inhibiting effect of estrogens disappeared in organ cultures but persisted in cell cultures. These results are in favor of a direct inhibitory effect of estrogens on Leydig cells in early fetal stages while in later stages intratesticular mechanisms could reverse the deleterious effects of estrogens. To investigate whether endogenous estrogens can affect fetal and neonatal testicular development we used mice in which the ERß or ERα gene was inactivated. The homozygous invalidation of ERß (ERß-/-) increased the number of gonocytes by 50% in 2 day old neonates but did not modify the numbers of Sertoli cells and Leydig cells nor the levels of testicular testosterone production. The increase in the number of gonocytes occurred just after birth when gonocytes resume mitosis and apoptosis, and resulted from a decrease in apoptosis rate and, to a lesser extent, from an increase of mitosis. Lastly, mice heterozygous for the ERß gene invalidation showed the same evolution of the gonocytes than their ERß-/- littermates suggesting that gonocytes are highly sensitive to the binding of endogenous estrogens to ERß. The homozygous invalidation of ERα (ERα-/-) had no effect on the number of gonocytes but the testosterone secretion was increased in the two studied ages: 13.5 dpc and 2 days post partum. This resulted from a higher steroidogenic activity of each Leydig cells and did not seem to involve any change in gonadotropin plasmatic levels. In conclusion, we evidenced periods where testis development is sensitive to estrogens, which seem to be species-dependant. Also, this study provides the first demonstration that endogenous estrogens can physiologically inhibit

germ cell growth in the male via ERß and testosterone secretion via ERα. These findings may have important implications concerning the potential action of environmental estrogens.

Study of aromatase gene expression and regulation in pituitary. Guillaume Galmiche[a], Sophie Corvaisier[b], Marie-Laure Kottler[b] ([a] Laboratoire Estrogènes et Reproduction, EA 2608-USC INRA, Université de Caen Basse-Normandie, France; [b] Département de Génétique et Reproduction, CHU de Caen, France).

The terminology "hypothalamic-pituitary-gonadal axis" masks our poor knowledge about the molecular mechanisms of the regulation of LH and FSH gonadotropins by sexual steroids. The negative feedback action of gonadal steroids on gonadotropin releasing is well documented during the follicular phase, however, the mechanisms of the stimulating action driving to the preovulatory gonadotropin releasing hormone surge are not completely understood. Increasing the frequency and the amplitude of GnRH pulses in the preovulatory period is admitted but the females deficient in GnRH and receiving GnRH pulses, ovulate whereas the frequency of pulses and the doses delivered remain identical during the cycle. Therefore other mechanisms must be involved within the pituitary. We hypothesise that a local production of estrogens could amplify the estrogen effect secreted by the preovulatory follicle. Estrogen biosynthesis is regulated by the cytochrome P450 aromatase, therefore it is necessary to study its expression. The rat aromatase gene is under the control of at least three tissue specific promoters: the hypothalamic type (Ph or P1.f), the ovarian type (PII) and the cortical type (Pcc). This latter is localised on the intron upstream of exon VI, encoding for a non functional variant and does not express exon I to exon III. Aromatase expression was studied under different physiological and pathological situations both in male and in female rats: during the estrous cycle (dynamic situation), in castration/estrogens replacement (20 $\mu g \cdot kg^{-1}$) models (chronic situation). The estrous cycle and the effect of estrogenic treatment were examined from vaginal smears for 20 days. After 20 d, pituitary tissues were removed for RNA extraction. Blood was collected for estrogen evaluation using RIA. The aromatase transcript detection was carried out by qualitative RT-PCR and the regulation of the aromatase expression by real time PCR (TaqMan®). The LHβ mRNA quantification was used as the control (SYBR Green®). PCR experiments reveal that all three transcript variants are expressed in the pituitary, showing a clear predominance of the brain transcript (1.f). From the castration/estrogen replacement studies, we also demonstrate that expression of the aromatase gene is under hormonal regulation. After ovariectomy, it increased by 2-fold compared to intact animals and this effect was reverted by estrogen administration. However this regulation appeared to be different in the male. Moreover, a significant variation exists during the estrous cycle, with a 2-fold increase in the amount of aromatase mRNA during the diestrus stage compared to the other stages. These results reinforce the hypothesis of estrogen pituitary production subject to hormonal regulation, implying the need for further investigations from in vitro models in order to understand the mechanisms involved.

Fertility breakdown in Tunisian chromosomal hybrid mice: structural effects or genetic incompatibilities I. Chatti Noureddine, Said Khaled (Laboratoire de Génétique, Institut Supérieur de Biotechnologie de Monastir, 5000 Monastir, Tunisia).

The reproductive features of wild all acrocentric and 2n = 22 Robertsonian (Rb) house mice (*M. m. domesticus*) from Tunisia were studied through laboratory crosses. The aim was to determine the existence of a reproductive selective advantage associated with chromosomal change as well as to measure the effect of heterozygosity for a large number of Rb fusions on the fertility of hybrids. The results showed that differences between the litter size of the two chromosomal races can be related to selective factors acting locally and not to different demographic strategies. The F1 hybrids showed a significantly reduced reproductive success and litter size. Histological analyses of F1 and backcrosses showed a breakdown of gametogenesis highly correlated to testis weight in males and leading to a significantly reduced oocyte number in females. The degree of this disturbance was not related to the level of chromosomal heterozygosity suggesting that genetic incompatibilities between the two genomes might be involved.

GnRH deficiency: genetic insights. Marie-Laure Kottler, Nicolas Richard, and the French network: "Phenotyping study, genetics and treatment of idiopathic hypogonadotrophic hypogonadism" (Department Genetics and Reproduction, CHU de Caen, avenue George-Clemenceau, 14033 Caen, France).

The acquisition of a sexually dimorphic phenotype is a critical event in mammalian development. Hypogonadotropic hypogonadism (HH) results from impaired secretion of GnRH. Male patients present delayed puberty micropenis and cryptorchidism, and females amenorrhoea reflecting gonadotropin insufficiency. Kallmann syndrome (KS) is defined by the association of HH and anosmia or hyposmia (absent of the sense of smell). Segregation analysis in familial cases has demonstrated diverse inheritance patterns, suggesting the existence of several genes regulating GnRH secretion. The X-linked form of the disease was associated with a genetic defect in the *KAL1* gene located on the Xp22.3 region. The *KAL1* gene encodes an extracellular matrix glycoprotein anosmin-1, which facilitates neuronal growth and migration. Abnormalities in the migratory processes of the GnRH neurons with olfactory neurons explain the association of HH with anosmia. Recently, mutations in the FGF receptor 1 (*FGFR1*) gene were found in KS with the autosomal dominant mode of inheritance. We also described a male patient homozygous for a mutation of FGFR1 presenting a cleft palate, corpus callosum agenesis, unilateral hearing loss, fusion of the 4th and 5th metacarpal bones. Interestingly, the "gain of function" mutations of this receptor are associated with a development defect called craniosynostosis, exhibited metacarpal abnormalities "in mirror" of those described in our patient. The role of *FGFR1* in the function of reproduction requires further investigations. Besides HH with anosmia, there are isolated HH. No human GnRH mutations have been reported so far, although hypogonadal mice due to a GnRH gene deletion exists. An increasing number of GnRH receptor (GnRHR) mutations have been described which represent about 50% of familial cases. The clinical features are highly variable and there is a good relationship between genotype and phenotype. A complete loss of function is associated with the most severe phenotype with resistance to pulsatile GnRH treatment, absence of puberty and cryptorchidism in the male. In contrast, a milder loss of function mutations causes incomplete failure of pubertal development. The preponderant role of GnRH in the secretion of LH by the gonadotrophs explains the difference of the phenotype between male and female with partial GnRH resistance. Affected females can have spontaneous telarche and normal breast development while affected males exhibit no pubertal development but normal testis volume, a feature described as "enuch-fertile". High-dose pulsatile GnRH has been used to induce ovulation. Another gene, called *GPR54*, responsible for idiopathic HH has been recently described by segregation analysis in two different consanguineous families. The GPR54 gene is an orphan receptor, and its putative ligand is metastin, the product of the *KISS-1* gene. Their roles in the function of reproduction are still unknown. However, mutations of *GPR54* appeared to be very low.

Use of organotypic culture to study human fetal testicular functions: effect of retinoic acid. Romain Lambrot[a], Hervé Coffigny[a], Catherine Pairault[a], René Frydman[b], René Habert[a], Virginie Rouiller-Fabre[a] ([a] INSERM U566/CEA/Université Paris 7, CEA/DSV/DRR, BP 6, 92120 Fontenay-aux-Roses, France; [b] Service de Gynécologie-Obstétrique, Hôpital Antoine Béclère, 92140 Clamart, France).

The two testicular functions, gametogenesis and steroidogenesis, are established during fetal and neonatal life. This onset is fundamental because adult fertility depends, partly, on the number of germ cells that are set up during fetal life. It is also the testosterone produced by fetal Leydig cells that is responsible for the masculinisation of internal and external genital organs. Retinoic Acid (RA) and its precursor, vitamin A, are essential for the maintenance of normal testicular functions in adult rodents. We have previously shown that RA also acts on gametogenesis and steroidogenesis in rat testis during fetal and neonatal development. Until now, no study has been conducted in humans, even though deficiencies in or excess of vitamin A are common occurrences. In this study, we developed an organotypic culture system of human fetal testis and studied the effect of RA on steroidogenesis and gametogenesis. The first step of our study was to characterise the two testicular functions

in this model. For steroidogenesis, the testosterone produced by human fetal testis in organotypic culture displays different profiles of secretion depending on the age of the fetuses. For the youngest fetuses (until the 7th week of development), testosterone secretion increases spontaneously during culture whereas it decreases for older fetuses, although, in vivo, it increases until the 14th week of development. So it lacks, in vitro, a factor which is necessary for the maintenance of secretion in fetuses over the 7th week of development. For the youngest stages, organotypic culture is a good tool for studying steroidogenesis and its regulation and we are trying to improve it for older ones. Gametogenesis is maintained in culture since the number of germ cells counted in a testis after three days of culture is nearly the same as that observed in the contralateral testis before culture. In the second part of our study we focused on the effect of RA on the two functions of the testis. This effect also depends on the stage of development of the fetus for steroidogenesis. Indeed RA stimulates testosterone production until the middle of the 7th week. So RA has a positive effect on the beginning of steroidogenesis. This effect is identical in mice but is the opposite of that observed in rats. For gametogenesis, the effect of RA seems to be close to that observed in rodents since RA decreases the number of germ cells in the testis. This effect is the result of an increase in proliferation of germ cells and an increase even stronger of their apoptosis. Thus RA appears as a regulator of both steroidogenesis and gametogenesis in human fetal testis during its early development.

Aromatase gene analysis in chromosomal hybrids of the two Tunisian chromosomal races of house mice *(Mus musculus domesticus).* Lamia Saïd[a,b], Sophie Lambard[b], Christelle Delalande[c], Brahim K. Ould[b], Ali Saâd[a], Khaled Saïd[b], Serge Carreau[c] ([a] Laboratoire de cytogénétique et de reproduction, Faculté de médecine Sousse, Tunisia; [b] Unité de recherche Génétique Biodiversité et Environnement, Institut Supérieur de Biotechnologie, Monastir, Tunisia; [c] Laboratoire de biochimie ISBIO, Université de Caen, France).

House mice (*Mus musculus domesticus*) in Tunisia have been characterised by the presence of two chromosomal races, one represented by mice carrying the 40-acrocentric standard karyotype and the other by a Robertsonian race (2n = 22) homozygous for nine centric fusions (Rb). The F_1 hybrids between the two chromosomal races showed a significant decrease of the reproductive success and litter size. Such results can be related to the formation of meiotic trivalents in these hybrids leading to the production of viable aneuploid gametes and post-zygotic elimination of embryos due to chromosomal non-disjunction events. Moreover, testicular histology of F_1 and backcross males showed in some cases a breakdown of spermatogenesis. The degree of this disturbance was not related to the level of chromosomal heterozygosity suggesting that genetic incompatibilities between the two genomes may be involved in addition to aneuploidy. The aromatase is a key enzyme for the irreversible transformation of androgens into estrogens. Several recent data showed that these hormones have an important role in the spermatogenetic process in addition to their well known female effects. In this study, we analysed the aromatase RNA messengers in the testis of the two parental races and their chromosomal hybrids by a semi-quantitative RT-PCR method. The results showed that the aromatase mRNA are as well present in hybrids as in the two parental races suggesting that this gene is not implicated in the breakdown of hybrid fertility. However, the hypothesis of a possible post-traductionnel modification of this enzyme deserves to be verified.

Effects of mono-(2-ethylhexyl) phthalate (MEHP) exposure on spermatogenesis in mice and changes in mSTI1 expression. Canan Mizrak[a], Dick G. de Rooij[a], Maria Paz Fernandez-España[b], Pedro P. Lopez-Casas[b], Jesus del Mazo[b] ([a] Department of Endocrinology, Faculty of Biology, University of Utrecht, Utrecht, The Netherlands; [b] Department of Cell and Developmental Biology, Centro de Investigaciones Biológicas, CSIC, Velazquez, Madrid, Spain).

Exposure to phtalates, which are commonly used in plastics, adversely affects testicular function. In the gut, phtalates are hydrolysed by esterases into active monoesters, which are the actual testicular toxicants. The corresponding esterase of di-(2-ethylhexyl) phthalate (DEHP), one of the most abundant phthalates, is called mono-(2-ethylhexyl) phthalate (MEHP). Both

DEHP and MEHP have been shown to cause reproductive toxicity in developing and adult animals. MEHP has been shown to affect germ cell development, impair fertility and to induce apoptosis in the testis after only a single dose administration. Also, MEHP adversely affects mitotically active neonatal rat gonocytes in vitro. This study was set up to analyse the effects of MEHP on testes function in more detail. Two weeks before mating, mice were exposed to drinking water containing either 1 mM, 500 µM or 100 µM MEHP. Control groups consisted of animals receiving plain water or water with vehicle (DMSO). Subsequently, for each treatment group exposure was ceased on the day of mating (Track A), day of birth of the offspring of treated mice (Track B) or at the end of postnatal week 4 (Track C). All mice were sacrificed on postnatal day 28 and the testes were removed, fixed in Bouin fluid and TUNEL analysis was performed. The percentage of tubuli containing TUNEL positive cells was statistically significant and increased in track B and track C. In a second set of experiments we studied the expression and localisation of possible target genes. We applied in situ hybridisation with a murine-stress inducible protein 1 (mSTI1) anti-sense and sense probe on Bouin fixed, paraffin embedded mouse testes. We were the first ones to localise mRNA of the mSTI1 gene in the testis. mSTI1 is a co-chaperone that is homologous to the human heat shock cognate protein 70 (hsc70)/heat shock protein 90 (hsp90)-organising protein (Hop) (6). mSTI1 has been described to have ten potential tetratricopeptide repeat (TPR) motifs, a putative nuclear localisation signal (NLS), six potential phosphorylation sites for casein kinase II and a central proline-rich region (6). mSTI1 was detected in major mouse organs such as lung, liver, skeletal muscle, spleen and brain with Western blot analysis. mSTI1 mRNA was mainly localised in the nuclei of spermatogonia and round spermatids uptill elongating spermatids. Comparing the testes of control animals and MEHP exposed animals, we observed a difference between the mRNA localisations of this gene. Similarly, human STI1 (hSTI1) expression was shown to be up-regulated approximately 2-fold following viral transformation and was reported to be localised primarily to the nucleus in SV-40 transformed MRC-5 fibroblasts, whereas in untransformed MRC-5 cells the localisation around the Golgi apparatus was observed. As a first result of our study, we can clearly say that MEHP induces apoptosis of germ cells in the testis. In our study we, for the first time showed the localisation of the mRNA of mSTI1 gene in testis with in situ hybridisation. When we looked at the effect of MEHP exposure on mSTI1 gene expression, we observed the up-regulation of mSTI1 after MEHP treatment. The possible mechanism could be a stress response caused by MEHP exposure and thereafter possible recruitment of heat shock proteins and consequently and eventually the up-regulation of mSTI1 expression. With further studies the cellular mechanism of mSTI1 expression will be revealed. This research has been carried out in the association of the GENDISRUPT project, supported by the European Commission, 5th Framework Programme of Research, Key action 4 "Environment and Health" within the "Quality of Life and Management of Living Resources".

Aromatase in the human adrenal: expression in the normal and tumoral adrenal cortex.

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The aromatase enzyme catalyses the final stage of the estrogen biosynthesis pathway from its substrate androgen. This enzyme is expressed in the ovary and testis, its expression in the normal adrenal is debated. A major aromatase overexpression has been demonstrated in case reports of estrogen-producing adrenal tumors. The aim of that work was to compare the protein and/or gene *CYP19* aromatase expression levels in (i) 2 adrenal feminizing tumors (FT) (ii) 10 adrenocortical adenomas with clinical hypercorticism (CH, 4/10) or sub-clinical hypercorticism (SCH, 6/10) (iii) 3 aldosterone-producing adenomas (APA) and (iv) 6 normal adrenals (NA). The tissue aromatase activity was determined by the tritiated (^{3}H)-water method. Aromatase mRNA quantification was achieved by competitive RT-PCR. A high aromatase activity was measured in the two feminizing tumors FT1 and FT2 (× 7 and × 40 compared to NA). Aromatase activity

was detected in all groups of adrenocortical adenomas at a level similar to that of the NA group, with a trend towards significance for a lower level in SCH vs. NA. Aromatase mRNA overexpression is major for FT2 but absent for FT1. Mean aromatase mRNA level in SCH was lower than in NA and APA adenomas, while no difference was observed between CH and APA compared to NA. Our results highlight the variability of aromatase overexpression in FT of identical phenotype. It confirmed the expression of the enzyme at low level in the normal adrenal. An expression of the aromatase enzyme has been demonstrated for the first time in hormone-producing adrenocortical adenomas, with a lower expression in SCH compared to NA and APA. Further studies will specify the regulation of aromatase expression and will characterize the aromatase promoter utilisation.

BMP-5 expression and action in the rat ovary. Alice Pierre, Claudine Pisselet, Martine Bontoux, Philippe Monget (Physiologie de la Reproduction et des Comportements, UMR 6175 INRA/CNRS/Université F. Rabelais de Tours/Haras Nationaux, 37380 Nouzilly, France).

Ovarian folliculogenesis is a complex process involving granulosa cell proliferation and differentiation. Growth factors are able to modulate the actions of FSH and LH during this process. Among them, the Bone Morphogenetic Proteins (BMP) were recently studied. Bone morphogenetic proteins comprise a large group of polypeptides from the transforming growth factor β superfamily, with essential physiological functions in morphogenesis and organogenesis in vertebrates and invertebrates. The role of BMP in ovarian folliculogenesis has recently been studied since the discovery of mutations in the genes of three members of this family. Several studies in the rat and the ewe using in situ hybridisation or immunohistochemistry have shown the expression of different elements of the BMP signalling pathway in the ovary. We have found in UniGene (http://www.ncbi.nlm.nih.gov/UniGene) that is one of a member of TGFβ, the Bone Morphogenetic Protein 5 (BMP-5) were highly represented in libraries from mouse egg and ovaries. So we studied the site of expression of BMP5 in the rat ovary by in situ hybridisation, and its putative role on granulosa cells in vitro. Here we show that BMP-5 is expressed by granulosa cells in the rat ovary from preantral to preovulatory follicles and at all stages of the sexual cycle. BMP-5 induced in vitro a marked decrease in basal and FSH induced progesterone but not estradiol production, BMP-5 also had a proliferative effect in granulosa cells with an increased accumulation of cyclin D2 in vitro. BMP-5 was also able to phosphorylate smad-1 in granulosa cells in vitro. In conclusion, BMP-5 is a new ligand expressed by the rat ovary, and might play a paracrine autocrine role in ovarian follicles by regulating proliferation and steroidogenesis of granulosa cells. Its biological action might be mediated by smad-1 phosphorylation.

Expression and regulation of the Stearoyl-coenzyme A (CoA) desaturase 2 (SCD2) in rat ovary. Céline Moreau, Pascal Froment, Virginie Moreau, Philippe Monget, Joëlle Dupont (Physiologie de la Reproduction et des Comportements UMR 6175 INRA/CNRS/Université de Tours/Haras Nationaux, 37380 Nouzilly, France).

Oocytes of all mammals contain an endogenous lipid reserve including mono and polyunsatured fatty acids. This feature reflects their ancestral origin, the yolk-rich amniote egg. Despite the significant role of the lipid reserve in cell structure and function, very few studies have provided detailed descriptions of the mono or polyunsatured fatty acid synthesis in the ovary. Here, we show by RT-PCR the mRNA expression of delta 5, 6 and 9 desaturase (SCD2 and SCD1) in the rat ovary. More particularly, we focused our study on SCD2 because of its high level of expression. By Northern-blot, two transcripts (3.9 and 5.2 kb) of SCD2 mRNA were detected in the rat ovary. Furthermore, we localised SCD2 mRNA by in situ hybridation mainly in granulosa cells of antral follicles, the cumulus oophorus (granulosa cells around the oocyte) and the corpus lutea. Interestingly, no SCD2 expression was observed in primordial follicles and oocytes. Moreover, we determined the SCD2 mRNA expression in vivo in rat ovaries during an artificially induced ovulatory cycle by using PMSG/hCG treatment on immature 22-day old rats. After PMSG injection for 48 h, the level of SCD2 mRNA in the ovaries increased by 4 fold as compared to those observed in the ovaries of untreated rats. Furthermore, this effect was enhanced with the hCG treatment for

48 h. Interestingly, these changes of SCD2 mRNA were correlated with those observed for the StAR protein. We also determined the SCD2 mRNA expression in the corpus lutea during gestation in the rat. We show a decrease in the SCD2 mRNA expression at day 15 of gestation, which is parallel to a fall in progesterone secretion. In order to investigate the molecular mechanisms involved in the SCD2 mRNA regulation in the ovary, we performed primary culture of rat granulosa cells. These cells were stimulated for 48 h with IGF-1 (10-8M) or FSH (10-8M). Northern-blot analysis revealed that both IGF-1 and FSH increased by about two fold the SCD2 mRNA expression. As previously shown, these two hormones were also able to increase protein levels of StAR and P450scc as well as progesterone secretion. In granulosa cells, IGF-1 is known to activate different signalling pathways including the MAPK (ERK1/2, JNK, p38) and the PI3K/Akt pathways. Using specific pharmacological inhibitors we demonstrated that the MAPK ERK1/2 pathway is involved in the IGF-1-induced SCD2 mRNA expression. The signalling pathways involved in the FSH-induced SCD2 mRNA expression remains to be determined. Taken together, the delta 9 SCD2 desaturase is expressed in the rat ovary and it may be involved in the regulation of follicular growth and/or oocyte maturation.

Effects of maternal undernutrition during lactation on the estrogen and androgen serum levels and on these receptor expression in rat testes at weaning. Cíntia Vilanova Teixeira, Alba Marcelly de Souza Santos, Francisco J.B. Sampaio, Cristiane da Fonte Ramos (University State of Rio de Janeiro, Brazil).

The goal of this study was to evaluate the effects of maternal malnutrition during lactation on testicular weight, testosterone and estradiol serum concentration, testosterone testis concentration, androgen and estrogen α receptor expression, by Western blot technique, in pups at weaning. From parturition until weaning, Wistar rats were separated into three groups: (C) control group, with free access to a standard laboratory diet containing 23% protein; (PER) protein-energy restricted group, with free access to an isoenergy and protein-restricted diet containing 8% protein; and (ER) energy-restricted group, receiving a standard laboratory diet in restricted quantities, which were calculated according to the mean ingestion of the PER group. All pups were sacrificed at weaning. Body ($C = 48 \pm 2.3$, $PER = 20 \pm 1.3$, $ER = 25.4 \pm 0.9$, $P < 0.01$) and testicular ($C = 0.15 \pm 0.02$, $PER = 0.05 \pm 0.007$, $ER = 0.06 \pm 0.0025$, $P < 0.001$) weights of both PER and ER groups were lower as compared to the C group. The testosterone serum concentration was significantly higher only in the PER group ($C = 0.09 \pm 0.012$, $PER = 0.45 \pm 0.04$, $ER = 0.15 \pm 0.03$, $P < 0.01$). Testosterone testicular concentration ($C = 2.1 \pm 0.43$, $PER = 6.5 \pm 0.7$, $ER = 13 \pm 2.3$, $P < 0.01$), AR ($C = 0.5 \pm 0.1$, $PER = 3.2 \pm 0.3$, $ER = 1.1 \pm 0.1$, $P < 0.01$) and ER ($C = 0.2 \pm 0.03$, $PER = 1.3 \pm 0.3$, $ER = 0.9 \pm 0.3$, $P < 0.05$) testis expression were also high in both PER and ER groups. The estradiol serum concentration was lower in both dietary groups ($C = 74 \pm 4.6$, $PER = 49 \pm 3.2$, $ER = 60 \pm 5.5$, $P < 0.01$). These data reinforce the concept that a nutritional state in early phases of development is important since maternal malnutrition during lactation led to changes in the urogenital system altering the estradiol and testosterone serum concentrations, the testicular testosterone concentration, AR and ER α receptor expression. Financial support: CNPq, FAPERJ, CAPES.

W16S substitution of GnRH peptide signal could be associated to a hypogonadropic hypogonadism phenotype without anosmia. Nicolas Richard[a], Peter Kamenicky[b], Adèle Hamel[a], Jacques Young[b], Marie-Laure Kottler[a], GIS Network ([a] Département Génétique et Reproduction, CHU de Caen, France; [b] Service Endocrinologie et maladies de la reproduction, CHU Kremlin-Bicêtre, France).

Deficiency of GnRH (Gonadotrophin Release Hormone) results in an inhibition of pituitary gonadotrophin secretion (LH and FSH) responsible for a hypogonadotropic hypogonadism phenotype (HH). Two aetiologies are well documented to date: a default of migration of GnRH neurons (Kallman Syndrome), or a default of binding to the GnRH receptor. We know of three main forms of GnRH, which differ in their affinity to the receptor and ability to stimulate gonadotrophin secretion but only GnRH type I and II are expressed in humans, with GnRH type I (GnRH-I) being essential. GnRH-I is a 10 amino acid (AA) peptide obtained from post transcriptional modification of a preprohormone of 92 AA

which includes a peptide signal and GAP (GnRH-Associated Peptide), the active form being released after cleavage by a proconvertase. The *GnRH* gene is mapped on chromosome 8 and constituted of 4 exons, 3 of them are coding, separated by large introns. The complete coding sequence of GnRh is included in exon 2. In the present study, we screened the *GnRH* gene (GnRH-I) mutation in 29 patients diagnosed with hypogonadotropic hypogonadism phenotype with neither anosmia nor a mutation of the GnRH receptor After DNA extraction from peripheral blood leukocytes, each exon was amplified by PCR and sequenced using a CEQ 8000 autosequencer (Beckman Coulter, France). We identified a G/C transversion at position 1120 (exon 2) responsible for a tryptophane No. 16 substitution by serine, this codon belonging to the signal peptide. We found this mutation in 12 patients, 2 at the homozygous state (C/C), the others at the heterozygous sate (G/C). So, allelic frequencies were 0.76 for G and 0.24 for C. Potential change of biochemical characteristics of the molecule is hypothesised to be important (a hydrophobic amino acid replaced by a polar amino acid) in a manner to disrupt GnRH secretion outside the cellular compartment. Thus, we screened this variation in a control population without the hypogonadotropic hypogonadism phenotype (n = 49). We tested a method based on allelic discrimination allowing fast screening of the population. We used 3 primer types, one common with the 2 types of the allele, one specific of normal allele with G at the 3'end, and one specific of the allelic variant with C at the 3'end. Thirteen people were heterozygous (G/C), and 2 were homozygous (C/C), with an allelic frequency of 0.83 for G and 0.17 for C. Statistical analysis showed no allelic frequency difference between the control population and patients with HH allowing us to conclude that W8S substitution is only a single polymorphism. In conclusion, no mutation in the *GnRH* gene has been described so far in humans whereas there is a model of *GnRH-I* deficient mice (Mason et al., 1986). Despite amino acid modification (a hydrophilic serine instead of a hydrophobic tryptophane) at the signal peptide, this change can not be involved in the hypogonadotropic hypogonadism phenotype.

Telomerase activity of adult mouse testis. Lydia Riou, Henri Bastos, Bruno Lassalle, François Boussin, Isabelle Allemand, Pierre Fouchet (Laboratoire de Gamétogenèse, Apoptose et Génotoxicité, UMR U566 INSERM/CEA/Paris VII, France).

In the adult, the testis is one of the organs in which the strongest telomerase activity is exhibited. This activity protects chromosomes from telomere attrition and ensures the transmission of full-length chromosomes to progeny. Late-generation (4th) of mTerc$^{-/-}$ (RNA component of mouse telomerase) mice is infertile, showing the importance of telomerase in spermatogenesis. The telomere hypothesis suggests that telomerase activity is high in embryonic cells and that it decreases in somatic tissues during development and differentiation. Previous studies have not detected telomerase activity in spermatozoa. However, little is known about telomerase activity in other specific testis cell types in the adult. As in other self-renewing tissues in the adult, spermatogenesis is a differentiation process which originates from a small pool of diploid stem cells and results in the production of haploid spermatozoa. In adult rodents, germ cell differentiation is continuous and each step is concomitantly present inside the seminiferous tubules of the testis. Consequently, the germinal cell population is highly heterogeneous and includes diploid pre-meiotic and meiotic cells, haploid postmeiotic cells, as well as somatic cells. Recently, our group clearly discriminated viable germinal populations by flow cytometry according to Hoechst 33342 DNA stain analysis. This method enables isolating spermatocytes I (meiotic population), rounds and elongated spermatids, as well as premeiotic spermatogonial and germinal stem cells assigned to the Side Population (SP). The phenotype SP is due to an active efflux of Hoechst 33342 depending on ABC transporter BCRP1. We took advantage of this method in order to analyse telomerase activity in these populations, especially in the Side Population. Cellular therapy based on transplantation of germinal stem cells is an interesting alternative for treatment of infertility. In cases of infertility following chemotherapy or radiation therapy of children or young men affected by cancer, stem cells should be sampled on the patient before treatment, cryopreserved and injected in the testis after recovery in order to restore spermatogenesis and fertility. Studies have shown that germinal stem cells of immature or adult mice initiated spermatogenesis after transplantation into the testis of infertile mice.

However, the molecular mechanisms regulating self-renewal and engagement in differentiation remain to be studied and especially the implication of telomerase in these mechanisms.

Regulation of the intronic promoter of rat estrogen receptor alpha gene. Diane Schausi, Christophe Tiffoche, Colette Vaillant, Marie-Lise Thieulant (Université de Rennes I, Interactions Cellulaires et Moléculaires, Équipe Information et Programmation Cellulaires, UMR 6026-CNRS, Campus de Beaulieu, 35042 Rennes Cedex, France).

A natural Estrogen Receptor (ER) variant, named TERP-1 (Truncated ER Product-1), has been shown to be specifically and transiently expressed in the rat lactotrope cells (Demay et al., Neuroendocrinology 1996, 63: 522–529). TERP-1 expression is variably expressed depending on the hormonal environment, dramatically increasing throughout the proestrous stage and the second half of gestation whereas the TERP-1 protein levels are abruptly inhibited by lactation. In both situations, TERP-1 expression increased concurrently to serum E2 level and decreased with increasing PRL (Vaillant et al., Endocrinology 2002, 143: 4249–4258). E2 seems to be the factor inducing TERP-1 expression. The signal(s) required to suppress abruptly the TERP-1 expression remain unknown. However, we showed that PRL dramatically decreases TERP-1 mRNA expression in MMQ cells, a pure lactotrope cell line. TERP-1 expression is depending on an intronic promoter of rat ERα gene (TERP-1 promoter) (Tiffoche et al., Endocrinology 2001, 142: 4106–4119), which is transcriptionally the most active in the lactotrope cells. A minimal –693 bp region encompassing the TATA box is sufficient to allow lactotrope-specific expression. Deletion and mutational analyses, and EMSA allowed us to demonstrate that the TERP-1 response to estrogen is mediated through an ERE located in the proximal region of the TERP promoter. In addition, ER acts in synergy with the pituitary-specific transcription factor Pit-1. The homeoprotein Pitx-1/2 is also able to activate the promoter but does not enhance E2 activity (Schausi et al., Endocrinology 2003, 144: 2845–2855). Search on the TERP promoter sequence has revealed no consensus GAS-response element for PRL signaling. However, a number of AP-1 response elements have been identified in the vicinity of the ERE. EMSA identified two functional AP-1 binding sites (–146 and –10). Site-directed deletion of these AP-1 sites reduced the basal activity of the TERP promoter in pituitary lactotrope cells. Surprisingly, mutating the –146 AP-1 site significantly enhanced ER/E2 transactivation, suggesting that the –146 AP-1 site plays a role in inhibiting ER gene expression in the presence of the hormone. Indeed, we demonstrated that the E2 activation of the promoter could be antagonized by PMA. Then, it appears that the –146 AP-1 site could be involved in mediating estrogen responsiveness of the TERP gene. The biological role of TERP is not very well understood. Although TERP-1 lacks the A/B-, DNA binding, -hinge region, and a portion of ligand binding domain, we show that the truncated protein can form heterodimers with ERα, and exert a role of a negative dominant. TERP-1 likely acts as a negative regulator of ERα activity in vivo, and could exert a protective role against the high level of E2 observed at the proestrous stage or in late pregnancy contributing to regulate the E2-induced activation of proliferation of lactotrope cells.

Detection of the mitochondrially encoded Cytochrome c Oxidase Subunit I in adult rat germ cells. Sonia Bourguiba, Ans M.M. Van Pelt, Dirk G. de Rooij (Department of Endocrinology, Faculty of Biology, University of Utrecht, The Netherlands).

Male offspring exposed in utero to antiandrogens often display alterations in androgen-dependent developmental markers (e.g. anogenital distance, nipple retention) together with clearly adverse responses such as genital malformations and reproductive tract lesions. The objectives of this study were to determine whether in utero exposure to flutamide results in permanent alterations in gene expression within the adult rat testis. Microarray analysis revealed that the Cytochrome C Oxidase subunit I (COX also called complex IV) gene is up regulated in the adult testis of parentally flutamide (10 $mg \cdot kg^{-1} \cdot day^{-1}$) treated rats compared to a control. To confirm this result, we analysed COX I mRNA expression by real-time RT-PCR. Expression of the COX I gene was detected in flutamide-treated testis, however this analysis was unable to confirm any alterations in COX I gene expression.

Western blot results, using a monoclonal antibody anti-COX I, displayed similar findings. A direct approach to identify the cellular source of COX I in the adult rat testis is the use of immunohistochemistry with a specific antibody that recognises this protein. In this study, we report a distinct expression pattern of COX I in rat spermatogenic cells. The highest levels of COX I were found in the cytoplasm of germ cells from mid to late pachytene spermatocytes onwards. RT-PCR with adult rat testis and purified germ cell fractions show high COX I mRNA expression in pachytene spermatocytes and round spermatids and weak expression in earlier spermatogenic cells. In mammals, Cytochrome C Oxidase, the terminal enzyme complex of the electron transport chain, consists of 13 subunits with a mixed genetic origin. The core of the COX complex is composed of its 3 largest subunits (I, II, and III), which are encoded by mitochondrial DNA and inserted into the inner membrane. The other 10 subunits are encoded by nuclear DNA. In male germ cells, mitochondria exhibit a highly complicated change in their structure during spermatogenesis. Taken together these results showed that flutamide in utero exposure could not imprint the mtDNA coding for COX I, since no significant differences in either mRNA nor protein expression were observed between the adult testis of flutamide–treated rats and the control. Because the COX I protein is specifically expressed in germ cells in the testis, it seems to be probable that COX I might be correlated with these structural and biochemical changes of the mitochondria in germ cells. Furthermore, the high levels of expression of COX I mRNA and protein in pachytene and round spermatids may reflect requirements for alterations in energy since these cells start meiosis and spermatid maturation. This study has been carried out with financial support from the commission of the European Communities, specific RTD programme "Quality of life and management of living resource", QLRT-2000-00684 "Identification of critical rat testicular genes altered after androgenic disruption by flutamide: Use of DNA microarray" (ENDISRUPT).

Radio-induced modification of the Sertoli genome in the prenatal period. Cécile Vissac-Sabatier, Évelyne Moreau, René Habert, Chrystèle Racine (INSERM U566/CEA/Université Paris 7, CEA/DSV/DRR, BP 6, 92120 Fontenay-aux-Roses, France).

During the development of the testis, the patterns of proliferation and differentiation of the germ cells and Sertoli cells are closely connected. So, Sertoli cells play a central role in the development of a functional testis, and hence in the expression of a male phenotype. Moreover, Sertoli cells have been shown to be sensitive to ionising radiations only during the prenatal and neonatal periods, when they are actively proliferating. The aim of this study was to evaluate the radiosensitivity of Sertoli cells in vitro to allow future studies of gene expression differences between control and irradiated Sertoli cells by cDNA microarray. One day post partum (dpp) NMRI mice testis cells were isolated by enzymatic digestion of seminiferous cords followed by selective depletion of contaminating cells either by sedimentation (Leydig cells) or by selective sorting on an alpha–6 integrin column (peritubulars cells). The remaining cells were then cultured for 24 h on a laminin support before the germ cells were eliminated by an osmotic choc. The purity of the remaining cells, as assessed by immunocytochemistry using the Stem Cell Factor (SCF) as a marker, showed 97% of Sertoli cells. We determined the cell radiosensitivity by testing different periods after a 5 Gray (Gy) irradiation. Two periods (4 and 48 h) were tested on three parameters: cell viability (blue trypan exclusion), proliferation (Brdu method) and apoptosis (Apo-Brdu Kit). Our first results show that the percentage of living cells after 4 and 48 h was roughly 92 and 82% respectively in both normal and irradiated cells. No apoptosis was detected after 4 or 48 h, neither in normal nor irradiated cells. Regarding the proliferation, however, a strong inhibition was noted but only for the longest period after irradiation. These results suggest that Sertoli cells have a good repair system, which will be investigated by the comet assay. Finally, cDNA microarray allows testing the expression of thousands of genes simultaneously and identifying the genes of interest after irradiation.

Spermicidal activity of dermaseptins. Amira[a] Zairi, Mounir[b] Ajina, Ali Saâd[b], Khaled[a] Hani ([a] Laboratory of Biochemistry, Faculty of Medecine, Sousse, Tunisia; [b] Laboratory of Cytogenetic and Biology of Reproduction, Hospital Farhat-Hached, Sousse, Tunisia).

The Spermicidal efficacy of two synthetic antimicrobial peptides, dermaseptin (DS1 and DS4),

were studied under in vitro conditions using human spermatozoa. The data showed that sperm motility was inhibited with various concentrations of dermaseptin at different intervals ranging from 2 to 240 min. The effective 100% inhibitory concentration (EC100) of DS4 in 30 min, of the sperm immobilisation assay was equal to 50 $\mu g \cdot mL^{-1}$, whereas sperm immobilisation EC100 of DS1 was equal to 100 $\mu g \cdot mL^{-1}$. The presence of 0.1% of chelating agent, EDTA, reduced EC100 of DS4 to 5 $\mu g \cdot mL^{-1}$, while less than a two fold enhancement in DS1 activity was observed upon combination with EDTA. The action of dermaseptins on sperm motility was observed to be dose-dependant. Besides, supplementation of pentoxifylline and calcium that are known to enhance the motility of the sperm, this could not prevent the spermicidal action of dermaseptins. In view of this fact, it is suggested that DS4, having anti-bacterial, anti-viral, antifungal and spermicidal activities, could be a potent vaginal contraceptive.

Histo-morphometric and biochemical effects of mercury chloride on testicular function in wistar rat. Abdelkader Oumeddour[a], Taharaoui Abdelkarim[a], Wafa Benchalel[a], Farida Ali-Rachdi[b], Hamid Amari[c], Mohamed Guelatti[a] ([a] Laboratoire de biologie animale appliquée, Université Badji-Mokhtar Annaba, Algeria; [b] Service d'anatomie pathologique, CHU Ibn-Roched Annaba, Algeria; [c] Service PMA, Clinique Al-Farabi, Annaba, Algeria).

The effect of mercury chloride on the reproductive capacity and the corticotropic response of male Wistar rats was studied. The Oral administration of mercury chloride (2.5, 5 and 10 $mg \cdot kg^{-1}$ during 10 days) induced a decrease of whole body as well as testicular and prostatic weights. In addition a significant diminution of blood testosterone and corticosterone concentrations were registered respectively in the three groups of treated rats and the two groups recieving 5 and 10 $mg \cdot kg^{-1}$ of mercury chloride. After histological studies of the testes, a blocage of spermatogenesis was observed especially at the stage of round spermatid. Germ cells are present in the lumen of seminiferous tubules. Leydig cells and germ cells show apoptotic features. These data demonstrated that mercury chloride alters the spermatogenesis via germ cell destruction and testosterone deficit.