The paternal sperm epigenome: implications for development and disease

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Despite the father transmitting half the heritable information to the embryo the focus on preconception health has largely been on the mother. New studies highlight the role of the father in disease transmission via non-genetic inheritance, through epigenetic mechanisms. Epigenetic mechanisms include, DNA methylation, post-translational modifications of histones and noncoding RNAs. Paternal effects have been linked to developmental abnormalities and complex diseases such as cancer, diabetes and obesity. Studies in humans and animals have linked epigenetic inheritance to the transmission of environmentally induced phenotypic traits from the father to the developing embryo and these have been associated with altered gene expression and developmental abnormalities in first and second offspring generationsThe genome in mature spermatozoa is packaged in a very unique manner. Whereas the majority of the genome is packaged by sperm-specific nucleoproteins, the protamines, regulatory regions of many genes retain nucleosomes. Moreover, such nucleosomes carry posttranslational modifications in a manner that is highly suggestive of a function in embryo development. In our previous study we show that paternal folate deficiency (FD) is associated with increased birth defects, minor alterations to DNA methylation in the sperm including at genes implicated in development and chronic disease (Lambrot *et al*., 2013. *Nat Commun*). We also showed folate deficiency altered global levels of histone methylation in sperm, suggesting their involvement in epigenetic inheritance.

To address the role of modified histones in sperm for embryonic development we generated transgenic mice over-expressing the human histone demethylase LSD1/KDM1A.

Offspring sired by such transgenic mice suffered from major developmental abnormalities and increased neonatal death. Remarkably, overexpression of KDM1A in heterozygote fathers affected wild type offspring as well, even for three following generations.

ChIP-Seq studies of sperm of transgenic fathers revealed that H3 lysine 4 dimethylation levels were reduced at promoters of over 2300 genes, many of which serve important functions during embryonic development. In contrast, nucleosome occupancy levels were unaltered at such genes, indicating that KDM1A overexpression affects homeostasis of H3K4me2 during spermatogenesis. Together, our data from environmental exposures and transgenicsstrongly support the idea that disruption of sperm epigenome homeostasis is extremely detrimental for embryonic fitness of offspring.

Our data reveals the potential of genetic mutations in chromatin modifiers and of environmental-induced alterations to the sperm epigenome as an underlying cause of birth defects and disease that may be traceable to the father.

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Concordance of the toxicity of endocrine disruptors in humans and in animals

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Over the last two decades, the scientific community has lifted the veil on the existence of the so-called "endocrine disruptors" (EDs) which may deleteriously affect hormonal balances in the body. These revelations have alarmed both the general population and public authorities. At first, considerable attention has been paid to the chemicals which display estrogenic properties (Toppari *et al*., 1996). In 1994, Kelce and collaborators used the term "anti-androgenic" to characterize the activity of the pesticide vinclozolin and its metabolites (Kelce *et al*., 1994). The effects of these EDs are susceptible to interfere with major physiological processes, such as masculinization, secondary sex characteristics establishment, and even bone metabolism.

In order to understand the likeliness of the human health impact of exposure to the chemicals of interest, various complementary disciplines have been mobilized which notably include epidemiology, biomonitoring, toxicology using both laboratory animals and human biological materials, and ecotoxicology (the "sentinel species" concept). While the latter concept has not really been yet validated, the development of *in vitro* and *in vivo* models in toxicology that are predictive for adverse effects in humans exposed to chemicals has represented a major task over the last two decades. The pertinence of these models is based on the assumption that the choice of animal models and the design of the studies are effectively predictive of human hazard. However, there has been a recent general rising concern of how well animals experiments, animal tests and tests using human biological materials are predicting human safety in the context of the screening of EDs.

Our presentation will provide a critical assessment of *in vivo*, *in vitro*/*ex vivo* and xenografting approaches which have been undertaken to explore the effects of EDs on the male reproductive health in rodents and in humans. Emphasis will be put on key issues such as *(i)* how the animal does predict human safety, *(ii) in vivo* and *ex vivo* models that are likely to be predictive for deleterious effects in humans exposed to EDs, *(iii)* how differences in the physiology of mice, rats and men can infer in the way these species, or the tests developed from these species, respond to the exposure to EDs, *(iv)* what are the usual, diverse and (hopefully) surmountable obstacles to EDs risk assessment.

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Determinants of adult male reproductive health – it's all in the womb

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Reproductive disorders of human males are remarkably common, and for some disorders at least the incidence has been increasing progressively in recent decades, indicating that (unknown) lifestyle/dietary/environmental factors must be responsible. These disorders comprise those that are evident at birth (cryptorchidism, hypospadias) and those that only emerge in young adulthood (low sperm counts, testicular germ cell cancer, altered Leydig cell function). It is common for two or more of the disorders to be associated, and overall there is now strong evidence that many cases of these disorders may comprise a testicular dysgenesis syndrome (TDS) with a common origin in fetal life. Moreover, there is increasing evidence that TDS disorders may stem from subtle deficiency in fetal androgen production/action. TDS disorders are important also in a wider health context, as several of them are also associated with reduced lifespan/poorer general health, especially during aging. For example, low adult testosterone levels are associated with most of the so-called 'Western diseases' related to obesity and the metabolic syndrome. Therefore, better understanding about the causes and origins of TDS disorders may have implications for improving general male health as well as reproductive health.

The big problem with the TDS hypothesis is that studying it in a meaningful way in humans is all but impossible, especially for adult-onset disorders, as this requires 'seeing back in time' several decades to try and discern how such a disorder may have arisen. Therefore, we have resorted to an animal model of TDS to (1) validate the TDS hypothesis, (2) identify if there is a critical period of fetal life when later TDS disorders are programmed by deficiency in fetal androgens, and (3) identify pathophysiological read-outs of (2) that can be used in humans to identify if individuals in whom a TDS disorder is diagnosed shows any measurable evidence for fetal androgen deficiency. In the longer term, it is hoped that this understanding will enable prevention or intervention to reduce the risk of TDS disorders, which may thus also improve general health during aging.

The biggest development from our TDS model has been to identify that androgen action within a discrete masculinization programming window (MPW; e15.5-e18.5 in rats) is what determines normality and ultimate size of all male reproductive organs. Anogenital distance (AGD) is also programmed by androgen action specifically within the MPW, and thus provides a lifelong means of 'seeing back in time' to read-out the level of androgen exposure in the MPW.In both experimental animal models and in humans with TDS disorders, occurrence of the disorders goes hand-in-hand with reduced AGD. Thus, reduced AGD is associated in adulthood with increased risk of cryptorchidism, hypospadias, smaller penis and testes, increased risk of low sperm counts and infertility and lower testosterone levels. Therefore, AGD measurement in humans offers the possibility of retrospectively measuring androgen exposure within the MPW; in humans the MPW is thought to be within the period of ~8-12 weeks' gestation.

Using our animal model of TDS, we are able to show that androgen deprivation during the MPW, but not at later stages of gestation, is responsible for all common male reproductive disorders. We also show that this is intimately associated with occurrence of focal dysgenetic features in the testis, both during fetal life and in adulthood; the features in adulthood are commonly seen in infertile men/those with low sperm counts and those with testicular germ cell cancer. The most recent development has been to show how fetal androgen exposure during the MPW programmes adult Leydig cell function via effects on the stem cells for adult Leydig cells, which are androgen targets in the fetal testis of man and rodents. Deficiency in fetal intratesticular androgens in rats during the MPW (but not later in gestation or after birth) results in compensated adult Leydig cell failure in adulthood (ie low/normal testosterone in the face of elevated luteinizing hormone levels). As compensated Leydig cell failure is a common finding in men with low sperm counts, even in young adulthood, these findings suggest that this condition may be another manifestation of TDS. If this is the case, it would be predicted that such men should have reduced AGD, as is the case in the animal model.

These studies show the benefits of a well-defined animal model for human disease, as it can identify endpoints that can be used clinically to improve diagnosis and understanding.

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Testicular function of normal European men - A matter of concern

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The debate whether semen quality in general has decreased reemerged by the publication in 1992 in BMJ showing that semen quality had decreased by 50% during a 50 years period (Carlsen *et al*., 1992). Several other publications followed, and some detected a temporal trend whereas other did not. The overall conclusion that semen quality has declined is mainly based on retrospective, historic data, which limits their significance, and the topic has been controversial (Jouannet *et al*., 2001). In contrast, the 4-5 times increased incidence rates of testicular cancers (Nordkap *et al*., 2012) in various populations during the same period are generally accepted.

To overcome some of the problems of the publications dealing with historical data several standardized and coordinated studies of semen quality of young men not selected due to fertility status and partners of pregnant women (i.e. fertile men) have been undertaken in various European countries, the US and Japan (Andersen *et al*., 2000; Jørgensen *et al*., 2001, 2002; 2011; Punab *et al*., 2002; Swan *et al*., 2003; Iwamoto *et al*., 2006; 2013a, b; Paasch *et al*., 2008; Mendiola *et al*., 2011; Fernandez *et al*., 2012). Some of the studies of the young men, which have been ongoing since the late 1990's, have shown a somewhat heterogeneous temporal pattern. A decrease of more than 20% in total sperm counts and sperm concentration has been shown for Finnish men between 1998 and 2006 (Jørgensen *et al*., 2011), and a decrease of approximately 15% has also been indicated for young Spanish men during the recent decade (Mendiola *et al*., 2013) whereas no changes were observed between 2000 and 2010 for Swedish men (Axelsson *et al*., 2011). In contrast, an increase in total sperm count and sperm concentration (approximately 14 and 12%, respectively) among Danish men in the period 1996-2010 was observed (Jørgensen *et al*., 2012). For the Danes the increase in median sperm concentration was from 43 to 48 mill/ml, but still compatible with a pronounced reduction in both total sperm counts and sperm concentration when compared with Danish data from the early 1940's despite that these data originated from male partners in infertile couples (Hammen *et al*., 1946). Furthermore, recently, a French study corroborated the previously indicated decreasing trend among French men (Auger *et al*., 1995; Rolland *et al*., 2013; Le Moal *et al*., 2014).

Several studies have shown that chances of conception increase when sperm concentration increases towards 40-60 mill/ml (Bonde *et al*., 1998; Slama *et al*., 2002) and the number of spermatozoa with normal morphology increases to 9-12% (Guzick *et al*., 2001). According to the current WHO guidelines (World Health Organization, 2010) a sample is classified as normal if the sperm concentration is 15 million per mill or more, the number of morphologically normal spermatozoa is 4% or more and more than 32% have good motility. There is little doubt that many men with semen parameters at that level or lower may be infertile with a need of ICSI (intracytoplasmic sperm injection) if they want to reproduce. On the other hand, there is no guarantee that a man with better semen quality, including higher sperm counts has normal fecundity (ability to reproduce; Skakkebaek and Jørgensen, 2010).

A striking feature of the semen quality of young normal men from the general population is the high frequency of men with poor semen quality in all countries despite the inter country differences. When interpreted against studies describing associations between pregnancy chances and sperm counts and frequencies of morphologically normal spermatozoa (Bonde *et al*., 1998; Guzick *et al*., 2001; Slama *et al*., 2002) it seems like only approximately 25% of men have an optimal semen quality, that 20-30% may be at risk of prolonged waiting time to pregnancy if they want to become fathers, and another 10-15% may have so low sperm counts that they may be at risk for need of fertility treatment. Thus, reduced semen quality seems so frequent that it may impair fertility and further increase the need for assisted reproduction in the future.

Humans are globally exposed to many classes of chemicals with endocrine disrupting potential via a variety of mechanisms. Exposure during fetal life may compromise testicular development, leading to reduced semen quality in adulthood, increased risk of testicular cancer and potentially also reduced capacity for testosterone production, besides an increased risk of being born with cryptorchidism and hypospadias as described by the Testicular Dysgenesis Syndrome (TDS) hypothesis (Skakkebaek *et al*., 2001). Since the first description of TDS hypothesis more and more evidence has emerged to support this hypothesis. Adverse effects caused by prenatal exposures do not exclude that adulthood exposures may also alter testicular function (Jørgensen *et al*., 2010); however, less focus has been on this aspect. Similarly, limited attention has been on the effect of life-style factors. However, recent publications have also indicated a possible contribution from such factors to the impaired human testicular function (Jensen *et al*., 2004a, b, 2007, 2010, 2011, 2013; Joensen *et al*., 2009, 2012, 2013; Kranich *et al*., 2014).

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Non-traditional animal models for advancing fertility preservation studies in humans

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Sustaining viable populations of any wildlife species requires a combination of adequate habitat protection as well as a good understanding of environmental and biological factors (including reproductive mechanisms) that ensure species survival. Thousands of species are under threat of extinction due to habitat loss/degradation, over-exploitation, pollution, disease, alien species invasions and urban sprawl. This has served as incentive for intensive management of animal populations, both ex situ (in captivity) and in situ (living in their natural habitat). Assisted reproductive technologies developed for addressing human infertility and enhancing livestock production have shown encouraging promise for wildlife species. However, species-specific physiological variations and a general lack of fundamental knowledge have limited how these tools can be used to help rapidly re-build sustainable populations of endangered species. Despite limitations, there is enormous potential in applying human-related fertility preservation strategies to wild animals, especially approaches that could assist managing or 'rescuing' the genomes of genetically valuable individuals. Indeed, one of the highest priorities in wildlife ex situ management is sustaining all existing genetic diversity to (1) preserve heterozygosity to avoid inbreeding depression and (2) ensure species integrity and the persistence of genomic adaptability to environmental changes. There are specific components of the rapidly emerging field of fertility preservation in men and women that are highly compatible with preserving valuable genomes of individuals or populations of threatened wildlife. Besides the more 'classical' approaches focusing on sperm and oocyte freezing, strategies associated with gonadal tissue cryopreservation and in vitro culture are especially attractive for better protecting and extending fertility for rare and endangered individuals. Likewise, lessons learned over the last decades in wildlife reproductive biology (either from wild or captive populations) are highly relevant to the advancement of human health and fertility. Additionally, studies conducted at the molecular or cellular level always are linked to physiological investigations in wild individuals or entire populations and take into account the interactions with the environment. The substantial amount of scholarly knowledge generated by multispecies and comparative approaches therefore is critical to better understand and mitigate complex issues affecting human beings (fertility, contraception, impact of the environmental changes). Comparative approaches in fertility preservation could benefit to the intensive and practical management of gene diversity in endangered species and lead to translational tools for human reproductive medicine.

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In vitro maturation of oocytes from small follicles: a field for active research interaction between veterinary and human reproductive medicine

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Although powerful assisted reproduction techniques (ART) have been developed over the last 35 years, access to treatment remains still problematic in most parts of the world due to its high financial burden on existing social security systems. Classical ART therapies and have a profound impact on women's professional life in part due to long hormonal treatments associated with physical discomfort and occasionally with severe complications. The high efficacy of IVF/ICSI has been obtained by improving the technologiesrelated to the treatment procedure (association of drug compounds, treatment monitoring, ICSI, longer embryo culture, embryo selection using genetic diagnosis, cryopreservation of embryos, etc.). While this chain of technologies is useful for the more complex cases of infertility, there might be a place for a first line ART as a "step-in" technology, where intervention on patients' normal life is kept minimal. In this context the ovarian follicular pool present throughout the menstrual cycle might is a source of immature oocyte-cumulus complexes which could be processed in-vitro up to developmentally competent oocytes. While the IVM technique has been pioneered by Bob Edwards, Alan Trounson and Carl Wood and further clinically developed by Scandinavianand Canadian Teams, it has not been broadly adapted in the ART clinics because of its lower efficacy in terms of implantation rate per transferred embryo compared to classical IVF/ICSI (10% vs 25% respectively).

One of the reasons for the slow implementation of IVM as a first-line technique for infertile patientsis that it has been worked out in a population of PCOS patients, who have an altered folliculogenesis, a compromised intrinsic oocyte quality and who demonstrate a higher miscarriage rate. While there is no doubt that IVM is the only technique that can totally avoid clinical hyperstimulation syndrome in PCOS, clinicians have remained hesitant to perform retrieval of oocytes out of small follicles (2-10 mm diameter), and most embryologists did not have precise and validated procedures to deal with a population of COC of diverse maturity grade.

In contrast, large breeding programs for several economically important animals are entirely based on the IVM technique and are already applied for several years. The source of immature oocytes are either slaughterhouse ovaries or ultrasound-guided retrievals in in-vivo monitored cycles. The embryos produced from IVM are cryopreserved and transported over continents to be transferred into receptive animals.The difference is that in animal breeding there is a large source of immature oocytes at the disposition of the lab and thus strong selection can be easily performed. The genetic background in animal breeding is also more homogenous than is the case between patients. In general in human IVM relatively few (5-15) COC are retrieved per cycle; and if HCG is injected the retrieved pool of oocytes is in a disparate stage of maturity (from GV stage with compact corona up to metaphase II oocyte with fully expanded cumulus). Having to deal with this large spectrum of oocyte development stages has implications in embryo culture practise, and the embryos obtained have to be replaced in the uterus of which endometrial receptivity is non-ideal due to the short growth span of the follicles, which have to provide sufficient estrogen and progesterone.

Despite all difficulties encountered with the introduction of IVM we believe that a suitable workmode could be set to guide the ART teams to a less invasive ART treatment procedure with competitive efficacy figures.

The IVM research program at the University Hospital (UZ Brussel) from the Free University Brussels (VUB) has been partnered by the Institute for early human development of the University of Adelaide (Adelaide, Australia) since 2009. The principal aim is to improve results obtained with currently commercialized IVM methods so that the efficiency gap with classical IVF/ICSI is narrowed, by improving the in-vitro culture procedure. This research has been supported over the last 5 years by IWT (the Flemish Institute for Innovation by Technology and Science) and FWO (Fund for Scientific Research Flanders) who are both governmental organisations.

The principal axes of translational research are based on recent findings emanating from experimental work in mouse (VUB) and other large mammals (Adelaide University) related to influences on intracellular cAMP levels in the COC and the use of recombinant factors of the EGF and TGF beta family.

The experimental conditions from animal models providing the most promising results are translated into experiments done on human oocytes from small follicles (2-10 mm).

Patients and volunteers have signed informed consent and the project works under approval of the Federal Commission for research on human embryos. Our clinical results on nearly 500 patients have been confirming the published efficacy data from the pioneering teams (McGill University Team; The Family Federation Team in Helsinki; Biogenesi atIstitutiCliniciZucchi from Monza).

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The IVM methodology from VUB team is based on oocyte retrieval without prior HCG injection. This approach allows to obtain uniformly GV oocytes from 2-6 mm follicles for setting new culture approaches.With the non-HCG approach the need for adequate luteal supplementation has been emphasized. The vitrification of day 3 embryos and their subsequent replacement in standard artificial cycles has demonstrated an attractive approach with high implantation figures per single embryo transferred. Associated research on imprinting establishment in human oocytes with the InstitutfürHumangenetik in Würzburg (Germany) has demonstrated that IVM technology per se is not associated with methylation alterations for 3 susceptible genes. An analysis by array CGH on 20 transferable good quality IVM embryos (all blastomeresanalysed) could not detect significant differences with control "classical" ICSI embryos obtained after conventional stimulation.

The clinical results obtained from September 2013 to March 2014 (50 cycles in PCO-like patients) with the non-HCG approach in combination with 'conventional' IVM culture method (Origio) in PCO-like patients (our control group) yields an ongoing pregnancy rate of 28% per oocyte retrieval (mainly single embryo transfer). Although losses by early miscarriage are still high in our series (44%) this figure is not different from the early pregnancy loss observed in our clinic in 2013 in PCO-like patients after regular IVF/ICSI.

In conclusion, IVM provides unique insights on early follicle development before luteinisationin human; our research should bring stepwise improvements in the performance of IVM to the benefit of our patients.

Fine-tuning the culture conditions will allow women to have a more simplified, short and safe infertility treatment. With the most recent technologies at reach we hope to transform IVM into a robust ART treatment with a much lower treatment burden for patient and savings for social security.

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