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Cross-talk during the periconception period

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Abstract

The cross-talk between gametes, embryos and female reproductive tract plays a crucial role in fine tuning of different reproductive events as well as influencing the epigenetic profile of offspring and their health in adulthood. Here, we describe some background to the recent investigations leading to the discovery of this cross talk. We will also point to important requirements for understanding the maternal communication with gametes and embryos. Finally we mention two probable hypotheses regarding how gametes and embryos are recognised by the female reproductive tract. It is clear that understanding this cross talk is leading to the production of new means for increasing fertility and potentials for affecting the epigenomic profile of an individual.

Keywords

Periconception; Oviduct; Fallopian Tubes; Embryo; Spermatozoa; Oocyte

The fall and the rise of research on cross-talk during the periconception period

Transport of the gametes, the final gamete maturation process, fertilization, early embryonic development and embryo implantation take place in the oviduct/Fallopian tubes and the uterus/uterine horns. These are all very important events that occur during the periconception period, leading to creation of new offspring. However, our knowledge of the periconception environment and how it is regulated is very limited. In the last forty years, the support for research in this field has been limited. Neglecting this area of reproductive research has not only been due to a lack of funding opportunities and limited financial support from the funders; the negligence has also originated from the scientific community. The dominant view in the scientific community has been rather dismissive of the importance of the periconception milieu and the important role that it may play in regulating important reproductive events. This attitude, at least for the last three decades of the twentieth century, was the dominant view in the scientific community even going as

far as rejecting grant applications based on the lack of importance in researching this area of reproductive sciences. One of the authors of the current paper (AF), once had a research grant application rejected because of a reviewer's comment, stating that the topic of investigation – the periconception environment - is "interesting", but not "important".

Probably the origins of this view - dismissing the importance of the periconception milieu – partially resulted from the success of in vitro fertilization (IVF) and other assisted reproductive technologies. The successful establishment of IVF as the method of choice for infertility treatment was not just a huge advance in helping infertility patients, but was a turning point for our understanding of the events taking place during the periconception period. IVF allowed detailed investigation of different events that take place in the maternal tract. Indeed, IVF contributed substantially to research findings in our field. However, at the same time it supported the view that the milieu of the oviduct/Fallopian tube and the upper parts of the female reproductive tract is replaceable by a simple combination of buffered salts called "IVF culture media". Hence, from the mid 1970's, the leading view gaining support between experts was that the oviduct/Fallopian tubes, and generally the upper parts of the female reproductive tract (that are the exact location/host of periconception events), are just passive contributors towards the events taking place during the periconception period. Their only function was regarded as providing a milieu with the right temperature, pH and nutrients, but without involvement/contribution in the fine tuning and regulation of different events taking place during this period.

This was the dominant view in the field until around the beginning of the 21st century several lines of evidence started to challenge this dogma. Better understanding of how events such as "sperm storage" in the female reproductive tract are mediated or the discovery of phenomena such as "large offspring syndrome", attracted the attention of scientists to the importance of the periconception milieu and the role that the periconception milieu plays in regulating fertility as well as the future health and development of offspring. Discovery of the sperm storage mechanisms, and the fact that majority of internally fertilising species are able to preserve sperm viability, not only by providing nutrients for spermatozoa, but by influencing diverse functional regulatory processes such as sperm plasma membrane fluidity, pointed to the presence of active sperm regulatory processes in the oviduct [1-4]. In cattle and sheep , embryos exposed to in vitro culture environments prior to the blastocyst stage had resulted in the development of unusually large offspring (large offspring syndrome) that also exhibited a number of organ defects [5]. The cause of large offspring syndrome was blamed on the presence of the serum in the in vitro culture media [6]. These interesting observations and the fact that these small changes in in vitro conditions can have such profound effects in the fate of the offspring, in addition to advances made in the field of epigenetics, attracted a lot of attention towards understanding how changes in the periconception milieu can affect the future health of the offspring, as well as how the periconception milieu is regulated and organised.

<u>Difficulties in the discovery of cross-talk mechanisms during the periconception period</u>

Early work on deciphering communication between the maternal tract, gametes and embryos was mainly focused on understanding the effect that the maternal tract components had on gametes or embryos. The majority of research in this field was driven by application and commercial interest to understand what molecules or components of the tract are responsible for improving the

preservation of sperm, supporting the maturation of oocyte and/or help with the development of embryos.

Seldom in the literature, was there a report, aimed at understanding whether the interactions between the maternal tract, gametes and embryos were truly cross-talks between the female reproductive tract from one side and gametes or embryos from the other side. Moreover, whether the cross-talk was directed from gametes and embryos towards the female reproductive tract. Part of the reason for this negligence may have been caused by a lack of a commercial interest or a practical application to drive the research in this field. For example, the discovery of molecules responsible for the maintenance of sperm viability in the female reproductive tract, and their use in commercial diluents for semen preservation, or finding the factors that promote the in vitro development of embryo to help infertile couples, were attracting big commercial interests and fuelling further research in understanding what is produced by the maternal tract in support of gametes or embryo function. However, at the same time, the main driver of research and discovery of the changes in the maternal tract responses to spermatozoa or embryo was pure basic scientific interest.

The other hindrance in research to understand the responses of the female reproductive tract to gametes and embryos was unavailability of an, easy to measure, so called “end point of assay” for evaluating the oviduct/Fallopian tube responses to gametes and embryo.

For example, in the case of measuring the sperm responses to oviductal factors, scientists were able to use viability or general andrology routine tests such as measuring the percentage of motile spermatozoa to check whether different components of oviductal fluid had any effects on sperm function. In the case of oocytes, several tests existed to check the effect of oviduct/ Fallopian tubes on the maturational stages of oocytes i.e., nuclear or cytoplasmic maturation or even zona pellucida hardening [7]. Even in the case of embryos, simple microscopy was enough to measure the rate of growth and development of an embryo. However, such proper and relatively easy to measure end points of assay were not available to the scientists investigating the maternal responses to gametes and embryo until the latter years of the previous century.

The other major issue that had stalled investigation in this field, was the subtlety of the reactions of the female reproductive tract to gametes and embryos. Today we know that changes happening in the maternal tract - for example at the transcriptomic level - in response to gametes and embryos only require small stimuli. Hence, it is very important to employ technologies that have a holistic ability and can detect the relatively minute changes between large and diverse populations of transcripts. Maternal responses to gametes and embryos are not major physiological events that produce huge transcriptomic or proteomic changes in the tissues and organs involved. They produce subtle modifications, and detecting these changes needs careful experimental design/planning as well as avoiding the background noise levels that can mask or hinder the detection of these reactions. Potential factors that may cause vast physiological transcriptomic and proteomic alterations in the female reproductive tract milieu e.g., changes in the reproductive tract milieu due to sex hormone alterations in the reproductive cycle, can themselves substantially alter the genome or proteome of the female reproductive tract and completely hide the minute responses of the female reproductive tract due to the arrival of gametes or embryos in the tract [8]. Hence, a need

exists to try to differentiate and recognise the fine responses of the maternal tract to gametes and embryo from the background noise.

Finally another major improvement, particularly in the in vivo analysis of periconception cross talk between gametes and embryos has been the application of in vivo models that provide both the test and the control within one female to check for the responses of the female reproductive tract to gametes and embryo. These in vivo models are the ultimate tools in investigation of the periconception milieu. They are very accurate and allow detection of minute changes in the transcriptomic and proteomic profile of the maternal tract. They have been successfully used in mice [9], pig [10, 11] and cattle [12].

A bit of history

The first reports indicating that there is cross talk happening between gametes, embryos and the maternal tract, appeared in the literature in the 1990's. This was the work done by Joanne Ellington et. al. [13] and Thomas et. al., [14] demonstrating *de novo* production of proteins in response to spermatozoa during *in vitro* co-culture of sperm-oviductal epithelial cells in cattle and mares respectively. Although these reports demonstrated the *de novo* production of oviductal proteins in response to spermatozoa, and as such the existence of a cross talk between sperm and oviductal epithelial cells, the identity of the proteins produced in response to spermatozoa was not known. But the fact remains that these were very intriguing reports. Although, these investigations were performed in vitro and may not have been as credible as those investigation that were later performed in vivo, they cracked the well-established dogmas that spermatozoa are inert cells and not recognised by the female reproductive tract. The evidence presented in these reports showed that spermatozoa could trigger a response in the female reproductive tract cells and intrigued many scientists regarding the nature of the sensory mechanisms involved in recognising spermatozoa and the identity and function of the molecules produced by the oviduct in response to spermatozoa.

Another seminal study was published by Lee et. al., [9] using an in vivo mouse model and comparing the genes that changed within the mouse oviduct in response to oocytes and embryos. This study employed suppressive subtractive hybridization (SSH) [15]. Lee's report was probably the first to identify genes in the oviduct that are upregulated in the presence of embryos during the periconception period. SSH was one of the initial technologies developed for high through-put transcriptomic analysis before microarray based technologies gained major popularity in the field of high throughput transcriptomic analysis. SSH was based on PCR amplification of cDNA fragments that differ between a control (driver) and the experimental transcriptome. Employing SSH, it was possible to highlight the differences in relative quantity of transcripts between the two samples. Hence, the report of Lee et. al., [9] was probably the first in vivo work using a high through-put genomic analysis technology and a controlled in vivo model, allowing the discovery of the responses of the maternal tract to oocytes and embryos. This was a seminal study that applied many principles that today we know are crucial for the detection of maternal responses to gametes and embryo.

Being inspired by Lee et al., paper [9], we tried to use the SSH technique to look at changes in the oviductal transcriptome in response to spermatozoa in porcine oviductal cells. Although, our attempts showed some signs of alterations in oviductal transcripts in response to spermatozoa, we were unable to produce concrete evidence of these effects of spermatozoa on porcine oviductal cells in vivo or in vitro. Part of the failure of these experiments was the fact that we were pushing

the technology of SSH to its limits and facing problems such as false positive identification of genes that were not differentially transcribed [16]. SSH did not have the ability to differentiate between the transcripts of the samples that were very similar to each other. The level of differences created in porcine oviductal genomes in response to spermatozoa was too small and it was nearly impossible to detect these differences with SSH.

Early in 2002, with the popularity of oligonucleotide arrays in the applications of high through-put gene expression analysis investigations [17], we tried to construct a murine oligonucleotide array to compare transcripts produced in mouse oviducts in response to spermatozoa. Part of the cDNA spotted on our homemade glass microarrays were made available through a collaboration agreement with Lee's lab in Hong Kong. These were mouse oviductal tissue specific transcripts as reported by Lee et. al. [18]. Unfortunately that attempt failed too. We had only around 240 genes spotted on our oligonucleotide glass arrays. Looking in hindsight, with our current knowledge of the amount of alterations in oviductal transcriptome in response to spermatozoa, we now know that with such low number of random transcripts spotted on our homemade glass oligonucleotide arrays, we had a very low chance (>1%) of discovery of any transcripts that might have been altered in oviduct in response to spermatozoa. Hence, this attempt failed too.

After nearly 5 years of trial and error, following many different protocols and trying to refine the techniques in our hands, finally in 2004 we published the first report describing alterations in oviductal transcriptomes in response to spermatozoa in mice mated to (a) fertile males and (b) mutant males unable to produce spermatozoa in their ejaculates [19]. This was probably the first report showing that the presence of spermatozoa in the female reproductive tract can itself send signals to the maternal tract and alter the oviductal transcriptome. The strategy we developed to discover transcripts altered in response to spermatozoa in oviduct involved two steps. First, using an Affymetrix high density oligonucleotide array, we screened transcripts of mouse oviducts that originated from two mouse populations, one at the onset of estrus and the other just 6 hours after mating. During this screening exercise, we looked at alterations in more than 12000 transcripts in these two groups and reduced the number of transcripts being potentially altered in response to spermatozoa arrival in the oviduct to just around 400 transcripts. In the next stage we utilised a quantitative PCR technique and compared the expression of two transcripts; adrenomedullin and prostaglandin endoperoxidase synthase 2 in the oviducts of two populations of mice, one mated to fertile males and the other to T145H mutant mice. The T145H mutant mouse is a sterile strain,, where males produce seminal plasma in their ejaculates without spermatozoa [20]. There were clear differences in the expression of adrenomedullin and prostaglandin endoperoxidase synthase 2 transcripts between oviducts obtained from females mated to fertile and mutant mice. Differences in transcription expression activity could only be attributed to the presence or absence of spermatozoa in the oviduct and not any other factors such as the act of mating. This report not only showed that spermatozoa are recognised by the female reproductive tract under physiological conditions, but allowed us to pinpoint the exact transcripts being altered in response to spermatozoa arrival in the female reproductive tract.

Since then a comprehensive list of publications from different labs worldwide have looked at this cross talk in different mammalian species and have documented the cross talk between maternal tract, gametes and embryos in both in vivo and in vitro model systems. Evidence for similar cross-talk has also been demonstrated in turkeys, where the arrival of spermatozoa in the sperm storage

tubules was shown to stimulate de novo gene transcription [21]. This paper cannot list all these investigations and we recommend the interested reader to recent reviews and papers published elsewhere [22-26]. What is of particular interest to our discussion here is to understand the mechanisms used by the maternal tract to recognise the gametes and embryos as well as the consequences of the cross talk and potential future research directions in this field.

How does the maternal tract recognise gametes and embryos?

It is still not known how the maternal tract recognises the presence of gametes and embryo. In the absence of concrete evidence to explain this phenomenon, we have put two hypotheses forward to explain how the maternal tract recognises and reacts to gametes and embryos.

Gametes and embryo pattern recognition receptors

One theory hypothesises the existence of an intrinsic ability/system in the maternal tract to recognise gametes and embryos associated molecular patterns and then respond to them accordingly. Examples of such pattern recognition mechanisms exist elsewhere in the body. For example Toll like receptors (TLRs) in the innate immune system are classed as pattern recognition receptors (PRRs). In the innate immune system, TLRs are responsible for the recognition of pathogen-associated molecular patterns (PAMPs). Hence, TLRs differentiate between self and non-self-entities and alert individuals to the presence of pathogens. In human 10 different TLRs exist where each is responsible for the recognition of particular pathogenic signature molecules. For example LPS (Lipopolysaccharide), is a major component of the outer membrane of Gram-negative bacteria, and takes part in the structural integrity of the bacteria. LPS is recognised by TLR4. Nearly all cells in the body that have TLR4 at their surface recognise LPS and respond to it.

It is now well known that several classes of PRRs exist and that each of these systems is responsible for the recognition of different associated molecular pattern molecules. Some, like TLRs, are responsible for recognition of PAMPs. Others have been found to alert and to respond to Damage-associated molecular pattern molecules (DAMPs), also known as danger-associated molecular pattern molecules. One can speculate that a comparable associated molecular pattern system may exist, or is produced by gametes and embryos, allowing gametes and embryos to be recognized by the maternal tract. Such a system if present should work in close collaboration with the innate immune system and, moreover should operate through ancient and conserved mechanisms present in all species that have an internal fertilization system [27].

Both spermatozoa and embryo are non-self-entities and should create a major immune reaction in the female reproductive tract, leading to the rejection of gametes and embryo from the female reproductive tract. However, in reality, spermatozoa and embryo are well received in the maternal tract. Sperm viability is maintained and embryos are allowed to implant. This cannot be achieved without a mechanism recognising their arrival and alerting the females to their existence within the reproductive tract. If gamete and embryo specific PRRs exist in the female reproductive tract, one of their functions would be to suppress the innate immune system as soon as it recognises the arrival

of spermatozoa and embryos within the female reproductive tract, thus allowing for sperm viability maintenance in the reproductive tract and embryo implantation.

Gametes and embryo produce exosomes and molecules capable of modulation of maternal tract responses

The other theory to explain the responses of the maternal tract towards gametes and embryo is that gametes and embryos produce molecules that can affect and modulate the function of the maternal tract cells. In this theory a need for the recognition of gametes and embryo by the female reproductive tract does not exist. The idea is that molecules produced by the gametes and the embryos themselves will take control of the reproductive cells and stimulate maternal responses towards gametes and embryos. Currently evidence of exosome production by different reproductive cell types (endometrial epithelial cells, embryo and…) as means of cell to cell communication is expanding (For a review see [28]). However, currently, direct evidence that gametes and embryos are capable of producing exosomes or molecules that can directly affect the function of the maternal tract is lacking. But as the field is growing and several reports of production of exosomes and microvesicles by different cell types is accumulating, such a chance is not improbable.

In conclusion, currently there is no substantial support for either of these theories or, indeed any credible opposition either. What is apparent is that the processes mediating potential recognition of gametes or embryos are very well tuned. It seems that the female reproductive tract is capable of recognizing and differentiating between the X and Y chromosome bearing spermatozoa, and is capable of responding to each of them in a different manner [29]. At the same time the maternal tract also responds to embryo and can differentiate between different developmental stages of embryos. How this recognition is achieved is currently a mystery.

The future of research

Understanding cross talk at the periconception period is gaining importance and is becoming attractive for many reasons. Partially, advances in understanding epigenomic is guiding us towards further research in understanding the periconception milieu. How the field will progress and where it will go is hard to predict. However, the general feeling is that the importance of the periconception milieu is no longer disputed and investigations in this field will raise more significant questions.

A crucial part of the periconception milieu is the maternal tract responses to gametes and embryos, which, at least at transcriptomic and proteomic level, are very diverse. Computational modelling (in silico models) that can combine different aspects of these interactions and define what would be the consequences of the cross-talk between gametes and embryos are very attractive routes for better understanding the modulation of the periconception milieu [30]. Our lab has initiated a number of investigations towards creating an in silico model of the oviduct [31-33]. However, it is already clear that these interactions are very diverse and complex. In the short term compared to other potential applications for modelling, the periconception milieu complexity seems to be a hindrance and is not

very attractive to modellers. Despite this fact, creating in silico models remains very important and looks inevitable for future progress of this field.

In summary, a research question initiated on the basis of scientific curiosity is leading to the production of new means for increasing fertility and potentials for affecting the epigenomic profile of an individual. Nature has used alterations in the periconception environment as a strategy to increase the adaptive ability of the offspring to survive in their new environment even before they are born. Understanding how the periconception environment affects the newborn will open a new window on the subtleties of reproductive processes.

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