Evidence for paternal effects transmitted through the germline

Author:
Douwe Grundel
Supervisor:
prof. dr. Jean-Christophe Billeter
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Abstract

Parental effects are recognised as an important regulator of phenotypic plasticity. Maternal effects are considered to be the most abundant and important parental effect. Paternal effects, on the other hand, are regarded as a rare phenomenon and only considered important in species with paternal care. Germline transmitted paternal effects (GTPE) were thought to be non-existent due to the limitations of the information carrying ability of sperm cells. However, by using IVF protocols recent studies showed compelling evidence for the existence of GTPEs. Furthermore, some light was shed on three epigenetic mechanisms that facilitate GTPEs: DNA methylation, chromatin structure modification and regulatory RNAs. Moreover, these three mechanisms are able to interact with each other, leading to more sophisticated ways of regulating offspring gene expression. An important step in better understanding these proximate regulatory mechanisms is creating an evolutionary framework. Evolutionary explanations of GTPEs often focus on maximizing the offspring's fitness. Although this is of importance, focussing on maximization of fitness can be misleading. It is likely that the mechanisms facilitating GTPEs also facilitate sexual conflict. Taking this into an account does not only help to establish a better evolutionary framework, but also provides a reason to further investigate proximate conflict mechanisms of paternal and maternal effects. In conclusion, it is very likely that GTPEs exist and mechanistic details are beginning to be unravelled. Furthermore, investigating the conflict in maternal and paternal mechanisms can be an important step in guiding experiments aimed at further unravelling the mechanisms of GTPEs.

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Introduction

Organisms must adapt to their environment in order to increase their chances of survival. An important mechanism of adaptation is phenotypic plasticity (West-Eberhard, 2003). Phenotypic plasticity is the ability of an organism to react to their internal or external environment by changing its phenotype, whilst its genotype remains unchanged (Agrawal, 2001). For example, the freshwater snail (*Physella virgate*) reduces growth and forms a more protective shell in the presence of predatory (molluscivorous) sunfish, thereby decreasing the chance of being predated (Langerhans & DeWitt, 2002). However, phenotypic plasticity is not always adaptive (West-Eberhard, 2003). The freshwater snails shows the same reaction in presence of a non-molluscivorous sunfish species, thereby impairing its growth rate by needlessly investing resources in a protective shell.

In the example above the phenotypic plasticity of the individual was induced by direct experience (environment) of the individual itself. This is not the only way phenotypic plasticity can be induced. The phenotype of the individual can also be influenced by the environment of its mother. This type of phenotypic plasticity is called a maternal effect (Mousseau & Fox, 1998). A maternal effect can be adaptive if the environment of the mother matches the environment of the offspring (Uller, Nakagawa, & English, 2013). It is a way of 'preparing the offspring to the environment'. An example of this is found in plants (*C. americanum*)(Galloway & Etterson, 2007). Plants that grew up in the same lighting conditions as their mothers had higher survival rates. Maternal effects can also be maladaptive, however. For instance, maternal malnutrition in rats can lead to irreversible effects in the neurogenesis of the offspring. (Morreale de Escobar, Jesus Obregon, & Escobar del Rey, 2004).
There are two main mechanisms through which mothers can transfer environmental information to their offspring (Mousseau & Fox, 1998). The first main mechanism is direct contact between mother and offspring. There, the phenotypic effects are transmitted through social interactions, most notably maternal care such as breast feeding or grooming. The amount of maternal care received in early life can have a profound impact on the offspring's phenotype. For example, in rats the amount of maternal care received in early life influences the expression of glucocorticoid receptors in the hippocampus, thereby changing the sensitivity of the hippocampus to glucocorticoids. As a result the HPA-axis mediated stress response changes, leading to different behavioural response to stress in adult rats (Champagne et al., 2003; Liu et al., 1997). The second main mechanism is phenotypic effects transmitted in absence of direct contact between mother and offspring. This can be done through RNAs, hormones and nutrients that are deposited in the egg cell or transmitted to the offspring during pregnancy through the placenta (Hsu et al., 2016; Mousseau & Fox, 1998; Schier, 2007).

In principle environmental information could also be transmitted to the offspring via the father. However, paternal effects are regarded as a rare phenomenon and only considered important in species with paternal care. There are two reasons for this (Crean & Bonduriansky, 2014). First, the male gametes. Compared to females, males invest little time and few resources in the production of each gamete. Therefore, males do not have as many opportunities to transmit environmental information to their gametes as females do. Moreover, sperm cells may not be able to carry environmental information at all, because they are much smaller than their female counterparts. Second, mammalian mothers have a direct physiological connection with the offspring during pregnancy, whereas fathers do not. Therefore, it has been assumed that paternal care is the only mechanisms for paternal effects (Crean & Bonduriansky, 2014). However, recent studies have shed new light on non-social paternal effects. It appears that information about the paternal environment can be transmitted through the male germline after all.

In this thesis I will investigate the plausibility of the claims made in these studies and try to answer two questions. First, do these paternal effects transmitted by germline exist? Second, what are the possible mechanisms to facilitate such effects? In this thesis I will mostly focus on mammals.

Experimental evidence

In this section several studies that provide evidence for germline transmitted paternal effects (GTPE) will be discussed. All of these studies have the same general set up: genetically identical (inbred strains) male test animals are divided over two, or more, groups. Each group is subjected to a different environment. Thereafter, the offspring of these males and control females (also genetically identical) are screened on specific aspects of their phenotype. This way paternal effects can be found. All the studies I will mention either use rats or mice, apart from two exceptions (Drosophila and C. elegans). In all studies the males were removed before the offspring was born in order to avoid direct contact between father and offspring.

In the first study the effect of paternal diet on female offspring is examined (Ng et al., 2010). Male rats were either given a high fat diet (HFD) or a control diet. Females consumed a control diet. As expected the HFD male rats showed an increase in body weight, adipose leptin, plasma leptin and liver mass. Furthermore, the HFD males were glucose intolerant and resistant to insulin. The female offspring of HFD males also showed impairment in insulin-glucose homeostasis, although the differences between the control female offspring was smaller than the difference between the HFD father and control fathers. The impaired insulin-glucose homeostasis in the female HFD offspring can be explained by the fact that their pancreatic isles were significantly reduced in size compared to the control group. Furthermore, the number of β-cells in the isles was significantly lower. In conclusion, paternal diet seems to affect the phenotype of female offspring.

There are many comparable studies that found paternal diet could influence offspring phenotype (Carone et al., 2010; Fontelles et al., 2016; Öst et al., 2014; Radford et al., 2014; Rechavi et al., 2014). Other paternal environments that influence offspring phenotype include stress (Franklin et al., 2010) and toxins (Tschurtschenthaler et al., 2016).

These results seem to support the existence of GTPEs. However, there is a problem with these experimental studies. These studies do not exclude the possibility of a maternally mediated paternal effect (MMPE) (Crean & Bonduriansky, 2014; Curley, Mashoodh, & Champagne, 2011; Van Otterdijk & Michels, 2016). In case of a MMPE the mother receives cues from the phenotype of the father. Theses cues are an environmental influence that may alter the phenotype of the mother, which in turn can influence the phenotype of the offspring. In other words, the offspring phenotype is not directly influenced by the father. Instead, the offspring phenotype is the result of a maternal effect.

Consider for instance the study by Ng et al. mentioned above. Here daughters of HFD fathers and the fathers had similar symptoms. The authors interpret these results as proof that the male germline carries information about the environment to the next generation. The alternative explanation is a MMPE. The females could have reacted differently to the obese males than to the control males. A possible mechanism for this is explained by the differential allocation hypothesis, which states that females allocate fewer resources to offspring of males of low quality (Curley et al., 2011). These resources can be in the form of maternal care, allocation of hormones to egg cells or some physiological effects during pregnancy. So, it could be that the symptoms of HFD offspring are a result of a maternal effect.

In order to investigate MMPEs, Mashoodh and colleagues looked at how the amount of maternal care a mother rat gave depended on the phenotype of her co-parent (Mashoodh et al., 2012). More specifically, they looked at how the paternal social environment influenced the grooming behaviour (licking and nursing of pups) of the mothers. In order to do this some male rats grew up in isolation, whereas the socially enriched male rats grew up in same sex groups of twelve individuals. When the males reached adulthood they were mated with control females. The amount of maternal care towards the offspring of these females was measured. The conclusion of the authors was that mothers that were mated with males that grew up in a socially enriched environment spend more time grooming and feeding their pups than mothers mated with males that grew up in isolation. Since the level of maternal care received can have a profound impact on the adult phenotype (Champagne et al., 2003), MMPE are a more likely than germline transmitted paternal effects.

However, there are some problems with the experiment from Mashoodh et al (2012). Firstly, the results are not as strong as the authors claim. Although there are significant differences between both types of mothers, these differences are not consistent over the first five postnatal days. Secondly, the authors do not examine whether these differences in maternal care indeed lead to different adult phenotypes in the offspring. Thirdly, the differences that were found could as well have been caused by information transmitted through the male germline. This possibility is not excluded. In conclusion, Mashoodh et al. set out to prove that paternal effects could be maternally mediated, but failed to provide sufficient proof.

In order to provide sufficient evidence for either a MMPE or a GTPE a rigorous method is required. In vitro fertilization (IVF) is the method that can differentiate between a MMPE and a GTPE, since IVF females do not receive any cues from the male phenotype. Only the information contained in the sperm cells can be of influence. I will discuss three studies that used IVF. All of them concluded that paternal effects can be transmitted via the germline.

The first study examined the inheritance of paternal traumatic exposure to specific odours (Dias & Ressler, 2014). There were three groups of male mice. Two groups received shocks in combination with a specific odour and a control group. The two odours used were acetophenone (which activates M71 receptors) and propanol (which does not activate M71 receptors). At high concentrations of either odour all three groups showed aversion behaviour (spending most of the time in the odourless compartment of cage). At lower concentrations the reactions differed between groups and were odour specific. Control males did not react to odours whilst the two odour groups only showed aversion behaviour towards the odour they were conditioned with. Furthermore, neuroanatomical differences were found in the olfactory system. The acetophenone males had larger dorsal and medial acetophenone-responding glomeruli (indicative of more M71 receptor neurons), whereas the other groups did not. These neuroanatomical differences could not have been a consequence of exposure to the odours during the behavioural tests, since the animals that were used for behavioural tests were not used to gather neuroanatomical data.

Dias & Ressler (2014) had two batch groups. In the first batch the males of the three groups were mated to control females, in the second batch offspring was produced by IVF. In the first batch the offspring behaved the same as their fathers in respect to odour. They only showed aversion of odours at high concentrations or if their father had been conditioned to that specific odour. The neuroanatomical differences were also found in the offspring. In the second batch the IVF offspring showed the same neuroanatomical differences as the offspring of the first batch. In the second batch the behavioural test could not be performed due to 'animal quarantine issues'.

Despite the fact no behavioural tests were performed in the IVF offspring, these results provide evidence for GTPEs. It is unlikely that odour specific aversion is transmitted by a MMPE. Both paternal experimental groups were subjected to shock conditioning, which resulted in stressed phenotypes that would not have differed depending on the odour the males were conditioned with. Yet, it is not unconceivable that the females were able to smell the odours on the males whilst mating. In combination with the stressed male phenotype this could potentially have resulted in a MMPE. Importantly however, the neuroanatomical differences between the experimental groups were still present in the IVF batch. This gives a strong indication of a GTPE.

The second study provided evidence that paternal diet can contribute to metabolic disorders in offspring (Q. Q. Chen et al., 2015). In this experiment male mice either received a high fat diet (HFD) or a normal diet (ND). As expected the HFD males became obese, glucose intolerant, and insulin resistant, whereas the ND males did not. Next IVF was used to create offspring. There was no difference in body weight between both offspring groups. However, offspring of HFD males showed impaired glucose tolerance and insulin resistance whereas the ND offspring did not. This provides strong evidence that paternal diet has consequences for the offspring that are transmitted via the male germline.

The third study provides evidence that paternal stress has consequences for the offspring that are transmitted via the male germline (Gapp et al., 2014). Male rats were either subjected to maternal separation (MS) during early life or not (control). MS rats showed behavioural differences compared

to the control group. MS rats stayed longer in light compartment during a light-dark test and MS rats had a lower latency to enter open spaces. Physiological differences were also found. MS rats had an impaired glucose-insulin metabolism, whereas control rats did not. IVF was performed to create offspring. In this offspring the same differences in behaviour were found between the two groups. The MS offspring had a normal glucose base line, but was hypersensitive to insulin. The control group was not.

Together these three studies give plausibility to the existence of GTPEs. Apart from performing more IVF studies, it would be insightful to perform studies that investigate whether MMPEs or GTPEs have a stronger effect if both types of transmission are present. A global set up for such studies would be as follows. Males are sterilized and subjected to a control or experimental environment. Thereafter control females are mated with either a control or an experimental male, because the males are sterile the females will not be fertilized. Next IVF is performed with the sperm of either a control or experimental male. The offspring phenotypes, shown in figure 1, can be compared. If the result is that females mated with experimental (E) males always had offspring with an E phenotype, independent which sperm was used for IVF, the conclusion would be that MMPEs are more important than GTPEs. To my knowledge studies using this general set up have not been performed. Nevertheless, the three IVF studies discussed above still provide robust evidence for the existence of GTPEs. The question then becomes: how do these effects work? In the next section possible mechanism that enable sperm cells to transfer information about the paternal environment will be discussed.

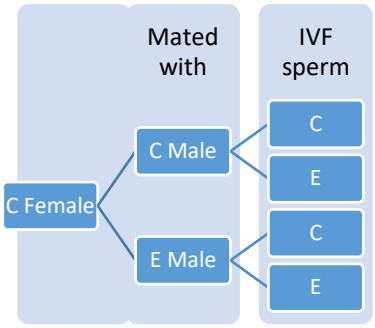

Figure 1. Experimental set up to investigate which has a bigger impact on the phenotype of the offspring: male phenotype trough maternally mediated paternal effects or the information in sperm cells trough germline mediated paternal effects. First a female is mated with a sterilized male that was submitted to a control (C) or experimental (E) environment. Thereafter, IVF is performed with either sperm form C or E males.

Mechanisms

In order to unravel the mechanisms of germline transmitted paternal effects three steps are required. First, it is necessary to establish how the information about the paternal environment is carried in the sperm cells. In other words, it is necessary to identify the information carriers of the process. The information is not contained in the male genome, since genetically identical individuals produce offspring with different phenotypes depending on the environment. Therefore, the information carriers that facilitate this stream of information through the male germline are epigenetic (Rando, 2016).

Second, it is necessary to understand by which mechanism epigenetic information carriers that originate in somatic cells arrive in the gametes. The paternal environment may affect epigenetic processes in the somatic cells of the father. For instance, a paternal high fat diet can alter gene expression levels of metabolic cells in the liver of the father (Carone et al., 2010). In case of a paternal effect these epigenetic changes are then transferred to the gametes (Q. Chen, Yan, & Duan, 2016). Alternatively, some environmental factors may affect the development of sperm cells directly leading to epigenetic changes. It is, for example, conceivable that high fat diet alters blood composition which directly affects the gametes.

Third, the information carriers need to influence the development of the embryo correctly. It is therefore necessary to understand the mechanisms that underlie this process.

The three epigenetic information carriers

There are three main types of epigenetic information carriers in mammalian sperm: 1) DNA methylation 2) Chromatin structure 3) RNAs (Dias et al., 2015; Rando, 2016; Soubry et al., 2014). It is possible that transcription factors and prions also are epigenetic information carriers. However, there is currently little evidence supporting this (Rando, 2016). Therefore, only the way the three main information carriers can influence gene expression will be discussed below.

DNA methylation

Methyltransferase enzymes can add methyl groups (methylation) to a cytosine base of a cytosine-guanine-dimer (a C next to a G in DNA sequence) (figure 2 A). Hereby the binding ability of transcriptional regulatory proteins is decreased. This results in a lower gene expression. However, methylation in an upstream region of the gene may in some cases lead to an increased gene expression. Ten-eleven translocation methylcytosine dioxygenase enzymes can remove methylation form cytosine (active demethylation). This usually leads to an increased gene expression (figure 2 B) (Dias et al., 2015).

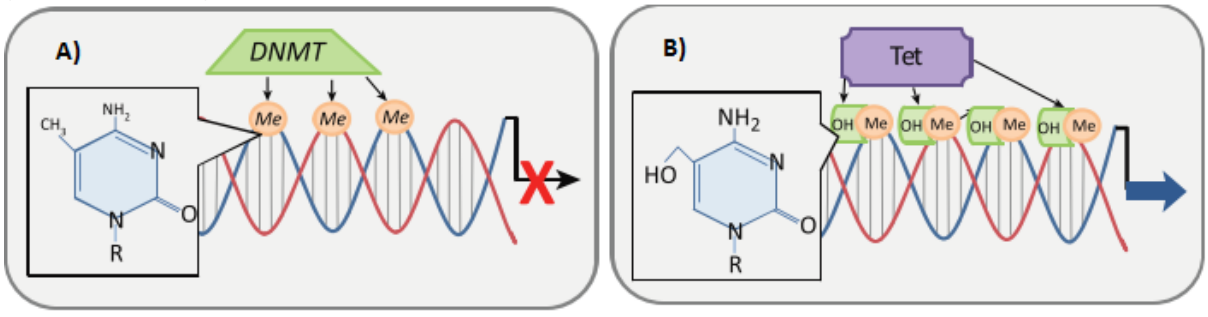

Figure 2. This figure was adapted from (Dias et al., 2015) *(fig. 1). A) Methylation of Cytosine-Guanine-dimers by methyltransferase enzymes usually leads to a lower gene expression. B) Active demethylation by Ten-eleven translocation methylcytosine dioxygenase enzymes usually leads to an increased gene expression.*

Chromatin structure

All eukaryote genomes are packaged in a nucleoprotein complex called chromatin. This is necessary to keep the DNA neatly foldup in the nucleus. The subunit in chromatin that is most important for this DNA folding is the histone protein. Histone proteins can be chemically modified. This modification results in a change in the 3D structure of the DNA. The DNA can be more tightly wrapped together, which decreases the binding ability of transcriptional regulatory proteins, thereby decreasing gene expression. Alternatively, the DNA structure might loosen up, leading to an increasing gene expression. Which of these two possibilities occurs depends on the type of chemical modification. If a acetyl group is added to the histone (acetylation) the DNA structure loosens up (figure 3 A,B). If the histone is methylated the DNA may either loosen or tighten (figure 3 C,D). Which of these two possibilities occurs depends on the number of methyl groups that is added (Dias et al., 2015; Rando, 2016).

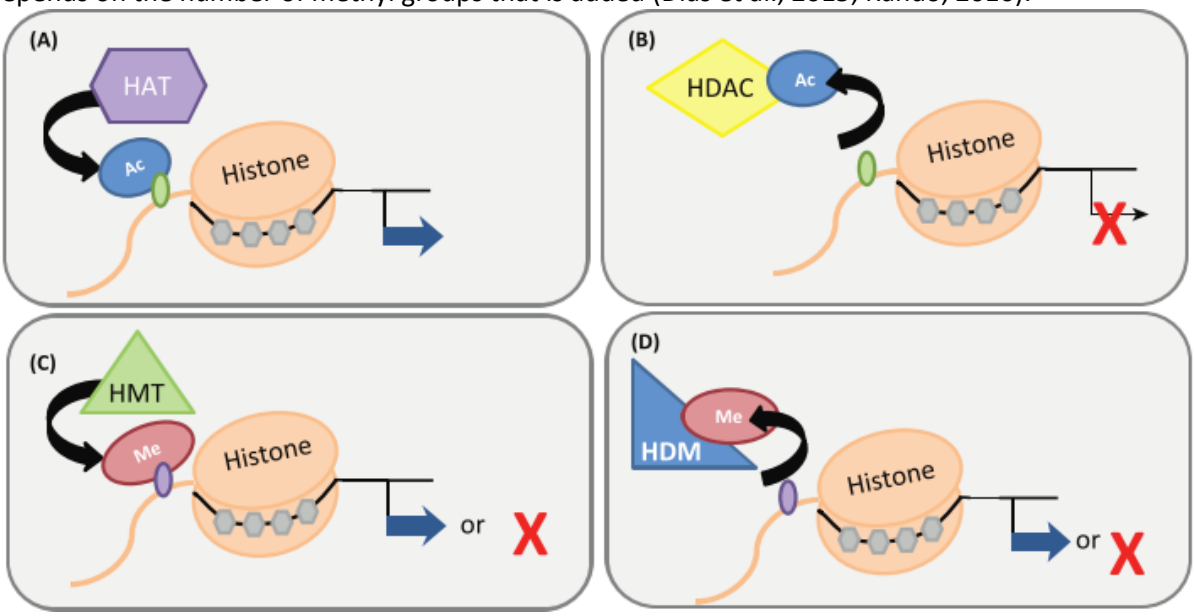

Figure 3. This figure was adapted from (Dias et al., 2015) *(fig. 1). A) Acetylation of histones by histone acetyltransferases (HATs) loosens up DNA structure thereby increasing gene expression. B) Active deacetylation of the histone by histone deacetylases (HDACs) tightens up the DNA structure thereby decreasing gene expression. C) D) Methylaiton of histones is mediated by histone methyltransferases (HMTs), which add methyl groups, this process is reversed by histone demethylases (HDMs). DNA structure can either loosen or tighten depending on how many methyl groups are still bound to the histone.*

RNAs

The two RNA types that are most important in regulating gene expression present in mammalian sperm are microRNAs (miRNAs) and tRNA-derived small RNAs (tsRNAs). Both RNA types influence gene expression through RNAi pathways in which a specific mRNA is targeted and degraded, thereby decreasing gene expression, however the details of the mechanisms of both RNA types are not known (Q. Chen et al., 2016; Dias et al., 2015; Holoch & Moazed, 2015). Figure 4 shows the miRNA pathway in mammals as an example. In addition to the RNAi pathways, both miRNAs and tsRNAs may in some cases stabilize mRNAs which leads to an increased gene expression (Q. Chen et al., 2016).

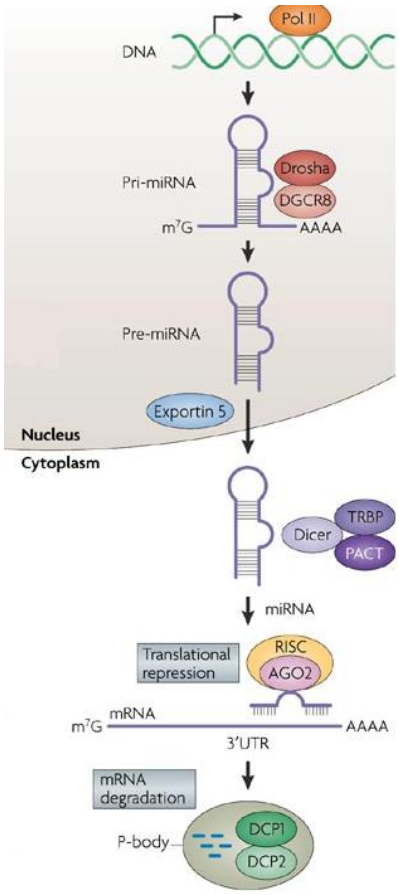

Figure 4. Figure and text adapted from (Kim & Rossi, 2007). The figure show the RNAi pathway for miRNA in mammals, not all details of this pathway are understood. RNA polymerase II (Pol 2) transcribes the DNA which results in a primary microRNA transcripts (pri-miRNAs). The Drosha–DGCR8 complex processes the pri-miRNA to generate precursor miRNAs (pre-miRNAs). These preRNAs are transported to the cytoplasm (by exporting 5) where the Dicer–TRBP–PACT complex is recruited. The Dicer–TRBP–PACT complex loads the preRNA into AGO2 and RISC. Now the miRNA recognizes target sites of mRNAs which will be degraded in processing (P)-bodies that contain the decapping enzymes DCP1 and DCP2.

Information from somatic cells enter germline through extracellular vesicles

Extracellular vesicles (EV) are important in intercellular communication and probably play a key role in the information flow from somatic cells to the germline in mammals, including humans, because EVs can deliver RNAs to developing sperm cells (Q. Chen et al., 2016; Vojtech et al., 2014). A study in mice shows that such RNA delivery probably takes place in the epididymis (Sharma et al., 2015). In this study, male mice either were either on a high protein diet, or a normal diet. Sperm collected from the caput and testis did not show differences in the RNA profiles between the two diet groups, however sperm

collected from the cauda showed a significant difference in RNA profile between the groups. Therefore, this difference in tsRNAs must have arisen somewhere between the caput and cauda, in other words: the epididymis. Next, EVs present in the epididymis were purified and analysed. The tsRNAs found in the EVs of each group corresponded with the tsRNAs found in the sperm of that group. Thus, tsRNAs delivered by EVs probably play a key role in paternal effects. However, the possibility that tRNAs that were present in the caput were cleaved during transit trough the epididymis cannot be excluded. The details of this RNA delivery mechanism by EVs remain unknown. It is, for example, counterintuitive to think that there is no selection in which RNAs are carried to the sperm cells, yet it is not known how EVs can select for specific RNAs (Q. Chen et al., 2016).

In c. elegans another mechanism has been discovered. Here, dsRNA produced in a neuron can enter the germline without transport in EVs, instead a mechanism involving RNA-interference defective protein-1 (SID-1) and SID-2 is in place. SID-1 can form a channel in the cell membrane trough which dsRNA can enter the germ cell. It is unclear whether mammalians homologues of SID family proteins exist (Q. Chen et al., 2016; Devanapally, Ravikumar, & Jose, 2015).

So, there are some pathways trough which RNAs can enter the germline. What about the other two epigenetic information carriers: DNA methylation and chromatin structure?

Almost all methyl groups are actively removed from the DNA during sperm development. Some methyl groups remain, but it is not clear why they are not removed (Q. Chen et al., 2016; Rando, 2016; Soubry et al., 2014). Thus, it would appear that even if DNA methylation patterns could enter the germline, most of them would be lost. However, the few DNA sites that are not demethylated might play a key role. The only known mechanism that explains the transition of methylation pattern from somatic cells to the germline is RNA guided DNA methylation. RNAs present in sperm cells, miRNAs and tsRNAs, recognize specific DNA sequences and then could induce a methylation process (Q. Chen et al., 2016; Holoch & Moazed, 2015). Almost the same can be said for chromatin structure. During sperm development most histones are replaced by protoamines. As a result the DNA is more tightly packed. Some histones remain, but it is not clear why they are not replaced (Q. Chen et al., 2016; Rando, 2016; Soubry et al., 2014). The histones that remain can again be modified by RNAs. (Q. Chen et al., 2016; Holoch & Moazed, 2015).

Effects of epigenetic information on the development of the embryo

The mechanisms by which the epigenetic information carriers present in the sperm cell eventually influence embryo development are the most elusive mechanisms discussed so far. Virtually nothing is known about them (Q. Chen et al., 2016). However, two studies that were discussed earlier provide strong evidence that both miRNA and tsRNA do influence embryo development.

The first study looked at the effect of paternal stress on offspring (Gapp et al., 2014). The IVF procedure of this experiment showed that offspring of stressed fathers also had stressed phenotypes (behavioural and physiological), whereas control offspring did not have stressed phenotypes. In addition to this, the authors injected the miRNA that was correlated with the changes in the sperm of the stressed fathers directly into a zygote that was fertilised with control sperm. This also resulted in offspring with the stressed phenotype.

The second study looked the effect of paternal diet on offspring (Q. Q. Chen et al., 2015). The procedure is very much the same as in the previous study. tsRNAs were injected into the zygote. The offspring that resulted from the RNA injections showed the same phenotypes for each diet group as the offspring per diet group produced by IVF.

Although the mechanisms trough which these RNAs influence embryo development are unknown, RNAi pathways are a likely candidate. By silencing specific genes embryo development can be steered. In addition to this DNA methylation and chromatin modification could also play important roles. Since DNA methylation patterns are erased again in the embryo (Q. Chen et al., 2016; Rando, 2016; Soubry et al., 2014) paternal RNAs could be vital in the creating of new methylation pattern in both the paternal and maternal genome through RNA guided DNA methylation. Furthermore, in the embryo the protoamines of the paternal DNA are swapped for histones, so the paternal RNAs could also guide chromatin modification processes. Moreover, interaction between the three epigenetic information carriers can lead to positive feedback loops, resulting in a permanently altered gene expression. However, it is uncertain whether these loops exist in mammals, since they have only been fully described in the yeast *Schizosaccharomyces pombe* and the plant *Arabidopsis thaliana* (BOX 1)(Q. Chen et al., 2016; Holoch & Moazed, 2015; Klosin & Lehner, 2016).

BOX 1 |Epigenetic positive feedback loops

Adapted from Holoch & Moazed, 2015. Small interfering RNAs (siRNAs) can guide enzymes that catalyse methylation to a specific DNA sequence resulting in either DNA methylation (DM) or histone methylation(HM), or in some cases both. In *S. pombe* and *A. thaliana* these methylation events are physically coupled to proteins that are involved in siRNAs amplification loops. As a result new siRNAs are created and a positive feedback loop is created.

Discussion

In order to show germline transmitted paternal effects exist, some scientist say that excluding MMPEs by looking at IVF produced offspring is not sufficient. In addition to this, the phenotype of the F2 offspring (also produced by IVF) should be examined (Curley et al., 2011). The reason being that the F2 generation is not directly exposed to the paternal environment that induced the change in phenotype, whereas the F1 generation is directly exposed to this environment, not because they themselves lived in that environment, but because they were exposed to it as sperm cells. As a consequence, the paternal environment could directly affect sperm development, eventually influencing the offspring's phenotype. Curley et al. say this direct influence of the paternal environment is not the same as a paternal effect and call it developmental plasticity instead. By looking at the F2 generation Curley et al. want to ensure the initial environmental stimulus is not necessary to affect the offspring phenotype. In other words, Curley et al. want to ensure that an environment leads to an epigenetic change in the somatic cells that can be transmitted to the germline.

I think, however, that it is not always necessary to look at the F2 generation in order to show a paternal effect. I have two reasons for this. Firstly, the direct effect of the environment on the sperm cells mentioned by Curley et al. is questionable. How direct are these effects? Although the mechanisms that deliver information to sperm are not known in detail, selection of information is likely. For instance, in rats the extracellular vesicles that deliver information to sperm carry specific RNA types (Sharma et al., 2015) and in C. elegans there the SIM-1 pathway specifically allows dsRNA to enter the germline (Devanapally et al., 2015). This means that many environmental stimuli, for example fatty acids from paternal diet, cannot directly affect sperm development. Only those stimuli that can pass the paternal selection process enter the germline. Therefore, I would argue that an effect observed in the F1 can still be a paternal effect. However, some environmental stimuli, such as toxins or temperature, might bypass the paternal selection process, allowing a more direct environmental influence. Even then this might still be called a paternal effect, since the environment of the father influences the phenotype of the offspring. Part of the problem here is the definition of paternal effect. In order to solve this definition problem parental effect that are passed to the F1 offspring are sometimes called intergenerational effects. Transgenerational or multigenerational effects are parental effects that span multiple generations (Van Otterdijk & Michels, 2016).

Secondly, from an evolutionary perspective it is unlikely that paternal effects will persist for multiple generations. The father 'prepares its offspring' to survive in the same environmental condition as he does. It is unlikely these conditions remain constant across multiple generations, therefore a multigenerational paternal effect would not increases the fitness of the offspring. As a consequence, it is unlikely that these multigenerational effects have evolved. Instead, the effects on the phenotype induced by the paternal environment should quickly disappear over the generations. The fact that proximate mechanisms are in place that erase most epigenetic signatures (DNA methylation and chromatin modification) every generation support this idea. Therefore, a paternal effect could be missed if only phenotypic effects seen in the F2 offspring are considered. However, two of the previously mentioned IVF studies in mammals did show that the phenotypic effects persisted in the F2 population. The first study showed that the physiological effects of stress do persist (Gapp et al., 2014), the second study showed that the behavioural aversion of specific odours could persist (Dias & Ressler, 2014). These results show that multigenerational paternal effects exist, but more research is needed to determine how frequent such multigenerational paternal effects are.

The existence of multigenerational paternal effects cannot be easily explained using the evolutionary framework described above. Consequently, this framework might be an oversimplification. An important indication of this oversimplification are the results of a meta-analysis that looked at parental effects (maternal and paternal effects) (Uller et al., 2013). This analysis combined the results of 58 studies with the following general method. Parent 1 grew up in one of two distinct experimental environments, while the parent 2 grew up in in a control environment. The offspring of those parents were randomly distributed over the two experimental environments. On basis of the oversimplified framework you would expect that growing up in the same environment as the experimental parent would be advantageous and not growing up in this environment would be disadvantageous. This is also what the analysis showed, however these (dis)advantages were very weak. This weak result was not due to big differences in the magnitude of the (dis)advantages between the 58 studies that were considered, but the cause was that within every study the (dis)advantages were very subtle. In other words, the parental environment hardly had an impact of the phenotype of the offspring. At first glance these results seem perfectly in line with the oversimplification. The (dis)advantages may be small, but they are there. Even these small effects may in some cases have a significant impact on the fitness of the offspring. So, the oversimplified evolutionary framework probably has a solid basis. Theoretical research also that shows parental effects evolve under certain conditions, for instance when the environmental change is relatively predictable. (English et al., 2015). However, there are some important aspects missing in the framework. Aspects that help explain why the magnitude of the (dis)advantages of parental effects are so small.

One aspect that could partly explain the small magnitude of the (dis)advantages are negatively correlated phenotypes (Kuijper & Johnstone, 2016). If an offspring has a phenotype that differs much from their parents, the offspring might be able to invade another environment and thrive there. This also means that such offspring would perform worse if placed in the same environment as their parent. However, negatively correlated phenotypes may only be advantageous in specific circumstances. For instance, in a situation where multiple specialist patches exist. The native patch me be overcrowded and invading another patch, where different phenotypic qualities are required, can be easier with a negatively correlated paternal effect in place. Furthermore, this explanation is also concerned with maximizing the offspring's fitness, which can be misleading (Gould & Lewontin, 1979). In reality the situation is probably more complex, since there is a conflict situation. There is direct sexual conflict where each parent's goal is to affect the phenotype of its co-parent to its own advantage. For instance, the seminal fluid protein Acp62F in Drosophila melanogaster increases egg storage, but decrease lifespan in females (Lung et al., 2002). There also is indirect sexual conflict. Here the conflict is about the amount investment in and phenotype of the offspring. For example, maternal and paternal interests differ concerning the amount of nutrients transferred to the embryo in mammals. The father could silence specific maternal alleles in the embryo, eventually influencing the placenta to provide more nutrients (Moore & Haig, 1991).

The epigenetic information carriers in sperm could be essential in paternal indirect sexual conflict mechanisms. There is experimental evidence to support this. In mice miRNA present in the sperm cell selectively degrade stored maternal mRNAs in the zygote after fertilization (Rodgers et al., 2015). It is very likely that such indirect sexual conflict mechanisms work both ways and adapt to each other over time. For example, Chen et al speculate EVs might also deliver maternal RNAs to sperm cells present in the female reproductive track, thereby reducing paternal influence on the offspring's gene expression (Q. Chen et al., 2016).

Although more research is needed to establish a more detailed and robust evolutionary framework for paternal effects, the oversimplified framework can now be extended to be more realistic by including sexual conflict mechanisms. The conflicting maternal and paternal interests can cancel each other out. This leads to the small magnitude of the (dis)advantages of paternal effect.
Considering this small magnitude of the paternal effect on offspring, these sexual conflict mechanisms might have been crucial for the evolution of paternal effect mechanisms. It could even be that the transmitters of environmental factors through the germline evolved later, making use of pathways that were involved in sexual conflict. This is speculation, but this can lead to new hypotheses in research in the proximate mechanisms that could eventually lead to a better understanding of both the ultimate and proximate aspects of paternal effects.

Another remark about the research done in the proximate mechanisms of paternal effects is that it often focusses on how the epigenetic modification of genes can lead to different phenotypes and therefore be involved in paternal effects. This is important, but epigenetic modification of noncoding DNA (ncDNA) could also play a crucial role in paternal effects. Although ncDNA does not encode for proteins, it is transcribed. The RNA transcripts of ncDNA can fulfil important roles in regulating gene expression (Q. Chen et al., 2016). Therefore, considering epigenetic modifications of ncDNA may be important in understanding paternal effect mechanisms.

Conclusion

Studies that want to investigate the germline transmitted paternal effects need to exclude the possibility that the paternal effects are maternally transmitted by using IVF. Only a handful of studies has used IVF so far, however all IVF studies showed that germline transmitted paternal effects do exist. Furthermore, miRNAs and tsRNAs have been found to play a key role in the epigenetic mechanisms of these paternal effects. In addition to the RNAs there are two other epigenetic information carriers involved in the mechanism: DNA methylation and chromatin modification. These three epigenetic mechanisms are likely to interact, leading to more sophisticated ways of controlling gene expression in the embryo. In addition to this, sexual conflict and interaction between maternal and paternal RNAs could play an important role. The details of all mechanisms remain unclear and further research is needed to unravel those. Furthermore, more IVF studies need to be performed to confirm the results found so far. Despite these uncertainties, the IVF studies provide strong evidence of the existence of germline transmitted paternal effects. In conclusion, it is very likely that germline transmitted paternal effects exist.

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