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Aberrant protamine content in sperm and consequential implications for infertility treatment

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Abstract

Human sperm express two types of protamine: protamine 1 (P1) and the family of protamine 2 (P2) proteins, with P1 and P2 normally existing in a ratio of approximately 1:1. Both the elevation and reduction of this ratio have been linked with male infertility suggesting that abnormalities in protamine expression, processing and replacement may be responsible for effects on semen parameters observed in infertile males affected by deficient protamination, along with abnormalities in associated regulatory processes. Abnormal protamination may result in insufficient condensation in the sperm nucleus, thus rendering paternal DNA susceptible to damage, which could have detrimental consequences upon embryogenesis. Consequently, it is imperative that Assisted Reproductive Technologies (ARTs) endeavour to utilise sperm devoid of protamine abnormalities, especially because retained histones are present in imprinted gene clusters. Emerging evidence indicates that abnormalities in protamine content may influence epigenetic signals transmitted via paternal DNA. Indeed, an increase in rare imprinting disorders has been observed in children conceived via in vitro fertilisation (IVF). This review examines the links between male infertility, abnormal protamine expression and replacement, the implications of abnormal sperm DNA packaging on fertility treatments and the potential iatrogenic effects of ART procedures on sperm function.

Keywords: Protamine, sperm, infertility, assisted reproductive technology, male infertility, epigenetics

Introduction

Sperm chromatin is a highly condensed, specialised structure comprising paternal deoxyribonucleic acid (DNA) bound to protamines (Ward & Coffey, 1991; Carrell et al., 2007; Kanippayoor et al., 2013; Montellier et al., 2013), a family of small, basic proteins which were first discovered in salmon sperm by Friedrich Miescher in 1872 (Miescher, 1874; Dahm, 2005). In many species, protamine is the most abundant nucleoprotein in the mature sperm nucleus.

During spermiogenesis, when spermatids mature into spermatozoa, nucleohistones, the nucleoproteins normally found associated with DNA in somatic cells, are successively replaced by nucleoprotamines, via a well-characterised transition mechanism (Boskovic & Torres-Padilla, 2013) (Figure 1). Firstly, testis-specific histones are incorporated into the DNA; the histone H2B variant, TH2B, may be solely responsible for nucleohistone displacement (Montellier et al., 2013). Hyper-acetylation of the histones reduces their DNA binding affinity and contributes to DNA relaxation via topoisomerase activation (Turner, 1991; Hong et al., 1993; Carrell et al., 2007), allowing replacement of histones with transition proteins 1 and 2 (TNP1 and TNP2). TPNs are then replaced by phosphorylated protamines, causing the formation of toroidal structures in the chromatin, resulting in increased levels of DNA packaging and chromatin condensation that are six times than those seen in somatic cells (Balhorn et al., 2000; Fuentes-Mascorro et al., 2000). Consequently, the integrity of the paternal genome is protected as the sperm progress through both the male and female reproductive tracts (Carrell et al., 2007). While the protection of paternal genetic information is the principal function proposed for protamine, experimental evidence also supports additional roles in epigenetic reprogramming of the paternal genome within the oocyte following fertilisation, and the generation of hydrodynamic nuclei (Oliva, 2006).

From the early spermatid stage, histones are systematically removed from the sperm DNA and replaced, in a species-dependent manner, by proteins such as...
sperm-specific histones, protamine-like proteins or true protamines during the final stages of sperm maturation. Comparative analyses across a variety of invertebrates and vertebrates suggest that these three types of proteins have arisen during the process of evolution, with evolutionary-advanced animals primarily utilising protamines for sperm DNA packaging (Balhorn, 2007).

In mammals, two types of protamine are present: protamine 1 (P1) and the family of protamine 2 (P2) protamines. P1 has been found in all vertebrates studied thus far, whereas P2 has only been identified in certain mammalian species, including humans and mice, indicating a conserved role for P1 (Corzett et al., 2002; Oliva, 2006; Balhorn, 2007). Protamines are characterised by...
the highest evolutionary variation among species, in terms of the total number of cysteine residues and amino acids present in sperm nuclei (Oliva, 2006). Such evolutionary diversity contributes to clear differential ratios of P1 and P2 across different mammalian groups (Corzett et al., 2002; Gosalvez et al., 2011). In humans, the P1 and P2 ratio is equivalent, or falls between a narrow range of 0.8–1.2. Infertility has been observed in males with undetectable P2 levels in sperm nuclei (de Yebra et al., 1993), and reduced fertility reported in males with abnormal P1:P2 ratio (Carrell & Liu, 2001; Mengual et al., 2003; Aoki et al., 2005a, 2005b; Torregrosa et al., 2006; Castillo et al., 2011; Depa-Martynow et al., 2012; Rogenhofer et al., 2013). The P1:P2 ratio in human sperm may therefore represent a useful indicator for determining fertility in a clinical scenario.

The aim of this review is to outline the structure and function of the protamine family with specific reference to the nucleohistone–nucleoprotamine transition, to discuss clinical links between abnormal protamine expression/replacement and male infertility, and consider the potential effects of abnormal sperm DNA packaging upon fertility treatment.

Protamine structure and function

Human males express P1 and the P2 protamine family (comprised of P2, P3 and P4 components). P1 (PRM1) and P2 (PRM2) genes are organised on chromosome 16 in a loop domain with the TNP2 gene and together with gene4, which was misleadingly referred to as ‘protamine 3 (P3)’ (Schluter et al., 1996; Oliva, 2006) (Figure 2a).
P3 is acidic, is not a protamine, and lacks introns, unlike P1, P2 and TNP2, and is therefore unlikely to be involved in DNA condensation (Martin-Coello et al., 2011). However, a role for P3 in the regulation of sperm motility has been proposed; gene4 knockout mice (PRM3−/−) exhibit reduced sperm velocity when compared to wild-type sperm (Grzmił et al., 2008).
P1 is translated as a mature protein, whereas components of the P2 family are generated via the processing of a precursor encoded by PRM2 (Yelick et al., 1987; Figure 2b). Immediately after translation, P1 is phosphorylated by serine/arginine protein-specific kinase 1 (SRPK1) (Papoutsopoulou et al., 1999) and a P2 intermediate is phosphorylated by calcium/calmodulin-dependent protein kinase 4 (CAMK4). Phosphorylation of the P2 intermediate is necessary for DNA binding and subsequent cleavage to mature P2 (Carrell et al., 2007).

Evolutionary selection has led to an increase in the number of positively charged residues within protamines, allowing tighter binding to negatively charged paternal DNA and the formation of highly condensed chromatin (Oliva & Dixon, 1990). Positively charged arginine residues in P1 appear to be under a form of purifying selection; their proportion is maintained at approximately 50% throughout taxonomic groups, highlighting the importance of this particular amino acid (Rooney et al., 2000) (Figure 2b). Nucleoprotamine structures are further stabilised by disulphide bonds which form between cysteine residues of adjacent protamines (Lewis et al., 2003).

Compaction of paternal DNA with the consequent generation of hydrodynamic nuclei and fast-swimming spermatozoa is one function proposed for the protamines (Oliva, 2006). Protamines may also play a role in the transmission of epigenetic information by imprinting DNA during spermatogenesis, thus conferring information that would permit reactivation of the paternal genome following fertilisation (Oliva, 2006). In addition, protamine provides protection to the paternal genome; condensation of DNA may render the paternal genetic message inaccessible to mutagens and nucleases, thus maintaining integrity (Oliva, 2006). It therefore follows that abnormal protamine expression and replacement in sperm chromatin could affect processes involved in any one or a combination of these functions, thereby disrupting the transmission of paternal genetic information, with adverse consequences upon embryo development.

Abnormal protamine content and male infertility

Alterations to the narrow range of protamine ratio (0.8–1.2) in mammalian sperm have been clearly linked with infertility. This has generated significant interest in evaluating whether the P1:P2 ratio may be a useful index for sperm selection for assisted reproductive technology (ART), given that conventional semen analysis provides only very limited and rather rudimentary information on sperm quality and function.

Infertile individuals have been identified who exhibit a significant increase in the P1:P2 ratio when compared to fertile men (Carrell & Liu, 2001). Furthermore, sperm from certain individuals undergoing infertility treatment have been identified as being devoid of P2 (de Yebra et al., 1993). In mice, mutation of one allele from the PRM1 or PRM2 genes leads to the production of non-functional sperm and infertility. Haploid spermatids carrying the mutant allele, and those carrying the wild-type allele, were both defective, demonstrating significant levels of haplo-insufficiency (Cho et al., 2001, 2003). Altered P1:P2 ratios have also been linked with abnormal sperm morphology and reductions in sperm count and motility (Aoki et al., 2005a). Low P1:P2 ratios (less than 0.8) are associated with increased levels of DNA fragmentation (Aoki et al., 2005b) and exert severe impact upon sperm quality (Aoki et al., 2005b).

Changes to P1:P2 ratios caused by reduced or altered P2 expression have also been linked with reduced progressive motility and penetration ability, along with abnormal sperm morphology (de Yebra et al., 1993; Carrell & Liu, 2001; Aoki et al., 2005a,
Increased expression of P2 precursors discovered in infertile individuals described as having low P2 levels, indicate that problems may arise during P2 processing (de Yebra et al., 1998). The pre-P2:P2 ratio may therefore influence the P1:P2 ratio (de Mateo et al., 2009), resulting in defective DNA compaction and detrimental changes in semen quality parameters if aberrant. Alternatively, altered P1:P2 ratios may arise from defective histone replacement during spermiogenesis; indeed, increased levels of histones have been reported in sperm from infertile men compared to fertile controls (Zhang et al., 2006; Montellier et al., 2013). Overall, deviation from the normal P1:P2 ratio appears to play a key role in male infertility, although the exact mechanisms by which this occurs may differ between individuals, and have yet to be fully elucidated. Research thus far has identified several potential mechanisms, as discussed herein.

Altered protamine expression

Links between altered P1:P2 ratios, or diminished P2 levels and infertility, underlie the notion that mutations in PRM1 and PRM2 genes may be responsible. Single nucleotide polymorphisms (SNPs) in both PRM1 and PRM2 have been identified in infertile males, although causal mutations have been difficult to elucidate. Tanaka et al. (2003) detected one SNP in the middle of the coding region of PRM2 which resulted in the formation of a stop codon and subsequently, premature termination of P2 translation. Consequential alterations to the functional structure of this protamine have therefore been suggested as a cause of azoospermia in patients identified with this SNP (Tanaka et al., 2003). Males whose sperm presented a similar phenotype to that of protamine-deficient knockout mice exhibited an SNP in PRM1; arginine to serine at position 34 (R34S), which
disrupts a highly conserved arginine cluster and created a new SRPK1 phosphorylation site (Iguchi et al., 2006; Jodar et al., 2010), both of which are essential for protamine-DNA binding. However, meta-analysis has revealed that this mutation does not appear to be a risk factor for male infertility (Jodar et al., 2010).

Nevertheless, protamine haplotype is suggested to exert an influence over various semen parameters. Tütelmann et al. (2009) reported higher sperm concentrations and counts in males with the haplotype ACC, formed by three SNPs: PRM1 230AA, and PRM2 298CC and 373CC. Males homozygous for these SNPs exhibited a two-fold higher sperm output compared to males without this haplotype (Tütelmann et al., 2009). In addition, an SNP in the PRM1 gene promoter region (−191 C>A) appeared to arise more frequently in individuals exhibiting abnormal sperm morphology in a Spanish population, with the c.-191AA genotype associated with an elevated P1:P2 ratio (Gazquez et al., 2008; Jodar et al., 2010). A further SNP in the PRM1 promoter region, 15 base pairs upstream from the transcription initiation site, has been identified in four males with severe unexplained oligozoospermia, suggesting that the region of DNA affected may influence P1 expression, although the precise effect of this mutation is unknown (Ravel et al., 2007). Protamine genotype may therefore influence male fertility (Tütelmann et al., 2009) and as a consequence, variations anywhere in the PRM1 and PRM2 open-reading frames may be risk factors for infertility via abnormal protamine expression (Jodar et al., 2010).

However, while mutations in PRM1, PRM2, and associated regulatory regions may have an impact upon protamine expression, they are unlikely to be a common cause of human infertility, and appear to differ greatly between individuals and populations.

Abnormal expression of P1 and P2 is also postulated to arise from defective regulation of transcription, translation and post-translational modifications (Carrell et al., 2007). Various elements are involved in controlling post-meiotic gene expression during spermatogenesis and have been implicated in disrupting normal protamine expression when defective (Table I). One such transcriptional regulator is cyclic adenosine monophosphate (cAMP) response element modulator (CREM) which is involved in transcribing several genes during the post-meiotic phase of spermatogenesis, including PRM1 and PRM2 (Ha et al., 1997). Sequencing of the promoter for the protamine 1 gene (P1) in 10 different species revealed a conserved sequence, referred to as ‘Prot 1C’, which demonstrated clear conservation of the CRE element within this consensus sequence (Queralt & Oliva, 1993). CREM tau, the testis form of CREM, has a binding site in the protamine gene promoter region known as cAMP response element (CRE) (Carrell et al., 2007). Activator of CREM (ACT) and KIF17B, a testis-specific kinesin and co-factor to ACT, can associate with CREM tau, allowing binding to CRE and subsequent gene expression. KIF17B is able to export ACT out of the nucleus, ceasing association with CREM, and thus repressing CREM-mediated transcription (Krausz & Sassone-Corsi, 2005). ACT-null mice exhibit dramatic reductions in sperm counts, with high numbers of sperm displaying severe head malformations caused by defective chromatin compaction and acrosome formation (Kotaja et al., 2004).

Human infertility could therefore result from the defective regulation of transcription and translation of post-meiotic genes, thus affecting the abundance and function of P1 and P2 required for DNA condensation. Indeed, protamine messenger ribonucleic acid (mRNA) levels are significantly correlated with protamine protein levels and consequently, with sperm concentration and potentially motility (Depa-Martynow et al., 2012). This point is particularly important when considering that protamine transcripts are stored in ribonucleoprotein particles in spermatocytes and round spermatids for later translation in elongating spermatids when transcription has ceased (Carrell et al., 2007). Altered protamine transcription may therefore be the origin of abnormal sperm protamine content (Depa-Martynow et al., 2012; Rogenhofer et al., 2013). The involvement of protamine transcription/translation regulators in the expression of other genes associated with spermatogenesis could also explain defective patterns of spermatogenesis detected in infertile individuals (Carrell et al., 2007).

### Abnormal protamine replacement

Alterations to P1:P2 ratios may result from the abnormal replacement of histones with protamines, leading to inadequate DNA packaging, and infertility. Usually, hyper-acetylation occurs during spermiogenesis to reduce histone–DNA binding affinity, inducing chromatin relaxation and allowing histone displacement (Hong et al., 1993). Sonnack et al. (2002) demonstrated a link between abnormal spermatogenesis and reduced/premature histone acetylation, highlighting the importance of appropriate temporal control for histone acetylation and deacetylation during spermatogenesis. In addition, Zhang et al. (2006) concluded that abnormal histone retention is associated with altered protamine replacement; sperm from infertile men exhibited a higher ratio of histone H2B:protamine than sperm from fertile men. Normal DNA compaction is thus prevented, increasing susceptibility to DNA damage (Zhang et al., 2006).

CAMK4, which phosphorylates P2 before the protamine undergoes proteolytic cleavage and DNA binding (Carrell et al., 2007), has also been implicated in abnormal protamine replacement. Targeted deletion of CAMK4 in mice caused infertility and a loss of P2 during late spermiogenesis, resulting in prolonged retention of TNP2 in the nucleus (Wu et al., 2000). Signalling via phosphorylation of P2 by CAMK4 is therefore proposed to be necessary for TNP displacement. Reduced levels of P2 found in humans may therefore prevent displacement, resulting in the retention of TNPs, disruption to DNA packaging, and consequential infertility (Wu et al., 2000). Cho et al. (2003) further highlighted the
importance of TNPs in nucleohistone-nucleoprotamine transition by revealing that TNP2-null mice exhibit incomplete chromatin condensation, and possess DNA that is more susceptible to denaturation compared to wild-type. TNP1-null mice exhibit elevated levels of P2 precursors and higher numbers of sperm with less condensed chromatin compared to wild-type controls, suggesting a relationship between TNP1 abnormalities and increased P2 precursors detected in infertile males (Yu et al., 2000).

Mechanisms of DNA damage
Abnormalities in the protamine content of sperm nuclei have been linked with increased susceptibility to DNA damage. Aoki et al. (2005b) found that individuals exhibiting a P1:P2 ratio within the normal range, but expressing reduced levels of both protamines, exhibited increased sperm DNA fragmentation compared to men with normal protamine levels. Simon et al. (2011) also reported increased sperm DNA fragmentation in males exhibiting both abnormally high and low P1:P2 ratios. Various mechanisms have been proposed to account for DNA damage in sperm, including the failure of germ cells to complete apoptosis, incomplete chromatin condensation during spermiogenesis, and oxidative stress induced by the release of reactive oxygen species (ROS) during post-testicular transport (Sakkas & Alvarez, 2010) (Figure 3). Sakkas et al. (1999) identified a higher frequency of ejaculated sperm exhibiting DNA breaks and the apoptotic marker, Fas, in men with abnormal semen parameters compared to males with normal sperm counts, and proposed that such sperm had undergone ‘abortive apoptosis’ in which sperm had escaped programmed cell death and been released normally for ejaculation. Signs of incomplete spermiogenesis such as DNA fragmentation and protamine abnormalities may therefore represent failed apoptosis (Sakkas et al., 1999). Alternatively, defective topoisomerase II activity between the round spermatid and elongating spermatid stage (Figure 3) may cause the persistence of double-stranded DNA nicks in mature sperm (McPherson & Longo, 1993; Carrell et al., 2007); removal of protamines in mouse sperm led to complete digestion of the DNA by topoisomerase and nuclease enzymes (Shaman et al., 2006), indicating that ineffective protamination may result in increased DNA damage and subsequent infertility.

ROS have also been implicated in sperm DNA strand breakage, cross-linking and base modifications (Twigg et al., 1998; Barroso et al., 2000). As highly condensed DNA packaging and seminal plasma antioxidants usually protect sperm DNA from oxidative stress, imbalance between ROS and antioxidant levels in the male reproductive tract may increase vulnerability to DNA damage (Saleh & Agarwal, 2002). Kodama et al. (1997) reported significantly elevated levels of oxidative damage in sperm DNA from infertile males compared to those of fertile controls. Recent research has also supported the clinical use of oral antioxidant therapies, given the clear role of ROS in sperm DNA damage (Abad et al., 2013). However, antioxidants such as vitamin C may also increase the susceptibility to DNA fragmentation by reducing the number of disulphide bonds in protamine (Menez et al., 2007).

Implications for infertility treatments and iatrogenic (medically-induced) damage
ART, developed to aid the 10% of couples worldwide facing infertility, has led to the birth of over 5 million babies globally (ESHRE, 2013). Male infertility is a major contributory factor to the requirement for ART. Protamine deficiency with associated reductions in sperm count, motility and penetration ability, strongly indicates the need for in vitro fertilisation (IVF) with
intra-cytoplasmic sperm injection (ICSI) (Aoki et al., 2006). However, the likelihood of ART being successful in such cases remains debatable, as low P1:P2 ratios have been linked with lower fertilisation and pregnancy rates following IVF (de Mateo et al., 2009). Furthermore, embryo quality appears to be negatively correlated with sperm protamine content (Simon et al., 2011; Depa-Martynow et al., 2012). In addition, males with increased sperm DNA fragmentation were less likely to achieve a clinical pregnancy following IVF than males with DNA fragmentation below a clinically prognostic threshold value, although this effect was not present in couples undergoing ICSI (Simon et al., 2011). Intra-sample heterogeneity observed in human sperm protamine content (Aoki et al., 2006) may allow ICSI to be successful in overcoming diminished sperm functional ability and DNA fragmentation associated with protamine deficiency, via sperm selection techniques such as density gradient washing (DGW). Such techniques may permit the identification of sperm with normal protamine content (Aoki et al., 2006), although a rapid test to reach such a conclusion for an individual viable sperm has yet to be developed. However, Rogenhofer et al. (2013) reported that males with aberrant protamine mRNA ratios exhibited a lower fertilisation capacity than normozoospermic men when undergoing both IVF and ICSI, indicating that ICSI may not always be helpful in cases of abnormal protamination. Nevertheless, measurement of protamine mRNA ratios may prove clinically useful in estimating the fertilisation potential of sperm (Rogenhofer et al., 2013), although such a procedure would not identify individual and selectable viable sperm for clinical application. However, questions have been raised regarding the clinical use of sperm exhibiting protamine abnormalities, especially given the role of highly condensed DNA in the epigenetic regulation of the paternal genome.

While 85% of histones are replaced by protamines during spermiogenesis in humans, the histones remaining are known to bear epigenetic modifications such as acetylation and methylation (Schagdarsurengin et al., 2012). Hammoud et al. (2009) demonstrated that modified nucleosomes are enriched in genes involved in embryonic development such as homeobox (HOX)
and imprinted gene clusters. Retention of up to approximately 15% of histones in the paternal genome may therefore be highly programmatic (Hammoud et al., 2009) and could affect the developmental capacity of the resulting embryo (Oliva, 2006). This finding is particularly important when considering the case of imprinted genes, which are only expressed from one allele in a parent-of-origin manner (Schagdarsurengin et al., 2012). The physical manipulation of gametes and embryos during ART may disrupt epigenetic marking, thus leading to imprinting errors. Indeed, the incidences of rare imprinting disorders such as Angelman, Beckwith-Wiedemann and Silver-Russell syndromes are increased in children conceived via ART (Amor & Halliday, 2008; Hiura et al., 2012). Sperm function may therefore encompass much more than the simple transfer of paternal DNA to the oocyte. The unique chromatin structure with epigenetic marks in genes involved in the regulation of transcription and developmental processes suggests that the paternal genome plays a key part in initiating embryonic development (Schagdarsurengin et al., 2012). In light of these findings, the use of sperm with protamine abnormalities during ICSI raises profound concerns for the developing embryo (Dada et al., 2012) and could predispose to epigenetic alterations (Kashir et al., 2012).

Laboratory techniques utilised during ART have also been implicated in gamete and embryo abnormalities, and such iatrogenic damage has been linked to the relatively low success rates seen in ART (Kashir et al., 2012). Concerns have been raised regarding the use of methods such as DGW and ‘swim-up’ for sperm selection which relies on centrifugation steps that could exacerbate oxidative stress and exert damage upon the sperm membrane and DNA (Tremellen, 2008). In addition, the common practice of incubating semen samples at 37°C has been associated with reduced sperm motility and increased DNA fragmentation; such effects were reduced when sperm was incubated at room temperature (Matsuura et al., 2010). Furthermore, commonly used sperm cryopreservation has been linked with increased levels of DNA fragmentation (Zribi et al., 2010) and ROS, with concurrent reductions in glutathione, an essential antioxidant (Gadea et al., 2011). Further studies are therefore required to determine whether the clinical use of sperm exhibiting abnormal protamination parameters during ART may have detrimental consequences for the embryo. It is also imperative that new bioassays are developed to identify viable and selectable sperm that are devoid of protamine-related abnormalities.

Conclusions

In summary, deviations from the expected P1:P2 ratio in human sperm have been clearly associated with infertility. The mechanisms by which this occurs require significant clarification; however, defective regulation of protamine expression, abnormal histone replacement, the presence of SNPs in genes encoding the protamines, and various other sources of protamine disruption, including interactions with metals and pesticides, are all potential explanations for the growing links between abnormal protamination and male infertility. Evidence suggests that a failure to generate a normal nucleoprotamine structure in the sperm nucleus renders paternal DNA vulnerable to nuclease and ROS damage, resulting in defective spermatogenesis and subsequent infertility. In these cases, ICSI can be successful as a clinical treatment. However, using sperm with known protamine abnormalities to fertilise an oocyte is highly likely to influence embryonic development. Given the role of retained modified histones in sperm chromatin in the transmission of epigenetic signals, abnormal protamination would be expected to have an adverse effect on these epigenetic marks (Carrell, 2012). The increased incidence of rare epigenetic disorders such as Angelman, Beckwith-Wiedemann and Silver-Russell syndromes in children born through IVF (Amor & Halliday, 2008; Hiura et al., 2012) could indicate that abnormalities in the paternal genome are becoming more prevalent. However, research also suggests that other iatrogenic effects may be contributing to such abnormalities. If ART is to be used in cases of protamine deficiency, it is imperative that further research targets the prevalence of such issues in patients attending infertility clinics, devises laboratory assays specifically to identify viable and selectable sperm that are devoid of protamine deficiency, and attempts to elucidate the specific role of protamines in transmitting epigenetic signals.

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