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Mammalian Sperm Chromatin as a Model for Chromatin Function in DNA Degradation and DNA Replication

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Abstract

Reproductive biology is considered a specialty field, however, an argument can be made that it is instead generally applicable to many fields of biology. The one-cell embryo is presented here as a model system for the study of eukaryotic DNA replication, apoptotic DNA degradation, and the study of signaling mechanisms between the cytoplasm and nucleus. Two unique aspects of this system combine to make it particularly useful for the study of chromatin function. First, the evolutionary pressure that lead to the extreme condensation of mammalian sperm DNA resulted in a cell with virtually inert chromatin. No DNA replication or transcription ongoing in the sperm cell, and all the cells are in a G₀ state. This chromatin is suddenly transformed into actively transcribing and replicating DNA upon fertilization. Therefore, the sperm chromatin is poised to become active but does not yet possess sufficient components present in somatic chromatin structure for all these processes. The second unique aspect of this system is that the one cell embryo houses two distinct nuclei, termed pronuclei, through the first round of DNA synthesis. This means the sperm cell can be experimentally manipulated to test the effects of the various treatments on the biological functions of interest. Experimental manipulations of the system have already revealed a certain level of plasticity in the co-ordination of both the timing of DNA synthesis in the two pronuclei and in the response to cellular signals by each pronucleus involved with the progression through the G₁/S checkpoint, including the degradation of DNA in the paternal pronucleus. The fact that two nuclei in the same cytoplasm can undergo different responses infers a level of autonomy in the nuclear control of the cell cycle. Thus, the features of mammalian fertilization provide unique insights for the normal biology of the cell cycle in somatic cells.

Keywords

DNA Replication; Apoptosis; DNA Degradation; Chromatin Function

Introduction: Fertilization as a Unique Model for Chromatin Function

Reproductive biology is usually considered a specialty field but a strong argument can be made that it is, in fact, generally applicable to many other fields in biology. The field has had uniquely important impacts in the area of DNA function. It encompasses a key element of evolution, the mixing of genes from two different organisms to provide greater variety for the progeny. The molecular mechanisms that control this genetic variety are found in meiosis. The unique chromatin structures of the synaptonemal complex, in which the two

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homologous chromosomes from each pair come together, have provided valuable insight into the structure of mitotic chromosomes. Studies of the mechanisms by which homologous chromosomes trade DNA sequences, crossing-over, have resulted in important insights into DNA repair, mutation research, and chromosomal translocations in cancer (Liang et al., 2010).

One important model reproductive biology has provided to the study of chromatin structure is fertilization. With no detectable transcription, DNA replication, or DNA repair mechanisms, sperm DNA is nearly functionally inert (Yanagimachi, 1994). Yet, when this genome is inserted into the oocyte at fertilization, the chromatin is reorganized and all these processes become activated *de novo*. This provides two set points for studying chromatin function. The first is the preparatory phase in the condensed sperm nucleus. The chromatin in these cells is not yet actively engaged in any of these processes but must be in a state that is able to participate in any of these pathways upon receiving the correct biochemical signals. The sperm nucleus therefore provides a model to identify the key elements that must be present for biologically viable chromatin. The second is the activation of the events after fertilization. The sperm chromatin thus represents a virgin state of active DNA in the sense that this DNA has not yet been involved in any functional process. This presents an opportunity to examine functions of interest from a different viewpoint than is often available in the somatic cell.

We discuss how our laboratory is using the sperm chromatin model to investigate two important chromatin functions, DNA replication and DNA degradation during apoptosis, and detail key questions in each area of study for which the sperm cell is an ideal model. The main hypothesis of this manuscript is that while many important differences between the one cell embryo and somatic cells exist, there are enough similarities that studying one can shed important insight on the other. One of the best examples of how the study of the reproduction can lead to important discoveries about somatic cell regulation is the discovery of cyclins controlling the cell cycle. Early studies on *Xenopus* oocytes identified an M-phase promoting factor, or MPF, that controlled the progression of oocytes that were arrested in meiotic prophase to activate the mitotic machinery (Reynhout and Smith, 1974; Wasserman and Smith, 1978). It was later discovered that MPF was composed of CDK and cyclin complexes (Maller et al., 1989; Moreno et al., 1989a) now known to control the progression of the cell cycle in somatic cell. In 1989, Moreno et al. stated that "Considerable advances have been made recently in our understanding of how the cell cycle timing of mitosis is regulated. This has come about because links have been established between two independent areas of research, one based on a genetic approach using the fission yeast *Schizosaccharomyces pombe* and the second based on a biochemical approach using *Xenopus* and starfish oocytes." (Moreno et al., 1989b). In this review we suggest that similar links between reproductive biology and somatic cell regulation can provide important insights into DNA replication and the degradation of DNA during apoptosis.

Regulation of Mammalian DNA Replication: The Need for Licensing

One of the important biological processes that seems to be initiated *de novo* in sperm DNA is the licensing of origins of replication. Before discussing the role of the sperm model in this process, we will first briefly review licensing. The 6 billion base pairs of the human (and mouse) genome are replicated in the cell with 10^3 to 10^5 different start sites, called replication origins, dispersed throughout the genome (Pardoll et al., 1980; Vogelstein et al., 1980). In most mouse and human cell lines in culture, the entire genome is replicated completely in a highly coordinated manner during S phase that lasts an average of 6 hrs, and each segment is only replicated once. Different parts of the genome can be replicated at independent times (Stubblefield, 1975), and in different structural foci in the nucleus

(Berezney, 1991; Berezney, 2002). DNA replication occurs at a fixed site within all cells, from bacteria (Lemon and Grossman, 1998) to mammals (Berezney and Coffey, 1975; Jackson and Cook, 1986; Pardoll et al., 1980). That is, the replication complex remains stationary while the template DNA moves through it during replication (Fig. 1). Because there are so many different replication origins that are replicated at different times, researchers in the field began to question how the cell efficiently prevented any of these origins from firing more than once during S phase, which would result in “rereplicating” parts of the genome (Fig. 2). In 1988, Blow and Laskey (Blow and Laskey, 1988) proposed that a “licensing factor” binds to DNA replication origins during G1 in the preparation for DNA synthesis, and subsequently provided experimental evidence for its existence (Blow, 1993; Leno et al., 1992). We now know that licensing is a highly regulated event with the coordinated sequential loading of several different proteins (Fig. 3, adapted from (Takeda and Dutta, 2005)). Licensing prevents rereplication of any segment of the genome, and failure to license can lead to checkpoint activation that halts DNA synthesis.

Licensing begins in early G1, soon after mitosis, when the origin recognition complex protein, ORC1L (origin recognition complex, subunit 1 – like (*S. cerevisiae*)), binds to a complex of five related proteins ORC2L-5L, which in mammals remains bound to the DNA throughout the cell cycle (Fig. 2) (DePamphilis, 2005; Thomae et al., 2008). The ORC complex recruits CDT1 and CDC6, which in turn recruits the MCM2-7 helicase complex. At this stage, the origin is considered licensed, and DNA synthesis can be initiated at this site in S phase. The CDK2/CCNA2 and CDK2/CCNE1 complexes activate the cell to progress to S-phase. At this point, replication begins, and all new licensing is inhibited, when CDK phosphorylates CDC6 releasing it and CDT1 from the DNA. PCNA and a host of DNA synthesis initiation proteins then bind, and finally DNA polymerase attaches to the origin complex. The MCM2-7 helicase may remain associated with the replication fork during replication. ORC2L-5L remain bound to the origin of replication, and ORC1L cycles off the complex, to return in the next round of DNA synthesis. Recent evidence suggests that some of these same factors are also important for licensing in the mammalian one cell embryo (Borsuk and Czolowska, 2010).

DNA Replication in the Mammalian One Cell Embryo

Mammalian fertilization has one attribute that makes it an excellent model to study DNA replication; the maternal and paternal genomes are segregated into to separate nuclei, called pronuclei, until the first mitotic division (Sirlin and Edwards, 1959). An important consequence of this segregation is that the two genomes are replicated in separate pronuclei in the same cytoplasm (Fig. 4A). After fertilization, the sperm chromatin decondenses to form the male pronucleus, while the metaphase II plate of the oocyte completes division and the maternal chromatin decondenses to form the female pronucleus. Both pronuclei undergo one round of DNA synthesis before the DNA condenses into mitotic chromosomes which join at a single meta-phase plate where the paternal and maternal genomes are united for the first time. Several reports have suggested DNA replication in mice begins in the male pronucleus up to 2 hr before it does in the female when embryos are generated by normal mating (Bouniol-Baly et al., 1997; Ferreira and Carmo-Fonseca, 1997; Howlett and Bolton, 1985; Luthardt and Donahue, 1973), while in IVF this asynchrony was less apparent (Aoki and Schultz, 1999; Howlett and Bolton, 1985). Specifically, Aoki and Schultz (Aoki and Schultz, 1999) reported that while male and female pronuclear DNA synthesis began synchronously, the male pronucleus completed DNA synthesis one hour before the female. Our recently published data using ICSI, have not detected any differences in the initiation of DNA replication within 1 hr windows (Yamauchi et al., 2009).

The simultaneous initiation of DNA synthesis in both pronuclei when IVF or ICSI are used suggests that either both nuclei have near identical molecular clocks for the timing of replication or that they both respond to a cytoplasmic signal to initiate DNA synthesis. Mammalian cell free DNA replication systems have revealed that the cytoplasm does exert some control over DNA replication in the nucleus (DePamphilis et al., 2006; Takeda and Dutta, 2005), supporting the latter model. However, the exact molecular mechanisms for this control are not well understood. One possibility is the CDK-cyclin complexes CDK2/CCNA2 and CDK2/CCNE1. At least one study has shown that these complexes shuttle between the cytoplasm and the nucleus (Jackman et al., 2002). Either possibility, complete nuclear autonomy or cytoplasmic control, would have implications for DNA synthesis studies in somatic cells, but the one cell embryo, which contains two nuclei in the same cytoplasm, is the most likely to provide a framework in which to distinguish them.

Experimental Manipulation of the Initiation of DNA Synthesis Initiation in Mouse Pronuclei

While evidence suggests the two pronuclei initiate replication together, there is some flexibility in the timing of DNA synthesis in the one cell embryo. For example, it is possible to experimentally manipulate fertilization such that the male pronucleus initiates DNA replication up to two hours after the female pronucleus. Two laboratories have demonstrated this by injecting mouse oocytes with spermatozoa after parthenogenic activation of the cell. In some cases delayed injection of spermatozoa into oocytes resulted in an embryo that does develop to the birth of a live pup (Kishigami et al., 2004; Maleszewski et al., 1999). We recently used the same technique to test whether DNA synthesis was initiated at the normal time when the sperm nucleus was injected after oocyte activation (Yamauchi et al., 2009). We found that in some of the oocytes injected with mouse sperm nuclei, the male pronucleus initiated DNA synthesis at least 2 hrs after the female pronucleus (as in Fig. 4B, except that sperm nuclei, not halos – see below – were injected at 2 hrs post activation). Asynchronous initiation of two nuclei in the same cytoplasm was also shown by another group by fusing immature oocytes with parthenogenetically activated oocytes (Borsuk and Czolowska, 2010).

These data suggest that even if the pronuclei normally initiate DNA synthesis together, as discussed above, there are some circumstances in which the two pronuclei can be in different phases, G1 and S, in the same cytoplasm. Understanding the mechanisms that control the plasticity of the cytoplasm, as well as the limits of that plasticity, will have important consequences for models of the cytoplasmic and nuclear communication during the G1/S transition.

The Sperm Matrix is Necessary for DNA Replication: Evidence that the sperm nucleus provides an origin replication demarcation

Mammalian sperm nuclei are remarkably resistant to mechanical stress, even when most of the DNA binding proteins are extracted with high salt and reducing reagent (Ward et al., 1989). This has allowed for the experimental manipulation of “sperm nuclear halos”, which are sperm nuclei that contain only a salt resistant nuclear skeleton termed the nuclear matrix, and naked 50 kp loops of DNA attached at their bases to it (Choudhary et al., 1995; Ward et al., 1989). Mouse sperm halos injected into oocytes can form normal paternal chromosomes (Mohar et al., 2002), indicating that one complete round of DNA synthesis is possible when the oocyte uses a sperm halo as the template for paternal DNA replication (Fig. 4B). More recently, we have demonstrated that when sperm nuclear halos are treated with restriction endonucleases to remove most of the loop DNA, the remaining DNA associated with the

sperm nuclear matrix still acts as a template for DNA synthesis (Shaman et al., 2007). Isolated sperm DNA injected into mouse oocytes does not replicate, nor does DNA reconstituted with isolated sperm nuclear matrices (Shaman et al., 2007). These data demonstrate that it is the in situ chromatin organization of DNA associated with the sperm nuclear matrix that is required for DNA replication in the male pronucleus.

This finding has many implications for the regulation of mammalian DNA replication. First, it supports many older reports that DNA replication occurs on the nuclear matrix in somatic cells (Berezney and Coffey, 1975; Dijkwel and Hamlin, 1995; Jackson and Cook, 1986; Pardoll et al., 1980). Second, the binding of the 6 ORC proteins is resistant to 50 mM KCl, but not to the 2 M NaCl, used to make sperm halos (Rowles et al., 1999). Thus, sperm halos are very unlikely to contain the earliest known origin binding proteins. However, the origins must have been identified by the replication machinery in the oocyte because the DNA was replicated completely. Mammalian replication origins are not determined by DNA sequence, therefore an epigenetic marker for DNA replication origins must be present in sperm nuclear halos to which ORC proteins bind after fertilization.

This suggests the possibility that the sperm nucleus and the events that occur in the paternal chromatin shortly after fertilization may provide the means through which we may answer one of the most perplexing questions in the field. Replication in bacteria and yeast initiates at defined DNA sequences, but mammalian origins are not dependent on DNA sequence (Costa and Blow, 2007; Gilbert, 2001; Robinson and Bell, 2005). ORC proteins, which remain on the origins throughout the cell cycle in mammals, are not likely to be the direct origin demarcation entities because in *Xenopus*, which also has sequence independent origins, all six ORC proteins cycle on and off the origins (DePamphilis, 2005; Sun et al., 2002). A major question in this field is “how do cells determine where and when to assemble pre-RCs, and how do they ensure sufficient sites are distributed throughout the genome to allow its complete duplication within a reasonable period of time?” (DePamphilis et al., 2006). In other words, what defines a mammalian origin of replication? Our data suggest that sperm halos contain this information in some form, possibly the attachment sites of DNA to the sperm nuclear matrix.

Nuclear Autonomy in DNA Degradation: Implications for Apoptosis

The plasticity of the relationship between the cytoplasm and the two pronuclei extends beyond simply the timing of the initiation of DNA replication. It is possible to experimentally manipulate mouse spermatozoa so that the nuclear responses of the cellular signals to cross the G1/S border are different (Fig. 4C). Mouse sperm nuclei that are treated with Mn^{2+} and Ca^{2+} degrade their DNA to 50 kb fragments in a reversible manner reminiscent of TOP2B cleavage (Shaman et al., 2006). The fragments can be religated by treatment with EDTA. Under certain conditions, an unidentified nuclease then irreversibly digests the sperm DNA. This TOP2 – nuclease interaction is similar to the normal apoptotic degradation of DNA in somatic cells, in which DNA is first fragmented by TOP2, then digested by nucleases (Durrieu et al., 2000; Gromova et al., 1995; Hars et al., 2006; Lagarkova et al., 1995; Li et al., 1999). When mouse spermatozoa are treated with Mn^{2+} and Ca^{2+} and then injected into oocytes, the male pronucleus appears to develop normally for the first 5 hrs (Yamauchi et al., 2007b). At the start of DNA synthesis, however, all of the DNA in the male pronucleus is degraded, while the DNA in the female pronucleus is replicated normally (Fig. 4C). The DNA degradation of the male pronucleus can be inhibited by the inhibitor of DNA replication, aphidicolin, (Yamauchi et al., 2007a), confirming the relationship between DNA degradation and the initiation of DNA replication.

These results have two implications for studies in the biology of the one cell embryo, in the relationship between the cytoplasm and nucleus in transitioning the G1/S border, and in apoptotic degradation of DNA. First, the data suggest that two nuclei in the same cytoplasm can respond differently to the cellular signals to cross the G1/S border. This indicates a certain level of autonomy in the two zygotic pronuclei which may have implications for the nuclear – cytoplasmic signaling in somatic cells. Second, in this case, at least, it appears that apoptosis-like DNA degradation can occur without the normal cytoplasmic signals because the female pronucleus was unaffected. Neither implication could have been easily identified in most other cells types that have only one nucleus. It must be emphasized that with regard to this last point we have not yet demonstrated that the DNA degradation observed in the one cell embryo is mediated by topoisomerase 2, so that it is not yet clear how it is mechanistically related to apoptosis in somatic cells. However, if a relationship can be established, it might suggest that DNA degradation and DNA replication share some common molecular pathways.

Finally, one other aspect of apoptotic DNA degradation in somatic cells may be more fully revealed by studying sperm chromatin. As mentioned above, sperm chromatin can be induced to degrade in the male pronucleus after fertilization by incubation with divalent cations, however, this degradation can also be induced in mature spermatozoa outside of the oocyte (Boaz et al., 2008). Both the TOP2B reversible degradation of DNA to 50 kb fragments, which we have termed sperm chromatin fragmentation or SCF, and the subsequent nuclease digestion of the DNA to very small DNA sizes occur when spermatozoa isolated from the vas deferens are treated with divalent cations. Spermatozoa isolated from epididymus only exhibit the TOP2B SCF degradation, suggesting that the nuclease is supplied by the vas deferens. We have recently provided evidence that the nuclease that is associated TOP2B DNA fragmentation is activated by EGTA chelated to calcium, a novel cofactor for nucleases given that most require divalent cations for activation and are actually inhibited by EGTA (Boaz et al., 2008; Dominguez and Ward, 2009). The existence of a nuclease that contributes to apoptotic-like DNA degradation in the male reproductive tract is somewhat controversial because it does not seem immediately consistent with the major biological goal of producing sperm cells with pristine copies of the paternal genome. We have suggested that this may represent a kind of checkpoint for the integrity of sperm DNA (Ward and Ward, 2004), but this has not yet been validated. However, if this mechanism can be more fully elucidated, it may contribute to the identification of the mechanism by which nucleases and TOP2 normally interact during apoptosis.

Conclusions

All of the experiments discussed in this manuscript have important implications for the field of reproductive biology, itself, but we have focused on how they also impact other fields of cell biology. Mammalian sperm chromatin represents the least functional DNA known, and the oocyte provides the mechanism for embellishing that inert chromatin with the components necessary for function. The combination of the sperm and activating oocyte provides a unique model for chromatin function through the window of this unique activation of inert DNA.

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Figure 1. DNA is Replicated at a Fixed Site

DNA origins are prepared for replication by licensing (blue circles, Figs. 2 and 3). During replication, DNA is pulled through the replication machinery, which rests at a fixed site on the nuclear matrix. Newly replicated strands of DNA emerge (red) and remain attached to the nuclear matrix at sites that are not licensed for DNA replication (green spots).

Figure 2. The Need for Licensing in Mammalian DNA Replication

DNA is replicated at several hundred thousand independent start sites called origins. To ensure that each origin only fires once during S-Phase, each origin is “licensed” by the ORC complex (Fig. 2). The license is removed when the origin is replicated, and cannot be added to any origins until the next G1 phase.

Figure 3. Licensing of Mammalian Replication Origins

(A) ORC2L-5L remain on the origin throughout the cell cycle (the fate of ORC6L is currently unknown). (B) Licensing begins with the binding of ORC1L, which (C) recruits CDC6 and CTD1. (D) This complex recruits the DNA helicases MCM2-7, and the origin is fully licensed. (E) CDK2/CCNA2 and CDK/CCNE1 initiate the entry into S-phase and the removal of ORC1L, CDC6 and CDT1, thereby preventing new licensing. At this point, a number of other proteins are loaded onto the replication fork.

A. Normal Fertilization

Figure 4. Experimental Manipulation of Mouse Fertilization to Study DNA Replication and Degradation

(A) Infertilization by ICSI or IVF, the female (F) and male (M) pronuclei are fully decondensed by about 4 hrs, and initiate DNA synthesis simultaneously at about 5.5 hrs. (B) When sperm nuclear halos are injected into oocytes 3 hrs after parthenogenetic activation of the oocyte, the male pronucleus initiates DNA synthesis after the female pronucleus. (C) When sperm nuclei are treated to induce fragmentation of the paternal genome prior to injection, the male pronuclei develop normally until the initiation of S-phase at which point the DNA is degraded.