Role of γ-H2AX in DNA Damage Response

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ABSTRACT

Little is known about the molecular mechanism of γ-H2AX in DNA damage response in mammalian embryos when compared to the somatic cells. An integrative review of the literature on the molecular mechanism of γ-H2AX in DNA damage response in somatic cells and in mammalian embryos was performed. The objective of the study is to summarize and synthesize the published databased literature over the last 10 years on molecular mechanism of γ-H2AX in mouse pre-implantation embryos and somatic cells using the key words – "γ-H2AX", "DNA damage", "somatic cells", "mouse embryo". An initial medline search was performed using our inclusion and exclusion criteria and then expanded by snowballing. The articles were compiled in the form of a Review Matrix.

The knowledge gap was investigated and the conceptual model was formulated to hypothesize the sequence of DNA damage responses occurring during the time of early mouse embryogenesis. **Major themes** drawn from the studies are - 1). Periodic appearance and disappearance of γ-H2AX in early mouse pre-implantation embryos, 2) Development of γ-H2AX foci in the pronuclei without imposing any replicative stress on the mouse embryo, 3) Occurrence of γ-H2AX in the S-phase of somatic cells under replicative stress and normal embryos, 4) Prevalence of prominent γ-H2AX signal in male pronuclei compared to the female pronuclei during mouse embryogenesis. The study will also show scopes of future research to explore the arena in clinical context.

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LIST OF ABBREVIATIONS

- DSB - Double stranded break
- IR - Irradiation
- HU - Hypoxyurea
- Hus-1 - hydroxyurea sensitive-1
- ATM - Ataxia Telangiectasia Mutated
- ATR - Ataxia Telangiectasia and Rad3 related
- GV - Germinal Vessicle
- ATRX - α-thalassemia/ mental retardation X-linked
- DNA-PK - DNA dependent Protein Kinase
- HR - Homologous recombination
- NHEJ - Non-homologous recombination end- joining
- PIKKs - phosphatidylinositol 39-kinase like kinase

INTRODUCTION

Eukaryotic chromatin is bound to nucleosomes, which consist of an octamer of four core histones: H2A, H2B, H3 and H4. H2A has four variants - macroH2A, H2A.Bbd (Nashun *et al.,* 2010), H2AZ and H2AX (Nashun *et al.,* 2010 & Furuta *et al.,* 2003). H2AX becomes phosphorylated when DNA sustains double stranded breaks (DSBs). DSBs appear when DNA undergoes damage during DNA replication, transcription, recombination and DNA repair (Furuta *et al.,* 2003).

To minimize DNA damage, somatic cells constantly maintain and monitor genetic integrity. Responses to the DNA damage are aided by cell cycle checkpoints. These checkpoints play a role of policing the DNA repair mechanism, and are also associated with cell cycle arrest or cell death/ apoptosis. They must activate the following mechanisms to ensure reliability in DNA replication (Bartek J. *et al.,* 2001) -

1. Induction of cell cycle delay
2. Activation of DNA repair
3. Maintenance of cell cycle arrest until repair is completed
4. Active re-initiation of cell cycle progression.

There are three major DNA damage sensors namely

1. Ataxia Telangiectasia Mutated (ATM),

2. Ataxia Telangiectasia and Rad3 related (ATR) and
3. DNA dependent Protein Kinase (DNA-PK).

These three sensors belong to the family of the phosphatidylinositol 39-kinase like kinase (PIKK), which are primarily serine/ threonine protein kinases (Pan Mei-Rei *et al.,* 2011,Cowell *et al.,* 2007, Bartek J. *et al.,* 2001).

In presence of damaged DNA, these sensors signal serine-139 to phosphorylate H2AX, thereby forming γ-H2AX. In somatic cells, γ-H2AX is present in the cells undergoing replicative stress. These replicative stresses are induced by double stranded DNA damage (DSB) and can be caused by radiation (X-rays & γ-rays), UV exposure, oxidative stress, and other DSB inducing chemical agents like hydroxyurea (Cowell *et al.,* 2007, Mah *et al.,* 2010). Hence, γ-H2AX is universally addressed as a DNA damage and repair marker for somatic cells (Furuta *et al.,* 2003, Hartwell *et al.,* 1994, Turner *et al.,* 2004, Lukas J *et al.,* 2001, Stiff T. *et al.,* 2004).

Clinical Relevance-

Dickey *et al.,* 2009 suggested that γ-H2AX could be an important therapeutic target, although no known drugs have been developed yet. Furthermore, through the γ-H2AX focus formation assay, it may be possible to further dissect the cellular response to DNA damage identifying other potential therapeutic targets that interaction through the γ-H2AX pathway.

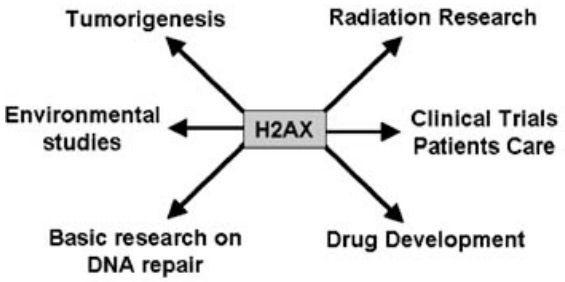

Figure.1. *Clinical application of γ-H2AX (Dickey J. S., 2009)*

Mammalian zygotes possess the ability to completely reprogram the entire somatic genome (Egli *et al.,* 2007 & Ziegler-Birling C. *et al.,* 2009). Studying the chromatin architecture and the patterns of γ-H2AX signal in the pronuclei of the zygote can help one understand DNA replication mechanisms during early mammalian embryogenesis. This can potentially reveal the role of γ-H2AX in pre-implantation embryos (Ziegler-Birling C. *et al.,* 2009), consequently improving the field of Assisted Reproductive Technology (ART).

γ-H2AX: Role in DNA damage & Repair-

The role of γ-H2AX is to facilitate DNA repair by rejoining DSBs and therefore, inhibiting illegitimate recombination events (Cowell *et al.,* 2007). Nucleosomes are repositioned at the damaged DNA sites to secure the broken DSBs together. γ-H2AX serves as a platform for recruiting DSB repair factors in the heterochromatin region. ATM, ATR & DNA-PKs have separate roles in phosphorylating H2AX. UV induced DNA damage follows ATR pathway whereas IR-induced DNA damage follows ATM pathways (Furuta *et al.,* 2003). The later is associated with DNA-PK.

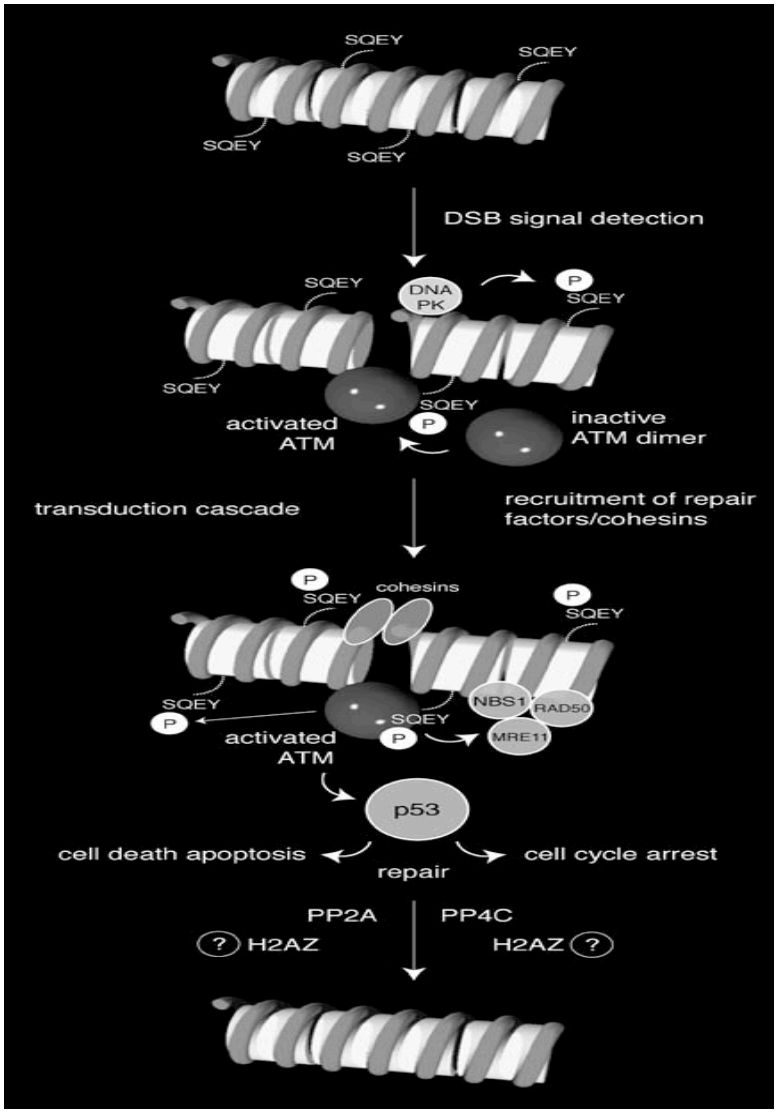

Figure 2. *Mechanism of Action to γ-H2AX (L-J Mah et al., 2010)*

1. Highly conserved SQEY tail of H2AX sends the signal and initiates DSB repair.
2. ATM activates due to autophosphorylated of Ser-1981 residues in inactive dimmer.
3. DNA PKcs & ATR phosphorylates γ-H2AX in reponse to UV mediated DSBs.
4. MRN complex & cohesin along with DSB repair protein are recruited.
5. Activates ATM which inturn targets p53.
6. p53 is responsible for cell death/ apoptosis repair or cell cycle arrest.
7. Dephosphorylation is removed by PP2A & PPAC.
8. γ-H2AX is replaced by H2AZ.

γ-H2AX: Role in mammalian embryos-

The appearance and disappearance of γ-H2AX signal in the early mouse embryo is not well understood (Ziegler-Birling *et al.*, 2009). The sperm after entering the oocyte cytoplasm is able to decondense it's chromatin and replaces protamines with

maternal histones (Perreault *et al.,* 1982). As a result, it undergoes extensive DNA transformation/shearing to form a paternal pronucleus required for mammalian embryogenesis. Derijck A.A.H.A *et al.,* 2006, Wossidlo M. *et al.,* 2010 & Ziegler-Birling *et al.*, 2009 observed higher levels of γ-H2ax in paternal pronuclei as compared to maternal pronuclei. In the mouse, H2AX has been found in the germinal vesicle of the mature oocytes and in the condensed chromatin of mature MII stage oocytes (Nashun *et al.,* 2010). There is evidence that the H2AX, present in Xenopus eggs, have the ability to remodel the sperm nucleus to form the paternal pronucleus after fertilization (Santenard A *et al.,* 2009 & Ziegler-Birling *et al.*, 2009). This remodeling is also associated with the phosphorylation of H2AX (Dimitrov *et al.*, 1994 & Santenard A. *et al.*, 2009). (Figure. 3)

Wossidlo M. *et al.,* 2010 reported that γH2AX foci overlap with the patterns of DNA methylation in early to mid zygotic stages. Branco *et al.*, 2008, Reik *et al.,* 2007 & Ziegler-Birling *et al.*, 2009 reported separately that there exists a direct relationship between the DNA methylation patterns and phosphorylation of H2AX.

Ziegler-Birling *et al.,* 2009 found high levels of γ-H2AX in zygotes after fertilization and 4-cell stage. They also mentioned that this occurrence persisted throughout the preimplantation development. Their work had no information showing the correlation between phases of the cell cycle and the occurrence of γ-H2AX.These high levels of γ-H2AX observed throughout the cell cycle in the embryo might not be directly related to the DNA damage response in the embryo and may potentially possess multiple

roles in mouse embryogenesis. Adiga *et al.*, 2007 stated that DNA damage response is significantly suppressed during early embryogenesis and does not undergo apoptosis until the morula stage. They also reported the presence of γ-H2AX without induced DNA damage in early fast dividing embryos, suggesting that they might have a role in mitosis (Ziegler-Birling *et al.,* 2009). However, *the role of γ-H2AX in mammalian zygotes is not well understood*.

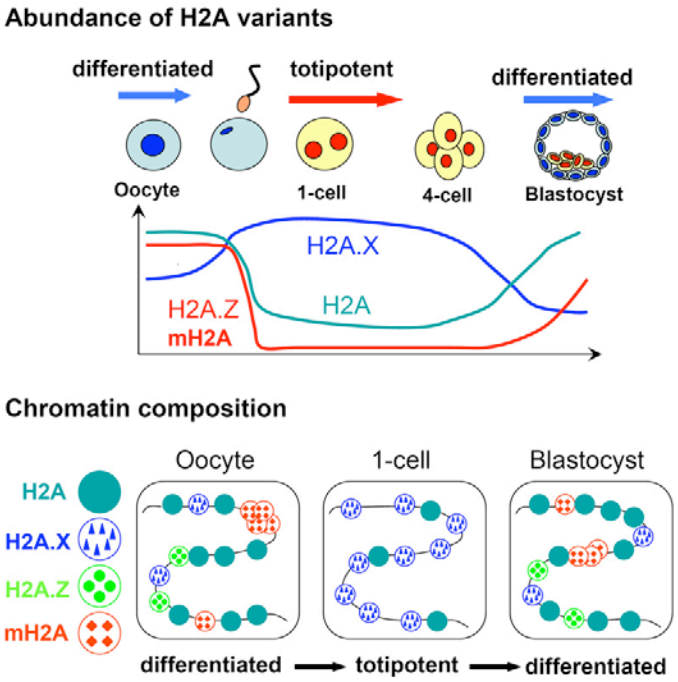

Figure 3. *Abundance of H2AX in oocytes and totipotent embryos (Nashun et al., 2010).*

In differentiated oocytes, chromatin contains the canonical histone H2A and its variants H2A.X, H2AZ and macroH2A (mH2A). However, after fertilization, the quantity of H2A, H2A.Z and macroH2A almost disappear, while the amount of H2A.X increases. Thus, chromatin mostly contains H2A.X during the early pre-implantation stage till the embryos are totipotent and genome reprogramming occurs. At the late preimplantation stage, when differentiation begins to occur, H2A and variant histones reappear in the nucleus, and the chromatin again contains all the histone H2A species.

PROBLEM

Little is known about the molecular mechanism of γ-H2AX in early mammalian embryos.

PURPOSE

1. To perform an integrative review of the existing literature on the molecular mechanism of γ-H2AX in mammalian embryos and in somatic cells.
2. To propose a model for phosphorylation of H2AX during DNA replication of the first zygotic cell cycle.

SPECIFIC AIMS

The goal is to identify and synthesize all published databased literature on molecular mechanism of γ-H2AX in DNA damage.

Significance- The molecular mechanism of phosphorylation of H2AX in somatic cells occurs only during DNA damage. γ-H2AX foci are also noticed in normal mouse embryos immediately after fertilization and 4-cell stage (Ziegler-Birling *et al.,* 2009). This phenomenon perhaps suggests that γ-H2AX signal is not associated with DNA damage response and repair in mammalian zygotes.

<u>Specific Aim 1. To perform a conceptual meta-analysis of the databased literature on the molecular mechanism of γ-H2AX in DNA damage response in somatic cells.</u>

Our aim is to identify the literature that focuses on the molecular mechanisms involved in phosphorylation of H2AX in eukaryotes. The thesis will investigate the

DNA damage response triggered by ATR and ATM pathways (Shiloh Y., 2001). The keywords and the selection criteria will be used to obtain suitable articles. All the selected literature will be organized in the form of medline table in order to extract and critically evaluate the information. Finally, a conceptual meta-analysis will be synthesized based on the findings.

Specific Aim 2. To perform a conceptual meta-analysis of the databased literature on the molecular mechanism of γ-H2AX in DNA damage response in mammalian embryos.

Our current focus is to explore the databased studies on appearance of γ-H2AX signal throughout the first cell cycle in mouse embryos and to understand its function in reprogramming embryonic genome. The keywords and the selection criteria will be used to obtain suitable articles. All the selected literature will be organized in the form of medline table in order to extract and critically evaluate the information. Finally, a conceptual meta-analysis will be synthesized based on the findings.

Specific Aim 3 To propose a model showing the association between phosphorylation of H2AX during DNA replication and the first zygotic cell cycle.

Based on the findings from Specific Aim 1 and Specific Aim 2, a model will be developed to conceptualize the potential relationship between the occurrences of γ-H2AX signal and early mammalian zygotes. There exists a clear gap in our knowledge on distribution, appearance, and disappearance of γH2AX throughout the cell cycle (Wossidlo *et al.,* 2010 & Ziegler-Birling *et al.,* 2009).

METHODS

Design- Review and systematic analysis of the selected data based publications covered over a period of 10 years.

Sample- Data based literature search in NCBI pubmed using search terms “γ-H2ax” AND “mammalian embryos”, “γH2AX” AND “somatic cells”, “γ-H2AX”, “H2AX” AND/OR “DNA damage”.

Procedure- An initial search on the basis of the keywords mentioned above will be performed, and then a follow-up search will be done by snowballing, which will lead to an increase in the sample size. The information obtained will be organized in the format of a Medline / Review Matrix table. In order to optimize the extraction of information, the papers will be dissected under 6 categories - author/journal/year, title, article type, concept, methods and conclusion.

The selection of the pertinent research studies will be done using the following inclusion criteria - English text, γ-H2AX, Somatic Cells, Reproductive Biology, DNA damage, Developmental Biology, and Mammalian Embryos. The exclusion criteria will include non-English text, abstract unavailability, articles not focused on H2AX & DNA damage, editorials, letters to the editor, case reports, opinion articles, and op-eds.

Analysis - Constant comparative analyzes (Glaser 1965) will be used to generate themes on γ-H2AX, somatic cells and embryos. Finally, the results will be organized to formulate a conceptual model of the pathways associated in phosphorylation of H2AX during DNA replication and the first zygotic cell cycle.

RESULTS

Close to 100 papers were identified, but only the ones that satisfied our selection criteria, were analyzed. There were about 25 papers that were found using the search terms “γ-H2AX” and “mammalian embryos” and 50 were found using the search terms “γ-H2AX”and “somatic cells, (19 papers) and “H2AX”, “DNA damage” and “somatic cells” (31 papers). A few additional articles were identified using the snowballing technique derived from our initial literature search. The major issues and motivations driving this body of research are determined by the lack of understanding of the role of γ-H2AX in early mouse embryo. Methods used to investigate this scientific question included basic science research articles on cancer therapeutics, DNA damage responses, and studies on γ-H2AX and pre-implantation mouse embryos. There are several questions in this arena of clinical embryology that need answering, in order to develop new interventions in embryogenesis and Assisted Reproductive Technology.

Major themes drawn from the studies include-

1). Periodic appearance and disappearance of γ-H2AX in early mouse pre-implantation embryos.

2) Development of γ-H2AX foci in the pronuclei without imposing any replicative stress on the mouse embryo.

3) Occurrence of γ-H2AX in the S-phase of somatic cells under replicative stress and normal embryos.

4) Prevalence of prominent γ-H2AX signal in male pronuclei compared to the female pronuclei during mouse embryogenesis.

A major knowledge gap has been identified and a conceptual model will hypothesize the sequence of DNA damage events that might be occurring during the time of early mouse embryogenesis.

Critical Analysis

Several studies have shown that DNA double and single-stranded breaks are indicative of the presence of γ-H2AX (Ward *et al.,* 2001, Furuta *et al.,* 2003, Derijck *et al.,* 2006, Cowell *et al.,* 2007). γ-H2AX, thus, has become an excellent candidate for measuring DNA damage in a cellular environment (Dickey *et al.,* 2009). The DNA single stranded breaks can be produced by a variety of exogenous and endogenous DNA lesions and by the action of DNA topoisomerase I (top1) in DNA containing base damages (abasic sites, mismatches, oxidized bases, carcinogenic adducts, and UV lesions) and pre-existing single-strand breaks (Furuta *et al.,* 2003).

1. Appearance of γ-H2AX in DNA damaged cells /cell lines during S phase.

DNA damage can be of the following types: Single stranded or double stranded breaks, inter and intrastrand DNA crosslinks, DNA–protein crosslinks, and chemical modification such as generation of 8-hydroxy guanosine residues or polycyclic aromatic hydrocarbon adducts (Kinner *et al.,* 2008, Mu X.F. *et al.,* 2011). During DNA replication at S phase of the cell cycle, there is widespread DNA damage in the cell. For instance, the cells treated with camptothecin (Topo1 inhibitor), in S-phase had

the highest γ-H2AX contents, whereas, γ-H2AX was lowest in the cells in the G1 and G2/M phases of the cell cycle (Furuta *et al.,* 2003). Cowell *et al.,* 2007 also reported on the same lines suggesting that DNA replication occurs towards the end of S-Phase in heterochromatin regions, and in the early to mid stage of S phase in euchromatin regions. There is a clear difference in the environment of chromatin organization in heterochromatin and euchromatin. Hence, there exists a possible difference in their susceptibility to DNA damage or processing of DSBs and can be determined by γ-H2AX content in the cells using laser-scanning cytometery. The reason for the delayed heterochromatin DNA replication may be caused by the ongoing replication of the euchromatin region in the cell, which may mask the presence of heterochromatin to stimulate DNA damage and produce γ-H2AX foci. Interestingly, γ-H2AX and HP1-α (Heterochromatin protein) signals in cells and embryos coincide with each other (Arney *et al.,* 2002 & Cowell *et al.,* 2007). However, Kruhlak *et al.,* 2006 & Cowell *et al.,* 2007 separately introduced the notion that the appearance of γ-H2AX signal at the site of DSBs may be due to the local chromatin decondensation.

1.1. Association of ATR & γ-H2AX in somatic cells

ATR is a critical player in the replication block pathway (Ward *et al.,* 2001). It is not only responsible for H2AX phosphorylation but also replication inhibition. It also has access to heterochromatin at least during S-phase (Cowell *et al.,* 2007). Furuta *et al.,* 2003 stated that γ-H2AX is produced primarily by ATR, secondly by DNA-PK, and to a lesser degree by ATM in response to topoisomerase1-induced replication-mediated

DNA damage caused by camptothecin. Further, Ward *et al.,* 2001 found evidence suggesting that ATR plays a central role in controlling downstream responses to replicative stress, while ATM primarily controls the cellular response to IR-induced DSBs.

Ward *et al.,* 2001 showed that phosphorylation of H2AX forms nuclear foci at the sites of stalled replication forks in response to hypoxyurea (HU) or UV radiation. Hus1 is a protein that surfaces in response to replicative stress. H2AX phosphorylation requires ATR, however, this phosphorylation event is independent of Hus1 (hydroxyurea sensitive-1). The replication block induces ATR-dependent γ-H2AX formation. Ward *et al.,* 2001 surmised that Hus1 probably act in conjunction with ATR in the DNA replication checkpoints.

2. Role of γ-H2AX in germ cells

γ-H2AX has been detected at the developmental stages of adult germ cells. H2AX phosphorylation in testis sections shows several foci of H2AX phosphorylation/ de-phosphorylation coupled to various developmental phases of spermatogonia and spermatocytes, as well as to spermatid differentiation. During spermiogenesis, numerous DNA strand breaks can be detected at the time of nuclear elongation caused in response to chromatin remodeling. Derijck *et al.,* 2006 demonstrated that topoisomerase 2 plays a role during sperm decondensation in the zygote. Rodriguez B. 2009 was able to show an association between DNA replication and γ-H2AX in spermatogonia.

At the time meiosis, the oocyte assumes two roles in nuclear organization (Sasaki *et al.,* 2008, Egli *et al.,* 2007 & Fuente *et al.,* 2006) –

1. Controlling the events of nuclear reprogramming mechanisms.

2. Spatio-temporal regulation of gene expression during development and differentiation.

There are a range of activities involved in this mechanism extending from a). the unfertilized egg through b). cells of the pre-implantation embryo, and further c). to the ES cells derived from them. De La Fuente *et al.,* 2004 addressed the fact that the patterns of ATRX expression and nuclear compartmentalization in mammalian oocytes are not known. Baumann C. *et al.,* 2010 and coworkers defined the ATRX protein as a chromatin-remodeling factor known to regulate DNA methylation at repetitive sequences of the human genome. Their study investigated the mechanisms associated with centrometric heterochromatin and ATRX in mammalian oocytes and during female meiosis.

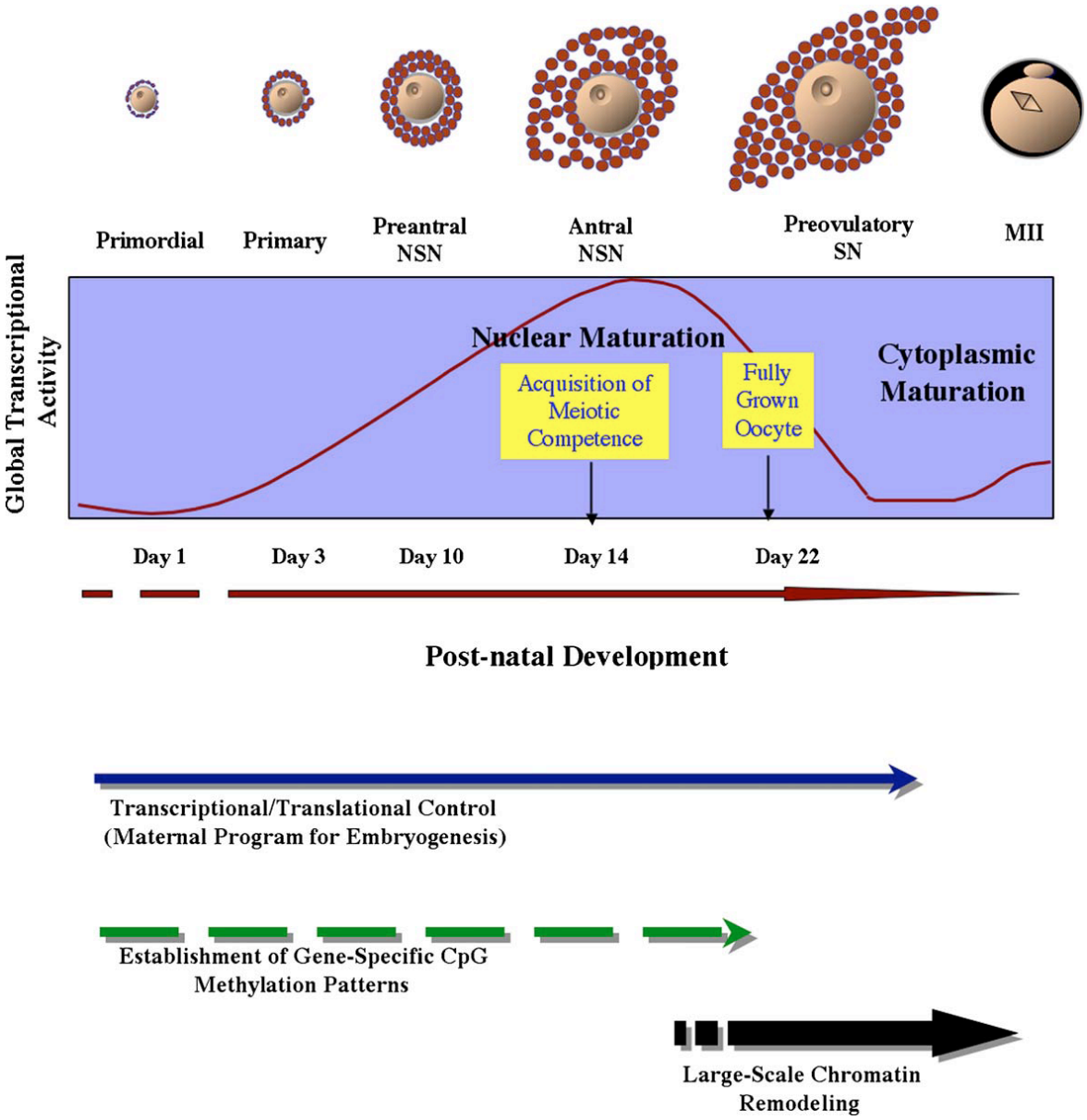

Figure 4. *Functional differentiation of chromatin structure during mouse oocyte growth (De La Fuente et al., 2006)*

Meiotic oocytes and mitotic zygotes possess the potential to reprogram the entire cellular genomes, however, the clones (oocyte enucleated and replaced with a somatic nucleus) are unable to do so (Sasaki *et al.,* 2008 & Egli *et al.,* 2007). The

clones follow the developmental patterns dictated by the somatic genome introduced in the enucleated oocyte. This suggests that the ability of the embryonic cytoplasm to control the cell cycle (Figure 5).

Histones and chromatin organization play a major role in programming that occurs during mammalian oogenesis and embryogenesis. Nashun *et al.,* 2010 characterized histone H2A and its variants into H2AZ, H2AX and macro H2A in mouse oocytes and early embryos. DNA repair mechanisms involving ATM and DNA PKs were detected as early as GV-stage oocytes during oogenesis (Nashun *et al.,* 2010 & Yukawa *et al.,* 2007). Yukawa *et al.,* 2007 reported that ATM is involved in p53-dependent and -independent cell death. They were also able to show that ATM was associated with DNA repair mechanisms. In the ovary, oocytes are arrested at the G2 phase and to monitor the genomic integrity, DNA repair and cell death mechanisms are engaged for the purpose. The findings by Yukawa *et al.,* 2007 also suggested that the DNA repair mechanisms and the G2/M checkpoints work inefficiently in one- and two-cell pre-implantation mouse embryos.

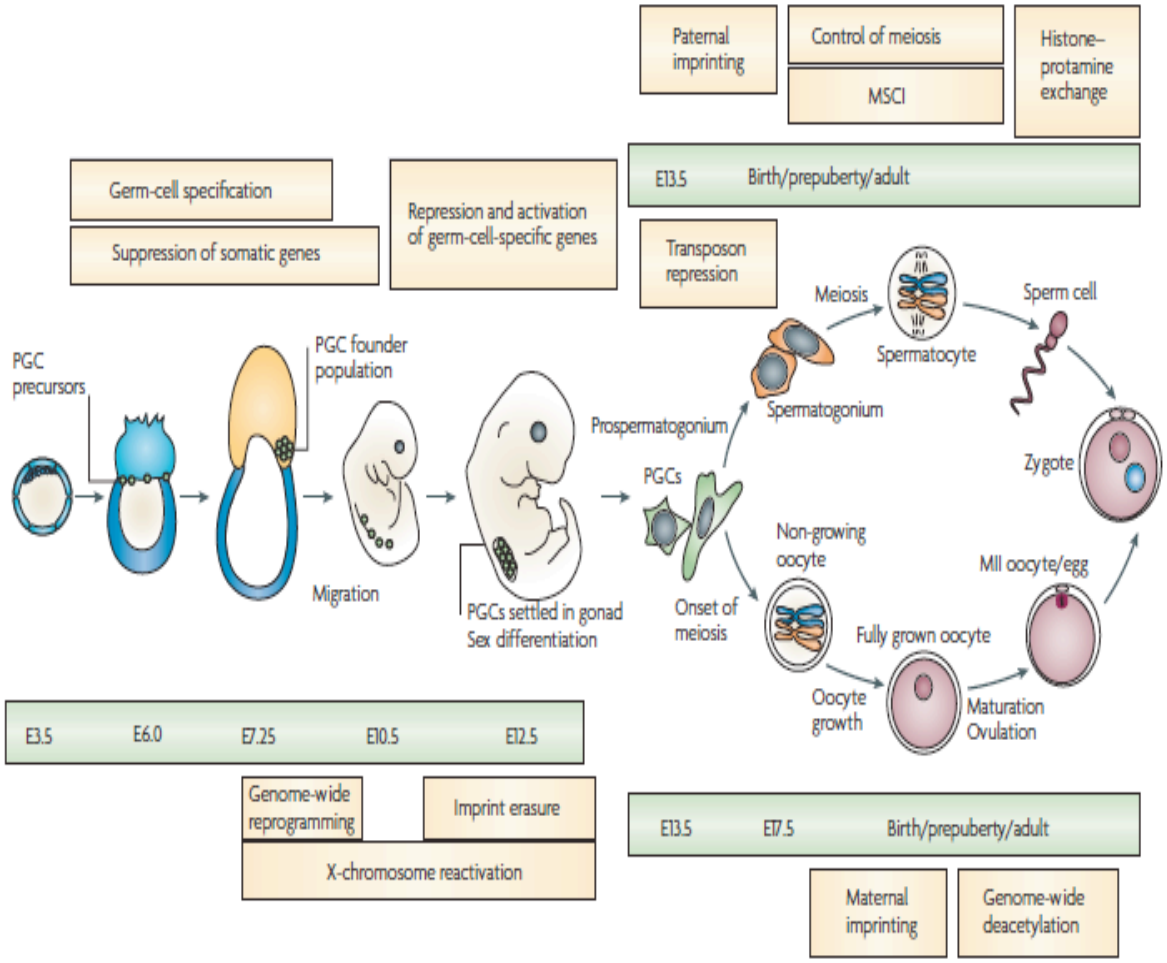

Figure 5. *Germ cell development and associated epigenetic events in mice (Sasaki et al., 2008).*

3. Role of γ-H2AX in pre-implantation embryos

The incongruence in the appearance of γ-H2AX and phases of DNA methylation were reported in the early to mid zygotic stage (Wossidlo *et al.,* 2010). The partial functioning of the G2/M checkpoint and the DNA repair mechanisms at the one and two-cell stage may leave a clue in solving the puzzle (Yukawa *et al.,* 2007). During the S and G2 phase, the somatic cells tend to differentiate faster but not as fast as

the cells in an early pre-implantation mammalian embryo (Ziegler *et al.,* 2009). Histones lay the ground for regulating DNA-mediated processes including transcription, replication and DNA repair. Several repair proteins like 53BP1, BRCA1, and MDC1 interact with γ-H2AX (Yukawa *et al.,* 2007). There are two mechanisms of DNA repair have been reported in the cells (Yukawa *et al.,* 2007)-

- Homologous recombination (HR) mainly functions in the late S and G2 phase.
- Non-homologous recombination end- joining (NHEJ) functions throughout interphase.

The appearance of γ-H2AX after γ-irradiation did not occur suggesting that neither HR nor NHEJ was functional at the G2 phase in one-cell embryos. The occasional presence of γ-H2AX in embryos depends on some factors other than those involved in the somatic cells. Ziegler *et al.,* 2009 was able to support this by reporting that the phosphorylation of H2AX in embryos increases during mitosis due to the absence of DNA damage pathways.

3.1. Deficiencies in the function of checkpoints or DNA repair mechanisms in pre-implantation embryo.

In the absence of the DNA repair mechanism, pre-implantation embryos either arrest or die, as the DNA damage mechanism doesn't function properly. DNA damage is not repaired in X-irradiated zygotes. Patterns of γ-H2AX in the embryo have been previously analyzed in response to DSBs caused by γ-irradiation in *in-vitro* fertilized embryos.

Phosphorylated H2A.X is directly linked to the recruitment of 53BP1 into DSB foci, suggesting that they mediate DNA repair in similar pathways. 53BP1 is a mediator of DNA damage checkpoint. However, in some embryos, the γ-H2AX signal can be detected at 1-2 cells next to the forming blastocoelic cavity, which displayed both cytoplasmic and nuclear localization of 53BP1. 53BP1 and γ-H2AX only rarely co-localize, suggesting that these high levels of phosphorylation of H2AX are not directly linked to the DNA damage process in the early embryo. This opens the possibility of γ-H2AX being involved in some other mechanism in the early mouse embryogenesis (Ziegler *et al.,* 2009).

The G2/M checkpoint in mice is functional in an irradiated mouse one-cell embryo. The embryos arrested in G2 phase, can escape this arrest to enter the M phase. Mouse zygotes fertilized with X-irradiated spermatozoa are stalled at the G1/S phase of the first cell cycle in the p53 dependent manner. Thus, NHEJ is not functional during the G1 and early S phases (Yukawa M. *et al.,* 2007).

The insufficient function of G2/M checkpoint in one and two cell embryos may be caused due to a lack of transcription of the genes involved in the G2/M checkpoint mechanism upon zygotic activation. DNA repair pathways are active during this early pre-Zygotic Gene Activation (ZGA) stage of life and transcripts for repair genes are more abundant.

Adiga *et al.,* 2007 & Shimura *et al.,* 2002 showed that mouse zygotes fertilized with irradiated sperm do not show G1/S and G2/M checkpoint responses. Caffeine, which is an inhibitor of ATR and ATM, can restore X-irradiated arrest (Mu X.F. *et al.,* 2011). ATM and ATR regulate the G2/M and G1/S cell cycle checkpoints in the early embryo differently in response to genotoxic stress (Mu X.F. *et al.,* 2011). The inductions of G1/S & G2/M checkpoints are activated by ATM & ATR respectively (Abraham RT., 2001, Liu QH *et al.,* 2000, & Mu X.F. *et al.,* 2011). X- irradiation of the zygote or two-cell embryo induces G2/M checkpoint arrest. These embryos have increased micronuclei and reduced developmental viability.

CONCLUSIONS –

In Xenopus eggs, Santenard *et al.,* 2007 found that H2AX is the most abundant H2A variant and has the ability to remodel the sperm nucleus to form a paternal pronucleus after fertilization is directly associated with its phosphorylation status. Ziegler-Birling *et al.,* 2009 indicated that H2AX potentially plays multiple roles during mouse embryogenesis. Moreover, unusually high levels of phosphorylated H2AX are present throughout mouse pre-implantation development (Ziegler-Birling *et al.,* 2009), showing the possible role of the variant in chromatin assembly and remodeling during these early stages. We suspect that the paternal chromatin may have a role in producing DNA damage response in the early mouse embryo.

Various mechanisms involved in the rapid removal of histone modifications have recently been identified, however, the mechanism of DNA demethylation still needs to

be determined in embryos (Wossidlo *et al.,* 2010). Wossidlo *et al.,* 2010 & Santenard *et al.,* 2007 suggested in their study that the epigenetic inheritance is linked to the methylation of the paternal genome after fertilization. *Our preliminary unpublished data on Androgenotes, Parthenotes, ICSI embryos, IVF embryos suggests that the induction of γ-H2AX in early mouse pre-implantation embryo is dependent on the presence of male pronuclei.*

In absence of the male pronuclei, γ-H2AX signal does not show up even during S phase of the cell cycle. Thus, there could be a possible factor or factors associated with the male chromatin (Ward 2010) and the DNA damage response in the early embryos. It will be interesting to follow through with this theme of the sporadic appearance and disappearance of γ-H2AX during the S phase and report the association between DNA replication and appearance of γ-H2AX.

On the basis of our intense literature search and preliminary data, we are able to generate the hypothesis that the paternal genome is the inducer of various epigenetic factors and chromatin modifications like DNA methylation/ DNA demethylation/ DNA acetylation/ DNA deacetylation etc., which triggers DNA damage pathways related to γ-H2AX in mouse pre-implantation embryos.

Recommendations-

To support and further validate our hypothesis we suggest the following investigations-

1). To observe the pattern of γ-H2AX appearance and DNA replication at G1 and S phases of the cell cycle in Androgenotes, mock injected Parthenotes, Parthenotes, IVF and ICSI embryos.

2) To compare the intensity of the γ-H2AX signal in IVF and Parthenotes.

3) Closely follow the works of Mu X.F. *et al.,* 2011, Ziegler-Birling *et al.,* 2009, Nashun *et al.,* 2010, Derijck *et al.,* 2006, Santenard *et al.,* 2007 as these groups have been actively involved in the studies associated with γ-H2AX and mouse embryos.

APPENDIX 1. - Review Matrix

Author, Journal & Year	Title	Article type	Concepts	Methods	Conclusions
Ward I.M. & Chen J., The Journal of Biological Chemistry, 2001	Histone H2AX Is Phosphorylated in an ATR-dependent Manner in response to replicational Stress	Experimental	Inhibition of DNA replication by hydroxyurea or ultraviolet irradiation also induces phosphorylation and foci formation of H2AX.	Cell culture and genotoxic agents, Immuno staining & western blotting	1. γ-H2AX forms nuclear foci at the sites of DSBs. 2. γ-H2AX foci colocalize with proliferating cell nuclear antigen (PCNA) and BRCA1 at the arrested replication fork in S phase cells. Cells lacking wild-type ATM (ataxia-telangiectasia-mutated) showed no difference in HU-induced γ-H2AX foci formation nor was the response impaired in Hus1 (hydroxyurea sensitive)-deficient cells. 3. There are evidences suggesting that ATR plays a central role in controlling downstream responses to replicational stress, while ATM primarily controls the cellular response to IR-induced DSBs. 4.Phosphorylated H2AX forms nuclear foci at the sites of stalled replication forks in response to HU-mediated replication arrest or exposure of S phase cells to UV radiation. 5. ATR is a critical player in the replication block pathway. Chromatin changes or modifications play a role in the replication block pathway. Hus1 probably acts in conjunction with ATR in the DNA replication checkpoints. While H2AX phosphorylation requires ATR, this phosphorylation event is independent of Hus1. Thus, the phosphorylated H2AX possibly functions up-stream of Hus1 in the transduction of DNA damage/replication block signals or in a parallel repair

					pathway.
Furuta, T., et al., (2003). Journal of Biological Chemistry, 278(22), 20303–20312.	Phosphorylation of histone H2ax and activation of Mre11, Rad 50, and NBS in response to replication – dependent DNA double stranded breaks induced by mammalian DNA topoisomerase 1 cleavage complexes.	Experimental	DSB causes various exo- & endogenous DNA lesions and by the action of DNA topoisomerase 1 in DNA containing base damage and pre-existing single stranded breaks.	1. Generation of γ H2AX by camptothecin in HCT 116 cells, 2. Camptothecin induced γ-H2AX is restricted to S phase.	1. DNA single-strand breaks can be produced by a variety of exogenous and endogenous DNA lesions and by the action of DNA topoisomerase I (top1) in DNA containing base damages (abasic sites, mismatches, oxidized bases, carcino- genic adducts, and UV lesions) and preexisting single-strand breaks. 2. The cells treated with camptothecin, in S-phase had the highest γ-H2AX contents, whereas γ-H2AX was lowest in the cells in the G1 and G2/M phases of the cell cycle and was examined by laser scanning cytometery. 3. γ-H2AX is produced primarily by ATR, secondly by DNA-PK, and to a lesser degree by ATM in response to top1-induced replication- mediated DNA damage induced by camptothecin.
De La Fuente et al.. Dev Biol (2004) vol. 272 (1) pp. 1-14	ATRX, a member of the SNF2 family of helicase/ ATPases, is required for chromosome alignment and meiotic spindle organization in metaphase II stage mouse oocytes.	Experimental	1. ATRX functions to regulate key stages of meiosis in mouse oocyte. 2. Global/ genome wide histone deacetylation is essential for the binding of ATRX to centromeric domains during	Oocyte collection & culture, Western blotting, immunochemistry, Antibody microinjection	1. At GV stage, the ATRX was found associated with perinucleolar heterochromatin rim in transcriptionally quiescent oocytes. 2. ATR has been mapped to the long arm of the human X chromosome and contains an open reading frame encoding 280 kDa proten with plant homeodomain (OHD) region at the amino terminus responsible for interaction with HP1 and a helicase domain at the carboxyl terminal region.

			mammalian meiosis and provides functional evicence indicating that ATRX . a centromeric heterochrom atin protein, regulates key stages of meiosis in that it plays an important role in establishing proper chromosome s alignment and a bipolar meiotic spindle at metaphase II in mouse oocytes.		3. The patterns of ATRX expression and nuclear compartmentalization in mammalian oocytes are not known. 4. The study was taken- 1. To determine the mechanisms involved in regulating the binding of ATRX to centrometric heterochromatin in mammalian oocytes and 2. To test the hypothesis that the ATRX protein plays a role during female meiosis. 5. Genome-wide epigenetic modifications such as global histone acetylation at the onset of meiosis are essential for the binding of ATRX to centromeric heterochromatin in mouse chromosomes. 6. ATRX associates with heterochromatin domains in mammalian oocytes are not known. Experiments were thus conducted to determine the subnuclear localization of ATRX in fully-grown mouse oocytes. 7. These results indicate that ATRX is a component of centromeric heterochromatin domains in the germinal vesicle. 8. ATRX phosphorylation is under the control of an alternative signaling pathway, oocytes were treated with KN-93, a specific inhibitor of the calcium calmodulin kinase II (CamKII) pathway. Mouse oocytes treated with KN-93 interfere with meiotic resumption. 9.Global deacetylation at the onset of meiosis is required for continuous binding of ATRX to

					centromeric heterochromatin. 10. Functional ablation of ATRX protein by antibody microinjection and RNAi indicates that ATRX plays an essential role in the organi-zation of proper chromosome alignment on the meiotic MII spindle. 11. The role of ATRX in nuclear compartmentalization and heterochromatin formation during meiosis is not known. 12. In this study, induction of histone hyper-acetylation with TSA did not prevent meiotic chromosome condensation. 13. Cell signaling pathway involved in ATRX phosphorylation is not known. 14. Two nature of ATRX isoform appears in MI and MII of meiosis is not known. These two ATRX isoforms correspond to different amino acid residues, or they are residues of alternatively spliced transcript in mouse oocytes. 15. Epigenetic modification such as Histone deacetylation during meiosis may thus be essential for recruitment of heterochromatin binding proteins involved in centromere structure and function.
Derijck, A. A. H. A., van der Heijden, G. W., Giele, M., Philippens, M. E. P., van Bavel, C. C. A. W., & de Boer, P. (2006 DNA repair, 5(8), 959–971.	γH2AX signalling during sperm chromatin remodelling in the mouse zygote.	Experimental	γ-H2AX foci were the paternal and maternal chromatin were analysed separately.	Irradiation, Gamete collection and IVF, timing of zygote development, DNA	1. DNA repair pathways are active during this early pre-Zygotic Gene Activation (ZGA) stage of life and transcripts for repair genes are more abundant then in later preimplantation stages. 2. Reversal to nucleosomes based chromatin is a necessity to enable

				damaging agents, Antibodies, fixation, immunofluorescence (IF) & foci quantification, Collections of Images, statistics,	progression into a pronucleus that can enter S-phase in conjunction with the maternal pronucleus. 3. Irradiated and non-irradiated male nuclei did not show qualitative differences in γ-H2AX. 4. Maternal chromatin in zygotes was only significantly affected by bleomycin only in these pulse-based treatments indicating a difference in sensitivity between paternal and maternal chromatin for these two clastogenic compounds. 5. Mitotic H2AX phosphorylation in somatic cells is apparently mechanistically conserved in the female gametes of mice. 6. DSB in nuclei of non-radiated non-cycling somatic cells is about 0.05. Non-irradiated sperm shows an average DSN level of 0.9 DSBs per male nucleus. 7. During spermiogenesis, numerous DNA strand breaks can be detected at chromatin remodeling during nuclear elongation. 8. Topoisomerase 2 plays a role during sperm decondensation in the zygote. 9. DNA DSB signaling pathway via γ-H2AX in the mouse zygote.
De La Fuente. Dev Biol (2006) vol. 292 (1) pp. 1-12	Chromatin modifications in the germinal vesicle (GV) of mammalian oocytes.	Review article		-	1.Meiotic arrest is maintained until puberty when the luteinizing hormone (LH) surge stimulates the resumption of meiosis in one or more oocytes depending on

					the species. In the mouse ovary, the first wave of oocyte growth and differentiation is synchronous and is also the time at which maternal-specific genomic imprints are established on a locus by locus basis. 2. Oocytes undergo a dramatic change in nuclear organization in which chromatin becomes progressively condensed, forming a heterochromatin rim in close apposition with the nucleolus, thus acquiring a configuration termed Surrounded nucleolus (SN). 3.Transition into the SN configuration and transcriptional repression in mammalian oocytes is regulated by HDACs. . 4.Timing of transcriptional repression is critical for subsequent embryonic development.
Egli D. et al., Nature, 2007	Developmental reprogramming after chromosome transfer into mitotic mouse zygotes.	Experimental	1. Animal cloning, 2.Production of embryonic stem cell lines by somatic cell nuclear transfer into meiotic oocyte.	1.Nuclear transfer 2.Expression of doc inducible histone H2B cherry by donor ES cells	1. Manipulated embryo cleaves 30-60%. Oocytes, zygotes and ES cells each harbor reprogramming activities suggesting that a continuum of activity may extend from the unfertilized egg through cells of the preimplantation embryo and further to the ES cells derived from them. 2. Allowed spindle polymerization and the determination of chromosome position while preventing progression out of mitosis. 3. Meiotic oocytes and mitotic zygotes can reprogram somatic genomes whereas zygotes enucleated in interphase cannot. This

					suggests that the ability of the embryonic cytoplasm to support reprogramming fluctuates with the cell cycle. 4. Some reprogramming factors are destroyed and renewed with each cell cycle, but it seems more likely that 1 or more factors critical for embryonic development and reprogramming localize to the pronuclei during interphase.
Wolf Reik., Nature 2007.,	Stability and flexibility of epigenetic gene regulation in mammalian development	Review article	Exploring the linear relationship between acquisition of epigenetic marks and developmental progression. It has been observed that there are some key restrictions in developmental potential that are brought about by epigenetic regulation might occur very early in development.	-	1 It is unclear whether there is a linear relationship between acquisition of epigenetic marks and developmental progression. 2 Some key restrictions in developmental potential that are brought about by epigenetic regulation might occur very early in development. 3 Judging from the results of somatic-cell nuclear-transfer experiments, it is unclear whether more-differentiated cells have more epigenetic marks or if they have marks that are more difficult for the oocyte to reprogram. 4 Judged by the substantial loss of immunofluorescence signal, together with the considerable loss of methylation of *Line1* elements as determined by bisulphite sequencing, the paternal genome loses a significant amount of methylation, although more precise measurements and more information about which sequences are

					affected and unaffected would be valuable. 5 Many of the basic molecular building blocks for epigenetics, such as the enzymes for DNA methylation and histone modifications, are highly conserved in vertebrates, but the regulation of epigenetic modifiers might evolve more rapidly together with specific developmental strategies. 6 During differentiation, genes that are crucial for pluripotency are silenced by histone modifications, as well as by DNA methylation.
Angèle Santenard and Maria-Elena Torres-Padilla., Epigenetics ., 2007	Epigenetic reprogramming in mammalian reproduction Contribution from histone variant	Review article	The specific pathways through which H3.3 could regulate different chromatin conformations at different loci and the identification of specific proteins responsible for this deposition are an important challenge for future investigation. The set of variants incorporated within the nucleosome can have important consequences in the	-	1. Variants of H2A are also involved in wide-scale chromatin remodeling events and they have acquired specialized functions during development. 2. Among these, phosphorylation of the H2A variant - H2A.X was implicated in the initiation of the MSCI. 3. H2A.X is the most abundant H2A variant in Xenopus eggs, and the ability to remodel the sperm nucleus to form a paternal pronucleus after fertilization is directly associated with its phosphorylation status. 4. Moreover, unusually high levels of phosphorylated H2A.X are present throughout mouse preimplantation development (Ziegler-Birling *et al.,* in press), showing the possible role of the

			regulation of epigenetic mechanisms during development .		variant in chromatin assembly and remodelling during these early stages. 5. MacroH2A is an H2A variant that possesses a long C-terminal domain and is conserved in vertebrates.6. 6. The mechanism of active demethylation in the zygote is still unknown. 7. Another DNA-damage-responsive gene, the mouse gene *Gadd45* (growth arrest and DNA-damage-inducible 45), might also have a role in demethylation. 8. Epigenetic inheritance is 'broken' by erasure of methylation of the paternal genome after fertilization. 9. Although various mechanisms for the rapid erasure of histone modifications have recently been identified, the mechanism of DNA demethylation still needs to be determined.
Adiga, S. K., Toyoshima, M., Shimura, T., Takeda, J., Uematsu, N., & Niwa, O. (2007). Reproduction, 133(2), 415–422.	Delayed and stage specific phosphorylation of H2AX during preimplantation development of irradiated mouse embryos.	Experimental	Phosphorylation of H2AX was readily induced by radiation in post-compaction stage embryos. It is possible that phosphorylation of H2AX is inefficient in early stage embryos. phosphorylated H2AX	Immunostaining, λ-phosphatase treatment, TUNNEL assay	1. Aphidicolin treatment of Xenopus and drosophila embryos decreases their DNA content, but they nevertheless progress to the midblastula transition stage. 2. The mouse zygotes fertilized with irradiated sperm do not show G1/S and G2/M checkpoint responses. 3. Damage response is composed of 3 components- damage sensors, signal transducer and effectors to transmit signals from DNA damage.

			exists in the dispersed chromatin structure of early stage embryonic pronuclei, so that it cannot be readily detected by conventional immunostaining method.		4. γ-H2AX focus formation is visible at breaks in irradiated cells and visible even at break and exchange points. 5. Irradiated embryos showed delayed phosphorylation of H2AX when they progressed to the morula stage. 6. ATM kinase phosphorylates H2AX and therefore, poor staining for γ-H2AX might be due to absence of the ATM kinase activity in one-cell stage mouse embryos. 7. The preimplantation stage mouse development has long been known to lack the G1 Phase. 8. Formation and disappearance of the nuclear γ-H2AX foci after irradiation is related to the embryonic stage. 9. The embryos do not undergo apoptosis unless they undergo first differentiation at compaction.
Cowell, I. G., Sunter, N. J., Singh, P. B., Austin, C. A., Durkacz, B. W., & Tilby, M. J. (2007).., PLoS ONE, 2(10), e1057.	γ-H2AX Foci Form Preferentially in Euchromatin after Ionising-Radiation.	Experimental	.γ-H2AX is regarded as forming a platform for the recruitment or retention of other DNA repair	Cell culture, cell irradiation and drug treatment, immunofluorescence microscopy	1. Histone modifications such as histone H3 lysine 9 trimethylation, the presence of heterochromatin-specific proteins such as HP1α, or structural features of heterochromatin may prevent access of ATM and/or limit the extent of the domain over which H2AX is phosphorylated. However, ATR, which is responsible for H2AX phosphorylation following replication inhibition, appears to have access to heterochromatin at least

					during S-phase. 2. Greater number of nuclei exhibit at least some overlapping γ-H2AX and HP1α signals when cells are irradiated in late S phase compared to G1, suggesting that transient decondensation of heterochromatin or depletion of heterochromatin proteins during replication allows H2AX phosphorylation. 3. Epigenetic or packaging properties of heterochromatin, preventing efficient H2AX phosphorylation. γ-H2AX is regarded as forming a platform for the recruitment or retention of other DNA repair and signaling molecules at DSBs, this implies that the processing of DSBs in heterochromatin differs from that in euchromatic regions.
Yukawa M. *et al.,* 2007 Biochemical & Biophysical Research communications	Deficiency in the response to DNA double-strand breaks in mouse early pre-implantation embryo	Experimental	Pre-implantation embryo, check point, DNA double strand break, DNA repair & γ-H2AX. To investigate the mechanisms responding to DNA damage at G2 in mouse pre-implantation embryos, we	Collection and culture of oocyte, Immuno cytochemistry.	1. γ-H2AX was detected in one cell and 2 cell embryos, causing hypersensitivity to γ irradiation. G2/M checkpoint and DNA repair mechanism have insufficient function in one- and two cell embryos, causing hypersensitivity to γ-irradiation. 2. Absence of γ-H2AX in one and two-cell embryos depends on some factors other than the DNA damage kinases. 3. γ-H2AX interacts with several repair proteins that have BRCA1 COOH-terminal (BRCT) domains,

			examined G2/M checkpoint and DNA repair mechanisms		including 53BP1, BRCA1, and MDC1. 4. 2 mechanisms of DNA repair- • Homologous recombination mainly functions in the late S and G2 phase. • Non-homologous recombination end-joining (NHEJ) functions throughout interphase. 5 Deficiencies in the function of checkpoints or DNA repair mechanisms. In absence of the DNA repair mechanism, pre-implantation embryos either arrest or die as the DNA damage mechanism doesn't function properly. 6 DNA damage is not repaired in X-irradiated zygotes. 7 G2/M checkpoint in mice is functional in an irradiated mouse one-cell embryo. These embryos are arrested in G2 phase but escapes this arrest and enters M phase. 8 Phos of γ-H2AX after γ-irradiation did not occur suggesting that neither HR nor NHEJ was functional at the G2 phase in One-cell embryo. 9 Mouse zygotes fertilized with X-irradiated spermatozoa are arrested at the G1/S phase of the first cell cycle in the p53 dependent manner. Thus NHEJ is not functional during the G1 and early S

					phase. 10 The insufficient function of G2/M checkpoint in 1 and 2 cell embryos may be caused by a lack of transcription of the genes involved in the G2/M checkpoint mechanism upon zygotic activation. 11. The γ-H2AX and phosphorylated ATM and DNA- PKcs were detected in GV-stage oocytes (Figs. 2 and 3) suggesting that DNA repair mechanisms function in the oocytes. ATM is involved in both p53-dependent and -independent cell death, as well as DNA repair mechanisms. 12. P63-dependent death of the oocyets in the primordial follicle [30] and Bax-dependent death of MII-stage oocytes have been also reported. 13. In the ovary, oocytes arrested for a long period at the G2 phase seem to be monitored through DNA repair and cell death mechanisms to ensure genome stability. Our findings suggest that the mechanisms of the G2/M checkpoint and DNA repair function insufficiently in one- and two-cell embryos, causing the hypersensitivity of mouse pre-implantation-stage embryos to γ-irradiation.
Kinner, A., Wu W., Staudt, C., & Iliakis, G. (2008)., Nucleic Acids Research, 36(17), 5678–5694.	γ-H2AX in recognition and signaling of DNA double-strand breaks in the context of chromatin.	Experimental	The role of c-H2AX in DNA damage response in the context of chromatin. The use of this modification	Pulsed-field gel electrophoresis	1. 2 types of DNA lesions- a. Base and nucleotide damages as well as single interruptions of the sugar phosphate backbone, does not overly jeopardize chromatin integrity or function, and error-free repair can be accommodated with limited

			as a surrogate marker for mechanistic studies of DSB induction and processing.		local modification of the chromatin structure using the complementary DNA strand as a template. b. DNA double- strand breaks (DSBs) may also include some types of DNA-protein crosslinks, and can bring chromatin to a state severely undermining its integrity and function. 2. The most profound effect of histone tail modifications is their ability to attract specific proteins. 3. On the other hand, γ-H2AX is unable to predict the efficacy of antioxidant radioprotective compounds. 4. γ-H2AX has the potential of developing into a useful predictor of cellular radiosensitivity to killing and may find clinical application during treatment of human tumors with ionizing radiation, in the evaluation of interindividual variations in radiosensitivity in the analysis of the endogenous DSB load and even in the prediction of dose in nuclear accidents or terrorist attacks involving radioactive materials. 5. γ-H2AX fluorescence intensity is taken as a sensitive test for the diagnosis of AT syndrome. γ-H2AX foci formation has been used as a measure for whole body dose after radiotherapy.
Kobayashi, J., Tauchi, H., Chen, B., Bruma, S., Tashiro, S., Matsuura, S., Tanimoto, K., et al. (2009). Biochemical	Histone H2AX participates the DNA damage-induced ATM activation through	Experimental	1. H2AX is important for effective activation of ATM-dependent	Cell culture, Knockdown experiment by	1. DNA double-strand breaks (DSBs) caused due to the exposure to ionizing radiation, DNA damaging agents or the stall or

and Biophysical Research Communications, 380(4), 752–757.	interaction with NBS1.		signaling pathways. 2. ATM is recruited to DNA damage sites in a c-H2AX-dependent manner. 3. H2AX functions in DSB-induced cell cycle checkpoints.	siRNA, immunofluorescent staining, ATM kinase assay, Measurement of radiation-resistant DNA synthesis.	collapse of DNA replication forks. 2. The generation of DSBs triggers the re-localization of many DNA damage response (DDR) proteins such as MRE11/NBS1/RAD50, MDC1 and BRCA1 to nuclear foci that co-localize with c-H2AX. 3. H2AX is one of essential component for active ATM complex and participates in the activation of ATM-dependent cell cycle checkpoints. 4. Complex formation with γ-H2AX through NBS1 might be indispensable for the DSB-dependent activation of ATM. 5. ATM cooperates with both NBS1 and γ-H2AX for regulation of ATM-dependent cell cycle checkpoint in response to DNA damage. 6. Role of these H2AX modifications in ATM-dependent pathways to clarify the detail of H2AX/ATM/NBS1-related DNA damage response is still unknown. 7. The interaction between ATM and γ-H2AX is through NBS1 and MDC1 may be dispensable for initial ATM activation. 8. MDC1 may be important for initial ATM activation in NBS1-defective cells. 9. NBS1 is also known to function parting some IR-induced cell cycle checkpoints such as intra-S checkpoint. 10. Hence, H2AX could function for cell cycle

					checkpoint through the regulation of ATM complex. Both AT and NBS cells exhibit radiation-resistant DNA synthesis (RDS) after IR, which is due to a defect in intra-S checkpoint. **11.** 5. H2AX-complemented ES cells showed decrease of 70% in DNA synthesis after 10 Gy of c-irradiation, but H2AX-knockout ES cells displayed about 50% decrease, which suggests that H2AX-knockout ES cells are defective in intra-S checkpoint following irradiation.
Birling Celine Ziegler et al., The International Journal of Developmental Biology., 2009	Distribution of p53 binding protein 1 (53BP1) and phosphorylated H2AX during mouse pre-implantation development in the absence of DNA damage	Experimental	γ-H2AX occurs in the mouse pre-implantation embryos in the absence of induced DNA damage. These levels may vary throughout the cell cycle, suggesting a role in mitosis during periods of fast division.	Embryo collection and culture, immuno staining, confocal analysis	1. The cells in the pre-implantation mammalian embryo proliferate with a high rate compared to somatic, differentiated cells and spend most of their cell cycle in the S and G2-phases. 2. Histones are essential targets for regulation of DNA-mediated processes including transcription, replication and DNA repair. 3. Loss of the H2A variant H2AX in mice results in genome instability and male infertility. 4. H2AX potentially plays multiple roles during mouse embryogenesis. 5. Phosphorylated H2AX is directly linked to the recruitment of 53BP1 into DSB foci, suggesting that they mediate DNA repair in similar pathways. 6. Phosphorylation of H2AX persists throughout preimplantation development, with particularly high levels occurring in the zygote right after fertilization and after the 4-cell stage. Phosphorylation

					of H2A.X increases during mitosis in the absence of induction of DNA damage. 7. 53BP1 and γH2A.X only rarely co-localize, suggesting that these high levels of phosphorylation of H2A.X are not directly linked to the DNA damage process in the early embryo. 8. However, in some embryos, we could detect 1-2 cells next to the forming blastocoeilic cavity which displayed both cytoplasmic and nuclear localization of 53BP1. 9. Patterns of γ-H2A.X in the embryo have been previously analyzed in response to DSBs caused by γ- irradiation in *in vitro* fertilized embryos. 10. Since phosphorylated H2A.X plays an important role in the generation of nucleosome arrays with physiological spacing prop-erties (Kleinschmidt and Steinbeisser, 1991) we suggest that the high levels of γH2A.X in the male pronucleus might reflect the fact that this constitutes newly assembled chromatin that needs to achieve a proper nucleosomal configuration. 11. H2A.X is highly phosphorylated throughout preimplantation development in the absence of any induced DNA damage. Moreover, γ-H2AX levels vary significantly throughout the cell cycle. After the 4-cell stage, we detected high levels of H2A.X phosphorylation in mitosis, where telomeres appeared focally enriched with γ-H2A.X.

Dickey, J. S., Redon, C. E., Nakamura, A. J., Baird, B. J., Sedelnikova, O. A., & Bonner, W. M. (2009). Chromosoma, 118(6), 683–692.	H2AX: functional roles and potential applications.	Review article	Potential clinical applications of γ-H2AX detection in cancer and in response to other cellular stresses.		1. H2AX in preventing genomic instability associated with cancer. 2. Female null mice are capable of breeding and males are infertile indicating a role for H2AX in spermatogenesis. 3. H2AX functions as a genome caretaker and the expression of both gene alleles is required for optimal protection against tumorigenesis. 4. H2AX might be required for the repair of ROS-induced DNA damage and preventing oxidative stress-related genomic instability. 5. Aging-associated γ-H2AX foci are caused by both dysfunctional telomeres as well as non-telomeric DNA double- strand damage that may play a causal role in mammalian aging.
Ashok Agarwal, Reda Z Mahfouz, Rakesh K Sharma, Oli Sarkar, Devna Mangrola, Premendu P Mathur., Reprod Biol Endocrino 2009 vol. 7 pp. 143	Potential biological role of poly (ADP-ribose) polymerase (PARP) in male gametes.	Review article	Cleaved PARP (cPARP) may be considered a marker of apoptosis. The presence of higher levels of cPARP in sperm of infertile men adds a new proof for the correlation between apoptosis and male infertility.	-	Poly (ADP-ribose) polymerase (PARP), a nuclear enzyme has a particularly well-researched role in base excision repair; it is one of the primary repair mechanisms to resolve DNA lesions caused by endogenous processes as well as those caused by exogenous chemical exposure and irradiation. A recent classification system by Hassa and Hottiger groups PARPs on the basis of their catalytic domain sequences
Nashun et al., 2010 Development and	Changes in the nuclear	Experimental	One hour after	RT-PCR,	1. Variants- H2A.Z, H2A.X, macroH2A and H2A.Bbd.

Stem cell.	deposition of histone H2A variants during pre-implantation development in mice		insemination, Flag-H2A.X was not yet incorporated into the paternal genome, although it was already detected in condensed maternal chromosome.	plasmid construction, production of transgenic mice carrying the flag-tagged histone H2A variant gene, Immunoblotting, Semi-quantitative RT-PCR, immunocytochemistry	Mouse during oogenesis and pre-implantation development when genome remodeling occurs. 2. H2AZ is highly conserved around the species (Zlatanova and Thakar, 2008). 3. Chromatin incorporation and deposition of the canonical histone H2A (*Hist3h2a* – Mouse 4. Genome Informatics) and its variants H2A.Z (*H2afz* – Mouse Genome Informatics), H2A.X (*H2afx* – Mouse Genome Informatics) and macroH2A (*H2afy* – Mouse Genome Informatics). 5. Histone H2A was detected in the GVs of full-grown oocytes and in the condensed chromosomes of mature MII-stage oocytes. 6. Strong H2A signal comparable with that in GV-stage oocytes was observed in blastocysts.
Wossidlo, M., Arand, J., Sebastiano, V., Lepikhov, K., Boiani, M., Reinhardt, R., ler, H. S. O., et al. (2010). The EMBO Journal, 29(11), 1877–1888.	Dynamic link of DNA demethylation, DNA strand breaks and repair in mouse zygotes.	Experimental	The same correlations are found in cloned embryos obtained after somatic cell nuclear transfer. Together, the data suggest that (1) DNA-methylation reprogramming is more complex and extended as anticipated	Mouse oocytes, IF staining, EdU and BrdU incorporation experiment, Somatic cell nuclear transfer, modified nick translation assay.	1. DNA methylation reprogramming includes loss of 5 methyl cytosine (5 mc) independent of DNA replication. 2. The first phase is found during early germ cell development and the second in the zygote shortly after fertilization. 3. γ-H2Ax and phase of DNA methylation in the early to mid zygotic stage. 4. Marking repairs particularly in paternal pronuclei at time points when DNA methylation is lost. 5. Asymmetric appearance of

			earlier and (2) DNA demethylation, particularly the rapid loss of 5mC in paternal DNA, is likely to be linked to DNA repair mechanisms.		γ-H2AX foci in mouse zygotes at PN2 (corresponding to late PN3 in our classification) and PN4. 6. PN1- this nucleotide incorporation completely disappears in both pronuclei and reappears at early PN3 again only in paternal pronuclei. 7. The dynamic changes of γH2AX foci at early, middle and late zygotic stages reveal several waves of appearing and disappearing DNA strand breaks.
Liu, H., Takeda, S., Kumar, R., Westergard, T. D., Brown, E. J., Pandita, T. K., Cheng, E. H.-Y., et al. (2010). Nature, 467(7313), 343–346.	Phosphorylation of MLL by ATR is required for execution of mammalian S-phase checkpoint .	Experimental	The discovery of MLL in executing the S-phase checkpoint provides new mechanistic insights into not only normal cell biology but also the pathology underlying MLL leukaemias.	Plasmid construction, antibodies and western blots, cell culture and synchronization.	1. Human chromosome-11 band-q23 translocations disrupting the MLL gene lead to poor prognostic leukaemias. 2. MLL acts as a novel effector in the mammalian S-phase checkpoint network and identifies checkpoint dysfunction as an underlying mechanism of MLL leukaemias. 3. MLL accumulates in the S phase on DNA insults and MLL dysfunction results in S-phase checkpoint defects.

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