Running Title:

Elimination of mismatches in mouse and human blastocysts

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Functional assessment of elimination of mismatches in nuclear extracts and whole cell extracts obtained from mouse and human blastocysts

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Abstract

The preimplantation embryos may have an increased risk of having mismatches due to the rates of cell proliferation and DNA replication. Elimination of mismatches in human gametes and embryos have not been investigated previously. In this study we developed a sensitive functional assay to investigate the repair or elimination of mismatches in both commercially available cell extracts and extracts obtained from preimplantation embryos.

Heteroduplex molecules were constructed using synthetic oligonucleotides. Efficiency of the repair of mismatches was semi-quantitatively analysed by exposure to nuclear/whole cell extracts (as little as 2.5µg) and extracts obtained from pooled mouse and human blastocysts to investigate the repair capacity in human embryos.

A cell free *in vitro* assay was successfully developed to analyze the repair of mismatches using designed heteroduplex complexes. The assay was further optimised to analyse repair of mismatches in cell extracts obtained from oocytes and blastocysts using minute amounts of protein. Repair of mismatch efficiency was observed in both mouse and human blastocysts (2.5µg). The blastocysts were observed to have lower repair efficiency compared to commercially available nuclear and whole cell extracts.

In conclusion, a sensitive, easy and fast *in vitro* technique was developed for the first time to detect the repair of mismatch efficiency in embryos. This study showed for the first time that the MMR was active in human embryos at the blastocyst stage.

Keywords: functional assay; DNA repair; repair of mismatch; embryo; human

Introduction

The rates of cell proliferation and DNA replication are high in the early developing embryos that may increase the risk of mismatches in gametes and preimplantation embryos (Baarends et al., 2001). Importance of correct repair or elimination of mismatches is shown by anomalies during gametogenesis and preimplantation embryo development. The main pathway of elimination of mismatches is mismatch repair (MMR). Aberrant expression or lack of MMR genes have been associated with abnormal spermatid phenotypes (Richardson et al., 2000), failure of meiosis I completion in oocytes (Lipkin et al., 2002), growth retardation and embryo fatality (van de Vrugt et al., 2009) and infertility in both male and female mice (Baker et al., 1996, Baker et al., 1995, Wang et al., 1999). Therefore, it is suspected that mismatches are repaired either by MMR genes and proteins or by another mechanism in gametes and preimplantation embryos.

One of the main difficulties in assessing repair capacity in human oocytes and embryos is the small amount of protein present in these samples, between122ng to 0.1µg in cattle oocytes and 162ng to 50µg in cattle preimplantation embryos (Grealy et al., 1996, Thompson et al., 1998). In Xenopus and Drosophila, repair of mismatches have been investigated by microinjecting a heteroduplex construct into oocytes and embryos or by using protein extracts obtained from these oocytes (Oda et al., 1996, Petranovic et al., 2000, Varlet et al., 1996, Labhart, 1999). Generally, cells are transfected with plasmid/bacteriophage circular DNA substrates and the efficiency of repair is assessed by restriction endonuclease cleavage *in vivo* (Zhou et al., 2009) or *in vitro* by exposure of nuclear or whole cell extracts (Wang and Hays, 2002, Thomas et al., 2002). The drawback of these techniques is that they require use of plasmids where the preparation is technically demanding and large number of cells is

required. They are also time consuming and can be expensive (Tsai-Wu et al., 1999, Lei et al., 2004).

The aim of our project was to develop a sensitive and simple functional assay to detect the efficiency of mismatch repair or elimination using mismatched oligonucleotide constructs and small amount of protein extracts. Exposure to endogenous and exogenous metabolites, such as reactive oxygen species, carcinogens or chemicals used in assisted reproduction treatments, could lead to DNA damage in embryos. Therefore, repair or elimination of mismatches is crucial during embryonic development; especially since apoptosis of a even a single cell could be detrimental to the overall development of early stage embryos. The results of this study provide a tool for future studies to evaluate the activity of different repair mechanisms in embryos.

Results

Repair or elimination of mismatches were functionally assessed by exposure of homo/heteroduplexes to the nuclear and whole cell extracts. A number of research studies suggest that repair of mismatches is strand specific and directed by the presence of a nick on one strand (Thomas *et al.*, 1991, Holmes *et al.*, 1990, Fang and Modrich, 1993, Umar *et al.*, 1994, Miller *et al.*, 1997, Taghian *et al.*, 1998). Therefore nicked homo/heteroduplex molecules were also constructed to investigate if repair or elimination of mismatches is nick-directed.

Repair or elimination of mismatches using nuclear and whole cell extracts

Different concentrations of nicked and non-nicked homoduplex (G-C) / heteroduplex (G-T) constructs (3.33-0.33µM) were exposed to MMR efficient nuclear extract HeLa S3 (1.33-14µg) and MMR deficient nuclear extract LoVo with an *MSH2* mutation. The repair of G-T

heteroduplex to G-C homoduplex was detected in the presence of both HeLa and LoVo nuclear extracts by mini-sequencing analysis. The repair or elimination of mismatches detected in the presence of HeLa nuclear extract was at a higher efficiency compared to the LoVo nuclear extract. In the absence of any nuclear extract or buffer, the mismatch was persistent (Table 1). The repair or elimination efficiency was semi-quantitatively determined by comparing the peak heights of the corrected alleles at different incubation time intervals (1, 3, 6 and 15 hours) (Figure 1 a, b, c and d, respectively). As the time of incubation increased from 1 hour to 3 hours, the repair or elimination efficiency was increased by 500-fold in the presence of HeLa. Exposing the mismatched heteroduplexes to the nuclear extracts for 6 and 15 hours improved the correction of the mismatched bases, but the efficiency was not improved as significantly. Exposing the homoduplex constructs to the same buffer solutions showed that bias was not introduced upon the reaction.

In the second part of the project, the nicked and non-nicked homoduplex molecules and heteroduplexes were exposed to commercially available HeLa whole cell extract. Different concentrations of G-Tn and G-T heteroduplexes (0.83-0.33μM) were exposed to 5-2.5μg HeLa whole cell extract for 23 hours (Table 2). It was observed that as the concentration of G-Tn decreased, the repair or elimination efficiency of the mismatched heteroduplex was also decreased (Figure 2).

Repair or elimination of mismatches in oocytes and blastocysts

In the final part of this study, repair or elimination efficiency of the mismatched heteroduplexes was assessed using whole cell extracts obtained from mouse/human oocytes and blastocysts. For all the reactions, repair or elimination of mismatches was assessed in two controls, one positive with commercially available HeLa whole cell extract and one negative with no extract. In the presence of HeLa whole cell extract, repair or elimination of the G-Tn

heteroduplex to G-C was observed in all the reactions; whereas mismatched bases were still detected in the absence of whole cell extract. In a subset of samples, repair or elimination of the mismatches was assessed in an additional negative control where buffer involving dNTPs, ATP and glutathione was excluded from the analysis. Similarly mismatched bases were observed in the absence of dNTPs, ATP and glutathione.

Whole cell extracts were obtained from five and ten pooled mouse oocytes, and four and eight pooled mouse blastocysts, respectively (Table 3). The mismatched bases were not repaired or eliminated upon exposure to whole cell extract obtained from pooled oocytes. Repair or elimination of G-Tn heteroduplex to G-C homoduplex was observed in extracts from four and eight pooled mouse blastocysts (Table 3). Although there was G-C homoduplex detected, the presence of G-Tn heteroduplex was still detectable indicating the incomplete repair or elimination of G-Tn heteroduplex in the reaction mixture.

Repair or elimination of G-Tn heteroduplex at 0.33µM was detected by exposure of WCE obtained from eleven pooled human blastocysts (Table 3). However, this correction of mismatches was considerably less compared to the repair observed for the pooled mouse blastocysts (Table 3).

Discussion

The repair or elimination of mismatches in gametes and preimplantation embryos has not been investigated thoroughly due to the small amount of protein present in these samples. Current techniques measure the repair or elimination efficiency *in vitro* (Thomas et al., 1991, Holmes et al., 1990) and *in vivo* (Wang and Hays, 2002) involve exposure of mismatched DNA substrates to large amounts of nuclear extracts and they require the construction of plasmids. Few studies assessed the mismatch repair in Xenopus oocytes by microinjecting the

heteroduplex molecule into the oocytes or by using the egg extracts (Oda et al., 1996, Petranovic et al., 2000, Varlet et al., 1996, Labhart, 1999). However similar to the other studies, these also require construction of plasmids to form the heteroduplex molecules. We therefore developed a simple assay that enables assessment of repair or elimination of mismatches without the need of large amounts of protein and construction of plasmids. This study forms the basis of future analyses to understand the repair or elimination of mismatches in human oocytes and embryos and to assess the embryonic development potential, especially in the presence of DNA damage.

Nicked and non-nicked G-T heteroduplex molecules were constructed using oligonucleotides designed to incorporate the rs1981929 SNP in the *MSH2* gene. Results of this study showed repair or elimination of both nicked and non-nicked G-T heteroduplexes to G-C homoduplexes in the presence of commercially available nuclear extracts of both MMR efficient HeLa and MMR deficient LoVo (1.33µg), commercially available whole cell (2.5µg) and whole cell extracts obtained from pooled mouse and human blastocysts. In theory, any nuclear extract that is MMR deficient should not repair the mismatches. However, studies have reported repair or elimination of mismatches in the presence of LoVo nuclear extracts in *Saccharomyces cerevisiae* showing that *MSH3* can function as an alternate for *MSH2* (Marsischky et al., 1996, Strand et al., 1995) in endometrial cancer cell lines (Umar et al., 1994), human colon carcinoma cell lines (Lei et al., 2004) and in mouse embryo fibroblasts (Edelmann et al., 1996). Repair or elimination of the mismatches could also be performed by other DNA repair mechanisms, such as base excision or nucleotide excision repair (O'Regan et al., 1996, Huang et al., 1994). Studies also identified a thymine DNA glycosylase that is specific for repairing the G-T mismatches to G-C in bacteria (Sohail et al., 1990) and in mammalian cells (Wiebauer and Jiricny, 1989, Wiebauer and Jiricny, 1990, Bill et al., 1998)

that could be responsible for the repair in MMR deficient cells. Any of these possibilities may have contributed to the repair of G-T to G-C homoduplexes in LoVo nuclear extracts.

Studies suggested that nicks may improve the repair efficiency and the repair is initiated from the nick (Thomas *et al.*, 1991, Holmes *et al.*, 1990, Fang and Modrich, 1993, Umar *et al.*, 1994, Miller *et al.*, 1997, Taghian *et al.*, 1998). Conflicting studies in monkey COS-7 (CV-1 in Origin and carrying the SV40 genetic material) cells suggested that nicks did not have an effect in directing the strand repair (Heywood and Burke, 1990a, Heywood and Burke, 1990b). Additional studies supported this by showing equal mismatch repair efficiency with nicked and intact plasmid DNA in *in vivo* studies (Lei *et al.*, 2004). The similarity of the mismatch repair activity between nicked and intact plasmid DNA could be due to fast ligation of the nick followed by the repair of the mismatch. Studies supported this hypothesis such that mismatch repair was more efficient in the presence of DNA ligase 3 with nicked plasmid DNA (Tomkinson *et al.*, 1993).

In mouse oocytes, although mRNA transcripts involved in DNA repair are present (Jaroudi et al., 2009), repair or elimination of mismatches was not detected. Repair or elimination of mismatches was detected in human blastocysts with lower repair efficiency compared to the MMR observed in commercially available nuclear and whole cell extracts. This could be due to the small amount of protein present in mammalian blastocysts (0.16-50μg in preimplantation embryos) (Grealy et al., 1996).

In conclusion, we described a method for monitoring repair or elimination of mismatches using commercially available nuclear and whole cell extracts as well as whole cell extracts obtained from pooled preimplantation embryos. This study is particularly important since there has not been any data on the repair or elimination of mismatches in human embryos and with this study it can be concluded now that there is a mechanism that eliminates mismatches

during preimplantation embryo development. This study further serves as proof of principle for a plasmid-free, *in vitro* functional assay allowing the semi-quantitative assessment of repair or elimination of mismatches . The technique is more sensitive, easier and faster than previously described methods, since it does not necessitate plasmid production, restoration of restriction endonuclease sites or reporter sequences. This modified protocol facilitates the efficient assessment of repair or elimination of mismatches in different/multiple mismatches with different lengths in addition to easily modifying the construct for the assessment of insertion deletion loops (IDLs) of variable sizes. Compared to previous assays, this method is superior, since small amounts of proteins can be used, allowing the investigation of repair or elimination of mismatches in human preimplantation embryos and evaluating the potential of embryonic development.

Methods

This study was licensed by the Human Fertilization and Embryology Authority (Reference: RO113) and ethical approval was granted by the National Research Ethics Service, Research Ethics Committee (Reference: 10/H0709/26).

Formation of mismatched DNA substrate

Oligonucleotides were designed around a single nucleotide polymorphism (SNP) site rs1981929 in the *MSH2* gene on chromosome 2 (2p22-p21) to construct mismatched substrates (heteroduplexes with a G-T mismatch). These substrates were constructed by denaturing the synthetic G and T oligonucleotide strands (Eurogentec, Belgium) at 95˚C for 5 minutes followed by 16 hour incubation at 37˚C. Similarly, homoduplex constructs (A-T duplexes) were formed by incubating two complementary strands (Supplemental table 1). Nicked homo/heteroduplex constructs were formed, by combining A or G strands with the

nicked T (Tn = Ta + Tb) strands, to test if the repair was nick-directed (see Supplemental table 1for sequence details). The details of the methodology are shown in figure 3.

The successful formation of the constructs was confirmed by mini-sequencing analysis (SNaPshot™, Applied Biosystems) detecting the allele at the SNP site rs1981929 using two different cycling conditions. The sequences of the primers detecting the allele for the A and G strands were 5'-GGTACAAATAGTACAG-3' and T strand was 5'-TAAATAGTAACTTTGGAGACCT-3', respectively. The first set of cycling conditions involved detection of both strands forming the homo/heteroduplex constructs by 25 cycles of denaturation at 96˚C for 10 seconds, annealing at 30˚C for 5 seconds and elongation at 37˚C for 30 seconds. The second set of conditions was used to verify that no excess oligonucleotides were present in the reaction mixture by the same cycling conditions excluding the denaturation step (only annealing at 30˚C for 5 seconds and elongation at 37˚C for 30 seconds for 25 cycles).

Repair or elimination of mismatches using commercially available cell extracts

The homo/heteroduplex constructs were subjected to commercially available nuclear extracts, HeLa S3 (MMR efficient) and LoVo (MMR deficient), and whole cell extracts (HeLa whole cell extract, WCE, Active Motif). The reaction was performed as previously described with some modifications (Wang and Hays, 2002). Briefly, 1.67-3.33µg/µl DNA constructs were mixed with 1.33-13.35µg nuclear extract or 5-2.5 µg/µl WCE in the presence of 3µl buffer (20mM Tris-HCl at ph 7.6, 1.5mM ATP, 1mM Glutathione, 0.1mM dNTP mix, 5mM $MgCl_2$ and 110mM KCl). To assess the Repair or elimination of mismatches, two negative controls were included in the analysis where no nuclear extract or no buffer was included in the reactions, respectively. The reaction mixture was incubated with the homo/heteroduplex constructs at 37˚C for 23 hours. The reaction was terminated by addition of 30µl stop solution

(25mM EDTA, 0.67% SDS, 90µg/ml Proteinase K) and incubated at 37˚C for 30 minutes and 75˚C for 15 minutes (Figure 3).

DNA constructs were purified immediately using DNA clean and concentrator™ (Zymo research corporation, USA) following the manufacturer's protocol. The purified repaired/non-repaired DNA constructs were analysed by mini-sequencing analysis (SNaPshot™, Applied Biosystems). The peak sizes were compared to obtain an estimate of the repair or elimination of mismatches for each allele.

Repair or elimination of mismatches using whole cell extracts from mouse blastocysts, human oocytes and human blastocysts

Whole cell extraction from MF1 strain mouse blastocysts, human oocytes and human embryos was carried out using the whole cell extraction kit (Millipore, UK) following the manufacturer's protocol with slight changes. Briefly, 30µl of extraction buffer mixture was added to each pooled oocyte and blastocyst samples. This mixture was mixed and incubated on ice for 15 minutes. The whole cell extract was obtained by centrifugation. The reaction was performed as previously described using 70% (v/v) oocyte and embryo extract, respectively.

Conflict of Interest

The authors have no conflicts of interest.

Author contributions:

Pinar Tulay performed the experiments, completed the optimizations and performed the analysis part on oocytes and embryos, wrote the manuscript.

Souraya Jaraoudi started the project, involved in the major part of design, performed the initial experiments for the optimization and revised and approved the final paper.

Alpesh Doshi performed the embryology part of the experiments and revised and approved the final paper.

Sioban SenGupta supervised the project, provided support for the design of the project, optimization process and analysis. She revised and approved the final paper.

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Figure Legends

Figure 1 Result from GeneScan analysis™ on ABI Prism™ 310 examining the MMR efficiency of G-Tn after HeLa nuclear extract exposure for 1, 3, 6 and 15 hours. Black, blue and green peaks represent the C, G and A alleles, respectively. The peak heights (corresponding to the fluorescence intensity) are shown in parenthesis. G-Tn constructs were observed by the presence of C and A peaks following mini-sequencing analysis, as reverse mini-sequencing primers were used. The detection of a G peak, equivalent to homoduplex G-C molecules formation, is indicative of repair. (A)-(D) i. SNaPshot™ analysis of G-Tn exposure to HeLa nuclear extract for 1, 3, 6 and 15 hours, respectively. (A)-(D) ii. SNaPshot™ analysis of G-Tn exposure to LoVo nuclear extract for 1, 3, 6 and 15 hours, respectively. (A)-(D) iii. SNaPshot™ analysis of G-Tn incubated in the absence of any nuclear extracts for 1, 3, 6 and 15 hours, respectively. As the time of incubation increased, the MMR efficiency was also increased observed by the higher peak heights. Repair in the presence of HeLa nuclear extract was increased compared to the MMR in the presence of LoVo nuclear extract.

Figure 2 Result from GeneScan analysis™ on ABI Prism™ 310 examining the MMR efficiency of the heteroduplex after exposure of HeLa whole cell extract. Black, blue, red and green peaks represent the C, G, T and A alleles, respectively. The alleles and the peak heights in parenthesis (corresponding to the fluorescence intensity) are shown. G-T constructs were observed by the presence of C and A peaks following mini-sequencing analysis, as reverse mini-sequencing primers were used. The detection of a G peak, equivalent to homoduplex G-C formation, is indicative of repair. (A)-(C) i. shows the SNaPshot™ analysis of 0.83µM, 0.67µM and 0.33µM G-T after 23 hours of HeLa whole cell extract exposure, respectively. T strands are shown to be repaired to C strand. (A)-(C) ii. shows the SNaPshot™

analysis of 0.83µM, 0.67µM and 0.33µM G-T that was incubated for 23 hours in the absence of any whole nuclear extracts showing the G (C) and T (A) alleles, respectively.

Figure 3 The homoduplex constructs were formed with two complementary A and T sequences. Mismatched heteroduplex constructs were formed with two complementary sequences; G and T except at the SNP site rs1981929. Nicked heteroduplex constructs were formed using the G sequence and Ta and Tb sequences that are complementary to the G sequence. Successful formation of homo/heteroduplex constructs was confirmed by SNaPshot™ assay using two different cycling conditions by detecting the allele at the SNP site rs1981929. Each allele fluorescences with a different colour; A in green, T in red, C in black and G in blue. Denatured SNaPshot reaction showed all the alleles at the SNP site within that reaction, whereas in the non-denatured samples the alleles that are present in the mixture belonged to the excess sequences following the formation of homo/heteroduplex constructs. Mismatch repair reaction was carried out in the presence and absence of mismatch repair proteins. The mismatch repair efficiency was evaluated by SNaPshot™ assay by detecting the allele at the SNP site rs1981929.

Table Legends

Table 1 Summary of all the mismatch repair reactions by HeLa and LoVo nuclear extracts.

a) Summary table of repair efficiency of heteroduplex construct to homoduplex after HeLa nuclear extract exposure

b) Summary table of repair efficiency of heteroduplex construct to homoduplex after LoVo nuclear extract exposure

a) Summary table of repair efficiency of heteroduplex construct to homoduplex after HeLa nuclear extract exposure. b) Summary table of repair efficiency of heteroduplex construct to homoduplex after LoVo (mismatch repair deficient) nuclear extract exposure. In order to find the optimal concentrations of heteroduplex constructs and nuclear extracts with correct incubation times, series of optimisation experiments were performed. These tables summarise the final concentrations of nicked and non-nicked heteroduplex constructs, (a) HeLa and (b) LoVo nuclear cell extract concentrations and time of exposure for the mismatch reaction. The efficiency of mismatch analysed by SNaPshot™ reaction was shown as a percentage of the ratio of repaired/unrepaired sequence of the heteroduplex. GT represents the non-nicked heteroduplex complex and GTn represents the nicked heteroduplex constructs.

Table 2 Summary table of repair efficiency of heteroduplex construct to homoduplex after HeLa whole cell extract exposure.

This table summarises the final concentrations of the nicked and non-nicked heteroduplexes, final concentrations of whole cell extracts and time of exposure for the mismatch repair reaction. The efficiency of mismatch repair analysed by SNaPshot™ reaction was shown as a percentage of the ratio of repaired/unrepaired sequence of the heteroduplex. GT represents the non-nicked heteroduplex complex and GTn represents the nicked heteroduplex constructs.

Table 3 Summary of the mismatch repair reactions by whole cell extracts obtained from pooled mouse oocytes, blastocysts and human blastocysts.

This table summarises the concentrations of the nicked heteroduplex constructs, number of oocytes and the initial concentrations of the whole cell extract obtained from mouse and human oocyte/blastocyst samples and time of exposure for the mismatch repair reaction. The efficiency of mismatch repair analysed by SNaPshot™ reaction was shown as a percentage of the ratio of repaired/unrepaired sequence of the heteroduplex. It was shown that there was no repair in pool of 5 and 10 mouse oocytes. Repair of G-T heteroduplex to G-C homoduplex was observed in whole cell extracts obtained from pool of 8 and 4 mouse blastocysts. Mismatch repair of G-T heteroduplex to G-C homoduplex was also observed in 11 pooled human blastocyst whole cell extract. However the repair efficiency in the human blastocysts was considerably lower compared to the mouse blastocysts.

Supplemental Table 1 Oligonucleotide sequences used to make homo/heteroduplexes. The oligonucleotide sequences around the SNP site rs1981929 showing A and G strands and the complementary T strand, including the nicked version: Ta and Tb. The alleles at the SNP site rs1981929 are shown in red.