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Research article

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Essential role for poly (ADP-ribosyl)ation in mouse preimplantation development

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Abstract

Background: Poly (ADP-ribosyl)ation is a covalent modification of many nuclear proteins. It has a strong chromatin modifying potential involved in DNA repair, transcription and replication. Its role during preimplantation development is unknown.

Results: We have observed strong but transient synthesis of poly ADP-ribose polymers on decondensing chromosomes of fertilized and parthenogenetically activated mouse oocytes. Inhibition of this transient upregulation with a specific enzyme inhibitor, 3-aminobenzamide, has long-term effects on the postimplantation development of the embryos. In addition, inhibition of poly (ADP-ribosyl)ation at the 4–8 cell stage selectively blocks morula compaction.

Conclusion: These observations suggest that poly (ADP-ribosyl)ation is involved in the epigenetic chromatin remodeling in the zygote.

Background

Poly (ADP-ribosyl)ation is a covalent modification of proteins catalyzed by the poly (ADP-ribose) polymerases (PARPs) (for detailed reviews see [1-3]). The modification involves the serial transfer of ADP-ribose moieties from co-enzyme NAD⁺ to an aspartate, glutamate or lysine residue on the surface of the acceptor protein. The reaction results in an ADP ribose polymer chain of variable length attached to the protein surface. Known natural targets of poly (ADP-ribosyl)ation include many proteins that participate in nuclear and chromatin structure (histones, HMG proteins, lamin B, many transcription factors, DNA replication factors etc). The best characterized mouse enzyme, PARP-1 also controls its own activity by auto-

modification. The negative electric charge of the polymers increases with length of the chain and causes the modified proteins to dissociate resulting in disruption of chromatin structure. The effect is transient, since the poly (ADP-ribose) polymers are rapidly degraded by poly (ADP-ribose) glycohydrolase, thus, restoring the initial charge of the protein. It is well established that poly (ADP-ribosyl)ation is implicated in DNA repair, and this function is based on the strong chromatin modifying potential of the modification. The two major poly (ADP-ribose) polymerases, PARP-1 and PARP-2 are strongly activated by single-strand DNA breaks. The chromatin proteins around the site of breakage are rapidly modified and dissociate from the DNA, making it accessible to repair enzymes.

Observations made with mice which carry a targeted mutation of the *Parp-1* or *Parp-2* gene have unambiguously confirmed the role of poly (ADP-ribose)ation in the cell response to DNA damage and base excision repair [4-8] and have validated earlier data obtained by using pharmacologic enzyme inhibitors [9].

In addition to their role in DNA repair, PARP-1 and PARP-2 have additional functions, since numerous physical and functional interactions of PARP-1 with specific transcription factors, including AP-2, DF1-4, E47, NF-kB, p53, PC1, Oct-1, RXR, TBP, TEF-1 and YY1 have been described. This suggests a direct role in gene expression. PARPs may also play a role in the maintenance of normal chromosome structure, since *Parp-2* deficiency leads to chromosomal instability, with breaks preferentially occurring in the centromeric region. Immunofluorescence analysis of human and sheep metaphase chromosomes revealed centromeric localization of the PARP enzyme [10].

In addition to *Parp-1* and *Parp-2*, at least three other mouse genes encoding distinct poly (ADP-ribose) polymerases with related catalytic domains have been identified. For example, Tankyrase, another enzyme with PARP activity, was identified through its interaction with the telomeric repeat binding factor-1 (TRF-1) [11]. The existence of a large PARP-coding gene family suggests that, in addition to known functions of poly (ADP-ribose)ation, some so far unrecognized, non-redundant functions may also exist. Given the strong chromatin structure-modifying activity of these enzymes and the transient nature of the modification, it is possible that poly (ADP-ribose)ation is involved in the initial steps of epigenetic remodeling of the chromatin required for the extensive change in gene expression patterns during development and cell differentiation. Disruption of the pre-existing chromatin structure could make the DNA and the chromatin proteins accessible for other modifications and facilitate the establishment of a new epigenetic modification pattern. A number of observations support this view. poly (ADP-ribose)ation is involved in local loosening of polytene chromatin structure associated with gene induction in *Drosophila* salivary glands [12]. The mutation of the single *Drosophila Parp* gene is lethal at early stages [13]. Mice carrying a double *Parp-1/Parp-2* mutation die at the onset of gastrulation [8], suggesting that these two enzymes are responsible for the majority of poly (ADP-ribose)ation in murine cells. These observations are compatible with the supposed role of poly (ADP-ribose)ation in the initiation of epigenetic chromatin remodeling. Direct interaction between poly (ADP-ribose)ation and epigenetic modifications, such as DNA methylation and histone acetylation, were also observed [14-16].

Extensive chromatin remodeling occurs in the zygote during the first cell cycle after fertilization. These changes involve disruption of chromatin structure inherited from the gametes and are accompanied by alterations in DNA methylation, histone modification and recruitment of various chromatin proteins into the pronuclei [17-19]. However, nothing is known about poly (ADP-ribose)ation in oocytes and preimplantation embryos. To gain further insight into the physiological role of PARlation, we analyzed the distribution of poly (ADP-ribose) polymers in oocytes and preimplantation embryos using an immunocytochemical approach and investigated the immediate and long term effects of the inhibition of PARP activity with a known inhibitor, 3-aminobenzamide (ABA). Our observations suggest that poly (ADP-ribose)ation plays an essential role in preimplantation development.

Results

Localization of poly ADP-ribose polymers in the early mouse embryos

To investigate the ontogeny of poly (ADP-ribose)ation during early mouse development, we performed immunodetection of poly ADP-ribose polymers. Fig. 1A shows the distribution of poly (ADP-ribose)ation in the oocytes before and after fertilization. A faint and diffuse cytoplasmic staining was detected in metaphase II-arrested oocytes. In contrast, poly ADP-ribose polymers were specifically detected on the anaphase chromosomes in the zygote. Both the compact sperm chromatin and the oocyte chromosomes in the process of segregation were strongly labeled. However, the accumulation of poly ADP-ribose polymers on the chromosomes was transient. In telophase- and pronuclear-stage embryos, only diffuse cytoplasmic staining was again detected. This indicates that the chromosomes are transiently poly (ADP-ribose)ated at the early stages of chromatin reorganization during the transition from metaphase to interphase. In parthenogenetically activated oocytes (Fig. 1B), 3 h after the activation by strontium chloride, transient and extensive labeling was observed around the oocyte chromatin. The labeling was stronger than that in fertilized oocytes and appeared to be localized around the forming pronucleus and second polar body instead of being strictly colocalized with the chromatin. 5 h after activation the labeling returned to the background cytoplasmic level. These observations clearly indicate that it is the activation of metaphase II arrested oocytes that induces transient upregulation of the poly (ADP-ribose)ation of the chromatin and other proteins that are recruited into the chromatin during its reorganization into interphase structures. All factors necessary for this process are already present in the oocyte.

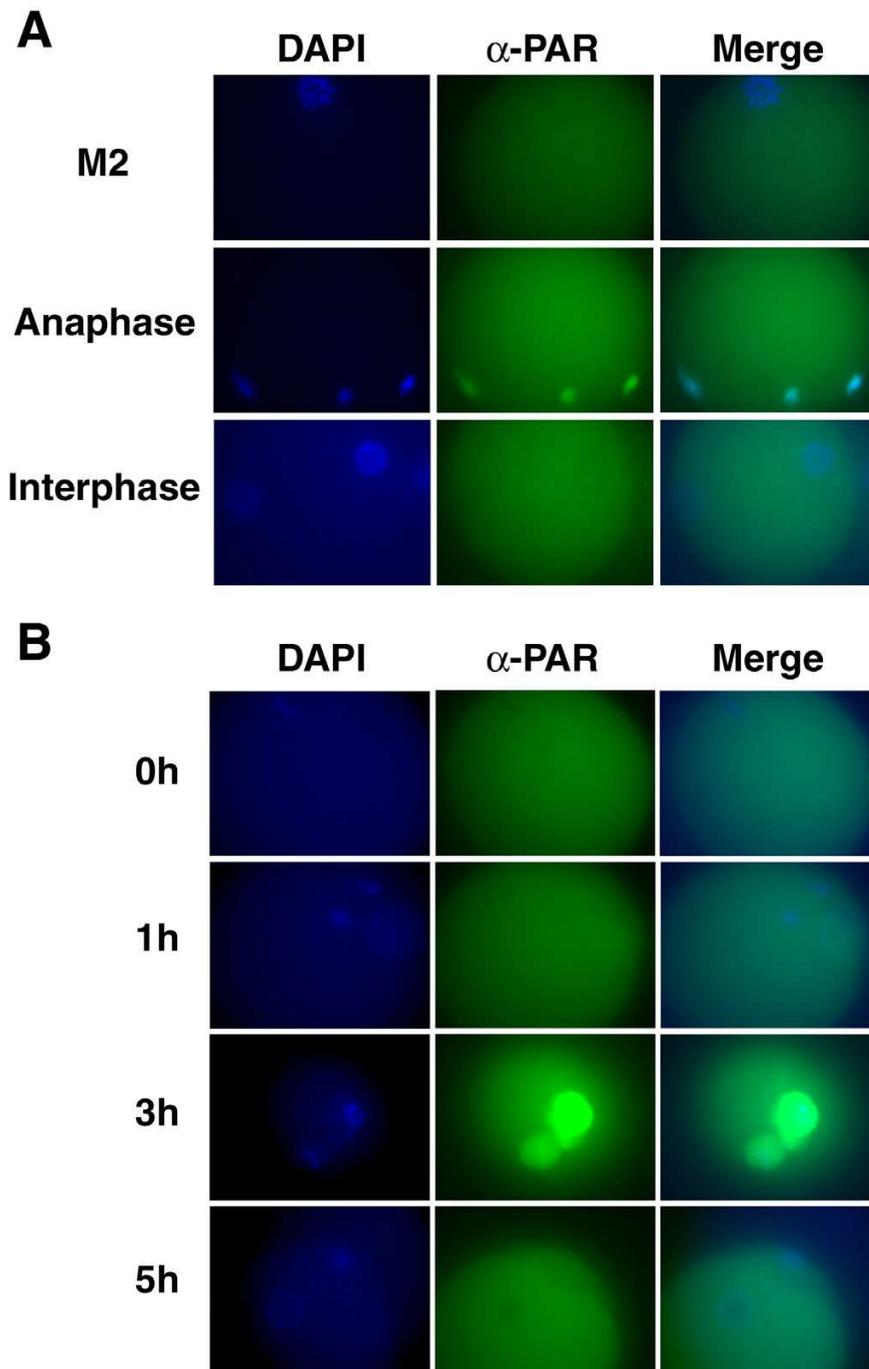


Fig. 1

Figure 1
Transient upregulation of poly (ADP-ribosylation) in fertilized and parthenogenetic 1-cell embryos A: fertilized embryos, B: parthenogenetic embryos. Poly ADP-ribose polymers were detected with an anti-PAR antibody and a fluorescein-labeled (green signal) second antibody, as described in Methods section. Nuclei were counterstained with DAPI.

As shown in Fig. 2, the distribution of poly ADP-ribose polymers remained diffuse and cytosolic during preimplantation development. The signal was the lowest in 2-cell stage embryos with the exception of the polar body, where the labeling was maintained. At the 4-cell stage, it increased again, and then, was stable in the 8-cell and the morula stage. No preferential association with the cell nucleus was observed. Some intercellular heterogeneity appeared in the blastocyst. These observations show the dynamic nature of poly (ADP-ribosyl)ation during preimplantation development.

The antibody used in this study detected ADP-ribose polymers composed of more than 25 monomers. Therefore, our observations reflect only the substantial upregulation of the poly (ADP-ribosyl)ation. Smaller fluctuations resulting in the synthesis of shorter polymers may also occur during preimplantation development, but would go undetected by the antibody. Controls for PAR immunofluorescence that were run in the presence of the inhibitor ABA were negative (not shown).

Effects of transient PARP inhibition on preimplantation development

To investigate the potential physiological consequences of the observed upregulation of poly (ADP-ribosyl)ation in the zygotes, we incubated the freshly fertilized oocytes in the presence of ABA. Pronuclear formation was recorded every hour and compared to the control oocytes that were cultured under the same conditions but without ABA. We started the incubation 17 h after the hCG treatment of the donor females. As shown in Fig. 3A, in the embryos incubated with 5 mM ABA, pronuclear formation was completed at 21 h after hCG treatment. In contrast, only 60% of the control oocytes formed pronuclei during the same period, with the pronuclear formation being completed between 22 and 23 h after hCG. Therefore, inhibition of PARP activity accelerated pronuclear formation by 1–2 h. This effect was confirmed in parthenogenetically activated oocytes. As shown in Fig. 3B, incubation with ABA accelerated pronuclear formation by ~30 min. These results indicate that ABA accelerates pronuclear formation, suggesting that inhibition of poly (ADP-ribosyl)ation may interfere with correct chromosomal rearrangement during the formation of the interphase nucleus in activated oocytes.

To further investigate the function of poly (ADP-ribosyl)ation in preimplantation development, we prolonged the *in vitro* culture in the presence of ABA. All the oocytes were able to cleave into two cells. However, 51-h incubation of the embryos with ABA resulted in an increased fragmentation rate; 32% of the embryos became fragmented compared to the only 7% of the control embryos (Fig. 4A). 75-h incubation in the presence of ABA further

increased the fragmentation rate to 45%, while only 9% of the control embryos became fragmented (Fig. 4A). In the presence of ABA the embryos were unable to undergo compaction and no blastocyst formation was observed. The embryos remained arrested at the 8-cell stage with round cells of slightly reduced size as compared to the controls (Figs. 4B and 4C). Under similar culture conditions, the control embryos became compacted and 64% of them reached the blastocyst stage.

These observations suggest that poly (ADP-ribosyl)ation may play a role in certain critical events during compaction and blastocyst formation. To examine this hypothesis, we cultured the embryos in the presence of ABA during periods of varying length. As expected, the continuous incubation with ABA completely inhibited the development to blastocyst stage (Fig. 4D). When the embryos were transferred into normal M16 medium after 10 h, 27 h or 51 h incubation with the inhibitor respectively 50%, 41% and 24% of them was able to reach the blastocyst stage (Fig. 4D). The loss was mainly due to fragmentation during the incubation period. When the embryos were initially cultured without ABA and the inhibitor was added to the culture medium only at the 2-cell stage (27 h), the compaction was completely inhibited. We found that 24-h incubation between 51 h and 75 h is sufficient for the complete inhibition of compaction and blastocyst formation. However, 13% of the embryos developed to blastocyst when ABA was transiently removed between 51 h and 75 h of culture.

These observations show that inhibition of poly (ADP-ribosyl)ation by ABA has a cumulative effect on development before the 4-cell stage and some critical events occurring around the time of compaction require poly (ADP-ribosyl)ation. The main conclusion is that poly (ADP-ribosyl)ation is essential for normal preimplantation development.

Effects of transient PARP inhibition on postimplantation development

We next addressed the question of whether transient inhibition of poly (ADP-ribosyl)ation has an influence on postimplantation development of the fetus. We cultured the freshly fertilized zygotes obtained 17 h after hCG in the presence of ABA overnight. On the next morning the 2-cell stage embryos were transplanted into the oviduct of pseudopregnant mothers. Development of the fetuses was analyzed on day (d) 13.5 of pregnancy (the day of transplantation was considered as d0). The implantation rate of ABA-treated embryos was slightly lower than for the controls. As shown in Table 1, the resorption rate was two times higher in the ABA treated group (29.6%) than in the control group (15.6%) (Table 1), suggesting that the inhibition of poly (ADP-ribosyl)ation during the first cell

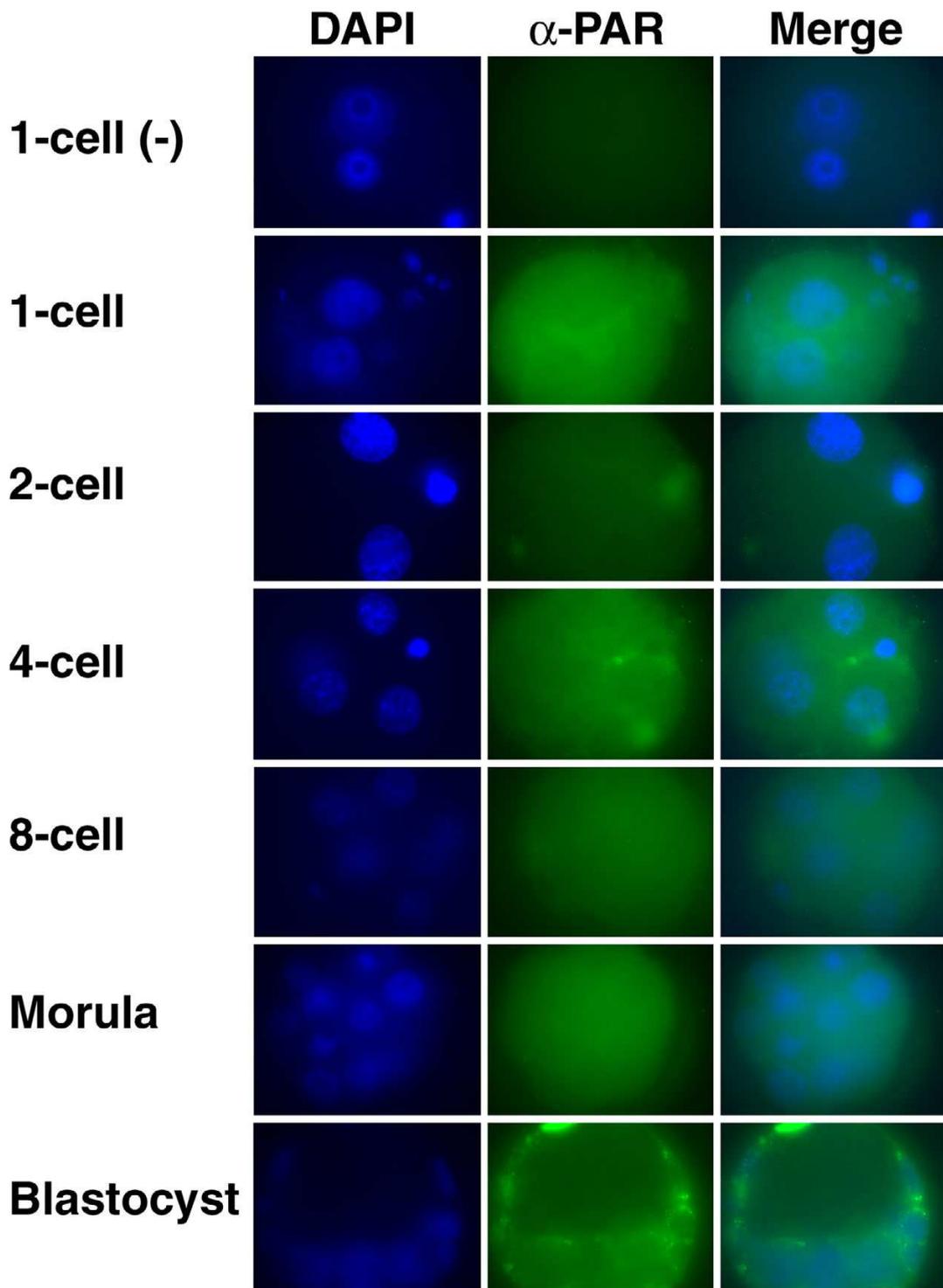
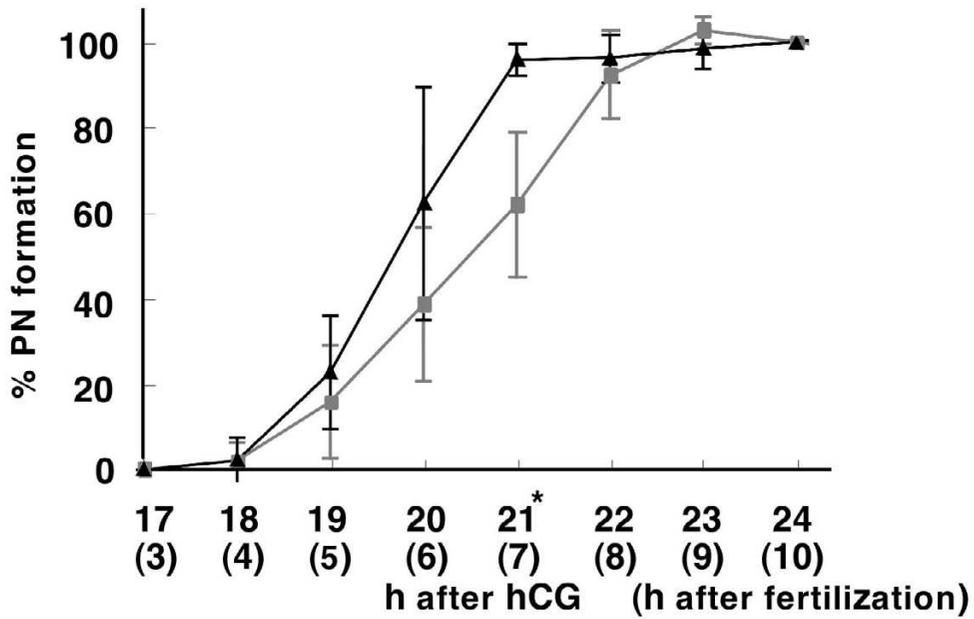


Fig. 2

Figure 2
Immunodetection of poly ADP-ribose polymers in preimplantation development Poly ADP-ribose polymers were detected with an anti-PAR antibody and a fluorescein-labeled (green signal) second antibody and the nuclei were counter-stained with DAPI.

A



B

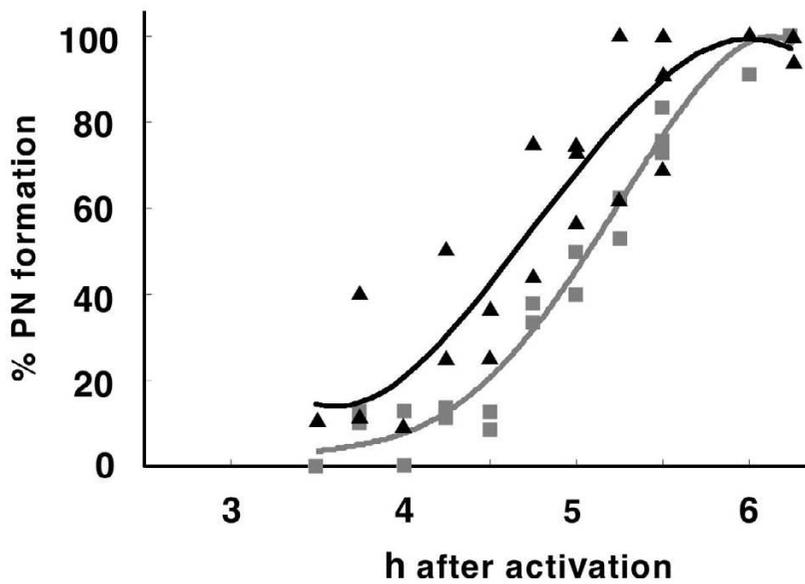


Fig. 3

Figure 3
The effect of PARP inhibition on pronuclear formation Acceleration of pronuclear formation *in vitro* in the fertilized (A) or parthenogenetic (B) oocyte in the presence of ABA, a PARP inhibitor. Triangles and squares indicate the fraction of oocytes that formed pronuclei in the presence or absence of ABA, respectively.

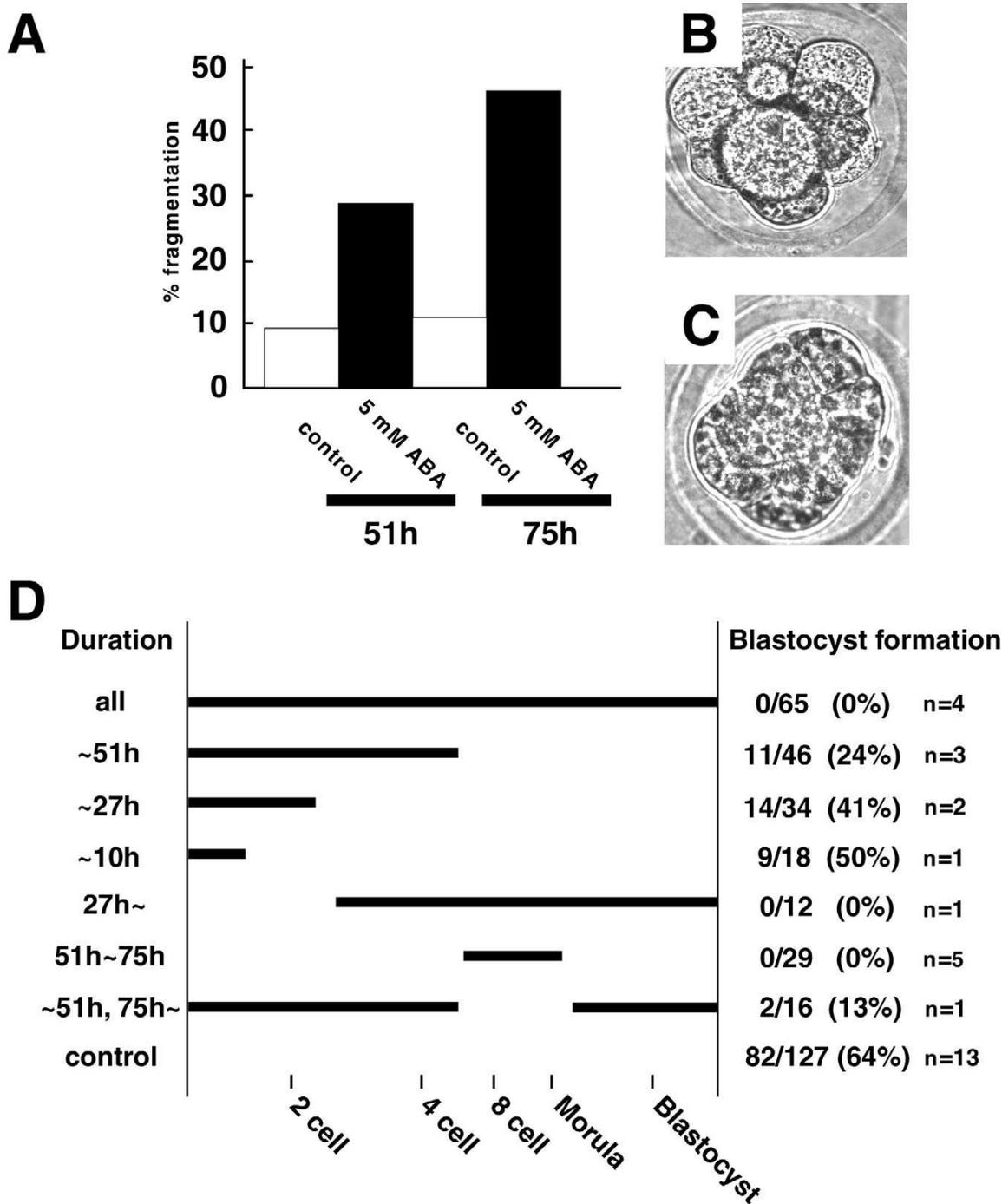


Fig. 4

Figure 4

The effect of ABA on preimplantation development A: Cell survival of the embryos cultured in the presence or absence of ABA for 51 or 75 h after fertilization. B and C: Absence of compaction of the embryos cultured in the presence of ABA (B) in contrast to controls (C) 75 h after fertilization. D: Preimplantation development of embryos cultured *in vitro* for various periods in the presence of ABA. The horizontal black line shows the presence of the inhibitor in the culture medium for the time length indicated on the left. Numbers on the right side indicate blastocysts/total number of embryos, (% of blastocyst formation); n indicates the duplicate experiments.

Table 1: High postimplantation developmental failure of ABA-treated embryos on d13.5

	N° of mothers	N° viable fetuses	N° of resorptions
ABA	8	38 (70.4%)	16 (29.6%)
control	7	38 (84.4%)	7 (15.6%)

Table 2: Average size of the recovered fetuses and placentas

Treatment	fetuses (mm)	placentas (mm)
ABA	10.1 ± 1.2	7.5 ± 0.6
control	10.2 ± 0.4	7.6 ± 0.5

cycle after fertilization has a long term deleterious effect that frequently results in developmental failure after implantation. We measured the size of the surviving fetuses and found no difference in the average size of the embryos and placentas. However, the size variation of the embryos in the ABA treated group was 3 times higher than in the control group (Table 2 and Fig 5). The increased size variation is statistically highly significant ($p < 0.01$ by F-test). It reflects the long-term effect of the transient inhibition of poly (ADP-ribosyl)ation in zygotes.

Discussion

The present study demonstrates that the level of poly (ADP-ribosyl)ation undergoes dynamic changes during preimplantation development. A rapid and strong accumulation of ADP-ribose polymers on the still condensed oocyte chromosomes and sperm genome can be detected within the first hour after fertilization. In accordance with the rapidly reversible nature of poly (ADP-ribosyl)ation, this modification is transient (Fig. 1). Two hours later the labeling is back to the background level. Transient upregulation of poly (ADP-ribosyl)ation is also observed in oocytes activated parthenogenetically by incubation in strontium chloride solution (Sr^{2+}). There are several differences between the oocytes activated naturally by fertilization or parthenogenetically by Sr^{2+} . The labeling in parthenotes is stronger than that in fertilized oocytes. In addition, there is a clear difference in the timing, since the upregulation occurs during the formation of pronuclei. These observations raise two questions: first, what is the biological importance of poly (ADP-ribosyl)ation in zygotes; and secondly, what is the mechanism of the transient upregulation? The synthesis of poly ADP-riboses is enzymatically catalyzed by the poly (ADP-ribose) polymerase (PARP). PARPs constitute a large family of proteins. The best-characterized enzymes, PARP1 and PARP2, are strongly activated by binding to single strand

breaks in the DNA damaged by genotoxic agents [1]. No high incidence of DNA breaks is known to occur in the activated oocyte to explain the rapid increase of poly (ADP-ribosyl)ation in oocytes. Therefore, we hypothesize that the transient upregulation of poly (ADP-ribosyl)ation in fertilized oocytes is independent of DNA repair, but is related to its capacity to modulate chromatin structure. Histones and other chromatin forming proteins are known substrates of poly (ADP-ribosyl)ation. Synthesis of ADP-ribose polymers on the surface of the target molecule introduces substantial changes in the charge of the protein, and thus, induces dissociation of the chromatin making the DNA accessible to the repair enzymes. A moderate level of chromosomal protein poly (ADP-ribosyl)ation might therefore loosen the chromatin structure enough to make it accessible to other possible epigenetic modification, such as histone acetylation or methylation or DNA methylation/demethylation. Indeed, both sperm and oocyte chromatin undergo complete remodeling during the metaphase to interphase transition of the zygote. This process involves not only widespread changes in histone acetylation and methylation, but also DNA methylation [17-19]. The transient upregulation of poly (ADP-ribosyl)ation is observed precisely during the same period. The role of poly (ADP-ribosyl)ation in pronuclear formation is not essential, since ABA treatment influences only the rapidity of the process. We postulate that due to its capacity to decondense compact chromatin, poly (ADP-ribosyl)ation plays the role of "facilitator" in chromatin remodeling by promoting other epigenetic modifications. It is worth mentioning that a link between chromatin compaction, DNA methylation and poly (ADP-ribosyl)ation has been recently reported in somatic cells [12,14,16]. The high incidence of postimplantation developmental failure and the high size variation of surviving midgestational fetuses observed after ABA treatment during the first cell cycle indicate that, in the absence

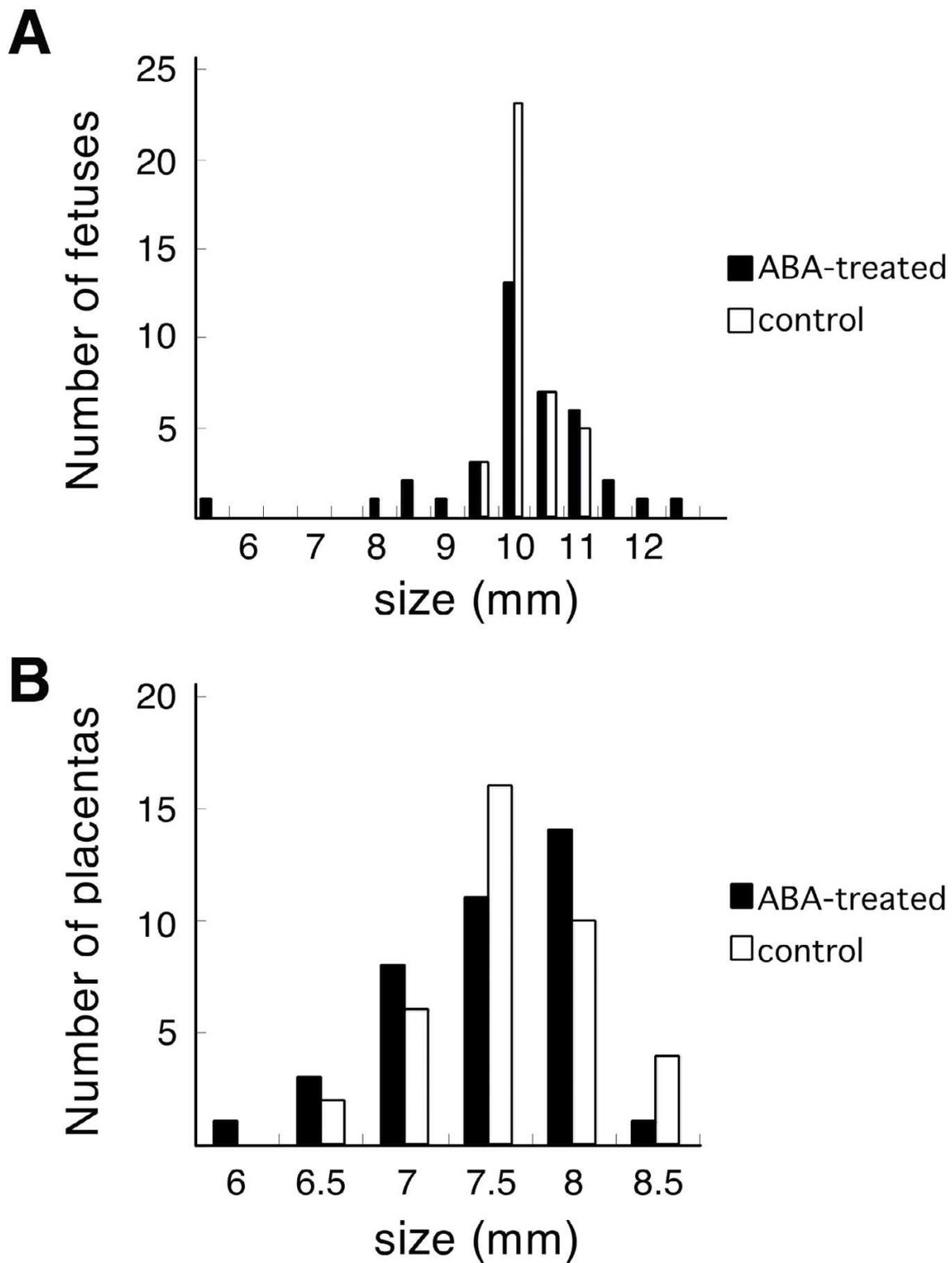


Figure 5
Effect of transient ABA treatment of the zygotes on postimplantation development after transfer to pseudo-pregnant mothers A: Size distribution of the d13.5 fetuses obtained with or without ABA inhibition. B: Size distribution of the placentas. Black: ABA treated, white: control

of poly (ADP-ribosyl)ation, the epigenetic chromatin remodeling is not always normal. The randomly occurring epigenetic variations generated in the zygote may be conserved and transmitted through the cell divisions and could influence development. The proposed role as "facilitator" of epigenetic remodeling is supported by a number of recent observations. Poly (ADP-ribosyl)ation has been found to play a fundamental role in modulating chromatin structure in *Drosophila* [12]. The mutation of the *Drosophila* poly (ADP-ribose) glycohydrolase that leads to accumulation of poly (ADP-ribose) polymers is also lethal in the larval stages [20]. The mutation of the *Drosophila* *Parp* gene is developmentally lethal [13]. In mice, the two major enzymes, PARP1 and PARP2 were shown to be involved in maintaining chromosomal stability [8]. Other members of the PARP family could also be involved in the epigenetic regulation of the genome function [21].

Parp-1^{-/-}/*parp2*^{-/-} double mutant mice are not viable and die at the onset of gastrulation [8]. This observation implies that, in addition to PARP-1 and PARP-2, other PARP enzymes are also acting in the oocyte. In fact, double mutants are able to develop beyond the compact morula stage, while total inhibition of poly (ADP-ribosyl)ation arrests development at this stage. Compaction is the first cell differentiation event in the life of the embryo and leads to two morphologically different cell types. This process requires differential activation of genes between the two cell types. Activation of previously silent genes typically involves epigenetic changes of the chromatin, and inhibition of poly (ADP-ribosyl)ation clearly might disturb this process.

Our observations were obtained using ABA, a commonly used enzymatic inhibitor. This approach proved to be of considerable value in the study of poly (ADP-ribosyl)ation and the conclusions obtained were corroborated by genetic studies [9]. In spite of its high specificity, it is impossible to exclude that some of the observations are the consequence of potential side effects of the inhibitor unrelated to PARlation.

Activation of poly (ADP-ribosyl)ation associated with chromatin of the zygote is likely to be independent of DNA damage. Unfortunately, little is known about the possible alternative routes of PARP activation which do not involve DNA breaks. One such mechanism was described recently in neurons [22] and theoretically could also be functional in oocytes. Fast activation of PARP is induced by inositol 1,4,5-triphosphate (IP3) dependent Ca²⁺ mobilization. Rapid, IP3-dependent increase of the intracellular Ca²⁺ concentration is a characteristic feature of oocyte activation [23]. One possibility is that the rapid and transient increase of poly (ADP-ribosyl)ation in activated oocytes is triggered by an IP3-dependent mecha-

nism similar to that found in neurons. In fertilized oocytes, the initial rapid increase of the intracellular Ca²⁺ is followed by oscillations during the period of transition to metaphase. Parthenogenetic activation induces a single wave of Ca²⁺ influx in the oocytes. Thus, the difference in the timing of PARP upregulation may reflect the different kinetics of oocyte activation after natural fertilization and parthenogenetic activation. This hypothetical mechanism suggests that PARP upregulation in the zygote is under cytoplasmatic control. Poly (ADP-ribosyl)ation is highly dependent on the concentration of the substrate, NAD⁺, a major regulator of the cellular metabolism. If our hypothesis on the role of poly (ADP-ribosyl)ation in epigenetic remodeling is correct, its dependence on metabolism could represent a potential mechanism by which extracellular stress due to experimental manipulations can induce epigenetic abnormalities [24].

Conclusions

The observations reported here show the essential role for poly (ADP-ribosyl)ation in preimplantation development. The long-term developmental effect of the inhibition of poly (ADP-ribosyl)ation suggests its involvement in the epigenetic remodeling of chromatin in the fertilized zygote.

Methods

Preparation of oocytes and embryos

MII oocytes were obtained after superovulation of 6–8 week old (C57BL/6 × CBA)F1 female mice by i.p. injection of 5IU of PMSG (Intervet, Angers), followed 48 h later by injection of 5IU of hCG (Intervet, Angers). Fertilized embryos were obtained after mating the superovulated females with F1 males. Oocytes and zygotes were recovered 17 h after the hCG injection and the cumulus cells were removed with 0.03% hyaluronidase (Sigma) in M2 medium (Sigma). The oocytes were either fixed immediately for immunodetection or were cultured in M16 medium (Sigma) in the presence or absence of 5 mM 3-aminobenzamide (ABA: Sigma-Aldrich) at 37°C under 5% CO₂/air.

MII oocytes collected 17.5–18 h after hCG treatment were used for parthenogenetic activation. These oocytes were incubated for 1 hr in an activation medium containing 10 mM strontium chloride as described (Wakayama et al., 1998). Then, the oocytes were placed into M16 medium in the presence or absence of 5 mM 3-aminobenzamide at 37°C under 5% CO₂/air. (A 100 mM ABA stock solution was prepared by dissolving the product directly in M16 culture medium.)

Immunocytochemistry

Poly ADP-ribose polymers were detected using a rabbit anti-poly ADP-ribose polymer antibody (Trevigen; Gaith-

ersburg, MD). Oocytes or embryos were fixed with a 4% paraformaldehyde-PBS solution containing 1% Triton-X100 for 10 min. They were permeabilized with PBS containing 2% Triton-X100 for 12 min. Blocking was done with serial application of 5% goat serum containing 0.2% Tween20 (buffer A) for 15 min and 5% skim milk containing 0.05% Tween20 (buffer B) for 15 min. Samples were incubated with anti-poly ADP-ribose polymer antibody (1:50) in buffer B at 37°C for 1 h. Blocking was done again as described above. Goat fluorescein-conjugated anti-rabbit IgG antibody (Vector Laboratories; 1:250) was applied at 37°C for 1 h. Nuclei were stained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; Sigma). The samples were mounted on glass slides with the aid of fibrinogen (Calbiochem) polymerized with thrombin (Sigma). Fluorescence was detected by LEITZ DMRB microscopy (Leica).

Transplantation

Two cell stage embryos were transplanted into the oviduct of pseudopregnant (C57BL/6 × CBA)F1 female mice on the morning following the mating with a vasectomized male at day (d) 0. The number of implantation sites, resorptions and the size of the embryos and placentas were recorded on d13.5 of pregnancy.

Authors' contributions

TI carried out the cell culture, immunocytochemical, and transplantation studies. TN and CT participated in the cell culture studies. AP participated in the design of the study and coordination, performed statistical analysis, and drafted the manuscript. All authors read and approved the final manuscript.

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