Pak2 Depletion Induces a Failure of Early Embryonic Development in Mice

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Research

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Abstract

Background: The quality of the early embryo is vital to embryonic development and implantation. As a highly conserved serine/threonine kinase, p21-activated kinase 2 (Pak2) participates in diverse biologic processes, especially in cytoskeleton remodeling and cell apoptosis. However, the role of Pak2 in preimplantation embryos remains unelucidated.

Methods: In the present work, Pak2 was depleted using a specific small interfering RNA in early mouse embryos, validating the unique roles of Pak2 in spindle assembly and DNA repair during mice early embryonic development. We also employed immunoblotting, immunostaining, in vitro fertilization (IVF) and image quantification analyses to test the Pak2 knockdown on the embryonic development progression, spindle assembly, chromosome alignment, oxidative stress, DNA lesions and blastocyst cell apoptosis.

Results: We found that Pak2 deletion significantly reduced blastocyst formation of early embryos. In addition, Pak2 depletion led to dramatically increased abnormal spindle assembly and chromosomal aberrations in the embryos. We noted the overproduction of reactive oxygen species (ROS) with Pak2 knockdown in embryos. Pak2 knockdown also resulted in the accumulation of phosphorylated γ H2AX, indicative of increased embryonic DNA damage. Commensurate with this, a significantly augmented rate of blastocyst cell apoptosis was detected in Pak2-KD embryos compared to their controls.

Conclusions: Collectively, our data suggest that Pak2 may serve as an important regulator of spindle assembly and DNA repair, and thus participate in the development of early mouse embryos.

Background

The early embryonic development of mammals is activated when a mature oocyte (*MII*) is fertilized by a mature spermatozoon [1]. Uzun A, Rodriguez-Orsorio N, Kaya A, Wang H, Parrish JJ, Ilyin VA and Memili E [1]. After fertilization, the zygote undergoes cleavage divisions from the 2-cell to blastocyst stages, at which point the embryos are implanted into the mother's uterus on embryonic day 4.5 in mice [2–4]. We now appreciate that embryonic development depends upon precise spatiotemporal regulation of gene expression [5].

Paks (p21-activated kinase) comprise an evolutionarily conserved group of serine/threonine kinases that regulate diverse cellular activities [6]. The mammalian Pak family consists of six members and is divided into two groups: group I is composed of Pak1, Pak2, and Pak3—with Pak1 and Pak3 being tissue-specific and showing the highest levels in brain—whereas Pak2 is ubiquitous [7]; group II is composed of Pak4, Pak5, and Pak6 [8]. The most fundamental and vital function of Pak2 is to regulate the remodeling of the cytoskeleton [9]. Pak2 can be activated by Cdc42 (GTP) and under various stress conditions, and cleaved caspase 3 also constitutively activates Pak2 during the apoptotic process [10]. Under low-amplitude physiologic forces, Pak2 is protected from proteolysis so as to ensure cellular survival, but under higher-amplitude forces Pak2 is left unprotected and stimulates apoptosis [11]. In addition, Pak2 has been

reported to be an important regulator of cellular senescence and organismal aging [11]. Moreover, Pak2 acts as a molecular switch for cytoostasis and apoptosis in response to different types and levels of stress, with broad physiologic and pathologic relevance [10]. Pak2 cardiac-deleted mice (Pak2-CKO) manifested endoplasmic reticulum stress, cardiac dysfunction, and severe cell death [12]; and Pak2 knockout mice showed embryonic lethality on embryonic day 8.5 (E8.5) due to multiple developmental abnormalities [13].

A role for Pak2 in early mouse embryonic development, however, remains unclear. In the current study, we explored Pak2 function during the development of early mouse embryos by using a small interfering RNA (siRNA) to silence the Pak2 gene.

Methods

Mice

We used ICR mice, 6–8 weeks of age, in the present study. Experiments were approved by the Third Affiliated Hospital of Guangzhou Medical University Animal Care and Use Committee and conducted in accordance with the guiding principles of the institution.

Antibodies and chemicals

All the chemicals and reagents were purchased from Sigma unless stated otherwise. Rabbit polyclonal anti-Pak2 antibody (Cat#: ab76293) and anti- γ -H2AX (phosphor S139) antibody (Cat#: ab81299) were obtained from Abcam; mouse monoclonal anti- α -tubulin-FITC antibody (Cat#: F2168) from Sigma; Alexa Fluor 488 goat anti-rabbit IgG (H + L) (Cat#: A11008) and CM-H2DCFDA (Cat#: C6827) from Thermo Fisher Scientific; an In Situ Cell Death Detection Kit (Cat#: 11684817910) was purchased from Roche; and HRP-conjugated Affinipure Goat Anti-Rabbit IgG (H + L) (Cat#: SA00001-2), HRP-conjugated Affinipure Goat Anti-Mouse IgG (H + L) (Cat#: SA00001-1), and GAPDH Monoclonal Antibody (Cat#: 60004-1g) were purchased from Proteintech.

In vitro fertilization and embryo culture

In vitro fertilization (IVF) was performed according to methods described previously [14]. Human tubal fluid fertilization (HTF) medium containing 1% bovine serum albumin (BSA) was used as IVF culture medium. Briefly, female mice were injected with 7.5 IU of equine chorionic gonadotropin (eCG), and 48 hours later they were injected with 7.5 IU of human chorionic gonadotropin (hCG). Thirteen hours after hCG injection, the cumulus-oocyte complexes (COC) were isolated from the oviduct, and capacitated spermatozoa were placed into HTF-droplets containing COCs for co-incubation of at least 5 hours. Fertilized zygotes were washed and incubated in potassium simplex optimization medium (KSOM) under paraffin oil.

siRNA knockdown

To explore the functions of Pak2 in early mouse embryos, specific Pak2-siRNA and negative control siRNAs were obtained from Shanghai GenePharma Co, Ltd. The siRNAs were diluted to 1 mM with RNase-free ddH₂O and stored in a - 80°C refrigerator. Microinjections of small interfering RNAs (siRNA) with a Narishige microinjector were used to knockdown Pak2 in zygotes. The Pak2-siRNA pairs that we used were as follows: forward strand, siRNA#1, 5'-CCGUGUGCAGAGAGUGUUUTT- 3'; reverse-strand, 5'-AAACACUCUCUGCACACGGTT- 3'; siRNA#2, 5'-AAUCACAGUUUGAAACCUUTT- 3'; reverse-strand, 5'-AAGGUUUCAAACUGUGAUUGG- 3'). A nonspecific siRNA was used as a negative control: forward strand, 5'-UUCUCCGAACGUGUCACGUTT- 3'; reverse-strand, 5'-ACGUGACACGUUCGGAGAATT- 3').

Western immunoblotting analysis

A total of 70 two-cell embryos were lysed in 12 µL of Laemmli sample buffer (95 µL of loading buffer contained 5 µL of β-mercaptoethanol) and denatured at 100°C for 5 min. Protein samples were separated on 12% SDS-PAGE gels and transferred to PVDF membranes, blocked in 5% skim milk diluted with PBS-Tween 20 for 1 h, and then incubated with primary antibody overnight at 4°C (Pak2, 1:1000; GAPDH, 1:2000). After at least three washes, membranes were incubated with secondary antibody for 1 h at room temperature. After an additional three washes, the protein bands were detected with ECL Plus Western Blotting Detection System (GE Healthcare, Piscataway, NJ, USA).

Immunofluorescence

Early mouse embryos were fixed in 4% paraformaldehyde (30 min), permeabilized with 0.1% Triton X-100 (20 min), and then blocked in 1% BSA (60 min). Embryos were incubated with the primary antibodies anti-Pak2 (1:200) and anti-γH2AX (1:300), and anti-α-tubulin FITC-labeled antibodies (1:200) overnight at 4°C. After three washes, samples were incubated with secondary antibodies (1:100) for 1 hour at room temperature, and chromosomes were co-stained with PI (propidium iodide) or Hoechst 33342 for 10 min. Finally, samples were mounted on glass slides with antifade medium (Vectashield, Burlingame, CA) and then examined via laser-scanning confocal microscopy (LSCM, Leica SP8, Germany).

Measurement of ROS levels

Changes in intracellular ROS content were determined using CMH2DCFDA (Cat#: C6827; Life Technologies, Invitrogen TM). Two-cell embryos were incubated for 20 minutes at 37°C in M2 medium containing 5 mM CMH2DCFDA, and after three washes in M2 medium, embryos were placed on a confocal dish with a microdrop of medium. Images of embryonic fluorescence emission were captured under LSCM and analyzed using ImageJ software.

Statistical analysis

All experiments were repeated at least three times. Results are presented as means ± one standard deviation and analyzed by Student's *t* test. We employed SPSS20.0 for statistical analyses, and *P* < 0.05 was considered to be statistically significant.

Results

Subcellular localization of Pak2 in early embryos

The subcellular localization of Pak2 in zygotes and pronuclear-, two-cell-, four-cell-, eight-cell-, and blastocyst-stage embryos was investigated by immunofluorescence staining. Our results revealed that Pak2 signals were distributed throughout the entire embryo, with strong accumulation in the nucleus relative to the cytoplasm (Fig. 1). This particular pattern of Pak2 protein localization implied that it may function in early embryonic development.

Pak2 deletion compromises early developmental potential of embryos

We next aimed to illustrate the role of Pak2 in early embryonic development of mice. We microinjected specifically designed siRNAs into zygotes to investigate the function of Pak2 during embryonic development, and demonstrated that endogenous Pak2 proteins were reduced by approximately 80% or 90% as verified by western blotting analysis (Fig. 2A and 2B), since the siRNA#2 knockdown is more efficient, we chose it for subsequent experiments. There were no obvious differences in the morphology of the early mouse embryos between control and Pak2- KD groups (Fig. 2C). However, Pak2-deletion in embryos showed significant developmental delays or cytoplasmic fragmentation (red asterisk) in 4-cell-, 8-cell-, and blastocyst-stage embryos (Fig. 2C and 2D). The above observations suggested that the developmental potential of Pak2-KD embryos was impaired during in vitro culture.

Attenuated Pak2 adversely affects spindle assembly and chromosomal congression in mouse embryos

Since Pak2 has been implicated in regulating cytoskeleton dynamics [15], we herein explored the role of Pak2 in mitosis by treating early embryos with small interfering RNAs, and immunostaining them with an anti- α -tubulin antibody to show spindle morphology and counterstaining with PI to observe chromosomes. Most embryos in the control group showed complete bipolar spindles and well-aligned chromosomes (Fig. 3A). However, the spindles of embryos in the Pak2-KD group revealed multiple defects, such as multipolar and non-polar spindles (Fig. 3A). Moreover, the majority of embryos in the Pak2-KD group exhibited severe chromosomal aberrations (Fig. 3A). The incidence of embryonic spindle defects in the Pak2-KD group was significantly higher than that in the control group (Fig. 3B), as was the incidence of chromosomal aberrations (Fig. 3C).

Reduced Pak2 induces elevated ROS levels in mouse embryos

It was demonstrated that Pak2 inhibition induced reactive oxygen species overproduction and mitochondrial-JNK pathway activation [16]. As production of ROS is a major measure of mitochondrial function [17], we therefore asked whether Pak2 knockdown influences mitochondrial status in the early embryos of mice. To address this question, 2-cell embryos were collected from control and Pak2-KD groups and stained with CM-H₂DCFDA fluorescent dye for the assessment of ROS generation. Compared

with the control group, Pak2-KD treatment significantly increased the levels of ROS in 2-cell embryos (Fig. 4A) as determined by mean fluorescence intensity (Fig. 4B). These findings imply that Pak2 participates in the regulation of redox homeostasis in mouse preimplantation embryos.

Decreased Pak2 causes the DNA damage in early embryos

Histone H2AX phosphorylation (γ H2AX) can be triggered by DNA double-strand breaks (DSBs) [18]. When cellular DSBs occur, H2AX is rapidly phosphorylated in the damaged chromatin, and this activity is localized in nuclear foci [19]. In the present study, γ -H2AX-recognizing antibodies were used to quantify DSBs, and we found that γ -H2AX foci in Pak-KD embryos were significantly increased compared to control embryos (Fig. 5A and 5B). These results imply that Pak2 is essential for genomic integrity of the early embryo.

Pak2 knockdown enhances apoptosis of blastocyst in mouse embryos

Considering the elevated DNA damage in Pak2-KD embryos, we conducted TUNEL assays to evaluate the apoptotic status in blastocyst-stage embryos. As shown in Fig. 6A, TUNEL-positive nuclei were almost undetectable in control embryos, but we readily observed TUNEL-positive cells in PAK2-KD blastocysts (Fig. 6A, arrowheads). Quantitative analysis further revealed a significantly increased percentage of Pak2-KD embryos with TUNEL-positive nuclei relative to controls (Fig. 6B).

Discussion

Pak2, as a highly conserved serine/threonine protein kinase, plays a significant role in cell motility, survival, mitosis, and apoptosis [20]. In view of the subcellular localization pattern in early embryos of mice (Fig. 1), we speculated on its involvement in chromatin-related cellular events. To validate our hypothesis, early mouse embryos treated with a Pak2-specific siRNA exhibited a significant increase in abnormal spindle assembly and chromosomal aberrations that contributed to their abnormal early development (Fig. 3). Pak2 has been reported to regulate cytoskeletal dynamics in diverse cell types [9, 15, 21, 22]. In a recent study investigators noted that inactivation of Pak2 caused oxidative stress [23], and that Pak2 was highly activated when mammalian cells were treated with hydrogen peroxide [10]. In our study, ROS levels were dramatically increased in early embryos when Pak2 activity diminished (Fig. 4), which suggested mitochondrial dysfunction. ROS exert detrimental effects on DNA, RNA, proteins, lipids, and other cellular components—consequently disturbing multiple biologic events that include cellular metabolism, apoptosis, and senescence [24]. Excessive ROS is thus detrimental to normal embryonic development [25].

It was reported that ROS comprised an important factor causing intracellular DNA lesions [26]. In the present study, the accumulation of phosphorylated γ H2AX was observed in the Pak2-depleted embryos (Fig. 5), which indicated increased DNA damage; and continuous DNA lesions compromise the integrity of the genome [27], with genome stability critical for the survival, growth, and normal functioning of

organisms [28–30]. ROS induce DNA-base damage, and single- and double-stranded breaks (DSBs) [31]; with DSBs constituting the most dangerous type of DNA lesion in cells [32–35]. To maintain stability, then, the delay or arrest of the cell cycle must occur to allow sufficient time for effective DNA repair [36]. For example, Pak2 dysfunction-induced cell-cycle arrest at the G1 phase caused p27Kip1 accumulation [37]. Our results also revealed that Pak2 deletion resulted in delayed embryonic development and reduced blastocyst-formation rate (Fig. 2)

When the extent of DNA lesions exceed the capacity for recovery, mitosis does not occur and cells undergo apoptosis, senescence, or death [19]. Pak2 is a kinase that can be cleaved by caspase 3 during apoptosis and occupies a dual role in apoptosis: full-length Pak2 then inhibits pro-apoptotic events by phosphorylating Bad28, whereas proteolytic activation of Pak2 p34 leads to apoptosis [11, 37]. In adult endothelial cells, Pak2 loss leads to severe apoptosis and acute angiogenic defects, and the absence of Pak2 in the endothelium leads to early embryonic lethality due to flawed blood vessel formation [7]. Similarly, we noted an increased number of apoptotic blastocysts in the Pak2-KD group in the current study (Fig. 6). Collectively, these data signified that DNA lesions and apoptosis induced by Pak2 knockdown might contribute to a developmental delay in Pak2-KD embryos.

Conclusions

In summary, our data indicated that Pak2, as a regulator of spindle assembly and DNA repair, plays a role in the developmental competence of mouse preimplantation embryos.

Abbreviations

Pak2, p21-activated kinase 2; Pak2-KD, Pak2 knockdown; ROS: reactive oxygen species; PI: propidium iodide; TUNEL: terminal deoxynucleotidyl transferase dUTP nick-end labeling; BL: blastocyst.

Declarations

Ethics approval and consent to participate

Experiments were approved by the Third Affiliated Hospital of Guangzhou Medical University Animal Care and Use Committee and conducted in accordance with the guiding principles of the institution.

Consent for publication

Not applicable.

Availability of data and materials

The data that supports the findings of this study are available in the supplementary material of this article

All data generated or analyzed during this study are available from the corresponding author on reasonable request.

Conflicts of interests

The authors declare that they have no conflicts of interest.

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Authors' contributions

Juan Zeng and Xiaofang Sun conceived and designed the study. Juan Zeng, Nengqing Liu, Yinghong Yang, Yi Cheng, Yuanshuai Li, Xiaoxia Guo, Qian Luo, Lifen Zhu, Hongmei Guan, and Bing Song collected, arranged, and analyzed the data and wrote the manuscript. All authors read, revised, and finally approved the manuscript.

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References

1. Uzun A, Rodriguez-Orsorio N, Kaya A, Wang H, Parrish JJ, Ilyin VA, Memili E: Functional genomics of HMGN3a and SMARCA1 in early mammalian embryogenesis. *BMC Genomics* 2009, 10:183.
2. Grycmacher K, Boruszezwska D, Sinderewicz E, Kowalczyk-Zieba I, Staszkiwicz-Chodor J, Woclawek-Potocka I: Prostaglandin F2alpha (PGF2alpha) production possibility and its receptors expression in the early- and late-cleaved preimplantation bovine embryos. *BMC Vet Res* 2019, 15:203.
3. Wennekamp S, Mesecke S, Nedelec F, Hiiragi T: A self-organization framework for symmetry breaking in the mammalian embryo. *Nat Rev Mol Cell Biol* 2013, 14:452-459.
4. Fujishima A, Takahashi K, Goto M, Hirakawa T, Iwasawa T, Togashi K, Maeda E, Shirasawa H, Miura H, Sato W, et al: Live visualisation of electrolytes during mouse embryonic development using electrolyte indicators. *PLoS One* 2021, 16:e0246337.
5. Harder MJ, Hix J, Reeves WM, Veeman MT: Ciona brachyury proximal and distal enhancers have different FGF dose-response relationships. *PLoS Genet* 2021, 17:e1009305.
6. Arias-Romero LE, Chernoff J: A tale of two Paks. *Biol Cell* 2008, 100:97-108.
7. Radu M, Lyle K, Hoeflich KP, Villamar-Cruz O, Koeppen H, Chernoff J: p21-Activated Kinase 2 regulates endothelial development and function through the Bmk1/Erk5 Pathway. *Mol Cell Biol* 2015, 35:3990-4005.

8. Kumar R, Sanawar R, Li X, Li F: Structure, biochemistry, and biology of PAK kinases. *Gene* 2017, 605:20-31.
9. Phee H, Au-Yeung BB, Pryshchep O, O'Hagan KL, Fairbairn SG, Radu M, Kosoff R, Mollenauer M, Cheng D, Chernoff J, Weiss A: Pak2 is required for actin cytoskeleton remodeling, TCR signaling, and normal thymocyte development and maturation. *Elife* 2014, 3:e02270.
10. Huang J, Huang A, Poplawski A, DiPino F, Jr., Traugh JA, Ling J: PAK2 activated by Cdc42 and caspase 3 mediates different cellular responses to oxidative stress-induced apoptosis. *Biochim Biophys Acta Mol Cell Res* 2020, 1867:118645.
11. Campbell HK, Salvi AM, O'Brien T, Superfine R, DeMali KA: PAK2 links cell survival to mechanotransduction and metabolism. *J Cell Biol* 2019, 218:1958-1971.
12. Binder P, Wang S, Radu M, Zin M, Collins L, Khan S, Li Y, Sekeres K, Humphreys N, Swanton E, et al: Pak2 as a novel therapeutic target for cardioprotective endoplasmic reticulum stress response. *Circ Res* 2019, 124:696-711.
13. Hofmann C, Shepelev M, Chernoff J: The genetics of Pak. *J Cell Sci* 2004, 117:4343-4354.
14. Han L, Wang H, Li L, Li X, Ge J, Reiter RJ, Wang Q: Melatonin protects against maternal obesity-associated oxidative stress and meiotic defects in oocytes via the SIRT3-SOD2-dependent pathway. *J Pineal Res* 2017, 63.
15. Reddy PN, Radu M, Xu K, Wood J, Harris CE, Chernoff J, Williams DA: p21-activated kinase 2 regulates HSPC cytoskeleton, migration, and homing via CDC42 activation and interaction with beta-Pix. *Blood* 2016, 127:1967-1975.
16. Xing J, Wang Z, Xu H, Liu C, Wei Z, Zhao L, Ren L: Pak2 inhibition promotes resveratrol-mediated glioblastoma A172 cell apoptosis via modulating the AMPK-YAP signaling pathway. *J Cell Physiol* 2020, 235:6563-6573.
17. Ramalho-Santos J, Varum S, Amaral S, Mota PC, Sousa AP, Amaral A: Mitochondrial functionality in reproduction: from gonads and gametes to embryos and embryonic stem cells. *Hum Reprod Update* 2009, 15:553-572.
18. Bulat T, Keta O, Koricanac L, Zakula J, Petrovic I, Ristic-Fira A, Todorovic D: Radiation dose determines the method for quantification of DNA double strand breaks. *An Acad Bras Cienc* 2016, 88:127-136.
19. Vitale I, Galluzzi L, Castedo M, Kroemer G: Mitotic catastrophe: a mechanism for avoiding genomic instability. *Nat Rev Mol Cell Biol* 2011, 12:385-392.
20. Molli PR, Li DQ, Murray BW, Rayala SK, Kumar R: PAK signaling in oncogenesis. *Oncogene* 2009, 28:2545-2555.
21. Wang Y, Zeng C, Li J, Zhou Z, Ju X, Xia S, Li Y, Liu A, Teng H, Zhang K, et al: PAK2 haploinsufficiency results in synaptic cytoskeleton impairment and autism-related behavior. *Cell Rep* 2018, 24:2029-2041.
22. Kosoff RE, Aslan JE, Kostyak JC, Dulaimi E, Chow HY, Prudnikova TY, Radu M, Kunapuli SP, McCarty OJ, Chernoff J: Pak2 restrains endomitosis during megakaryopoiesis and alters cytoskeleton

- organization. *Blood* 2015, 125:2995-3005.
23. Wang S, Bian W, Zhen J, Zhao L, Chen W: Melatonin-mediated pak2 activation reduces cardiomyocyte death through suppressing hypoxia reoxygenation injury-induced endoplasmic reticulum stress. *J Cardiovasc Pharmacol* 2019, 74:20-29.
 24. Dumollard R, Carroll J, Duchen MR, Campbell K, Swann K: Mitochondrial function and redox state in mammalian embryos. *Semin Cell Dev Biol* 2009, 20:346-353.
 25. Bedaiwy MA, Falcone T, Mohamed MS, Aleem AA, Sharma RK, Worley SE, Thornton J, Agarwal A: Differential growth of human embryos in vitro: role of reactive oxygen species. *Fertil Steril* 2004, 82:593-600.
 26. Srinivas US, Tan BWQ, Vellayappan BA, Jeyasekharan AD: ROS and the DNA damage response in cancer. *Redox Biol* 2019, 25:101084.
 27. Palou R, Palou G, Quintana DG: A role for the spindle assembly checkpoint in the DNA damage response. *Curr Genet* 2017, 63:275-280.
 28. van Gent DC, Hoeijmakers JH, Kanaar R: Chromosomal stability and the DNA double-stranded break connection. *Nat Rev Genet* 2001, 2:196-206.
 29. Maciejowski J, de Lange T: Telomeres in cancer: tumour suppression and genome instability. *Nat Rev Mol Cell Biol* 2017, 18:175-186.
 30. Niedernhofer LJ, Gurkar AU, Wang Y, Vijg J, Hoeijmakers JHJ, Robbins PD: Nuclear genomic instability and aging. *Annu Rev Biochem* 2018, 87:295-322.
 31. Shoji T, Masumoto S, Moriichi N, Ohtake Y, Kanda T: Administration of apple polyphenol supplements for skin conditions in healthy women: a randomized, double-blind, placebo-controlled clinical trial. *Nutrients* 2020, 12.
 32. Burma S, Chen BP, Chen DJ: Role of non-homologous end joining (NHEJ) in maintaining genomic integrity. *DNA Repair (Amst)* 2006, 5:1042-1048.
 33. Bhattacharjee S, Nandi S: Choices have consequences: the nexus between DNA repair pathways and genomic instability in cancer. *Clin Transl Med* 2016, 5:45.
 34. Sibanda BL, Chirgadze DY, Blundell TL: Crystal structure of DNA-PKcs reveals a large open-ring cradle comprised of HEAT repeats. *Nature* 2010, 463:118-121.
 35. Symington LS, Gautier J: Double-strand break end resection and repair pathway choice. *Annu Rev Genet* 2011, 45:247-271.
 36. Imreh G, Norberg HV, Imreh S, Zhivotovsky B: Chromosomal breaks during mitotic catastrophe trigger gammaH2AX-ATM-p53-mediated apoptosis. *J Cell Sci* 2016, 129:1950.
 37. Koo KH, Kwon H: MicroRNA miR-4779 suppresses tumor growth by inducing apoptosis and cell cycle arrest through direct targeting of PAK2 and CCND3. *Cell Death Dis* 2018, 9:77.

Figures

Figure 1

Subcellular localization of Pak2 in early mouse embryos. Zygotes; pronuclear-stage, 2-cell, 4-cell, and 8-cell embryos; and blastocysts were labeled with a Pak2 antibody (green) and counterstained with propidium iodide (PI; red) to visualize DNA. Arrowheads indicate the accumulated Pak2 signal. Scale bars, 25 μ m; Pak2, p21-activated kinase 2.

Figure 2

Pak2 knockdown disrupts the developmental potential of early embryos. In vitro culture of zygotes microinjected with Pak2-siRNA and analysis of developmental progression. A, With GAPDH as a loading control, the efficiency of Pak2 knockdown was confirmed by western blot analysis. B, Band intensity was calculated using ImageJ software (NIH, USA), and the ratio of Pak2/GAPDH expression was normalized and the values are indicated. C, Representative bright-field images of 2-cell embryos, 4-cell embryos, 8-cell embryos, and blastocysts from control and Pak2-KD groups. Black asterisks indicate examples of normal morphology; red asterisks indicate examples of abnormal morphology (scale bars, 100 μ m). D, Quantification of normal embryos. Total number of cells analyzed: 2-cell embryos, n = 118 for controls and n = 127 for Pak2-KD; 4-cell embryos, n = 103 for controls and n = 82 for Pak2-KD; 8-cell embryos, n = 89 for controls and n = 33 for Pak2-KD; blastocysts, n = 63 for controls and n = 18 for Pak2-KD. Data are expressed as the mean $\pm$ SD from three independent experiments. *Significantly different ($p < 0.05$). Pak2, p21-activated kinase 2; SD, standard deviation; Pak2-KD, Pak2 knockdown; BL, blastocyst.

Figure 3

Pak2 knockdown affects spindle morphology and chromosomal alignment in early mouse embryos. (A) Early mouse embryos at metaphase were stained with anti- α -tubulin (green) and counterstained with propidium iodide (PI) to visualize chromosomes (red). In contrast to the control group, embryos in the Pak2-KD group showed a variety of defects, including multipolar and nonpolar spindles. The chromosomes in embryos of the Pak2-KD group were severely misaligned (bar = 25 μ m). (B) A significantly higher proportion of embryos in the Pak2-KD group exhibited spindle defects relative to the control group ($37.0 \pm 3.89\%$, $n=64$ vs. $17.9 \pm 1.24\%$, $n = 56$; respectively). (C) The incidence of chromosomal misalignment was also higher in embryos in the Pak2-KD treatment group compared to the control group ($38.8 \pm 11.77\%$, $n=67$ vs. $19.5 \pm 4.62\%$, $n = 73$; respectively). Data are expressed as the mean $\pm$ SD from three independent experiments. *Significantly different ($p < 0.05$). Pak2, p21-activated kinase 2; SD, standard deviation; Pak2-KD, Pak2 knockdown.

Figure 4

Pak2 knockdown elevates ROS levels in embryos. (A) Representative images of CM-H2DCFDA fluorescence (green) in 2-cell embryos from control and Pak2-KD groups (n = 24 for control group and n = 28 for Pak2-KD group; scale bars, 80 μ m). (B) Quantitative analysis of fluorescence intensity in control and Pak2-KD embryos (7.9 ± 1.39 , n = 24 vs. 15.8 ± 1.41 , n = 28, respectively). Data are expressed as the mean $\pm$ SD from three independent experiments. *Significantly different (p < 0.05). Pak2, p21-activated kinase 2; Pak2-KD, Pak2 knockdown; SD, standard deviation; ROS, reactive oxygen species.

Figure 5

Pak2 knockdown results in the accumulation of γ H2AX during preimplantation embryonic development. (A) Two-cell embryos were immunostained with an anti- γ H2AX antibody to detect DNA damage (green) and counterstained with PI for DNA (red). Arrowheads indicate the increased DNA damage in embryos (scale bars, 25 μ m). (B) Quantification of the numbers of γ H2AX foci in control and Pak2-KD group embryos (94.3 ± 2.59 , n = 32 vs. 234.0 ± 4.31 , n = 37; respectively). Data are expressed as the mean $\pm$ SD from three independent experiments. *Significantly different ($p < 0.05$). Pak2, p21-activated kinase 2; Pak2-KD, Pak2 knockdown; SD, standard deviation; PI, propidium iodide.

Figure 6

Pak2-KD induces apoptosis of early mouse embryos. (A) TUNEL analysis of control and Pak2-KD embryos. Embryos were labeled with Hoechst 33342 (blue) for DNA and TUNEL used to assess fragmented DNA (red). Arrowheads point to apoptotic cells in blastocysts (scale bars, 25 μ m). (B) Quantification of control and Pak2-KD group blastocysts with TUNEL- positive nuclei (8.9 ± 0.32 , n = 50 vs. 32.8 ± 1.25 , n = 37; respectively). Data are expressed as mean percentage $\pm$ SD from three independent experiments. *Significantly different ($p < 0.05$). Pak2, p21-activated kinase 2; SD, standard deviation; Pak2-KD, Pak2 knockdown; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling.