Letter to the Editor

The effect of phosphoinositide 3-kinase inhibitors on programmed nuclear degradation in *Tetrahymena* and fate of surviving nuclei

Cell Death and Differentiation (2004) 11, 1146–1149. doi:10.1038/sj.cdd.4401473

Published online 16 July 2004

Dear Editor,

Programmed nuclear degradation (PND) and programmed cell death (PCD) are essential for tissue development and homeostasis in multicellular eucaryotes. PND may be a primitive form of PCD, in which nuclear degradation is uncoupled from plasma membrane events associated with cell death.^{1,2} PND occurs in a subset of differentiating mammalian cells (for example, lens fibers), and like PCD is activated by caspases.^{3,4} PND and PCD are found in ancient lineages, such as the ciliated protozoan, *Tetrahymena thermophila*,^{5,6} and fundamental underlying mechanisms are conserved. Fas ligand, for example, activates PCD in higher eucaryotes and *Tetrahymena*,⁷ and caspase inhibitors effectively block *Tetrahymena* PND.^{2,8} *Tetrahymena* PND is associated with chromatin condensation, formation of TUNEL-positive nuclei, and DNA cleavage into nucleosome-sized fragments.^{6,8} Autophagy ensues as apoptotic nuclei fused with lysosomes.⁹

PND is an integral part of development in ciliates, which harbor genetically related, but functionally distinct micro- and macronuclei within the same cytoplasm.¹⁰ The diploid micronucleus is transcriptionally silent and serves as the reservoir of genetic material that is transmitted during conjugation. Four haploid pronuclei are generated during meiosis in *T. thermophila*, three of which undergo PND (Figure 1a, upper panel). The remaining nucleus divides to generate two genetically identical pronuclei. Pronuclei are reciprocally exchanged and fuse to form a diploid 'zygotic' micronucleus in each progeny cell. The micronucleus undergoes two post-meiotic divisions, after which two of the four progeny micronuclei differentiate into polyploid, transcriptionally active macronuclei. Hallmarks of macronuclear development include site-specific chromosome fragmentation, DNA rearrangement, selective gene amplification, and extensive chromatin remodeling.¹⁰ The commitment to new macronuclear anlagen formation triggers the destruction of the old parental macronucleus by an apoptotic-like autophagic mechanism.^{6,8} Thus, PND, nuclear division, and nuclear differentiation are temporally regulated during development, thereby determining the fate of different nuclei that share a common cytoplasm.

Since metazoan PCD and *Tetrahymena* PND utilize the same downstream effectors (caspases),^{2,3} these processes may respond to similar signaling molecules. Phosphoinositide 3-kinase (PI 3-kinase) catalyzes the formation of PIP3, and

induces autophagic PCD in mammals.¹¹ PI 3-kinase inhibitors block PCD in mammals; however, a role for this pathway in mammalian PND has yet to be reported. Whereas PIP3 has not yet been identified in *Tetrahymena*, inositol phospholipid signaling is well documented,¹² and there is compelling evidence for a PI 3-kinase gene family in the *T. thermophila* genome.¹³

To assess the potential role of PI 3-kinase in PND, we examined the effect of well-characterized PI 3-kinase inhibitors on *Tetrahymena* development. Mating cultures were treated with the PI 3-kinase inhibitors, wortmannin (Calbiochem), 3-methyladenine (3-MA, Sigma), or LY294002 (Calbiochem) at various times during development, and assayed for nuclear acidification and degradation by the pH-sensitive apofluor-staining method.⁹ Acidified, apoptotic nuclei stain orange/red, whereas healthy, nonacidic nuclei are blue/green (Figure 1a, top panel). Acidification of the (old) parental macronucleus was inhibited when 250 nM wortmannin was added to mating cells prior to pronuclear exchange (Figure 1a, bottom panel). In addition to blocking macronuclear acidification, additional staining structures were detected. The staining compartments correspond to nuclei, since they reacted with the DNA-specific dye DAPI alone (Figure 1b, lower right panel). The sizes and abundance of DAPI/Apofluor staining nuclei suggest that additional micronuclei and macronuclei are generated when PND is blocked.

Since high concentrations of wortmannin inhibit myosin light chain kinase (MLCK) in other organisms,¹⁴ we assayed macronuclear acidification and nuclear overproliferation in cells treated with the highly specific MLCK inhibitor ML-7. ML-7 (1–10 μ M) had no effect on nuclear acidification or proliferation (Figure 1b). Higher concentrations (50–100 μ M) were toxic and therefore uninformative. Conversely, when we reduced the wortmannin concentration from 250 to 25 nM, a comparable block in macronuclear acidification was observed. A similar block in macronuclear acidification was seen with two additional PI 3-kinase inhibitors, 3-MA (10 mM) and LY294002 (100 μ M), at concentrations comparable to those used with cultured mammalian cells (Figure 1b). 3-MA and to a lesser extent LY294002 also induced nuclear overproliferation. Wortmannin and LY294002 bind to the catalytic subunit of PI 3-kinase, while 3-MA interacts with the regulatory subunit.¹⁵ The consistent results obtained with three different PI 3-kinase inhibitors argue that PI 3-kinase

Figure 1 (a) Top: Simplified schematic diagram of normal development in *T. thermophila*. See text for details. Micronuclei undergo meiosis, producing four haploid meiotic micronuclei (4–5 h), three (shown in red) of which acidify and subsequently undergo PND. The old parental macronucleus (shown in red) is acidified (8–15 h) and degraded. The micrograph at the end shows the apofluor staining pattern of micro- and macronuclei from representative cells harvested at 15 h. The acidified parental macronucleus stains red/orange, while new micro- and macronuclei stain blue/green. Bottom: the effect of wortmannin (250 nM) (Calbiochem) added at 5 h post-mating on nuclear acidification and PND. Comparable results were observed with 25 and 50 nM wortmannin (see panel (b)). The three pronuclei that are not involved in exchange (shown in green) fail to undergo PND, but instead exhibit pronuclear retention (PNR). The micrograph at the end is representative of the subpopulation of wortmannin-treated cells that contain extra micro- and macronuclei (15 h post-mating). For apofluor staining, a 0.1-ml sample of conjugating cells was concurrently stained with 0.001% acridine orange and 5 μg/ml Hoechst 33342 (Sigma). After mixing briefly, the stained cells were observed immediately with fluorescence microscopy using filters for blue fluorescence. (b) Effect of PI 3-kinase inhibitors (wortmannin, 3-methyladenine (Sigma), and LY294002 (Sigma)) and the MLCK inhibitor ML-7 (10 μM) (Calbiochem) on macronuclear acidification and nuclear overproliferation. Inhibitors were added at 5 h post-mating and cells were harvested for analysis at 15 h. Dye staining: Apofluor or diamidinophenolindole (DAPI) (Sigma) (lower right panel). (c) Analysis of radiolabeled phospholipids. Vegetative cells were incubated with [³²P]-orthophosphate for 24 h and starved for 18 h prior to inducing pair formation. Lipids were extracted at various times during development and resolved by thin-layer chromatography¹⁶

activates programmed degradation of the parental macronucleus and unexchanged pronuclei.

In an effort to visualize the PI 3-kinase substrate (PIP2) and product (PIP3) directly, mating *Tetrahymena* cells were radiolabeled with [³²P]-orthophosphate, and extracted lipids were separated by thin-layer chromatography.¹⁶ No labeled phospholipids were detected when the labeling period was restricted to starved mating cells (data not shown). However, when growing cells were labeled and then starved in preparation for mating, robust phospholipid labeling was observed throughout development. Although no definitive PIP3 signal was evident, its precursor, PIP2, was readily detected (Figure 1c). These results are consistent with data from mammalian cells, where PIP2 and PIP3 represent 5 and

0.005% of the total phosphatidylinositol pool.¹⁷ We conclude that the precursor for PIP3 is generated in actively growing cells and maintained throughout development. In combination with strong bioinformatic evidence for a PI 3-kinase gene family and PTEN phosphatase gene in *Tetrahymena* (PTEN converts PIP3 back into PIP2) and the inhibitor studies described above, these results provide further evidence for a functional PI 3-kinase pathway in *Tetrahymena*.

Since PI 3-kinases regulate a diverse set of pathways in higher eucaryotes, we examined the effect of adding inhibitors at different times during development. The highest percentage of conjugants with extra nuclei occurred when wortmannin was added immediately after mixing cells of opposite mating type (Figure 2a, *T* = 0 h; criteria: mating pairs with

Figure 2 (a) Early treatment with wortmannin enhances the production of extra micro- and macronuclei. Wortmannin (50 or 250 nM) was added at various times after mating (h) (U: mock-treated cells). Cells were harvested at 15 h and the number of mating cells containing extra nuclei was determined by apofluor staining. The mean and standard deviation for three independent experiments are shown (200 cells counted for each time point in each experiment). Note that 250 nM wortmannin blocked pair formation when added at $T = 0$ h, as did the high final concentration of carrier (1% dimethylsulfoxide (DMSO)). Control experiments: DMSO alone (0.1–2 %) added at 5 h or later did not block old macronuclear acidification or result in the production of extra nuclei. (b) Nuclear overproliferation dose response curve. Wortmannin was added at $T = 5$ h and cells were harvested at 15 h. (c) The extra macronuclei in wortmannin-treated cells actively synthesize DNA. Wortmannin (250 nM) was added at 5 h after mating. BrdU (100 μ M) was added 30 min later and cells were harvested at 18 h for immunofluorescence detection with anti-BrdU antibodies (Amersham): (1) DAPI staining, (2) BrdU labeling, (3) light, (4) DAPI/light/BrdU merge. (d) Proposed model for the role of PI 3-kinase in micro- and macronuclear acidification and degradation during *Tetrahymena* development (see text for details). The wild-type developmental stage at the time of early and late addition of 3-MA or wortmannin in denoted in Figure 1, panel (a) (*1, early; *2, late)

more than 10 nuclei – 4 micronuclei, 4 new macronuclear anlagen, and 2 parental macronuclei). The frequency of extra-nucleate cells decreased when wortmannin was added at later time points during development (Figure 2a, 5–9 h; 12–18 h, data not shown). The temporal loss in drug-induced nuclear ‘overproliferation’ correlated with the time period for degradation of unexchanged pronuclei. As expected, the effect of wortmannin on nuclear overproliferation was dose dependent (Figure 2b). However, the block in nuclear acidification was manifested at lower inhibitor concentrations (wortmannin, 3-MA and LY294002, data not shown), suggesting that the signaling pathway that regulates nuclear acidification and nuclear proliferation diverges.

The involvement of the PI 3-kinase pathway in programmed pronuclear degradation is supported by the following obser-

vations. (1) The accumulation of extra ‘micronuclei’ was maximal when inhibitors were added prior to degradation of unexchanged pronuclei. (2) The parental macronucleus was not acidified or degraded in drug-treated cells, so the additional nuclei cannot be apoptotic fragments of the parental macronucleus. (3) Since fewer extra nuclei were observed when inhibitors were added later during macronuclear anlagen development, they are not likely to be derived from fragmentation of the developing macronucleus or formation of macronuclear extrusion bodies.¹⁰ (4) The maximal number of total apofluor-staining nuclei detected in mating pairs (21) approached the theoretical maximum of 22, predicted if all pronuclei escape PND.

To determine whether the additional nuclei actively synthesize DNA, cells were exposed to bromodeoxyuridine (BrdU,

Sigma) 30 min after the addition of wortmannin or 3-MA, and subjected to immunolabeling with anti-BrdU antibodies (Amersham). When control cells were labeled with BrdU at 5.5 h post-mating and harvested at 18 h, only macronuclear-specific labeling was detected (Figure 2c). This level of sensitivity allowed us to ask whether surviving nuclei undergo endo-replication, a hallmark of macronuclear development. Multiple BrdU-positive nuclei were detected in wortmannin and 3-MA-treated cells (Figure 2c and data not shown). Thus, a subset of surviving pronuclei not only divide, but also replicate their DNA to a level that is qualitatively similar to that of a differentiated macronucleus.

Tetrahymena exhibits a remarkable degree of plasticity during development. Matings between normal and 'functionally amiconucleate' strains result in nonreciprocal pronuclear exchange. Alternative developmental programs can be induced in wild-type crosses by microtubule inhibitors or osmotic shock.¹⁰ In these cases, the unfused pronucleus undergoes a single endo-replication cycle to generate a new diploid germline micronucleus, or alternatively differentiates into a macronucleus. Although deviating from the normal developmental program, the correct number of micro- and macronuclei are produced. In contrast, all the three PI 3-kinase inhibitors induced nuclear overproliferation. Thus, nuclei that escape PND are refractory to 'counting mechanisms' that regulate the number of micro- and macronuclei per cell. The abundance of extra nuclei varied considerably in the respective mating partners, indicating that the final nuclear makeup is determined in a cell autonomous manner.

These experiments provide a new insight into signaling pathways that operate during *Tetrahymena* development and reveal the following: (1) The PI 3-kinase pathway is involved in PND in *T. thermophila*, and is required for acidification and degradation of both non-exchanged pronuclei and the (old) parental macronucleus. (2) Pronuclei that are normally degraded can be re-programmed to differentiate into micro- and macronuclei when PND is blocked. Figure 2d shows a model for the role of PI 3-kinase in micro- and macronuclear PND in *T. thermophila*. We propose that PI 3-kinase activates PND of three haploid pronuclei early in development and degradation of the parental macronucleus at a later time. Inhibition of PI 3-kinase early in development leads to pronuclear survival, replication, and differentiation. Late-stage inhibition blocks degradation of the parental macronucleus alone, as pronuclei have already been eliminated.

Although these two types of nuclei reside in the same cytoplasm, they undergo PND at different times during development. Uncoupling these events may provide a safety net to assure that progeny that fail to produce a functional macronucleus retain their parental copy. Since 'maternal' gene expression drives many of the events in macronuclear development, premature destruction of the parental macronucleus could affect macronuclear differentiation, including site-specific chromosome fragmentation, *de novo* telomere addition, and DNA rearrangement. Whereas PI 3-kinase regulates PND of pronuclei and parental macronuclei, the signaling pathway must activate different downstream effectors to achieve the appropriate temporal regulation. The identification of downstream molecules in *Tetrahymena* PND could provide insight into developmental programs that distinguish PND from PCD in other eucaryotes.

Acknowledgements

We thank Dorothy Shippen for careful reading of the manuscript. This work was supported by NIH grant GM53572 to GMK.

JS Yakisich¹ and GM Kapler^{*1}

¹ Department of Medical Biochemistry and Genetics, Texas A&M University System Health Science Center, College Station, TX 77843-1114, USA

^{*} Corresponding author: GM Kapler, TAMUHSC, Reynolds 446, College Station, TX 77843-1114, USA; E-mail: gkapler@tamu.edu

1. Davis MC *et al.* (1992) *Dev. Biol.* 154: 419–432
2. Kobayashi T and Endoh H (2003) *Cell Death Differ* 10: 634–640
3. Ishizaki Y, Jacobson MD and Raff MC (1998) *Cell Biol.* 140: 153–258
4. Grutter MG (2000) *Curr. Opin. Struct. Biol.* 10: 648–655
5. Christensen ST *et al.* (1995) *Cell Death Differ.* 2: 301–308
6. Mpoke S and Wolfe J (1996) *Exp. Cell Res.* 225: 357–365
7. Jaso-Friedmann L, Leary JH and Evans DL (2000) *Exp. Parasitol.* 96: 75–88
8. Lu E and Wolfe J (2001) *Cell Death Differ.* 8: 289–297
9. Mpoke SS and Wolfe J (1997) *J. Histochem. Cytochem.* 45: 675–683
10. Karrer KM (2000) In *Methods in Cellular Biology* Vol 62, Asai DJ and Forney JD (eds) (San Diego: Academic Press) pp. 128–189
11. Petiot A *et al.* (2000) *J. Biol. Chem.* 275: 992–998
12. Kovacs P and Pallinger E (2003) *Cell Biochem. Funct.* 21: 169–175
13. <http://www.tigr.org/tdb/e2k1/ttg/>
14. West KA, Castillo SS and Dennis PA (2002) *Drug Resist. Updates* 5: 234–248
15. Walker EH *et al.* (2000) *Mol. Cell* 6: 909–919
16. Serunian LA, Auger KR and Cantley LC (1991) *Methods Enzymol.* 198: 78–87
17. Leslie NR and Downes CP (2002) *Cell Signal.* 4: 285–295