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Letter to the Editor

Histological analyses of normally grown, fertile Apaf1-deficient mice

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Dear Editor,

Apaf1, the mammalian homologue of *Caenorhabditis elegans* CED-4, is the critical component in the mitochondria-dependent pathway of apoptosis, and its deficiency has severe impacts on the development of central nervous system (CNS).[1–4] Most Apaf1-deficient mice die perinatally, due to craniofacial abnormalities, with hyperproliferation and accumulation of neural progenitor cells. Besides in apoptosis of developing neurons, Apaf1-mediated apoptosis plays roles such as in apoptosis of thymocytes and embryonic fibroblasts. Despite the hitherto described critical roles of Apaf1 in development, a small percentage of the mutants, however, are viable and come to maturity.[1,3] In addition, Honarpour *et al.* have reported that the adult male *Apaf1* knockout mice exhibited the absence of spermatogenesis, resulting in male infertility, whereas the female mutants were fertile.[3] We recently identified a number of Apaf1-deficient mice that normally grew up into adulthood. To our surprise, the adult mutant male mice possessed the ability to reproduce offspring. We, therefore, examined the histological phenotypes of organs, whose development and function may largely depend upon apoptosis, in the mutant mice.

Apaf1 heterozygous mutant mice were maintained in our facility and were backcrossed into C57BL/6 background more than five times (continual crossing). To generate homozygous mutant mice, intercross of the heterozygotes was performed. As we identified a male mutant mouse, which survived to adulthood with no apparent abnormality, we crossed the mouse with a female heterozygote. In the first litter born to the breeding, five out of 10 pups were homozygous for *Apaf1* mutation (Figure 1a). Three animals of the mutants showed no apparent craniofacial abnormalities and survived to adulthood. In the second litter born to the breeding, four out of 10 were homozygous for *Apaf1* mutation. In 60 pups born to female heterozygote and male homozygote crossing, 27 (45.0%) *Apaf1*$^{-/-}$ mice were born alive and 12 out of the 27 survived more than 8 weeks; five out of the 12 had hydrocephalus revealed by histological analysis. Interestingly, these rates were different in heterozygote intercrosses. In 45 pups born to heterozygous intercrosses, only five (11.1%) *Apaf1*$^{-/-}$ mice were born alive. However, four of the five survived more than 8 weeks; two of the four with hydrocephalus. Most of the mutant survivors, either male or female, without apparent behavioral abnormalities were fertile (see below). During their development, the mutants were obviously smaller in both body length (from muzzle to anus) and weight (Figure 1b). First opening of eyelids delayed in the mutants by a day as compared to the heterozygous littermates. Of these mutants, the smallest one (female) died at 22 days old, whereas the remainder (one male and two females) survived to adulthood. The adult mutants (from the first and the second litters) were indistinguishable in appearance from the heterozygous littermates, except the difference in body size (data not shown). Some male mutants (for instance, progeny 4 and 5 in Figure 1a) succeeded in impregnating the heterozygous females within 7 days of mating. These results revealed that Apaf1 is not necessarily essential for normal development, and also that male infertility was not the inevitable phenotype in the *Apaf1* knockout mice.

Histological analyses were carried out for the surviving adult homozygous and heterozygous mutants (8 weeks of age) in the first litter (Figure 1c–r). Progeny 4 and 5 in Figure 1a had no macro anatomical abnormality in brains, lungs, livers, jejuna, kidneys, and testes (data not shown). Although the spleens of the mutant mice were slightly larger as compared to those of the heterozygous littermates (0.1025 ± 0.035 *versus* 0.0675 ± 0.015 g), the mutant mice showed no apparent splenomegaly or lymph adenopathy. Histological analyses also revealed that the development of these organs was apparently normal and indistinguishable from ones of heterozygous control (Figure 1c–h, m, n, p, and q). Organ architectures, such as lobular structure in the liver or laminar structure with villi in the intestine, were also normal in the mutant mice. In the undamaged stomach and intestine, spontaneous apoptosis occurs in the epithelial cells for homeostatic turnover of the epithelium. TUNEL assay revealed that the occurrence of apoptotic cells in the epithelia of the mutant stomachs and jejuna was comparable to that in the heterozygous littermates (compare Figure 1i and j or k and l, respectively). Seminiferous tubular structure in the mutant testis was also normal and normal spermatogenesis was observed in the mutant seminiferous tubules (Figure 1m and n). Macro anatomical and histological analyses revealed no apparent abnormality in the brain of the progeny 5. On the other hand, striking expansion of lateral ventricles (hydrocephalus) was observed in the brain of the progeny 6 (Figure 1r), which showed marked behavioral abnormalities such as locomotor hyperactivity and whirling on a point. Hydrocephalus in the postnatal *Apaf1* knockout mice has also been described previously.[1,3] Since the progeny 6 had no other abnormalities, particularly in the spermatogenesis (Figure 1o), the failure of progeny 6 to impregnate females might result from neurological problem accompanied by the

serious behavioral abnormalities rather than from its sexual function. These results reiterated that Apaf1 is not an absolute requirement for normal development.

As most of the *Apaf1*-mutant survivors were smaller than wild-type or heterozygous littermates, we measured the serum levels of growth hormone and leptin. Unexpectedly,

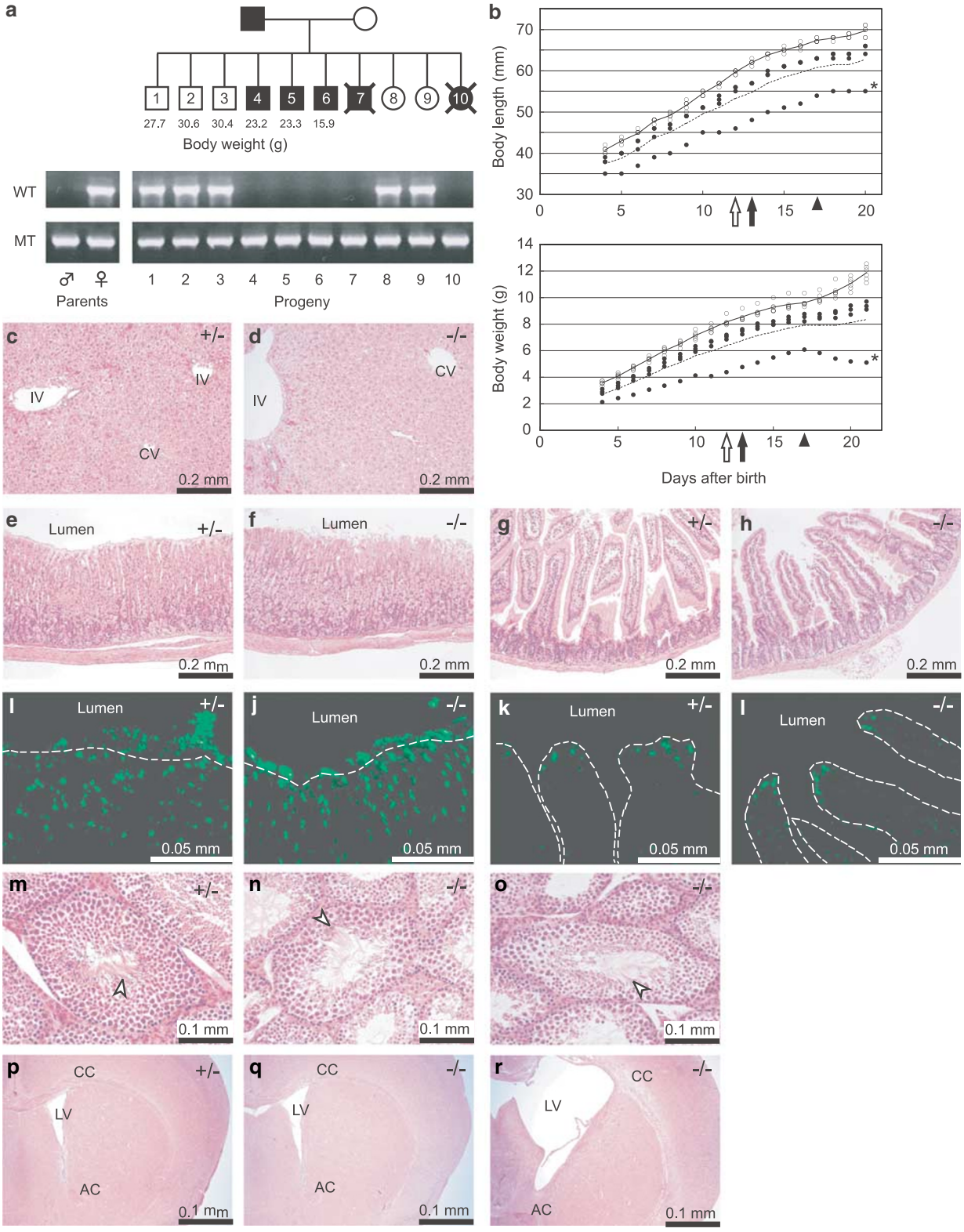

Figure 1

Apaf1$^{-/-}$ mice showed higher growth hormone and lower leptin than wild-type mice of the same sex (Table 1). Neither pituitary tumors nor apparent hypothalamus abnormalities were seen in these mice by gross histological analyses (data not shown). Although not formally excluded, involvement of inappropriate production of growth- or appetite-regulating hormones thus seems unlikely as a cause of runting of the mice. It is rather assumed that a compensatory mechanism is working, by which the mutant mice are trying to catch up in their growth by secreting more growth hormones and taking up more calories. Although histologically normal, the function of epithelial cells in the digestive organs, hepatocytes, adipocytes, and/or myocytes of the mutant mice may be abnormal due, for instance, to senescence of the cells as a result of Apaf1 deficiency, so that normal growth of the body is disallowed. Alternatively, although seemingly normal eye and ear development was observed in the *Apaf1*$^{-/-}$ mice and the mice appeared to respond to light and sound, Apaf1 deficiency may have resulted in malfunctioning of the sensory (and possibly, motor) neuron network and hence the mice have behavioral disadvantages for milk and food. Further metabolic and/or neurological analyses await sufficient numbers of *Apaf1*$^{-/-}$ mice to be examined.

Despite the ubiquitous tissue distribution of *Apaf1* expression during embryogenesis,[5] Apaf1 deficiency showed exclusive impact on the developmental apoptosis of the neurons. Except for that, Apaf1-dependent apoptosis appears to play essential roles in none of sculping, deleting structures, and adjusting cell numbers during development.[1–3] In the current study, we reported *Apaf1*$^{-/-}$ mice with no developmental abnormality in the brain as well as in other organs including testis. In our first report on the generation and analyses of Apaf1-deficient mice, mice were in the mixture of the genetic background of 129 and ICR strains. As we have empirically felt that the more times the mice are crossed to C57BL/6 background, the less frequent and the less drastic the developmental brain abnormality becomes, we assume that a genetic input(s) has influence on the developmental apoptosis in the brain. Given the significant contribution of Apaf1 to apoptosis induced by various apoptotic stimuli, including anticancer drugs or irradiation,[1,2] Apaf1-dependent apoptosis may be a way of deleting damaged cells in emergency. As such, much less cells that are damaged or unwanted during the brain development in C57BL/6 genetic background may be produced. Of note, neuroepithelial cells isolated from normally looking *Apaf1*$^{-/-}$ embryos are more resistant to genotoxic stimuli than wild-type cells (manuscript in preparation), demonstrating that Apaf1 is still required for apoptosis induced by some apoptotic stimuli in the cells of the current genetic background.

Apaf1 has been the sole adaptor molecule that mediates caspase activation upon mitochondria damage. The current study clearly demonstrated the presence of physiological cell death independent of Apaf1. As have been reported, CNS phenotypes observed among Casp3-deficient,[6,7] Casp9-deficient,[8,9] Apaf1-deficient,[1,2] and cytochrome *c*-deficient,[10] or mutated cytochrome *c*-knockin[11] mice are essentially the same. Nevertheless, slight variation in the phenotypes and the existence of animals with histologically normal brain among these mutants implicate redundant and compensatory apoptotic pathways. For example, autoprocessing of Casp9[12] or TNF-α-mediated apoptosis[13] may circumvent the Apaf1 requirement. Marsden *et al.*[14] have actually reported apoptosome-independent caspase activation. In addition, necrotic cell death may contribute to removal of unnecessary cells.[15,16] Presumably, the dependency on Apaf1-mediated apoptosis as well as on the cell death machinery described above may vary in distinct developmental stages, in distinct

Table 1 Serum growth hormone and leptin levels in the wild-type and *Apaf1*$^{-/-}$ mice[a]

	Male				Female			
	Apaf1$^{+/+}$		*Apaf1*$^{-/-}$		*Apaf1*$^{+/+}$		*Apaf1*$^{-/-}$	
	#1	#2	#1	#2	#1	#2	#1	#2
Growth hormone (ng/ml)	384.5	296.2	1803.2	804.4	15.6	16.5	23.2	77.9
Leptin (ng/ml)	3.64	2.58	1.92	1.74	2.42	3.68	1.10	1.28
Body weight (g)	16.88	16.71	8.43	11.25	16.83	13.99	8.52	6.54

[a]Four mice of *Apaf1*$^{+/+}$ or *Apaf1*$^{-/-}$ genotype (two males and two females) were killed at 4 weeks of age and sera were collected. Serum growth hormone and leptin concentrations were measured using ACTIVE Mouse/Rat growth hormone ELISA kit (Diagnostic Systems Laboratories, Inc., Webster, TX, USA) and Quantikine mouse leptin immunoassay kit (R & D Systems, Inc., Minneapolis, MN, USA), respectively

Figure 1 Developmental and histological comparison between surviving *Apaf1* knockout mice and their heterozygous littermates. (**a**) Genotyping of parent mice and their litter. DNA extracted from the tail of each individual was analyzed for the *Apaf1* wild-type allele (WT) or mutant allele (MT). Squares, male; circles, female. Open symbols, heterozygotes; closed symbols, homozygotes. The body weights of progeny 1–6 at 8 weeks old are denoted under the pedigree. Progeny 7 and 10 with extremely small body sizes and marked behavioral abnormalities died by 5 weeks after birth. (**b**) Growth curves for body length and weight. Six heterozygous (○) and four knockout (●) pups in a litter born to a mutant and heterozygous mating were measured during the first 3 weeks after birth. Solid and broken lines indicate averages of the values of the heterozygous and knockout pups, respectively. Open and closed arrows point the days when the heterozygous and mutant pups first opened their eyelids, respectively. Arrowheads point the day when the pups were weaned. *The smallest pup died 22 days after birth. (**c–h** and **m–r**) Hematoxylin and eosin staining of livers of progeny 1 (**c**) and 5 (**d**), stomachs of progeny 1 (**e**) and 5 (**f**), jejuna of progeny 1 (**g**) and 4 (**h**), testes of progeny 1 (**m**), 4 (**n**), and 6 (**o**), and brains of progeny 1 (**p**), 4 (**q**), and 6 (**r**). (**i–l**) The results of TUNEL assay in stomachs of progeny 1 (**i**) and 5 (**j**) and in jejuna of progeny 1 (**k**) and 4 (**l**). White broken lines indicate borders of the epithelia. +/−, heterozygous mice; −/−, knockout mice. Arrowheads in M, N, and O show mature sperms. CV, central vein; IV, interlobular vein; AC, anterior commissure; CC, corpus callosum; LV, lateral ventricle

environments, and/or in distinct genetic background of the mice. Our work thus offers the indication that requirement for Apaf1 during development may be context-dependent and other mechanisms may exist independently or interdependently for physiological apoptosis during development.

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