Biochemical and Biophysical Research Communications **278,** 665–670 (2000)
doi:10.1006/bbrc.2000.3864, available online at http://www.idealibrary.com on IDEAL®

Disruption of *klotho* Gene Causes an Abnormal Energy Homeostasis in Mice

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Received October 23, 2000

***klotho* mice, which genetically lack *klotho* gene expression, are characterized with various systemic phenotypes resembling human aging, and also with growth retardation. Here we show that *klotho* mice have a barely detectable amount of the white adipose tissue but their brown adipose tissue (BAT) is comparably preserved. Glucose tolerance and insulin sensitivity in *klotho* mice are increased compared to those in wild-type mice as revealed by intraperitoneal glucose and insulin tolerance tests. Uncoupling protein-1 gene expression of BAT and body temperature in *klotho* mice are lower than those in wild-type mice, suggesting that *klotho* mice have less energy expenditure than wild-type mice. Histological examination suggests that *klotho* mice possess less energy storage than wild-type mice with respect to glycogen in the liver and lipid in BAT. All these changes of parameters for energy homeostasis in *klotho* mice are very similar to those reported under food-restricted conditions. However, the amount of food intake is not different between *klotho* and wild-type mice when normalized for body weight. The present study elucidates the importance of *klotho* gene expression for the maintenance of normal energy homeostasis.**

***Key Words: klotho;* energy homeostasis; brown adipose tissue; white adipose tissue; glucose tolerance; insulin sensitivity; food restriction; food intake; phospho*enol*-pyruvate carboxykinase; uncoupling protein-1.**

A novel mouse model for human aging was recently established (1). Mice with homozygous disruption of a novel gene, termed *klotho,* manifest multiple systemic phenotypes such as arteriosclerosis, ectopic calcification, osteoporosis, skin and gonad atrophy, and pulmonary emphysema. Appearance of these mice (*kl/kl* or *klotho* mice) is normal as early as 3 weeks of age, after which, they almost stop growing and gradually die around 8–9 weeks of age. *klotho* mice also have low blood glucose level and barely detectable amount of subcutaneous fat. Here we investigated energy homeostasis in *klotho* mice which may be related to growth retardation in these mice.

klotho gene, which is expressed predominantly in the kidney, encodes secreted and membrane-bound forms of the protein containing one and two β-glucosidase-like domains, respectively (2). Almost all phenotypes in *klotho* mice are rescued by ectopic overexpression of the membrane-bound form of *klotho* cDNA (1). Endothelial dysfunction in heterozygous *klotho* (*kl/+*) mice is restored by parabiosis with wild-type mice, which allows exchange of soluble factors in the body fluid through the connected peritoneal surfaces (3). These findings have suggested that *klotho* gene product may function as an enzyme, a secreted ligand, or a soluble receptor. To date, no direct action of *klotho* gene product has been demonstrated *in vitro.* The present study elucidates new aspects of phenotypes in *klotho* mice, which may bring novel insights into our understanding of the molecular mechanisms underlying the actions of *klotho* gene product.

MATERIALS AND METHODS

Animals. Generation of *klotho* mice has been described elsewhere (1). Male homozygous *klotho* (*kl/kl*) mice and their male wild-type (+/+) littermates were used in the present study. Animals were housed with free access to tap water and ground standard chow (CE-2, Japan CLEA) on a 12 h light/12 h dark cycle. Food intake of

Abbreviations used: BAT, brown adipose tissue; WAT, white adipose tissue; PEPCK, phospho*enol*pyruvate carboxykinase; UCP-1, uncoupling protein-1; BW, body weight.

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0006-291X/00 $35.00

TABLE 1

Body and Tissue Weights of *klotho* and Wild-Type Mice

Genotype	BW	Liver	Kidney	Pancreas	BAT
Wild-type	16.16 ± 0.61	910 ± 51 (56 ± 2)	130 ± 11 (8.0 ± 0.6)	68 ± 4 (4.2 ± 0.2)	46 ± 5 (2.8 ± 0.3)
klotho	6.70 ± 0.20	318 ± 31 (47 ± 3*)	58 ± 4 (8.7 ± 0.6)	30 ± 4 (4.5 ± 0.6)	26 ± 2 (3.9 ± 0.3*)

Note. Tissue weight (mg) per BW (g) is shown in parentheses. BW and tissue weights of *klotho* mice were all statistically smaller than those of wild-type mice ($P < 0.01$ for BAT and $P < 0.0005$ for the others, $n = 5$).

* $P < 0.05$ vs wild-type.

mice, which had been kept in metabolic cages for 3 days, was measured for two consecutive days and the mean value was calculated. Anal body temperature was measured 4 times for each mice to determine the mean value, using Digital Thermometer (TD-300, Shibaura Electronics). All animal experiments were conducted in accordance with the Guidelines for Animal Research in our institute.

Histological examination. Liver was fixed by immersion in Carnoy's solution followed by 10% buffered formaldehyde, embedded in paraffin, sectioned in 2 μm, and stained with periodic acid Schiff reagent (4). Pancreas was fixed with 10% buffered formaldehyde, embedded in paraffin, and sectioned in 2 μm. Slices were deparaffined, refixed with Bouin's solution, and further oxidized with permanganate before Gomori's aldehyde–fuchsin staining, to increase the staining intensity of β cell granules in islets (4, 5). Cryosections of brown adipose tissue (BAT) in 6 μm were stained with Sudan III.

Blood glucose and serum insulin measurements. Basal blood samples were collected at 9:00 AM from the retro-orbital sinus of mice fed *ad libitum.* Blood glucose and serum insulin concentrations were determined by the glucose oxidase method using a reflectance glucometer (One Touch II, Lifescan) and by a radioimmunoassay kit with rat insulin standards (Linco).

Glucose and insulin tolerance tests. For glucose tolerance test, mice were injected intraperitoneally with 1.5 mg/g glucose after 6 h fasting. For insulin tolerance test, mice were ip injected with 0.48 mU/g human regular insulin (Nobolin R, Novo Nordisk) after 2.5 h fasting.

Northern blot analysis. Total RNA was extracted from tissues with Trizol reagent (GibcoBRL). By reverse transcription-PCR, partial cDNA fragments of rat phospho*enol*pyruvate carboxykinase (PEPCK, 91% identity with the mouse cDNA) (6) and uncoupling protein-1 (UCP-1, 94% identity with the mouse cDNA) (7) were generated from liver and BAT of Sprague–Dawley rat with the following primers: PEPCK sense, 5′-gatgacattgcctggatgaagtttg-3′; PEPCK antisense, 5′-tgccgaagttgtagccaaagaagg-3′; UCP-1 sense, 5′-tattcattgggcagcccacagagg-3′; UCP-1 antisense, 5′-acacaaacatgatgacgttccagg-3′. In each lane, 20 μg of total RNA was loaded. Hybridization was performed by [^{32}P]dCTP-labeled cDNA probes for PEPCK and UCP-1. Relative mRNA levels were normalized for the ethidium bromide staining intensities of 28S ribosomal RNA.

Statistical analysis. All values were expressed as means ± SE. Statistical significance of differences was assessed by analysis of variance with repeated measures analysis and Student's t test, where applicable. P values less than 0.05 were considered statistically significant.

RESULTS

Body and Tissue Weights

After 3 weeks of age, *klotho* mice hardly gained body weight (BW), and at 5 weeks BW of *klotho* mice was as small as 41% of that of wild-type littermates ($P < 0.0005$, Table 1). The weights of kidney and pancreas of *klotho* mice were proportional to their small size. *klotho* mice possessed no visible white adipose tissue (WAT) such as subcutaneous, mesenteric, retroperitoneal, or epididymal fat (not shown), whereas their interscapular BAT was preserved. The BAT weight of *klotho* mice was even statistically heavier than that of wild-type mice when normalized for BW ($P < 0.05$). On the other hand, the normalized liver weight of *klotho* mice was slightly but significantly smaller than that of wild-type mice ($P < 0.05$). Further studies were done using *klotho* and wild-type mice at 5–6 weeks of age.

Histology of Liver, Pancreas, and BAT

The reactivity of periodic acid–Schiff staining in the liver was weaker in *klotho* mice compared with wild-type mice, suggesting smaller glycogen storage in *klotho* mice (Fig. 1) (4). The sizes of pancreatic islets were reduced in *klotho* mice. The staining intensity with Gomori's aldehyde–fuchsin, which specifically detects islet β cells by reacting at least partly with insulin (4, 5), was also reduced in *klotho* mice. These findings are consistent with the decreased insulin content of the pancreas in *klotho* mice (1). Sudan III staining of BAT revealed that BAT in *klotho* and wild-type mice both possess lipid, but BAT in *klotho* mice tended to contain smaller lipid droplets, suggesting less lipid content of BAT in *klotho* mice compared with wild-type mice.

Basal Blood Glucose and Serum Insulin Levels

klotho mice had significantly lower blood glucose level than wild-type mice when fed *ad libitum,* which was 76% of the wild-type level ($P < 0.05$, Table 2). Serum insulin level of *klotho* mice was also significantly lower (56% of the wild-type, $P < 0.05$).

Glucose and Insulin Tolerance Tests

In glucose tolerance test, *klotho* mice at 6 weeks of age showed lower peak glucose level than wild-type mice at 15 min after glucose injection (58% of the

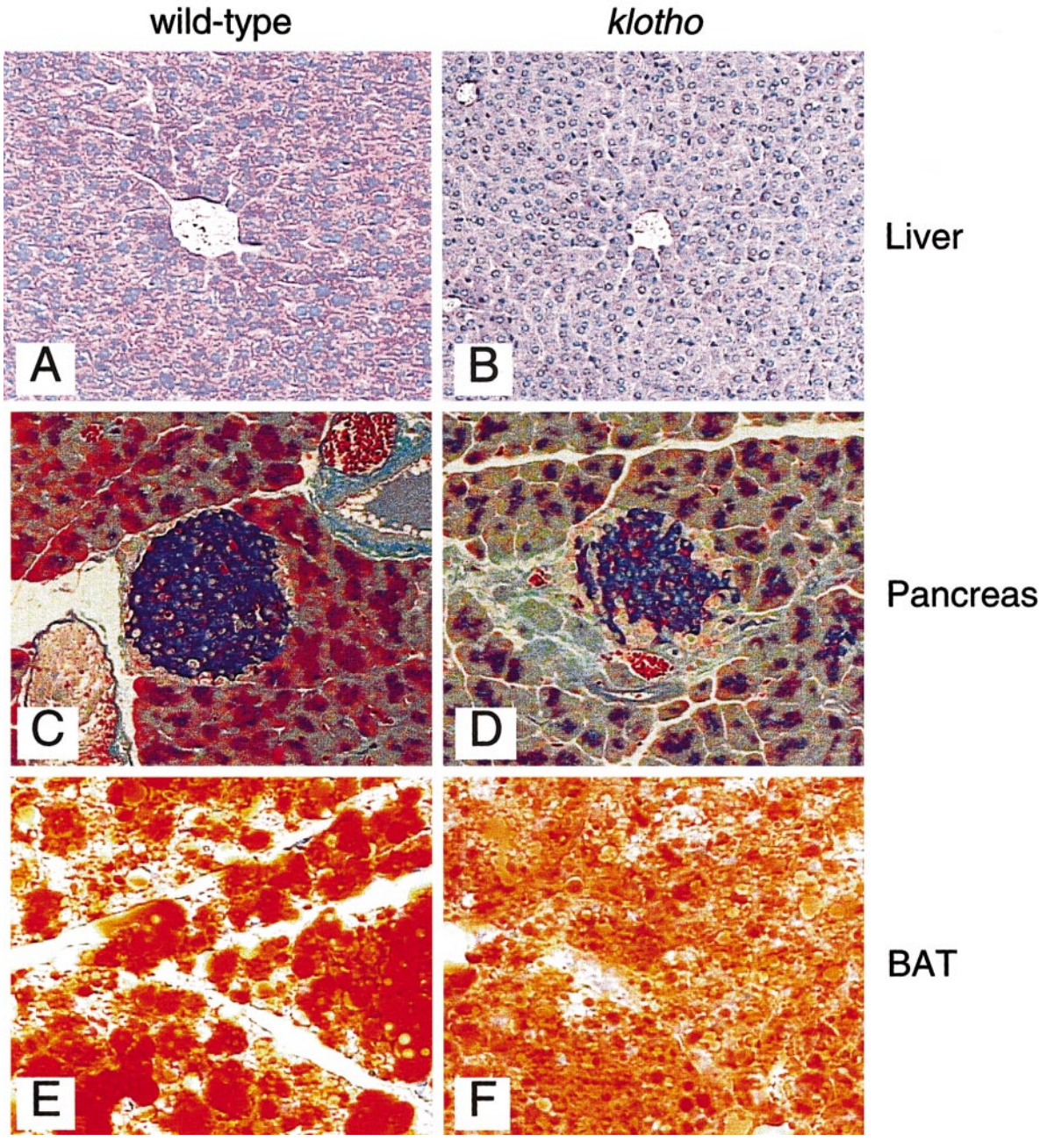

FIG. 1. Histological examination of the liver, pancreas, and BAT in *klotho* and wild-type mice. Hepatic glycogen (A, B), pancreatic islet β cells (C, D), and BAT lipid droplets (E, F) in *klotho* (B, D, F) and wild-type mice (A, C, E) were detected as red–purple by periodic acid–Schiff staining, as purple by Gomori's aldehyde–fuchsin staining, and as orange by Sudan III staining, respectively. Figures are the representative of three mice analyzed. Original magnification: A–D, ×200; E and F, ×400.

wild-type level), and the glucose level at 30 min was statistically lower in *klotho* mice ($P < 0.05$, Fig. 2). In insulin tolerance test, *klotho* mice exhibited statistically stronger response than wild-type mice at 15 min after insulin injection (−69% vs −49% of initial value, $P < 0.05$) and also at 30 min ($P < 0.05$). These findings indicate that glucose tolerance and insulin sensitivity are increased in *klotho* mice, and are in good agreement with low insulin content of the pancreas in *klotho* mice (1), which can be explained by less requirement of insulin in *klotho* mice.

Hepatic Gene Expression of PEPCK

PEPCK is one of key enzymes which control gluconeogenesis in the liver, and the level of PEPCK gene expression correlates well with its enzyme activity (8). During *ad libitum* feeding, hepatic PEPCK mRNA

TABLE 2

Basal Blood Glucose and Serum Insulin Concentrations in *klotho* and Wild-Type Mice

Genotype	Glucose (mg/dl)	Insulin (pg/ml)
Wild-type	173 ± 6	323 ± 35
klotho	132 ± 10*	180 ± 31*

* $P < 0.05$ vs wild-type ($n = 4$).

level of *klotho* mice was 2.2-fold higher than that of wild-type mice ($P < 0.01$, Fig. 3). These findings are consistent with much lower serum insulin level in *klotho* mice, since insulin is known to inhibit potently PEPCK gene expression and its enzyme activity (8). After overnight fasting, PEPCK mRNA level of *klotho* mice increased only 1.3-fold but that of wild-type mice increased 3.0-fold, thereby coming to very similar levels, when blood glucose levels were almost the same in *klotho* and wild-type mice (95 ± 13 vs 116 ± 13 mg/dl). These findings suggest that low blood glucose level in *klotho* mice is not caused by the downregulation of PEPCK activity as is the case with homozygous CCAAT/enhancer-binding protein α (C/EBPα) gene-deficient mice (9), and that hepatic PEPCK gene expression in *klotho* mice is regulated rather normally.

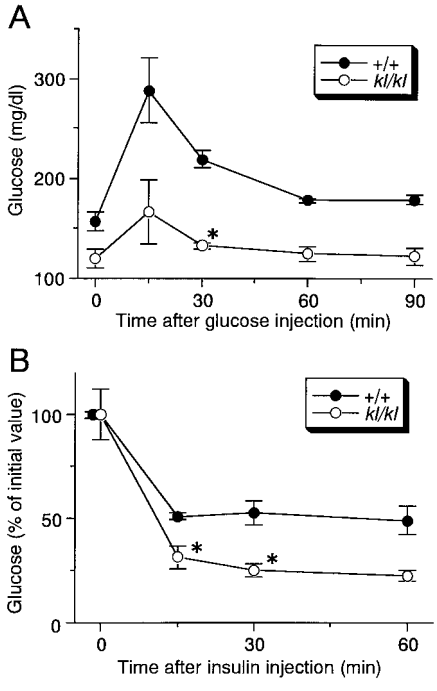

FIG. 2. Glucose tolerance and insulin sensitivity in *klotho* and wild-type mice. (A) Glucose tolerance test ($n = 4$). (B) Insulin tolerance test ($n = 5$). In insulin tolerance test, initial glucose levels in *klotho* (*kl/kl*) and wild-type (+/+) mice were 104 ± 13 and 184 ± 3 mg/dl, respectively. *$P < 0.05$ vs wild-type.

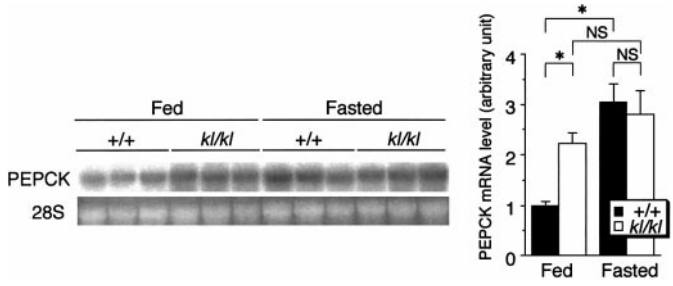

FIG. 3. Hepatic gene expression of PEPCK in *klotho* and wild-type mice and effects of feeding status. Livers were sampled from both mice ($n = 3$ each) when either fed *ad libitum* (Fed) or fasted overnight (Fasted). 28S, ethidium bromide staining of 28S ribosomal RNA.

BAT Gene Expression of UCP-1 and Body Temperature

UCP-1 plays a critical role in nonshivering thermogenesis in BAT (10, 11). UCP-1 mRNA level in *klotho* mice was significantly lower compared with wild-type mice (53% of the wild-type level, $P < 0.05$, Fig. 4), suggesting less nonshivering thermogenesis in BAT. Body temperature in *klotho* mice was significantly lower compared with wild-type mice (35.8 ± 0.1 vs 37.0 ± 0.2°C, $P < 0.0005$, $n = 5$).

Food Intake

The above-described metabolic phenotypes of *klotho* mice are very similar to those reported in food-restricted conditions (12–15). Therefore, we examined food intake in *klotho* and wild-type mice at 5 weeks of age (Table 3). Daily food intake in *klotho* mice was significantly smaller than that in wild-type mice (53%, $P < 0.005$), but when food intake was normalized for BW, the amounts were almost the same.

DISCUSSION

In the present study, we have revealed that severe growth retardation in *klotho* mice is accompanied by marked reduction in WAT mass, but not such reduction in BAT mass, and histological changes suggesting less glycogen content in the liver, less insulin in the pancreas, and less lipid in BAT compared with wild-type littermates. Furthermore, basal blood glucose and insulin levels are decreased, and glucose tolerance and

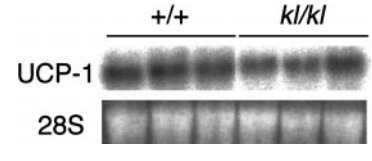

FIG. 4. UCP-1 gene expression of interscapular BAT in *klotho* and wild-type mice during *ad libitum* feeding ($n = 3$).

TABLE 3

Food Intake in *klotho* and Wild-Type Mice

Genotype	FI (g/day)	BW (g)	FI/BW (mg/day/g)
Wild-type	2.74 ± 0.32	17.3 ± 0.6	157 ± 13
klotho	1.45 ± 0.07*	8.2 ± 0.1**	178 ± 7

Note. Food intake (FI) in each mouse was expressed as an amount per head every day, or normalized for BW. *$P < 0.005$ vs wild-type; **$P < 0.0001$ vs wild-type ($n = 5$).

insulin sensitivity are increased in *klotho* mice. These findings are associated with compensatory upregulation of hepatic PEPCK gene expression to enhance gluconeogenesis in the lack of glycogen storage. Furthermore, body temperature and BAT thermogenic activity as suggested by UCP-1 gene expression level are decreased in *klotho* mice as if to reduce thermogenesis. Thus, *klotho* mice have characteristic metabolic features in the blood, liver, pancreas, adipose tissue, and body temperature, which closely resemble those observed in lean mice under food-restricted condition (12–15).

Are these metabolic phenotypes caused directly by the lack in *klotho* gene expression? *klotho* gene is expressed predominantly in the kidney, and also the gene expression is detectable in the pancreas but not in the liver (1). Therefore, *klotho* gene expression in the pancreas might affect insulin secretion, but this does not explain the whole phenotypes elucidated in the present study. Furthermore, *klotho* gene product has been shown to function in a non-cell-autonomous fashion (1, 3), making the interpretation very difficult. The present study adds new aspects of phenotypes in *klotho* mice, strengthening the pleiotropic roles of *klotho* gene product to maintain homeostasis in the body.

The above-described leanness-associated phenotypes in *klotho* mice may not be caused directly by the lack in *klotho* gene expression but secondary to reduced food intake. We therefore compared food intake in *klotho* and wild-type mice and found that *klotho* mice has similar food intake as wild-type mice when normalized for BW at 5 weeks of age. The amount in *klotho* mice is even slightly larger by 13%, but the difference is not statistically significant. To note, food intake per BW in normal mice gradually decreases after weaning, and the food intake per BW at 3 weeks of age is 20–30% larger than that at 5 weeks of age (by our calculation from the data described) (16). Therefore, we cannot exclude a possibility that food intake in *klotho* mice is mildly reduced. However, this degree of food reduction unlikely explains the observed phenotypes in *klotho* mice, since even 50% food reduction does not cause a severe decrease in WAT weight per BW at least in adult rats (12). To summarize, feeding status of *klotho* mice does not seem to be the main cause of the metabolic phenotypes in *klotho* mice.

Not only food restriction but also increased energy expenditure lead to leanness. As described above, body temperature and BAT thermogenic activity are decreased in *klotho* mice. Furthermore, spontaneous locomotor activity of *klotho* mice as revealed by behavioral analysis is much reduced than that of wild-type mice (1). These findings suggest, but not demonstrate, that energy expenditure in *klotho* mice is reduced compared with wild-type mice.

With respect to glucose tolerance and insulin sensitivity, abnormal blood electrolyte levels in *klotho* mice may have a contribution. *klotho* mice have slightly elevated blood calcium level and markedly elevated phosphorus level (1). Since hypophosphatemia decreases glucose tolerance and insulin sensitivity by inhibiting the secretion and action of insulin (17, 18), hyperphosphatemia, inversely, may play a role in the increased glucose tolerance and insulin sensitivity in *klotho* mice.

Here we show that *klotho* mice have various metabolic abnormalities which closely resemble those in food-restricted condition. These abnormalities seem to be caused by unknown mechanism other than reduced food intake. The present study demonstrates that *klotho* gene expression plays a crucial role for the maintenance of normal energy homeostasis, and implies the importance of *klotho* gene product as a novel factor which affects glucose tolerance and insulin sensitivity in the body.

Note: during preparation of our manuscript, Utsugi *et al.* reported that insulin production is decreased and insulin sensitivity is increased in *klotho* mice (19).

ACKNOWLEDGMENTS

This work was supported in part by research grants from the Japanese Ministry of Education, Science, Sports and Culture, the Japanese Ministry of Health and Welfare, Research for the Future (RFTF) of Japan Society for the Promotion of Science, the Japan Foundation for Aging and Health, the Salt Science Research Foundation, and the Smoking Research Foundation.

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