Genetic loss or pharmacological blockade of testes-expressed taste genes causes male sterility

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TAS1R taste receptors and their associated heterotrimeric G protein gustducin are involved in sugar and amino acid sensing in taste cells and in the gastrointestinal tract. They are also strongly expressed in testis and sperm, but their functions in these tissues were previously unknown. Using mouse models, we show that the genetic absence of both TAS1R3, a component of sweet and amino acid taste receptors, and the gustducin α-subunit GNAT3 leads to male-specific sterility. To gain further insight into this effect, we generated a mouse model that expressed a humanized form of TAS1R3 susceptible to inhibition by the antilipid medication clofibrate. Sperm formation in animals without functional TAS1R3 and GNAT3 is compromised, with malformed and immotile sperm. Furthermore, clofibrate inhibition of humanized TAS1R3 in the genetic background of *Tas1r3*$^{-/-}$, *Gnat3*$^{-/-}$ doubly null mice led to inducible male sterility. These results indicate a crucial role for these extraoral "taste" molecules in sperm development and maturation. We previously reported that blocking of human TAS1R3, but not mouse TAS1R3, can be achieved by common medications or chemicals in the environment. We hypothesize that even low levels of these compounds can lower sperm count and negatively affect human male fertility, which common mouse toxicology assays would not reveal. Conversely, we speculate that TAS1R3 and GNAT3 activators may help infertile men, particularly those that are affected by some of the mentioned inhibitors and/or are diagnosed with idiopathic infertility involving signaling pathway of these receptors.

phenoxy compounds | spermiogenesis

A family of three G protein–coupled receptor (GPCR) genes, the TAS1Rs, encode type 1 taste receptors that mediate perception of sweet and umami (amino acid) tastes. TAS1R receptors assemble into heterodimers: TAS1R2+TAS1R3 form a sweet receptor, and TAS1R1+TAS1R3 form an umami receptor. TAS1R3 is the common subunit and may also serve as a low-affinity sweet receptor alone, perhaps as a homodimer or homomultimer (1–3).

Sugars and noncaloric sweeteners (e.g., saccharin and sucralose) are known ligands and agonists of the sweet TAS1R2+TAS1R3 receptor (1, 2, 4, 5). Likewise, amino acids such as monosodium glutamate (MSG) are ligands of the umami TAS1R1+TAS1R3 receptor. Pharmacologically blocking or genetically eliminating *Tas1r3* results in loss of both sweet and umami taste in animals (2, 6, 7), indicating that heteromers require the TAS1R3 subunit for function in vivo. Small differences in the primary amino acid sequence of TAS1R receptors among species are responsible for species selectivity toward many sweeteners. For example, aspartame and cyclamate taste very sweet to humans, but are tasteless to mice (4, 8).

A number of natural and synthetic anti-sweet or sweet-modifying substances/antagonists exist. They are suspected or proven ligands of the sweet receptor (7, 9, 10), and similar to sweeteners, many are specific to human receptors. We recently identified a group of potent antagonists of the human sweet receptor that share a common phenoxy motif (10) also present in lactisole, one of the first sweet taste antagonists described (5, 11). Lactisole is in common use as a food additive for its anti-sweet properties (12). Among these widely used lactisole analogs are fibrates used to treat certain forms of hyperlipidemia (13–16). Although the therapeutic effect of fibrates on lipid metabolism is mediated via nuclear peroxisome proliferator-activated receptor-α (PPAR-α), our results (10) show that clofibric and bezafibric acids also inhibit TAS1R3 receptors at drug concentrations similar to those needed to activate PPAR-α (14). Thus, in humans, TAS1R3 receptors may be important biological targets of fibrates and could mediate some of their effects. Although no effects of fibrates on reproduction or fertility have been reported, no study has specifically examined fertility in patients receiving antilipid treatment. A second group of compounds structurally similar to lactisole are phenoxy-auxin herbicides, which are used extensively in crop agriculture and in landscape turf management (17, 18). Approximately 55 million pounds of phenoxy herbicides are used annually in the United States (18). We have shown that these herbicides are the most potent TAS1R3 blockers known to date (10). The long-term biological effects of these compounds in humans are not well known, although some reports point to male infertility (19–21).

Although once thought to function only in taste buds, TAS1R receptors are also expressed in gut enteroendocrine cells and pancreatic islet cells, where they contribute to nutrient sensing and regulation of glucose metabolism (22–25). Additional reports have shown their expression in brain, although their functional role there is unclear (26). The endogenous ligands of TAS1R receptors in extraoral tissues remain unknown.

Expression of Tas1rs and the associated "taste" G protein α-subunit (α-gustducin, encoded by the *Gnat3* gene) has been noted in testes and in spermatozoa (27–31). However, before our present study, no functional role has been determined for these testes-expressed taste genes. During our studies of these receptors and G proteins, we found that male, but not female, gametes with nonfunctional *Tas1r3* and *Gnat3* genes were unable to transmit their genes to progeny. Using existing KO animals and unique transgenic mice expressing a humanized form of *Tas1r3* that, unlike the mouse form of *Tas1r3*, can be pharmacologically blocked, we determined that inhibition of TAS1R3 in the absence of *Gnat3* results in male selective sterility. We report here that such treatment results in oligospermia, testis pathology with giant cells, and exfoliation of seminiferous epithelium along with functional, yet reversible, sterility. Similar pathologies in testis are seen in the genetic absence of these two molecules in *Gnat3* and *Tas1r3* double KO males. In addition, spermatozoa of these double KO males are immotile and mostly morphologically abnormal.

Male infertility is a complex disorder that affects a significant proportion of the population. It is estimated that about 7% of men experience problems in conceiving a child because of sperm defects (32). Fertility is decreasing worldwide, with the greatest effects in Western countries (32–34). In most cases, the causes are unknown, although environmental factors are suspected (35–38). In ~30–50% of infertile men, oligozoospermia or azoospermia is

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present despite unimpaired reproductive hormone secretion and a lack of other known determinants of infertility (35, 39, 40). We hypothesize that compounds present in the environment may act to inhibit testes-expressed taste receptors and/or G proteins to cause infertility.

Results

***Tas1r3* and *Gnat3* Are Expressed in the Male Reproductive Organs.** We found that *Gnat3* and *Tas1r3* were both expressed in testis, primarily in developing haploid spermatids (Fig. 1; Fig. S1). Expression of both molecules is strongest in the latest stages of development, in the elongating and elongated spermatids (Figs. S1 and S2). In agreement with a previous study (28), we found that *Tas1r2* mRNA is also expressed in the same cells (Fig. 1 *B* and *E*; Fig. S2). Interestingly, *Tas1r2* and *Tas1r3* mRNAs are present in epididymal sperm (Fig. 1 *G* and *H*). *Tas1r2* expression in testis and *Gnat3* and *Tas1r1* expression in testis and sperm have been previously reported (28–30). It thus appears that many of the initial components of the sweet/umami taste signaling cascade are expressed in testis and are present in sperm. We did not detect expression of *Tas1r3* and *Gnat3* in epithelial cells in epididymis, or in ovaries, although *Tas1r1* expression in ovaries has been reported (30).

Transmission Ratio Distortion Implicates *Tas1r3* and *Gnat3* in Sperm Function or Development. The first indication that the taste receptor TAS1R3 and the taste G protein α-subunit α-gustducin (GNAT3) are crucial in male reproduction came from crosses designed to produce *Tas1r3* and *Gnat3* double-null homozygotes. Because each KO line can be propagated without any reproductive or health problem, and in some taste cells α-gustducin functions downstream of TAS1R receptors, it was surprising to find that double KOs were not born, despite repeated attempts, from crosses of double heterozygotes and compound homo/heterozygotes. When we could not detect any late or early double KO embryos, we sought to identify what genotypes are passed to the next generation.

We found that only in males was there a failure to transmit the double-null haplotype; transmission of this haplotype from females occurred at the expected frequency (Table 1). We obtained the same failure to transmit in males by crossing compound homo/heterozygotes (e.g., $Gnat3^{+/-}$, $Tas1r3^{-/-}$ crossed with $Gnat3^{-/-}$, $Tas1r3^{+/-}$) and by crossing double heterozygotes $Gnat3^{+/-}$, $Tas1r3^{+/-}$ with WT animals. In each case, the *Gnat3*–, *Tas1r3*– haplotype was not transmitted from males but was transmitted from females (Table 1). Because the nontransmission from the double-heterozygous parents seems unlikely to be caused by altered physiology or hormonal levels (all other genotypes are produced normally, and animals are fertile), and double-heterozygous pups are born from double-heterozygous females but not from double-heterozygous males, we concluded that the haploid double-null sperm was either defective or not produced. This phenomenon is known as transmission ratio distortion (41, 42).

Single KO lines lacking either *Tas1r3* or *Gnat3*, or compound homo/heterozygotes (e.g., $Tas1r3^{-/-}$, $Gnat3^{+/-}$ or $Tas1r3^{+/-}$, $Gnat3^{-/-}$ mice) displayed normal gross testicular histology, and their sperm parameters and mating behavior were normal (Table S1). Two compound homo/heterozygote ($Tas1r3^{-/-}$, $Gnat3^{+/-}$) males were assessed at Charles River laboratory and compared with C57BL/6 males for total sperm concentration, motility, rapid cells, and abnormalities (e.g., no tail or head, bent midpiece, and head abnormalities). Based on the above criteria, one male had average sperm parameters and one had slightly above average sperm parameters.

Humanized *Tas1r3* Expressed in Transgenic Mice Is Blocked by Fibrates. To determine whether *Tas1r3* and *Gnat3* are necessary during spermatogenesis or in sperm function (e.g., fertilization), we first developed a mouse model in which we could pharmacologically inhibit the activity of TAS1R3-containing receptors (*SI Materials and Methods*). For this purpose we generated $Tas1r3^{-/-}$ mice expressing as a transgene *mhTas1r3-IRES-Gfp*, a humanized form of the TAS1R3 receptor that, unlike the native mouse receptor, is inhibited by lactisole, fibrates, and phenoxy compounds (7, 10). In transgenic mice of two independent lines, the humanized receptor functioned in the taste system to restore responses to sugars of the *Tas1r3* KO mice (Fig. S1*C*), and these taste responses were blocked by fibrates and phenoxy herbicides (Fig. S1*D*), in contrast to WT animals, which are insensitive to these blockers (7, 8). The GFP marker downstream from the humanized receptor gene was expressed in testes (Fig. S1 *E* and *G*), indicating that the *Tas1r3* promoter properly drives transgene expression to testes.

***Tas1r3*$^{-/-}$, *Gnat3*$^{-/-}$ Double-Null Mice with the *mhTas1r3* Transgene.** In the presence of the *mhTas1r3-IRES-Gfp* transgene, we were able to generate mice that were homozygous null for both endogenous *Tas1r3* and *Gnat3* genes. The genotype of these mice is $Tas1r3^{-/-}$, $Gnat3^{-/-}$, *mhTas1r3-IRES-Gfp*, hereinafter referred to as doubleKO-*mhTas1r3*. That these mice could be generated without any difficulty indicates that the humanized *Tas1r3* transgene expressed in testis functioned to complement the defect(s) underlying the transmission ratio distortion we had previously found in our attempts to generate doubly null mice without the transgene. Male and female double KO-*mhTas1r3* mice were both fertile (Table S1).

Pharmacological Block of the mhTAS1R3 Receptor Produces Reversible Sterility in Double KO-*mhTas1r3* Males. To determine the effects of pharmacologically blocking the humanized receptor in the double KO background, we placed double KO-*mhTas1r3* males and control WT males for a month on a diet containing 5 mg/g clofibrate (13, 14, 20, 43). No adverse health effects were noted with any of the males on the clofibrate diet. After 1 mo on the clofibrate diet, we housed each of the males with two females while continuing the clofibrate diet. The males displayed normal copulative behavior. Each female became obviously pregnant within 2 wk in cages with WT males on clofibrate and with double KO-*mhTas1r3* males on regular diet without clofibrate. However, there were no pregnancies and no litters from the double KO-*mhTas1r3* males

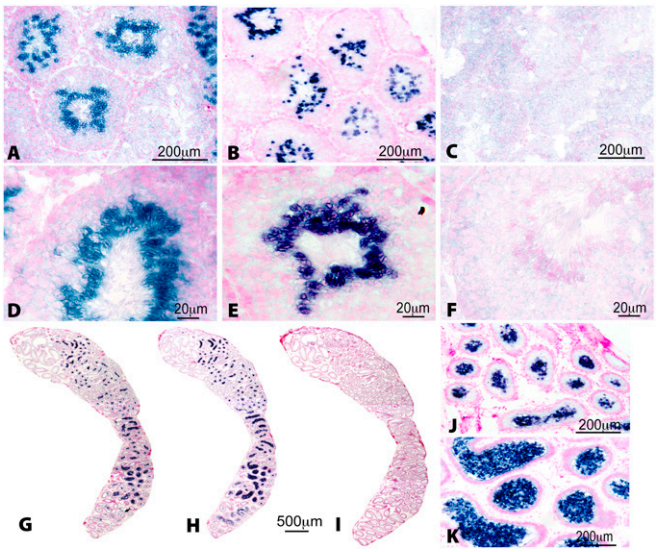

Fig. 1. Detection of taste gene mRNAs in male germ cells. (*A–F*) In situ hybridization to sections of seminiferous tubules from WT C57BL/6 males with antisense probe to *Tas1r3* (*A* and *D*), antisense probe to *Tas1r2* (*B* and *E*), sense probe control to *Tas1r3* (*C*), and sense probe to *Tas1r2* (*F*). (*G–K*) In situ hybridization on sections of epididymides from WT C57BL/6 males with antisense probe to *Tas1r3* (*G* and *J*), antisense probe to *Tas1r2* (*H* and *K*), and sense probe to *Tas1r2* (*I*).

Table 1. Transmission Ratio Distortion

Donor haplotypes	Female transmission		Male transmission	
	Observed (%)	Predicted (%)	Observed (%)	Predicted (%)
Cross 1				
Gnat3+ *Tas1r3*–	45	50	100	50
Gnat3– *Tas1r3*–	55	50	0	50
Cross 2				
Gnat3+ *Tas1r3*+	24	25	32	25
Gnat3– *Tas1r3*+	24	25	33	25
Gnat3+ *Tas1r3*–	25	25	35	25
Gnat3– *Tas1r3*–	27	25	0	25

Cross 1: compound hetero/homozygous mice ($Gnat3^{+/-}$, $Tas1r3^{-/-}$) were crossed with gustducin-null partners ($Gnat3^{-/-}$, $T1R3^{+/+}$), and their progeny were genotyped. Both haplotypes were transmitted from the females in expected Mendelian ratios, whereas the double-null haplotype (*Gnat3*–, *Tas1r3*–) was not transmitted from males. Cross 2: Double heterozygotes ($Gnat3^{+/-}$, $Tas1r3^{+/-}$) were crossed with WT partners ($Gnat3^{+/+}$, $Tas1r3^{+/+}$), and their progeny were genotyped. All four possible haplotypes were transmitted from females in expected Mendelian ratios, whereas the double-null haplotype (Gnat3–, Tas1r3–) was not transmitted from males.

on clofibrate even after 6 wk. We repeated this experiment three times with comparable results (Table 2).

Although sterile on the clofibrate diet, double KO-*mhTas1r3* males regained fertility after discontinuation of clofibrate, with first progeny appearing ~1 mo after clofibrate withdrawal. The short time sufficient for the onset of sterility and its recovery after clofibrate treatment suggests that only the latest stages of spermiogenesis are affected.

In an additional control experiment, $Gnat3^{-/-}$ single-null males (without the *mhTas1r3* transgene and with the intact endogenous mouse TAS1R3 receptor) and $Tas1r3^{-/-}$ males on the clofibrate diet also produced progeny (Table 2; Table S1). Thus, specifically blocking the humanized TAS1R3 receptor in the $Tas1r3^{-/-}$, $Gnat3^{-/-}$ background causes male sterility.

Pharmacological Block of the mhTAS1R3 Receptor in Double KO-*mhTas1r3* Males Induces Pathological Changes in the Male Reproductive Organs. Paraffin sections of testes and epididymis from WT males on clofibrate showed normal histology (Fig. 2 *A* and *B*). Likewise, testes from $Tas1r3^{-/-}$ males, $Gnat3^{-/-}$ males, and double KO-*mhTas1r3* males on a regular diet displayed normal histology (Fig. S3). In contrast, the testes and epididymides of double KO-*mhTas1r3* males after 1 mo on clofibrate showed signs of testicular degeneration and the presence of immature cells and periodic acid-Schiff (PAS)-positive material in the epididymis (Fig. 2 *C* and *D*). Paraffin sections of testes from double KO-*mhTas1r3* mice on clofibrate displayed abnormal spermiogenesis with multiple giant and pyknotic cells in many tubules; the germinal epithelium containing the pyknotic and giant cells appeared disorganized. Additionally, several tubules per each histological section contained exfoliated germinal epithelium in the lumen (Fig. 2 *E* and *F*). The exfoliated epithelium appeared to be of the same stage as the surrounding tubular epithelium and contained mostly round and elongated spermatids. Occasionally, however, spermatocytes were also present (see detailed images in Fig. S3). In epididymides of double KO-*mhTas1r3* males on clofibrate, we found immature precursor cells. Compared with WT males, the epididymis of double KO-*mhTas1r3* males also contained significantly more PAS-positive material (Fig. 2*D*). TUNEL staining of the testes sections from double KO-*mhTas1r3* males on clofibrate showed no increase compared with WT in the number of apoptotic cells present in some tubules. The numerous giant cells seen in the tissue of clofibrate treated double KO-*mhTas1r3* were not TUNEL positive, suggesting a necrotic process rather than apoptosis (Fig. S4). These giant cells may represent germ cell syncitia.

Sperm retrieved from cauda epididymis from double KO-*mhTas1r3* males on the clofibrate diet for 1 or 2 mo were, respectively, approximately one-half and one-quarter the number of those from WT (Table S1). About 18% of spermatozoa from double KO-*mhTas1r3* males on clofibrate were abnormal (flipped heads, amorphous heads, flagella with tight loops). These are at least twice as many abnormalities as typically found in WT C57BL/6 males (44) (Table S1). The most significant abnormalities noted were flipped heads (~8%) and amorphous heads (~8%). The motility was normal.

Rare $Tas1r3^{-/-}$, $Gnat3^{-/-}$ Double-Null Males Have Nonmotile Sperm and Testis Pathology. While breeding the double KO-*mhTas1r3* line on a single occasion, we obtained two $Tas1r3^{-/-}$, $Gnat3^{-/-}$ double-null mice without the *mhTas1r3* transgene. Both animals were males that by gross examination appeared normal, healthy, and without any obvious abnormalities. Despite many crosses during the last 9 mo, we have not detected any more of these animals, so we cannot explain their initial occurrence or the failure to replicate this rare event.

One male was euthanized at 6 wk of age, and the reproductive tract was examined. The testes and epididymides appeared of normal size. However, on dissection of the vas deferens and cauda epididymis, we recovered only immotile material that slowly disintegrated in the media. This pathology was in striking contrast to sperm retrieved from WT males, where vigorous sperm motility was readily seen under a dissecting microscope. Histological examination (H&E and Christmas tree stains) of the recovered material from the double KO male without the *mhTas1r3* transgene revealed that >75% of the sperm had multiple abnormalities such as detached heads, amorphous heads, tails flipped over heads, and multiple kinks and loops in the sperm tails (Fig. 3 *A*–*C*). The second male, euthanized at 10 wk of age, also displayed sperm immotility. There was only this single phenotypic change; otherwise, the male appeared normal, well nourished, and of normal weight and size.

Paraffin sections of the testes and epididymides of both double KO males revealed a phenotype similar to that of the clofibrate-treated double KO-*mhTas1r3* males: large (giant) cells with pyknotic nuclei in the spermatid cell layer in testicular tubules (Fig. 3 *D*,*G*, and *H*) and exfoliated germinal epithelium in the lumen (Fig. 3 *E* and *F*). Sections through the epididymides showed pathology similar to that seen in double KO-*mhTas1r3* males on clofibrate: occasional immature cells, PAS-positive material, and cellular debris (Fig. 3*I*).

Table 2. Male infertility due to clofibrate inhibition of humanized *Tas1r3* transgene

Male breeder genotype*	Males mated†	Clofibrate‡	Litters§
$Gnat3^{-/-}$, $Tas1r3^{-/-}$, $mhTas1r3^{+/-}$¶	6	–	12
	6	+	0
$Gnat3^{-/-}$, $Tas1r3^{+/+}$	2	–	4
	2	+	4
$Gnat3^{+/+}$, $Tas1r3^{-/-}$	2	–	4
	2	+	4
$Gnat3^{+/+}$, $Tas1r3^{+/+}$ (WT)	6	–	12
	6	+	12

*The $Gnat3^{-/-}$, $Tas1r3^{-/-}$, $mhTas1r3^{+/-}$ mice differ from the $Gnat3^{-/-}$, $Tas1r3^{+/+}$ animals only in the nature of the TAS1R3 receptor (*mhTas1r3* humanized transgene vs. endogenous mouse *Tas1r3* gene).

†Mating groups consisted of a single breeder male of the indicated genotype and two WT females. Results of three independent experiments are summarized here.

‡Males after 1 mo on a clofibrate diet were mated with young WT females and maintained on the clofibrate diet.

§Generation of progeny was scored after 6 wk.

¶Animals were +/– or +/+ for the *mhTas1r3* transgene.

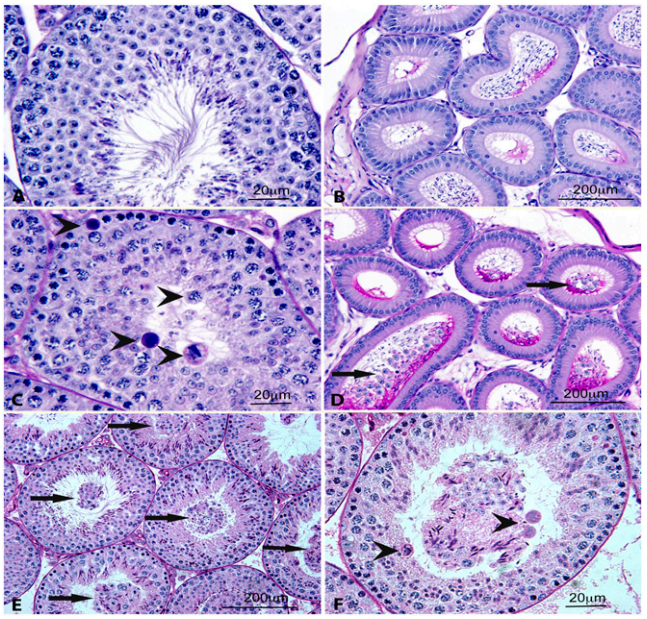

Fig. 2. PAS-stained paraffin sections of testicular tubules and epididymides from WT or transgenic mice treated with clofibrate. (*A* and *B*) WT male mice treated with clofibrate. (*C*–*F*) DoubleKO-*mhTas1r3* males treated with clofibrate. Arrows in *D* mark abundant PAS-positive material and immature cells in the epididymis. Arrowheads in *C* and *F* mark giant and pyknotic cells; arrows in *E* mark exfoliated spermatids in the testicular tubule lumen.

Impaired cAMP-Regulated Gene Transcription May Be Involved in the Infertility Mechanism. Both TAS1R receptors and GNAT3 are known to signal via phospholipase Cβ2 and cAMP in taste cells (1, 2, 28, 45). Because cAMP-regulated transcription is critical for spermatid development, we used quantitative PCR (qPCR) to analyze expression of several genes regulated by the transcription factor CREM (cAMP response element modulator) (46). We found down-regulation of *Prm1*, *Tnp1*, *Gapdhs*, *Spem1*, *Smcp*, and *Akap4* gene expression in testis of the double KO-*mhTas1r3* males on the clofibrate diet, as well as in the rare *Gnat3*$^{-/-}$, *Tas1r3*$^{-/-}$ double-null males (Table S2). As discussed in more detail below, we hypothesize that multiple pathways may be affected by the lack of these two signaling molecules.

Discussion

We found that *Tas1r3* and *Gnat3* are expressed in haploid spermatids in testis. We also detected mRNAs for *Tas1r3* and *Tas1r2* in mature spermatozoa stored in the epididymis. These data are in agreement with previous reports showing GNAT3, TAS1R1, TAS1R2, and TAS1R3 in testis and in sperm (27–30). It is noteworthy that TAS2R receptors that respond to bitter compounds in taste cells also are expressed in these postmeiotic cells (47, 48). It thus appears that many signaling molecules originally identified in taste cells are expressed also in male reproductive organs, where their function is currently unknown.

We found that the combined absence of *Tas1r3* and *Gnat3* genes results in male transmission ratio distortion, where the double-null haplotype is not transmitted to progeny from males, although the same haplotype is freely transmitted from females. Although this phenomenon can be due to the absence or functional impairment of sperm of a particular haplotype, our data indicate that TAS1R3 and GNAT3 are functionally important in spermiogenesis and that their absence causes a disruption of sperm development and/or maturation at the level of spermatids. We speculate that TAS1R3 and GNAT3 are strictly compartmentalized and do not cross the cytoplasmic bridges between spermatids. The cell without both genes/proteins then does not develop properly. It could be that such a cell may be lost due to an adhesion problem, but that does not seem likely at the stage of joined cells, unless the defect also impairs the development/maintenance of these bridges themselves. Pharmacologically blocking humanized TAS1R3-containing receptors on the *Gnat3*-null background results in a relatively rapid onset of male-specific sterility/infertility, accompanied by pathological signs of disruption in spermiogenesis, oligospermia, spermiogenic epithelium exfoliation, and the presence of immature cells in the epididymis. The sterility induced by pharmacologically blocking humanized TAS1R3 in mice of the *Gnat3*$^{-/-}$, *Tas1r3*$^{-/-}$ background is fully reversible, with males recovering normal fertility shortly after withdrawal of the TAS1R3 inhibitor.

Importantly, the TAS1R3 inhibitor we used, clofibrate, is a known antilipid medication from the fibrate family, which includes fenofibrate and bezafibrate currently on the market. Chemically similar compounds, all with the same phenoxy motif, have been developed into herbicides that are widely used (e.g., 2,4-DP, 2,4,5-TP, and dicamba). Most of these compounds are potent inhibitors of human TAS1R3 but have no effect on rodent TAS1R3 (10). As a practical matter, biological activity mediated through human-type TAS1R3 receptors would not be seen in toxicology tests using rodent assays and thus would be missed in many animal studies on the safety of herbicides, fibrates, and chemically similar compounds. Although clofibrate and other phenoxy compounds act on PPAR-α and perhaps several other off-targets (49, 50), we think that the block of TAS1R3 is the site of action that is important in male reproduction. WT males, WT females, and *Gnat3*$^{-/-}$ single-null males (all expressing the mouse form of TAS1R3) are not affected by clofibrate treatment, in contrast to double KO-*mhTas1r3*

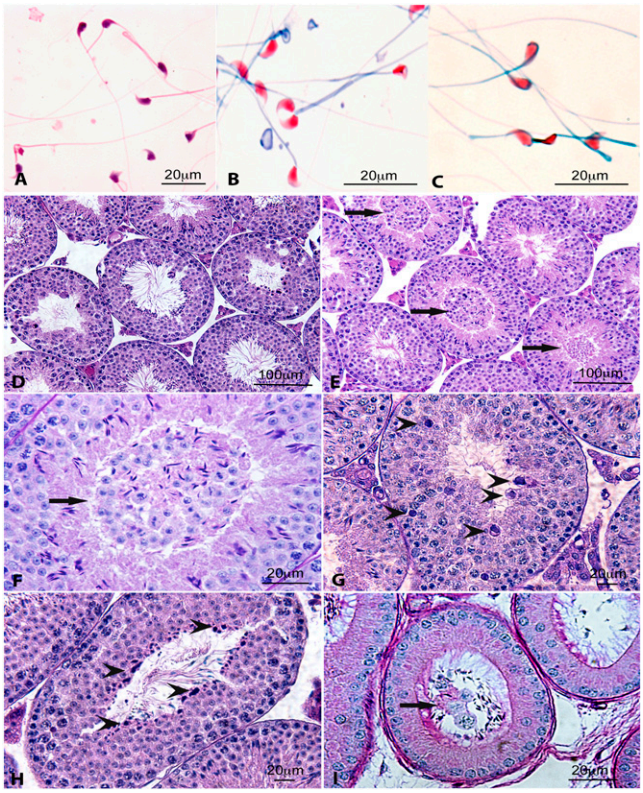

Fig. 3. Histological examination of sperm material and testis from rare *Gnat3*$^{-/-}$, *Tas1r3*$^{-/-}$ double-null mice. (*A*–*C*) H&E staining (*A*) and Christmas tree staining (*B* and *C*) of immotile sperm material. Note flipped heads, multiple tail loops, and amorphous hammer-shaped heads. Double and detached heads were frequent. (*D*–*I*) PAS staining of paraffin sections. Note the exfoliated spermatids (*E* and *F*, arrows) and numerous giant and pyknotic cells (*G* and *H*, arrowheads) in testicular tubule lumen, and PAS-positive material and immature cells in epididymis (*I*, arrow).

male mice expressing the humanized *mhTas1r3* transgene. Thus, many of these phenoxy compounds could potentially affect human reproduction, particularly if environmental agents or physiological circumstances interfere with the function of GNAT3 (or its downstream targets). Although *Gnat3* mutations in the human population have not been reported to date, natural variations and SNPs in the *Gnat3* gene and its promoter exist and have been shown to account for different sensitivities to sucrose in human population (51). Thus, a low-sensitivity/activity allele of *Gnat3* could be a risk factor if the TAS1R3 receptor is blocked by fibrates or herbicides.

Interestingly, we were able to obtain two *Tas1r3*$^{-/-}$, *Gnat3*$^{-/-}$ double KO mice from initial crosses of transgenic animals expressing the humanized *mhTas1R3* transgene. Sperm immotility was the single obvious phenotypic distinguishing feature of these males. Unfortunately, no additional double KO males have been born, and we have not yet identified the enabling conditions. We speculate that the chimeric transgene may behave differently or be expressed differently than the endogenous *Tas1r3* and thus be able to partly rescue the transmission ratio distortion. Perhaps the transgene mRNA or protein is less compartmentalized and able to provide sufficient TAS1R3 protein to the developing spermatid tetrads that do not express their own *Tas1r3* gene. Finding true *Tas1r3*$^{-/-}$, *Gnat3*$^{-/-}$ double KO males indicates that a doubly negative sperm can be produced and, once produced, can be functional to contribute to the progeny.

The pathology in the rare *Tas1r3*$^{-/-}$, *Gnat3*$^{-/-}$ double-null males is very similar to, but more severe than, that of the clofibrate-treated double KO-*mhTas1r3* males. Perhaps the pharmacological block of TAS1R3 with clofibrate is incomplete due to poor penetration through the blood-testis barrier or by the relatively short half-life of clofibric acid. In addition, clofibric acid acts as an inverse agonist on the TAS1R3 receptor (10, 52, 53), and therefore, the receptor may display rebound activation after the inhibitor is removed (10, 54). Thus, it is possible that during treatment of the animal, the receptor alternated between the inhibited and activated state. Furthermore, the *Tas1r3*$^{-/-}$, *Gnat3*$^{-/-}$ double-null males entirely lack *Tas1r3* from birth, which is likely to be a more severe deficit than that achieved by pharmacological manipulation of TAS1R3 activity in adults. For example, lack of TAS1R3 may affect membrane transport, cellular trafficking, or oligomerization with other GPCR receptors (55).

What are the functions of TAS1R3 and GNAT3 in testis, and how does their absence lead to male sterility? In taste cells, TAS1R receptors and GNAT3 are frequently coexpressed in the same cell and are thought to operate in the same signaling cascade. However, GNAT3 is also found in TAS1R-negative taste cells that express TAS2R bitter receptors. *Gnat3*$^{-/-}$ mice have deficits in their responses to both sweet and bitter compounds (56). In spermatids, TAS1R3 and GNAT3 may be expected to signal through different cells or pathways given the severe effect of the double null that is not displayed by either single-null mutant alone. In spermatids, TAS1R3 may signal through a G protein other than or in addition to GNAT3, whereas GNAT3 may be critical for coupling to spermatid-expressed TAS2R receptors (48). We hypothesize that in spermatids their activities converge on a common second messenger that controls spermatid differentiation and maturation. In taste cells, TAS1Rs are known to trigger Ca^{2+} signaling pathways and affect cAMP levels (3, 4, 24). GNAT3 is closely related to the α-subunits of retinal transducins (GNAT1 and GNAT2) (53, 56) that activate retinal (type 6) phosphodiesterases (PDEs) (53, 56, 57). Indeed, lack of GNAT3 in *Gnat3*$^{-/-}$ mice produced a significant increase of cAMP levels in taste cells (45). TAS1R1 was recently reported to function in regulation of Ca^{2+} and cAMP levels in spermatozoa (30). We thus hypothesize that in spermatids, TAS1R3 and GNAT3 may affect cAMP levels and Ca^{2+} signaling independently of each other.

Alteration of cAMP level in spermatids may result in disruption of several critical pathways required for normal sperm development and can explain the complex phenotype we observed. The exfoliation of spermiogenic epithelium in the *Tas1r3*$^{-/-}$, *Gnat3*$^{-/-}$ double KOs, as well as in the double KO-*mhTas1r3* mice on clofibrate, suggests that the exchange protein activated by cAMP (EPAC) pathway and its effectors required for cell adhesion may be involved. Males with a mutation of the EPAC downstream effector RAP1 develop significantly reduced fertility and oligospermia, with a high number of abnormal spermatozoa having sluggish movement (58). Their testis histology shows exfoliated epithelium due to the poor adhesion between Sertoli cells and spermatids (58), a pathology virtually indistinguishable from what we observed in our experimental animals.

Disruption of another cAMP-regulated pathway may be even more critical. In spermatids that express TAS1R3 and GNAT3, CREM is the master transcriptional factor regulated by cAMP. *Crem* KO males are sterile, with a total block in spermiogenesis. Testis histology in *Crem* KO mice (59, 60), with giant cells and spermiogenic arrest, is also remarkably similar to the histopathological findings in our double KO-*mhTas1r3* males and the rare *Tas1r3*$^{-/-}$, *Gnat3*$^{-/-}$ double-null males. Accordingly, we found down-regulation of *Prm*, *Tnp*, and other CREM-regulated genes in the double KO-*mhTas1r3* on clofibrate and the *Tas1r3*$^{-/-}$, *Gnat3*$^{-/-}$ double-null males. Even modest reduction of protamines is known to cause male sterility (61, 62) and therefore could substantially contribute to the observed pathologies in our mouse models.

The nature of the endogenous ligands of TAS1R (and TAS2R) receptors in testis remains to be determined. Because spermatids also express TAS1R2 and TAS1R1 receptors that can form heteromeric sweet and/or amino acid receptors with TAS1R3, these ligands are likely to be, respectively, sugars and amino acids. Alternatively, TAS1R receptors may function through their constitutive activity and nonspecifically lower the baseline resting cAMP levels and thus serve as a dampening mechanism so that the cells are better able to respond to stimuli, as was demonstrated in *Gnat3*-null taste cells (45). Because TAS1Rs and GNAT3 are present also in mature sperm, their signaling can also be used there.

We hypothesize that inhibitors of human TAS1R3 together with nonspecific G protein inhibitors, or mutations in these genes, could result in infertility or sterility and be responsible for some forms of idiopathic male infertility. These effects would be exaggerated by additional drugs, medications, or even dietary components that affect cAMP and Ca^{2+} signaling pathways, such as phosphodiesterase and Ca^{2+} channel blockers. With better knowledge of the function of these signaling pathways in spermatids, it may be possible to design medications composed of two or more compounds, each individually without a systemic effect, that together would selectively target spermatids and provide a fertility block with few side effects. Importantly, because there is significant species variation in the *Tas1r3*/*TAS1R3* sequence, it may be possible to develop species-selective contraceptives. Conversely, TAS1R3 and GNAT3 activators may help infertile men, particularly those that are affected by some of the mentioned inhibitors and/or are diagnosed with idiopathic infertility, especially those with abnormally low or altered CREM expression.

Materials and Methods

In situ hybridization was carried out using the TSA Plus DNP (AP) kit by Perkin-Elmer, with full-length *Tas1r2* and *Tas1r3* cDNAs as probes. Standard histology techniques were used on paraffin sections of tissues fixed with the Davidson's fixative; immunohistochemistry was as previously described (6, 56, 57). Epididymal sperm count and sperm evaluation was as described in ref. 63 and in *SI Materials and Methods*. Further details about generation of transgenic mice, behavioral testing, pharmacology experiments, TUNEL staining, and qPCR techniques are described in *SI Materials and Methods*.

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