To the editor:

Male fertility is dependent on dipeptidase activity of testis ACE

Testis angiotensin-converting enzyme (ACE) is an isozyme exclusively expressed by developing sperm1,2. This protein has only a single catalytic domain containing the HEXXH consensus-site motif typical of zinc metallopeptidases3. The exact role of testis ACE is unknown, but male mice lacking the protein reproduce poorly, despite normal numbers of sperm and normal motility4,5. Kondoh et al. recently analyzed glycosylphosphatidylinositol (GPI)-releasing activity in the testis and suggested that the source of this activity is ACE6. They further characterized the role of the ACE GPI-releasing activity in sperm-egg binding assays and concluded that this activity is crucial for mammalian fertilization. Finally, they provided evidence suggesting that the dicarboxypeptidase activity of testis ACE is dispensable for sperm-egg binding.

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Using targeted homologous recombination, we prepared mice with selective genetic inactivation of the dicarboxypeptidase activity of testis ACE. Animal use was approved by the Emory University Institutional Animal Care
and Use Committee. We achieved this preparation by introducing genomic point mutations converting the active site of testis ACE from HEMGH (amino acids 413-417) to KEMGK (Fig. 1a). These genetic changes had no effect on the expression levels of testis ACE, as measured by western blot (Fig. 1b) and immunohistochemistry (data not shown), but reduced the ACE catalytic activity within testis to 3% of that found in wild-type tests. This small residual activity is the result of the presence in testis of some somatic ACE with retained N-terminal catalytic activity.

To determine whether sperm isolated from wild-type or homozygous mutant mice contain a GPIase activity, we analyzed the partitioning of SPAM1 (PH-20), a GPI-anchored protein present on the sperm membrane, into water-soluble or detergent-soluble fractions. The water-soluble fraction contains cytoplasmic proteins and soluble components of the acrosome, whereas the detergent-soluble fraction contains membrane-anchored proteins. If the sperm contained an active GPIase activity, then cleaved SPAM1 would partition into the water-soluble fraction. The results show no difference in SPAM1 partitioning between sperm from wild-type and homozygous mutant mice (data not shown). We verified proper fractionation with Erk1/2-specific and fertilin β-specific antibodies. These data suggest that the inactivation of dipeptidase activity in homozygous mutant mice had no effect on whatever GPIase activity is present in sperm.

To directly assess whether ACE can cleave GPI-anchored proteins, we transiently coexpressed a somatic ACE expression vector with an enhanced green fluorescent protein (EGFP)-GPI-anchored expression construct in HEK293 cells. We monitored release of the GPI-linked EGFP by epifluorescence (Fig. 1c). The fluorescent signal in the ACE-cotransfected cells was equivalent to the fluorescent signal seen in cells transfected with only the EGFP-GPI construct. The addition of filipin, to disrupt the lipid rafts, did not promote the release of EGFP. Also, filipin (5 μg/ml) did not affect EGFP release (performed in triplicate, bars represent s.d.).

We incubated capacitated sperm from wild-type or homozygous mutant mice with wild-type eggs, washed and counted them. The binding of homozygous mutant sperm to eggs was nearly abolished as compared to wild-type (WT, 33.2 ± 1.2 sperm/egg versus homozygous mutant, 1.2 ± 0.1 sperm/egg). The number in parentheses represents the total number of eggs counted for each genotype over three experiments (bars represent s.d.). Two-cell embryos were included in all assays as a control for nonspecific binding (insert).
CORRESPONDENCE

Table 1 Testis ACE dipeptidase activity is required for fertility

<table>
<thead>
<tr>
<th></th>
<th>Total number of plugs</th>
<th>Total number of litters</th>
<th>Total number of embryos</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type (n = 6)</td>
<td>19</td>
<td>15</td>
<td>153</td>
</tr>
<tr>
<td>Homozygous mutant (n = 7)</td>
<td>22</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

We caged wild-type or homozygous mutant males individually with a CD1 female and then assessed presence of copulatory plugs. Females were killed during the last week of gestation and the embryos counted. The average litter size for wild-type males was 10.2 ± 0.6 embryos. Homozygous mutant mice produced only a single embryo.

We observed no difference in the sperm count, morphology or motility after capacitation. We incubated capacitated sperm with wild-type unfertilized eggs and, as a control, two-cell embryos for 45 min, after which we washed eggs and embryos until one to two sperm remained bound to the two-cell embryos. We counted the remaining sperm bound to the zona pellucida of unfertilized eggs. As expected, sperm from wild-type males readily bound to the zona pellucida (33.2 ± 1.2 sperm/egg). However, sperm from homozygous mutant mice showed background levels of binding (1.2 ± 0.1 sperm/egg; Fig. 1d).

Our data are consistent with the results of previous studies that ACE-dipeptidase activity is required for male fertility, the hypothesis that ACE diacarboxypeptidase activity is required for male fertility. We previously assessed the sensitivity of the sperm-egg binding assay to assess the functionality of the sperm. We collected cauda epididymal sperm from homozygous mutant or wild-type male mice and capacitated the sperm for 45 min. We observed no difference in the sperm count, morphology or motility after capacitation. We incubated capacitated sperm with wild-type unfertilized eggs and, as a control, two-cell embryos for 45 min, after which we washed eggs and embryos until one to two sperm remained bound to the two-cell embryos. We counted the remaining sperm bound to the zona pellucida of unfertilized eggs. As expected, sperm from wild-type males readily bound to the zona pellucida (33.2 ± 1.2 sperm/egg). However, sperm from homozygous mutant mice showed background levels of binding (1.2 ± 0.1 sperm/egg; Fig. 1d).

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Kondoh replies:
Leisle et al. and Fuchs et al. argue that, contrary to our published findings1,1, angiotensin-converting enzyme (ACE) does not directly release GPI-anchored proteins in vitro and in vivo. Several reasons could account for the differences between the studies.

The two groups coexpressed ACE and several GPI-anchored proteins in cell lines, measured released product in the media and compared the results with those of mock controls. But as can be seen from the data of Fuchs et al., spontaneous release of EGFP-GPI was extremely high, a phenomenon that we have also seen before2. As HKEK293 cells do not express endogenous ACE, this spontaneous release might be caused by other factors, such as endogenous GPI-PLD3, GPI-PLC-like activity4 or secretion of unanchored products. So, the background release of GPI-anchored proteins should not be compared with the results after adding ACE; instead, it should be subtracted from them.

As ACE is not the only factor with GPIase activity, the intracellular localization of these other factors might be relevant to the differences between our studies. For example, GPI-PLD acts at the level of intracellular organelles5. As ACE acts at the cell surface, substrates may not reach it in sufficient amounts to yield a substantial signal.

In our experiments, we managed to detect the initial amount of substrate and the magnitude of the background. By using PI-PLC as a control, the rate of enzymatic activity can be simply indicated as percent shedding. As the reaction takes place at the cell surface, we could ignore intracellular events. Last, we could evaluate the effect soon after performing the enzymatic reaction, excluding the possibility of subsequent modifications that could affect the size of the signal.

Leisle et al. showed that GPI-anchored alkaline phosphatase was not released from the cell surface by purified porcine kidney and insect ACEs even after filipin treatment. It is possible that cofactors required for efficient GPIase activity were lost during their purification of the ACEs, but remained in the rabbit lung ACE sample that we used. We previously assessed the sensitivity of the shedding assay by applying various doses of rabbit lung ACE and found that it was 20-fold weaker than that of the in vitro PLAP conversion assay.

By using rabbit lung ACE, Leisle et al. found limited but significant release of alkaline phosphatase from filipin-treated porcine brush-border membrane, but not from MDCK cells. In contrast, we showed shedding of six GPI-anchored proteins in three different cell types using the same enzyme. So, this difference might be the result of differences in the cells used. We have also found that serum inhibits the shedding activity of ACE. Complete inhibition of EGFP-GPI shedding from F9 cells occurs in the presence of very small amounts of serum (unpublished data).

Third, we reported that GPI-anchored proteins were not released from ACE-null sperm and that the egg-binding defect of mutant sperm was rescued by peptidase-inactivated ACE or PI-PLC treatments, and by wild-type ACE. We concluded that the GPIase activity of ACE has a crucial role in fertilization. Fuchs et al. created a mutant mouse that car-