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# Comment on ‘Atlas of Fshr expression from novel reporter mice’.

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| SPECIAL REQUESTS<br><br>In place of a cover letter, you may enter specific comments or requests to the editors here. | Dear Editor,<br>this is an invited Commentary. Thanks for giving us this opportunity.<br>I declare that some of my co-authors are members of the endocrine Society.<br>Best regards,<br>Livio Casarini. |
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# Comment on 'Atlas of Fshr expression from novel reporter mice'.

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**Commentary**

In recent years, there have been controversial claims of extragonadal effects of follicle-stimulating hormone (FSH). A paper by Chen et al. recently published in *eLife* further muddies the waters, though its intent was to provide clarity (1). Chen et al. developed a new transgenic reporter mouse designed to map FSH receptor (FSHR) expression throughout the body. Using CRISPR/Cas9, the authors reportedly inserted a DNA fragment encoding a P2A-ZsGreen fluorescent protein between the end of the *Fshr* coding sequence and its 3'-UTR. These mice were expected to express a bicistronic *Fshr*/ZsGreen transcript under the control of the endogenous *Fshr* regulatory sequences. If successful, FSHR and ZsGreen should be co-expressed in cells as two distinct proteins via ribosomal skipping driven by the P2A sequence, with the former faithfully reporting the endogenous expression of the latter. The ZsGreen signal was unexpectedly observed in nearly all extragonadal tissues and cells examined. Even within the gonads, the reporter was more abundant in non-canonical FSH targets than in Sertoli or granulosa cells.

Though provocative, in our opinion, these results more likely represent misleading technical artifacts than a true reflection of *Fshr* expression in the mouse. The conclusions of this paper, if accurate, would overturn decades of convergent evidence in reproductive medicine on selective expression and function of FSHR in the gonads. Moreover, Chen et al. did not fulfill established criteria to conclusively demonstrate extragonadal FSHR expression (2).

Here, we highlight several limitations of the Chen et al. study that must be addressed for any firm conclusions to be drawn regarding the putative extragonadal expression of the FSHR:

1) The authors attempted to validate their reporter gene data (ZsGreen) with orthogonal methods, including immunofluorescence with an FSHR antibody, single molecule in situ hybridization (smISH), and droplet digital RT-PCR (ddRT-PCR) for the *Fshr* mRNA.
    a. Immunostaining for FSHR (as in Fig. 14A) is notoriously fraught by the inadequacy of most, if not all, FSHR antibodies, leading to nonspecific signals (3). The authors should

have provided the source and validated the specificity of their antibody staining in *Fshr* knockout mice (4).

b. The smISH results from testes (Fig. 2C) contradict prior ISH analyses using radiolabeled probes. These results, too, should have been validated in *Fshr* knockout mice.

c. The multiple tissue mRNA analysis excluded testes and ovaries, precluding assessment of relative *Fshr* expression levels in extragonadal tissues to the classic FSH target organs (Fig. 14B).

d. The *Fshr* expression data for adult testes and ovaries (Fig. 2G) raise concerns about the accuracy of the ddRT-PCR analyses. *Fshr* expression in the mouse testis decreases as animals age. In adulthood, levels are higher in the ovary than testis. The Chen et al. results suggest far greater levels in testes.

2) Assuming the ZsGreen signal truly reflects expression from the *Fshr* locus, Chen et al. did not exclude the possibility that their genetic manipulation altered the chromatin landscape of the gene-edited *Fshr* locus. That is, the genetic alteration may have led to the ectopic activation of the *Fshr* gene. This could have been assessed with analyses of chromatin accessibility, such as ATAC-seq, in extragonadal tissues of these reporter mice compared to wild-type controls. Analyses of publicly available data show that the *Fshr* locus is compacted in extragonadal tissues of wild-type mice.

3) As far as we can determine, no data were presented to show that the mice express the *Fshr*-P2A-*ZsGreen* bicistronic mRNA in the gonads or any other tissues. This is important as the authors did not exclude the possibility that the P2A-ZsGreen cassette was inserted elsewhere in the genome. Indeed, this might provide a more parsimonious explanation for the ubiquitous expression of the reporter. Genotyping was done with locus specific primers. The Southern blot analysis, however, shows at least one animal with what appears to be an additional integration site (Fig. 1Cb; first lane). It is not clear if multiple founder lines were examined and how many backcrosses were performed to eliminate off-target integration. There is only reference to the F1 generation, which would be insufficient. Fluorescence ISH could be used to establish a single integration site in Chr. 17.

In addition to these technical considerations, we would note that expression does not necessarily indicate function. It is therefore surprising, considering the data shown, that Chen et al. did not examine or report FSH actions in any of the extragonadal tissues purportedly expressing the FSH receptor. This could have been done in primary cultures of many of the tissues examined.

Finally, the authors did not explain how their results can be reconciled with >50 years of experimental, clinical, and physiological evidence indicating the selectivity of FSH actions to Sertoli and ovarian granulosa cells. Historical experiments with radioligands in rodent tissue homogenates revealed FSH binding only in gonadal tissues. Extragonadal FSHR expression appears to be detectable only in the context of clinical conditions, such as cancer. In normal physiology, FSHR is expressed transiently at relatively low levels and only in gonadal cells, likely due to overall weak promoter activity and the detrimental impact of aberrant long-term expression on cell metabolism (5). On the contrary, the transgenic mice described by Chen et al. lack any pathological phenotype, although they suggest having found almost ubiquitous and relatively high FSHR protein levels using an approach not allowing to detect lower-level signals than previously defined.

In light of these concerns, we advise extreme caution and skepticism when interpreting the data from Chen et al. Unless and until the above concerns are addressed and/or the data are independently confirmed, it is our opinion that this paper should be added to a growing series of controversial studies on putative extragonadal FSHR expression, based on inconclusive or unconvincing evidence (6). Hence, it is not yet time to rewrite endocrinology textbooks.

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