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Warman *et al.* reply

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Humans with specific missense mutations in the Wnt co-receptor *LRP5* have a high bone mass (HBM) phenotype similar to that caused by loss-of-function mutations in *SOST*, the gene encoding the LRP5 inhibitor sclerostin (reviewed in ref. 1). HBM-causing mutations of *SOST* cluster in the region corresponding to the receptor's sclerostin-binding site, a finding that is consistent with results from *in vitro* experiments showing that HBM-causing mutant LRP5 receptors are poorly inhibited by sclerostin (reviewed in ref. 1). Conversely, humans and mice genetically deficient in LRP5 have low bone mass, and *Lrp5* knockout mice have inadequate local anabolic responses to increased mechanical load (reviewed in ref. 1). Together, these findings suggest that LRP5 functions in bone cells to enable bone to respond to alterations in mechanical load. We provided further support for this hypothesis by generating mice with knock-in *Lrp5* alleles (*Lrp5*^{ΔA214V} and *Lrp5*^{ΔG171V}, referred to as A and G, respectively) that recapitulate the human HBM phenotype and mice with conditional knock-in alleles (A_N and G_N) that recapitulate the HBM phenotype when activated by Cre-mediated recombination in osteocytes².

Karsenty and colleagues created their own *Lrp5* mutant mice³, which they used to generate a hypothesis that LRP5 affects bone by regulating tryptophan hydroxylase (*Tph1*) expression and serotonin synthesis in the intestine, rather than by functioning locally in bone as we suggest. They now claim that their study of mice with the A and G_N alleles, which we generated and donated to the Jackson Laboratory (JAX) in 2010 and which we described in our 2011 publication², supports their hypothesis (ref. 4). Karsenty and colleagues performed assays on our mice and obtained markedly different results from ours. We reported that HBM-causing *Lrp5* alleles (e.g., A) did not alter *Tph1* mRNA expression in the duodenum or serotonin levels in the blood (Fig. 4a,d in ref. 2). In contrast, Karsenty and colleagues report that duodenal *Tph1* expression and blood serotonin are both lower in mice carrying A

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alleles than in wild-type mice (Fig. 1a in ref. 4). We reported that conditional HBM-causing *Lrp5* alleles (i.e., A_N and G_N) required Cre-mediated recombination to become fully functional (Fig. 2a,c in ref. 2) and that bone mass increased significantly after Cre-mediated recombination in bone cells but not in intestine cells (Fig. 2b–e in ref. 2). In contrast, Karsenty and colleagues report that the G_N allele exhibited leaky expression in duodenum and not in bone (Fig. 1g in ref. 4) and that this leaky expression resulted in lower whole-blood serotonin levels and greater bone mass than are seen in wild-type mice (Fig. 1e,f in ref. 4). Karsenty and colleagues also report that villin-Cre-mediated recombination of the G_N allele in intestine cells caused an even greater increase in bone mass (Fig. 1f in ref. 4), although they did not compare this with the effect of G_N recombination in bone cells. In sum, the results reported by Karsenty and colleagues, using mice we generated, are contrary to our results.

We are pleased that Karsenty and colleagues have studied some of the strains of mice we donated to JAX, and we encourage other investigators to do so as well. However, we find several potential methodological errors in Karsenty and colleagues' measurements of serotonin and allele-specific gene expression that raise questions about their conclusions. We², and others⁵, have suggested that serum serotonin measurements are unreliable, particularly when blood is permitted to clot on ice for only 5 min as was done by Karsenty and colleagues (Fig. 1a,d in ref. 4). We included ascorbic acid in our assays of whole-blood serotonin² because it prevents hemolysis from destroying serotonin by oxidation⁶, whereas Karsenty and colleagues measured serotonin in frozen heparinized blood without ascorbic acid (Fig. 1e in ref. 4). Better serotonin assays might have helped Karsenty and colleagues avoid inconsistencies in their data, such as their observation in $+G_N$ mice of reduced whole-blood serotonin but unchanged serum serotonin levels (Fig. 1d,e in ref. 4). Also, we think RNA-seq is preferable to qRT-PCR for determining whether, and to what extent, a conditional allele exhibits leaky expression. The qRT-PCR as performed by Karsenty and colleagues (Fig. 1g in ref. 4) does not differentiate between the expression of conditional G_N and wild-type alleles. RNA-seq could have directly compared the expression of each allele in $+G_N$ mice, just as we used it to show equivalent expression of A and wild-type alleles in tibia cortical bones from $+A$ mice⁷. RNA-seq might have provided Karsenty and colleagues better insight into their incongruous finding of an increase in bone formation in $+G_N$ mice that is comparable to the increase in $+G_N$; *Villin-Cre* mice (Fig. 1f in ref. 4). We believe these methodological issues undermine Karsenty and colleagues' claims, and we remain confident of our data and our conclusions.

Separately from the experiments described above, we reported normal serotonin levels in three independently generated global *Lrp5* knockout mouse strains that had low bone mass², whereas Karsenty and colleagues reported a sixfold increase in serotonin levels in their one *Lrp5* knockout strain³. Moreover, Karsenty and colleagues generated their own *Lrp5* conditional knockout and knock-in alleles and reported changes in bone mass when they altered *Lrp5* expression in the intestine, and no changes when they altered *Lrp5* expression in bone³.

Using animal models we created, Karsenty and colleagues have claimed results that are different from ours². However, as discussed above, the experiments performed by Karsenty

and colleagues, in particular those related to serotonin measurements and analysis of the expression pattern of the G_N allele, have potential methodological shortcomings. As independent testing of research findings is fundamental for the proper advancement of science, we call on Karsenty and colleagues to donate their mice to JAX (or an equivalent distribution center with public access), as we have done, so that other investigators can independently test each of our groups' results and conclusions.

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References

1. Baron R, Kneissel M. *Nat Med.* 2013; 19:179–192. [PubMed: 23389618]
2. Cui Y, et al. *Nat Med.* 2011; 17:684–691. [PubMed: 21602802]
3. Yadav VK, et al. *Cell.* 2008; 135:825–837. [PubMed: 19041748]
4. Kode A, et al. *Nat Med.* 2014; 20:1228–1229. [PubMed: 25375916]
5. Brand T, Anderson GM. *Clin Chem.* 2011; 57:1376–1386. [PubMed: 21859903]
6. Blum JJ, Ling NS. *Biochem J.* 1959; 73:530–535. [PubMed: 13801689]
7. Ayturk UM, et al. *J Bone Miner Res.* 2013; 28:2081–2093. [PubMed: 23553928]