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Comment on Levanon *et al.*, '*Runx3* knockouts and stomach cancer', in *EMBO reports* (June 2003)

In this issue, Y. Groner and colleagues discuss the possible involvement of the transcription factor Runx3 in stomach cancer (Concept, pp.560–564). The fundamental point of the article is that, although both of our groups observed the same neurological and T-cell phenotypes in Runx3 knockout (KO) mice, the C57BL6 strain that we used (type I KO; Li *et al.*, 2002) developed gastric abnormalities, whereas the ICR strain used by Groner (type II KO; Levanon *et al.*, 2002) did not. Although careful analysis to find reasons for the differences in these studies is necessary, we feel that important points were not made in this Concept and need to be addressed here.

We acknowledged the differences between the two strains in one of our subsequent papers (Inoue *et al.*, 2002), but as the ICR strain is outbred, we did not further analyse its phenotype. However, we have shown that Runx3 is involved in the transforming growth factor-β (TGF-β) signalling pathway, and it is well known that responses to TGF-β vary in different strains of mice (Kallapur *et al.*, 1999), which could explain the difference in the gastric phenotypes of the type I and type II KO mice. The other main query raised over the use of the C57BL6 mice was that they are more susceptible to *Helicobacter felis* infection. However, we examined the stomach epithelium of *Runx3*$^{-/-}$ C57BL6 mice before the mice drank milk, ruling out the possibility of *Helicobacter* involvement in the phenotype we described.

Groner's group was unable to detect the Runx3 protein in the gastric epithelium of mouse embryos and therefore question whether the gastric abnormalities seen in the type I KO mice are due to a lack of *Runx3*. On the basis of their results, they conclude that *Runx3* is not expressed in mouse stomach epithelial cells at any time during their life cycle. This is the most direct contradiction between the two groups and therefore merits careful investigation. Genes involved in development and differentiation, such as the *Runx* genes, change their expression patterns during development. We have shown that *Runx3* is expressed in the glandular stomach epithelial cells of 10-week-old mice and also in the embryonic epithelial cells, albeit at much lower levels (Li *et al.*, 2002). Thus, the levels at this early stage might have been too low to be detected in Groner's study. Importantly, specific antibodies were used for detection in Groner's studies, whereas we tested for the presence of *Runx3* RNA. The titre of the antibody might not have been high enough to detect such low levels of protein and, in addition, the Runx3 protein could be more labile in stomach than in other tissues. As the main issue here is whether *Runx3* is expressed in stomach epithelial cells, we suggest that they analyse adult mouse stomach. Conversely, we agree with the finding of Groner's group that *Runx3* is expressed in mesenchymal tissues of mouse embryo stomach. However, we reported that this *Runx3* expression in mesenchyme is low compared with that in epithelial cells. Therefore, there seems to be a marked change in the relative expression levels of *Runx3* in epithelial cells and mesenchymal cells from embryo to adult.

As doubts have been cast on the expression of Runx3 in the stomach, it is interesting to consider the roles of *Runx3* from an evolutionary perspective. *Runx3* is thought to be the most ancient form of the three mammalian *Runx* genes and is involved in the neurogenesis of the monosynaptic reflex arc. But it is also known that *Caenorhabditis elegans* and sea urchins contain only one *Runx* gene and, in these animals, this is expressed in the intestine and foregut, respectively (Nam *et al.*, 2002; Robertson *et al.*, 2002). Thus, *Runx3* might have had an important role in controlling growth and differentiation of gut epithelial cells throughout evolution.

Several crucial observations that were made in the original paper describing the type I KO mice have not been mentioned in the Concept. For example, the growth of tumours in nude mice, induced by a human gastric cancer cell line that does not express *RUNX3*, was strongly inhibited by exogenous expression of *RUNX3*. This observation suggests that *RUNX3* has a tumour-suppressive effect. Although rare, we also found a loss-of-function mutation in *RUNX3*, termed RUNX3(R122C), in one gastric carcinoma patient. RUNX3(R122C) did not have the tumour-suppressive effect mentioned above. Finally, although cell lines isolated from the gastric epithelia of *p53*$^{-/-}$ *Runx3*$^{+/+}$ mouse embryos did not induce tumours in nude mice, those from *p53*$^{-/-}$ *Runx*$^{-/-}$ mice induced adenocarcinoma (Li *et al.*, 2002). These data alone are sufficient to suggest strongly that there is a causal relationship between the loss of expression of *RUNX3* and gastric cancer.

The construction of the target vector used to generate the type I KO mice is also cited as a possible source of the discrepancy between the gastric phenotypes. However, we feel that the method was not sufficiently clear in our original paper and readers may have interpreted that LacZ was directly fused at the *Sma*I site in exon 4 of *Runx3* (designated exon 3 in the original paper). This would eliminate only a small part of the carboxy-terminal end of the Runt domain and the resulting protein product might still interact with polyomavirus enhancer-binding protein 2β (PEBP2-β)/core-binding factor β (CBF-β). In fact, although the DNA was cleaved at the *Sma*I site, the 3′ end was digested and a *Kpn*I site was inserted. Therefore, the final construct is lacking 24 amino acids from the C terminus of exon 4 and has only the 12 remaining amino acids fused in-frame to LacZ; this is unlikely to bind to PEBP2-β/CBF-β.

Another point raised in the Concept is that, in type I KO mice, the inserted phosphoglycerate kinase (PGK)–*neo* gene could

also drive the expression of *Clic4*, 50 kb downstream, which has been shown to abrogate apoptosis. The PGK promoter can activate the expression of neighbouring genes when they are clustered and of distantly located genes when the promoter is inserted in the locus control region (Scacheri *et al*., 2001). Neither of these applies to our targeted locus. Furthermore, the finding by Scacheri and colleagues that a gene located 2 kb from the PGK promoter was not affected, suggests that a gene 50 kb away would also not be affected.

Runx3 has two promoters, P1 and P2, and the latter is silenced by hypermethylation in human gastric tumours. However, Groner and colleagues suggest that *Runx3* expression could then be driven by P1. We have previously performed RT–PCR (PCR after reverse transcription) with two primer sets, Ps-N for P2-specific messenger RNA and Ps-C for common mRNA, and did not observe any product in either of the reactions in P2-methylated cell lines (Li *et al*., 2002). So, in the case of gastric cancer cell lines, a promoter switch of Runx3 from P2 to P1 was not observed.

Finally, the Concept highlights the fact that *Runx1* is expressed in stomach epithelium, with which we agree. A potential regulatory role of *Runx1* in the stomach will be an interesting subject for future study.

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