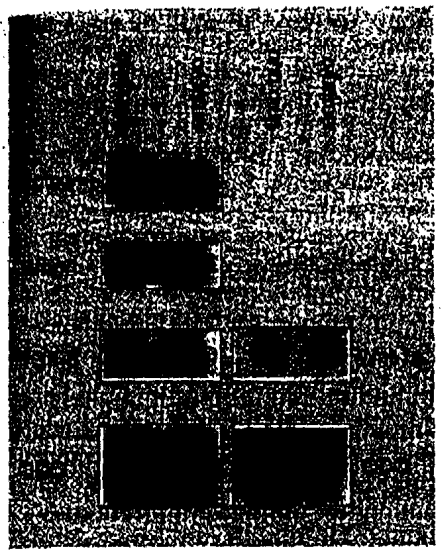

Figure 2 RNase protection analysis of stage 50/52 *Xenopus* limb buds showing the three diffusely expressed gene products together with FGF-8. Results for the same genes in stage 33 embryos are shown as positive controls. Ornithine decarboxylase (ODC) is a loading control. Detailed methods are available from the authors.

activity and the AER. These observations suggest that the mechanisms in proximodistal and anteroposterior axes are indeed conserved.

Amphibia was the first tetrapod class to diverge from the vertebrate lineage, so our results suggest that the proximodistal and anteroposterior patterning systems have indeed been conserved across the tetrapods. The same cannot be said of the dorsoventral system, however. Our results show that En-1 is not repressing the dorsal genes, and that it is not necessary to have restricted expression of *Wnt-7A*, *Rfng* or *Notch-1* to make a limb. Because all these genes are expressed diffusely, it remains possible that there is a permissive requirement for their products, but a mechanism depending on interaction across boundaries[4,5] does not seem likely.

Some differences between amphibian and amniote limb development that might account for a difference in the mechanism of dorsoventral patterning are known[7], including a more complex muscle pattern in amniotes and the regenerative ability shown by many amphibians, including *Xenopus* in the larval stage[8,9]. We have examined the expression of the same eight genes in regeneration blastemas and find very similar results to the original limb buds (data not shown). Whatever the reason for the difference, our results show that the accepted mechanism for dorsoventral patterning is not a necessary feature of limb development but is, at its most general, a derived feature shown by the amniotes.

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Does vitamin C have a pro-oxidant effect?

The conclusions of the Scientific Correspondence by Podmore *et al.*[1] are undermined by some unaddressed scientific issues. Podmore *et al.* gave vitamin C supplements of 500 milligrams to healthy volunteers, and measured levels of vitamin C in plasma and of oxidative DNA adducts in lymphocytes before, during and after supplementation.

Because the reported changes in levels of 8-oxoguanine and 8-oxoadenine occurred in lymphocytes, the relevant vitamin C concentrations are not those in plasma but those in lymphocytes; however, these data were not presented. Because millimolar concentrations of ascorbate are found in lymphocytes, measurements of vitamin C are practical using as few as 0.25×10^6 cells and a sensitive electrochemical high-performance liquid chromatography assay[2–4]. Available cell numbers should not have been a limiting problem because few cells are needed for the measurement compared with the number that can be isolated from peripheral blood[2,3,5].

Although the data on plasma vitamin C concentrations presented by Podmore *et al.*[1] were expressed as percentage changes only, true intracellular vitamin C concentrations can still be calculated. Because the vitamin C supplement produced a 60% increase in plasma concentrations of vitamin C, and because 500 mg is a saturating dose of vitamin C, the initial plasma vitamin C concentration was roughly 50 μM (refs 5,6). However, at this concentration lymphocytes are already saturated, with an intracellular ascorbate concentration of approximately 3 mM (refs 5,6). Increasing the extracellular plasma concentration of ascorbate above 50 μM will not affect the intracellular concentration further.

If the reported changes in oxidative DNA adducts could not be due to changes in intracellular lymphocyte vitamin C concentrations, what is responsible? The concentrations of 8-oxoguanine reported by Podmore *et al.* are 25–120 times more than those reported by others[7–9]. These measurements are notoriously difficult to make and are easily increased artefactually by oxidation[9]. The possibility of oxidation is increased further if mononuclear cells rather than pure lymphocytes were isolated, because activated contaminating monocytes can produce superoxide and other oxidants. The purity of the isolated lymphocytes was not indicated by Podmore *et al.* In addition, without proper precautions ascorbate can act as a pro-oxidant. By increasing plasma ascorbate levels with supplements, the authors may have increased the probability of an oxidation artefact occurring.

Because Podmore *et al.* used an experimental design that did not take into account the fact that cells become saturated with vitamin C at lower doses than does plasma, an inappropriate choice of tissue sampled for vitamin C, unclear lymphocyte-isolation procedures, and assays that may be biased by oxidation artefacts, we believe that their conclusions are not justified by the data.

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Podmore *et al.*[1] reported a decrease in 8-oxo-7,8-dihydroguanine and an increase in 8-oxo-7,8-dihydroadenine in lymphocyte DNA after intake of 500 milligrams of vitamin C daily for 6 weeks. In our view, four issues compromise the validity of their findings.

First, there are severe problems resulting from increased oxidation during the DNA isolation and extraction necessary[2,3] for gas chromatography mass spectrometry. Podmore *et al.* reported high values in lymphocyte DNA of 30 molecules of 8-oxo-7,8-dihydroguanine per 100,000 guanines, but did not acknowledge these problems or refer to related published studies (see, for example, ref. 4). Artefactual oxidation *ex vivo* in their study cannot be excluded and hence their results are difficult to interpret.

Second, the study design is without randomization or true placebo control, nor

is it double-blinded; thus, time and other effects unrelated to those of vitamin C cannot be excluded. Third, Podmore *et al.* did not mention whether any of their subjects smoked. Fourth, the authors did not reference relevant previous studies.

We have reported a 59-fold variation in guinea-pig liver vitamin C concentrations as a result of dietary manipulation, without any change in oxidized guanine levels in the liver[5], for example. In a two-month randomized, placebo-controlled trial in which we gave 38 smoking men 500 mg vitamin C every day, we found no change in oxidative DNA damage measured by 24-hour urinary excretion of 8-oxo-7,8-dihydro-2′-deoxyguanosine, as compared with 19 smoking men who received a placebo[6]. Urinary excretion of 8-oxo-7,8-dihydro-2′-deoxyguanosine is interpreted as a total-body average measurement of the rate of DNA oxidation, a theory supported by recovery of 2-nitro-propane-induced excess organ 8-oxo-7,8-dihydro-2′-deoxyguanosine in excreted urine in animals[7].

Measurements of the oxidized bases 8-oxo-7,8-dihydroguanine and 8-oxo-7,8-dihydroadenine, or of the corresponding nucleosides 8-oxo-7,8-dihydro-2′-deoxyguanosine and 8-oxo-7,8-dihydro-2′-deoxyadenosine, in lymphocytes represents concentrations of oxidized bases in those cells only and their relevance to other organs, for example target organs, is not known. Neither is it known whether one or both of the two measurements is relevant for risk assessment.

We think it is too soon to say whether supplemental doses of vitamin C exert pro-oxidant or mutagenic effects. Further well-designed trials with an analysis that would eliminate artificial *ex vivo* oxidation are needed to resolve this question.

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Podmore et al. *reply* — In our Scientific Correspondence[1] we reported changes in lymphocyte DNA to two established 'markers' of oxidative DNA damage after daily supplementation of healthy volunteers with 500 mg vitamin C for six weeks. One marker, 8-oxoadenine, increased, whereas the other, 8-oxoguanine, decreased. On this basis, vitamin C seems to act as both a pro-oxidant and an antioxidant *in vivo*. Our contribution has stimulated many comments, most of which were addressed in our original manuscript to *Nature*, which was shortened for publication at the editors' request. The full details are available from us on request.

The suggestion made above by Poulsen *et al.* and Levine *et al.* that artefactual oxidation has occurred during lymphocyte isolation and DNA extraction is based entirely on the measurement of a single marker, 8-oxoguanine. As founder members of the European Standards Committee on Oxidative DNA Damage (ESCODD), we are aware of the discrepancies between 8-oxoguanine measurement by different techniques. Indeed, with this in mind we have measured 8-oxoguanine in DNA samples from healthy volunteers by two additional assays using high-performance liquid chromatography: one measuring the base, and the other, widely used, its 2′-deoxynucleoside derivative, 8-oxo-2′-deoxyguanosine (Cooke, M. S. *et al.*, manuscript in preparation). All three assays show the same decrease in 8-oxoguanine on vitamin C supplementation.

We disagree with Poulsen *et al.* about the need for randomization or a double-blind study, as we were not reporting a clinical trial requiring a subjective interpretation of efficacy. The design of our study included a correct placebo treatment. Methods for improving DNA extraction are currently evolving through ESCODD. The important question remains of why, if 8-oxoguanine and 8-oxoadenine are generated solely as artefacts, one increases and the other decreases on supplementation but both return to baseline/placebo values following washout.

We do not consider the references cited by Poulsen *et al.* to be relevant to our study on human volunteers (all of whom were non-smokers, as smoking is known to reduce levels of plasma ascorbate). One citation refers to a study measuring 8-oxoguanine in guinea-pig liver on "dietary manipulation"[2]. The other used only measures of 8-oxo-2′-deoxyguanosine in urine[3], which has not yet been shown to derive directly from DNA. However, its relevance as a marker of the total-body average rate of DNA oxidation cannot be ruled out. Using a monoclonal antibody to detect 8-oxo-2′-deoxyguanosine, we observe an increase in urine following supplementation of the volunteers with vitamin C, a finding that corresponds to loss of 8-oxoguanine from DNA (Cooke, M. S. *et al.*, manuscript in preparation).

The mean plasma ascorbate concentration for 30 healthy volunteers (14 males and 16 females, aged between 17 and 49) in our study was 51.3 μM, increasing to 80.4 μM on vitamin C supplementation (Cooke, M. S. *et al.*, manuscript in preparation). Levine *et al.* argue that lymphocytes saturate when plasma ascorbate levels reach 50 μM. We do not find their argument compelling as it seems to be based on a depletion–repletion study of seven healthy volunteers, all males aged between 20 and 26 (ref. 4). In addition, we find considerable individual variation of plasma ascorbate with presupplementation values as low as 8 μM, equivalent to an intracellular ascorbate concentration of approximately 1.5 mM (less than 50% of the stated saturation value)[4]. It is therefore reasonable to conclude that saturation of plasma with vitamin C does not occur with at least 50% of our volunteers before supplementation.

Furthermore, we observe highly significant positive and negative correlations between plasma ascorbate concentration and levels of 8-oxoadenine and 8-oxoguanine, respectively, which we believe suggests that vitamin C is influencing levels of both markers in a divergent manner (Cooke, M. S. *et al.*, manuscript in preparation). This differential effect, which cannot be explained as methodological artefact, is related to the possible induction of specific repair of 8-oxoguanine. There is a precedent for redox regulation of repair of this lesion[5].

In conclusion, our results show a definite increase in 8-oxoadenine after supplementation with vitamin C. This lesion is at least ten times less mutagenic than 8-oxoguanine[6], and hence our study shows an overall profound protective effect of this vitamin.

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