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Left-right patterning: conserved and divergent mechanisms

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Summary

The left-right (LR) asymmetry of visceral organs is fundamental to their function and position within the body. Over the past decade or so, the molecular mechanisms underlying the establishment of such LR asymmetry have been revealed in many vertebrate and invertebrate model organisms. These studies have identified a gene network that contributes to this process and is highly conserved from sea urchin to mouse. By contrast, some specific steps of the process, such as the symmetry-breaking event and situs-specific organogenesis, appear to have diverged during evolution. Here, we summarize the common and divergent mechanisms by which LR asymmetry is established in vertebrates.

Key words: Nodal, Cilia, Left-right asymmetry

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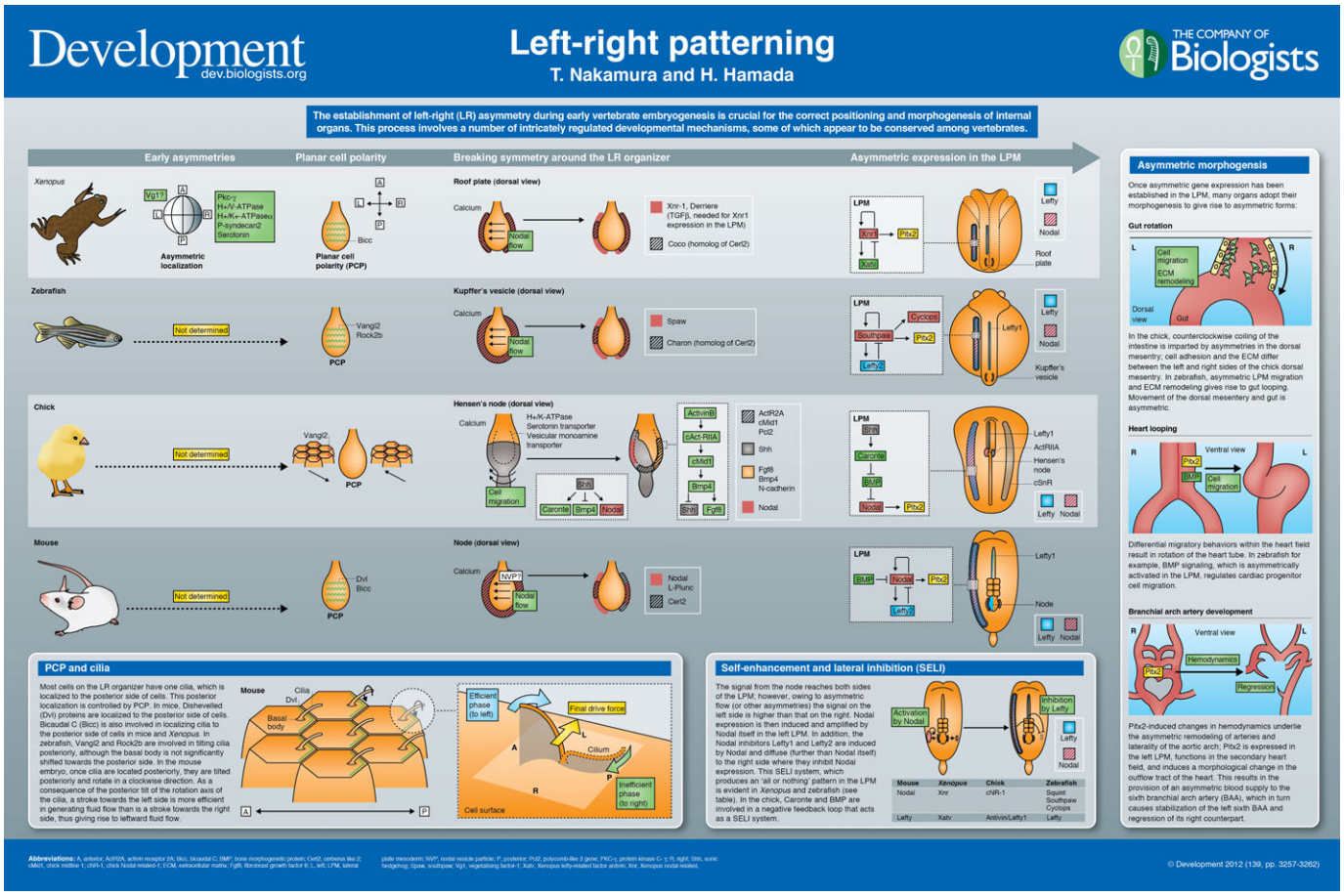
Introduction

The establishment of left-right (LR) asymmetry during early embryogenesis is crucial for the correct positioning and morphogenesis of internal organs. This process involves a number of intricately regulated developmental mechanisms, some of which appear to be conserved among vertebrates. For some organisms, LR symmetry breaking takes place around the left-right organizer (LR organizer; the node in mice, Hensen's node in the chick, the gastrocoel roof plate in *Xenopus*, and Kupffer's vesicle in zebrafish). In these animals, except for the chick and pig, asymmetric fluid flow produced by rotation of multiple cilia on the LR organizer results in asymmetric gene expression around the LR organizer. Earlier asymmetries in the localization of some molecules are observed in *Xenopus* and chick embryos and contribute to the establishment of LR asymmetry, but the roles of these molecules in establishing LR asymmetry in other organisms still need to be established (Spéder et al., 2007; Vandenberg and Levin, 2010). Following the symmetry-breaking event, asymmetric information is transmitted to surrounding tissues and induces the asymmetric expression of *Nodal* and *Lefty*. Nodal and Lefty,



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members of the transforming growth factor β (TGFβ) superfamily, act as diffusible activator and inhibitor molecules, respectively, and constitute a self-enhancement and lateral-inhibition (SELI) system, which is highly conserved among vertebrates and some non-vertebrates, and provides robustness to LR determination. Finally, asymmetric Nodal signals induce the left-sided expression of other genes, such as pituitary homeobox 2 (Pitx2), which in turn contribute to the LR asymmetric morphogenesis of various visceral organs. The cellular mechanisms underlying situs-specific organogenesis, by contrast, seem to vary among vertebrates.

Early asymmetries

In *Xenopus* embryos, several genes exhibit asymmetric patterns of gene expression at early stages of development (Kramer et al., 2002; Fukumoto et al., 2005). The earliest LR asymmetry in *Xenopus* embryos described to date is the localization of H^+- and K^+-dependent ATPaseα mRNA and protein in the two-cell embryo (Aw et al., 2008); a few hours after fertilization, the amount of these mRNAs and protein is higher in one cell than in the other. The mechanisms responsible for the unequal distribution of H^+/K^+-ATPaseα mRNA and protein are unknown but probably involve unidirectional transport by a motor protein along the cytoskeleton. In addition, directionality of the embryonic cytoskeleton itself, that is the position of the '+' ends and '–' ends, might play a role in moving the motor protein in one direction and further analysis of this role is expected. At the 32-cell stage, the neurotransmitter serotonin is localized asymmetrically in *Xenopus* embryos, and this LR asymmetric localization requires gap junctions and H^+/K^+-ATPaseα (Fukumoto et al., 2005). Furthermore, pharmacological inhibition of serotonin receptors R3 and R4 and injection of mutant serotonin receptor mRNAs in *Xenopus* embryos result in heterotaxia (Fukumoto et al., 2005). These observations thus suggest that H^+/K^+-ATPaseα may determine the distribution of serotonin through gap junctions in the *Xenopus* embryo, and that this might in turn influence LR patterning. Other molecules, such as PKC-γ and phosphorylated syndecan 2, have also been shown to be distributed asymmetrically in *Xenopus* embryos (Kramer et al., 2002). Furthermore, H^+-V-ATPase is also indispensable for left-right asymmetry in *Xenopus* and chick (Adams et al., 2006). In the chick, asymmetric localization of H^+/K^+-ATPaseα mRNA is apparent at Hensen's node and is required for the subsequent establishment of Sonic hedgehog (Shh) asymmetric gene expression (Aw et al., 2008). However, in general, such early asymmetries in mRNA or protein localization have not been studied in detail in other vertebrate embryos.

Symmetry-breaking events and fluid flow

In the mouse, gastrulation in a symmetric embryo results in the formation of the primitive streak at the future posterior side. The formation of the organizer (the node) at the anterior tip of the primitive streak leads to the breaking of symmetry as a consequence of the concerted rotation of cilia present in the organizer (Essner et al., 2005; Nonaka et al., 2005; Schweickert et al., 2007). These cilia rotate in a clockwise direction (when viewed from a ventral position) and subsequently generate a leftward flow of extra-embryonic fluid. The precise mechanism by which the fluid flow results in symmetry breaking is unknown, but it has been suggested that it transports small molecules or vesicular particles to the left side of the embryo (Hirokawa et al., 2006) or exerts mechanical pressure on the left wall of the organizer (McGrath et al., 2003; Tabin and Vogan, 2003).

Two types of cilia, motile and immotile, have been identified in the mouse node, with the latter type, which are present at the edge of the node, having been proposed to sense mechanical force (McGrath et al., 2003). The basal body, an organelle located at the base of each cilium, is located on the posterior side of mouse node cells (Nonaka et al., 2005; Okada et al., 2005). This localization results in a posterior tilt of the cilium, because the surface of node cells is curved. As a consequence of the posterior tilt of the rotation axis of the node cilia, a stroke towards the left side is more efficient in generating fluid flow than is a stroke toward the right side (Cartwright et al., 2004; Nonaka et al., 2005; Okada et al., 2005).

The gastrocoel roof plate (GRP) in *Xenopus* embryos and Kupffer's vesicle (KV) in zebrafish and Medaka embryos are the cilitated organs of asymmetry generation in these organisms. Thus, the GRP and KV similarly possess motile cilia and their movement generates unidirectional flow. In *Xenopus*, fluid flow is necessary only in the left portion of the GRP (Vick et al., 2009), suggesting that the cells on the left side of this structure sense the signal, whether it be mechanical or chemical. In zebrafish, KV acts as the LR organizer but its architecture is different from that of the mouse node. Thus, motile cilia are present at the ventral and dorsal surface inside this vesicle, but they are preferentially found in the anterior region of KV. Although cilia in the anterior region are posteriorly tilted, the basal body is not significantly shifted towards the posterior side. Nonetheless, unidirectional flow (counterclockwise as observed from the dorsal side) is formed in the anterior-dorsal region of KV (Kreiling et al., 2007; Okabe et al., 2008).

The posterior localization of the basal body within node cells can be regarded as a type of planar cell polarity (PCP) and is regulated by the noncanonical Wnt signaling pathway in the mouse and *Xenopus*, and in zebrafish, the distribution of cilia inside KV is also regulated (Maisonneuve et al., 2009; Zhang and Levin, 2009; Antic et al., 2010; Borovina et al., 2010; Hashimoto et al., 2010; Song et al., 2010; Wang et al., 2011). Whereas Dishevelled proteins, mediators of Wnt signaling, are localized to the posterior end of node cells (Hashimoto et al., 2010), the PCP protein Prickle2 is found at the anterior end (Antic et al., 2010). However, the initial anterior-posterior cue responsible for polarizing of the distribution of these proteins is unknown.

Recent studies have shown that calcium might also play a role in the symmetry-breaking event. Imaging of intracellular Ca^{2+} with a Ca^{2+} indicator has revealed an asymmetry in Ca^{2+} signaling on the two sides of the vertebrate organizer (McGrath et al., 2003; Tanaka et al., 2005; Garic-Stankovic et al., 2008; Francescatto et al., 2010). Furthermore, polycystin 2 (polycystic kidney disease 2; Pkd2) and polycystin 1 like 1 (polycystic kidney disease 1 like 1; Pkd1l1), which form Ca^{2+} channels, are required in the mouse for asymmetric *Nodal* expression in the lateral plate mesoderm (LPM) (Pennekamp et al., 2002; Field et al., 2011), and Ca^{2+}/calmodulin-dependent kinase II (CamKII) is required in KV for LR patterning in zebrafish (Francescatto et al., 2010). However, *curly up* (*pkd2*) zebrafish mutant shows bilateral expression of *southpaw* (Schottenfeld et al., 2007), which is different from the phenotype of the *Pkd2* mouse mutant, which lacks Nodal expression in the LPM. These observations implicate Ca^{2+} influx in the establishment of LR asymmetry, but the precise role of such influx remains unknown. Given that the gene for the Nodal inhibitor Cerberus like 2 (Cerl2; also known as Coco) is the earliest known target of the leftward fluid flow in *Xenopus* embryos (Schweickert et al., 2010), it is possible that Ca^{2+} may regulate the expression of this gene directly or indirectly.

In the chick embryo, symmetry breaking does not appear to involve cilia-generated flow. Instead, asymmetric cell migration

around the LR organizer is an early event that has been observed in birds (Cui et al., 2009; Gros et al., 2009); cells migrate from the right to the left around Hensen's node (anticlockwise), resulting in the generation of asymmetric gene expression around this structure. The directionality of this movement is determined by H^+/K^+ ATPase. Intriguingly, the pig embryo appears to lack cilia at the LR organizer (Gros et al., 2009), suggesting that it also uses a cilia-independent mechanism for breaking symmetry. Interestingly, it is known that some migrating cells, including neutrophils, possess intrinsic polarity, which could be generated by a subcellular chiral structure such as the centrosome (Xu et al., 2007; Wan et al., 2011), and it might be possible that such a chiral structure determines the direction of cell migration for LR patterning. In the mouse embryo, however, cells at the periphery of the node move symmetrically around the node (Yamanaka et al., 2007). Furthermore, *Nodal* expression at the node is indispensable for *Nodal* expression in the LPM of the mouse (Brennan et al., 2002). Therefore, symmetry breaking by directional migration might be a mechanism that is specific to only some organisms.

Asymmetric gene expression around the LR organizer

The cilia-generated flow detected in many vertebrates, or the directional cell migration observed in the chick embryo, induces asymmetric gene expression around the organizer. The gene for Cerl2 (Dand5 – Mouse Genome Informatics), which encodes a Nodal antagonist, shows an asymmetric (R>L) expression pattern around the node of the mouse embryo. Cerl2 thus inhibits the activity of Nodal preferentially on the right side and is required for normal LR patterning (Marques et al., 2004). In *Xenopus*, *Coco* is the ortholog of *Cerl2* and is the gene thought to respond earliest to leftward flow; it thus manifests asymmetric expression around the organizer and plays a role similar to that of *Cerl2* (Schweickert et al., 2010), although histone deacetylase activity at earlier stages might be involved in epigenetic silencing of *Nodal* (Carneiro et al., 2011). In zebrafish and Medaka, Charon (Dand5 – Zebrafish Information Network), an ortholog of mouse Cerl2, is asymmetrically expressed (R>L) in KV and is indispensable for left and right asymmetry (Hashimoto et al., 2004; Schneider et al., 2010; Hojo et al., 2007; Lopes et al., 2010).

Asymmetric signal generated in the node is transmitted to the surrounding tissues. Current data collectively suggest that, in the mouse, Nodal protein generated by perinodal cells at the node is transported via the internal route of the embryonic tissue, that is between the endoderm and mesoderm, to the left LPM where it induces its own expression (Oki et al., 2007; Kawasumi et al., 2011). Alternatively, calcium signals may be transmitted from the node to the LPM, given that a calcium signal is detected at the left margin of the node (McGrath et al., 2003). Furthermore, as a result of the R>L asymmetric expression of *Cerl2*, Nodal activity is preferentially inhibited on the right side. Indeed, the distribution of *Nodal* mRNA around the node shows a small asymmetry, whereas the activity of Nodal (as reflected by the level of Smad2/3 phosphorylation) is highly L>R asymmetric (Kawasumi et al., 2011).

In the chick embryo, the establishment of asymmetry around the node seems to involve different signaling pathways. Thus, at the right side of the node, ActivinB, which belongs to the TGFβ superfamily, stimulates the expression of bone morphogenetic protein 4 (Bmp4), which in turn represses Shh expression (Levin et al., 1995; Monsoro-Burq and Le Douarin, 2001). At the left side of the node, where ActivinB is downregulated, Shh is induced, which represses Bmp4 expression and induces expression of *cNR-1* (a Nodal ortholog). This gene network establishes asymmetric gene expression around the node and the resulting information (probably asymmetric Nodal activity, as for the mouse) is transmitted to the left LPM where it activates *cNR-1* expression.

Asymmetric gene expression in the left lateral plate

After symmetry-breaking in the LR organizer, asymmetric gene expression arises in the lateral plate on the left side (Meno et al., 1999; Saijoh et al., 2005; Shiratori et al., 2006). In the mouse, *Nodal*, *Lefty2* and the transcription factor *Pitx2* are expressed in the LPM exclusively on the left side. Whereas Nodal functions as a left-side determinant, Lefty is a feedback inhibitor of Nodal. Nodal induces its own expression via a Nodal-responsive enhancer of *Nodal*, and the operation of this positive feedback loop underlies the expansion of the Nodal expression domain. Nodal also simultaneously induces the expression of Lefty2. This combination of positive and negative feedback loops constitutes a SELI system that is able to amplify a small difference between the left and right of the node into a robust difference in the LPM, because *Nodal* expression in the right LPM is inhibited by Lefty diffusing from the left LPM (Nakamura et al., 2006). Mathematical modeling indicates that the SELI system can establish correct LR patterning only if the diffusion velocity of Lefty is higher than that of Nodal. That Lefty travels faster than Nodal was suggested by an early study (Sakuma et al., 2002), but recent work has directly revealed such a difference in the diffusion velocities of Lefty and Nodal (Marjoram and Wright, 2011). The diffusion of Lefty and Nodal also appears to be facilitated by sulfated proteoglycans of the extracellular matrix (Oki et al., 2007; Marjoram and Wright, 2011).

The SELI system composed of Nodal and Lefty seems to be conserved among vertebrate species (Nakamura et al., 2006; Marjoram and Wright, 2011); however, other genes are also expressed asymmetrically in the LPM. In the chick embryo, bone morphogenetic protein (BMP) represses *Nodal* expression in the LPM, whereas BMP signaling positively upregulates CFC (a member of EGF-CFC: Cripto/FRL-1/Cryptic) expression, which is indispensable for Nodal positive feedback loop (Piedra and Ros, 2002). However, caronte, an inhibitor of BMP signaling that is expressed in the paraxial mesoderm on the right, induces *Nodal* expression in the left LPM by inhibiting BMP activity (Yokouchi et al., 1999). Similarly in the mouse embryo, chordin and noggin, inhibitors of BMP signaling, are expressed at a higher level in the left LPM than in the right LPM, suggesting that BMP signaling might repress *Nodal* expression on the right side (Mine et al., 2008). In addition, the transcriptional repressor snail is preferentially induced by fibroblast growth factor 8 (Fgf8) in the right LPM and represses *Nodal* expression in the chick embryo, whereas mutant mice that conditionally lack *Snail* exhibit bilateral *Nodal* expression (Boettger et al., 1999; Patel et al., 1999; Murray and Gridley, 2006). In the zebrafish embryo, a different mechanism appears to inhibit *Nodal* expression on the right side. Thus, inhibition of *Nodal* on the right side involves a BMP signal at the posterior tip of the LPM and Lefty2 at the anterior side (*Lefty2* expression is present only in the anterior region of LPM in the zebrafish) (Bisgrove et al., 2000; Lenhart et al., 2011).

Asymmetric Nodal signaling in the left lateral plate induces expression of *Pitx2*, which encodes a transcription factor with a bicoid-type homeobox, in the same region. Whereas Nodal induces Pitx2 expression transiently and asymmetrically by activating FoxH1, which is a transcription factor of the TGFβ pathway and

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binds to ASE (asymmetric enhancer), asymmetric *Pitx2* expression is maintained as a result of the activity of Nkx2-dependent enhancer after the transient activation by FoxH1. FoxH1 is responsible for inducing transcription of *Pitx2* whereas Nkx2 maintains *Pitx2* expression once it has been induced (Shiratori et al., 2001).

Asymmetric morphogenesis

Left-sided expression of Pitx2 induces asymmetric morphogenesis of most of visceral organs, including the heart, lungs, stomach, intestine and kidneys. However, the cellular mechanisms responsible for asymmetric organogenesis in vertebrates remain largely unknown. Several studies have provided insight into the mechanism of gut looping. Looping of the chick gut is determined by LR asymmetries in the cellular architecture of the dorsal mesentery, the structure that connects the developing gut to the body wall (Davis et al., 2008; Kurpios et al., 2008; Plageman et al., 2011). Furthermore, the overlying epithelium shows LR asymmetries in cellular morphology that are under the control of Pitx2. Subsequent morphogenesis of gut looping can be explained by the relative growth between the gut tube and the anchoring dorsal mesentery (Savin et al., 2011). In the case of zebrafish, the differential migration of LPM cells on the left and right sides gives rise to gut looping (Horne-Badovinac et al., 2003). This asymmetric migration of LPM cells depends on Nodal signaling but is independent of the overlying endoderm, and it involves cell rearrangements associated with remodeling of the extracellular matrix (Yin et al., 2010). Whether such mechanisms exist in the mouse and in *Xenopus* is under investigation.

During morphogenesis of the heart, leftward looping of the aortic arch results from asymmetric regression of the sixth branchial arch, which is induced by asymmetric blood flow triggered by the spiral rotation of the outflow tract in the mouse embryo (Yashiro et al., 2007). In zebrafish, asymmetric migration of cardiac progenitor cells is responsible for this cardiac rotation. This asymmetric migration is regulated by BMP signaling, which is preferentially activated in the LPM on the left side and is controlled by early asymmetric Nodal signaling (Baker et al., 2008; de Campos-Baptista et al., 2008; Rohr et al., 2008; Smith et al., 2008). However, it remains unknown whether this migration depends on Pitx2 or not. In fact, some of the asymmetric morphogenesis events are independent of Pitx2. For example, heart looping and embryonic turning remain normal in the mutant mouse lacking asymmetric *Pitx2* expression (Shiratori et al., 2006). Interestingly, recent work suggests that the direction of embryonic turning depends on blastomere compositions at the 8-cell stage (Gardner, 2010). This pivotal finding also suggests that a part of asymmetry in the mouse may in fact be determined prior to node formation. It remains to be determined whether the cellular mechanisms proposed for the morphogenesis of individual organs in individual model animals are shared among vertebrates.

Perspectives

In summary, multiple mechanisms contribute to the generation of LR asymmetry in vertebrates and some of these appear to be conserved. The gene network comprising *Nodal*, *Lefty* and *Pitx2*, for example, is highly conserved among animals. Asymmetric expression of *Nodal* also appears to be conserved among deuterostomes (Chea et al., 2005). In the deuterostome sea urchin embryo, for example, these genes are expressed asymmetrically, albeit exclusively on the right side (Duboc and Lepage, 2008), which is possibly owing to the inversion of the dorsal-ventral axis during evolution of bilaterian animals. Nodal is also found in non-deuterostomes, for example in snails that belong to the Lophotrochozoa (Grande and Patel, 2009; Kuroda et al., 2009). Lefty is similarly conserved although its precise expression domains might vary.

By contrast, the cellular mechanisms underlying asymmetric morphogenesis have probably diverged among species. Furthermore, there is considerable diversity in initial symmetry breaking even among vertebrates; whereas many vertebrates rely on unidirectional flow generated by cilia, the chick and pig do not adopt this strategy, and both the frog and chick exhibit asymmetries prior to organizer formation. Furthermore, chiral blastomere arrangement controls LR asymmetry in snails (Kuroda et al., 2009), whereas H^+/K^+-ATPase determines the asymmetry in *Ciona* (Shimeld and Levin, 2006). The different strategies deployed for symmetry breaking (reviewed by Spéder et al., 2007) may originate from the divergence of morphologies or developmental processes (or both) among organisms. A key challenge is to determine to what extent each step of the LR pathway is conserved among species. It will be interesting to know the precise mechanism of symmetry breaking in diverse organisms. Another challenge is to clarify the cellular mechanisms underlying asymmetric morphogenesis, including those occurring during heart looping, branching and turning, which are largely unknown at present. Further development of various approaches, genetic, cellular, biophysical and mathematical, will undoubtedly help to clarify these issues.

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Competing interests statement
The authors declare no competing financial interests.

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