Review

Human Corneal Endothelial Cells Expanded *In Vitro* Are a Powerful Resource for Tissue Engineering

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Abstract

Human corneal endothelial cells have two major functions: barrier function mediated by proteins such as ZO-1 and pump function mediated by Na-K-ATPase which help to maintain visual function. However, human corneal endothelial cells are notorious for their limited proliferative capability *in vivo* and are therefore prone to corneal endothelial dysfunction that eventually may lead to blindness. At present, the only method to cure corneal endothelial dysfunction is by transplantation of a cadaver donor cornea with normal corneal endothelial cells. Due to the global shortage of donor corneas, it is vital to engineer corneal tissue *in vitro* that could potentially be transplanted clinically. In this review, we summarize the advances in understanding the behavior of human corneal endothelial cells, their current engineering strategy *in vitro* and their potential applications.

Key words: cornea, endothelial, progenitor, regenerative medical application.

Introduction

The human corneal tissue consists of the epithelium, Bowman's layer, the stroma, Descemet's membrane, and the endothelium. The epithelium is a well-characterized self-renewing layer with stem cells at its peripheral areas. The stroma cells are usually a group of small quiescent cells, which play an important role in maintenance of corneal functions [1]. In contrast, the endothelial cells form a single hexagonal monolayer located at the Descemet's membrane in the posterior cornea [2], and play a significant role in maintaining visual function [3]. As a result of aging, diseases, injury or surgeries, corneal blindness may occur due to dysfunctional human corneal endothelial cells (HCECs), such that there are insufficient numbers and density of HCEC in a

disease so called "bullous keratopathy" (reviewed in [4]) or fibroblast metaplasia due to endothelial-mesenchymal transition (EMT) [5]. Such EMT also occurs in *in vitro* culture of HCECs if the cell-cell junctions are disrupted. Interestingly, the conventional approach of isolating HCECs using trypsin-EDTA to break intercellular junctions might cause EMT and change the HCEC phenotype to fibroblastic-like shape [6]. Such change of phenotype has been shown to be due to activation of canonical Wnt signaling in the presence of EGF and/or bFGF, especially when TGF-β1 is added which activates canonical TGF-β signaling resulting in nuclear translocation of pSmad2/3 and Zeb1/2 [7]. Other groups also showed that TGF-β1/2 inhibits expansion

induced by bFGF in trypsin-EDTA treated HCECs [8], rat CECs [9], and bovine CECs [10]. Interestingly, the use of SB431542, a selective inhibitor of the TGF-βR, may block EMT in HCECs [11]. Since the result of EMT is loss of the normal HCEC phenotype, the inhibition of canonical Wnt-Smad2/3-Zeb1/2 signaling is necessary during the expansion of HCECs. Although HCECs may proliferate *in vitro*, unfortunately, HCECs do not normally proliferate *in vivo* [12] due to arrest at the G1 phase in the cell cycle [13]. Hence, corneal blindness may develop as mentioned previously [4, 5]. Until now, the only effective medical treatment is by corneal transplantation from healthy donors. In this review, we will review *in vitro* expansion of corneal endothelial cells, their potential application in the treatment of human blindness and other corneal diseases.

Contact Inhibition of HCECs

The corneal endothelium form a single hexagonal monolayer located at the Descemet membrane of the posterior cornea and face the TGF-β2-containing aqueous humor [14]. Through tight junction ZO-1 and adherent junction Na-K-ATPase, human corneal endothelial cells (HCECs) mediate corneal stromal hydration and transparency via barrier and pump functions, respectively (reviewed in [15]). Unlike murine, rabbit, and bovine cells, HCECs have limited proliferative capability *in vivo* after aging, injury and surgery [12]. HCECs' limited proliferative capability has been shown to be caused by "contact inhibition" at the G1 phase of the cell cycle (reviewed in [16]). Contact inhibition is also reported in human corneal explants [17], cat explants [18] and rat corneal endothelial cultures [19] when the cells reach confluence.

Expansion of HCECs *in Vitro*

Substrates

The substrates for culturing HCECs have been reported by several groups around the world [17, 20-23]. The attachment and growth of HCECs ex vivo and *in vitro* can be facilitated by artificial matrices, such as collagen I and fibronectin [24], collagen IV [25, 26], chondroitin sulfate and laminin [27], laminin-5 [28], matrigel [23] and FNC coating mix [29]. Although the substrates such as matrigel, laminin and fibronectin have been widely used for the expansion of HCECs, it has been reported that collagen IV is optimal for the expansion of HCECs for tissue engineering purposes [30].

Media

Several culture media have been proposed for the expansion of HCECs, for example, Dulbecco's Modified Eagle Medium (DMEM), DMEM/F12, Ham's F12/M199 and Opti-MEM-I [31]. The effect of the four culture medias in the isolation and growth of HCECs were compared by Peh and his associates [32]. They noted that HCECs cultured in these four media quickly attached and expanded when cultured on FNC-coated dishes. Nevertheless, HCECs cultured in DMEM and DMEM/F12 could not be expanded more than the first and second passage. In contrast, HCECs cultured in Opti-MEM-I and Ham's F12/M199 were cultured far beyond the first and second passage (reviewed in [31]). The HCECs cultured in Opti-MEM-I and Ham's F12/M199 within the third passage expressed human corneal endothelial markers such as Na+K+/ATPase and ZO-1 (reviewed in [31]), however the cultured HCECs were not hexagonal beyond the third passage. Furthermore, five culture medias were compared for their effect on the survival of corneal endothelium [33]. The five different medias included HCEC growth medium (F99), MEM with 2% fetal calf serum (FCS), MEM with 5% FCS, and serum-free medium (SFM). Their conclusion was that the counts of apoptotic cells in the untreated controls was significantly higher when cultured in MEM than those when cultured with F99 and SFM [33]. Zhu et al recommended a medium composed of OptiMEM-I plus 8% fetal bovine serum (FBS), 20 ng/mL nerve growth factor (NGF), 5 ng/mL epidermal growth factor (EGF), 100 μg/mL bovine pituitary extract, 20 μg/mL ascorbic acid, 200 mg/mL calcium chloride, and 0.08% chondroitin sulfate for corneal endothelial expansion [29]. Once the cells were confluent, the cultures were switched to a culture medium without fibroblast growth factor (FGF), EGF, NGF, or pituitary extract, for a few days in order to stabilize the morphology of the monolayer which was shown to be similar to that *in vivo* [29]. They claimed that with this method, primary HCECs might reach confluence within ten days. Recently, a success was reported in the culture and maintenance of HCECs in a serum-free medium called MESCM [34], which is advantageous due to serum-free medium avoiding potential contamination by viruses, bacteria and other infectious agents.

Growth Factors and Cytokines

Several growth factors have been proposed to promote the growth of HCECs, for example, FGF [17, 20, 22, 27, 35, 36], LIF [34, 37], EGF [6, 20, 22, 25, 26, 36, 38], NGF [20] and endothelial cell growth supplement [22, 39]. It has been suggested that LIF delays contact-inhibition and more effectively promotes

HCEC growth with bFGF without serum and without change of the phenotype [34, 37].

Endothelial Mesenchymal Transition due to Disruption of Cell-Cell Junctions Is the Obstacle for *in Vitro* Growth of HCECs

Contact-inhibition is present in non-transformed normal cells when neighboring cells are in contact with one another. This fact causes low regenerative capability of *in vivo* human corneal endothelial cells. The conventional approach to grow HCECs *in vitro* is to break the cell-cell junctions by trypsin-EDTA and culture them in a medium containing growth factors such as FGF [27]. However, such a culture method may cause "endothelial-mesenchymal transformation" (EMT), which is a pathologic process that may eventually result in corneal blindness (reviewed in [5]). It is believed that the use of trypsin-EDTA to break the cell-cell junctions and then to culture HCECs in a bFGF-containing medium causes EMT via activation of the canonical Wnt signaling, especially when TGF-β1 signaling is also activated [6].

In contact-inhibited cultures of HCEC monolayers [25, 26], a short exposure of the HCEC monolayers to 5 mM EDTA for 1 h results in significant disruption of intercellular junction [6]. Without FGF, cells recover their pre-treated monolayer morphology in 2 days. However, when 20 ng/ml FGF is present for 2 days, 10 ng/ml TGFβ1 is present for 3 days, or 20 ng/ml FGF is present for 2 days followed by 10 ng/ml TGFβ1 for 3 days, HCECs turn into fibroblastic-like cells because of EMT. This phenomenon is closely associated with activation of canonical Wnt signaling and canonical TGF-β-Smad2/3 signaling [6]. A similar result is also noted in contact-inhibited ARPE-19 epithelial cells [7] [40]. Remarkably, a 5-minute treatment of trypsin/EDTA caused fibroblastic shape change of appearance in contact-inhibited HCEC monolayers within 24 h, which failed to fully recover the hexagonal shape 28 days later in EGF-containing SHEM [34]. This morphological alteration was accompanied by activation of canonical Wnt signaling as evidenced by nuclear localization of β-catenin and LEF1, 4- and 6-fold increase of transcript expression of β-catenin and LEF1, 13- and 15-fold elevation of nuclear β-catenin and LEF1, and 17-fold increase of TCF/LEF promoter activity [34].

Novel Expansion of HCECs with Normal Phenotype by Preserving Cell-Cell Junctions

An *in vitro* model system of HCEC monolayers has been established that exhibit a mitotic block regulated via contact inhibition, which preserves cell-cell junction and cell-matrix interaction during isolation and subsequent expansion [25] [26]. Remarkably, the contact inhibition of HCEC monolayers can safely be perturbed by transient knockdown with p120 catenin and Kaiso siRNAs, which activates non-canonical BMP-NFkB signaling in SHEM without disrupting the intercellular junction and without causing EMT after collagenase digestion of HCECs [6] [34]. This p120-Kaiso signaling is linked to activation of RhoA-ROCK signaling, which destabilizes microtubules, and inhibits Hippo signaling, but not Wnt signaling. As a result, human corneal endothelial cells maintain a hexagonal shape with junctional expression of N-cadherin, ZO-1, and Na-K-ATPase during their growth [6]. Such engineering technology has successfully produced HCEC monolayers with a hexagonal shape and *in vivo* cell density [6] [34].

Without p120-Kaiso knockdown, MESCM promoted growth of HCEC monolayers in diameter from 1.4 mm in SHEM to 4.4 mm after 6 weeks of culture. With p120-Kaiso knockdown, MESCM promoted growth of HCEC monolayers from 5.0 mm in SHEM to 11.0 mm in diameter, i.e., a good size for clinical transplantation. BrdU labeled nuclei were only found in MESCM [6, 34]. The proliferative effect regulated by non-canonical BMP signaling in SHEM was not linked to higher mRNA expression of embryonic stem cell and neural crest cell markers. In contrast, such dramatic proliferative effect was associated with higher transcript expression of embryonic stem cell markers such as Nanog, Nestin, Oct4, Sox2, SSEA4 and neural crest markers, for example AP2β, FOXD3, and SOX9, which was completely blocked by BMP inhibitor noggin, indicating that the reprogramming is controlled by the canonical BMP signaling [34]. Correspondingly, immunostaining staining of FOXD3, Nanog, Nestin, Oct 4, SOX2, SOX9 and SSEA4 was found in p120 or p120-Kaiso siRNA treated HCECs [34]. In addition, Nanog, Oct 4 and SOX-2 were translocated to the nucleus with a significant increase in the expression of miRNA 302b* and miRNA 302c* only in p120 or p120-Kaiso siRNA treated HCECs [34], indicating that the cells were in active reprogramming. Furthermore, such over-expression and nuclear translocation of ESC markers and neural crest markers was attenuated by noggin, an extracellular BMP inhibitor [34]. These findings support the notion that the switch from non-canonical to canonical BMP signaling results in the reprogramming of HCECs to embryonic- or neural crest-like progenitors. Because such a reprogramming process was completely blocked by Noggin, which also blocked BrdU labeling, the

effective growth of HCEC monolayers is successful due to the activation of canonical BMP signaling in MESCM that reprograms HCECs into their progenitor status [6] [34]. Remarkably, withdrawal of p120 siRNA in SHEM maintains *in vivo* morphology, density and phenotype [6, 34], which is also true when the cells are cultured in MESCM [34]. Compared to the *in vivo* HCECs, the resultant cell shape retained hexagonal shape during the entire experimental period (i.e. 5 weekly treatments of p120-Kaiso siRNAs followed by withdrawal for one week) for both scrambled(sc)RNA and p120-Kaiso siRNA [34]. The final HCEC monolayer maintained expression of acetylate-α-catenin (a marker of cilium), cytoplasmic expression of γ-tubulin and p75NTR, junctional expression of α-catenin, β-catenin, F-actin Na-K-ATPase, N-cadherin, p120 and ZO-1 (the markers of HCECs), and without enhanced transcript expression of LEF1 and S100A4 (the markers of EMT) [34]. The expression pattern is similar to that *in vivo,* reported previously [26].

Human Corneal Endothelial Progenitors Are Powerful Resources of HCEC Regeneration

Hatou et al (2013) reported that functional corneal endothelium can be obtained from corneal stromal stem cells of neural crest origin, called cornea derived precursors (COPs) [41]. Hatou et al isolated human COPs by 400 U/ml type I collagenase digestion, expanded them on plastic at a density of 1×10^5 cm^2 in a medium containing 1:1 DMEM/F-12 supplemented with 20 ng/ml EGF, 20 ng/ml bFGF, B27 and N2 for 1 week [41]. The researchers then reprogrammed these single progenitor cells into HCECs by seeding at the density of 2×10^5 cm^2 on 0.1% gelatin or 1.0 mg/ml laminin- or type I collagen –coated dishes in MEM supplemented with 1% FBS, 1 mM all-trans retinoic acid, 1 mM GSK 3β inhibitor (6-bromoindirubin-3'-oxime), 5 ng/ml TGFβ2, 10 mM ROCK inhibitor Y-27632, and 1 mM insulin for 1 week. The authors used endothelial markers such as Atp1a1, Slc4a4, Car2, Col4a2, Col8a2, and Cdh2 (and Pitx2, a homeobox gene involved in the development of the anterior segment of the eye) to demonstrate the endothelial characteristics, and showed the Na-K-ATPase activity expressed by endothelial cells. However, p120, ZO-1, F-actin, N-cadherin, β-catenin and Na-K-ATPase were also used to characterize the HCEC phenotype [6]. In contrast, Hara et al (2013) reported that HCECs can be generated from human corneal endothelial progenitors (HCEPs) with neural crest phenotype [42]. This group of scientists used enzyme cell detachment medium (accutase, Life

Technology) to detach the cells from Descemet membranes, expanded the cells in a medium containing 1:1 DMEM/F12, 20% knockout serum, 4 ng/ml bFGF on laminin 511, differentiated the cells in a low glucose DMEM with 10% FBS on FNC coated dishes in 3-4 weeks, and used neural crust markers such as P75NTR, AP2β, SOX-9, Snail, Slug and PITX2 to demonstrate the neural crust phenotype of HCEPs. The authors also used COL8A1 and COL8A2 as HCEC markers, FOXC-2 as a mesenchymal marker to demonstrate their mesenchymal characteristics and colony formation assay to demonstrate their cells have stem-cell like characteristics, and determined Na-K-ATPase activity to demonstrate that the cells have pump function similar to endothelial cells. It is unclear whether neural crest like cells generated from HCF can differentiate into HCECs in MEM supplemented with 1% FBS, 1 mM all-trans retinoic acid, 1 mM GSK 3β inhibitor (6-bromoindirubin-3'-oxime), 5 ng/ml TGFβ2, 10 mM ROCK inhibitor Y-27632, and 1 mM insulin, or in a low glucose DMEM with 10% FBS on FNC coated dishes. It is also unclear whether the neural crest cells generated from HCF can differentiate into keratocytes, if so, whether culture of the neural crest like cells generated from HCF in Hatou expansion medium could expand the neural crest like cells, and if so, whether the neural crest like cells can differentiate into HCECs or other type of cells.

The culture methods from Hatou (2013) [41] and Hara (2013) [42] are summarized in Table 1.

Although a number of groups have reported the presence of human corneal endothelial progenitor cells [43-47], the detailed features of the progenitors, including whether the progenitors can be reprogrammed into neural crest progenitors and whether such reprogrammed progenitors have multi-plasticity and more proliferative potential have not been revealed until recently. It has been reported that transient knockdown with p120 catenin (p120) and Kaiso siRNAs activates p120-Kaiso-RhoA-ROCK-canonical BMP signaling when cultured in LIF and bFGF-containing MESCM [34], which results in effective growth of HCEC monolayers because of reprogramming adult HCECs into their progenitor status [34]. It has also been reported by the same group that without p120-Kaiso knockdown, transit activation of LIF-JAK1-STAT3 signaling may promote growth of human corneal endothelial progenitor cells by delaying contact-inhibition but not reprogramming [37], suggesting that LIF-mediated signaling acts synergistically with BMP signaling to promote the reprogramming and expansion of HCEC monolayers.

Table 1. Methods for isolation and expansion of human corneal progenitors and their differentiation to corneal endothelial cells.

Purpose	Cell Name	Isolation	Culture Cell Density	Growth Medium				Substrate	Time	References
				Basal Medium	Serum (%)	Growth Factors	Supplements			
Expansion	HCEPs	Cell detachment Medium (Accutase)	100-300 cells/cm²	1:1 DMEM/F12	20% KO Serum	4 ng/ml bFGF		20 µg/ml Laminin 511	To sub-confluence	Hara, 2013
Differentiation	HCECs	Accutase	Passaged 1:2	Low Glucose DMEM	10%			FNC Mix	3-4 Weeks	Hara, 2013
Expansion	COPs	400 U/ml type I collagenase	1×10⁵ Cells/cm²	1:1 DMEM/F12		20 ng/ml EGF, 20 ng/ml bFGF	B27 and N2	None	Not found in the Paper	Hatou, 2013
Differentiation	HCECs	?	2×10⁵ Cells/cm²	MEM	1%	5 ng/ml TGFβ2	1 mM all-trans retinoic acid, 1 mM GSK 3β inhibitor (6-bromoindirubin-3'-oxime), 10 mM ROCK inhibitor Y-27632, 1 mM insulin.	0.1% gelatin; 1 µg/ml laminin; 1 µg/ml type I collagen	1 week	Hatou, 2013

Interestingly, the delay of contact-inhibition is via inhibition of nuclear translocation of p16INK4a, important cyclin-dependent kinase inhibitors (CKIs) in the cell cycle regulation [37].

In mammalian cells, the G1/S transition is blocked through contact-inhibition. The cell cycle progression is negatively controlled by contact inhibition but facilitated by E2F, of which the activity is negatively regulated by non-phosphorylated retinoblastoma tumor suppressor (Rb) [48]. Release of inhibition mediated by phosphorylation of Rb is controlled positively by cyclin D1/cyclin-dependent kinase-4 (CDK4) and cyclin E/CDK2 complex, but negatively by cyclin-dependent kinase inhibitors (CKIs) such as p16INK4a, p15INK4b, p18INK4c, p19INK4d, p21CIP1, p27KIP1, and p57KIP2 [49]. Without p120-Kaiso knockdown, it has been reported that LIF-JAK1-STAT3 signaling delays contact inhibition but not reprogramming of HCEC monolayers [37], suggesting that canonical BMP signaling is indeed critical for reprogramming induced by p120 and Kaiso siRNAs in MESCM [34]. In this case, the miR-302 cluster acts on multiple targets to promote human somatic cell reprogramming [50], nucleus-translocated Oct4, Sox2, and Nanog may activate expression of this miR-302 cluster [51, 52] and miR-302 may indirectly mediate expression of Oct4, Sox2, and Nanog [53, 54], suggesting that miR 302 plays a significant role in such reprogramming. Although more work is needed to illustrate the detailed mechanism of reprogramming of HCECs and other cells into their progenitor status, more evidence has indicated that p16INK4a plays an important role in the reprogramming process, which can be negatively mediated by Bmi1 [55-57]. Still there is a controversial report that states Rho kinase inhibitor Y-27632 enables cell-based therapy for corneal endothelial dysfunction [58].

Potential Clinical Application of Human Corneal Endothelial Grafts after Pre-Clinical Animal Studies

In past decades, several laboratories have reported a number of carriers for the construction of HCEC sheets. Initially, full-thickness corneal transplantations of reconstructed grafts with cultured human CECs [59-61] and animal CECs were performed in rabbit [62, 63], bovine [64], cat [65] and murine [66]. As early as 1979, the CEC sheets constructed with cultured CECs were tested in bovine and rabbit models (i.e. bovine corneal endothelial cells were transplanted onto bovine and rabbit corneas denuded of their endothelium) [62, 65]. Subsequently, cultured HCECs were tested on human corneas denuded of the endothelium [17, 67, 68].

Due to rapid hydration of the grafted PIPAAm, adherent culture of the cells might be separated spontaneously from these surfaces by decreasing culture temperature without the need for proteolytic enzymes [69]. The cells in the HCEC sheets were mostly hexagonal with a lot of microvilli and cilia, resembling the native corneal endothelium under electron microscopy. Lai and his associates also expanded HCECs on a thermoresponsive type of PNIPAAm as a carrier of cultivated HCEC grafts [70]. Choi and his associates used decellularized thin-layer human corneal stroma as a carrier [71]. Liang and his associates also developed a chitosan-based membrane made of hydroxyethyl chitosan, gelatin, and chondroitin sulfate as a new carrier of cultured HCEC sheets [72]. In addition, Nishida and his associates created gelatin hydrogels as a carrier for HCECs [73]. Gelatin hydrogel discs was also tested as a carrier of cultured HCEC sheets [70]. Decellularized thin-layer human corneal stroma was also tested as a carrier [71]. A chitosan-based membrane consisting of gelatin,

hydroxyethyl chitosan and chondroitin sulfate was used as a carrier of HCEC grafts *in vitro* [72]. Similarly, gelatin hydrogels were tested as carrier sheets of HCEC grafts [73] and collagen I carrier used for *in vitro* monkey CEC grafts [74, 75]. A comprehensive review has been published recently to guide tissue engineering of human corneal endothelial grafts (reviewed in [30]).

Despite this progress, there still remain some major challenges in this field. For instance, there is no clear established animal model to test these engineered HCEC grafts that will correlate to clinical success in humans. It has been reported that Dr. Tseng's group have been testing their HCEC grafts generated from HCEC progenitors in a mini-pig model recently. Mini-pigs have reported similar anatomical ocular characteristics to humans and swine CECs do not proliferate *in vivo* which may make it a viable option. Such test is promising because if successful, the grafts can be used for curing the blindness due to deficiency of human corneal endothelial cells, a common disease in this world. In fact, a clinical trial for endothelial cells is currently in progress, suggesting the cultivated ocular cells are a promising alternative in the future (reviewed in [76]). However, such transplantation of HCECs may be dependent on their surgical manipulation and further testing will be needed on their stability, sterility, purity and viability to fulfill the rigorous demands of notified bodies for approval.

Conclusion

New techniques of endothelial keratoplasty surgery with healthy HCEC have quickly replaced conventional penetrating keratoplasty surgery as a preferred procedure for clinical treatment of endothelial diseases. This review has highlighted the latest discoveries and innovations in engineering HCEC grafts to overcome the worldwide shortage of donor corneas. The novel techniques presented in this article are good examples for clinical treatments of CEC dysfunction. Since CECs from the peripheral cornea contain more CEC precursors than CECs from the central cornea in a rabbit model [77] and a human model [46], expansion of peripheral cells by MESCM and p120-Kaiso knockdown may eventually be successful in transplantation of HCEC grafts [34]. Such reprogramming of HCEC progenitors should facilitate engineering of HCEC grafts for repair and regeneration of human corneal endothelium. Innovative breakthroughs of engineered HCEC grafts *in vitro* is now promising to bring cultivated HCECs from bench to bedside.

Abbreviations

bFGF: Basic fibroblast growth factor
BMP: Bone morphogenic protein
BrdU: Bromodeoxyuridine
DLEK: Deep lamellar endothelial keratoplasty
DMEK: Descemet membrane endothelial keratoplasty
DSAEK: Descemet stripping automated endothelial keratoplasty
EK: Endothelial keratoplasty
EDTA: Ethylenediaminetetraacetic acid
EMT: Endothelial-mesenchymal transition
EGF: Epidermal growth factor
HCECs: human corneal endothelial cells
LEF1: lymphoid enhancer-binding factor 1
LIF: Leukemia inhibitory factor
NGF: Nerve growth factor
MESCM: modified embryonic stem cell medium
P120: p120 catenin
RPE: retinal pigment epithelial cells
siRNA: Small interfering ribonucleic acid
SHEM: supplemental hormonal epithelial medium
TGF: Transforming growth factor

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Competing Interests

The authors have declared that no competing interest exists.

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