Conversion of embryonic stem cells into neuroectodermal precursors in adherent monoculture

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Mouse embryonic stem (ES) cells are competent for production of all fetal and adult cell types[1]. However, the utility of ES cells as a developmental model or as a source of defined cell populations for pharmaceutical screening or transplantation is compromised because their differentiation *in vitro* is poorly controlled[2]. Specification of primary lineages is not understood and consequently differentiation protocols are empirical, yielding variable and heterogeneous outcomes. Here we report that neither multicellular aggregation[3,4] nor coculture[5] is necessary for ES cells to commit efficiently to a neural fate. In adherent monoculture, elimination of inductive signals for alternative fates is sufficient for ES cells to develop into neural precursors. This process is not a simple default pathway, however, but requires autocrine fibroblast growth factor (FGF). Using flow cytometry quantitation and recording of individual colonies, we establish that the bulk of ES cells undergo neural conversion. The neural precursors can be purified to homogeneity by fluorescence activated cell sorting (FACS) or drug selection. This system provides a platform for defining the molecular machinery of neural commitment and optimizing the efficiency of neuronal and glial cell production from pluripotent mammalian stem cells.

The task of directing ES cell commitment represents a challenge to understanding of germ layer diversification in vertebrate embryos. The mechanism of neuroectoderm formation from pluripotent founder cells is controversial. Studies in *Xenopus laevis* and chick embryos have given rise both to a permissive, or default, model[6] and to an instructive model[7]. For ES cell differentiation it has been shown that neural fates emerge in the absence of serum or added growth factors in multicellular aggregates[4] or in suspension culture of single cells[8]. However, in the latter case this occurs in only a small fraction (0.1–0.2%) of the cells[8]. Higher levels of neural differentiation are achieved by treatment of aggregates with retinoic acid in the presence of serum[3] or by coculture with a particular stromal cell line, PA6 (ref. 5). The action of retinoic acid is pleiotropic and of indeterminate physiological relevance, whereas the effect of PA6 cells is attributed to an undefined neural inducing activity, stromal cell–derived inducing activity (SDIA)[5].

We developed a green fluorescent protein (GFP) knock-in reporter ES cell line to examine the process by which ES cells acquire neural identity. The open reading frame of the *Sox1* gene was replaced with the coding sequence for GFP and an internal ribosome entry site (IRES)-linked puromycin resistance gene. Sox1 is the earliest known specific marker of neuroectoderm in the mouse embryo[9]. It is first expressed in the neural plate and subsequently maintained in neuroepithelial cells throughout the entire neuraxis, but is downregulated during neuronal and glial differentiation. This highly restricted expression was recapitulated by the GFP reporter following germline transmission (Fig. 1A). Fidelity of the *Sox1*-GFP reporter is likewise maintained *in vitro*. GFP was not detectable in undifferentiated ES cells (Fig. 1B) but becomes evident in a significant proportion of cells after induction of neural differentiation by aggregation and treatment with retinoic acid[10].

We used *Sox1*-GFP knock-in (46C) ES cells to investigate whether multicellular aggregation was necessary for activation of *Sox1* expression and neural determination. ES cells were plated on gelatin-coated tissue culture plastic, and differentiation was triggered by withdrawal of leukemia inhibitory factor (LIF). In serum-containing medium, *Sox1*-GFP positive cells did not appear. In the absence of serum, however, withdrawal of LIF resulted in the emergence of GFP-positive cells in the rosette conformations typical of neuroepithelial cells (Fig. 1C). When 46C cells were plated on gelatin-coated plastic in a conventional serum-free culture medium with N2 and B27 supplements, more than 60% of cells expressed GFP by 4 days (Fig. 1D, E). Plating efficiency in N2B27 medium was high (>50%), and the exponential increase in cell numbers throughout the culture period (Fig. 1F) was evidence of high cell viability. Moreover, when the ES cells were plated on laminin or fibronectin, they produced large numbers of differentiated flattened cells that were *Sox1*-GFP negative, demonstrating that N2B27 medium does not select against other cell lineages.

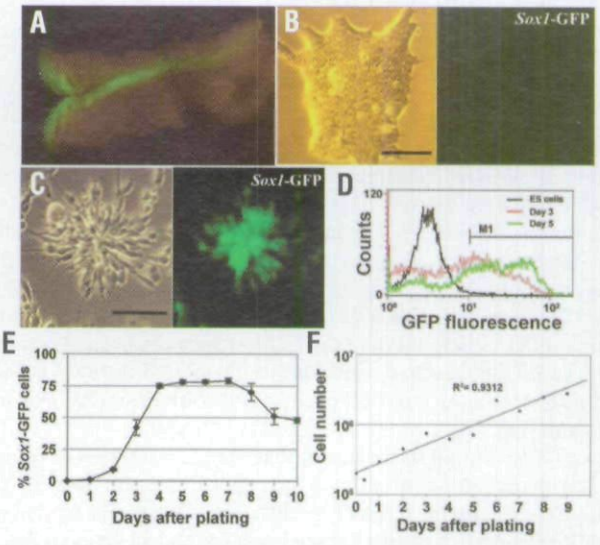

Figure 1. *Sox1*-GFP reporter of neural specification. (A) GFP fluorescence restricted to the neural tissues of embryonic day 8.5 mouse embryo following germline transmission of $Sox1^{GFP}$ allele. (B) Undifferentiated 46C ES cells do not express GFP. (C) Expression of GFP in rosette of neural precursors derived from 46C ES cells plated in N2B27. (D) Flow cytometry profiles of *Sox1*-GFP activation in N2B27. (E) Kinetics of *Sox1*-GFP positive cell population during monolayer differentiation. Gates were set at 10 units of fluorescence, excluding 99% of the undifferentiated ES cell population. Data are means ± s.d. for triplicate determinations from two independent experiments. The decline in GFP positive cells after day 8 occurs because differentation into neurons and glia is accompanied by downregulation of *Sox1*. (F) Plot of cell numbers during monolayer differentiation of 46C cells. Data are means from the same experimental series as shown in (E). Bar, 50 μm.

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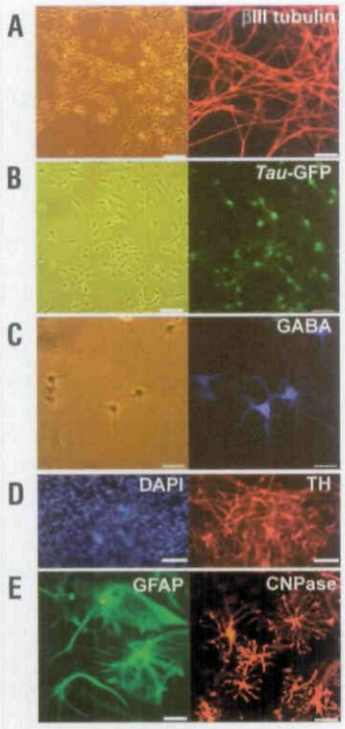

Figure 2. Neuronal and glial differentiation in monolayer culture. (A) Phase contrast and type III β-tubulin immunostained images of neurons generated by monoculture of 46C ES cells in N2B27. (B) Phase contrast and GFP expression from *Tau* knock-in after monolayer differentiation of TK23 ES cells with replating on fibronectin in N2B27. (C) Phase contrast and GABA immunostaining of neurons generated by monolayer differentiation with replating on fibronectin in N2B27. (D) DAPI and tyrosine hydroxylase immunostaining after replating of monolayer neural precursors and exposure to FGF8 and SHH. (E) GFAP and CNPase immunostaining of astrocytes and oligodendrocytes, respectively, generated 10–14 d after replating monolayer precursors in N2B27 (plus 1% serum for oligodendrocytes). Bar, 50 μm.

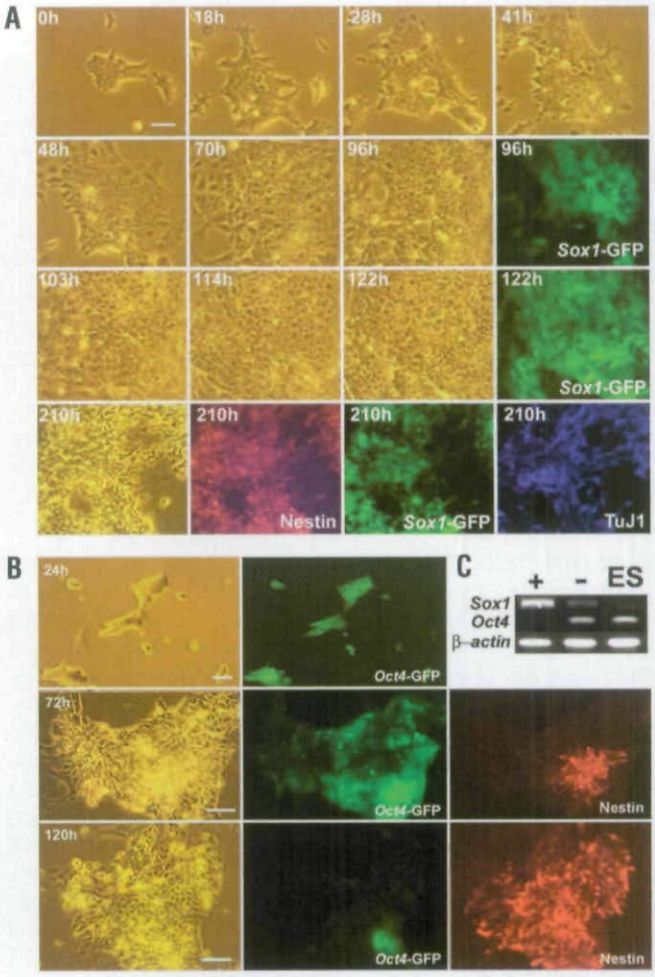

Figure 3. Visualization of neural determination of ES cells. (A) Sequential images of a single colony during 46C monoculture differentiation. Cells were plated at low density in ES cell medium containing serum and LIF and transferred to N2B27 without LIF after overnight incubation (Time 0). After 210 h cells were fixed and immunostained for nestin and class III β-tubulin (TuJ1). GFP fluorescence was captured at the indicated times. (B) *Oct4*-GFP and nestin expression in Oct4GiP cells 24, 72, and 120 h after plating in N2B27. There was no detectable nestin immunoreactivity at 24 or 48 h. Immunostaining for Oct4 protein gave comparable results to the distribution of *Oct4*-GFP. (C) Separation of *Sox1*- and *Oct4*-expressing cells by FACS. RNAs were prepared from undifferentiated 46C ES cells or from GFP-positive (58.5%) and GFP-negative (17.5%) populations on day 4 of monoculture differentiation and analyzed by RT-PCR. Bar, 50 μm.

Cells with overt neuronal morphology and marker expression became apparent from 5 days after LIF withdrawal (Fig. 2A). To monitor the production of neurons during monolayer differentiation, we engineered a second ES cell line, TK23, in which GFP is integrated into the *Tau* locus and is therefore expressed only in neurons[11]. *Tau*-GFP-positive cells first appeared after 4 days of monoculture differentiation. When the cultures were dissociated between day 6 and 8 and replated onto fibronectin-coated dishes, ~60% of cells developed into *Tau*-GFP-positive cells with extended neuronal processes (Fig. 2B).

TK23 differentiation illustrates that the phenomenon of monoculture neural differentiation is not specific to the 46C ES cell clone. To date we have tested 15 different ES cell clones, derived from 3 independent primary ES cell isolates, and all generated neurons efficiently with this protocol. Many of the neurons formed in N2B27 are immunopositive for γ-aminobutyric acid (GABA) (Fig. 2C), with few tyrosine hydroxylase–positive cells. However, replating and addition of FGF8 and sonic hedgehog[12] resulted in significant numbers of tyrosine hydroxylase–immunoreactive neurons (Fig. 2D). Therefore, neural precursors generated by monolayer differentiation are malleable and can be directed into particular neuronal fates. They can also produce both astrocytes and oligodendrocytes (Fig. 2E).

The monolayer protocol facilitates visualization of the process of neural conversion. Figure 3A presents sequential images of a representative colony of 46C cells during the transition from undifferentiated ES cells to *Sox1*-GFP-positive neural precursors. It is evident that neural identity is acquired throughout the colony without significant cell loss but also that the activation of *Sox1*-GFP is not synchronous in all cells.

We examined the profile of the ES cell transcriptional organizer Oct4 (ref. 13). Immunostaining for Oct4 protein and GFP fluorescence from an *Oct4-GiP* transgene[14] gave comparable results. Figure 3B shows the time course of downregulation of *Oct4*-GFP coincident with upregulation of the neuroepithelial marker nestin. GFP expression is evident uniformly in all cells 24 hours after plating. Oct4 began to be lost from the population only after 48 hours, coincident with the emergence of nestin-positive cells. The loss of Oct4 was also asynchronous within the culture. Furthermore, some clusters of cells escaped differentiation and retained Oct4 expression indefinitely.

We used FACS purification to verify that *Sox1*-GFP-expressing 46C cells are distinct from ES cells and constitute neural precursors.

RT-PCR analysis of sorted GFP-positive and GFP-negative populations confirmed that the former expressed *Sox1* mRNA as expected and lacked detectable levels of *Oct4* transcript (Fig. 3C). Conversely, the GFP negative population had very low levels of *Sox1* mRNA and appreciable *Oct4* mRNA, indicating persistence of undifferentiated ES cells. We next plated the sorted cells in the presence of serum and LIF to test for ES cells. ES cell colonies were readily generated from GFP-negative cells but arose only at very low frequency (<0.1%) from the GFP-positive population. Contamination of the FACS could account for the latter result, although it is also possible that very primitive neural cells may retain the capacity to regenerate pluripotent cells[8].

We replaced the N2B27 supplements in a stepwise fashion (see Supplementary Table online). Removal of B27 did not reduce the frequency of conversion to GFP-positive cells, indicating that the various components of this supplement do not have specific inductive activity. Furthermore, although plating efficiency and viability were reduced on removal of albumin and insulin, *Sox1*-GFP cells could be generated in fully defined medium in which transferrin was the sole protein component. We conclude that exogenous inductive stimuli are not essential for neural specification of ES cells.

In *Xenopus laevis*, bone morphogenetic protein (BMP) antagonists such as noggin promote neural development[6]. However, we did not detect any effect of noggin on neural conversion of ES cells (Fig. 4A). The addition of BMP4, in contrast, suppressed the development of neural precursors and neurons and induced the appearance of non-neural differentiated cells (Fig. 4B). These large, flattened cells resembled the predominant cell types generated in the presence of serum or in N2B27 on laminin or fibronectin. They were viable in N2B27 medium, confirming that this medium is not intrinsically inhibitory to non-neural cell types. We conclude that although BMPs act as potent stimulators of alternative differentiation, endogenous BMP activity does not limit neural determination in ES cell cultures. This may be explained, at least in part, by expression in the cultures of the BMP antagonists noggin and follistatin (Fig. 4C).

Addition of LIF increased the proportion of undifferentiated ES cells and consequently reduced the production of *Sox1*-GFP cells (Fig. 4A). This contrasts with a suggestion that LIF promotes the generation of primitive neural precursors from ES cells[8], but is consistent with the well-established role of gp130 cytokines in promoting self-renewal of undifferentiated ES cells[2]. Interestingly, LIF appears rather less effective at blocking ES cell differentiation into neural derivatives in defined medium than at inhibiting development of non-neural cell types in serum-containing medium[2].

Studies in planaria[15] and in frog[16] and avian[17,18] embryos have suggested a primary requirement for FGF signaling in neural specification. ES cells express appreciable amounts of FGF4 (ref. 13; Fig. 4C). We therefore plated cells at low densities under which an autocrine factor might become limiting. Under these conditions, addition of FGF4 modestly but reproducibly increased the initial frequency of *Sox1*-GFP-positive 46C cells on day 3 (Fig. 4D). This effect is unlikely to be attributable to selective amplification of neural precursors because FGF4 was withdrawn on day 2, at which point very few *Sox1*-GFP positive cells had emerged (Fig. 1E). Furthermore, FGF2 had no stimulatory effect in this assay, although it is a potent mitogen for Sox1-positive cells.

We used a pharmacological inhibitor of FGF receptor tyrosine kinases, SU5402 (ref. 19), to test whether autocrine FGF signaling is an obligate requirement for neural specification of ES cells. SU5402 (5 μM) virtually eliminated the development of *Sox1*-GFP-positive 46C cells without observable effect on cell viability or proliferation (Fig. 4E). Notably, SU5402 prevented the initial formation of *Sox1*-GFP-positive cells on day 3, indicating that it acts directly on pluripotent cells. This action was not reversed by addition of noggin at levels that completely block the action of 50 ng/ml BMP4 (Fig. 4A, E), and is therefore unlikely to be mediated via derepression of BMP expression as reported in the chick[18]. Independent evidence that FGF signaling is necessary for neural specification was obtained by expression of dominant-negative FGF receptor (DN-FGFR)[20]. Under ES cell maintenance conditions, DN-FGFR transfectants showed viability and growth rate comparable to those of parental cells or cells transfected with a control plasmid. However, on withdrawal of LIF and serum from N2B27, ES cells expressing the DN-FGFR remained viable but specifically failed to generate appreciable numbers of either nestin-positive neural precursors or type III β-tubulin (TuJ)-positive neurons (Fig. 4F, G).

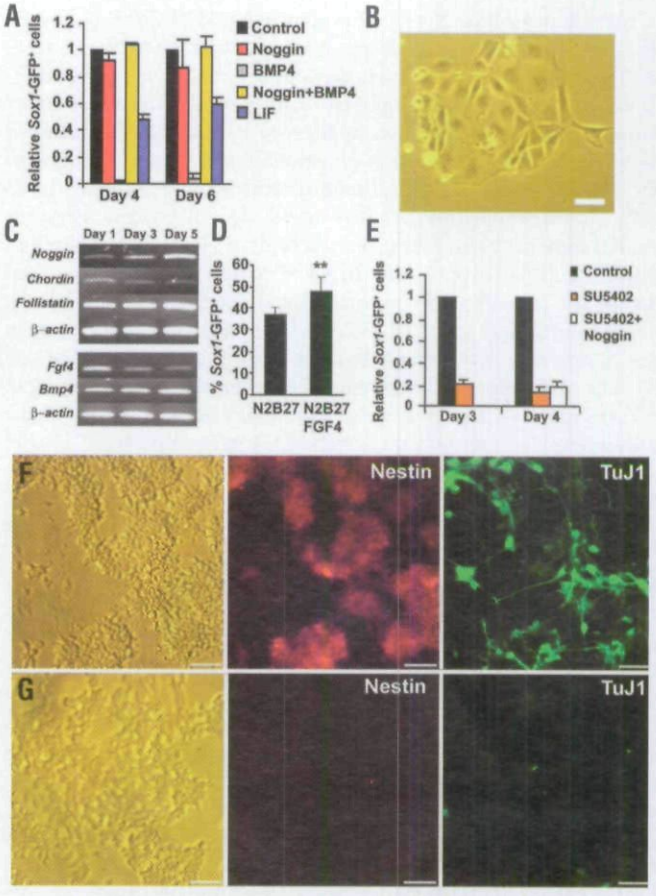

Figure 4. Effects of growth factors and antagonists on neural determination. (A) Effect of BMP4, noggin, and LIF. 46C cells were cultured in N2/B27 medium with recombinant proteins: 10 ng/ml BMP-4, 0.1 μg/ml noggin/Fc chimera (all from R&D Systems), or 100 U/ml LIF, as indicated. Data are means ± s.d. of triplicate determinations from two or three independent experiments, normalized relative to the frequency of GFP positive cells obtained in N2B27 alone. (B) Non-neural differentiation induced by exposure of 46C ES cells to BMP4 in N2B27. (C) RT-PCR analyses of mRNA expression during monoculture differentiation. (D) FGF4 increases the frequency of *Sox1*-GFP positive cells emerging on day 3. For each condition, data are from a total of 17 wells in four independent pairwise experiments. The increased frequency of GFP-positive cells after FGF4 treatment is significant, $P < 0.001$ (ANOVA). In parallel assays, addition of FGF2 produced no significant difference from N2B27 only. (E) Effect of FGF receptor inhibitor SU5402 (5 μM). Assays performed as in (A). (F, G) Suppression of neural development from ES cells expressing DN-FGFR. Cells transfected with control vector (F) or DN-FGFR expression vector (G) were plated in N2B27, allowed to differentiate for 6 d, then immunostained for nestin and the neuronal marker class III β-tubulin. Bar, 50 μm.

Elsewhere we have provided evidence that Wnt signaling directs ES cells into non-neural lineages[10], and here we show that serum, BMPs, and extracellular matrix components also induce non-neural differentiation. Thus, suppression of neural determination by induction of alternative fates appears to be a principle conserved between the differentiation of pluripotent cells in embryos[6] and of ES cells. However, the critical requirement for FGF signaling in ES cells indicates that this is not a simple default process. Previous studies have provided evidence for a role for FGFs in neural induction in planaria[15], frogs[16], and chicks[17,18]. Whether FGFs are required for neural specification in mammalian epiblast is not known because both *Fgf4* (ref. 21) and *Fgfr2* (ref. 22) knockouts result in pre-gastrulation lethality. Germ layer commitment in embryos and ES cells may follow the same rules. However, the culture setting of ES cells may also confer distinct responses compared with epiblast cells *in vivo*. For example, the action of LIF in sustaining self-renewal is particular to cultured ES cells, as this pathway serves a purely facultative function in the early embryo[23].

An intriguing feature of the monoculture system, also evident in PA6 cocultures[5], is that not all cells behave identically. The onset of *Sox1*-GFP expression is not synchronous throughout the cultures (Fig 3A). Furthermore, a minority of cells differentiate into non-neural cell types and 10–15% persist as clusters of undifferentiated ES cells. The latter are fully competent for differentiation if trypsinized and replated. These observations might indicate that there is a stochastic component, a community effect, or both in lineage commitment. The *Sox1*-GFP-expressing neural precursors can readily be purified from other cells, however, by FACS or by transient selection with puromycin.

Understanding how to generate appropriate cell types robustly and under defined conditions from ES cells will be essential for functional applications including the development of cell replacement therapies. The present findings provide a simple, rational system for conversion of ES cells into neuroepithelial precursors and thence into neurons and glia. Furthermore, the elimination of multicellular aggregation, coculture, or conditioned medium extracts provides for direct assay of lineage determination factors and a foundation for truly directed differentiation of ES cells.

Experimental protocol

Embryonic stem cell culture. ES cells were routinely propagated without feeders in LIF-supplemented medium[2]. Cell lines were CGR8 or E14Tg2a.IV and derivatives thereof, or Oct4GiP, derived from strain 129Ola mice carrying an *Oct4-GFPiresPac* transgene[14]. 46C ES cells were generated by gene targeting in E14Tg2a.IV ES cells. The open reading frame of the *Sox1* gene was replaced by *GFPiresPac*, with excision of the *hytk* selection cassette being accomplished via transient expression of Cre recombinase.

Differentiation protocol. For monoculture differentiation, undifferentiated ES cells were dissociated and plated onto 0.1% gelatin-coated tissue culture plastic at a density of $0.5–1.5 \times 10^4/cm^2$ in N2B27 medium. Medium was renewed every 2 d. N2B27 is a 1:1 mixture of DMEM/F12 (Gibco, Paisley, UK) supplemented with modified N2 (25 μg/ml insulin, 100 μg/ml apotransferrin, 6 ng/ml progesterone, 16 μg/ml putrescine, 30 nM sodium selenite and 50 μg/ml bovine serum albumin fraction V (Gibco)) and Neurobasal medium supplemented with B27 (both from Gibco). Tyrosine hydroxylase–positive neurons were induced with FGF2 (20 ng/ml), Sonic Hedgehog (400 ng/ml), and FGF8 (100 ng/ml)[12]. For assay of FGF effects on emergence of neural precursors, cells were plated at 2.6×10^3 cells/cm^2 and cultured for 24 h in N2B27 plus LIF. Cells were then transferred to N2B27 alone or containing FGF2 or FGF4 (2 ng/ml). After 48 h medium was replaced with non-supplemented N2B27 for a further 24 h prior to FACS analysis. The dominant-negative FGF receptor construct encodes a truncated version of murine FGFR2 IIIc lacking the cytoplasmic domain[20]. This construct was inserted into the pPyCAGiP episomal expression vector and introduced by supertransfection into E14/T ES cells harboring polyoma large T protein[10].

Immunostaining and RT-PCR. Details of antibodies are given in the Supplementary Experimental protocol online. For RT-PCR analyses, 1 μg total RNA was reverse transcribed using random hexamer primers and PCR performed on 1/20 of the final cDNA volume. Primers and PCR details are available in Supplementary Experimental protocol online.

Note: Supplementary information is available on the Nature Biotechnology website.

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Competing interests statement

The authors declare competing financial interests: see the Nature Biotechnology website (http://www.nature.com/naturebiotechnology) for details.

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