Pluripotency can be rapidly and efficiently induced in human amniotic fluid-derived cells

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Direct reprogramming of human somatic cells into pluripotency has broad implications in generating patient-specific induced pluripotent stem (iPS) cells for disease modeling and cellular replacement therapies. However, the low efficiency and safety issues associated with generation of human iPS cells have limited their usage in clinical settings. Cell types can significantly influence reprogramming efficiency and kinetics. To date, human iPS cells have been obtained only from a few cell types. Here, we report for the first time rapid and efficient generation of iPS cells from human amniotic fluid-derived cells (hAFDCs) via ectopic expression of four human factors: OCT4/SOX2/KLF4/C-MYC. Significantly, typical single iPS cell colonies can be picked up 6 days after viral infection with high efficiency. Eight iPS cell lines have been derived. They can be continuously propagated in vitro and express pluripotency markers such as AKP, OCT4, SOX2, SSEA4, TRA-1-60 and TRA-1-81, maintaining the normal karyotype. Transgenes are completely inactivated and the endogenous OCT4 promoter is adequately demethylated in the established iPS cell lines. Moreover, various cells and tissues from all three germ layers are found in embryoid bodies and teratomas, respectively. In addition, microarray analysis demonstrates a high correlation coefficient between hAFDC-iPS cells and human embryonic stem cells, but a low correlation coefficient between hAFDCs and hAFDC-iPS cells. Taken together, these data identify an ideal human somatic cell resource for rapid and efficient generation of iPS cells, allowing us to establish human iPS cells using more advanced approaches and possibly to establish disease- or patient-specific iPS cells.

INTRODUCTION

Direct reprogramming of human somatic cells into pluripotency using defined factors is an exciting turning point in stem cell biology with broad implications for generating patient-specific induced pluripotent stem (iPS) cells for disease modeling and cellular replacement therapies. Since the first report of generation of iPS cells in 2006 (1), a great deal of effort has been made to address the low efficiency and the safety issues associated with initial generation of mouse and human iPS cells obtained through viral delivery of four transcription factors (Oct4, Sox2, Klf4 and c-Myc) (2,3). Among parameters considered, methods of delivering reprogramming factors have been most investigated and various modified approaches have been reported, including use of transient transfection of plasmids (4), non-integrating adenoviruses (5), chemical inhibitors (6,7), epismal expression vectors (8), a piggyBac transposition system (9,10) and Cre-exciscable viruses (11). Recently, Zhou et al. (12) reported generation of mouse iPS cells using recombinant proteins and small molecules: the first iPS cells generated without the use of any genetic manipulation and a step...
forward towards clinical application of iPS cells. More recently, generation of human iPS cells by direct delivery of reprogramming proteins was also reported (13). However, the efficiency with use of recombinant proteins is very low and 30 days are needed before mouse iPS cell colonies can be picked up. Approximately 56 days are required to induce pluripotency in human somatic cells using this approach. Previous investigations have indicated that the cell types have significant influence on reprogramming efficiency and kinetics of the process (14). For example, mouse neural progenitor cells (NPCs) could be more efficiently reprogrammed than fibroblasts and reprogramming occurs by the expression of only two or even one exogenous factor due to their endogenous expression of Sox2 and c-Myc (15,16). Moreover, it was reported that human embryonic stem (ES) cell-like colonies appeared 10 days after transduction of human keratinocytes with the four factors, which is a substantially shorter time than the ~16–30 days required for human fibroblasts (17). Obviously, exploration of cell types easier to reprogram with shorter time required and higher efficiency for induction of pluripotency in human ES cells. After normalization and clustering analysis, we did not find expression of any of these markers against these three antibodies uniformly. In contrast, we were not able to detect significant expression of pluripotency markers using antibodies against OCT4, SOX2 and TRA-1-60 in this cell line (Fig. 1B). To further characterize the hAFDCs, we carried out microarray analysis and compared the expression levels of well-known pluripotency markers, such as CDH1, FGFR2, SOX2, LIN28, JARID2, NANOG, TDGF1, DNMT3B, ZFP42, LEFTY1, DPPA4, SALL4, POU5F1, ZIC3 and DPPA2, between hAFDCs and human ES cells. After normalization and clustering analysis, we did not find expression of any of these markers (Fig. 1C). At last, in order to exclude the possibility that a small number of pluripotent cells exist in the hAFDCs, we also carried out teratoma formation assay using primarily cultured hAFDCs derived from six independent patients. Teratoma was not found in any samples after long-term observation, indicating that the hAFDCs were not pluripotent (Supplementary Material, Table S1).

RESULTS

Derivation and characterization of hAFDCs

To find cell types suitable for induction of pluripotency, we isolated hAFDCs from human amniotic fluids with the consent of subjects and of the Ethical Review Board of the Xinhua Hospital. Human amniotic fluids were obtained through amniocentesis under sterile conditions during routine prenatal diagnosis. We selected the adhesive short and spindle-like cells and discarded the rest of the adherent and non-adherent cells under the culture conditions described in the Materials and Methods section. After long-term culture (more than 10 passages) in vitro, hAFDCs displayed homogeneous morphology and were used to induce iPS cells. To learn more of the cells molecularly, we compared expression levels of several marker genes among human ES cells, hAFDCs and hFSF. Data from quantitative real-time PCR (qPCR) revealed strong expression of C-MYC and moderate expression of REX1 and KLF4 in hAFDCs. Trace amounts of NANOG, OCT4A, FGF4 and SOX2 were detected, being similar in hAFDCs and hFSF, but much lower than that in human ES cells. Interestingly, weak expression of OCT4B can also be detected in hAFDCs, although the function of OCT4B remains unknown up to now (Fig. 1A). To further characterize the hAFDCs, immunofluorescence staining was performed using antibodies against specific lineage markers, including VIMENTIN (mesoderm), NESTIN (ectoderm) and GATA4 (endoderm). All of the hAFDCs had positive signals against these three antibodies uniformly.

Generation of iPS cells from hAFDCs

Human cDNAs for OCT4, SOX2, C-MYC and KLF4 in the pMXs vector were transduced using recombinant retroviruses. hAFDCs (~200 000 cells) were infected with a 1:1:1:1 mixture of the four viruses immediately after their attachment to gelatin-coated dishes (Fig. 2A). Using this protocol, almost 100% infection efficiency was obtained. Twenty-four hours after infection, virus-infected hAFDCs were plated onto irradiated mouse embryonic fibroblast (MEF) cells. One day later, the medium was changed to the human ES cell culture medium. Interestingly, small cell clusters with high nucleus-to-cytoplasm ratio appeared 3 days after reprogramming typical undifferentiated human ES cell-like colonies formed and could be picked up on day 6 post-infection (Fig. 2B). To compare reprogramming efficiency, the same induction procedure was conducted in hFSF derived from human foreskin tissue. On day 6 after infection, AKP staining was performed. We carefully counted the number of colonies having cells with a high nucleus-to-cytoplasm ratio as well as being AKP staining positive. In five independent experiments, we obtained 118, 980, 2496, 1560 and 3050 colonies, respectively, according with our selection criteria mentioned above. Therefore, the frequencies for induction of pluripotency in hAFDCs were between 0.059 and 1.525% (Fig. 2C). The inter-experimental variability in
the reprogramming efficiency could be attributed to different batches of viral packaging and random integration of reprogramming factors (22). In contrast, we did not find any AKP positive or human ES cell-like colonies at the same time using hFSF as starting cells. Interestingly, 16 days after four-factor infection of hFSF, iPS cell colonies emerged when co-cultured with the cell extract from mouse ES cells. All colonies (66) from $1 \times 10^5$ hFSF were picked up and five iPS cell lines were established (Supplementary Material, Fig. S1).

Figure 1. Characterization of hAFDCs. (A) qPCR was performed on human ES cells (hES), hFSF and hAFDCs using primers against $OCT4A$, $OCT4B$, $NANOG$, $SOX2$, $KLF4$, $C-MYC$, $FGF4$ and $REX1$. Values are shown as means ± SD of results from three independent experiments. (B) Immunostaining was performed on hAFDCs using antibodies against VIMENTIN, NESTIN, GATA4, OCT4, SOX2, NANOG and TRA-1-60. The scale bars are 25 μm in (a)–(i) and 50 μm in (j)–(u). (C) Clustering analysis of pluripotency marker genes detected by the microarray assay in hAFDCs and human ES cells. Green panels denote low expression levels, whereas red panels denote high expression levels.
Figure 2. Derivation of iPS cells from hAFDCs. (A) The flow diagram of hAFDC-iPS cell generation based on retroviral transduction of four factors. (B) Morphology of hAFDCs at low (a) and high (b) magnifications. Typical undifferentiated colonies at low (c) and high (d) magnifications in culture dishes at day 6 post-viral infection are shown. The scale bars are 1000 μm in (a) and (c) and 100 μm in (b) and (d). (C) At day 6 post-infection, the number of colonies having human ES cell-like cells as well as being AKP positive was counted and calculated in hAFDC-OSKM (a), hAFDC-GFP (b) and hFSF-OSKM (c) groups. The efficiency of iPS cell induction from hAFDCs by retroviral transduction of the four factors was compared with that from hFSF cells or that hAFDCs transduced by GFP virus alone. Five independent experiments were performed using different batches of viruses and starting cells. The quantitative data are shown in (d). The ‘OSKM’ denotes the mixture of the four retroviruses. The scale bars are 50 μm. (D) At day 6 post-infection, hAFDC-iPS cells were immunostained by antibodies against OCT4 and NANOG, and then restained using the AKP kit. Representative iPS cell colonies and non-iPS colonies were analyzed for OCT4, NANOG and AKP expression and shown in (a). For AKP positive colonies, the percentage of OCT4 or NANOG positive colonies was calculated and is shown in (b).
To verify whether the colonies selected represent fully reprogrammed cells, we double-immunostained iPS cell colonies with antibodies against OCT4 and NANOG and subsequently re-stained the colonies using AKP staining kit. As shown in Figure 2D, all selected colonies were also OCT4-positive, whereas 90.5% of selected colonies were NANOG-positive, implying that AKP staining combined with cell morphology could serve as a faithful parameter for evaluating the reprogramming efficiency. Interestingly, the same kinetics of rapid reprogramming was obtained in these five independent experiments, although the reprogramming efficiency was variable among different experiments. As a negative control, transduction of hAFDCs with the viral vector containing the cDNA sequence of GFP did not result in formation of any colonies, indicating that pluripotency was induced by expression of the four transcription factors. Moreover, the rapid and efficient induction of iPS cells was well repeated using additional two independently derived hAFDC lines. In addition to four factors, we could observe iPS cell colonies 6 days post-infection using three factors (OCT4/SOX2/KLF4), although the efficiency was much lower than that using four factors (Li et al., unpublished data).

Characterization of hAFDC derived-iPS cells

Thirty-six single human ES cell-like colonies were picked up mechanically and eight of them were successfully expanded on a large scale (more than forty passages). Six lines were further characterized in detail (Supplementary Material, Table S2). Cells in the colonies displayed typical human ES cell morphology with a high nucleus-to-cytoplasm ratio and strong AKP expression (Fig. 3A). Immunofluorescence staining shows that they all expressed OCT4, SOX2, SSEA4, TRA-1-60 and TRA-1-81 (Fig. 3B). Moreover, successful activation of endogenous pluripotency marker genes OCT4, SOX2, REX1, GDF3 and NANOG was verified by RT–PCR analysis (Fig. 3C). Interestingly, differential mRNA levels of endogenous KLF4 and C-MYC were found in different iPS cell lines. On the other hand, the four exogenously transduced transcription factors were silenced in iPS cell lines such as 16, 19, 35 and 36, whereas iPS cell line 26 retained strong expression of exogenous OCT4. The finding suggests that a differential extent of reprogramming occurred in individual lines.

Bisulfite genomic sequencing analysis demonstrated the demethylation status of cytosine guanine dinucleotides (CpG)
in the promoter region of *OCT4* for hAFDC derived-iPS (hAFDC-iPS) cells lines 4 (94.9%), 6 (95.8%) and 7 (97.5%), as well as in human ES cells (SHhES1, 97.5%) (23) (Fig. 4). In contrast, parental hAFDCs exhibited a low-demethylated status at the *OCT4* promoter (25.5%). This result suggests that the *OCT4* promoter was released to be active in fully reprogrammed human iPS cells.

Karyotype analysis was also conducted for hAFDC-iPS cells during long-term culture. The hAFDC-iPS cell lines 4, 6, 7 and 19 maintained a normal karyotype of 46 XX for more than forty passages (Supplementary Material, Fig. S2 and Table S3). To rigorously confirm that hAFDCs exhibited a low-demethylated status at the *OCT4* promoter (25.5%). This result suggests that the *OCT4* promoter was released to be active in fully reprogrammed human iPS cells.

In vitro and in vivo differentiation

To evaluate the *in vitro* differentiation ability, we suspended hAFDC-iPS cells of lines 4, 6, 7, 19 in low-attachment dishes for embryoid body (EB) formation. Typical well-shaped EBs formed after 6 days in suspension culture and attached onto Matrigel-coated dishes for further differentiation. After 2 days in adhesive culture, expression levels of differentiation markers were examined using RT–PCR analysis. As shown in Figure 6A, activation of all three embryonic germ layer markers was observed in all lines tested. However, the markers activated during differentiation processes varied among different iPS cell lines, implicating that the lines responded to differentiation signals differentially. Moreover, results from immunostaining assays illustrated expression of NESTIN (ectoderm), GATA4 (endoderm) and VIMENTIN (mesoderm) at a protein level in differentiated hAFDC-iPS cells. To exclude the possibility that the expression of these markers in differentiated cells was from contamination of starting hAFDCs in hAFDC-iPS cells as the parental hAFDCs also expressed these three markers, we stained hAFDC-iPS cells using the three antibodies. As shown in Supplementary Material, Figure S3, we did not find any positive signal. Moreover, we detected the positive cells using antibodies against CK7 (mesoderm), CK14 and TUJ1 (ectoderm), AFP and SOX17 (endoderm), revealing that hAFDC-iPS cells possess the ability to differentiate into cell types originated from all three germ layers *in vitro*.

To determine the potential of the hAFDC-iPS cells to differentiate *in vivo*, we injected the cells into NOD-SCID mice. Well-shaped teratomas were detected and harvested 6–10 weeks post-transplantation of hAFDC-iPS cell lines 6 (data not shown) and 36. Histochemical analysis indicated the existence of various tissues from all three germ layers, such as neural epithelia (ectoderm), intestinal epithelia (endoderm) and muscle (mesoderm) tissues (Fig. 6C).

**DISCUSSION**

Here, we report for the first time that pluripotency can be rapidly and efficiently induced in hAFDCs. Our data show that iPS cell colonies can be picked up at the sixth day after infection with four key factors, a time frame which, to the best of our knowledge, is the most rapid reported for human iPS cell formation. Generally, direct reprogramming of
human fibroblasts to pluripotency requires several weeks (24),
although Aasen et al. (17) recently reported that 10 days was
sufficient for reprogramming human keratinocytes. Practically,
rapid reprogramming makes it possible to transfer individual
iPS cell colonies to new culture dishes before the widely used MEF cells break up, usually in a week. In addition, a frequency of \(1.525\%\) could be obtained with hAFDCs, this being at least 100 folds higher than that found with human fibroblasts. Oct4 or Nanog promoter-driven GFP reporter cell lines have been often used as a convenient tool for identification of fully reprogrammed colonies in mouse cells (22,25). However, it is very difficult to obtain such lines for human cells. Therefore, identification and evaluation of genuine reprogrammed human pluripotent cells are a challenge. AKP staining has been widely used for identification of pluripotent cells. However, AKP staining alone could not represent fully reprogrammed cells. In this study, we employ a unique strategy, which combines AKP staining with the cell morphology. Our data show that all colonies identified by such criteria express OCT4 and 90% express NANOG. It is possible that we are able to identify fully reprogrammed cells solely based upon the morphology eventually, which would greatly facilitate propagation of genuine iPS cells during reprogramming human somatic cells. Furthermore, the rapid and efficient generation of iPS cells in hAFDCs provides a useful platform to allow not only in-depth dissection of the underlying molecular and biochemical processes of nuclear reprogramming, but also broad testing of new approaches, such as those using chemicals and recombinant proteins, for induction of pluripotency in human cells.

Amniotic fluid represents a very heterogeneous population which contains cells from the developing fetus (26,27). So far, various types of fetal cells have been detected in amniotic fluid, including cells derived from all three embryonic germ layers (28). Interestingly, a subpopulation of hAFDCs was shown to express pluripotency marker OCT4 as well as the surface antigen c-Kit (CD117), the receptor for stem cells (29), suggesting the possibility that hAFDCs contain pluripotent stem cells. Recently, De Coppi et al. (30) reported isolation of human amniotic fluid-derived stem (AFS) cells which express certain embryonic as well as adult stem cell markers and can be induced to differentiate into cell types representing each germ layer. However, they do not form teratomas \textit{in vivo}, as do pluripotent stem cells, which is consistent with our finding. In the current study, using a unique strategy, we isolated adherent hAFDCs, which expressed REX1, KLF4 and C-MYC and stained positively for RESTIN, VIMENTIN and GATA4. Long-term cultured hAFDCs display homogeneous morphology and they do not express typical pluripotency markers such as OCT4, SOX2, NANOG and TRA-1-60. However, the correlation coefficient between hAFDC and SHhES2 was high (0.8866), suggesting that hAFDCs may represent a precursor state and providing the genetic basis for efficient and rapid conversion of
hAFDCs to a genuine pluripotent state. The evidence for the pluripotent state of hAFDC-iPS cells includes the following: (i) an ES cell-like morphology and expression of pluripotency markers, such as OCT4, SOX2, SSEA4, TRA-1-60 and TRA-1-81; (ii) demethylation at the endogenous OCT4 promoter sequence; (iii) production of teratomas containing various cell types of three embryonic germ layers in immunodeficient mice; (iv) a global gene expression profile closely resembling that of human ES cells, but distinct from that of parental hAFDCs. The failure to generate iPS cell colonies from hAFDCs infected with the viral vector containing the cDNA sequence of GFP excludes the possibility that hAFDC-iPS cells might originate from rare stem cells existing in parental hAFDCs. In addition, the hAFDC-iPS cells can be

**Figure 6. In vitro and in vivo differentiation abilities of hAFDC-iPS cells.** (A) RT–PCR analysis of expression levels of various germ layer markers was performed for EB samples of hAFDC-iPS cell lines 4, 6, 7, 19. Representative results of two independent experiments are shown. (B) Expression of germ layer markers in differentiated iPS cells was examined using immunostaining. The scale bars are 25 μm in (a)–(i), 10 μm in (j)–(l) and 250 μm in (m)–(o). (C) Teratomas were harvested 6–8 weeks after intramuscular injection of undifferentiated cells from hAFDC-iPS cell line 36 into SCID-NOD mice. Using histochemical staining, low magnification of a teratoma was shown in (a). Various tissues, such as neural epithelia (b); cartilages (c); smooth muscle and blood vessels (d); intestinal glandular tissues (e) and lung epithelia (f) were found. The scale bars are 500 μm in (a) and 50 μm in (b–f).
expanded easily and maintain a normal karyotype after being passaged forty times, suggesting that they have the capacity to proliferate for a long time in vitro. Our data point out that hAFDCs can serve as an attractive cell source for derivation of iPS cells. Advantages of the use of hAFDCs for iPS cell generation include: (i) routine isolation from amniocentesis specimens which are obtained for prenatal genetic diagnosis and would be otherwise discarded; (ii) generation of disease- or patient-specific iPS cells when a genetic disorder is detected in the hAFDCs; (iii) use of cells differentiated from hAFDC-iPS cells as an ideal source for autologous cell-replacement therapy in the later life of the fetus and (iv) banking of hAFDC-iPS cell lines allowing matching of histocompatible donor cells with recipients. Obviously, hAFDCs represent a very promising resource for human iPS cell research. However, it is worthy to note that hAFDCs isolated in independent studies may represent different cell types due to the heterogeneous nature of cells in human amniotic fluid. Therefore, further definition and characterization of hAFDCs are important. We have profiled global gene expression patterns of the derived hAFDCs, hAFDC-iPS cells and human ES cells. In-depth analysis and comparison are in process to provide more information about the molecular nature of these cell types. It is tempting to speculate on the existence of certain subpopulations of hAFDCs which may be more rapidly and more efficiently converted to a pluripotent state.

MATERIALS AND METHODS

Derivation and culture of hAFDCs

Human amniotic fluid was obtained by ultrasound-guided amniocentesis performed on pregnant women for routine prenatal diagnosis purposes at gestational ages ranging from 18th to 22nd weeks. All of the human samples were obtained after approval from the Ethical Review Board of the Xinhua Hospital (Shanghai, China) and after obtaining written informed consent from subjects. The fluids were filtered using 100 μm filters and were then centrifuged at 400 g at 4°C for 10 min. The supernatants were discarded and the precipitates were seeded in 60 mm tissue culture dishes which were pre-coated with 0.2% gelatin. The dishes were incubated at 37°C under 5% humidified CO₂. The culture medium consisted of α-MEM medium (Gibco, Invitrogen) containing 15% heat-inactivated fetal bovine serum (Hyclone), 2 mM L-glutamine (Gibco, BRL, Rockville, MD, USA), 0.1 mM β-mercaptoethanol (Sigma), 50 U/ml penicillin (Gibco, BRL) and 50 μg/ml streptomycin (Gibco, BRL). Cells clusters emerged 7 days after seeding. Non-adherent cells were discarded. The adherent epithelial-like cells as well as long fibroblast-like cells were mechanically removed with a cell scraper, leaving cells displaying a short and spindle-like shape and possessing a large nucleus. These cells were cultured and passaged routinely at 70–80% confluence.

Derivation of hAFDC-iPS cells

For retrovirus production, cDNAs encoding human OCT4, SOX2, KLF4 and C-MYC in pMXs vector were co-transfected with packing plasmids GAG, POL and VSVG into 293 cells using the Fugene 6 kit (Roche). Virus-containing supernatants were collected 48 h after transfection. For viral transduction, 2 × 10⁵ hAFDCs were seeded on gelatin-coated 60 mm dishes. After the cells spread well on culture dishes, a mixture of four viruses was added into the medium with polybrene for infection overnight. Twenty-four hours later, infected hAFDCs were replated onto irradiated MEF cells with the standard human ES cell culture medium as previously reported (23). Three days later, small colonies emerged in the culture dish. Single colonies were picked up 2 days later and cultured until they grew to large colonies.

RT–PCR and qRT-PCR

Total RNA was extracted from cells using TRIzol (Invitrogen) and was transcribed into cDNA using oligo(dT)₁₆ and RevertAid reverse transcriptase (Toyobo). PCR reactions were carried out by mixing 1 μl of cDNA template, 250 nM of each primer, 200 μM dNTP mixture and 1 U of Taq DNA polymerase in a volume of 20 μl. Samples were amplified in a thermocycler. For qPCR, each sample was analyzed in triplicate with GAPDH as the inner control. Amplification data were collected using the ABI PRISM 7900 and analyzed using the Sequence Detection System 2.0 software. Primer information is provided in Supplementary Material, Table S4.

Immunofluorescence staining

Cells on the glass cover slips were fixed in 4% paraformaldehyde in PBS supplemented with 0.1% Triton X-100 for 15 min at room temperature (RT), followed by permeabilization with 0.1% Triton X-100 in PBS for 5 min at RT. Cells were blocked for 30 min in 3% BSA in PBS. All primary antibodies were diluted in the same blocking buffer and incubated with samples overnight at 4°C. Cells treated with fluorescently coupled secondary antibody (anti-rabbit Cy3, Jackson ImmunoResearch, 1/200) were incubated for 1 h at RT. The nuclei were stained with DAPI (Sigma) for 3 min at RT. All images were captured using a confocal microscopy. Antibody information is provided in Supplementary Material, Table S5.

Bisulfite sequencing

Genomic DNA from hAFDC-iPS cells, human ES cells and hAFDCs was restricted with EcoRV and treated with sodium bisulfite as previously described (31). Treated DNA was subjected to nested PCR. Primer information is provided in Supplementary Material, Table S4. The PCR products were cloned into T-vectors (Promega) and individually sequenced.

Teratoma formation

For teratoma induction, 5 × 10⁶ cells of each hAFDC-iPS cell line were harvested and injected intramuscularly into NOD-SCID mice with matrigel. Six to ten weeks later, teratomas were harvested and processed with hematoxylin and eosin staining.
Microarray analysis

Affymetrix U133 2.0 gene chips were used for this study. All experiments were performed and analyzed at the Shanghai Chip Center. Three biological repeat samples from human ES cells (SHiES2), hAFDCs, hAFDC-iPS cell lines 4 and 7 were collected and analyzed as described in the Affymetrix Technical Manual.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

Conflict of Interest statement. None declared.

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