Complement targeting of nonhuman sialic acid does not mediate cell death of human embryonic stem cells

To the editor:

Human embryonic stem cells (HESCs) represent a promising cell source to replace damaged or diseased tissue. However, the recent observation that one of the US federally approved HESC lines[1] contains a potentially immunogenic nonhuman sialic acid, Neu5Gc (ref. 2), indicates that there may be limitations on their use. Neu5Gc is found on the surface of most nonhuman mammalian cells and is targeted by circulating antibodies found in human sera, which results in complement-mediated death of cells bearing this molecule[3]. Based on this report[2], a broader investigation of HESC lines and human sera is required to determine the extent of Neu5Gc incorporation and the subsequent likelihood of HESC cell death induced by recipient sera.

Accordingly, we directly cocultured two NIH-approved HESC lines (H1 and H9)[1] on mouse embryonic fibroblasts (MEFs; protocol approved by the animal ethics board of McMaster University) as previously reported for H1 (ref. 2), and additionally in feeder-free conditions using media containing an animal-derived serum replacer (SR) alone[4] or conditioned by MEFs (MEF-CM)[5] containing Neu5Gc (**Supplementary Methods** online). Both HESCs incorporated Neu5Gc similarly whether cultured in feeder-free media or grown on MEFs[2] (**Supplementary Table 1** online), consistent with the range (2.5–10.5%) previously reported for H1 cultured on MEFs[2]. However, Neu5Gc content of MEFs themselves (7.4%) was similar to that in HESCs grown in SR or on MEFs, versus 20% previously reported[2], prompting us to investigate whether HESCs in feeder-free formats would be similarly targeted for killing upon exposure to human sera. When H1 and H9 cells were exposed to PBS (no serum) or serum from 18 independent healthy donors (**Fig. 1a**,**b**), the average percentage of cell death in the presence of serum was no different from that in its absence (13.7% versus 16.7% for H1, 11.9% versus 13.4% for H9), in contrast with the 40–70% previously described[2]. To control for human serum activity and variability, we tested the cell lysis activity against MEFs. Nearly all human sera induced cell killing of MEFs (**Fig. 1b**), and this activity was abolished by heat inactivation (**Supplementary Fig. 1** online), indicating that mediation by heat-sensitive elements within complement had occurred[6]. To provide a positive control for complement-mediated lysis of HESCs, HESCs were labeled with a mouse IgG_3 antibody against the SSEA-4 embryonic antigen expressed on the surface of the majority of HESCs (**Fig. 1c**). Subsequent exposure to human sera caused cell death of the labeled (average of 36.6%) but not unlabeled (average of 8.1%) HESCs (**Fig. 1c**), indicating that HESCs can be targeted by human serum complement in the presence of xenoantibodies.

As the previously published human serum killing was limited to H1 cocultured directly on MEFs[2], we evaluated whether the presence of MEFs could account for the HESC susceptibility to serum-induced killing. After 2 h or 7 d of coculture at various ratios of GFP^+ H9 HESCs and unmarked MEFs, the frequency of dead HESCs remained similar to that seen in HESCs not treated with serum, whereas the frequency of dead MEFs increased proportionally to the ratio of MEFs (**Fig. 1d**,**e**), indicating that HESCs and MEFs did not reciprocally influence each other's killing.

We next investigated whether the absence of serum-induced killing of HESCs was the result of insufficient binding of complement C3. With the exception of H9 in MEF-CM, in which C3 bound to fewer cells after serum exposure than it did in the unexposed control, all samples— H1 in MEF-CM, H1 in SR and H9 in SR—showed higher frequencies than the controls did, reaching frequencies close to those observed for the MEFs (**Supplementary Fig. 2** online). No linear correlations were observed between Neu5Gc content and cell killing ($R^2 = 0.284$) or C3 binding ($R^2 = 0.5446$). Even though a strong correlation between C3 binding and cell killing ($R^2 = 0.9244$) was observed for the MEFs, this correlation was not observed for the HESCs ($R^2 = 0.0015$ for H1 in MEF-CM; $R^2 = 0.4049$ for H9 in MEF-CM; $R^2 = 0.6275$ for H1 in SR; $R^2 = 0.0615$ for H9 in SR). Similar results were seen when HESCs were cultured on MEFs (**Supplementary Fig. 3** online), indicating that C3 deposition may not reliably reflect susceptibility to complement-mediated cell death of HESCs as previously inferred[2].

By analyzing a large pool of human sera and culture conditions of HESC lines, we demonstrate that serum-induced killing of currently available HESC lines is limited and does not correlate with Neu5Gc content. In the only previous report, Neu5Gc content, C3 deposition and cell death were presented in mutually exclusive experiments but were suggested to be related[2], and HESC killing may have been observed as a result of the selection of a rare serum containing high titers of Neu5Gc antibodies. Although there are several crucial reasons for the derivation of new HESC lines[7] under new culture conditions[8] completely devoid of xenoantigens, incorporation of Neu5Gc is unlikely to be of any consideration for future basic or clinical practice.

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1. Ready, T. *Nat. Med.* **7**, 1262 (2001).
2. Martin, M.J., Muotri, A., Gage, F. & Varki, A. *Nat. Med.* **11**, 228–232 (2005).
3. Nguyen, D.H., Tangvoranuntakul, P. & Varki, A. *J. Immunol.* **175**, 228–236 (2005).
4. Wang, L., Li, L., Menendez, P., Cerdan, C. & Bhatia, M. *Blood* **105**, 4598–4603 (2005).
5. Xu, C. *et al. Nat. Biotechnol.* **19**, 971–974 (2001).
6. Muller-Eberhard, H.J. *Annu. Rev. Biochem.* **44**, 697–724 (1975).
7. Rao, M.S. & Civin, C.I. *Stem Cells* **24**, 1412–1413 (2006).
8. Ludwig, T.E. *et al. Nat. Biotechnol.* **24**, 185–187 (2006).

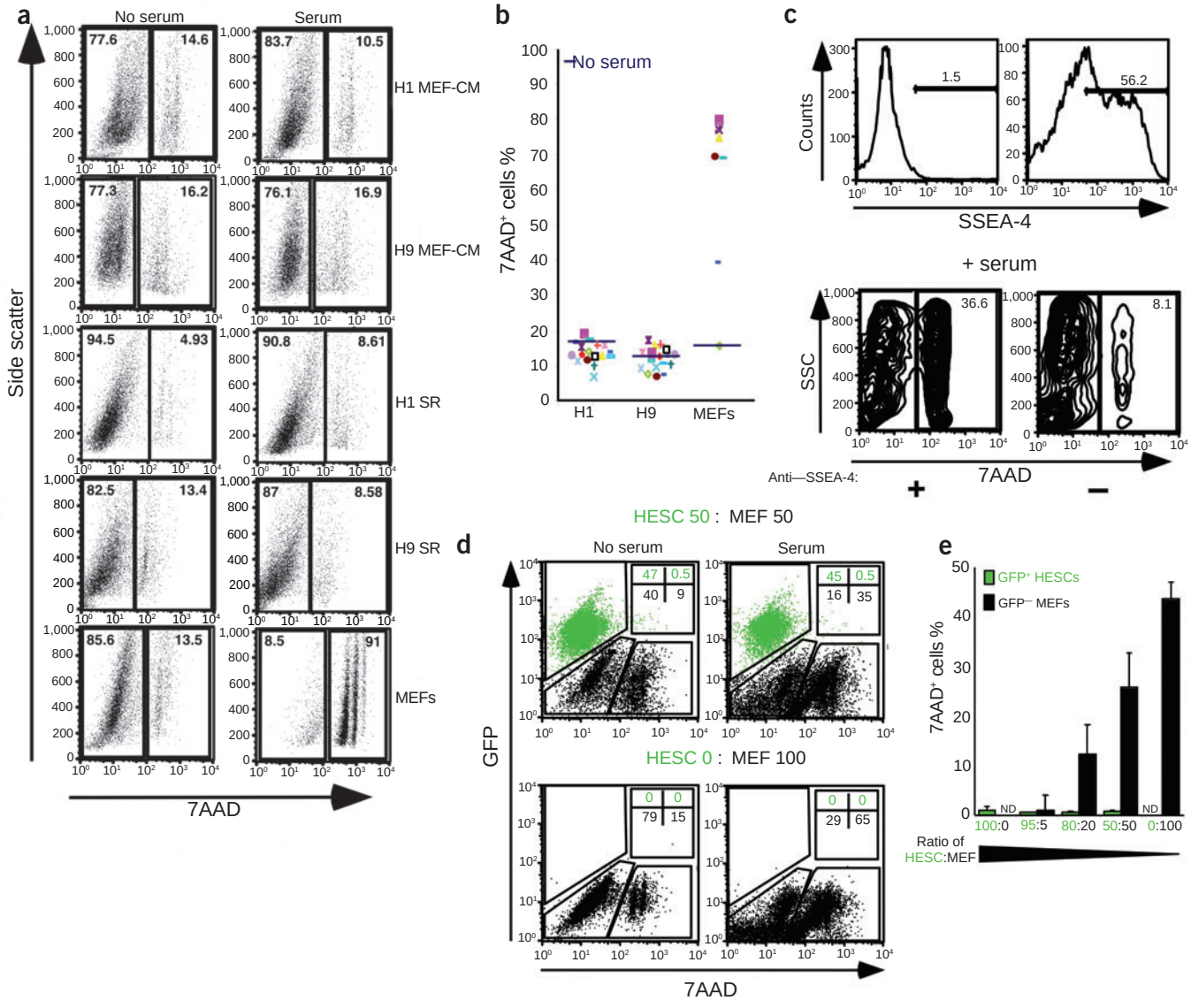

Figure 1 Flow cytometric analysis of HESC and MEF cell death in response to sera. H1 and H9 HESC lines cultured with either MEF-CM, SR or MEFs, dissociated, washed and incubated for 2 h at 37 °C with 25% vol/vol PBS (no serum) or normal human sera (+ serum), then stained with 7AAD viability dye to estimate the frequencies of live (7AAD$^-$) and dead (7AAD$^+$) cells. (**a**) Representative staining ($n = 4$ for MEFs; $n = 3$ to 5 for each HESC line in each medium). (**b**) Effects of 18 individual human sera on HESC killing. Each color-coded dot represents an individual serum sample. Horizontal bar line represents the average cell death induced by PBS (no serum). (**c**) HESCs incubated (+) or not (–) with SSEA-4 antibody, followed by serum exposure (+ serum) and 7AAD staining. Histograms represent SSEA-4 staining performed in parallel, using sequential incubation with SSEA-4 (right) or isotype control (left) antibodies, and FITC-conjugated goat antibody to mouse IgG. (**d**) GFP$^+$ H9 and unmarked (GFP$^-$) MEF cells mixed at 50:50 and 0:100 ratios for 2 h in the presence or absence of serum and then stained with 7AAD to determine the frequencies of dead HESCs (GFP$^+$7AAD$^+$) and MEFs (GFP$^-$7AAD$^+$); $n = 3$. (**e**) Frequencies of 7AAD$^+$ cells for both the HESC (GFP$^+$ H9) and MEF (GFP$^-$ MEFs) populations mixed at the indicated ratios, after serum exposure ($n = 3$). ND, not detected, as MEFs or HESCs were not added where indicated.