RESEARCH ARTICLE

Generation of definitive endoderm from human embryonic stem cells cultured in feeder layer-free conditions

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Purpose: The purpose of these studies was twofold: to reduce the level of nonhuman, potentially immunogenic sialic acid *N*-glycolylneuraminic acid (Neu5Gc) in human embryonic stem cells (hESCs) through culture of the cells in the absence of feeder layers; and to determine whether directed differentiation was preserved under these conditions, that is, using exclusively human-derived products. **Methods:** Using a technique developed in our laboratory to culture hESCs in the absence of feeder layers, all nonhuman cell culture reagents were replaced with recombinant or human-derived reagents. The level of the nonhuman sialic acid (Neu5Gc) was measured by high-performance liquid chromatography and monitored over many passages. Subsequently, the cells were subjected to *in vitro* differentiation into definitive endoderm by lowering the serum concentrations and elevating the amount of activin A. **Results:** Under standard tissue culture conditions using mouse and other animal products, the basal levels of Neu5Gc were measured between 7 and 10%. After the cell culture reagents were changed to all human products, Neu5Gc levels decreased steadily before leveling below 2%. Upon initiation of the differentiation protocol under these cell culture conditions, we observed robust endoderm formation, as measured by fluorescence-activated cell sorting analysis and the appearance of mRNA for markers of definitive endoderm (*Sox17*, *CXCR4*, Goosecoid and *FoxA2*). **Conclusion:** Consistent with other findings, elimination of nonhuman products in cell culture of hESCs decreases the levels of nonhuman and potentially immunogenic sialic acid levels. Furthermore, our studies demonstrate that in this feeder layer-free system, hESCs undergo directed differentiation into definitive endoderm.

Keywords: cell culture, definitive endoderm, human embryonic stem cells, Neu5Gc

As human embryonic stem cells (hESCs) become a more common research tool, the need for a better understanding of the basic biochemistry behind maintenance of pluripotency and differentiation of these cells becomes more important. Over the past few years, our laboratory and others have begun to understand how activin-mediated signaling maintains pluripotency in the absence of contaminating mouse feeder layers, allowing advances in the analysis of global transcription and proteome analysis of hESCs [1–5].

A recent report describes the incorporation of the nonhuman, and possibly immunogenic, sialic acid *N*-glycolylneuraminic acid (Neu5Gc) from animal-derived tissue culture products on the surface of cultured hESCs [6]. The presence of Neu5Gc on hESCs diminishes enthusiasm for therapeutic use of the cells for the treatment of disease owing to fears of xenogenic rejection through an immune response. One possible solution to the problem is to develop and culture new hESC lines on human feeder layers and exclusively human-derived culture products. Another is to determine whether Neu5Gc levels can be decreased in existing hESC lines through manipulation of cell culture conditions.

In this report, we demonstrate that culture of hESCs in the absence of contaminating animal products is associated with a decrease in Neu5Gc levels. Additionally, we show for the first time that directed differentiation of hESCs into definitive endoderm can occur in the absence of feeder layers.

Materials & methods

hESC culture

Initial culture of the hESC line HSF6 on feeder layers was performed on mitomycin C-treated CF1 mouse feeder layers at 37°C, 5% CO_2 in DSR (high-glucose DMEM-containing knockout serum replacer, glutamine, nonessential amino acids, 0.1 mM β-mercaptoethanol) media, as previously described [7]. Cells were transferred to feeder layer-free conditions by plating on dishes coated with laminin (20 µg/ml, Chemicon, MA, USA [101]). Three different feeder layer-free culture conditions of hESCs were used in this study. First, hESCs

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were cultured under our standard feeder layer-free cell culture conditions in the presence of conditioned medium (CM) from mouse embryonic fibroblasts (mEFs) supplemented with 10 ng/ml basic fibroblast growth factor (bFGF2; Preprotech Inc., NJ, USA [102]). Second, hESCs were also grown in the presence of DSR + knockout [KO] serum + ANK (A: recombinant activin A, 50 ng/ml; K: human recombinant keratinocyte growth factor [KGF], 50 ng/ml, both from Preprotech; and N: nicotinamide [NIC], 10 mM from Sigma Corp., MO, USA). Finally, hESCs were also grown in DSR containing normal human serum in the presence of ANK. Medium was changed every other day and cells were passaged weekly at 1:3 or 1:4 dilution by gentle treatment with 1 mg/ml collagenase IV (Gibco BRL, CA, USA) for 5 min, followed by scraping.

Sialic acid measurements

Sialic acids were released from cells by mild acidic treatment pf cells followed by derivatization with 1,2 diamino-4,5 methylene dioxybenze and analysis by high-performance liquid chromatography (HPLC), as previously described [8].

Differentiation protocol

To generate definitive endoderm, hESCs grown in DSR containing normal human serum and ANK, as described previously, were switched from DSR to RPMI media containing 100 ng/ml activin A in the absence of serum. After 1 day, the medium was changed to RPMI containing 100 ng/ml activin A in the presence of 0.2% serum and incubated for another 48 h. Subsequently, cells were lysed and mRNA was prepared.

FACS analysis

hESCs were originally sustained in DMEM/F12 with 20% knockout serum replacement (KO-SR), 4 ng/ml bFGF and 10 ng/ml activin A. At passage, 61 cells were transferred in condition media from mEFs supplemented with 8 ng/ml bFGF on BD growth factors-reduced matrigel and split every 4–5 days. Differentiation was started when cells reached confluence. After a brief wash with RPMI, cells were differentiated for 4 days in media containing activin A (100 ng/ml) and a 0.2% human serum. On day 1, hESCs were first exposed for 24 h to Wnt3A (25 ng/ml) and activin A (100 ng/ml) in RPMI media. For the next 2 days, the cells were incubated with 0.2% human AB serum and activin A (100 ng/ml). On day 4, the concentration of human AB serum was increased to a final concentration of 2%, while the activin A concentration was held constant.

At day 4, cells were washed with phosphate-buffered saline (PBS) and dissociated with 0.05% trypsin/EDTA (Invitrogen, CA, USA). Cells were washed twice, pelleted and resuspended in normal human serum to block non-specific antibody binding. Cells were labeled with CXCR4-PE (R&D Systems, MN, USA) at 20 µl for 0.5×10^6 cells for 45 min on ice. Cells were washed twice and resuspended in 200 µl PBS buffer. Cells were analyzed using a BD FacsScan instrument and CellQuest software (Cytometri Research LLC, CA, USA).

Reverse transcription-PCR

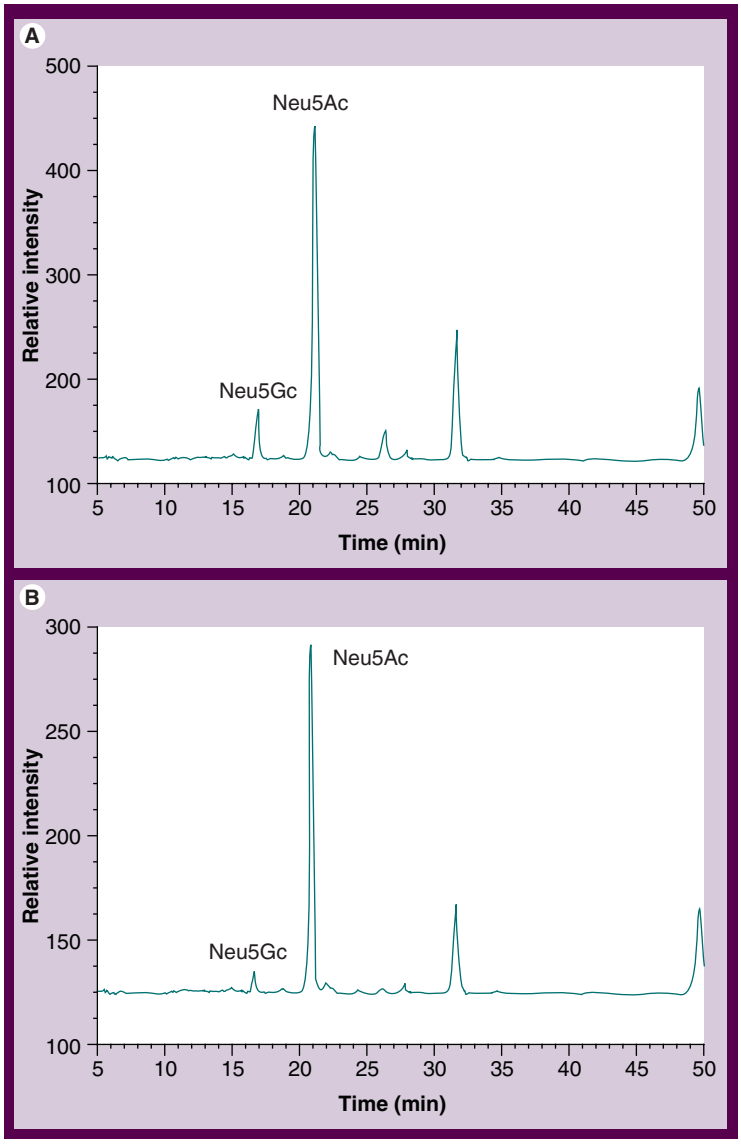
RNA was purified using the RNeasy minikit, including DNase treatment (Qiagen, CA, USA [103]) and reverse transcribed using avian myeloblastosis virus with 3.2 µg of random primer (Roche, IN, USA [104]) and 1 µg of total RNA in a reaction volume of 20 µl. A total of 1 µl of cDNA was used for each PCR reaction in a total volume of 50 µl. The following primers were used: *Sox17*, forward primer 5´GGCGCAGCAGAATCCAGA3´, reverse primer, 5´CCACGACTTGCCCAG-CAT3´; *CXCR4*, forward primer 5´CACCG-CATCTGG-AGAACCA3´, reverse primer 5´GCCCATTTCCTCGGTGTAGTT3´; *FoxA2*, forward primer 5´GGGAGCGG-TGAAGATGGA3´, reverse primer 5´TCAT-GTTGCT-CACGGAGGAGTA3´; Goosecoid forward primer 5´GAGGAGAAAGTGGAGG-TCTGGTT3´ and reverse primer 5´CTCT-GATGAGGA-CCGCTTCTG3´.

The PCR products were loaded onto a 1.2% agarose gel, run at 100 volts and and stained with ethidium bromide.

Results

We have previously developed a protocol to culture hESCs in the absence of mouse feeder layers [1]. Accordingly, the cells were grown on laminin-coated tissue culture dishes in the presence of DSR media supplemented with ANK, a mixture of growth factors containing recombinant human activin A, NIC and recombinant human KGF. Starting with these cell culture conditions, we wanted to determine whether we could adjust our cell culture conditions and lower the amount of Neu5Gc expressed on cultured hESCs.

Figure 1. Total levels of Neu5Gc in human embryonic stem cells are reduced upon culture in the absence of animal products.



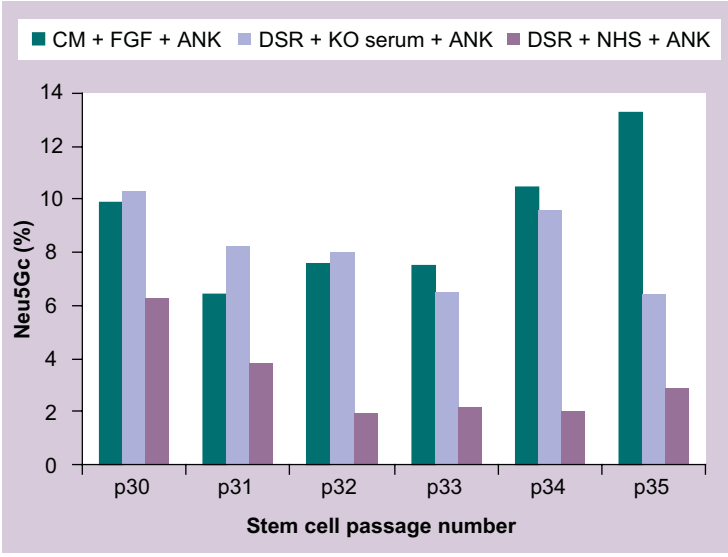
Sialic acids from washed human embryonic stem cells (hESCs) were extracted in mild acid and derivatized with 1,2-diamino-4,5-methylene dioxybenzene and analyzed by high-performance liquid chromatography. The peaks corresponding to Neu5Gc and Neu5Ac are labeled. **(A)** hESCs cultured in the presence of DSR media supplemented with serum derived from mouse.
(B) hESCs after 4 weeks in culture with exclusively human products.

In initial experiments, we wanted to determine whether the total amount of Neu5Gc could be reduced when hESCs were cultured under feeder layer-free conditions in the presence of human serum. Both Neu5Gc and the ubiquitous sialic acid *N*-acetylneuraminic acid (Neu5Ac) content on hESCs was measured by HPLC [8]. Under standard hESC culture conditions [1], 908.8 pmols of Neu5Gc were detected per microgram of total protein (pmol/µg) by HPLC (Figure 1A). After 4 weeks in DSR supplemented with ANK and human serum, the amount of Neu5Gc dropped fivefold to 182.4 pmols (Figure 1B), suggesting that a change in cell culture conditions could dramatically alter Neu5Gc content. In control experiments, human laminin alone was not identified as a source of Neu5Gc (data not shown). Taken together, these results indicate that removal of nonhuman cell culture products reduces Neu5Gc expression on hESCs after multiple passages.

Next, we wanted to systematically examine hESC culture conditions to determine which components contributed to Neu5Gc expression on hESCs and to optimize cell culture conditions. To address these questions, HSF6 cells were grown under three different conditions for up to six passages and membrane-bound Neu5Gc and Neu5Ac content was measured by HPLC. Cells were passaged weekly and a portion from each passage was retained for protein determination and measurement of sialic acid content. In all experiments, the picomoles of Neu5Gc were normalized to total proteins levels (Figure 2). In the first set of experiments, cells were grown in CM from mouse fibroblast feeder layers supplemented with human bFGF and ANK (dark bars). This conditioned media DSR contains growth factors released from mouse fibroblasts and commercially available KO-SR (GibCo). The normalized amounts of Neu5Gc remained relatively constant over the course of the experiments, with a range of 6.3 to 9.9% Neu5Gc and an average of 7.9% Neu5Gc.

In the second set of experiments, HSF6 cells were incubated in complete DSR media supplemented with ANK (red bars). Again, this media contained potential nonhuman sialic acids derived from serum. Over five passages during a 5-week period, the amount of Neu5Gc was again relatively constant, ranging from 7.7 to 10.3%, with an average of 8.5% Neu5Gc. In the final experimental condition, HSF6 cells were grown in DSR media supplemented with normal human serum and ANK to maintain pluripotency. After the initial passage, the amount of Neu5Gc dropped from 10.3 to 6.3%. Subsequent passages of cells in the same media saw a continued drop in the amount of Neu5Gc to 2%, where it stabilized (blue bars). These results indicate that the amount of Neu5Gc can be reduced through the elimination of animal products in tissue culture conditions. One potential source of Neu5Gc contamination, the KO-SR, was tested and found to have a consistently low, but detectable, level of sialic acid. However, the amount detected was lower than the 2% observed in hESCs.

Figure 2. Analysis of *N*-glycolylneuraminic acid content of human embryonic stem cells grown under three different culture conditions.



Human embryonic stem cells were grown under feeder layer-free conditions in the presence of CM-mouse + FGF + ANK (green bars), DSR + KO serum + ANK (red bars), or in DSR + NHS + ANK for four passages (blue bars). During each passage, a portion of the cells was removed for protein determination (BCA assay, Pierce) and for high-performance liquid chromatography analysis of Neu5Ac and Neu5Gc.
ANK: Activin, nicotinamide, keratinocyte growth factor; CM: Conditioned media; FGF: Fibroblast growth factor; KO: Knockout; NHS: Normal human serum.

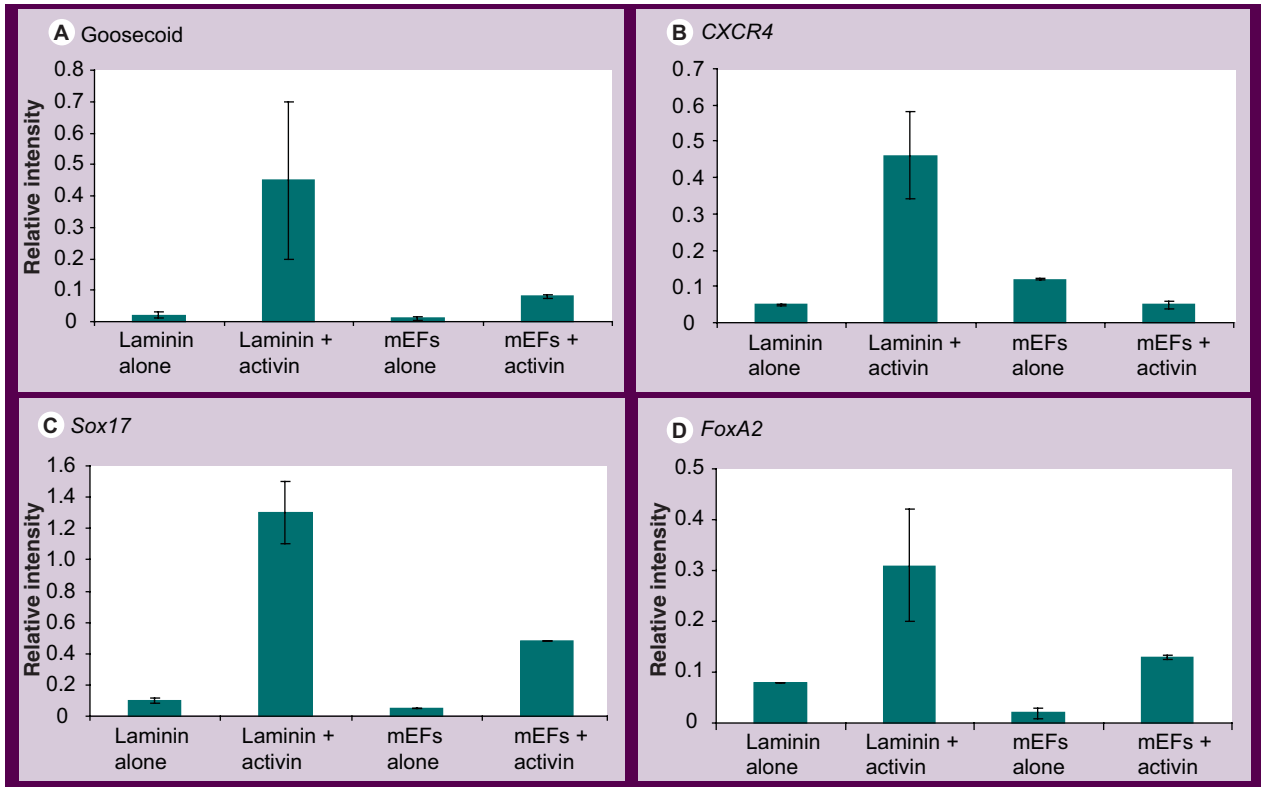
Previous studies have found that Neu5Gc levels have little impact on the differentiation of hESCs into neural cell precursors [9]. We wanted to determine whether reduced Neu5Gc levels and hESCs culture in feeder layer-free conditions interfered with the generation of definitive endoderm from hESCs. hESCs were plated either on mEFs or on laminin in the presence of low (0.2%) human serum in the presence or absence of high concentrations of activin A (100 ng/ml) for 5 days. Sialic acid levels did not increase under these conditions. To determine the efficiency of differentiation, fluorescence-activated cell sorting (FACS) analysis was performed on cells expressing CXCR4, a marker for definitive endoderm. In initial experiments, undifferentiated hESCs were found to express low levels of CXCR4 receptor (<4%). After the 4-day differentiation protocol, the number of cells expressing CXCR4 had increased to 62%, while mock differentiated cells showed no change in CXCR4 levels after 4 days in culture. To confirm differentiation into definitive endoderm, cells were harvested, and mRNA was generated using the Qiagen RNeasy kit, and cDNA was prepared by reverse transcription using iScript cDNA Synthesis Kit (Bio-Rad, CA, USA), and reverse transcription-PCR was performed as previously described [4]. Levels of the definitive endodermal markers *Sox17*, *FoxA2*, Goosecoid and *CXCR4* were quantified (Figure 3). Consistent with results previously published by D'Armour *et al.* [4], hESCs grown on mEFs in the presence of high activin A levels showed a significant increase in *Sox17* and *FoxA2* transcript levels. In these experiments, neither the Goosecoid nor the *CXCR4* levels were significantly different from the control cells grown on mEFs in the absence of activin A. A significant increase in message for *Sox17*, *FoxA2*, Goosecoid and *CXCR4* was observed when hESCs were cultured on laminin under conditions previously demonstrated to reduce Neu5Gc levels. These results demonstrate that reduced nonhuman sialic acid levels and hESC culture in feeder layer-free conditions do not interfere with the endodermal differentiation program.

Discussion

Here, we describe the reduction of the nonhuman sialic acid Neu5Gc through the elimination of animal-derived cell culture products. Specifically, much of the Neu5Gc is associated with the serum used during culture, not with the laminin or recombinant protein cocktail used to maintain pluripotency.

During the preparation of this report, other investigators have helped to establish a strong foundation for the reduction of Neu5Gc levels through changes in cell culture conditions [10,11]. Heiskanen *et al.* published a report demonstrating that Neu5Gc levels could be decreased in both mesenchymal and hESCs using a mass spectrometry and immunohistochemical approach [10]. Cell death resulting from compliment-mediated targeting was demonstrated to be reduced upon removal of hESCs from mEFs [12], although the results of these experiments have been called into question [13]. Our results are similar to those obtained by Nasonkin *et al.*, who observed substantial decreases in Neu5Gc (<1%) during neural differentiation using media composed of B27/N2/noggin [9]. Taken together, these studies provide strong evidence that culture of hESCs on animal feeder layers is a significant source of Neu5Gc and stem cell death, but that the nonhuman sialic acid levels can be decreased through the manipulation of cell culture conditions.

Figure 3. Decreased levels of *N*-glycolylneuraminic acid do not impair human embryonic stem cell differentiation into definitive endoderm.



HSF6 cells were either plated on laminin or on mouse embryonic fibroblast feeder layer in the presence or absence of 100 ng/ml activin A. After 5 days, cells were harvested for quantitative PCR analysis, as previously described [3].
mEF: Mouse embryonic fibroblast.

We also demonstrate that hESCs can undergo directed differentiation to definitive endoderm lineage, when cultured under feeder layer-free conditions. Differentiation occurs whether cells are grown under standard conditions (DSR + KO serum + ANK; data not shown), or in the presence of exclusively human/recombinant-derived cell culture products. Interestingly, cells grown in the absence of feeder layers showed a stronger expression of definitive endodermal markers. Goosecoid and *CXCR4* receptor message RNA was increased fourfold, while *Sox17* and *FoxA2* message RNA was increased threefold over cells differentiated on mEFs. Whether this represents a more robust expression of markers or larger population of cells undergoing differentiation is currently under investigation.

Executive summary

- The therapeutic use of human embryonic stem cells (hESCs) may be complicated by the presence of the nonhuman and potentially immunogenic sialic acid *N*-glycolylneuraminic acid (Neu5Gc).
- Changing cell culture conditions of hESCs to a feeder layer-free system containing exclusively recombinant- and human-derived products decreased the detectable amount of Neu5Gc fivefold.
- The culture of cells in animal serum appears to be a major source of Neu5Gc.
- hESCs were demonstrated to undergo directed differentiation to form definitive endoderm when grown under feeder layer-free conditions in the presence of human serum.
- The appearance of markers of definitive endoderm (Goosecoid, *CXCR4* receptor, *FoxoA* and *Sox17*) was more robust than cells cultured on feeder layers, suggesting that feeder layer-free conditions may be a better system for differentiation of hESCs into definitive endoderm.

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Financial & competing interests disclosure

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No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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