bFGF Supports Human ES Cell Self-Renewal

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SUMMARY

Basic fibroblast growth factor (bFGF) is an important cytokine used to support the growth and self renewal of human embryonic stem cells. Stemgent's Stemfactor™ FGF-basic, Human Recombinant provides a high-quality product for human embryonic stem cell culturing applications. Here we show that when added to cell culture media, Stemfactor bFGF is capable of maintaining human embryonic stem cells long-term in feeder and feeder-free conditions.

INTRODUCTION

Embryonic stem (ES) cells are pluripotent cells capable of self-renewing and generating different functional cell types of the body[1,2]. Traditionally, they are grown on feeder cells with other factors added to support self renewal. Basic fibroblast growth factor (bFGF, FGFb, or FGF2) is a member of the FGF superfamily and has important roles in development[3]. Traditionally, human ES cells have been isolated and maintained in media supplemented with bFGF[2,4]. Interestingly, bFGF was found to be sufficient by itself to support human ES cell self-renewal in feeder-free culture[5].

Stemgent provides **Stemfactor FGF-basic, Human Recombinant (Stemgent Cat. No. 03-0002)**, a human recombinant bFGF for human ES culturing applications. Here, we show that Stemfactor bFGF can maintain the self renewal of human ES cells in both feeder and feeder-free conditions. We also determine the purity of Stemfactor bFGF and its ability to stimulate NIH3T3 cells.

RESULTS

bFGF was expressed in a bacterial strain and purified using an affinity-tag system. After an overnight dialysis, endotoxins were removed and the proteins were filter-sterilized. To determine purity, 1 µg of the final product was evaluated using SDS-PAGE. Following electrophoresis, the protein bands were visualized by staining with Coomassie® Blue (**Figure 1**). Only one predominant band was observed. The size of the band is around 17.3 kDa which matches the predicted molecular weight for human bFGF proteins based on its amino acid sequence.

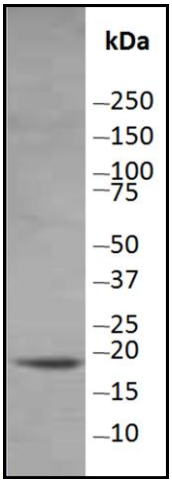

Figure 1. Analysis of the purity of Stemfactor bFGF. 1 µg of Stemfactor bFGF was loaded onto an SDS-PAGE gel. Following electrophoresis, the gel was stained with Comassie blue.

Since bacterial endotoxins carried over during the purification steps can have detrimental effects on cell culture, we used the LAL[6] method to determine the endotoxin level for the purified bFGF. The results show that the endotoxin level is 0.26 EU/µg, well below the widely accepted parameter of 1.0 EU/ µg[6].

To determine the biological activity of Stemfactor bFGF, we tested its ability to promote NIH3T3 fibroblast cell proliferation. Briefly, cells were cultured overnight in standard media and then transferred to a low serum media overnight. The following day, Stemfactor bFGF was added to the starved cells at a final concentration range of 0.02 to 5 ng/ml. The cells were cultured for 3 days, and the final cell numbers were determined. The standard curve was generated (**Figure 2**) and the ED_{50} value was calculated using the GraphPad Prism® 5 software. The results show that the ED_{50} value is 0.53 ng/ml, which is comparable to the best products that are on the market today.

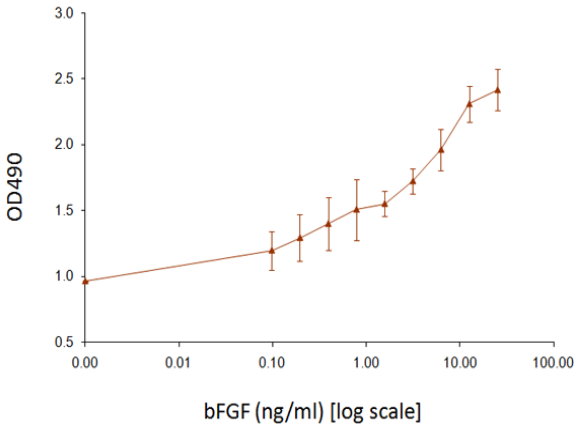

Figure 2. NIH3T3 proliferation assay analysis of biological activity of Stemfactor bFGF. All tests were performed in triplicate. The ED_{50} is 0.5 ng/ml.

Stemfactor bFGF was further tested in the application of human ES cell culture. Two human ES cell lines, H1 and HuES9, were cultured on gamma-irradiated mouse embryonic fibroblasts (MEFs) in culture medium supplemented with Stemfactor bFGF at 4 ng/ml. After three passages, the cell colonies that were supplemented with Stemfactor bFGF maintained their human ES morphology and had positive alkaline phosphatase (AP) staining, while the cell colonies cultured without Stemfactor bFGF began to differentiate (**Figure 3**). ES cells were maintained for ten passages, and then analyzed for pluripotency markers by immunocytochemistry (ICC) and flow cytometry (FC). The ICC results showed high expression levels of Oct4, Sox-2, SSEA-4, and Tra-1-60 (**Figure 4**). The FC analysis showed that more than 90% of the cells expressed SSEA-3, SSEA-4, Tra-1-60, and Tra-1-81 (**Figure 5**).

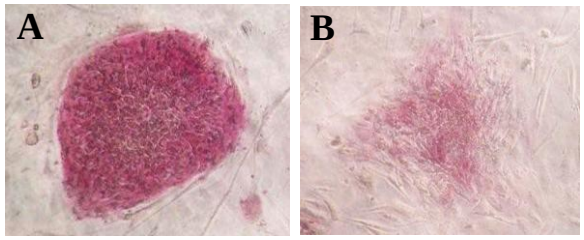

Figure 3. AP staining of H1 cells passaged three times on MEF feeder cells. A) H1 cells cultured with Stemfactor bFGF at 4 ng/ml show strong AP staining. B) H1 cells cultured without Stemfactor bFGF begin to differentiate and loose AP staining. Similar results were obtained using HuES9 cells (data not shown).

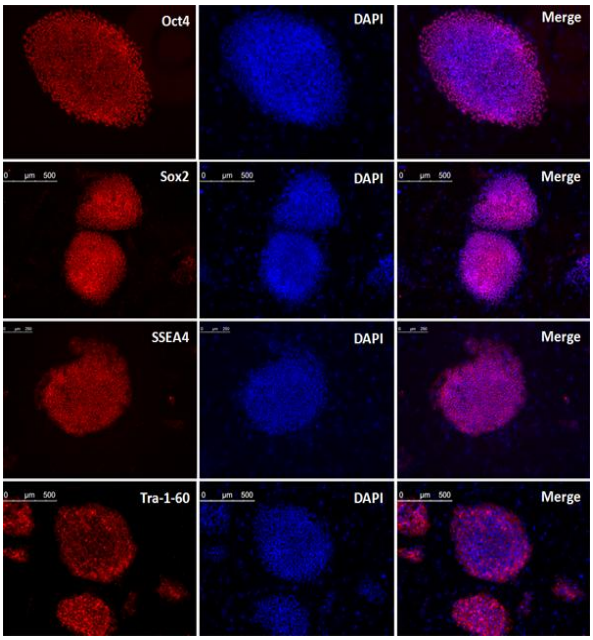

Figure 4. Pluoripotency marker analysis of H1 cells cultured for ten passages on MEF feeder cells. The left column represents Oct4-, Sox2-, SSEA-4- or Tra-1-60-specific antibody staining, the middle column represents DAPI-stained nuclei, and the right column represents the merged images of the DAPI- and antibody-stained cells.

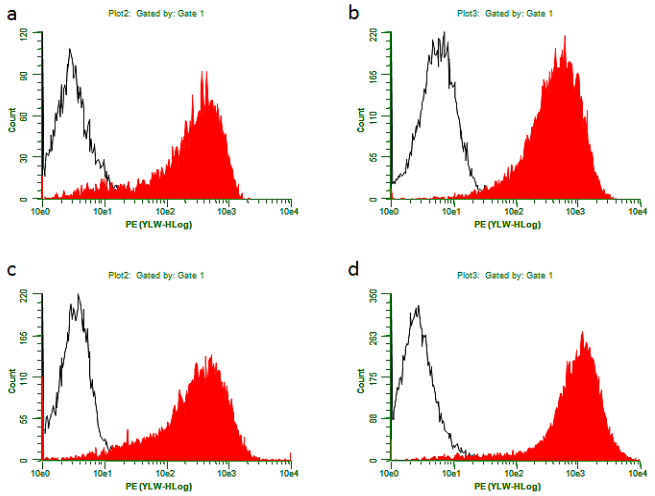

Figure 5. Flow Cytometry analysis of the H1 cell line. H1 cells maintained for ten passages using Stemfactor bFGF express high levels of (a) Tra-1-60, (b) Tra-1-81, (c) SSEA-3, and (d) SSEA-4 cell surface pluripotency biomarkers.

To test Stemfactor bFGF under feeder-free conditions, both H1 and HuES9 cells were cultured without MEF feeder cells in media containing 20 ng/ml of Stemfactor bFGF for at least ten passages. **Figure 6** shows that HuES9 cells maintained in the presence of Stemfactor bFGF have high expression levels of typical ES cell pluripotency cell surface markers. Similar results were seen for the H1 cell line (data not shown). Taken together, these data show that Stemfactor bFGF is sufficient to maintain the hES cell lines H1 and HuES9 in a pluripotent state, even in feeder-free conditions[5].

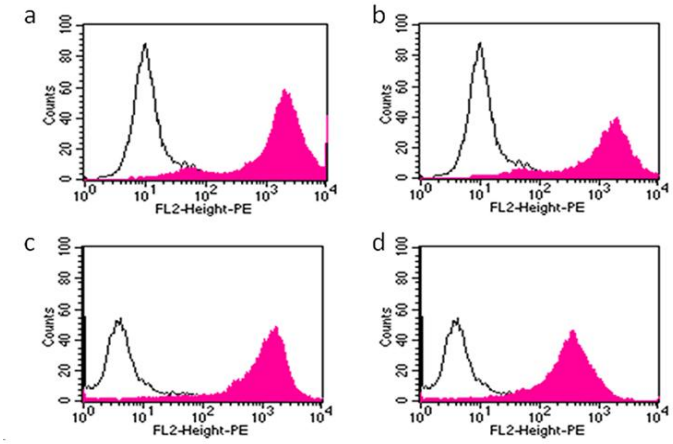

Figure 6. Flow cytometry analysis of the HuES9 cell line. HuES9 cells maintained in feeder-free medium supplemented with 20 ng/ml Stemfactor bFGF for ten passages express high levels of (a) Tra-1-60, (b) Tra-1-81, (c) SSEA-3, and (d) SSEA-4 cell surface pluripotency biomarkers.

EXPERIMENTAL PROCEDURES

Cell Culture Procedure

The H1 and HuES9 human ES cell lines were maintained on irradiated CF-1 MEFs in hES Medium (400 ml DMEM/F12 supplemented with Stemfactor bFGF, 100 ml Knockout™ Serum Replacement, 5 ml non-essential amino acids, 5 ml 200 mM L-Glutamine, and 0.9 ml of 55 mM β-mecaptoethanol) or on Matrigel™ in Feeder-Free Medium (500 ml DMEM/F12 supplemented with 5 ml N-2 Supplement, 10 ml B-27 Supplement, 5 ml non-essential amino acids, 5 ml 200 mM L-Glutamine, and 0.9 ml β-mecaptoethanol). The cells were passaged once every week. To passage, the cells were treated with 1 mg/ml of Collagenase IV (for the cell lines on feeder cells) or Dispase (for the cell lines in feeder-free culture) and then split at a 1:3 to 1:6 ratio.

In Vivo Activity Assay

NIH3T3 cells (ATCC) were seeded at 4 x 10^3 cells per well in a 96 well plate and cultured in DMEM supplemented with 10% calf bovine serum (CBS) overnight. The medium was replaced with low serum medium (DMEM supplemented with 0.1% CBS) and the cells were incubated overnight. After an overnight starvation, the cells were treated with DMEM supplemented with 1% CBS and Stemfactor bFGF (from 0 to 25 ng/ml) for 72 hours. After incubation with CellTiter 96® AQueous One Solution Reagent according to manufacturer's instructions at 37°C for 2 hours, the plate was measured using a plate reader at OD_{490} to determine the bFGF activity.

AP staining

AP staining was performed using the Stemgent Alkaline Phosphatase Staining Kit (Cat. No. 00-0009) (reference *Protocol: Alkaline Phospatase Staining Kit* online at www.stemgent.com /support/protocols).

Immunocytochemistry and Flow Cytometry

Immunocytochemistry and flow cytometry were performed using standard procedures (reference *Protocol: Immunocytochemistry and Protocol: Flow Cytometry* online at www.stemgent.com /support/protocols).

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