Application Note

Stemfactor® Fibroblast Growth Factor-basic (Human Recombinant) Supports Human ES Cell Self-Renewal

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SUMMARY

Basic Fibroblast Growth Factor is an important cytokine used to support the growth and self renewal of human embryonic stem cells. Stemgent's Stemfactor™ Fibroblast Growth Factor-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, Stemgent's 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™ Fibroblast Growth Factor-basic (Human Recombinant) (Stemgent Cat. No. 03-0002), a human recombinant bFGF for human ES culturing applications. Here, we show that bFGF can maintain the self renewal of human ES cells in both feeder and feeder-free conditions. We also determine the purity of Stemgent's bFGF and its ability to stimulate NIH3T3 cells.

RESULTS

Stemfactor™ Fibroblast Growth Factor-basic (Human Recombinant) 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 bFGF 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.

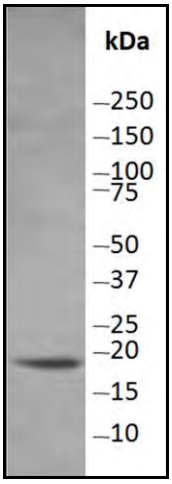

Figure 1. Analysis of the Purity of Stemfactor® Fibroblast Growth Factor-basic (Human Recombinant)
1ug of bFGF was loaded onto an SDS-PAGE gel. Following electrophoresis, the gel was stained with Comassie® blue.

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 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, 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.

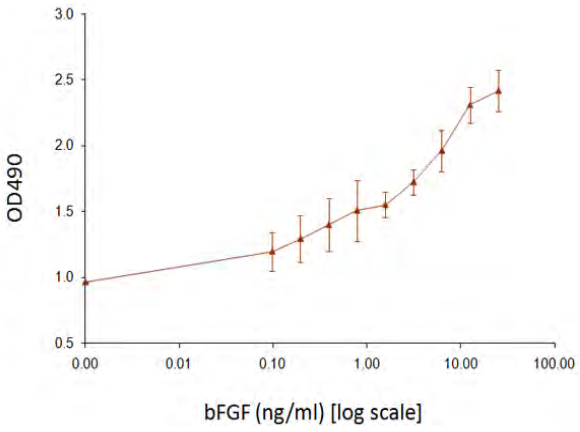

Figure 2. NIH3T3 Proliferation Assay Analysis of Biological Activity of Stemfactor bFGF (Human Recombinant)
All tests were performed in triplicate. The ED_{50} for this lot of Stemfactor bFGF is 0.5 ng/ml.

bFGF was further tested in the application of human ES cell culture. Two hES cell lines, H1 and HuES9, were cultured on gamma-irradiated mouse embryonic fibroblasts (MEFs) in culture medium supplemented with bFGF at 4 ng/ml. After 3 passages, the cell colonies that were supplemented with bFGF maintained their hES morphology and had positive Alkaline Phosphatase (AP) staining, while the cell colonies cultured without bFGF began to differentiate (Figure 3). ES cells were maintained for 10 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).

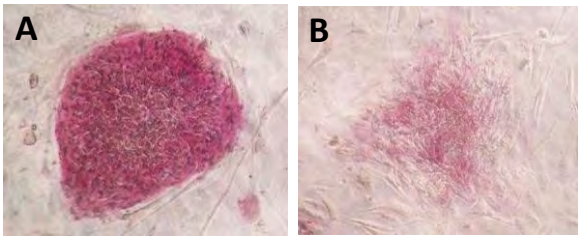

Figure 3. AP Staining of H1 Cells Passaged 3 Times on MEF Feeder Cells
H1 cells cultured with Stemfactor bFGF (Human Recombinant) at 4 ng/ml show strong AP staining. H1 cells cultured without Stemfactor bFGF (Human Recombinant) begin to differentiate and loose AP staining. Similar results were obtained using HuES9 cells (data not shown).

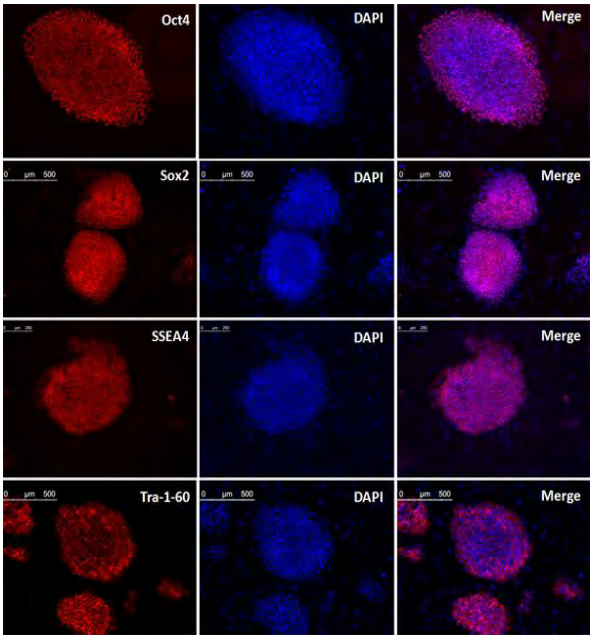

Figure 4. Pluoripotency Marker Analysis of H1 Cells Cultured for 10 Passages on MEF Feeder Cells
The left column represents Oct4-, Sox2-, SSEA-4- or Tra-1-60-specific antibody staining, the middle column represents DAPI-stained nuclei, and the right column represents the merged images of the DAPI- and antibody-stained cells.

To test bFGF under feeder-free conditions, both H1 and HuES9 cells were cultured without MEF feeder cells in media containing 20 ng/ml bFGF[5] for at least 10 passages. Figure 6 shows that HuES9 cells maintained in the presence of 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® Fibroblast Growth Factor-basic (Human Recombinant) is sufficient to maintain the hES cell lines H1 and HuES9 in a pluripotent state, even in feeder-free conditions[5].

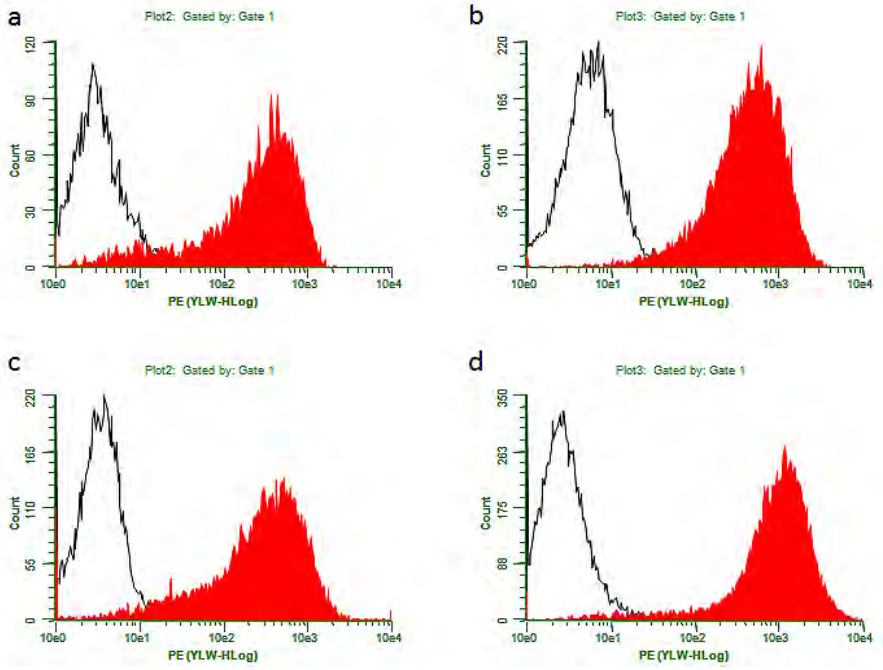

Figure 5. Flow Cytometry Analysis of the H1 Cell Line
H1 cells maintained for 10 passages using Stemfactor® Fibroblast Growth Factor-basic (Human Recombinant) express high levels of (a) Tra-1-60, (b) Tra-1-81, (c) SSEA-3, and (d) SSEA-4 cell surface pluripotency biomarkers.

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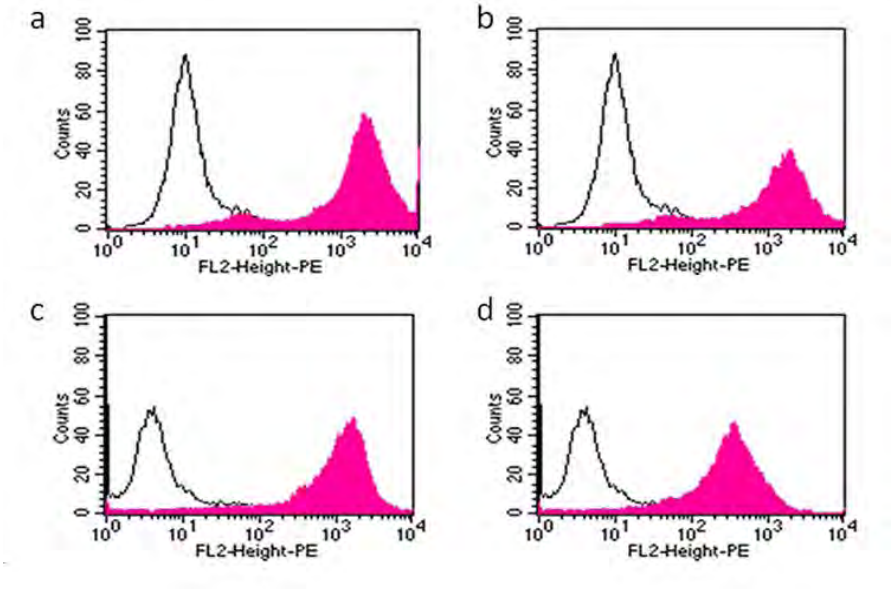

Figure 6. FC Analysis of the HuES9 Cell Line
HuES9 cells maintained in feeder-free medium supplemented with 20 ng/ml Stemfactor® Fibroblast Growth Factor-basic (Human Recombinant) for 10 passages express high levels of (a) Tra-1-60, (b) Tra-1-81, (c) SSEA-3, and (d) SSEA-4 cell surface pluripotency biomarkers.

MATERIALS AND METHODS

Materials

- Stemfactor™ Fibroblast Growth Factor-basic (Human Recombinant) (Stemgent Cat. No. 03-0002)
- Knockout™ Serum Replacement (Invitrogen Cat. No. 10828-028)
- Dispase (Invitrogen Cat. No. 17105-041)
- Collagenase, Type IV (Invitrogen Cat. No. 17104-019)
- Matrigel™ hESC-Qualified Matrix (BD Cat. No. 354277)
- CellTiter 96® AQueous One Solution Reagent (Promega Cat. No. G3582)
- Stemgent® Alkaline Phosphatase Staining Kit (Stemgent Cat. No. 00-0009)

Preparation of Human ES Culture Medium

400 ml DMEM/F12 (1:1) 1X supplemented with Stemfactor™ bFGF (Human Recombinant), 100 ml Knockout™ Serum Replacement, 5 ml MEM non-essential amino acids (100X solution), 5 ml L-Glutamine (200 mM), and 0.9 ml of 55 mM β-mecaptoethanol.

Preparation of Feeder-Free Culture Medium

500 ml DMEM/F12 (1:1) 1X supplemented with 5 ml N-2 Supplement (100X), 10 ml B-27 Supplement (50X), 5 ml MEM non-essential amino acids (100X solution), 5 ml L-Glutamine (200mM), and 0.9 ml or 55 mM β-mecaptoethanol.

Cell Culture Procedure

The cell lines H1 and HuES9 were maintained on irradiated CF-1 MEF feeder cells in Human ES Culture Medium or on Matrigel™ in Feeder-Free Culture Medium. The cells were passaged once every week. To passage, the cells were treated with 1 mg/ml Collagenase IV (for the cell lines on feeder cells) or Dispase (for the cell lines in feeder-free culture) for an optimal time period, 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 at 37°C and 5% CO_2. The medium was

replaced with low serum medium (DMEM supplemented with 0.1% CBS) and the cells were incubated overnight at 37°C and 5% CO_2. After an overnight starvation, the cells were treated with DMEM supplemented with 1% CBS and bFGF (from 0 to 25 ng/ml, 3 wells for each concentration) 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.

Immunocytochemistry (ICC) Analysis

The ICC staining procedure was performed as instructed in the ICC general protocol (Stemgent Cat. No. 00-0017). Purified antibodies against Oct4, Sox2, SSEA-4, and TRA-1-60 were diluted following the manufacturers' instructions.

Flow Cytometry (FC) Analysis

Flow Cytometry was performed by following the Stemgent general protocol: Immunofluorescent Staining of Cell Surface Biomarkers using Flow Cytometry. The PE Mouse anti-Human SSEA-4 Antibody, PE anti-Human TRA-1-60 Antibody , PE Mouse anti-Human TRA-1-81, PE Mouse IgG_3, κ Isotype Control, PE Mouse IgM, κ Isotype Control, and PE Rat IgM, κ Isotype Control antibodies were diluted following the manufacturers' instructions. The data were collected and analyzed using the Guava EasyCyte™ Plus System.

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*The ED_{50} value is the dose of a drug that is pharmacologically effective for 50% of the population exposed to the drug or 50% response in a biological system that is exposed to the drug.

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