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Establishing Optimal Seeding Density and Ideal Starvation Conditions of Sf9 and NIH/3T3 Cell Lines

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Establishing Optimal Seeding Density and Ideal Starvation Conditions of Sf9 and NIH/3T3 Cell Lines

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Abstract

Fibroblast Growth Factors (FGFs) are a family of structurally related polypeptide cytokines. 22 FGF genes (FGF1-14, FGF16-23) are found in the human genome. FGFs have various known intracrine, paracrine, and endocrine functions and are essential for the development and wound repair in organisms through these mechanisms. The role of FGFs in viruses has become an area of piqued interest in the field of pathology as several virus families have genomes that encode one or more growth factor homologues. It has been suggested that a virally encoded ortholog of FGF (vFGF) identified in the viral families of Baculoviridae and Iridoviridae is involved in the movement of these viruses across the basal lamina in the midgut of insect hosts to shift from primary infection to systemic infection (Means and Passarelli 2010). It was found that the Baculoviridae encodes a viral fibroblast homolog (vfgf) expressed as an early gene in the beginning stages of viral infection (Katsuma et al. 2006). Despite the evidence of the involvement of vFGF in cell migration, there is no published research on its role in cell proliferation, even though many FGFs are known to be mitogens. The purpose of our research is to produce recombinant FGF from two baculoviruses, AcMNPV and CfMNPV, and test their effect on cell proliferation of multiple cell lines. A part of this process includes finding the optimal seeding densities and optimal starvation periods of each cell line used. The optimal seeding density was found by seeding 96-well plates of SF9 and NIH/3T3 cells at various ranges of seeding densities correlated to their growth curves. The data was then analyzed using CCK-8 and crystal violet proliferation assays to observe where cells appeared to begin to plateau, indicating that they were overgrown. We found the optimal minimal seeding densities of SF9 cells to be about 50,000 cells/well, and about 4,000-4,500 cells/well for NIH/3T3 cells. We then took 96-well plates seeded at 3,000, 5,000, and 7,000 cells/well and treated them with media containing a range of 0-10% and 0-2% newborn calf serum to observe which concentration of serum allowed for cells to remain viable while being starved. It was found that serum containing 1% newborn calf serum allowed cells to remain the most viable during starvation at a seeding density of around 5,000 cells/well. This data will be used to set up plates of SF9 and NIH/3T3 cells to be starved and then treated with various concentrations of vFGF to observe the effects on cell proliferation.

Introduction

Fibroblast Growth Factors (FGFs)
- Polypeptide cytokines.
- 22 FGF genes found in the human genome.
- Intracrine, paracrine, and endocrine functions.
- Essential for embryonic development and wound repair.
- Several virus families have genomes that encode one or more growth factor homologs.

Baculoviridae and Viral FGFs
- Baculoviridae is a family of DNA viruses infectious to arthropods.
- Hold the ability to produce large quantities of recombinant proteins, leading to their extensive use as protein expression systems in pharmaceutical applications.
- Diptera, Hymenoptera, and Lepidoptera are well-documented hosts.
- Involved in the control of natural insect populations and have been used to control agricultural pests of human food crops.
- Occlusion bodies (polyhedra (NPVs) or granules/capsules (GVs))
- Increase stability and protect from the environment, causing indefinite virulence.
- Suggested that a virally encoded ortholog of FGF (vFGF) is involved in the movement across the basal lamina in the midgut of insect hosts.
- Shift from primary infection to systemic infection (Means and Passarelli 2010).
- Encodes a viral fibroblast homolog (vfgf) expressed as an early gene in the beginning stages of viral infection (Katsuma et al. 2006).

Importance of Research
- No published research on its role in cell proliferation despite known mitogen properties.
- Will give insight to baculovirus pathogenesis, similar viruses, and induced cell lines.
- Misexpression of FGF in humans has been linked to cancer.
- Aim to produce recombinant FGF from two baculoviruses, AcMNPV and CfMNPV, and test their effect on cell proliferation with multiple cell lines.
- This portion involves finding the optimal seeding density and starvation concentrations of Sf9 and NIH/3T3 cell lines to be treated with vfgf.

Methods

Optimal Seeding Density:
Sf9
A 96-well plate was set up with a seeding density range of 0-110k cells/well in ESF-921. The plate was incubated at 27C for 5 days. The plate was treated with CCK-8 reagent and allowed to digest the reagent for 2h. Absorbance readings were collected every hour for 4h. Plate was then fixed with glutaraldehyde 12.5%, treated with .01% and then treated with crystal violet.
NIH/3T3
Two 96-well plates were set up with a seeding density range of 500-10k cells/well in DMEM + NCS 9% and incubated at 37C for 3 and 5 days. Wells were fixed and then treated with crystal violet.
Starvation:
Two 96-well plates were set up with seeding densities of 3k, 5k, and 7k cells/well and incubated for 12h. Medium was removed to waste, cells were washed with DPBS, and medium containing 0, .1, .15, .2, .25, .5, 1, or 2% concentrations of newborn calf serum (NCS) was added to the plate and incubated for another 12h. Plates were fixed with 12.5% glutaraldehyde and treated with crystal violet.

Optimal Seeding Density

Sf9

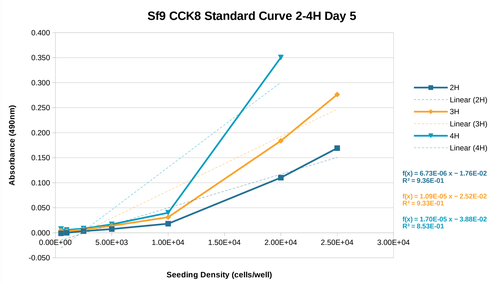

Figure 1. Sf9 Optimal Seeding Density Assay analyzed with CCK-8 at 2, 3, and 4H on Day 5. 96-well plate was seeded at 500-25k cells/well and incubated for 2H. The cells did not plateau, so a higher seeding density was needed.

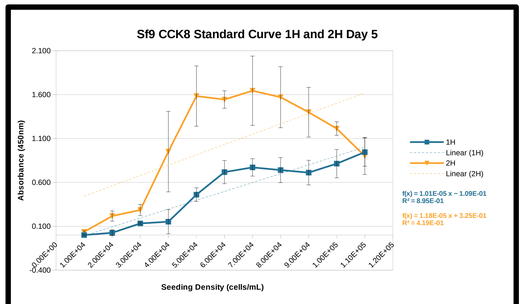

Figure 2. Sf9 Optimal Seeding Density Assay analyzed with CCK-8. 96-well plate was seeded at 10-110k cells/well and incubated for 5 days. Peak is observed at about 70k cells/well, indicating optimal seeding density.

NIH/3T3

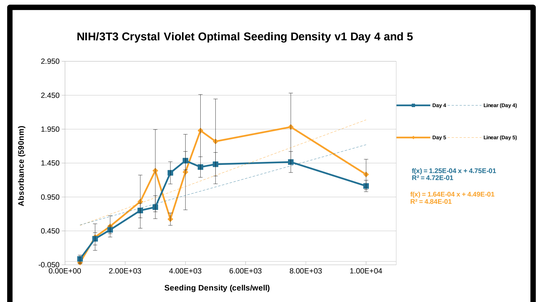

Figure 3. NIH/3T3 Optimal Seeding Density Assay. 96-well plates were seeded at 500-10k cells/well and incubated for 4 and 5 days. Peak is observed at about 8k cells/well on both plates, indicating optimal seeding density.

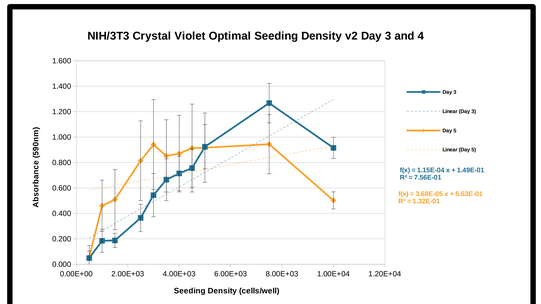

Figure 4. NIH/3T3 Optimal Seeding Density Assay. 96-well plates were seeded at 500-11k cells/well and incubated for 3 and 5 days. Peak is observed at about 8k cells/well on both plates, indicating optimal seeding density.

NIH/3T3 Ideal Starvation Conditions

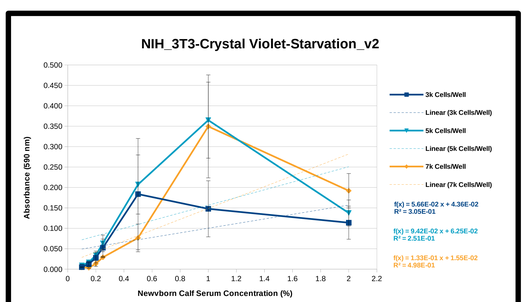

Figure 5. NIH/3T3 Starvation Assay. 96-well plate was seeded at densities of 3k, 5k, and 7k cells/well. Cells were treated with NCS concentrations of 0, .1, .2, .25, .5, 1, and 2%. Results suggest 1% is the ideal NCS concentration at which cells continued to thrive.

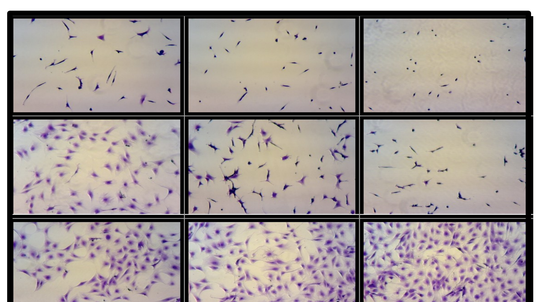

Figure 6. Images of NIH/3T3 Starvation Assay. Images show seeding densities of 3k, 5k, and 7k from left to right, and NCS concentrations of .25, .5, and 1% from top to bottom. Observed denser cells in 1% NCS row. Cells were the optimal density in the 5k column.

Results and Discussion

Optimal Seeding Density – Sf9
In this experiment, we aimed to create a standard curve for use in determining the absorption and concentration of vfgf treated cells after starvation. We wanted to focus on the seeding density range that will allow cells to grow while producing readable absorbances. Our first experiment produced graphs that did not plateau on either Day 0 or Day 5, so we repeated the experiment with a new Day 0 plate with higher seeding densities of 25k-225k cells/well and a Day 5 plate with seeding densities of 10k-110k cells/well. The growth curve began to plateauing at around 70k cells/well. Sf9 cells double every 24-30 hours, so this is about 225k cells/well after 5 days (Figure 2). These results suggest this is the point of optimal seeding density for both cell growth and readable CCK-8 absorbances for Sf9 cells.

Optimal Seeding Density – NIH/3T3
The aim of this experiment was to determine the optimal seeding density of NIH/3T3 cells. This was done by seeding 2 96-well plates; one read on day 4 of incubation, and the other on day 5, both seeded at 500-10k cells/well. We analyzed the proliferation of the cells using crystal violet assay. The cells' growth peaked at about 4k and 4.5k cells/well on day 4 and day 5 plates, respectively (Figure 3). There was a skewed data point, so we redid the experiment, seeding again at a range of 500-10k cells/well. These plates were analyzed on days 3 and 5 with crystal violet. The cells' growth peaked at about 7.5k cells/well for the day 3 plate, and 3.5k cells/well on the day 5 plate (Figure 4). Because NIH/3T3 cells double every 20-26 hours, the second experimen day 3 plate likely peaked at 7.5k instead of 4.5k due to being incubated for approximately 24 hours less. 4.5k doubled to 36k over 4 days, and 7.5k doubled to 30k over 3 days. Had we let the plate incubate for one more day we suspect it would have peaked in the 4-4k range again. The optimal seeding density was then used to come up with an efficient cell density range for our starvation experiment using the NIH/3T3 cells.

Starvation Conditions– NIH/3T3
The aim of this experiment was to observe the lowest concentration of newborn calf serum that allowed for the starvation of cells without causing cell death. Our first experiment included 3 96-well plates, each seeded with 4 columns of 3k, 5k, and 7k cells/well in DMEM + NCS concentrations of 0-10%. All plates were incubated for approximately 2h in DMEM + 9% NCS, and was then removed and replaced with the correlating medium containing different serum concentrations. The plates were then starved for about 12, 24, and 48 hours, respectively, and then fixed and treated with CV to be analyzed. We realized that NIH/3T3 cells needed more time to adhere to the 96-well plates as we noticed splotchy distribution of the cells in different wells that should have been nearly uniform (no data shown). We suspect that due to the cells not being properly adhered to the plate, many of the cells were washed away during the removal of DMEM+9% NCS. We replicated the experiment by taking 1 plate with 4 columns of 3k, 5k, and 7k cells/well that was incubated overnight to allow for proper attachment before changing the medium. The cells were then fed with DMEM + NCS concentrations of 0, .1, .15, .2, .25, .5, 1, and 2%. The results show that there was a stark contrast between 1% serum medium versus the other concentrations and that 5k cells/well seeding density appeared to thrive between the 3 (Figure 5, 6). We concluded that a 1% starvation condition at 5k cells/well is ideal for starving NIH/3T3 cells in 96-well plates

Future Research
This data will be used in future vFGF assays to set up experimental plates of Sf9, NIH/3T3, EPC, and BG cell lines to be treated with various concentrations of vFGF purified from AcMNPV and CfMNPV. The cell proliferation of each cell line will be measured via CCK-8 and crystal violet assays. We hope to show evidence that vFGF induces cell proliferation in each cell line and use this research to open an area of interest in the roles of other FGFs on proliferation.

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