Genomic and Proteomic Tools for the Development of Cell Culture Media

Daniel W. Allison, Kathryn A. Aboytes, Terrell K. Johnson, Heather N. Loke, Danny K. Fong, Stacy L. Leugers, Anne E. Dennett, and Laurel M. Donahue

Cell Culture R&D, Sigma-Aldrich, St. Louis, MO, United States.

Abstract

Cell culture media are currently developed based largely on prior knowledge of cell nutritional biochemistry, as well as literature searches to identify additional components that have been shown to have a desired effect on the cell type of interest. In addition, an investigator must undertake some amount of inefficient, random screening of factors that may have a positive effect on the cellular process being optimized. Understanding the biochemical pathways involved in the regulation of cellular processes is important when trying to optimize the *in vitro* environment for a given cell-based system. Many processes are important to the success or failure of cell culture systems, including proliferation, adhesion, apoptosis, and protein production. The objective of optimization is to enhance, prevent or maintain these processes, often in changing conditions (i.e. reduced serum levels). In order to facilitate a more targeted approach to cell culture medium development, we have employed high-throughput genomic and proteomic approaches to identify candidate factors to screen. For example, by using microarray analysis we can identify the receptors for growth factors, hormones and cytokines, cell adhesion molecules and other components of cell signaling pathways that are expressed by the cell type of interest. Once the proteins of interest are identified, effector molecules (largely ligands) are used in culture to test for the desired effect on given cellular processes (such as proliferation, adhesion, etc.). This approach has allowed us to successfully identify factors that affect both proliferation and adhesion. In addition, the identification of these pathways allows for the use of small molecules to bypass the receptor ligand interaction and stimulate or inhibit the pathway of interest. Several different genomic and proteomic based experiments will be discussed as examples of how they can be used to improve the *in vitro* environment of different culture systems.

Introduction

One of the biggest concerns for the development of new cell culture media is the investment of time required to optimize these products for the intended culture. It is not atypical for the development of a new cell culture product to take more than one year. In order to allow customers to get their end product to market faster, Sigma-Aldrich is working to develop new tools to expedite this labor-intensive process.

Historically, cell culture media development has been based on the developer's knowledge of nutritional biochemistry and some amount of random screening of potentially beneficial molecules. While it has been effective, it is by no means an efficient process. In recent years the adaptation of many statistical approaches has allowed researchers the ability to test more components in a shorter time, but there is still a degree of randomness to these methods. We have tried to provide a more targeted/focused approach to the design of cell culture products, by using more rational methods of identifying beneficial candidates for inclusion in a medium.

The influx of genomic and proteomic research into the scientific community has led to an increased number of tools that can be applied to the development of cell culture products. These tools allow us to better predict the behavior of cells in culture by asking what the cells are poised to respond to *in vitro.* These methods could include genomic tools such as microarrays and quantitative PCR or proteomic tools such as antibody based arrays. These approaches allow us to look either at mRNA or protein levels within a cell culture, and predict based on the expression patterns what might elicit a response from the culture. We can examine the expression patterns of receptors (for growth factors, hormones or cytokines), adhesion molecules, or cell signaling components that might indicate important pathways for us to explore. These pathways could have beneficial effects on a wide range of functions, such as regulation of proliferation, apoptosis, differentiation, adhesion, or production.

In order to improve the efficiency, and thereby reduce the cycle time required to develop products for our customers, we have implemented these technologies to create better medium development tools. These tools allow us to provide a more targeted approach, that is both reproducible and higher-throughput and will have a significant impact on the development time.

Materials & Methods

Cell Preparation

All materials are from Sigma-Aldrich unless otherwise noted. For all cell types, cells were grown in tissue culture treated T-flasks to 60-80% confluence in order to maintain log phase growth. Cells were grown in medium containing the lowest amount of FBS required to still allow reasonable growth rates. Cells were then scraped into RNA*later*™ and frozen.

RNA Isolation, cDNA Labeling, Microarray Hybridization and Detection

RNA, either total or mRNA, was isolated using the GenElute™ Mammalian Total RNA Miniprep Kit or GenElute™ Direct mRNA Miniprep Kit. Labeled cDNA was prepared using dendrimer based 3DNA Array 350™ (or 900™) Expression Array kits utilizing the Cy3™ and Cy5™ dyes (Genisphere Inc., Hatfield, PA). Separate Cy3 and Cy5 reverse transcription reactions were prepared using the appropriate oligo dT primer (each containing a specific 3DNA capture sequence) and RNA. Subsequently, the reactions were pooled and run over SigmaSpin Post-Reaction Clean-Up Columns to remove free primers, salts and nucleotides. The cDNA was then concentrated using a Microcon YM-30 centrifugal filter device (Millipore, Billerica, MA) prewashed with Tris-EDTA Buffer. The cDNA hybridization solution was prepared by adding concentrated cDNA, water, LNA™ dT blocker, human repetitive sequence DNA (ID Labs, London, Ontario, Canada) and 2X formamide based hybridization solution. The solution was then applied to a prewarmed human cytokine microarray (TaKaRa Mirus Bio Inc., Madison, WI) under a 22X25I stan-

dard or m-series LifterSlip™ (Erie Scientific, Portsmouth, NH). Hybridizations were performed overnight for 16-24 hours. Following hybridization, the arrays were washed extensively and dried. The Cy3 and Cy5 Capture Reagents were combined in the formamide based hybridization buffer prepared with Anti-Fade reagent. The 3DNA hybridization mixture was added to a prewarmed array under a LifterSlip. Following a thorough wash, the arrays were dried and then scanned using a PerkinElmer ScanArray Express (PerkinElmer, Wellesley, MA) using the Cy3 and Cy5 channels at Laser and PMT settings that yielded the maximum signal with minimal saturated pixels.

Resazurin Assay

Cells were plated in each well of a 24-well tissue culture treated plate containing 1 ml of a base medium. The base medium contained the lowest amount of FBS required to maintain approximately half-maximal growth of the given cell type. Test conditions were performed in triplicate, with each test compound added to the base medium at three different concentrations. The cells were allowed to grow until they reached approximately 33% confluent. At this point, 100 µl of the resazurin solution from our *In Vitro* Toxicology Assay Kit (Product Code TOX-8) was added to each well. After sufficient incubation time to convert some of the resazurin, the fluorescence was measured on a HTS 7000 Plus BioAssay Reader (Perkin-Elmer, Boston, MA). Readings were taken once a day until the culture was confluent (typically about 4 days). A plate with base medium only (no cells) was used as a blank and subtracted from the RFU reading to establish the final RFU values.

Adhesion Assay

Cells were plated in the base medium at low density on various substrates using the BD BioCoat extracellular matrix coated plates (BD BioSciences, San Jose, CA). Starting 24 hours post plating, the cells were observed for both number of cells attached and morphology (i.e. degree of cell-spreading).

Receptor	Ligand
AXL receptor tyrosine kinase	gas6
neuropilin 1	VEGF*
EGF receptor	EGF*
macrophage stimulating 1 receptor	MSP
chemokine (C-X3-C) receptor 1	Fractalkine
PDGF receptor, α polypeptide	PDGF AB*
PDGF receptor, β polypeptide	PDGF AB*
nerve growth factor receptor	NGF*
interleukin 15 receptor, α	IL-15
interleukin 11 receptor, α	IL-11*
interleukin 2 receptor, α	IL-2*
interleukin 10 receptor, β	IL-10*
interleukin 2 receptor, β	IL-2*
FGF receptor 4	aFGF*
chemokine (C-C motif) receptor 2	MCP1
bone morphogenetic protein receptor, type II	BMP-2*
interleukin 2 receptor, γ	IL-2*
TGF, β receptor II	TGFβ*
interleukin 18 receptor 1	IL-18
FGF receptor 1	bFGF*
colony stimulating factor 1 receptor	CSF-1
chemokine (C-X-C motif), receptor 4	SDF1α*
oncostatin M receptor	OSM*
interferon γ receptor 1	IFNγ*
interleukin 4 receptor	IL-4*
interferon γ receptor 2	IFNγ*
vitamin D3 receptor	Vitamin D3*

**denotes growth factors tested in vitro*

Table 1: List of positive receptor:ligand pairs screened from microarray. Can we add the ligand for a given expressed growth factor or cytokine receptor and see an effect (positive or negative)? These 27 receptors were deemed positive after screening on the microarray. This list was pared down to 16 growth factors/cytokines to test in a "medium-throughput" *in vitro* format.

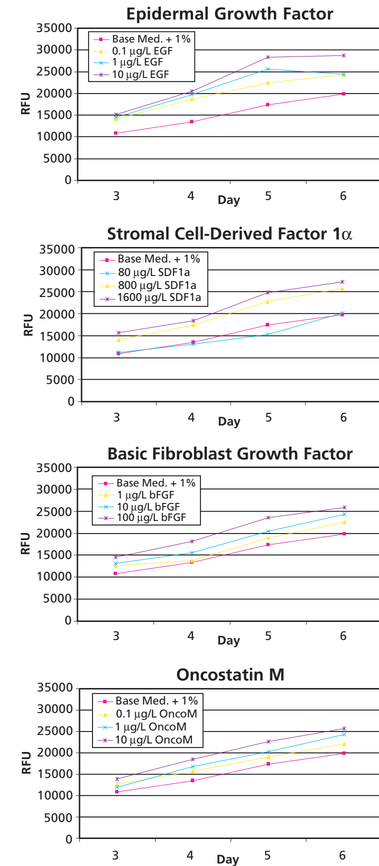

Figure 1: Proliferation of the cells treated with the growth factors/cytokines can be significantly enhanced. Addition of some of the growth factors showed a positive effect on the proliferation of the cells as represented by relative fluorescence units (RFU) from a resazurin assay performed in a 24-well tissue culture plate. Conversion of resazurin to a fluorescent form, represents metabolic activity which correlates to an increased number of cells. For these assays, the base medium contains 1% FBS (pink line), to which each growth factor is added at various test concentrations. Each of these 4 growth factors/cytokines showed increased proliferation when compared to the base medium alone.

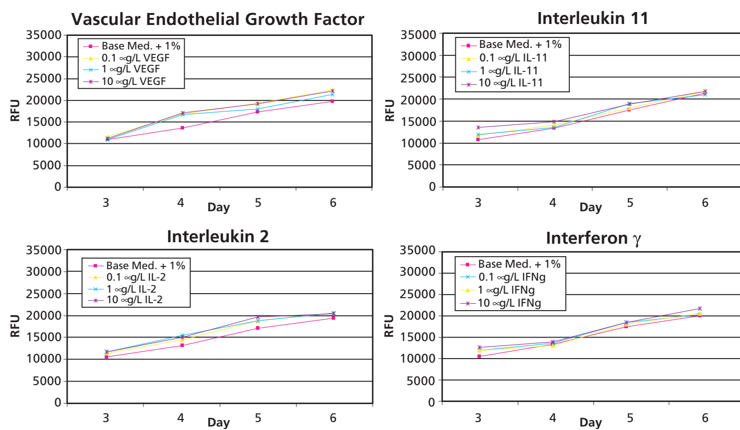

Figure 2: Even in the presence of the receptor, proliferation of the cells treated with the growth factors/cytokines is often unaffected. As might be expected, addition of some of the growth factors did not have an effect on the proliferation of the cells. Each of these 4 growth factors/cytokines showed no effect on proliferation when compared to the base medium alone. It is possible that the addition of these factors had other positive effects, but they did not stimulate proliferation. Some factors even had inhibitory effects on the proliferation of the cells (data not shown).

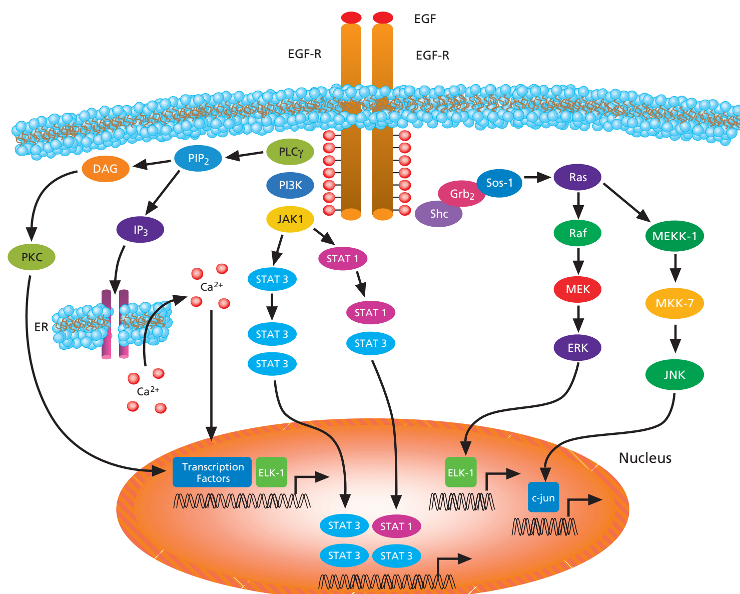

Figure 3: The binding of these ligands to their growth factor/cytokine receptors can have various downstream effects. The addition of the growth factor or cytokine to the cell culture medium can have a variety of downstream effects, from the control of the phosphorylation state of proteins to the regulation of gene expression. Due to the expense involved with the addition of these factors, it may be reasonable to add putative downstream activators or inhibitors to mimic the effect of the ligand. Endogenous intermediates such as inositol-1,4,5-phosphate (IP_3) and diacylglycerol (DAG), as well as many synthetic activators, can be tested for the desired effect.

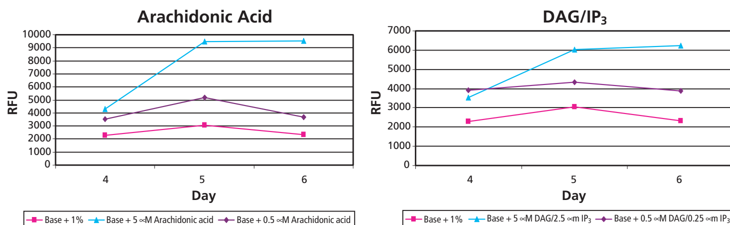

Figure 4: Various compounds can be used instead of growth factors/cytokines to bypass receptor:ligand interactions and activate the same pathways. As an alternative to the addition of growth factors to cell culture media, we added other compounds that would activate the same pathways. Protein kinase C (PKC) is activated by several of the positive receptors. The addition of arachidonic acid, which has been found to stimulate PKC in some systems, had a positive effect on proliferation. Endogenous intermediates such as IP_3 and DAG (produced by the activation of phospholipase C) act via stimulation of PKC and release of intracellular Ca^{++}. This combination also led to increased proliferation.

Receptor:Ligand Conclusions

We selected 16 growth factor/cytokine receptors from the data collected from the microarray

- 4 ligands enhanced proliferation
- 2 ligands we would have probably tried, based on experience or the published literature (bFGF and EGF)
- 2 ligands we would probably have never discovered on our own (Oncostatin M and Stromal Cell-Derived Factor 1 α)
- Compounds that mimic downstream effects of the receptor:ligand interaction are an alternative

Protein	Function
integrin, α8	Cell to Substrate interaction
integrin, α9	Cell to Substrate interaction
integrin, α3	Cell to Substrate interaction
integrin, α1	Cell to Substrate interaction
integrin, β5	Cell to Substrate (vitronectin) interaction
integrin, α7	Cell to Substrate interaction
Integrin, αV	Cell to Substrate (vitronectin) interaction
integrin, αE	Cell to Cell (E-cadherin) interaction
E-cadherin	Cell to Cell interaction; epithelial
P-cadherin	Cell to Cell interaction; placental
OB-cadherin	Cell to Cell interaction; osteoblast
N-cadherin	Cell to Cell interaction; neuronal
VCAM 1	Binds integrins α4β1 and α4β7
ICAM 2	Binds integrin αLβ2
ALCAM	Binds CD6 on leucocytes
MMP 9	Cleaves collagen IV
MMP 10	Cleaves collagen IV
MMP 15	Cleaves fibronectin
TIMP 2	Inhibits MMP2 (cleavage of collagen I,IV)
TIMP 3	Inhibits MMP3 (cleavage of collagen III, IV)

Table 2: List of positive adhesion related molecules screened from microarray. Can we predict, based on adhesion molecule expression, which substrates a cell will or will not attach to? These 20 adhesion related molecules were deemed positive after screening on the microarray. Based on this expression pattern we predicted that the cells would adhere to collagen I based on integrin expression and not adhere to collagen IV based on MMP expression.

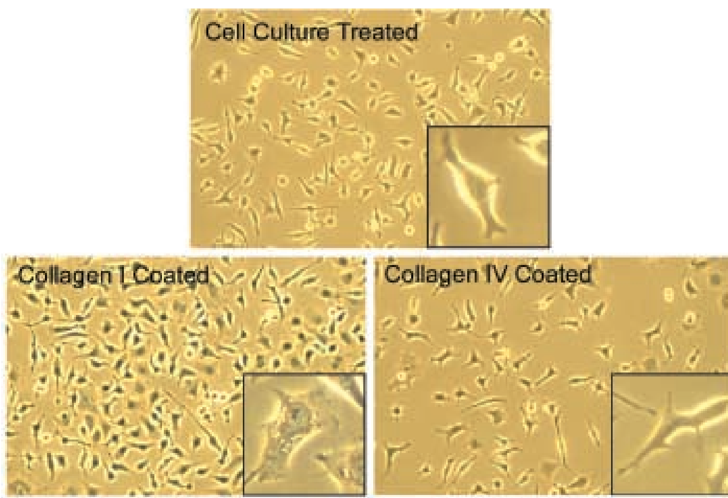

Figure 5: Different adhesive substrates had different effects on adhesion. As was predicted by the adhesion molecule expression pattern, the cells exhibited variable affinity for different adhesion surfaces compared to normal cell culture-treated surfaces. As expected based on integrin expression, collagen I provided better overall adhesion, showing both more total cells attached to the surface and better spreading of the cells once attached. The cells also expressed high levels of the matrix metalloproteinase responsible for cleaving collagen IV. Therefore, we predicted adhesion on collagen IV would not be any better than standard cell culture-treated plates. Cell attachment and morphology on collagen IV was identical to cells plated on cell-culture treated plates.

Adhesion Data Conclusions

- We identified 2 possible adhesion molecule-substrate interactions
- Results of plating on the 2 substrates correlated with prediction from array data

Conclusions

- Microarrays appear to be a powerful tool for use in medium development
    - Speed development
    - Reduce cost
    - Some products can now be developed where previously the chance of success using standard methods was slim
- Use this tool in every medium development project where applicable
- Many genomic or proteomic tools will probably work for this application
- Design our own cell culture development tools (macroarray, qPCR primer sets, antibody arrays, etc.)
- Patent filed for the use of this assay in cell culture development

SIGMA-ALDRICH