

Using Genomic Tools for the Identification of Important Signaling Pathways in Order to Facilitate Cell Culture Medium Development

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Abstract

Given the diversity of cells grown in *ex vivo* culture and the fact that each of these cultures is poised to respond to a variety of stimuli, we set out to establish a method to identify these important pathways to accelerate the design and development of cell culture media. Many of the pathways that are necessary for important cell functions (i.e., cell proliferation, protein production, cell adhesion, differentiation, etc.) have been elucidated in great detail, but there are undoubtedly many unknown pathways which may also be involved. Identification of these untapped resources allows us to better understand these processes and potentially manipulate them to our advantage.

In order to validate this concept, we identified the mRNA expression profile for a variety of proteins within cells in culture using cDNA microarrays. In one example, we identified a specific growth factor receptor not previously known to be expressed *in vitro*. When the ligand for this receptor is added to the culture, the cells are poised to respond and proliferate at an increased rate under reduced fetal bovine serum (FBS) conditions. As an alternative, these same pathways can also be stimulated using various endogenous intermediates, agonists or antagonists. This method has been used on a variety of culture systems (CHO, HEK-293, WI-38, neural stem cells, etc.), to either reduce (or eliminate) FBS requirements or to improve the performance of already serum-free formulations. The ability to identify the presence of components of key pathways within a culture allows us to focus our efforts to develop better cell culture media. This more targeted approach to medium development allows us to perform less of the random screening approaches of the past, thereby decreasing the investment of time and resources into this endeavor.

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, with 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. Adherent cell types were grown in tissue culture treated T-flasks to 60–80% confluence in order to maintain log phase growth. Cells grown in attached culture (HEK-293, WI-38) were grown in medium containing the lowest level of FBS necessary to maintain reasonable growth rates. Cells grown in suspension (CHO-AP; CHO producing recombinant alkaline phosphatase) were cultured in serum-free media. Cells were collected by scraping into RNeasy Lysis Buffer 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) pre-washed 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 pre-warmed human cytokine microarray (TaKaRa Mirus Bio Inc., Madison, WI) under a 22X25I standard 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 kit (Sigma Product 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 (PerkinElmer, 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.

Alkaline Phosphatase Assay

Alkaline phosphatase activity was assayed based on conversion of the fluorescent substrate methylumbelliferyl phosphate using Sigma's Alkaline Phosphatase Reporter Gene Assay Kit (Product Code AP-F). Briefly, 1 mL of cell suspension was taken from each sample and centrifuged at 3,500 rpm for 5 minutes. The supernatant was collected and stored at -20 °C until assayed for the presence of alkaline phosphatase. Samples were diluted 1:10 with dH₂O before being assayed using the AP-F Kit.

Receptor	Ligand	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	TGF β *
Interleukin 18 receptor 1	IL-18	FGF receptor 1	bFGF*
Colony-stimulating factor 1 receptor	CSF-1	Chemokine (C-X-C motif), receptor 4	SDF1 α *
Oncostatin M receptor	OSM*	Interferon γ receptor 1	IFN γ *
Interleukin 4 receptor	IL-4*	Interferon γ receptor 2	IFN γ *
Vitamin D ₃ receptor	Vitamin D ₃ *		

*denotes growth factors tested *in vitro*

Table 1. HEK-293. List of positive receptor:ligand pairs identified 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.

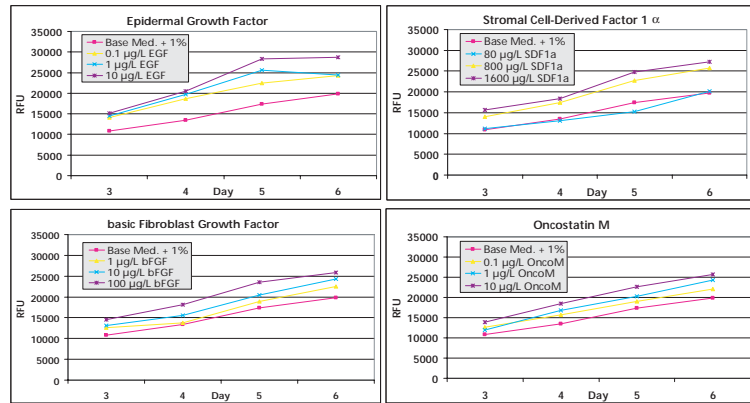


Figure 1. Proliferation of the HEK-293 cells treated with the growth factors/cytokines can be significantly enhanced. Addition of some of the growth factors showed a positive effect on the proliferation of the cells as represented by relative fluorescence units (RFU) from a resazurin assay performed 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 four growth factors/cytokines showed increased proliferation when compared to the base medium alone.

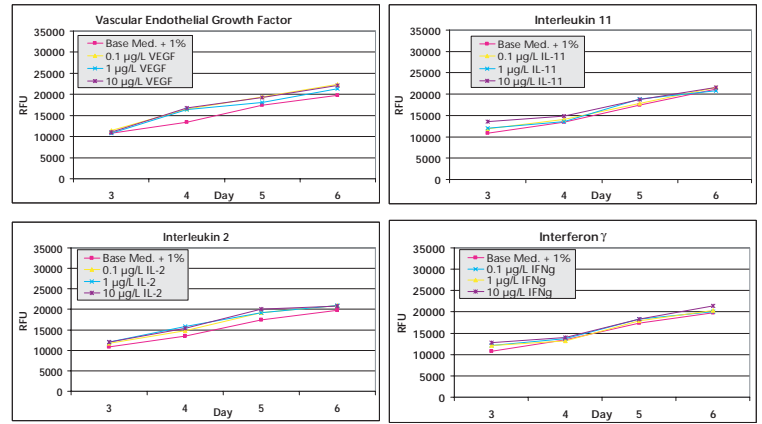


Figure 2. Even in the presence of the receptor, proliferation of the HEK-293 cells treated with the growth factors/cytokines is often unaffected. As might be expected, addition of some of the growth factors did not have an effect on the proliferation of the cells. Each of these four growth factors/cytokines 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).

Receptor	Ligand
Interferon γ receptor 2	IFN γ *
GDNF family receptor α	Artemin*
Interleukin 1 receptor associated kinase 1	IL-1*
Chemokine (C-X-C motif), receptor 4 (fusin)	SDF1 α *
Transforming growth factor, β receptor II	TGF β *
Interferon γ receptor 1	IFN γ *
Platelet-derived growth factor receptor, β	PDGF AB*
Fibroblast growth factor receptor 1	bFGF*
AXL receptor tyrosine kinase	gas6
Insulin-like growth factor 1 receptor	IGF1*
Interleukin 13 receptor, α	IL-13*
Bone morphogenetic protein receptor, type II	BMP-2*
fms-related tyrosine kinase 1	VEGF*
Fibroblast growth factor receptor 4	aFGF
Interleukin 2 receptor, γ	IL-2
Interleukin 7 receptor	IL-7*
Interleukin 2 receptor, α	IL-2

*denotes growth factors tested *in vitro*

Table 2. WI-38. List of positive receptor:ligand pairs identified from microarray. Can we reduce the amount of FBS required for the culture of these human fibroblasts by adding the ligand for a given expressed growth factor or cytokine receptor? These 17 receptors were deemed positive after screening on the microarray. This list was pared down to 12 growth factors/cytokines to test in a "medium-throughput" *in vitro* format.

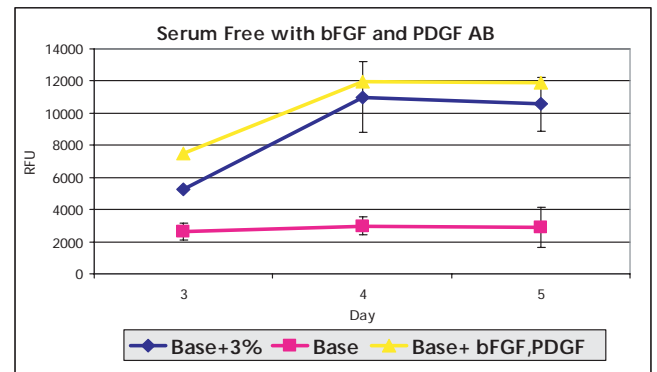


Figure 3. Development of a serum-free formulation for WI-38 cells, with the addition of two growth factors. bFGF and PDGF AB were identified as factors that, when added to a select base medium, permitted the successful development of a serum-free medium for WI-38 fibroblasts.

Receptor	Ligand	Receptor	Ligand
Interleukin 12 receptor, β 2	IL-12	Transforming growth factor, β receptor II	TGF β^*
Colony-stimulating factor 1 receptor	CSF1	Activin A receptor, type II	Activin A *
Burkitt lymphoma receptor 1	BLC *	Macrophage-stimulating 1 receptor	MSP *
Chemokine receptor 9	TECK *	Interferon γ receptor 2	IFN γ^*
Interleukin 11 receptor, α	IL-11	Bone morphogenetic protein receptor, type II	BMP2 *
Bone morphogenetic protein receptor, type IA	BMP2 *	G protein-coupled receptor 9	MIG *
Fibroblast growth factor receptor 4	aFGF	Activin A receptor, type I	Activin A *
Interleukin 1 receptor-like 1	IL-1 *	Fibroblast growth factor receptor 1	bFGF *
Chemokine receptor 4	MDC *	Insulin-like growth factor 2 receptor	IGF2 *
Chemokine receptor 1	MCP3 *	Autocrine motility factor receptor	PGI *
Platelet-derived growth factor receptor, β polypeptide	PDGF AB *	GDNF family receptor α 3	Artemin *

*denotes growth factors tested *in vitro*

Table 3: CHO-AP: List of positive receptor:ligand pairs identified from microarray. Can we increase the recombinant protein production for this CHO cell line by adding the ligand for a given expressed growth factor or cytokine receptor? These 22 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. From those original 16, several were chosen to be tested in spinner culture in order to more closely replicate the normal environment for these cells.

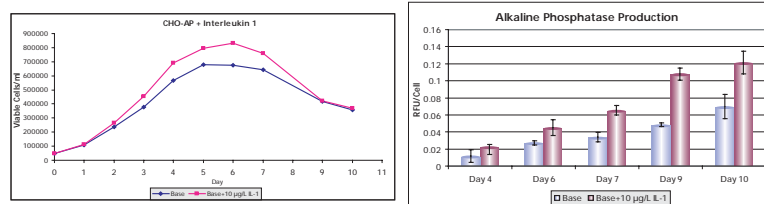


Figure 4. Growth of CHO-AP cells in spinner culture is enhanced with the addition of interleukin 1. A. The addition of 10 μ g/L of interleukin-1 leads to an increase in the proliferation of the CHO-AP cells in spinner culture. B. The increased growth also corresponds to a significant increase in the production of recombinant alkaline phosphatase by the cells.

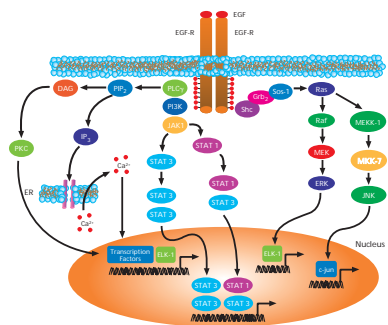


Figure 5: The binding of these ligands to their growth factor/cytokine receptors can have various downstream effects. The addition of the growth factor or cytokine to the cell culture medium can have a variety of downstream effects, including the control of the phosphorylation state of proteins to the 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.

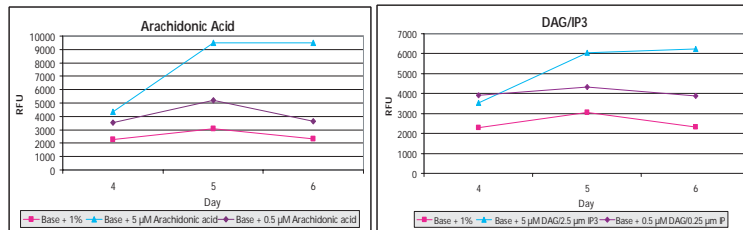


Figure 6: Various compounds can be used instead of growth factors/cytokines to bypass receptor:ligand interactions and activate the same pathways. As an alternative to the addition of growth factors to cell culture media, we added other compounds that would activate the same pathways. Protein kinase 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 in HEK-293 cells. 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.

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
- Based on the accumulation of data from various cell lines, we can design cell culture focused development tools (microarray, macroarray, qPCR primer sets, antibody arrays, etc.)
- A patent application has been filed for the use of this assay in cell culture medium development

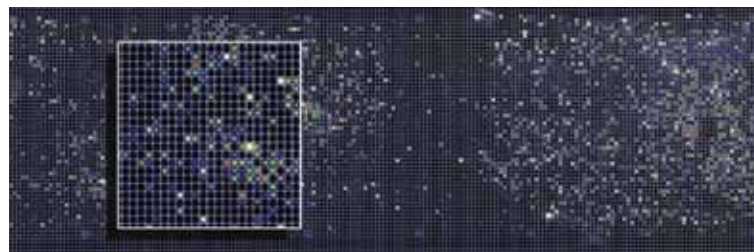


Figure 7: Future directions for the development of new tools for rational cell culture medium design. Using the many tools at our disposal, we can generate new and improved tools to help us more efficiently design cell culture media, including the approximately 12,000 spot array seen here which is in the process of being designed to screen various CHO lines. This image represents our continued efforts to provide improved products for our customers.