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REVIEW

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Stable expression of human proteins in cultured cells

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ABSTRACT • Functional proteome analysis involves elucidation of the roles of proteins in the cell. To perform functional proteome analysis, the protein of interest has to be either deleted or mutated from the genome to create a loss of function mutants or exogenously expressed to mimic the effect of physiological events caused by the protein of interest. One way to exogenously express proteins is to create a stable cell line in which a DNA vector harboring a gene of interest is integrated into the genomic DNA. The protein expression is then achieved from the genomic DNA. Information e.g., localization, activity, stability and interaction partners, regarding the expressed protein may be obtained using the created cell line. Although creation of stable cell line is a challenging task, the investment of the time and finance is well worth considering the importance of the data gathered. In this communication, experimental approaches regarding creation and the pitfalls of stable cell lines were discussed.

INTRODUCTION

Doing life science research is a challenging task (1). For many occasions, it involves characterization of gene sequences, transcripts and the proteins. There are many questions can be asked regarding the physiological function of a protein. Among those questions, the major ones come to mind are *"How much of a protein is being made in the cell? Where is a protein located in the cell? How long does a protein stay in the cell? What type of activity a protein carries out in the cell? and What other molecules does a protein interacts within the cell?"*. Based on the answers one can make a strong prediction about the physiological role of a protein.

To answer those questions, researchers use two different approaches; one is that they delete or mutate or knockdown a gene sequence or its transcript that they are interested in and look for the effects of deletion or the mutation (2,3). The results of such a gene deletion or mutation experiment would tell us about how important the protein of interest coded by the deleted/mutated gene is for the cell and whether or not the cell would survive in the absence of the deleted/mutated gene (4). The second approach involves introduction of a gene that encodes for the protein of interest (5). In this approach, contrary to the gene deletion studies, many of the aforementioned questions can be answered.

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INTRODUCTION OF A GENE OF INTEREST INTO THE HOST CELLS FOR PROTEIN EXPRESSION

The introduction of a gene of interest into the host cells may be achieved in two ways; transient and stable (6-8). Transient gene expression fulfils many of the needs for researchers. The vector carrying the gene of interest is transfected into the cultured cells and the protein of interest is expressed for 24 hrs or more before the cells are collected and used. The advantage of transiently performed transfection is that it does not take much time and effort. However, despite of these appealing advantages, transiently transfected cell populations present a mixture of cells formed by the cells to which the gene of interest is delivered and the cells to which the gene of interest is not delivered. The percentage of the cells to which the gene is delivered depends on the cell type and the method of choice used for transfection. In transient transfection experiments, researchers generally use cationic liposomes (lipofectamine) to transfect their cells with the gene of interest (9). There are commercially available kits to transfect various cell types with lipofectamine reagent. The reagent, for most cell types, works well and produces transfection efficiencies more than 70%. However, for some cell types, unfortunately, the transfection efficiency is very limited and requires an effort for optimization. A best example for hard-to transfer cell line with which our lab had experience in the past is 3T3-L1 cells. For some other cell types, lipofectamine creates toxic effects and causes undesired cell death. Therefore, the researchers have to watch out for the toxic effects of lipofectamine reagent on the cells.

Electrotransfection is usually the method of choice for creation of cell lines that can stably express the protein of interest (10,11). Compare to lipofectamine-based transfection methods, the efficiency of electrotransfection is much lower. However, electrotransfection is a mild way of transfecting DNA and the cells transfected with electrotransfection can recover and survive indefinitely. There are several commercially available instruments for electrotransformation of various cell types that provide excellent satisfaction.

SELECTION OF THE CELLS TRANSFECTED WITH A VECTOR HARBORING THE GENE OF INTEREST

After a population of cells is electrotransfected with a gene of interest, a selection process using an appropriate antibiotic is taken place to create a stable cell line (12). The selection allows survival of the cells which take the vector and integrates it into its genomic DNA (gDNA). The choice for the type of antibiotic used depends on the type of vector chosen. The concentration of the antibiotic for selection of the cells depends on the type of cells being used. In reality, different laboratories can use different antibiotic concentrations for the same cell type because even the number of passages the cell line underwent can affect its resistance to antibiotics. Therefore, the first experiment for a stable cell line creation should always be the one that determines the sensitivity of the cell line to the antibiotics. In other words, whichever antibiotic is used, its “killing curve” has to be determined for the specific cell line that is planning to be used (Figure 1). There is a list of ranges given for various cell types in the literature (Table 1). The rang-

Table 1 Suggested concentration ranges for some of the antibiotics recommended for HeLa and HEK293T cells.

Antibiotic	HeLa	HEK293
Blasticidine S	1-3 µg/mL	5-10 µg/mL
Zeocine	150 µg/mL	200-400 µg/mL
Geneticine	200-400 µg/mL	600-800 µg/mL
Hygromycin B	550 µg/mL	1000 µg/mL

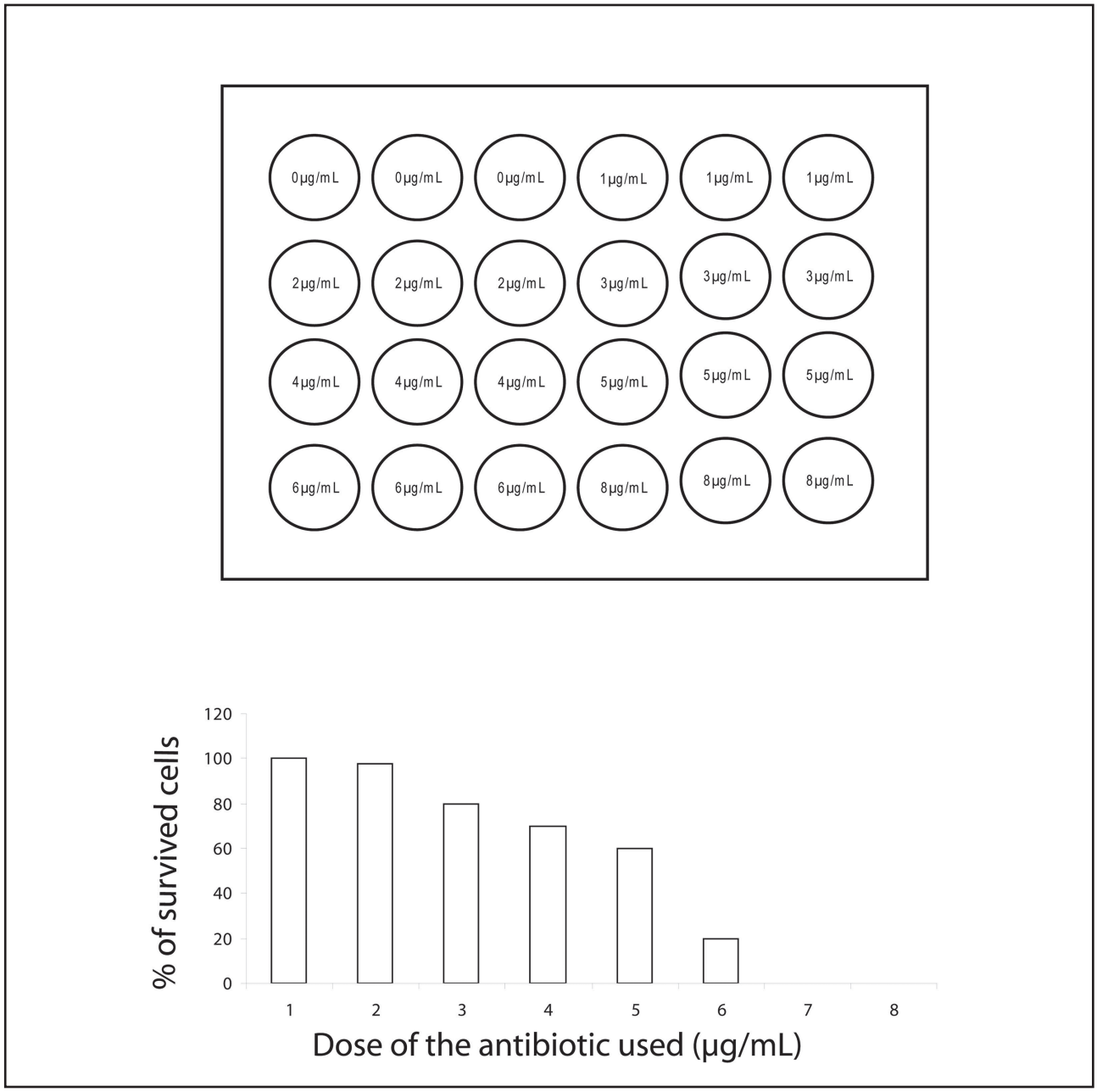

Figure 1 Creation of a killing curve for determination of antibiotic selection concentration.

es are only good for determination of the starting and the end concentration of the antibiotics used for selection. The precise value can only be determined empirically.

After selection-concentration is determined, the cells are challenged to survive in a medium containing the antibiotic. The surviving cells are the ones which take the vector harboring the gene of interest. While some of the cells survive, majority of the cells die. However, the dying of the cells take time and the duration of the time depends on the antibiotic used (Figure 2).

For example, when blasticidin is used during the selection process, the cells die relatively quick-

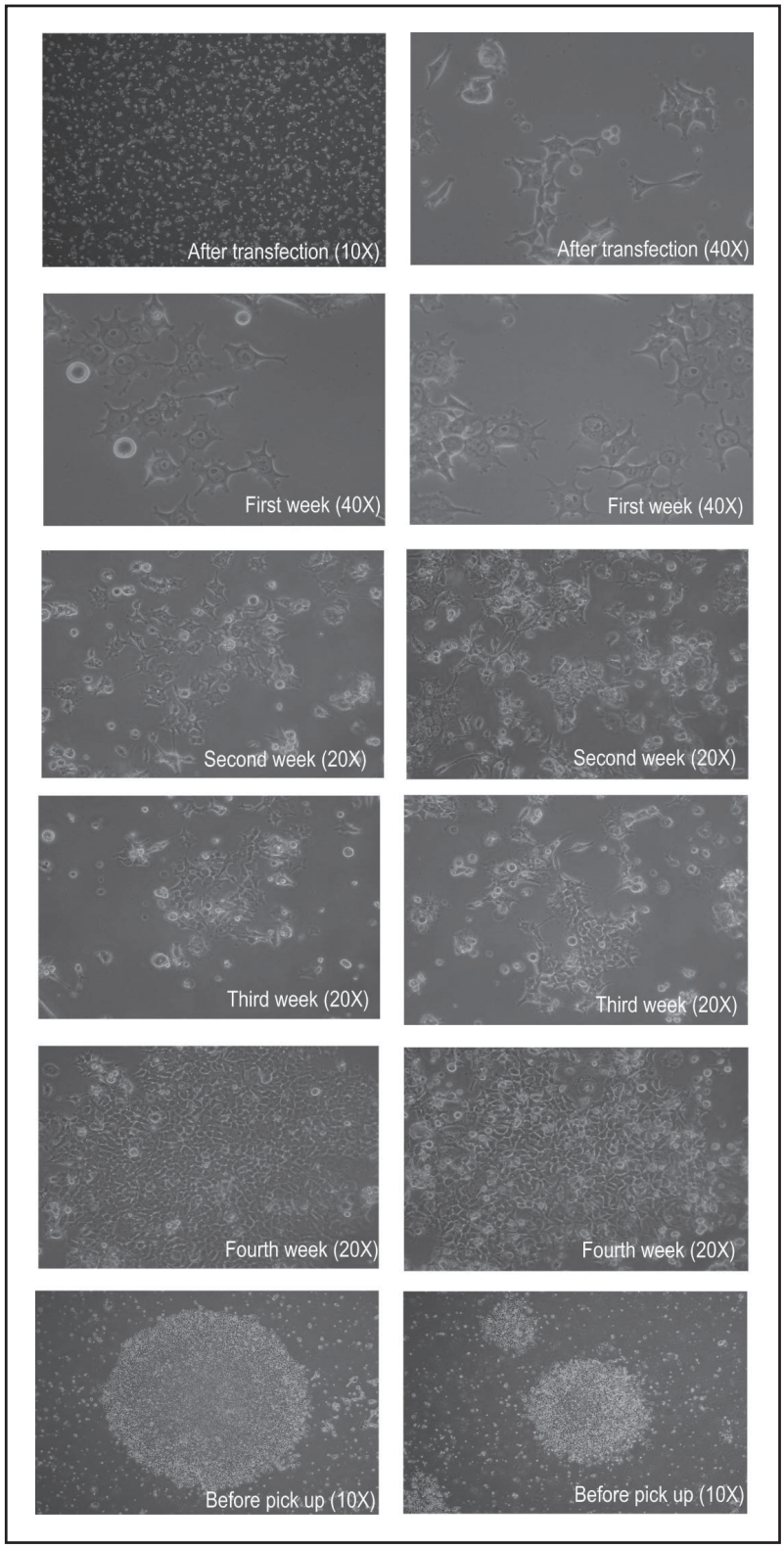

Figure 2 Formation of single cell colonies during antibiotics selection process. The images were taken with an inverted microscope.

ly (1-2 weeks). On the other hand, when zeocine is used during the selection process, the cells die relatively slowly (3-5 weeks). Therefore, researchers should be patient during the selection process and do not feel the selection process is failing.

At the end of the selection process, the plates should contain visible colonies that are ready for pick up (Figure 2). To pick up the colonies, the colonies are carefully located under a microscope and the location of each colony is marked with a permanent marker. The colonies are then picked up by using specifically designed plastic cylinders and transferred into 16, 24 or 96-well plates (Figure 3).

The plates containing the individual colonies are then incubated under 5% CO_2 environment until they are 80-90% confluent. They are then passaged into two 5-mL culture plates and allowed to divide until they are again 80-90% confluent. At this point, the cells are ready to screen for expression of the protein of interest.

SCREENING THE COLONIES FOR EXPRESSION OF THE PROTEIN OF INTEREST

The antibiotic resistant colonies should normally express the protein of interest at similar levels. However, in practice, it does not work that way. Some colonies do express the protein of interest but some others completely lacked the expression (Figure 4).

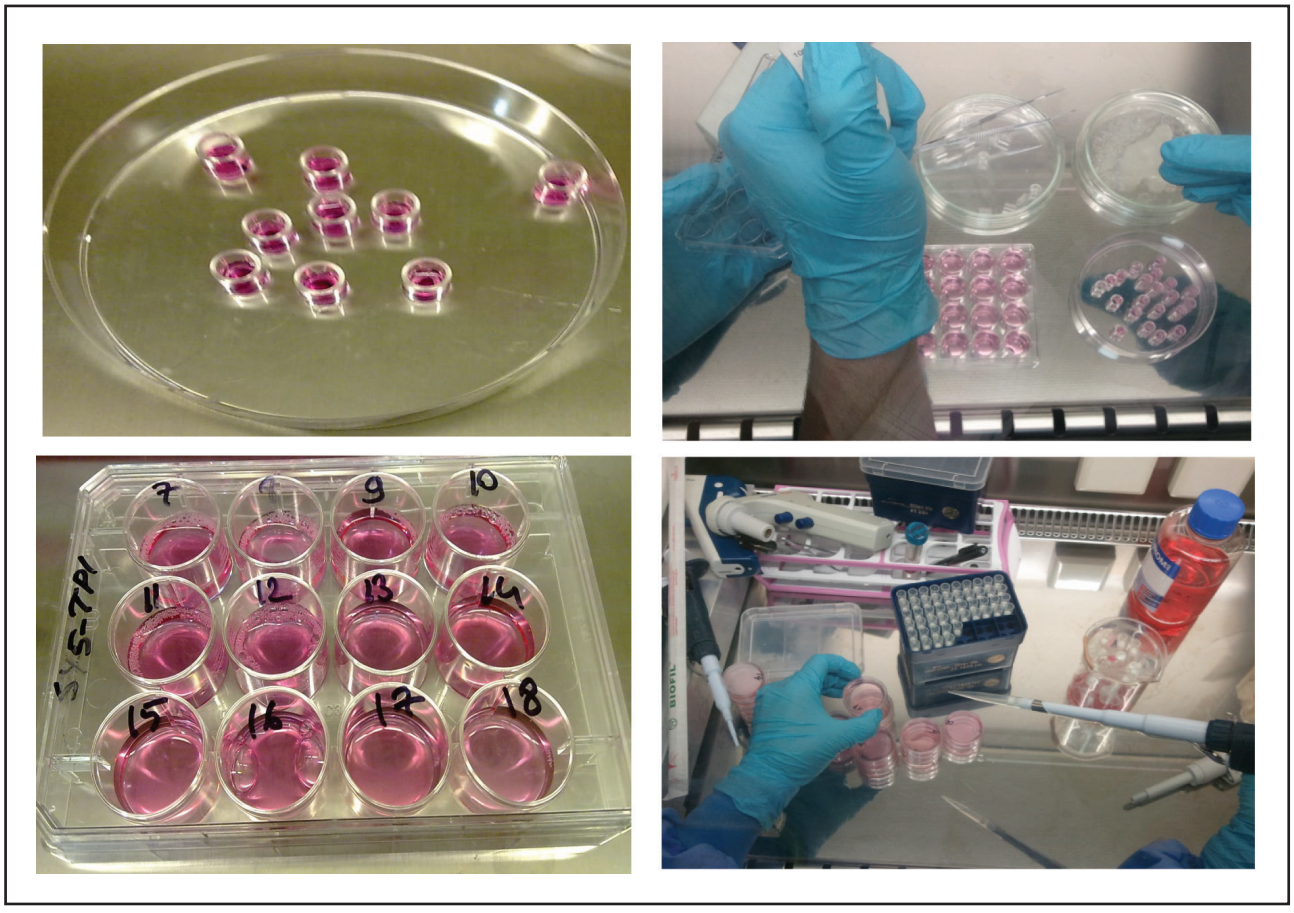

Figure 3 Demonstrative images for single colony pick-up.

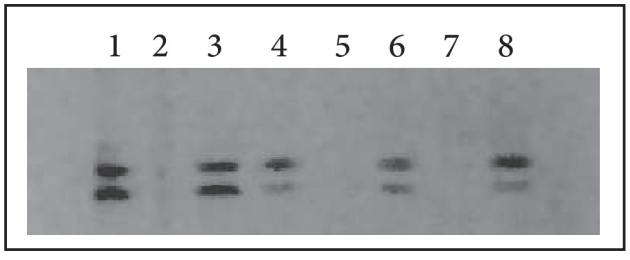

Figure 4 Western blot analysis of the expressing and non-expressing colonies of SH-SY5Y cells. The cells were screened for the expression of wild-type Parkin protein. Lanes 2, 5 and 7 are non-expressing colonies.

This is due to the fact that the gene of interest is inserted into genome and then being silenced. After determination of the protein expressing colonies, at least three protein expressing colonies are selected and their cultures are continued and used for further work. The rest of the plates containing non-expressing or the weakly expressing colonies are discarded.

After the appropriate colony expressing the protein of interest is selected cultures from these cells can be used to extract information regarding the protein of interest.

PROTEIN EXPRESSING CELLS FOR LOCALIZATION STUDIES

The cells stably expressing the protein of interest can be used for localization studies. For that purpose, the cells are allowed to express the protein of interest for at least 12 hr from a strongly expressing colony and then are fixed and examined

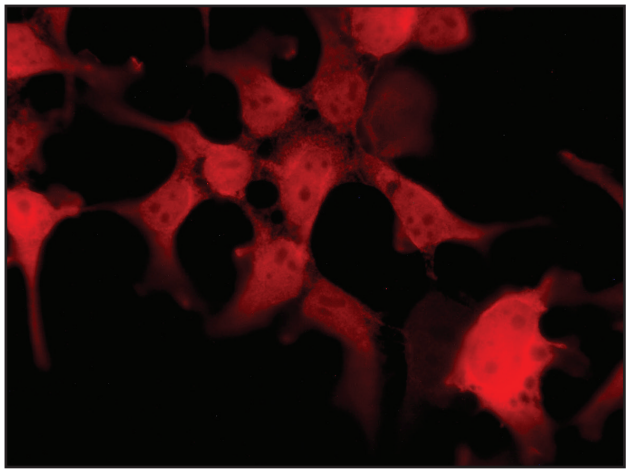

Figure 5 Immunofluorescence microcopy analysis of Parkin expressing cells. A Texas red conjugated seconder anti-mouse antibody was used for detection.

for protein localization using immunofluorescence microscopy (Figure 5).

PROTEIN EXPRESSING CELLS FOR STABILITY STUDIES

The cells expressing the protein of interest can be used to determine the stability of the protein of interest. In other words, the answer to the question of how long the protein stays in the cell can be answered. For that purpose, the protein of interest is expressed at a minimum level (around 5 to 10 hr) and then cyclohexamide is added to the culture to stop new protein synthesis. The cells are then collected at certain time intervals and the incline in the level of protein of interest is studied by western blotting.

PROTEIN EXPRESSING CELLS FOR ACTIVITY STUDIES

If the protein of interest has certain enzymatic activity, the activity may be measured using the cells stably expressing the protein of interest. For example, in our laboratory we routinely measure the activity of Parkin protein, an E3 ubiquitin ligase. To assay Parkin's ubiquitylation activity, the cells are allowed to express the protein of interest for 24 to 48 hr and then collected for cell-free extract preparation. The cell-free extracts are then used to assay the enzyme's activity (13).

PROTEIN EXPRESSING CELLS FOR INTERACTOME STUDIES

Sometimes, a researcher would like to know about the interaction partners for a specific protein. In such cases, the protein of interest can be expressed stably using a tap-tag and the cells can be collected for cell-free extract purification (14,15). The tap-tagged protein of interest can then be used to elucidate the interaction partners of the protein of interest.

In other cases, the researcher would like to know the effect of expressing either a wild type or a mutant form of a protein on the cellular proteome. For that purpose, the protein profile of cells expressing the protein of interest can be compared with the protein profile of cells that do not express the protein of interest. The protein profiles can be compared in two ways. One way is to use the tools of two-dimensional gel electrophoresis-based (2DE) approach (16). In a routine 2DE experiment, changes in the levels of 700 to 1000 proteins can be compared (Figure 6).

After comparison, proteins whose abundance displays changes may be identified via MALDI-TOF/TOF analysis. The identified proteins and their regulation ratios can then be used for bioinformatics analysis to elucidate the associated pathways.

The second approach involves label-free quantification of protein abundances using LC-MS/MS (17). In such an experiment, the proteins extracted from the cells expressing the protein of interest are compared with the proteins extracted from the cells that are not expressing the protein of interest. A huge quantity of data can be obtained from an LC-MS/MS experiment. Several different bioinformatics approaches may be used to interpret the data (18).

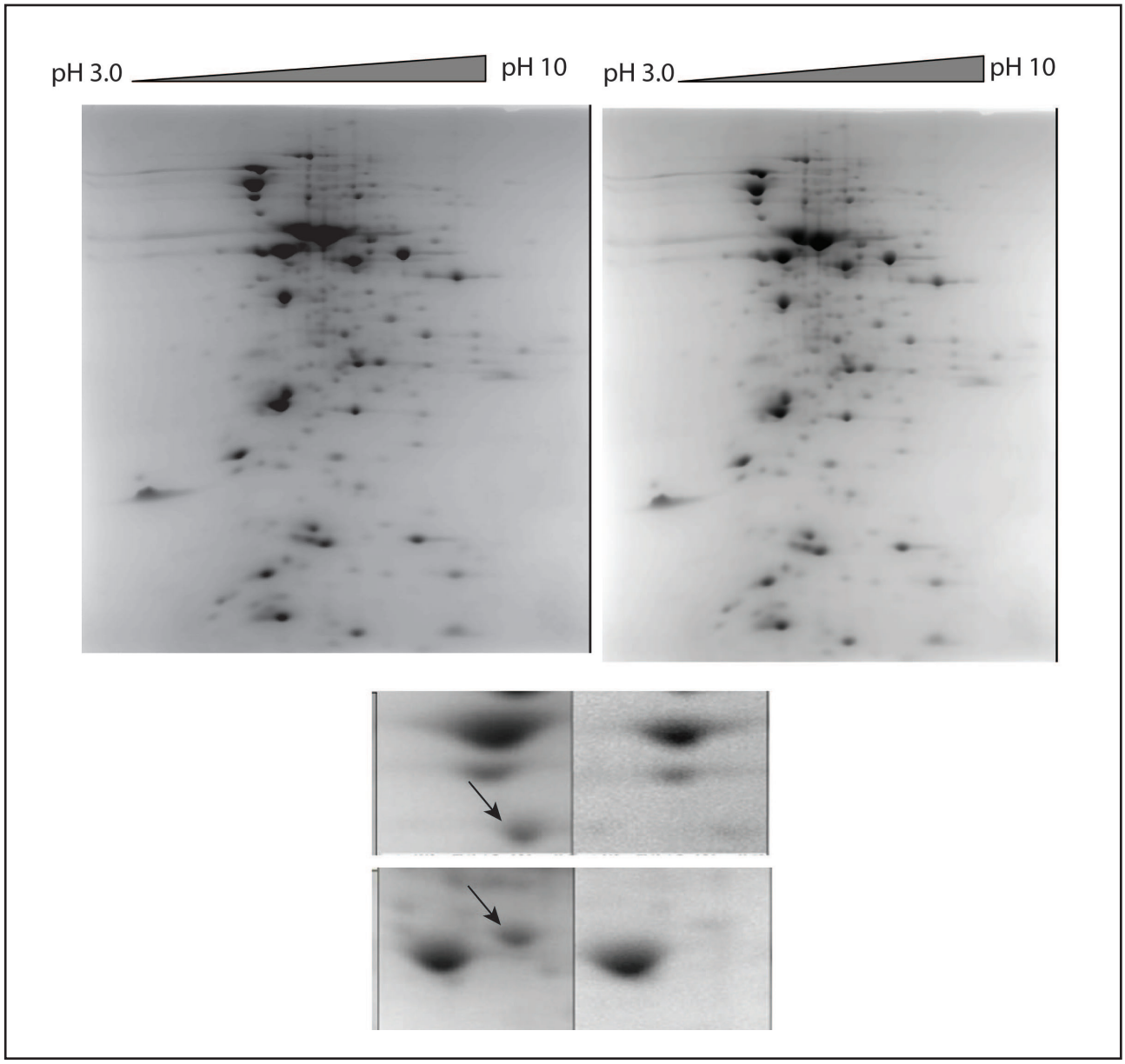

Figure 6 Comparative 2D gel analysis of protein extracts obtained from omentin-1 expressing and non-expressing cells.

PROTEIN EXPRESSING CELLS FOR PRODUCTION OF MEDICALLY IMPORTANT PROTEINS

The commercial values of certain human proteins that are used for therapeutic purposes are immense (19). The production of those commercially important proteins may be achieved in several different ways. One way, probably the easiest way, is to use bacteria e.g., *Escherichia coli* for production purposes. However, because most human proteins are post translationally modified such as glycosylated their production in *E. coli* can sometimes be very challenging. Although certain optimization strategies can be used to improve production of human proteins in *E. coli,* they most often stay short (20).

As an alternative to *E. coli* expression systems, yeast expression systems (e.g., *Pichia pastoris*) are more preferable since the organism yeast is phylogenetically more close to humans and can post translationally modify the proteins. The most common problem with the yeast system for production of human proteins is that its glycosylation pattern vastly differs from the glycosylation pattern of human proteins. Therefore, in some cases, although the protein can be expressed in yeast systems, it may not have any biologic activity.

The last system is the cell cultures utilizing either human originated cancer cell lines or the cell lines of close origin. There are two cell lines commonly used for this purposes. One of the cell lines is formed by HEK293T cells that are originated from human embryonic kidney. The other cell line is formed by CHO cells that are originated from a Chinese hamster ovary. Both cell types can produce human proteins with appropriate post translational modifications. The only disadvantage of using these cell lines is that they are expensive to cultivate and large scale protein productions are financially challenging.

CONCLUSIONS

Protein expression in stably transfected cell lines is a powerful tool for researchers. There are many questions regarding the physiological role of a protein that can be answered using cultured cells. Considering the fact that many of the proteins in human genome are assigned functions by doing bioinformatics predictions and many others still have no known function, it is imperative to utilize the available power of cell cultures for functional protein studies. However, it is also important to note that cell culture studies are only the beginning for a study and the data produced in cell culture studies should be carefully approached. There is always a need for *in vivo* studies for verification of the data produced.

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