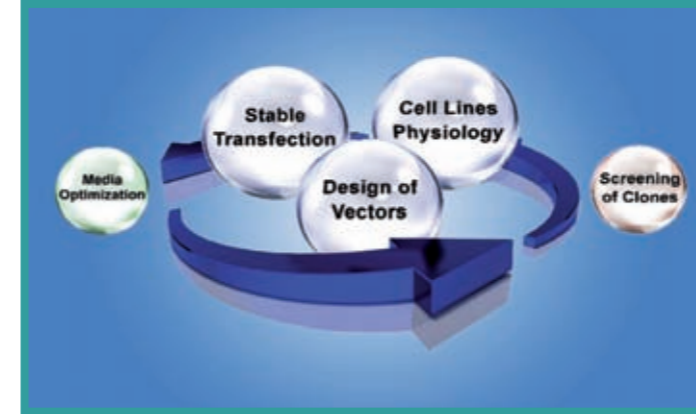




Anne-Laure Gilquin,
Cellular Engineering Manager.



Biopolis Biopark
located at the heart of Grenoble City in South-Eastern France.



A figure,
the fields of competence of the cellular engineering.



Pierre-Yves Perche,
President and co-Founder.

Creating Stable and Reliable Cell lines

by Pierre-Yves Perche and Anne-Laure Gilquin

Today more than ever, in the history of pharmaceutical industry and biotechnology, stable cell lines (or the recombinant mammalian cell lines) find their application in the compounds' screening on the one hand and in the recombinant protein production on the other hand.

In the first case, the stable cell lines are to be considered as cellular models of first choice: they allow us to identify new compounds or to control their biological activity directly on a living cell (efficacy and safety tests). The above named compounds may be molecules arising from chemical synthesis or from bio-pharmaceutics such as DNA, RNA, recombinant proteins, monoclonal antibodies, etc.

In the second case, stable cell lines are used as a tool for producing recombinant proteins (r-proteins) that contain post-translational modifications, which are essential for an optimal biological activity.

of highly productive cell lines that preserve their stability in time and a quick process of development at a convenient purchase rate. This balance must nonetheless respect the imposed standards and in the same time be able to guarantee the production of perfectly sterile and homogenous batch of cells.

Whether it's about screening of compounds or producing of r-proteins, the engineering of stable cell lines needs to sum up three levels of competence: the designing of expression vectors, the stable transfer of genes and the mastering of cell lines physiology (confer the A figure above). These specialisations are both related to the formalisation of the users' needs and, in the same time, to controlled facilities. What are the main steps in the engineering of a stable cell line? What are the critical aspects that need to be considered? What are the new strategies available nowadays? Here are some answers to these questions.

The expression vector would like to state something

The first step in developing a stable cell line corresponds to the conceiving of the expression vector, which usually is a circular or linear plasmid. This DNA contains several cassettes that are essential to assuring a strong and constant expression of the protein. The expression's intensity is primarily related to the choice of the viral or mammalian promoter. It can reach a physiological level of expression or even grossly overpass it. Then, the presence of polyadenylation signals is essential in order to avoid the degradation of the produced RNA and insure its functioning within the translation machinery. To this first expression cassette of the r-protein, one adds a selection cassette into the vector. This particular DNA allows the expression of a resistance gene to an antibiotic and therefore, the selection of

cells that contain several copies of the vector that is integrated in their genome. A highly expanded resistance gene is the neo gene that can encode for an aminoglycoside 3'-phosphotransferase. This enzyme neutralises the G418, that is an inhibitor antibiotic of the protein synthesis.

The minimum expression vector is now designed, but several improvements may be considered, especially at the level of the nucleic sequence of the r-protein. Indeed, it has been proven that by optimising the codons and by reducing the percentage in the nitrogenous bases (G and C), the productivity of the expression can highly be improved. The same effect can be noticed when adding an intronic sequence in the upstream of the protein's coding sequence. In order to assure a constant expression in time, other genetic elements can add up to the system, such as the Insulators that protect the vector from the position effect, that is, of an eventual transcriptional suppression imposed by the chromatin; or, even more, the MAR (Matrix Attachment Region), that is, highly rich elements in A and T bases, that bind themselves to the periphery of the cell nucleus in active chromatin domains. Another process uses an IRES sequence (Internal Ribosome Entry Site) and allows the result of a single RNA that will code for the gene of the r-protein, on the one hand, and for a resistance gene to an antibiotic, on the other hand. Thus, the selection process is strengthened when integrating the vector into the host genome. This improved vector is already sufficient for creating stable cell lines that will be used for screening the compounds. However, in the case of stable cell lines that are created for the overproduction of the r-proteins, the protein synthesis machinery has to be reinforced. This is the reason why the chosen selection marker is no longer a resistance gene

to an antibiotic, but a coding gene for an enzyme capable of re-establishing a metabolic process. A common example would consist in using the DHFR gene (dihydrofolate reductase) in association with the CHO DHFR cells. In these particular cells, the biosynthesis of purines and pyrimides is lacking. The expression of the DHFR, always associated to the expression of the r-protein, re-establishes this biosynthesis and allows the integration of the vector into the host cell. Therefore, the treatment of cells by an inhibitor of the DHFR (the methotrexate) in increasing proportions causes a growth in number of the DHFR gene copies and in the same time a growth in number of the interest genes copies that are associated to them. From this regard, the productivity of the expression can be multiplied by 100!

The improved vector ended and its quality controlled (sequence and purity), its transfer to the cell nucleus becomes imminent. Nevertheless, a maximum number of cells need to receive this vector in order to increase the chances of isolating a clonal population in conformity with the expected specifications.

An efficient transfer in the cell nucleus depends mainly on the nature itself of the cell line and also on the non viral gene delivery agent used for the

The Cellular Ecosystem

The mammalian cell holds a dynamic balance and auto-regulates its vital functions. It is a perfect micro and nanoscopic ecosystem that, by a multitude of coordinate chemical reactions and of fine regulatory mechanisms between its millions of components, guarantees a durable structure and functions. The mammalian cell engineering applied to the creation of stable cell lines consists in modifying and exploiting a part of this cellular ecosystem, all in preserving its balance.

Who is CreaCell ?

The company was founded in 2003 by two brothers : Pierre-Yves Perche, PhD in molecular and cellular biology, graduate of the Joseph Fourier University in Grenoble, and Guillaume Perche, graduate of the Business School in Bordeaux.

CreaCell is specialised in the engineering of mammalian cells. Its offer consists of products, particularly of stable cell lines that express ion channels, and also of different services in the field of cell biology, one of which being the proper development of custom-made stable cell lines. Several dozens of cell lines have been developed to this day. Their services are performed in a P2 confinement laboratory and include the complete cell lines history.

As searched for within other pharmaceutical industry professions, the conceiving of a stable cell line by means of cellular engineering aspires to reducing costs and processing time. The major issue here resigns in finding the appropriate balance between the conception

transfection, which in most cases is a lipid or a cationic polymer. The nature of the cell culture media may also have an influence on this transfer. One thing to bear in mind is that the chosen transfection agent would have to assure not only an efficient transfer of the vector towards the core, but also to preserve the cellular viability, and, generally speaking, the cell's physiology. Regarding the CHO or the HEK293 cell lines, various agents are available. **The B figure** shows the efficiency of the transfer obtained with a PEI (polyethylenimine) on a CHO cell line.

The host cell line: he who can do best can do even better.

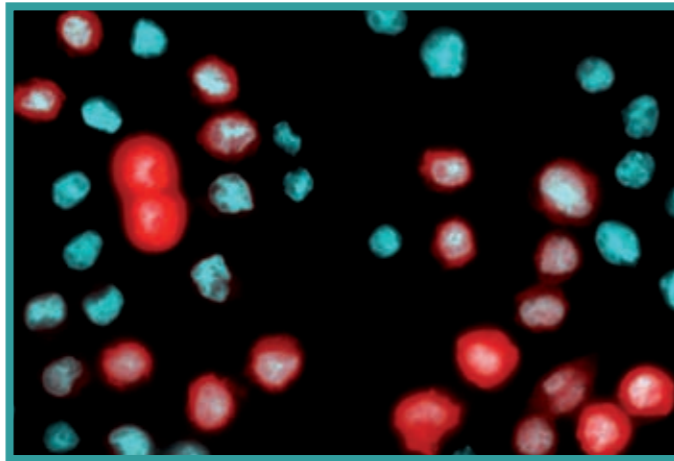
No matter what the fields of application are; whether it is cellular pharmacology or production of the r-proteins, the choice of the host lineage is of major importance. This choice is perfectly related to the nature of the interest protein and to the cell culture conditions assured for a medium and a long period of time. If the CHO cell line and its derivatives are most often used for the development of a stable cell line, other traditional host cells are available: HEK293, NS0, BHK-21, etc. These cells have the advantage of being well known in the fields of the industry and of the regulatory affairs. From a scientific point of view, the understanding of their physiology has been most successful. Some cell lines may be cultivated alternatively: in adherence or in suspension. This would give its user a greater flexibility in manipulating them. Moreover, they can adapt themselves to serum free or protein free culture conditions. And in this way, they become particularly appropriate for processes of purification of the r-protein. Lastly, their size and doubling time are compatible with most of the new processes of development of stable cell lines (**confer the C figure**).

If the chosen cell line must be adequate for

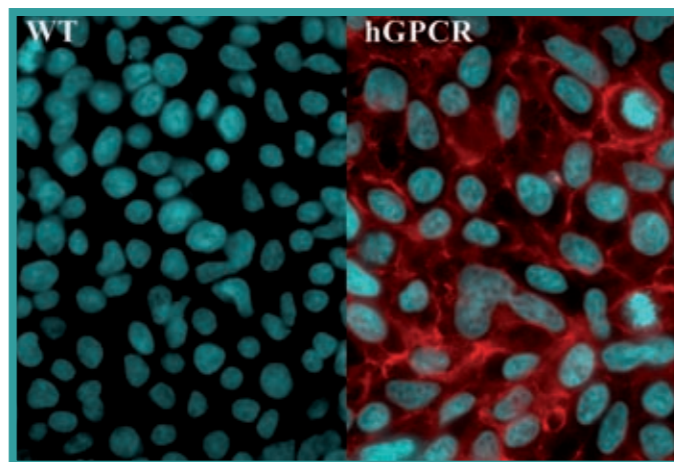
the future environment that awaits it, it must also insure the synthesis of a quality r-protein by well performing during the processes of transcription, translation, folding of the r-protein and of other post-translational modifications. Furthermore, the presence of suitable biosynthesis pathways is another significant point in the development of stable cell lines. Nevertheless, serious questions arise: which is the degree of toxicity of the r-protein compared to the host lineage? Does the presence of some endogenous proteins endanger the cellular productivity? And vice-versa, do the signalisation pathways or protein maturation pathways that are involved in the protein biosynthesis contain all the elements necessary to their proper functioning? Is the intervention of the chaperone proteins really necessary? Is the co-expression of one or several subunits necessary to the stability and good functioning of the protein? Does a peptide signal need to be fused in order to insure proper delivery and maturation?

To answer these questions, we should take into consideration the characteristics proper to each protein family. We would insist on the fact that a proven toxicity may eventually be avoided by carefully choosing an inducible system of expression, that is, a system that allows us to modulate the intensity of the recombinant expression in time. In the presence of unwanted endogenous proteins, the technology of RNA interference may be proven to be efficient. Lastly, a multiple expression of subunits may be obtained by using plasmids that have different selection genes, for instance.

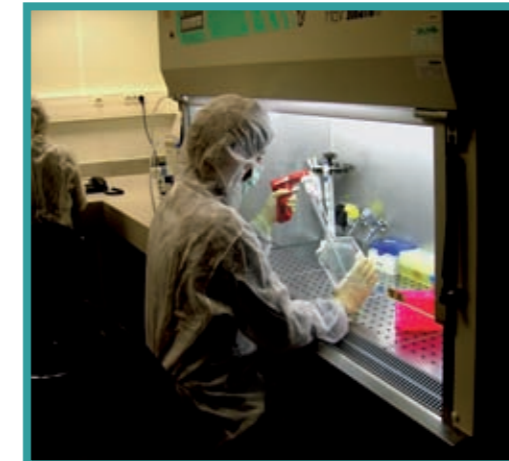
The quality of the r-protein is now assured. Nonetheless, is this protein produced in sufficient quantity? The control and optimization of productivity have been subject to many researches,



B figure, the polyethylenimine-transfected CHO-S cell line (the r-protein is visible in red and the cell nuclei are coloured in blue)



C figure, on the left the HEK 293 host cell line before the transfer of an expression vector. On the right an HEK 293 monoclonal stable cell line expressing a GPCR (the r-protein is visible in red and the cell nuclei are coloured in blue)



D figure, cellular engineering in the CreaCell's P2 confinement laboratory

majority of the confined clones is likely to produce the interest protein in the desired amounts. A third method would be by using an equipment that is able to detect the presence of cellular colonies possessing a stable expression of the protein (adherent cells) and in the same time that is able to detach specifically the colonies before submitting them individually in culture for their phenotype.

After the clones' magnification (**confer the D figure**), an analysis of the expression's yield confirms the biological activity which is characteristic of the purified r-protein or its activity in the cell's physiological context. The phenotype may eventually include a global analysis of the clones at a molecular level (the "omic" approaches) for an even more precise selection of the best champions.

Conclusion

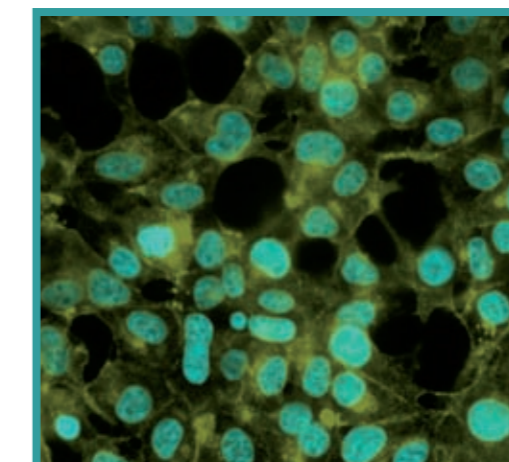
Four or five months have passed since the launching of the conceiving of a new stable cell line. The performances obtained are the result of various factors, but above all, are determined by a rational mammalian cell engineering approach that combines a general strategy of design with a specific strategy that dependent on the nature itself of the interest protein. Even if in the beginning, the cellular engineering was intended to be an exploration based on fragmentary scientific knowledge that exploited only the results of local initiatives, the increasing inputs of the researches in cellular biology and the access to innovative technological processes make it possible nowadays for more and more integrated and efficient strategies. These new researches and technologies helped cellular engineering to definitively enter the era of rationalization. Therefore, they have multiplied the chances of success in the development of high performance stable cell lines. ■

Text translated by A. Dragomir

but we will enumerate here only a few of them that have already been tested: on a transcriptional level, the RNA synthesis may be multiplied by processing the cells with the HDAC inhibitors (Histone Deacetylase). Even so, this can have an impact on the quality of the r-protein. On the level of the cellular cycle, its stoppage by chemical treatment or over-expression of an oncogene may have a very good influence on its productivity. On the other hand, on the level of the apoptosis, and especially when cells are cultivated at a high density, the over-expression on oncogenes may be beneficial. Lastly, more generally speaking, the culture conditions established all along of the stable lineage's "career" are extremely critical and one should master several points: the composition of the culture medium, the choice of the containers (flasks, bioreactors), the technique of feeding the cells (fed-bath, perfusion), the physical and chemical parameters adjusted all along the cellular growth and in the compounds' screening phases or the r-protein production phases. The facts speak for themselves: the same stable cell line can present a productivity that may vary from 10 to 100 from a culture environment to another.

The clones' screening or how to isolate the "champions"

The former steps have lead to a stable cell line whose performances have been confirmed by a pertinent phenotype. The most complex operations are now over, but there is still a major inconvenient left : the cellular population resulted is polyclonal and therefore presents heterogeneity. It is high time to identify and then isolate the best cellular clones. The simplest approach (and the longest) for obtaining a monoclonal population of stable cell lines consists in creating a limited dilution of a polyclonal population into multi-well cell culture plates. Another method would be by highlighting the interest protein, directly or indirectly with a fluorochrome, so that we can analyse the polyclonal population by means of an analyzer-sorter. In this case, the initial analysis precedes the cellular confinement so that a very big



The **hERG HEK293** cell line is one of the ion channel cell lines developed by CreaCell. It conforms to the terms of the ICH S7B directives that relate to the evaluation of the prolongation of the QT interval (the hERG potassium channel is visible in yellow and the nuclei are coloured in blue).