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Transposon-based technology enhances the generation of stable and high-producing CHO clones for industrial production of recombinant proteins and antibodies

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Abstract

Recombinant proteins and antibodies are the key reagents for development of diagnostic immunoassays. Recombinant proteins are commonly produced in both prokaryotic and eukaryotic microorganism because they allow high productivity with rapidity and low costs. However, complex proteins that contain posttranslational modifications, several disulphide bonds or multiple subunits, such as antibodies, are challenging to be expressed in these hosts. Indeed, to obtain properly folded and functional complex biomolecules it is required the posttranscriptional metabolic machinery only available in mammalian cells. Although different approaches for gene transfer have been developed in the last 15 years, it is still difficult to obtain stable, high-producing cell lines for industrial applications. Conventional methods, based on spontaneous integration of episomal DNA, often result in low efficiency of clone establishment and in low transgene expression mainly due to plasmid concatemers silencing and/or positional effects. To overcome these limitations, in my thesis project, I evaluated the potentiality of using an improved PiggyBac (PB) transposon system as new molecular tool for transgene delivery. Transposon-based approaches rely on the ability of transposase enzyme to catalyze single transgene integration into actively transcribed regions of genome. In order to assess the suitability of PB transposon vectors compared to conventional methods, two different model proteins, the human fibroblast growth factor 23 (hFGF23) and one mouse recombinant antibody, have been cloned into both expression plasmids and produced in CHO (Chinese Hamster Ovary) cells.

A preliminary comparison between the two expression systems demonstrated that PB transposition increased the frequency of stable cell lines generation up to 10-15 fold compared to standard plasmid transfection. Cell lines establishment was faster and the frequency of high-producing clones was enhanced, thus reducing the extent of clones screening to recover the best performing cell lines. In addition, I also evaluated that changing PB promoter strength affected the frequency of high-producing clones. Taking advantages from these results, I was able to generate CHO cell lines expressing hFGF23 protein with an average yield of 35 mg/L in batch culture. The obtained purified protein was correctly detected by an automated chemiluminescence immunoassay (DiaSorin) with results comparable to a commercial available mammalian recombinant hFGF23 protein and it resulted biologically active when tested in a cell proliferation assay.

Then I evaluated the application of PB transposon system for the generation of recombinant antibodies. After identification of heavy and light chain variable regions from an IgG2a mouse immunoglobulin developed by hybridoma technology, I have generated a chimeric IgG1 antibody by cloning mouse variable regions upstream of mouse heavy and light chain constant sequences. The ensuing full length sequences were cloned into standard vectors and transposon for co-expression in CHO cells. In these set of experiments, my results highlighted the advantages of using PB transposon to stably integrate, in one transfection step, two different transgenes with an appropriate molar ratio (light/heavy chain ratio unbalanced in favour of light chian), as required for proper antibody assembly. Random integrations, typical of standard plasmid transfections, showed difficulties in fine tuning of co-transfected transgenes expression, resulting in 80% of clones with very low productivity. In contrast, integrations mediated by PB transposase increased the number of high producing clones. The chimeric IgG1 immunoglobulin, purified from the best producing clone, showed affinity and immunochemical performances comparable to that of the parental hybridoma IgG2a antibody, confirming the potentiality of our system.

In conclusion, my work demonstrates that the PB transposon system is a quick and powerful alternative to standard method for generation of stable, high-producing recombinant mammalian cell lines to generate critical reagents useful for diagnostic applications.

Riassunto

Le proteine ricombinanti e gli anticorpi sono reagenti chiave per lo sviluppo di saggi immunodiagnostici. Le proteine ricombinanti sono comunemente prodotte sia in microrganismi procariotici che eucariotici poiché consentono di ottenere un'elevata produttività a bassi costi. Tuttavia in questi sistemi eterologhi è difficile esprimere proteine che hanno più subunità, come gli anticorpi, o modifiche post-transcrizionali complesse. Infatti per ottenere la loro corretta conformazione sono necessarie le modifiche post-trascrizionali tipiche delle cellule di mammifero. Negli ultimi 15 anni sono stati sviluppati diversi metodi per il trasferimento genico, ma nonostante ciò è ancora difficile ottenere linee cellulari stabili e altamente produttive per applicazioni industriali. Le strategie convenzionali si basano sull'integrazione spontanea di DNA episomale e perciò hanno una bassa efficienza di generazione di cloni e, spesso, una scarsa espressione del transgene a causa di effetti posizionali e del silenziamento di transgeni integrati come concatameri. Per superare questi limiti, nel mio progetto di tesi, ho valutato le potenzialità dell'utilizzo dei trasposoni piggyBac come nuovo metodo per veicolare transgeni. I sistemi basati sui trasposoni sfruttano la capacità dell'enzima trasposasi di catalizzare l'integrazione del transgene in singola copia e in regioni genomiche attivamente trascritte. Per studiare questo sistema e confrontarlo con i vettori tradizionali ho clonato, in entrambi i plasmidi, due proteine modello, il fibroblast growth factor 23 (hFGF23) e un anticorpo ricombinante, per la loro produzione in cellule CHO (chinese hamster ovary).

I risultati ottenuti hanno dimostrato che la trasposizione aumenta la frequenza di generazione di linee cellulari stabili di circa 10-15 volte rispetto alla trasfezione di plasmidi standard. Inoltre, lo screening per ottenere i cloni migliori è facilitato poiché la generazione di linee cellulari stabili è più rapida e la frequenza di cloni produttivi è maggiore. Infine ho dimostrato che la frequenza di cloni altamente produttivi è influenzata dalla forza del promotore clonato nel PB. Grazie a questo sistema, ho generato linee cellulari in grado di esprimere l'hFGF23 con una resa media di 35 mg/L in colture batch. La proteina purificata è stata correttamente riconosciuta da un test immunodiagnostico (DiaSorin) con risultati paragonabili a quelli di un hFGF23 ricombinante commerciale ed inoltre è biologicamente attiva, come dimostrato da un saggio di proliferazione cellulare.

In seguito ho valutato l'utilizzo del sistema dei trasposoni per la generazione di anticorpi ricombinanti. Dopo l'identificazione delle regioni variabili della catena pesante (HC) e leggera (LC) di una immunoglobulina IgG2a murina sviluppata con la tecnologia dell'ibridoma, ho generato un anticorpo chimerico IgG1. A tal scopo le sequenze variabili della catena leggera e pesante sono state clonate a monte della sequenza costante della rispettiva catena. Tali sequenze sono state inserite nei vettori standard o nei trasposoni per la co-espressione in cellule CHO. I risultati ottenuti hanno evidenziato i vantaggi dell'uso del sistema dei trasposoni PB per l'integrazione stabile di due transgeni con un'unica trasfezione, mantenendo un rapporto molare appropriato. Infatti questa è una caratteristica utile per la corretta formazione degli anticorpi che richiede un rapporto LC/HC sbilanciato a favore della catena leggera. Le integrazioni casuali, tipiche delle trasfezioni con plasmidi standard, hanno creato difficoltà nella co-espressione dei transgeni, infatti l'80% dei cloni aveva una produttività molto bassa. Al contrario le integrazioni mediate dalla trasposasi PB hanno aumentato il numero di cloni altamente produttivi. Infine, l'immunoglobulina IgG1 chimerica è stata purificata e ha mostrato affinità e prestazioni immunochimiche paragonabili a quelle dell'anticorpo IgG2a prodotto dall'ibridoma originale, confermando così le potenzialità del nostro sistema.

In conclusione, questa tesi dimostra che il sistema basato sui trasposoni PB può essere considerato una rapida e potente alternativa al metodo standard per la generazione di linee cellulari stabili, capaci di produrre reagenti utili per applicazioni diagnostiche.

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Introduction

CHAPTER 1: Recombinant protein production

1.1 Recombinant proteins: from discovery to industrialization

Modern biotechnology has its main purpose in developing products with therapeutic, diagnostic and industrial potential. Among these, the most interesting class of biologics are recombinant proteins which emerged as a fundamental corner stone of life sciences research and drug development, with a world-wide market exceeding 100 billion US dollars in 2012 (Carter et al., 2013) (Fig. IA). Recombinant proteins are not only important for biopharmaceutical industry, but also for other applications such as diagnostics, agriculture and all areas that involve enzyme processing.

It has been more than thirty years since the first recombinant protein was obtained paving the way to industrialization of recombinant products. This biotechnological revolution found its origins in 1970's with the invention of recombinant DNA technology (Cohen et al., 1973). The molecular cloning of a DNA fragment allowed the production of foreign proteins in hosts that naturally do not express them. Few years later (1982), exploiting this discovery, Genentech company created the first FDA-approved recombinant protein in *Escherichia Coli* (E.coli), the human insulin "Humulin" (Goeddel et al., 1979). The same company obtained soon, in 1986, the market approval for the first recombinant protein produced in an eukaryotic host, the tissue-type plasminogen activator (tPA, Activase) from Chinese hamster ovary (CHO) cells. Their success resulted in an exponential growth production of recombinant proteins from academic field to industrial area, sector still in continuous expansion. Currently, over 140 therapeutic recombinant proteins are available on the world market and several hundreds more are in clinical trials (Rader, 2013). Moreover, thousand of recombinant proteins used in industrial processes, diagnostics and research have been commercialized since the advent of this technology.

1.2 Host expression systems

The production of recombinant proteins begins with 2 steps: cloning the DNA of interest in a suitable vector and genetically engineering the organism chosen as expression host. The ultimate use of the protein is an important factor in the selection of the host system

because it will eventually determinate amount, purity, tolerance, functionality and production costs.

There is a wide variety of heterologous expression systems available, because the protein production often requires not only the synthesis of peptide backbone, but also its proper modifications and folding, processes influenced by physiological and biochemical properties of the host. Depending on dimension, complexity and post-translational modifications, recombinant proteins can be expressed both by prokaryotic, such as bacteria, and eukaryotic organisms, for examples yeast, insect, plant and mammalian cells (Fig. IB). Each of these systems presents limitations or advantages in relation to costs, ease to handle and yields. Although general conclusions can be drawn, expression has to be optimized for every new protein because of its unique amino acid sequence.

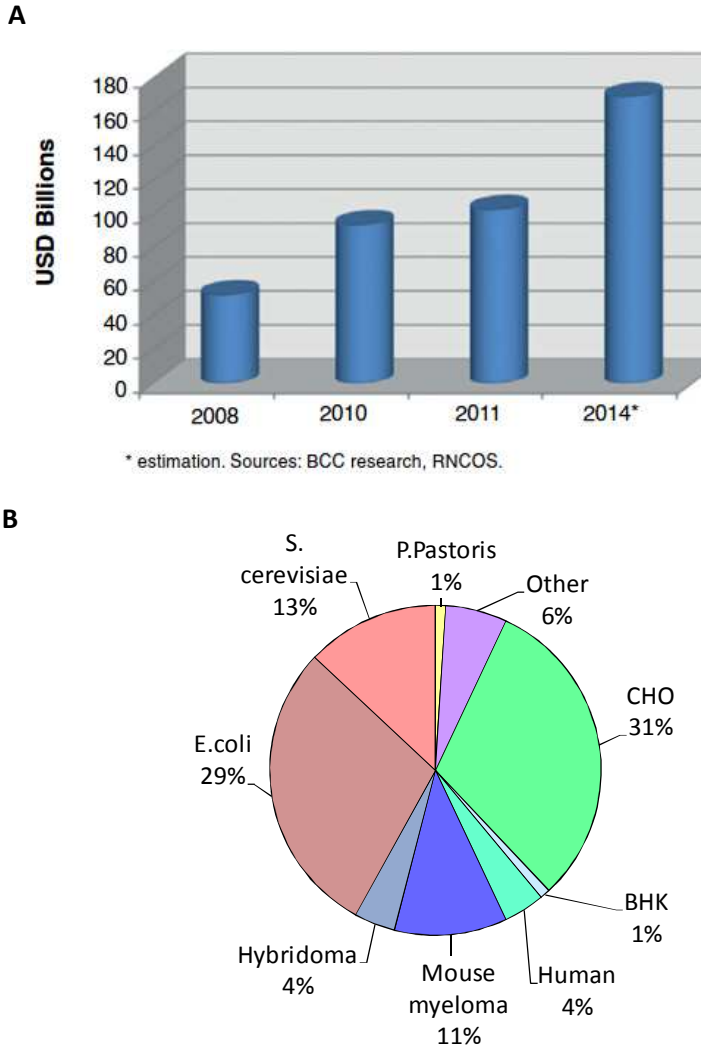


Figure I. A) Global market for recombinant protein drugs and B) percentage of protein-based recombinant pharmaceuticals produced by different hosts. CHO: Chinese hamster ovary, BHK: baby hamster kidney (Martínez et al., 2012; Kantardjieff and Zhou, 2014)

1.2.1 Prokaryotic expression hosts

Prokaryotic expression systems are often the first option since they are inexpensive and achieve high protein yields in short time. Bacterial cells require basic nutrition media, have a short doubling time and they are easy to manipulate, facilitating large scale productions. Thus, they are especially indicated for the manufacturing of small, soluble proteins with no post-translational modifications.

Among the various prokaryotic hosts, the gram-negative bacterium *E. coli* is the most widely used. It is the best genetically characterized microorganism and nowadays several manipulations and expression tools are available for its engineering. However, the use of *E. coli* as host has some drawbacks. First of all, more than 50% of *E.coli* expressed proteins are insoluble (Braun and LaBaer, 2003). A limiting factor in expressing a soluble protein in prokaryotic system is represented by its molecular weight, because correct protein folding decreases considerably above 60 kDa (Structural Genomics Consortium et al., 2008). Insoluble proteins accumulate in inclusion bodies and require complicated and costly denaturation and refolding processes to recover their functionality. Strategies to overcome these shortcomings include co-expression with chaperones (Baneyx and Mujacic, 2004) and fusion with solubility enhancing molecules (Kapust and Waugh, 1999). In addition, formation of disulfide bonds is problematic in the reducing environment typical of *E. coli* cytoplasm. Therefore researchers have developed methods to target proteins to other compartments, such as periplasm (Schlegel et al., 2013). Periplasmic protein localization also allows to avoid cells eruption in the downstream process, a step that usually leads to release of endotoxins causing restrictions in therapeutic applications. Finally, the major limitation of this host is its lack of ability to glycosylate proteins, preventing in some cases their proper conformation and activity. In recent years, the discovery of a N-glycosylation pathway in bacterium *Campylobacter jejuni* opens up to the possibility to produce glycoproteins in engineered *E.coli* (Wacker et al., 2002). Although prokaryotic systems are being continuously improved, production of complex and glycosylated proteins still requires expression in eukaryotic hosts.

1.2.2 Eukaryotic expression hosts

Eukaryotic expression systems can secrete the protein of interest into the media and post-translational modifications can be carried out, including N- or O-linked glycosylation, phosphorylation, acetylation, palmitation, proteolytic-processing and subunit assembly. These modifications are essential for correct folding, solubility and biological activity of expressed proteins. In spite of these advantages, eukaryotic cells grow more slowly than bacterial cells and costs are higher than those for the prokaryotic system.

1.2.2.1 Yeast

Among the simplest eukaryotic hosts, there is yeast, a unicellular organism. Its main advantage is that it is both a microorganism and an eukaryote. In fact, it couples some prokaryotic advantages like easiness in gene manipulation and fast growth to an eukaryotic environment where it is possible to perform many eukaryote-specific post-translational modifications. However, there are several complex modifications, such as prolyl hydroxylation and amidation, as well as some types of phosphorylation and glycosylation that yeasts cannot perform (Cereghino and Cregg, 1999). Another important advantage of yeast compared to *E.coli* is the secretion of proteins into extracellular broth when a signal sequence is cloned in frame with the transgene. Given that only few yeast endogenous proteins are present in the culture media, the heterologous proteins are in a relative pure matrix that simplifies the downstream purification process (Romanos et al., 1992).

Since the 1980s, *Saccharomyces cerevisiae* has been the most commonly used yeast for recombinant protein production. As a model organism, it is the best characterized yeast expression system because of the deep knowledge of its molecular and biochemical properties, as well as manipulation techniques. However, the baker's yeast has some drawbacks. Its proteins are differently and excessively glycosylated in comparison to those of higher eukaryotes. For examples, the O-linked oligosaccharides contain only mannose moieties instead of sialic acid, galactose, and N-acetylgalactosamin residues. In addition, thought N-linked glycosylation patterns are more conserved, the yeast expresses over-glycosylates proteins with structures harboring up to 100 mannose residues in different linkages (Gemmill and Trimble, 1999). These atypical glycan profiles can change proteins epitopes or functional sites and may arise immunogenic response in case of therapeutic applications. Furthermore, *S. cerevisiae* has limited secretion capacity and lacks suitable

strong inducible promoters (Romanos et al., 1992). To overcome these limitations, other yeast expression hosts were developed and, among all, *Pichia Pastoris* has emerged as the most popular system. *P. pastoris* is a methylotrophic yeast, in fact it can use methanol as its sole carbon and energy source in the absence of a repressing carbon source. It retains all the advantages of *S. cerevisiae*, plus its protein expression achieves higher levels. The superiority of *P. pastoris* is linked to the use of alcohol oxidase I (AOX1) promoter, which is one of the strongest inducible promoters known (Cregg et al., 1989). Moreover, this yeast prefers the respiratory growth, thus facilitating high-density culture. The combination of these last two characteristics allows high protein production when expression is induced shifting to a methanol medium, able to activate AOX1 promoter. Finally, *P. pastoris* has a great capability to secrete heterologous proteins and it performs a lower hyperglycosylation compared to *S. cerevisiae. P. pastoris* mannose oligosaccharides chains usually contain only 5-15 residues instead of 50–150 residues present into those of *S. cerevisiae* (Cummings and Doering, 2009). Recently, the potential of this yeast has been further expanded by the development of a genetically modified *P. pastoris* that can produce glycoproteins with humanized glycosylation pattern (Choi et al., 2003; Hamilton et al., 2006). Even if the development of *P. pastoris* system has hugely increased the yeast protein expression performances, big sized, multimeric and structurally complex proteins still require expression in hosts more similar to mammalian cells.

1.2.2.2 Insect cells

Among higher eukaryotic expression systems, insect cells are the closer host to mammalian cells in evolutionary term. They are able to carry out more complex post-translational modifications than yeast, even thought those are slightly different from the mammalian ones. High recombinant protein expression levels are effortlessly obtained and their cultures are easier and cheaper than those of mammalian cells (Drugmand et al., 2012).

Since its first application in 1983, baculovirus expression vector system (BEVS) has been the most commonly used tool to produce large amounts of active recombinant proteins in this host (Smith et al., 1983). The system is based on the lytic infection of insect cells with a recombinant baculovirus, usually *Autographa californica*, which is naturally pathogenic for lepidopteran cells. In particular, the virus is routinely amplified in insect cell lines derived from the fall armyworm *Spodoptera frugiperda* (Sf9, Sf21) or from the cabbage looper *Trichoplusia ni* (High-Five, Invitrogen). Native baculovirus double strand DNA contains a gene encoding for polyhedrin that is normally produced at high level in the very late phase

of infection, but it is not essential for viral replication. Therefore, exploiting the strong promoter of this gene, it is possible to express the heterologous protein up to 20-50% of cell proteins (Matsuura et al., 1987). The extensive use of BEVS is based on several considerations. Firstly, this system can achieve high yield of recombinant protein with almost all eukaryotic post-translational modifications, including proper folding, correct signal peptide cleavage, proteolytic processing, phosphorylation, N- and O-glycosylation, acylation, palmitylation and myristylation (Liu et al., 2013). Secondly, baculovirus has a high gene cargo capacity, because its genome (130 kb) can accommodate long DNA fragments. Moreover, the system is based on the engineering of the vector and not of the host cell line, thus reducing dramatically time requirements for protein production. Another advantage is the biosafety, because baculovirus does not infect human cells. Finally, insect cells do not require CO_2 regulation for growth and can be readily adapted to a high-density suspension culture for large-scale expression in simple bioreactor devices. However, there are some drawbacks. The first one is that virus-infected cells have a limited life span and cannot be used for continuous expression cultures. In addition, proteases released during lytic phase could degrade recombinant proteins. Furthermore, though the N-linked glycosylation sites are the same of mammalians, insect cells show some differences in the oligosaccharide chains. Even in this case, some researchers engineered insect cells to produce proteins with a mammalian-like glycosylation pattern (Wagner et al., 1996; Aumiller et al., 2012).

As stated previously, BEVS is a powerful and successful tool to express proteins similar to those produced in mammalian cells but, in some kinds of applications, especially those that require high reproducibility and long term production, mammalian cell cultures still remain the first option.

1.2.2.3 Mammalian cells

Mammalian cells are the favorite host for production of complex eukaryotic recombinant proteins, especially when their biological activity and pharmacokinetic are relevant. This system has the unique ability to express and secrete high quality proteins with correct folding, disulfide bonds formation and all required post-translational modifications, in particular glycosylations. Since these proteins are highly human compatible, mammalian cells have become the dominant host used to produce therapeutic proteins and antibodies.

In addition, mammalian proteins are required for functional analysis and structural studies by academic researchers.

Although cultures of mammalian cells have been performed since 19th century, their application in recombinant protein production were delayed due to complexity of their genomic manipulation and cell culture fragility. Even if they are approximately 10- to 50-fold larger than microbial cells, they are more breakable since they lack the protective microbes cell wall. Furthermore, they are more affected by impurities and they have complex nutritional requirements, such as hormones and growth factors, usually provided by bovine serum (Glacken et al., 1983). However, the serum components are not chemically defined, leading to yield inconsistency among different batches. Moreover, this kind of cultures increases the risk of virus or prions contaminations, especially critical for the production of therapeutic proteins (Shah, 1999). These shortcomings, coupled to labourios purification process from serum containing media, have pushed the development of serum-free media to facilitate production scale-up and reproducibility, and to simplify the downstream processes. Another mammalian cells constraint is that adherent cell lines cannot reach the high densities required for industrial production, therefore they are usually adapted to grow in suspension cultures. Large-scale bioreactor productions mostly expose cells to stressful conditions, such as shear forces and aeration, emphasizing once more the complexity of this culture system. Unfortunately, these are just some of the hurdles to deal with animal cells. Indeed, the major disadvantages are lower production yields, slower growth rate and higher costs compared to other eukaryotic systems. In the last 20 years, significant improvement of expression vectors, selectable markers and transgene delivery have allowed to obtain cell lines with high specific productivities and a consequent reduction of the time needed for cell line development. One of the most considerable advances in recent years has been the genetic manipulation of mammalian hosts to improve their survival. For example, apoptotic pathway have been engineered to prevent or slow the onset of apoptotic cell death through overexpression of Bcl-2 family members (Mastrangelo et al., 2000).

Despite significant progress has been achieved, recombinant protein production in mammalian hosts still remain challenging and time consuming. Nonetheless, it is often the only way to produce active biomolecules with all the mammalian specific modifications required.

1.2.3 Other expression systems

Besides single cell systems, more rarely, entire organisms such as transgenic plants or animals can be used for recombinant protein production.

Plants are currently used as bioreactors for production of enzymes both for industrial or research applications (Kusnadi et al., 1997; Hood, 2002). They are one of the safest and cheapest system for large-scale protein production because they are easy to scale up requiring only water, minerals and sunlight. Proteins can be expressed and stored in leaves or in seeds. Moreover, the risk of human pathogen contaminations is low, making transgenic plants an attractive system, especially for production of biopharmaceutical products. Although both therapeutic proteins and antibodies have been produced in transgenic plants since late 80s (Barta et al., 1986; Hiatt et al., 1989), it was only in 2012 that a protein produced by carrot cells received FDA approval (Fox, 2012). Despite that, the major concern about transgenic plants remains the environment biosafety issues due to the risk of transgene dissemination and contamination of our ecosystem or food supply.

Transgenic animals are another useful system for the growing need of complex and biologically active recombinant proteins. They assure high yield with low costs of production and maintenance compared to those of mammalian culture facilities. Possible sources of recombinant protein are milk, egg white, blood, urine, seminal plasma and silk worm cocoon. Anyway, at the moment, mammary gland is the most mature expression system because large quantity of milk can be collected daily (Wang et al., 2013). Among the drawbacks, firstly, there is the risk of product contaminations with animal pathogens active in human. Secondly, current methods of generating transgenic animal founders are relatively inefficient and time-consuming. Finally, ethical and environmental aspects of animal transgenesis are gaining an increased interest from the public opinion.

CHAPTER 2: Mammalian cells as expression system

The possibility to produce biologically active recombinant glycoproteins in mammalian cells has been the major driving force for the rapid improvement of methods, tools and technologies to provide more and more effective mammalian expression systems. Currently, about 51% of FDA approved biologics are produced in mammalian cells (Kantardjieff and Zhou, 2014), thus confirming the substantial investment of biotech companies in this expression system in spite of its costs. The development of new vectors, host cells transfection methods and improved bioreactors has had a deep impact in many areas of basic and applied research, as well as in industrial recombinant protein production.

2.1 Cell lines

A key element in the initial steps for the generation of high producing cell lines is the selection of a suitable cell type. Since they have to support production at industrial scale, cells must be capable of achieving high-level protein expression maintaining high viable cell density and genetic stability over long periods of time. In addition, they have to perform desired post-translational modifications with uniform product characteristics. Several mammalian cell lines have been reported for industrial bioprocesses, for example chinese hamster ovary (CHO) cells, baby hamster kidney (BHK) cells, non secreting mouse myeloma cells, including NS0 and SP2/0, and human cell lines such as embryonic kidney 293 cells (HEK293) and human-retina derived cells (PER.C6) (Jones et al., 2003; Kantardjieff and Zhou, 2014). Despite the availability of such a variety of cell lines, nearly 60% of biopharmaceutical proteins are produced in CHO cells (Kantardjieff and Zhou, 2014).

CHO

The popularity of CHO cells is due to their robustness and flexibility. This cell line was established in 1957 as an immortalized line from a primary culture of Chinese hamster (*Cricetulus griseus*) ovarian cells. The CHO-K1 cell line is a subclone derived directly from the original CHO line and it requires proline for growth (Kao and Puck, 1968). CHO-S cell line, a separate subclone of the parental CHO cells, is characterized by the capacity to grow as single-cell suspension culture. Due to their low chromosome number (2n=22), these cell lines are functionally hemizygous for many gene and, for this reason, they were extensively

used, from 1957 to the late 1970s, to study cell genetics (Puck et al., 1958). Although, initially, they were not intended for the purpose of recombinant protein production, they shortly become the gold standard for manufacturing biologics. The step from laboratory benches to industrial processes was facilitated by the availability of auxotrophic CHO mutants created for research study. The nutritional requirements of these mutants could be exploited as a method to select for genome integration and stable expression of exogenous DNA. Historically, the emerged metabolic marker was dihydrofolate reductase (*dhfr*) gene. DHFR enzyme reduces folate, a necessary precursor for nucleotide synthesis when thymidine and hypoxanthine (a purine derivative) are absent in the culture medium. CHO DXB11 and CHO DG44 are two *dhfr* deficient cell lines, generated in 1980s, that are widely used for recombinant protein production (Urlaub and Chasin, 1980; Urlaub et al., 1983). Transfection of these cell lines with *dhfr* gene plus the gene of interest facilitates the screening of high producing clones grown in thymidine and hypoxantine depleted medium. Moreover, the availability of DHFR inhibitors, such as methotrexate (MTX), applies an additional selective pressure and promotes the transgene copy number amplification (Nunberg et al., 1978). This system was exploited for the production of the first FDA approved therapeutic protein, i.e. the tPA (Wurm, 2004).

Another metabolic marker frequently used for generating recombinant CHO cell lines is the glutamine synthetase gene (*gs*). The enzyme coded by *gs* gene produces glutamine in absence of any exogenous sources. This selection system was initially developed for murine NS0 cell lines since their endogenous GS activity is low, but it has been recently extended to CHO lines and other mammalian cells (Bebbington et al., 1992). Indeed, it is a dominant selectable marker independent on lineage. However, CHO cells have a higher endogenous GS activity than NS0 cells, and require the use of the inhibitor methionine sulphoximine (MSX) in order to maintain a sufficient genetic pressure and to induce transgene amplification (Bebbington et al., 1992). This approach has also the advantage of avoiding glutamine addition to the medium, lowering ammonia accumulation (Zhang et al., 2006). Recently, to further increase the selection stringency of this system, GS-knock out CHO cell lines have been generated by zinc-finger nucleases (Liu et al., 2010).

Beyond the availability of these powerful gene amplification systems, CHO cells have other features that facilitate their use in industry production. Firstly they can be easily manipulated and adapted to grow in serum-free suspension cultures at very high density, an ideal characteristic for large-scale processes. Secondly, there is less risk of pathogen

contaminations, because the majority of human viruses are not infective in CHO cells (Xu et al., 2011). The safety of this cell line is demonstrated by three decades of commercial therapeutics production and makes it easier to obtain market approval from regulatory agencies, such as FDA. Thirdly, CHO post translational modifications, glycosylations in first place, generate active human-like glycoforms. Finally, the CHO-K1 genome has been sequenced (Xu et al., 2011), therefore it would be possible to improve CHO cell lines performances by targeting specific genomic loci. However, this opportunity could be complicated by genomic heterogeneity between the different CHO cell lineages (Xu et al., 2011).

Nowadays, optimization of culture upstream processes as well as progress in cell line engineering have allowed to increase productivity, reaching the gram/liter range, a 100-fold improvement compared to protein titers of 1980s.

NS0

NS0 myeloma cells were derived from the plasmacytoma tumor MOPC21 induced in a Balb/c mouse by peritoneal injection of mineral oil. The tumor cells were then cloned and selected until they no longer produced any endogenous antibody (Galfrè and Milstein, 1981). All murine myeloma cell lines used commercially today originate from the MOPC21 tumor cells (Barnes et al., 2000). NS0 were initially used as fusion partner for generation of hybridoma cells, as well as SP2/0 myeloma cells. Currently, NS0 cell line is exploited as an important host system for the production of recombinant proteins, in particular antibodies, because the original cell type was a differentiated B cell, capable of high levels Ig production. Their success is mainly derived from the development of the above described GS selection system, since these cells are GS deficient (Bebbington et al., 1992). This system is commonly used for recombinant antibodies production. In addition, NS0 cell line is a native suspension culture, which made serum-free adaptation and growth in bioreactors more straightforward than CHO cells. Nevertheless, their scale up processes could be more complicated than other mammalian cell lines since they are auxotroph for cholesterol. Until recent years, the only source of cholesterol was serum because animals were the only possible source of it. Nowadays, synthetic cholesterol has become available (Talley et al., 2005), but its high cost is still a concern for large-scale cell cultures. Furthermore, it is not water soluble hence its supplementation into bioreactors is a complicated step. To

overcome these hurdles, recently, researchers have developed cholesterol independent NS0 cell lines (Hartman et al., 2007).

HEK293

The HEK293 cell line was established by transformation of human embryonic kidney cells with sheared adenovirus DNA fragments of the Ad5 serotype (Graham et al., 1977). Although it was generated almost 40 years ago, it is only in recent years that it has been exploited for recombinant protein production. This cell line is easy to handle likewise CHO cell lines, and many variants of the original clone, adapted to suspension growth and serum free conditions, are available. In addition, HEK293 cells are widely used as hosts for transient gene expression (TGE, discussed in next section) because of their high transfection efficiencies with most of gene delivery system. In order to improve protein production in TGE, other cell lines have been derived from the original one such as the HEK293-T line expressing the SV40 large T-antigen (DuBridge et al., 1987), and the HEK293-E line expressing the Epstein-Barr virus EBNA1 protein (Invitrogen, Carlsbad, CA). Both cell lines support the episomal amplification of plasmids containing the SV40 or the EBV origin of replication respectively, thereby extending the transgene expression after transient transfection. Recently, it is becoming clear that certain recombinant proteins necessitate production in human cells to assure a high similarity to their natural counterpart in terms of post translational modifications. The widely used CHO cell line is indeed unable to perform some types of human glycosylations, since it lacks specific sugar transferring enzymes such as α(2–6) sialyltransferase and α(1–3/4) fucosyltransferases (Grabenhorst et al., 1999). Moreover, it has happened that some post-transitional modifications performed by CHO cells were found to be inadequate (Durocher and Butler, 2009) (Fig. II). For these reasons, HEK293 cells, along with other human cell lines such as PER.C6 (Fallaux et al., 1998), have been growing in prominence in industrial protein production.

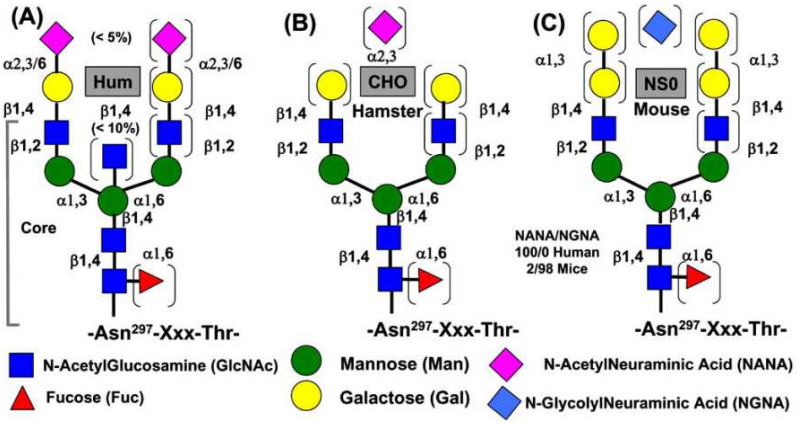


Figure II. Glycosilation patterns observed in recombinant antibodies produced in A) human cells B) CHO cell line and (C) murine cells (NS0 or SP2/0 cell lines and hybridoma) (Beck et al., 2008).

2.2 Transgene expression

Different methods are available for the expression of recombinant proteins in mammalian cells. Depending upon economical and technical issues, the protein expression could be stable or transient.

2.2.1 Stable and transient gene expression

Development and manufacturing of recombinant proteins for therapeutic and commercial purposes are usually produced by stable gene expression system because it is usually assured reproducibility, consistency, homogeneity and quality of the produced proteins. In this system the transgene expression is prolonged over time because the vector is stably integrated into the host cell genome. During transfection, the transgene is always accomplished by a marker gene that allows to isolate and select cells in which the gene of interest has been integrated. As mention before, several selection markers have been developed, such as DHFR or GS systems, along with the classical use of antibiotic selection drugs. The successfully transfected cells are then cloned by limiting dilution and evaluated for the level of recombinant protein expression. The highest productive clones are further characterized for protein production to avoid instability over time. Many could be the issues causing instability problem. First of all, since the transgene expression is affected by genome flanking regions and the exogenous DNA integration is a random event that occurs by non homologous recombination, a phenomena called “positional effect” can strongly influence the transgene expression levels. This phenomena frequently occurs when genes

are integrated in heterochromatin regions, composed by tightly packed DNA. These regions, indeed, prevent the transcriptional machinery from binding to DNA sequences. However, even thought the integration happens in actively transcribed euchromatin regions, the long term transgene expression is not ensured since it can be silenced by epigenetic modifications (Mutskov and Felsenfeld, 2004). To overcome this problem, the transgene can be flanked with DNA sequences able to block the formation of condensed chromatin and to assure stable transgene expression irrespectively of the chromosomal integration site. Several genetic elements have been developed, such as insulators, scaffold or matrix attachment regions (S/MAR) and ubiquitous chromatin opening elements (UCOEs) (Lai et al., 2013). Additionally, site-specific recombination, mediated by different recombinases, can integrate the transgene into actively transcribed and stable regions of the genome called hotspots (Turan et al., 2010). Besides "positional effect", it often happens that plasmid vectors are rearranged head-to-tail by nuclear ligases to form long concatemers (Perucho et al., 1980). These repeated sequences cause transgene silencing (McBurney et al., 2002) or loss of transgene copy number due to homologous recombination between copies within the array (Weidle et al., 1988). Given these issues, candidate cell lines must be cultivated and monitored for at least 35 passages to guarantee protein expression stability over time (Chusainow et al., 2009). Even though stable gene expression platform is well established, it remains a labor-intensive and time-consuming process that involves considerable investments and usually takes more than six months. Therefore, the need for quicker and more economical approaches for protein production has pushed the evaluation of alternative methods, like transient transfection.

Transient expression technology has been developed to rapidly produce milligrams of protein for high-throughput characterization. Nevertheless, in recent years it has become a tempting tool for large scale protein production (Baldi et al., 2007). In transient gene delivery, DNA is introduced into cells and maintained as an extrachromosomal unit. Hence, the expression of the transgene typically dissipates after a short period (usually 2 weeks) because the expression plasmid is either degraded or "diluted" at each cellular division. The main difference with stable gene expression is that genetic selection is not applied, deleting time required for screening and isolation of successfully transfected clones. Transient transfection technology is based on a bulk cell culture system in which the key element for high level protein production is the plasmid copy number per cell. For this reason HEK293

and CHO cell lines have been improved by expressing the Epstein-Barr virus (EBV) nuclear antigen 1 (EBNA1) or the SV40 large-T antigen, that allow to support the episomal replication of vectors carrying the EBV or the SV40 origin of replication, respectively (Daramola et al., 2014). The process can be scaled up to 100L bioreactor owing to 1) media optimization for high density suspension culture and 2) use of inexpensive transfection agent, such as CaPi or PEI (Girard et al., 2002). Besides these reagents, the amount of recombinant plasmid required for large-scale transfection is a cost that should be taken into consideration. Although it is undeniable that transient expression technology is time saving (Dalton and Barton, 2014), the moderate productivity of a transient pool cannot compete with that of stable cell lines (Liu et al., 2008). However, recently, after a deep investigation of many factors that influence transient gene expression, researchers have developed highly optimized protocol that has allowed 1 g/L production of recombinant antibodies in 14 days (Backliwal et al., 2008). Another disadvantage is the difficulty to scale up transient gene expression as a routine method for 100-1000 L range bioreactors. In such volumes, transfection efficiency could vary between experiments, leading to protein heterogeneity from batch to batch. Finally, repetition of transient gene expression procedure for continual protein production is laborious and could be expensive over time compared to culture of a stable cell line.

2.2.2 Transgene delivery

Gene transfer technologies allow the introduction of foreign DNA molecules into eukaryotic cells without killing them. The challenge in this delivery is the electrostatic conflict between the negatively charged nucleic acid molecules and the negatively charged plasma membranes. Several methods are now available, they can be classified as physical, chemical or virus-mediated methods (Fig. III). All the advances in these techniques have led to simplified procedures, reduced toxicity, enhanced reproducibility and increased success rate.

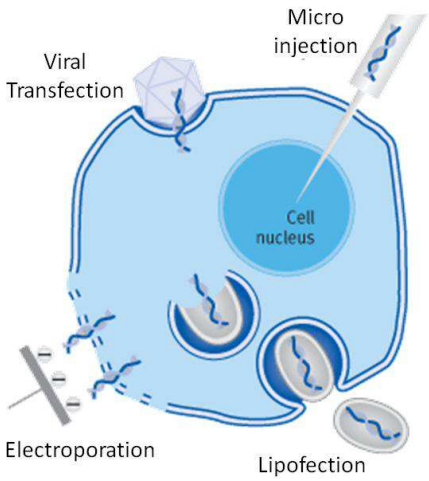


Figure III. Schematic overview of transgene delivery methods: microinjection, lipofection, electroporation and viral transfection

2.2.2.1 Physical methods

Physical methods of transfection allow the introduction of plasmid DNA into cells avoiding the use of chemical substances or viruses. The principal techniques available are electroporation, microinjection and gene gun delivery.

Electroporation employs a rapid high-voltage electrical field to temporarily form pores in the cell membrane. This facilitates the passage of DNA into the cell. There is practically no limit to the size of DNA that can be delivered. It is one of the most efficient technique, especially for delivering exogenous DNA to suspension cells. However, the electroporation method is difficult to optimize and always causes a high incidence of cell death, as a result of the intrusive nature of the procedure.

Microinjection entails the direct injection of DNA into the nuclei of cells using fine glass needles under a microscopy (Capecchi, 1980). Although it is highly efficient, it is labor-intensive and require operator skills. Moreover, only one cell at a time can be microinjected and consequently it is incompatible with industrial bioprocess.

Finally, the gene gun method, also known as biolistic, achieves gene delivery by shooting metal microparticles coated with DNA into cells, using either electrostatic force or gas pressure (Klein et al., 1987). This technique is fast, simple and safe, but it is so far mostly applied for plant cells due to is low efficiency.

2.2.2.2 Chemical methods

Chemical methods rely on positively charged reagents that create a layer around the negatively charged DNA, thus neutralizing their charges. They are the most used transfection methods in gene delivery for industrial applications and include calcium phosphate co-precipitation, cationic polymers or cationic lipids based transfection reagents.

Calcium phosphate (CaPi) co-precipitation was invented by Graham and colleagues in 1973 and they subsequently used it for the generation of HEK293 cell line (Graham and van der Eb, 1973). It is the most renowned and widely used inorganic compound for the transfection of mammalian cells. The CaPi method involves mixing DNA with a calcium chloride solution, followed by the addition of a phosphate solution to precipitate CaPi-DNA complexes. These DNA-containing precipitates are then dispersed onto the cell layer and enter into cells via endocytic vesicles (Graham and van der Eb, 1973). It is a simple and inexpensive technique applicable to a wide range of cell types. Due to its economical nature it could be applied in large-scale transient transfections of cell cultures up to a 100 L (Girard et al., 2002). However, the calcium phosphate method is difficult to reproduce because of its sensitivity to slight changes in precipitate formation conditions. The transfection efficiency depends on these preparation parameters and, in general, is low compared to new methods, especially for suspension cells (Khosravi-Darani et al., 2010). Moreover, to improve transfection efficiency, CaPi method requires serum in the growth medium in order to limit the aggregation of the CaPi-DNA precipitates into cytotoxic complexes (Girard et al., 2001). As stated before, the use of serum is strongly avoided in industrial bioprocesses. As a consequence, an effort in condition optimization is necessary, thus limiting the use of this method.

Polyfection and Lipofection are the most recently developed and simple methodologies for gene delivery in a variety of cells. Polyfection employs cationic polymers such as diethylaminoethyl-dextran (DEAE-Dextran) (McCutchan and Pagano, 1968), polyethylenimine (PEI) (Boussif et al., 1995) and dendrimers. They form a polyplex interacting with the anionic phosphodiester backbone of DNA. The polyplex has an overall cationic charge that allows the binding to negatively charged cell membrane surfaces, where they are internalized by endocytosis. DEAE-dextran is one of the first chemical reagents used for polyfection (McCutchan and Pagano, 1968). Although it is simply and

appropriate for transient transfection, its high toxicity and low efficiency have limited its use compared to other polymers. Alternatively, polyethylenimine (PEI) can achieve higher transfection efficiency because it protects DNA from lysosomal degradation, though the mechanistic details of intracellular transport from endosomes to the nucleus remains elusive (Benjaminsen et al., 2013). This method has been extensively used for transient transfection in serum free suspension cells culture because it successfully provides a low cost gene delivery technique at large scale (Raymond et al., 2011).

Lipofection refers to cationic lipid-mediated gene transfer into the cell. In aqueous solutions cationic lipids form vesicles with a lipid bilayer sheet, known as liposomes. Electrostatic interactions between head group of lipids and negatively charged DNA generate a lypoplex that enters into cells by means of endocytosis, as described for polyfection, or via membrane fusion (Felgner et al., 1987). Liposomes usually contain a combination of amphipathic lipids, which are positively charged at physiological pH, and neutral ones, which are needed as "helper" to stabilize formation of micelles. Moreover, neutral lipids increase transfection potency because allow entrapped DNA to escape from endosomes (Hui et al., 1996; Xu and Szoka, 1996). Different mixtures with high transfection efficiency are commercially available such as Lipofectamine (Invitrogen, Carlsbad, CA) and Fugene (Roche, Basel, Switzerland). The main advantages of lipofection are reproducibility, simplicity and high transfection efficiency with very low cytotoxicity either in the presence or absence of serum, as well as using both adherent and suspended cells (Rosser et al., 2005). As a consequence, lipofection is one of the most used gene transfer method for stable transfection. On the contrary, due to its high costs it is unfeasible for large-scale transient transfections.

2.2.2.3 Viral transduction

Virus-mediated transfection, also known as transduction, is based on engineered mammalian virus as carrier for gene delivery. It was first developed in 1970s (Goff and Berg, 1976). This system is extremely efficient since virus naturally infect cells to integrate their genetic material into host genome. Indeed, scientists address their attention to this tool in order to increase efficiency, especially in cell types resistant to other transfection methods. Transduction can assure that up to 100% of cells are infected without cytotoxicity. Even if virus gene delivery is better compared to non-viral systems in term of effectiveness, several drawbacks limit its use in clinical applications. The major concern is safety, since virus

preparations require biosafety level 3 facilities, even though they are engineered to be replication-defective. Moreover, the production of viral particles is a time-consuming preparation that requires transfection of packaging cell lines expressing viral genes necessary for virus replication. In addition, due to physical space boundary, viral particles can carry a limited amount of DNA, usually 5-10 kb depending on virus type (Walther and Stein, 2000). Due to these hurdles, as well as its costs, viral transduction system has not been embraced by industrial communities. However, in recent years, optimized lentiviral vectors have been successfully exploited to achieve high-level, long-term production of milligrams of protein in short time, thus becoming an attractive system for industrial protein production (Oberbek et al., 2011; Dhamne et al., 2014). To this purpose modified baculovirus has been demonstrated a safer alternative to mammalian virus (Dukkipati et al., 2008).

2.3 Small scale protein production

The production of therapeutic and diagnostic recombinant proteins in mammalian cells employs techniques similar to those used for microbial cultures, even if mammalian cells have a slower growth rate and lack the protective cell wall. As a consequence, they are much more sensitive to shear than microbial cells. This characteristic makes it necessary to develop suitable bioreactors.

There are three cell culture mode operations used in industry applications: batch, fed-batch and perfusion (Kantardjieff and Zhou, 2014). In the batch system, all nutrients are supplied in the beginning of the culture and only aeration, pH and temperature are controlled. The growth curve of a batch process includes four phases. A lag phase, in which cells adapt themselves to the new environment and do not grow; an exponential growth phase, where cells increase rapidly in cell density; a stationary phase, in which cells death is balanced by cells division and finally a death phase when all nutrient are depleted. The length of lag phase depends on the physiological state of cells and on concentration of the inoculum, whereas the final phase depends upon medium components depletion and toxic metabolic products accumulation, such as ammonia and lactate (Butler, 2005). Batch cultivation is the simplest system of culture, but results in low product yield because nutrients depletion limits cell density. To reduce this problem it is possible to partially harvest a portion of cells

along with product and replace it with fresh medium. However, the repeated batch system is expensive and does not allow to achieve high product concentrations.

A different production system is fed-batch cell culture. In this operation mode, additional nutrients are supplied when needed during the cell culture process, thus lengthening the growth phase of cell, hence, the protein production. The feeds are added as concentrated solutions in order to limit increase in culture volume and product dilution. The fed batch culture consist of the four phases described previously, but the growth rate can be modulated by nutrient supply, reducing production of metabolites and extending culture lifetime. This strategy allows to achieve higher cell concentrations and, as a result, higher yields, up to 2g/L (Butler, 2005). It is frequently applied in protein production for its convenience, even thought equipment for parameter monitoring is required.

Perfusion culture is the third operation mode and consists of a continuous cell culture process. After an initial cells accumulation phase, cells are retained in the bioreactor, while new culture media is constantly added and supernatant removed at the same time. This allows to obtain very high cell density because cells continue to grow until they reach an intrinsic limit of the bioreactor, such as cell retention capacity or aeration rate. However, this system is more complex than others and the continuous replenishment of medium is expensive. Due to longer time required for harvesting, batch and fed-batch cultures are usually employed to produce stable molecules, while perfusion cultures are more useful to produce labile molecules (Kantardjieff and Zhou, 2014). Fed batch and perfusion cultures can be scaled up for large-scale productions that are almost exclusively performed in stainless steel stirred-tank bioreactors.

2.3.1 Small-scale production devices

There are many different devices for small scale protein production that can be classified on the basis of the mixing, oxygenation and nutrient supply technologies (Eibl and Eibl, 2009). Flask cultivation on orbital shaker is one of the most employed technique for microorganism culture and, nowadays, thanks to the use of disposable shake flasks is also broadly used for mammalian cell lines. However, the working volume necessary for good aeration is only 10-15% of total flask volume, limiting its scale up.

Another successful technology is the hollow fiber reactor which was developed in early 1970s (Knazek et al., 1972). It is a continuous perfusion culture system that permits to attain high cell density. It consists of a cartridge with a series of stacked capillary tubes in which medium is pumped. Cells are grown in the extracapillary space (ECS) of the cartridge.

Nutrients and waste products diffuse through the barrier while cells and recombinant proteins are retained in the ECS due to hollow fiber molecular weight (MW) cut-off. The hollow fiber also protects cells from shear, because they are continuously oxygenated trough fresh medium and not by vigorous agitation.

The use of a semi-permeable membrane for growing cells at high density is at the basis of two-compartment systems. Cells are cultivated in a small chamber separated by a low MW cut-off barrier from a larger compartment that contains medium. As mention above, the compartmentalization allows to culture cells at high density in repeated batch mode, with production of recombinant protein at very high concentration. Two systems are commercially available: CELLine (Integra Biosciences), a T-flask based vessel, and MiniPerm (Greiner Bio One), a roller bottle based system. However, both of them provide small volume of products and the basic parameters for bioproduction cultures, like temperature, pH and dissolved oxygen, cannot be monitored.

A completely different single use bioreactor is Wave Bag (GE Healthcare) that uses rocking motion to provide mixing and oxygen transfer (Singh, 1999). This system utilizes an inflated plastic bag as cultivation chamber, filled up to 50% of volume. In this case, process parameters are monitored and controlled, facilitating the scale-up of the process. Different volumetric bags, ranging from 500 mL to 1000L, are available.

Recently, disposable stirred-tank bioreactors have become also available for small scale production. 3L Mobius CellReady (Millipore) bioreactor is the first bench-scale single use reactor of this class. Aeration is guaranteed by a microsparger and culture is mixed by a marine impeller, in order to reduce shear sensitive. Stirred-culture vessels are easily scalable, well characterized and provide a completely automated control of the culture environment.

Thanks to this growing availability of different types of bioreactors, the small scale production process is more easily regulated.

CHAPTER 3: Transposon: a powerful approach for efficient transgene delivery

3.1 Transposable elements

Transposable elements (TE) comprise a wide array of DNA segments with the distinctive ability to move from one genetic location to another. They were first discovered in maize genome by the geneticist Barbara McClintock in the 1940s. Nevertheless, the idea of mobile DNA was largely dismissed and her work was ignored for thirty years. Finally, she was awarded a Nobel Prize in Medicine and Physiology in 1983.

TEs were found in almost all organisms, both prokaryotes and eukaryotes, and typically they contribute to large portions of the genome, for example 10% in fish species, 37% in mouse, 45% in humans and more than 80% in maize (Muñoz-López and García-Pérez, 2010). Although TEs are wide spread into genomes, far long time they were considered junk DNA and molecular parasites, with no other purpose than to propagate themselves. Nowadays, it is well known that their ability to move and replicate contributed to genomic evolution by insertion of additional DNA into genes, thus promoting gene inactivation, gene expression modulation and random recombination (Feschotte and Pritham, 2007).

TEs fall into two major classes according to whether their replication intermediate is an RNA molecule (class I) or a DNA segment (class II) (Fig. IV). Elements of class I, also called retrotransposon, transpose by a replicative “copy and paste” mechanism. The RNA intermediate is reverse transcribed into DNA by TE-encoded reverse transcriptase and then integrated at a new genome site. This process creates an additional copy of the retrotransposon, leaving intact the TE at its original genomic position. This class includes long terminal repeat (LTR) retrotransposons or non-LTR ones, a subclass that comprises long interspersed elements (LINEs) and short interspersed elements (SINEs). In all LTR retrotransposons, the inner coding region of the element is flanked by long terminal direct repeats, that carry transcriptional regulatory elements. Among the non-LTR transposons, LINEs represent the autonomous forms, whereas SINEs depend on LINEs machinery for transposition.

Class II elements, also referred to as DNA transposons, move directly as DNA segments. Eukaryotic DNA transposons can be divided into three major subclasses: 1) those that excise as double stranded DNA and reinsert elsewhere in the genome, 2) *Helitrons* that utilize a mechanism probably related to rolling-circle replication using a single-stranded DNA intermediate, and 3) *Mavericks* that are supposed to replicate as single-strand DNA using a self-encoded DNA polymerase (Feschotte and Pritham, 2007). The first subclass, standard DNA transposons, move by a conservative "cut and paste" mechanism of transposition. In this process the TE DNA fragment is excised from its original genomic locus and reintegrated elsewhere. Conversely to retrotransposons, DNA transposons leave behind none or only a short footprint consisting of a few nucleotides duplicated during integration or generated by DNA repair machinery repaired after excision.

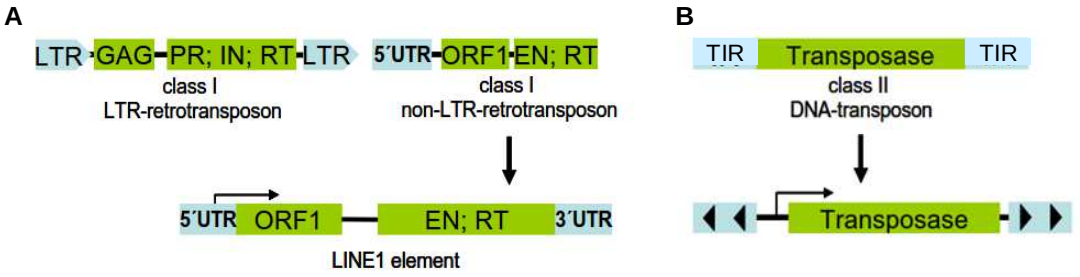


Figure IV. Schematic structure of main transposon types in eukaryotes: A) Class I of TEs that comprises LTR-retrotransposons and non-LTR retrotransposons. LTR: long terminal repeats, GAG: group-specific antigen; PR: protease, IN: integrase, RT: reverse transcriptase; UTR: untranslated region, ORF1:DNA-binding protein, EN: endonuclease. Below, it is reported a LINE1 element, a well-studied class I non-LTR-retrotransposon. B) Class II or DNA transposons that contain two terminal inverted repeats (TIR) flanking the transposase enzyme. Below, black arrowheads represent transposase binding sites within the TIRs

3.2 DNA transposon

"Cut and paste" DNA transposons consist of a single gene encoding the transposase enzyme, which is flanked by terminal inverted repeats (TIRs), typically composed of sequences that are imperfect palindromes. TIRs vary in length between different subclasses, but always contain binding sites recognized by transposase (Skipper et al., 2013).

The mechanism of transposition starts with the transposase that binds to TIRs and forms a circular pre-excision synaptic complex from which the transposon is excised. This complex is created by the tetramerization of transposase that brings together the transposon ends. Then the synaptic complex binds to a new region of genomic DNA, known as target site,

and the transposase mediates the DNA opening and the transposon integration. Upon insertion, target site DNA could be duplicated, resulting in target site duplications (TSDs), which represent a unique hallmark for each DNA transposon (Muñoz-López and García-Pérez, 2010). Depending on their sequence, TIRs and TSDs, eukaryotic DNA transposons are classified into different superfamilies of which the most important are Tc1/*mariner*, *hAT*, and *piggyBac* (Fig. V).

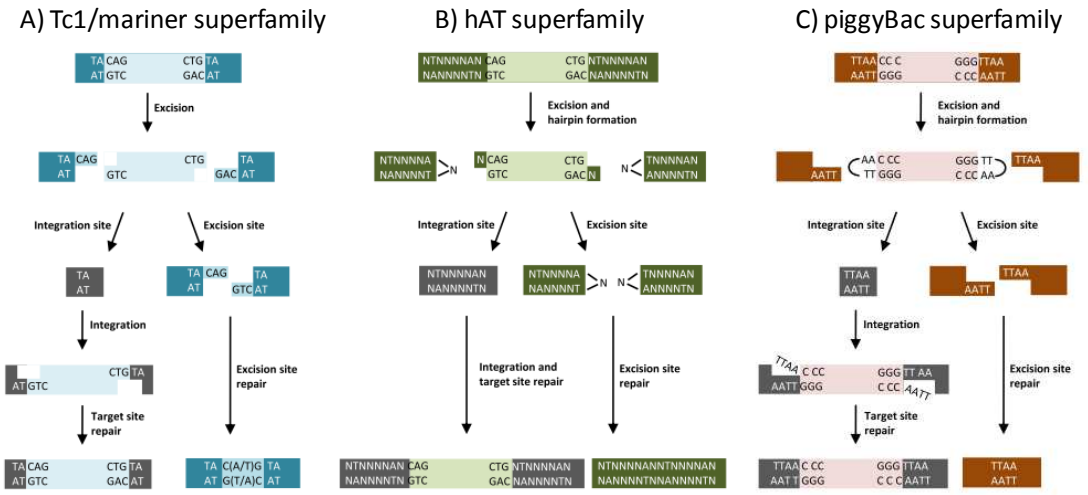


Figure V. Schematic representation of cut and paste transposition. A) Transposition of Tc1/*mariner* elements leads to double-stranded breaks and formation of a 3′-overhang at the excision site. DNA repair by host-encoded enzymes creates a characteristic footprint at the excision site. Integration occurs at TA dinucleotides which are duplicated upon transposition. The single-stranded gaps are repaired by host-encoded enzymes. B) *hAT* transposition creates hairpins at the ends of the flanking donor DNA. Integration is targeted to NTNNNNAN target sites, and creates target-site duplication. Excision site repair leaves a random footprint. C)*PiggyBac*-mediated excision is followed by hairpin-formation at the transposon ends. After integration into TTAA target sites that are consequently duplicated, the single-stranded breaks are repaired by ligation. The 5′ TTAA overhangs created at the excision site anneal, thus repairing the double-stranded break without leaving any footprint. (Skipper et al., 2013)

The elements from the superfamily Tc1/*mariner* are probably the most widely distributed family of TEs in nature, having been identified in *C. elegans,* rotifers, fungi, plants, fish and mammals (Plasterk et al., 1999). However, several transposons of this group harbor inactivating mutations and only few are naturally active, including *minos*, *Mos1* and Tc1 (Muñoz-López and García-Pérez, 2010). Tc1/*mariner* elements are between 1 and 5 kb in length, and encode a transposase of 282 to 345 amino acids which is flanked by two TIRs that can vary between 17 to 1100 bp in length (Plasterk et al., 1999). The transposase protein has two characteristic domains: a N- terminal region containing the helix-turn-helix

motif necessary for recognition and binding of TIRs, and a C-terminal domain that includes the catalytic motif constituted by three amino acids, DDD in the case of mariner-like elements, or DDE in the case of Tc1-like elements. The transposase sequence also contains the Nuclear Localization Signal (NLS), indispensable for transposase transport through the nuclear membrane (Brillet et al., 2007; Ivics et al., 1996). Tc1/*mariner* transposon mediates a double-strand break, leaving 2 or 3 bp 3'-overhangs at the transposon ends after excision. The repair of the 3'-overhang, generates a characteristic transposition footprint (Luo et al., 1998). The TE is inserted exlusively into TA target-site with TSDs that flank the integrated element (Skipper et al., 2013).

The superfamily *hAT* (hobo/Ac/Tam3) comprises DNA transposons of 4 to 12 kb that encode a transposase with a catalytic DDE motif and a zinc finger DNA binding domain. These transposons generate 8 bp TSD (NTNNNNAN) as a result of the transposition event and they have TIRs between 5 and 27bp in length. The excision site repair leaves a random footprint (Kempken and Windhofer, 2001).

DNA transposon of the *piggyBac* (PB) superfamily have been found in plants, fungi and animals, including humans, even though they are probably inactive due to mutations (Sarkar et al., 2003). *PiggyBac*, the founder member of this superfamily, was originally isolated from the cabbage looper moth *Trichoplusia ni* and is a 2472 bp transposon that contains 13 bp identical TIRs and additional asymmetric 19 bp internal inverted repeats (Cary et al., 1989; Fraser et al., 1996). It codifies for a single open reading frame of 1.8 kb that encodes a functional transposase, although the DNA-binding domain and catalytic core have not yet been identified. Despite it has a completely different sequence, it shares the same transposition mechanism of Tc1/*mariner* elements, apart from the target insertion site that is TTAA and the excision of the element. Indeed, the transposase action results in hairpin formations at the excised transposon ends and in 5′-TTAA overhangs creation in the flanking DNA. After excision, those segments anneal in the absence of DNA synthesis, leaving an intact excision site without any transposition footprint (Elick et al., 1996). The double strand break at the integration target site are repaired by ligation (Mitra et al., 2008).

3.3 DNA transposons as gene delivery system

In invertebrate model organisms, such *Drosophila* species or *C. elegans,* active DNA transposons of that particular species have been widely used as genetic tools (Rubin and Spradling, 1982; Rushforth et al., 1993). On the contrary, no active class II transposon was known in vertebrates until the discovery of the Tol2 transposon (*hAT* superfamily) in medaka fish in 1995 (Koga et al., 1996). Considering the potential applications of DNA transposons as molecular tools in vertebrate genetics, Ivics and colleagues created an active transposon, named *Sleeping Beauty*, re-awakening a fossil transposon found in the salmonid fish genome. It belongs to the Tc1/*mariner* superfamily and its reconstruction, based on molecular phylogenetic data, has allowed to exploit DNA transposon in mammalian cells for the first time (Ivics et al., 1997). Subsequently, several other transposons, were shown to be active in mammalian cells such as *piggyBac*, isolated from the cabbage looper moth *Trichoplusia ni* (Ding et al., 2005); *Frog Prince*, reconstructed from inactive transposons of frog *Rana pipiens* (Miskey et al., 2003); *Hsmar1* reconstructed from an ancient human transposon (Miskey et al., 2007) and *Passport* a native transposon isolated from fish *Pleuronectes platessa* (Clark et al., 2009). Among them, *Sleeping Beauty* (SB) and *piggyBac* (PB) are the most extensively evaluated and the best characterized DNA transposon systems for vertebrate genome manipulation.

The simple structure of DNA transposon makes it easy to establish a controllable molecular tool, called transposon system, by physically separating the transposase gene from its target DNA sequences, the TIRs (Ivics et al., 1997). Therefore the system consists of two fundamental components: 1) a donor plasmid carrying the artificial transposon with the gene of interest, instead of transposase gene, flanked by TIRs, and 2) a helper vector that encodes for transposase. After transfection, transposase enzyme is produced and acts in trans to catalyze the excision of transposon from donor molecule and its re-integration elsewhere in the host genome, as a single copy (Fig. VI). Thanks to these features, transposon systems have been harnessed for non-viral transgenes delivery into cultured mammalian cells.

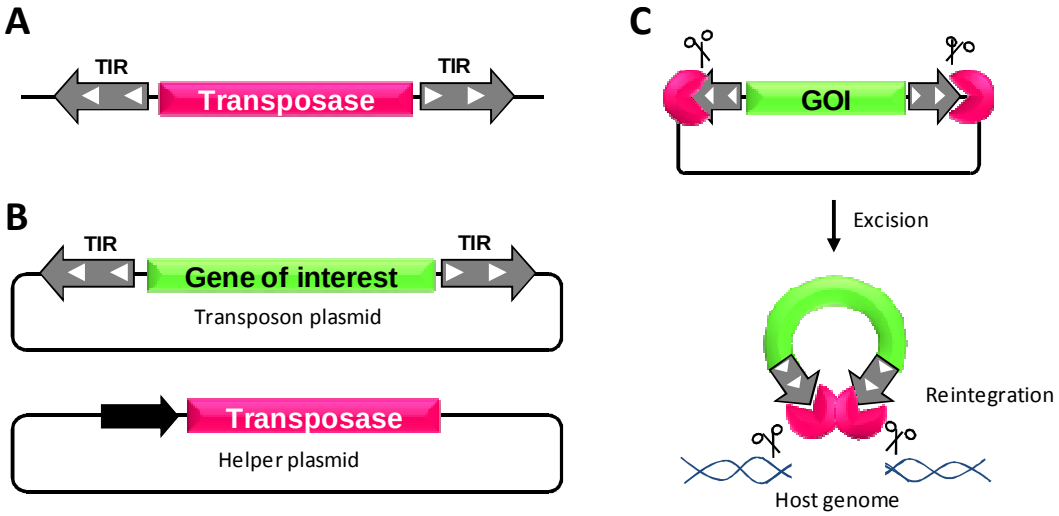


Figure VI. The transposon system. A) Native DNA transposon structure, B) Gene delivery toolbox: the transposase coding region is replaced by a gene of interest within the transposable element and inserted in a plasmid. This transposon is non-autonomous and can be mobilized in trans by transposase if it is supplied on a separate plasmid (helper). C) Co-transfection of these two plasmid into cells provides the platform for transposition into host genome . Adapted from Ivics and Izsvak 2010.

3.3.1 Parameter to consider in the use of DNA transposon

As transposon numbers are growing, some parameters should be considered in choosing the correct type of transposon to be used in relation to the final application. The basic criteria for their applicability in any given model organism are 1) a sufficient level of transpositional activity in the given species, and 2) a lack of endogenous copies in the targeted genome, in order to avoid mobilization of resident copies. Moreover, this choice is also influenced by specific features of each transposon system, such as transposition efficiency, stability, cargo capacity, integration site preference and transposition to linked chromosomal sites (“local hopping”).

Trasposition efficiency

Efficiency of transposition is perceived as a bottleneck to efficient gene delivery. Therefore, during years, several hyperactive versions of the SB and PB transposon systems were created by successive mutations in TIRs and in the transposase coding sequence. These attempts have led to the generation of SB100X, a more robust SB transposase than the originally resurrected or previously described hyperactive SB versions (Mátés et al., 2009), and to hyPBase transposase that has been shown to have 2 to 3 fold more activity in human cell lines than SB100X or native codon-optimized PB (Doherty et al., 2012; Yusa et al., 2011).

Another critical issue for the transposition process is the amount of transposase expressed in cells , since efficiency of transposition decreases in the presence of excess of transposase, a phenomenon called “overproduction inhibition” (OPI). The mechanism of OPI is not clearly understood, but it has been suggested that it acts on the post-translational level. Given that transposase works in multimeric complexes, the high number of available transposase molecules can shift the equilibrium towards less active oligomers (Lohe and Hartl, 1996). This phenomenon has been described for several class of TE, although *piggyBac* appears to be generally less sensitive to OPI (Izsvák et al., 2000). Indeed it was reported that molar PB transposon to transposase ratio as high as 1:43 did not induce OPI in HEK-293 cells, in contrast to a similar ratio for SB (Wilson et al., 2007).
Therefore, in the molecular tool, the number of transposition events can be regulated by modulating the concentration of donor and helper plasmids, i.e. transposon and transposase.

Stability of integration

Any transgene introduced into the host genome is a potential target for the positional effect that causes transgene silencing. This phenomenon is observed particularly in viral vectors but also appears in random integrations of plasmid, especially when they occur as a tandem array. In addition, the vector backbone, co-integrated into the host genome with the transgene, contributes to this silencing. Since transposons are natural genetic elements existing in the mammalian genome, they may be less prone to be silenced. Furthermore, a transposon can insert the transgene flanked by TIRs into the host genome without useless plasmid backbone. Recently it was demonstrated that transgene silencing occurred only in 1,7-3,8% of clones generated by SB100X and PB transposition, a very low level compared with 26,5% of clones derived from standard random integration (Grabundzija et al., 2010).

Cargo Capacity

Some applications, such as genomic integration of extensive genes or complex constructs, can require mobilization of large DNA fragments. The efficiency of transposition of transposon vectors can vary depending on the size of the gene to be transferred and to the type of TE used. Tc1/*mariner* elements are markedly affected by this factor, since transposon insertion efficiency decreases as the cargo size increases. In a mammalian cell culture, additional 1 kb over the original 1.7 kb *Sleeping Beauty* transposon results in 30% reduction of transposition efficiency (Izsvák et al., 2000). In contrast, *piggyBac* transposons are more tolerant in their cargo capacity. *piggyBac* transposon system can deliver up to 9.1 kb of foreign DNA into the host genome without significantly reduction in transposition activity, whereas sequences up to 14 kb are transposed with a considerable decreased frequency (Ding et al., 2005). Despite of these considerations, the *piggyBac* system has been successfully used for mobilizing transposons as large as 200 kb both *in vitro* and *in vivo* (Li et al., 2011, 2013). To overcome the cargo limitation in the SB system, it was developed a "sandwich SB vector", which consists of two complete SB elements flanking the transgene of interest. In this configuration, the number of SB binding sites is increased, ameliorating the transposition efficiency for transgenes longer than 10 kb (Zayed et al., 2004). Although SB was improved in this aspect, piggyBac is recognized more suitable than SB for delivery of large DNA molecules.

Integration site preference

Most transposable elements do not integrate randomly into target DNA and display some degree of specificity in target site utilization.

As regards the primary DNA sequence, all Tc1/*mariner* transposons, including *SB,* integrate into TA dinucleotides, while *piggyBac* transposon targets the sequence TTAA. However, computational analyses on integration site preferences of SB revealed that target selection is not only determined by primary sequence but also by secondary DNA structure (Geurts et al., 2006). On the contrary *piggyBac* integration sites lack such consistent, clear-cut structural patterns (Hackett et al., 2007), even though it targets only the TTAA tetranucleotide in a particular sequence context. Indeed, at least the first 100 nucleotides on either side of piggyBac target sites seem to be important, since subtle change in the primary sequence within this 200 bp interval may result in loss of potential for piggyBac targeting (Meir et al., 2011).

In the context of chromatin, SB prefers heterochromatin instead of transcription units and 5' regulatory regions, and when it integrates into genes most of the hits are localized within intronic sequences, since they are the largest components of transcription units (Yant et al., 2005). On the other hand, *piggyBac* targets preferentially transcription units, with insertions primarily localized into introns and upstream of regulatory sequences (Galvan et al., 2009). In addition, *piggyBac* exhibits striking preference in targeting to the CpG island (Meir et al., 2011).

In light of these considerations, the preferred site of integration is an important feature to consider when choosing a transposon system for a given application. Thus, integration into genes is desirable for mutagenesis screening and functional genomic studies, whereas it should be avoided for human gene therapy.

Local Hopping

In addition to the primary target site sequence, the genomic distance from the transposon donor site is another important feature. “Cut and paste” DNA transposons, already integrated, tend to reintegrate to target sites that are physically close to the donor locus, a phenomenon called “local hopping”. Although it is a shared features, each transposon has a specific chromosomal window that varies depending on host species and genomic location of the donor site. Local hopping was first described for *Sleeping Beauty* thanks to observation of co-segregation of new transposon insertions with their donor sites. The total transposition interval of local hopping is between 5 and 15 Mb (Fischer et al., 2001; Muñoz-López and García-Pérez, 2010). Interestingly, the *piggyBac* transposon seems to exhibit no evidence of local hopping (Liang et al., 2009), suggesting a different way of target site selection compared to the majority of “cut and paste” TEs.

This phenomenon could be exploited in mutagenesis to produce insertions in a limited chromosomal region.

3.3.2 *piggyBac* transposon system for recombinant protein production

DNA transposon systems represent an important alternative to viruses to enhance gene delivery in mammalian cells, and they have several advantageous properties that make them very promising tools for a wide variety of applications. In last 15 years, transposon system evaluation has been especially focused on human gene therapy, but recently their application in recombinant protein production has also been investigated (Matasci et al., 2011).

Recombinant proteins are traditionally produced by stable mammalian cell lines generated by conventional transfection methods that relay on random and spontaneous transgene integrations into host genome. This process often results in low level of protein production due to insertion in no transcribed regions that causes the previously described "positional effect" (Henikoff, 1992). To overcome this shortcoming, epigenetic regulatory elements could be added to transposon sequence in order to enhance their expression (Wang et al., 2012). Moreover, the integrated transgenes are often inserted as concatamers generated by DNA repair and recombination enzymes, thus inducing transgene silencing and instability (McBurney et al., 2002). A powerful solution could be viral transgene delivery, but it is not yet supported by biotech industries due to risks in viral particles handling, difficulties in their preparations and also regulatory concerns. Therefore, transposon systems are a very attractive alternative, considering that many copies of the transgene of interest could be actively integrated as single copy into the genome of the cell. These integrations are spread into the genome, in contrast to random integration of plasmids that usually occurs in the same genomic locus (Girod et al., 2007). Moreover, the transposase integrates the transgene of interest without unnecessary DNA plasmid backbone, thus reducing transgene silencing.

For recombinant protein expression, *piggyBac* is more suitable compared to *Sleeping Beauty*. *PiggyBac* specifically targets transposition into active areas of the mammalian genome resulting in greater levels of protein expression. On the contrary, SB transposon prefers integrations within heterochromatin and, as a consequence, has the disadvantage of producing low levels of transgene expression (Muñoz-López and García-Pérez, 2010). In addition, as previously described, *piggyBac* has large cargo capacity and is able to accommodate up to 14 kb of DNA without compromising transposition efficiency. Finally, since overproduction inhibition has not been reported using *piggyBac* system, the number of transposition events can be regulated by balancing the molar ratio of donor to helper

plasmids. In general, the PB system integrates up to 10 copy per cell line (Ding et al., 2005; Grabundzija et al., 2010).

Exploiting this method, it is possible to achieve high levels of stable protein production, as successfully reported by Matasci and colleagues (Matasci et al., 2011).

3.3.3 Other transposons applications

Transposon-mediated forward genetic approaches

Genome-wide, forward insertional mutagenesis provides a powerful and high-throughput means to investigate gene function. Insertional mutagenesis using engineered transposable elements can be one of the most productive and versatile approaches to disrupt and manipulate genes, followed by easy identification of the mutated gene through sequencing of DNA-junction fragments between transposon-known sequence and surrounding genome.

Transposon-based insertional mutagenesis requires integration of transposable element into transcriptional units in order to promote their disruption or significantly alter their expression. However, even if a transposable element inserts into a gene, it may not have a mutagenic effect. For example, intronic insertions are likely spliced out without having an effect on gene expression. Thus, various transposon-based constructs have been established to enhance the mutagenicity as well as 'trap' transcription units (Ivics and Izsvák, 2010). These transposons typically contain a reporter gene, whose expression is dependent on the genetic context of the integration. As an example, a conventional "gene-trap" vector consists of a promoterless reporter gene flanked by an upstream splice acceptor (SA) and a downstream poly(A) signal. Reporter activation occurs if the vector is integrated into an expressed gene, and splicing between the reporter and an upstream exon takes place, hence providing a promoter (Ivics and Izsvák, 2010). This system offers the possibility to visualize spatial and temporal expression pattern of the mutated genes by using LacZ or fluorescent proteins as a reporter gene.

Cancer gene discovery

Since the discovery that some types of tumors can be induced by insertional mutations due to retrovirus integrations into the host cell genome (Furth, 1934), the possibility to use inserted virus as sequence tag to identify oncogenic mutations has been estabilished, and

has led significant contributions especially for blood and mammary cancers (Kool and Berns, 2009). Through an approach similar to that of retroviruses, transposons can be engineered as an “oncogene trap” vector to perform cancer gene screens in different somatic cells (Ivics and Izsvák, 2010). In 2005, several publications described the generation and application of an SB “oncogene trap” transposon called T2/Onc. This transposon contained splice acceptors in both orientations followed by a polyadenylation signals, as well as strong viral enhancers/promoters that could drive transcription outwards from the vector (Collier and Largaespada, 2007). Thereby, this mutagenic transposon was able to induce loss-of-function mutations in tumor suppressor genes as well as promote gain-of-function over-expression of proto-oncogenes near the genomic insertion sites. *Sleeping Beauty* and *piggyBac* vectors harboring oncogene traps have been successfully used in large-scale cancer gene discovery screens in experimental animals (Dupuy et al., 2009; Rad et al., 2014). Thus, this approach can be successfully employed not only to identify novel cancer genes, but also combinations of cancer genes that act together to transform a cell.

Transgenesis

Classical methods to express foreign genes in vertebrates rely on microinjection of nucleic acids into oocytes or fertilized eggs. Two main drawbacks of these approaches are the low rates of genomic integration, and that the injected DNA generally integrates as a concatemer. Both drawbacks can be circumvented by transposition-mediated gene delivery because it can increase the efficiency of chromosomal integration and facilitates single-copy insertion events that are presumably less prone to transgene silencing than concatamers. In case of transgenesis, insertions away from endogenous genes are clearly desirable, for this reason, transposable elements that do not show pronounced bias for integration into genes are more suitable for this type of applications, such as *Sleeping Beauty*. Another particular problem concerning transgenesis is that founders that develop from the injected oocytes or eggs are predominantly mosaic for the transgene, because integration generally occurs relatively late during embryonic development. Therefore, in order to potentiate successful transmission of the transgene through the germline to the next generation, it is necessary to shift the window of integration events as early as possible. The injection of in vitro synthesized mRNA as a transposase source can further enhance the efficiency of this technique thanks to the more rapid availability of the transposase, resulting in reduced transgene mosaicism in the embryo.

Transposons have been used for efficient germline transgenesis in zebrafish, Xenopus, chicken, mouse and rat (Dupuy et al., 2009; Koga et al., 1996; Sato et al., 2007; Sinzelle et al., 2006).

Gene Therapy

Application of transposon-mediated gene transfer to gene therapy has been explored because it avoids several disadvantages of viral delivery systems. These disadvantages include: (1) viral preference for integrating into genes or promoter regions with the risk of altering the expression of the hit or surrounding genes; (2) the difficulty and expense associated with viral production in large quantities; (3) the difficulty with purification to eliminate toxic or infectious agents; (4) viral potential to elicit unwanted immune or inflammatory responses (5) the constraint on therapeutic cargo size (Ivics and Izsvák, 2010).
In contrast, preparations of non-viral plasmid-based transposons can be assembled in cell-free systems from well-defined components, resulting relatively inexpensive and largely non-immunogenic. Moreover, transposons have less cargo constrains compared to viral vectors and they can accommodate fairly large transgenes (10-14 Kb). On the other hand, one negative aspect with DNA vectors is the less efficient delivery into the cells of interest.
One of the first demonstration that transposons could be used to correct patient's cells was obtained by transfecting keratinocytes from patients with junctional epidermolysis bullosa with a transposon encoding laminin along with a selectable marker gene (Ortiz-Urda et al., 2003). The corrected cells were selected and able to regenerate human skin on immunodeficient mice. Furthermore, it has been published that SB transposons can efficiently (up to 40%) and stably transfer genes in hematopietic stem cells (HSC) or progenitors opening the possibility to achieve gene corrections in this cell type without use of viral vectors (Baus et al., 2005).
Direct *in vivo* gene delivery of transposon/transposase constructs has been achieved by polyethylenimine (PEI) in lungs and in brain tumors, to promote the expression of nitric oxide synthase and antiangiogenic factors respectively (Liu et al., 2006a; Ohlfest et al., 2005), or by hydrodynamic injection to transfect hepatocytes in mouse models (Bell et al., 2007). Nowadays successes in gene therapy have been limited, but transposons still seem overwhelmingly promising for the future.

CHAPTER 4: Model proteins

In this thesis the PiggyBac transposon toolbox has been applied for the generation of stable CHO cell lines for recombinant protein production. In order to assess the potentiality of this gene delivery system, two different model proteins have been chosen: fibroblast growth factor 23 (FGF23) and a mouse recombinant antibody. The former has been selected as an example of complex protein because previously attempts to produce it in other heterologous systems had failed. The latter has been chosen to prove the feasibility of chimeric immunoglobulin production using transposon system and to investigate the efficiency of transgenes co-transfection.

4.1 FGF23: a difficult protein to express in heterologous system

Fibroblast growth factor 23 (FGF23) is a recent discovered regulator of phosphate homeostasis and vitamin D metabolism and it is involved in the pathogenesis of several human diseases, becoming a novel emerging biomarker in the diagnostic field of bone metabolism disorder.

Human FGF23 has been expressed in *E.coli* both in inclusion body or as a fusion protein (Plotnikov et al., 2000; Liu et al., 2012). As stated before, this heterologous system lacks the ability to perform post translational modifications and proper disulfide bonds, thus the protein resulted almost completely insoluble, not correctly folded or inactive. Therefore, in order to obtain a soluble and biologically active protein, Shimada and colleagues (2001) have stably expressed hFGF23 in CHO mammalian cells with success. However, poor information about the recombinant protein production process are available.

4.1.1 hFGF23 gene and protein features

FGF23 is the most recently discovered member of fibroblast growth factor (FGFs) family, which consists of 22 molecules with different function. Together with FGF19 and FGF21, FGF23 belongs to the FGF19-subfamily of endocrine FGFs, distinguished from other non-circulating and paracrine FGFs-family members (Itoh and Ornitz, 2011).

Fgf23 gene was first identified in 2000 by homology to *Fgf15* in mouse embryos (ortholog of the human *FGF19*) and human *FGF23* gene was also identified, in the same year, by positional cloning as the gene responsible for autosomal dominant hypophosphatemic

rickets (ADHR) (Yamashita et al., 2000; ADHR Consortium, 2000). The *FGF23* gene is located on chromosome 12p13 and composed of three exons encoding a 251 amino acid glycoprotein (ADHR Consortium, 2000). The first 24 amino acids act as a signal peptide suggesting that the mature form of FGF23 is a secreted protein of 227 amino acid working as an hormone. It has a 32 kDa molecular weight that is evidently larger than the calculated one (MW 25331.05) because it undergoes several O-glycosylations. Those modifications occur in the 162-228 region and are mediated by polypeptide N-acetylgalactosaminyl transferase 3 (GALNT3) (Shimada et al., 2002). In particular, to be transported from the Golgi apparatus through the cytoplasm and finally secreted, FGF23 requires O-linked glycosylation of the threonine residue at position 178 (Kato et al., 2006). For inactivation, the full-length protein is cleaved immediately after the consensus sequence ^{176}RXXR^{179} by an unknown subtilisin-like pro-protein convertase into 18 kDa N-terminal and 12 kDa C-terminal fragments (Benet-Pagès et al., 2004). The O-glycosylations at Thr^{178} has been also shown to protect FGF23 from the pre-term proteolytic cleavage (Kato et al., 2006).

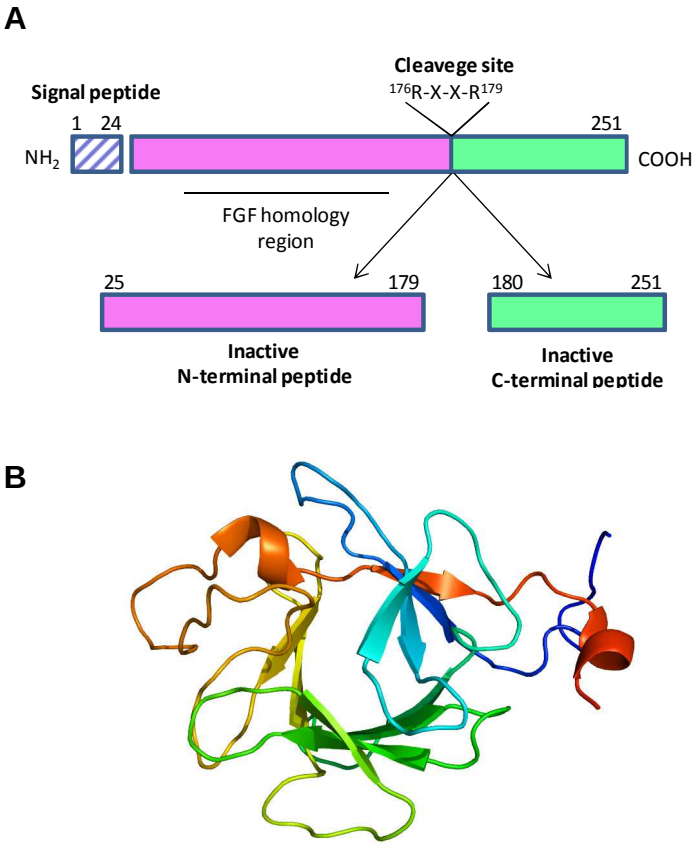


Figure VII. hFGF23 protein. A) Representation of FGF23 protein. *FGF23* gene produces a polypeptide with 251 amino acids. FGF23 protein has a signal peptide (24 amino acids) that is cleaved off to produce the biologically active FGF23 hormone. A FGFs family homology region is present in its N-terminal portion. Proteolytic cleavage occurs at the end of the RXXR motif between R179 and S180 and produces the biologically inactive N- and C-terminal fragments. B) Structure of FGF23 protein. It has a β-trefoil structure and a unique C-terminal portion.

FGF23 shares with other FGFs a core homology region of about 120 amino acids in its N-terminal portion, consisting of twelve antiparallel β-strands arranged into a β-trefoil structure. Unlike paracrine FGFs, endocrine FGFs contain a disulfide bond in the core region that is probably required to stabilize the structure for its transport in the blood stream (Harmer et al., 2004). Additionally, it has a unique and longer C-terminal sequence compared with the other members of FGF family. This structural differences might be important to exert FGF23 specific functions (Harmer et al., 2004). For example, the endocrine FGFs affinity to glycosaminoglycans (GAGs), such as heparin, is low compared to that of the canonical FGFs. This enables them to avoid capture in the extracellular matrices, thus allowing their circulation in the blood stream as endocrine molecules (Goetz et al., 2012).

4.1.2 Physiology

4.1.2.1 FGF23 signaling

The physiological effects of FGFs are mediated by FGF receptors (FGFRs), which are tyrosine kinase receptor encoded by four distinct genes (Itoh and Ornitz, 2011). Paracrine FGFs entail the presence of heparin sulfate GAGs to allow signal transduction, whereas the FGF19 subfamily has a reduced affinity for FGFR due to their weak heparin-binding ability (Mohammadi et al., 2005). FGFRs are ubiquitously expressed, but the target organs of FGF23 are limited to the kidney and parathyroid (Ben-Dov et al., 2007). These specificity is due to αKlotho, a protein that acts as cofactor of the FGFR, instead of heparin (Urakawa et al., 2006). αKlotho protein is a single-pass transmembrane glucosidase, mostly expressed in the renal proximal tubules, parathyroid gland and choroid plexus of the brain (Matsumura et al., 1998). αKlotho forms a complex with multiple FGFR that binds FGF23 with a higher affinity than FGFR or αKlotho alone (Kurosu et al., 2006). It has been also reported that this complex is stabilized by heparin, thus enhancing the activity of FGF23 (Urakawa et al., 2006; Wu et al., 2007). The binding of FGF23 to its receptor leads to a downstream cascade that includes phosphorylation of ERKs (extracellular signal-regulated kinases) belonging to MAPK signaling pathway, and consequent induction of early growth response genes like *Egr-1* (early growth response 1) (Urakawa et al., 2006). Only the full-length FGF23 activates this signal, since the FGFR binding domain resides within the FGF23 N-terminus while the αKlotho binding region is in the specific C-terminus. FGF23 proteolytic cleavage disrupts the binding site and the C-terminal peptide alone exerts also a direct inhibitory effect on this

complex (Goetz et al., 2010). However, the physiological relevance of the FGF23 fragments is still unknown.

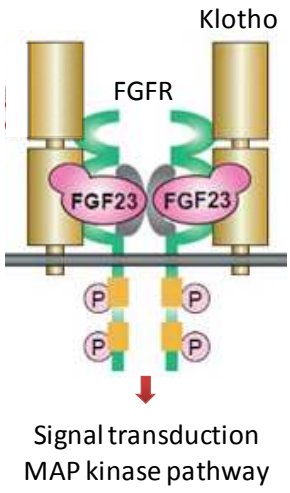


Figure VIII. FGF23 signaling: FGF23, in presence of Klotho, can bind to FGF receptors with high affinity to trigger downstream signaling, especially mitogen activated protein (MAP) kinase pathway. This induces the expression of genes regulating cellular homeostasis and mineral ion homeostasis. In gray is indicated heparin, which stabilized the Klotho-FGF23-FGFR complex (Urakawa et al., 2006; Fukumoto, 2012)

4.1.2.2 FGF23 function and regulation

FGF23 is produced by both osteoblasts and osteocytes in the bone and it is secreted in the blood stream, where it works like a hormone (Liu and Quarles, 2007). It is involved in mineral metabolism where it carries out three key functions.

(1) FGF23 acts mainly as a phosphaturic factor in the kidney. It inhibits the expression and endocytosis of type IIa and IIc sodium-phosphate cotransporters (NaPi2a and 2c) on the apical membrane of proximal tubular cells, thus leading to an inhibition of phosphate reabsorption (Saito et al., 2003).

Moreover, (2) FGF23 inhibits 1-α-hydroxylase activity, an enzyme that converts 25-hydroxyvitamin D (25(OH)D) to its active metabolite 1,25-dihydroxyvitamin D (1,25(OH)2D). It also stimulates the activity of 24-hydroxylase enzyme which degrades both forms of vitamin D (calcitriol). These actions result in a decreased calcitriol serum levels (Shimada et al., 2004).

In addition, (3) FGF23 has been also recently described as a regulating factor of parathyroid hormone (PTH) in the parathyroid (Ben-Dov et al., 2007). Whether FGF23 increases or decreases PTH remains a matter of controversy. Nevertheless, recent findings have reported that FGF23 decreases PTH expression and secretion (Krajisnik et al., 2007). It also increases 1α-hyroxylase mRNA levels, in discrepancy with the negative effects of FGF23 on

the renal expression of this enzyme (Krajisnik et al., 2007). The general physiological effect of FGF23 is the reduction of phosphate absorption from the intestine and the increase in phosphate excretion by the kidney.

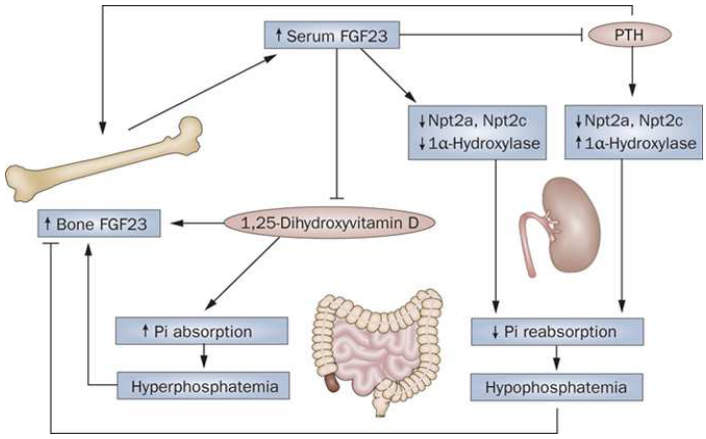


Figure IX. FGF23 function and regulation: FGF23 is produced in bone and secreted into the blood stream, potentially in response to increased phosphate, 1,25(OH)2D, and PTH. FGF23 acts in the kidney to decrease Npt2a and Npt2c expression and also 1,25(OH)2D production, resulting in hypophosphatemia. Potentially, in a novel feedback loop FGF23 may reduce PTH mRNA and protein. (Farrow and White, 2010)

Regulation of FGF23 is managed by several factors and it is still poorly understood. All key components of mineral metabolism, such as phosphate, vitamin D and PTH, stimulate the expression of FGF23. Calcitriol stimulates FGF23 transcription through a vitamin D responsive element (VDRE) into the *FGF23* promoter (Liu et al., 2006b). Moreover, inhibition of vitamin D receptors signaling disrupts FGF23 secretion from osteoblasts (Masuyama et al., 2006). As a result, FGF23 and calcitriol are part of a classical endocrine bone-kidney feedback loop, where FGF23 production in bone is stimulated by calcitriol levels and vitamin D production in kidney is suppressed by FGF23.

Dietary phosphate has been shown to regulate FGF23 in mice, although the effects in healthy individuals are less robust (Nishida et al., 2006). Furthermore, intestine appears to play a part in this regulation because FGF23 levels are unchanged by non-dietary interventions (Ito et al., 2007). Thus, it is still unclear whether phosphate has a direct regulation or not.

In addition, also PTH regulation of FGF23 is still controversial since it is context dependent. However, the most substantial theory is that PTH stimulates FGF23 expression in bone through direct and indirect mechanisms (López et al., 2011).

Recently, also iron has been implicated in FGF23 expression alterations in humans, since its infusion leads to an increase in serum FGF23 levels (Imel et al., 2011). A final remark is that

numerous other factors that modulate bone metabolism have been shown to regulate FGF23 expression, such as leptin, estrogen and glucocorticoid (Tsuji et al., 2010).

4.1.3 FGF23 a novel biomarker for human metabolic bone disorders

Abnormal circulatory levels of FGF23 can impair regulation of phosphate homeostasis, causing several metabolic bone diseases.

Elevated FGF23 results in several hereditary and acquired hypophosphatemic disorders. As stated before, FGF23 was originally identified as the causative factor of autosomal dominant hypophosphatemic rickets (ADHR), a rare phosphate wasting disorder characterized by reduced bone mineralization (ADHR Consortium, 2000). Three possible missense mutations at 176Arg or 179Arg were found in patients with ADHR. Their presence destroys RXXR motif, thus protecting FGF23 from the proteolytical cleavage and resulting in accumulation of FGF23 in serum (ADHR Consortium, 2000).

Almost simultaneously, FGF23 was shown to be over expressed in patients with tumor-induced osteomalacia (TIO), which causes an acquired form of hypophosphatemia (Shimada et al., 2004). Moreover, FGF23 levels are abnormally elevated also in X-linked hypophosphatemia (XLH) and autosomal recessive hypophosphatemic rickets (ARHR). Those disorders are caused by loss-of-function mutations in FGF23-regulating genes *Phex* and *Dmp1* (or *Enpp1*) respectively, though the molecular mechanisms behind these diseases are still unknown (Bhattacharyya et al., 2012). On the contrary, alterations that cause low levels of bioactive circulating FGF23 result in familial hyperphosphatemic tumoral calcinosis, a syndrome characterized by hyperphosphatemia and deposition of calcium-phosphate crystals in the soft tissues. The causative factors of this disease are biallelic inactivating mutations in genes encoding for FGF23, Klotho and GALNT3 (Benet-Pagès et al., 2005; Ichikawa et al., 2007; Topaz et al., 2004).

As described above, direct alterations of FGF23 action are the primary causes of all these disease. Conversely, in chronic kidney disease (CDK) FGF23 levels change as a consequence of the disorder. CDK is a progressive loss of kidney function over time, typically due to hypertension, diabete, polycystic kidney disease and various inflammatory and systemic disorders. As renal function declines, a gradual imbalance in mineral homeostasis occurs. FGF23 levels have been shown to be high in patients with CKD (Larsson et al., 2003), and their increase precedes the raising of PTH or phosphate and the decrease in calcitriol

(Isakova et al., 2011a). Early FGF23 increase, indeed, is an attempt to compensate for the reduced viability of nephrons in order to maintain serum calcium and phosphate within normal ranges. Together with PTH, FGF23 postpones the onset of hyperphosphatemia (Gutierrez et al., 2005). However, as the disease worsen, this adaptive response is insufficient to counterbalance the loss of glomerular filtration rate and phosphate retention, resulting in hypocalcemia and, at the end stage, renal disease (ESRD) (Wolf, 2012).

Recent publications have highlighted the great potential for use of FGF23 as a early biomarker in CDK since it could accelerate the elucidation of the complex mineral homeostatic disorders, hence directing more effective therapies that could significantly retard progression to the end stage of renal failure. During the early and intermediate stages of CKD, FGF23 levels are often 2–5 fold above normal one (10-50 pg/mL) and can reach more than 200 times the normal level in cases of ESRD (Jimbo and Shimosawa, 2014). Numerous epidemiological studies have reported a robust association between higher plasma FGF23 concentrations and poor CDK patient outcome (Isakova et al., 2011b). Moreover high levels of FGF23 have been associated with increased cardiovascular risk and mortality in dialysis patients (Jimbo and Shimosawa, 2014). Currently, commercial kits for FGF23 measurement in plasma or serum consist of sandwich ELISA to detect either intact FGF23 (iFGF23) or C-terminal portion. This latter assay can detect also intact FGF23 because binds two distinct epitopes within the C-terminal region. However, these tests showed calibration and consistency issues (Smith et al., 2013).

4.2 Recombinant mouse IgG: a model for engineered antibody

Antibodies are one of the most important molecules in mammals due to their essential function in defence from pathogens and other foreign substances. The high specificity of these molecules for their target has highlighted their potential as laboratory and therapeutic tools since 1900s, when Paul Ehrlich proposed them as “magic bullets”, able to find their targets by themselves (Strebhardt and Ullrich, 2008).

4.2.1 Antibody structure and function

Antibodies, also known as immunoglobulins, are glycoproteins produced by plasma cells as part of the immune response against foreign antigens. Typically, an antibody molecule is a heterotetramer that consists of two identical light (L) chains, polypeptides of about 25 kDa and two identical heavy (H) chains, larger polypeptides of 50 kDa or more. A disulfide bond connects a cysteine at the C-terminus of each light chain to a cysteine of a heavy chain. The two heavy chains are associated through several disulfide bonds in the hinge region and non covalent interactions, depending on the immunoglobulin subtype (Edelman et al., 1969). In both chains, the N-terminal region of about 110 amino acid discloses great sequence diversity among different antibodies. For this reason, they are called variable regions VH and VL for heavy and light chain, respectively. The remaining parts of each chain are referred to as constant regions (CH or CL) and the limited differences in their amino acid sequences define the immunoglobulin classes. Both heavy and light chains are folded in compact globular domains typical of the immunoglobulin superfamily. Each domain consists of about 110 amino acid residues and an interchain disulphide bond that fold into two β-pleated sheets with a sandwich-like structure. Thanks to their hydrophobic interior, these domains facilitate the antibody quaternary structure formation.

Antibodies have a three dimensional Y-shape and are responsible for two different biological activities: (1) to bind an antigen epitope and (2) to trigger immune response. The former function is carried out by a binding site present in each arm of the Y molecule, called antigen binding fragment (Fab). In contrast, the biological functions such as activation of natural killer cells, complement pathway and opsonization are provided by the Fc fragment (Fragment, crystallizable) (Fig. X) (Lipman et al., 2005).

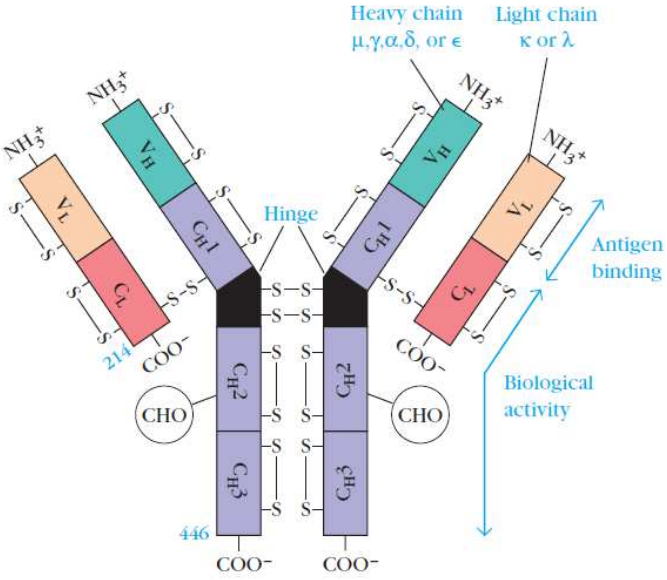


Figure X. Schematic diagram of antibody molecule: the general structure consist of two light chains (LC) and two heavy chains (HC). Each LC consists of a variable domain (VL) and a constant domain (CL1), and each HC consists of one variable domain (VH) and three constant domains (CH1-3). The Ig structure is stabilized by disulfide bonds (indicated as S-S) connecting LC and HC and also linking HC to each other. There are disulfide bonds inside each domain. The VH and VL domains bind to specific targets, while the biological functions reside in the CH and CL domains (Kindt et al., 2006).

The antigen binding site resides in the variable region of H and L chains. Alignment of the amino acid sequence of a large number of these domains disclosed that main sequence variations occur in three short portions, named complementarity determining regions (CDRs) (Wu and Kabat, 1970). These hypervariable regions correspond to the loops between βstrand of VH or VL domains that, in the quaternary structure, form a pocket complementary to the epitope structure. The infinite combinations of amino acid residues and also different sequence lengths of the six CDRs in each Fab fragment are at the origin of the wide range of antibody specificities. The CDRs are separated by four conserved sequences, known as framework regions (FR) that result in the β-sheet structural folds. Although they have a limited number of main chain conformations, recently, researchers have demonstrated that also alterations in the FR backbone influence the antigenic specificity of antibodies (Chothia and Lesk, 1987; Honegger and Plückthun, 2001).

As mention before, the heavy chain type defines the immunoglobulin class. In human there are 5 heavy chains α, γ, μ, δ, or ε and the respective immunoglobulin isotypes are IgA, IgG, IgM, IgD or IgE. For light chains there are only two types of constant regions, kappa (κ) and lambda (λ), but each immunoglobulin class can have either. The unique amino acid sequence in the CH chain bestows structural and functional properties that differ between

classes. IgG is the predominant antibody isotype, about 80% of the total serum immunoglobulins. There are four IgG subclasses (IgG1, IgG2, IgG3 and IgG4) that diverge for number of disulfide bonds and length of the hinge region. The main role of this class is the complement activation, which eliminates foreign elements. IgM class is the first Ig secreted by B cells after primary antigen stimulation, it is a pentamer and is the third most common serum Ig. Due to their pentameric nature, IgM possess high valency and are more effective at complement activation than other Ig classes. IgA is the second most common serum Ig, accounting for 10-15% of total, and circulates as a monomer. However it is also the major class of Ig in external secretions, where it is found as a dimer and prevents attachment of pathogens to mucosal cells. IgD and IgE are found in low levels in serum. IgD role is uncertain, while IgE are involved in the hypersensitivity or allergic reactions, binding very tightly to Fc receptors on basophils and tissue mast cells.

4.2.2 Organization and expression of immunoglobulin genes

The capacity of immune system to respond to every antigen that could be encountered is guaranteed by a highly controlled gene segment recombination, a process that generates antibody diversity during B-cell development. Without this process, the genome should have been incredibly larger in order to accommodate this huge amount of genomic information. The phenomenon of this DNA rearrangement was first discovered by Tonegawa in 1976 (Hozumi and Tonegawa, 1976). The light and heavy chains are encoded by multigene structures situated on different chromosomes. The heavy chain gene family contains four different types of segments named V (variable), D (diversity), J (joining) and C (constant), whereas the light chain family contains only three types: V, J and C (Early et al., 1980). There are some differences in the number of gene segments of each type between species, but their number is very high. For example, human V_H regions contain 51 different V segments, 27 D segments and 6 J segments (Kindt et al., 2006). During B cell development the gene rearrangements of variable region take place, firstly for the heavy chain, then for the light one. This process creates mature B cells committed to produce a single antibody molecule with unique binding sites. The generation of heavy chain gene requires a first join between D_H and J_H gene segments, followed by rearrangement of one of the V_H segment. This results in a $V_HD_HJ_H$ unit that encodes the entire variable region in the same genetic locus of the constant region (Fig. XI). Finally, the variable region is transcribed and spliced in

frame to a constant region (μ or δ). The same process occurs for light chain, generating a $V_\lambda J_\lambda$ or $V_k J_k$ rearrangements. If those recombinations create a non-productive rearrangement, for example out of frame joining or stop codon, it begins the rearrangement of gene segments on the other allele, which was previously inactive (Alt et al., 1984).

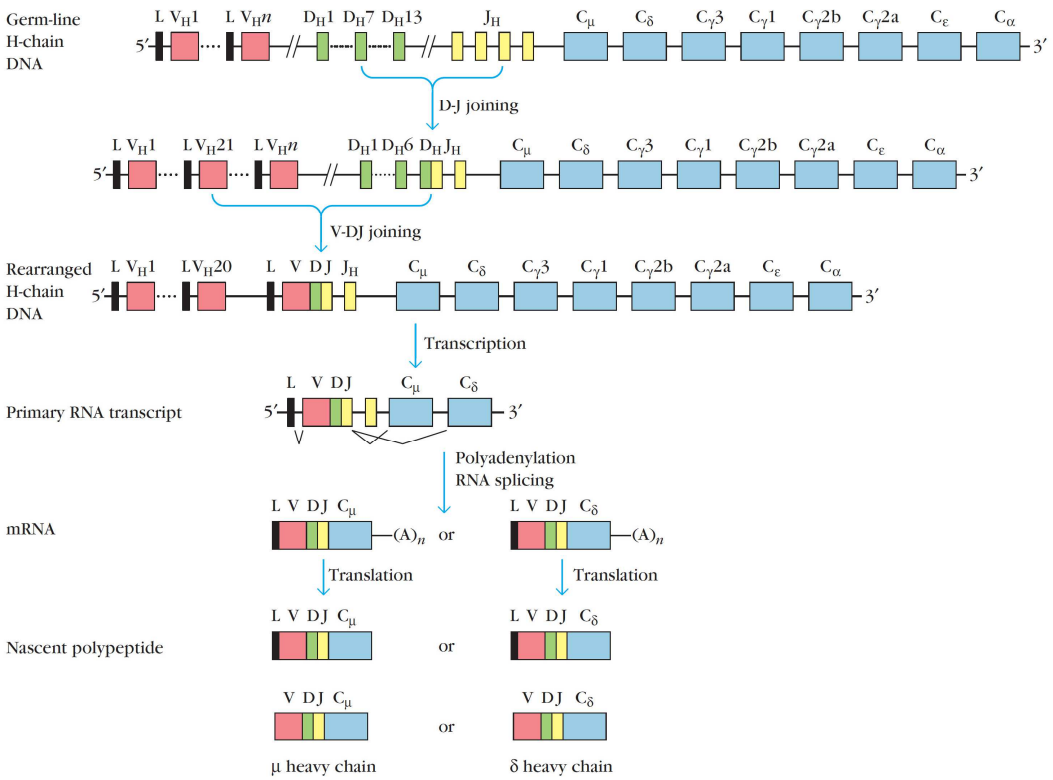


Figure XI. Heavy-chain gene rearrangement and RNA processing events: Two DNA joinings are necessary to generate a functional heavy-chain gene: a DH to JH joining and a VH to DHJH joining. In this example, VH21, DH7, and JH3 are joined together. Expression of functional heavy-chain genes involves RNA splicing with C sequences. (Kindt et al., 2006)

The incredible wide diversity in the variable region of antibodies are enhanced by other numerous mechanisms in addition to multiple germ-line gene segment recombination and combinatorial V-(D)-J joining. Junctional flexibility during the recombinase action and nucleotides addition give a major contribution. This further diversification of the antibody repertoire has been shown to fall within the CDR3, which is the most important in the antigen binding domain. Additional variability is due to affinity maturation that naturally improve antibody affinities after repeated exposure to the antigen. This process is called

somatic hypermutation and consists of mutations introduction at a high rate into V-(D)-J coding region of rearranged Ig genes (Papavasiliou and Schatz, 2002). Finally, combinatorial association of light and heavy chains lead to an enormous antibody repertoire.

Furthermore, B cells maturation induces isotype switching, a process that combines the V-D-J unit with a different constant heavy chain (γ, ε and α) in response to unknown regulatory proteins, such as interleukins (Kindt et al., 2006). As a result, immune system can cope with an unlimited number of antigens.

Once V region gene rearrangement is accomplished, heavy and light chains transcription starts and, after splicing, the mature mRNA is transported out of the nucleus and translated. The newly synthesized polypeptides enter in the endoplasmic reticulum (ER), where the chains are assembled and glycosylated. They are retained in the ER until they acquire a so-called export-competent conformation. Free heavy chains are not exported in absence of light chains (Leitzgen et al., 1997). In contrast, light chains can be secreted as free monomers or dimers. The ratio between H and L chains in the ER influences the folding and assembly into a complete immunoglobulin (Li et al., 2007). Following the formation of disulfide-linked immunoglobulin molecule, it is transported to the Golgi apparatus where glycosylation is completed and the mature glycoprotein is secreted by exocytosis (Kindt et al., 2006).

Glycosylation has a critical role for antibody functional properties (Arnold et al., 2007). All antibodies are glycosylated at conserved positions in heavy chain constant region, whereas glycosylation of variable regions is less common since it is due to variations in amino acid sequence that can lead to the introduction of N-linked glycosylation sites. A survey of cDNA sequences indicated that 9% of variable regions had a potential N-linked glycosylation site (Zhu et al., 2002). Glycosylations in the constant regions are essential for antibody effector functions and molecular stability, but antibody efficacy depends on the type of oligosaccharide attached, instead of its presence (Wright and Morrison, 1994). On the other hand, glycosylation of the variable regions has a strong impact on antigen binding properties. Indeed, as recently reported, glycosylation of asparagine residues within a CDR is implicated in changing the loop conformation, thereby modulating the antigen binding properties of the antibody (Arnold et al., 2007).

4.2.3 Antibody production

Antibodies are useful tools in research, diagnostics and therapy due to their wide ability to interact with any possible antigen. In 1900s, the only way to produce antibodies was animal immunization and subsequent polyclonal antibodies isolation from serum. These polyclonal antibodies had a high sensitivity since they recognized several different epitopes of the same antigen. However, polyclonal antisera were heterogeneous and often not so specific because only a small percentage of antibodies recognized the immunogen. Another disadvantage was the variability between different antiserum, even if they were obtained from an identical animal and immunogen. Moreover, each antiserum had a finite availability, limiting its use in long comparative experiments, clinical test and industrial applications.

4.2.3.1 Hybridoma technology

In 1975, Köhler and Milstein devised the hybridoma technology, which allowed the production of antibodies with specificity for a single antigen epitope (Köhler and Milstein, 1975). These antibodies, known as monoclonal antibodies (mAbs), are produced by a single clone of cells, called hybridoma. It is generated by the somatic cell fusion between spleen cells from an immunized mouse and myeloma cells, an immortal cell line selected for inability to produce immunoglobulins. The somatic cell fusion produces an heterogeneous mix of cells, among which are present hybrid cells. These cells possess the lymphocytes B ability to secrete an antibody directed to a single epitope, in combination with the unlimited growth capacity of myeloma cells. Myeloma cells lack also the hypoxanthine-guanine phosphoribosyltransfer enzyme (HGPRT), which is essential in the salvage purine synthesis pathway. Therefore, hybridoma cells are selected using HAT (Hypoxanthine, Aminopterin, Thymidine) containing medium. Aminopterin blocks the normal nucleic acid synthesis and only hybridomas and spleen cells can survive. As a primary cell culture, unfused spleen cells cannot survive in long term culture and hybrid cells, not derived from fusion with lymphocytes, are excluded during the screening of antigen specific hybridomas. Those clones with high sensitivity and specificity are cloned by limiting dilution to select for a single monoclonal antibody-producing hybridoma cell.

Nowadays, the hybridoma technology procedure is the same invented by Milstein and Köhler, except for the use of different myelomas and the introduction of chemically

synthetized polyethylene glycol (PEG) as a fusing agent instead of Sendai virus (Davidson and Gerald, 1976). The procedure is relatively simple and, once obtained, hybridoma cultures provide an unlimited source of identical antibodies that can be easily purified. Otherwise, the production of mAbs can be done by injecting hybridoma cells in the peritoneal cavity of mice, where they produce tumors that secrete an antibody-rich fluid called ascite.

Although, hybridoma technology is able to assure good amount of a specific mAb, it has several drawbacks. It is a long procedure since it includes mice immunization and different cloning steps to ensure monoclonality; it is based on a mammalian expression system, and it doesn't allow to optimize the antibody affinity and properties.

Nevertheless, murine monoclonal antibodies are widely used in basic research and diagnostics. On the contrary their clinical applications are limited by immunogenic reactions, usually referred as human anti mouse response (HAMA) (Shawler et al., 1985). They are also ineffective in activation of effector functions in humans. Therefore several efforts have been done to create human hybridomas, but the lack of suitable myeloma cells and the ethical issue in immunization of human subjects had prevented to obtain efficient procedures until recent years (Traggiai et al., 2004). Consequently, thanks to the advent of recombinant DNA technology, alternative strategies have been developed to generate antibodies with desired properties.

4.2.3.2 Recombinant antibodies

The first attempt to reduce immunogenic reaction to murine mAbs was the generation of antibodies, called chimeric mAbs, that retained the antigen-binding characteristics of murine mAbs but resemble the isotype properties of human antibodies (Fig. XIIA). Through recombinant DNA technology, the variable regions of a murine mAb were joined to constant domains of a human immunoglobulin and were expressed in myeloma cells (Boulianne et al., 1984). As expected, chimeric antibodies turned out to be a more successful therapeutic agents than murine mAbs and have been applied for cancer therapy. However, as 35% of these molecules was derived from mouse antibody sequences, chimeric antibodies were still immunogenic in humans (LoBuglio et al., 1989). For this reason, researchers have further improved recombinant antibodies with CDR-grafting on human framework regions. The ensuing antibody, called humanized or reshaped mAb, has a human immunoglobulin scaffold with only the CDRs of a high-affinity mouse antibody

(Jones et al., 1986). The 90-95% of the humanized antibody sequence is of human origin and carries a lower risk of triggering an immunogenic response than chimeric and murine antibodies. Nevertheless, this approach sometimes reduces the mAb affinity because, as mention before, the CDR loops, that define the antigen binding domain, have a conformation that strictly depends on nearby framework residues (Sato et al., 1994). In addition, the development of this antibody type is demanding because it is necessary to develop a murine antibody specific to the antigen before obtaining a chimeric or humanized antibody. Those procedures are time consuming and expensive, and it is also needed a careful molecular docking to ensure the same biological activities of the parental murine mAbs. Besides their human scaffolds, repeated treatments over time with humanized mAb engender an immune response.

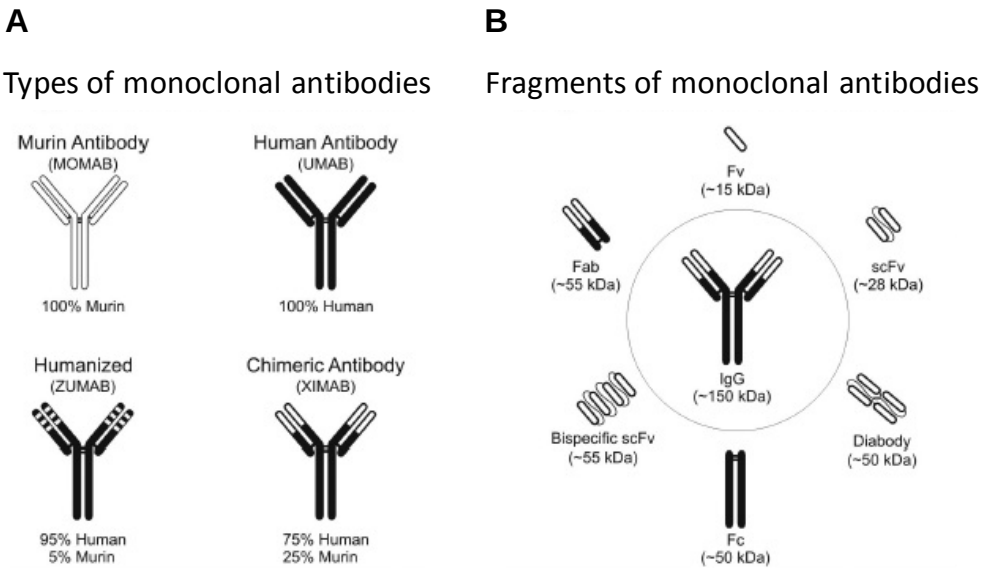


Figure XII. Recombinant monoclonal Antibodies: Schematic representation of different A) Types and B) active fragments of mAbs. Fab (fragments antigen-binding) is the Ab region that binds to antigens, Fv (variable fragment) consists of only variable region, scFv (single chain Fv) is formed by light and heavy chain variable fragments connected by a polypeptide linker, diabody and bispecific scFV consist of two identical or different scFv linked together, Fc (crystallizable fragment).

For this reason, nowadays, different technologies are used to directly generate human antibodies. Among these, the most widely exploited are phage display and transgenic mice. The phage display technology provides tools for creating and efficiently isolating high affinity recombinant mAbs. It was firstly reported by Smith in 1985 and consents to present on phage surface a selected protein or peptide by fusing the corresponding gene to the minor coat protein III gene of the filamentous bacteriophage M13 (Smith, 1985). The major

advantage of this elegant method is the direct link between phenotype and genotype in the phage particle. Naïve, immune-derived or synthetic variable V_H and V_L gene repertoire can be cloned into phage to construct large antibody fragment libraries. The antibody fragments can be displayed as single chain variable fragments (scFv), where V_H and V_L are joined by a flexible spacer or as Fab fragments, in which the association of the variable regions is stabilized by the first constant domains (Fig. XII). After library construction, phage display technology can be used to indentify in vitro high affinity antibody fragments against a variety of antigens, circumventing hybridoma technology. Phages that display antibody fragment of interest are selected by binding to the antigen in a process called panning that is repeated until a significant enrichment of antigen specific phages is achieved (Parmley and Smith, 1988). Finally, selected phages are used to infect *E. coli* in order to express soluble antibody fragments and identify individual binders. The gene sequence of the best binder is then used to generate different antibody active fragments depending on the application, such as full length IgG or scFv-Fc fusion fragment (Hairul Bahara et al., 2013).

Another approach to obtain directly human antibodies is the use of transgenic mice in which immunoglobulin genes have been replaced by human antibody genes (Lonberg et al., 1994). After immunization, these knock-out/knock-in mice have B cells that produced antigen-specific human antibodies. Thus their spleens are used to generate monoclonal antibodies with standard hybridoma technology. Even though the human heavy and light chain gene segments inserted in these mice are incomplete, these miniloci undergo normal combinatorial V(D)J rearrangement and other processes for diversity generation, such as somatic hypermutation and nucleotide addition, in order to construct high specificity and high affinity antibodies (Xu and Davis, 2000). In addition, researchers have recently managed to engineer immunoglobulin loci with an entire human immunoglobulin variable-gene repertoire (Lee et al., 2014). However, these transgenic mice are usually created by biotechnological industries, hence they aren't available to the scientific community.

4.2.4 Diagnostic applications of recombinant antibodies

During the past thirty years, monoclonal antibodies have had a central role in targeted therapy and diagnosis. The initial efforts to develop recombinant antibodies were aimed to overcome immune system response and modulate pharmacokinetics in order to increase their stability in clinical use. However, in recent years the molecular engineering of mAbs

has opened to new perspectives in designing antibodies more suitable not only for therapeutic purposes but also for in vivo and in vitro diagnostic applications.

In medicine, agriculture and food industries, monoclonal antibodies are used in vitro for the detection of a variety of analytes. Those include a completely different range of antigens such as protein, peptides, hormones, drugs and small chemicals. There are three options to use antibodies in immunodiagnostics. The first type of assay detects antigens using antibodies, while the second one, called reversed method, detects serum antibodies using antigens. The third option is the competition assay, where serum antibodies compete with a defined tracer antibody for antigens detection.

At the beginning recombinant antibodies didn't have a great impact on immunoassay technology because in many cases it is not necessary to have human antibodies, which in contrast are required in immunotherapy. However immunoassay kits for the detection of patient antibodies against infectious agents usually contain a specific human antibody as reference, in order to establish cutoff values (calibrators) and to confirm test integrity (positive controls). These antibodies typically derived from human plasma and serum samples collected from infected patients. Since their finite source, those specimens are constantly recollected and require recalibration to ensure that test parameters remain identical besides lots variability. Another disadvantage is the difficulty in finding antibody-positive serum/plasma samples for rare or exotic diseases. In addition, it is often difficult to obtain specimens with high titers of immunoglobulin subclasses different from IgG. For example, specimens with IgM are complex to collect because high level of these Ig are present only in acute phase of diseases, usually before diagnosis. Even more difficult is sourcing large volumes of IgA-rich samples, because IgA are a small percentage of total immunoglobulin in the serum/plasma. Moreover, collection of blood from individuals, sometimes children, who are sick arise ethical problems and regulatory concerns. The advent of DNA recombinant technology and the development of chimeric antibodies have allowed to overcome these serological limitations. The variable regions cloned from specific mouse hybridomas ensure the binding to the antigen under detection, while the human constant regions assure the recognition by the immunoassay tracer, an anti-human antibody. Those recombinant antibodies has been proven as successful calibrator and positive control in different immunoassays (Hackett et al., 1998; Huang et al., 2003; Thibodeaux et al., 2011).

The opportunity to engineer antibodies, especially to modulate their binding properties, has also other massive consequences on in vitro immunodiagnostic, since it allows the development of more sensitive and specific assays. Affinity maturation of antibodies has been aided by phage (or yeast) display due to its simplicity and high throughput (Schier et al., 1996; Chowdhury and Pastan, 1999). Applying this technology it is possible to generate recombinant antibodies even able to distinguish between slightly antigens differences, for example phosphorylation of amino acid residues or changes in functional group of chemical compound (Stoevesandt and Taussig, 2013; Bradbury et al., 2011). Moreover, using in vitro evolution technologies or other non-immunoglobulin molecular scaffolds it is possible to select mAbs stable in stringent conditions (Jermutus et al., 2001; Gebauer and Skerra, 2009), which are often necessary for the immunoassay success.

Aim of the work

Recombinant proteins and antibodies are the key reagents for development of diagnostic immunoassays. Recombinant proteins are commonly produced in both prokaryotic and eukaryotic microorganisms because they allow high productivity with rapidity and low costs. However, complex proteins that contain posttranslational modifications, several disulphide bonds or multiple subunits, such as antibodies, are challenging to be expressed in these hosts. Indeed, to obtain properly folded and functional complex biomolecules it is required the posttranscriptional metabolic machinery only available in mammalian cells.

To achieve high-level expression in stable cell lines, the choice of the gene delivery method and the type of expression vector are critical. Among the non viral gene delivery methods, transposon-based vectors are a new promising molecular tool.

In this thesis the PiggyBac transposon system has been evaluated and optimized for the generation of stable CHO cell lines for recombinant proteins and antibodies production. In order to assess the potentiality of this gene delivery system, two different model proteins have been considered:

a) **human fibroblast growth factor 23 (hFGF23):** hFGF23 protein has a β-trefoil structure stabilized by an intramolecular disulfide bond. Its structural complexity cannot be properly reproduced in prokaryotes, or lower eukaryotes, making it an ideal candidate for the evaluation of our mammalian cell culture system.

b) **mouse recombinant antibody:** antibodies are heterotetrameric proteins. This second protein has been chosen to prove the feasibility of producing chimeric immunoglobulin by using transposon-based vectors. The main issue of this part of the project is related to stably integrate, in one transfection step, both light and heavy chain transgenes required for proper antibody assembly and function.

Materials and Methods

Constructs

All enzymes used for plasmids construction were purchased from New England Biolabs (NEB, Beverly, Massachussets) unless indicated otherwise.

pBlueScript (LifeTechnologies, Carlsbad, CA) was an empty vector used to maintain the DNA amount constant during transfections.

hFGF23

-Standard vector

The sequence of both wild-type or mutated R179Q version of hFGF23 were synthetically obtained from GeneArt (LifeTechnologies, Carlsbad, CA). The sequences were extracted by *NheI*/*XhoI* enzymes from the pMA plasmid (GeneArt) and cloned into pcDNA3.1 plasmid (LifeTechnologies, Carlsbad, CA) digested with the same restriction enzymes, generating pcDNA3.1-hFGF23_wt and pcDNA3.1-hFGF23_R179Q.

-Transposon system

The transposon vector and the transposase PB200 plasmid were purchased from System Biosciences (Mountain View, CA). The sequence of mutated hFGF23 obtained from GeneArt were extracted by *NheI*/*EcoRI* enzymes from the pMA plasmid (GeneArt) and cloned into transposon plasmid (System Biosciences, Mountain View, CA) digested with the same restriction enzymes to generate PB_CMV_hFGF23R179Q. Then, CMV promoter was replaced with promoter 1, 2, or 3. Their sequences were synthetically obtained from GeneArt and extracted from pMA plasmid (GeneArt) by digestion with *StuI* and *NheI* restriction enzymes. Each sequence was cloned into transposon vector containing hFGF23_R179Q sequence after removal of CMV promoter by *StuI*/*NheI* digestion. We obtained PB_prom1_hFGF23R179Q, PB_prom2_hFGF23R179Q and PB_prom3_ hFGF23R179Q.

Chimeric antibody

-Standard vector

The sequence of both hTg5 variable heavy (VH) and light (VK) chains were obtained from GeneArt (LifeTechnologies, Carlsbad, CA). Briefly, hTg5VK sequence was extracted from sequence pMA_T plasmid (GeneArt) by digestion with *BstAPI* and *EcoRI* restriction enzymes and cloned in frame with murine kappa constant region into pFUSEssCLIg_mK vector

(Invivogen, Toulouse, France), previously digested with the same restriction enzymes. We obtained pFUSEssCLIg_mK_hTg5VK.

hTg5VH sequence was extracted from pMA_T plasmid (GeneArt) by *AfeI*/*EcoRI* restriction enzymes and cloned in frame with murine IgG1 constant region into pFUSEssCHIg_mG1 vector (Invivogen, Toulouse, France) digested with the same restriction enzymes. We obtained pFUSEssCHIg_mG1_hTg5VH.

-Transposon system

The coding sequence of variable and constant light chain, plus signal sequence, was recovered from pFUSEssCLIg_mK_hTg5VK by digestion with *KasI* and *NheI* restriction enzymes and its ends were blunted by Klenow enzyme. After removal of hFGF23R179Q sequence from PB_prom3 vector by digestion with *NheI*/*EcoRI* enzymes, vector ends were blunted by Klenow enzyme and dephosphorylated by calf intestinal alkaline phosphatase (CIP, Promega, Madison, WI). Then, hTg5VK_mK sequence was cloned into PB_prom3 for the generation of PB_prom3_ ssCLIg_mK_hTg5VK.

The coding sequence of variable and constant heavy chain, plus signal sequence, was recovered from pFUSEssCHIg_mG1_hTg5VH by a first digestion with *KasI* restriction enzymes, and blunting by Klenow enzyme and a second digestion with *BsmI* restriction enzyme, followed by 3' overhang removal by T4 DNA polymerase. After PB_prom3 vector preparation as described before, the hTg5VH_mG1 sequence was cloned into this vector for the generation of PB_prom3_ ssCHIg_mG1_hTg5VH.

Mammalian Cell Culture

- CHO-K1 Cell-line

The attached CHO-K1 cell line was obtained from American Type Culture Collection (ATCC ID: CCL-61). Cells were cultured in Ham's F12 medium (Lonza, Verviers, Belgium) supplemented with 10% FBS, 2mM L-glutamine and 100 UI/ml pen/strep (Lonza, Verviers, Belgium). Cells were grown at 37°C, 5% CO_2 in either 10 cm plates, 6-well or 96-well cell culture dishes.

Passaging Adherent Cells

When cell density was greater than 90% confluency, cells were subcultured. Briefly, the media was aspirated and the cells were rinsed with Ca2+/Mg2+ free Dulbecco's phosphate-buffered saline (D-PBS, Lonza, Verviers, Belgium). In order to detach cells from the surface,

the cells were incubated with approximately 0,1 volumes of Trypsin/EDTA (Lonza, Verviers, Belgium) at 37°C for 5 minutes. The vessel was gently tapped to ensure all the cells were detached, and then, 0,9 volumes of complete medium were added to inactivate trypsin activity with FBS. After cell clumps disrupting with gentle pipetting, cells were centrifuged at 100 x g for 8 minutes to completely remove trypsin. The cell pellet was resuspended in fresh culture medium and cells were counted with a haemocytometer. The trypan blue exclusion method was used to determine cell viability and total cell count. Finally cells were aliquoted for sub-culturing into new flasks or for future experimental analysis.

Cryopreservation of Adherent Cells

Established cell-lines or transfected pools were cryopreserved to create working cell banks or backup stocks. Appropriate number of cells was harvested at 100 x g after trypsinization. Cell pellets were resuspended at a density of approximately 15 x 10^6 cells/ml in 10% Hybri-Max™ DMSO (Sigma-Aldrich, St. Louis, MO), 90% Ham's F12 added with 20% FBS in 1,5 mL polypropylene vials (Nalgene). Vials were slowly frozen at -80°C and then transferred for long-term storage in a liquid nitrogen tank (at -180°C).

Thawing procedure for adherent cells

Recovery of cells stored in liquid nitrogen must be completed in minimum time, since the cryopreservant DMSO is toxic. A vial of cells removed from the liquid nitrogen storage vessel was quickly thawed at 37°C, followed by transferring the cells into 5 ml of growth medium in a 15 ml centrifuge tube, and centrifuged at 100 x g for 8 minutes. The supernatant was removed and the cell pellet resuspended in 10 ml of fresh medium supplemented with 20% FBS and dispensed into a 10 cm^2 tissue culture plate.

Transfection of CHO-K1 adherent cells

Standard plasmids or transposon system vectors were transfected into CHO-K1 cells using Lipofectamine 2000 transfection reagent (Life Technologies, USA) as described in manufacturer's protocol. Briefly, cells were seeded into 6-well cell culture plates at 1x10^6 cells per well in 2 ml Ham's F12 complete medium containing 10% FBS and incubated at 37˚C overnight until reached 90-100% confluency. The transfection reagents were prepared in separated tubes as follows: a) 4,2 µg (or 5,9 µg in the case of chimeric antibody) of total DNA (table I) in 250 µl OPTIMEM medium (Gibco, Life Technologies, USA), b) 10 µl of

Lipofectamine 2000 in 250 µl OPTIMEM medium. The two solutions were then combined, mixed gently by inverting up and down the tube and incubated for 30 minutes at room temperature in order to form the DNA-lipid complex. Confluent cells were washed twice with D-PBS before medium replacement with 1 ml of Ham's F12 medium without FBS. The DNA-lipid complex was then added to the cells and incubated at 37°C for 4 hours. Finally 1,5 ml Ham's F12 medium with 20% FBS was added. One day post transfection, cells were detached, counted and seeded at low density, from 500 to 50000 cells/dish, in Ham's F12 medium containing appropriate concentration of G418 (800 µg/ml) (Sigma-Aldrich).

A

Ratio transposon: transposase	Plasmids	DNA amount
0	Standard plasmid or transposon pBlueScript	3 µg 1,2 µg
2,5:1	Transposon Transposase PB200	3 µg 1,2 µg
5:1	Transposon Transposase PB200 pBlueScript	3 µg 0,6 µg 0,6 µg

B

Ratio LC:HC	Plasmids	DNA amount
1:1	Standard plasmid or transposon LC Standard plasmid or transposon HC pBlueScript or pBlueScript	2,1 µg 2,1 µg 1,7 µg
3:2	Standard plasmid or transposon LC Standard plasmid or transposon HC pBlueScript or pBlueScript	2,5 µg 1,7 µg 1,7 µg
4:1	Standard plasmid or transposon LC Standard plasmid or transposon HC pBlueScript or pBlueScript	3,4 µg 0,8 µg 1,7 µg

Tabe I. DNA mix prepared for transfection: quantity used for each plasmids in different experimental conditions: A) hFGF23 vectors; B) chimeric antibody vectors.

After 4 days the medium was replaced with fresh medium and lasted for other 3 days. Following 7 days of G418 selection, 100 neomycin resistant clones on the plate were picked up and transferred in a 96 well tissue culture dish containing selective media. To favour cells detachment, 1 ml of trypsin/EDTA were added to dish and incubated for 1 minutes at 37°C. Five plates, not used for clone picking, were stained by crystal violet (Sigma-Aldrich).

Crystal Violet staining

The method was performed for the quantitative analysis of clones. Cells were fixed in the fixing/staining solution (10% ethanol, 1% Crystal violet) for 10 minutes with gentle agitation. Then the dishes were destained until only clones were colored by performing repeated D-PBS wash for 5 minutes with vigorous agitation. The stained plates were then photographed.

-CHO-K1-S Suspension Cell Line

Cell lines adapted to growth in suspension and serum free media were grown in ProCHO4 medium (Lonza, Verviers, Belgium) supplemented with non essential aminoacid MEM 1X (Gibco, Life Technologies, Carlsbad, CA), anti-clumping agents 1:250 (Invitrogen, Life Technologies, Carlsbad, CA), 2mM L-glutamine (Lonza, Verviers, Belgium) and 100 UI/ml pen/strep (Lonza, Verviers, Belgium). Cells were grown at 37°C, 5% CO_2 in 125 ml Flask (Corning, Corning, NY) on an orbital shaker at 100 rpm.

Suspension adaptation of CHO-K1 established cell lines

The best producing adherent CHO-K1 clones and the not transfected CHO-K1 cell line were gradually adapted from growth in serum-containing medium to serum-free suspension conditions following the protocol below:

Passages in culture	**Adhesion Medium:** Complete Ham's F12 + 10% FBS	**Suspension Medium:** Supplemented ProCHO4	**Type of culture**
-	100%	0%	Static culture
2	75%	25%	Static culture
2	50%	50%	Static culture
2	25%	75%	Static culture
2	0%	100%	Static culture
At least 10 passages	0%	100%	On orbital shaker at 100 rpm

Table II. Serum free suspension adaptation protocol: Complete Ham's F12: Ham's F12, 2mM L-Glutamine and 100 UI/ml Pen/Strep. Supplemented ProCHO4: MEM 1X, Anti clumping agents 1:250, 2mM L-Glutamine and 100 UI/ml Pen/Strep.

The serum-containing medium was reduced stepwise from the initial 100% to the stages of 75, 50, 25, and 0 %, while the ProCHO4 serum free suspension adaptation medium increased specularly. The medium changed every two passages in static cultured. During the medium shift, cells growing in the supernatant and in adhesion (detached with Trypsin/EDTA) were collected by centrifugation at 100 x g for 8 minutes, counted and plated in 10 cm dish at the final density of 2,5-5 x 10^5 cells/ml. After the last passage in static culture, cells were moved to a 125 ml Flask (Corning, Corning, NY) and cultured in 10 ml of completed ProCHO4 medium at the density of 0,8 x 10^6 cells/ml following protocol described in "Passaging suspension cells". The number of passages required for suspension adaptation is clone-dependent and sometimes clones could be discarded during adaptation due to poor growth or decreasing productivity. Cells were considered adapted to suspension growth when their doubling time was below to 35 hours (Nielsen et al., 2010). To calculate doubling time an online calculator was used (*www.doubling-time.com*). Adapted cells were named CHO-K1-S.

Passaging suspension cells

For cell lines maintenance, cells were plated in 10 ml of fresh medium at the density of 0,8 x 10^6 cells/ml and after 3-4 days sub-cultured. Briefly, the suspension of cells was harvested and centrifuged at 100 x g for 8 minutes. The cell pellet was resuspended in 1 mL of Trypsin/EDTA (Lonza, Verviers, Belgium) and warmed at 37°C for 5 minutes to minimize cell clumps. In this case, D-PBS rinse is not necessary because completed medium is FBS free. Then, 9 mL of complete medium were added in order to dilute trypsin. After cell clumps disrupting with gentle pipetting, cells were centrifuged at 100 x g for 8 minutes to completely remove trypsin. The cell pellet was resuspended in fresh culture medium and cells were counted with a haemocytometer. The trypan blue exclusion method was used to determine cell viability and total cell count. Finally cells were aliquoted for sub-culturing into new flasks or for future experimental analysis.

Cryopreservation of suspension Cells

Cell lines were cryopreserved to create working cell banks as described before. Approximately 15-20 x 10^6 cells/ml in 10% Hybri-Max™ DMSO (Sigma-Aldrich, St. Louis, MO), 90% supplemented medium in 1,5 mL polypropylene vials (Nalgene). Vials were slowly

frozen at -80°C and then transferred for long-term storage in a liquid nitrogen tank (at -180°C).

Thawing procedure for suspension cells

The thawing procedure is the same described for adherent culture, but the medium used is not supplemented with serum (ProCHO4 complete medium) and the tissue culture vessel is a 125 mL flask placed on an orbital shaker platform rotating at 100 rpm. Since cell viability could decrease at 24-48h after thawing, cells were kept in culture for 3-4 days before subculturing.

Nucleofection of CHO-K1-S suspension cell line

CHO-K1-S cell line was stably transfected applying Amaxa Nucleofector apparatus (Lonza Group, Basel, Switzerland) and the CHO-S cells specific nucleofection protocols. The day before nucleofection, cells were sub-cultured 1:2 in fresh medium. The day of transfection cells were collected and counted in order to prepare a cell pellet containing 2 x 10^6 cells by centrifugation at 100 x g for 8 minutes. The pellet was resuspended in 100 µl of previously prepared Nucleofector solution (solution V). Then, the cell suspension was combined with 4,2 µg of DNA (prepared as described for transfection in adherent cells) and transferred into a proper cuvette. Once inserted in the Nucleofector 2b Instrument, the U-023 program was applied. After nucleofection, cell suspension was diluted with 500 µl of ProCHO4 and gently transferred into a 6-well plate containing 2 ml of supplemented ProCHO4. Transfected cells were incubated at 37°C in agitation (100 rpm) on orbital shaker. 48 hours post transfection cells were diluted with complete ProCHO4 medium supplemented with 800 µg/mL G418 (Sigma Aldrich) and transferred into a 125ml flask. Selective medium was replaced every 3-4 days over 2 weeks during routine cell passage with cell inoculation at 8×10^5 cells/mL, obtaining a neomycin resistant pool of CHO-K1-S.

Adaptation to other serum-free media

Selected clones were adapted to one of the following media: HyClone SFM4CHO (GE Healthcare Life Sciences, Logan, UT), ProCHO5 (Lonza, Verviers, Belgium) and CellVento CHO100 (EMD Millipore, Woburn, MA). All media were supplemented with non essential aminoacid MEM 1X (Gibco, Life Technologies, Carlsbad, CA), anti-clumping agents 1:250 (Invitrogen, Life Technologies, Carlsbad, CA), 2mM L-glutamine (Lonza, Verviers, Belgium)

and 100 UI/ml pen/strep (Lonza, Verviers, Belgium). The protocol used recapitulated that applied for the transition from adherent to suspension culture and is reported below:

Passages in culture	ProCHO4	New Medium	Type of culture
-	100%	0%	On orbital shaker at 100 rpm
2	75%	25%	On orbital shaker at 100 rpm
2	50%	50%	On orbital shaker at 100 rpm
2	25%	75%	On orbital shaker at 100 rpm
2	0%	100%	On orbital shaker at 100 rpm

Table III. New serum free media adaptation protocol

Cells were sub-cultured in the new medium every 3-4 days, until their doubling time was shorter than 35 hours and they were considered adapted (Nielsen et al., 2010). To calculate doubling time an online calculator was used (*www.doubling-time.com*).

Clones screening

Liaison Platform

Supernatants obtained from clones after 4 days of culture were diluted 1:100 in PBS-BSA 0,1% and analyzed with Liaison Platform (Diasorin, Saluggia, Italy). It is an automated instrument that perform chemiluminescent immunometric analysis of biological matrices (serum, plasma and feces). The chemiluminescent molecule used in Liaison immunoassay is ABEI (N-(4-Amino-Butyl)-N-Ethyl-Isoluminol), a derivative of luminol, that is conjugated with the antibody or antigen used as tracer. The light signal is measured by a photomultiplier as relative light units (RLU).

Different immunoassay configurations were used regarding the analyte to identify:

Analyte	Solid phase	Tracer	Assay type
hFGF23 recombinant protein	mAb 1 anti-hFGF23	mAb 2 anti-hFGF23	Quantification in µg
hFGF23 transmembrane protein (on cells)	mAb 1 anti-hFGF23	mAb 2 anti-hFGF23	Quantification in RLU
Chimeric antibody anti hTg	Ab anti mouse IgG	hThyroglobulin	Quantification in RLU

Table IV. Immunoassay formats used: mAb anti-hFGF23 were generated at DiaSorin Research Center (Gerenzano, Italy); polyclonal anti mouse IgG antibody (Abcam, Cambridge, UK), hThyroglobulin (CalbioChem, San Diego, CA)

Specific productivity rate (SPR) for screening of clones producing hFGF23

To determine the specific productivity of clones during screening, the cells were counted using cell counting kit CCK8 (Dojindo, Kumamoto, Japan), a colorimetric assay that exploit WST-8 molecule. Briefly, once harvested cell culture supernatants, cells were washed, detached form 96well tissue culture dish surface by trypsin/EDTA and diluted 1:15 into final 100 µl of fresh culture medium. A standard curve was also preparated using CHO-K1 cell line plated at the following density 0, 250, 500, 1000, 5000, 10000 cells/well. After 24 hours, 10 µl of CCK-8 solution was added to each well and incubated at 37°C in the dark for 2 hours. Then, absorption was measured at 450 nm using a microplate reader (BioTek, Winooski, VT). Cell numbers for each well was calculated based on standard curve. Taking into account the hFGF23 quantification for each supernatant, the specific productivity rate was calculated by the following formula:

$$SPR = \frac{\text{ng of protein}}{10^6 \text{ cells x incubation days}}$$

Specific RLU rate (SRR) for screening of clones producing the recombinant antibody

In order to screen clones that produce the chimeric antibody we had not developed an immunoassay for absolute quantification, therefore to determine the specific productivity of clones we considered RLU instead of ng of protein. The previous formula was modified as follows:

$$SRR = \frac{\text{RLU}}{10^6 \text{ cells x incubation days}}$$

Clone characterization

hFGF23 quantification

hFGF23 protein quantification in the supernatant or after purification was performed using a commercial ELISA assay (KAINOS Laboratories, Kasama, Japan) following the manufacturer's instructions.

Specific productivity rate (SPR) value

To determine the specific productivity of candidate clones, the cells were seeded in 10 mL of ProCHO4 or CellVento CHO100 with supplements at a concentration of 8×10^5 cells/ml in 125 ml flask. The cells were cultivated for up to 4 days and samples were collected every day to determine the viable cell density and the hFGF23 concentration. The former was

determined with the Trypan blue method, while hFGF23 concentration in the cell culture medium was determined by ELISA assay (KAINOS Laboratories, Kasama, Japan). Cell-specific productivity during the exponential phase of cell growth was calculated with the following formula:

$$\text{SPR (pg/cell/day)} = \frac{\text{Titre2-titre1}}{\text{(cell density2-cell density1)}} \text{ x daily growth rate}$$

where daily growth rate was calculated with

$$\text{daily growth rate} = \frac{\ln(\text{density2}) - \ln(\text{density1})}{(\text{time2} - \text{time1})} / 24$$

Glucose measurement

Glucose concentration in cell supernatant was measured using an Accu-Chek Aviva Plus glucose meter (Roche Diagnostics, Indianapolis, IN). Briefly, cell supernatant was diluted in physiologic water 1:10 to fit the instrument range of measurement. Then, a drop of this dilution was used for the measurement with a disposable Accu-Chek strip (Roche Diagnostics, Indianapolis, IN), according to manufacturer's instructions. 2 fold serial dilution of a commercial glucose solution in PBS was used to design a calibration curve, in which Accu-Chek data were correlated to real glucose concentration. Finally, value obtained from cell supernatant with the Accu-Chek system were converted based on calibration curve.

RNA extraction

For RNA collection, candidate clones were seeded at the density of 8 x 10^5 cells/ml in 10 ml of supplemented ProCHO4 medium and cultured for 4 days. Next, 5 x 10^6 cells from each clone were harvested and total RNA was extracted by using RNeasy MiniKit (Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions. Before reverse transcription, RNA was treated with DNaseI (Ambion) and purified using the RNeasy kit (Qiagen, Hilden, Germany). Following elution, the integrity of the RNA was confirmed by 1% agarose gels using Gel Red staining (Biotium, Hayward, CA). The concentration of RNA was determined by Nanodrop1000 spectrophotometer (NanoDrop, Wilmington, USA) by measuring light absorption at 260 nm. Nucleic acid purity was checked by the determination of absorption at a wavelength of 230 and 280 nm, respectively.

cDNA sythesis

The cDNA was was produced from 1 µg total RNA with SuperScript II Reverse Transcriptase (LifeTechnologies, Rockville, MD). Oligo(dT)$_{12-18}$ primers (500 ng) (LifeTechnologies, Rockville, MD) were used to prime the cDNA synthesis. The synthesis was completed according to the manufacturer's recommendations, with an incubation temperature of 42°C. The resulting cDNA was diluted to 50 ng/µl and was stored at -20°C. The ensuing cDNA was used both for PCR or Real Time amplification. As a negative control, RNA for CHO not-transfected cells or Ag8 myeloma cells was also collected and treated in the same way.

PCR

PCR was performed using 4U GoTaq DNA Polymerase (Promega, Leiden, Nederland) and 1X Green GoTaq® Reaction Buffer, 0.5 mM of each oligonucleotide primer, 1.5 mM $MgCl_2$, 0.2 mM of each deoxyribonucleoside triphosphate (dATP, dCTP, dGTP, dTTP) were also added to the reactions. PCR was performed for 30 cycles as follows: denaturing for 30s at 94°C; annealing for 30s at T_{ann}; and extension for 30s at 72°C. PCR products were resolved on a 2% agarose gel. Primer sequences and annealing temperatures were listed in table V.

Gene	**Forward**	**Reverse**	**T_{ann}**
βactin	GGCCCAGAGCAAGAGAGGTATCC	ACGCACGATTTCCCTCTCAGC	62
hFGF23	TGAAGCCCAGAGCCAGAATGA	ATGGTGGTGGTGATGGCTGC	62
OVKJ	39 forward primers based on Essono et al. 2003	GTTTGATCTCCAGCTTGGTCCC	68
OVKcost		TCAATCTTCCACTTGACATTGATGTC	62
OVHJ	26 forward primers based on Essono et al. 2003	TGACCGTGGTCCCTTGGCCCCAG	68-62
OVHcost		GTTCCAGGTCACTGTCACTGGCTC	62

Table V. List of PCR primers: T_{ann}: annealing temperature

Real time-PCR

Quantitative PCR (qPCR) analysis was performed using iTaq SYBR Green Supermix with Rox on a Dyad Disciple Peltier Thermal Cycler (Bio-Rad). All reactions were performed in a total volume of 20 µl containing 0,8 ng cDNA, forward and reverse primers as indicated in the table below. Amplification cycles consisted of an initial denaturing cycle at 95°C for 10 min, followed by 45 cycles of 15 s at 95°C and 30 s at 62°C. Fluorescence was quantified during the 62°C annealing step, and specificity of the amplification product was confirmed by

examination of dissociation reaction plots (55–94°C). A distinct single peak indicated that a single DNA sequence was amplified during PCR. Each sample was tested in triplicate and samples obtained from three independent experiments were used for analysis of relative gene expression using the Pfaffl ΔΔCt method. Amounts of target gene mRNA were normalized to β2-microglobulin (β2m), a reference gene. Primer sequences and their concentration in the reaction are listed below.

Gene	**Forward**	**Reverse**	**T_{ann}**
β2m	CCTGAACTGCTATGTGTCTC 5 µM	GTGGGTGTAAACTCCGTGTG 5 µM	62
hFGF23	TGAAGCCCAGAGCCAGAATGA 1,5 µM	ATGGTGGTGGTGATGGCTGC 1,5 µM	62
hTg5 VK	AGCATTGTGATGACCCAGACTCA 5 µM	TCAATCTTCCACTTGACATTGATGTC 5 µM	62
hTg5 VH	GAAGTGAAGCTGGTGGAGTCTGA 5 µM	GTTCCAGGTCACTGTCACTGGCTC 5 µM	62

Table VI. List of qPCR primers T_{ann}: annealing temperature

NIH/3T3 proliferation assay

NiH/3T3 cell line was obtained from American Type Culture Collection (ATCC ID: CRL-1658). Cells were cultured in DMEM medium (Lonza, Verviers, Belgium) supplemented with 10% FBS, 2mM L-glutamine and 100 UI/ml pen/strep (Lonza, Verviers, Belgium). Cells were grown at 37°C, 5% CO_2 in either 10 cm plates.

The day before the test, cells were plated at 2000 cells/well in a 96-well plate and cultured in DMEM supplemented with 10% FBS, 2mM glutamine, 100 UI/ml pen/strep, without phenol red. In the evening, medium was exchanged with DMEM supplemented with 0,2% FBS , 2mM glutamine, 100 UI/ml pen/strep, without phenol red. After overnight serum starvation, 20 µl of controls or samples were added to cells to obtain a final volume of 120 µl in each well. Dilution of controls and samples were prepared at a 12X concentration, in order to obtain a final 1x concentration. For each experimental condition 6 replicates were prepared. hFGF23 protein were diluted at different concentration (0,3 µg/ml, 0,6 µg/ml, 1,2 µg/ml, 2,4 µg/ml) and mixed with Klotho at 0,3 µg/ml (R&D system, Minneapolis, MN) and heparin at 10 µg/ml (Sigma-Aldrich) in DMEM 0,2% FBS. Control samples were: 1) Klotho at 0,3 µg/ml and heparin at 10 µg/ml in DMEM 0,2% FBS, 2) hFGF23 protein at 1,2 µg/ml or 3)

at 2,4 µg/ml in DMEM 0,2% FBS and 4) medium alone. Cells were incubated for 24 hours at 37°C. The day after, cell counting kit CCK8 (Dojindo, Kumamoto, Japan) (described for "clone screening" session) was applied for the determination of number of cells. Briefly, 12 µl of CCK-8 reagent were added to each experimental well and to standard curve wells, and incubated at 37°C for 2 hours in the dark. Then, absorption was measured at 450 nm using a microplate reader (BioTek, Winooski, VT). Cell numbers for each well was calculated based on standard curve.

Recombinant antibody enrichment in the supernatant with Protein A

In order to perform western blot analysis on supernatant from adherent CHO-K1 clones, it is important to eliminate BSA protein derived from FBS. For this reason, superantants were treated with Protein A-Sepharose 4 Fast flow (GE Healthcare, Mississauga, Canada). Briefly, the resin was vigorously agitated and 1.5 mL was harvested and centrifuged at 1000 rpm for 2 minutes in order to eliminate the slurry (20% EtOH). Protein A resin was washed 2 times with PBS and centrifuged 1000 rpm for 2 minutes. Finnaly it was resuspend with 500 µl of PBS. Clones originated from transfection with murin heavy and light chain anti-hTg (PB_CAGssVL_CL/VH_CH mIgG1 anti-hTg ratio 4:1) were grown in 2 mL HAM'S F12 10%FBS on a MW6. 4 mL of supernatant were harvested from each clones and centrifuged in order to eliminate cells debris. 40 µl of Protein A-sepharose (50% PBS buffer) were added to samples and incubated on a rotating wheel at 4°C overnight. The day after, samples were centrifuged and resin pellets were washed twice with PBS-Tween20 0,1% buffer. After the second wash, each sample was divided into 2 aliquots to be used in reducing and non-reducing conditions analysis. Samples were centrifuged, supernatants were discharged and 30 µl of sample buffer were added to the resins pellets and boiled at 99°C for 5 minutes before loading on SDS-PAGE gel.

Protein Gel Electrophoresis

Polyacrylamide gel electrophoresis (PAGE) with denaturant sodium dodecyl sulfate (SDS) was used to separate proteins by size from cell culture supernatants. The running buffer Tris/Glycin (Bio-Rad, Hercules, CA) was diluted 1:10 and used to run the gel electrophoresis. 15µl of clarified supernatant samples (or protein A treated samples) were combined with 15µl of sample buffer (reducing or non reducing) followed by 5 minutes at 99°C. The samples were centrifuged briefly and were then loaded into Criterion™ TGX Stain-Free™

Precast 10% polyacrylamide gels (Bio-Rad, Hercules, CA). Precision Plus molecular weight markers (Bio-Rad, Hercules, CA) were also included. The peculiarities of Criterion™ gels are fast run times (30-40 minutes at 240V) and the possibility to visualize protein with a ChemiDoc imagers (Bio-Rad, Hercules, CA) without the comassie staining. This ability is due to the trihalo compounds incorporated into the gel matrix that react with tryptophan residues in a UV-induced reaction to produce fluorescence, which can be easily detected by the ChemiDoc imagers. The reaction occurs within gels or on a PVDF membrane after transfer. In our case, UV light was applied for 5 minutes to obtain the best sensitivity.

Western Blotting

Proteins were transferred from the gel to a PVDF membrane employing the Trans-Blot Turbo Transfer System (Bio-Rad, Hercules, CA). This system provide a rapid blot transfer thanks to Transfer Packs that include an optimized buffer, a PVDF membrane and filter paper combination. The process is a semi-dry transfer and occur in 7 minutes on the Trans-Blot Turbo blotting apparatus (Bio-Rad, Hercules, CA).

-hFGF23 immunoblotting:

The membrane was blocked in 0,5% BSA in PBS buffer at room temperature for 2 hr. After blocking, blot was immunostained with anti-hFGF23 mouse serum (1:10000) for 16 hrs at 4°C. The mouse serum was originated by mice immunization for the generation of monoclonal antibodies directed to hFGF23. Post incubation, blot was washed three times with PBS-Tween20 0,1% and probed with goat anti-mouse IgG (Fc) conjugated with horseradish peroxidase (1:1000) (Thermo Fisher Scientific) for 2 hr at room temperature on a rocking platform. Then, the membrane was washed three times with PBS-Tween20 0,1% before the blots were subjected to detection using the 4CN (4-Chloro-1-naphthol) method.

-Recombinant IgG immunoblotting:

The membrane was blocked in 0,5% BSA in PBS buffer at room temperature for 2 hr. After blocking, blot was immunostained with goat anti-mouse IgG (H+L) conjugated with horseradish peroxidase (1:1000) (Thermo Fisher Scientific) for 2 hours at room temperature on a rocking platform. Then, the membrane was washed three times with PBS-Tween20 0,1% before the blots were subjected to detection using the 4CN (4-Chloro-1-naphthol) method.

4-chloro-1-naphthol method of detection

This substance is a chromogenic peroxidase substrate and works in a solution composed by 20 ml of PBS, 5 ml of 4-chloro-1-naphthol in 3mg/mL methanol, and 20 µL of hydrogen peroxide to give an insoluble black/purple stain. The developed membrane was fixed in water, air-dried and recorded by Chemidoc instruments (Bio-Rad, Hercules, CA) using ImageLab Software (Bio-Rad, Hercules, CA).

Immunofluorescence

- Adherent CHO-K1 cells

Parental CHO-K1 cell line and pool of cells expressing hFGF23 on surface were stained as follows: cells were washed with PBS and blocked with 5% FBS in PBS for 1 hours at room temperature on orbital shaker. After blocking, cells were incubated for 2 hours at room temperature with the primary antibody chicken anti-HA tag, 1:250 (Abcam, Cambridge, UK) diluted in PBS containing 2% FBS. After three washes in PBS, goat anti-chicken AlexaFluor 488 antibody, diluted 1:1000 in PBS containing 2% FBS was applied for 1 hour at room temperature. Cells were then washed three times with PBS and fixed with a solution of paraformaldehyde 4% in PBS for 15 minutes on an orbital shaker at room temperature. After three washes in PBS, cells were analyzed with a fluorescence microscope (Zeiss Observer A1 AXIO; GFP Filter).

- Suspended CHO-K1-S cells

Not transfected CHO-K1-S cell line and pool of cells expressing hFGF23 on surface were stained as follows. Each protocol step was performed by centrifuging cells at 100 x g for 8 minutes in order to collect them. Cells were washed with PBS and blocked with 3% BSA in PBS for 1 hour at room temperature on a rotating wheel. After blocking, cells were incubated for 2 hours at room temperature on the rotating wheel with the primary antibody chicken anti-HA tag, 1:250 (Abcam, Cambridge, UK) diluted in PBS containing 1% BSA. After three washes in PBS, goat anti-chicken AlexaFluor 488 antibody, diluted 1:1000 in PBS containing 1% BSA was applied for 1 hour at room temperature. Cells were then washed three times with PBS and fixed with a solution of paraformaldehyde 4% in PBS for 15 minutes on a rotating wheel at room temperature. After three washes in PBS, cells were seeded in a 6-well tissue culture plate and analyzed with a fluorescence microscope (Zeiss Observer A1 AXIO; GFP Filter).

Immunization strategies

Three-10 week old female NZB/BJNG mice were immunized with the following protocol:

Days	Task	Immunogen
1	I immunization	$5x10^6$ cell/mouse
21	II immunization	$5x10^6$ cell/mouse
41	bleeding	-
42	III immunization	$5x10^6$ cell/mouse
63	IV immunization	$10x10^6$ cell/mouse
73	bleeding	-
79	V immunization	$3x10^6$ cell/mouse
169	VI immunization	$5x10^6$ cell/mouse
179	bleeding	-

Table VII. Immunization protocol

Stable pool of CHO-K1-S cells expressing hFGF23 on membrane surface were centrifuged and suspended in PBS before each immunization. The cells suspensions were injected intraperitoneally without any adjuvants. Bleeding stand for the collection of a blood sample from each mouse to evaluate serological responses to the antigen of interest. After each bleeding, blood samples were treated 2 hours at 37°C to favor clot formation, incubated 16 hours at 4°C and than centrifuged for 5 minutes at 1000 rpm to separate sera from clot. Sera were conserved at -20°C.

Assesment of mouse serological response: ELISA assay

Mouse sera were tested for reactivity against hFGF23 in a solid phase ELISA. Animals sera were tested at different dilutions to assess which mouse was the best responder to the immunizations. The ELISA plate (NUNC) was coated with hFGF23 (our protein) at 5 µg/ml overnight, followed by saturation with PBS-BSA 0,5% for 2 hours at room temperature. First incubation was carried out by adding 100 µl of diluted sera (1:100, 1:1000, $1:10^4$, $1:10^5$, $1:10^6$, $1:10^7$) for 3 hour at 37°C. After washing three times with washing buffer (DiaSorin), 100 µl of HRP-conjugated anti-mouse IgG (Fc) antibodies (1:10000) (Thermo Fisher Scientific) were incubated for 1 hour at room temperature. After three washes with washing buffer (DiaSorin), the chromogen/substrate solution (DiaSorin) was added and OD was read at 450 nm.

Antibody affinity assessment by Surface Plasmon Resonance (SPR)

Samples were analyzed by Proteon XPR36 System (Bio-Rad, Hercules, CA) using a NLC sensor chip. This chip has NeutrAvidin bound to the carbossilic groups of modified alginate molecule (NLC) for the capturing of biotinylated molecules. The chip was conditioned with 30 µL injections of 50 mM NaOH and 1.0 M NaCl at a flow rate of 30 µL/min in both vertical and horizontal paths. Running buffer was PBS, pH 7.3, and Tween-20 (0.005% v/v), and the chip surface was maintained at 25°C. Biotinylated anti-mouse IgG antibody (Abcam, Cambridge, UK) was individually immobilized in six parallel channels by injecting, at a rate of 30 µl/min, 150 µl of antibody in PBST, a solution of PBS and the surfactant Tween 20 (0.005%). About 2000 RU of biotinylated anti-mouse IgG was typically immobilized under these conditions. The chip was rotated into the horizontal direction and stabilized with a 100 µL pulse of running buffer at a flow rate of 100 µL/min, followed by a 50 µL pulse of 1.0 M NaCl at a flow rate of 100 µL/min. Purified parental monoclonal antibody (hTg5) and recombinant antibody (Rec hTg5) concentrated 1,6 nM were injected as ligands at a flow rate of 50 µL/min for two minutes. Two channels for each antibody was used and the remaining were injected with running buffer as blank. About 60-80 RU of tested antibody was typically immobilized under these conditions. The chip was stabilized with a 100 µL pulse of running buffer at a flow rate of 100 µL/min and then the hThyroglobulin analyte was injected. Serial two-fold dilutions of analyte in PBST starting from 20 nM were applied across the horizontal path. The association step was performed at a flow rate of 50 µL/min for two minutes with a dissociation time of 600 seconds. Response data were analyzed using the ProteOn Manager™ software with a Langmuir fit.

Purification protocols

hFGF23 purification

Cell supernatant was thawed at 4°C in a cold room for about 60 hours. Cellular debris were eliminated from the hFGF23 supernatant by mean of a peristaltic pump and a high flow rate filter (PALL Corporation, Port Washington, NY). Sample volume was reduced with tangential flow filtration technique (TFF) using a hollow fiber cartridge with a MW cut off of 5 kDa (GE Healthcare, Uppsala, Sweden) and a QuixStand™ benchtop system (GE Healthcare, Uppsala, Sweden). Finally, reduced sample underwent a dialysis step using dialysis flasks with a MW cut off of 10 kDa (Thermo Scientific) against 5 liters of "Buffer CEC" ($NaHPO_4$*$2H_2O$ 50 mM pH 7,5) over night at 4°C. Sample after dialysis was ready to be loaded in a cationic

exchange chromatographic resin (volume 50 mL), exploiting high isoelectric point of hFGF23 protein, pI=8,72. Loading and washing buffer was “Buffer CEC”. Flow rate was of 24,4 cm/h during sample loading and of 61,1 cm/h during washing and elution. Washing step was done with 4 column volumes (CV). Protein elution was done with a linear gradient from 0% to 100% of a phosphate-sodium chlorite based buffer: $NaHPO_4*2H_2O$ 50 mM NaCl 1 M pH 7,5 in 2 column volumes. Elution was harvested in fraction of 5 ml. Chromatographic profiles were recorded at 280 and 260 nanometers of wavelengths. Eluted fractions containing hFGF23 protein at higher concentration and relative pure were pooled together. This sample was loaded in a 5 milliliters pre-pached IMAC column (GE Healthcare, Uppsala, Sweden) previously activated with nickel and then equilibrated in the following buffer: $NaHPO_4*2H_2O$ 28 mM NaCl 0,1 M pH 8,0. After sample loading at 120 cm/h, washing and elution steps were performed at 300 cm/h. Column washing was done with 10 CVs of the previous described buffer. Protein elution was performed with a linear gradient from 0 to 100% of $NaHPO_4*2H_2O$ 28 mM, NaCl 0,1 M, Imidazole 500 mM pH 8,0 in 4 CVs. Eluted fractions had a volume of 2 mL. Chromatographic profile were monitored at 280 and 260 nanometers of wavelengths. IMAC eluted fractions containing hFGF23 protein at higher concentration were pooled together and dialyzed overnight against 2 liters of a specific buffer, named Buffer A and aliquoted. Eventually, aliquots were lyophilized and stored at -20°C.

Chimeric antibody purification

Chimeric antibody was purified by affinity chromatography using Protein A. Briefly, cell supernatant (50 ml) obtained from adherent culture of clone 15C.47.44 was filtered using a 0.2 µm syringe filters (Sartorius). Protein A column 1 ml-size (GE Healthcare, Uppsala, Sweden) was equilibrated with Tris 0,5 M, NaCl 3 M buffer. Samples loading had a flow rate of 0,4 ml/min. The washing step was performed with 20 CVs of Tris 0,5 M, NaCl 3 M buffer at a flow rate of 1,0 ml/min. Elution step was done with citrate acid monohydrate 0,1 M buffer at pH 3.0 at a flow rate of 1 ml/min. Eluted fractions had a volume of 0.5 ml. Chromatographic profile were monitored at 280 nanometers of wavelengths. Eluted fractions containing the chimeric antibody at higher concentration were pooled together and stored at -20°C.

Results

Part 1: Recombinant proteins: expression of secreted and transmembrane hFGF23 protein in CHO cells

In last 15 years, different approaches for gene transfer into mammalian cells have been developed, however it is still challenging to obtain stable, high-producing cell lines for industrial applications. Conventional methods, based on spontaneous integration of episomal DNA, often result in low efficiency of clone establishment and in low transgene expression mainly due to plasmid concatemers silencing and/or positional effects. These strategies generate clones with a great variability in transcription, therefore time-consuming and laborious steps are required to select high producing clones suitable for industrial production requirements. To overcome these limitations, we tested transposons as new gene transfer method (Matasci et al., 2011). In particular, we decided to compare the PiggyBac (PB) transposon technology to standard mammalian expression vectors in the generation of stable CHO cell lines expressing recombinant proteins. As standard mammalian expression vector we used pcDNA3.1. This vector carries the cytomegalovirus (CMV) immediate early promoter and the bovine growth hormone polyadenylation signal for the efficient expression of the gene of interest and, in a separated expression cassette, the selectable marker, which is the neomycin resistance gene under the control of SV40 early promoter control. On the contrary, PiggyBac Transposon vector uses the CMV promoter to drive the expression of a bicistronic cassette for the transgene of interest and the neomycin resistance gene. The entire cassette is flanked by genomic insulators elements, to stabilize transgene expression, and the inverted terminal repeats (TIR), that are recognized by the PiggyBac transposase, thus enabling transposition.

1.1 Secreted hFGF23 protein

In order to compare the two gene delivery methods, we chose, as a model protein, the human fibroblast growth factor 23 (hFGF23), a 251 amino-acid protein with a 24 amino-acid signal peptide at the N-terminal portion. To assure the expression of a secreted protein suitable for purification, hFGF23 sequence, including the natural secretion signal at the N-terminus, was codon optimized and a His-tag tail was added at the C-terminus. The sequence was synthesized by GeneArt (Life Technologies) and it was cloned in both expression vectors described above.

1.1.1 Comparison of wild type and mutated sequence of hFGF23

As a first step, in order to avoid physiological protein cleavage between 179Arg and 180Ser mediated by specific proprotein convertases, we mutated the wild-type sequence of hFGF23 in the ^{176}RHTR^{179} cleavage site. The mutation of arginine 179 to glutamine was selected for the production of cleavage-resistant full length protein, as reported in literature (Shimada et al., 2002).

To prove the maintenance of protein integrity by using the selected mutated form, we cloned both the wild-type and the R179Q mutated sequences of hFGF23 into pcDNA3.1 plasmid. Adherent CHO-K1 cells were transfected with both constructs and 3 clones for each transfection condition were selected and expanded in culture. Supernatants were collected and hFGF23 expression was analyzed by western blot experiment immunostained with a homemade mouse serum anti-hFGF23. As illustrated in figure 1, in samples expressing the mutated hFGF23 form, the immunostaining confirmed the presence of a band around 32 KDa that corresponds to the glycosylated full length protein (Shimada et al., 2002), while, in clones expressing wild-type transgene, two distinct degradation fragments were clearly visible at around 15 kDa.

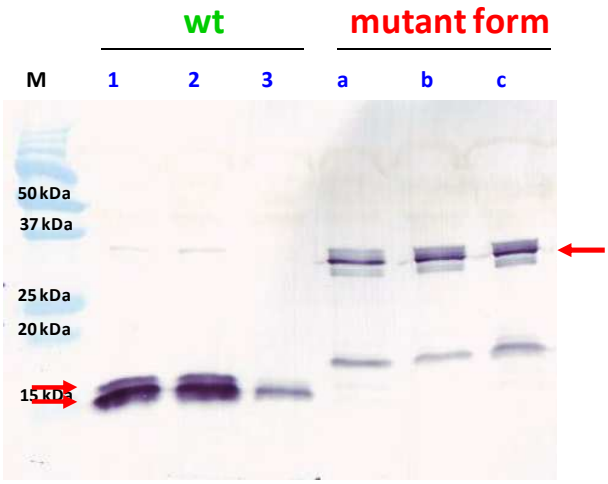


Fig 1. Wild type and mutated hFGF23 forms: western blot on hFGF23 expression in supernatants derived from CHO-K1 cell lines expressing hFGF23 wild-type (1, 2 and 3) or R179Q mutated form (a, b and c) immunostained with mouse serum against hFGF23 protein.

1.1.2 Comparison of standard transfection methods and transposons system

In order to assess the suitability of a transposon-based approach in the generation of stable producing clones, a characterization has been performed to compare standard expression plasmid (pcDNA3.1, Std) with the PiggyBac (PB) Transposon technology.

As a preliminary step, the selection strategy was optimized for the generation of CHO-K1 cell pools resistant to G418, an aminoglycoside antibiotic, similar in structure to neomycin. To define the sensitivity of CHO-K1 cells to G418, no-transfected cells were directly cultured in medium containing increasing concentrations of this antibiotic for 7 days and then stained with crystal violet. The same conditions were applied to cells transfected with pBlueScript plasmid, an empty vector used for balancing the total amount of DNA in transfections that lacks the neomycin resistance gene. Figure 2A and 2B show that CHO-K1 cells had no intrinsic resistance to G418 because cells died at all the concentrations tested. Subsequently, to determine the level of neomycin resistance conferred by pcDNA3.1 and PB vectors, transfected CHO-K1 cells were examined following the conditions described before.

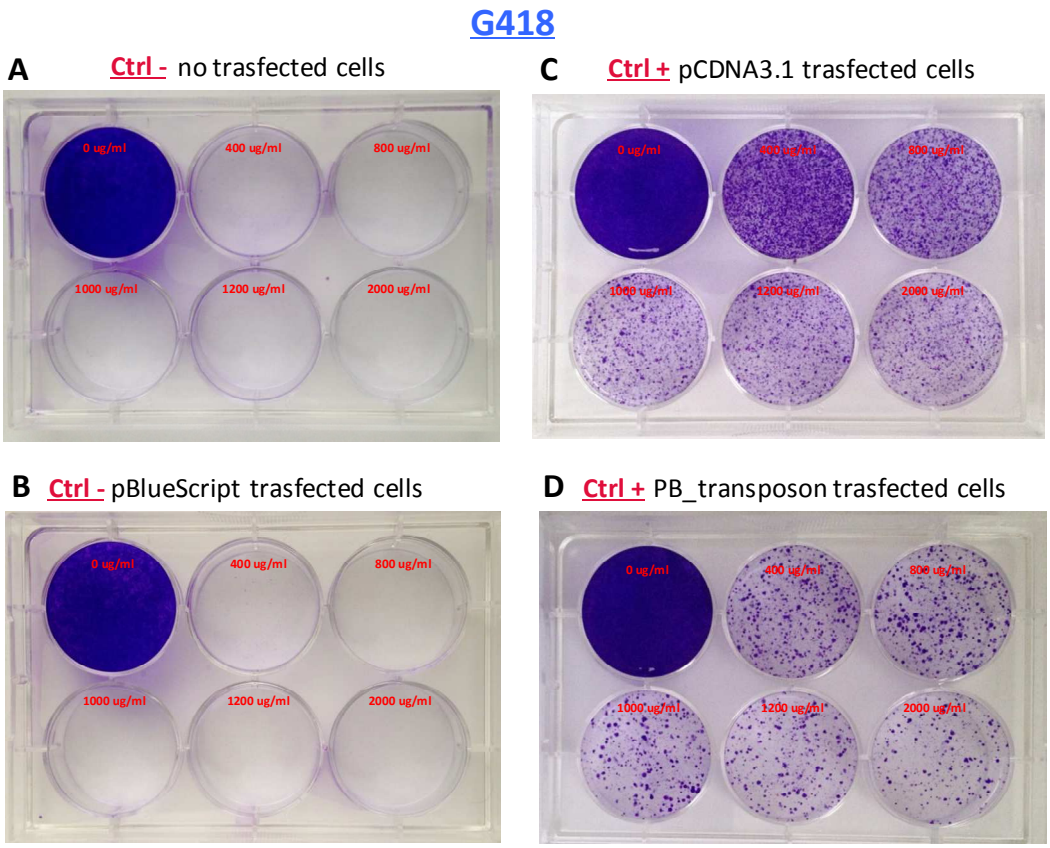


Fig 2. Neomycin selection strategy: G418 was added at six increasing concentrations (0, 400, 800, 1000, 1200 and 2000 µg/mL) to A) CHO-K1 no transfected cells, B) pBlueScript transfected CHO-K1 cells, C) pcDNA3.1 transfected CHO-K1 cells and D) PB_transposon transfected CHO-K1 cells. After 7 days of culture, cells were crystal violet stained. Ctrl -: negative control; Ctrl +: positive control.

As illustrated in figure 2C and 2D, after 7 days of culture in selective medium, cells died with different rates depending on G418 concentration. We concluded to use G418 at 800 µg/ml for following experiments because higher concentrations had generated slightly differences in number of clones and were uneconomical.

1.1.2.1 Generation efficiency of stable CHO-K1 clones

To properly work, the transposon system requires co-transfection of a transposon plasmid, carrying the transgene of interest, and a helper plasmid, carrying the transposase enzyme sequence. One of the parameters to be considered to optimize the efficiency of the system is the molar ratio between these two plasmids. Thus, as a first step in our experimental condition setting, two different transposon/transposase molar ratios (2.5:1 and 5:1) were tested. Adherent CHO-K1 cells were transfected using Lipofectamine 2000 (Life Technologies) with both pcDNA3.1-hFGF23_R179Q construct (Std) and PB-hFGF23_R179Q construct + transposase vector at the above mentioned molar ratios. In order to maintain constant the total amount of transfected DNA, we used an empty pBlueScript vector. Moreover, as an experimental control, cells were also transfected with PB_CMV_hFGF23R179Q alone (0). Transfected cells were plated at four different densities (500, 2500, 5000 and 50000 cells/dish) and cultured under neomycin selection (G418) for 7 days. After 4 days of selection, neomycin-resistant clones had become visible on the dishes. At the selection day 7, clones were stained with crystal violet and counted. Crystal violet staining revealed an outstanding improvement in the number of neomycin-resistant clones when CHO-K1 cells were transfected in the presence of transposon (PB) and transposase (PBase) plasmids at both molar ratio tested (Fig.3A), while the PB transposon alone performed in a way comparable to the standard (Std) vector pcDNA3.1. To facilitate an accurate quantification of the clone numbers, the count was carried out on the lowest cell plating density. At 500 cells/dish, the presence of transposase increased clone formation by about 8-9 times compared to cell transfected with Std vector or transposon alone (0), thus confirming that the great efficiency obtained using the transposon system is due to transposase action (Fig.3B). No statistically differences were observed between the two molar ratio tested, but, as transposon/transposase molar ratio 2,5:1 showed a trend toward greater improvement, has been selected as best condition for subsequent experiments.

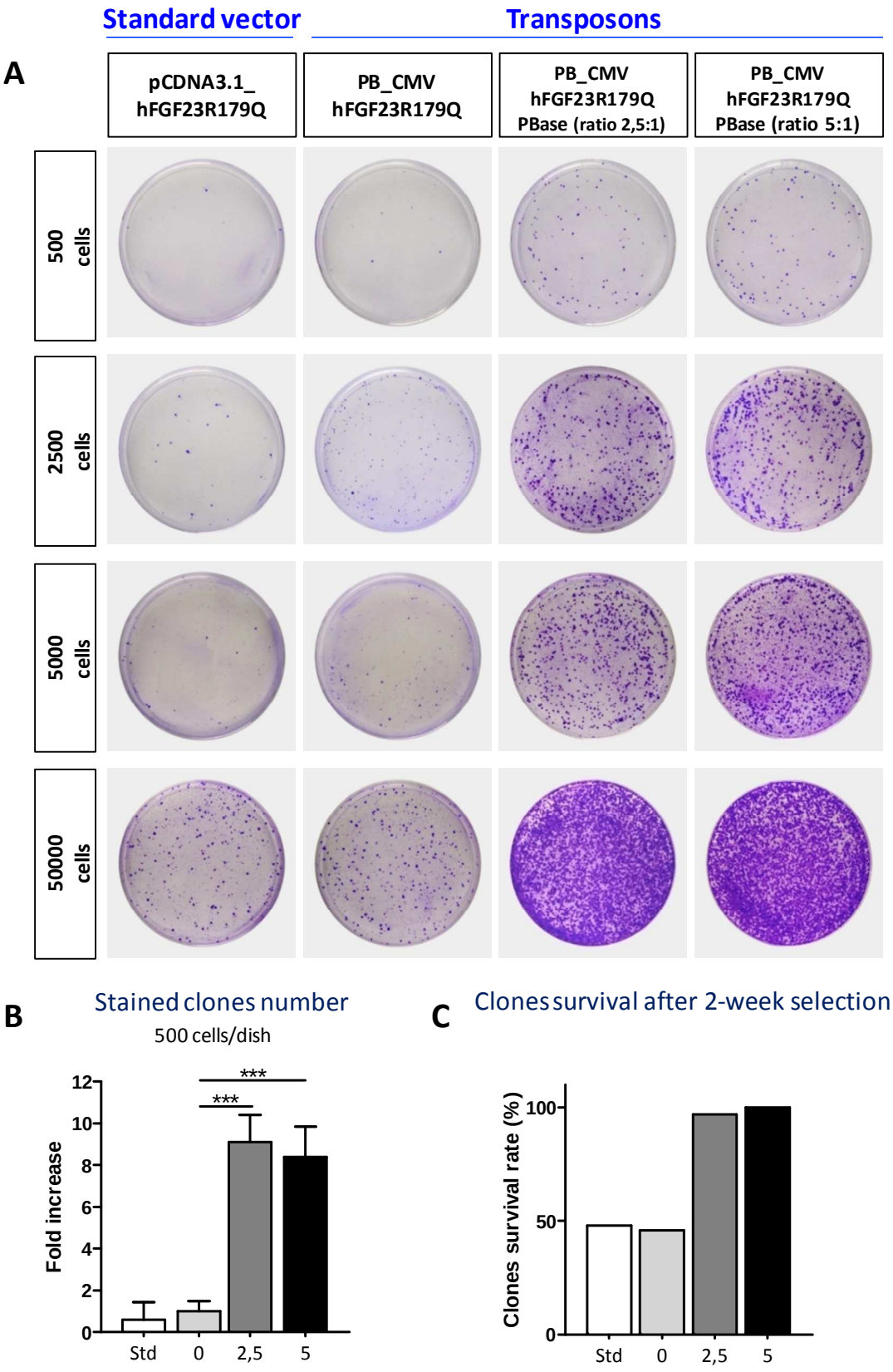


Fig 3. Clone formation assay: A) Crystal violet staining of clones obtained after 1 week neomycin selection. Transfection with standard vector was compared to transfection with different molar ratio of transposon to transposase plasmid (0:1, 2,5:1 or 5:1). Total amount of DNA in each transfection was constant. Cells were plated at different densities(500, 2500, 5000 and 50000 cells/plate). B) Count of stained clones obtained plating 500 cells/dish for each transfection condition (see text). Results was expressed as fold increase over clones originated from transposon alone (0) (n=5, ***p < 0.001). C) Percentage of survival clones after 2 weeks in selective medium. 100 clones were picked for each transfection condition.

Moreover, at the selection day 7, one hundred clones for each transfection condition, were picked up and maintained in culture for 15-20 days under G418 selection. Our analysis of clone survival rate (Fig.3C) revealed that only clones obtained from transposon/transposase co-transfection were almost 100% stable neomycin-resistant clones, while those derived from Std vector or transposon alone showed a survival rate of about 50%. This observed instability could be due to the short selection period (7 days) that is favorable to the active DNA integration process driven by transposase enzyme but it is insufficient for the stochastic integration of episomal plasmids.

These results underline that a transposon-based approach could be a powerful tool for faster generation of a high number of stable transfected clones compared to standard methods currently used, since it reduces of one week the time required for clone screening (Fig. 4) and it allows the generation of a greater number of clones.

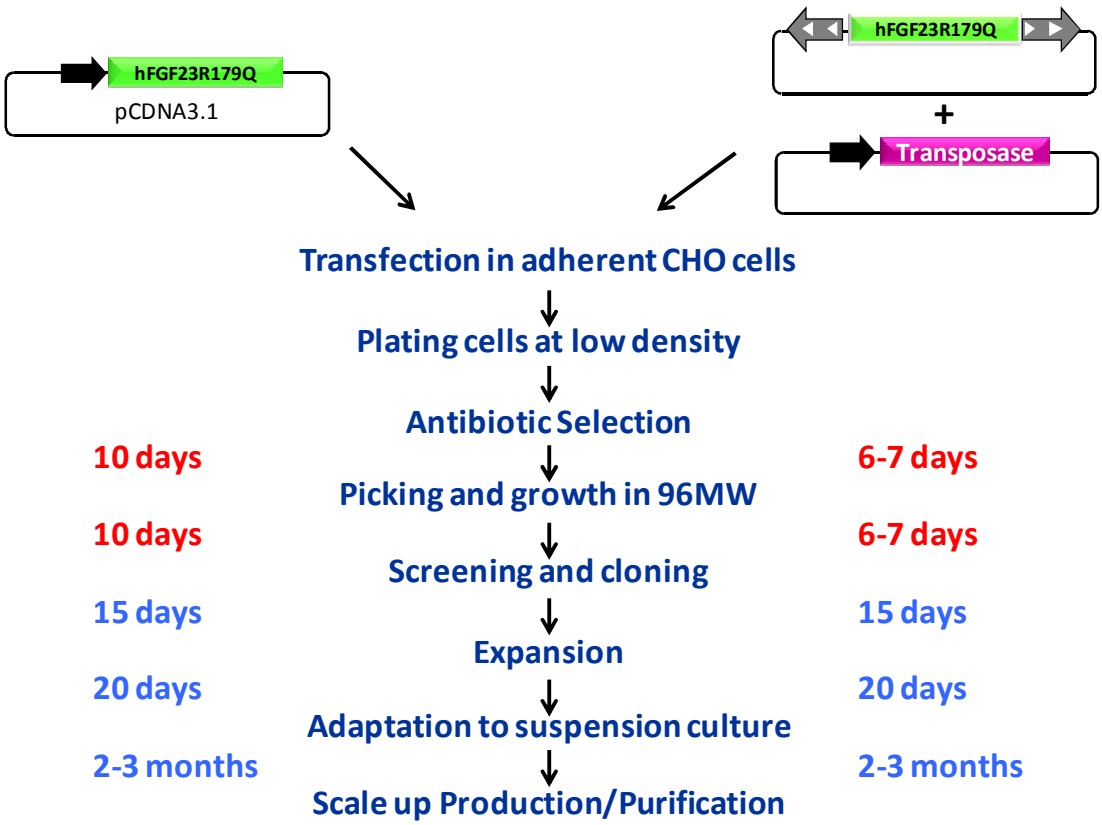


Fig 4. Schematic representation of steps required to obtain stable high producing CHO-K1 cell lines. Time necessary for each step is reported to compare standard transfection method and transposon system.

1.1.2.2 Enhancing frequency of high producing clones

As a next step, we evaluated the productivity of clones isolated from pcDNA3.1 and PB-transposon transfection experiments.

To better improve the possibility to generate high producing clones, we also decided to test the strength of different promoter elements, which is an approach traditionally used to maximizing level of protein expression. The selected promoters will be referred as promoter 1 (prom1), promoter 2 (prom2) and promoter 3 (prom3). The commercial transposon vector, in which we cloned the hFGF23_R179Q sequence, contains CMV promoter that was replaced with the sequences of selected promoters, synthetically obtained from GeneArt and cloned by restriction enzymes (see “Materials and Methods” section). The ensuing PB transposon vectors, expressing hFGF23R179Q sequence under the control of CMV, prom1, prom2 or prom3 promoters, were transfected into CHO-K1 cells using a transposon/transposase molar ratio of 2,5:1. As a comparative experiment, cells were also transfected with pcDNA3.1_hFGF23R179Q construct (Std), in which the hFGF23 sequence is expressed under the control of CMV promoter. Transfected cells were plated at the density of 500 cells/dish and cultured under G418 selection for 7 days. At the selection day 7, clones were stained with crystal violet and counted to assess the role of promoters in the clone establishment process. Crystal violet staining confirmed the efficiency of the transposon/transposase system in increasing the generation of stable transfected clones (Fig. 5A). PB vectors containing promoters 1, 2 and 3 performed even better than the previous observation, enhancing clone formation number of about 15-20 times compared to the pcDNA3.1 standard vector, whereas the PB-CMV construct resulted in an increase of approximately 8-9 times, confirming previous results (Fig. 5B).

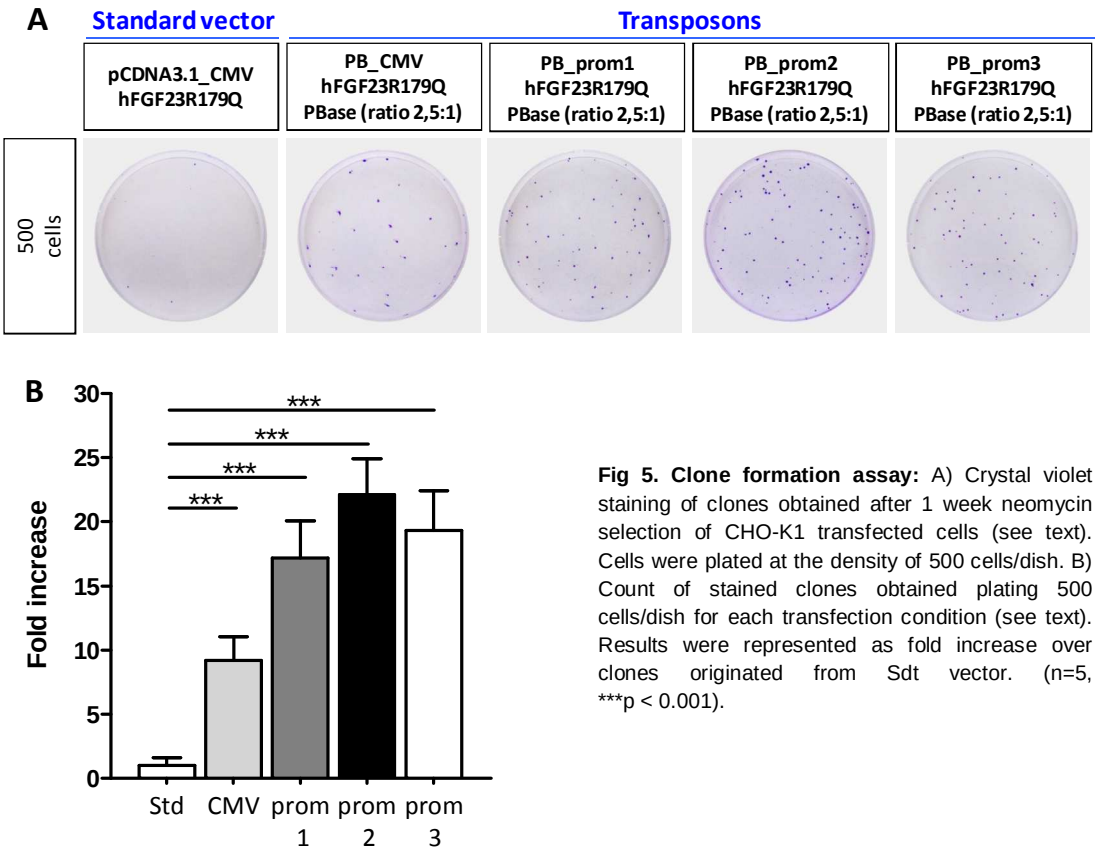


Fig 5. Clone formation assay: A) Crystal violet staining of clones obtained after 1 week neomycin selection of CHO-K1 transfected cells (see text). Cells were plated at the density of 500 cells/dish. B) Count of stained clones obtained plating 500 cells/dish for each transfection condition (see text). Results were represented as fold increase over clones originated from Sdt vector. (n=5, ***p < 0.001).

In order to evaluate clone productivity, about one hundred of clones for each experimental condition were picked up from plate at 500 cells/dish and maintained under neomycin selection for further 15 days to ensure stable transgene expression and to stabilize cell growth performance and metabolism. To perform a quantitative screening, supernatants obtained from 2 days cells culture, were collected and analyzed with the DiaSorin automated prototype immunoassay for the detection of hFGF23 in serum and plasma samples developed on Liaison platform. Quantitative values were normalized both to clone cell number and to days of culture, obtaining a final production rate value expressed as ng of protein produced by 10^6 cells per day (ng/ 10^6 cells /day). Results obtained from the quantitative screening were plotted arbitrarily defining four production categories (0-80, 80-500, 500-1000 and >1000 ng/ 10^6 cells /day) and estimating the percentage of clones for each experimental condition that fell in each category (Fig. 6).

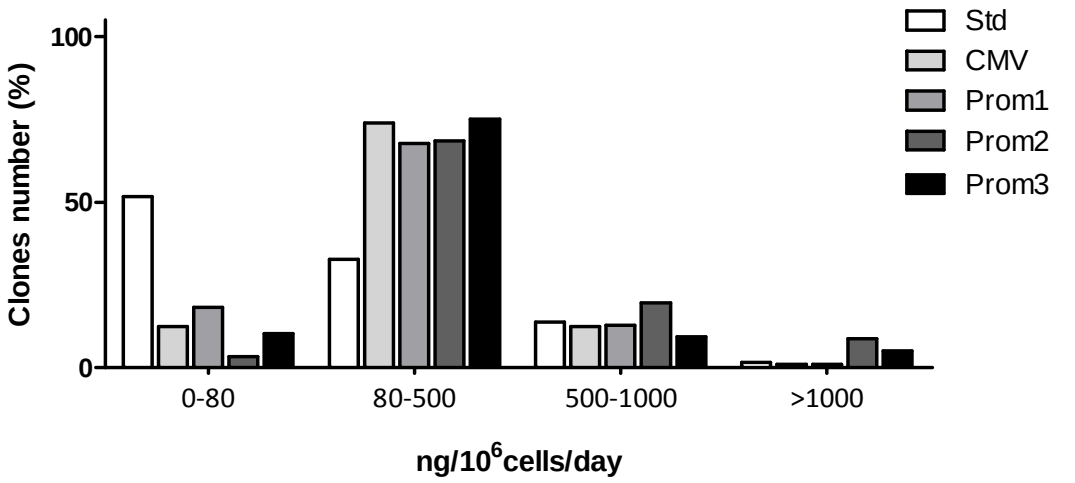


Fig 6. Classification of hFGF23 producing clones: Quantitative screening of clones generated with the different transposon plasmids. Clones were classified based on hFGF23 expression level. Cell supernatants were analyzed with an automated prototype assay for detection of hFGF23. Values were normalized based on clones cells number (Dojindo cell counting assay) and days of culture. Data were reported as percentage of clones classified into arbitrary categories of production (see text).

Our analysis revealed that productivity of most of the clones, irrespective of the transfected construct, fell into the 80-500 ng/10^6 cells/day category and that the higher percentage of high producing clones (>1000 ng/ 10^6 cells /day) was derived from cells transfected with PB_prom2 _hFGF23R179Q and PB_prom3_hFGF23R179Q plasmids. Moreover, transposon-derived clones were mainly distributed in the medium-high producing categories, while more than 90% of pcDNA3.1-derived clones fell into the two lower production categories. In particular, 50% of these clones were very low producers. Taken together, our results supported the potentiality of transposon system as a tool to generate an increased number of high producing clones.

We selected six clones from the highest producing categories (2 derived from PB_prom2 construct, 2 from PB_prom3 construct and 2 from pcDNA3.1 construct) that were cloned to assure monoclonal growth and, subsequently, they were expanded in adherent culture in absence of G418 selection.

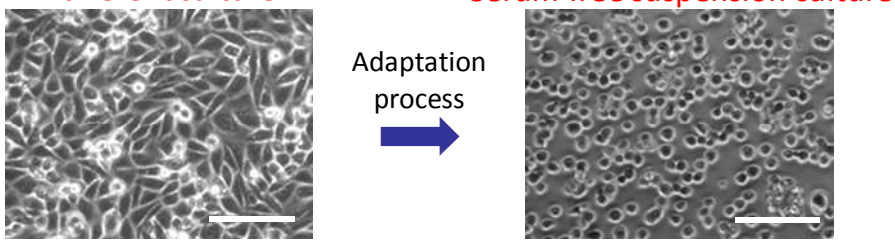
1.1.3 Recombinant hFGF23 production

1.1.3.1 Suspension adaptation

Serum-free suspension cell cultures are largely used in the recombinant protein production thanks to their advantages. They allow to obtain a higher cells density compared to adherent cultures, thus they are more easily scaled-up from small scale production to large volume bioreactors. In addition, the absence of serum in the medium promotes batch to batch reproducibility, and facilitates the downstream process for protein purification.

As stated before, the four best producing adherent CHO clones derived from transfection using PB_prom2_hFGF23R179Q and PB_prom3_hFGF23R179Q constructs plus two clones derived from transfection with pcDNA3.1_hFGF23R179Q plasmid were adapted to serum-free suspension culture using a specific medium, named ProCHO4 (Lonza), that progressively substituted the serum-containing medium. The adaptation process was cell-line dependent and it required about 2 months (see "Materials and Methods" section). Cells were repeatedly passaged until they grew as a single cell culture, without aggregates, and they reached a doubling time below 35 hours (Nielsen et al., 2010).

A



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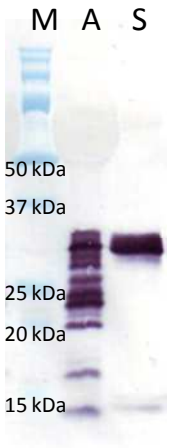


C

Vector used	Clone ID	Adherent culture yield (µg/ml)	Suspension serum-free culture yield (µg/ml)
pCDNA3.1_ hFGF23R179Q	a	6,2	13
	b	3,1	6
PB_prom2 hFGF23R179Q + Pbase (2,5:1)	a	3,4	8
	b	2,1	3
PB_prom3 hFGF23R179Q + Pbase (2,5:1)	a	6,9	11
	b	4,3	7

Fig 7. Serum-free suspension adaptation: A) Phase contrast microscopy of a CHO-K1 clone in adherent (A) and serum-free suspension (S) culture. Magnification 20x, scale bar 50 µm. B) Western blot of hFGF23 in supernatant from the same clone in adherent (A) or serum-free suspension (S) culture. C) hFGF23 quantification by KAINOS ELISA assay in adherent or serum-free suspension culture.

In figure 7A it is shown the appearance of CHO-K1 clones in adhesion and after the serum free suspension adaptation process. To verify the preservation of hFGF23 productivity during the adaptation protocol, we maintained clones also in adherent culture and we analyzed supernatants from both type of culture by western blot using a mouse serum anti-hFGF23. In both conditions the full length protein was visible at 32 kDa, other bands around it confirmed the variation in hFGF23 polypeptide O-glycosylation that was previously reported (Shimada et al., 2002). However, in adherent culture the hFGF23 protein appeared mostly degraded in fragments, while suspension cells showed only the full length polypeptide band at 32 kDa (Fig.7B). The observed degradation in adherent culture could be due to the reaching of a over-confluent growth that leads to cell death and, consequently, to proteases release.

After the adaptation process, in order to compare the productivity of adherent and suspension cultures of selected clones, we quantified hFGF23 protein in the supernatant using a commercial ELISA assay (KAINOS Laboratories). Cells were plated at same starting cell number (0,75 cells/ml) and cultured for 4 days in both conditions. Results are listed in figure 7C. Quantification revealed that all clones improved their productivity in suspension culture, confirming the best performances of this culture system compared to the adherent one. Clone (a) derived from pcDNA3.1 construct (4B.128.37) and clone (a) derived from PB_prom3 construct (8G.67.29) resulted the highest producing clones in both type of cultures. We focused on these clones for further characterization in term of stability and scale up processes.

1.1.3.2 Stability of clone transgene expression over time

One parameter to be considered for industrial recombinant protein production is the transgene expression stability over time. The two selected clones were maintained in suspension culture for over 30 passages (about 3-4 months) and hFGF23 expression was checked at the time points 11S and 30S (passages in suspension culture) by RT-PCR and Real Time PCR.

In order to amplify only the transgene of interest, PCR primers were designed on His-tag sequence (reverse primer) and on a sequence with low homology between endogenous CHO-K1 FGF23 and human FGF23 (forward primer). By RT-PCR analysis we qualitatively evaluated the hFGF23 transgene expression. Figure 8, reporting the agarose gel after electrophoresis run, showed a well-defined band at around 180 bp in samples derived from

clones 8G.67.29_S and 4B.128.37_S at the two different passages analyzed. Not-transfected CHO-K1-S sample were used as negative control, indeed only two faint and non-specific bands were detected. The RT-PCR results confirmed the long-term stable expression of hFGF23 mRNA in both selected clones.

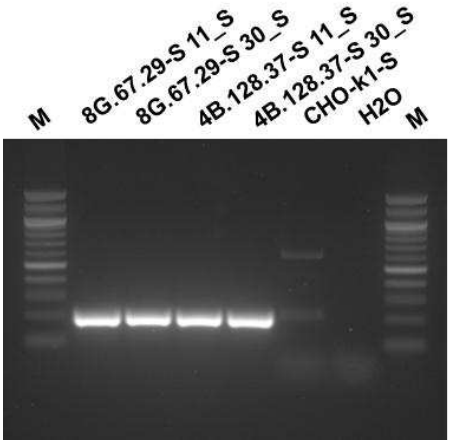


Fig 8. RT-PCR for detection of hFGF23 expression: RT-PCR analysis on RNA extracted from clones 8G.67.29_S and 4B.128.37_S after 11 and 30 passages in suspension culture. Not-transfected CHO-K1-S sample was used as a negative control.

To obtain a quantitative result, we performed also a Real Time PCR analysis, evaluating the increase or decrease of hFGF23 mRNA expression for each clone at the time point 30S compared to passage 11S. As a reference gene the CHO β2-microglobulin sequence (b2m) was selected (Ley et al., 2013). Our results (Fig.9 A and B) demonstrated the absence of statistically significant differences between the two selected passages for both clones, confirming clone expression stability. As expression level could be affected by optimizing growth conditions, we decided to continue our analysis on both clones.

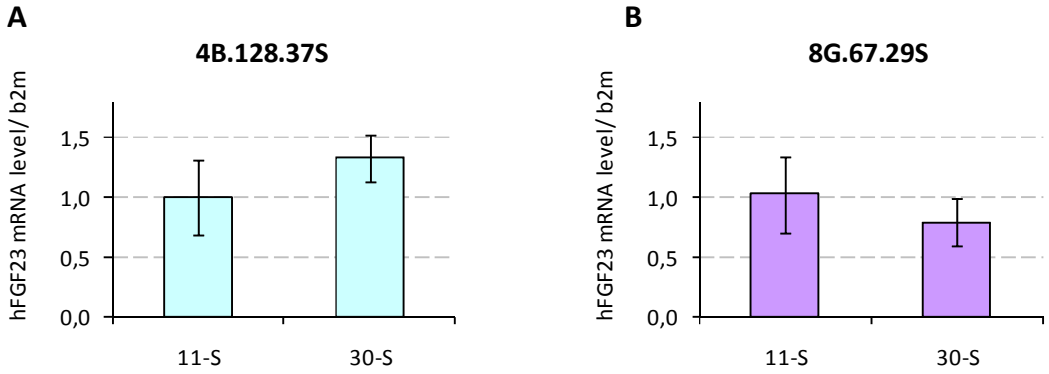


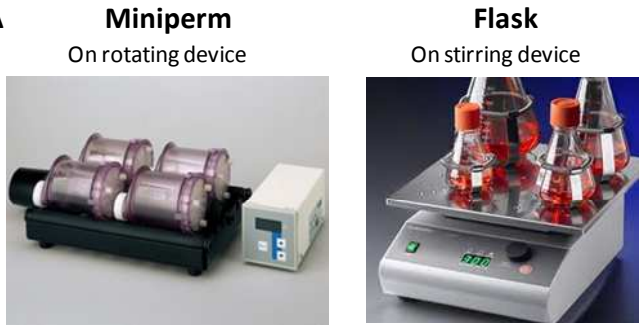
Fig 9. Real Time PCR for detection of hFGF23 expression: Real Time analysis on RNA extracted from clones 8G.67.29_S and 4B.128.37_S after 11 and 30 passages in suspension culture. A) Comparative analysis on hFGF23 expression of clone 4B.128.37_S at the time points 11_S and 30_S. Value of time point 11_S was equated to 1, while value of time point 30-S was calculated as relative increase/decrease to 11_S value. B) Comparative analysis on hFGF23 expression of clone 8G.67.29_S at the time points 11_S and 30_S. Value of time point 11_S was equated to 1, while value of time point 30_S was calculated as relative increase/decrease to 11_S value.

1.1.3.3 Small scale production of recombinant human FGF23

Generating a high producing clone through cell line selection and development makes it possible to obtain high productivity. However, to achieve cell line full potential, it is important to optimize the culture environment, providing the most favorable physical and chemical conditions for cells growth and production.
There are many different device types and media that could be used for optimizing protein productivity in culture. Thus, to select the best conditions for our clones, we decided to test some of them comparing growth and productivity performances of the two best candidate clones.

Firstly, we analyzed the effect of two devices on clone productivity. Clone 4B.128.37_S and clone 8G.67.29_S were grown in batch culture for 7 days using a Miniperm (Sarstedt) or a Flask system (Fig. 10A). The Miniperm vessel is characterized by two chambers, one with a small volume, in which are seeded the cells, and a second one in which is contained a large amount of fresh medium for the nutrient supplying. Chambers are divided by a semi-permeable membrane. In that device, gas exchange and stirring are guaranteed by means of a rotation system. Conversely, flasks are simple vessels maintained in agitation on an orbital shaker. During cultivation, both devices were kept in CO_2 incubator at the temperature of 37°C. After 7 days, clone supernatants derived from both type of cultures were collected and hFGF23 protein was quantified by KAINOS ELISA assay (Fig. 10B). Results clearly demonstrated that flask production gave the best performances for both clones, while they had a very low yield in Miniperm vessels. One possible explanation could be related to different rate of agitation and gas exchange supported by the two devices, which are greater in flask culture. As a result, flask was chosen as preferred for small scale production.

A



B

Vector used	Clone ID	Miniperm yield (µg/ml)	Flask yield (µg/ml)
pCDNA3.1_ hFGF23R179Q	4B.128.37-S	**2,3** ± 1,2	**22,1** ± 4,3
PB_prom3_ hFGF23R179Q + Pbase (2,5:1)	8G.67.29-S	**4,3** ± 1,5	**24,0** ± 5,1

Fig 10. Comparison between Miniperm and Flask devices for culture of suspension CHO cells: A) An exemplifying picture of the two different devices used for culturing hFGF23 producing clones. B) hFGF23 quantification by KAINOS ELISA assay on supernatants from clone 4B.128.37_S and 8G.67.29_S collected after 7 days of culture.

Secondly, we were interested in evaluating clone production performances in different commercial media, advertised to promote high cell density culture and to enhance protein productivity. Among all, we selected the three following media: ProCHO5 Protein-free CHO medium (Lonza) which is part of the product line of the medium used for serum free suspension adaptation; HyClone SFM4CHO (Thermo Scientific), which is formulated using a proprietary lipid complexing process for enhanced stability and growth promotion of various CHO cell lines and CellVento CHO-100 (Millipore), which is particularly rich in nutrients and suitable for batch and perfusion applications. Clones were adapted to the new media following a protocol that recapitulated the one used in the transition from adherent to suspension culture (see "Materials and Methods" section). After the adaptation period, the two selected clones were grown for 7 days in flask on orbital shaker at 100 rpm. For each culture condition, we seeded 8 x 10^5 cells/ml in 10 ml of selected medium added with supplements (125 ml Flask) (for details see "Materials and Methods" section). Cell growth was monitored every day by cell count (Fig. 11A) and hFGF23 productivity was detected by ELISA assay at the culture time points 96 and 168 hours (respectively 4 and 7 days). Results are listed in the figure 11B. As regards cell growth, ProCHO5 and Hyclone SFM4 media promoted in both clones a rapid increase in cell number

during the first days of culture, reaching a peak at around 96 or 120 hours of culture (day 4 or 5); but such increased cell density did not correlate with an augmented protein productivity compared to ProCHO4 and CellVento CHO-100 media (Fig. 11 and Table 1). Moreover, clones cultured in these two media showed a massive cell death at 168 hours of culture (7 days). On the contrary, ProCHO4 and CellVento CHO-100 media promoted a slower growth thus maintaining a higher number of viable cells for longer time. Results from KAINOS quantification, at the time point 168 hours, showed a similar productivity of the two clones in ProCHO4 and CellVento media. As both clones reached the highest productivity in CellVento CHO-100 medium, it was selected for further characterizations.

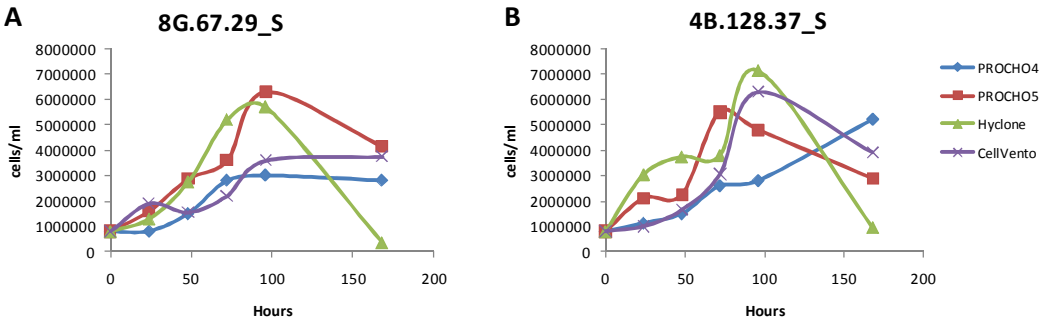


Fig 11. Comparison of clone growth performances in different media: cl. 4B.128.37_S and cl. 8G.67.29_S were grown in 125 mL flask in 4 different media (ProCHO4, ProCHO5, Hyclone SFM4 and CellVento CHO-100) for 7 days. For each culture condition, cells were counted every day and the resulting growth curves were plotted.

	ELISA hFGF23 quantification (ug/ml)							
ID clone	**ProCHO4 (Lonza)**		**ProCHO5 (Lonza)**		**HyClone SFM4 (Thermo Scien)**		**CellVento (Millipore)**	
time	96 h	168 h	96 h	168 h	96 h	168 h	96 h	168 h
8G.67.29-S	11,1	15,3	8,8	n/a	7,0	n/a	9,6	19,3
4B.128.37-S	20,7	20,9	17,9	n/a	19,9	n/a	15,5	20,7

Table 1. hFGF23 production in different media: Clone 4B.128.37_S and 8G.67.29_S were grown in 125 mL in 4 different media (ProCHO4, ProCHO5, Hyclone SFM4 and CellVento CHO-100) for 7 days. For each culture condition, supernatant were collected after 96 and 168 hours for hFGF23 quantification by KAINOS ELISA assay.

As last step of our optimization process, clones 4B.128.37_S and 8G.67.29_S performances were tested in a larger scale production. Both clones were seeded at the density of 5 x 10^5 cells/ml in 100 ml of ProCHO4 or CellVento CHO-100 supplemented media and grown for 7 days in 1L Flask on orbital shaker (100 rpm). At the time points: 0, 72, 96, 120, 144 and 168 hours, we monitored: cell growth by cell count, glucose consumption by AccuCheck glucose meter (Roche, Basel, Switzerland) and hFGF23 productivity by ELISA assay.

As regards clone performances in ProCHO4 medium, clone 8G.67.29_S cell number reached a peak at 96 hours of culture and remained constant during the following 72 hours, while clone 4B.128.37_S cells peaked at 120 and 144 hours (days 5 and 6), decreasing at day 7 of culture (Fig. 12 A and B). Since glucose consumption trend was similar, clone 8G.67.29_S seemed more resistant to nutrient depletion than clone 4B.128.37_S. However, at the growth peak, clone 4B.128.37_S cell density was higher compared to that of clone 8G.67.29_S (Fig.12 A and B). Moreover, at all analyzed time points clone 4B.128.37_S hFGF23 productivity was higher than that of clone 8G.67.29_S, as confirmed also by western blot analysis (Fig. 12C). In the western blot analysis, thanks to a highest protein concentration in 4B.128.37_S samples, it is visible not only the full length form of hFGF23 protein (32 kDa band), but also another form at 24 kDa, that probably correspondes to the not-glycosylated one (Fig. 12C).

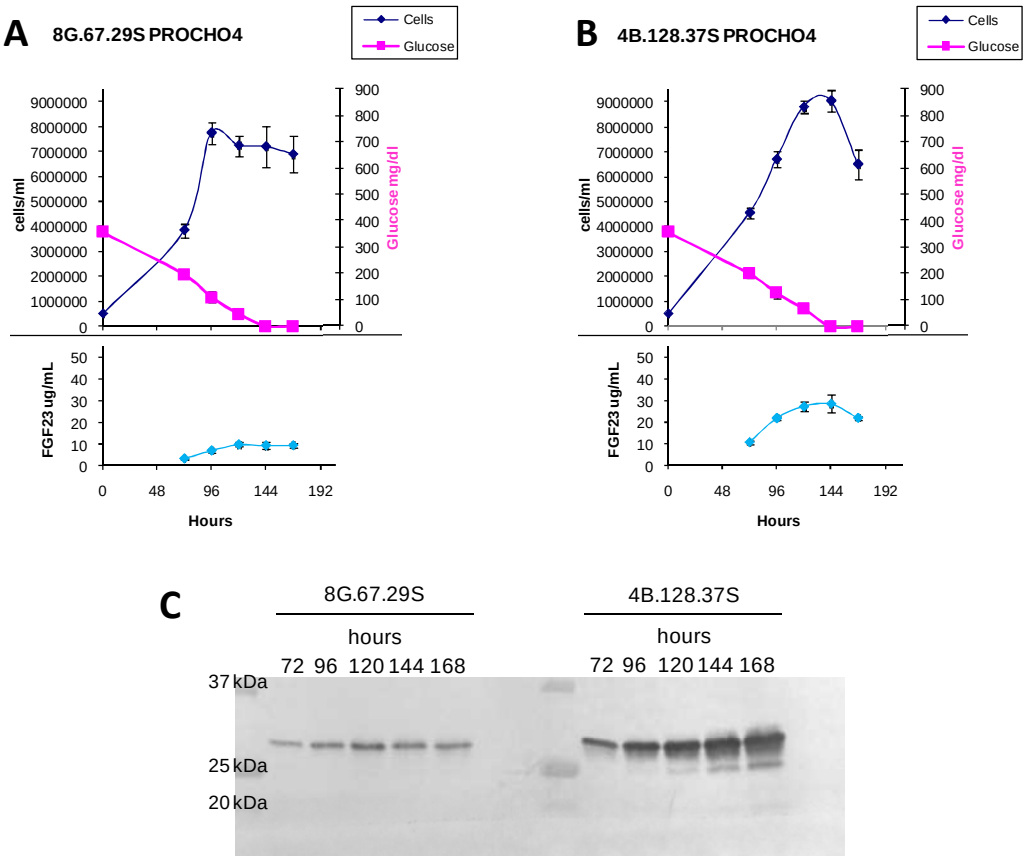


Fig 12. Growth curve and hFGF23 productivity of selected clones in ProCHO4 medium: A) cl. 4B.128.37 and B) cl. 8G.67.29 were grown for 7 days in ProCHO4 supplemented medium. For each clone cell number, glucose consumption and hFGF23 protein production were evaluated at the following time points: 0, 72, 96, 120, 144 and 168 hours. C) WB analysis of hFGF23 protein on the supernatants of cl 4B.128.37_S and cl. 8G.67.29_S at the above specified time points. Detection with mouse serum against hFGF23.

In CellVento CHO-100 medium, culture of both clones was extended at day 10 (240 hours) because the level of glucose at day 7 was still sufficient to support cell growth. In this medium, clone 8G.67.29_S cell number reached a peak at day 5 of culture, one day later compared to ProCHO4 medium, and fluctuated during the following 3 days starting to decrease at day 10 (Fig. 13A). Despite the greater glucose content in CellVento CHO-100 medium, clone 8G.67.29_S cells reached a cell density lower than that obtained in ProCHO4 medium (Fig. 12A). On the other hand, cl. 4B.128.37_S cell growth showed a sort of two waves trend, decreasing dramatically at 240 hours (day 10). In this medium, clone 4B.128.37_S attained the highest cell density (about 8.5 million cells/ml) at 168 hours of culture (7 days) (Fig. 13B). In addition, in CellVento CHO-100 medium, this clone produced higher level of full-length hFGF23 protein compared to clone 8G.67.29_S. Western blot analysis confirmed ELISA quantification data (Fig. 13C). As reported in figure 13B, clone 4B.128.37_S reached a productivity of about 50 µg/ml of protein in correspondence to the peak of cell density (day 7).

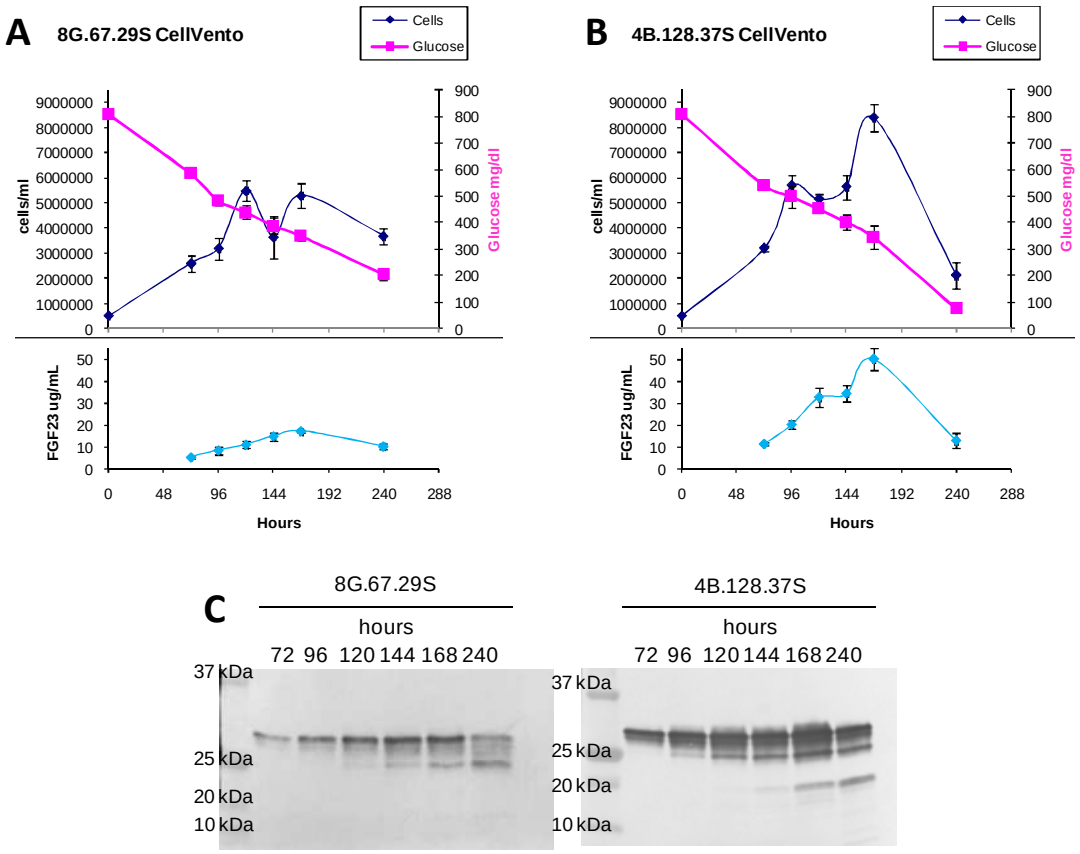


Fig 13. Growth curve and hFGF23 productivity of selected clones in CellVento CHO100 medium: A) cl. 4B.128.37_S and B) cl. 8G.67.29_S were grown for 7 days in CellVento supplemented medium. For each clone cell number, glucose consumption and hFGF23 protein production were evaluated at the following time points: 0, 72, 96, 120, 144, 168 and 240 hours. C) WB analysis of hFGF23 protein on the supernatants of cl 4B.128.37_S and cl. 8G.67.29_S at the above specified time points. Detection with mouse serum against hFGF23.

In order to further characterize selected clones we also calculated the specific productivity rate (SPR) (Table 2A). Values obtained confirmed previous data and highlighted that performances of clone 4B.128.37_S were better than those of clone 8G.67.29_S in both media tested. This observation underlines that repeated passages in media for serum-free suspension culture could affect productivity in a clone dependent manner, since the two clones, after the 2-months adaptation process, had a comparable productivity (Fig. 7C). As regard the two media, CellVento CHO-100 gave higher protein productivity than ProCHO4 medium. In particular clone, 4B.128.37_S grown in CellVento CHO-100 gave the best SPR value, 2,71 pg/cell/day, thus this combination was selected as the optimal one. In addition, we also evaluated the duplication time of both clones (Table 2B). Clone 4B.128.37_S does not change its duplication time in the two media, while clone 8G.67.29_S decreases its duplication time in CellVento CHO100 medium, which makes it not the best choice. To maintain the growth of this clone it will be necessary to test other commercial media.

A

SPR (pg/cell/day)		
	Clone	
Medium	8G.67.29S	4B.128.37S
ProCHO4	0,65	1,81
CellVento	1,35	2,71

B

Duplication time (h)		
	Clone	
Medium	8G.67.29S	4B.128.37S
ProCHO4	24,2	28,2
CellVento	37,9	27,6

Table 2. Selected clones characterization in ProCHO4 and CellVento-CHO100 medium: A) Specific productivity rate (SPR) and B) duplication time of clone 4B.128.37_S and clone 8G.67.29_S.

To support results on optimized culture conditions, 7 days batch culture of clone 4B.128.37_S in CellVento CHO-100 medium was repeated 2 more times, obtaining an average hFGF23 productivity around 35 mg/L. Since hFGF23 concentration increases/decreases with cell densities (clearly evidenced in Fig. 13B), we furthermore concluded that this protein was not accumulated during time, because of its continuous degradation. Therefore we also evaluated a 2 days batch culture inoculating a higher cell number (600 x 10^6) in CellVento CHO-100 medium. hFGF23 productivity was comparable to that observed in the 7 days batch culture, therefore we concluded that the best condition for batch culture in 1L flask was clone 4B.128.37_S grown for 2 days in CellVento CHO-100 medium.

1.1.4 Purification of recombinant human FGF23

In order to explore different strategies of hFGF23 purification, we produced several batch cultures of clone 4B.128.37_S as described before. The obtained supernatants were stored at -80°C after primary clarification by centrifugation.

The best protocol developed was based on four steps:

- **1.** Supernatant pretreatment

  CHO supernatant was thawed at +4° C for two days. Since supernatant was not clear, cellular debris were eliminated by filtration. Moreover, the sample volume was too large to be loaded on chromatographic resins therefore we reduced it down using a hollow fiber cartridge by Tangential Flow Filtration (TFF). The obtained volume was about one fifth of the starting one. Finally, sample buffer was exchanged by dialysis to a suitable buffer for the next purification step.

- **2.** Cationic exchange chromatography

  hFGF23 protein was firstly purified by cationic exchange chromatographic (CEC) resin exploiting its high isoelectric point (pI=8,72) and eluted by a sodium chlorite linear gradient (Fig. 14A). Eluted fractions containing the target protein were identified by SDS-PAGE analysis and pooled together (Fig. 14B).

- **3.** Immobilized metal ion affinity chromatography

  To further increase protein purity the pool of selected CEC fractions were purified by an immobilized metal ion affinity chromatography (IMAC), thanks to the His-Tag added at C-terminus of hFGF23 sequence. Protein elution was obtained with a linear gradient of imidazole (Fig. 15A). As already done in step 2, eluted fractions were characterized by SDS-PAGE analysis and those containing our target protein were pooled (Fig. 15B).

- **4.** Overnight dialysis and storage

  Finally, we performed an overnight dialysis to have hFGF23 protein in a suitable buffer for storage.

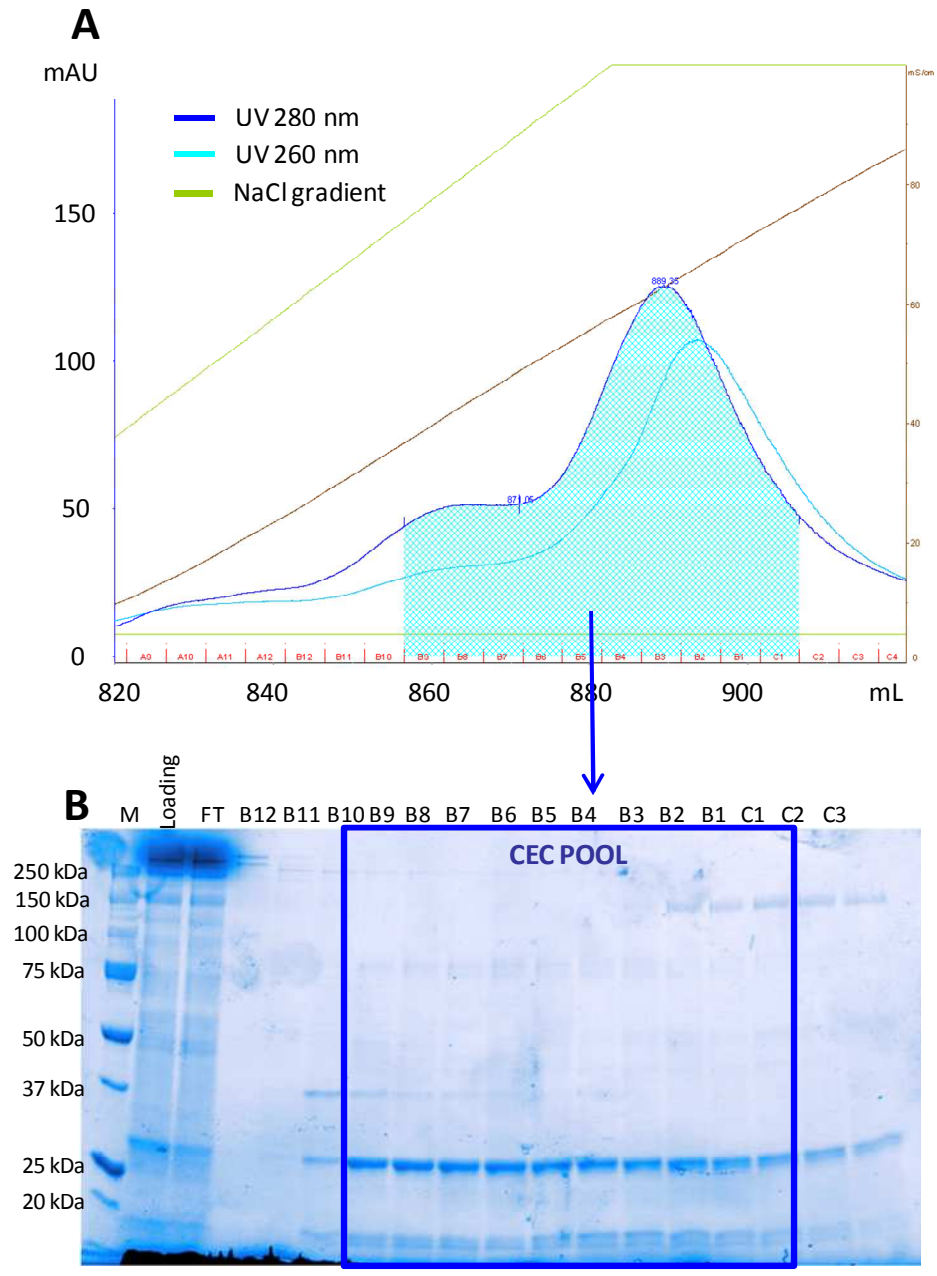


Fig 14. Cationic exchange chromatographic step: A) CEC profile, elution (magnification): in full azure are indicated eluted fractions used to establish the "*CEC pool*". B) SDS-PAGE analysis, image acquired by chemidoc instrument (Biorad). Blue rectangle indicates selected eluted fractions. On top of gel lanes are reported the name of fraction eluted by CEC. M: standard molecular weights; Loading: total sample loaded on CEC; FT: Flow through, impurities which did not bind to the CEC column

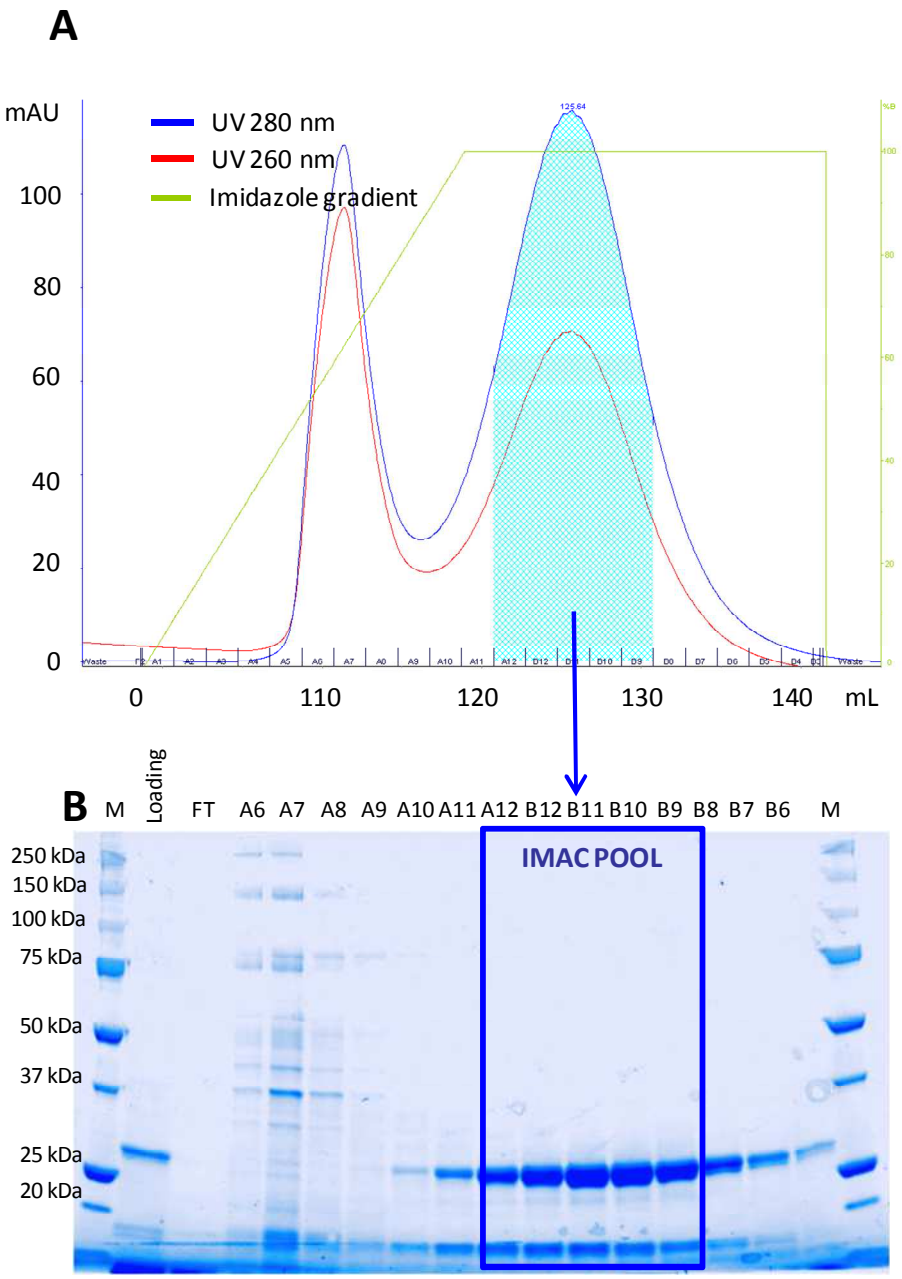


Fig 15. IMAC chromatographic step: A) IMAC profile, elution (magnification): in full azure are indicated eluted fractions used to establish the "*IMAC pool*". B) SDS-PAGE analysis, image acquired by chemidoc instrument (Biorad). Blue rectangle indicates selected eluted fractions. On top of gel lanes are reported the name of fraction eluted by IMAC. M: standard molecular weights; Loading: total sample loaded on IMAC; FT: Flow through, impurities which did not bind to the IMAC column

In order to evaluate the performance of the whole purification process, a time course analysis of each step was carried out by SDS-PAGE. Figure 16 shows that the purification steps succeeded in isolation, purification and maintenance of the full length forms of hFGF23 protein, visible at 32 kDa. The not-glycosylated protein was also present in all samples around 24-25 kDa as a faint band. Other bands, under 20 kDa, were the only

contaminants present in the purified sample. Since in western blot analysis those bands resulted immunostained by an anti-hFGF23 mouse serum (data not shown), we concluded that they were degraded hFGF23 forms. Despite these contaminant bands, the final hFGF23 protein had a good purity, estimated above 90% by SDS-PAGE analysis (Fig. 16).

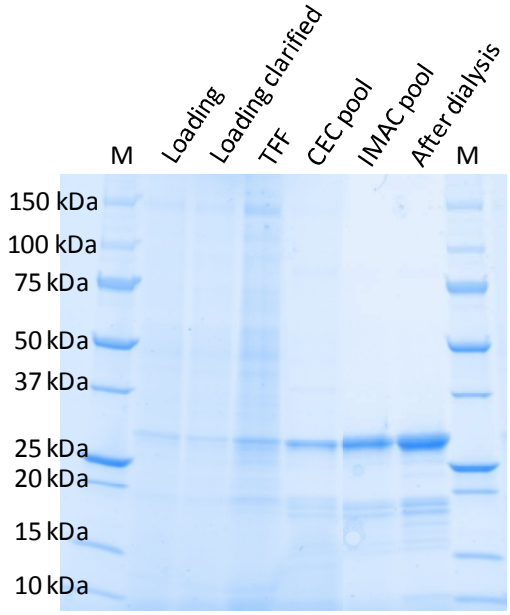


Fig 16: Purification steps: SDS-PAGE analysis, image acquired by chemidoc instrument (Biorad). M: standard molecular weights; Loading: total sample thawed; FT: Flow through, impurities which did not bind to the IMAC column; TFF: tangential flow filtration; CEC: cationic exchange chromatography; IMAC: Immobilized metal ion affinity chromatography.

In order to prove the robustness of our purification protocol, three different batches were processed. In table 3 are reported the hFGF23 recovery yields quantified by ELISA assay for each purification. We obtained 4-6 mg of hFGF23 purified protein, a sufficient amount for DiaSorin annual requirements. The purified protein will be used as control and calibrator in a chemiluminescent immunoassay (CLIA) for hFGF23 detection in serum and plasma specimens.

Batch	Supernatant Volume	Total hFGF23 (supernatant)	hFGF23 Purified	Yield
RMP024	1850 mL	72,2 mg	4,0 mg	5,5 %
RMP026 -27-28	1150 mL	32,8 mg	5,7 mg	15,4 %
RMP025	1850 mL	69,3 mg	4,4 mg	6,3 %

Table 3: Purification yield: Starting volume of supernatant and hFGF23 quantification by KAINOS ELISA assay on culture supernatant and on purified protein. Yields of purification were calculated on the basis of ELISA assay result.

1.1.5 Stability of hFGF23 recombinant protein

All recombinant proteins have a shelf life. Its extension depends on the nature of the protein and the conditions used for storage. In order to retain their original structure, integrity and activity, it is necessary to find additives to assure proteins stability and eventually aid the lyophilisation process. Many compounds can be added to protein solutions to lengthen shelf life such as metal chelators and reducing agents to maintain the protein in its reduced state, anti-microbial agents, protease inhibitors, cryoprotectants and commercial cocktail for protein stabilization (Back et al., 1979). Moreover it is often useful the addition of amino acids, sugars and polymers (Schein, 1990; Kaushik and Bhat, 2003; Hamada et al., 2009).

Taking into account literature advices, we developed a specific basal solution for hFGF23 recombinant protein storage, named Buffer A, that was tested for protein storage at 4°C or for lyophilization. As regards the protein stability at 4°C, Buffer A was tested alone or supplemented with the following additives: protease inhibitors cocktail, glycerol at 10% or 50%, a second buffer (Buffer B) and the amino acid glycin. hFGF23 was diluted at a final concentration of 1500 µg/ml in all the selected conditions and stored for 4 months at 4°C. At different time point, 0, 5, 14, 90 and 120 days of storage, hFGF23 quantification was performed by the prototype immunoassay on Liaison platform (Fig. 17). As shown in the graph, Buffer A was not sufficient to preserve hFGF23 protein at 4°C for long term, although it was able to maintain up to 60% of initial quantity for 90 days. The addition of protease inhibitors accelerated hFGF23 degradation, instead of protecting it, probably due to other components present in their buffer. The same behavior was observed when glycin was added to buffer A, in this case, about 60% of protein was already degraded after 5 days. On the contrary, the use of glycerol at both concentration tested or buffer B allowed to stabilize the recombinant protein in solution. In particular, hFGF23 stored in buffer A plus 50% of glycerol or Buffer B was maintained up to 80% for 90 days and decreased to 60% at 120 days. Therefore the solution composed by Buffer A added with Buffer B was chosen as best storage condition at 4°C.

Next, we evaluated the stability of hFGF23 after lyophilization process in buffer A, since lyophilization allows for long-term storage of proteins with the advantage of less difficulties in stocking and transport than temperature controlled kits (i.e. 4°C).

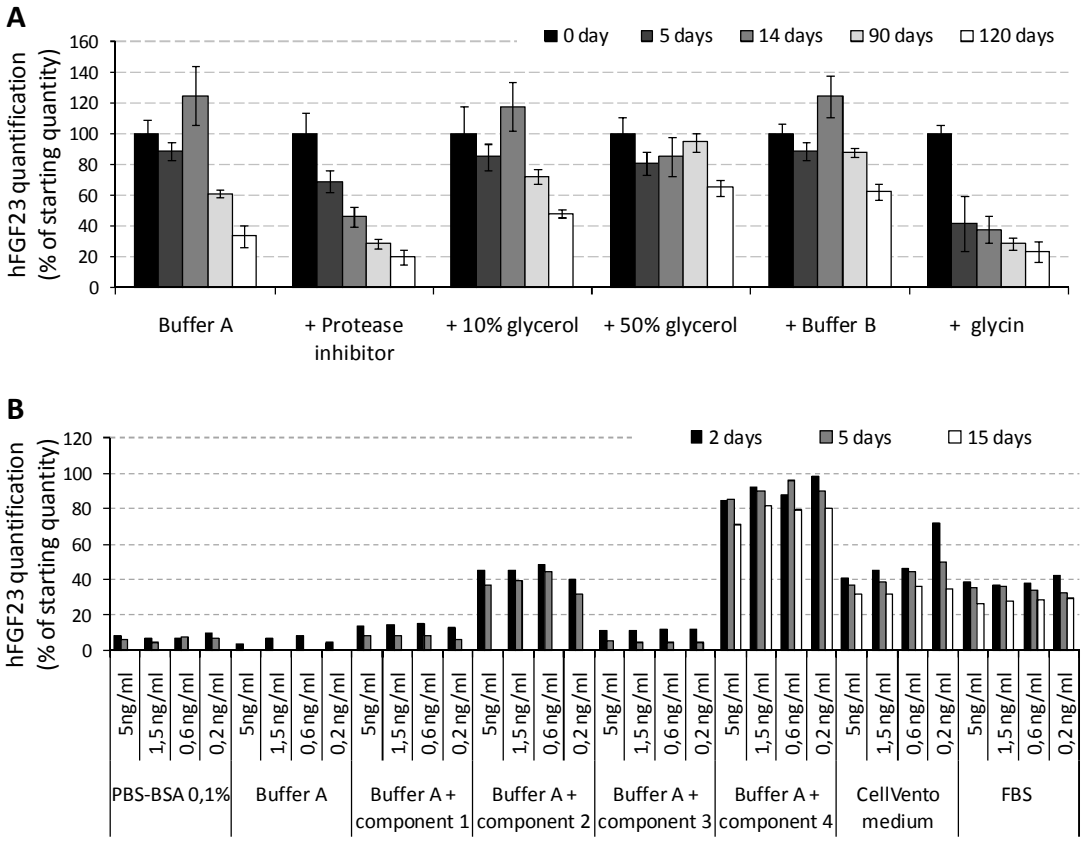


Fig 17. Recombinant hFGF23 protein stability: A) Quantification of purified hFGF23 stored at 4°C in several buffers. Buffer A or Buffer A plus one of the following substances: protease inhibitor, 10% glycerol, 50% glycerol, buffer B and glycin, were used to stabilized hFGF23 in solution. Protein quantifications were performed by the hFGF23 prototype immunoassay (DiaSorin) on Liaison platform at different time point: 0, 5,14, 90 and 120 days.
B) Quantification of lyophilized hFGF23 resuspended in several buffers at low concentrations. Buffers used were: buffer A, buffer A added with component 1, 2, 3 or 4, CellVento CHO100 medium or fetal bovine serum (FBS). Protein quantifications were performed by the hFGF23 prototype immunoassay (DiaSorin) on Liaison platform at different time point: 2, 5 and 15 days. Data were reported as percentage of the quantification at time point 0.

We lyophilized hFGF23 at the concentration of 1,5 µg/ml. After storage at -20°C, protein was thawed and resuspended in PBS at the same concentration. Then it was diluted at 5, 1.5, 0.6 and 0.2 ng/ml in different buffers, in order to create the dilution conditions that should be used in the final assay. The aim of this experiment was to identify the best buffer for protein stabilization after lyophilization process. The buffers evaluated were based on buffer A added with different components, referred to as 1, 2, 3, 4, or more complex solutions, such as CellVento CHO-100 medium and fetal bovine serum (FBS). In this case hFGF23 quantification was performed on Liaison platform at time points 2, 5 and 15 days of storage at 4°C after protein resuspension and dilution. As shown in figure 17B lyophilized protein was rapidly degraded in the majority of the tested buffers and the different dilutions didn't affect this behavior. Buffer A added with component 2, as well as CellVento

CHO100 medium and FBS, allowed to preserve about 40% of the protein after 15 days. Anyway, the condition that maintained hFGF23 at nearly 80% after 15 days of storage at 4°C was buffer A plus component 4. Although further aspects had to be optimized, we were able to identify preliminary conditions in which long-term storage of hFGF23 protein is possible.

1.1.6 Characterization of purified hFGF23

The automated prototype CLIA immunoassay for hFGF23 quantification in serum and plasma samples, developed at DiaSorin Research Center, was designed as a "sandwich" immunoassay, exploiting two antibodies directed to hFGF23 protein. As described before (Fig. 6), it was able to recognize and quantify recombinant hFGF23 protein in culture supernatant. In order to assure that the purification process did not affect the protein recognition by the antibodies used in the DiaSorin prototype, we performed the analysis on the Liaison automated platform. Our recombinant protein was tested in comparison with a commercial mammalian hFGF23. Different dilutions of the purified protein were compared to those of the commercial one (Fig. 18). The two proteins gave similar Liaison signal (RLU) and, most of all, the purified protein maintained linearity throughout the serial dilutions, thus confirming that it was correctly detected by Liaison analyzer.

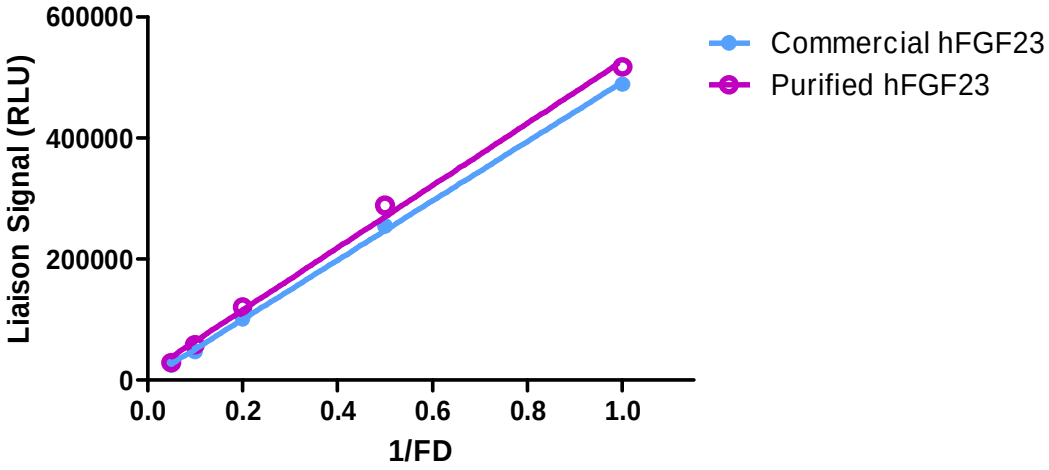


Fig 18: Characterization by a DiaSorin CLIA immunoassay: Purified hFGF23 (violet) and a commercial hFGF23 (light blue) were diluted and analyzed by an automated prototype CLIA immunoassay for hFGF23 quantification in serum and plasma samples developed at DiaSorin Research Center. FD: dilution factor; RLU: relative light unit.

Moreover, to further investigate the quality of the recombinant protein, we evaluated its functionality by a proliferation assay using NIH/3T3 mouse embryonic fibroblast cells. FGF23, in presence of its co-factor Klotho, induces a mitogenic response mediated by phosphorylation of extracellular signal-regulated kinase (ERK), and the consequent increase in Egr-1 (early growth response 1) gene expression (Medici et al., 2008).

To determine the biological activity of purified recombinant hFGF23, NIH/3T3 cells were serum starved at 0,2% FBS for 16 hours and then stimulated with different concentrations of the protein in presence of Klotho at 0,3 µg/ml (Fig. 18). We also added heparin at a relatively high concentration of 10 µg/ml because it has been previously reported to enhance the activity of FGF19 family members, in particular stabilizing hFGF23-Klotho-FGFR1 (FGF receptor 1) complex (Urakawa et al., 2006; Wu et al., 2007). At 24 hours, as expected, NIH/3T3 cell proliferation was induced by hFGF23 in a dose-dependent manner compared to control cells treated only with Klotho and heparin. In absence of Klotho, treatment with hFGF23 at 2,4 µg/ml surprisingly induced a response compared to not treated cells. The same effect didn't occur at a lower concentration. The reason of NIH/3T3 cells proliferation at the highest concentration of hFGF23, despite Klotho absence, could be due to hFGF23 interaction with soluble bovine Klotho protein, present in serum used for cell culture. However the cell proliferation induced by hFGF23 at 2,4 µg/ml in presence of Klotho was significantly higher respect to all controls.

In conclusion, our results indicate that we have obtained a properly folded protein, able to be recognized by specific antibodies and to have a biological activity.

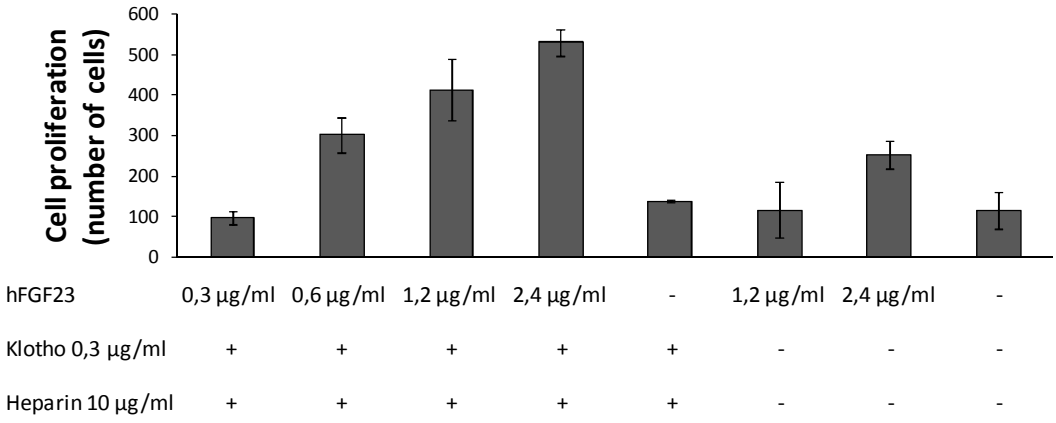


Fig.18 hFGF23 biological activity: cell proliferation induced by different doses of recombinant hFGF23 (0.3, 0.6, 1.2 and 2.4 µg/ml). NIH/3T3 fibroblast cells were serum starved at 0,2% FBS for 16hr and then stimulated with purified hFGF23R179Q in presence of Klotho (0.3 µg/ml) and heparin (10 µg/ml). After 24 hours, cells number was measured by a proliferation assay (Dojindo). Results were expressed as means ± SD (n = 5).

1.2 Transmembrane hFGF23 protein

Once demonstrated the feasibility of recombinant protein production using a transposon based technology, we also evaluated the possibility to exploit our optimized transposon system to target recombinant proteins to the surface of mammalian cells.

The final aim of this experiment was to bypass any need for recombinant protein purification required for mice immunization and hybridoma screening during monoclonal antibody generation (Dreyer et al., 2010). It is often difficult to purified recombinant protein in native conformation and in sufficient yield for mice immunization. Therefore, instead of purified proteins, it could be possible to inject a stable mammalian cell line expressing the antigen of interest on the cell surface. In particular, we decided to express mutated form of hFGF23 on CHO cells membrane. To this end, we cloned in the PB_prom3 vector the mutated hFGF23 gene in frame with a unique N-terminal secretion signal and the C-terminal transmembrane anchoring domain of a specific receptor (TM domain). We selected PB_prom3 vector because it was the transposon that allowed to obtain the highest productive clone in previous experiments. In order to localize protein expression, a hemagglutinin A epitope (HA) tag was inserted at the N-terminus of the protein and later used for protein detection.

CHO-K1 cell line, grown both as adherent (CHO-K1) and suspension (CHO-K1-S) culture, were transfected with PB_prom3_hFGF23_TM vector using a transposon/transposase molar ratio of 2,5:1. Once established a stable cell pool, the expression of recombinant hFGF23 on the cell surface was confirmed by immunofluorescence using anti-HA tag antibody (Fig. 19). In adherent cells, transmembrane hFGF23 expression was localized mainly in surface focused points, while in suspension CHO-K1-S cells we observed a more diffused surface expression, which appeared more evident on the edges. In order to further characterize these cell lines, we analyzed the expression of transmembrane hFGF23 by the prototype CLIA immunoassay described before. Cell suspension dilutions (both for CHO-K1 and CHO-K1-S) were used as samples. In both cases transmembrane hFGF23 protein was detected (Table 4), thus confirming that it is recognized by the antibodies used in the immunoassay and suggesting a proper protein folding. In addition, the linearity of dilution series were detected until 1:1000 point, therefore results obtained were quantitative. CHO-K1-S cells had the highest expression of transmembrane hFGF23 and were used as immunogen in mice immunization experiments.

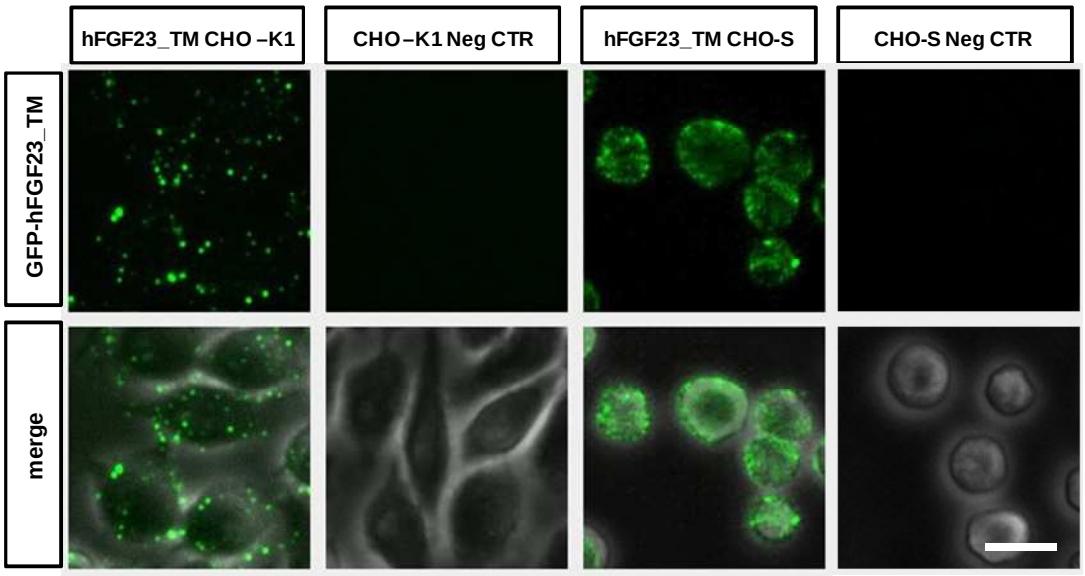


Fig 19: Cell-surface expression of hFGF23 on stably transfected CHO cells: First raw, immounofluorescence of adherent (CHO-K1) or suspension (CHO-K1-S) cells using chicken Ab anti-HA tag and AlexaFluor 488-labelled anti-chicken IgG antibodies. Second raw, merge between immounofluorescence images and phase contrast image. Magnification 20x, scale bar 10 um.

	RLU	
Cells dilution factor	hFGF23_TM CHO-K1	hFGF23_TM CHO-K1-S
1/10	614.633	916.412
1/100	51.180	91.563
1/1000	6.112	9.591
1/10000	1.738	2.189

Table 4: Quantitative analysis of transmembrane hFGF23 expression: CHO-K1 and CHO-K1-S cells were serial diluted and analyzed on Liaison platform with the automated prototype CLIA immunoassay for the hFGF23 quantification. RLU: relative luminescent unit

Three New Zeland black NZB/BJNG mice were treated following the immunization protocol described in Materials and Methods. Briefly, 5 x 10^6 CHO-K1-S cells expressing hFGF23 on their surface were resuspended in PBS and used directly as immunogen for subcutaneos injections. No adjuvants were added to the solution. The mice serological responses, shown in Figure 20, were tested by ELISA assay on our purified recombinant hFGF23. All sera showed a reactivity on purified hFGF23, with a good titer in comparison to serum from not immunized animal (NMS). After the second immunization, mouse #238 showed the highest titer (#238.1). Unexpectedly, after about 6 immunogen boosts in 6 months, the sera from this mouse (#238.3) displayed a binding activity lower than the first one. On the contrary,

mouse #236 showed an increased reactivity on the immunogen, reaching a titer of 1000. Those titers were not as high as that obtained from a mouse immunized with purified hFGF23 (AT fusion), in fact the dose-response curve didn't display a plateau for higher serum concentration.

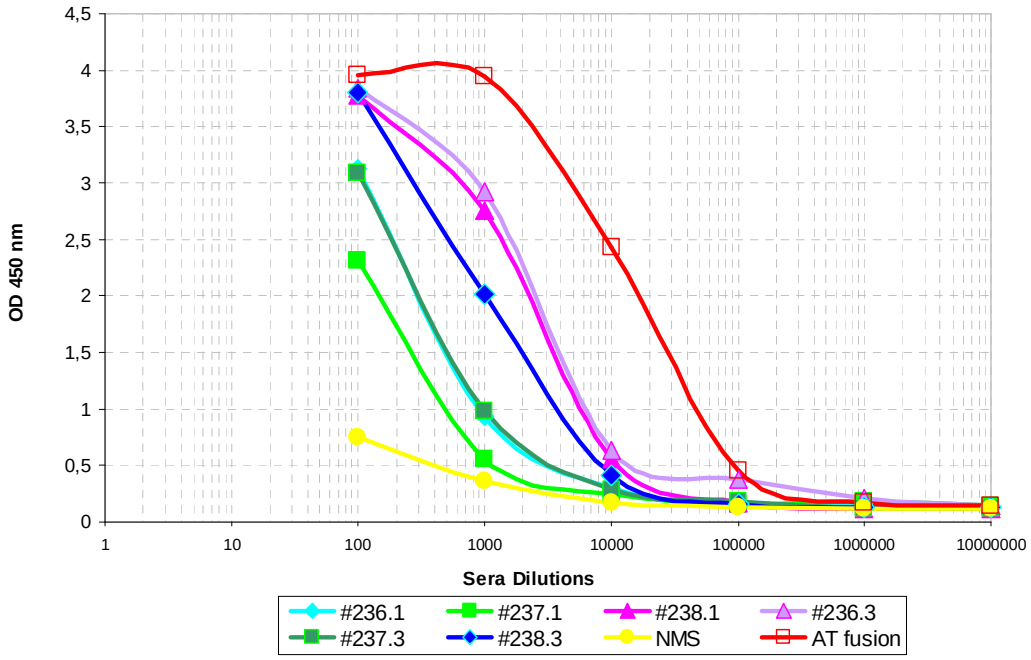


Fig 20. Evaluation of mouse serological responses: ELISA assessment of anti-hFGF23 antibody titer in serum of mice (#236, #237 and #238) immunized with CHO-K1-S cells expressing hFGF23 as a membrane protein. Data were reported as titration curves of the first (236.1, 237.1, 238.1) and third bleedings (236.3, 237.3, 238.3), respectively at 40 and 179 days from the beginning of the immunization protocol. NMS: normal mouse serum; AT fusion: serum from mice immunized with recombinant hFGF23 (positive control).

Although we have to improve the immunization protocol, results obtained were promising and demonstrated that this immunization strategy could offer a rapid and efficient method to obtain murine immune response for monoclonal antibodies generation, without the need of recombinant antigen purification.

Part 2: Recombinant antibodies: expression of a chimeric IgG in CHO cells

Recombinant antibody technology has tremendous application in the field of diagnostics. The generation of chimeric antibodies with human constant regions is a great improvement in immunoassay development because they can be used as calibrators or controls instead of human antibodies derived from seropositive patients. Moreover, chimeric antibodies can be exploited for isotype switching or to recover monoclonal antibodies produced by instable hybridomas.

Here is presented the feasibility of this latter case. As a model, it was used a mouse monoclonal antibody, named hTg5, directed against human thyroglobulin previously generated by hybridoma technology at DiaSorin Research Center.

2.1 Parental hTg5 mAb characteristic

hTg5 monoclonal antibody is an IgG2a/k immunoglobulin directed against human thyroglobulin. It is produced by H18.1.2 hybridoma cell line generated at DiaSorin Research Center by fusion of murine spleen cells with Ag8 myeloma cells with classical hybridoma technology (Köhler and Milstein, 1975). Its affinity was assessed by SPR technology resulting in a KD value in the picomolar range.

Evaluation of its performance on Liaison analyzer revealed that hTg5 mAb has remarkable potentiality if used as tracer in a prototype assay for the detection of human thyroglobulin in serum and plasma. Its superiority was due to a strong increase in the prototype sensitivity, reaching a functional sensitivity of 0,115 ng/ml way below the expected target (0.5 ng/ml). For this reason it was chosen as candidate tracer. Unfortunately, the production of this mAb was complicated by the instability of H18.1.2 hybridoma, which lost its expression over time. Several attempts of subcloning and different methods of production were evaluated without any improvement. Since the noteworthy properties of hTg5 mAb, we decided to express a recombinant hTg5 antibody in CHO-K1 cells.

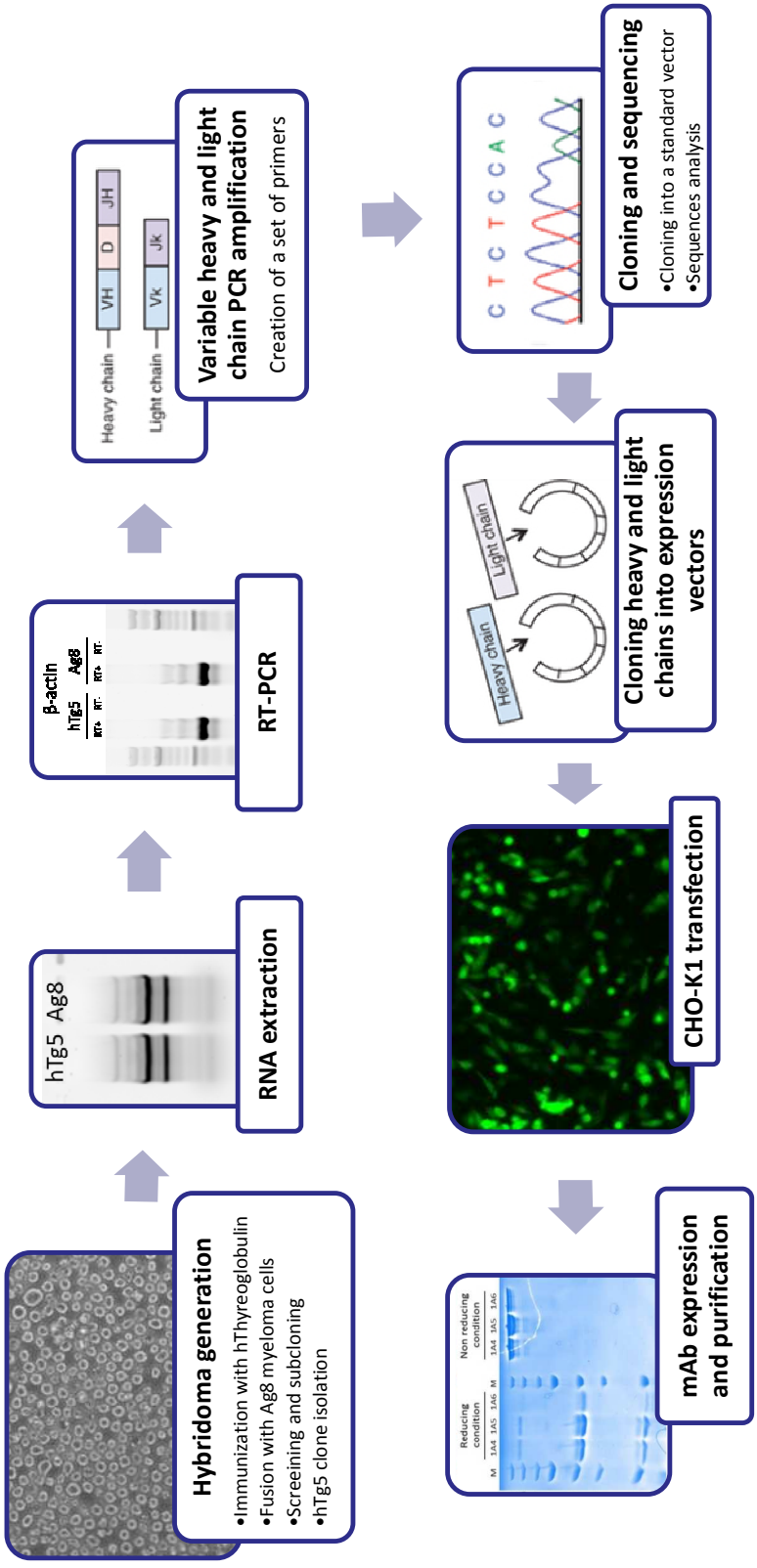


Fig 21: Scheme of the recombinant antibody development: Starting from murine hybridoma to chimeric antibody purification.

2.2 Cloning of hTg5 mouse antibody variable regions

The construction of chimeric antibody (Fig. 21) entails cloning and ligation of the variable region fragments of mouse monoclonal antibody with heavy and light chain immunoglobulin constant regions. For this purpose, it is necessary to isolate from hybridoma cells the light and heavy chain variable regions (V_L and V_H) in order to identify their sequences.

2.2.1 Hybridoma RNA Extraction and cDNA synthesis

Genomic DNA extracted from hybridoma can not be used for cloning V_L and V_H genes, because it contains also numerous non-transcribed germ-line variable gene segments that are not involved in the final rearranged genes. For this reason it is used total RNA.

In our attempt to isolate the sequences of hTg5 variable regions, total RNA was extracted from H18.1.2 hybridoma cells. It is well known that myeloma partners used for fusion procedure express aberrant light chain, even thought the transcripts are not translated (Carroll et al., 1988). Therefore, total RNA was also extracted from Ag8 myeloma cells, in order to exclude the amplification of aberrant chains in the following experiments. After RNA quantification, first-strand cDNA was synthesized by reverse transcription. β-actin gene was amplified as an internal control (data not shown). The cDNA template was used in the amplification of the mouse variable heavy and light chain genes applying proper designed primers.

2.2.2 PCR optimization for variable heavy and light chain amplification

The development of a PCR that can amplify mouse rearranged and expressed V_H and V_L sequences belonging to any V gene family is complicated by the degeneracy of the genetic code and by the number of V gene segments, which are more than 100 (Thiebe et al., 1999). The success to obtain a correct sequence for each immunoglobulin variable region mainly depends on the selection of a right primer set and on the optimization of PCR conditions. Many published studies have developed different primer sets for amplifying the immunoglobulin variable domains (Orlandi et al., 1989; Essono et al., 2003). In my work, in order to identify hTg5 V_H and V_L domains we designed two set of forward primers based on those described by Essono et al., 2003. These primers are complementary to sequences in

the framework one (FR1) region that is relatively conserved within each V gene family. 26 forward primers were designed to amplify heavy chain variable regions and 39 forward primers were designed to amplify light chain variable regions, called respectively OVHn and OVKn primers. Those were used in combination with a reverse primer complementary to sequences contained either in the framework 4 (FR4) regions (J_H or J_L fragment), or in isotype-specific constant regions. These regions have a high degree of conservation, hence a unique primer is sufficient to obtain amplification of any V_H or V_L fragment (Fig. 22).

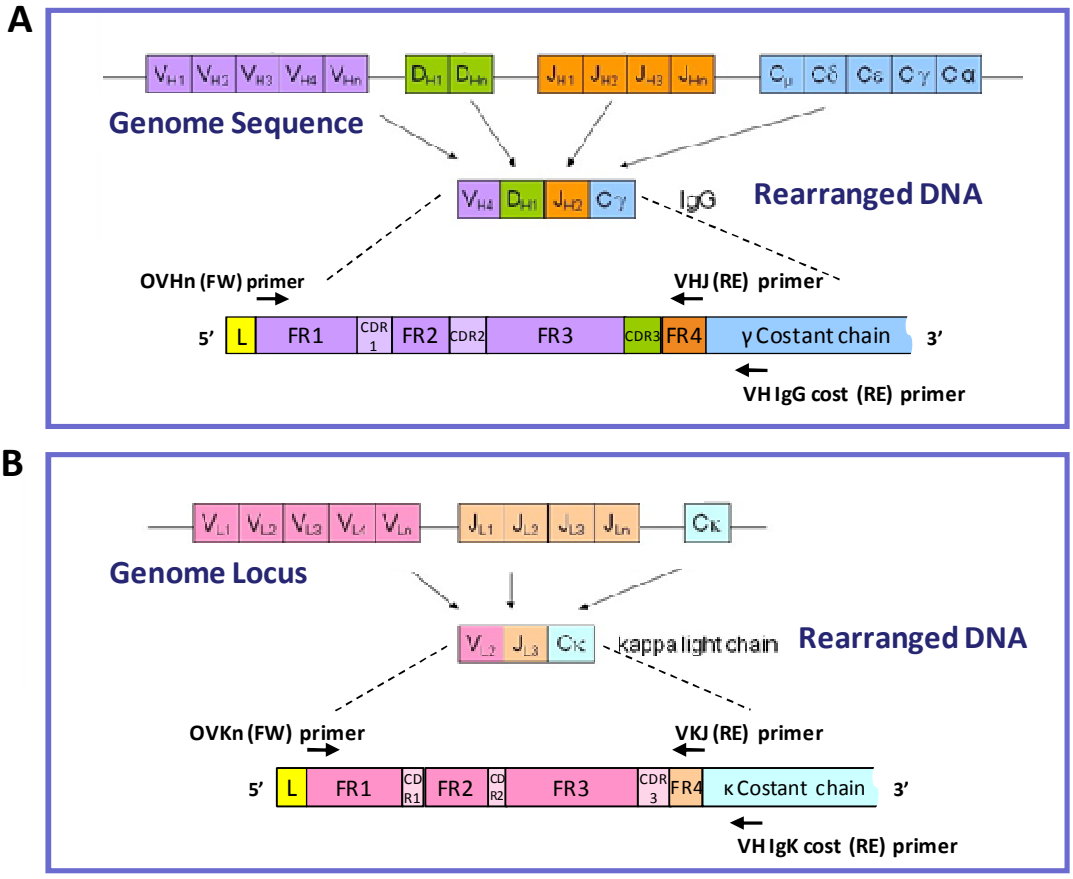


Fig 22: Schematic representation of antibody heavy and light chain variable region rearrangement with primer annealing sites: Organization of the (A) gamma heavy and (B) kappa light chain genes in the germ line and their final rearrangements in the mature lymphocyte. In order to amplify the variable regions, several primer pairs were designed. Primer annealing positions are shown as arrows. FW: forward primer, RE: reverse primer, FR:framework region, CDR: complementarity determining region.

As illustrated in figure 23 we performed PCRs at two different temperatures, 62°C or 68°C, using all possible combination of designed forward primers with the two reverse primers annealing on either FR4 (J region) or constant region, named respectively VHJ and VHcost (or VKJ and VKcost). Heavy chain variable regions could be successfully amplified with only 3 forward primers, among the 26 designed, with differences in their performances depending on the reverse primer and temperature used (Fig. 23A and B). In particular, VHJ

reverse primer allowed to amplify the 348 bp expected sequence only using OVH2 forward primer at 68°C. Moreover, the amplicon was present not only in hTg5 sample, but also in myeloma one (Fig. 23A). On the contrary, VHcost primer, complementary to constant IgG region allowed to obtain an amplicon only for hTg5 samples using OVH5 or OVH13 forward primers at 62°C (Fig. 23B).

The same scheme of PCRs was performed for kappa light chain variable region. However, amplification of this sequence applying reverse primer complementary to the FR4 region (VKJ) was not successful at either tested temperatures (data not shown). This problem was solved by using the reverse primer, complementary to mouse Ig kappa constant chain at 62°C (Fig. 23C). Among the 39 forward primers, eleven were able to yield amplification, although not all amplified a productive IgK fragment as demonstrated by sequence analysis (table 5B). In figure 23C were reported the most significant PCR products obtained.

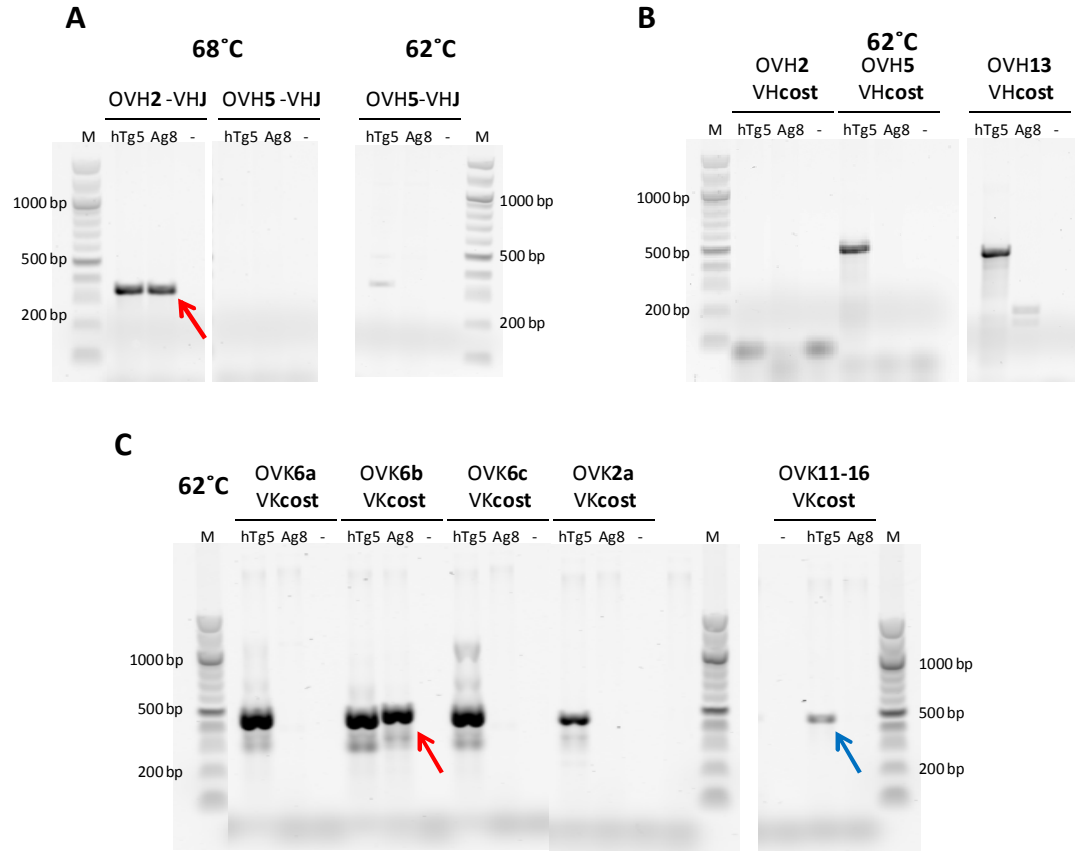


Fig 23. Amplified antibody variable regions: 2% agarose gel electrophoresis images of most significant PCRs performed for heavy chain (A and B) and light chain (C) variable gene amplification. PCRs were done at 62 or 68°C, exploiting a reverse primer on either J region (VHJ) or constant region (VHcost or VKcost). Ag8 aberrant chains are shown as red arrows, while hTg5 V_K unproductive sequence is indicated by a blue arrow. Amplicon lengths are VHJ: 348 pb, VHcost:484 bp, VKcost: 450 bp.

As described before, one problem in this process is the frequent amplification of aberrant variable regions, resulting from non productive allele rearrangements. This unsuccessful VDJ recombination is often due to aberrant chains originated from MOPC21 derived fusion partner, such as the Ag8 myeloma cells used in this work (Carroll et al., 1988). Furthermore, those sequences are usually more expressed than functionally rearranged antibody genes.

For that reason, generated amplicons for both V_H and V_K regions were cloned into the TOPO TA-cloning pCRII vector and sequenced. Sequence alignments revealed several different single base mutations, probably due to DNA polymerase-induced errors. Once identified the most frequent base for each position, IMGT online database (international ImMunoGeneTics information system) was exploited to find the VDJ fragment combination that mainly correspond to our sequence. Moreover, the sequence productivity was evaluated by IMGT tools in order to exclude the presence of frame shift mutations, stop codons, out-of-frame junction (in J fragment), defects in the splicing sites or other possible anomalies. In table 5 are reported information about each identified sequence. We concluded that the sequence identified for hTg5 heavy chain variable region codified for a productive rearranged sequence, constituted by V_H6-J_H4-D_H2 fragments. On the contrary, the sequence obtained from myeloma amplicons was an unproductive one, due to out-of-frame junction (Table 5A). On the other hand, light chain sequence identification was harder because three different results were obtained from sequencing. Through alignment analysis and IMGT tools we discovered that hybridoma cells not only expressed a productive sequence, but also an out-of-frame rearranged one (Table 5B). This latter sequence was amplified by different primers more frequently than the correct one, making difficult its identification. Furthermore, Ag8 myeloma cells also expressed an aberrant light chain variable region, which was different from that expressed by hTg5 hybridoma cells. After all these analysis, we were confident that the correctly rearranged fragments of the hTg5 light chain variable region were V_K6-J_k4.

Once identified the hTg5 V_H and V_K coding regions, their sequences were synthesized by GeneArt in order to be cloned in suitable vectors.

A

	hTg5 VH sequence		Ag8 VH sequence	
V-GENE and allele	Musmus IGHV6-6*01 F	94.90% (279/294 nt)	Musmus IGHV2-6*02 F	100.00% (285/285 nt)
J-GENE and allele	Musmus IGHJ4*01 F	81.48% (44/54 nt)	Musmus IGHJ3*01 F	80.56% (29/36 nt)
D-GENE and allele	Musmus IGHD2-2*01 F	D-REGION is in reading frame 2	Musmus IGHD5-5*01 ORF	D-REGION is in reading frame 3
FR lengths	[25.17.38.11]		[25.17.38.7]	
CDR lengths	[8.10.10]		[8.7.X]	
	Productive IGH rearranged sequence (no stop codon and in-frame junction)		**Unproductive IGH rearranged sequence** (out-of-frame junction)	

B

	hTg5 VK sequence			
V-GENE and allele	Musmus IGKV6-15*01 F	94.62% (264/279 nt)	Musmus IGKV4-57-1*01 F	98.23% (277/282 nt)
J-GENE and allele	Musmus IGKJ4*01 F	97.14% (34/35 nt)	Musmus IGKJ2*01 F	100.00% (35/35 nt)
FR lengths	[26.17.36.10]		[26.17.36.10]	
CDR lengths	[6.3.9]		[7.3.X]	
	Productive IGK rearranged sequence (no stop codon and in-frame junction)		**Unproductive IGK rearranged sequence** (stop codons,out-of-frame junction)	

	Ag8 VK sequence	
V-GENE and allele	Musmus IGKV3-12*01 F	97.25% (283/291 nt)
J-GENE and allele	Musmus IGKJ2*01 F	94.29% (33/35 nt)
FR lengths	[26.17.36.10]	
CDR lengths	[10.3.X]	
	Unproductive IGK rearranged sequence (out-of-frame junction)	

Table 5. Variable regions sequence analysis: IMGT tools were exploited to analyze (A) heavy chain and B) light chain sequences. Identified V, (D) and J fragment allele with nucleotide identity were reported. Framework region (FR) and complementarity determining region (CDR) lengths were also reported.

2.3 Construction of chimeric hTg5 antibody genes

Chimeric antibodies are usually composed by variable regions of one organism and costant regions from another species. Conversely, our recombinant antibody model was a mouse-mouse chimeric antibody due to its application in the diagnostic immunoassay for the quantification of human thyroglobulin in serum and plasma specimens.

In order to prove that the mAb gene cloning, engineering and expression technology in CHO-K1 cells works in our hands and it is applicable for the generation of any type of chimeric antibody, we decided to modify the recombinant hTg5 antibody by introducing an isotype switch from IgG2a to IgG1.

Our strategy for generation of chimeric antibodies involved insertion of the mouse V-region sequence into eukaryotic expression vectors containing mouse constant region coding elements. Among commercial plasmids, pFUSE vectors (InvivoGen) were chosen. Since the generation of a complete recombinant antibody requires the expression of both heavy and light chains, we selected one pFUSE plasmid containing mouse IgG1 constant heavy chain and another one containing mouse IgK constant light chain. In order to allow the efficient secretion of the recombinant antibody chains, we preferred pFUSE vectors that contain interleukin 2 (IL2) signal sequence. In fact, we were unable to determine the native sequence of leader peptide since our set of 5' primers were designed on the framework region 1. hTg5 variable region sequences, synthesized by GeneArt, were cloned in frame in the corresponding plasmid between the leader peptide and the constant region coding fragment, as described in "Materials and Methods" section. Obtained plasmids were named respectively pFUSEssCLIg_mK_hTg5VK and pFUSEssCHIg_mG1_hTg5VH. In both vectors, the expression of recombinant antibody chains was driven by hEF1-HTLV promoter, a composite promoter comprising the Elongation Factor-1α (EF-1α) core promoter1 and the R segment and U5 sequence (R-U5') of the Human T-Cell Leukemia Virus (HTLV) to assure a strong activity and enhance mRNA stability.

Subsequently, the whole coding sequence of each chain, comprising the signal peptide, was cloned in the transposon vector under the control of promoter 3 to obtain PB_prom3_ssCLIg_mK_hTg5VK and PB_prom3_ssCHIg_mG1_hTg5VH.

Our final aim was the assessment of the efficacy of transposon system for the generation of recombinant antibodies in comparison to standard vectors, as previously evaluated for recombinant proteins. In this case, the production of functional antibody heterotetramers was more complicated because it required the integration of both heavy and light chain

transgenes, as well as their expression with a ratio that assure proper antibody folding and secretion.

Before starting the comparison, it was necessary to devise a proper strategy for the selection of pFUSE transfected CHO-K1 cell. This is because pFUSE vectors contain selective markers different from neomycin resistance gene that was previously used. In particular, pFUSE light chain plasmid conferred resistance to blasticidin, while pFUSE heavy chain vector contained zeocin resistance gene. Therefore, to define CHO-K1 cells sensitivity to those antibiotics, they were transfected with both vectors or with pBlueScript vector as a negative control. After 7 days of culture in medium containing increasing concentration of one of the above described antibiotics, cells were stained with crystal violet (Fig. 24A and C). As illustrated in figure 24A blasticidin had a strong activity on not resistant cells, causing cell death even at low antibiotic concentrations. On the contrary, zeocin had a more gradual effect on cell viability (Fig. 24C). The same antibiotic concentrations were applied to cells transfected with pFUSE plasmids for 7 days. At day 7, we performed crystal violet staining to establish the selective conditions for following experiments (Fig. 24B and D). Cells transfected with pFUSE light chain plasmid decreased their viability only at 10 μg/ml of blasticidin concentration (Fig. 24C). Since it was very toxic for not resistance cells, we decided to use this antibiotic at the concentration of 10 μg/ml. On the other hand, cells transfected with pFUSE heavy chain vector died with different rate depending on zeocin concentration (Fig. 24D). Concentrations higher than 500 μg/ml imposed a selective pressure that was too harsh for these cells. As a result, for subsequent experiments zeocin was used at the concentration of 400 μg/ml.

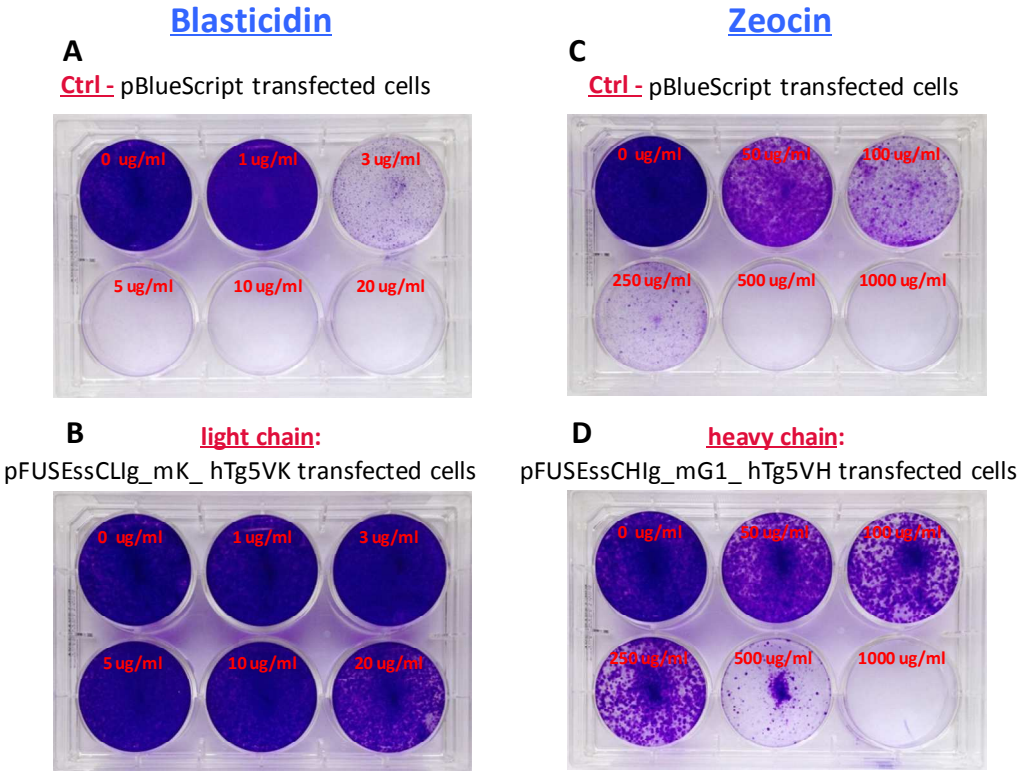


Fig 24. Antibiotic selection strategy for pFUSE vectors: Blasticidin was added at six increasing concentrations (0,1, 3, 5, 10 and 20 µg/mL) to A) pBlueScript transfected CHO-K1 cells or B) pFUSEssCLIg_mK_ hTg5VK transfected cells. Zeocin was added at six increasing concentrations (0,50, 100, 250, 500 and 1000 µg/mL) to C) pBlueScript or D) pFUSEssCHIg_mG1_ hTg5VH transfected cells. After 7 days of culture, cells were crystal violet stained. Ctrl: control.

2.4 Chimeric antibody expression in stably transfected CHO-K1 cells

Antibodies have a quaternary structure constituted by two identical heavy (HC) and two identical light chains (LC). As stated before, the major constrain in recombinant antibody production is the need to concurrently express, fold and assemble this two polypeptides. Consequently, the process of generating stable high-producing cell lines is complicated by the requirement to optimize genome integration and expression of both transgenes (Rita Costa et al., 2010). LC and HC genes are traditionally introduced by cotransfecting two separate vectors or, more recently, by transfection of a single vector carrying both transgenes. Since transposition efficiency is strongly influenced by the length of the transposon, we chose to apply the traditional approach, also to be unbiased in the comparison with pFUSE plasmids.

Moreover, the LC:HC peptide ratio plays an important role in the kinetics of mAb formation, in particular, excess in LC was reported to favor a higher antibody production (Schlatter et al., 2005). Therefore we have experimentally evaluated the effect on chimeric antibodies production of different LC:HC vectors ratios during transfection of CHO-K1 cells.

2.4.1 Comparison of commercial plasmids and transposons in the generation of stable CHO-K1 clones expressing chimeric antibody

In order to investigate the impact of transgenes co-transposition on our optimized transposon system, we firstly evaluated the efficiency of stable clone generation compared to standard transfection method. Adherent CHO-K1 cells were transfected using Lipofectamine 2000 (Life Technologies) with both LC and HC vectors at different molar ratios, i.e. 1:1, 3:2 and 4:1. Standard plasmids pFUSEssCLIg_mK_hTg5VK (pFUSE_LC) and pFUSEssCHIg_mG1_hTg5VH (pFUSE_HC) were cotransfected at the selected molar ratios, maintaining constant the total amount of DNA. The same molar ratios were used for PB_prom3_ssCLIg_mK_hTg5VK (PB_prom3_LC) and PB_prom3_ssCHIg_mG1_hTg5VH (PB_prom3_HC), and the ratio between transposons vector and transposase plasmid was maintained 2,5:1, as established in the previous experiments.

Transfected cells were plated at the density of 10000 cells/dish and cultured under appropriate antibiotic selection for 7 days. At the selection day 7, clones were stained with crystal violet and counted. Crystal violet staining revealed a remarkable improvement in the

number of resistant clones when CHO-K1 cells were transfected with transposon system at each LC:HC molar ratio tested (Fig. 25A). This observation was underscored by the count of resistant clones, in which is clear that the active transgene integration mediated by transposase allowed to obtain a number of clones 10 times greater than random integration promoted by standard plasmids (Fig. 25B). Moreover, no statistically differences were observed between the different LC:HC molar ratios tested in the clones generation efficiency.

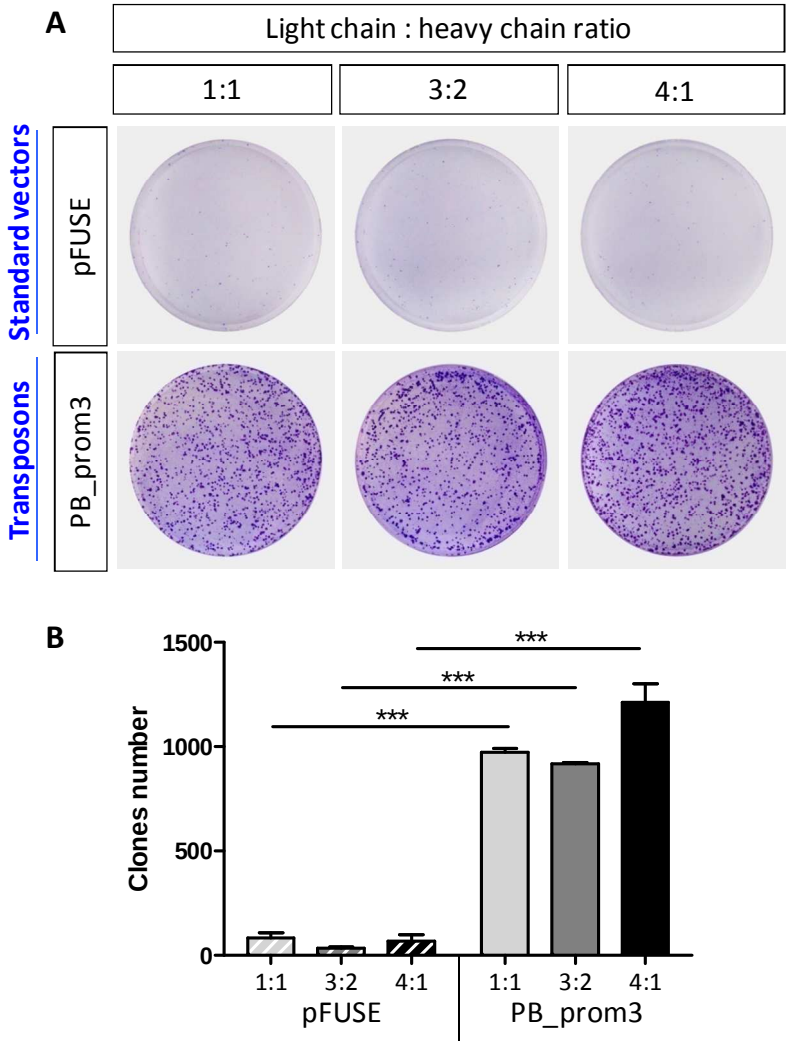


Fig 25. Clone formation assay: A) Crystal violet staining of clones obtained after 1 week in selective media. Blasticidin and zeocin were used for pFUSE transfected cells, while neomycin was used for transposon transfected cells. Several molar ratios between light and heavy chain vectors were used (1:1, 3:2 or 4:1). Total amount of DNA in each transfection was constant. Cells were plated at 10000 cells/plate. B) Count of stained clones for each transfection condition, ratio were indicated as LC:HC of either pFUSE plasmids or PB_prom3 transposon (n=5, ***$p < 0.001$).

2.4.2 Transfection ratios of heavy to light chain: impact on IgG production

To test whether the different light chain/heavy chain molar ratio used during transfection affected levels of IgG production, we performed a quantitative screening on about one hundred clones for each experimental condition. Those clones were randomly picked up from plates at 5000 cells/dish and maintained under suitable antibiotic selection for further 15 days. This period ensured stable transgene expression of selected clones and stabilization of cell growth and metabolism. Cell supernatants obtained after 2 days of culture were analyzed by the automated immunoassay prototype for detection of mAbs directed to hThyroglobulin in serum and plasma specimens on Liaison platform (DiaSorin). Since this assay detects antibodies with high affinity for hThyroglobulin, we supposed to quantify only correct folded recombinant antibodies. Relative light unit (RLU) values were normalized both to clone cell number and to days of culture, to calculate the production rate value, expressed as RLU obtained by 10^6 cells per day (RLU/ 10^6 cells /day). This value was called specific RLU rate (SRR). Results, obtained from the screening, were plotted estimating the percentage of clones for each experimental condition that fell in four production categories, arbitrarily defined (0-80000, 80000-400000, 400000-10^6 and >10^6 RLU/10^6 cells/day) (Fig. 26). Our results validated what previously observed about standard vectors in hFGF23 experiments. Clones originated from cotransfection of pFUSE plasmids were classified in the lowest categories of production, in particular 60-90% of clones fell into the 0-80000 RLU/10^6 cells/day category. Conversely, transposon system allowed to obtain a greater percentage of high producing clones. These data confirmed that the transposon molecular tool improved the efficiency in the generation of high producing clones compared to standard vectors, even in co-transfection experiments. Moreover, an excess in the molar amount of LC vector during transfection generated the highest mAb titers among clones, irrespective of the vector used, thus confirming literature data.

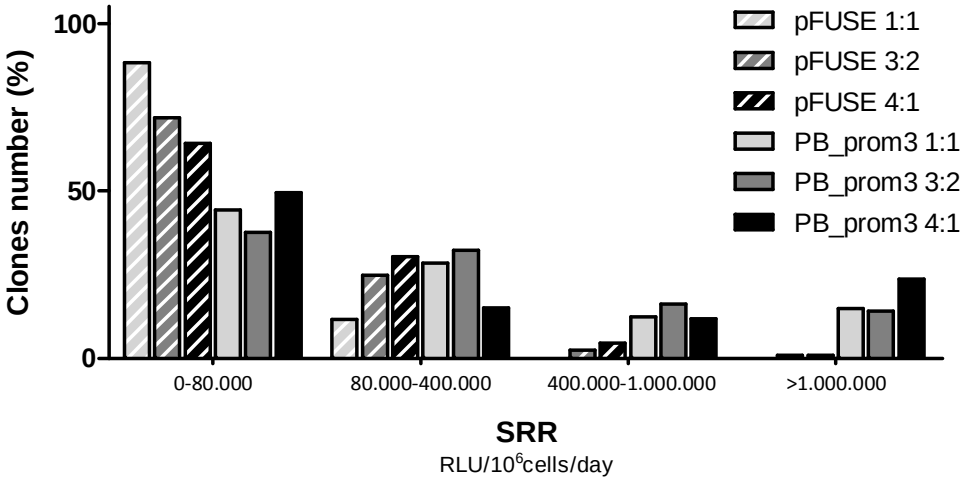


Fig 26. Classification of recombinant antibody producing clones: Quantitative screening of clones generated with pFUSE plasmids (patterned bars) or transposon system (filled bars) applying different LC:HC molar ratio (1:1, 3:2, 4:1). Clones were classified in categories based on mAb production level. Cell supernatants were analyzed with an automated immunoassay for detection of mAb directed to hThyroglobulin. Values were normalized based on clones cells number (Dojindo cell counting assay) and days of culture. SRR: specific RLU rate.

2.4.3 Characterization of high-producing clones

Recombinant antibody production requires not only high transgenes expression, but also the proper assembly between light and heavy chains that occurs in the endoplasmatic reticulum lumen. In order to further characterize IgG high-producing clones and investigate features of produced recombinant antibodies, the three best cell lines for each experimental condition were selected and cultured in adhesion for 3 weeks. Then cells were seeded at equal density in 6-well plates and cultured for 4 days in order to collect supernatants for SRR calculation and western blot analysis. Figure 27A reports SRR values of selected clones and shows that productivity of clones derived from PB_prom3 transposons was 3-5 times higher than that of standard vector derived clones. Moreover, in PB derived clones, HC:LC molar ratio of 4:1 gave the most productive clones. Nevertheless, few clones lose their productivity during long-term culture regardless of vector type. We speculated that the 2 clones originated from standard vectors had lost their productivity due to the common issues of transgenes expression silencing and genomic rearrangements. However, these couldn't be the reasons for the productivity lost in clones generated by transposition mechanism, because one of the features of this system is the avoidance of those shortcomings. Therefore, we supposed that the cause of transgenes instability in clones generated by transposon system was the selection strategy during cell line establishment. In fact it relied only on G418, instead of two antibiotics as used for pFUSE vectors.

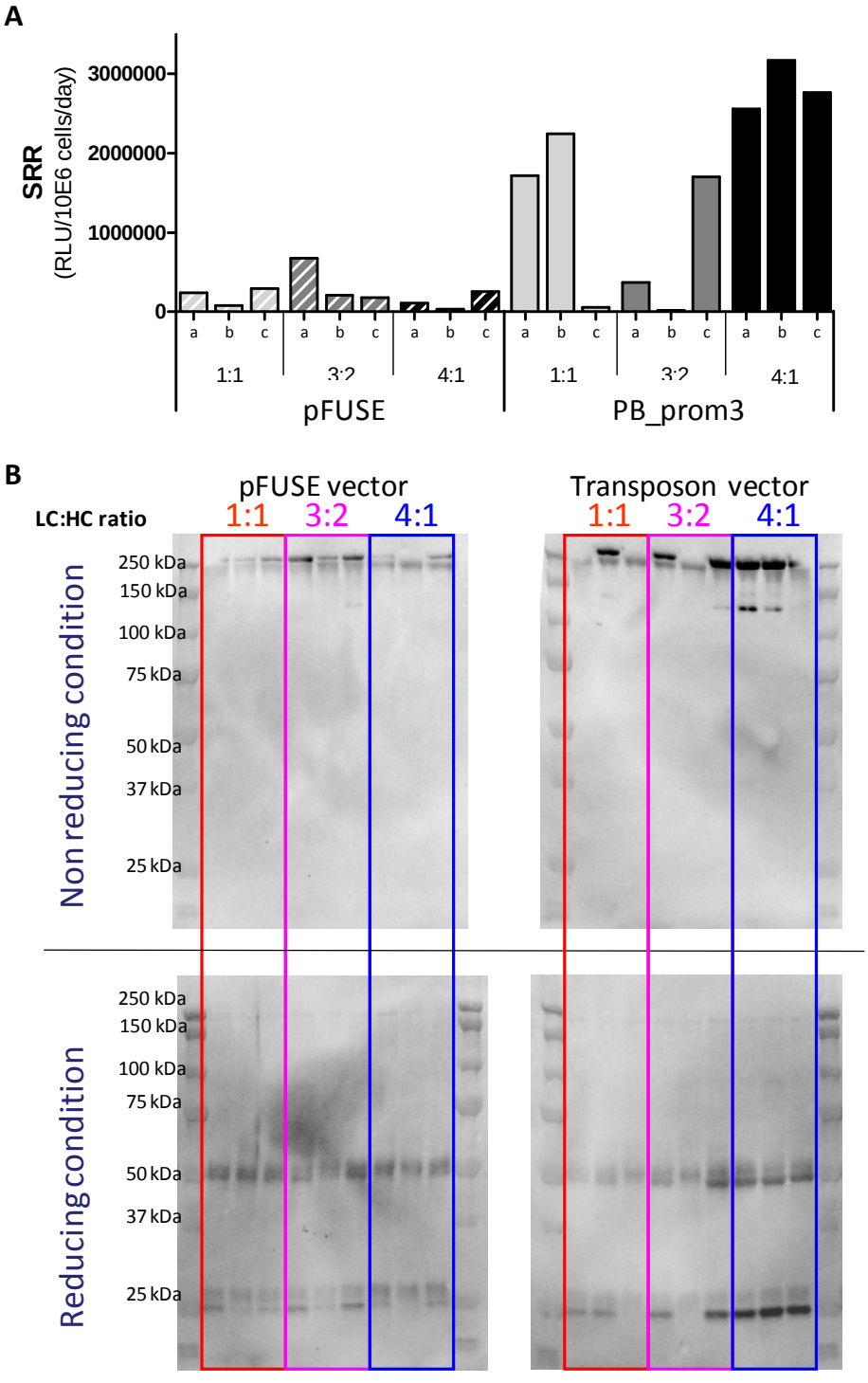
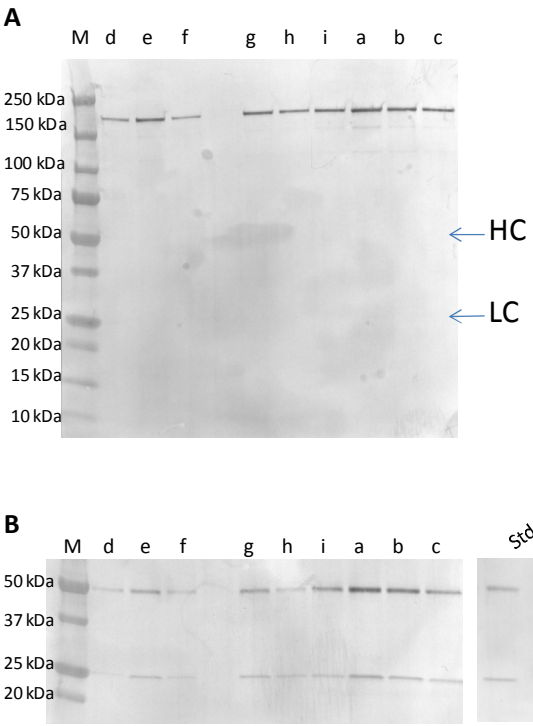


Fig 27. Characterization of high-producing clones: three high-producing clones generated with pFUSE plasmids or transposon system were selected for each LC:HC molar ratio used (1:1, 3:2, 4:1). A) Quantitative screening of clones. Cell supernatants were analyzed with an automated immunoassay for detection of mAb directed to hThyroglobulin. Values were normalized based on clones cells number (Dojindo cell counting assay) and days of culture obtaining the specific RLU rate (SRR). B) Western blot on samples enriched in recombinant antibodies by protein A binding, immunodecoration with anti mouse IgG antibody.

Next, we studied recombinant antibodies production by western blot analysis. Given that selected clones were cultured in medium containing serum, bovine serum albumin protein (BSA) should have prevented the analysis. Hence, harvested supernatants were enriched in recombinant antibodies concentration by protein A, also eliminating other serum contaminants. Samples were than separated by SDS-PAGE under either reducing or non reducing conditions, and analyzed by Western blot using a polyclonal anti mouse IgG antibody that allowed the detection of both heavy and light chains (Fig.27B). Transposon-derived clones, with the LC:HC transfection ratio of 4:1, were confirmed to be the most productive clones. Results were not directly comparable with SRR value, as the production rate was normalized to the number of cells. Nevertheless, clones that had low SRR value showed blurred bands in western blot analysis. Moreover, in reducing western blot, clones that had lost their productivity had a completely suppressed light chain production. Neither unpaired heavy chains nor aggregated molecules were detected in non reducing condition. We concluded that transfection with PB_prom3_LC and PB_prom3_HC transposons at 4:1 molar ratio was the best condition to obtain stable high producing CHO-K1 clones.

With the purpose to further evaluate high producing clones, we selected a panel of 9 clones generated from transposon transfection with LC:HC 4:1 molar ratio. After adaptation to serum free media in adhesion, clones were cultured for 5 days and supernatants harvested. The lack of BSA allowed to perform western blot analysis directly on supernatant samples. In order to confirm absence of secreted unpaired chains, supernatant were analyzed in non reducing conditions (Fig.28A). Immunostaining with a polyclonal anti mouse antibody didn't show any bands apart from the assembled heteroteramer. In the most productive clones, referred to as a and b, it was also present a faint band around 150 kDa that probably corresponded to the not glycosylated form. Next, we analyzed clone supernatants in reducing conditions to study the relative abundance of HC and LC secreted polypeptides (Fig. 28B). Densitometric analysis of discrete bands in the immunoblot images was done by meaning of ChemiDoc Image Lab software (Bio-Rad) and each chain quantification was normalized to the respective polypeptide chain derived from a purified murine monoclonal antibody, used as a reference. This step was necessary because the polyclonal anti-mouse IgG antibody showed different affinities for heavy chain and light chain. Secreted LC and HC polypeptide ratio was near 1 for each clone, corroborating proper assembly of the heteroteramer (Fig. 28C).



C

Clones	LC/HC
d	1,02
e	0,99
f	0,99
g	0,94
h	0,99
i	0,94
a	0,91
b	0,95
c	0,99

Fig 28. Light chain to heavy chain polypeptide ratio: supernatants of nine clones (PB_prom3, LC:HC ratio 4:1) cultured in serum free media for 6 days were analyzed by western blot both in non reducing (A) and in reducing conditions (B). Immunostaining was performed using a polyclonal anti-mouse IgG antibody. Densitometric analysis was applied on LC or HC bands in the digitized immunoblot. Data were normalized to a murine mAbs used as standard (Std 500 or 1000 ng). For each clone, ratio between the two secreted chains is reported in table C.

In addition, we also studied the relative LC and HC genes expression to investigate correlations with clones productivity. For each clones, CHO-K1 cells and their supernatant were harvested after 4 days of culture. RNA was extracted from cell pellets and RT-PCR was performed. Samples were used as templates in real-time PCR experiments for relative quantification of both chains expression by Pfaffl ΔΔCt method. Finally, the relative intracellular abundance of LC transcript compared to HC was determined as their ratio. Data obtained for each clone were plotted as crescent ranking value in figure 29. Moreover we analyzed clones IgG productivity in the supernatants using an automated immunoassay for the quantification of mouse IgG on Liaison platform (DiaSorin). Graph 29 correlates the antibodies concentration in the supernatants to the ranking values obtained by LC:HC relative expression ratio. As shown, the three clones (a, b, c) with the highest productivity (>9 µg/ml) had an excess of light chain greater than other ones. In contrast, low-producing clones (<4 µg/ml) had a smaller light chain excess. We concluded that an unbalanced

expression of the two chains in favor of light chain caused an increase in antibody productivity; however an excess of LC beyond a threshold led to difficulties in the antibody assembly, thus reducing clone productivity, as was observed for the clone with the highest ranking value (clone c) (Fig.29).

In conclusion, a 4:1 transfection ratio between light and heavy chain transposon vectors increased overall clones productivity, probably because it promotes a LC excess, even if it is difficult to obtain a reproducibility in transgene integrations and, consequently, in expression ratio.

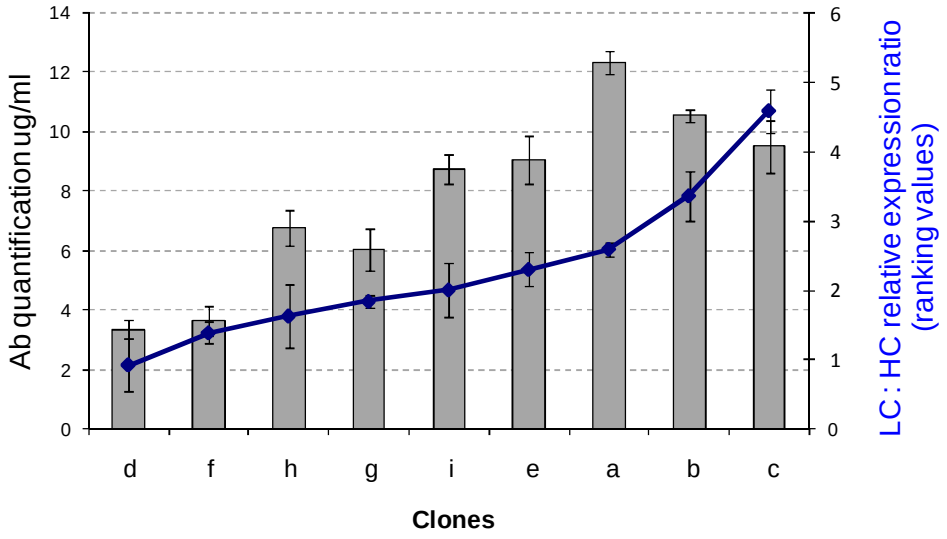


Fig 29. Light chain to heavy chain relative expression: supernatants of nine clones (PB_prom3, LC:HC ratio 4:1) cultured in serum free media for 4 days were quantified by an automated immunoassay for mouse IgG detection on Liaison platform (Diasorin) (grey bar, n=3). Light chain and heavy chain expression were analyzed by real-time PCR applying Pfaffl ΔΔCt method (n=3). Ratio between relative expression of LC to HC was determined. Data obtained are reported as ranking values and clones are ordered based on this information.

2.4.4 Chimeric antibody purification

Clone (a), named 15C.47.44, was the best producer with a recombinant IgG antibody average concentration of 12 µg/ml. It was cultured in adhesion for 4 days in presence of serum. Then, 50 mL of supernatant was harvested and the IgG1 recombinant antibody was purified with the standard protocol for affinity chromatography on protein A (Fig. 30A). The purified antibody was analyzed by SDS-PAGE under reducing and non-reducing conditions (Fig. 30B). Under reducing conditions, the heavy and light chains were visible respectively at about 50 and 25 kDa, as expected. Under non-reducing conditions, a band with a molecular mass higher than 150 kDa was stained. A faint band was present at around 150 kDa that

probably corresponded to not glycosilated form of the antibody. As already demostrated, unpaired light or heavy chain were not detected, suggesting that antibody chains were properly assembled into a whole IgG molecule. The final antibody concentration was 0,230 mg/mL in 1 mL total.

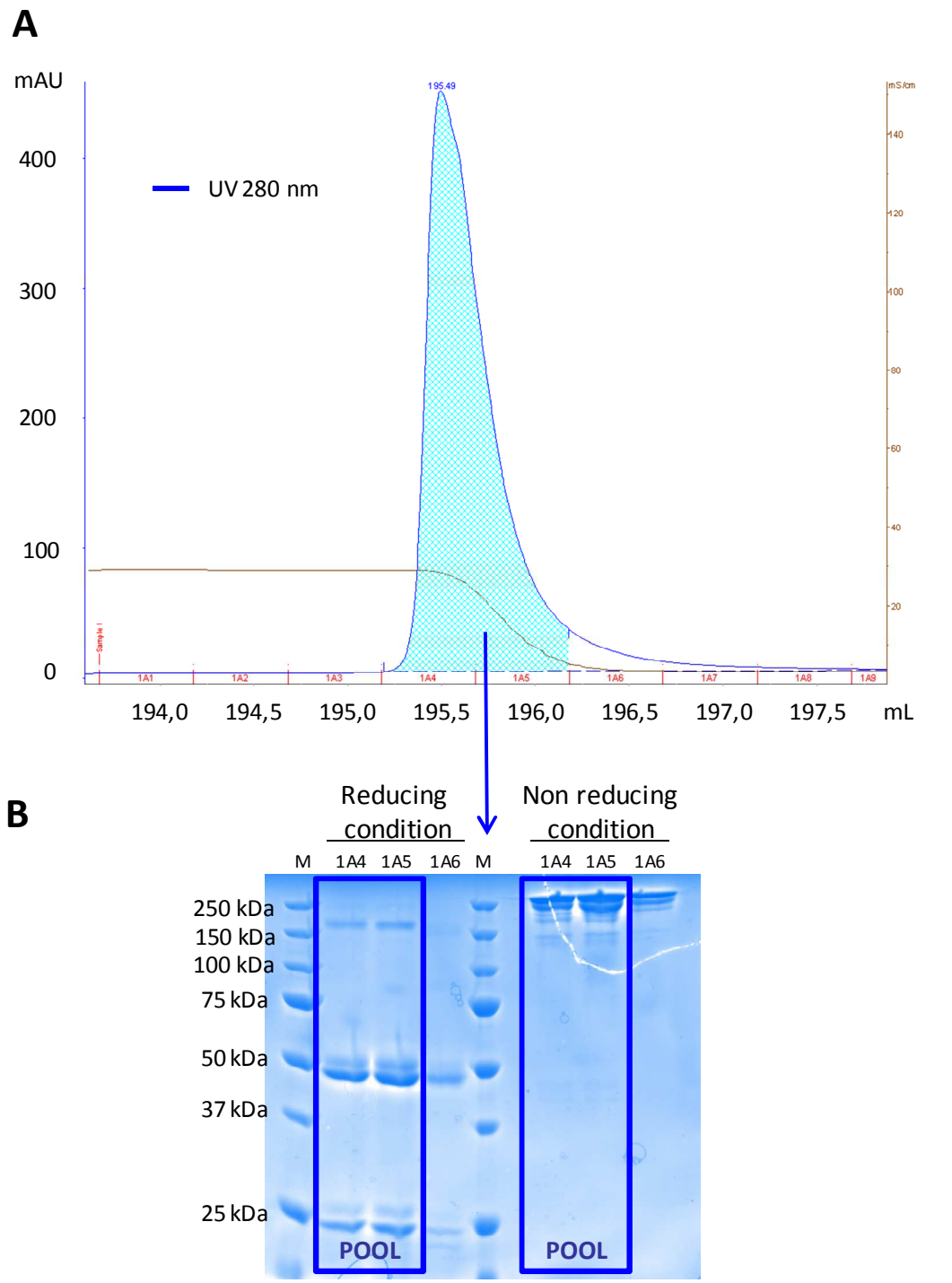


Fig 30. Purification of recombinant IgG1 anti-hThyroglobulin: A) Affinity chromatography on protein A column profile, elution (magnification): in full azure are indicated eluted fractions used to establish the "*pool*". B) SDS-PAGE analysis in reducing and non-reducing conditions. Blue rectangle indicates selected eluted fractions. On top of gel lanes are reported the name of eluted fraction. M: standard molecular weights.

2.4.5 Chimeric hTg5 vs hybridoma hTg5 antibody: characterization for diagnostic applications

Our aim in chimeric antibody generation was the production of a mAb with the same functionality of the parental one in order to substitute it as reagent in automated immunoassays. Therefore, purified IgG1 recombinant antibody, referred to as hTg5Rec, was compared to purified parental IgG2a hybridoma mAb (hTg5) in order to evaluate if reactivity against thyroglobulin was maintained.

2.4.5.1 Liaison analysis

The reactivity against human thyroglobulin was firstly evaluated on Liaison platform. The immunoassay developed for this evaluation combined a solid phase made of anti-mouse IgG antibody coated on tosyl-activated paramagnetic microparticles, and soluble human thyroglobulin, conjugated with ABEI (N-(4-Aminobutyl)-N-ethylisoluminol) that was used as tracers. Both recombinant hTg5 and parental hTg mAb were serial diluted and tested (Fig. 31). hTgRec showed a hThyroglobulin binding activity comparable to parental hybridoma mAb. Both antibodies had an hook effect at concentrations above 100 ng/ml. However, recombinant mAb produced lower RLU values. Since tested mAbs were respectively an IgG1 and IgG2a, we speculated that discrepancy in RLU values could be due to anti-mouse IgG different affinities for these two gamma immunoglobulin subclasses.

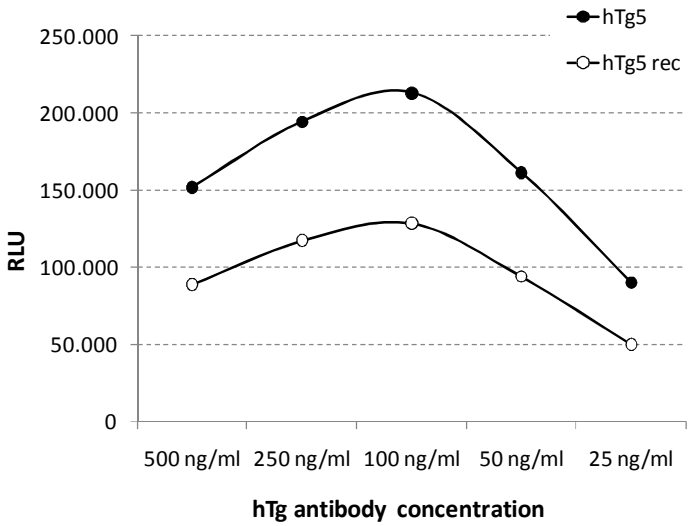
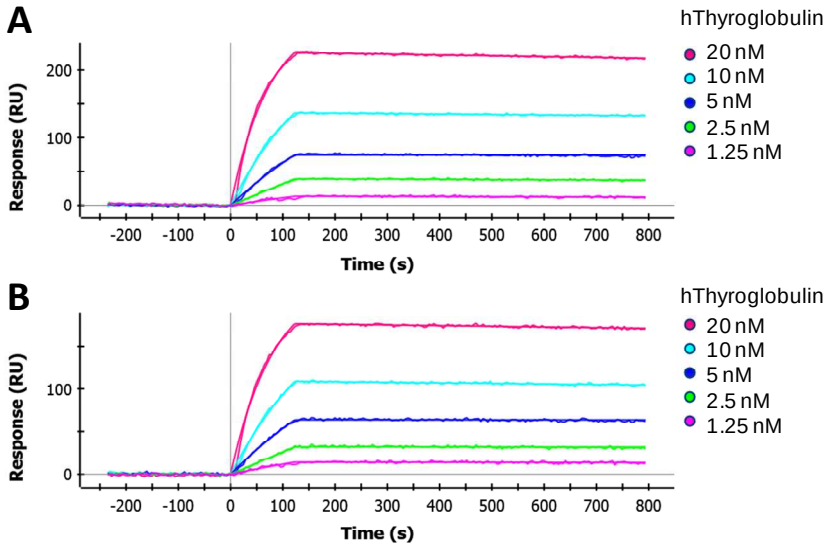


Fig 31. Comparison of hTg5 antibodies on Liaison platform: recombinant hTg5 antibody (white bullets) and hybridoma hTg5 (black bullets) were serial diluted (dilution factor 2) and tested with an automated immunoassay on Liaison platform (DiaSorin). The CLIA assay exploited anti-mouse IgG antibody coated on solid phase and human thyroglobulin, ABEI conjugated, as a tracer.

2.4.5.2 Surface plasmon resonance (SPR) analysis

To further investigate recombinant antibody features and dig a little deeper in RLU discrepancy observed on Liaison platform, we determined the affinity of our antibodies to the human thyroglobulin by surface plasmon resonance (SPR) analysis on Proteon XPR36 (Bio-Rad). The binding curves obtained using concentrations of purified human thyroglobulin from 20 nM to 1,25 nM (Fig 32A and B) were analyzed assuming a "one to one" interaction using Langmuir model. According to the antibodies binding curves, the equilibrium dissociation constant (KD) values were calculated to be 2,94 ± 0,64 x 10^{-11} M for parental mAb and 4,47 ± 1,02 x 10^{-11} M for recombinant mAb (Fig. 32C). Differences in these values were not significant, thus confirming that the binding affinity of hTg5 antibody was conserved in the chimeric form.



C

	KD (M)
hTg5 mAb (hybridoma)	2,94 ± 0,64 x 10^{-11}
Recombinant hTg5 mAb	4,47 ± 1,02 x 10^{-11}

Fig 32. Affinity determination by surface plasmon resonance (SPR) assay. Sensograms of binding interactions of parental (A) and recombinant hTg5 mAb (B) with human Thyroglobulin. SPR kinetic binding analysis were recorded using ProteOn XPR36 system (BioRad) and a NLC sensor chip immobilizing biotynilated anti-mouse IgG. hTg5rec or hTg5 mAbs were injected as ligands and different concentrations of hThyroglobulin (20, 10, 5, 2.5, 1.25 nM) were used as analytes. Binding curve were fitted using the Langmuir model describing a 1:1 binding stoichiometry. (C) Calculated values of the equilibrium dissociation constant (KD) of recombinant and parental hTg5 mAbs. Error values were estimated as the standard deviation of 3 individual experiments.

Overall, our results indicate that we achieved the production of a recombinant chimeric antibody with 1) the variable regions derived from a monoclonal antibody produced by an hybridoma, and 2) the constant region of murine origins that differs in isotype from the original mAb. Since the chimeric antibody that we have produced shows a comparable binding affinity for hThyroglobulin to that of parental one, it can be used in DiaSorin immunoassay for detection of hThyroglobulin in place of the parental mAb.

Discussion

Stable expressing mammalian cell lines are essential for the large-scale production of complex recombinant proteins and antibodies for industrial purposes, since they are reliable over time and crucial to ensure compliance with current biopharmaceutical regulations. Even if this aspect is not relevant in recombinant proteins production for *in vitro* diagnostic applications, the assurance of cell lines expression stability is an essential requirement to fulfill any industrial needs.

CHO cells are one of the most popular mammalian cell line used for the expression of recombinant proteins. Their wide application is due to well-characterized metabolism and growth kinetic, and also to their historical use as standard cell type for the production of therapeutic proteins. Among the factors that impact cell line final productivity, the most important are: the (i) transgene integration efficiency, (ii) the design of the expression construct to assure high level of transgene transcription, (iii) the development of high-throughput screening methods to allow the isolation of clones with the highest performances, and (iv) the optimization of culture conditions to improve viability and, hence, the productivity.

As regards the transgene integration strategies, during last 15 years several approaches have been developed to overcome limitations of conventional transfection methods. The most efficient strategy is based on viral vectors since viruses naturally infect cells to integrate their genetic material into host genome. However, due to the intrinsic risk in their handling and to regulatory concerns they have been poorly considered for pharmaceutical purposes (Oberbek et al., 2011). As a safe alternative, a gene delivery method based on DNA transposons has been developed for academic research applications (Ding et al., 2005) and, recently, it has been investigated for industrial recombinant protein production (Matasci et al., 2011). DNA transposons offer some interesting advantages: they are safe to handle and can be manipulated as conveniently as any plasmid vectors by standard molecular techniques.

Therefore, the purpose of this thesis was to develop and characterize a transposon based system for recombinant proteins and antibodies production in CHO cells, that was able to assure stable transgene integrations and high levels of expression, avoiding post-integration gene silencing.

Evaluation of transposons as a gene delivery system in CHO cells

Among the known transposable elements, *PiggyBac* transposon system represents the most suitable tool for the manipulation of the mammalian cell genome (Meir et al., 2011). We evaluated its potentialities as gene delivery method compared to standard vectors in adherent CHO-K1 cells by focusing on different features:

1) Transposon system enhances the generation efficiency of stable CHO-K1 cells

The first one is the generation efficiency of stable CHO-K1 cells obtained by co-transfecting the transposable element in the presence of transposase helper plasmid compared to standard plasmids or transposon without transposase. After the application of G418 selection, the increase in clones formation obtained by transposon system was about 8-9 fold compared to that obtained with standard vector. This highlights the potency of active transposition instead of episomal DNA random integration. Our results confirmed the 8 fold increase in clone generation efficiency obtained in HEK293 cells by Ding and colleagues at the 5:1 transposon to transposase ratio (Ding et al. 2005). The use of stronger promoters also contributed to enhance the efficiency, leading to an increase up to 20 fold. Moreover, transfection of transposon alone produced a number of clones similar to standard plasmid, corroborating that the efficiency of PB transposon system is due to transposase action. In order to further hone our system, we have optimized the PBase concentration by applying two different molar ratio between PB transposon and transposase (2,5:1 or 5:1). Since no differences have emerged, we choose the higher transposase concentration to maximize the transposition events. This result also confirms the lack of transposase over production inhibition phenomena at the selected concentrations for the PB system, as stated by previous works (Wilson et al., 2007).

2) Transposon system reduces time of clone establishment and increases the percentage of isolated stable clones

To isolate clone with stable genomic integrations, transfected CHO-K1 cells were grown for 6-7 days under G418 selection before clone picking. After picking, clones were expanded in G418 supplemented medium for further two weeks. Originally, all clones derived by both standard vector and PB transfection experiments were resistant to G418 medium, but only those derived from transfection with PB transposon system showed a 100% survival rate after prolonged culture. In contrast, 50% of clones generated by transfection with standard plasmid (or transposon alone) died in few days after picking. This result could be explained by two main causes: 1) a too short time of selection before clone picking (6-7 days) for

clones derived by standard vector transfection, resulting in the residual presence of episomal plasmids that confer initial antibiotic resistance lost during clone expansion; and 2) standard vector random integration that often results in transgene disruption and inactivation. On the other hand, thanks to the active DNA integration process mediated by transposase, 100% of clones derived from transposon transfection were stably isolated after 6-7 days of selection, reducing time necessary for this phase of the clone establishment process.

3) Transposon system enhances frequency of high producing CHO-K1 clones

Along with the previous advantages, the PB transposon system overcomes standard vectors also in the generation of clones with high protein productivity. Our results indicated that 50% of clones derived from standard plasmid transfection had a very poor productivity. On the contrary, frequency of high-producing clones was enhanced if PB transposon system was used, thus supporting the extensive literature about transposon site integration preference for actively transcribed genomic regions (Galvan et al., 2009). Moreover, we have assessed the effect of different promoters on protein expression. In particular, we have demonstrated that the percentage of high-producing clones is further improved by using a particular promoter, named prom3, upstream to the transgene into our transposon vector. Since the use of a stronger promoter allowed only a slight enrichment in high producing clones, a better approach to enhance the clone productivity may be the introduction of other epigenetic regulatory elements in the transposon vector. These could be scaffold or matrix attachment regions (SARs or MARs) and ubiquitous chromatin opening elements (UCOEs), which have been demonstrated to reduce heterogeneity and enhance transgene expression in CHO cell lines (Ley et al., 2013; Chang et al., 2014; Boscolo et al., 2012).

Our characterization has demonstrated that PB transposon features can appreciably contribute to reduce efforts necessary to produce and isolate clones with high productivity performances.

Secreted hFGF23 (human fibroblast growth factor 23)

As a first protein model to test the suitability of our optimized transposon system, we selected hFGF23, a recombinant protein with a structural complexity that cannot be properly reproduced in prokaryotes or lower eukaryotes. The protein is a regulator of

phosphate homeostasis and vitamin D metabolism recently suggested as an early biomarker for chronic kidney disease and other disorders. It is physiologically cleaved after the ^{176}RHTR^{179} motif by specific proprotein convertases in two inactive fragments. For this reason, we mutated the arginine 179 to a glutamine in the FGF23 wild-type sequence. This has allowed the production of cleavage-resistant full length protein, confirming literature data (Shimada et al., 2002). Applying both standard transfection and transposon system, we have generated FGF23 producing CHO clones, among which we have selected the best producers for further characterization. The first step in the cell line development process for industrial application is the adaptation of cells to serum-free suspension culture to achieve high cell densities and improve growth and production performances. During this process we have indeed observed that the adaptation to suspension culture led to higher clone productivities, affecting clone performances regardless of the vector used to integrate the transgene, thus the best producer clone in adherent culture was not necessary the same in suspension culture. This aspect has become even more evident when growing conditions have been optimized by testing different media for batch culture production. Although the two best clones, one derived from transfection with standard vector and one derived from transposon system, had a similar productivity after the adaptation process, adaptation to production media and additional months in serum free suspension culture further changed their performances, leading the productivity of the standard vector-derived clone exceed the productivity of the other clone, with a hFGF23 supernatant concentration of 35 mg/L. Taking into account the whole process of clone generation, standard plasmids generate less clones than PB transposon with a higher initial productivity, therefore further studies are required to investigate the performance variations observed during clone expansion and suspension adaptation, since this work is limited by the intensive labour of manual clone picking. This limit could be overcome using an automated colony picker that would allow to perform a wider study, increasing the probability to find even more productive clones. In addition, transfection of suspension CHO cell lines might be an alternative option to reduce variability in clone productivity due to the adaptation processes.

As regard the volumetric productivity, yields obtained are lower than those recently reported in literature for small scale batch cultures (Matasci et al., 2011). However, this could be due to intrinsic features of hFGF23 protein (Shimada et al., 2001). Nonetheless, different strategies could be pursued to increase the volumetric productivity. For example

the PB transposon could be engineered with UCOE sequences or other epigenetic regulators, as described before. In addition, chemical compounds, such as sodium butyrate and valproic acid, could be used to modify chromatin structure, favouring transcription factors access to the transgene sequence (Allen et al., 2008).

Another major concern in the generation of mammalian stable cell lines for industrial application is the loss of productivity that sometimes occurs upon extended time in culture. However, selected clones, regardless transfection vector, have exhibited a stable transgene expression up to 30 passages in suspension culture that correspond to about 4 months.

We also have developed a purification procedure for the hFGF23 protein produced in the supernatant of selected clone during batch culture. This process has several phases but yielded always more than 5 mg of purified protein, an amount sufficient to fulfil DiaSorin manufacturing requirements.

Even though the hFGF23 protein is muted to prevent physiological cleavage, it is still quite instable in its full-length form. During cell culture at 37°C, the protein was continuously degraded, since it didn't accumulate over time in the supernatant. Therefore, shortly after the purification we investigated several strategies to preserve hFGF23 protein. We concluded that protein is stable in an optimized buffer for 3 months at 4°C. On the contrary, lyophilization process deeply affects the stability of this protein, and only one condition, among those tested, avoids protein degradation 15 days after resuspension.

Once identified conditions for protein storage, we tested our hFGF23 protein compared to a commercial one as a calibrator in an automated immunoassays on Liaison analyzer (DiaSorin platform). No significant differences were observed between the two proteins, thus suggesting that our protein is correctly recognized by the set of antibodies present in the assay. As a different approach to characterize the purified protein, we also tested its biological activity in a proliferation assay on NIH/3T3 fibroblast cells. We observed a dose-dependent effect on cell growth compared to control cells treated only with the FGF receptor cofactors Klotho and heparin. However, treatment with hFGF23 at the higher concentration without cofactors also appeared to induce cell proliferation. We speculated that this action could be mediated by Klotho like proteins present in the fetal bovine serum, used for cell line maintenance. Despite of this detail, we concluded that our protein was biologically active, confirming the quality and proper folding of CHO-derived hFGF23.

Transmembrane hFGF23

Given the success in the production of secreted hFGF23, we tried to express it on CHO cells surface in order to use transfected cells for mouse immunization. This is highly desirable as it supersedes the laborious task of antigen purification and, as previously demonstrated, it ensures presentation of target proteins in their native conformation (Dreyer et al., 2010). Our experiments proved the feasibility of this cellular approach, in fact the protein was widely expressed on cell membrane of both adherent and suspended CHO cells. Moreover, after cells injection in mice, they were able to arise an immune response against hFGF23, even thought we did not use any adjuvant. The mouse serum titers obtained were pretty low; this is probably due to the high similarity between hFGF23 and the endogenous mouse protein that affects the protein immunogenic effect. However, our results underscore the potentiality of such an innovative immunization strategy and pave the way to further investigations.

Chimeric antibody against hThyroglobulin

As a second protein model, we selected the production of a chimeric antibody direct against hThyroglobulin. Chimeric antibodies sequences are typically engineered to have variable regions from one species, usually mouse, in frame with human constant regions. In this work, our aim was to prove the effectiveness of transposon system for the generation and production of chimeric antibody in CHO cells for diagnostic applications. In diagnostics, mouse-human chimeric antibodies can be used as control and calibrators instead of patient serum/plasma, while mouse-mouse chimeric antibodies can substitute monoclonal antibodies as reagents, for example in case of hybridoma instability or isotype switching. Here, it has been reported the “mouse-mouse chimeric” case in order to get a “proof of principle” that mAb gene cloning, engineering and expression technology work in our hands. To raise the stakes, we also changed the IgG isotype from 2a to 1.

Starting from an instable hybridoma clone expressing a mAb directed to hThyroglobulin, we succeeded in cloning variable heavy and light chains using an appropriated designed set of primers. Isolation of variable regions is a complicate step due to the presence of expressed aberrant chains derived by unsuccessful allele rearrangements in myeloma cells used as fusion partner (Carroll et al., 1988). After a laborious work of PCR amplification and sequencing, data were analysed by using the IMGT database to discriminate between

productive and non-productive chains. Productive sequences were cloned in both standard and transposon vectors for mammalian cell expression.

To produce recombinant IgG in mammalian cells, both chains have to be expressed and assembled in a single cell. Conventional co-transfection of multiple transgenes can be a very low efficient process and results in few productive clones. Consequently, bicistronic constructs or multiple rounds of gene delivery are required to obtain acceptable results. For this reason we investigated the efficiency of the transposition mechanism for the co-integration of two transgenes in CHO cells, as recently reported also in human cells and mouse embryonic stem cells (Kahlig et al., 2010; Lu and Huang, 2014). As expected, the transposon system allowed to obtain a 10 fold increase in the number of clones obtained after selection in comparison to conventional methods. Moreover, 30% of clones generated were medium-high IgG producers, in contrast to those generated by standard co-transfection that were very low producers (70-90% of total clones). The application of transposon system for the production of antibodies is even more advantageous if considering that the selective pressure applied is identical for both co-transfected transposons, while it is different for standard plasmids. We concluded that the PB transposon system was an efficient method for the co-integration of different genes.

Another essential feature for high-level production of mature antibody is a specific expression rate between light and heavy chains because this ratio controls the assembly and stability of the heterotetramer (Schlatter et al., 2005). In our study we confirmed previous data that describes better results when LC:HC ratio is unbalanced in favour of light chain during transfection, in particular we obtained the best clone producers with 4:1 LC:HC ratio. The transposon system probably contributes to convert the transfection transgenes ratio into a proper LC:HC integration ratio because of the active mechanism mediated by transposase that works on both constructs with a similar efficiency dependent only by their relative amount. On the contrary, standard vectors co-integration is a random event and nothing assures the compliance of transgenes ratio. Although such unbalance ratio of transfection, the ensuing recombinant antibody was constituted by 1:1 HC and LC polypeptides and no unpaired chain were detected. In order to further characterize the impact of transfection chain ratio, we also have analyzed the relative expression of LC to HC chains in the category of most productive clones (PB transposon system with a 4:1 LC:HC ratio). However, our results failed in the identification of a reproducible relative expression ratio between the two chains, even thought the amount of transcribed light chain was

always in excess, suggesting that this imbalance promoted the overall productivity favouring the assembly kinetics. The evaluation of a higher number of clones and the estimation of effective integrated copies for both chains could be an interesting field of studies to improve clone productivity.

The recombinant antibody produced is able to recognize hThyroglobulin in an automated chemiluminescence immunoassay, hence the reactivity of the parental mAb was maintained, highlighting the success of the developed process. However, our data clearly showed that the signal of recombinant IgG was lower than parental mAb. This was not due to a reduced affinity of the antibody for the hThyroglobulin, since SPR analysis did not reveal significant difference between affinities of the original and the recombinant mAb. Therefore, the reduced signal was probably due to a stronger affinity for IgG2a than for IgG1 of the anti-mouse antibody used in solid phase.

Taking into account this hypothesis, other projects involving the generation of human-mouse chimeric antibodies could be more relevant in evaluating if massive changes in the constant structure of the immunoglobulin can affect its affinity.

In conclusion, this work has demonstrated that PB transposon system can be considered a quick, powerful alternative to standard methods of transfection for generation of stable, high-producing recombinant mammalian cell lines during production of critical reagents useful for diagnostic applications.

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