Review

[Designing cell lines for viral vaccine production: where do we stand?]

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Abbreviations:

ADCC, antibody-dependent cell cytotoxicity; **ATCC,** American Type Culture Collection; **BHK,** baby hamster kidney cells; **CDM,** chemically defined medium; **CAP,** Cevec's amniocyte production cell line; **CEF,** chicken embryo fibroblast; **CHO,** Chinese hamster ovary; **CR,** Cairina retina; **CS,** Cairina somite; **CA,** Cairina amnion membrane; **ECACC,** European Collection of Animal Cell Culture; EMA, European Medicines Agency; **ERV,** endogenous retrovirus; **FDA,** Food and Drug Administration; **HCV,** hepatitis C virus; **HEK293,** human embryonic kidney cells; **HIV,** human immunodeficiency virus; **ISKNV,** infectious spleen and kidney necrosis virus; **MDBK**, Madin Darby bovine kidney; **MDCK**, Madin Darby canine kidney; **MDV,** Marek's disease virus; **MNNG,** N-methyl-N'-nitro-N-nitrosoguanidine; **MVA,** modified vaccinia Ankara; **PEG,** polyethylene glycol; **RSV,**

respiratory syncytial virus; **TBE**, tick-borne encephalitis; **$TCID_{50}$,** tissue culture infectious dose 50; **YF,** yellow fever; **WHO,** World health organization;

Abstract

Established animal cells, such as Vero, Madin Darby canine kidney (MDCK) or chicken embryo fibroblasts (CEFs), are still mainly used for viral vaccine production, although new "designer cells" have been available for some years. These designer cell lines were specifically developed as a cell substrate for one application and are well characterized. Later screening for other possible applications widened the product range. These cells grow in suspension in chemically defined media under controlled conditions and can be used for up to 100 passages. Scale-up is easier and current process options allow cultivation in disposable bioreactors at cell concentrations higher than 1 x 10^7 cells/mL.

This review covers the limitations of established cell lines and discusses the requirements and screening options for new host cells. Currently available designer cells for viral vaccine production (PER.C6, CAP, AGE1.CR, EB66 cells), together with other new cell lines (PBS-1, QOR/2E11, SogE, MFF-8C1 cells) that were recently described as possible cell substrates are presented. Using current process knowledge and cell line development tools, future upstream processing could resemble today's Chinese hamster ovary (CHO) cell processes for monoclonal antibody production: small scale bioreactors (disposable) in perfusion or fed-batch mode with cell concentrations above 1 x 10^8 cells/mL.

1 Introduction

Current animal cell culture in industry is typically cultivation of CHO cells for the production of recombinant proteins or monoclonal antibodies [1-3]. Additionally, in the academic field, human embryonic kidney (HEK293) or insect cells are of great interest for transient expression of proteins, for production of virus-like particles, or for capturing biochemical mechanisms. But there are many more cells available and new cell lines are constantly being developed. For viral vaccine manufacturing, very specialized cells are occasionally needed, and the variety of cell substrate mainly depends on the desired

characteristics of the viral product and the regulatory requirements. With new tools such as cell engineering and bioreactor technologies, the question emerges whether cells cannot be "designed" for their viral product. The aim of this review is to explore this vision by summarizing the traditional established cell lines for viral vaccine production, by defining the requirements for new cell substrates, and by comparing different "designer cells" that have been developed lately. The focus lies on whole virus replication for human and veterinary vaccines. Insect cell lines that are typically used for recombinant protein production or VLPs are not in the scope of this review. Throughout, new options that become available with such designed cell lines are highlighted.

2 Established cell lines used for vaccine production

Many of the cells used today have been developed in the 1960's and 1970's (Table 1). These cell banks are thus 40 to 50 years old. Whether growth properties are still equivalent to the growth properties seen in 1961 cannot be answered, as today's cultivation methods are different from that time. Modern cultivation technologies typically use plastic vessels, cells may be cultivated on microcarriers in disposable bioreactors, and cell culture media are clearly leaner than those used for the original cells. Attempts have been made to overcome serum-usage, and if possible, serum-free or chemically defined media are used. In general, the protein content of today's culture media is much lower. Hence, the motivation for developing new cell lines using these advanced cultivation conditions is self-evident [4]. In the case of CHO cells, which are mostly used for monoclonal antibody production, an immense effort led to the current product titers of 5-10 g/L. The authorities (Food and Drug Administration (FDA), European Medicines Agency (EMA)) accept these processes, and licensing procedures are straightforward. From an established CHO cell line, the required production cell lines are designed for their

specific applications, and are cultivated effectively in new bioreactor settings and culture media.

Obtaining a license for production as quickly and conveniently can be a deciding factor for vaccine manufacturers; therefore the change to new cell substrates and processes may prevail rather slowly. Old vaccine processes often still rely on chicken embryo fibroblasts and thus on primary cells. The reason these traditional methods are still used may also lie in the long primary life-span of human, primate and chicken cells compared to rodent cells [2].

Table 1: Overview on established cells for vaccine production

cells	first described	origin	source	immortalization	used for:	comments
MRC5	1970 [5]	human	embryonic lung	diploid cells	hepatitis A	adventitious agents? finite life span
WI38	1965 [6]	human	embryonic lung	diploid cells	polio	adventitious agents? finite life span
HEK293	1977	human	embryonic kidney or neuronal cells	transformation with adenovirus functions		ethical concerns? easy into suspension
BHK21	1961	hamster	kidney	spontaneous transformation	rabies, foot-and-mouth disease	not for human vaccines, easy into suspension
Vero	1962	monkey	kidney	spontaneous transformation	polio, rabies	only low passage nr, multilayer, bead-to-bead transfer difficult, limitation of WHO cell bank
MDCK	1958	dog	kidney	spontaneous transformation	influenza	only low passage nr, available as suspension cells
CEF	-	chicken	embryonic fibroblasts	primary cells	measles, mumps, rabies, tick-borne encephalitis	finite life span

(see also Merten et al. [1])

The following is a list of problems that can arise with traditional cell lines as they age:

- Cell lines such as MDCK, MDBK (Main Darby bovine kidney) or Vero cell lines were deposited at the American Type Culture Collection (ATCC) and European Collection of Animal Cell Culture (ECACC) cell line collection in the 1960's or earlier. The stocks of cell seeds are slowly being depleted. Hence, the viral vaccine production community should discuss and address this problem, because in order to maintain current levels of vaccine research and production, new seeds from next passages should be generated,

characterized, and certified anew. The World Health Organization (WHO) is currently conducting an internal discussion into this problem for the Vero cell line, recommended by the WHO for viral vaccine production [7]. This is especially also an issue as these cell lines can only be used for production up to a certain passage number (typically 20 additional passages for veterinary vaccines; for human vaccines this can be longer, insofar as stable production is shown for the covered passage levels (European Pharmacopoeia version 5.0)). Beyond this passage number, modifications in the genome are expected to become significant. However, if host-cell DNA removal is not an issue for the respective process, this discussion might become irrelevant.

- From the available MDCK and Vero seed stocks, for example, several subclones and further passages with different reference numbers were deposited at the cell culture collections. Although these cells often show different properties such as growth behavior or virus productivities or even glycosylation profiles, they are often referred as MDCK or Vero cell lines. Which is just similar to what is a regular practice for CHO cell line use. It is well known that each CHO clone is a new cell line. However, MDCK and Vero are handled as if this were not the case.

- HEK293 cells are widely used for viral vector production. The cell line was generated in 1977 by Graham et al. [8]. For the first time, immortalization was obtained by transformation with Ad5 E1A and E1B gene functions. It was later shown that the integration site of the adenoviral DNA was at the chromosome region 19q13.2 [9]. As the acronym “HEK” stands for “human embryo kidney”, many refer to this cell line as to a kidney cell line. However, Shaw et al. demonstrated clearly that this cell line shows more neuronal cell line markers than kidney markers [10]. It seems that immortalization of embryonic tissue by adenovirus functions is preferably resulting in immortalized neuronal cells. This has also been shown for the cell line AGE1.HN that was recently generated. However, for the amniocytes CAP (Cevec’s amniocyte production cells), the retinoblasts

PER.C6, and the duck cell lines AGE1.CR and AGE1.CR.pIX, also other tissues were successfully immortalized with these functions.

Finally, traditional cell lines still connotes adherent cells with a slow and tedious scale-up into bioreactors with microcarriers. Cells are often still grown under serum-containing conditions, and only for purposes of virus production are conditions changed to serum-free (examples for these concepts can be found under [11, 12]). Cell concentrations of 2 x 10^6 cells/mL are typically reached before infection. Higher concentrations up to 1 x 10^7 cells/mL are also possible, but this is not straightforward. The benefit of using adherent cells lies in the easy medium exchange, but comes at the cost of the growth surface being a limiting factor. As long as there are no better alternatives this form of production may be maintained. 50 years of experience has demonstrated it is robust enough for viral vaccine processes.

3 What are "designer cells"?

The word "designer cell line" may be misleading, because at least two definitions of "designer cells" hold.

On the one hand there are ideas on "designing" cells similar to electronic circuits by stably implementing synthetic gene switches into multicomponent circuits [13]. In a certain sense, synthetic biologists aspire to use the designed cells as living biocomputers that process manually applied signals into metabolic or therapeutic functions. The switches can be triggered by different inputs, such as blue light, radio waves, temperature change, antibiotics, hormones, other chemicals, or even oncolytic viruses Applications are especially seen in diagnostics via biomarkers but also in cell therapy.

The second meaning of "designer cell line" addresses the directed development of cells for specific production processes such as monoclonal antibodies, recombinant proteins or viral vaccines [1, 14]. Organism, tissue, immortalization strategy, and growth conditions

are specifically chosen for the product in mind. In another statement, “designer” cells are restricted to human cell lines obtained by transformation using defined oncogenes or immortalizing cellular genes (Vaccines and related biological products advisory committee (2001): http://www.fda.gov/ohrms/dockets/ac/01/briefing/3750b1_01.pdf, access date Jan 2015).

However, especially the second definition is unclear. Typically, the cell lines were developed for a certain application, but are then used for many other applications. Cell line development today uses all available genetic tools to improve for example, metabolism, cell physiology or virus productivity. However, not all key factors of these desired functions are yet understood and therefore afterwards often a thorough screening is required. Currently, high producer cell lines are still identified by combining genetic modification with selection pressure and screening (Figure 1). Nevertheless, the contents of the genetic tool box are growing; developments in synthetic biology [2, 13] have contributed greatly. In the future, hopefully this tool box and the knowledge base on virus replication in producer cell lines will become broad and deep enough to allow the direct design of a high producer cell line for a specific virus. For now, the term “designer cell” implies unfulfilled promises. The existing so-called “designer cells” (PER.C6, CAP, EB66 and AGE1.CR) have been developed for a specific application, but then actual applications were selected from screenings (for PER.C6 see [15] and Table 2, for example).

Following the scheme in Figure 1, one could take the MDCK suspension cell line used by Novartis Vaccines for the first licensed cell culture based influenza vaccine as an example of a “designer cell line”[16, 17]. In this case however, selection pressure was used to adapt an established cell line to grow in suspension with only the production of influenza virus in mind.

Publications from China and India, where many new vaccine processes are currently established, show vaccine producers clearly prefer established cells such as Vero and

MDCK to obtain licensure more quickly [18, 19]. To the author's knowledge, there are currently no examples of licensed vaccines produced with the so-called new designer cells (PER.C6, CAP, EB66 or AGE1.CR).

Finally, the development of a specific cell line for a single viral vaccine application is clearly a high risk prospect with respect to costs and regulatory requirements. Therefore, cell lines should be developed as a platform, similar to the CHO cell line. Then again, this undertaking will be a costly, complex, and time-consuming challenge, which is very likely why established cell lines are still of such a great importance in vaccine manufacturing.

4 How to develop new cell substrates for viral vaccine production

In retrospective, the successful establishment of cells for viral vaccine production offers some insight that might help identify new potential cell lines. Whether these new cells are competitive against the established cell lines must be evaluated. Apart from being excellent virus producers, these cell lines of course need to fulfill regulatory and biosafety requirements [20].

The difficulties involved in the development of a new cell line for a specific demand is evident in the production of viral vaccines for the shrimp industry [21]. There exist at least nine viruses that pose serious problems for the shrimp industry, and for the last 25 years, and despite industry efforts, no continuous cell line for virus production has been established. Until now, shrimp species were chosen based on availability and personal choice. Cells were taken from ovary and lymphoid tissue, but no specifically adapted cell culture medium was available. Additionally, experience in molecular approaches for immortalization is lacking.

4.1 Selection of host

The choice of host (animal or human) depends on the virus. For example, for attenuated viruses that have been cultivated in CEF's or chicken eggs over many passages, such as yellow fever (YF), mumps, measles [11] or MVA (Modified Vaccinia Ankara), avian hosts are a better choice. Chicken cells have barely been used, as these cells are difficult to immortalize. They have a relatively long primary life-span, during which they are genetically quite stable [22]. However, it seems that ducks are better hosts than chickens, as chickens are at more risk for endogenous viruses, and people allergic to chicken proteins may encounter problems.

Endogenous retroviruses (ERVs) can become a high risk as they can be integrated in genomes of all mammalian species as DNA proviruses, and if they are replication-competent, they can be produced as fully infectious viruses. This requires special attention in live-attenuated vaccines. Therefore, current risk assessment requires strict vetting process for known retroviruses. This issue has, for example, slowed down the licensing process for the FluBlok vaccine from Protein Sciences produced in insect cells, as the FDA demanded additional assays on retroviruses before granting the license.

New approaches are being developed to overexpress tetherin in new cell substrates. Tetherin, a type II integral membrane protein, was shown to inhibit the release of several types of retroviruses, so it could also actively inhibit any potential endogenous retroviruses of cells [23]. Whether humans cell lines are of benefit or not remains a controversial topic. Pros for human cells are that then the produced virus is replicating in its correct host and highest immune response can be expected. Glycosylation of glycoproteins is adequate, and the product resembles best the “real” virus. For new therapeutic vaccines especially, such as vaccines against Alzheimer's, diabetes, or nicotine addiction it may be advantageous to not switch to a different host [24]. Using human cells

for the production of recombinant therapeutic proteins was recently reviewed by Swiech et al. [25].

On the other hand, replication in human cells carries the risk that other pathogens, such as ERV's, find an opportunity to replicate, increasing the risk of contamination [26]. Complications can also occur when the rights of the tissue donor must be clarified. Today, several organizations, networks, and cell line repositories exist that help to guarantee quality standards and traceable documentation for the delivery of tissues and cell lines. The vaccinees may encounter an ethical problem with regard to the origin of the vaccine. Human embryonic cells, perhaps from an abortion, as in the case of PER.C6, are not always accepted by the patient. Media exposure may also be a factor. It can be stated that, in general, a variety of cells/hosts will be needed to efficiently respond to variations of the viruses or virus subtypes. Such an approach offers potential solutions to problems such as lower performance for some subtypes, or if allergies or contamination issues occur.

4.2 Selection of tissue

The choice of tissue depends on what immortalization strategy is used, and for what virus production the tissue will be selected. It seems, for example, that kidney tissue is not only good for influenza virus replication, but also for other viruses (Vero, BHK, MDCK, and MDBK cells are often good virus producers). No lung tissue has been chosen so far. Probably some embryonic tissue is easier to obtain than others, such as retinoblasts. Embryonic tissue is clearly preferred over carcinoma tissue or adult tissue.

4.3 Immortalization

Many ways to immortalize cells are described [4, 27-31]. If transformation leads to immortalization, then typically these cells will develop into a tumor when transplanted into immunocompromized mice. But immortalization can also be obtained spontaneously

by induction with certain cultivation conditions, treatment with carcinogens, or by transfer of certain genes that regulate proliferation. These non-transformed immortalized cells can of course equally undergo transformation and then become tumorigenic [30].

With this immortalization, cells can proliferate indefinitely. However, the cells typically accumulate genetic abnormalities, such as polyploidy/aneuploidy. Immortalized cells can show telomere length stabilization, epigenetic gene silencing, oxidative DNA damage, inactivation of cell cycle regulatory genes such as p53, overexpression of cellular oncogenic proteins such as c-MYC, or expression of viral oncogenes. For example, HEK293 cells can be nearly triploid with 62-70 chromosomes/cell by immortalization with the adenovirus E1A and E1B genes, whereas Vero cells have a normal chromosome number of 60 and can be found hypodiploid as well as hyperdiploid [27]. When cells are then further cultivated and karyotype changes occur, they are often seen together with changes in tumorigenicity. Heterogeneity in structure and number of chromosomes between cells in the same cell line is, thus, not surprising.

Transformation by small DNA tumor viruses; e.g. adenoviruses or SV40

When cells are transformed by small DNA tumor viruses, they can produce a tumor when injected into isogenic or immunocompromised animals [31]. Typically, only a selected part of the integrated viral genome can be found expressed as mRNA (small and large T-antigen of SV40 virus and E1A and E1B proteins for adenoviruses). Very often these transformations for immortalization only function if the DNA tumor viruses insert two genes. For adenovirus this means for example that E1A activates cellular and viral DNA synthesis (by binding to the cellular Rb protein) and activates p53 together with apoptosis signaling initiation, whereas E1B inactivates the p53 function. When tumor cells are transformed, these cells typically need less or no growth factors. Overall, it seems that

rodent cells are more easily immortalized than other species, while human cells require slightly different strategies [30].

Immortalization by UV irradiation

The company Baxter has described a method to generate continuous cells by UV irradiation (AU2013206102 (A1) — 2013-06-20). This was explicitly used for a cell line named QOR/2E11 derived from quail embryos (*Colinus virginianus virginianus*). In this method, UV light from 100 – 400 nm can be used, depending on the cell line (254 nm in the example for quail cells). Irradiation was conducted upon 6 well plates with low liquid level for 4 min. Additionally, photosensitizing agents can be added to enhance the mutation frequency. The method can be seen as a spontaneous transformation induced by UV irradiation. Those clones that then continued to grow in the selected chemically defined medium in suspension were later chosen as a new cell line [32].

Chemical carcinogens for immortalization

There are different carcinogens described for the chemical immortalization of cells. One carcinogen that seems to be more efficient than others is N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) [28]. It was successfully used for the generation of the chick embryo PBS-12SF cell line (see also below) [33, 34]. What exactly is involved in this immortalization is not described. However, MNNG can also be used to induce senescence in cancer cells [35]. In this case, DNA damage and activity towards p53, alpha-tubulin and APC protein were described when high concentrations of MNNG were used.

4.4 Adaptation to growth in a single cell suspension

Some viruses need cell-to-cell contact to spread, and some viruses that spread via syncytia need an orientation of cells. In these cases, a process with adherent cell growth is

advantageous. For all other viruses, suspension cell growth allows for easier and faster scale-up, and limits costs and the risk of contamination by microcarrier handling. Suspension cells could then grow to cell concentrations above 1 x 10^7 cells/mL, and high-cell-density freezing for direct inoculation of bioreactors becomes possible. However, production of viruses with cell concentrations above 1 x 10^7 cells/mL is still in its infancy. Single cell suspension is not always obtainable, and cells form small aggregates of more than 10 cells. Cell aggregation is influenced by osmolality, salt concentration and/or addition of antifoam for example. Medium development could certainly help to improve towards single cell growth.

In many cases, viruses demonstrate an undefined "cell-density-effect" with reduced cell-specific productivities at higher cell concentrations. Cultivation strategy and media need fine tuning to ensure cell-specific productivities as high as at low cell concentration batch cultivations [36, 37]. Naturally, downstream processing will then require modifications. At higher cell and virus concentrations, contaminants (such as protein, DNA) may be found at higher concentrations, or chemical precipitations or instabilities could occur at different viscosities that were not seen for lower concentrations (for an example of downstream processing of a high cell density process, see [38]).

4.5 Adaptation to growth in serum-free medium

Any new cell line should be able to grow in serum-free medium to meet regulatory requirements to reduce the risk of prion or adventitious agent contamination.

In 2000, adaptation to serum-free medium was described, for example, to be best conducted in a three phase process: 1) adaptation to growth in suspension, 2) adaptation to growth in serum-free media, 3) adaptation to "high-cell-density" conditions (up to a maximum of 4 x 10^6 cells/mL) [39]. Change of medium was conducted by stepwise reduction of serum and stepwise increase of serum-free medium. This was described for

CHO, BHK and hybridoma cells, which are adapted to suspension fairly easily. For other cells this can be much more difficult and other adaptation protocols may need to be used.

Currently the media are animal component free and chemically defined, thus even protein-free. The media used in the early years for such adaptations to serum-free conditions were often still very undefined, and contained hydrolysates or animal proteins like BSA. The available knowledge on CHO cell media is certainly helpful for the development of media for suspension cells. Especially for MDCK and Vero cells, it seems there is still a high demand for new media that support adherent growth on microcarriers. These new media are still being developed and tested only with a single available cell lines (e.g. only one MDCK cell line) and only in T-flasks. Growth in roller bottles or on microcarriers is not evaluated in the early stage of medium development, and so often these media do not optimally support cell attachment for adherent cells. Media today must perform not only in stainless steel vessels, but also in disposable plastic vessels with possible influence of the plastic on media components.

4.6 Adaptation to growth in chemically defined medium (CDM)

Ultimately, an adaptation to chemically defined medium is desirable. However, this means that hydrolysates, proteins, and some growth factors that may be vital for cell proliferation are missing. Some cell lines may simply not be so demanding, while others definitely are, hence this step can be critical. Most media appear to function well in the beginning, but they need to support cell growth with high viability and virus productivity for many passages. Some media need additional optimization for large scale production, as cell adaptation is typically performed in shakers with different shear, pH and oxygen characteristics than in large scale stirred tank bioreactors.

Currently not many CDMs are available, and experience with medium quality in our lab were highly variable: even these defined media showed batch to batch variations, and

switching from one medium supplier to another resulted in different growth performance. It seems that in these lean media, any slight inconsistency can have enormous impact on cell growth and virus replication performance. Finally, modified formulations of these media (including cryoprotectant) to support freezing and thawing of the cells are required.

4.7 Improving cell growth

After adapting to growth in chemically defined medium in suspension, cells should be able to grow within 4-6 days from 5-8 x 10^5 cells/ml up to 1 x 10^7 cells/ ml in batch mode. If cells are not growing at this level of performance, high improvement potential lies in media development. For adherent cells, this can be even more difficult. Here, high performance cell growth is a quick cell attachment onto microcarriers with about 10 cells/microcarrier inoculation, then achievement of confluency on the microcarrier within 3-4 days. Attachment onto the microcarriers is essential for optimal growth and this must be supported by the medium. Unfortunately, many of the chemically defined media do not support attachment, but rather are starting media for adapting cells to grow in suspension. However, some viruses do replicate better in attached cells, as cell-to-cell contact or cell orientation for syncytia formation may be necessary for virus spreading [40, 41].

By further improvement of cell properties or introducing helper functions for the virus replications, the current designer cells could be further developed for specific applications (one example is the AGE1.CR cell line with stable transfection of structural genes of alphaviruses to produce alphavirus particles [42]).

4.8 Freezing and thawing of optimized cell line

Master and working seed should be frozen in an adequate freezing medium that allows easy thawing with good growth performance afterwards. Many suspension cell lines

demonstrate a slow start of cell growth after thawing. Several passages are needed before growth performance is equal to the performance before freezing. Some cell lines have at times great difficulties in starting up after thawing, and multiple attempts are required (which demonstrates the requirement of optimized freezing media). For now, large stocks of cell seeds are frozen under different conditions to meet the demand for back-up variations. There exists a clear trend towards freezing working seeds at high cell density for a quicker scale-up.

5 Screening for a good virus producing cell line

Some viruses are lytic, while some are not; some replicate slowly, and others, quickly. Those with membranes typically build in membrane components from the host cell. Some viruses replicate in the nucleus, others in the cytoplasm. Some require a freeze-thaw cycle for virus harvest; still others only replicate in dividing cells, and some viruses require cell-to-cell contact for virus spreading. Thus, it is not easy to give just one definition of a good virus producing cell line. By looking at the points covered in the previous chapter, some important issues for a screening can already be considered up front; other issues can only be verified when a new candidate cell line is available.

- Unadapted seed virus: The seed virus that is used is typically not adapted to the cells of the screening. Virus titers might increase after several passages within the new cell line, and then be clearly better than the titers obtained in the first passage.
- Non-optimized parameters (moi, trypsin, temperature): If the screening is carried out at only one moi, one temperature, and/or one pH, the best cell line may be screened for the wrong parameters, and the cell line performance will not be seen correctly.

- Moi should be as low as possible to avoid defective interfering particles that will reduce the virus titer [43, 44].
- Screening at one time point, or from a time course: Each cell line will show a different performance of virus replication with different dynamics that will depend on the susceptibility for the unadapted virus. If only one point in time is used to judge performance, those cell lines that reach maximum titers earlier or later than this time point may be missed
- Screening for several subtypes: Especially for influenza, several subtypes need to be produced with one cell line. But some cell lines, such as Vero cells, are only good producers for some virus subtypes, while others do not replicate at all. To account for this factor, screening should contain „difficult“ virus subtypes.
- Comparison of cell-specific productivity: Even under identical growth conditions and with biological replicates, the maximum cell number at time of infection is typically unequal. Therefore, the comparison should rely on cell-specific productivity, and not on a virus amount per mL of cell culture volume.
- On the basis of infectious or total virus titer: Depending on the vaccine, infectious or total virus will be essential; this must be considered for the screening.
- Biological replicates: Of course, biological replicates are required, but some of the virus assays are very tedious (e.g. $TCID_{50}$ assay (tissue culture infectious dose 50)). Therefore, screening is often performed without replicates. Nevertheless, cell line performance should be reevaluated at different passage numbers, which will deliver at least some biological replicates.
- DNA and host cell protein content, possible time of harvest: Some important features of downstream processing may be important during the screening process and can influence the decision of whether or not a cell line is a candidate for the process in mind.

6 Currently available "designer cell lines"

Even now, not many "designer cell lines" are available. For vaccine production, especially for emerging diseases, there is not always enough time and money to develop or evaluate a new cell line, as time to market becomes a more critical factor in this field. However, for viruses where old cell substrates are not leading to process relevant virus titers, new cell substrates will have a chance. Additionally, many issues of the established cell substrates such as restricted passage number, adherent growth, and limitation of the master cell bank may continue the development of new cell lines. Once the safety for one cell line has been demonstrated within a licensing process, such as the suspension MDCK cell line from Novartis Vaccines, further manufacturers will be encouraged to develop their own cell lines in a similar fashion. Today's methods in cell line development and genetic engineering, together with process intensification, should clearly convince to use "designed cell lines".

6.1 PER.C6 cells

Several PER.C cell lines were first developed and described in 1998 by Fallaux et al. [15] from the same lineage as the 911 cells obtained from human embryonic retinoblasts [45]. On the basis of these cell lines Falloux founded the firm Crucell with the idea to provide a cell platform, initially for adenovirus production in gene therapy applications. Later, the platform was also applied to protein expression and viral vaccine development. For one specific application, the customer can obtain a license for the use of the cells and the process. Later, Pau et al. described the application of the PER.C6 cell line for influenza and other viral applications [46] (see Table 2).

In 2006 a master cell bank for the production of a recombinant E1 deficient adenovirus type 5 vaccine vector for HIV-1 (human immunodeficiency virus) was developed [47]. The cell bank with growth in suspension and serum-free medium was rigorously tested and characterized. On the basis of this platform (called the AdVac® platform), further approaches to develop vaccines for Ebola, Marburg, hepatitis C, tuberculosis or malaria, have been pursued. This approach is also applicable for the human respiratory syncytial virus (RSV) to which the PER.C6 cells do not seem to be susceptible [48].

After having used the PER.C6 cells in suspension for some years, Crucell decided to file a patent on the growth of their cell line in adherent form. This could allow further applications of this cell line, especially towards blood coagulation factors and viral vaccine production [49].

The suspension cell line was further improved by selection and medium development with the aim of high cell density cultivation in perfusion [50]. Using hollow fiber based cell retention, cell concentrations of more than 1.5 x 10^8 cells/mL were obtained [51]. This was successfully used for an exemplary mAb production [38]. However, when evaluating production of adenovirus at these high cell concentrations, a high cell density effect allowed only cell concentrations up to 1.6 x 10^7 cells/mL without loss of cell specific productivity [52].

Table 2: Overview on production options of viruses in PER.C6 cells

virus	reference
adenovirus (vectors)	[51, 53, 54]
influenza	[55, 56]
rotavirus	[56]
herpes simples	[56]
measles	[56]
polio	[57]
west nile fever	[58, 59]

6.2 CAP cells

Another human designer cell line that was developed shortly after the PER.C6 cell line is Cevec's aminocyte production cell line (CAP). In contrast to the HEK293 and PER.C6 cells, these cells do not originate from an abortion and are therefore ethically more acceptable for the patients. The primary cells were isolated from an amniocentesis and then immortalized similar to PER.C6 or HEK.293 cells via E1A/E1B adenoviral functions [60]. The cells grow in suspension in serum-free or chemically defined medium up to 5 x 10^6 cells/mL. When grown in perfusion mode, up to 3,3 x 10^7 cells/mL were reached within 6.5 days [37]. The authors have demonstrated, together with Cevec, that these cells can be used for influenza virus production [37, 61]. In contrast to PER.C6 cells, CAP cells are susceptible to RSV [62]. Additionally, evaluation of polio 1-3 virus replication showed acceptable virus titers [62].

6.3 AGE1.CR cells

While the company Crucell pioneered designer cells, companies such as Vivalis or ProBioGen followed suit by introducing their own new cell lines. However, the preferred host for cell lines from these firms was avian, as they were working towards an application for modified vaccinia Ankara (MVA) production. MVA has been adapted to chicken embryo fibroblasts (CEF's) by over 500 passages, which led to its attenuation. The attenuated MVA virus can be used as a vector to produce vaccines against HIV or tuberculosis, for example, or for gene therapy purposes. Initially, both companies evaluated the use of chicken as a follow up on CEF's. However, the risk of adventitious agents, and especially endogenous retroviruses, convinced them to switch to ducks as host instead.

ProBioGen together with IDT Biologika developed several duck cell lines by immortalization with the human adenovirus serotype 5 E1A and E1B functions [14, 63]. Starting material was derived from Muscovy duck neurospheres (Cairina moschata ST4) of

primary retina (CR), somite (CS) and amnion membrane cells (CA). Later, they continued only with the retina based cells, originating from the cell line NC5T11 leading to AGE1.CR and AGE1.CR.pIX cells (first named CR.HS and CR.MCX, respectively). In the variant called AGE1.CR.pIX, the human adenovirus serotype 5 structural gene pIX is additionally added to increase possible virus titers by the action of the pIX protein [64]. The cells were grown in suspension first in serum-free medium, and later in CDM [65], reaching up to 9 x 10^6 cells/mL in batch mode [66]. Cell numbers of up to 5 x 10^7 cells/mL in this CDM in perfusion have recently been reached [36, 37]. Although ProBioGen focuses on the use for MVA production, the replication of other viruses has also been described (Table 3).

Table 3: Overview on virus production options in AGE1.CR cells

virus	reference
influenza	[36, 37, 67, 68]
MVA	[14, 65, 67, 69]
fowlpox	[65]
ALVAC-GFP	[65]
attenuated alphaviruses[a]	[70]
duck circovirus, duck hepatitis A virus 1, goose parvovirus, goose haemorrhagic polyomavirus	[71]

[a] Cells are modified and stably contain additional structural genes of alphaviruses in the nucleus (named AGE1.CR pool C cells).

6.4 EB66 cells

The cell line EB66 is derived from stem cells of Peking ducks (*Anas* sp.), which makes the immortalization procedure dispensable. However, the generation of the cell line was highly cumbersome: the cells were obtained from the egg yolk of about 22 000 eggs. Compared to the AGE1.CR cells, only a few publications can be found for these cells [72, 73]. Some more details, however, mainly on the EB14 cells (chicken derived), are given in patent applications [74-77]. EB14 cells were first developed, but then avian endogenous retroviruses were found, and the same cell line generation was used for ducks to obtain the EB66 cell line. Mainly, these cells are known through intensive communication by the company Vivalis (now Valneva SE) directly with the customer and at conferences.

Virologists doing research on MVA use neither AGE1.CR nor EB66 cells. They still however use CEF's, most likely preferring the comparability to previous data and the use of diploid fibroblasts.

Currently, Glaxo Smith Kline (GSK) is building a new production facility together with Valneva SE at Texas A&M (USA) to produce influenza vaccine in EB66 cells. In March 2014, Kaketsuken, a co-development partner with GSK, obtained the license to produce pandemic H5N1 influenza vaccine in EB66 cells in Japan. The production site has a production capacity of 80 million doses.

Described maximum cell numbers for EB66 cells are about 3 x 10^7 cells/ml with growth at 37°C [72], and good virus titers are reported for several influenza subtypes, MVA and r-MVA, canary pox, polio, measles, herpes type 1 & 2, alphaviruses and others. Valneva reports on ongoing cooperation with many other companies for different vaccines on the EB66 cell platform. The upcoming years will certainly reveal more details on the new options this cell line introduces to this field. Unfortunately, strict license agreements restrict many academic researchers from investigating these cells.

Furthermore, it is described that EB66 cells perform a typical avian-like glycosylation of glycoproteins with clearly lower fucose content than, for example, CHO cells. This might become interesting also for vaccine production, as proteins with lower fucose content enhance the antibody-dependent cell cytotoxicity (ADCC) [72, 73].

7 Other new cell lines proposed for vaccine production

7.1 Chick embryo cell lines

The original chick cell line, CHCC-OU2, was obtained and immortalized by N-methyl-N´-nitro-N-nitrosoguanidine (MNNG) by Ogura and Fujiwara [33]. Later, the PBS-1 cell line was derived from CHCC-OU2 as a faster growing subpopulation [34, 78]. After adaptation to serum-free medium (Optipro), the cell line was named PBS-12SF. It shows both Sial2-

3Gal and Sial2-6Gal moieties important for influenza replication and does not need trypsin for good titers. Virus titers for A/New/Caledonia/20/1999 (H1N1), A/Wisconsin/67/2005 (H3N2) and an H5N1 reassortant were described. After adaptation to the cell line, comparable titers as with MDCK cells could be reached. The cell line is non-tumorigenic.

Another chicken embryo cell line was derived from liver tissue by Lee et al. [79] . Here, gene expression for cell cycle regulatory factors and telomerase activity was monitored. Adequate genetic stability could be shown for up to 100 passages, indicating a potential to become a candidate for vaccine production.

7.2 Human liver cells

The production of hepatitis C virus (HCV) is still very difficult. If cells are found that replicate this virus, the titer is so low that only 1-5 virions are produced per cell. Thus, cells are constantly screened for possible candidates for large scale production of this virus. Lately, it was found that exogenous expression of microRNA-122 facilitates the replication of HCV in HepG2 and Hep3B cells (Hep3B/miR122 cells) [80]. Other strategies have also been evaluated to modify existing cell lines to obtain better virus replication [81]. Clearly, upcoming strategies of cell-line development and cell engineering can be seen that will contribute to future development of “designer cells”. This is especially true for those vaccines where no classical cell lines are available that produce reasonable titers. Now, new cell lines were identified that produce HCV from a screening of human cancer cells that express liver-specific alpha-fetoprotein [82]. As these cells are derived from cancer cells, they will not be good candidates for vaccine production. However, they are interesting to study the HCV life cycle as well as for studies on therapeutic agents for chronic hepatitis C. These insights may support the design of possible new producer cell lines.

7.3 Quail cells QOR/2E11 & SOgE:

There are two approaches described for the use of quail cells. One is by the company Baxter: QOR/2E11 cells derived from quail embryos immortalized by UV-irradiation [1, 32, 83], and the other is by the Friedrich-Loeffler Institutes: SOgE cells derived from the permanent muscle quail cell line QM7 (ATCC CRL-1632) [84].

After immortalization via UV-irradiation (see above), the selected clone was adapted to grow in chemically defined medium in suspension and was named QOR/2E11. Reasonable virus titers were described for MVA, r-MVA (TroVax), tick-borne encephalitis (TBE), and influenza New Caledonia. It seems that Baxter is mostly interested to use the cell line for production of r-MVA [83].

The SOgE cells, in contrast to the QOR/2E11 cells, were specifically generated or "designed" to produce Marek's disease virus (MDV). Into an existing permanent cell line (QM7) the glycoprotein E (gE) of the MDV-1 vaccine strain CVI988 was introduced under the control of a human cytomegalovirus promoter. Marek's disease virus is difficult to produce in cell culture. Even in Vero cells, the virus requires a great deal of time to adapt to the cell line. By using the SOgE cell line, this process of adaptation is not required, and thus SOgE cells represent an alternative to primary chicken embryo fibroblasts.

7.4 Fish cell lines:

The production of vaccines for fish in aquaculture has great relevance to industry and different approaches are followed [85]. Several cell lines for viral replication towards vaccine production and diagnostics have been developed, and are being evaluated for vaccine production. For example, infectious spleen and kidney necrosis virus (ISKNV) seems to be a major problem in aquacultures, and no fish cell lines are available that can replicate this virus. Therefore, the cell line MFF-1 was derived from mandarin fish fry by spontaneous transformation. Cells were cultivated for more than 60 passages [86].

Later, MFF-8C1 cells were obtained from MFF-1 cells by single cell cloning and were found to be good for the replication of megalocytiviruses that are problematic in finfish aquaculture [87]. Other available fish cell lines were tested by Sarath Babu et al. for betanodavirus replication [88]. The used cell lines are possible candidates for large scale vaccine production. Lakra et al. described three new diploid cell lines from an Indian major carp [89]. Furthermore, these cell lines were discussed for use in viral disease diagnostic. Another five cell lines were evaluated by Lopez-Doriga et al. for replication of salmon pancreas disease virus [90].

7.5 Human somatic hybrid cell line HKB11 cells:

The cells called HKB11 are derived from a fusion of HEK293S cells with human 2B8 cells (a Burkitt's lymphoma derivative) using polyethylene glycol (PEG). They were proposed as a cell line that grows in single cells for the production of recombinant proteins [91]. Due to their origin from lymphoma, rigorous testing on tumorigenicity will certainly be required, and therefore this cell line is currently not a candidate for vaccine production.

8 Summary and outlook: Designed cells offer new options for vaccines

Having new cell lines that grow in suspension, are stable up to 100 passages [50, 65, 92], and produce viruses of interest to high titers now allow to reconsider vaccine production processes. For influenza, it may be advantageous to isolate virii and prepare the virus seeds directly in continuous cell lines, and not in chicken eggs. This could reduce the time to adapt the virus seeds to the production cell lines, and may keep some properties of the vaccine virus seed more similar to the virus isolate.

Further cell modifications like the introduction of the adenovirus pIX gene, changes in apoptosis (HIF gene), or overexpression of the protein tetherin to inhibit the release of

endogenous retroviruses [23] might lead to more stable processes with higher productivities. Further leveraging the growing genetic tool box, for example in the implementation of synthetic gene switches, enables assessment of the key factors required to develop high producer host cell lines for specific viruses. This could then lead to the directed design of new cell lines with less time needed for screening (Figure 1). However, work to modify the viruses and adapt them to the new cell lines should be also pursued [40, 93]. With chimeric viruses, more viral platforms next to the adenovirus, MVA, and baculovirus platform will become available and process optimization towards high cell density will become decisive. If new media are developed in parallel that meet up to these changes and challenges, further improvements will be seen. Nevertheless, the rate of advancement is difficult to predict. For bacteria and yeast, more tools and understanding of control points within the cells are already available, yet still many challenges remain [94].

Clearly, these newly designed cell lines allow process intensification with shorter scale-up times due to high cell density seeds, and with continuous manufacturing at high cell density, the option to use smaller disposable bioreactors is developed. Vaccine production processes become more flexible and simpler to configure, even for small companies or in countries with low production capacities. Thus, the coming years will show if a new cell line platform will meet many of the named criteria to generate high yielding producer cells for different viruses.

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Conflict of interest

The authors declare no financial or commercial conflict of interest.

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Figure 1.

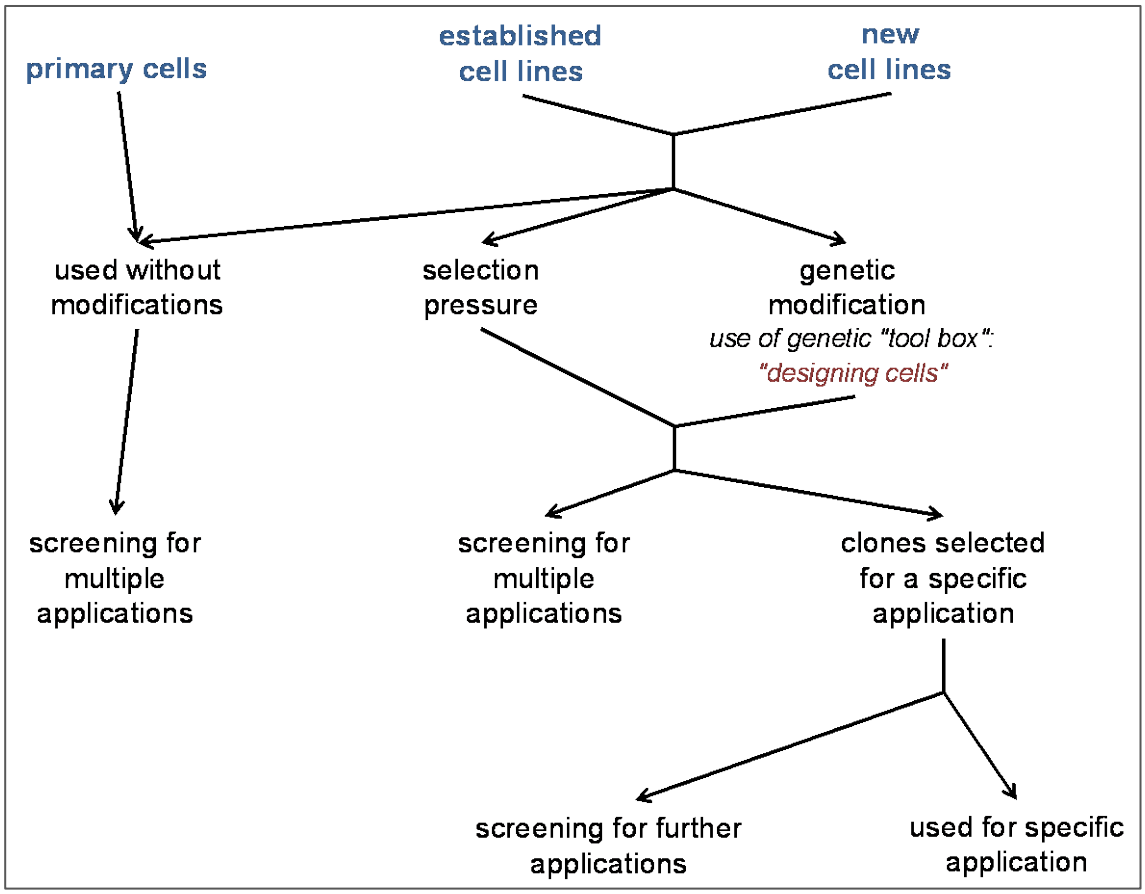

Figure 1. Strategies to develop new cell substrates for viral vaccine production

Table 1. Overview on established cells for vaccine production

cells	first described	origin	source	immortalization	used for:	comments
MRC5	1970 [5]	human	embryonic lung	diploid cells	hepatitis A	adventitious agents? finite life span
WI38	1965 [6]	human	embryonic lung	diploid cells	polio	adventitious agents? finite life span
HEK293	1977	human	embryonic kidney or neuronal cells	transformation with adenovirus functions		ethical concerns? easy into suspension
BHK21	1961	hamster	kidney	spontaneous transformation	rabies, foot-and-mouth disease	not for human vaccines, easy into suspension
Vero	1962	monkey	kidney	spontaneous transformation	polio, rabies	only low passage nr, multilayer, bead-to-bead transfer difficult, limitation of WHO cell bank
MDCK	1958	dog	kidney	spontaneous transformation	influenza	only low passage nr, available as suspension cells
CEF	-	chicken	embryonic fibroblasts	primary cells	measles, mumps, rabies, tick-borne encephalitis	finite life span

(see also Merten et al. [1])

Table 2. Overview on production options of viruses in PER.C6 cells

virus	reference
adenovirus (vectors)	[51,53,54]
influenza	[55,56]
rotavirus	[56]
herpes simples	[56]
measles	[56]
polio	[57]
west nile fever	[58,59]

Table 3. Overview on virus production options in AGE1.CR cells

virus	reference
influenza	[36,37,67,68]
MVA	[14,65,67,69]
fowlpox	[65]
ALVAC-GFP	[65]
attenuated alphaviruses[a]	[70]
duck circovirus, duck hepatitis A virus 1, goose parvovirus, goose haemorrhagic polyomavirus	[71]

[a] Cells are modified and stably contain additional structural genes of alphaviruses in the nucleus (named AGE1.CR pool C cells).