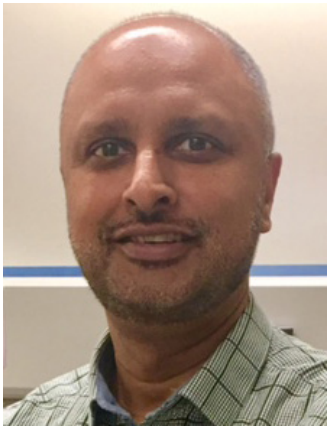
INTERVIEW

State of the art lentiviral vector manufacturing



Dr. Chetan (Chet) Tailor is the Founder and CEO of Tailored Genes. He has over 20 years of experience in molecular biology, cell culture and generating retrovirus/lentivirus vectors. Dr. Tailor obtained his Ph.D in the field of Virology from the University of London UK in 1995, under the supervision of Prof. Robin A. Weiss and Prof. Mary K. Collins, two of the top Scientists in the field of retroviruses/lentiviruses and gene therapy. He then spent 5 years (1996-2001) as a post-doctoral Fellow at the Oregon Health Sciences University in Portland, Oregon, USA under the supervision of Prof. David Kabat. Dr. Tailor next established his own research laboratory at The Hospital for Sick Children Research Institute, Toronto, Canada from 2001-2012. From 2012-2014, Dr. Tailor was Scientific Director of the Vector Core Facility at the University Health Network, Toronto, Canada generating custom-made lentivirus vectors for Canadian and US customers before finding Tailored Genes.

Q What are the key considerations when assessing which viral vector platform to use for your potential therapy?

Target cell and the size of gene you want to introduce are the two key factors for determining the vector you want to use. Another factor includes the duration of gene expression. There are different types of virus vectors currently being used, predominantly lenti-virus, adeno-associated virus (AAV), adenoviruses (AV), and retroviruses but also a number of other types. So, looking at the first consideration, the target cell: depending on what cell you're going to target will determine which type of vectors you want to use as they all have different affinities and expression in different tissue/cell types. For example, if you want to target lung

cells, AVs would be a good vector of choice simply because you can make it as an inhaler/aerosol to get it down to the lungs. If you want to target brain cells, AAVs and herpes simplex virus vectors are good vectors of choice. And if you want to target stem cells or blood cells, a good vector to use would be the lenti viral vectors.

The second consideration is the gene itself. Some viruses, such as AAV can only incorporate small pieces of genes, with the maximum capacity being around 4–4.5 Kb. For lentiviruses, it is 8–9 Kb but for AV you can insert up to almost 30 Kb of DNA.

Duration of gene expression is also an important consideration for virus vector choice. Lentiviruses and retroviruses vectors provide long-term gene expression as they integrate the transgene into the target cell genome. For shorter-term expression, AAV and AV vectors can be used as the transgene is not integrated in the target cell genome.

And then there's the issue of whether the target cells are dividing or non-dividing cells. Retroviruses can only infect dividing cells, which can be limiting factor to any gene therapy utilizing these viruses as a delivery tool. As such, a lot of researchers have reduced the use of retroviruses and are now using lentivirus, AAV and AV so they can infect dividing and non-dividing.

Q You specialize in the production of lentiviral vectors. Could you describe the main differences between 2nd and 3rd generation LV?

Lentivirus vectors are a class of retroviruses that are derived predominantly from a modified version of HIV where the disease-causing genes, and replication ability, are removed thus enabling it to become a viral vector for delivering genes to target cells.

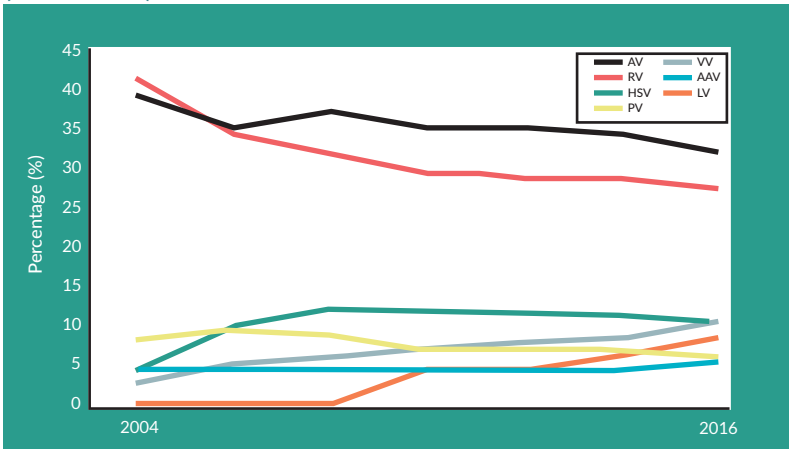
There are different generations of lentivirus vectors that have been produced, and the whole reason for this was to improve their safety profile.

The 1st generation lentivirus vectors involved taking the full genome of HIV, deleting non-essential genes for virus production, in addition to removal of a signal called the packaging signal. A second plasmid with your gene of interest – the transgene – containing the packaging signal is also required, therefore creating a 2-plasmid system.

One safety consideration with viral vectors concerns the frequency of recombination events. Recombination happens between DNA that have similar sequences. So certain sequences such as a packaging signal on the gene of interest will get

FIGURE 1

Viral vector use in gene therapy clinical trials worldwide (2004-2016).



Viral vectors account for 67% of the total vectors used for gene therapy clinical trials worldwide. This graph depicts the % of each viral vector used. AV: adenovirus; RV: retrovirus; HSV: herpes simplex virus; PV: parvovirus; VV: vaccinia virus; AAV: adeno-associated virus; LV: lentivirus. Data extrapolated from *The Journal of Gene Medicine*.

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transferred to the viral plasmid. So in first generation vectors only one recombination event is required to obtain a fully replication-competent virus. Therefore, later-generation lentiviral vectors were created that involved splitting up these plasmids in order to reduce the chances of developing replication-competent lentivirus vector.

Second generation lentivector production involves a 3-plasmid transfection: the main viral plasmid, which produces all the viral proteins to make the virus particle; a second plasmid required to make the envelope of the virus vector (usually the vesicular stomatitis virus [VSV] G envelope), and the third plasmid contains the transgene.

A 3rd generation vector was then generated to increase the safety profile further by using a 4-plasmid approach. Basically the first plasmid that encodes the viral genes is split, resulting in 2 plasmids that have the viral genes, the 3rd plasmid expresses the VSV-G envelope gene, and the 4th plasmid is the transgene plasmid. That's the big difference between the 2nd and 3rd vectors.

There's also a 4th generation lentiviral vector that involves a 6-plasmid transfection. For that you need to amplify and transfect 6 plasmids which I believe leads to loss in efficiency. If you consider the efficiency factor, to get virus produced using a 4th generation system you have to make sure the packaging cell gets all 6 plasmids, which is much more difficult than the 4 plasmid required with 3rd generation lenti vectors. I've utilized the 2nd, 3rd and 4th generation lentivirus vectors and my preference is 3rd generation. In my hands I have found that the viral titer is not high enough in the case of 4th generation vectors and there are safety issues for 2nd generation vectors.

So that's the difference between 2nd and 3rd generation vectors. In terms of what is better, most researchers are going with 3rd generation now, because of the safety element. Moving to the 4th generation has been slow because there are too many plasmids involved.

Q What are the main production steps in manufacturing clinical-grade lentiviral vector?

The first steps in manufacturing research-grade and clinical-grade vectors are very similar. You take a plasmid, transfect the cells and produce a virus. It's the next step, the downstream processing and purification, that's the key difference in the case of clinical-grade vectors.

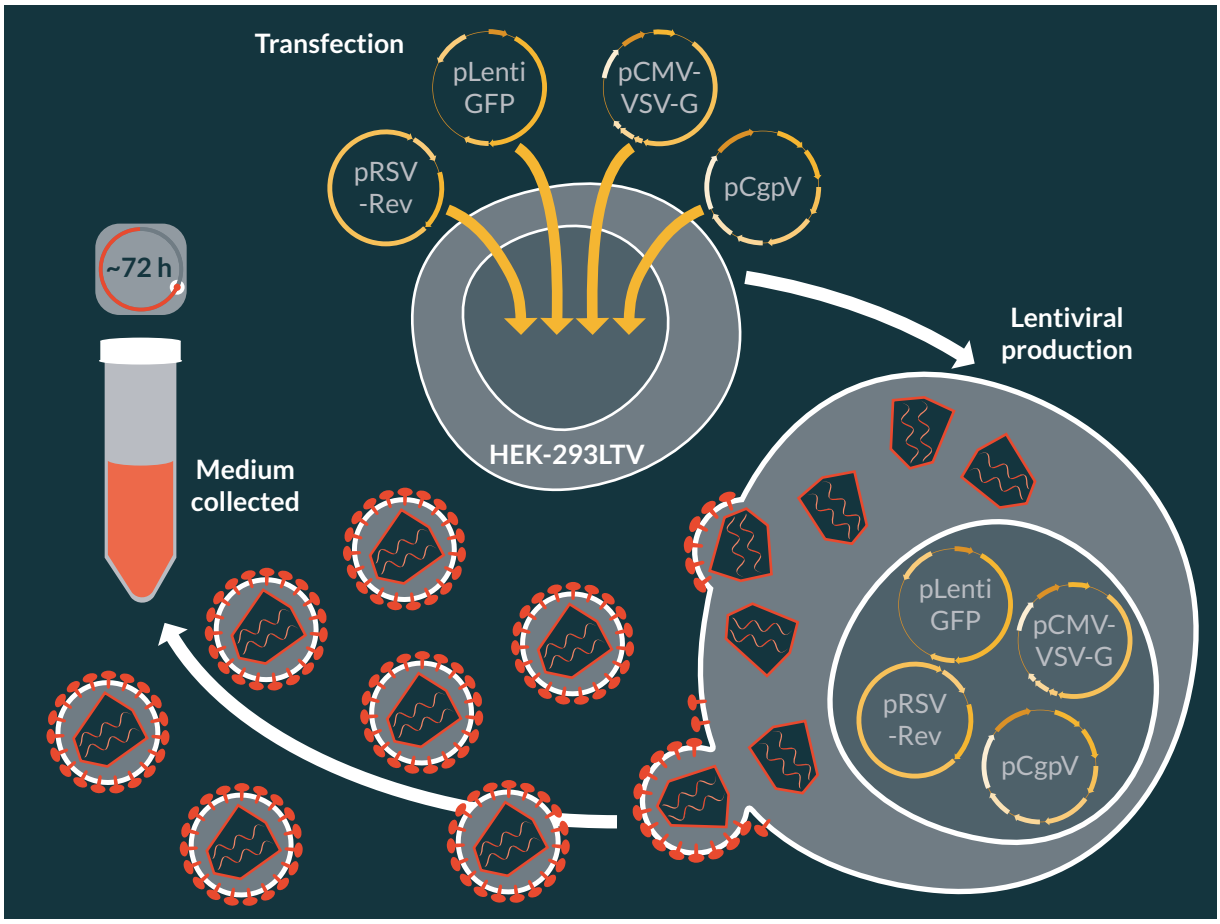
The first requirement is removing the plasmid DNA in the viral preparation. Plasmid DNA is added to the packaging cells to make the virus vector, and so in your virus medium you're going to have plasmid DNA freely floating around. Removal of the free DNA requires treatment of the virus containing medium with enzyme **(Figure 2)**.

The second requirement is the elimination of cellular debris or host cell proteins. The cells you use for virus production are human embryonic kidney cells, HEK293 cells. You're going to get cell debris, and protein as well, that could be toxic to the individual when you administer to the patient.

There are standards set by each health agency, which has the limits you can have within a preparation for clinical grade lentivirus vector, whereas for research grade you don't have to go through all these purification steps.

FIGURE 2

Overview of lentiviral vector manufacturing.



Q Why do we use HEK293 cells for virus production?

HEK293T cells are a cell type that are easily transfectable with plasmids and can express plasmids/proteins at very high levels and that's the key advantage. There are additional advantages like the HEK293T have a hexamer protein, called the SV40 large T antigen, which actually helps increase the overall plasmid expression and hence protein expression. This results in really robust expression of proteins, which is an advantage if you want to make high quantity of virus.

Another quality of the 293T cells is that there are certain components in HEK293T that help are essential in making adenovirus and AAV vectors. The HEK293T cells contain E1A and E1B, two genes with helper function for making adeno virus vectors and AAV's.

Q There are a number of bottlenecks within the manufacture of cell and gene therapies including vector production. What do you see as the main production challenges behind these?

If we take it stepwise, the first challenge is one of scalability. On a small scale you're making maybe 1-2 litres of virus. When you scale up to say

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50 litres, the amount of plasmid you need is much higher and you have to make sure it's clean and doesn't contain contaminants such as endotoxins.

One way to get around it, is to make stable cell lines. This requires the expression plasmids to be stably introduced within the packaging cell, and the way you express those plasmids is you induce them with a drug, and they will start expressing the specified viral genes/proteins.

In this case, you still have to amplify and transfect the transgene plasmid each time you are making lentivirus vector. Another way to make a stable cell line that has all your plasmids and so each time you want to make a scale-up version of the virus, you don't have to do additional transfections, you just scale up the cells

A downside is you're adding drugs to the stable cells to induce expression of plasmids. So when you go through the purification process you need to make sure you get rid of the selectable drug so that there's no trace of it or it meets the requirements set by health agencies.

Another key challenge is that you lose a lot of the virus during the whole downstream purification process, potentially up to 70%. That's a huge problem in manufacturing.

The key to this is make sure you make a really good batch of virus, because you're going to expect some losses in the purification process. You can do a sample test during your downstream purification to see how much virus is there and estimate your loss within a batch, but obviously from one batch to the next there will be variations.

This is definitely a crucial bottleneck within manufacturing and I know the industry is trying to improve the equipment used for downstream processing to reduce this inefficiency.

Q One possible approach to reduce vector manufacturing costs is to create stable cell lines as you mentioned. What does this process involve?

It's not too difficult and in fact there are some labs that have already produced stable cell lines and are going through the process now of testing the stability. For example if you want to make a stable cell line using a third-generation vector, you take the 4 plasmids, introduce them individually to HEK293T cells, and then you select for the cells that express all 4 plasmids at a very high rate.

The first plasmid will have a drug resistance gene encoded in its sequence. Addition of drug will kill off HEK293T cells that don't have the plasmid and will maintain the cells that do have it. Each cell will express the drug-resistant gene at different rates and therefore a screening process needs to be performed where you can select for the cells that have the high rate of plasmid expression. Following this, the cells need to be transfected with the second and third plasmid to make a stable packaging cell line. Although it is time consuming to make this initial stable packaging cell line, once you have it, you can freeze it down and thaw it to use it whenever you want. And when you do need to use this cell line, you now only have to transfect one additional plasmid – your transgene.

Some groups are now moving towards making a stable packaging cell line that has all the four plasmids: 2 viral plasmids, VSV-G envelope plasmid, and the transgene plasmid. So if someone wants a clinical-grade lentivirus, you just need to thaw out the cells and transfer them to a bioreactor to start obtaining the virus. Even after a few years, if you want to scale up and make more of the same lentivirus vector you don't have to go through the whole transfection process. You would just simply thaw out the cells and start producing virus vector, and that's going to be a crucial advancement to improving manufacturing efficiencies and therefore cost.

In Europe, the group headed by Luigi Naldini in Italy, are now manufacturing clinical-grade lentivirus and have successfully utilized it in two disease indications. There are more companies that are coming on board now to manufacture clinical-grade lentivirus as demand for virus vectors increases. In fact, the demand is outstripping current manufacturing volumes. One of the goals we are trying to achieve here in Canada is to build up manufacturing capabilities and capacity to produce clinical-grade lentivirus and other virus vectors.

Q And what about the move to closed systems and automation in tackling some of the challenges and bottlenecks in vector manufacturing?

In the context of vector manufacturing, closed system refers to the use of bioreactors. Right now a lot of what we do is based on adherent cell cultures. For example, on a small scale we use a tissue culture flask and the cells stick to the surface. In some larger scale processes you can use bioreactors that have what's called surface membrane that the cells attach to.

One of the advancements moving forward is to utilize suspension cell cultures technologies. This involves the use of large flask/container that have stable packaging cells swirling around. You add nutrients and media to grow the cells. The process results in a high production rate with increased efficiency and reduced cost as well, because you don't have to make these bioreactors with multiple surface areas

Q What opportunities are on the horizon in terms of new developments in vector manufacturing?

I think some exciting developments in lentiviral and viral vector use in general is gene editing. You can package the CRISPR gene editing elements into a lentivirus vector and target those cells that you want to correct. This is the beauty of lentiviruses and whilst there is still a lot of work to be done in increasing CRISPR gene editing efficiencies, it's going to be exciting to see how this CRISPR-lentivirus vector and other CRISPR virus vector technologies pushes the field forward.

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