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Review

Concise review on optimized methods in production and transduction of lentiviral vectors in order to facilitate immunotherapy and gene therapy

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ABSTRACT

Lentiviral vectors (LVs) have provided an efficient way to integrate our gene of interest into eukaryote cells. Human immunodeficiency virus (HIV)-derived LVs have been vastly studied to become an invaluable asset in gene delivery. This abled LVs to be used in both research laboratories and gene therapy. Pseudotyping HIV-1 based LVs, abled it to transduce different types of cells, especially hematopoietic stem cells. A wide range of tropism, plus to the ability to integrate genes into target cells, made LVs an armamentarium in gene therapy. The third and fourth generations of self-inactivating LVs are being used to achieve safe gene therapy. Not only advanced methods enabled the clinical-grade LV production on a large scale, but also considerably heightened transduction efficiency. One of which is microfluidic systems that revolutionized gene delivery approaches. Since gene therapy using LVs attracted lots of attention to itself, we provided a brief review of LV structure and life-cycle along with methods for improving both LV production and transduction. Also, we mentioned some of their utilization in immunotherapy and gene therapy.

1. Introduction

Viruses are an intriguing conveyor to transfer genes into various host cells. Among them, retroviruses have gained more attention due to the ability to integrate their genome into the target cell genome [1]. Retroviruses have been utilized as a vehicle for gene delivery since 1996 by Naldini et al. [2,3]. Although there are different types of LVs, including HIV-2, Murine Leukemia Virus (MLV), feline immunodeficiency virus (FIV), equine infectious anemia virus (EIAV), and bovine immunodeficiency virus (BIV), the HIV-1 based LV has earned more heed than others as an efficient vector [4–9]. HIV-1 based LVs have been the vectors of choice due to the HIV's capability to integrate specific genes into dividing and non-dividing cells irreversibly, in order to express the gene of interest permanently [10,11]. Mainly, four generations of HIV-1 based LVs have been developed until now [12,13]. Producing and transducing clinical-grade LV into target cells often faces limitations because clinical strategies require safe, efficient, and sufficient LVs. To address those challenges, different production and transduction enhancers, including some specific reagents, adjuvants, and mechanical-based systems such as microfluidic devices, have been applied [14]. Gene delivery approaches can be improved potentially due to the controlled environment provided by microfluidics systems [15]. Recently, lots of progress have been made in the microfluidics field toward both viral and non-viral gene delivery [14,16]. The unique features of these striking LV have entered them into the field of gene therapy and immunotherapy [12,17]. Large amounts of investment have been drawn to the subject of gene medicine in order to overcome the most challenging diseases [12]. Nowadays, LV serves as a phenomenal tool in both immunotherapy and gene therapy by transducing chimeric antigen receptor (CAR) into leukocytes, delivering gene-editing tools like CRISPR/CAS9 into target cells, or integrating a healthy gene in order to rectify a disease [18]. It is worthy of mentioning to say that gene delivery and gene therapy have some limitations in both in vivo and in vitro, including innate and adaptive immune responses, anatomical obstacles, transgene activation, size of the vector, production of large-scale clinical-grade vectors, disruption of host genes, costs of materials and equipment, limited strategies toward optimizing gene delivery [19–23]. In this concise review, we demonstrated some optimized methods that have taken place in the matter of LV production and transduction, along with describing their role in clinical approaches.

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2. Lentivirus

Lentivirus is a single-stranded RNA (ssRNA) virus belongs to the family of Retroviridae (retroviruses), which contains two copies of positive-strand RNA encompassed by capsid and envelope with a diameter between 80–120 nm [24]. All retroviruses encode three equivalent genes, including *gag, pol*, and *env*, which are flanked by long terminal repeats (LTRs). Both ends of the viral genome have LTRs with 600–900 nucleotides needed for virus replication, integration, and expression. Unlike simple retroviruses (y-retroviruses), complex retroviruses (lentiviruses) possess *vif, vpr, vpu*, and *nef* as accessory genes and *tat* and *rev* as regulatory genes that can encode proteins contributing to infection, binding, and releasing [25–27].

2.1. The virus and its structure

The provirus (the viral cDNA that integrates to host genome) has a length, about 9–10 kilobases. The proviral LTRs are located in both ends of the lentivirus genome, and each is divided into three regions, including U3, R, and U5, in which correspondence to transcription initiation is related to the first nucleotide of the R region. The interaction of cellular factors with the U3 region that contains elements of basal, enhancer, and modulatory is requisite for transcriptional activity, although additional regions in both R and U5 are required. The R region comprises particular sequences able to form stable stem-loops, which are pivotal in the Tat-mediated transactivation. Eventually, LTRs also possess sequences needed for RNA capping and polyadenylation in the R region [28,29].

Lentiviruses hold three primary genes, namely *gag, pol,* and *env,* which are responsible for encoding the viral core, reverse transcriptase (RT), integrase (IN) and protease, and viral envelope proteins, respectively [30]. Also, there are regulatory and accessory genes, including *vif, vpr, vpu, tat, rev,* and *nef*.

After the virus integration into the host genome, transcription complexes owned by the host assemble at the promotor site located in the 5′ LTR and produce short incomplete viral transcripts. In order to generate full-length viral mRNAs, a viral transcription factor called Tat recruits the host's RNA polymerase II (RNAP II) and increases its activity. The host's cyclin T1 (CCNT1) is a requisite cofactor for Tat [31–33]. Additionally, Tat manipulates the expression of cellular genes to promote viral replication [34]. Viral transcripts need to be transferred to the cytoplasm. In this manner, the Rev possess shuttling properties involved in the translocation of lentiviral transcripts from the nucleus to the cytoplasm [35].

The Nef protein has been credited with some functions, including downregulating BST2/Tetherin, MHC-I, CD4, and other cell surface receptors [33]. Nef is able to bind with endosomal sorting complexes required for transport machinery (ESCRT), and AP-1 and AP-2 clathrin adaptor complexes involved in coated vesicle budding [36].

The Vif protein has an antagonizing role against the host antiviral agent, APOBEC3 (A3), which results in the degradation of A3 [37]. The secondary function of Vif is the ability to bind with the CBF-β transcription factor, which further leads to a lower expression of A3 [38,39].

The Vpr has several functions, including nuclear import of the pre-integration complex (PIC), cell cycle arrest at G2 phase, LTR transactivation, cellular apoptosis, and activation of the DNA damage response [40,41]. Mostly, the presence of these phenotypes relies on the interaction of Vpr with $CRL4^{DCAF1}$ ubiquitin ligase complex [42–44].

The protein that Facilitates the viral release is Vpu, which contributes to the inhibition of BST-2/Tetherin. The Vpu also induce immunosuppression by downregulation of the NF-κB pathway through inhibiting BST-2/Tetherin [45–47] (Fig. 1).

2.2. The virus life-cycle

The lentivirus life cycle consists of two steps, the early and late phases. Entry to the cell is the first step in the initial stage; simple retroviruses gain entry to the cell via endocytosis mechanism following interaction of cognate cell receptors with the viral glycoproteins within the envelope. So too, complex retroviruses use direct fusion or receptor-mediated endocytosis [48]. After virus attachment to the cell receptor for example binding of CD4 on T lymphocyte (T-cell) to gp120 on the surface of HIV's envelope, multiple conformational changes happen to viral envelope glycoproteins which give access the virus gp120 to bind with other co-receptors such as CCR5 (or CXCR4) chemokine receptor. These conformational changes result in proceeding the membrane fusion of the virus [49,50].

Following initial fusion, the viral capsid transports by microtubule system in a process called intracellular trafficking. During the entry of the capsid, reverse transcription of viral RNA by RT occurs in the cytoplasm through a complicated multistep process to synthesis the viral double-stranded DNA, which is accompanied by capsid disassembling [48,51]. The RT has enzymatic features, including RNA-dependent DNA polymerase, DNA-dependent DNA polymerase, and RNase H [52].

Accordingly, the capsid converts to the PIC that contains provirus DNA, Vpr, integrase, and matrix p17 (MA) [52,53]. The nuclear entrance of PIC is facilitated by the interaction of specific sequences on both integrase and MA with importin α and β [54,55]. Herein, the Vpr protein expedites nuclear transportation by increases the affinity of importin α to sequences on both IN and MA. Also, it has been demonstrated that Vpr interaction with human Nup153 can facilitate nuclear translocation of the lentivirus genome [28,56]. Opposite to simple retroviruses, complex retroviruses are able to pass through nuclear pores. Given that, this potent them to infect not only dividing but also quiescent cells in the G0 phase [57,58].

The provirus integration to the host DNA is mediated by PIC, which is a precursor for chromosomal integration. This process takes place by chance with respect to the preserved sequence located at the end of LTR. Even some endogenous transcription factors belong to host cells, such as LEDGF involves in the integration process [59,60]. As the integration completed, RNAP II recruited by Tat starts to use the U3 element located at 5′ as the promoter for transcription [61]. Through alternative splicing, retrovirus extends the protein-coding potential, encoding two open reading frames (ORFs) from a solitary promoter without neglecting the “one mRNA, one gene” rule. This ability allows the retroviruses to have a fixed ratio of viral proteins (high gag to pol ratio) [48,62]. After transcription and translation, capsid assembly in the cytoplasm initiates by Gag binding to the genomic RNA. All in all, the viral progeny uses a process termed budding to be released into the extracellular space [48,63].

2.3. Generations of HIV-1 based lentiviral vector

Stripping down the HIV-1 genome to its bare bones provides a potential primary LV; nonetheless, a significant breakthrough achieved by Naldini et al. was segregation of the viral vector sequences into distinct plasmids, which invented a safe and reliable LV [64,65]. The first-generation contained all of the HIV-1 genome except *env*; instead, another viral envelope was replaced. The G glycoprotein of the vesicular stomatitis virus (VSVG) on a separate plasmid included in the design of first-generation to encode a more effective envelope [64]. Pseudotyping LVs with VSVG protein provided a broader breadth of tissue tropism [66]. The VSVG envelope has been proven to have higher stability, resulting in lengthy storage periods and effective concentration rates by ultracentrifuge. The gene expression rate was intensified by using the human cytomegalovirus (CMV) promoter in the 5′ LTR [67].

The *vif, vpr, vpu,* and *nef* genes have shown to augment the virulence effect of viral vector in the first generation. For example, Vpu, Vpr, Vif, and Nef are potentially able to induce immunosuppression, cell cycle

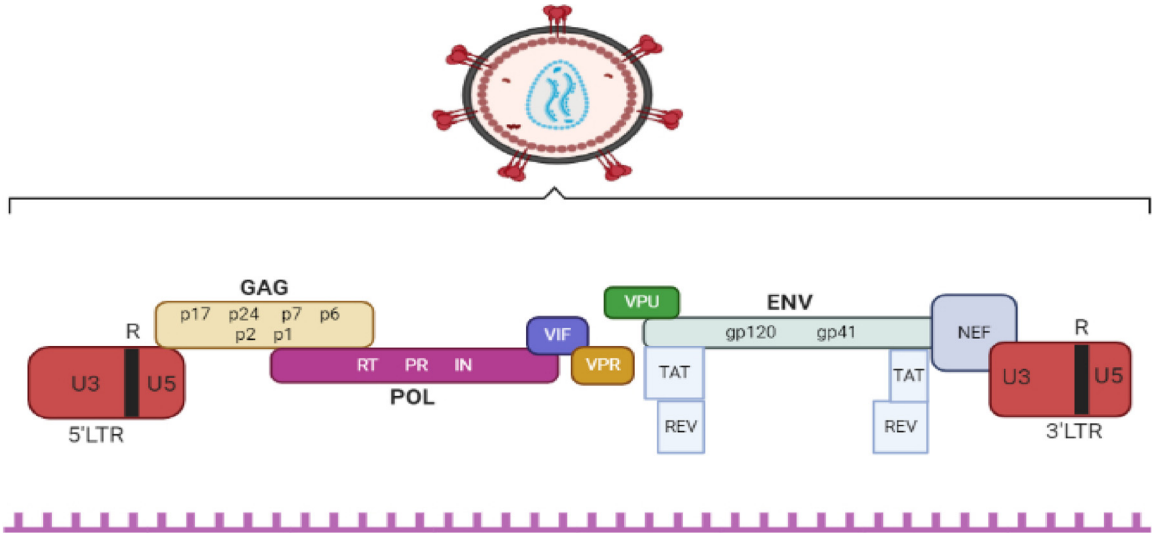

Fig. 1. Schematic representation of HIV-1 structure and the corresponding genes. HIV-1 genome is flanked by 5′LTR and 3′LTR. *GAG, POL*, and *ENV* genes encode structural proteins. *GAG* gene encodes 6 proteins (p17, p24, p7, p6, p2 and p1), *POL* gene encodes 3 essential proteins (reverse transcriptase (RT), protease (PR), and integrase (IN)), *ENV* gene encodes 2 proteins (gp120 and gp41). *TAT* and *REV* are 2 regulatory genes. *VIF, VPU, VPR*, and *NEF* are 4 accessory genes [27].

arrest, cell growth inhibition, and provoking apoptosis, respectively [68–71]. The second generation of LVs became safer by eliminating these four viral accessory genes; however, the LV gene transferability rate remained considerably efficient [72].

Unlike the second-generation construction that *tat, rev, gag*, and *pol* genes were placed along together, in the third-generation system, *tat* was deleted from packaging construct due to its role in intensifying HIV replication and exceedingly increasing the transcriptional activity [73]. Besides, Tat has been considered as a potential key factor in developing Kaposi's sarcoma and inducing adverse cell responses [73,74]. By deleting *tat* from the packaging construct, an active promotor sequence such as CMV promoter was added into a construct containing the transgene, which could be considered as a further enhancement in the system [72,73]. The third-generation of LV can be produced by three- or four-plasmid systems, which former codes for gag-pol, env and gene of interest, and later codes for gag-pol, env, rev, and gene of interest. These plasmids can be transfected into the human embryonic kidney 293 (HEK293) cell line, which is being utilized widely to produce LVs [75]. Recently, a novel and more stable lentiviral packaging cell line termed LentiPro26-A59 was developed that can provide viral titer above 10^6 TU. mL^{-1}. day^{-1}, which shows more adaptability for large-scale viral production [76]. However, still, over time, the viral productivity becomes less that could be due to the epigenetic alterations in the CMV promoter [76,77]. In addition to separating viral particles into distinct plasmids, self-inactivating (SIN) LVs were developed to achieve safer transduction and less likely recombinant virus generation [78].

Compared to other generations of LVs that contain 19.6 % similarity to the genome of wild type HIV-1, the fourth generation of the LV termed LTR1 has been designed by incorporating quintessential RNA signal sequences to reduce the similarity of the LV to 4.8 % [79]. Since these signal sequences are placed outside of the viral backbone, they would be subsequently lost throughout the reverse transcription process, which hampers them to be copied into the transduced transgene. Given that, the LTR1 lentiviral vector might be a safer armamentarium toward gene therapy [13].

2.4. Optimizing vector production

In brief, adherent cell lines such as HEK293 T or HEK293 are generally considered as a proper packaging cell line for plasmids (transgene, gag-pol, rev, and env) containing codes for vector packaging construct. The expression of SV40 T-antigen on the HEK293 T is the main difference between these cell lines. This antigen showed to lead to yield higher titers [80]. After HEK293 T reached 70 % confluency, the plasmids would be transfected transiently into HEK293 T cells with transfection reagents such as polyethylenimine (PEI) or Calcium Phosphate (CaPO4). Transfection mixture should be removed from culture media after several hours by replacing the media with a fresh one. Then, 48–72 hours post-transfection, the virus should be harvested. Then, an ultracentrifuge can provide adequate amounts of vector for small-scale or research purposes [81]. In order to capture large-scale of the vector from the supernatant, anion exchange chromatography and tangential flow filtration (TFF) might be a proper call. The typically achieved titer fluctuates between 1×10^8–1×10^9 viral particles per mL. However, Tinch et al. introduced a method using Mustang Q chromatography, TFF and diafiltration that might yield even higher titer [82]. Other mentioned methods for LV production are electroporation and nucleofection [83,84].

The HEK293 T is a serum-dependent cell line, so producing the current Good Manufacturing Practices (cGMP)-grade LVs through this cell line consist of some limitations due to the consumption of animal-derived serum for growth and maintenance, which is costly and also prone the culture media to a higher risk of contamination by unpremeditated viruses. Furthermore, the need to use multi-stack plastic tissue culture vessels demands more production time and requires further costs [85]. To address these limitations, Bauler et al. developed an enhanced adapting HEK293 T-derived cell line, called SJ293 TS, with the ability to grow in suspension using serum-free media (SFM). Compared to the adherent serum-dependent HEK293 T cell line, the SJ293 TS cell line showed the equivalent value of the LV generation in SFM. Importantly, an efficient transduction rate was achieved, following to transduction of SJ293 TS-derived LVs into human T cells and CD34 + cells. Although other HEK293-derived cells have been developed to grow and produce LVs in suspension with SFM, the SJ293 TS demonstrated at least 10-fold higher LV titers [85–89].

Suzuki et al. showed that intensifying promotor activity in HEK293 T would results in a significant enhancement in the production of LVs. They found out that LV production will be enhanced by co-expression of SPRY domain-containing SOCS box protein 1 (SPSB1), along with higher *gag* and *env* (VSVG) expression and increased activation of the promoter (CMV promotor) [90]. Subsequently, they have figured that Tax protein from human T-lymphotropic virus type 1 (HTLV-1) can activate essential transcription factors, including AP-1, NF-κB, and CREB/ATF, which binds to their recognition sites on the

CMV promotor. All that eventually leads to higher production of LVs [90–94].

The harvested LVs could play different roles in research experiments or therapeutic strategies through transducing cells with either a protein-coding gene or an RNA interference (RNAi) gene such as short hairpin RNA (shRNA) [95–97].

2.5. Optimizing vector transduction

LVs can be pseudotyped to transduce a vast spectrum of tissue tropism, including lymphocytes, myocytes, hematopoietic cells, fibroblasts, neural cells, hepatocytes, lung epithelial cells, pancreatic islet cells, muscle cells, and retinal pigment epithelium. However, the VSVG pseudotyped LVs fulfilled this purpose [98–101]. Note that, the VSVG protein recognizes a ubiquitously expressed receptor called low-density lipoprotein (LDL) receptor, enabling the vector to transduce a vast range of cells [102].

Some studies explicated that prior to binding of enveloped viral vectors with their established receptor on target cells, there are other binding events independent of those specific interactions [103,104]. Fundamentally, enveloped viral vector interaction with the target cell might not be straightforward due to the electrostatic repulsion forces between negatively-charged cells and the viral vector [105,106]. In this regard, poly-L-lysine, diethylaminoethyl (DEAE)-dextran, polybrene, protamine sulfate, or cationic liposomes are applied as positively-charged polycationic agents to diminish those repulsion forces [105,107–114]. Though utilizing polybrene in viral transduction indicates adverse effects on the proliferation and senescence of stem cells, especially mesenchymal stem cells (MSC), inhibiting the activity of p38 mitogen-activated protein kinases (MAPK) revealed to be effective in attenuating those adverse effects of polybrene. Intriguingly, the p38 MAPK inhibition elucidated no weakening in transduction efficiency [115]. Albeit, data suggest that DEAE-dextran has a preferable aptitude over other aforementioned polycations in heightening LV transduction rate [114]. The amphiphilic poloxamer synperonic F108, also called lentiBOOST, is a non-ionic substance that diminishes the microviscosity of the cell membrane, escalates lipid exchange, and amplifies transmembrane transport [116]. In several studies, lentiBOOST provided high transduction efficiency to hematopoietic stem cells (HSCs), hematopoietic stem and progenitor cells (HSPC), and T cells, and also have not shown any specific toxicity or alteration in cell viability and phenotype [116–121].

Other enhancers, including Vectofusin-1, FuGENE 6, PEI, Lipofectamine 2000 and 3000, demonstrated the capability to improve transfection and subsequently viral transduction efficiency [122–125]. Also, results provided by some studies showed that adjuvants such as staurosporine, poloxamer 407, and prostaglandin E2 (PGE2) are potent to increase LV transduction in human CD34+ cells either alone or in combination together [126–128].

Intrinsically, LV with a VSVG envelope would be recognized as an invader to cells. Since all cells possess innate intracellular immune barriers, they would hinder the transduction process [129,130]. Measures like attenuating intracellular responses by using immunosuppressors can be taken into account to increase the transduction rate. Several studies showed that immunomodulatory compounds such as Cyclosporine A and H, BX795, and corticosteroids like Dexamethasone (DEX) would result in a heightened viral vectors transduction rate [130–136]. Furthermore, using Cyclosporine H (IFITM3 inhibitor) in comparison to Cyclosporine A, resulted in a remarkably higher LV transduction rate in HSPCs derived from the human cord blood [135].

Retronectin (recombinant human fibronectin fragment) has a great potential in juxtaposing the target cell and lentivirus. It possesses C-, CS1-, and H-domains, which C- and CS1-domains can bind to the target cell via cell integrins (VLA4 and VLA5), and H-domain absorbs lentiviruses [137]. Many studies stated, either adding LVs prior to introducing target cells or adding a mixture of LVs and cells to a Retronectin-precoated well plate could boost transduction efficiency, but in comparison to other novel methods using Retronectin requires a relatively high multiplicity of infection (MOI) to reach the desired transduction efficiency [14,137–143]. Although Retronectin is GMP grade transduction enhancer, the high MOI needed for Retronectin-mediated transduction and specific coating protocols might be count as limitations in clinical strategies [139]. According to that, a targeted and specific single-chain variable fragment (scFv) was fused to VSVG, and low MOI required for safe and efficient transduction was achieved [144]. However, the cells that are hard to transduce, such as human CD30+ lymphoma cells and EGFR + tumor cells, could be efficiently transduced by fusing scFv antibody to VSVG along with using spinoculation, lentiBOOST [139,144].

Extracellular vesicles (EVs) can carry various types of RNAs, and it has been implied that EVs are biocompatible transporters for gene delivery [145]. Somewhat, the biological pathway of producing LVs from adherent cells is parallel to the biogenesis of these putative EVs [146–148]. A transmembrane protein called CD9 is required for EV biogenesis; besides, CD9 has different roles in cell adhesion, cell-cell fusion, and somehow CD9 might be linked with HIV-1 infection [149–151]. Based on that, O. Böker et al. found out that if they amplify CD9 expression in the LV producer cells, in the absence of VSVG protein on LVs, which is required for binding to LDL receptor of cells, the produced EV-based LVs containing viral proteins, transgene and the CD9 on their surface could minorly transduce the cells, without need to interact with any specific receptor. Afterward, they produced VSVG-LVs containing high amounts of CD9 on the vector's surface, and transduced five distinct cell lines, including B cells (Raji), T cells (Jurkat), HeLa, HEK293, and SH-SY5Y neuroblastoma. All in all, Utilizing CD9-VSVG-LVs resulted in achieving faster and enhanced transduction efficiency [152] (Fig. 2).

3. Microfluidic approach toward LV transduction

New and efficient approaches toward the improvement of gene delivery and therapy are always required to overcome different obstacles in these areas. The microfluidic approach in the field of gene delivery and therapy promises a remarkable breakthrough by providing a micro-environment to obtain better control and precise optimization over both transfection and transduction processes [15]. Using LVs as a gene delivery tool through microfluidic devices would benefit in different directions, including less LVs usage for transduction, and faster transduction rate. Thus, it can prevail over some limitations and extra costs [153]. Not only the microfluidic system would allow enhancing transduction, but it also provides an additional feature like microfluidic-based electroporation to manipulate a cell leading to improved transfection efficiency and higher cell viability [154–156]. For instance, a study used microfluidic-based electroporation to transfect 100 million human T cells with CAR RNA, and process only took thirty minutes, afterward the cells reached to their full efficiency in 24 h [157]. It worth mentioning that cells naturally operate in a micro-scaled environment, so according to that, A study by Houshmand et al. showed that to some degrees, culturing the living hematopoietic bone marrow in microfluidic systems is able to simulate and mimic a bone marrow environment like (BM) in a 2D and 3D in vitro conditions [158]. All of which indicate great potentials of microfluidic systems.

By allocating an ideal environment at 37 °C for LVs, their half-life could be considered to be in a mean range of 600 μm through Brownian motion from their initial point to their endpoint [159,160]. Conventional transduction procedures and even real-time transduction with co-culturing LV producer cells with target cells take place in standard cell culture vessels [154]. However, traveling in these standard cell culture vessels requires millimeters or centimeters of distances. It brings up that most LV particles degrade before reaching their target. Microfluidic devices overcome that obstacle by miniaturizing the environment to

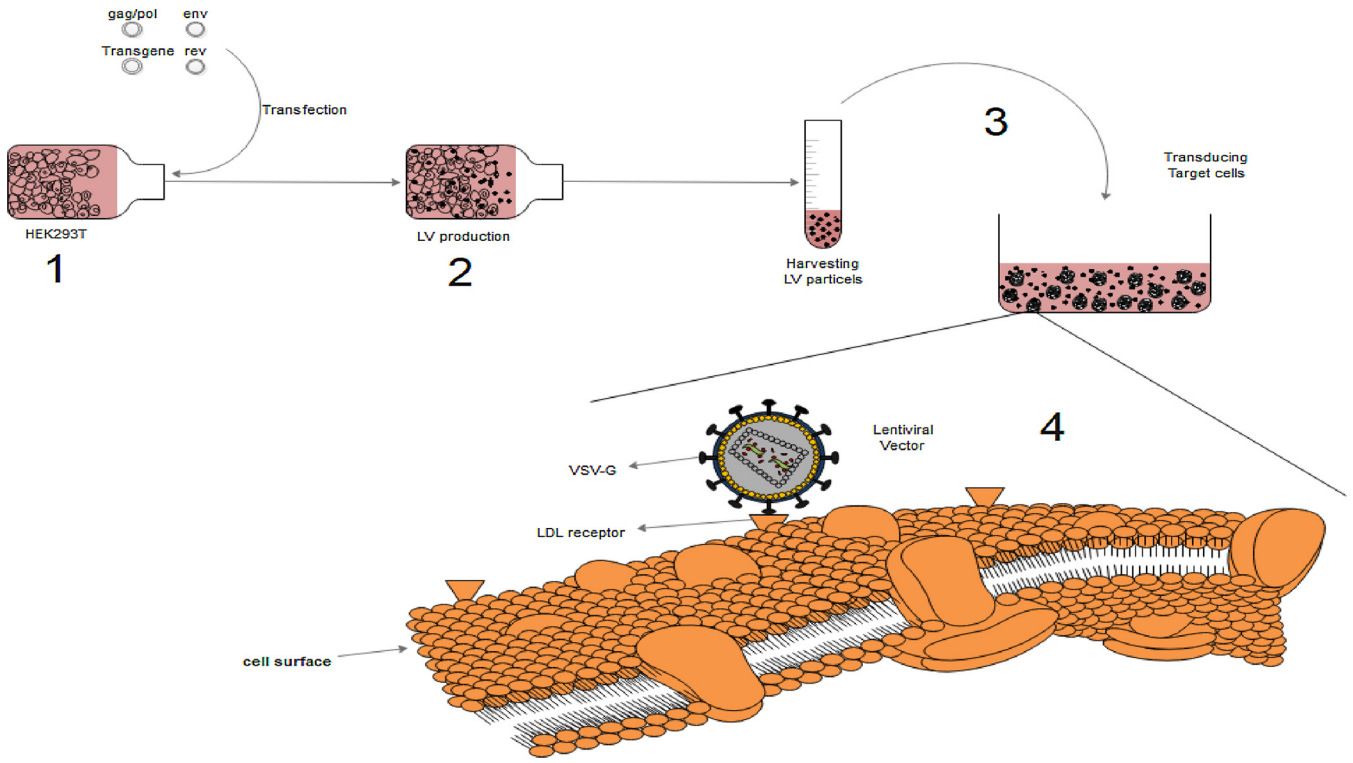

Fig. 2. Schematic events of lentiviral vector production and transducing target cells. 1- The required plasmids, including gag/pol, env (VSV-G), rev, and transgene in a mixture with transfection reagents, would be added to HEK293 T cells. 2- After the incubation and transfection process completed, the HEK293 T cells start to produce the viral particles containing transgene and their necessary viral proteins. 3- Then, the separated supernatant containing viral particles from the HEK293 T cells would be added to well plates containing the target cells. 4- The viral vectors by their VSV-G protein would bind to the LDL receptors of target cells in order to transduce the target cells.

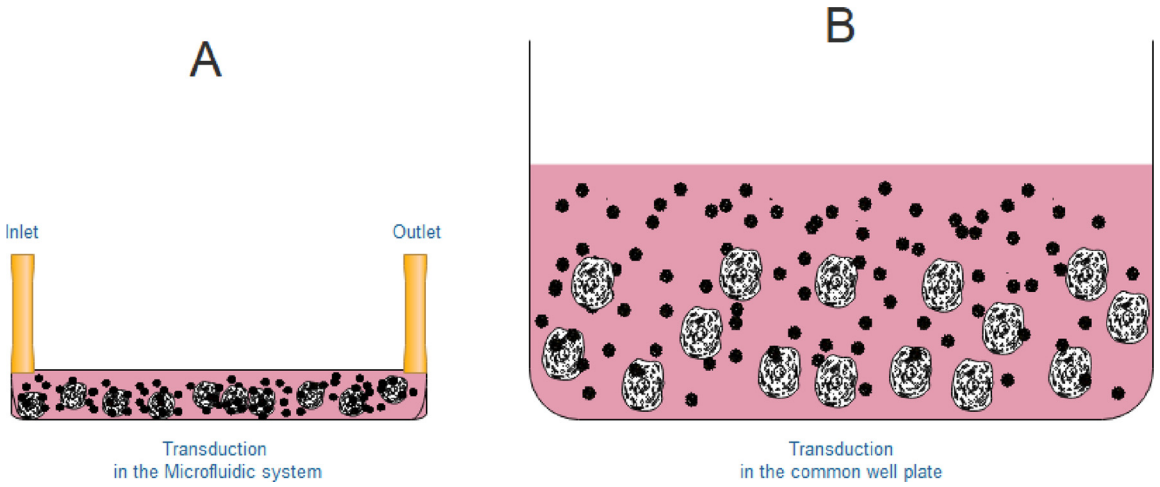

Fig. 3. Schematic of LV transduction in Microfluidic system vs. conventional well-plate. In an ideal environment at 37 °C for LVs, their half-life could be considered to be in a mean range of 600 μm through Brownian motion from their initial point to their endpoint. (B) Traveling in these standard cell culture plates requires millimeters or centimeters of distances. It brings up that most LV particles degrade before reaching their target. (A) Microfluidic systems provide a far more controlled micro-scaled environment, which gives the viral particle enough time to reach and transduce the target cell efficiently.

provide a micro-scale system, which allows having less viral particles wasted. In addition, microfluidic systems possess advantages by consuming a fewer amount of reagents, reduced transduction time, and low LV concentrations (~one-twentieth) usage over other aforementioned methods used as an enhancer of transduction [14,161,162]. Microfluidic approaches toward ex vivo gene therapy to producing effective factor VIII for hemophilia A as a challenging disorder showed to be promising. As a result of that, transduced HSPCs showed to produce curative amounts of factor VIII [14,163,164] (Fig. 3) (Table 1).

4. LVs application in research and clinical approaches

LV utilization toward cell manipulation provided an armamentarium to immunotherapy, gene therapy, and clinical research. LVs convoying an RNAi (i.e., shRNA) code to alter gene expression or a protein-coding gene like green fluorescent protein (GFP) have been vastly used in a multitude of clinical research areas, especially in achieving better comprehension of cell signaling pathways [18,169–175].

Using LV in immunotherapy through designing CAR-T cells and

Table 1
Approaches toward optimizing lentiviral production and transduction.

Lentiviral Vector	Enhancers class	Name	Description and Mechanism of action	Ref
Vector production	Plasmid transfection reagents	Polyethylenimine (PEI)	PEI condenses DNA into positively-charged particles, enhancing DNA binding to negatively-charged cell surface followed by endocytosis	[165,166]
		Calcium Phosphate (CaPO4)	CaPO4 forms a complex with DNA plasmid and facilitates its binding to the cell surface followed by endocytosis	[166,167]
		FuGENE 6	Non-liposomal reagent that creates a complex with DNA and enhance transfection, which may subsequently increase the viral transduction	[124]
		Lipofectamine	Transfection agents, Lipofectamine 2000 and 3000 can form a liposome, entrapping the transfection payload, which results in high transfection rate and subsequently higher viral transduction efficiency	[124]
	Direct DNA introduction	Electroporation	High-voltage electric shocks permeable the cells in order to the introduce DNA	[84]
		Nucleofection	Combination of electroporation and transfection reagents	[83,168]
	Enhanced producer cell line	SJ293 TS cell line	SJ293 TS cell line, unlike HEK293 T, have the ability to grow in suspension using serum-free media and produces at least 10-fold higher LV titers.	[85]
	Production accelerator proteins	SPSB1	Co-expression of this protein with other viral plasmids causes higher gag and env (VSVG) expression and increased activation of the promoter (CMV promotor)	[90]
		Tax protein	It is a HTLV-1 derived protein which activates essential transcription factors that binds to CMV promoter and enhance its activity. Co-expression of Tax markedly increased LV particles	[90]
	Harvesting methods	Ultracentrifuge	Ultracentrifuge can precipitate adequate amounts of vector for research purposes	[81]
		Combination of Mustang Q chromatography, TFF and diafiltration	Combination of high-quality chromatography and filtration technique for harvesting large-scale lentiviral particles	[82]
Vector transduction	Transduction enhancer reagents	positively-charged polycationic agents	Diminish repulsion forces between negatively-charged cells and the viral vector	[114]
		lentiBOOST	Promoting the interaction between viral particles and the host cell membrane	[118]
		Prostaglandin E2	Promoting the interaction between viral particles and the host cell membrane	[118]
		Vectofusin-1	Enhances both processes of the adhesion and fusion of LVs with the plasma membrane	[125]
		Polyethylenimine (PEI)	Positively-charged PEI forms a LV/PEI complex to enhance LV interaction with cell membrane	[123]
		Poloxamer 407	Promoting the interaction between viral particles and the host cell membrane	[128]
		Retronectin	It has receptor for both lentiviral and most cells, which can juxtapose them together to enhance transduction rate	[141]
		CD9 plasmid	Increase cell-cell fusion and alongside with VSVG leads to faster transduction and more efficient transduction	[152]
	Enhanced environment	Microfluidic system	Provide a controlled micro-scaled environment that increases the interaction of LV with target cells, and also due to the limited half-life of LVs, this system allows the LV particles to reach their targets, while they are still sustained in culture media.	[14]
	Intracellular shuttling enhancer	Dexamethasone	Dexamethasone promote shuttling of the HIV-1 pre-integration complex to the nucleus by increasing the cytoplasmic Importin α levels.	[131]
	Higher transfection/ higher transduction	FuGENE 6	Non-liposomal reagent that creates a complex with DNA and enhance transfection, which may subsequently increase the viral transduction	[124]
		Lipofectamine	Transfection agents, Lipofectamine 2000 and 3000 can form a liposome, entrapping the transfection payload, which results in high transfection rate and subsequently higher viral transduction efficiency	[124]
	Inhibitor of intracellular barriers	Staurosporine	It is a serine/threonine kinase inhibitor that overcomes barriers of CD34+ cells against LV entry, and it also increases the vector copy number	[126]
		Cyclosporine A and H	In comparison to Cyclosporin H, Cyclosporin A is less potent in enhancing LV transduction Cyclosporine H inhibits IFITM3 in HSPCs, which is an immune barrier against LV transduction.	[135]
		BX795	An inhibitor of the TBK1/IKKε, which weakens the antiviral responses of the cell against LVs	[133]
	Targeted LV	scFv-VSVG	A targeted and specific single-chain variable antibody fragment fused to VSVG can enhance the transduction. This method requires low MOI.	[139]

CAR-natural killer (NK) cells paved a way to prevail upon challenging hematological malignancies (HMs) such as acute lymphoblastic leukemia (ALL), non-Hodgkin's lymphoma, and chronic lymphocytic leukemia (CLL) [176–178]. Although at first CAR-T cells were designed to wipe out the CD19+ cells, lentiviral vector features enabled the further enhancement of CAR-T cells against a vast range of cell markers. Along with CD19 CAR-T cell, other tested CAR-T cells in HMs are against CD5, CD33, CD70, CD123, CD38, and B cell maturation antigen (BCMA) [179,180]. In this regard, CD5 would be a potential target in T-ALL and malignancies involving the subpopulation of B cells called B1 cells [179,181,182]. CD33 is a target in myeloid malignancies, especially acute myeloid leukemia (AML), and CD123 is expressed in different HMs, including blastic plasmacytoid dendritic cell neoplasm, hairy cell leukemia, B-ALL, and AML [179,183,184]. Moreover, CD38 and BCMA are mostly expressed on myeloma cells, and CD70 has a broad spectrum of expression in both HMs and solid tumors [179,185–187]. Successful application of CAR-T cell against HMs brought the idea to use CAR-T cell against various solid tumors, for example, liver cancer, pancreatic cancer, brain tumors, breast cancer, ovarian tumor, colorectal cancer, lung cancer, and currently numerous clinical trials are up to attain this end [179,188]. LV-based immunotherapy stepped up even more than before by designing CAR-NK cells. Most recently, Liu et al. produced allogenic anti-CD19 CAR-NK cells to use on 11 patients, in which all were in the relapsed phase of non-Hodgkin's lymphoma and CLL. Following that, the results were promising, in which seven patients showed evidence of complete remission within 30 days [177]. Among all CAR-T cells designed using LVs, CD19 CAR-T cells, also known as Kymriah (Tisagenlecleucel) achieved both EMA and FDA approval, and others are yet in development or under clinical trial [189]. The future of CAR-T cell shows to be promising.

Moreover, LV-based gene therapy is an ongoing field in treating different genetic diseases; for example Sickle cell disease (SCD), transfusion-dependent β-thalassemia, Wiskott-Aldrich Syndrome (WAS), metachromatic leukodystrophy, two types of severe combined immunodeficiency (SCID), Fanconi anemia, cerebral adrenoleukodystrophy, X-linked adrenoleukodystrophy (XLA) and X-linked chronic granulomatous disease (CGD) [18,190–195]. Not only can LVs contribute to integrating intact and healthy genes into target cells, but also they can be a part of the gene-editing process by delivering the CRISPR/CAS9 system [196]. It is worth mentioning that LV-based gene therapy toward curing transfusion-dependent β-thalassemia gained a magnificent achievement by introducing genetically modified EMA-approved cells called Zynteglo, which is an autologous CD34+ cell encoding βA-T87Q-globin [189]. Until the end of 2019, There were 22 EMA- or FDA-approved gene therapy using various kinds of vectors, which Cui-Cui Ma et al. reviewed all [189].

5. Conclusion

Various systems have been developed for gene delivery, which also includes viral vectors such as LVs, but all systems have some disadvantages of their own. Recently researches on gene delivery systems through viruses have introduced some methods for enhancing LV production and transduction. Now new approaches have been applied to produce large-scale of these vectors, and also multiple systems have been added to the field of viral-based gene delivery to achieve a high transduction rate. Microfluidic systems could be mentioned as one of the greatest achievements in the field of gene delivery and gene therapy. Based on what we have mentioned, microfluidic systems are potential in mimicking *in vivo* environments, enhancing transfection and viral transduction. Also, gene therapy and gene delivery techniques are not cost-effective usually, so the microfluidic approach can reduce the costs of viral gene delivery due to using lesser reagents and materials in comparison to other methods, which subsequently reduce the gene therapy expenses. We think, according to the recent arrival of the microfluidic system in the area of gene therapy, it promises more potential that yet to be found.

All in all, exceptional attention has been drawn to LVs in order to produce clinical-grade vectors. Although significant advancements have been made by LVs, including gene therapy for some diseases and easing the way toward immunotherapy by turning T cells and NK cells to CAR-T cells and CAR-NK cells, yet there are also challenges ahead that should be taken into consideration. Clinical trial success with LV-based gene therapy shows promising results, but it should be mentioned that one of the most significant drawbacks of gene therapy is the cost that comes with it. Gene therapy advances in a frantic pace that could be promising to overcome challenges ahead.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

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Authors' contributions

All Authors' equally contributed to this study.

Declaration of Competing Interest

The authors have no conflicts of interest.

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