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Novel Platforms to Create Labeled Stem Cells

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1. Introduction

Stem cell research offers unlimited potential for applications in regenerative medicine. Much of the excitement surrounding this area of research comes from the cells inherent qualities; the ability to self renew and generate a variety of cells within the body. In the past two decades, research on stem cells has progressed exponentially providing a vast array of tools for a large breadth of areas. As research persists, there is a growing need for faster and more effective tools to analyze the many unknown processes that will unlock the power within these cells.

Delivery of genes to create labeled stem cells vastly enhances their utility as tools for basic research and drug screening but also as potential therapeutic agents. Gene transfer has been a routine method in stem cell research since scientists first began culturing stem cells *in vitro*. Genetic manipulation has accelerated research enabling the creation of *in vitro* models for drug discovery, use as a tool for dissecting basic stem cell biology and potential development of *in vivo* cell-based delivery strategies. Efficient gene transfer into stem cells is a critical step in the creation of engineered stem cells. Several viral and non-viral methods exist for the modification of stem cells, albeit with varying efficiencies (Colosimo et al., 2000). Each platform has its advantages and disadvantages that can be effectively utilized for specific applications.

Here, we review gene delivery, cloning, and modification methods and describe in detail three platforms that enable the user to create modified stem cells that transiently or stably express one or multiple genes of interest. The three platforms described here each offer a unique feature and advantage that may be ideal for a particular cell type or application. The first is a non-integrating insect virus that is ideal for the transient short term expression of genes of interest. Second is an episomal EBV based vector method that allows for populations of transgene expressing cells to be stably maintained without genomic insertion. The third platform is a site specific integrating platform that can be clonally selected and is not subject to silencing.

2. Gene delivery

Genes carried on plasmid DNA can be introduced into cells with one of the methods listed below. The choice of the method largely depends on the size of the DNA plasmid and target cell type. Working with stem cells provides a new set of challenges to gene delivery requiring substantial optimization. Human embryonic stem cells (hESC) are typically grown in tight compact colonies to maintain pluripotency thus requiring transfection methods that do not disrupt this. Apart from cell conditions, applications of the gene delivery can direct

the method of choice for gene delivery. For cells with downstream clinical applications it is essential to use platforms that are “footprint-free” of transgene material. For overexpression of specific proteins or knockdown of pathways integrating virus may be ideal and required for high copy number insertion. When working with hard to transfect cell types, it is crucial to consider all options available.

2.1 Chemical methods

Chemical methods utilize lipids, polymers or proteins that form a complex with DNA. This condensed complex fuses with the cell membrane thus enabling the entry of the DNA into the cells.

Liposomes remain a popular chemical vehicle for gene delivery in many cell types. In stem cells, lipid based methods have been reported with varying efficiencies depending on the gene type and cell line (Strulovici et al., 2007). Products like Lipofectamine 2000 (Life Technologies) and FuGENE (Roche) are liposomes that utilize the characteristics of chemical compounds to form complexes with the DNA/RNA and fuse with the negatively charged cell membrane. Lipid methods show reduced toxicity as compared to CaPO_4 precipitation and earlier transfection protocols. Precipitation methods vary depending on pH and uniformity of the precipitate making them an unfavorable mechanism for stem cells. Synthetic polymers face similar hurdles. Cell toxicity of the reagent in the target cell type remains a limiting factor for chemical methods requiring extensive adaptations and optimization.

2.2 Physical methods

These methods involve delivery of plasmid across cell membrane using electroporation, sonoporation or particle bombardment. Electroporation utilizes electric pulses to transiently disrupt the cell membrane to create pores that allows delivery of charged RNA and DNA molecules into the targeted cells. This method is highly efficient and primary choice for delivery of large constructs into cells. In the recent years specialized electroporation based units have emerged. The Nucleofector™ from Lonza utilizes a combination of set programs with specific electric parameters and cell-type specific solutions to achieve high levels of transfection in several cell types including embryonic stem cells (Lakshmi et al., 2004). The Neon™ Transfection System from Life Technologies is an open system that allows optimization of electrical parameters and the flexibility of using cells from 2×10^4 to 6×10^6 per reaction. This technology avoids the use of standard electroporation cuvette and uses an electronic pipette tip as a transfection chamber (Kim et al., 2008). This method has been used to create labeled cells with diverse vector platforms in human embryonic stem cells (Thyagarajan et al., 2009; Liu et al., 2009). Sound waves or sonoporation has also been used to transiently create pores in the cell membrane to deliver nucleic acids into cells (Bao et al., 1997). Other mechanical methods include manual microinjection of gene into cells (King et al., 1994) or particle bombardment using gene guns (Guo et al., 1996) that are effective for certain cell types but harsh with the risk of damaging cells.

2.3 Viral methods

The use of viral vehicles for gene delivery in mammalian cells has become common practice due to the ability of virus to bypass cell membrane with ease. While viral systems retain their ability to infect the cells they have been modified to be replication incompetent. This

allows for the generation of a recombinant virus that cannot proliferate within the target cells. Viral systems infect by a variety of pathways reaching a variety of targets allowing the user to choose a virus that best suits the end need.

Unlike chemical gene transfer, some viral vectors provide a means to directly deliver the DNA to the nucleus and in some cases integrate to the genome. While this enhances the ability to create a stable cell line, it is also highly variable in copy number and genomic site of integration which may cause detrimental effects. Retrovirus and Lentivirus are integrating viral systems with the ability to infect a variety of cell types providing stable expression of the gene of interest. However, these systems have the ability to recombine with genetic material creating replication competent virus. Similarly, Adeno associated virus (AAV) provides a mechanism for genomic insertion with the added benefit of site specific integration. Conversely, adenovirus has the capability to provide a means of transient expression without genomic integration. Adenovirus and AAV are limited by genetic capacity of transgene size compared to other viral systems. (Giordano et al., 1999) Regardless of adaptations, viral systems remain unfavorable in cells for downstream clinical applications. This factor is due to the nature of virus to infect mammalian cells, thus limiting their use in cell therapy and other clinical models for stem cells.

3. Cloning strategies

Traditional cloning using restriction endonuclease and ligase procedures can be cumbersome for large and complex expression vectors. With advances in developmental biology there is a need for more intricate cloning systems that can handle customizable and high throughput screening through multiple platforms. The cloning procedures described below provide a faster and more efficient workflow for directional cloning for a variety of uses.

3.1 TOPO[®] TA cloning

The TOPO[®] TA technology is an efficient method for directionally recombining PCR (polymerase chain reaction) products into platform specific vectors. Rather than using ligase, the system utilizes vaccinia virus topoisomerase 1, an enzyme involved in the digestion and ligation of DNA during replication. TOPO[®] TA requires a modified plasmid equipped with the enzyme covalently bonded to a phosphate on a thymidine at the three prime end of the linearized vector. PCR products produced using Taq polymerases have three prime and five prime adenine overhangs. This overhang is utilized for the recombination with the linearized vector allowing for efficient directional recombination within five minutes at room temperature. TOPO[®] TA is limited by the necessity to create a TA adapted linearized vector stock for the TOPO[®] reactions to occur. TOPO[®] is not ideal for larger inserts showing decreasing efficiency with inserts larger than 5kb. The TOPO[®] reaction is irreversible and the inserted gene cannot be cloned out by the enzyme. This limits the ability to use this gene for other platforms without repeating the PCR (Katzen et al., 2007).

3.2 MultiSite Gateway[®] cloning

Each of the cell modification platforms below has been outfitted with MultiSite Gateway[®] Technology. Otherwise known as Gateway[®], this technology is an efficient and relatively easy way to clone multiple gene configurations into a variety of expression vectors. This

improves the speed of the cloning process eliminating the need for restriction endonuclease cloning procedures. This system harnesses the power of the lambda integrase enzyme to recombine multiple fragments in an orientation specific manner with high accuracy.

The lambda integrase recognizes sequence specific recombination sites. Each gene must be amplified using Gateway® specific primers containing the proper recombination sites. As shown in Figure 1, the building process begins by cloning components of your cassette of interest into intermediate vectors. These vectors are recombined with the destination vector belonging to your platform of choice. The cloning steps are simple incubation reactions speeding up the overall process.

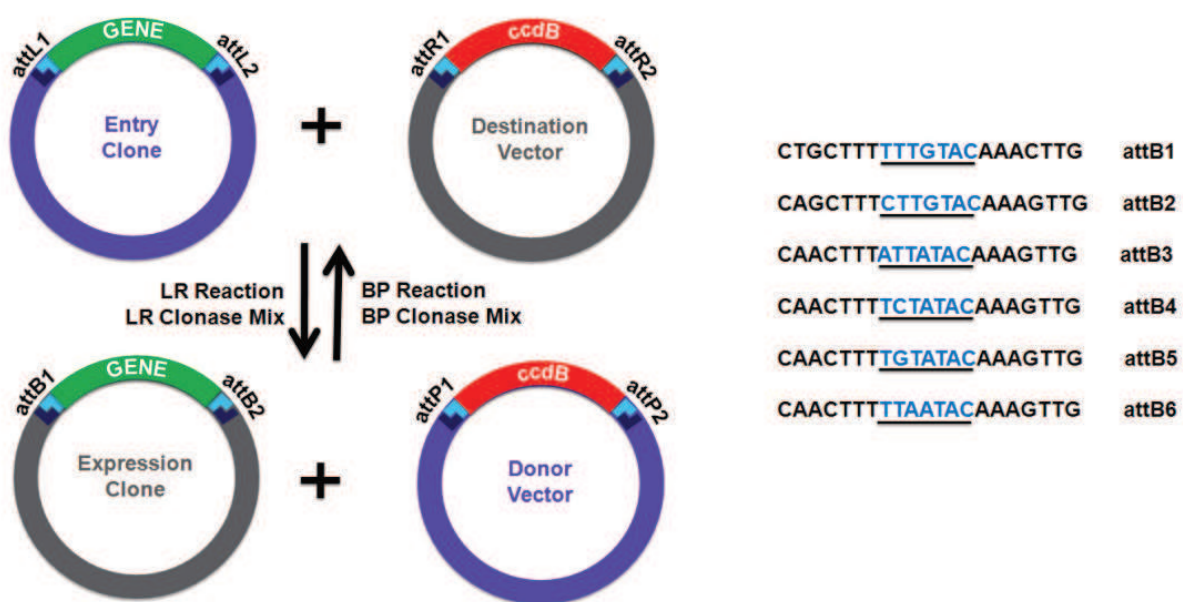


Fig. 1. Multisite Gateway® Cloning

MultiSite Gateway® Technology is a site specific recombinational cloning system where the different att specificities allows assembly of 2-4 gene fragments in a defined order and orientation. The difference in the att site specificity is determined at the single base level (underlined sequence) with virtually no cross recombination between the different sites. Here entry clones carrying the gene elements of choice are constructed into a donor vector via BP reaction to result in entry clones. Donor vectors with appropriate flanking att sites for single or multiple fragment cloning are remixed with the right Destination vector and LR reaction performed to yield final expression vectors.

Outfitting a variety of platforms with Multisite Gateway® technology makes the transition between technologies a simple cloning reaction. Each expression cassette can be cloned into a variety of destination vectors expanding your ability to modify cells based on specific needs. Thus, the user can create a library of gene cassettes that can be readily used for a variety of applications. The dual arrows in Figure 1 indicate that Multisite Gateway® provides a reversible reaction (under different conditions) that retains the rapid cloning

features of the TOPO® TA system. Using Multisite Gateway® we traverse a variety of cell modifying systems, based on need, with ease and speed.

4. Cell modification platforms

Besides efficient gene transfer into stem cells, a critical step is the choice of platform used to create labeled cells. Traditionally, naked plasmid DNA is used to create stable clones, but is less favored in stem cells due to varied expression levels depending on the context of its genomic integration. (Eiges et al., 2001) To overcome this issue, lentiviral methods that offer higher transduction rates and multiple integrations have been used to generate stable transgene expressing cells (Jang et al., 2006). A major limitation of lentiviral systems is the size or payload of DNA that can be packaged. Recently, methods that result in efficient random integration via transposases and site-specific genomic integration mediated via adeno-associated viruses (AAV), and site-specific integrases, have been reported as a means to modify stem cells (Surosky et al., 1997, Kowabata et al, 2006).

Site-specific integration offers an elegant method to generate clones with one copy of the gene at a specific genomic location that can be further screened to identify ideal sites that support sustained gene expression. This method requires the isolation of clonal population of cells followed by rigorous characterization of the genomic integration site to ensure sustained transgene expression and is best suited for cell types that can be cultured for long periods of time. Embryonic stem cells and other cell lines, with unlimited proliferative potential, can be subjected to such methods to create engineered cells expressing the gene of interest. JumpIn™ platform utilizes phiC31 and R4 integrases to target pseudo sites in the mammalian genome that are known to support higher and longer-term expression of inserted transgenes. This method requires prolonged culture and is manipulation and ideal to create labeled pluripotent stem cells for sustained gene expression both in pluripotent state as well as differentiation.

EBV-based vectors support episomal maintenance of large genomic fragments and hence are an appealing alternative to rapidly generate labeled cells. Since the plasmid is not integrated into the host genome, expression of the transgene is relatively free from chromosomal effects associated with genomic integration. This method requires drug selection but not clonal isolation. Pooled clones can therefore be rapidly generated and suited for expression of genes or knocked down in the undifferentiated state. Since there is a risk of loss of transgene/plasmid in the absence of selective pressure, labeled pluripotent stem cells will result in attenuated transgene expression with long term differentiation.

Both the methods highlighted above require transfection of plasmid into cells and prolonged manipulation and culture, a feature not amenable to most primary and adult stem cell types due to their limited proliferation. BacMam offers an easy and fast method to deliver transgenes into a wide spectrum of stem cells with the least toxicity. The introduced transgene is diluted with passage and therefore ideal for transient expression to create assay-ready cells. Figure 2 displays a comparison of transient (BacMam), stable non-integrating (EBV-Vector), and integrating platforms (Jump-In™).

The three platforms discussed here are; Episomal vectors that results in stable retention of the plasmid (green circles) without integration into the genome; JumpIn™ platform that allows site-specific insertion of the gene (shown in green) into the host genome for stable expression and; BacMam, a viral method where the transgene does not integrate into the host genome (Green circles) but dilutes out as cell divides and hence transient.

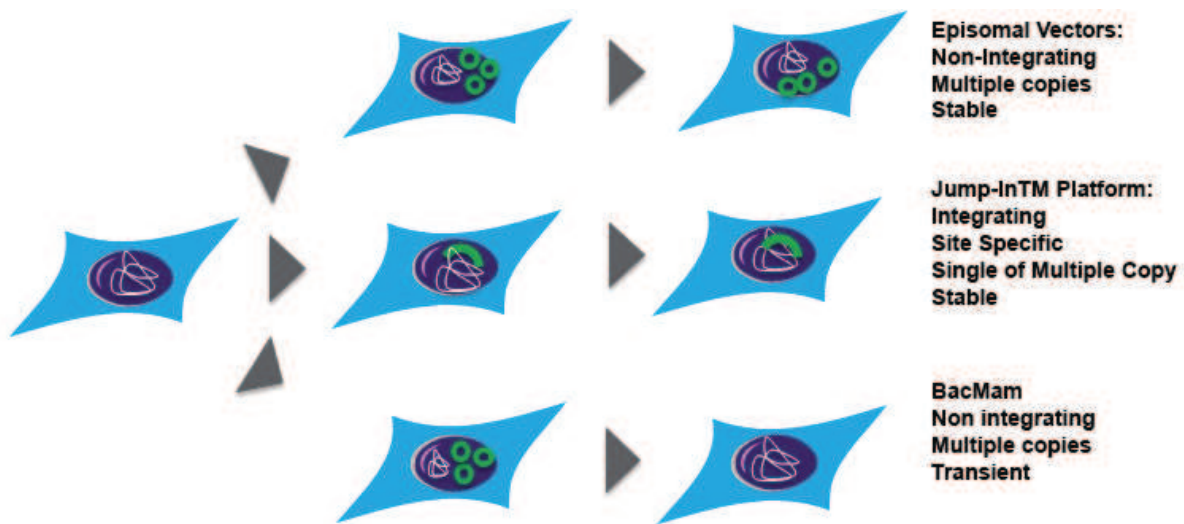


Fig. 2. Labeling Platforms

4.1 BacMam

Transient expression systems are ideal for rapidly creating labeled cells for immediate utilization in downstream applications such as cell tracking, identification, enrichment, drug screening and other types of high throughput assays. Traditionally lipid-based methods have been used to introduce DNA carrying the gene of interest. This method, however, is not amenable to certain cell types, especially primary and stem cells that are generally hard to transfect. Baculoviruses are known to enable high efficiency labeling of hard to label cells with minimal toxicity (Zeng et al., 2009, Ho et al., 2005). These viruses are non-replicating in human cells and are stable at 4°C and thus easy to generate and use.

The BacMam platform, named for the ability of Baculovirus to transfect Mammalian cell types, is a double stranded DNA virus capable of infecting over 500 insect species. The DNA is packaged in a rod-shaped particle 40-50 nm in diameter and 200-400 nm in length. Gp64, a major glycoprotein on the envelope, is proposed to play a key role in virus attachment and entry into mammalian cells. The viral particles are endocytosed and are released into the cytoplasm before migrating to the nucleus.

BacMam is non-replicating in mammalian cells without any additional modifications. This is contrast to other viral methods such as lenti, adeno, or retro that integrate into the host genome and requires inactivation of key attributes of the virus to prevent unwanted replication within cells. In addition, integrating viral constructs also have the chance of recombining with endogenous sequences in the human genome resulting in instability. The ease of use, high efficiency of labeling and relatively low toxicity renders BacMam an ideal choice for primary, stem and progenitor cells. In addition, their ability to carry high load capacity reaching upwards of 30kb sequences adds to its appeal for simultaneous delivery of single or multiple genes of interest.

The most commonly used method to generate BacMam virus is the Bac-to-Bac® method where the gene of interest is first cloned into a transfer vector. The cloning and production process are shown in Figure 3A. This transfer vector could be created by either by Restriction endonuclease mediated cloning (pFastBac™) or MultiSite Gateway® adapted (pDest). Once the expression vector is constructed, it is transformed into a modified E coli that contains a baculovirus genome. Recombination of the transfer vector with the baculovirus genome results in BacMid which is ready for transfection into insect cells for

virus production. BacMid is transfected into Sf21 (*Spodoptera frugiperda*) insect cells. The production is limited by variability of the virus which results in testing a variety of clones. Despite the selection of multiples clones, there is a necessity for plaque purification of the virus to ensure a clone is selected with the expression cassette.

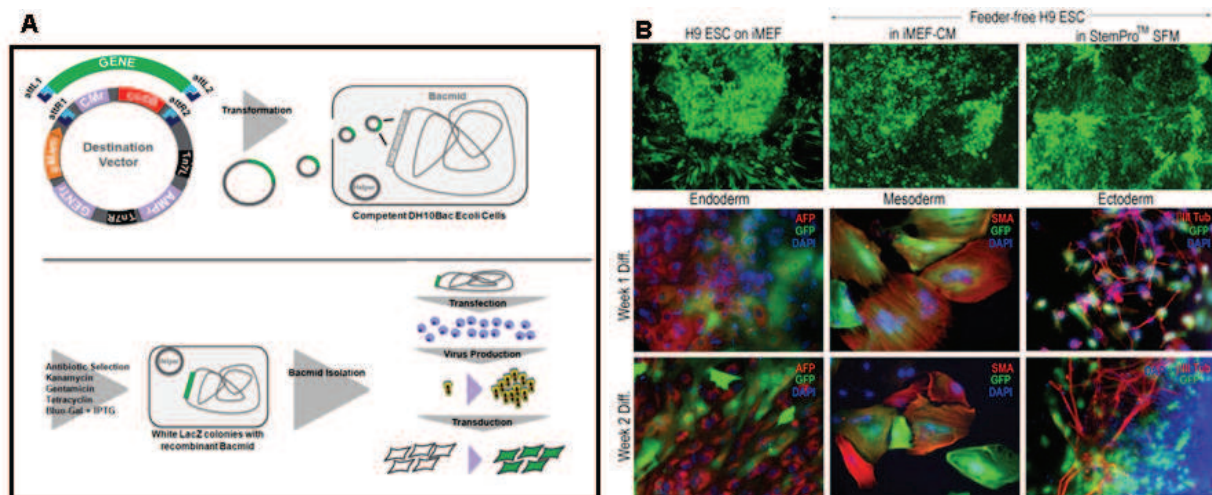


Fig. 3. BacMam

(A) Gene of interest is cloned into the baculovirus transfer vector using MultiSite Gateway® cloning. The resulting expression vector is transformed into DH10Bac™ to generate BacMid via site-specific transposition of the expression elements on the transfer vector to the baculovirus genome present in DH10Bac cells. Transfection of BacMid DNA into Sf9 or Sf21 insect cells results in BacMam virus carrying the expression cassette that can be further amplified in insect cells to generate high titer stocks. (B) BacMam carrying the expression cassette CMV-GFP can be transduced into undifferentiated H9 hESC either on feeders or feeder-free conditions. Cells that are induced to differentiate for 1-2 weeks can also be transduced by BacMam and GFP labeled cells colocalize with lineage specific markers.

The ability to transduce a variety of cells types makes BacMam an ideal system for expression in partially or terminally differentiated cells derived from embryonic stem cells (Figure 3B). These cells have a limited proliferation preventing their use with selectable markers that require clone establishment. Transduction of differentiating ESC with GFP BacMam and overlap with differentiation markers has been demonstrated.

BacMam provides an easy to clone, easy to use, non-integrating transfection system. However, this system may not be ideal for long term gene expression in dividing cell types. The inability to replicate may be a limitation of the technology since the signal will dilute out in rapidly dividing cells. Additionally, for downstream clinical applications it may undesirable to use a viral system despite its inability to infect mammalian cells.

4.2 Episomal vectors

With the ability of stem cells to divide indefinitely it is essential to have a platform that can express long term. Generating labeled stem cell lines provides a valuable tool for research. However the creation of stable lines is complicated by stability and copy number of the loci. Technologies for this purpose have been reviewed using viral, homologous recombination and integrase specific integrations (Yates et al., 2006). Plasmid DNA alone has been utilized

to randomly integrate for a fast way to create a labeled line. Lentivirus works efficiently due to multiple copy insertion into the genome. However, this can have negative effects. Thus, there is a need for a stable line that does not have any genomic alterations or silencing issues. To overcome these problems, an episomally maintained DNA vector for stably expressing stem cell lines is used.

The Epstein Bar Virus (EBV), a member of the herpes virus family, is known to infect multiple cells types and remains one of the most common virus in humans. EBV based episomal vectors have been successfully used to stably express gene of interest in multiple types of cells both in vitro and in vivo since 1985 (Yates et al., 1985). The crucial components of the viral genome that have been added to vector systems are the latent origin of replication (OriP) and the EBV Nuclear Antigen-1 region (EBNA). These elements are required in combination for stable maintenance of episomal plasmid DNA. The trans acting element, EBNA, cannot function without the cis acting element, OriP. Additionally, the EBNA-OriP combination is only functional in human, primate and canine cells. Reproduction of extrachromosomal replication using the EBNA1 has also been reported in murine models, however, this requires the additional expression of transgenes (Habel et al., 2004).

In hESC these vectors have had relative levels of success using a two step system first creating a stable EBNA1 expressing cell line and transfecting a OriP vector (Ren et al., 2006). This however does not mitigate the problem of silencing from genomic integration. The EBNA oriP destination vector (Figure 4A) is a one step vector system that includes both elements eliminating the need for an EBNA cell line step. The ability to express high levels of transgenes without genomic integration provides a useful system for downstream clinical applications. Recently, episomal vector systems using the EBNA OriP elements have been reported for the generation of induced pluripotent stem cells (Yu et al., 2009). Reprogramming by the forced expression of multiple transcription factors is an ideal example of the power of the episomal vector outfitted with MultiSite Gateway® technology. Multiple configurations can be created in a high throughput manner allowing for the adaptations of expression, stoichiometry and exchange of alternate genes.

Episomal vectors replicate once per cell cycle with activation of replication by binding of multiple EBNA1 homodimers to OriP. Similar to BacMam, this system offers an appealing alternative to integrating technologies since they are relatively free from chromosomal effects associated with genomic integration. These vectors, being rather larger in size (over 10 kb) can be transfected into embryonic and adult stem cells such as MSC albeit with lower efficiencies. Since the distribution of episomal vectors with cell division is unequal between the daughter cells, it is best to keep cells under selective pressure for sustained gene expression. It is also not necessary to generate individual clones; drug resistant colonies obtained can be pooled to rapidly create labeled cells for downstream applications. Episomal vectors have been used to generate stable hESC pooled clones that maintain transgene expression for long periods of time. Genes driven by constitutive or lineage specific promoters have been shown to express in a context specific matter (Figure 4B). In addition, majority of the cells retain transgene expression when induced to differentiate in the absence of selective pressure for 3 weeks (Thyagarajan et al., 2009).

Episomal vectors provide an easy platform to rapidly create stable cells expressing transgene of interest. A major limitation of this method is the requirement for efficient transfection method and pooled clones that have varying copy numbers per cell thereby creating a heterogeneity in expression levels that may be undesirable for certain applications.

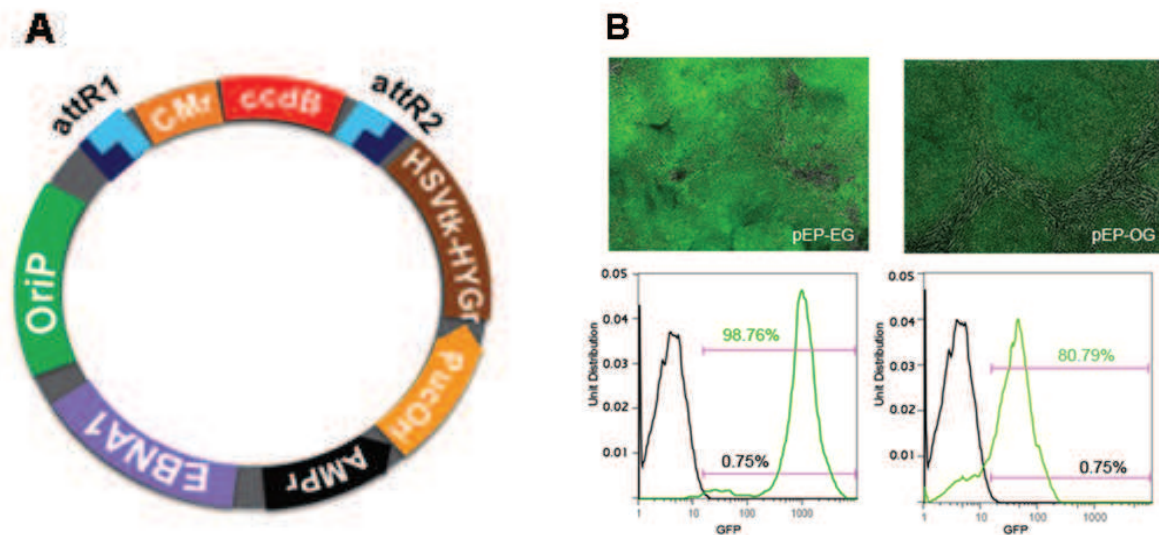


Fig. 4. Episomal Vectors.

(A) Epstein Barr virus based vectors contain Epstein-Barr virus nuclear antigen (EBNA1) and the latent origin of replication OriP that help in episomal maintenance of the vectors by facilitating the replication of the vectors once per cell cycle by binding of the multiple EBNA1 homodimers to OriP. Mutlitsite Gateway[®] adaption of these vectors allow rapid assembly of single or multiple gene elements and a Hygromycin resistance gene provides a means to maintain selective pressure on cells carrying these vectors. (B) Human ESC can be transfected with episomal vectors to create stable pooled clones expressing GFP or gene of interest driven by constitutive promoters such as Efla or lineage specific promoter such as the pluripotent cell specific Oct4.

4.3 Jump-In[™] technology

As research uncovers the potential of stem cell research there are many applications that require a clonal population expressing a custom cassette. Transgene cassettes can vary from lineage reporter lines, to multicistronic elements or even systems useful for higher content analysis. These complex tools will require single copy integration of the expression cassette with resistance to silencing. Single copy cell lines have been reported in human embryonic stem cells using homologous recombination, cre/lox mediated integration and zinc finger site directed integration (Costa et al., 2007, Lombardo et al., 2007, Soldner et al., 2009). Homologous recombination can lead to random integration as well as silencing depending on the locus. Unlike homologous recombination, non-viral integrases have the potential to recombine two non-identical sites. Zinc finger has been proven as an accurate method, however, require designing sequence specific zinc finger for recombination (Lombardo et al., 2007).

The Jump In system uses non-viral integrase-mediated site-specific integration to develop a platform stem cell line. The two integrases phiC31 and R4 are both members of the Streptomyces family with the potential to integrate transgenes to native attP (attachment P site) sites that are present in the bacterial genome or pseudo attP sites that are present in other species such as human, rat and mouse. Although there are a variety of pseudo attP sites within the human genome, certain hot spots have high affinity for recombination. This

results in a very low background of non site specific targeting. A pseudo attP hot spot on chromosome 13 has provided an ideal location due to the known intronic areas of the chromosome. Many other gene delivery methods are affected by chromatin remodeling during differentiation. The chromosome 13 loci has been shown to remain active throughout differentiation to multiple lineages remaining resistant to silencing. The system has a much higher efficiency for large complex fragments.

Delivered via electroporation, the vectors introduce large complex gene cassettes to a specific genomic locus validated for efficiency, stability, transcriptional activity and resistance to silencing (Liu et al., 2009). The phiC31 integrase is employed to insert a native R4 attP site into the genome that can be accessed for future recombination. R4 integrase mediated integration occurs at the new R4 attP site activating the expression of a selection marker. Figure 5 shows stepwise creation of the target line and subsequent retargeting that results in activation of the Zeomycin gene. Stable clones can be selected and expanded for further validation.

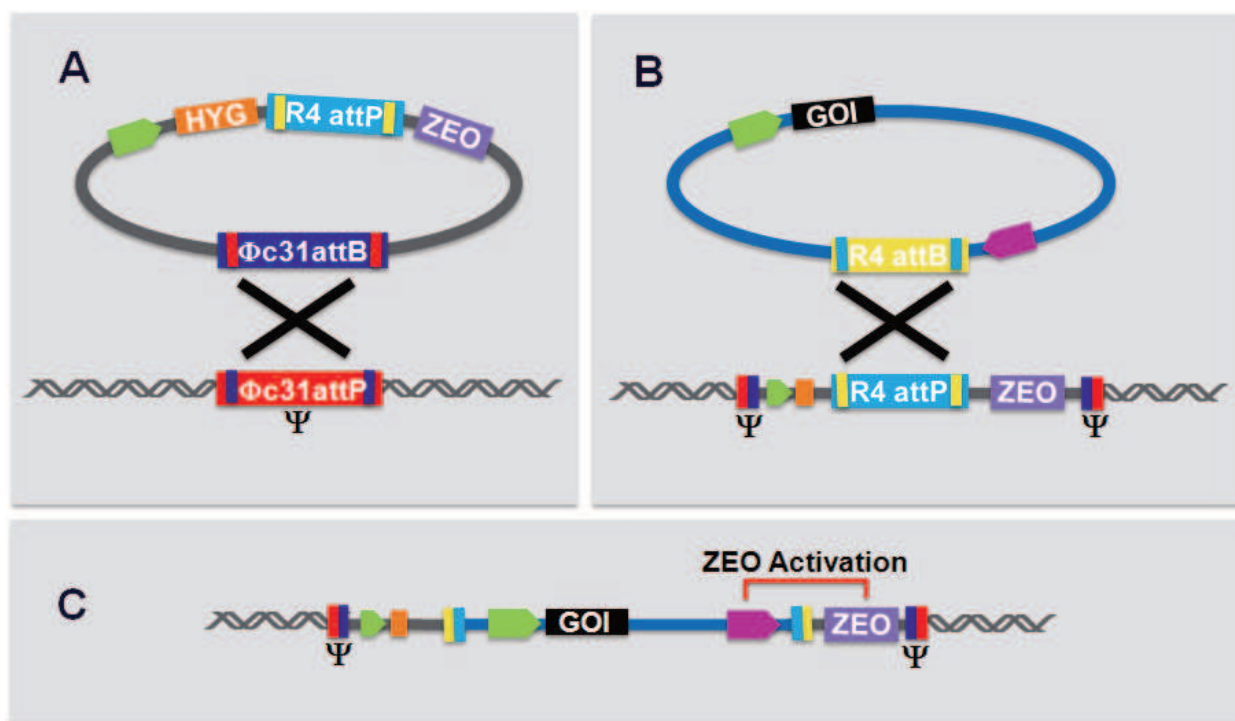


Fig. 5. Site Specific Integration

(A) A Target plasmid containing the wild type R4 integrase recognition (attP) site carrying a constitutive expressed antibiotic gene with a PhiC31 integrase attB site is transfected into cells along with a plasmid expressing phiC31 integrase. The enzyme catalyzes the integration of the target plasmid into "Pseudo attP sites" within the host cellular genome. Also contained on the Target plasmid is a promoterless antibiotic gene. The constitutive expressed antibiotic is used to select stable drug resistant clones and screened for single copy and genomic site of integration. (B) Clones carrying a single copy of the target plasmid in a desired genomic locus are retargeted. A retargeting plasmid carrying the promoter-gene expression cassette, a promoter adjacent to a R4 attB site and a plasmid expressing R4 integrase is transfected. (c) R4 integrase mediated retargeting will result in positioning of the promoter adjacent to the drug resistance gene placed in the original Target cells to activate the antibiotic gene which can then be utilized to generate drug resistant clones.

With single copy integration there is less variability between copy number and expression patterns if trying to express multiple genes. The technology has been reported for potential lineage tracking by expressing GFP from an Ef1alpha promoter throughout differentiation without silencing.

Although this platform has proven to be an accurate and efficient way to modify stem cells there are still improvements that can be made. The technology still shows a tendency to randomly integrate plasmid sequences leaving the need to verify copy number and integration sites via molecular techniques.

5. Conclusion

Gene modification provides researchers with tools to accurately dissect cellular function. The techniques described offer a toolbox with interchangeable components to create specific assays for specific applications. Utility of the various available platforms depends on several factors. First is to determine the desired length of expression needed for the desired application. Second, it is important that the cell type to be modified is amenable to gene delivery methods either via transfection or via specific viral methods. Third, the proliferative capability of cells that are being modified is important to determine if these cells can sustain long periods of manipulation and drug selection and if the resulting modified cells will retain their original stem cell characteristics.

The three platforms summarized in this chapter each have their advantages and disadvantages. The most notable difference between the three is the length of time and amount of manipulation required for generation of the labeled cells (Table 1). Starting from a vial of frozen pluripotent stem cells, 2-3 week is needed to recover and expand enough cells, typically at least 6-10 million cells for the first step. For transient expression using BacMam, cells are ready for use 24-48 h post transduction. With EBV-episomal vector, cells after transfection are subjected to drug selection and resulting pooled clones to be characterized. This entire process of generating stable clones is approximately 8 weeks long. The Jump-In™ platform that requires two rounds of transfection, drug selection and subsequent characterization requires approximately 15 weeks to obtain modified stem cells. Choice of platform to use is not merely based on ease or length of creating labeled cells. It is important to understand the advantages and disadvantages of each method so that features ideal for the application of interest is used to create modified stem cells (Table 2). BacMam enables efficient delivery into diverse cell types and is the fastest method to create labeled cells. However the expression is transient and since workflow does not involve drug selection resulting cells will have variable expression depending on the number of copies they carry. This method is therefore ideal for delivery of target to create assay ready cells for experiments that can be completed within 24-48 h after creation of the labeled cells. This method however is not suitable for expressing genes and monitoring its long term effect on the cells. Episomal vectors offer the advantage of creating stable pooled clones in a relatively short period of time. Disadvantages of this method are that transfection into hard to transfect cells remains an issue and since copy number is variable expression can be heterogeneous and expression level may be attenuated with long term differentiation. Finally Jump-In™ system is ideal for creation of stable pluripotent stem cells for sustained expression both in its undifferentiated state and with differentiation. The ability of this system to create clones with a single copy of the gene at a known genomic locus makes this an ideal choice for use of this line as a Platform line into which diverse targets can be inserted for comparison all from the same genomic context.

Recently efforts are focused on creating hybrid systems to generate novel engineering method which overcome some of the limitation for the system. EBV-elements EBNA and OriP cloned into BacMam transfer vectors to generate EBNA BacMam has easy delivery but also sustained gene expression. The ultimate goal is to create labeled cells that can be used for the end application.

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BACMAM	EBV-vector	JUMP-IN™	STEP	Duration #Days	Total Duration #Weeks
■	■	■	Thaw and Culture cells	14	2
			Feeder-free expansion	7	3
			Transfect and recover	2	3.2
			Drug select	14	5.2
			Expansion	14	7.2
			Characterize	7	8.2
			Feeder-free expansion	7	9.2
			Retarget and recover	3	10
			Drug select	14	12
			Clonal Expansion	14	14
			Characterize	7	15

Table 1. Steps and Duration of the Cell Engineering Methods
The length in time to generate labeled cells varies between the three methods (highlighted in the color bars)

	PROS	CONS	POTENTIAL APPLICATION
BACMAM	<ol style="list-style-type: none"> 1) Ease of use 2) Efficient infection 3) Broad host range 	<ol style="list-style-type: none"> 1) Transient expression 2) heterogeneous expression 3) long viral generation process 	Assay ready cells
EPISOMAL VECTORS	<ol style="list-style-type: none"> 1) Rapid engineering 2) Stable expression 3) no genomic integration 	<ol style="list-style-type: none"> 1) need for optimal transfection 2) Heterogeneous expression 3) Variable silencing 	Expression of genes and RNAi
JUMP-IN™	<ol style="list-style-type: none"> 1) Stable integration 2) Homogeneous expression 3) defined locus, sustained expression 	<ol style="list-style-type: none"> 1) need for optimal transfection 2) clonal selection and screening 3) integrational method 	Developmental reporters

Table 2. Summary of cell engineering methods
Advantages and disadvantages of each method and potential application of each method

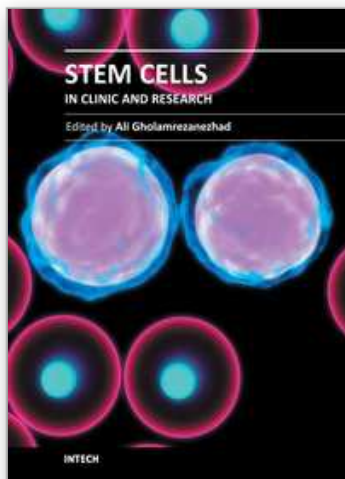
6. Acknowledgement

We thank Stem Cell team at Life Technologies for discussions and suggestions.

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Stem Cells in Clinic and Research

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ISBN 978-953-307-797-0

Hard cover, 804 pages

Publisher InTech

Published online 23, August, 2011

Published in print edition August, 2011

Based on our current understanding of cell biology and strong supporting evidence from previous experiences, different types of human stem cell populations are capable of undergoing differentiation or trans-differentiation into functionally and biologically active cells for use in therapeutic purposes. So far, progress regarding the use of both in vitro and in vivo regenerative medicine models already offers hope for the application of different types of stem cells as a powerful new therapeutic option to treat different diseases that were previously considered to be untreatable. Remarkable achievements in cell biology resulting in the isolation and characterization of various stem cells and progenitor cells has increased the expectation for the development of a new approach to the treatment of genetic and developmental human diseases. Due to the fact that currently stem cells and umbilical cord banks are so strictly defined and available, it seems that this mission is investigational more practical than in the past. On the other hand, studies performed on stem cells, targeting their conversion into functionally mature tissue, are not necessarily seeking to result in the clinical application of the differentiated cells; In fact, still one of the important goals of these studies is to get acquainted with the natural process of development of mature cells from their immature progenitors during the embryonic period onwards, which can produce valuable results as knowledge of the developmental processes during embryogenesis. For example, the cellular and molecular mechanisms leading to mature and adult cells developmental abnormalities are relatively unknown. This lack of understanding stems from the lack of a good model system to study cell development and differentiation. Hence, the knowledge reached through these studies can prove to be a breakthrough in preventing developmental disorders. Meanwhile, many researchers conduct these studies to understand the molecular and cellular basis of cancer development. The fact that cancer is one of the leading causes of death throughout the world, highlights the importance of these researches in the fields of biology and medicine.

How to reference

In order to correctly reference this scholarly work, feel free to copy and paste the following:

Andrew Fontes, Rene Quintanilla and Uma Lakshmipathy (2011). Novel Platforms to Create Labeled Stem Cells, Stem Cells in Clinic and Research, Dr. Ali Gholamrezanezhad (Ed.), ISBN: 978-953-307-797-0, InTech, Available from: <http://www.intechopen.com/books/stem-cells-in-clinic-and-research/novel-platforms-to-create-labeled-stem-cells>

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