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# Application of phiC31 integrase system in stem cells biology and technology: a review

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## ABSTRACT

Two specific characteristics of stem cells, self-renewal and their differentiation capacity have made them important for vast scientific areas, particularly in the molecular and cell biology of stem cells, as well as regenerative medicine. Gene targeting plays a pivotal role in creating modified stem cells, which are considered the mainstay in regenerative medicine. The phiC31 integrase system, as a site-specific recombinase, could be a straightforward and efficient approach for this aim. According to the high potential of the application of phiC31 integrase system in stem cells, the present paper reviews previous studies, placing the focus on stem cell types and their application.

## ARTICLE HISTORY

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## KEYWORDS

phiC31 integrase; stem cells; gene targeting; regenerative medicine

## Introduction to stem cells

Stem cells are progenitor cells capable of self-renewal and differentiation into multiple different cell lineages. These cells have been used for treating a wide range of diseases (Fontes and Thomson 1999). When a stem cell divides, each daughter cell has the potential either to remain a stem cell or to become another type of cells with a more specialized function, such as blood, brain, heart muscle or bone cells.

Most of the advances in stem cell research have been concentrated on understanding the etiology of diseases, regenerative medicine and drug screening.

Mammalian embryonic and adult stem cells are two types of stem cells, which can be isolated from the inner cell mass of the blastocyst, and various adult tissues, respectively. Adipose tissue, bone marrow and blood are three different sources of adult tissue, which are currently used for the isolation of stem cells. Stem cells can also be harvested from umbilical cord blood, just after birth.

## Overview of genome modification in stem cells

Genetic engineering techniques have been applied in stem cell genome modification by several techniques (Giudice and Trounson 2008; Tenzen et al.

2010; Fontes and Lakshmipathy 2013; Nowakowski et al. 2013; Zhou and Zeng 2013). These techniques are capable of modifying stem cell genome in random or site-specific manners. Random gene integration method is a popular method for random genomic integration of an exogenous gene which in several cases, provides stable gene expression. One of the main drawbacks for this strategy is the limitation in transfection efficiency, which could be overcome by using viral vectors as a vehicle in the genome modification technology (Ma, et al. 2003). These viral-based vectors are useful research tools for disease models and basic research studies due to their ease of use and high reprogramming efficiency. However, their appliance is limited for eventual therapeutic uses.

Non-viral-based vectors systems, such as the Sleeping Beauty, Tol2 and piggyBac have been successfully used to generate modified human stem cells (Wilber et al. 2007; Orban et al. 2009). These systems integrate the gene of interest randomly and, usually, in transcriptionally inactive regions (Huang et al. 2010).

Non-integrating systems, such as episomal and minicircle vectors, also provide safe engineering platforms in the vast range of studies, particularly in gene therapy (Fontes and Lakshmipathy 2013). On the other hand, variable expression patterns and copy number

variation can be considered as disadvantages for these approaches. Zinc-finger nucleases (ZFNs), transcription activator like effector nucleases (TALENs), as well as clustered regulatory interspaced short palindromic repeat (CRISPR)/Cas-based RNA-guided are newly introduced systems which have been successfully used for gene targeting via homologous recombination technology (Nieminen et al. 2010; Fontes and Lakshminipathy 2013). Despite the widespread use of ZFNs, it is restricted due to the challenges associated with designing DNA sequences for finger design, which confer sequence specificity, therefore eliminating the target effects (Holt et al. 2010). The TALENs recognize DNA in a modular manner. This method holds a huge potential for the modification of induced pluripotent stem cells for various downstream applications (Hockemeyer et al. 2011). The CRISPR/CAS, which has superior advantages compared to the previous gene

targeting systems especially in terms of specificity and efficiency, allows the simultaneous editing of multiple target loci in the mammalian genome (Wang et al. 2013). However, these techniques are technically challenging, expensive and may sometimes result in potential genome off-target (Gaj, et al. 2013).

For site-specific genomic integration; Adeno-associated virus (AAV) and phiC31 integrase systems have been used for stem cells. Several studies investigating the application of phiC31 integrase system in both human and animal models have been summarised in Tables 1 and 2, respectively.

### Introduction to phiC31 integrase system

The phage of *Streptomyces* soil bacteria starts its life cycle by inserting its whole genome into the host bacterial genome (Kuhstoss and Rao 1991; Rausch and

**Table 1.** Summary of some application of phiC31 integrase system for stem cell in human.

Cell type	Description	Result	Transgene	Reference
Human epidermal progenitor cells	Regeneration of human skin on immunodeficient mice for junctional Epidermolysisbullosa (JEB) patients	Produced human skin tissue with restored laminin 5 expression	laminin unctionalepidermolysisbullosa (JEB gene)	Ortiz-Urda et al. (2003)
Human mesenchymal stem cells (hMSCs)	Using phiC31 integrase systems for genomic modification of Human mesenchymal stem cells (hMSCs)	PhiC31 integrase represents an efficient and practical non-viral approach for genetic modification of hMSCs	Integrase has been used to achieve genomic integration in human keratinocytes, mouse liver, rat retina and rabbit joints	Keravala et al. (2005)
Mouse muscle-derived stem cells and normal human myoblasts	Introducing eGFP-dystrophin fusion protein and the phageC31 integrase into myogenic cells and to integrate these genes into a limited number of sites in the genome	Produced fluorescent human myoblasts	Dystrophin gene	Quenneville et al. (2004)
Human muscle precursor cells (MPCs)	Obtaining modified cells as a cure for mouse Duchenne muscular dystrophy (DMD)	Correction of MPCs with phiC31 integrase is a possible approach to treat DMD	eGFP	Quenneville et al. (2006)
Mouse embryonic fibroblasts and human amniotic fluid cells	Reprogram mouse embryonic fibroblasts and human amniotic fluid cells into iPS cells	This method was applied successfully	Two polycistronic constructs containing the transcription factor genes OCT3/4, SOX2, KLF4 and cMYC (O,S,K,M) joined by 2A peptides together with the GFP gene (G) at the 3' end	Ye et al. (2010)
Human cardiac progenitor cells and human endothelial cells	Feasibility of site-specific genetic integration in human cardiac progenitor cells and human endothelial cells	Stable expression of the reporter gene from its unique chromosomal integration site resulted in no discernible genomic instability or adverse changes in cell phenotype	The triple fusion reporter gene encodes firefly luciferase (Fluc), monomeric red fluorescent protein (mRFP), and HSVtk	Lan et al. (2012)
Mouse Adipose tissue-derived mesenchymal stem cells and fibroblasts	Murine iPS cell generation using a non-viral vector	Efficient generation of rat iPS cells using a single non-viral vector that allows tight control over reprogramming factor expression	Oct4, Sox2, c-Myc and Klf4	Merkel et al. (2013)
Human embryonic stem cells (hESCs) and pluripotent stem cells (iPSCs)	Creation an H9 ESC and iPCS lines with ability of expression of mCherry and neural transcription factors	Transgenic Esc and iPSC lines successfully expressed transgene and differentiated to neurons	mCherry, LMX1, FOXA2 and OTX2	Zhu et al. (2014)

**Table 2.** Summary of some application of phiC31 integrase system for stem cell in animal models.

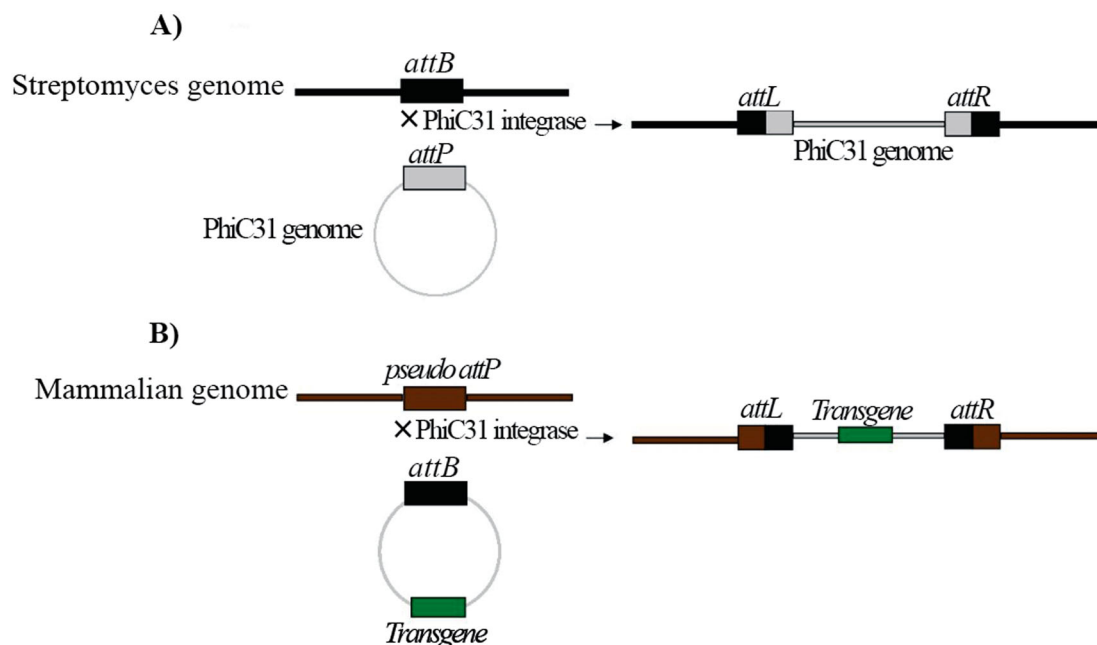
Cell type	Description	Result	Transgene	Reference
Mouse embryonic stem cells	Creation of chimer transgenic mouse with ability to transfer ectopic transgene to next generation	This method was applied successfully	Mouse phosphoglycerate kinase-1 (Pgk-1)	Belteki et al. (2003)
Mouse embryonic stem cells	Quantifying and comparing the activities of nine ubiquitous promoters in mouse embryonic stem cells	Insertion of promoter sequences at single copy within defined chromosome position	Nine ubiquitous promoters (ROSA26, CAG, CMV, CMVd1, UbC, EF1a, PGK, chicken b-actin and MC1)	Chen et al. (2011)
Mouse embryonic stem cells	Creation mouse embryonic cell line with endogenously controlled expression of mutants	Achieved at > 60% efficiency without selecting against random selection	Endogenously controlled expression of mutants p53	Wei et al. (2011)
Bovine fibroblasts	Creating transgenic cattle by somatic cell nuclear transfer	This method was applied successfully	Identification of pseudo- <i>attP</i> sites for phage $\phi$ C31 integrase in bovine genome	Ma et al. (2006)
Chicken primordial germ cells	Stable integration of reporter gene obtained using phiC31 integrase at the immunoglobulin light chain locus	The results of this study indicated that phiC31 integrase could be used for generation of transgenic chicken	Vectors consisting of the CAG promoter (the chicken b-actin promoter with human CMV enhancer)	Leighton et al. (2008)
Mouse neural progenitor cells	Promoting stable transgene expression in mouse neural progenitor cells	Sustained transgene expression was demonstrated in genetically modified NPCs	Luciferase marker gene	Keravala et al. (2008)
Mouse neural stem/progenitor cells	Transgenic mice that express codon-optimized phiC31 integrase in neural stem/progenitor cells or tyrosine hydroxylase expressing catecholaminergic neurons	PhiC31 integrase was functional in transgenic mice and was suitable for mosaic recombination in restricted cell populations	Nestin gene	Imayoshi et al. (2012)

Lehmann 1991). The phiC31 integrase is a member of serine-catalyzed enzymes encoded by this phage and mediates the aforementioned phage genome insertion (Smith and Thorpe 2002). This enzyme inserts exogenous DNA into a particular location by using the recognition of two sequences. These sites are termed *attB* and *attP* and have been found to be 34 and 39 base pairs long and naturally exist into phage and bacteria, respectively (Kuhstoss and Rao 1991; Rausch and Lehmann 1991). The phiC31 integrase-mediated recombination has been illustrated in Figure 1. Unlike the other site-specific integrase families, the phiC31 integrase system recombines two recognized sites in a unidirectional manner and it requires no host-specific co-factors. This feature turns phiC31 integrase into a good candidate for gene targeting and in vitro site-specific integration (Thorpe and Smith 1998; Ishikawa et al. 2006). Groth et al. (2000) showed that phiC31 integrase may be able to recognize a series of sites in the mammalian genome which have a partial identity to native *attB* (Figure 2). These sites, named pseudo-*attP* sites, have been found to be extended throughout the mammalian genome (Thyagarajan and Calos 2005; Chalberg et al. 2006). The location of pseudo-*attP* sites, place in safe positions and free of chromatin blockage, and they are transcriptionally active in the host mammalian genome (Thyagarajan et al. 2001) (Figure 3). A more interesting issue for this system was

presented when scientist showed that pseudo-*attP* also exist in the human genom (Groth et al. 2000; Thyagarajan et al. 2001; Bi et al. 2013), which opened a new window for applying this system to several areas such as production of human recombinant proteins (Thyagarajan and Calos 2005), gene therapy (Chalberg, et al. 2005; Ishikawa et al. 2006; Olivares et al. 2002; Quenneville et al. 2004; Keravala et al. 2011), cell therapy and human embryonic stem cell bioengineering (Olivares et al. 2002; Kong et al. 2009; Liu et al. 2009; Sivalingam et al. 2010). One of the main concerns of gene transfer methods in all the above mentioned areas is random ectopic gene integration (Thyagarajan and Calos 2005; Chalberg et al. 2006). Random gene integration elicits several questions concerning gene silencing, variable gene copy number per cell, unpredictable gene expression patterns or altered regulation of gene expression (Thyagarajan and Calos 2005; Kong et al. 2009). This problematic issue might be overcome appropriately by the phiC31 integrase system.

### Recombinase-mediated cassette exchange by phiC31 integrase in stem cells

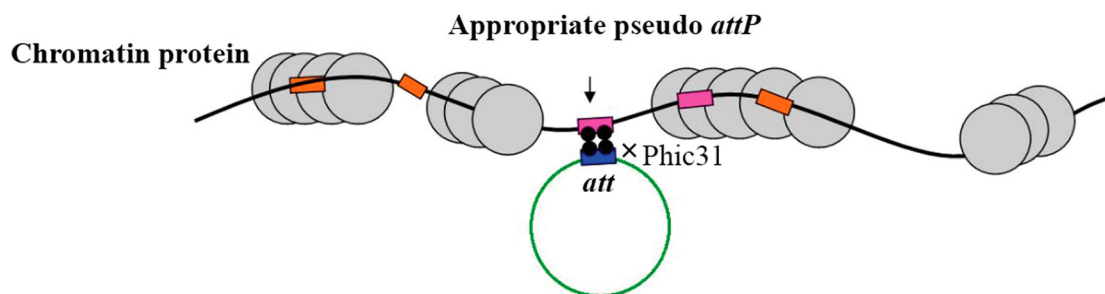
Recombinase-mediated cassette exchange (RMCE) is a potent method for achieving gene targeting at a single mammalian locus, which could be mediated by the phiC31 integrase system. In this method, the gene of



**Figure 1.** Schematic diagrams of phiC31 integrase-mediated recombination. (A) phiC31 integrase determines the two keys (*attB* and *attP*) in *Streptomyces* and phage genome recombined these two sites. Right and left recombinant sites which create by phiC31 integrase termed 'attR' and 'attL' and do not mediate enzymatic reaction by phiC31 reversibility. (B) One of the recognition sites by phiC31 could exist in mammalian which named pseudo-*attP* site. If the other key (*attB*) has been located in a construct harboring gene of interest and pseudo and *attB* sites exposed to phiC31 integrase, the site-specific integration has been conducted in the same manner take placed naturally.

	Core	% identity in central 28 bp related to attB sequences
attB	GTG <b>CC</b> AG GCGTG <b>CC</b> CT TGGCTCC <b>CC</b> G-GGCGCG	(28/28) 100%
Mouse	ACCA <b>TC</b> TGA GATGTA <b>CC</b> CT GGCTTT <b>CC</b> TA TTGACAC <b>CC</b> A AAGG <b>CC</b> CT	(16/28) 57%
Bovine	GT <b>TT</b> CT <b>CC</b> CT AGA <b>TA</b> CT <b>TT</b> AGGG <b>TT</b> TT <b>TC</b> AGAG <b>TC</b> CTT	(12/28) 42%
Human	<b>CC</b> TT GGTTAA <b>CC</b> TT TAGGTTAT <b>CC</b> ATGG	(15/28) 53%
attP	CCCCAACTGG GGTA-AC <b>CT</b> T TGAGTT <b>CT</b> CT CAG----TTG GGGG	(12/28) 42%
Consensus	..... g.t... <b>CC</b> .t gG...t.C <b>CT</b> t t.Gg.t.tcc a.gG...c..	

**Figure 2.** Sequence alignment between wild *attB/attP* sites with some mammalian pseudo-*attP* sites. Most of the matches occur within a 28-bp region, which was used to calculate a percent identity between *attB/attP* and mammalian pseudo-*attP* sites.

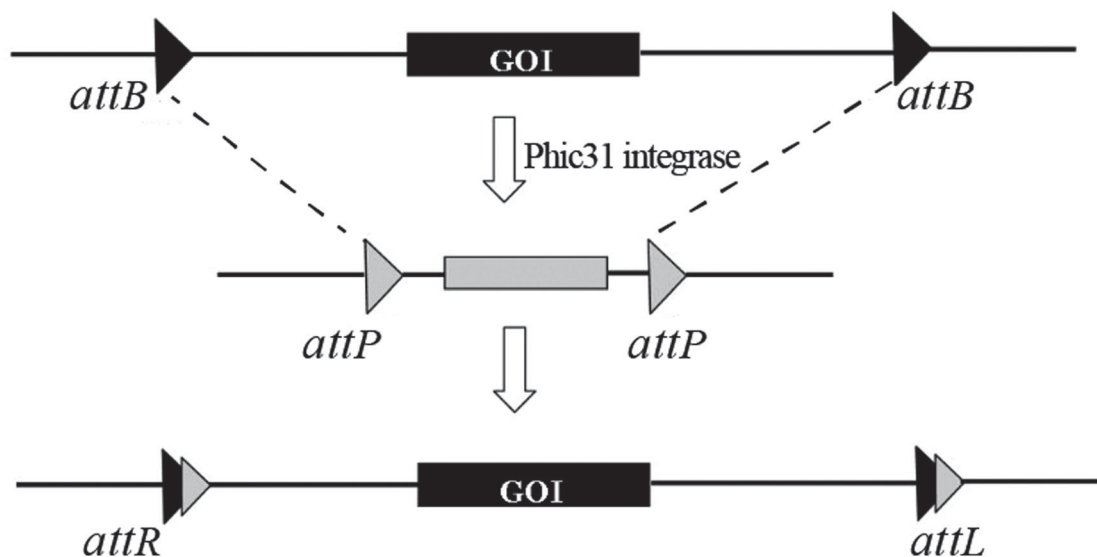


**Figure 3.** Schematic picture which illustrates the particular location of pseudo-*attP* sites in mammalian genome. As indicated by an arrow the appropriate pseudo-*attP* sites exist in open chromatin region in the chromosome. These sites are accessible for transcription machinery which allow over integrated gene expression.

interest (GOI) could be replaced in the donor predetermined genome sequence by flanking GOI at the two identical *attB* sites in the same direction (Figure 4).

Belteki and his colleagues used the cassette exchange approach by phiC31 integrase system for the creation of mouse embryonic stem cells. This method was





**Figure 4.** Exchanged constructs harboring the gene of interest (GOI) flanked in two *attB* sites was targeted to the sequence *attP* via phiC31 integrase-mediated cassette exchanged.

successfully applied for the creation of chimeric transgenic mice with the ability to transfer ectopic transgenes to the next generation (Belteki et al. 2003). In addition, this method has been performed efficiently for the insertion of promoter sequences at single-copy sites within defined chromosome positions in mouse embryonic stem cells (Chen et al. 2011). In another study, with the aim of creating mouse embryonic cell lines with endogenously controlled expression of p53 mutants, the phiC31 RMCE achieved > 60% efficiency without selecting against random selection (Wei, et al. 2011). Recently, Zhu et al. (2014) described a novel system for precise genetic modification of human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs). This system, mediated by phiC31 and Bxb1 integrases, was named dual integrase cassette exchange (DICE). The authors suggested that the DICE system is rapid, efficient and the precise genome modification approaches in ESC and iPSC is particularly well suited for repeated modification of the same locus (Zhu et al. 2014).

### Genetic engineering to create animal model

There is a growing need for site-specific recombinases that function in the mammalian environment to catalyze genomic integration and excision. For example, in the development of transgenic organisms and the study of gene function in vivo, phiC31 integrase could be a straightforward and efficient approach in the

field of biotechnology. In this regard, mRNA encoding phiC31 integrase could be injected into mouse embryos during the unicellular stage, leading to the integration of co-injected *attB*-carrying plasmid DNA into pseudo-*attP* sites (Hollis et al. 2003). The phiC31 integrase system has also been applied in primary bovine fibroblasts, with the expected application of creating transgenic cattle by somatic cell nuclear transfer (Ma et al. 2006). The focus of this section is to survey the application of phiC31 integrase system for the achievement of transgenic stem cells. The first study was conducted by stable integration of a reporter gene obtained using phiC31 integrase at the immunoglobulin light chain locus in chicken primordial germ cells (PGCs). Despite a common transcriptional repression in transgenic PGCs, targeted PGCs by phiC31 integrase system showed considerable levels of expression. The results of this study indicated that phiC31 integrase could be used for the generation of transgenic chicken (Leighton et al. 2008). The phiC31 integrase system-based approach was applied to produce transgenic mice via a pronuclear injection, whereby an intact single-copy transgene could be inserted into predetermined chromosomal loci with high efficiency (up to 40%) and faithfully transmitted through generations (Tasic et al. 2011). In another study, Imayoshi and his colleagues (2012) generated transgenic mice that express the codon-optimized phiC31 integrase in neural stem/progenitor cells or tyrosine hydroxylase expressing catecholaminergic neurons. PhiC31

was functional in these cells and capable of excising a transcriptional stop cassette flanked by phiC31-specific *attP/B* recognition sites. The results of this study have shown that phiC31 integrase was functional in transgenic mice and was suitable for mosaic recombination in restricted cell populations (Imayoshi et al. 2012).

### Cell and gene therapy

In this regards, a study was concentrated on junctional epidermolysis bullosa (JEB) patients. An *attB*-containing laminin 5 beta3 expression plasmid was integrated into the genomes of primary keratinocytes from genetically characterized JEB patients. PhiC31 integrase supported genomic integration into epidermal progenitor cells. Regeneration of human skin on immune deficient mice, using these cells, produced human skin tissue with restored laminin 5 expression. Furthermore, corrected JEB tissue restored hemidesmosome formation and abolished histologic evidence of subepidermal blistering. These findings provide an approach to durable non-viral correction of JEB (Ortiz-Urda et al. 2003). Keravala and her colleagues (2005) used phiC31 integrase systems for the genomic modification of human mesenchymal stem cells (hMSCs). The hMSCs have emerged as a promising new tool in gene therapy/cell therapy strategies due to their potential use in a wide variety of applications, including both short- and long-term regeneration. For genomic integration, integrase has been utilized in different species including human keratinocytes, mouse liver, rat retina and also rabbit joints. Their results suggested that phiC31 integrase as a non-viral method shows an efficient approach for genetic modification of hMSCs, could be a good candidate for preclinical trials and also clinical applications. (Keravala et al. 2005). Quenneville and his co-workers showed co-nucleofection of the phiC31 integrase plasmid and a large plasmid containing the *attB* sequence and the gene for an eGFP – full-length dystrophin fusion protein 1.

In 2012, Lan and colleagues reported for the first time the feasibility of site-specific genetic integration in human cardiac progenitor cells and human endothelial cells using the phiC31 integrase system. They found that the unique chromosomal integration site with stable expression of the reporter gene is accompanied with no noticeable genomic

instability or adverse changes in cell phenotype. Namely, phiC31-modified human cardiac progenitor cells were unchanged in their differentiation propensity, cellular proliferative rate, and global gene expression profile (Lan et al. 2012).

### Evaluation of biosafety and efficacy in transgenic cells

There are several studies regarding the phiC31 integrase system safety in different cell lines. In this aspect, the first research on stem cell was conducted by Liu et al. in 2009. The goal of their research was to study the mechanisms involved in possible chromosomal abnormalities in human adult fibroblasts. They showed that the phiC31 integrase induces chromosome rearrangements in primary human adult cells (Liu et al. 2009). The results confirmed their previous data obtained by using primary human embryonic fibroblasts (Liu et al. 2006). Interestingly, they showed that the phiC31 integrase, in contrast to the SB transposase, leads to significant up-regulation of H2AX phosphorylation, and H2AX phosphorylation leads to recruitment of repair factors for the damaged DNA (Celeste et al. 2002). Moreover, the dosage dependent effects of integrase are still unknown and need to be further clarified.

Sivalingam et al. (2010) evaluated the genotoxic potential of phiC31 bacteriophage integrase-mediated transgene integration in cord-lining epithelial cells (CLECs) readily cultured from the outer membrane of human umbilical cords. The main reason to use this cell line was the ready availability and potential of CLECs, as cellular carriers of therapeutic transgenes. The results demonstrated that phiC31 integrase induced minimal genomic and transcriptomic alterations in CLECs. Using hemophilia as a model for cell therapy revealed that phiC31 integrase-mediated integration of a human factor VIII (FVIII) transgene in CLEC induced stable FVIII secretion and corrected the phenotype of FVIII-deficient hemophilic mice (Sivalingam et al. 2010).

### Generation of engineered and modified stem cell lines

The first study in the field of stem cells engineering was conducted by Thyagarajan et al. in 2007. The work was meant to set a foundation for a broader cell engineering effort that combines rapid assembly of complex

expression vectors and their site-specific insertion into the cells. In this study, they have shown that the phage phiC31 integrase can be used to successfully target sites in the genome of hESC and lead to the generation of transgenic lines. They have also shown that integration into pseudo-*attP* sites leads to sustained expression of the introduced transgene without significant silencing and that these lines retain their pluripotency. Moreover, the results indicate that the profile of the pseudo-*attP* site in hESC is clearly distinct from that seen in differentiated cells (Thyagarajan et al. 2008).

Another study, which is reported by Pauline et al. in 2009, is focused on the development of cell-based assays in human embryonic stem cells using the phiC31/R4 system. They described a system that enables genetic material to be targeted at a specific and predetermined locus and demonstrated the expected protein function in three cell lines: CHO-S, HEK293, and hESC/BGO1 V. The overall process allows the generation of reproducible and high expression clones and pools in a very short time frame due to the specificity and efficiency of cell engineering and the generation of expression vectors via Multisite Gateway technology. The adoption of this technique will allow researchers to assemble multiple reporters and promoters, as well as deliver multiple genetic elements at a specific locus, thereby enhancing the process of stable cell lines development for drug screening, bio-production, and cell-based therapies (Lieu et al. 2009).

In 2011, Wilber and colleagues suggested phiC31 (as well as the SB transposon) as an alternative to viral methods of gene transfer into multipotent adult progenitor cells, as well as other types of stem cells. They used a plasmid-encoding GFP and neomycin phosphotransferase, along with recognition sequences for both phiC31 and SB integrating systems, to demonstrate that both systems effectively mediated integration in cultured human fibroblasts and in rat multipotent adult progenitor cells (Wilber et al. 2011).

Recently, a new mESC cell reporter for cell imaging has been created. This new reporter system is functional and can be studied by both fluorescence microscopy and flow cytometry (Ghorbani et al. 2013). Zhu et al. (2014) used phiC31 and Bxb1 integrase systems spontaneously to create H9 ESC and iPSC lines derived from a Parkinson's disease patient. These cell lines successfully expressed series of the neural transcription factors LMN1a, FOXA2 and OTX2 (Zhu et al. 2014).

## PhiC31 integrase system for reprogramming

Ye et al. (2010) showed that the phage integrase system can be used to generate iPSC from mouse and human fetal somatic cells. They indicated that integrase-mediated reprogramming was similar to the piggyBac transposon method in a way that both methods yield stable transformants without viral vectors (Ye et al. 2010). Ye and his co-workers described a method using bacteriophage phiC31 integrase to reprogram mouse embryonic fibroblasts and human amniotic fluid cells into iPSC. These iPSCs showed all ESC features and successfully formed teratomas with three germ layers. Importantly, these iPSCs have only a single integration site in each cell line. The locations of integration favor the intergenic regions, and their distances from the adjacent genes extended from several hundred to > 1 million bp (Ye et al. 2010).

Recently, Merkl et al. (2013) reported iPSC generation using a non-viral vector containing the murine reprogramming factors Oct4, Sox2, c-Myc and Klf4, controlled by a bidirectional doxycycline-inducible promoter and equipped by the bacteriophage phiC31 attB site. A significant advance in rat iPSC technology is the efficient generation of rat iPSC using a single non-viral vector that allows tight control over reprogramming factor expression. The established iPSC lines were self-sustaining and had activated the endogenous pluripotency sequence. Methods have been developed to improve rat iPSC viability and successful generation of differentiated iPSC derivatives in vitro (Merkl et al. 2013).

## Future perspective for application of phiC31 integrase system in stem cells biology and technology

To compare available alternatives for genome modification in stem cells, methods relying on random integration have the serious limitation of lack of control over the genomic target site, which leads to position effects on endogenous gene expression. The use of a homologous recombination to control the target site will relieve these problems. This approach is safe, but relatively inefficient. Recombination stimulated by ZFNs, TALENs and (CRISPR)/Cas-based RNA-guided technology increases recombination efficiency, although off-targeting effects, introduction of mutations near the target site, as well as the required high



technical support level, are several potential drawbacks of this system (Gaj et al. 2013). These techniques could be replaced or at least combined in many stem cell research areas with the simpler, less technology demanding phiC31 integrase system. The phiC31 integrase system provides a relatively quick, simple, economical and reliable method for stem cell genome modification.

## Disclosure statement

No potential conflict of interest was reported by the authors.

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