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# Use of Transposon-Transposase Systems for Stable Genetic Modification of Embryonic Stem Cells

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## 1. Introduction

Human stem cells provide new hopes in the clinical treatment of a number of diseases, are excellent models for tissue and cell differentiation, and serve as the basis of new screening systems for drug development and toxicity. Regenerative medicine makes use of cells that can grow and differentiate to replace a damaged tissue. Hematopoietic stem/progenitor cells are successfully applied for bone marrow transplantation in otherwise lethal clinical conditions, while many other cell-based treatments are still experimental.

Based on their basic features, we distinguish two major kinds of stem cells. Pluripotent stem cells, capable of differentiating to all types of the cells of human body, were first derived from early human embryos (HuES) and could be grown to provide cell lines with preserved pluripotent characteristics (Thomson et al., 1998). A recently discovered method of generating induced pluripotent stem (iPS) cells from differentiated cell types (Takahashi & Yamanaka, 2006) provides a potential to obtain autologous human stem cell lines without using embryonic tissues. In both cases a major concern, regarding therapeutic applications, is the formation of teratomas, consisting of numerous types of partially differentiated tissues (Reubinoff et al., 2000).

Another major source of human stem cells is our own body. Although in a relatively small number, the so called “tissue-derived stem cells”, sometimes referred to as “adult stem cells”, are present throughout our life in various tissues and organs. The “stemness” of these cells, that is their actual stage of differentiation, depends on age, tissue origin and many other still unrecognized conditions. We know that the bone marrow or the cord blood of the neonate contains mostly hematopoietic progenitor cells, the skin, the liver or the intestine has a large number of repopulating epithelial progenitors, and even the muscle or the brain have dormant cells capable of tissue regeneration. Whether our body also contains early cell types similar to the embryonic stem cells, capable of differentiating into any tissue, is still an

unanswered question (Vieyra et al., 2005; Bussolati & Camussi, 2006; Bussolati & Camussi, 2007; Satija et al., 2007).

Under laboratory conditions undifferentiated cells from HuES or iPS cell lines grow in small clumps, but retain their undifferentiated form only under special conditions. They require media which contain a specific combination of growth factors, and in most cases the presence of appropriate feeder cells. The passage of the pluripotent cells can be performed by mild protease treatment and each cell type may require individual culturing and passaging conditions.

The differentiation of the HUES or iPS cells is initiated spontaneously, as soon as these cells are removed from the special media and/or feeder cells, and placed into normal tissue culture plates or flasks. If the attachment of the cells is prevented, pluripotent stem cells generate so called embryoid bodies (EBs), which are complex, teratoma-like tissue structures with highly variable forms and tissue elements. If these EBs are placed onto tissue culture plates, they attach to the surface and start a further spontaneous differentiation process, by forming in many cases well recognizable tissue-types. Under these conditions, the formation of endothelial, epithelial, and neuronal cells, as well as of fibroblasts or cardiomyocytes can be observed.

Tissue formation greatly depends on the culture conditions, which can be relatively well adjusted to obtain a specific enrichment of a desired tissue type. By applying protease-based cell separation, specific protein and nucleic acid extractions, or even by studying the formed tissues in situ, the pattern of protein expression can be followed during the stages of differentiation of stem cells.

Well characterized human pluripotent stem cells thus indeed represent a great new tool for developmental studies, drug screening as well as cell- and gene-therapy applications. However, all these approaches usually require the development of efficient, stable gene delivery, and proper progenitor cell and tissue separation methods. Following of cell fate of differentiation by stably expressed marker proteins, or the introduction of new or corrected genes into stem cells, greatly facilitate and expand their research and therapeutic potential.

Currently, the most widely applied methods for gene delivery into stem cells are based on the use of viral vector constructs. There are numerous efficient retrovirus- or lentivirus-based methods which allow stable genomic incorporation of the foreign DNAs with high gene product expression levels. However, virus-based gene therapy technologies also have serious drawbacks, including safety concerns of virus production, and the preferential incorporation of foreign genes into active host gene loci, which may cause uncontrolled proliferation of the gene-modified stem cells (Schroder et al., 2002; VandenDriessche et al., 2003). Non-viral gene delivery techniques are usually considered to be less efficient, however, with the emergence and refinement of the transposon based methods, they represent a valid alternative to viral applications (Ivics & Izsvak, 2004; Izsvak & Ivics, 2004; Ivics & Izsvak, 2006). In the following sections we detail the basic features, advantages and concerns of using transposon-based gene delivery into human stem cells.

## 2. Transposon systems as genetic tools

Transposons are “selfish” genetic elements that can move from one DNA locus to another either by a replicative or a non-replicative manner. They are widely present in the genome of all organisms and are also believed to be important driving forces for evolution (Kazazian, 2004; Hedges & Batzer, 2005; Feschotte & Pritham, 2007). The human genome

carries a significant proportion of transposable elements: it is estimated that ca. 45% of our genetic material is made up of transposons (Biemont & Vieira, 2006; Mills et al., 2006; Wicker et al., 2007; Goodier & Kazazian, 2008). The majority of these belong to the Class I retrotransposons which contain currently active elements moving in our genome by the replicative “copy and paste” mechanism (Mills et al., 2007). Although applications using certain retrotransposons as genetic tools exist (Uren et al., 2005; Ostertag et al., 2007), their obvious disadvantages (the potential remobilization of the delivered transgene and the high mutational rate resulting from reverse transcription) make them less favorable as genetic delivery vehicles.

As opposed to retrotransposons, the Class II DNA transposons make up a relatively small portion (~3%) of the human mobile elements (Feschotte & Pritham, 2007) and currently none of them have been shown to be active in our genome (Collier & Largaespada, 2007; Izsvak et al., 2010). They move by the non-replicative “cut and paste” mechanism and their active and modified forms have been widely used in genetic analysis of lower (mostly invertebrate) model organisms including *Drosophila* species and *Caenorhabditis elegans* (Mates et al., 2007). Various gene trapping or insertional mutagenesis experiments proved that transposon based gene delivery is an efficient system, however, lacking similar genetic methods in mammalian cells was an obvious disadvantage.

The first breakthrough in this field was the resurrection of *Sleeping Beauty* (SB), an artificial Tc1/Mariner-type transposon system “remastered” from old fish transposon fossils, and the

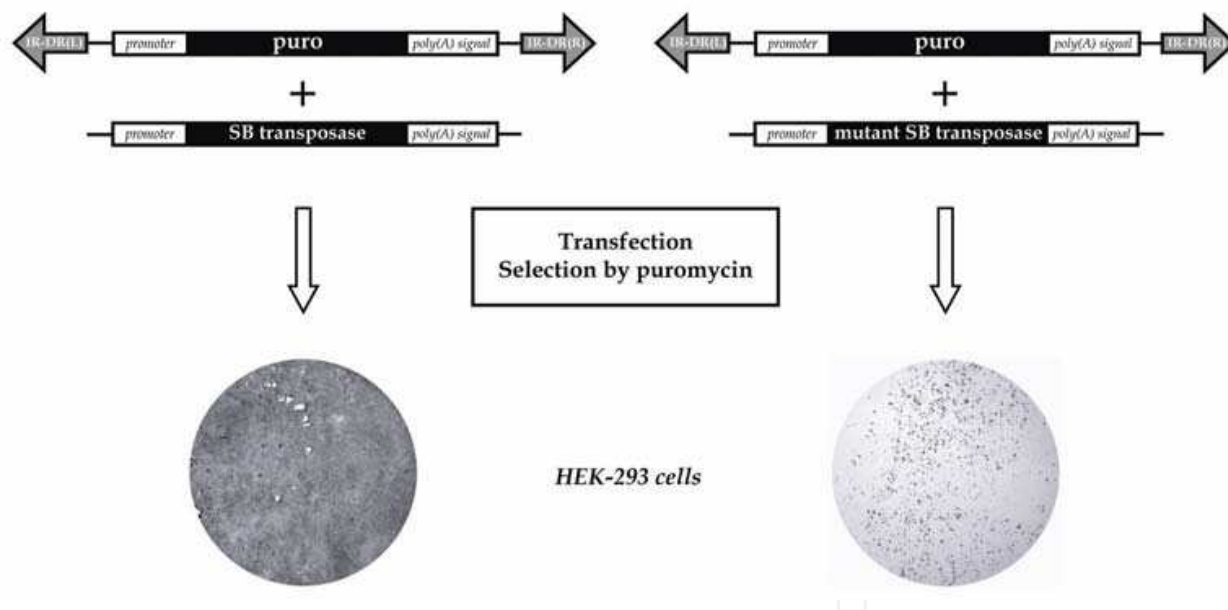


Fig. 1. The principle of gene delivery by the *Sleeping Beauty* transposon system. The gene of interest in a transcription unit (here a puromycin resistance gene, “puro”) is placed between the transposon sequences (IR-DR(L) and-(R): inverted repeat – direct repeat Left and Right sequences). Such an engineered transposon vector is co-transfected with a transposase expressing plasmid into the cells (left panel) and selected by puromycin for 10 days. Following selection, live cells are visualized by Giemsa-staining. The control reaction is carried out using a mutant transposase (right panel) to detect the level of random integration without transposition. As shown in this experiment, transposon based gene delivery is several orders of magnitude higher than the background random integration.

proof of principle that it was active in mammalian cells (Ivics et al., 1997). Its structure is relatively simple (**Figure 1**) and an elegant way of creating a non-autonomous version by separating the transposase from its targets (the terminal inverted repeat sequences) made it an easily controllable system and therefore an attractive tool for functional genetics (Izsvak et al., 2000). Nevertheless, its efficiency in gene delivery at that stage was still behind that of viral vectors, the canonical genetic vehicles used in human applications.

Following the reconstruction of SB, other DNA transposons were shown to be active in mammalian cells, including transposons isolated from other species (such as *piggyBac* from the insect *Trichoplusia ni* (Ding et al., 2005) and *Tol2* from medaka fish (Balciunas et al., 2006)), and another “awakened” Tc1/Mariner transposon called *Frog Prince* (Miskey et al., 2003). However, transposons are naturally not selected for maximal activity in order to minimize the insertional mutagenesis in the host genome and the reconstructed ones were not expected to be the most active forms either. It was then shown that it is possible to create hyperactive versions of these transposons by molecular engineering (Zayed et al., 2004; Baus et al., 2005; Pledger & Coates, 2005), and such variants would likely represent more efficient genetic vehicles.

Recently, a 100 times more active form of SB (SB100x) was created, and its activity was already comparable to the most efficient viral vectors (Mates et al., 2009). The system could also tolerate practically any inserted sequence and the cargo size capacity is less limited than that of viral vectors: it can efficiently move inserts of >8 kb, although transposition efficiency decreases with larger cargo size (Izsvak et al., 2010). Unexpectedly, the amount of the transposase seemed to be a more critical issue: the transposition efficiency paradoxically decreases when the amount of transposase raises beyond a certain level, a phenomenon called overproduction inhibition (Lohe & Hartl, 1996). All of the used DNA transposons seem to share this feature but the careful titration to set up the optimal transposase level provided evidence that among them, SB was the most efficient system in conditions when the amount of transposon DNA is limiting (Grabundzija et al., 2010). This finding made the SB system attractive to many applications where transgene delivery into hard-to-transfect cell types is required, including embryonic stem cells (see **Part 3**). In addition, the lack of endogenous copies in vertebrate (particularly in human) genomes represents an important safety issue as it ensures that the integrated transgenes are not being remobilized (Ivics et al., 1997; Ivics et al., 2004). Such attractive characteristics prognosticated that the SB100x transposon version would likely represent a method of choice when carrying out gene delivery into mammalian cells.

The “technology transfer” from invertebrates to use transposons for genetic manipulations had an immediate effect on mammalian forward genetic screens: SB transposon was successfully used in cancer genetics in order to identify genes involved in certain malignant phenotypes (Carlson et al., 2005; Collier et al., 2005; Collier & Largaespada, 2005; Starr et al., 2009). In addition, various insertional mutagenesis screens could be set up, often exploiting the phenomenon of “local hopping” when the transposon preferentially moves in the vicinity of the donor chromosomal locus (Dupuy et al., 2005; Lu et al., 2007; Takeda et al., 2007; Takeda et al., 2008). This characteristic seems to be a common feature of “cut and paste” transposons and could be very useful for saturation mutagenesis of particular chromosomal regions (Luo et al., 1998; Fischer et al., 2001; Carlson et al., 2003; Carlson & Largaespada, 2005; Keng et al., 2005). For SB, however, this “local hopping” does not appear to be very stringent since the transposition intervals are higher than that of other DNA transposons (Carlson et al., 2003). Nevertheless, the SB transposon was successfully

established as an efficient genetic tool for forward genetics in mammals, similarly to the P element based applications in *Drosophila* (Ryder & Russell, 2003; Miskey et al., 2005).

When considering gene therapy applications in human, however, efficiency is only one of the important issues that must be addressed before a genetic system becomes approved. For example, an important drawback of the efficient viral methods are their non-random integration profile: they integrate favorably into transcription units, often preferably into 5' regions of active genes (Schroder et al., 2002; Bushman, 2003; VandenDriessche et al., 2003; Wu et al., 2003; Narezkina et al., 2004). To a lesser extent, this adverse site preference is also the characteristic of some transposons, including *piggyBac* (Wilson et al., 2007) and *Tol2* (Grabundzija et al., 2010). On the other hand, *Sleeping Beauty* seems to be a favorable system from this point of view: the integration profile was revealed to be very close to random on the genomic level (Vigdal et al., 2002; Liu et al., 2005; Yant et al., 2005). This important feature significantly lowers the risk of insertional mutagenesis which is beneficial for gene therapy applications.

Other issues include the potential silencing of the transgene which could hinder the applicability of viral vectors (Ellis, 2005). Embryonic stem cells, for instance, have clear molecular defense mechanisms against viral promoter sequences (Meilinger et al., 2009; Rowe et al., 2010). When addressing this question for the SB transposon, it was revealed that the effect of silencing depends rather on the cargo sequence and not on the vector itself (Garrison et al., 2007; Zhu et al., 2010). All these encouraging characteristics further supported the use of SB as a tool for gene therapy and provided the basis for the first clinical trial initiated by a non-viral vector: SB is used in the treatment of a B-lymphoid malignancy by ex vivo genetically modified autologous T-cells (Williams, 2008). The outcome of the trial will apparently provide valuable information on the efficiency and biosafety of transposon based gene delivery and could potentially set new standards in gene therapy application, especially since the use of the SB transposon system was recently shown to be applicable also in embryonic stem cells (Wilber et al., 2007; Orban et al., 2009). This promising scenario undoubtedly played a role in that the SB100x transposase version was recently nominated as the "Molecule of the Year" in 2009 (<http://www.biotechniques.com/news/Sleeping-Beauty-named-Molecule-of-the-Year/biotechniques-187068.html?autnID¼191663>).

### 3. Selecting transgene expressing stem cells after transposition

The basis of any successful gene therapy applications is undeniably an efficient and stable gene delivery into stem cells. For this purpose, viral based applications were traditionally favored as viruses have been selected through evolution to efficiently deliver genetic material (DNA or RNA) into their host cells. However, two negative aspects of their usage made non-viral approaches favorable despite their lower efficiency: the biased integration profile of viral vectors, and the limitation of the cargo size due to the packaging constraint of the virus particles. The latter one is an important issue when considering relatively large cDNAs of certain human genes, or the need for simultaneous delivery of more cDNAs into one sample. Although potentially overcoming these problems, the uses of first generation non-viral vectors were clearly several magnitudes less efficient than their peer viral counterparts. With the emerging hyperactive transposons, however, gene delivery efficiency was partly resolved. Nevertheless, the use of any transposon system requires the

transfection of DNA into the host cells, and several applications involve cell types that are generally difficult to transfect, including embryonic stem cells. Albeit various transfection protocols are currently available, it is infrequent to achieve higher than 50-80% transfection efficiency without severely affecting cell survival, therefore efficient and preferably non-invasive selection protocols should always be worked out to establish homogenous transgene expressing stem cells following transfection and transposition.

One widely used selection method is to apply chemical selection (e.g. antibiotics) to enrich for transgene expression. This approach usually serves well if cell source is not limiting and when the chemical selection does not significantly perturb cell physiology. Embryonic stem cells, however, represent much more sensitive cell types and if later clinical applications are taken into account, it has to be borne in mind that the precious cells on which the therapy can begin with will most likely come from a limited source. Therefore, a selection must be efficient and at the same time, the least invasive procedure. The commonly used drugs, however, could induce undesired gene expression profiles, or initiate partial differentiation of the stem cells. If chemical selection is inescapable, the cells must always be examined carefully whether they retained their pluripotency status at least by immunostaining for accepted surface markers or preferably also by scrutinizing their differentiation potential (Duan et al., 2007; Tomescot et al., 2007; Orban et al., 2009). Moreover, since the use of a marker gene (eg. an antibiotic resistance gene) evidently means a use of a larger cargo, the overall gene delivery efficiency will decrease. An elegant way of reducing this problem is the use of viral linker peptides between cDNA sequences instead of independent transcription units (see **Part 4**), but the potentially altered genetic profiles still disfavors such selection approaches, if possible.

Another unexpected problem of chemical selection originates from the multidrug resistance phenotype, more precisely, from the presence of MDR-ABC transporters, especially the ABCG2 protein. It is now well established that this multidrug transporter is present in embryonic stem cells and is responsible for the so called "side population" phenotype of a wide variety of tissue-derived stem cells (Zhou et al., 2001; Sarkadi et al., 2010). The exact function of ABCG2 in these cell types is not fully elucidated yet but numerous evidence points to its role to protect these valuable sanctuaries against various noxae by extruding undesired drugs out of the cells (**Figure 2**). Clearly, the presence of such defense mechanism can work against chemical selection since the increase of endogenous ABCG2 expression could work against the enrichment of transgene expressing stem cells, e.g. by pumping out puromycin from stem cells (Takenaka et al., 2007). Also, antibiotic selection may greatly increase MDR-ABC protein expression as a stress-related response (Theile et al., 2010).

Another approach for the enrichment of transgene expression is the use of fluorescent proteins as markers. The genetically modified, fluorescent stem cells can be separated by FACS (Fluorescent Activated Cell Sorting) analysis or positive clumps can be sequestered in sterile conditions using fluorescent microscopy. In our laboratory, we have routinely established different transgene expressing human embryonic stem cell clones, usually combining both methods (**Figure 3**). Although less invasive than chemical selection, this procedure can be more laborious and time consuming. Moreover, as strongly expressing cells have a higher chance of being selected, this method inherently favors stem cells with higher copy numbers which should be considered if a certain application requires low copy number (or even single copy number) clones. Nevertheless, this approach is still less

invasive as a chemical selection, even though the problem of delivering another transgene additionally to the selection marker again places a burden by the increase of the cargo size, therefore lowering the efficiency.

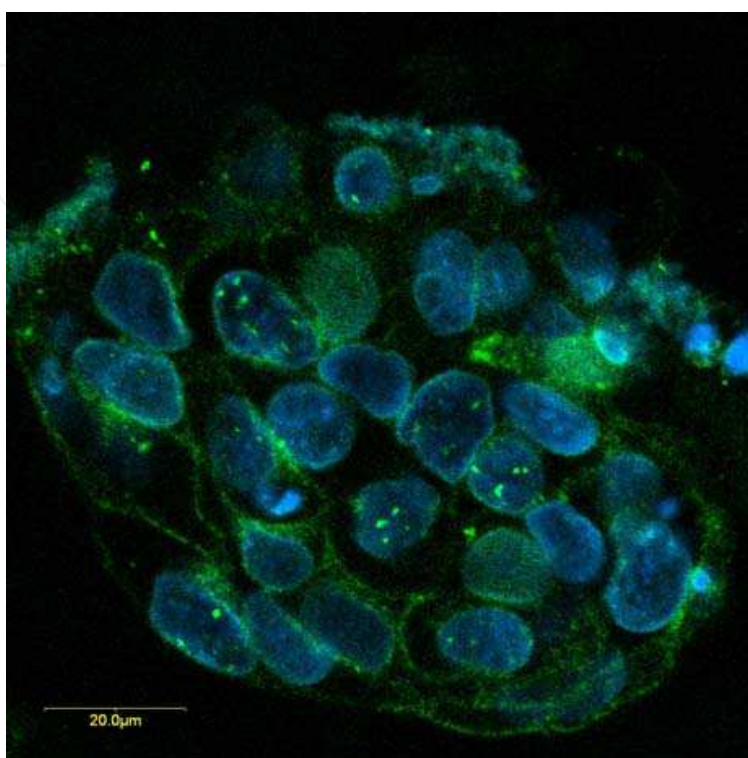


Fig. 2. Expression of the ABCG2 membrane transporter in a human embryonic stem cell clump.

HUES9 cells were immunostained with the 5D3 anti-ABCG2 antibody (green); the Hoechst 33342 dye (blue) was used to visualize cell nuclei. The confocal microscopy image clearly shows that ABCG2 is localized in the plasma membrane of all examined cells. Scale bar represents 20  $\mu\text{m}$ .

To combine the advantages of viral vectors and transposons, several groups attempted to create genetic chimera vehicles, using non-integrating virus forms with the SB system (Bowers et al., 2006; Staunstrup et al., 2009; Vink et al., 2009; de Silva et al., 2010b). This approach can overcome the inefficient delivery often associated with transfection of DNA into certain cell types, and presents the favorable transgene integration profile provided by the SB transposase. Such hybrid vectors certainly open new vistas in gene therapy, although rigorous testing should still be carried out to carefully examine the safety and efficiency of these methods. There is one study claiming that the chimera vector of a Herpes simplex virus and a hyperactive version of SB loses its transposition “hyperactivity” in vivo (de Silva et al., 2010a). However, as this study used an earlier version of the transposase, the results should be carefully repeated with the new SB100x system which provides a far more robust gene delivery in vivo than any previous transposons, therefore could potentially overcome this negative side effect.



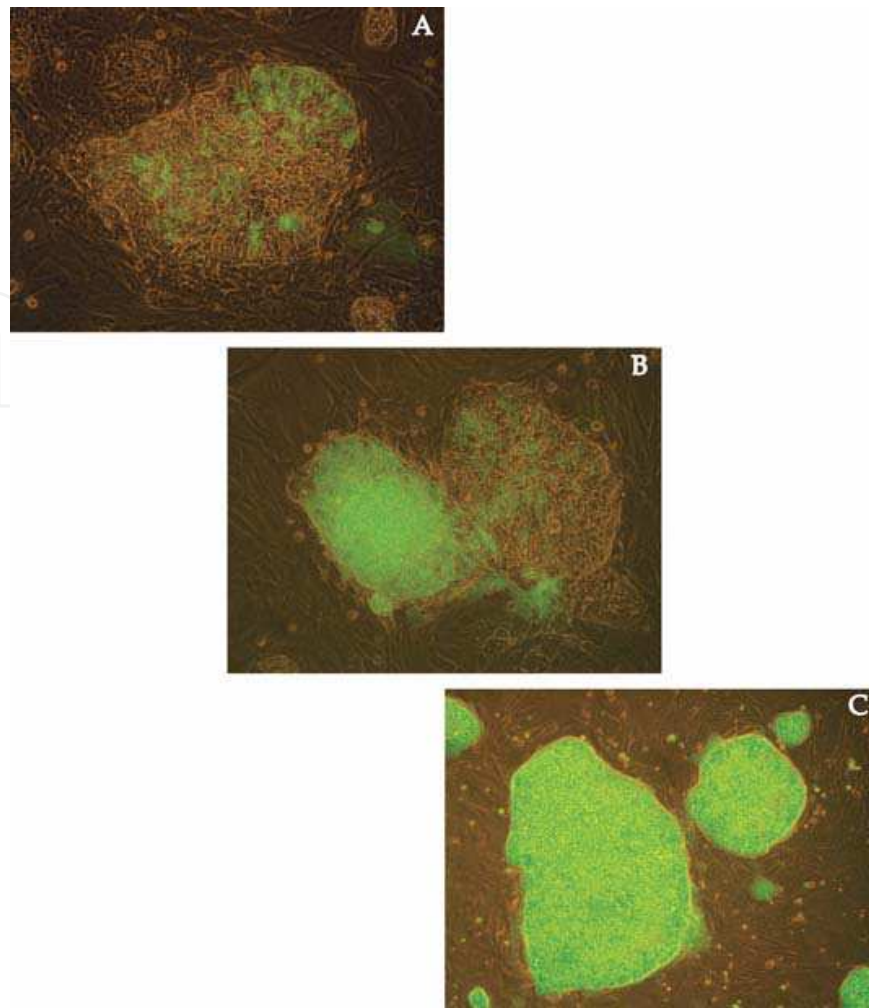


Fig. 3. Cloning of the transgene expressing embryonic stem cells following transposon mediated gene delivery.

(A) GFP expression cassette is delivered into HUES9 cells by the SB transposon system. The image is taken 48 hours post-transfection, showing a heterogeneous cell population of a clump on mouse embryonic feeder cells. (B) Enrichment of transgene expressing cells by manual sequestration of GFP positive cells using fluorescent microscopy. (C) The results of cloning GFP expressing stem cells by FACS from clumps in stage (B). Phase contrast fluorescence microscopy images, x40 magnification.

#### 4. Applications of transposon based gene delivery in embryonic stem cells

HuES cells represent excellent models for cell and tissue differentiation, however, directing the genetic program toward a certain lineage is often challenging. For various applications, such as pharmacological screening models, the aim is to achieve a reasonable enrichment of a given tissue type among the various progeny of cell types. Current methods often apply endogenous morphogenic proteins or invasive chemicals to obtain the tissue(s) of interest, however, the use of such artificial chemical cocktails could have serious side effects, including undesired gene expression profiles and/or distorted differentiation pathways. On the other hand, the alternatively used spontaneous differentiation of HuES cells (e.g. via the embryoid body pathway) is a stochastic process and the efficiency of obtaining a particular

cell type is often very low. In addition, some tissue types are difficult to recognize solely by morphological signs, and other – often invasive – molecular identification methods are necessary to apply.

In our laboratory, we are examining cardiovascular differentiation, with an obvious advantage of having a clearly recognizable phenotype at the end of differentiation. Such contracting cell populations can be found following spontaneous differentiation, however, their number can vary significantly. To increase the efficiency of cardiac cell detection, we have developed a method using a specific “double-feature” promoter (Orban et al., 2009). It is based on the unprecedented characteristic of a CAG promoter variant: as being a constitutive promoter, it is expressed in all tissue types which was the reason why it was chosen to drive the expression of a fluorescent reporter gene. On the other hand, the transcriptional activity of this CAG variant becomes extremely high in differentiated cardiomyocytes, providing an excellent platform of selecting these cell types based on the intensity of the fluorescent signal. This behavior is also very useful when a transposon based gene delivery is applied: the “double-feature” promoter is used to identify the transgene expressing undifferentiated cells after transfection and later on, it offers the possibility to select for differentiated cardiomyocytes (**Figure 4**). This approach represents a great advantage because up to our knowledge, no commercially available antibodies against cardiac specific cell surface markers exist which would allow gentle separation of these cell types. Moreover, as this promoter is less prone for silencing (Chung et al., 2002; Xia et al., 2007), the loss of cells containing inactive transgene copies is also significantly reduced. The background of this “double-feature” phenomenon is still under investigations, and deciphering its structural basis could lead to a promising scenario of creating promoters with different tissue specificities. Such achievement would represent a great technological

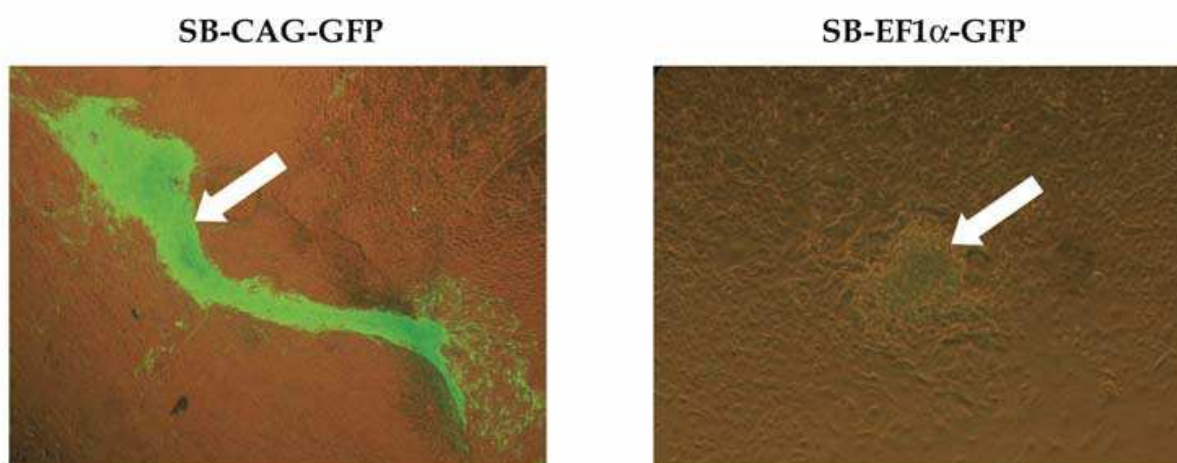


Fig. 4. Using the “double-feature” CAG promoter to visualize cardiomyocytes.

White arrows depict contracting cardiomyocytes arising from spontaneously differentiated HUES9 cell clones expressing either CAG promoter- (SB-CAG-GFP) or EF1 $\alpha$  promoter-driven (SB-EF1 $\alpha$ -GFP) transgene. Note that in SB-EF1 $\alpha$ -GFP cells, the entire population expresses GFP at low level almost uniformly, whereas in SB-CAG-GFP cells, GFP expression in cardiomyocytes is extremely high as compared to the surrounding tissues; see further details in text. Phase contrast fluorescence microscopy images, x40 magnification.

breakthrough as tissues lacking easily recognizable morphological signs could be separated without invasive identification protocols; and the SB transposon based transgene delivery would ensure the lowest possible risk of mutagenesis by its random integration profile.

The technology of generating iPS cells is an obvious example where the use of transposons as genetic vehicles is clearly beneficial due to the large cargo size. For efficient reprogramming of fibroblasts, at least 4 transcription factors need to be overexpressed (Takahashi & Yamanaka, 2006) which places a heavy burden on the otherwise also not very efficient method itself. The use of independent transcription units are clearly represent too large cargos; the use of IRES (Internal Ribosome Entry Site) sequences instead also faces the problem of inefficient and non-equimolar expression of several cDNAs. An elegant way of overcoming this issue is the use of the 2A viral linker peptides: it basically allows to establish a polycistronic mRNA from which separate peptide chains can be translated equimolarly in eukaryotic systems (Szymczak et al., 2004). However, when considering an additional selection marker, the length of such a cargo (~7 kb or higher) still pushes the packaging limits of most viral vectors so the need to deliver such a long transgene calls for the use of transposons. Indeed, the *piggyBac* system was applied to successfully establish pluripotent iPS cell lines (Kaji et al., 2009; Woltjen et al., 2009). Nevertheless, some disadvantages of the *piggyBac* system, e.g. the non-random integration profile (Wilson et al., 2007) or the presence of endogenous elements potentially capable of remobilizing the transgene (Newman et al., 2008) clearly awaits for the use of another, technically safer system such as the SB transposon. In addition, for any gene therapy application constrained by the size of a large human transgene cDNA calls for the application of transposons, preferably the SB system.

## 5. Conclusion

Embryonic stem cells represent promising new tools in the clinical treatment of various diseases, and in the meantime, they provide emerging new systems in modeling tissue differentiation and pharmacological screens for drug development and toxicity. With the development of laboratory protocols for the maintenance of these cell types, it is also important to work out efficient and biologically safe methods for gene delivery as it often represents the “take-off” point of any successful work with HuES cells. Among the non-viral gene delivery techniques, the hyperactive *Sleeping Beauty* transposon-transposase system represents a particularly attractive method with several advantages. It is a powerful gene delivery methodology with the least currently known genotoxic effects mainly due to its random integration profile at the genomic level. Also, it is a favorable genetic vehicle in terms of cargo capacity, tolerating relatively long transgene sequences. Moreover, as opposed to most viral vectors, the transposon sequences are less prone to epigenetic silencing which also provides the background for a long-term stable transgene expression. The last but not the least argument for the use of this transposon system is the cheaper and easier production of vector DNA, especially when clinical-grade applications are considered. Taken together, all these favorable characteristics definitely make the hyperactive SB system an attractive alternative for any gene therapy purposes, although, as it is the case with any other newly developed techniques, further investigations are still necessary to validate its biological safety in clinical applications.

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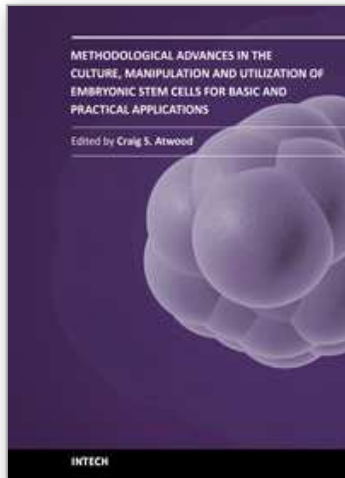
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