



**This is a postprint of an article published in**  
**Schucht, R., Coroadinha, A.S., Zanta-Boussif, M.A., Verhoeyen, E.,**  
**Carrondo, M.J.T., Hauser, H., Wirth, D.**  
**A New Generation of Retroviral Producer Cells: Predictable and Stable**  
**Virus Production by Flp-Mediated Site-Specific Integration of Retroviral**  
**Vectors**  
**(2006) Molecular Therapy, 14 (2), pp. 285-292.**

**A new generation of retroviral producer cells:  
Predictable and stable virus production by FLP mediated site-specific integration of  
retroviral vectors**

R. Schucht<sup>1</sup>, A. S. Coroadinha<sup>2</sup>, M.A. Zanta-Boussif<sup>3</sup>, E. Verhoeyen<sup>4</sup>, M. Carrondo<sup>2</sup>, H. Hauser<sup>1</sup>, and D. Wirth<sup>1\*</sup>

<sup>1</sup> Dept. Gene Regulation and Differentiation, GBF - German Research Centre for Biotechnology, Braunschweig, Germany

<sup>2</sup> Instituto de Biologia Experimental e Tecnológica/ Instituto de Tecnologia Química e Biológica (IBET/ITQB), Apartado 12, P-2780-901 Oeiras, Portugal

<sup>3</sup> Genethon, 1 bis, Rue de l'Internationale-BP60, F-91002 Evry Cedex, France

<sup>4</sup>Laboratoire de Vectorologie Retrovirale et Therapie Genique, Inserm, 46 allée d'Italie, 69364 Lyon Cedex 07, France

\* Corresponding author

Dagmar Wirth, PhD

GBF- National Research Centre for biotechnology

Mascheroder Weg 1

D-38124 Braunschweig

Tel. ++49-531-6181-252

FAX: ++49-531-6181-262

Email: dagmar.wirth@gbf.de

**Abstract**

A new strategy was developed that provides well-defined high-titer producer cells for recombinant retroviruses in a minimum of time. The strategy involves the targeted integration of the retroviral vector into a chromosomal locus with favourable properties. For proof of concept we established a novel HEK293 based retroviral producer cell line, called Flp293A, with a single copy retroviral vector integrated in a selected chromosomal locus. The vector was flanked by non-interacting FLP-recombinase recognition sites (FRT) and was exchanged for different retroviral vectors via FLP mediated cassette exchange. All analyzed cell clones showed correct integration and identical titers for each of the vectors, confirming that the expression characteristics from the parental cell were preserved. Titers up to  $2.5 \times 10^7$  ip/ $10^6$  cells were obtained. Also, high titer producer cells for a therapeutic vector that encodes the 8.9 kb collagen VII cDNA in a marker free cassette was received within three weeks without screening.

Thus, we provide evidence that the precise integration of viral vectors into a favourable chromosomal locus leads to high and predictable virus production. It is compatible with other retroviral vectors including SIN vectors and marker free vectors. Further, it provides a tool for evaluation of different retroviral vector designs.

**Keywords**

Retroviral vector production, cassette exchange, FLP mediated site-specific recombination

## **Introduction**

Retroviral gene transfer is a well-established and highly efficient technology for the transduction of genes and has been exploited for both, research and gene therapy purposes [1]. A number of packaging cell lines were developed, mainly based on mouse or human cell lines that stably express the retroviral helper genes and support high titer virus production [2-9]. The integration of modified envelope proteins (env) or envs from different viruses (pseudotyping) allowed to modify and broaden the host spectrum [4, 8, 10], to label virus particles [11] and to generate virus particles with an improved stability with respect to purification [12] but also stability towards human serum [9, 13-15].

Although high titer virus production could be established, the procedure for producing a new therapeutic virus is still time and cost intensive [16]. The procedure requires the establishment of the vector into the chromosomal DNA of the packaging cell and is accompanied by random integration. The properties of cell clones generated in this way and in particular the titer is largely determined by the nature of the integration site and the influence of neighboring chromosomal elements. Screenings have to be undertaken in order to identify clones that lead to a high and stable titer virus production. The need for screening for appropriate expression levels of the viral vector indicates that this parameter is critical for virus production. This is supported by previous work indicating that optimal virus production depends on a high retroviral vector transcription and balanced expression of the retroviral helper genes encoding gagpol and env [17]. Once having isolated a cell clone, it has to be optimized with respect to large scale culture conditions, including the adaptation to production media [18]. This again has to be conducted for each producer cell clone independently. For clinical applications, production under GMP conditions has to be established including a detailed safety testing and an accurate and precise characterization of the producer cell and the stability of recombinant virus production. In total, the process starting from transfection of a packaging cell line to a

suitable clone usually takes six months or more [16]; even then, the performance of virus production is not guaranteed.

The strategy for the work reported here is based on two assumptions: 1. Retroviral vector transcription is the major limiting factor for maximal virus production. 2. Expression levels of gagpol and env must serve the extent of vector expression.

Accordingly, we first focused on an extensive screening for a highly expressed and stable chromosomal locus that particularly supports retroviral vector transcription. In a second step, we screened for adjusted expression of gagpol and env.

Such a strategy would necessarily rely on the re-use of the retroviral vector integration site for a therapeutic vector of choice and thus excludes its random integration into the hosts' chromosomal DNA. Site specific recombination systems like the Cre/loxP and the flp/FRT system were shown to allow precise integration of genes of interest into precharacterized loci in mammalian cells [reviewed by Baer and Bode in ref. 19]. To improve targeting, spacer mutants have been developed [20, 21]. The use of spacer mutants allows establishing tag-and-target strategies that preclude excision of cassettes, thereby overcoming the major side-reaction of this technology. This enables to tag and reuse chromosomal integration sites even in mammalian cells [19, 22]. Moreover, it can be combined to stringent selection procedures to apply this technology in cells that support frequent random integrations like most differentiated cell lines [23, 24]. Here, we demonstrate that the use of recombinase mediated cassette exchange (RMCE) allows the rapid generation of recombinant viruses with a high and predictable titer. In this way a potent virus production cell line in which the retroviral integration site is flanked by a set of non-interacting FRT sites can be generated. The therapeutic vector of interest is targeted into the same precharacterized genomic locus. The performance of the technique is demonstrated by the creation of a new broadly applicable amphotropic producer cell line Flp293A.

## Results

### *Tagging HEK293 cells with a single copy retroviral tagging vector*

The strategy for the construction of retrovirus producer cells is outlined in Fig. 1A. It involves the tagging and screening for integration sites with high level retroviral vector expression, stable transfection of the helper functions gag/pol and env and subsequent targeted integration of the therapeutic vector of interest by Flp mediated cassette exchange.

To identify chromosomal sites which support high level retroviral vector expression we first screened HEK293 cells using a retroviral tagging vector pTAGeGFP (Fig. 1B). This vector transduces a bicistronic cassette of eGFP and a hygromycin phosphotransferase/thymidine kinase fusion protein. In its 3' U3 region a tag-and-target cassette is integrated comprising both a wild type and a mutant FRT site [25] as well as a 5'-truncated, ATG deficient and thus inactive neomycin phosphotransferase gene ( $\Delta$ neo in figure 1B). This gene is complemented upon targeted cassette exchange [23]. Upon reverse transcription the 3' U3 including the tag-and-target cassette is duplicated to the 5' LTR, thereby flanking the reporter genes with two tag-and-target cassettes.

HEK293 cells were infected with the pTAGeGFP tagging vector under conditions that preferentially lead to single copy integration events. Pools of approximately 50.000 randomly tagged cells were generated upon selection towards hygromycin B. To identify clones that have the capacity to produce maximal amounts of the retroviral vector RNA clones with highest levels of eGFP expression were sorted. Fifty cell clones with comparable GFP expression were isolated in a second single-cell sorting step. Southern Blot analysis was performed to check the copy number of vector (not shown). Five of the clones showed more than one copy and were thus discarded. Out of the 45 single copy vector clones, clone 1B2 showed high level and stable eGFP expression for at least 25 weeks in the absence of selection pressure (Fig 1C). This clone was chosen for establishment of a master virus producer cell line. The other clones were not considered for further evaluation since expression was lower or stability was not guaranteed.

### *Establishment of a master retrovirus producer cell line*

For the establishment of a master virus producer cell clone 1B2 was transfected with plasmids encoding the helper genes gag/pol and amphotropic env (see Materials and Methods for details). More than 80 independent cell clones were generated that differed in the level of

gag/pol and env expression. Screening was performed to find a single clone with high titer production of recombinant retrovirus. Among the clones generated by stable transfection of the helper functions, 80% produced recombinant viruses with a titer below  $10^4$  ip/ $10^6$  cells. Three clones produced more than  $1 \cdot 10^5$  ip/ $10^6$  cells and from these clones 1B2-8-F produced  $4.1 \cdot 10^6$  eGFP transducing viruses per  $10^6$  cells. Virus production was stable for more than 12 months in absence of any selection for the retroviral vector and the helper function, respectively (Fig. 1D). This clone is called Flp293A in the following. From an independent approach Flp293A\* was isolated with a titer of  $2 \times 10^6$ /ml. This clone provides the same properties as the Flp293A (not shown).

To evaluate the efficiency of integrating retroviral vectors into the preselected chromosomal site we prepared different targeting vectors. In these vectors a wild type and mutant FRT site flank a complete retroviral vector including both LTRs and the transgene of interest. An ATG start codon preceding the 3' FRT site and either a CMV promoter or an EMCV IRES element was integrated to complement the inactive  $neo^R$  gene in Flp293A cells upon targeting (Fig 1B and Fig 2). While for the CMV construct resistance to G418 is obtained from a second, independent mRNA, IRES mediated activation requires a certain level of readthrough transcription of the mRNA initiated at the 5'LTR. For the CMV mediated activation of neomycin resistance gene both the sense and the reverse orientation of the retroviral vector was used.

The targeting vectors were cotransfected with the Flp recombinase expression construct pFlp. Screening for G418 resistance identifies cells that have undergone site specific cassette exchange which results in complementation of the inactive  $neo^R$  gene. In addition, this eliminates cells that have only undergone excision of the tagging construct.

Table 1 shows the number of G418 resistant clones generated from a representative experiment. While the efficiency of generating targeted clones was comparable for integrating the retroviral vectors in a sense orientation, we were not able to establish G418 resistant clones with vectors that integrate the retroviral cassette in reverse orientation (pMLCMeGFPprev and pMSCMcolVIIrev). The reason for this remains obscure. G418<sup>R</sup> clones were isolated and confirmed for site specific integration of the targeted vector cassette by Southern blot analysis (Fig3A and data not shown). In total, 71/72 of the analysed clones showed excision of the tagging vector and correct integration of the targeting cassette (Table

1). One clone showed the parental band in addition to the signal for targeted exchange, indicating that the excision of the tagging vector had not occurred. Importantly, no random integration of the targeting cassette was found in any clone in addition to the specific integration.

Overall, correct cassette exchange took place in 98% of clones analysed, indicating that no screening has to be performed.

#### *Flp293A supports high level virus production from different types of retroviral vectors.*

We asked if the high virus production of the tagging virus pTAGeGFP applies also for retroviral vectors upon targeted cassette exchange. Single G418<sup>R</sup> clones obtained after targeting were evaluated for retrovirus production. Generally, no indication for clonal variation was found and titers of independent subclones were homogenous and stable over time (Fig. 3B and data not shown). Interestingly, while the IRES containing targeting vectors supported high retroviral titers the corresponding CMV containing vectors did not result in recombinant retrovirus production. Possibly, the close proximity of the retroviral promoter to the CMV promoter interferes with efficient retroviral transcription as previously reported for similar designs [26].

For the eGFP transducing targeting vectors pMSIREGFP and pMLIREGFP the titers were elevated two-fold and six-fold, respectively, if compared to the parental cell line Flp293A. A similar difference in titer was confirmed in transient production assays (data not shown). The different titers could be due to differences in vector design. Generally, the increase may be explained by an enhanced stability of mRNA resulting in an increased steady state level. Further, the elimination of one of the two copies of the neo<sup>R</sup> gene could be expected to enhance viral transcription since the npt coding sequence silences nearby promoters [27]. The neo<sup>R</sup> gene within the 3'LTR of the tagging vector could also reduce reverse transcription. Finally, other changes in vector design including different strength of the LTRs could contribute to enhanced transcription or reverse transcription in the targeted vectors.

#### *Production of a therapeutic viral vector encoding collagen VII*

For proof of concept, we evaluated the capacity of the helper cell line Flp293A for production of a therapeutically relevant retroviral vector. We chose human ColVII that is a candidate

gene for treatment of epidermolysis bullosa [reviewed by Bauer and Laimer in ref. 28]. The hColVII cDNA comprises 8.9 kb which is not compatible with an additional integration of a marker gene that would facilitate screening for high titer producer cells.

An MSCV based hColVII transducing retroviral vector was modified for targeted integration according to the above described scheme resulting in pMSIRcolVII and pMSCMcolVII for the IRES and CMV mediated activation of neoR, respectively, and pMSCMcolVIIrev for the reverse orientation. In addition, we evaluated the self-inactivating hColVII retroviral vector pSINcolVII for targeted integration. In this vector, the promoter activity of the 3' U3 is eliminated and hColVII transcription is controlled by an internal EF1 $\alpha$  promoter in the infected cells (Fig. 2B).

The different targeting vector designs confirmed the results described above: the CMV promoter interfered with targeting and/or virus production, while the IRES containing vectors allowed efficient targeting and high level virus production (Table 1). The targeted subclones were confirmed for correct integration and analyzed with regard to hColVII transgene expression and virus production. Immune fluorescence staining of the virus producer as well as the infected cells showed hColVII accumulating in the cytoplasm (Fig. 4a and b, respectively). Importantly, also the SIN-retroviral vector could be efficiently expressed in the pre-selected integration site. High level recombinant virus production was observed. Based on the ColVII immune fluorescence the retroviral titer was calculated to  $1.2 \cdot 10^6$  ip/ $10^6$  cells  $\cdot$  24h (Table 3).

## Discussion

Recombinant retroviruses are the most frequently used vectors for *ex vivo* transduction in gene therapy trials. Accordingly, optimisation of retroviral vector expression was an important aim of a number of independent approaches [29-31]. In contrast, the production technology for retroviral vectors is in an early state and still relies on methods established some 25 years ago. Production of therapeutic virus is hampered by the time- and cost-effective procedure that is needed for isolation of high titer and stable producer cells and the establishment of optimal production conditions [16]. These steps have to be conducted for any therapeutic vector individually.

To facilitate this screening many therapeutic viruses additionally contain a (selectable) marker gene. This marker gene allows screening for stable integration and/or high level, long term expression. This approach is often needed in settings where the therapeutic transgene itself does not support high throughput screenings. However, in the last years evidence has been arising that such marker genes can impose significant problems in therapeutic settings. One of the major concerns is that an immune response against the foreign gene(s) (e.g. selection markers) is provoked. This can result in efficient elimination of transduced cells, thereby limiting or excluding the therapeutic success. Even more concern is raised since insertional leukemogenesis was observed in a mouse model due to the transduction of a marker gene that was considered before to be biologically inert [32, 33]. Thus, it is now state of the art to exclude any coding sequences beside the therapeutic gene. Several approaches have addressed this point. One strategy is to express the resistance marker from an independent expression cassette outside the retroviral vector cassette [34]. Alternatively, a loxP flanked marker gene is integrated into the retroviral vector to be excised upon Cre mediated recombination [35, 36]. While the latter approach allows efficient selection of producer clones it is not compatible with therapeutic vectors of large size. Further, both approaches do not overcome the problem of screening for optimal vector integration.

The novel type of retroviral producer cell, exemplified by the here described Flp293A is based on a previously published highly efficient tag and targeting principle [23]. The producer cell line has been established upon an initial screening for appropriate chromosomal sites that support optimal expression of a retroviral marker vector. This step is most critical for virus production since the level of retroviral vector expression and thus the titer significantly depends on the nature of the chromosomal integration site. Apart from a maximal level of RNA to be packaged a balanced expression of gag/pol and env is required. These requirements have been taken into account in the strategy presented here.

Upon site specific targeting, a precise exchange of the marker vector with a therapeutic vector of interest results in producer cells. The approach presented here should be valid for any therapeutic vector of choice. Importantly, it also allows to switch the retroviral LTR and to transduce any vector type, including SIN vectors. Further, site specific integration is the method of choice for comparative evaluation of different retroviral vector designs since it overcomes position effects. With a frequency of more than 98% of correctly targeted cells it might also overcome the need for subcloning in certain applications.

Site specific integration of retroviral vectors into predefined sites is characterized by several advantages. i) It is a high speed method that overcomes the need for screening. ii) It is safe due to the defined integration of the vector within the packaging cell line. In contrast to multiple integrations, it excludes the integration of the recombinant vector next to oncogenes and the risk of cotransduction. iii) Finally, the production conditions for targeted clones are expected to correspond to that of the master cell line. The adaption to culture conditions and media can be performed with the master cell line. Overall, therapeutic virus production from bench to bed-side is safer, faster and cheaper.

On the basis of the cells preselected for optimal vector transcription virus producer cells with other envelopes can be established (e.g., Corroadinha et al., submitted). In this way helper cell lines for all types of target cell receptors can be produced. Further, the strategy as it is presented here is highly flexible and can be adapted to the construction of other types of stable producer cell including lentiviral producer cell lines.

## **MATERIAL AND METHODS**

### **Plasmids**

**pTAGeGFP.** The tagging vector pTAGeGFP is based on the vector pIRESGALEO [23] in which the lacZ reporter gene was NotI/SalI excised and exchanged for eGFP. A wild-type FRT site and a spacer mutant FRT site [23] were integrated in tandem into the 3'LTR, followed by an ATG-deleted neomycin phosphotransferase gene.

**Targeting vectors.** Retroviral vectors have been integrated into two different precursor targeting vectors containing the FRT wild-type and FRT mutant site that flank a multiple cloning site and a CMV promoter (pTARDKL) or an EMCV IRES element (pEMTAR), respectively. In both vectors, an ATG start codon is positioned upstream the FRT mutant site to complement the inactive neoR gene after targeting. The retroviral vector cassette of MFG-eGFP [37] was integrated into the precursor vectors resulting in pMLIReGFP, pMLCMeGFP and pMLCMeGFP<sub>rev</sub>. The MSCV targeting vector pMSIReGFP was derived from pMSIRcolVII by exchanging the collagen VII cDNA for eGFP.

pMSCVhColVII encodes the human collagen VII gene on an EcoRI fragment excised from pTOPO-XL-hCol7 (kindly provided by Prof. A Hovnanian, Toulouse, France) integrated into the single EcoRI site of pMSCVneo [38] in which the PGK-npt cassette was previously eliminated by BglII/ BamHI digestion and religation. The retroviral cassette was integrated into the precursor vectors to obtain pMSCMcolVII, pMSCMcolVII<sub>rev</sub> and pMSIRcolVII.

Further, human the ColVII cDNA was integrated into a SIN retroviral vector pCMS with an internal EF1alpha minimal promoter. The retroviral cassette was integrated into pEMTAR to obtain pSINcolVIII.

All vector constructions were confirmed by virus production upon transient transfection into PG13 packaging cells. Maps are available on request.

### **Mammalian cell culture and transfection**

HEK 293 cells (BioReliance), NIH 3T3 cells, and PG13 packaging cell line were cultivated at 37°C in a humidified atmosphere with 5% CO<sub>2</sub> in DMEM (Gibco-BRL) with 10 % fetal calf serum (Cytogen), 2 mM L-glutamine, penicillin (10 U/ml), streptomycin sulfate (100 µg/ml). Selection was performed in medium supplemented with Hygromycin B (200U/ml), Histidinol (11 mM) and Blasticidin (2.5 µg/ml), respectively.

PG13 packaging cells were seeded in a six-well plate ( $1 \cdot 10^5$  cells/well). 10µg of pTAGeGFP was stably transfected using calcium phosphate coprecipitation as described earlier [15] and selected. The supernatant was used to infect HEK 293 cells with an MOI of 0.1 and subjected to selection with hygromycin B. Clone 1B2 was used for all further studies.

Tagged clones were transfected with 10 µg of a gagpol encoding plasmid pCeb [9] and an amphotropic envelope encoding plasmid (pENVAhis, [15]) and selected for resistance towards Blasticidin and Histidinol. A high titer clone designated Flp293A\* was isolated. Independently, the 1B2 tagged cells were transfected with the gagpol encoding plasmid pSVgp-1 and pENVAhis. Histidinol resistant clones that showed suboptimal titers were additionally transfected with pCeB and selected for blasticidin. From the second approach Flp293A was isolated.

Both Flp293A\* and Flp293A have comparable properties concerning virus production and efficiency of cassette exchange. The absence of RCRs and mycoplasma was proven for both lines (not shown).

### **Targeted cassette exchange**

For site-specific cassette exchange  $1 \cdot 10^5$  Flp293A cells were cotransfected with 10µg Flp recombinase-expressing vector (pFlpe; K. Maaß, unpublished, in which the FlpE recombinase is expressed from an SV40 promoter) and 3 µg of targeting plasmid using calcium phosphate

coprecipitation. The medium was replaced 18 hours post transfection and the cells cultivated for 4 days to allow cassette exchange. On the fifth day the cells were transferred to a 60-mm culture plate and G418 (1500 µg/ml) and Gancyclovir (10µg/ml) containing medium to select for targeted subclones.

### **Flow Cytometry**

FACSCalibur (Becton Dickinson) was used for isolation of high EGFP expressing clones and for the virus titration. The cells were washed, trypsinized and stained with Propidium iodide (50µg/ml) to exclude dead cells from the analysis.

### **Virus titration**

The virus titration of the Flp293A master cell line as well as eGFP encoding targeted subclones was done based on flow cytometry. NIH 3T3 cells were seeded on 12-wells ( $8 \cdot 10^4$  cells/well) one day before infection. The cells were infected with 300 µl of serial dilutions of 24 hours produced virus producer supernatant. The titer was calculated based on the percentage of eGFP positive NIH3T3 cells. Infections resulting in 2-20% of infected cells were considered for titer calculation.

The titration of human collagen VII producer clones was done based on immune staining of infected cells according to a previously described protocol [39]. Briefly, infected HEK 293 cells were plated on glass cover slides and incubated for 16 hours. The cells were washed twice with phosphate buffered saline (PBS), fixed with acetone/methanol (1:1), washed twice with PBS supplemented with 3% bovine serum albumin (BSA) and washed once with PBS/3%BSA/0.1% Saponin (Sigma). The slides were covered with 50 µl of 1:500 primary antibody solution (anti-human collagen type VII, Sigma, C6805), incubated for 60 min at room temperature and washed three times with PBS/0.1% saponin. The secondary goat anti-mouse IgG antibody (FITC labeled, Dianova) was applied (1:200). The air-dried cover slides

were mounted onto glass slides with moviol. Collagen VII positive cells were counted and the titer was calculated.

### **Southern blot analysis**

The analysis of cassette exchange was done as described previously [23]. The genomic DNA was prepared according to Ramirez-Solis et al. [40]. The 518 bp *BamHI/SacI IRES* fragment were used as a hybridization probe.

### **Acknowledgements**

We thank the members of the EU consortium 'InsertAGene' for discussion. We thank A. Hovenanian for generously providing the human collagene VII cDNA and K. Maaß for the pFlpe Plasmid.

This work was supported by the Volkswagen Foundation (1/77112), the European Community (QLG2-CT-2000-00345 and QLG2-CT-2000-00930 and QLK3-CT-2002-01949) and the Deutsche Forschungsgemeinschaft (Wi 2648).

## References

1. Pages, J.C. & Bru, T. (2004). Toolbox for retrovectorologists. *J. Gene Med.* **6** Suppl **1**: S67-S82.
2. Danos, O. & Mulligan, R.C. (1988). Safe and efficient generation of recombinant retroviruses with amphotropic and ecotropic host ranges. *Proc. Natl. Acad. Sci. U. S. A.* **85**: 6460-6464.
3. Miller, A.D. & Buttimore, C. (1986). Redesign of retrovirus packaging cell lines to avoid recombination leading to helper virus production. *Mol. Cell Biol.* **6**: 2895-2902.
4. Miller, A.D. *et al.* (1991). Construction and properties of retrovirus packaging cells based on gibbon ape leukemia virus. *J. Virol.* **65**: 2220-2224.
5. Miller, A.D. & Chen, F. (1996). Retrovirus packaging cells based on 10A1 murine leukemia virus for production of vectors that use multiple receptors for cell entry. *J. Virol.* **70**: 5564-5571.
6. Davis, J.L. *et al.* (1997). Retroviral particles produced from a stable human-derived packaging cell line transduce target cells with very high efficiencies. *Hum. Gene Ther.* **8**: 1459-1467.
7. Markowitz, D., Goff, S., & Bank, A. (1988). Construction and use of a safe and efficient amphotropic packaging cell line. *Virology* **167**: 400-406.
8. Cosset, F.L. *et al.* (1995). Retroviral retargeting by envelopes expressing an N-terminal binding domain. *J. Virol.* **69**: 6314-6322.
9. Cosset, F.L., Takeuchi, Y., Battini, J.L., Weiss, R.A., & Collins, M.K. (1995). High-titer packaging cells producing recombinant retroviruses resistant to human serum. *J. Virol.* **69**: 7430-7436.
10. Lavillette, D., Russell, S.J., & Cosset, F.L. (2001). Retargeting gene delivery using surface-engineered retroviral vector particles. *Curr. Opin. Biotechnol.* **12**: 461-466.
11. Spitzer, D, Dittmar, K.E. J. Rohde, M., Hauser, H. and Wirth, D. (2003). Green fluorescent protein-tagged retroviral envelope protein for analysis of virus-cell interactions. *J. Virol.* **77** (10): 6070-6075.
12. Burns, J.C., Friedmann, T., Driever, W., Burrascano, M., & Yee, J.K. (1993). Vesicular stomatitis virus G glycoprotein pseudotyped retroviral vectors: concentration to very high titer and efficient gene transfer into mammalian and nonmammalian cells. *Proc. Natl. Acad. Sci. U. S. A.* **90**: 8033-8037.
13. Pensiero, M.N., Wysocki, C.A., Nader, K., & Kikuchi, G.E. (1996). Development of amphotropic murine retrovirus vectors resistant to inactivation by human serum. *Hum. Gene Ther.* **7**: 1095-1101.
14. Rigg, R.J. *et al.* (1996). A novel human amphotropic packaging cell line: high titer, complement resistance, and improved safety. *Virology* **218**: 290-295.

15. Spitzer, D., Hauser, H., & Wirth, D. (1999). Complement-protected amphotropic retroviruses from murine packaging cells. *Hum. Gene Ther.* **10**: 1893-1902.
16. Sinn, P.L., Sauter, S.L., & McCray, P.B., Jr. (2005). Gene therapy progress and prospects: development of improved lentiviral and retroviral vectors--design, biosafety, and production. *Gene Ther.* **12**: 1089-1098.
17. Yap, M.W., Kingsman, S.M., & Kingsman, A.J. (2000). Effects of stoichiometry of retroviral components on virus production. *J. Gen. Virol.* **81**: 2195-2202.
18. Merten, O.W. (2004). State-of-the-art of the production of retroviral vectors. *J. Gene Med.* **6** Suppl **1**: S105-S124.
19. Baer, A., Bode, J. (2001). Coping with kinetic and thermodynamic barriers: RMCE, an efficient strategy for the targeted integration of transgenes. *Curr. Opin. Biotechnol.* **12**: 473-480.
20. Schlake, T. & Bode, J. (1994). Use of mutated FLP recognition target (FRT) sites for the exchange of expression cassettes at defined chromosomal loci. *Biochemistry* **33**: 12746-12751.
21. Lee, G. & Saito, I. (1998). Role of nucleotide sequences of loxP spacer region in Cre-mediated recombination. *Gene* **216**: 55-65.
22. Seibler, J., Schubeler, D., Fiering, S., Groudine, M., & Bode, J. (1998). DNA cassette exchange in ES cells mediated by FLP recombinase: an efficient strategy for repeated modification of tagged loci by marker-free constructs. *Biochemistry* **37**: 6229-6234.
23. Verhoeyen, E., Hauser, H., & Wirth, D. (2001). Evaluation of retroviral vector design in defined chromosomal loci by FLP-mediated cassette replacement. *Hum. Gene Ther.* **12**: 933-944.
24. Wirth, D. & Hauser, H. (2004). FLP-mediated integration of expression cassettes into FRT-tagged chromosomal loci in mammalian cells. *Methods Mol. Biol.* **267**: 467-76.
25. Karreman, S., Hauser, H., & Karreman, C. (1996). On the use of double FLP recognition targets (FRTs) in the LTR of retroviruses for the construction of high producer cell lines. *Nucleic Acids Res.* **24**: 1616-1624.
26. Emerman, M. & Temin, H.M. (1986) Quantitative analysis of gene suppression in integrated retrovirus vectors. *Mol. Cell Biol.* **6**: 792-800.
27. Artelt, P., Morelle, C., Ausmeier, M., Fitzek, M., & Hauser, H. (1988). Vectors for efficient expression in mammalian fibroblastoid, myeloid and lymphoid cells via transfection or infection. *Gene* **68**: 213-219.
28. Bauer, J.W. & Laimer, M. (2004). Gene therapy of epidermolysis bullosa. *Expert. Opin. Biol. Ther.* **4**: 1435-1443.
29. Kraunus, J. *et al.* (2004). Self-inactivating retroviral vectors with improved RNA processing. *Gene Ther.* **11**: 1568-1578.

30. Schambach, A. *et al.* (2000). Context dependence of different modules for posttranscriptional enhancement of gene expression from retroviral vectors. *Mol. Ther.* **2**: 435-445.
31. Riviere, I., Brose, K., & Mulligan, R.C. (1995). Effects of retroviral vector design on expression of human adenosine deaminase in murine bone marrow transplant recipients engrafted with genetically modified cells. *Proc. Natl. Acad. Sci. U. S. A* **92**: 6733-6737.
32. Li, Z. *et al.* (2002). Murine leukemia induced by retroviral gene marking. *Science* **296**: 497.
33. Baum, C. *et al.* (2003). Side effects of retroviral gene transfer into hematopoietic stem cells. *Blood* **101**: 2099-2114.
34. Xu, L. *et al.* (2004). A convenient method for positive selection of retroviral producing cells generating vectors devoid of selectable markers. *J. Virol. Methods* **118**: 61-67.
35. Wildner, O. *et al.* (1998). Generation of a conditionally neo(r)-containing retroviral producer cell line: effects of neo(r) on retroviral titer and transgene expression. *Gene Ther.* **5**: 684-691.
36. Loew, R. *et al.* (2004). Simplified generation of high-titer retrovirus producer cells for clinically relevant retroviral vectors by reversible inclusion of a lox-P-flanked marker gene. *Mol. Ther.* **9**: 738-746.
37. Verhasselt, B., De Smedt, M., Verhelst, R., Naessens, E., & Plum, J. (1998). Retrovirally transduced CD34<sup>++</sup> human cord blood cells generate T cells expressing high levels of the retroviral encoded green fluorescent protein marker in vitro. *Blood* **91**: 431-440.
38. Hawley, R.G., Lieu, F.H., Fong, A.Z., & Hawley, T.S. (1994). Versatile retroviral vectors for potential use in gene therapy. *Gene Ther.* **1**: 136-138.
39. Niedick, I. *et al.* (2004). Nucleolar localization and mobility analysis of the NF-kappaB repressing factor NRF. *J. Cell Sci.* **117**: 3447-3458.
40. Ramirez-Solis, R. *et al.* (1992). Genomic DNA microextraction: a method to screen numerous samples. *Anal. Biochem.* **201**: 331-335.

## Figure legends

### Figure 1. Strategy for the establishment of the master cell line and principle of targeted cassette exchange

A) Flow chart describing the process for generating high level, single copy tagged retroviral producer cells: HEK 293 cells were infected using the eGFP encoding tagging vector pTAGeGFP under conditions that ensure single copy integration (MOI < 0.01). High eGFP expressing cells were enriched twice by sorting and single cells were isolated in a final third sorting step. A selection of single copy clones was transfected with retroviral helper functions gagpol and amphotropic env and screened for optimal virus production.

B) Strategy of the targeted cassette exchange: The retroviral tagging vector (pTAGeGFP) (I) contains a tag-and-target cassette consisting of two FRT sites in tandem, a wild-type (open triangle) and a non-interacting FRT site (filled triangle), followed by an ATG deficient neomycin phosphotransferase ( $\Delta$ neo) gene [23]. Infection with this vector results in duplication of the tag-and target cassette within the 3' U3 region to the 5' LTR (II). Tagged parental clones are G418 sensitive. Cotransfection of the Flp recombinase-expressing plasmid (pFlp) and the targeting vector carrying corresponding FRT sites (III) results in sequence-specific recombination as indicated by crosses. In targeted state (IV) the resulting subclones are G418 resistant due to the complementation of the  $\Delta$ neo gene in the 3' LTR with an IRES element or a CMV promoter (I/C) and an ATG positioned in frame. C) Long term eGFP expression of the retroviral tagged locus FLP293A. The clone was cultivated without hygromycin selection pressure for more than 50 passages and eGFP expression was measured by flow cytometry. D) Virus production of the master cell line FLP293A. The productivity was followed for 12 months without the use of any selection pressure.

### **Figure 2. Vector setups for targeted cassette exchange**

Schematic representation of the retroviral targeting vectors. Both, MSCV and MLV retroviral vector cassettes are flanked by a wild type and a mutant FRT site and carry an eGFP reporter gene or human collagenVII. An EMCV IRES element or a CMV promoter together with an ATG start codon provides G418 resistance upon targeting. An EF-1 $\alpha$  internal promoter drives the collagenVII transcription of the SIN-vector pSINcolVII.

### **Fig. 3 Analysis of pMSIREGFP targeted subclones.**

A) Schematic representation of the proviral integrate and pMSIREGFP after site-specific integration. The SacI digested genomic DNA of the master cell line FLP293A and pMSIREGFP targeted subclones (1-8) was subjected to Southern blot analysis and hybridized to the IRES sequence. A 3.83 kb DNA fragment is expected for the parental clone and a 1.35 kb fragment for the targeted subclones. Lane M,  $\lambda$  HindIII/EcoRI, <sup>35</sup>S-labeled marker; Lane 1, Master cell line Flp293A. B) Virus production of the master cell line Flp293A and G418-resistant subclones obtained after pMSIREGFP targeting. No additional bands were observed excluding unspecific integrations of the targeting vectors.

### **Fig. 4 Human collagenVII expression in virus producer cells and upon infection**

Flp293A cells were targeted with pMSIRcolVII and selected for G418 resistance. Single cell clones (A) and NIH3T3 cells infected with the supernatant (B) were analysed by indirect immunostaining using a monoclonal antibody directed against human collagenVII and a FITC labelled secondary antibody. (C) shows a magnification of infected cells.



Table 1

Vector	Targeting efficiency [clones obtained]	Analyzed/correctly targeted
pMSIReGFP	80 - 100	12/12
pMLIReGFP	20 - 30	10/10
pMLCMeGFP	40 - 50	5/6*
pMLCMeGFP <sub>rev</sub>	0	-/-
pMSIRcoVII	40 - 50	15/15
pMSCMcoVII	15 - 20	11/11
pMSCMcoVII <sub>rev</sub>	0	-/-
pSINcoVII	40 - 50	18/18

\* One of the analyzed clones showed the parental band in addition to the signal for targeted exchange, indicating that the excision of the tagging vector had not occurred.

Table 2

Clones	Average Titer [ip/1*10 <sup>6</sup> cells * 24h]
1B2-8-F	4.1*10 <sup>6</sup> ± 1.1*10 <sup>6</sup>
-pMSIReGFP*	8.1*10 <sup>6</sup> ± 1.5*10 <sup>6</sup>
-pMLIReGFP*	2.5*10 <sup>7</sup> ± 1.3*10 <sup>7</sup>
-pMLCMeGFP*	< 1.0*10 <sup>3</sup>

\* The average titer was calculated based on the eGFP expression upon infection using least 5 targeted subclones

Table 3

Clones	Average Titer* [ip/1*10 <sup>6</sup> cells * 24h]
-pMSIRcoVII*	1.2*10 <sup>5</sup> ± 2.1*10 <sup>4</sup>
-pSINcoVII*	1.2*10 <sup>6</sup> ± 2.0*10 <sup>5</sup>
-pMSCMcoVII*	< 1.0*10 <sup>3</sup>

\* The average titer was calculated based on the CoVII expression upon infection using least 5 targeted subclones

**A new generation of retroviral producer cells:  
Predictable and stable virus production by Flp mediated site-specific integration of retroviral  
vectors**

R. Schucht<sup>1</sup>, A. S. Coroadinha<sup>2</sup>, M.A. Zanta-Boussif<sup>3</sup>, E. Verhoeyen<sup>4</sup>, M. Carrondo<sup>2</sup>, H. Hauser<sup>1</sup>,  
and D. Wirth<sup>1\*</sup>

<sup>1</sup> Dept. Gene Regulation and Differentiation, GBF - German Research Centre for  
Biotechnology, Braunschweig, Germany

<sup>2</sup> Instituto de Biologia Experimental e Tecnológica/ Instituto de Tecnologia Química e Biológica  
(IBET/ITQB), Apartado 12, P-2780-901 Oeiras, Portugal

<sup>3</sup> Genethon, 1 bis, Rue de l'Internationale-BP60, F-91002 Evry Cedex, France

<sup>4</sup>Laboratoire de Vectorologie Retrovirale et Therapie Genique, Inserm, 46 allée d'Italie, 69364  
Lyon Cedex 07, France

\* Corresponding author

Dagmar Wirth, PhD

GBF- National Research Centre for biotechnology

Mascheroder Weg 1

D-38124 Braunschweig

Tel. ++49-531-6181-252

FAX: ++49-531-6181-262

Email: dagmar.wirth@gbf.de

**Abstract**

A new strategy was developed that provides well-defined high-titer producer cells for recombinant retroviruses in a minimum of time. The strategy involves the targeted integration of the retroviral vector into a chromosomal locus with favourable properties. For proof of concept we established a novel HEK293 based retroviral producer cell line, called Flp293A, with a single copy retroviral vector integrated in a selected chromosomal locus. The vector was flanked by non-interacting Flp-recombinase recognition sites (FRT) and was exchanged for different retroviral vectors via Flp mediated cassette exchange. All analyzed cell clones showed correct integration and identical titers for each of the vectors, confirming that the expression characteristics from the parental cell were preserved. Titers up to  $2.5 \times 10^7$  ip/ $10^6$  cells were obtained. Also, high titer producer cells for a therapeutic vector that encodes the 8.9 kb collagen VII cDNA in a marker free cassette was received within three weeks without screening.

Thus, we provide evidence that the precise integration of viral vectors into a favourable chromosomal locus leads to high and predictable virus production. It is compatible with other retroviral vectors including SIN vectors and marker free vectors. Further, it provides a tool for evaluation of different retroviral vector designs.

**Keywords**

Retroviral vector production, cassette exchange, Flp mediated site-specific recombination



Figure 2

[Click here to download high resolution image](#)

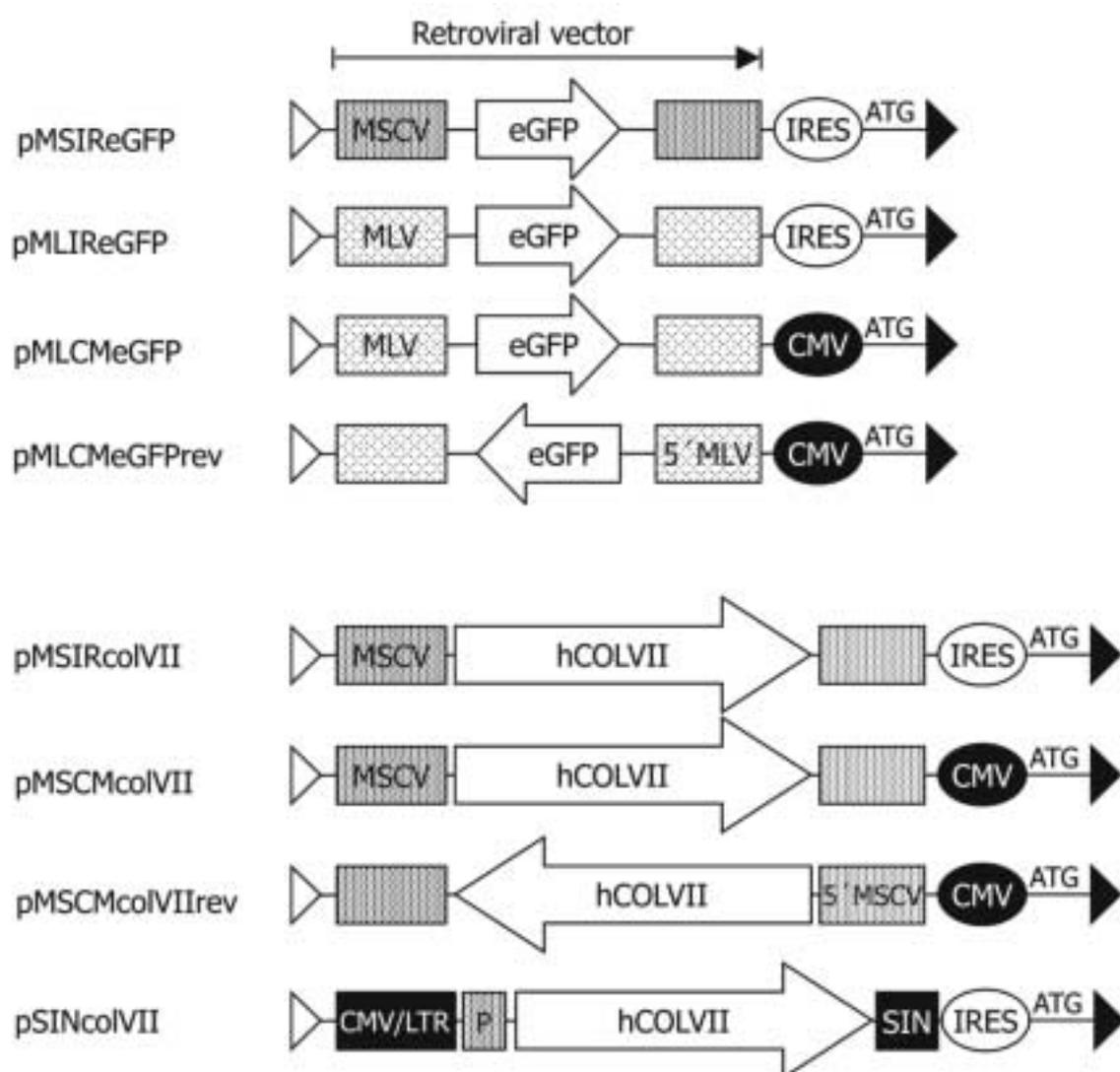
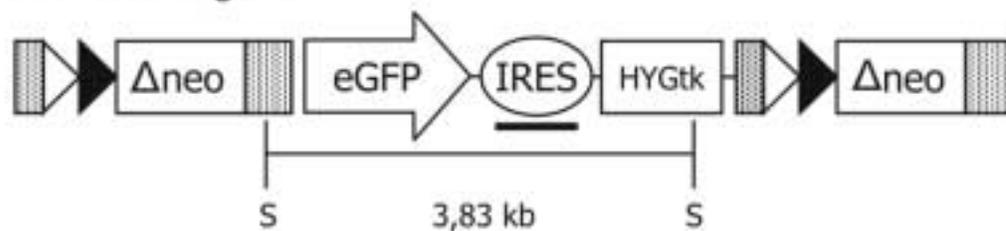


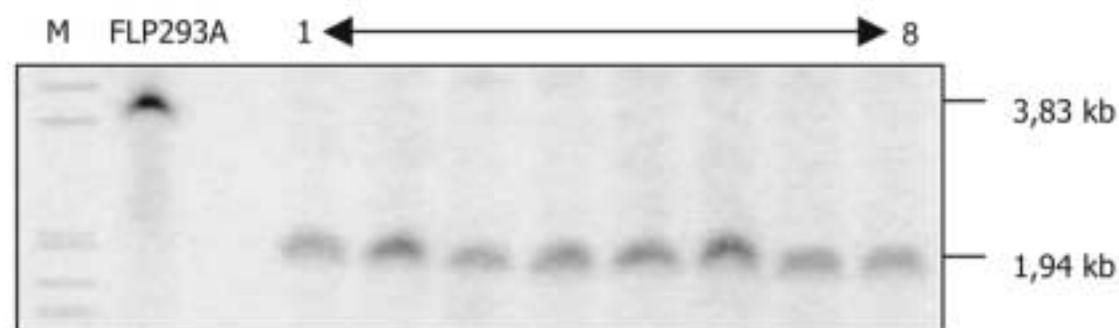
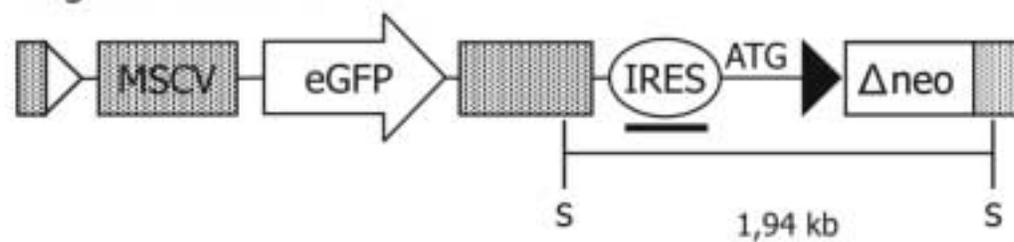
Figure 3  
[Click here to download high resolution image](#)

A

Proviral integrate



Targeted subclone



B

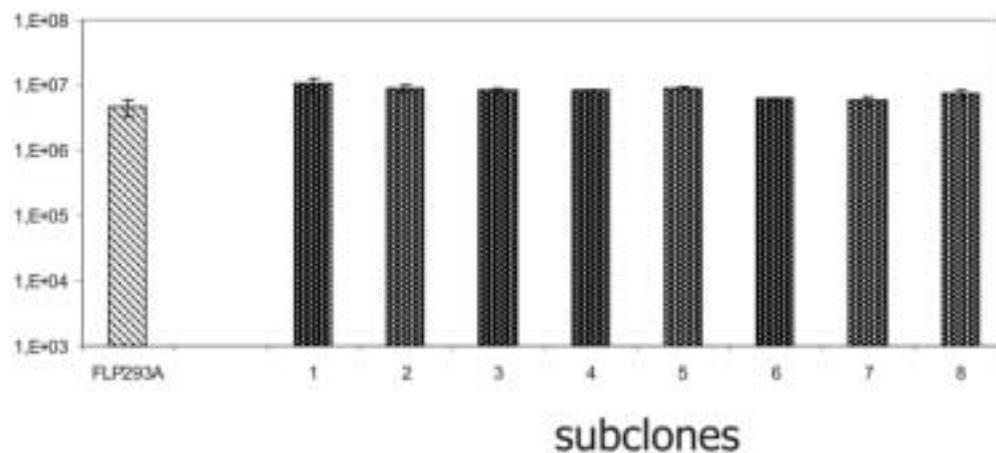


Figure 4  
[Click here to download high resolution image](#)

