Rapid establishment of GPCR expressing cell lines by Site specific integration

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Abstract

The establishment of mammalian cell lines reliably expressing G-protein-coupled receptors (GPCR) can be a tedious and often time-consuming process. A strategy has been developed to allow the rapid production of such cell lines. The first step of this approach was the generation of a specialized master cell line, characterized by optimized stable expression of a membrane bound reporter protein. In the second step this reporter gene was exchanged for that of the GPCR of interest by a DNA recombinase “cut and paste” engineering step. It has been demonstrated that the resulting GPCR cell lines inherit the advantages of the master cell line, expressing the GPCR in a homogenous and stable manner. The case studies presented demonstrate the functionality of the established GPCR cell lines and most importantly, due to the highly efficient integration event, these recombinant GPCR-expressing cell lines were generated within a timeframe of two to four weeks. The advantages of this “cut and paste” approach versus other strategies like Flp-In or Jump-In are compared.

Introduction

The conventional drug development process is expensive and time-consuming, and despite large investments in research and development activities the success rate of bringing a new drug to the market is low. The most prominent target class is that of GPCRs which cover about 60–70% of recently developed drugs.[1]

Upon modulation by extra-cellular ligands these cell surface receptors mediate the transduction of extra-cellular stimuli into intra-cellular signals. The hydrophobic core composed of seven-transmembrane α-helices (7TM) is common to all GPCRs.[2] This large class comprises approximately 800 different receptors which are grouped in three families (Classes A, B and C).

In general the generation of cell lines stably expressing a transgene requires transduction of the host cell line and subsequent integration of the expression cassette into the host cell genome. This leads to a random distribution of integration sites.[3] It is a fact that most genomic loci do not support high or consistent transgene expression. Both the level and stability of transgene expression is strongly dependent on the genomic surroundings of the integration sites. This “position effect” renders the generation of stable cell lines a cumbersome and often lengthy task involving extensive screening to identify those cell clones that display the desired properties.[4]

The recombinase mediated cassette exchange (RMCE) strategy was introduced to overcome this limitation of cell line engineering for review see.[5] RMCE can be best described as a two step molecular “cut and paste” mechanism.[6] In the first step a reporter gene cassette is randomly integrated into a host cell line of interest. The expression of the reporter gene serves as a marker that permits screening for the desired expression characteristics (e.g. high and stable expression). Once isolated and confirmed, those “tagged” clones function as master cell lines. A prerequisite for the use of such a master cell line is a single copy integration of the tagging cassette. With the help of DNA recombinases such as the Cre,[7-9] the Flp recombinase[10-12] or the phiC31 integrase[13,14] the tagged genomic loci of the master cell lines can be recycled by the integration of any gene of interest. Since the recombinase driven cassette exchange itself is a highly site-specific event, all positive expression characteristics of the master cell line are transferred to the resulting subclonal producers.

The aim of the study presented here is to employ the RMCE strategy for the establishment of GPCR expressing cell lines. The main application area of these cell lines is the drug discovery process. Therefore the cell lines should meet the following requirements: (i) consistent expression, (ii) stable expression without a need of persistent use of selection agents and (iii) sufficient expression of the receptors to monitor pharmacological responses. The most significant feature of the approach described here is that the GPCR expressing cell

lines should be rapidly established (within two to four weeks) while still showing the abovementioned properties.

The RMCE strategy has been proven useful for the flexible generation of various production cell lines expressing e.g. antibodies or retroviral vectors. Although the principle itself can be adapted to virtually any desired gene it has become clear, that there can be no universal master cell line. This is because the expression characteristics of a cell line are mainly influenced by the position effect, the design of the expression cassette and last but not least by the transgene itself.[15] In approaches described previously, cytosolic reporters (GFP, lacZ) were utilized for the establishment of the master cell lines.[11,12]

The present study follows the hypothesis that a master cell line established through expression of a protein that recapitulates the complex maturation process of a GPCR is better suited to stably and consistently express the GPCR of choice. Therefore novel CHO master cell lines have been generated dedicated for GPCR expression. The key features of these master cell lines are (i) the selection for stable expression of membrane bound proteins and (ii) a highly efficient targeting process that ensures site-specific cassette exchange in a significant number of master cells. The RMCE process utilized was extremely precise with 100% positive recombination events in the selected clones.[11,12] However, previously described master cell lines gave rise to few recombined clones making RMCE a rare event. Improvement in the frequency of the RMCE process would lead to more clones. In addition, due to their isogenic nature the clones can be pooled enabling a further shortening of the time needed for establishment of a stable line from 4 to 2 weeks.

Materials and Methods

Plasmids

The vector for tagging chromosomal sites harbors a truncated version of the nerve growth factor receptor (NGFR). This gene mutant acts as a reporter gene and therefore lacks the internal domain of the NGFR to avoid any signaling from this receptor. In addition, the tagging vector harbors the selection marker hygromycin phosphotransferase, thymidine kinase whose expression is mediated by a poliovirus internal ribosomal entry site (IRES). Both genes are driven by an SV40 promoter. The cassette is flanked by a wild-type FRT site and F5 spacer mutant FRT site,[16] followed by an ATG-deficient neomycin phosphotransferase gene (neoR).

All RMCE cassettes coding for the GPCRs of interest are also driven by a SV40 promoter and are flanked by FRT wild-type and the F5 FRT mutant sites. The vectors carry an IRES element and an ATG start codon upstream of the FRT F5 mutant site to complement the inactive neoR gene upon successful RMCE process. A successful RMCE process was verified by a PCR employing primers CGTCAAGAAGGCGATAGAAGGC (NeoRev) and ccatgggacgctagttgtgaa (Polio1) which bind in the ATG-deficient neomycin (from the master cell line) and in the IRES from the targeting plasmid respectively. For monitoring the modulation of the GPCRs the reporter construct pGL4.29 (Promega Corp.) was used. pGL4.29 harbors a hybrid promoter composed of a minimal promoter with CRE binding elements and drives the expression of the reporter gene luciferase.

Mammalian Cell Culture and Transfection

CHO-K1 cells (ECACC, Salisbury, UK) were cultivated at 37°C in a humidified atmosphere with 5% CO_2 in HamF12 medium (Gibco) containing 10 % fetal calf serum and 8 mM L-glutamine. For transduction of the CHO-K1 cells the respective vectors were electroporated using a standard protocol (amaxa AG; Cologne; Germany; Nucleofector™ Kit V). For the tagging procedure $1 \cdot 10^6$ cells were transfected with the NGFR expression plasmid. The transduced cells were selected with medium supplemented with hygromycin B (150U/ml). The RMCE process in the CHO-K1 master cell line was facilitated by a co-transfer of Flp recombinase-expressing vector and the targeting plasmid.[17] For the reporter assays the pGL4.29 was electroporated into the respective GPCR expressing cell lines and as a control also into the master cell line. Cryo-preserved “Assay-Ready” reagent for high throughput analysis prepared in multilayer bioreactors (Corning Cellstack™). Cryo-preservation was carried out using rate controlled freezer (Planer™).

Cellular analysis

FACSCalibur and FACSVantage SE (Becton Dickinson) were used for flow cytometry analysis and cell isolation. The analysis and sorting of the NGFR expressing cells was done as previously described.[18] Briefly, the transduced cells were incubated for 30 min with a primary mouse monoclonal antibody against NGFR (Becton Dickinson) at room temperature. Afterwards the cells were washed twice with PBS supplemented with 2% fetal bovine serum (PBS*). As a secondary antibody an anti-mouse PE-conjugated antibody (Jackson ImmunoResearch Laboratories) was used (30 min at room temperature). Afterwards the cells were washed with PBS*, stained with propidium iodide (50 µg/ml) to exclude dead cells from the analysis and then analysed by flow cytometry. For the analysis of the ADRB2 expressing cells a fluorescently labeled ligand specific for ADRB2 (CellAura, Nottingham, UK) was utilized. The cells were incubated with the ligand (0.4 nM) at room temperature for 5 min. Afterwards the cells were washed with PBS* and analyzed by flow cytometry or by immunofluorescence. All chemicals used in this study were purchased from Sigma-Aldrich unless otherwise stated. For determination of the reporter plasmid activity a luciferase assay was performed as detailed in.[19] Briefly, cell lysates were created by repeated freeze-thaw cycles. An aliquot of the protein lysate was mixed with of reaction buffer (25 mM glycylglycine, 15 mM $MgSO_4$, 1 mM ATP pH 7.8) and luciferin (Promega Corp.). The resulting luciferase activity was measured in a luminometer (Lumat LB9507 (Berthold)) and corrected for protein content (RLU/µg protein).

High-Throughput analysis

The analysis of the pharmacological characteristics of the muscarinic M_3 receptor cell line was performed using the FLIPR$^{TETRA®}$ system (Molecular Devices (UK) Ltd). Cells in continual culture were plated into Corning CellBIND black/clear base 384-well plates at 10,000 cells/well in 50 µL Hams F-12, left on the bench for 60 min at room temperature then grown overnight at 37°C, 95% humidity and 5% CO_2. Dye loading buffer was prepared by dissolving the contents of one vial of FLIPR® Calcium 5 Reagent completely with a final volume of 20 mL Hanks Balanced Salt Solution, 20 mM HEPES, 2.5 mM probenecid adjusted to pH 7.4. Cell plates were removed from the incubator, growth media removed and 50 µL dye loading buffer was added to each well. Dye loaded plates were incubated for 45 min at 37°C, 5% CO_2 and allowed to cool to room temperature for 15 min prior to reading on the FLIPR$^{TETRA®}$ system (E_x 470-495 nm and E_m 515-575 nm). Plates were not washed after dye loading. Compounds were added as 12.5 µL of a 5X ligand stock. For IC_{50} determinations, an EC_{80} concentration of acetylcholine was used as the standard agonist. Data analysis was performed using GraphPad Prism software. The data were normalised as % change over baseline.

Results

Development of GPCR optimized master cell lines

Following the hypothesis that the reporter protein for the tagging step should reflect the properties of the later protein of interest, a novel tagging cassette was constructed coding for the membrane bound nerve growth factor receptor (NGFR).

The tagging cassette was electroporated into CHO cells and after a selection period of 14 days the cells were analyzed and the highest yielding 10% of NGFR-expressing cells was cloned by flow cytometry (**Fig. 1**). In total 219 clones were cultivated for 4 weeks without selection pressure and then reanalyzed for NGFR-expression. Of these clones approximately 10% (25 clones) displayed a stable and homogenous NGFR-expression (see **Fig. 1** for representative clone). Subsequently, these clones were analyzed for "targetability" (ability to perform RMCE) and long term stability of NGFR expression (> 4 months without selection pressure). This resulted in the generation of 2 cell lines showing stable NGFR expression for 50 passages (more than 20 weeks). In a standard RMCE procedure these master cell lines typically gave rise to 200-300 clones, which were approximately a 10 fold higher efficiency than in previously published reports.[11,12] Thus, these cell lines meet the above described requirements and can serve as novel master cell lines (SCREENflex-GR1 and SCREENflex-GR2). For the subsequent experiments SCREENflex-GR1 was used.

Establishment of various GPCR expressing cell lines

For the generation of the GPCR expressing cell lines, targeting vectors were cloned that allow site-specific integration into the NGFR tagged locus of SCREENflex-GR1 (**Fig. 2A**).

The cDNAs of 13 different GPCR were cloned into this targeting vector (**Tab. 1**) and used for RMCE experiments. After electroporation of both a flipase encoding vector and a targeting vector selection with G418 was applied to screen for those cell clones that complemented the defective neomycin. For all GPCR targeting experiments performed neomycin resistant colonies were obtained within 7-10 days of selection. Clones of G418 resistant cells were randomly picked and expanded. The average time required to expand the GPCR expressing cells (expansion to 5 Mio. cells) was within 4 weeks. G418 resistant colonies were pooled in order to obtain a mixture of GPCR expressing cells. Both pools and clones were analyzed by PCR for correct cassette exchange. The expected band (1 kb) was detected for both clones and pool in all established GPCR cell lines (representative data shown for ADRB2 targetings in **Fig. 2A**).

Expression of the ß2-adrenergic receptor

The expression of the ß2-adrenergic receptor (ADRB2) shows, as an example, the performance of the above described procedure. ADBR2 is a prototypic and well characterized member of the GPCR family which falls into the class of adrenoreceptors with

adrenaline being the natural ligand.[20] In parallel to the RMCE approach recombinant ADBR2 expressing cell lines were generated by co-transfection of the ADBR2 expression plasmid and a selection plasmid into CHO-K1 wild type cells. After selection 18 clones were randomly picked and expanded for further analysis.

To compare the expression of the ADRB2 receptor of targeted pool, targeted subclones and randomly integrated clones a fluorescent ligand was used that specifically recognizes ADBR2 receptor. The docking of the ligand was followed by fluorescence microscopy (**Fig. 3A**) as well as flow cytometry (**Fig. 3B**). The analysis demonstrated that the cell clones and the pool derived by RMCE were composed of 100% ADBR2 expressing cells, showing a homogenous expression pattern of the GPCR. This is in line with the genetic analysis showing isogenicity of the targeted subclones. Therefore the generated pools can be considered as "pure clones". This result is important as a pooling step could lead to heterogeneously expressing cell lines, which could complicate the analysis of further tests.

The cells generated by random integration were also analyzed with the fluorescent ligand using flow cytometry. This analysis showed a different picture if compared to the RMCE approach. The 18 individual clones could be divided into three different categories concerning their expression characteristics (i) 3 clones that express ADBR2 homogenously (ii) 4 clones that showed a mixed expression of ADBR2 and (iii) 11 clones that express no or only low amounts of ADBR2 (**Fig. 3C**). This suggests that for the generation of recombinant cell lines by random integration, a time consuming screen for those cells that express the transgene in the desired fashion is required.

Functional characterization of targeted GPCR cell lines

Cell lines generated by RMCE were further analyzed to determine if they follow typical pharmacological responses after challenge with corresponding ligands. For this analysis the ADRB2 cell line (see above) and also a SCREENflex-GR1 based cell line expressing the human glucagon receptor (GCGR) were included (**Tab. 1**). The main downstream activity of both GPCRs is primarily coupled to the Gs pathway leading to the stimulation of adenylyl cyclase activity.[21] Therefore the established cell lines were investigated to determine if there was an increase in the production of cAMP upon stimulation. For this purpose a reporter construct was used in which luciferase expression is driven by a promoter that harbors cAMP responsive element-binding protein (CREB) binding sites (**Fig. 4**). CREB is a downstream effector of cAMP and therefore any change of cAMP level is indirectly measured. This expression construct was stably integrated in the established ADRB2 and glucagon receptor expressing cell lines (clones and pool). These cells were then treated respectively with either isoprenaline, a known inducer of ADRB2, and with glucagon, respectively. Both receptor cell lines exhibited a strong increase in luciferase expression upon induction with their ligands (**Fig. 4**).

HTS compatibility of the cell lines established by RMCE

The identification of novel modulators of GPCRs is a major task of current drug discovery programs, for this purpose large compounds libraries are typically screened in a high throughput fashion. To achieve this in an acceptable time frame the corresponding assays are performed in 384 or 1536 well format. Therefore one of the prerequisites of a GPCR expressing cell line is the demonstration of the correct and consistent pharmacological response profile. In a pilot study a human muscarinic receptor 3 (CHRM3) cell line was generated with the RMCE approach (**Tab. 1**) and assayed in a 384 well format using a FILPR^{tetra} ™ system. In a series of experiments four known muscarinic agonists (acetylcholine, oxotremorine M, carbachol and bethanechol) and three known antagonists (ipratroprium, p-F-HHSiD M, pirenzipine) were used to characterize the cell line (**Fig. 5A, B**). The assay is based on an increase of cytosolic Ca^{2+} ions upon induction with specific ligands (due to Gq protein coupling of the respective receptor). The increase of fluorescence signal due to the binding of calcium to a calcium-sensitive dye (FLIPR® Calcium 5 Assay) served as a read-out. The different compounds produced the expected pharmacological response profiles (**Fig. 5A, B**) and the rank order of potency is the same for the agonists and antagonist as compared to those described in the IUPHAR database (**Tab. 2, 3**). The values measured for the agonists differ slightly from those described in IUPHAR. This is possibly due to the fact that the IUPHAR values describe affinity binding estimates whereas the pEC_{50} data presented here is based on functional response. What is significant, however, is that the hierarchy of potency is the same as described in IUPHAR. The antagonist data on the other hand are equivalent to those in the IUPHAR database (**Tab. 2, 3**).

Discussion

In the present study the generation of novel master cell lines was verified, specifically designed for the expression of membrane bound proteins. For this purpose, the neuronal growth factor receptor (NGFR) was used to screen for chromosomal integration sites which support a stable and appropriate expression of membrane spanning proteins. The implementation of the recombinase based cassette exchange principle allowed the reuse of such master cell lines (tagged loci). Because no laborious testing for proper expression features was necessary, all cell lines were able to be generated in parallel. This was validated by rapidly establishing 13 different GPCR expressing cell lines with the help of the novel master cell line SCREENflex-GR1 (**Tab. 1**). All cell lines were generated in a time frame of two to four weeks; this was possible because of the high number of RMCE events. The exchange of the NGFR reporter gene in the master cell line to the respective GPCR is 100% efficient which is in accordance with previous studies.[11,12] In addition, the approach of the development of novel master cell lines ensured that the RMCE event occurs frequently. It was demonstrated that the number of correct targeting events is up to 10 fold higher than in previously established master cell lines. This allowed us to further reduce the time for RMCE based cell line development down to two weeks, as described by.[11]

For site specific integration also other strategies have been employed. Amongst them are e.g. the Flp-In system, the Jump-In system (both from Invitrogen) and meganuclease based approaches (cGPS system from Cellectis) for review see.[5] These technologies enable the site-specific integration of the desired gene expression cassette into the genome of a previously tagged host cell line (the respective master cell line). Recently, the Jump-In as well as the meganuclease approach has been successfully used for the establishment of HTS-compatible cell lines.[14, 22]

All these systems differ significantly from the RMCE approach presented here. In contrast to the cut-and-paste mechanism performed by the RMCE, the other systems (Flp-In, the Jump-In, Meganuclease) are best described as “paste” steps into chromosomal sites that were previously tagged by a first tagging vector. This pasting leads to the integration of two expression cassettes into the same genomic locus – the one used for the establishment of the master cell line (tagging cassette) and the one carrying the gene of interest. Such configurations of the expression units are prone to interferences for review see.[23] For example promoter interferences can arise when two transcription starts are in close proximity to each other. These promoter interferences can lead to an unpredicted expression to the point that the expression of the gene of interest is completely abolished.[11] Further, this leads to the integration of vector backbone sequences that are CpG rich and thus have been shown to silence gene expression.

Indeed, it was shown that upon Flp-In mediated transgene integration the expression of the respective transgene was negatively influenced.[24] This report demonstrated that cell clones established with the Flp-In System display a high degree of variance despite their isogenicity. The authors found in isogenic clones different expression pattern ranging from homogenous to mosaic or even silenced expression of the transgene.

The RMCE approach circumvents these problems as it is a molecular cut-and-paste mechanism. For the establishment of the master cell line an expression cassette is employed which facilitates the tagging of a suitable genomic locus. In this study a construct was used that expresses NGFR. This tagging cassette is completely removed when the desired expression construct is pasted in. Thereby RMCE excludes phenomena as the mentioned promoter interference. Apart from this, the benefit of the approach described here is that the master cell line was specifically developed for the expression of trans-membrane proteins. To enable robust screening campaigns or to facilitate purification of GPCRs e.g. for crystallization studies, recombinant expression is advantageous. Recombinant expression of GPCRs has been accomplished in all major expression systems ranging from *Escherichia coli*, yeast, and insect to mammalian cells.[25] In bacteria like *Escherichia coli* over-expression of GPCRs often results in the accumulation of the receptor in inclusion bodies, and is hampered by the lack of posttranslational modification.[26] Yeast provides a eukaryotic environment and ease of handling while still leading to high level expression of the recombinant protein. However, mammalian cells are most likely to preserve structural and functional integrity when expressing mammalian proteins and are therefore the expression system of choice for drug development campaigns. Importantly, all recombinant cell lines presented here are stable in their expression characteristics and support drug screening efforts.

One major bottleneck in research is that GPCRs are difficult to express in mammalian cells. Strategies to solve this obstacle include e.g. optimization of codon usage[27-29] or inducible expression of the respective receptors.[28,30] Here it is shown that this limitation can be overcome by RMCE. With this approach genomic loci are identified (and reused) that favor the expression of the recombinant gene.

The over-expression of trans-membrane spanning proteins such as GPCRs depends on a complex maturation process within the host cell, and this is known as a rate limiting step to obtaining appropriate cell lines.[31,32] The correct folding and post-translational modification leads to stress which counter-selects for non-expressing cells. On the other hand, a few cells amongst a bulk of transduced cells are capable of performing high and stable expression. In order to specifically screen for those cells, the hypothesis was followed that expression is depending on the integration site, or in other words, that certain chromosomal regions

support the expression of certain classes of proteins. In order to establish GPCR expressing cell lines it was decided to use a receptor (NGFR) to screen for correct tagging sites. The results presented here demonstrate the validity of the hypothesis as the established GPCR expressing cell lines did indeed show a robust and reliable expression pattern.

Beside GCPRs, other drug targets have become of high interest in the field of drug discovery, most importantly, ion channels and protein kinases. The current work and previous published approaches have shown that the RMCE strategy is flexible and can be adapted to various cells (of diverse origins) but also to various protein classes. Especially with respect to drug discovery it is worth to include the promising drug target classes of ion channels and kinases into RMCE based cell line establishment efforts.

Furthermore it has been shown that oligomeric proteins can be expressed from one targeted locus upon single-step integration.[11,33] Such an approach would suggest that also two different protein classes can be introduced at the same time, for example the drug target and a specific reporter for monitoring the compound induced target modulation.

In the field of GPCR research other options are also imaginable. For example; in order to study heterodimerization, two different GPCRs can be integrated simultaneously into the same genomic locus which should facilitate the expression of similar levels of both GPCRs. Alternatively, the desired GPCR could be potentially coupled to different arrestins or to different G-proteins to investigate the differential effects of these downstream effectors. In addition to the expression of heterologous proteins the authors have used the RMCE strategy to integrate siRNAs to knock down the expression of cyclinD and reporter genes (unpublished data). The use of siRNAs can be used in this context to specifically knock down the expression of downstream factors of in/activated drug targets and to validate novel drug targets. Such approaches would maximize the potential of RMCE to become a flexible tool for the establishment of cell lines for drug discovery.

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References

1. Hassaine G, Wagner R, Kempf J, Cherouati N, Hassaine N, Prual C, Andre N, Reinhart C, Pattus F, Lundstrom K: Semliki Forest virus vectors for overexpression of 101 G protein-coupled receptors in mammalian host cells. *Protein Expr Purif* 2006; 45:343-351.
2. Pierce KL, Premont RT, Lefkowitz RJ: Seven-transmembrane receptors. *Nat Rev Mol Cell Biol* 2002; 3:639-650.
3. Hansen J, Floss T, Van Sloun P, Fuchtbauer EM, Vauti F, Arnold HH, Schnutgen F, Wurst W, von Melchner H, Ruiz P: A large-scale, gene-driven mutagenesis approach for the functional analysis of the mouse genome. *Proc Natl Acad Sci U S A* 2003; 100:9918-9922.
4. Wurm FM: Production of recombinant protein therapeutics in cultivated mammalian cells. *Nat Biotechnol* 2004; 22:1393-1398.
5. Wirth D, Gama-Norton L, Riemer P, Sandhu U, Schucht R, Hauser H: Road to precision: recombinase-based targeting technologies for genome engineering. *Curr Opin Biotechnol* 2007; 18:411-419.
6. Schucht R, Wirth D, May T: Precise regulation of transgene expression level and control of cell physiology. *Cell Biol Toxicol*; 26:29-42.
7. Araki K, Araki M, Yamamura K: Site-directed integration of the cre gene mediated by Cre recombinase using a combination of mutant lox sites. *Nucleic Acids Res* 2002; 30:e103.
8. Toledo F, Liu CW, Lee CJ, Wahl GM: RMCE-ASAP: a gene targeting method for ES and somatic cells to accelerate phenotype analyses. *Nucleic Acids Res* 2006; 34:e92.
9. Wong ET, Kolman JL, Li YC, Mesner LD, Hillen W, Berens C, Wahl GM: Reproducible doxycycline-inducible transgene expression at specific loci generated by Cre-recombinase mediated cassette exchange. *Nucleic Acids Res* 2005; 33:e147.
10. Coroadinha AS, Schucht R, Gama-Norton L, Wirth D, Hauser H, Carrondo MJ: The use of recombinase mediated cassette exchange in retroviral vector producer cell lines: predictability and efficiency by transgene exchange. *J Biotechnol* 2006; 124:457-468.
11. Schucht R, Coroadinha AS, Zanta-Boussif MA, Verhoeyen E, Carrondo MJ, 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. *Mol Ther* 2006; 14:285-292.
12. Verhoeyen E, Hauser H, Wirth D: Evaluation of retroviral vector design in defined chromosomal loci by Flp-mediated cassette replacement. *Hum Gene Ther* 2001; 12:933-944.
13. Belteki G, Gertsenstein M, Ow DW, Nagy A: Site-specific cassette exchange and germline transmission with mouse ES cells expressing phiC31 integrase. *Nat Biotechnol* 2003; 21:321-324.
14. Lieu PT, Machleidt T, Thyagarajan B, Fontes A, Frey E, Fuerstenau-Sharp M, Thompson DV, Swamilingiah GM, Derebail SS, Piper D, Chesnut JD: Generation of site-specific retargeting platform cell lines for drug discovery using phiC31 and R4 integrases. *J Biomol Screen* 2009; 14:1207-1215.
15. Gama-Norton L, Herrmann S, Schucht R, Coroadinha AS, Low R, Alves PM, Bartholomae CC, Schmidt M, Baum C, Schambach A, Hauser H, Wirth D: Retroviral vector performance in defined chromosomal Loci of modular packaging cell lines. *Hum Gene Ther*; 21:979-991.
16. Branda CS, Dymecki SM: Talking about a revolution: The impact of site-specific recombinases on genetic analyses in mice. *Dev Cell* 2004; 6:7-28.

17. Wirth D, Hauser H: Flp-mediated integration of expression cassettes into FRT-tagged chromosomal loci in mammalian cells. *Methods Mol Biol* 2004; 267:467-476.
18. May T, Butueva M, Bantner S, Markusic D, Seppen J, MacLeod RA, Weich H, Hauser H, Wirth D: Synthetic gene regulation circuits for control of cell expansion. *Tissue Eng Part A* 2009; 16:441-452.
19. Nehlsen K, Herrmann S, Zauers J, Hauser H, Wirth D: Toxin-antitoxin based transgene expression in mammalian cells. *Nucleic Acids Res* 2009; 38:e32.
20. Foord SM, Bonner TI, Neubig RR, Rosser EM, Pin JP, Davenport AP, Spedding M, Harmar AJ: International Union of Pharmacology. XLVI. G protein-coupled receptor list. *Pharmacol Rev* 2005; 57:279-288.
21. Bylund DB, Eikenberg DC, Hieble JP, Langer SZ, Lefkowitz RJ, Minneman KP, Molinoff PB, Ruffolo RR, Jr., Trendelenburg U: International Union of Pharmacology nomenclature of adrenoceptors. *Pharmacol Rev* 1994; 46:121-136.
22. Cabaniols JP, Ouvry C, Lamamy V, Fery I, Craplet ML, Moulharat N, Guenin SP, Bedut S, Nosjean O, Ferry G, Devavry S, Jacqmarcq C, Lebuhotel C, Mathis L, Delenda C, Boutin JA, Duchateau P, Coge F, Paques F: Meganuclease-driven targeted integration in CHO-K1 cells for the fast generation of HTS-compatible cell-based assays. *J Biomol Screen* 2010; 15:956-967.
23. Gill DR, Pringle IA, Hyde SC: Progress and prospects: the design and production of plasmid vectors. *Gene Ther* 2009; 16:165-171.
24. Liu W, Xiong Y, Gossen M: Stability and homogeneity of transgene expression in isogenic cells. *J Mol Med* 2006; 84:57-64.
25. Sarramegna V, Talmont F, Demange P, Milon A: Heterologous expression of G-protein-coupled receptors: comparison of expression systems fron the standpoint of large-scale production and purification. *Cell Mol Life Sci* 2003; 60:1529-1546.
26. Loll PJ: Membrane protein structural biology: the high throughput challenge. *J Struct Biol* 2003; 142:144-153.
27. Bradel-Tretheway BG, Zhen Z, Dewhurst S: Effects of codon-optimization on protein expression by the human herpesvirus 6 and 7 U51 open reading frame. *J Virol Methods* 2003; 111:145-156.
28. Cook BL, Ernberg KE, Chung H, Zhang S: Study of a synthetic human olfactory receptor 17-4: expression and purification from an inducible mammalian cell line. *PLoS One* 2008; 3:e2920.
29. Mirzabekov T, Bannert N, Farzan M, Hofmann W, Kolchinsky P, Wu L, Wyatt R, Sodroski J: Enhanced expression, native purification, and characterization of CCR5, a principal HIV-1 coreceptor. *J Biol Chem* 1999; 274:28745-28750.
30. Chelikani P, Reeves PJ, Rajbhandary UL, Khorana HG: The synthesis and high-level expression of a beta2-adrenergic receptor gene in a tetracycline-inducible stable mammalian cell line. *Protein Sci* 2006; 15:1433-1440.
31. Stanasila L, Pattus F, Massotte D: Heterologous expression of G-protein-coupled receptors: human opioid receptors under scrutiny. *Biochimie* 1998; 80:563-571.
32. Tate CG, Grisshammer R: Heterologous expression of G-protein-coupled receptors. *Trends Biotechnol* 1996; 14:426-430.
33. Nehlsen K, Schucht R, da Gama-Norton L, Kromer W, Baer A, Cayli A, Hauser H, Wirth D: Recombinant protein expression by targeting pre-selected chromosomal loci. *BMC Biotechnol* 2009; 9:100.

Figure legends

Fig. 1 Scheme for the establishment of novel master cell lines

Flow chart describing the process for generating the novel master cell line optimized for the expression of membrane bound proteins. CHO-K1 cells were electroporated using a NGFR expression vector to tag genomic loci supporting high level expression of a membrane bound protein. After transduction cells were selected and single cells were sorted. The resulting clones were expanded for four weeks without selection pressure and reanalyzed for NGFR expression. The NGFR positive clones were assessed for their capability to perform the RMCE process and for long term stability (>4 months) of the NGFR expression.

Fig. 2 RMCE in the novel master cell lines

a) Strategy of the targeted cassette exchange. The tagging vector contains two FRT sites, a wild-type (filled triangle) and a non-interacting FRT site (open triangle) which flank the NGFR expression cassette. The site is followed by an ATG-deficient neomycin phosphotransferase (Δneo) gene. Therefore the CHO master cell lines are G418 sensitive. The integration of the GPCR of interest is facilitated by co-transfection of the flipase along with the GPCR expressing targeting vector carrying corresponding FRT sites. The flipase cuts out the NGFR expression construct and at the same time pastes in the GPCR expression construct. Clones that underwent the correct RMCE process are selected with G418 as the neomycin resistance gene is complemented by the RMCE process. For this purpose an ATG-start codon was cloned upstream of the F5 FRT site in such a way, that it comes in frame with Δneo upon cassette exchange.

b) Scheme of the PCR detecting correct RMCE. For this purpose primers were used that bind in the genomic locus and within in the incoming GPCR targeting cassette. A representative PCR of generated ADRB2 cell lines is shown.

Fig. 3 Characterization of cell lines expressing ADRB2

ADRB2 expressing cell lines were established with the RMCE process (a) and (b) and by random integration (c). The expression of the GPCR was assessed with a fluorescent ligand specifically recognizing ADRB2. (a) Fluorescence microscopy demonstrated that the RMCE established ADRB2 cells showed a strong membrane bound expression. As a control the master cell line was included in the analysis which showed no fluorescence signal. (b) Flow cytometry revealed a homogenous expression of the ADRB2 in the cells generated through RMCE. The master cell line was used as a negative control. (c) ADRB2 expressing cell lines established by random integration were assessed by flow cytometry for the expression of the GPCR.

Fig. 4 Pharmacological properties of GPCR cell lines

The pharmacological properties of the established GPCR expressing cell lines were assessed by the transfection of a reporter plasmid in the respective cell line. The reporter plasmid contains a hybrid promoter that is composed of CRE binding sites and a SV40 minimal promoter. The hybrid promoter drives the expression of the reporter gene luciferase. After transduction of the reporter construct the GPCR expressing cell lines was cultivated with and without a known agonist and the activity of luciferase was determined. The diagrams show the response of ADRB2 expressing cell line to isoprenaline (clone/pool) and of GCGR to glucagon (10^{-6} M each).

Fig. 5 Analysis of HTS compatibility of a CHRM3 cell line

Four known agonists (a) and three known antagonist (b) of the CHRM3 receptor were used to obtain dose response curve in a HTS-like experiment using the FLIPR$^{TETRA®}$ system and FLIPR® Calcium 5 Assay 5.

Tab. 1 List of GPCRs targeted into the SCREENflex-GR1 master cell line.

Targeted GPCRs	Clones analyzed/ correct targeted	Familiy
ADRB2	12/12	Gs
CHRM1	12/12	Gq/G11
CHRM2	14/14	Gi/G0
CHRM3	11/11	Gq/G11
FFAR2	12/12	Gq/G11
GPR120	24/24	Gq
CXCR4	8/8	Gi/G0
HRH2	12/12	Gs
SSTR1	12/12	Gi/G0
CNR1	24/24	Gi/G0
GCGR	12/12	Gs
NPY1R	6/6	Gi/G0
PTGER3	6/6	Gi/G0

Tab. 2 Agonists and the corresponding pEC50 values of the CHRM3 receptor analysis.

Agonist	pEC_{50} (experiment)	Hill coefficient	pK_i (literature)*
AcetylCholine (●)	9.12	0.98	5.6
Oxotremorine M (●)	8.65	0.93	5.1
Carbachol (●)	8.18	0.89	4.4
Bethanechol (●)	6.76	0.87	4.2

* Literature - IUPHAR database

Tab. 3 Antagonists and the corresponding pIC50 values of the CHRM3 receptor analysis.

Antagonist	pK_B (experiment)	Hill coefficient	pK_B (literature)*
Ipratropium (●)	10.2	- 0.98	9.8
p-F-HHSiD (●)	7.7	- 1.3	7.6
Pirenzepine (●)	6.8	- 1.2	6.8

* Literature - IUPHAR database