Animal Cells and Systems 13: 97-103, 2009

Animal Cells
and
Systems

http://www.AnimalCells.or.kr

Considering Cell-based Assays and Factors for Genome-wide High-content Functional Screening

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Abstract: Recently, great advance is achieved in the field of genome-wide functional screening using cell-based assay. Here, we briefly introduce well-established and typical cell-based assays of GPCR and some parameters which should be considered for genome-wide functional screening. Because of characters and importance of GPCR as drug targets, several ways of assay systems were devised. Among them, high-content screening (HCS) that is based on the analysis of image by confocal microscope is becoming favorite choice. The advances in this technology have been driven exclusively by industry for their convenience. Now, it is turn for academy to define more detail signaling networks via HCS using cDNA or siRNA libraries at genome-wide level. By isolating novel signaling mediators using cDNA or siRNA library, and postulating them as new candidates for therapeutic target, more understanding about life science and more increased chances to develop therapeutics against human disease will be achieved.

Key words: cell-based assay, screening, siRNA library, cDNA library

INTRODUCTION

Cell biologist had been in difficulties to explain what they observed under microscope, because it contains too much information. As results of efforts to solve this, technologies have been evolved as a ‘high-content screen (HCS)’ system and those highly contented information is accumulated as a valuable data. Especially, it has been very useful tool to researchers especially in drug discovery field (Garippa et al., 2006; Ghosh et al., 2005). Meanwhile, new research field, RNA interference (RNAi) which is a natural mechanism to silence individual gene very specifically was opened (Bernstein et al., 2001; Hammond et al., 2000; Hannon, 2002; Hannon et al., 1992). RNAi technology has facilitated to identify new group of genes in signaling pathways at genome level. Because of their powerful advantages, there have been efforts to combine these two high technologies and the results are rather successful (Krausz, 2007).

G protein-coupled receptors (GPCRs) are members of 7 transmembrane receptor families (Pierce et al., 2002); over 700 GPCRs were identified (Jacoby et al., 2006) and more than 200 receptors were matched with their ligands. Because these proteins are known to be related with many diseases, they have been dealt as important targets for drug discovery. About a quarter of drugs show their effects through GPCR (Overington et al., 2006). Therefore, there have been extensive researches to identify novel modulators of GPCR in the pathogenesis of diseases with various tools. Among them, siRNA technology has been proved as a helpful tool to screen new player (Ge et al., 2009; Tsutsumi et al., 2009; Wang et al., 2009; Zou et al., 2009). In those trials, however, there has been not much usage of genome-wide screening and more over, recent trials have focused on cell cycle and cell survival (Aza-Blanc et al., 2003; Poon et al., 2008; Silva et al., 2008).

In this review, typical cell-based assays for GPCRs that were developed and utilized in HCS systems for drug discovery will be briefly described and several parameters for HCS screening using siRNA and cDNA libraries will be discussed.

GPCR ASSAY SYSTEMS: EVOLUTION OF METHODS

Receptor redistribution and binding assays

When GPCRs are activated by its ligands, heterotrimeric guanine nucleotide-binding proteins (G proteins) mediate

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the signal. Classical assay just focused on the interaction and tried to develop better methods to calculate the replaced ratio of radio-labeled ligands using membrane fraction. If GPCRs recruit Gai or Gas and activate it, the intracellular levels of cAMP would change and that can be detected by displacement assays using labeled cAMP tracers and anti-cAMP antibodies (Williams, 2004). When Gaq is involved, there would be influx of calcium ions that can be observed using cell-permeant dyes (Monteith and Bird, 2005).

Another typical assay principle for GPCRs is measuring the internalization of receptor induced by ligands. Tracking this process is suitable for cell-based analysis for HCS (Kohout and Lefkowitz, 2003). In this process, GPCR kinases (GRKs) are recruited to and result in the phosphorylation at C-terminus of GPCR. Then, β-arrestin binds to the phosphorylated GPCR and acts as an adaptor to target the receptor to endosomes for internalization (Luttrell and Lefkowitz, 2002; Pierce et al., 2002). This internalization can be tracked by several ways and four of them have been widely used for cell based assays.

(1) Enhanced green fluorescent protein (EGFP)-tagged GPCR is usually employed as a tool (Fig. 1A) (Haasen et al., 2006). However, there is a concern that due to the size of EGFP, the internalization of the fusion protein may not be efficient enough in some cases.

(2) EGFP-tagged β-arrestin was used successfully in HCS assays to screen a small peptide library for V2R agonists (Fig. 1B) (Ghosh et al., 2005).

(3) Bioluminescence resonance energy transfer (BRET) uses EGFP-tagged β-arrestin and a Renilla luciferase fused to the GPCR, and signals can be recorded upon GPCR-β arrestin interaction (Fig. 1C) (Vrecl et al., 2004). When a ligand activates the receptor, chemiluminescent is transferred to GFP molecule and the activation of GPCR is monitored by GFP signal.

(4) Tango™ assay (Invitrogen) is based on a transcription factor fused to a GPCR via a protease cleavage site and coexpression of a β-arrestin-protease fusion protein (Fig. 2). Cleavage of this transcription factor induces the expression of reporter gene, such as a luciferase or GFP. Because of the nature of this method, there have been concerns on the possibility of higher incidence of false-positive. Nonetheless, a screening looking for agonist for kappa opioid receptor was very successful (Doucette et al., 2009), suggesting that it is a very useful and powerful method that can replace classical binding assay.

Reporter gene system

There are more suitable assays to identify new modulator that acts on intracellular signaling pathway. Those are reporter gene assays employing transcription factors, such as CREB, NF-AT, and NF-κB. Gαs stimulates its adenylyl cyclases activity, and Gαi, Gαo, and Gαz inhibit the activity (Birnbaumer and Birnbaumer, 1995; Offermanns, 2003). Increase of cellular cAMP causes activation of PKA and results in phosphorylation of cAMP-responsive element (CRE)-binding protein, CREB, and induces transcription of CRE-dependent genes (Baker et al., 2004). Then, activation of Gαs can be monitored by using a reporter system that contains CRE elements and a reporter gene. By measuring reporter activity, the changes of cAMP concentration can also be monitored (Fan and Wood, 2007; Wang et al., 2009). In addition, Gq family proteins activate PLCβ (Offermanns, 2003) and PLCβ activity is then measured with reporter assays using NFAT or NF-κB (Oosterom et al., 2005; Siehler et al., 2001). Nowadays, DNA chip technology makes it possible to identify complex expression pattern of groups of genes in rather simple way than before and systems biology lets us understand what the meaning of the plethora of data is, providing us enough information to establish reporter gene assay systems for GPCR.

Other alternative ways for assays

Although we described several ways of assay methods for GPCRs, there are still many alternative ways and new methods are under developing. For example, impedance-

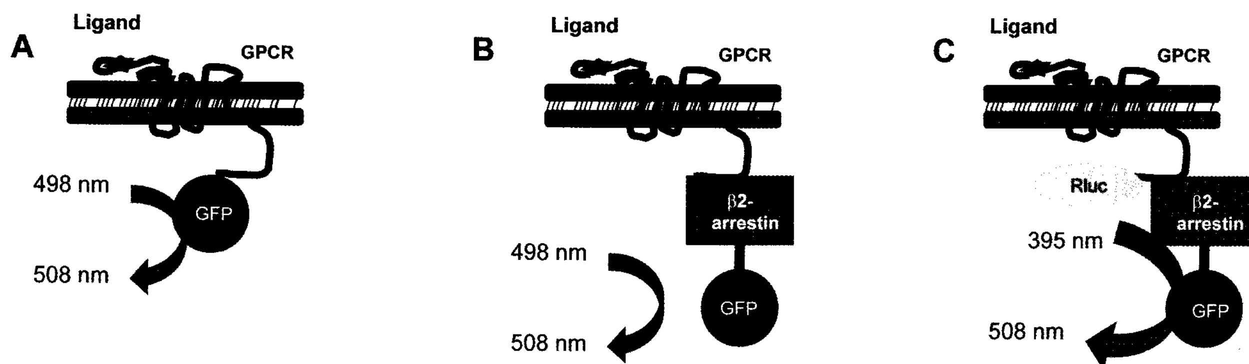


Fig. 1. Assays for GPCR using GFP. (A) By fusing GPCR to GFP, redistribution of the receptor can be monitored. (B) β-arrestin is labeled with GFP (TransFluor assay). (C) Receptor is fused with Renilla luciferase and β-arrestin is labeled with GFP (BRET^2). When a ligand activates the receptor, chemiluminescent is transferred to GFP.

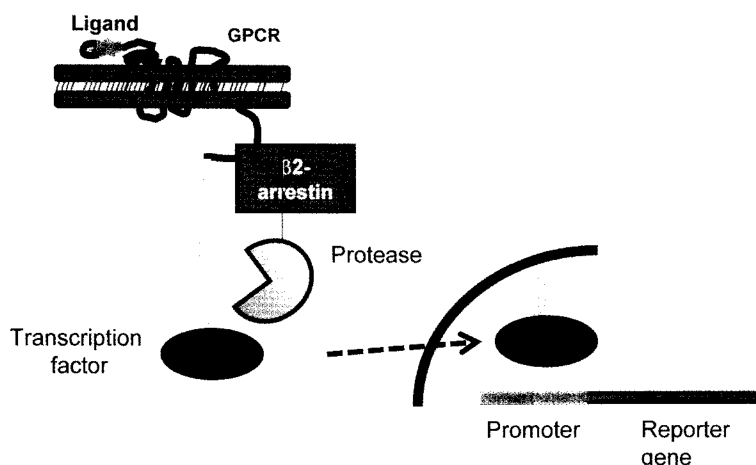


Fig. 2. Tango™ assay (Invitrogen) is based on a transcription factor fused to a GPCR via a protease cleavage site and coexpression of β-arrestin-protease fusion protein.

based cellular assay has been tried with new technology, such as CellKey™ system from MDS Analytical Technologies. That is a label-free and non-invasive method and is universal for nearly all classes of GPCRs (McGuinness, 2007), but it seem that is good for electrophysiological changes.

PARAMETERS FOR GENOME-WIDE HIGH-CONTENT SCREENING

Recent advance in this area has been driven mostly by pharmaceutical companies for drug discovery (Heilker et al., 2009). These kinds of functional assays using mammalian cells were adopted to screen drug-like chemicals in large scale, but nowadays it has also been introduced into academic field. Not only compound libraries, but also cDNA and siRNA libraries are commercially available from several companies, such as Ambion, Sigma, Open Biosystems, and so on (Rines et al., 2006). Thus, many groups are developing diverse cell-based assays and identifying new genes involved in various signal pathways and diseases using the cell-based assays. For example, using siRNA library and melanocyte-based assay, 92 candidate genes that are involved in the pathway for melanin production had been isolated (Ganesan et al., 2008). Similarly, genome-wide HCS with GPCR assay were reported (Garippa et al., 2006; Ghosh et al., 2005; Lee et al., 2006). There are several factors to be considered for HCS, especially using siRNA or cDNA.

HCS assay system and optimization

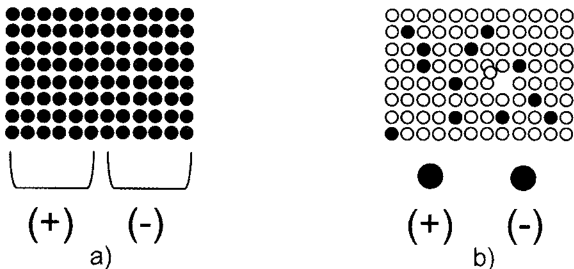
Cell culture condition: For HCS, optimization of assay fit to 96 well or 384 well formats are essential. Although use of 96 well/384 well plate saves time and cost, miniaturization in multi well plates can produce unexpected results. Factors affecting this result include cell culture condition, edge well effect, and assay readout specificity. Because 96 well/384 well culture dishes can contain only limited number of cells and volume of culture media, small changes of cell culture condition can affect the result with producing improper output. To bypass this, it is needed to check whether cell density can affect the result or not. Seeding small number of cells can decrease output signal, and this output can be easily deteriorated by small change. Conclusively, adequate cell culture condition to maintain optimal cell growth within the time line of screening is very important.

Edge well effect: Edge well effect refers the artifact readout resulting from the position of wells in plate, not from biological effect. Edge effect is thought as widespread factors that affect the deterioration of result in high throughput screens. It is not identified yet why edge effect occurs in multi well plates without any unified rule (Lundholt et al., 2003). Typically, control wells of experiments are located on outer columns of multi well plates and generate reasonable output signals compared to inside column of multi well plates, thus causing experimental wells to be higher (or lower) than their real value. Some recommendations to escape edge effect include 1) preincubation of plates at room temperature before incubation in cell culture incubator, 2) avoiding of bubble formation in seeding, 3) minimizing the number of move in and out of culture incubation, and 4) place control wells randomly in plates.

Assay stability analysis by z factor calculation: z factor is a measure of statistical effect size suggested for use in high throughput screen to decide whether the result of assay is reliable enough for further analysis (Zhang et al., 1999). For best result, z factor should be between 0.5 and 1. Before applying designed assay to the screening procedure, test plate can be made by following schemes.

a) Use half of plate for positive control and half for negative control, calculate z factors, analyze, and estimate assay stability in assay optimization before screen.

b) Use random positioning of positive control and negative control in the plate of use and calculate z factors with analysis as indicated in the reference (Zhang et al., 1999).



Genome-wide HCS with RNAi

Profound progress was made in the usage of siRNA as a tool to reduce specific protein expression *in vivo* and *in vitro* (Elbashir et al., 2001; Elbashir et al., 2002; Xia et al., 2004; Bernards et al., 2006). There are several factors to be considered for the screening procedure using siRNA library.

High throughput transfection: Transfection of siRNA can induce cell death in certain cases because of use of antibiotics in siRNA transfection. Use of antibiotics-free medium and care for possible contamination are needed. To acquire maximum transfection efficiency, optimization of small scale siRNA transfection in the decided cell line is essential. siRNA tranfection efficiency is affected by cell types, transfection reagent, cell density, media volume, and final concentration of siRNA/transfection reagents. During optimization of assay step, cytotoxicity of transfection reagent and optimal concentration of siRNA need to be checked (Borawski et al., 2007; Xin et al., 2004). The way of transfection is another factor. Some types of cells are more easily transfected by reverse transfection, while others are not. Best is to compare both methods in cell lines of use. Many siRNA manufacturers are operating database websites to introduce most efficient ways of transfection for each cell line. For 'Hard to transfect by lipid' cell lines, short hairpin RNAs in viral vector (shRNA library) give a possible alternative way.

Specificity of siRNA: It is repeatedly reported that siRNA can reduce expression of non-related genes. If researchers use single kind of siRNA to knock down the expression of specific genes, they will meet big portion of false positive. To escape this, multiple siRNA approach is preferred. For single gene, at least 3 different siRNAs are in use to knockdown the expression in different wells. Pool of siRNA to knockdown a single gene is not recommended because of same reason (Haney, 2007).

Window of assay readout: Readout of general factors, such as cellular ATP content for the cell survival, is vulnerable to off target effect of siRNAs than more narrow-window assays like translocation of downstream protein in the intended signaling. Naturally, many assays of high specificity are image-based assays reflecting cause-result relationship directly (Haney, 2007).

Phenotype transfer after isolation of hit: After siRNA library screening by appropriate procedures, researchers will collect 'real hit' with collecting out false positive. All the isolated genes are tested again to eliminate false positives. At this stage, secondary assays can be accompanied with different methods related to the biological process of interest. When the phenotype transfer is considered, different set of siRNAs from different sources (commercial companies or custom designed) will help to confirm the phenotypes. Following facts are a few more considerations.

(a) Size of library: Genome-wide or selected library. If you don't know exactly where the target gene is located and what is its function, you should adopt a genome-wide siRNA library. Then, it will cost a lot, but may not miss a gene. When you just focuse on an event or organelle, you can choose a focused library but may be disappointed by the results.

(b) siRNA, shRNA, or cDNA: Whether gain-of-function or lose-of-function is observed should be well determined based on the phenomenon or pathway. When a screen adopts siRNA library, it is recommended to use cDNA expression in a validation step or *vise versa*.

(c) Cell type: It is very clear that quality of every cell-based assay is dependent on the type of cells used. Except for study about cancer, normal cell is better than cancerous cell. When you should use carcinoma cell line, usage of more than two cell line is needed for verification *in vitro* as shown above. Recent advances in inducible pluripotent stem cell (iPS) technology make it possible that the generation of patient specific stem cell line without killing embryo (Hyun et al., 2007; Takahashi and Yamanaka, 2006; Webb, 2009; Yamanaka, 2009). Therefore, in near future, we can do HCS in a neuronal cell lines that was derived from skin cell of patients who suffered by neurodegenerative diseas, such as Alzheimer's disease or Huntington's disease.

(d) Point of analysis: For better assay, it is need to use as much parameters as possible and define its characters more specifically. Although the reporter gene assay system or trafficking of receptor internalization is well established methods, it tells just a simple fact. To take advantage of HCS, it is recommended to observe additional factors, such

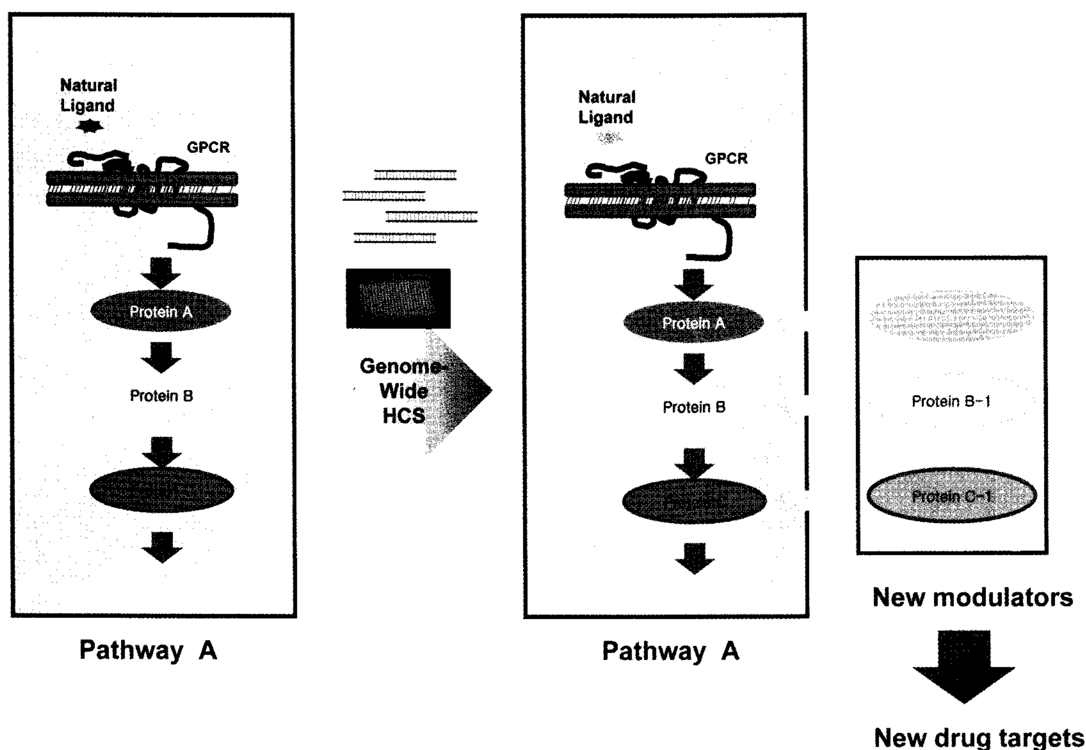


Fig. 3. Finding new pathway modulators from old pathway. When already known signal pathway (Pathway A) is not suitable for small chemical ligands, it is recommended to identify new modulators by genome-wide functional screening using siRNA, shRNA, or cDNA libraries.

as structure of intracellular organelle, shape of cell surface, function of mitochondria, and so on. For this, collaboration with IT part is necessary.

CONCLUSION

Although GPCR has been known to be related with several disease, sometimes its ligands are too small to give a selectivity or too big to replace with small chemicals. That hindered the progress of drug discovery targeting GPCRs. Therefore, introduction of new genes into a signaling pathway that is already known to be related with certain disease but hard to make a drug with proteins in the cascade might give a breakthrough for drug discovery.

In imaging analysis-based HCS using siRNA or cDNA, we can get a set of compositional data that has statistical properties rather than single representative image. Those data will give hints what are strong and weak points of the genes as a drug target and reduce time and cost for drug development. As shown in above example, genome-wide HCS with siRNA, shRNA, or cDNA library will introduce discovery of novel genes as new drug target candidates (Fig. 3), increasing our understanding various signal pathways and facilitating drug discovery process associated with the pathway.

ACKNOWLEDGMENTS

This work was supported by CRI-Acceleration Research from the Korea Science and Engineering Foundation (KOSEF) of the Korean Ministry of Science and Education.

REFERENCES

Aza-Blanc P, Cooper CL, Wagner K, Batalov S, Deveraux QL, and Cooke MP (2003) Identification of modulators of TRAIL-induced apoptosis via RNAi-based phenotypic screening. *Mol Cell* 12: 627-637.

Baker JG, Hall IP, and Hill SJ (2004) Temporal characteristics of cAMP response element-mediated gene transcription: requirement for sustained cAMP production. *Mol Pharmacol* 65: 986-998.

Bernards R, Brummelkamp TR, and Beijersbergen RL (2006) shRNA libraries and their use in cancer genetics. *Nat Methods* 3: 701-706.

Bernstein E, Caudy AA, Hammond SM, and Hannon GJ (2001) Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature* 409: 363-366.

Birnbaumer L and Birnbaumer M (1995) Signal transduction by G proteins: 1994 edition. *J Recept Signal Transduct Res* 15: 213-252.

Borawski J, Lindeman A, Buxton F, Labow M, and Gaither LA (2007) Optimization procedure for small interfering RNA transfection in a 384-well format. *J Biomol Screen* 12: 546-

559.

Doucette C, Vedvik K, Koepnick E, Bergsma A, Thomson B, and Turek-Etienne TC (2009) Kappa Opioid Receptor Screen with the Tango™ beta-Arrestin Recruitment Technology and Characterization of Hits with Second-Messenger Assays. *J Biomol Screen* 14: 381-394.

Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, and Tuschl T (2001) Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* 411: 494-498.

Elbashir SM, Harborth J, Weber K, and Tuschl T (2002) Analysis of gene function in somatic mammalian cells using small interfering RNAs. *Methods* 26: 199-213.

Fan F and Wood KV (2007) Bioluminescent assays for high-throughput screening. *Assay Drug Dev Technol* 5: 127-136.

Ganesan AK, Ho H, Bodemann B, Petersen S, Aruri J, Koshy S, Richardson Z, Le LQ, Krasieva T, Roth MG et al. (2008) Genome-wide siRNA-based functional genomics of pigmentation identifies novel genes and pathways that impact melanogenesis in human cells. *PLoS Genet* 4: e1000298.

Garippa RJ, Hoffman AF, Gradl G, and Kirsch A (2006) High-throughput confocal microscopy for beta-arrestin-green fluorescent protein translocation G protein-coupled receptor assays using the Evotec Opera. *Methods Enzymol* 414: 99-120.

Ge X, Loh HH, and Law PY (2009) {micro}-Opioid receptor cell surface expression is regulated by its direct interaction with RibophorinI. *Mol Pharmacol.*

Ghosh RN, DeBiasio R, Hudson CC, Ramer ER, Cowan CL, and Oakley RH (2005) Quantitative cell-based high-content screening for vasopressin receptor agonists using transfluor technology. *J Biomol Screen* 10: 476-484.

Haasen D, Schnapp A, Valler MJ, and Heilker R (2006) G protein-coupled receptor internalization assays in the high-content screening format. *Methods Enzymol* 414: 121-139.

Hammond SM, Bernstein E, Beach D, and Hannon GJ (2000) An RNA-directed nuclease mediates post-transcriptional gene silencing in Drosophila cells. *Nature* 404: 293-296.

Haney SA (2007) Increasing the robustness and validity of RNAi screens. *Pharmacogenomics* 8: 1037-1049.

Hannon GJ (2002) RNA interference. *Nature* 418: 244-251.

Hannon GJ, Maroney PA, Yu YT, Hannon GE, and Nilsen TW (1992) Interaction of U6 snRNA with a sequence required for function of the nematode SL RNA in trans-splicing. *Science* 258: 1775-1780.

Heilker R, Wolff M, Tautermann CS, and Bieler M (2009) G-protein-coupled receptor-focused drug discovery using a target class platform approach. *Drug Discov Today* 14: 231-240.

Hyun I, Hochedlinger K, Jaenisch R, and Yamanaka S (2007) New advances in iPS cell research do not obviate the need for human embryonic stem cells. *Cell Stem Cell* 1: 367-368.

Jacoby E, Bouhelal R, Gerspacher M, and Seuwen K (2006) The 7 TM G-protein-coupled receptor target family. *Chem Med Chem* 1: 761-782.

Kohout TA and Lefkowitz RJ (2003) Regulation of G protein-coupled receptor kinases and arrestins during receptor desensitization. *Mol Pharmacol* 63: 9-18.

Krausz E (2007) High-content siRNA screening. *Mol Biosyst* 3: 232-240.

Lee S, Howell B, and Kunapuli P (2006) Cell imaging assays for G protein-coupled receptor internalization: application to high-throughput screening. *Methods Enzymol* 414: 79-98.

Lundholt BK, Scudder KM, and Pagliaro L (2003) A simple technique for reducing edge effect in cell-based assays. *J Biomol Screen* 8: 566-570.

Luttrell LM and Lefkowitz RJ (2002) The role of beta-arrestins in the termination and transduction of G-protein-coupled receptor signals. *J Cell Sci* 115: 455-465.

McGuinness R (2007) Impedance-based cellular assay technologies: recent advances, future promise. *Curr Opin Pharmacol* 7: 535-540.

Monteith GR and Bird GS (2005) Techniques: high-throughput measurement of intracellular Ca(2+) -- back to basics. *Trends Pharmacol Sci* 26: 218-223.

Offermanns S (2003) G-proteins as transducers in transmembrane signalling. *Prog Biophys Mol Biol* 83: 101-130.

Oosterom J, van Doornmalen EJ, Lobregt S, Blomenrohr M, and Zaman GJ (2005) High-throughput screening using beta-lactamase reporter-gene technology for identification of low-molecular-weight antagonists of the human gonadotropin releasing hormone receptor. *Assay Drug Dev Technol* 3: 143-154.

Overington JP, Al-Lazikani B, and Hopkins AL (2006) How many drug targets are there? *Nat Rev Drug Discov* 5: 993-996.

Pierce KL, Premont RT, and RJ L (2002) Seven-transmembrane receptors. *Nat Rev Mol Cell Biol* 3: 639-650.

Poon SS, Wong JT, Saunders DN, Ma QC, McKinney S, Fee J, and Aparicio SA (2008) Intensity calibration and automated cell cycle gating for high-throughput image-based siRNA screens of mammalian cells. *Cytometry A* 73: 904-917.

Rines DR, Tu B, Miraglia L, Welch GL, Zhang J, Hull MV, Orth AP, and Chanda SK (2006) High-content screening of functional genomic libraries. *Methods Enzymol* 414: 530-565.

Siehler S, Wang Y, Fan X, Windh RT, and Manning DR (2001) Sphingosine 1-phosphate activates nuclear factor-kappa B through Edg receptors. Activation through Edg-3 and Edg-5, but not Edg-1, in human embryonic kidney 293 cells. *J Biol Chem* 276: 48733-48739.

Silva JM, Marran K, Parker JS, Silva J, Golding M, Schlabach MR, Elledge SJ, Hannon GJ, and Chang K (2008) Profiling essential genes in human mammary cells by multiplex RNAi screening. *Science* 319: 617-620.

Takahashi K and Yamanaka S (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* 126: 663-676.

Tsutsumi R, Fukata Y, Noritake J, Iwanaga T, Perez F, and Fukata M (2009) Identification of G protein alpha subunit-palmitoylating enzyme. *Mol Cell Biol* 29: 435-447.

Vrecl M, Jorgensen R, Pogacnik A, and Heding A (2004) Development of a BRET2 screening assay using beta-arrestin 2 mutants. *J Biomol Screen* 9: 322-333.

Wang CJ, Hsu SH, Hung WT, and Luo CW (2009) Establishment of a chimeric reporting system for the universal detection and high-throughput screening of G protein-coupled receptors. *Biosens Bioelectron* 24: 2298-2304.

Webb S (2009) iPS cell technology gains momentum in drug discovery. *Nat Rev Drug Discov* 8: 263-264.

Williams C (2004) cAMP detection methods in HTS: selecting

the best from the rest. *Nat Rev Drug Discov* 3: 125-135.

Xia H, Mao Q, Eliason SL, Harper SQ, Martins IH, Orr HT, Paulson HL, Yang L, Kotin RM, and Davidson BL (2004) RNAi suppresses polyglutamine-induced neurodegeneration in a model of spinocerebellar ataxia. *Nat Med* 10: 816-820.

Xin H, Bernal A, Amato FA, Pinhasov A, Kauffman J, Brenneman DE, Derian CK, Andrade-Gordon P, Plata-Salaman CR, and Ilyin SE (2004) High-throughput siRNA-based functional target validation. *J Biomol Screen* 9: 286-293.

Yamanaka S (2009) A fresh look at iPS cells. *Cell* 137: 13-17.

Zhang JH, Chung TD, and Oldenburg KR (1999) A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays. *J Biomol Screen* 4: 67-73.

Zou H, Thomas SM, Yan ZW, Grandis JR, Vogt A, and Li LY (2009) Human rhomboid family-1 gene RHBDF1 participates in GPCR-mediated transactivation of EGFR growth signals in head and neck squamous cancer cells. *Faseb J* 23: 425-432.

[Received May 29, 2009; accepted June 15, 2009]