Take it personal! Genetic differences in G protein-coupled receptors as studied with label-free technology

Hillger, J.M.

CHAPTER 7

Conclusions and future perspective

This thesis delves into examining the influence of genetic variation on GPCR function within the human individual. In this concluding chapter, insights gained from case studies at three different GPCRs are elaborated on, and suggestions for future investigations around precision medicine for GPCRs are presented.

Conclusions

Assay methodology and model systems

GPCRs are traditionally investigated in reporter-based assays performed on heterologous cell lines, which offer only limited translational value [1-6]. Physiologically more appropriate model systems and assays are thus required (Chapter 2). LCLs, which are among the most frequently biobanked samples used for storing genetic material [7-13], could form a highly valuable resource for investigating genetic effects on drug action and receptor function. In addition, label-free cellular assays offer increased physiological relevance over the assays used traditionally in GPCR research, as discussed in Chapter 1 and 2 [4, 13-15]. Unfortunately, these were originally deemed incompatible with suspension cells such as LCLs due to the detection mechanism positioned at the bottom of the well [16]. In this thesis I present a methodology with increased translational value by employing personal cell lines (the LCLs) as a model system, in combination with a physiologically more appropriate label-free cellular assay (the xCELLigence) to investigate GPCR function (Chapter 3). Adaptation to suspension cells drastically widens the realm of application for label-free assays, while investigating GPCR functionality in LCLs opens up an avenue for exploring precision medicine for GPCRs.

Genetic variation in GPCRs

Genetic variants in drug targets affect pathology and drug action [17]. Despite GPCRs being the largest group of drug targets to-date [18], studies on their genetic variation are sporadic, often only statistically associative and focus on one single target. Investigations generally work with one consensus form of a receptor, the so-called Wild-type, hereby ignoring the naturally occurring genetic variation in the population. However, other receptor variants may be more relevant for certain diseases or drug effects. Three separate cases of common polymorphisms that affect GPCR signaling are presented in this thesis, each revealing different properties including the sensitivity to agonist type, chemical scaffold and variant position in the gene.

Throughout this thesis I present examples that show genetic variations at different positions in GPCRs can be of influence. Logically, single nucleotide polymorphisms (SNPs)

most likely to have profound effects on receptor function are those that alter the amino acid sequence of the receptor, the so-called non-synonymous SNPs. Indeed, the variants that affected the Cannabinoid Receptor 2 (CB_2R) and the glucose-dependent insulinotropic polypeptide receptor (GIPR), Q63R and E354Q respectively, both changed an amino acid (**Chapter 5** and **6**). In fact, many cases presented in the literature fall into this category [2, 19, 20]. However, changing amino acid sequence is not the only way in which a receptor can be affected by polymorphisms. **Chapter 4** presents the case of the Adenosine A_{2A} receptor, in which responses differ between individuals in the absence of any non-synonymous SNPs. Genotype comparison revealed differences in two intron SNPs, one of which associated with caffeine-induced sleep disorders [21-23]. Such SNPs could have regulatory potential, for instance in affecting receptor expression which in turn may affect G protein coupling efficiency [23, 24].

Interestingly, this particular $A_{2A}R$ SNP altered partial agonist potency, but not that of full agonists or antagonists (**Chapter 4**). In a similar manner, the partial agonists for the CB_2R showed higher efficacy in a Q63R minor homozygote (**Chapter 5**). While either potency or efficacy of partial agonists can be affected, it overall appears that partial agonists may be more receptive to polymorphism-induced changes. This concurs with the theory that deems partial agonists more sensitive to system-related differences in receptor function, for instance in receptor expression or downstream coupling, than full agonists or antagonists [25]. The nature of the ligand thus influences its sensitivity to e.g. polymorphisms. In addition, the chemical scaffold of a ligand is likewise important. **Chapter 5** presents how compounds of different chemical classes show more or less modulation due to CB_2R genetic variation. Non-classical cannabinoid CP55940 showed the most pronounced personal effects, while aminoalkylindole compounds showed fewer individual differences. Taking both ligand nature and chemical scaffold effects into account could allow early identification of compounds prone to personal differences ('precision medicine') or compounds that would be more suited as drugs for the general population.

Besides affecting drug action, SNPs can also alter the physiological function of a receptor with potentially pathological consequences. **Chapter 6** focusses on the investigation of the GIPR, in which E354Q influenced endogenous agonist effects, in particular with respect to potency in LCLs and duration of response when the receptor was expressed in recombinant HEK293 cells. This SNP has previously been linked with various pathologies including insulin resistance, diabetes and cardiovascular disease [26-29]. Interestingly, endogenous agonists are not necessarily more sensitive to receptor polymorphisms than synthetic ligands, as the study of adenosine on the $A_{2A}R$ and various endocannabinoids on CB_2R show (**Chapter 4** and

5). While some SNPs are of pathological consequence, others may be more relevant for drug effects. For instance, the $A_{2A}R$ SNP has been associated with caffeine effects and pharmacotherapy-related toxicities in acute lymphoblastic leukemia as well as pathological conditions including anxiety in autism [21-24]. Similarly, Q63R in the CB_2R has been linked to various pathological disorders [5, 30-35] as well as synthetic ligand effects (Chapter 5, [36]). It is important to note that if a polymorphism affects an endogenous agonist, this may not directly leading to pathology. However it can still drastically alter a system's sensitivity to drug treatment, even if the synthetic compounds are not directly affected themselves. In conclusion, it is undoubtedly necessary to take physiology and pathology into account when selecting ligands and conditions to study the influence of GPCR polymorphisms.

Finally, it could be argued that SNPs with profound effects on receptor function are likely less frequent in the population due to evolutionary pressure. It is a common misconception that a frequent SNP has likely little effect [2]. The frequencies of the SNPs in this thesis however tell a different story, as the SNPs in the GIPR and CB_2R with a global Minor Allele Frequency of approx. 35% and 16%, respectively [37-39], are in fact quite frequent, regardless of any functional effects. Many disease-related SNPs are quite rare, but some common SNPs are also known to contribute to or cause certain disease phenotypes [2, 17].

In summary, the cases presented in this thesis demonstrate that for every GPCR, there appears to be at least one polymorphism candidate to affect receptor function. The particularities of each polymorphism can however differ, depending on the nature of the ligand such as endogenous vs. synthetic, partial vs. full agonist, chemical scaffold as well as the number of individuals potentially affected.

LCLs as model system for genetic effects on GPCRs

The examples summarized in this thesis (Chapter 3-6) demonstrate that LCLs are a suitable model system to study genetic effects on GPCRs, and the applied methodology facilitates phenotypic measurements of personal responses. LCLs thus enable direct measurement of polymorphism effects in a physiological environment, without having to generate and introduce a receptor mutant into a heterologous cell line as is generally done in the GPCR field. Any such alterations can affect receptor pharmacology and decrease translatability (Chapter 2). It is therefore unsurprising that the results presented in this thesis agree with previous investigations to some degree, while contradicting others. In chapter 5 for instance, Q63R influences on CB_2R contrasted previous reports obtained in recombinant overexpressing cell systems, while confirming findings in a more physiological cell type [5, 36, 40, 41]. E354Q effects on GIPR differed between LCLs and HEK293 cells even in our hands

(Chapter 6), and results of either cell type were both in accordance and contrast with previous studies [28, 29, 42]. Overall, it appears that LCLs are a well-suited system to measure personal polymorphism effects on GPCRs in a physiological setting, and enable explorations into the realm of GPCR precision medicine. While they increase translatability in comparison to traditional cell systems, the relevance of effects established in this thesis depends on further replication in e.g., more individuals for genotype effects, and/or primary cell types directly involved in pathology.

Future perspectives

Altogether a variety of impact factors for GPCR research including model systems, assay technology and genetic variation have been detailed throughout this thesis. The following section will discuss the future perspectives precision medicine for GPCRs involving some of these findings and additional aspects for further consideration.

Genetic variation landscape in druggable GPCRs

With the rise of personalized or precision medicine concepts, it is increasingly recognized that genetic differences between individuals can affect both drug action and susceptibility to diseases [17]. Examples of influential genetic variants of various types, frequencies and physiological consequences are accumulating. However, which variants are pathogenic, collateral of inconsequential is still largely undefined and subject of tremendous ongoing research efforts.

When regarding any two unrelated individuals, 99% of their genomic DNA sequences are identical. The other 1%, however, signifies in fact 38 million different genomic variations. In turn, 90% of these variations are formed by SNPs, which makes these the most common source of genetic variation in the human population [12, 32]. On average, around one SNP occurs per 300 bases, meaning that each GPCRs should contain several SNPs, which occur more or less frequently in the population [2, 43]. During our annotation process of SNPs in druggable GPCRs (Chapter 1, Fig. 3), we noted several trends.

First, the total amount of SNPs is related to gene size (Fig. 1). The largest GPCR genes, which belong mostly to Class C and Adhesion GPCRs, generally have the most genetic variation. Table 1 shows the top and bottom 5 genes with most or least SNPs. Based on these, SNPs of any kind occur within a GPCR gene on average around every 140 bp in the largest genes and every 413 bp in the shortest genes. This increased distance in shorter genes is unsurprising as, the shorter a gene, the larger the relative part that is coding sequence, which is more evolutionary conserved.

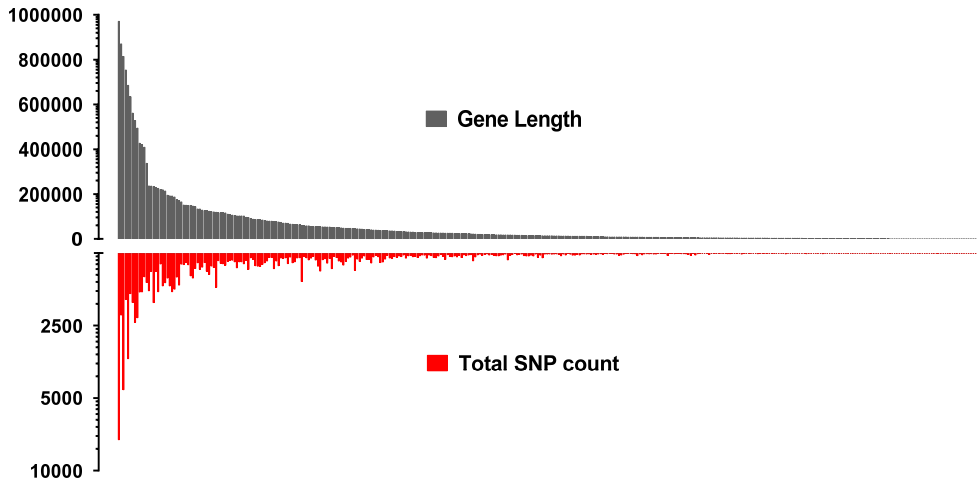

Figure 1. Total SNP count vs gene length for all 370 druggable GPCRs. A list of all druggable non-olfactory GPCRs was downloaded from the IUPHAR database. The gene length and SNP information were exported from Ensembl Biomart archived page (April 2013, version 71) corresponding to genome build GCRh37.p10.

Secondly, most SNPs are located in non-coding regions and/or do not affect amino acid sequence in any way. Non-synonymous SNPs only represented 0.7% of all SNPs found in druggable GPCRs (total 152.000 SNPs on 370 GPCR genes). Synonymous SNPs which are located in the coding region but do not change the amino acid sequence made up 25%. Hence, GPCRs contain an abundance of SNPs predominantly in non-coding regions, with 42.5% in UTR and intron regions. This is common for any intron-containing GPCR due to the evolutionary conservation of the different regions [43, 44].

Finally, SNPs with possibly profound effects on receptor function i.e. by altering the amino acid sequence of the receptor, are more abundantly occurring than one might expect. While the overall amount of SNPs increases with gene size (**Fig. 1**), on average each druggable GPCR contains at least 1-5 non-synonymous SNPs, independent of gene size (**Table 1**). The bottom line is that for each GPCR, there appear to be genetic differences which may impact receptor and drug functionality. Hence it is paramount not to ignore the potentially influential natural variation occurring in any GPCR or drug target for future pharmacological research.

Although SNPs form the major source, there are other types of genetic variants present in the human genome. These include bi-allelic short insertions or deletions, large deletions, short repeats such as micro- and minisatellites, and copy number variants (CNV) which can extend to repeats of entire genes [32]. While most have no detrimental clinical

Table 1. GPCR genes with the top five most and least SNPs in total. The gene length and SNP information were exported from Ensembl Biomart archived page (April 2013, version 71) corresponding to genome build GCRh37.p10. The column labelled altering SNPs entails the number of SNPs that are either missense-, start and stop codon, or frameshift variants for the respective receptor.

Gene	GPCR class	Receptor type	Gene lenght (bp)	Average SNP distance (bp)	SNPs	
					Total	Altering
GRM7	Class C	Metabotropic glutamate receptor 7	971527	118	8216	2
LPHN2	Adhesion	Latrophilin receptor 2	686275	126	5430	5
LPHN3	Adhesion	Latrophilin receptor 3	871208	160	5430	2
GRM8	Class C	Metabotropic glutamate receptor 8	814696	151	5388	5
BAI3	Adhesion	Brain-specific angiogenesis inhibitor	754144	144	5248	2
TAS2R16	Taste	Type 2 taste receptor 16	995	332	3	2
GPR32	Class A	Orphan receptor	1268	423	3	1
CHRM4	Class A	Muscarinic acetylcholine receptor 4	1467	489	3	0
MC3R	Class A	Melanocortin receptor 3	1083	361	3	1
FFAR1	Class A	Free fatty acid receptor 1	922	461	2	1

consequences, some form a pathological risk. For instance, several repeat polymorphisms in the Arginine vasopressin receptor 1A have been associated with altered social, sexual and reproductive behavior [45-47]. Also, the TAS2R receptor family that detects bitter taste of compounds such as caffeine contains about 25 GPCRs, but the exact number per individual varies due to copy number variation. Individual experiences of bitterness are altered by genetic variation in these receptors [48, 49]. Finally, duplication of orphan receptor GPR101 has been shown to lead to X-linked acrogigantism [50]. Thus next to SNPs, it would be an important addition to study other forms of genomic variations too, as these can also account for a difference in GPCR response [51].

Of note, the Netherlands Twin Registry (NTR; *http://www.tweelingenregister.org/en/*) [7] from which the collection of LCLs utilized in this study originated, has served as data source for many genetic studies, including SNPs as well as CNVs already [52-55]. Given the appropriate samples are available, utilizing the set-up of LCLs and label-free technology could offer additional insights into the functional influences of such other types of variants too.

LCLs and emerging cellular model systems for drug research

Two of the major challenges in today's drug development are the lack of understanding inter-individual variability in drug effectiveness, and the translatability of preclinical results. Inappropriate model systems have contributed to both issues, and consequently to lack of reproducibility in preclinical research, lack of clinical effectiveness and high attrition rates [6, 56].

In this thesis I have presented a methodology utilizing LCLs from the NTR [7] as a model system to investigate genetic effects on GPCR functionality. Applications of LCLs are however by no means limited to the three exemplary GPCR cases discussed in this thesis (Chapter 4-6), as LCLs express many more GPCRs as well as other drug targets [11, 57, 58].

In general, renewable *in vitro* cell sources have been essential in facilitating drug discovery and pharmacogenomic studies. In fact, much of our understanding of the influences of genetic variation in humans is based on studies utilizing LCLs [59]. LCLs are already easily available in large variety as LCL repositories exist in abundance, some representing specific disease populations or ethnicities [7-11, 59-61]. Hence they are utilized in many aspects of pharmacogenomics, and examples include general genotype-phenotype association, many genome-wide association studies (GWAS) for drug-induced phenotypes and even follow-up studies of clinical findings [11, 57].

Notwithstanding the convenience and usefulness of LCLs as a cellular model system, there are concerns that their immortalization and cell line maintenance could obscure genetic findings [59, 62-64]. Certainly, it is well known that a large number of genes are differentially expressed between primary cells and cell lines [59]. Opposed to this, primary cells express signaling pathways and retain many cellular functions that are seen *in vivo*, thus providing a more relevant context (Chapter 2). Over the past decades, numerous biobanks have been set up to support medical research by programmed storage of biological material and corresponding data. These biomaterials include LCLs as well as primary material such as tissues, (stem) cells and blood, all of which are actively used from translational and personalized medicine to target and drug discovery [65, 66]. Several approaches applying label-free technology to utilize patient primary cells as model system are discussed in Chapter 2. While such cell types have increased translational value, the materials are often limited due to culture and sampling issues. On the other hand, LCLs are a renewable source that is already widely available, and offer genotypic and phenotypic information and stability that is absent in many other renewable sources. How appropriate either model system is depends largely on the application and question at hand.

An alternative that could incorporate renewability, primary tissue properties and patient origin are stem cells, which offer great potential as physiologically more relevant models. In particular induced pluripotent stem cells (iPSC), which can maintain the disease genotype and phenotype indefinitely, provide a source of models for an expansive range of adult differentiated cells, possibly even for each individual patient. The ability to reprogram cells of patients into disease-relevant cell types could provide more representative and predictive cellular models for both disease modelling and drug discovery [60], and has the potential to personalize pharmacological research [67, 68]. iPSC have already been used for drug screening and disease modelling, particular as neural cells, haematopoietic cell types, hepatocytes and cardiomyoctes [69, 70]. For some of such cell types, hiPSC-cardiomyocytes in particular, there are also examples of their application in label-free assays (Chapter 2, Table 1) [71-73]. Interestingly, iPSC can be derived from a variety of cellular sources, including LCLs. This taps into the invaluable resources of the already available, vast collections of LCLs. iPSC derived from LCLs retain their disease mutation, exhibit identical characteristics as iPSC derived from more common sources such as fibroblasts, and can be differentiated into various cell types including neurons and even intestinal organoids [60, 61]. Organoids constitute near-physiological 3D models of an organ with realistic micro-anatomy, and as such enable more accurate study of many physiological processes [74]. Furthermore, iPSC from LCLs even recover their donor-specific gene expression signature [59, 60]. While it is unlikely that the lack of donor signature on gene expression in LCLs themselves would cause false-positive findings of genetic influence, such as the ones presented in this thesis in Chapter 3-6, regaining this signature in iPSC increases the ability to study inter-individual differences in gene expression [59].

In summary, as these developments show, LCLs offer an enormous bioresource for both drug discovery and disease modelling [60, 61].

Comeback of phenotypic assays for drug research

In addition to the need for more representative model systems, a preference is emerging for minimally invasive, time-resolved and thus pharmacologically more relevant assays. As principal criteria, new assay approaches for pharmaceutical drug discovery are to be more efficient and multidimensional [19]. Amongst these are label-free cellular assays. As discussed in Chapter 2, these assays offer a wide range of applications and have similarly been applied to many important classes of drug targets, which include besides GPCRs also receptor tyrosine kinases and nuclear receptors [75-78]. Their realm of application is large and constantly expanding.

This preference is part of a general trend back towards phenotypic screening. Phenotypic screens were in fact the norm for drug discovery prior to the 1980s. Following the advent of molecular cloning then, target-based screening became the standard approach for drug discovery. This strategy includes cloning and functional expression of potential drug targets in recombinant cell lines for study and screening of drug candidates (**Fig. 2**). While this approach has delivered many drug candidates over the years, there were relatively few new drugs. One reason is that this approach may work very well for monogenic diseases, however, most human diseases are likely multifactorial. Rather than caused by a single genetic change, they are complex diseases originating through an interplay of a multitude of genetic and environmental factors. Hence they may require engagement of multiple targets to achieve clinical efficacy [79-82].

In such instances, target-agnostic approaches as utilized in phenotypic screening assays can offer advantages. In fact, significantly more small-molecule first-in-class drugs were discovered through phenotypic screening than target-based approaches [83]. Instead of focusing on engaging a specific target, phenotypic assays rely on finding molecules with a particular biological effect in cell-based or animal models (**Fig. 2**) [80-82]. This approach does however have its own hurdles to overcome, which include the need to identify a phenotypic

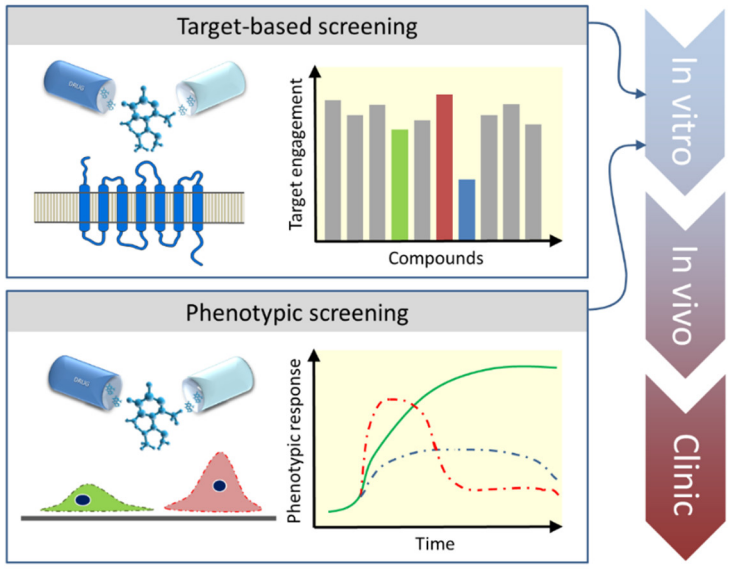

Figure 2. Phenotypic assays versus target-based approach. Target-based drug discovery approach focuses on engaging a specific target, often using molecular cloning and recombinant techniques. Phenotypic assays identify molecules with a particular biological effect in cell-based or animal models. Phenotypic assays can provide context that is closer to the clinical situation.

endpoint appropriately associated with the disease of interest. Label-free assay technologies offer additional advantages here, as they do not require assumptions about molecular mechanisms and pathways but rather allow for a multidimensional and less biased investigation [80]. In summary, label-free assay technology provides phenotypic assays that are able to acquire molecular-level understanding of complex biological processes in their native environment [6, 84]. When combined with the appropriate cellular model systems, as discussed in this thesis in e.g. **Chapter 2**, the combination offers a powerful approach for pharmaceutical research in general and precision medicine in particular.

Precision medicine prospective for GPCRs

The human genome mapping, the resulting pharmacogenetic discoveries and the ongoing movement towards precision medicine have influenced drug development in general, and hence also for GPCRs. It is increasingly recognized among the GPCR research community that tailoring a drug candidate for a particular genetic variant of a GPCR could offer various benefits [19] (**Fig. 3**). There are numerous examples of genetic variants in GPCRs altering pharmacology or pathology. In 2001, Sadee *et al.* published an exemplary catalogue of genetic GPCR variants and possible implications for drug therapy [64]. More than a decade later, the tailoring of GPCR targeting drugs based on genetic variation in patients is still deemed to be in the early stages of feasibility [34]. To name a particular example, the α_{2A} adrenergic receptor antagonist yohimibine improved insulin secretion in type II diabetes patients that were carriers of a particular SNP in this receptor [20]. Other forms of genetic variation besides SNPs have also been found to be of influence, for instance GPCR expression

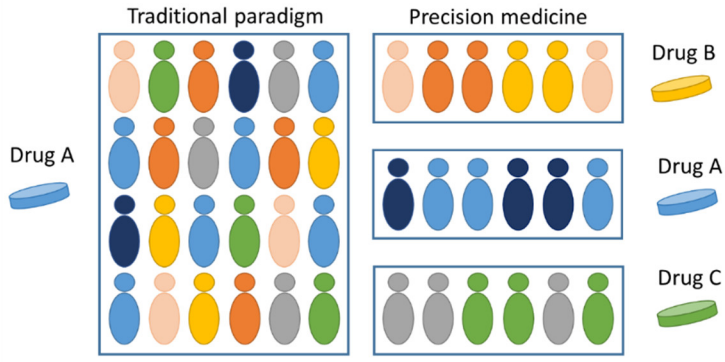

Figure 3. Precision medicine versus traditional treatment paradigm. Tailoring a drug (candidate) to patient characteristics such as genetic information can offer several benefits including decreased risks of ineffective treatment, of inappropriate dosing or of side effects [91-93].

as a biomarker for the clinical efficacy of the A_3 adenosine receptor agonist IB-MECA, or the conversion of $P2Y_{12}$ receptor prodrug Plavix being impaired by the 2C16 isoform of CYP450 enzymes [85, 86] .

The progress in precision medicine for GPCRs has come in large part through pharmacogenomic advances. Over the past two decades, the Human Genome Project, HapMap project and 1000 Genomes project have been instrumental in identifying human genetic variants contributing to common diseases [8, 79, 87]. The emergence of GWAS in 2005 has led to a surge in the successful identification of numerous disease-associated genetic loci. However useful, with GWAS genetic variants are mostly associated, not necessarily correlated with disease, as there is no clue to the underlying mechanism [79, 88]. Finally, in the past couple of years, whole-exome sequencing experiments which specifically focus on coding regions related to proteins have become available [79]. The costs of such techniques are decreasing, while patient willingness to participate is on the rise [79, 89]. Continuing these trends, first personalized whole-genome sequencing and finally, with gaining the appropriate pharmacological understanding, various forms of precision medicine may become standard clinical practice (**Fig. 3**). Before this becomes clinical reality however, there are hurdles to be overcome such as the existing skepticism by clinicians, mostly related to ethical concerns about privacy and potential discrimination of patients [79, 90]. First and foremost however remains the appropriate identification of disease-related genetic variants and corresponding implications for medical treatment. To deliver the required molecular-level understanding of genetic influences on pathology and pharmacology, more representative model systems and assay techniques are becoming available. Now is the time to employ these tools to become more familiar with the key contributing factors, establish the necessary key concepts, integrate these into target discovery and drug development and hereby lay the path towards precision medicine for GPCRs, drug targets and patients in general.

Final Notes

Altogether a novel cellular approach towards studying genetic effects on GPCR function has been explored and detailed throughout this thesis. Several GPCRs and different types of genetic variations were investigated, demonstrating together that personal cell lines in combination with label-free technology are an appropriate tool to enable GPCR pharmacogenetic studies. Incorporating aspects such as genetic variation in drug targets,

representative model systems and appropriate assay technology are important factors for advancing GPCR drug discovery. The data presented in this thesis contributes towards the progress of applying precision medicine concepts to this class of drug targets.

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