CHAPTER ELEVEN

Measuring Circadian Clock Function in Human Cells

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Abstract

Circadian clocks are present in most cells and are essential for maintenance of daily rhythms in physiology, mood, and cognition. Thus, not only neurons of the central circadian pacemaker but also many other peripheral tissues possess the same functional and self-sustained circadian clocks. Surprisingly, however, their properties vary widely within the human population. In recent years, this clock variance has been studied extensively both in health and in disease using robust lentivirus-based reporter technologies to probe circadian function in human peripheral cells as proxies for those in neurologically and physiologically relevant but inaccessible tissues. The same procedures can be used to investigate other conserved signal transduction cascades affecting multiple aspects of human physiology, behavior, and disease. Accessing gene expression

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variation within human populations via these powerful *in vitro* cell-based technologies could provide important insights into basic phenotypic diversity or to better interpret patterns of gene expression variation in disease.

1. INTRODUCTION

Enormous advances in molecular biology and genetics during the last few decades helped scientists to understand the nature and the role of biological rhythms, especially endogenously generated circadian rhythms. In mammals, a broad spectrum of physiology, metabolism, and behavior is orchestrated by this highly complex circadian system, whose architecture is organized into a hierarchy of oscillators (Gachon, Nagoshi, Brown, Ripperger, & Schibler, 2004; Reppert & Weaver, 2002). The suprachiasmatic nuclei (SCN) in the rostroventral hypothalamus—a tiny paired structure containing approximately 16,000 neurons—were identified as the "master pacemaker" that is responsible for the generation and maintenance of mammalian circadian rhythmicity. Lesions of this structure resulted in complete loss of circadian rhythmicity of laboratory animals, and its retransplantation from fetal tissue restored circadian activity rhythms with the properties of the donor (Aguilar-Roblero, Morin, & Moore, 1994; Ralph, Foster, Davis, & Menaker, 1990; Silver, Lehman, Gibson, Gladstone, & Bittman, 1990; Silver, LeSauter, Tresco, & Lehman, 1996).

Although sustained circadian rhythmicity of behavior requires an intact SCN, multiple studies proved that other parts of the brain such as thalamic nuclei, amygdala, olfactory bulbs, hippocampus, or cerebellum possess circadian oscillations in the gene expression (Guilding & Piggins, 2007; Storch et al., 2002). In fact, transcriptome analysis revealed that almost 10% of all detected genes in a variety of tissues possess circadian patterns of expression (Akhtar et al., 2002; Valnegri et al., 2011). Thus, circadian clocks are ticking not only in neurons of the brain but also in other peripheral tissues including lungs, liver, kidneys, spleen, pancreas, heart, stomach, skeletal muscle, cornea, thyroid, and adrenal gland (Balsalobre, Damiola, & Schibler, 1998; Yamamoto et al., 2004; Yamazaki et al., 2000; Yoo et al., 2004).

The molecular makeup of these peripheral oscillators is composed of interlocked positive and negative transcription/translation feedback loops and seems to be the same as the one operating in the SCN (Shearman et al., 2000; Yagita, Tamanini, van der Horst, & Okamura, 2001). In brief,

the transcriptional activators CLOCK and BMAL1 of the primary feedback loop heterodimerize and promote the transcription of target genes containing E-box *cis*-regulatory elements, including Periods (*Per1*, *Per2*, *Per3*) and Cryptochromes (*Cry1*, *Cry2*). Negative feedback is secured by PER:CRY heterodimers that translocate back into the nucleus and repress their own transcription. In a second regulatory loop, CLOCK:BMAL1 heterodimers activate the transcription of the retinoic acid-related orphan nuclear receptor *Rev-erbα*, that consequently competes with its sister RORα to bind retinoic acid-related orphan receptor response elements (RREs) in the *Bmal1* promoter. RORs activate transcription of *Bmal1*, whereas REV-ERBs repress the transcription process (Bell-Pedersen et al., 2005; Lowrey & Takahashi, 2004). In turn, the same *cis*-acting elements (E-boxes and RREs) are sufficient to promote precisely phased circadian transcription at other "clock output genes" throughout the genome (Ukai-Tadenuma et al., 2011). These transcriptional rhythms also persist in explanted and cultured tissues (Plautz, Kaneko, Hall, & Kay, 1997; Yamazaki et al., 2000) as well as in cultured cells (Balsalobre et al., 1998).

2. STUDIES OF CIRCADIAN CLOCK PROPERTIES USING REPORTERS

Since the discovery of peripheral circadian clocks, researchers have developed increasingly sophisticated tools to measure clock properties like circadian period length, phase, and amplitude, as well as coherence among clocks in different cells and tissues. While approximations of some of these parameters can be obtained by traditional biochemistry and molecular biology in serially sampled cells and tissues, such methods are seriously hampered by the resolution of manual sampling and the effort required to achieve it. For this reason, higher-throughput methodologies were rapidly developed. One such possibility, and the one principally considered in this chapter, is so-called "reporters" of transcription. Technological advances in molecular biology have afforded the opportunity to monitor eukaryotic gene expression selectively and in real time in living cells via enzymatic reactions or fluorescence. By using circadian promoters to drive expression of these reporters, rapid and high-resolution quantification of circadian rhythms *in vivo*, *ex vivo*, and *in vitro* in cultured cells can be achieved. Thus, expression of green fluorescent protein from a circadian promoter in *Drosophila* first allowed researchers to realize that independent photoreceptive clocks were present throughout the fly body (Plautz et al., 1997).

Since then, among a variety of available "reporter genes," high sensitivity, excellent dynamic range, and low toxicity have made luciferase the most commonly used bioluminescent reporter in the analysis of eukaryotic transcriptional regulation (Alam & Cook, 1990; Gould & Subramani, 1988; Williams, Burlein, Ogden, Kricka, & Kant, 1989). In the presence of oxygen, magnesium, and ATP, luciferase catalyzes the oxidative decarboxylation of beetle luciferin, resulting in the light emission in the 560 nm wavelength that can be easily quantified directly from living cells. Therefore, circadian promoter-luciferase reporters have proven useful tools for circadian biologists, reporting such basic clock properties as a period length, phase, and amplitude via bioluminescent reporting of circadian gene expression (Cuninkova & Brown, 2008).

Using this technology, it was rapidly apparent that circadian rhythms in peripheral tissue cultures dampen after a few cycles, whereas SCN explants possess self-sustained oscillators (Yamazaki et al., 2002). However, a closer look at cultured cells in single-cell resolution revealed that dampening of rhythmicity in peripheral cell types is rather a question of intercellular desynchrony than the loss of individual cellular rhythms (Nagoshi et al., 2004; Welsh, Yoo, Liu, Takahashi, & Kay, 2004). Even apparently low amplitude of transgenic reporter expression in some tissues like the liver and lungs can persist over 20 daily cycles in individual cells (Yoo et al., 2004). Not only various mammalian tissue explants but also many cultured primary and immortalized cell lines maintain robust circadian oscillation. Cells like rodent and human primary and embryonic fibroblasts, NIH/3T3-immortalized fibroblasts, some human cancer lines like U2OS osteosarcoma cells, and human peripheral blood mononuclear cells all reveal robust circadian gene expression *in vitro* (Akashi & Nishida, 2000; Balsalobre et al., 1998; Boivin et al., 2003; Brown et al., 2005a; Hughes et al., 2009; Keller et al., 2009).

Under normal *in vitro* conditions, these oscillations are mostly desynchronized. However, these oscillations can be transiently synchronized by treatment with a high concentration of serum or a full spectrum of chemical compounds (e.g., forskolin, dexamethasone, retinoid acid, glucose, prostaglandin) acting on variety of signaling pathways (protein kinase A, protein kinase C, mitogen-activated protein kinase) via both transmembrane G-protein-coupled receptors and nuclear hormone receptors (Balsalobre et al., 2000; Balsalobre, Marcacci, & Schibler, 2000; Yagita & Okamura, 2000). Thus, circadian clocks in cultured cells also display a full range of input pathways from the environment to the oscillator itself.

Beyond an ability to be synchronized by outside stimuli, most other features of circadian clocks in organisms have also been demonstrated in cultured cells. One such property is temperature compensation. While many biochemical processes increase with rising temperature, the circadian period length remains mostly constant or even slightly faster with lower temperature both in organisms and in cultured fibroblasts (Dibner et al., 2008; Izumo, Johnson, & Yamazaki, 2003; Reyes, Pendergast, & Yamazaki, 2008). In both contexts, these rhythms can also be entrained by rhythmic temperature variations. In culture, even simulated body temperature rhythms of mice and humans, with daily temperature variation of only 3 °C, will gradually synchronize circadian gene expression in cultured fibroblasts (Brown, Zumbrunn, Fleury-Olela, Preitner, & Schibler, 2002; Saini, Morf, Stratmann, Gos, & Schibler, 2012).

Thus, at a cell-autonomous level, circadian clocks in cultured cells, in tissues, and in whole organism display surprisingly homogeneous properties. However, one key difference lies in "rigidity," the ability of circadian clocks to adapt to imposed environmental stimuli or else to resist them. Comparing circadian clocks in cultured SCN slices to those from other tissues or from cells, the SCN is considerably more rigid and adapts to a much narrower range of environmental period lengths. This property is directly dependent upon the tight coupling of SCN neurons to each other by synaptic and neurochemical connections. By contrast, peripheral tissues and cultured cells will adapt to a much broader range of periods (Abraham et al., 2010). While this is a highly artificial situation—a normal day is always 24 h long—the same rigidity and coupling also affect resilience to mutation. Thus, SCN cultures from mice with genetically impaired clocks, or the intact animals themselves, typically show much smaller changes in clock properties than do peripheral tissues or cultured cells from the same animals (Brown et al., 2005a; Liu et al., 2007).

This tight coupling between neurons of the SCN also explains why SCN explants show much less dampening of circadian oscillations in culture than do peripheral tissues or cultured cells. Dispersal of neurons in these explants results in weakening of rigidity as well as more rapid dampening (Liu et al., 2007). In fact, it has been postulated that SCN neurons possess intrinsically less robust clocks than peripheral cells. Indeed, dispersed cultures of SCN neurons can be largely arrhythmic in culture, and individual cells gain and lose rhythmicity in a process likely dependent upon cell-intrinsic "noise" (Webb, Angelo, Huettner, & Herzog, 2009).

3. *EX VIVO* AND *IN VITRO* STUDIES OF HUMAN CIRCADIAN CLOCKS

For most of the studies outlined above, transgenes were used to obtain laboratory animals containing the desired reporters. However, the same technologies can also be used to look at circadian clocks in cells from organisms not easily accessible to transgenic manipulation, including humans. To deliver such reporters into cells for stably sustained gene expression, lentivirus-mediated gene transfer offers many advantages. Beside their heritable and potentially long-lasting gene expression, these vectors possess a large packaging capacity and the ability to infect a variety of dividing and nondividing cells. Since human primary cell cultures are quite resistant to transient transfection technologies and the large amount of introduced DNA can itself alter gene expression, a lentiviral delivery system became a reasonable choice for many circadian as well as noncircadian experiments (Salmon & Trono, 2001).

Conservation of circadian oscillators in the periphery gives enormous advantages in determining circadian properties using tissue explants as well as cultured cells. Almost a decade ago, our laboratory developed lentiviruses containing a luciferase gene whose expression is driven by the promoter and 3′-untranslated regions of the mouse *Bmal1* circadian gene that allows the measurement of interindividual variation in circadian rhythms directly in human skin biopsy fibroblasts (Fig. 1). By introducing a stably integrated lentiviral luciferase circadian reporter, we showed large interindividual differences in the circadian period length. The same reporter system was employed in mouse circadian mutants and revealed the correlation between the circadian period length from mouse fibroblast cultures and behavioral

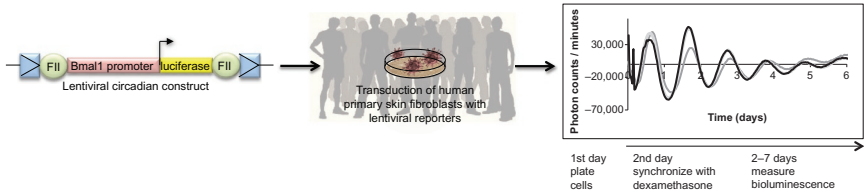

Figure 1 Measurement of human circadian properties *in vitro*. Human primary fibroblasts isolated from skin biopsies are transduced by lentiviruses containing a luciferase gene whose expression is driven by the promoter and 3′-untranslated regions of the mouse *Bmal1* circadian gene. After chemical synchronization of circadian clocks in these cells, real-time bioluminescence measurements are performed in subsequent days.

rhythms of these clock mutants (Brown et al., 2005b). Moreover, it was revealed that there exist individual differences in circadian period, phase-shifting responses and amplitude in fibroblasts from human subjects, and these differences are correlated with diurnal phase preferences in individual subjects (Brown et al., 2008; Hida et al., 2013). Later, Pagani and colleagues measured circadian period in human primary fibroblasts and for the first time showed the positive correlation between the circadian period length in physiology and the gene expression of fibroblasts measured via lentivirally delivered circadian reporters (Pagani et al., 2010). The same technology has been used in cells from reindeer showing little circadian activity in the arctic summer in order to suggest a cellular basis for this circadian suppression (Lu, Meng, Tyler, Stokkan, & Loudon, 2010).

Reporters can also be used in reverse, as a screening tool to identify factors affecting circadian function in cells. For example, circadian reporters in primary fibroblasts were used to screen serum from older and younger individuals with the aim to identify serum factors contributing to circadian disturbances in elderly individuals (Pagani et al., 2011). On a much larger scale, optimized U2OS reporter lines have been screened with RNA interference "hairpins" that reduce expression of each endogenous RNA in order to determine the set of genes important for circadian function (Maier et al., 2009; Zhang et al., 2009), or with small-molecule drugs in order to find possible targets of pharmacological intervention within the circadian clockwork (Chen et al., 2012; Hirota et al., 2010).

Recently, circadian reporter technologies, as well as conventional transcriptional measurements, have also been applied to primary cells from various patient groups such as those with bipolar disorder (Bamne et al., 2013; McCarthy et al., 2013; Yang, Van Dongen, Wang, Berrettini, & Bucan, 2009) and used to measure circadian effects of treatments such as lithium or valproic acid (Abe, Herzog, & Block, 2000; Johansson, Brask, Owe-Larsson, Hetta, & Lundkvist, 2011; Li, Lu, Beesley, Loudon, & Meng, 2012; Osland et al., 2011). In the future, differences observed in these cellular readouts could furnish important endophenotypes in these heterogeneous diseases.

4. SIMILAR TECHNOLOGIES TO STUDY OTHER MAJOR SIGNALING PATHWAYS

Altogether, luciferase reporters have found applications in many different circadian studies, some using the simplest microorganisms and others

in mammalian species. However, the direct circadian clock circuits targeted by existing reporters are only a fraction of the many signaling cascades contributing to the diurnal regulation of human physiology and behavior. The same technologies developed for the circadian clock could be equally applied to other signaling cascades with transcriptional outputs: for example, MAP-kinase pathways, the inflammatory responses (IFN-γ), immune system responses (NFAT and NFkB), as well as apoptosis (p53). For this purpose, we recently developed a novel autonormalized lentivector-based system which allows one to detect real-time drug-induced activation of various signaling pathways directly in human primary cell lines (Fig. 2) (Gaspar et al., 2014).

With this system, surprisingly large interindividual differences in several major signaling pathways were uncovered, and these reporter-based measures correlated with cellular measures of genome-wide transcription and drug toxicity. Moreover, these differences also correlated with actual human physiology: the study of cAMP/CREB signaling, known to be important to circadian hormonal variation and to synaptic plasticity, revealed that the magnitude of forskolin-activated CREB signaling in human primary skin fibroblasts correlates with both light-dependent suppression of the circadian hormone melatonin in healthy subjects and bipolar disorder in affected individuals (Gaspar et al., 2014). Overall, these data suggest that genetic differences in major signaling pathways including circadian clocks can be reliably

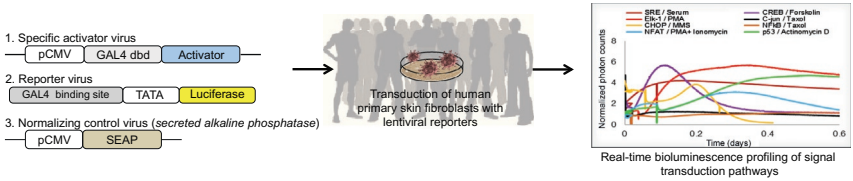

Figure 2 Real-time bioluminescence monitoring of signal transduction pathways. Human primary skin fibroblasts are transduced with the three different lentiviral constructs—a *specific activator virus* containing the human cytomegalovirus (CMV) immediate early promoter and GAL4 DNA-binding domain fused with a pathway—specific transcriptional activator (CREB, Elk1, CHOP, c-jun, NFAT, etc.); a *reporter virus* carrying five tandem repeats of the yeast GAL4 DNA-binding site that controls the expression of the luciferase gene; and a *normalizing control virus* encoding secreted alkaline phosphatase produced under the control of the CMV promoter. Cotransduction of mammalian cells with a set of these lentiviruses and subsequent addition of a specific extracellular stimulus results in activation of a cellular signaling cascade, finally potentiating the transcriptional activation of the *Reporter* by the *Specific Activator*, resulting in the transcriptional activation of the luciferase gene.

unearthed by sensitive viral-based reporter profiling and that these differences can be conserved across tissues and most probably predictive of physiology and disease susceptibility.

5. CELL-BASED APPROACHES TO STUDY GENE EXPRESSION VARIATION AND HUMAN INTERINDIVIDUAL DIFFERENCES IN DRUG RESPONSES

Unlike various model organisms that can be repeatedly exposed to drugs or toxins in the laboratory and subsequently phenotyped, humans pose much greater difficulties to determine either natural or disease-induced differences in pharmacological response. Besides ethical and safety issues, there is usually an inability to control different *in vivo* environmental factors as well as difficulty to manipulate the *in vivo* system to evaluate biological changes. Due to these restrictions, *in vitro* cell-based approaches became a rational solution to overcome these problems. Various human cell lines have been already used for human disease modeling as well as in preclinical drug development to determine drug-induced cell growth inhibition or cell death, or to identify interactions between the target compounds and transporter proteins or enzymes involved in drug metabolism.

Moreover, in recent years, there has been a rising utilization of lymphoblastoid cell lines (LCLs) as accessible and renewable resources for functional genomic studies in humans. Although, LCLs were originally established as DNA sources (Carl, Kroll, Bux, Bein, & Santoso, 2000), they are now extensively employed in studies of the genetic and epigenetic determinants of gene regulation (Cheung et al., 2005; Dixon et al., 2007; Stranger, Forrest, et al., 2007; Stranger, Nica, et al., 2007; Veyrieras et al., 2008), as well as for the exploration of the host responses to different perturbations or treatments, such as transcript depletion (Badhai et al., 2009), radiation (Correa & Cheung, 2004; Niu et al., 2010), and drugs (Duan et al., 2007; Huang et al., 2007). Since genetic factors have been shown to play an important role in differential drug response among human individuals, the availability of extensive genotype data for many panels of human LCLs allows easy study of genetic variants contributing to interindividual variation in susceptibility to drug (Wheeler & Dolan, 2012).

Unfortunately, in our own experience, LCLs show both very poor transfectability and infectability and lack robust circadian oscillations at a population level. Therefore, they are decidedly less suitable for real-time

lentivirus-based studies of human circadian biology than other non-immortalized primary cell types. Although circadian gene expression of primary B lymphocytes or peripheral blood mononuclear cells (of which B cells comprise the major population) was reported in several studies (Archer et al., 2014; Silver, Arjona, Hughes, Nitabach, & Fikrig, 2012), our findings suggest that biological clocks might have been attenuated by simple Epstein–Barr virus-mediated transformation of B cells. This transformation technique creates easy to maintain highly proliferative LCLs that showed significant genetic instability with a somatic mutation rate of 0.3% (Mohyuddin et al., 2004). Either this high mutation rate, or the high proliferation *per se*, or a combination of the two may have significant effects on the function of circadian clock genes. Regardless of the ultimate cause, however, circadian studies of these cells in our hands have proven unfruitful.

Along with the cell lines derived from healthy human subjects, cells obtained from diseased tissues such as NCI-60 bank of cancer cell lines have proven to be useful models for the investigation of direct effects of drugs on a variety of tumor tissue types. As with LCLs, mRNA expression (Ross et al., 2000; Scherf et al., 2000), SNP genotype data (Garraway et al., 2005), and proteomic data (Nishizuka et al., 2003) are available for these cell lines. Usage of this cell-based disease model has resulted in the identification of transcription factors that predict sensitivity to chemotherapeutics as well as proteomic and microRNA profiles predicting drug response (Dai, Barbacioru, Huang, & Sadee, 2006; Shankavaram et al., 2007; Staunton et al., 2001; Weinstein et al., 1997). Polymorphisms in candidate genes associated with drug response *in vitro* have been successfully identified as well (Le Morvan et al., 2006; Puyo, Le Morvan, & Robert, 2008; Sasaki, Kobunai, Kitayama, & Nagawa, 2008). For example, a combination of SNP data and gene expression profiles for this panel of tumor cell lines revealed that cytotoxicity to the alkylphospholipid analog *perifosine* is associated with MAPK and apoptotic pathways (Zhang, Liu, Poradosu, & Ratain, 2008). In principle, circadian factors could easily be factored into such cellular model systems.

Self-sufficiency in growth signals, insensitivity to the growth inhibitory signals, evasion of apoptosis, limitless replicative potential, tissue invasion, and metastases are all hallmarks of human tumorigenesis that have been found to be major signatures of deregulated cellular signal transduction cascades. Although these interconnected signaling events are being slowly elucidated, understanding the alterations that lead to cancer still remains a substantial challenge. Therefore, the same reporter technologies could be equally applied to trace and study the behavior of signaling pathways within developing and progressing tumors (Fig. 3). Since cancer progression is a multilevel process that

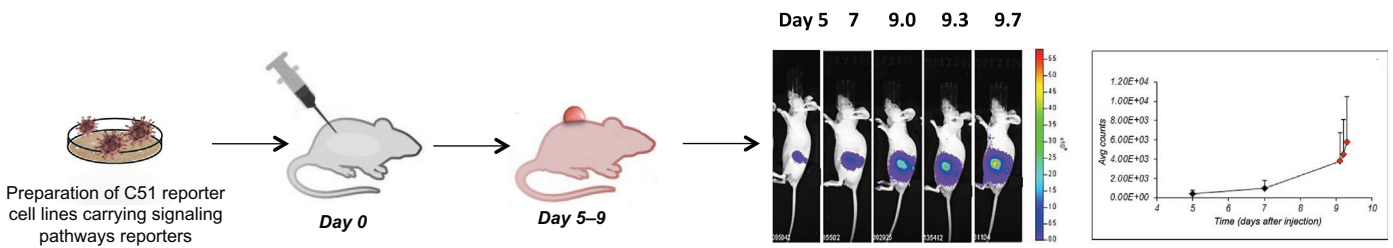

Figure 3 Longitudinal monitoring of signaling transduction pathways behavior within growing tumors. Mouse colon carcinoma cell lines are initially transduced with signaling pathways reporters and used for the inoculation of subcutaneous tumors in nude mice. Within the range of 15 days, *in vivo* bioluminescence measurement of signaling pathway behavior is monitored in anesthetized mice after injection of luciferin substrate.

involves highly organized signal transduction pathways, better understanding the basis of the signal transduction alteration will certainly bring more insights into the variability of cancer pathogenesis among the human populations as well as improve current approaches for cancer therapy.

The final source of cells used to study human expression variation that we shall consider here is represented by induced pluripotent stem cells (iPSCs) (Lowry et al., 2008; Takahashi et al., 2007; Takahashi & Yamanaka, 2006; Yu et al., 2007). Recently, iPSCs have opened a new avenue of cell-based approaches applicable in drug discovery, in *in vitro* disease modeling, as well as in cell replacement therapy. Recent studies demonstrated that the recapitulation of *in vitro* disease phenotype via iPSCs seems to be feasible for numerous monogenic diseases (Carvajal-Vergara et al., 2010; Ebert et al., 2009; Lee et al., 2009; Rashid et al., 2010; Urbach, Bar-Nur, Daley, & Benvenisty, 2010; Zhang et al., 2011). The availability of such disease-relevant pathological cells obtained directly from the patient might benefit drug discovery. Thus, "a patient in a dish" represented by iPSCs seems to have a promising perspective in the discovery of various drug effects and drug toxicity among the population of patients. Although iPSCs and embryonic stem cells possess no functional circadian oscillators, differentiated or pluripotential stem cells derived from them, like neural precursor cells, already possess functional circadian clocks (Kowalska, Moriggi, Bauer, Dibner, & Brown, 2010; Yagita et al., 2010). Therefore, these cells too could serve as potential systems in which to study variations in circadian clock function in different groups.

6. PROMISE OF *IN VITRO* GENE EXPRESSION PROFILING

Given the wide variations in the human genome, it is not surprising that for any individual tissue, variations have also been observed in the transcriptome (the sum of all RNAs transcribed from cellular DNA), the proteome (the sum of all proteins made from the RNAs), and the metabolome (the sum of all small molecules catalytically created by the proteins). Recently, investigators have taken such analyses one step farther, treating the expression levels of transcripts, proteins, and metabolites among various individuals as quantitative traits in themselves, and relating them to particular genetic polymorphisms. Such "eQTLs" (expression quantitative trait loci) have been heralded to hold great promise for the elucidation of the causes

of disease, based upon the assumption that polymorphisms in regulatory proteins, binding sites, or genes are likely to be responsible for heritable variation in gene expression (Monks et al., 2004; Petretto et al., 2006). eQTLs mapping has been already applied to a number of species ranging from yeast to humans as a powerful tool for determining various regulatory associations (Brem & Kruglyak, 2005; Bystrykh et al., 2005; Chesler et al., 2005; Cheung et al., 2005; DeCook, Lall, Nettleton, & Howell, 2006; Jin et al., 2001; Petretto, Mangion, Pravanec, Hubner, & Aitman, 2006; Schadt et al., 2003; Stranger, Nica, et al., 2007). Nevertheless, due to limitations in sample size coupled with often very small effects, such studies have been notoriously variable and often yield quite large eQTL regions (up to several centimorgans) (Michaelson, Loguercio, & Beyer, 2009), leading some prominent investigators even to question their diagnostic potential (Altshuler, Daly, & Lander, 2008). For this reason, several strategies have been used to improve accuracy and narrow down the candidate causal gene regions, including traditional but frequently infeasible "fine mapping" (Bing & Hoeschele, 2005; Stuart, Segal, Koller, & Kim, 2003; Visscher, Thompson, & Haley, 1996), likelihood-based selection (Kulp & Jagalur, 2006), and pathway-based approaches founded on the assumption that target gene expression correlates with the activity of other genes in the same pathway (Tu, Wang, Arbeitman, Chen, & Sun, 2006). Such pathway-based approaches have been particularly useful for eQTL-based pharmacology, where drugs applied to cells are known to elicit responses in particular signaling cascades (Wen et al., 2012). eQTL approaches in humans, though promising, are subject to a serious limitation that only a few tissues are readily available, such as blood and skin, except in certain clinical situations where biopsy is routine (in cancer, for example). Nevertheless, several recent eQTL studies revealed that the gene expression levels generated from various human samples including LCLs (Dixon et al., 2007; Stranger, Nica, et al., 2007), liver tissues (Schadt et al., 2008), or primary lymphocytes (Goering et al., 2007) could be vigorously associated with *cis*-acting genetic variants. However, usage of immortalized versus primary cell lines for eQTL mapping has raised significant contradictions. While some studies highlighted cell type-specific effect, others found a significant amount of shared eQTLs across tissues (Dimas et al., 2009; Emilsson et al., 2008). Other studies comparing the overlap of eQTLs found in LCLs and primary tissues revealed that some (Bullaughey, Chavarria, Coop, & Gilad, 2009; Ding et al., 2010; Nica et al., 2011) but

not all (Min et al., 2011) eQTLs detected in LCLs can be detected in even the same primary tissue. Hence, even though the same signaling pathways are widely conserved in all tissues, application of eQTL technologies to human populations effectively remains an important challenge. In principle, use of reporter-based technologies such as those that we describe could provide important tools for lessening the noise inherent in single gene-based expression quantitative traits, allowing for more significant imputations of functional genetic variation.

7. SPECIFIC PROTOCOLS

7.1. Cell lines for the measurement of human circadian properties

Since the characterization of human circadian properties *in vivo* under laboratory conditions is quite laborious and expensive, the conservation of circadian oscillators provides the convenient possibility to study human circadian properties directly in primary cells. Various cell lines such as blood monocytes (Keller et al., 2009), hair follicle cells (Akashi et al., 2010), and keratinocytes have been used; we prefer human primary skin fibroblasts due to their easy isolation, cultivation, and high amplification potential. Surprisingly, adult skin fibroblasts appear to have higher amplification potential than those derived from umbilical cord. As described previously (Brown et al., 2005a), we derive human primary fibroblasts from 2 mm round skin biopsies taken from the upper arm. After partial tissue digestion (performed during 4–7 h in 2 ml of DMEM/high-glucose medium containing 10% FBS, 1% penicillin/streptomycin, 1% gentamycin, and 0.325 WU/ml Liberase Blendzyme3), all tissue fragments are washed in 10 ml of warm 1× PBS and centrifuged for 5 min at low speed. 0.2 ml of normal growth DMEM/high-glucose medium containing 20% FBS, 1% penicillin/streptomycin, 1% gentamycin, and amphotericin B is used to transfer the tissue to a 4-cm Petri dish treated for cell culture. Tissue is subsequently overlaid by a Millicell CM membrane disc (MILLIPORE POCMORG50) without spacer "feet," and 1.5 ml of the growth medium is added to the interior and 0.5 ml to exterior of the disc. The plate is then incubated without disturbance at 37 °C, 5% CO_2. The medium should be changed every 3–4 days. Amphotericin B can be omitted from the medium and the Millicell membrane gently removed 1 week after biopsy. Length of the culture will vary based upon the number of viable fibroblast foci. Once fibroblasts cover approximately 25% of the culture surface,

they can be trypsinized and replated to fresh dishes normally, being careful not to oversplit them. Isolated fibroblasts are usually split once or twice per week 1:2 by trypsinization.

7.2. Generation and production of lentiviral vectors

The construction of lentiviral circadian reporter constructs that carry circadian reporters such as *Bmal1*-luciferase has been described previously in Brown et al. (2005b), and the cell-based reporter system used for the measurement of the transcriptional activation of multiple signaling pathways is described in Gaspar et al. (2014).

All viruses are produced, concentrated 10-fold by ultracentrifugation, and used for transduction according to standard protocols (Cepko, 2001; Salmon & Trono, 2001). In brief, the production of VSV-G pseudotype lentivirus vectors is based on the transient transfection of 293T cells. These cells are usually split so that they are semiconfluent at the day of transfection. Plates should contain 9 ml of DMEM medium containing 10% FBS and 1% penicillin/streptomycin. For one 10-cm plate to be transfected, the DNA mixture comprises 14 μg coding plasmid, 10 μg of packaging plasmid psPAX2, 6 μg of coat protein plasmid pMD2G, 100 μl of $CaCl_2$, and water buffered by HEPES (pH 7.05) within the final volume of 500 μl. 0.5 ml of DNA mixture is added to 0.5 ml of HeBS solution (0.283 *M* NaCl, 0.023 *M* HEPES acid, 1.5 m*M* Na_2HPO_4, pH 7.05) and the mixture is incubated for 30 min at room temperature. When fine translucent precipitate is formed, the mixture is added dropwise to the plate of cells. Twenty-four hours after transfection, medium is aspirated, cells are washed once with 1 × PBS, and 10 ml of normal growth DMEM medium containing 10% FBS, 1% penicillin/streptomycin, and supplemented by 20 m*M* HEPES is added. The virus is harvested twice, at 48 and 72 h posttransfection. Ultimately, supernatant containing viral particles is concentrated by ultracentrifugation 30,000 rpm for 90 min at 4 °C (in Beckman SW28 rotor) and resuspended overnight in the desired volume of normal growth medium by medium speed vortexing in an automatic vortexer at 4 °C.

7.3. Transduction of primary skin fibroblasts and real-time bioluminescence measurement of human circadian properties *in vitro*

One day before transduction, human primary skin fibroblasts are split to be 50% confluent. On the day of transfection, growth medium is removed

and warmed, and 10-fold concentrated viral supernatant is added to cells for 6–24 h in the presence of 8 μg/ml protamine sulfate. Antibiotic selection can be started 3–4 days posttransduction to select stable transformants.

To measure circadian properties such as the period length, phase, and amplitude, cells are synchronized with synthetic glucocorticoid dexamethasone (1 μ*M*) for 20 min or with 50% FBS for 30 min. Medium containing dexamethasone is aspirated, and cells are washed twice with 1 × PBS. The real-time bioluminescence measurements are done for up to 7 days in normal culture medium (DMEM medium, 10% FBS, 1% penicillin/streptomycin) lacking phenol red but supplemented with the 0.2 m*M* substrate luciferin and 25 m*M* HEPES as described previously (Nagoshi et al., 2004).

7.4. Bioluminescence measurement of the transcriptional activation of different signaling pathways

Semiconfluent plates ($2.5–5 \times 10^5$ cells in 35-mm Petri dish) of primary fibroblasts transduced and selected for the lentiviral luciferase reporter construct carrying puromycin resistance (pLDEST-FRLuc-Puro) are additionally cotransfected with the activator (pLDEST-CREB-Hygro, pLDEST-Elk-1-Hygro, or pLDEST-CHOP-Hygro) and normalizing control lentivirus vector (pLDEST CMV-SEAP Hygro) without selection as described for the circadian lentiviral construct. Prior to bioluminescence measurement of the transcriptional activation of signal transduction pathways, aliquots of the medium taken from the fully confluent samples are used to detect levels of secreted alkaline phosphatase (we use the Phospha-Light™ Secreted Alkaline Phosphatase Reporter Gene Assay System, Cat. No. #T1015). Ultimately, DMEM/high-glucose medium, without phenol red, 10% KnockOut™ Serum Replacement, 1% penicillin/streptomycin, 1% gentamycin, and 0.1 m*M* luciferin, is applied 24 h prior to measurement and used to measure basal levels of the light emission during 20–30 min on the day of induction. A suitable serum-free medium is required prior to measurement due to the large number of signaling molecules present in serum. Subsequently, a pathway-specific drug stimulus is added to the tested samples. Each obtained signal is normalized by dividing the activator activity (maximal photon count value of pathway induction) by the control reporter activity (photon counts corresponding to secreted alkaline phosphatase values).

7.5. Use of lentiviral reporters in longitudinal *in vivo* imaging of signaling pathways within a growing tumor

The induction of signaling pathways within cancer is often the result of genomic alterations such as mutations, translocation, and copy number gains or losses in crucial components of signaling pathways. Many studies have already demonstrated the potential of gene expression profiling for the analysis of oncogenic pathways, and these data have helped to define cancer subtypes, likelihood of disease recurrence, and response to specific therapies (Huang et al., 2003; Ramaswamy & Golub, 2002; Segal, Friedman, Koller, & Regev, 2004). However, these DNA microarray-based gene expression signatures suffer from the fact that most of them reflect activity of an entire tissue at a single point in time. Therefore, reporter systems and optical imaging technologies are becoming more often used to study tumorigenesis (Lehmann et al., 2009). In our own studies, we combined a lentiviral cell-based reporter system with a long-term *in vivo* imaging technique to reveal the behavior of principal signal transduction pathways within developing tumors. The big advantage of this noninvasive imaging technique arises from longitudinal and dynamic monitoring of cellular and molecular processes in the same subject. In the experiment presented in Fig. 3, semiconfluent plates ($2.5–5 \times 10^5$ cells in 35-mm Petri dish) of C51 mouse colon adenocarcinoma cells were transduced with ELK1 signaling pathway reporters. We prefer to infect a large volume of cells to create most of the quantity needed for each experiment, rather than infecting a small number of cells and running the risk of clonal amplification or enrichment. Eight-week-old BALB/C nude mice (Janvier, France) were gas anesthetized by isoflurane, and 1×10^6 C51 cells stably transduced with lentiviral reporters were injected subcutaneously on the left flank of the mouse. The subsequent imaging of tumor transcriptional activity 5–14 days later was done as previously described by Lehmann et al. (2009). Briefly, mice were anesthetized by isoflurane. Each mouse was i.p. injected with 100 μl of luciferin in PBS (15 mg/ml; Caliper Life Sciences). Ten minutes later, the animals were placed into the light-tight chamber consisting charge-coupled device imaging camera, IVIS 100; Xenogen. Bioluminescence measurement was done in 5 and 300 s intervals. Collected images were analyzed with Living Image software (Xenogen) and IGOR image analysis software (Xenogen). Total photon counts were determined by drawing a region of interest (ROI) around the peak of photon emission. The border of an ROI is formed by those pixels whose signal intensity was 5% of the maximal

signal in the ROI. To correct for the loss of signal associated with bigger tumor volumes, total counts from tumors were normalized to pcDNA3.1-luciferase control tumor counts (cytomegalovirus (CMV)-expressed luciferase devoid of pathway-specific responses).

The same techniques have also been used to monitor circadian function within tumors and in surrounding tissues (Geusz, Blakely, Hiler, & Jamasbi, 2010; Gross, Abraham, Prior, Herzog, & Piwnica-Worms, 2007; Hiler, Greenwald, & Geusz, 2006). However, these experiments are somewhat confounded by the need to anesthetize animals for imaging, since anesthetics themselves can alter clock function (Anzai et al., 2013). Recently, bioluminescent reporter measurement has also been performed in freely moving animals using photomultiplier tubes placed above highly darkened cages. Although signal intensity varies with source distance, a simple moving average creates stable values from even freely moving animals (Saini et al., 2013).

8. PERSPECTIVES

The importance of natural gene expression variation to differences in human behavior and physiology is by now undisputed, and eQTL studies have shown not only that gene expression levels differ among individuals, but have also revealed unexpected and interesting aspects of gene regulation (Stranger, Nica, et al., 2007; Yan, Yuan, Velculescu, Vogelstein, & Kinzler, 2002). The profiling technique for the analysis of cellular circadian, as well as other major signaling pathways, could have a significant impact not only for basic research but as well for its application in medicine. In the area of basic research, identification of genetic modifier loci that might correlate with a variety of difficult-to-access behavioral phenotypes (daily behavior, memory consolidation, or mood) will bring more insights into the knowledge of natural human variability regulated by the complex operation of these pathways.

Apart from basic research, however, *in vitro* cell-based screening technologies could be directly applicable to the fields of pharmacology and medicine. The last century has been devoted to the discovery of new medical treatments of scores of human diseases. Nevertheless, most drugs do not act equally upon all people. Increasing evidence of interindividual differences in pharmaceutical drug treatment has greatly increased interest in the field of so-called personalized medicine. Until now, two main factors are thought to be responsible for variability in drug toxicity and treatment efficacy: first is a matter of timing (e.g., chronopharmacology; timed

treatment) and the second is the actual existence of interindividual variation in human physiology (e.g., inherited metabolic differences, genetic polymorphism in drug-metabolizing enzymes or target receptors, etc.) (Evans & Johnson, 2001).

Results obtained from the studies using cell-based signaling pathway reporter systems suggested trait-like behavior or interindividual differences in signal transduction pathways in peripheral cells. Since the behavior or expression pattern of these pathways is, in fact, a response to various pharmacological stimuli, it is possible that the signals obtained from these assays could be used equally as valuable biomarkers for individual therapeutic response, and for the importance of variations in circadian clock function in aspects of disease-related processes.

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