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SPECIAL ISSUE REVIEW

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Steven A. Brown Special Issue: Dynamic Interactions of Biological Clocks, Sleep and Metabolism

Human primary cells can tell body time: Dedicated to Steven A. Brown

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Abstract

The field of chronobiology has advanced significantly since ancient observations of natural rhythms. The intricate molecular architecture of circadian clocks, their hierarchical organization within the mammalian body, and their pivotal roles in organ physiology highlight the complexity and significance of these internal timekeeping mechanisms. In humans, circadian phenotypes exhibit considerable variability among individuals and throughout the individual's lifespan. A fundamental challenge in mechanistic studies of human chronobiology arises from the difficulty of conducting serial sampling from most organs. The concept of studying circadian clocks in vitro relies on the groundbreaking discovery by Ueli Schibler and colleagues that nearly every cell in the body harbours autonomous molecular oscillators. The advent of circadian bioluminescent reporters has provided a new perspective for this approach, enabling high-resolution continuous measurements of cell-autonomous clocks in cultured cells, following in vitro synchronization pulse. The work by Steven A. Brown has provided compelling evidence that clock characteristics assessed in primary mouse and human skin fibroblasts cultured in vitro represent a reliable estimation of internal clock properties in vivo. The in vitro approach for studying molecular human clocks in cultured explants and primary cells, pioneered by Steve Brown, represents an invaluable tool for assessing inter-individual differences in circadian characteristics alongside comprehensive genetic, biochemical and functional analyses. In a broader context, this reliable and minimally invasive approach offers a unique perspective for

Abbreviations: ADHD, attention deficit hyperactivity; BMAL1, Basic Helix–Loop–Helix ARNT Like 1; cAMP, cyclic adenosine monophosphate; CLOCK, Circadian Locomotor Output Cycles Protein Kaput; CREB, response element-binding protein; CRY, cryptochrome; DSWPD, delayed sleep–wake phase disorder; FCS, foetal calf serum; IH, idiopathic hypersomnia; LUC, luciferase; MCTQ, Munich Chronotype Questionnaire; N24SWD, non-24-h sleep–wake rhythm disorder; PER, period; SAD, seasonal affective disorder; SCN, suprachiasmatic nucleus; T2D, type 2 diabetes; TTFL, transcription/translation feedback loop.

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unravelling the functional inputs and outputs of oscillators operative in nearly any human tissue in physiological contexts and across various pathologies.

KEYWORDS

circadian bioluminescence, circadian clockwork, human diseases, human primary skin fibroblasts, Steven A. Brown

In Memoriam

This review is dedicated to the memory of Steven A. Brown, a brilliant scientist, whose originality, scientific liberty and creative ingenuity pushed the boundaries of the circadian field. To Steve, for whom neither scientific challenges nor mountain peaks were beyond reach. To Steve, who, along with circa diem, embraced the spirit of carpe diem. To Steve, who brought a novel twist to science and a new taste to the art of winemaking with talent and grace, raising both scientific and "douceur de vivre" experiences to extraordinary heights. To Steve, whose aristocratic, generous and magnetic personality captivated those who had the privilege of orbiting in his sphere, ensuring they remained there for good. Remembering and grateful forever, for scientific and personal inspiration, and for the wonderful friendship.

1 | INTRODUCTION

A year after the tragic disappearance of Steven A. Brown, a very dear colleague and friend to many of us, it feels incredibly sad and surreal to write a review in Steve's honour dedicated to his scientific endeavours, instead of doing science together with Steve. Despite the sadness and immense void that Steve's disappearance has left in our hearts and in the field of chronobiology altogether, his scientific and personal inspiration will stay with us for good. In this review, we highlight one of the numerous research lines pioneered by Steve, based on his initial discovery that primary skin fibroblasts cultured and synchronized in vitro serve a reliable proxy for animal and human clock studies in vivo, and discuss its wide implementations for chronobiology.

By the time Steve joined the laboratory of Ueli Schibler in Geneva for his postdoctoral training, the fundamental discovery of the cell-autonomous nature of the peripheral clocks by Aurelio Balsalobre had been under the way (Balsalobre et al., 1998), followed by uncovering the mechanisms of these cellular clock entrainment, where Steve took an active part (Balsalobre et al., 2000). Further, the development of fluorescence- and luminescence-based circadian reporters allowing to continuously follow the molecular clocks in cultured cells with high temporal resolution at the population and single-cell levels opened a new horizon for the circadian field (Nagoshi et al., 2004). Steve was thrilled to tackle the implementation of this newly discovered fundamental property of the peripheral oscillators' cell autonomy, allowing the disentangling of the clock properties in the cultured cells synchronized in vitro for measuring the whole-body clockwork in vivo (Brown et al., 2005). This research line was one among several creative scientific directions developed by Steve during his postdoctoral work. The latter is highlighted in the review by Ueli Schibler "Steven A. Brown and the synchronization of circadian rhythms by body temperature cycles" in this issue.

While Steve's extraordinary intellect and scientific creativity shaped diverse fundamental research directions within the fields of chronobiology and sleep, his generous way of collaborating allowed to widely implement his discoveries. Indeed, this approach has been further developed by Steve's own group and by many other researchers and applied for the wide variety of fundamental and clinical research questions, in rodents and in humans. We thus summarize the extensive implementation of Steve's initial discovery made during his postdoc by his own group and by numerous other groups in the field for a vast variety of research questions.

The authors feel privileged and humbled to either have the opportunity to collaborate with and learn from Steve (C. D. and G. K.), or to develop their research inspired by the initial discovery by Steve presented here (A. D. B. and C. J.-S.).

2 | HIERARCHY AND MOLECULAR ORGANIZATION OF THE MAMMALIAN CLOCKS

Chronobiology, the discipline focusing on biological rhythms and their impact on living organisms, traces its

roots back to antiquity when the first observations of tree leaves closing at dawn were made in the 4th century BC (McClung, 2019), followed by experiments in *Mimosa pudica* (de Mairan, 1729) that demonstrated for the first time the presence of plant intrinsic circadian rhythms. The term "circadian", derived from the Latin words "circa" (approximately) and "*diem*" (day), was introduced in the late 60s to describe biological processes with a periodicity of about 24 h (Halberg, 1969; Lee et al., 2015). Circadian clocks play a crucial role in regulating cellular processes, physiology and behaviour in almost all living organisms, allowing them to anticipate and adapt to environmental changes according to the solar day (Kuhlman et al., 2018).

Circadian clocks are present in almost all cell types throughout the mammalian body, forming a hierarchical network of self-sustained, cell-autonomous oscillators capable of measuring time independently of external cues (Balsalobre et al., 1998; Finger et al., 2020; Nagoshi et al., 2004; Partch et al., 2014; Rijo-Ferreira & Takahashi, 2019). The master clock situated in the suprachiasmatic nuclei (SCN) of the hypothalamus ensures phase coherence among peripheral clocks in the rest of the body (Moore & Eichler, 1972; Stephan & Zucker, 1972; Welsh et al., 2010).

At the molecular level, the main core clock architecture is conserved across organisms. It consists of a network of interconnected transcription/translation negative feedback loops (TTFL) regulating the rhythmic expression of core clock genes and clock output genes (Doherty & Kay, 2010; Pett et al., 2018; Ueda et al., 2005). In mammals, the main loop of the circadian clock includes transcription activators Basic Helix–Loop–Helix ARNT Like 1 (BMAL1) and Circadian Locomotor Output Cycles Protein Kaput (CLOCK), which regulate the expression of repressors Periods (PER) and Cryptochromes (CRY). The negative feedback loop is completed when PERs and CRYs inhibit their own synthesis by obstructing CLOCK:BMAL1 activity. An auxiliary loop involving REV-ERB and ROR nuclear receptors contributes further to the complexity of the molecular core-clock (Cox & Takahashi, 2019; Partch et al., 2014).

The SCN maintains the alignment in phase synchrony with the external light–dark cycle through various direct and indirect mechanisms. Peripheral clocks respond to additional synchronization cues (Zeitgebers) such as feeding (Damiola et al., 2000), body temperature fluctuations (Saini et al., 2012), neural and humoral signals, metabolites (Schibler et al., 2015), oxygen levels and more (Adamovich et al., 2017; Sinturel et al., 2020).

Circadian oscillators play pivotal roles in regulating nearly all the aspects of body physiology via temporal orchestration of gene transcription, post-transcriptional events, protein synthesis and post-translational modifications (reviewed in (Bass, 2017; Finger et al., 2020)). Furthermore, lipids and metabolites follow diurnal profiles in blood and tissues, highlighting the critical importance of circadian rhythmicity for metabolic landscape (Chua et al., 2013; Held et al., 2020; Petrenko et al., 2022; Petrenko et al., 2023; Sinturel et al., 2023). The organ-specific fluctuations observed in the circadian landscape emphasize the fine-tuning required for each organ and even each cell type to perform its biological role (Gachon et al., 2017; Petrenko et al., 2018; Sinturel et al., 2020).

The field of chronobiology has come a long way since ancient observations of nature's rhythms. The intricate molecular organization of circadian clocks, their hierarchical arrangement in the mammalian body and their critical role in organ physiology highlight the complexity and importance of these internal timekeeping mechanisms. As research continues, unravelling the multilevel circadian orchestration of body physiology provides invaluable insights into health, disease and the fundamental principles governing life's temporal order.

3 | MOLECULAR CLOCKWORK STUDIES IN HUMAN PRIMARY FIBROBLASTS SYNCHRONISED IN VITRO

Prior to the advent of studying human molecular clocks "in the dish", in the primary cells derived from fresh biopsies and transduced with lentiviral circadian reporters, studies of circadian rhythm in humans required prolonged subject observation, which was laborious, organizationally challenging, costly and lacking precision and temporal resolution. Furthermore, getting mechanistic insights into the molecular clocks operative in most of the organs and tissues (liver, pancreas, lung, etc.) was simply out of reach, due to the inability to obtain serial biopsies. There remained an urgent need for modelling human circadian rhythmicity in vitro. This brought about the first characterization of human molecular circadian rhythmicity in vitro, which was published in 2005 by Steven A Brown in the laboratory of Ueli Schibler in Geneva (Brown et al., 2005). As an initial step, Steve engineered a circadian reporter that allowed for longitudinal recording of circadian rhythms in human primary cells cultured in vitro. Specifically, he created a lentiviral vector that introduced a stably integrated construct containing the firefly luciferase coding region under the mouse promoter of the core clock gene *Bmal1*. Importantly, Steve demonstrated that primary mouse skin fibroblasts derived from *Per1* deficient mice that were transduced with *Bmal1-luciferase (Bmal1-luc)*

lentivectors and subjected to in vitro synchronization exhibited shorter oscillation period, concordant with the shortened activity period recorded for *Per1*KO mice in vivo. In fact, the period shortening measured in vitro was more pronounced as compared to the in vivo recorded phenotype (Brown et al., 2005). At the next step, Steve decided to employ the same approach for studying human clockwork. Following several optimization steps in different tissue sources (cell lines, primary human cells), fibroblasts from skin biopsies were deemed the most appropriate tissue for human circadian studies in vitro. Several (2–5) skin biopsies of 2 mm diameter were collected from 19 healthy participants from areas of the abdomen, buttocks, foreskin and other sources (Figure 1a). The expression rhythms of the *Bmal1-luc* reporter in primary skin fibroblasts synchronized in vitro revealed a circadian period of 24.5 h with a standard deviation of 45 mins. A high variability with a maximum difference of 4 h was observed across individuals. However, despite the significant variability between participants, the individual period length was reproducible across replicate biopsies from the same subject, highlighting this way the robustness of the experimental approach (Figure 1b) (Brown et al., 2005).

A few years later, during his Humboldt Research Fellowship in the laboratory of Achim Kramer at the Charité University of Berlin, Steve and colleagues in Achim Kramer's group employed the same innovative approach for in vitro studies of the circadian rhythmicity in subjects with extreme chronotypes (the term "chronotype"

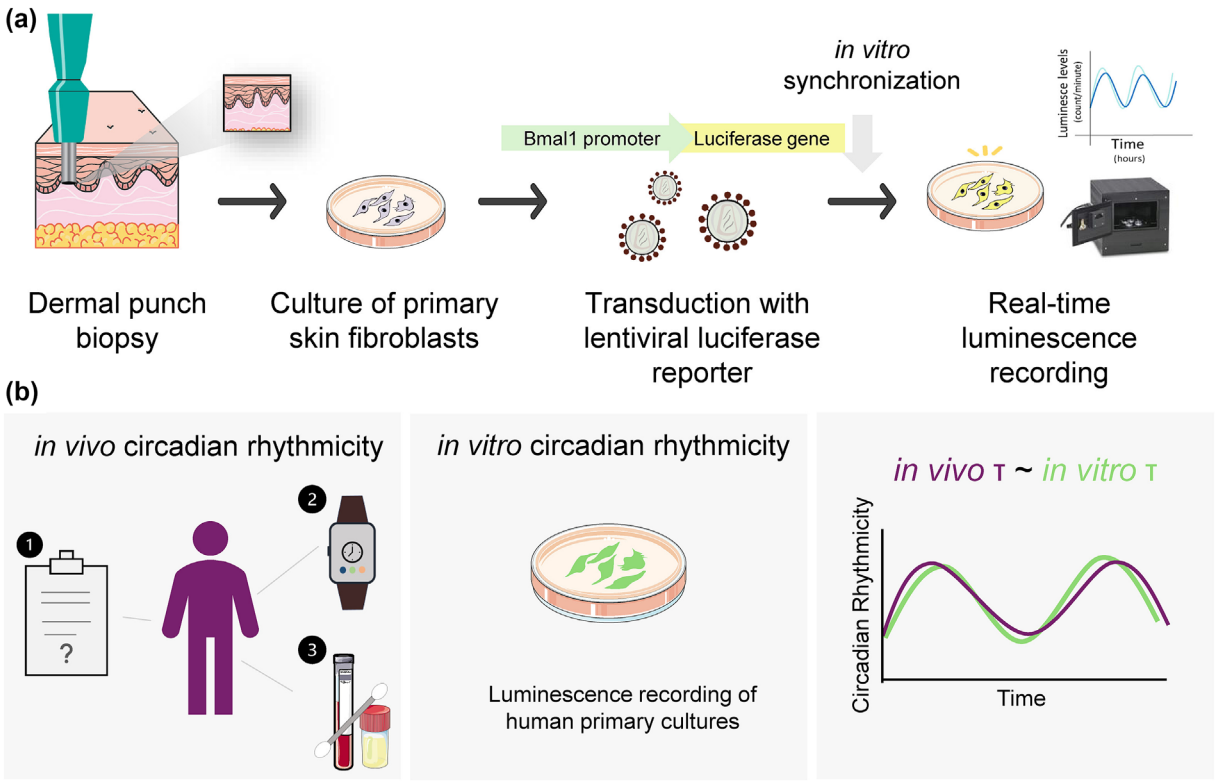

FIGURE 1 Human primary skin fibroblasts synchronized in vitro represent an invaluable tool to study molecular circadian rhythmicity. (a) Schematic representation of the experimental protocol designed by Steven A. Brown. Two mm dermal punch biopsies were obtained from human volunteers to establish primary skin fibroblast cultures. Skin fibroblast cells were transduced with lentivectors expressing the luciferase gene driven by regulatory sequences of BMAL1 or other core-clock genes. Following in vitro synchronization pulse, circadian bioluminescence levels were continuously recorded in real-time with a high temporal resolution during several consecutive days, allowing to assess molecular oscillations with high precision (Brown et al., 2005; Brown et al., 2008; Pagani et al., 2010). (b) Circadian bioluminescence recording in human primary cells synchronized in vitro reflects body rhythms in vivo. Human circadian rhythmicity was traditionally studied with the use of self-assessment questionnaires (shown as number 1 in the figure), such as the Munich Chronotype Questionnaire (MCTQ) (Roenneberg et al., 2003) and the Horne-Östberg's Morningness-Eveningness score (Horne & Ostberg, 1976). Actigraphy watches (number 2 in the figure) or multiple blood, urine or saliva samples (number 3 in the figure) around the clock have also been extensively used to study human diurnal preferences. The pioneering work of Steve Brown made possible the study of human circadian rhythmicity using cultures of human fibroblasts transduced with circadian lentivectors. Human primary cultures retain, up to a certain degree, the circadian phase coherence of the source individual evaluated by the chronotype (Brown et al., 2008) and the period length, if compared to the one evaluated by salivary melatonin profile (Pagani et al., 2010).

describes a person's diurnal preference, namely the body's natural sleep–wake cycle (Roenneberg, 2012)). Skin biopsies were collected from 11 participants with early ("larks") and 17 with late ("owls") chronotypes. In vitro fibroblast cultures were transduced with the *Bmal1-luc* reporter (Brown et al., 2005), their rhythmicity was recorded for several days, and circadian features, including period length and ability to phase-shift and entrain to environmental and chemical cues, were evaluated (Brown et al., 2008). Fibroblasts from subjects with early chronotypes exhibited a shorter circadian period (~24.33 h) compared to the ones with late chronotypes (~24.74 h). A correlation was thus established between the period length measured in cultured skin fibroblasts in vitro and human behaviour in participants with extreme chronotypes. Interestingly, the authors identified three subjects with seasonal affective disorder (SAD) exhibiting an early behavioural phase but a longer fibroblast period. Furthermore, important differences were observed in the amplitude and phase-shifting features of the circadian oscillator among individuals with identical period lengths (Brown et al., 2008). This study provided the first evidence of differences not only in the period length but also in the amplitude and phase-shifting characteristics of primary fibroblasts among individuals with extreme chronotypes. Additionally, it suggested a correlation between affective disorders and the misalignment of circadian clocks (Brown et al., 2008). In the past, consecutive gene expression measurements (for example multiple qPCRs around the clock) were required to gain circadian insights, with limited space for dynamic studies such as response to synchronizing agents (Lippert et al., 2014).

The initial studies on primary human fibroblasts performed by Steve and colleagues not only introduced the new protocol for circadian luminometry in primary human fibroblasts (Figure 1) but also provided important insights into inter-individual circadian differences, such as the wide variance of period length between participants (Brown et al., 2005; Brown et al., 2008; Cuninkova & Brown, 2008). Later on, Lucia Pagani in the newly established Brown lab investigated the correlation between the clock's period length in living organisms and human primary fibroblasts in vitro, further validating the reliability of the experimental protocol (Pagani et al., 2010). The study utilized behavioural interventions followed by around-the-clock melatonin measurements complemented by luminometry of cultured human fibroblasts from the same participants. Similarly to the previous studies, dermal fibroblasts were obtained by cylindrical 2-mm diameter cutaneous biopsies from the buttocks or upper arm, transduced by the luciferase-based circadian reporter *Bmal1-luc*, synchronized with dexamethasone pulse a few days later, with subsequent recording of circadian bioluminescence for at least five consecutive days. Another very interesting aspect of this study was the inclusion of a group of eight blind subjects whose circadian pacemakers cannot be entrained by the environmental light–dark cycle (Pagani et al., 2010). The cellular oscillator recordings revealed a prolonged physiological period ascertained by melatonin rhythms for the blind participants compared to the sighted ones, however, the average fibroblast period measured in vitro was comparable for both populations. Thus, cellular fibroblast rhythms were insensitive to the periodicity differences between blind and sighted individuals (Pagani et al., 2010). Similar inter-individual reproducibility of fibroblast period length, with lower correlation between in vitro and physiological periodicity, was identified in a subsequent study indicating significant differences in molecular mechanisms between the central and peripheral oscillators (Hasan et al., 2012).

The pivotal innovation of longitudinal bioluminescence recording in primary human skin fibroblasts in vitro as a reliable proxy for circadian behaviour in vivo was pioneered by Steven A. Brown (Brown et al., 2005; Brown et al., 2008; Cuninkova & Brown, 2008; Nagoshi et al., 2005; Pagani et al., 2010) established a foundational framework for subsequent studies on circadian properties and their relationship to a wide variety of diseases, many of which are not traditionally considered as circadian clock-related.

4 | IMPACT OF CIRCULATING SERUM FACTORS ON THE CELLULAR CLOCKWORK CAN BE MEASURED IN VITRO

In 2011, Steve Brown in collaboration with Anne Eckert compared the rhythmicity between young and elderly adults (Pagani et al., 2011). The study, which implemented a total of 32 healthy young (age 21–30 y) and older men and women (age 60–88 y) reported no differences in the skin fibroblast circadian properties in vitro, in contrast to the observed behavioural changes. However, incubation of fibroblasts from both groups with the culture medium that contained 15% of serum from older subjects (instead of usually employed foetal calf serum, FCS) led to the shortening of the cellular circadian period and shift to an earlier phase, compared to the counterpart cells incubated in the presence of serum from younger counterparts. This work suggested that age-related attenuation of the behaviour rhythm and shorter oscillation period observed in the skin fibroblasts cultured and

synchronised in vitro might be driven by blood-born factors (Pagani et al., 2011). Importantly, this finding highlights that molecular oscillators are plastic, and may change their properties according to their environment. The impact of blood-born factors on the cellular clockwork in the context of metabolic diseases was further unravelled by Ngoc-Hien Du and Nora Nowak in the Brown lab (see the publication by Ngoc-Hien Du "Multi-omics correlates of insulin resistance and circadian function mapped directly from human serum" in this issue).

5 | STUDIES IN SKIN FIBROBLASTS IN SLEEP AND CIRCADIAN DISORDERS

Human primary skin fibroblasts established from fresh biopsies and cultured and synchronized in vitro have been widely implemented as a unique model for studying human sleep and circadian disorders (Figure 2b). Hida and colleagues (2017) used in vitro culture of dermal punch fibroblasts as a tool to study circadian rhythm in the context of sleep disorders. The researchers assessed

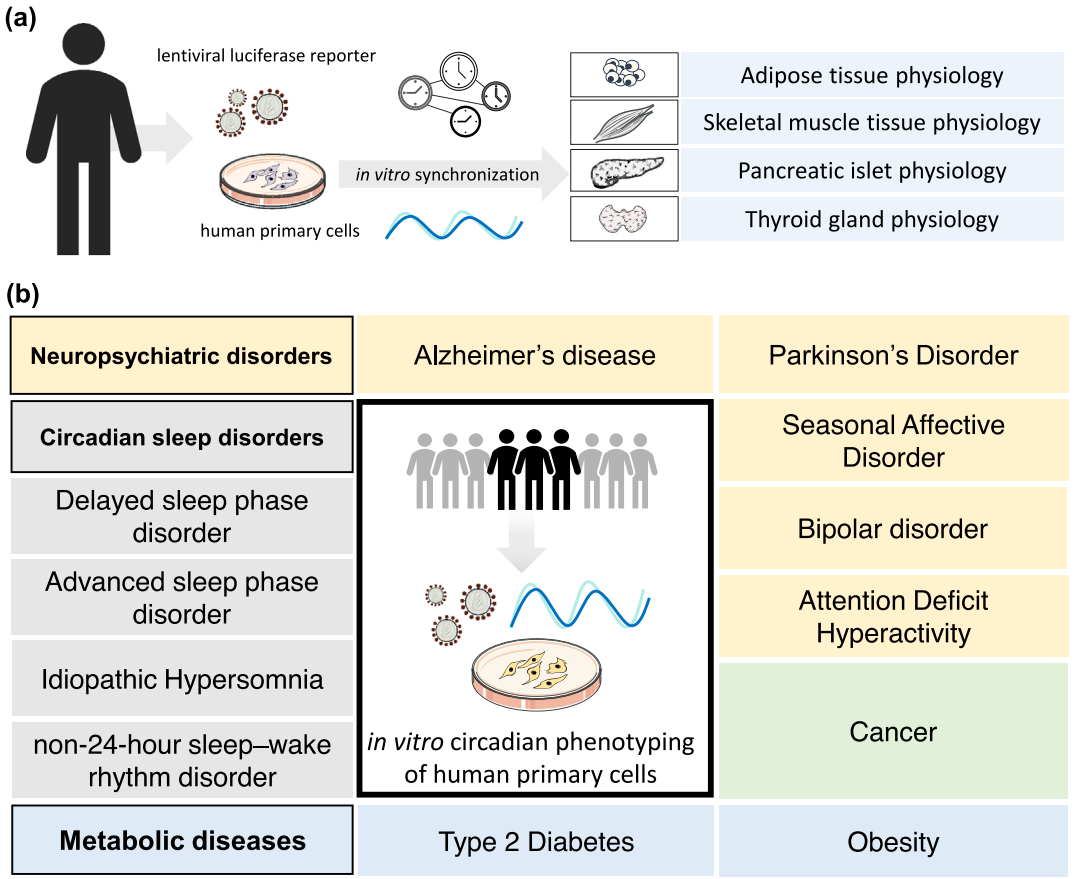

FIGURE 2 Human primary cells synchronized in vitro as a tool to study molecular circadian rhythmicity across the organs (a) and diseases (b). (a) Human tissue biopsies obtained from surgical procedures or post-mortem donation are used to establish respective primary cultures. Protocol adaptations are required for each tissue type. As with fibroblasts, primary cells are transfected with lentiviral vectors expressing a circadian luciferase reporter such as Bmal1-luc. Following in vitro synchronization, bioluminescence levels are recorded in real-time for several days with subsequent assessment of molecular rhythmic features. For most of the tissues, repetitive sampling across 24 h is impossible. Thus, circadian phenotyping of human peripheral clocks and their functional outputs based on the primary cultures synchronized in vitro provides unique insight into the human organs' rhythmic physiology. (b) Primary human cultures, expressing a circadian luciferase reporter are synchronized in vitro and their luminescence levels are recorded longitudinally. In vitro circadian rhythmicity of human primary cells is used to assess molecular rhythmic features and disease characteristics in a wide range of human pathologies. Numerous diseases, including sleep disturbances, psychiatric and metabolic disorders or cancer, have been studied using circadian phenotyping of peripheral clocks in vitro. Longitudinal circadian recordings of primary human cells open a new avenue not only to study disease pathogenesis and molecular phenotypes but also to design new therapeutic approaches and predict their efficacy in a dish. The figures include images adapted from Servier medical art under creative commons license 3.0 (https://creativecommons. Org/licenses/by/3.0/).

the molecular circadian properties of subjects with delayed sleep–wake phase disorder (DSWPD) and non-24-h sleep–wake rhythm disorder (N24SWD) (Hida et al., 2017). Analysis of fibroblasts' circadian properties revealed an in vitro period length of approximately 22.8 h in healthy participants, while DSWPD-derived and N24SWD-derived fibroblasts had a period of around 22.67 h and 23.18 h, respectively (Hida et al., 2017). In addition, in the N24SWD group, the in vitro fibroblast period length correlated with response to chronotherapy, with longer periods observed in the non-responders (Hida et al., 2017). Similarly, an elegant study by Patke et al., (2017) addressed the genetic component of a hereditary form of DSWPD and identified a dominant coding variation of the core clock gene CRY1 (Patke et al., 2017). In vitro luminometry of dermal fibroblasts from DSWPD individuals carrying the CRY1 polymorphism displayed a prolonged period, providing a causal link between circadian genetics and perturbed sleep patterns (Patke et al., 2017). Using a similar experimental approach, Materna et al. (2018) studied the circadian properties of patients with idiopathic hypersomnia (IH), a disease characterized by excessive sleepiness, difficulty in waking up and fatigue (Materna et al., 2018). The researchers found an approximately 1-h prolonged circadian period in IH patients, indicating an important circadian component in the pathophysiology of this complex and poorly understood sleep disorder (Materna et al., 2018).

6 | STUDIES IN SKIN FIBROBLASTS IN NON-CIRCADIAN HUMAN DISEASES

At present, there are several reports highlighting the relationship between altered circadian rhythmicity and non-circadian human disorders, such as depression (Xu et al., 2005), Parkinson's disease (Marano et al., 2023) and type 2 diabetes (T2D) (Sinturel et al., 2019) (Figure 2b). In the initial study of Steven A. Brown and Achim Kramer (2008), the researchers identified three participants with prolonged in vitro fibroblast period, but with early behavioural chronotype, which was in contrast with the data from the rest of the participants. Interestingly, these three participants were retrospectively identified to suffer from SAD, an observation that indicates a relationship between perturbed circadian clocks and mood (Brown et al., 2008). At present, dermal fibroblasts from patients with psychiatric and neurological diseases are widely used to study disease pathogenesis, progression and potential treatments, as well as, circadian properties in vitro (Kálmán et al., 2016; Schmitt et al., 2017).

Bipolar disorder was one of the first diseases studied using in vitro human fibroblasts, initially via classic gene expression assays (qPCR, Western Blot) (Yang et al., 2009) and later by implementing a circadian luciferase reporter (Nudell et al., 2019). The viral introduction of the Period2-luciferase (*Per2-Luc*) or *Bmal1-luc* circadian reporters in fibroblasts from bipolar patients showed circadian abnormalities such as dampened amplitude under entrained conditions (Nudell et al., 2019), overall longer period (McCarthy et al., 2013) and higher period length variability (Sanghani et al., 2021). Furthermore, circadian analysis of primary fibroblasts allowed the investigation of benchmark pharmacotherapies, such as the effect of lithium on gene expression rhythmicity (McCarthy et al., 2013), and the prediction of lithium treatment response, which was inversely correlated with the patient's period length (Sanghani et al., 2021).

The Brown laboratory further developed the initial lentiviral circadian reporter approach by creating a system of luciferase-based signal transduction reporters in primary fibroblasts from healthy and bipolar participants (Gaspar et al., 2014). Their findings suggest that inter-individual variations in the cyclic adenosine monophosphate (cAMP) – response element-binding protein (CREB) pathway, which regulates brain processes underlying both circadian rhythmicity and affection disorders, could act as a mechanistic link between circadian clock disruption and bipolar disorder (Gaspar et al., 2014). Following an analogous experimental design, in vitro circadian studies in human dermal fibroblasts have identified altered circadian rhythmicity in adults with attention deficit hyperactivity (ADHD), such as differing responses to synchronizers (Palm et al., 2023), which is further affected by the currently available ADHD medication (Faltraco, Palm, Uzoni, Borchert, et al., 2021; Faltraco, Palm, Uzoni, Simon, et al., 2021; Palm et al., 2021). Lastly, cultures of human primary fibroblasts have been used to study circadian rhythmicity in neurodegenerative diseases (Figure 2b). Fibroblasts from patients with Alzheimer's (Cronin et al., 2017) or Parkinson's (Marano et al., 2023) disease exhibit altered circadian rhythmicity in vitro that was associated with aberrant *BMAL1* transcription, based on circadian bioluminescence and/or endogenous transcript analyses around-the-clock.

Currently, there is a plethora of publications that build upon the initial works by Steven A. Brown and further adapt the protocol of circadian bioluminescence recording in cultured human fibroblasts to study the circadian elements of pathological neuropsychiatric conditions (see Figure 2b and Palm et al., 2023, for an elaborate review).

Implementation of this powerful methodological approach was further extended into the studies of

perturbed circadian orchestration of human metabolism in the context of metabolic disorders (for review, see (Dibner, 2020; Dibner & Schibler, 2015; Finger et al., 2020; Saini et al., 2015; Sinturel et al., 2020)). In 2019, a collaborative work between the Brown and the Dibner groups, based on the initial work by Steve, addressed the interplay between clock dysregulation and metabolic impairment in obese and type 2 diabetic (T2D) participants, implying the link between glycemic control and the circadian clock machinery (Sinturel et al., 2019). In this study, circadian oscillations in primary human fibroblasts were compared between individuals with T2D and a non-diabetic control group. In vitro bioluminescence profiles of synchronized fibroblasts were investigated using the *Bmal1-luc* reporter and revealed a strong inverse correlation between the fibroblast period and HbA1c in the T2D group. Further mechanistic analysis through RNA-seq revealed changes in the *ICAM1* gene associated with poor regulation of glucose homeostasis in T2D patients and identified as a binding target of the CLOCK protein in dermal fibroblasts (Sinturel et al., 2019). These findings have been followed in a larger human cohort to determine if intrinsic cellular properties or circulating factors drive this relationship between HbA1c and period length in the context of obesity and T2D (see the publication by Ngoc-Hien Du in the Brown group "Multi-omics correlates of insulin resistance and circadian function mapped directly from human serum" in this chapter). Further investigation into this association will likely provide valuable information into the bidirectional link between metabolic dysfunction and circadian impairment. Moreover, primary human dermal fibroblasts transduced with the *Bmal1-luc* reporter have been used to study the effects of anti-obesity treatments in the context of cellular senescence (Heo et al., 2023), opening a perspective for employing this methodology for screening therapeutic approaches for metabolic diseases management.

The recent book Circadian Clocks (Springer Protocols) edited in 2021 by Steve, includes a chapter by Du and Brown providing an elaborate protocol for real-time longitudinal bioluminescence recording of circadian rhythmicity in primary dermal fibroblasts. The protocol provides essential information for skin punch biopsy collection, the culture of the primary fibroblasts, the generation of stable cells expressing a circadian luciferase reporter and finally measurement of cellular rhythms and analysis (Du & Brown, 2021). Thus, the use of in vitro circadian monitoring in fibroblasts from skin biopsies became an essential tool for studying human diseases in the intersection of circadian biology, metabolism, neurobiology and mental health (Figure 2b). Taken together, these observations highlight the necessity for a better understanding of the circadian machinery alterations in human pathologies, and the role of circadian misalignment in human health and provide a potential therapeutic window for chronotherapy.

7 | PASSING THE BATON: INVESTIGATING MOLECULAR CLOCKWORK AND DAILY FUNCTIONAL ORCHESTRATION IN HUMAN PRIMARY CULTURES ACROSS TISSUE TYPES AND PATHOLOGIES

The initial discovery by Steven A. Brown provided compelling evidence that cultured primary human skin fibroblasts expressing circadian bioluminescence reporters represent an excellent experimental system for the dissection of oscillator properties (Figure 1; Brown et al., 2005). Circadian oscillator characteristics measured in cultured skin fibroblasts correlate with rhythmic human behaviour, as evaluated on the basis of human subjects whose circadian physiology was examined under laboratory conditions (Pagani et al., 2010) or individuals completing a questionnaire (Brown et al., 2008; Juda et al., 2013; Zavada et al., 2005). Furthermore, the cultured cells provide substrates for biochemical or genetic analyses of the mechanisms underlying these properties (see the publication by Ngoc-Hien Du and colleagues "Multi-omics correlates of insulin resistance and circadian function mapped directly from human serum" in this issue (Gaspar et al., 2014; Gaspar et al., 2017; Gaspar & Brown, 2015)). Translational studies in humans have now expanded from the utilization of dermal fibroblasts to a wide variety of human primary cell types providing novel avenues for studying clock molecular details in the tissues that are otherwise challenging for dissecting the temporal organization (Figure 2a). Indeed, state-of-the-art approach developed by Steve has already been applied to primary cells established from fresh tissue biopsies such as primary human keratinocytes, pancreatic islet cells, skeletal myotubes, white adipocytes or thyrocytes (Figure 2a) (Kolbe et al., 2019; Mannic et al., 2013; Perrin et al., 2015; Pulimeno et al., 2013; Sporl et al., 2011; Sporl et al., 2012; Tuvia et al., 2021). Moreover, the effect of different physiologically relevant synchronization stimuli on tissue-specific clocks, as well as transcriptional, metabolic and functional outputs of the clockwork, are readily assessable by this approach (Kolbe et al., 2019; Loizides-Mangold et al., 2016; Loizides-Mangold et al., 2017; Perrin et al., 2018; Pulimeno et al., 2013; Sporl et al., 2011; Thurley et al., 2017; Tuvia et al., 2021). A comparison between

circadian transcriptomic and lipidomic analyses from serial skeletal muscle tissue biopsies collected in vivo and primary myotubes synchronized in vitro suggests a significant effect of the cell-autonomous muscle clock on the temporal regulation of the transcriptional and metabolic landscape (Loizides-Mangold et al., 2017; Perrin et al., 2018). Furthermore, siRNA-mediated clock disruption in human primary cells has been developed for studying the effect of clock perturbation on gene transcription, lipid metabolism, hormone or cytokine secretion (Loizides-Mangold et al., 2017; Perrin et al., 2015; Perrin et al., 2018; Petrenko et al., 2016; Saini et al., 2016).

Given that altered circadian characteristics were linked to various diseases, analyses of clock properties in diverse primary cell types can be used for diagnostic and treatment purposes (Figure 2b; Saini et al., 2015). The circadian system and body metabolism are tightly interconnected through behavioural and molecular pathways (Allada & Bass, 2021; Bass, 2016; Finger et al., 2020; Gachon et al., 2017; Munch & Kramer, 2019; Reinke & Asher, 2019; Sinturel et al., 2020). Increasing body of evidence imply that in humans, circadian clock perturbation is associated with islet dysfunction and T2D pathogenesis (Perelis, Marcheva, et al., 2015; Perelis, Ramsey, & Bass, 2015; Petrenko et al., 2020; Petrenko et al., 2022; Petrenko et al., 2023; Saini et al., 2016). Indeed, disruption of the clock machinery in primary human islet cells using a siRNA targeting the core clock gene *CLOCK* revealed that a functional circadian clock is required for proper absolute and temporal regulation of glucagon and insulin secretion via regulation of the islet hormone secretory granule exocytosis, and for regulating membrane fluidity. Noteworthy, alterations in the islet hormone secretion and lipid homeostasis associated with clock disruption in the islets from normoglycemic donors corroborated the phenotypes in the T2D islet counterparts, further highlighting that clock perturbation takes part in the pathophysiology of T2D (Petrenko et al., 2020; Petrenko et al., 2022; Saini et al., 2016). In the same line, a recent study on circadian rhythmicity in obesity by Kolbe et al. that utilised cultured mature adipocytes from obese participants obtained from subcutaneous and visceral adipose tissues during bariatric surgery reported age-related oscillator changes in human primary adipocytes (Kolbe et al., 2019). In vitro culture of human primary differentiated skeletal myotubes has further underscored the interconnection of the circadian timing system and metabolic health, with functional cell-autonomous myotube clocks regulating transcriptional and lipid landscape of these cells along with pro-inflammatory myokine secretion and glucose uptake (Loizides-Mangold et al., 2017; Perrin et al., 2015; Perrin et al., 2018). Furthermore, cultured myotubes from T2D individuals revealed altered transcriptional oscillations of the core clock genes *BMAL1*, *CLOCK* and *PER3*, reduced number and amplitude of cycling genes, disrupted mitochondrial function and oxygen consumption compared to the counterpart myotubes established from normal glucose-tolerant individuals (Gabriel et al., 2021).

The use of primary human cells for studying the reciprocal connection between circadian rhythmicity and pathophysiology has further been adapted in cancer studies. Breast cancer is one of the first cancer types that was associated with misalignment of internal clocks and geophysical time, with several studies indicating an increased cancer risk on female shift workers (Davis et al., 2001; Megdal et al., 2005; Schernhammer et al., 2001). Although epidemiological studies strongly suggested that circadian disruption may play a role in carcinogenesis, the molecular element between circadian mechanisms and tumour development remained elusive (Saha & Sassone-Corsi, 2007). Primary mammary epithelial cells, isolated from normal and cancerous regions from the same mastectomy patients were cultured and transduced with a *Per2-luc* lentivectors (Broadberry et al., 2018). While robust circadian cycling was identified in normal cells, the adjacent cancer-tissue-derived counterpart cells displayed dampened circadian amplitude and quickly lost their rhythmicity. In the same direction, circadian oscillations of *Bmal1-luc* reporter in synchronized human primary cancerous thyrocytes displayed altered circadian rhythmicity, which was quickly dampened in vitro (Mannic et al., 2013).

Primary human cells cultured and synchronized in vitro have emerged over the last two decades as a powerful, renewable and relatively non-invasive research tool opening the novel horizon for molecular and functional studies in human chronobiology. Indeed, primary human in vitro cultures bridge the experimental gap for translation between animal models and human subjects, paving the path to circadian clinical research and chrono medicine. Real-time bioluminescence recording using circadian luciferase reporters in primary cells from transgenic animals or immortalized cell lines are still widely used for fundamental chronobiology research, as it is also apparent from the latest publications/collaborations of Steve (Jagannath et al., 2021; Katsioudi et al., 2023). Furthermore, the experimental approach of longitudinal bioluminescence recording has been adapted for the design of other non-circadian luciferase reporters to study pathway dynamics and mechanistic interactions in mouse (Katsioudi et al., 2023) or human tissue (Gaspar et al., 2014) in vitro. Lastly, circadian luciferase reporters

have also adapted for longitudinal in vivo studies in rodents (Hoekstra et al., 2021; Katsioudi et al., 2022; Saini et al., 2013).

Animal and cell line studies are now further complemented with circadian recordings of primary human cells. A collaboration between the Brown and Eckert laboratories employed a combination of in vitro human fibroblast cultures and transgenic mice as in vivo models (Schmitt et al., 2018) to shed light on the crosstalk between the mitochondrial network and circadian rhythmicity. The data revealed a rhythmic regulation of the mitochondrial fission protein DRP1, which serves as the molecular mediator of the circadian control of mitochondrial morphology and oxidative metabolism, helping to understand metabolic homeostasis in human health and disease (Schmitt et al., 2018). In the same direction, employing pancreatic islet cells from transgenic mice and from human donors, both expressing the circadian reporter *Per2:luc*, Andersen et al. report that inflammatory stress perturb the intrinsic oscillators of islet cells with a potential link to diabetic phenotype (Andersen et al., 2021). Furthermore, in vitro circadian recordings of primary human cells provide important insights into disease prevention identified the perturbed clock components or downstream metabolic pathways as targets for potential therapeutic avenues for metabolic diseases (Petrenko et al., 2020; Petrenko et al., 2022; Petrenko et al., 2023; Sinturel et al., 2020).

Today the use of circadian bioluminescence reporters in human dermal fibroblasts and across various human primary cell types has been well established. It is widely implemented for providing new insights into the roles of circadian clocks in human pathologies, ranging from affective and neurological disorders to metabolic diseases and cancer (Figure 2).

8 | CONCLUSIONS

Human circadian traits, such as chronotype, exhibit considerable variation among individuals and throughout their lifespans. The use of circadian bioluminescence lentivectors in primary human cells along with a tight correlation between the circadian rhythms of human skin fibroblasts and the circadian phenotype of their donors, proposed by Steven A. Brown, revolutionised the research of human molecular oscillators under physiological situations, and in context of human pathologies (Figures 1 and 2). Considering the challenges associated with studies of circadian phenotypes in humans, investigating the properties of molecular oscillators in cultured primary cells presents an invaluable and unique opportunity to elucidate molecular intricacies that are otherwise elusive. Ranging from basic chronobiology research to dissecting complex diseases and potential therapies, in vitro human circadian phenotyping represents a powerful tool for mechanistic research along with the improvement of diagnostics and management tools for a wide variety of human diseases, allowing to access inter-individual differences in circadian characteristics, in-depth genetic, biochemical, metabolic and functional analyses. In a broader context, this highly reliable and minimally invasive approach offers a unique perspective for unravelling functional inputs and outputs of the oscillators operative in nearly any human tissue in a physiological context, and across various pathologies.

AUTHOR CONTRIBUTIONS

Conceptualization: G.K. and C.D., Visualization: G.K., and A. D. B., Supervision: C.D., Writing original draft: G.K., A. D. B., C.J.-S and C.D., Writing, review, and editing: G.K., A. D. B., C. J.-S and C.D., Funding acquisition: C.D. and G.K.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

PEER REVIEW

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DATA AVAILABILITY STATEMENT

No new data were created or analysed in this study. Data sharing is not applicable to this article.

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