Charles University in Prague

Faculty of Science

&

Institute of Physiology, Academy of Sciences of the Czech Republic, v.v.i.

Ph.D. study program: Animal Physiology

Matúš Soták

Role of intestinal circadian clock in epithelial transport, proliferation, and tumourigenesis

Úloha črevných cirkadiánnych hodín v epiteliálnom transporte, proliferácii a tumorigenéze

Ph.D. thesis

Supervisor: Prof. Jiří Pácha, Ph.D., D.Sc.

Praha, 2014

I declare that presented submission is my own work under supervision of Prof. Jiří Pácha, and to my best knowledge it contains no material previously published or written by another person neither material which to a substantial extent was used to award of any other degree or diploma title. All used materials are properly cited in reference section. I give the permission to make the thesis publicly available at Charles University thesis repository.

I declare that the results presented in section 6.1.1 were made with substantial contribution of Lenka Polidarová, M.Sc. and the results presented in section 6.2.6 were made with substantial contribution of Miroslav Hock, Ph.D.

ACKNOWLEDGEMENTS

Chcem poďakovať Prof. RNDr. Jiřímu Páchovi, DrSc. za perfektné a obetavé vedenie práce počas celého štúdia, za pomoc, nápady, podnetné diskusie pri dizajnovaní experimentov, analýze dát i príprave výsledného textu práce a za odovzdanie vzoru poctivej, systematickej a efektívnej vedeckej práce. Zároveň ďakujem kolektívu *odd. Funkce epitelu* (Fyziologický ústav AV ČR, v.v.i.) za inšpiratívne pracovné i ľudské prostredie a všestrannú nezištnú pomoc. Mgr. Miroslavovi Hockovi, Ph.D. ďakujem za pomoc pri elektrofyziologických meraniach a Višni ďakujem za technickú asistenciu pri práci s experimentálnymi zvieratami a spracovaním vzoriek.

Ďakujem PharmDr. Alene Sumovej, Ph.D. a Mgr. Lenke Polidarovej (*odd. Neurohumorálních regulací*, Fyziologický ústav AV ČR, v.v.i.) za intenzívnu a plodnú spoluprácu a za vrelé sprostredkovanie metodického i intelektuálneho know-how v oblasti výskumu cirkadiánnych rytmov. Ďakujem Mgr. Martinovi Sládkovi, Ph.D. za dizajn sekvencií PCR primerov.

Ďakujem svojim rodičom a rodine za trvalú morálnu podporu a pochopenie pre rehoľu intelektuálnej vedeckej práce. Rovnako ďakujem svojej priateľke Mgr. Dite Kašparovej za trpezlivú a nekončiacu oporu a metodické konzultácie.

Nakoniec ďakujem všetkým, ktorých sa to týka, za dlhé, vytrvalé, neoblomné a intenzívne povzbudzovanie pri dokončovaní finálneho textu tejto práce.

Then God said, "Let there be light"; and there was light. God saw that the light was good; and God separated the light from the darkness.

Genesis 1, 3-4.

1 TABLE OF CONTENT

2 ABSTRACT

The molecular circadian clock enables anticipation of environmental changes. In mammals, clocks are ubiquitously present in almost all tissues and they are comprised of transcriptional-translational feedback loops of the so-called clock genes. The central clock represents the intrinsic pacemaker which is located in suprachiasmatic nuclei (SCN) of hypothalamus and synchronizes peripheral clocks. Clockwork system in alimentary tract and its regulatory link to intestinal functions are poorly understood. Therefore the objective of the thesis was to characterize molecular clock in particular parts of the rat intestine and to elucidate its link to the intestinal transport, regulation of cell cycle and neoplastic transformation in colonic tissue.

We used quantitative RT-PCR (qPCR) to determine circadian profiles of mRNA expression of clock genes in the epithelium of duodenum, jejunum, ileum, and colon of rat. Furthermore, we analysed the expression of genes coding sodium chloride transporters and channels as well as cell cycle regulators in colon. To focus more precisely on different structures of intestinal epithelia we used laser capture microdissection. In addition, we performed Ussing chamber measurements to determine the colonic electrogenic transport. To study the contribution of circadian colonic clock to colon tumourigenesis, we used the model of colitis associated azoxymethane-induced colorectal carcinoma, where we studied circadian expression of clock and clock-controlled genes in colorectal tumours of mice.

The experiments demonstrated functional molecular clock in all studied intestinal segments, as we observed robust rhythmic expression of main clock genes, *Per1*, *Per2*, *Revrbα*, and *Bmal1*. We found circadian expression also in the genes *Nhe3*, *Dra*, *Ae1*, and *Atp1a1* coding proteins involved in sodium chloride transport, particularly Na^+/H^+ exchanger (*Nhe3*), Cl^-/HCO_3^- exchangers (*Dra, Ae1*), and a subunit of Na/K ATPase (*Atp1a1*). Furthermore, the genes coding NHE3 regulatory factor 1 (*Nherf1*) and γ regulatory subunit of sodium channel ENaC (*Scnn1g*) exhibited rhythmic pattern of mRNA expression. These rhythms were affected by feeding regime suggesting direct involvement of the peripheral clock. Moreover, we showed diurnal changes in the rate of electrogenic sodium transport via ENaC channels which indicated circadian regulation of sodium transport on the functional level.

To dissect the role of the clock in epithelial cell proliferation we measured circadian expression of the main cell cycle regulators. We observed apparent rhythm in expression of the gene coding WEE1 kinase (*Wee1*), which withholds G2/M progression. This finding was also supported by laser capture microdissection experiments, where we found rhythmic expression of

Wee1 both in crypt base and crypt mouth even if we observed significant differences in total amounts of transcripts between both crypt compartments. In addition, the genes coding the cyclins A, and B1, and CDK inhibitor p27 exhibited rhythmic expression. We did not detect any significant rhythm of the gene coding c-Myc protooncogene, which was documented as rhythmic in some other tissues previously. Likewise, neither the genes *p21*, *p16*, nor *cyclin D1* met the criteria of significant rhythm. We can hypothesize, that (i) the intestinal clock is at least partially involved in the regulation of proliferation of colonic epithelium, even if additional regulatory stimuli might play a substantial role and (ii) involvement of the clock is probably tissue specific.

In the model of azoxymethane-induced colorectal carcinoma associated with colitis, we found substantially depressed rhythms of expression of the main clock genes *Per1*, *Per2*, and *Revrbα*, and non-rhythmic expression of *Bmal1* in tumour tissue comparing to control counterparts or healthy-looking adjacent colon tissue. Interestingly, the significant clock alteration was detected not only in neoplastic tissue but also in healthy-looking adjacent tissue. These data demonstrate involvement of circadian clock in processes of colorectal carcinogenesis.

In summary, we characterized the circadian clock in the intestinal epithelium and revealed its involvement in the regulation of sodium chloride transport, epithelial proliferation, and the process of colonic epithelium tumourigenesis. These findings significantly advance understanding of the physiological output of the circadian clock with potential clinical use in future.

3 LITERATURE REVIEW

"Without being sure of something, we can not begin to think about everything elses."

-Kathryn Schulz, 2010-

3.1 Circadian rhythms and clock

3.1.1 Circadian rhythms

Earth rotation around the Sun as well as around the inner axis generates periodically changing environment for almost all living organisms. Correspondingly, most physiological processes and behaviour including activity/resting phase, body temperature, heart rate, hormonal levels, enzyme activities and other parameters exhibit rapid circadian (from Latin *"circa diem"* - "about a day") changes, which are not just responses to relevant environmental cues, but rather intrinsically anticipated variations. Strong evolutionary pressure for adjusting organism according to changing environment and anticipating particular conditions during the year or within the day is evidenced by evolution of "time awareness" system, which appears early in phylogenetic history and governs numerous cellular and physiological processes. In mammals, nearly all cells are virtual clocks, hence, the whole body synchronization is required. Recently, a great progress has been made in circadian rhythms investigation and molecular basis of intrinsic pacemaker system was revealed. In this chapter, I review up-to-date knowledge of central and peripheral circadian clock mechanism and regulation, particularly in relation to transepithelial transport, cell cycle regulation, and tumourigenesis.

3.1.2 The mechanism of molecular clock

Studies during the last 15 years revealed molecular mechanism of clockwork system. From cyanobacteria, via fungi and plants to most complex metazoan including humans, there were reported studies describing molecular clock core comprised of the so called clock genes. Homologues genes operate in various types of organisms (e.g. *Neurospora*, *Drosophilla, Xenopus, mouse, rat, human*) with some particular specificities and differences, but the main principle is the same among all organisms (Dunlap, 1999). Basic molecular clockwork system is built up of specific genes, whose temporal transcription and translation regulation is orchestrated in circadian manner within transcriptional-translational feedback loop comprising of a set of clock genes (Fig 3.1). Positive elements *Bmal1* and *Clock* encode proteins which are members of basic helix-loop-helix transcription factors containing PAS (Period-Arnt-Single-minded) domain. While expression of *Clock* mRNA is mostly constant, *Bmal1* transcription fluctuates within

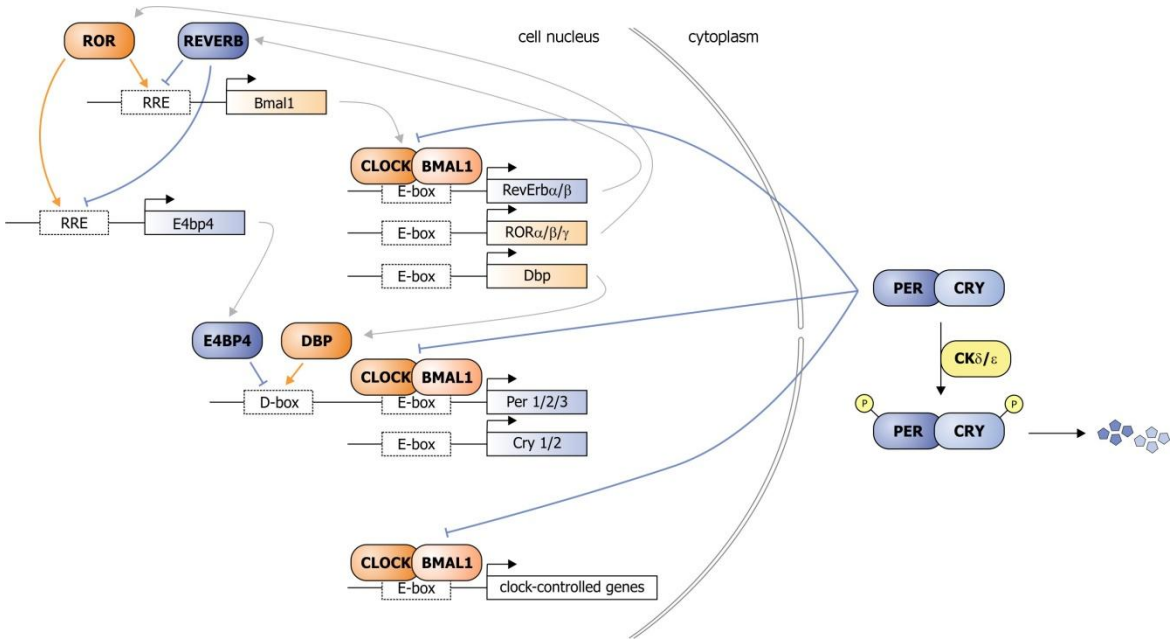

Figure 3.1 Molecular mechanism of main transcriptional-translational feedback loops comprising intrinsic circadian pacemaker. Heterodimer CLOCK/BMAL1 enhances expression of *Per* and *Cry* genes via E-box binding. Subsequently, the protein products of these genes negatively regulate CLOCK/BMAL1 activity, while they are being phosphorylated by casein kinase (CK) δ and ε for degradation. Furthermore, protein products of *ROR* and *Reverb* genes up-regulate and down-regulate, respectively, the expression of *Bmal1* and *E4bp4*. Furthermore, DBP activates expression of *Per* genes via D-box sequence binding, while E4BP4 counteracts this action by competing binding position at D-box. In addition, CLOCK/BMAL1 regulates expression of various clock-controlled genes whose protein products constitute clock output. Enhancement is depicted as an orange line, while inhibition is depicted as a blue line. Positive elements are coloured orange; negative elements are coloured blue.

internal period lasting close to 24 hours. After translation in cytoplasm, CLOCK and BMAL1 proteins heterodimerize and translocate to nucleus, where they act as transcription enhancers by binding to E-box elements in promoter sequences of Period genes (*Per1*, *Per2*, *Per3*), Cryptochrome genes (*Cry1*, *Cry2*), nuclear receptors *RevErbα*, *RevErbβ*, and receptor-related orphan receptors *ROR* (α, β, γ). E-box elements are partially conserved sequences in promoter region, mostly with pattern CACGTG termed conservative E-boxes, though additional non-canonical E-box-like sequences (CANNTG) seem to be also important for proper circadian expression (Nakahata *et al.*, 2008; Ripperger & Schibler, 2006). Resulting protein heterodimer PER/CRY acts as a negative regulator, which inhibits CLOCK/BMAL1 activity. Simultaneously, REVERB proteins suppress, while ROR factors enhance *Bmal1* transcription by binding to *REV response element* (RRE) in its promoter region (Ko & Takahashi, 2006; Lowrey & Takahashi, 2004; Reppert & Weaver, 2001; Takahashi *et al.*, 2008). This results in approximately 24 hours lasting autoregulatory periodic oscillations of positive as well as negative regulators activity.

Concurrently, CLOCK/BMAL1 enhances transcription of various downstream clock-controlled genes by acting at E-box sequences in their promoter regions. Many of them are transcription factors (*Dbp*, D-box binding protein; *Tef*, thyrotroph embryonic factor; *Hlf*, hepatic leukemia factor) or transcription repressors (E4BP4, E4 promoter binding protein 4) and they act in protein signalling cascade to spread the signal of "actual time" as well as participate in co-regulation of clock core feedback loop (Bozek *et al.*, 2009; Zhang & Kay, 2010).

Direct regulatory action of the clock component CLOCK/BMAL1 in regulation of physiological output was observed in transcription orchestration of gene coding vasopressin (*Avp*), whose peptide product is the hormone regulating salt and water homeostasis both in periphery and in central nervous system. CLOCK/BMAL1 binds directly to E-box elements of *Avp* gene promoter and enhance its rhythmic transcription (Jin *et al.*, 1999).

The whole clockwork mechanism involves broader palette of genes participating in the final circadian output. Particularly, casein kinase ε (CKε) and δ (CKδ) are able to alter stability of PER/CRY complex by specific phosphorylation, the signal for degradation of protein complex in proteasome. Recently there has been discovered the modulation of molecular clock by basic helix–loop–helix transcription factors *Dec1* and *Dec2*, which repress CLOCK/BMAL1-induced transactivation of the *Per1* promoter through direct protein–protein interactions with BMAL1 (Honma *et al.*, 2002). This action can be tissue specific, as expression of *Dec1* and *Dec2* is differently altered in various tissues of *Clock*$^{/-}$ mutant mice (Noshiro *et al.*, 2005). Moreover specific *Dec2* mutation is linked to human short sleep phenotype suggesting its role in sleep homeostasis (He *et al.*, 2009). Furthermore, F-box protein with leucine-rich repeats *Fbxl3* has been shown to take part in regulation of the length of period by directing the degradation of the cryptochrome proteins (Busino *et al.*, 2007; Godinho *et al.*, 2007).

3.1.3 Master clock in SCN

Location of the central clock has been discovered by lesions of suprachiasmatic nuclei of hypothalamus (SCN). Animals with such lesions lost their rhythmic locomotor activity patterns (Stephan & Zucker, 1972) and corticosterone rhythm (Moore & Eichler, 1972), while their locomotor activity has been restored by implanted SCN cells with the period of the donor (Silver *et al.*, 1996). Similarly, genetically arrhythmic animals with implanted SCN were able to recover behavioural rhythms (Sujino *et al.*, 2003) suggesting central role of SCN in keeping rhythmic physiological output. Subsequent analyses revealed, that SCN neurons isolated and cultured *in vitro* sustain autonomous circadian rhythms in electrical firing rate (Groos & Hendriks, 1982)

without needs of external stimuli, what indicates their role as intrinsic pacemaker. Period length is individual and genetically determined. In completely non-periodic conditions, such as total darkness, the clock would be free running according to intrinsic period length. Nonetheless, it is perfectly entrainable by environmental cues in order to be synchronized to ambient conditions, which can change during a year. The most prominent entraining signal (called also *Zeitgeber* – "time giver") is light. Interestingly, light perceived at late subjective day/early subjective night causes phase delay of the clock, while in contrast light pulse during late subjective night/early subjective night causes phase advance (Golombek & Rosenstein, 2010; Lowrey & Takahashi, 2004). Light is sensed via retina by non-image forming photoreceptors – photosensitive retinal ganglion cells, which express photosensitive pigment melanopsin (Hankins *et al.*, 2008). The signal is spread via neurons of retino-hypothalamic tract (RHT) to SCN, where electric signals are converted to chemical, finally leading to altered regulation of core feedback loop (Berson *et al.*, 2002). RHT projects not only to SCN, but also to intergeniculate leaflets (IGL), which projects back to SCN via geniculohypothalamic tract, so SCN can perceive light signal also indirectly. Additionally, projections from dorsal raphe nucleus, and median raphe nucleus can mediate non-photic signals to SCN (Dibner *et al.*, 2010). As SCN function as pacemaker, timing signal has to be transmitted to other brain and peripheral structures. Additional projections lead timing signal to dorsomedial hypothalamus (DHM), and the arcuate nucleus. In the thalamus, axons from the SCN innervate the paraventricular nucleus and possibly the IGL (Dibner *et al.*, 2010). Moreover, clock genes are expressed within these particular brain structures, although their expression level and/or pattern, and response to environmental stimuli like light and/or feeding are distinct from the SCN master clock (Abe *et al.*, 2002; Feillet *et al.*, 2008; Namihira *et al.*, 1999).

3.1.4 Extra-SCN pacemakers

3.1.4.1 Methamphetamine sensitive circadian oscillator - MASCO

Interestingly extra-SCN autonomous pacemakers have been described recently. SCN-lesioned mice continually administered methamphetamine were able to restore clear rhythmic locomotor behaviour, although with longer period (Tataroglu *et al.*, 2006). Furthermore, methamphetamine lengthened period of locomotor activity also of intact mice and desynchronized phase of *Per2* gene in peripheral tissues (Pezuk *et al.*, 2010). Analyses with various transgenic clock genes-defective mice did not abolish entrainment ability of

methamphetamine, suggesting presence of SCN-independent pacemaker – methamphetamine sensitive circadian oscillator (Mohawk *et al.*, 2009). These findings are important in the view that there might be environmental cues and conditions, in which other oscillator beside central clock is able to govern particular physiological rhythms.

3.1.4.2 Food-entrainable oscillator - FEO

In fact, there is also another potent entrainment stimulus, which seems to be able to elicit central driven oscillations – it is food availability. When mammals are periodically exposed to food availability in a time frame limited to a few hours per day in the resting phase, they alter their physiological responses and behaviour including locomotor activity, body temperature, and corticosterone secretion in a way to anticipate food accessibility (Boulos & Terman, 1980; Mistlberger, 1994; Nelson *et al.*, 1975; Stephan, 2002). Although locomotor activity in rodents is dominantly present in the active night phase, the daytime restricted feeding can invoke locomotor activity just in a short time frame before food availability, what is called food-anticipatory activity (FAA). FAA appears also in SCN-lesioned rats, documenting existence of an extra SCN food-entrainable oscillator (FEO) (Stephan *et al.*, 1979). Until now, there is still unknown anatomical substance of FEO. Experiments to elucidate this issue have been conducted (Davidson *et al.*, 2000; Mistlberger & Mumby, 1992) with no conclusive results. DHM was suggested as site of FEO niche based on *Per2* mRNA oscillation and early response *c-fos* gene expression in region of DHM exclusively under restricted feeding. Furthermore, blocking of FAA behaviour in DHM-lesioned animals (Mieda *et al.*, 2006) supported the hypothesis. However, following experiments did not confirm particular structure as definitive primary site of FEO (Landry *et al.*, 2006; Moriya *et al.*, 2009). In addition, participation of other brain parts, such as hippocampus and cerebral cortex has been demonstrated (Wakamatsu *et al.*, 2001).

Results of recent experiments suggest that complex processes and more brain structures participate in generating of the FAA behaviour (Mitra *et al.*, 2011) and/or peripheral organs take a substantial role (LeSauter *et al.*, 2009). Though it is not clear which anatomical structure is definite site of FEO, molecular analysis revealed partial role of clock genes in the process. *Per2* knockout mice exhibited altered anticipation of mealtime, while sustained peripheral synchronization (Feillet *et al.*, 2006). In contrast, normal feeding time anticipation was observed in *Per1*, *Clock* and *Bmal1* knockouts (Feillet *et al.*, 2006; Pendergast *et al.*, 2009; Pitts *et al.*, 2003). Interestingly, knockouts of *Npas2* gene, *Clock* paralog, which plays the same role instead

of CLOCK in some forebrain structures, exhibit alteration in FAA behaviour (Dudley *et al.*, 2003) as well as *Cry1/Cry2* doubleknockouts (Iijima *et al.*, 2005).

3.1.5 Peripheral clocks

While molecular basis of clockwork system was revealed in the central nervous system, further experiments identified functional peripheral clocks in various peripheral tissues. Cultured fibroblasts were found to exhibit expression of clock genes *Per1*, *Per2* in circadian manner accompanied by rhythmic expression of genes encoding transcription factors *Rev-Erbα*, *Dbp* and *Tef* after serum shock (Balsalobre *et al.*, 1998). Also, pineal gland cells rhythmically express numerous clock genes (Namihira *et al.*, 1999). To date, molecular clockwork machinery was identified in almost all tissues, including retina (Tosini & Menaker, 1996), liver, lung, skeletal muscle (Yamazaki *et al.*, 2000), kidney, heart, pancreas (Damiola, 2000), and recently, colon (Hoogerwerf *et al.*, 2007; Sládek *et al.*, 2007). The same molecular mechanism of the self-sustaining feedback loop of clock gene transcription and translation is employed in peripheral tissues. However, these "slave" clocks are synchronized by central clock with neural and humoral signals (Dibner *et al.*, 2010).

In vitro experiments showed that cultured fibroblasts are acutely reset by corticosterone analogue, dexamethasone, which induces *Per1* mRNA expression. Similarly, *in vivo* studies demonstrated that dexamethasone induced resetting of liver, kidney and heart clocks, while SCN was not affected by glucocorticoid application (Balsalobre, 2000). Interestingly, liver, lung and skeletal muscle responded differentially to light-induced phase shifting, when liver adapted to phase delay more slowly than lung and skeletal muscle. Response to the phase advance took more days with distinct adaptation rate for all studied tissues, however it was completed after 6 days (Yamazaki *et al.*, 2000). Humoral signals are not the only possible candidates for mediation of entrainment, as neural projections from SCN via sympathetic and parasympathetic nervous system are also suggested to play a role in resetting of periphery (Cailotto *et al.*, 2009; Logan *et al.*, 2011; Shibata, 2004).

As seen, unequivocal dissection is still missing and it seems that harmony of both regulatory principles participate. Moreover, regulation is much more complex, as emerging from experiments showing uncoupling of peripheral clock phase from the central clock under specific physiological conditions, particularly reverse restricted feeding (RF), when animals has limited access to food only for a few hours during the resting light phase (Damiola, 2000; Stokkan *et al.*, 2001). Liver clock is being entrained by scheduled feeding independently of the central clock,

when acrophase of clock genes is shifted according to mealtime in liver, while it remains unchanged in SCN. This is the first evidence that peripheral clocks can be uncoupled and RF can entrain peripheral clock independently of the central clock. Interestingly, only 4-hour, but not 8-hour, RF was able to entrain peripheral clock in lung (Stokkan *et al.*, 2001). Moreover, process of resetting clocks in kidney, heart, and pancreas was slower than that in the liver (Damiola, 2000) suggesting tissue specific response to external stimuli. Recently it has been shown, that restricted feeding shifts also the phase of colonic clock in the same way and the same phase as in liver, while SCN clock remains unshifted. This demonstrates partially independent functional clock, which might govern rhythmical changes in intestinal epithelium (Hoogerwerf *et al.*, 2007; Sládek *et al.*, 2007).

3.2 Intestinal rhythms and clock

3.2.1 Rhythms of intestinal enzymes

Simultaneously with general circadian rhythm observations, a lot of findings documented rhythmic changes in intestinal functions. Marked circadian variations in cholesterol synthesis (Edwards *et al.*, 1972) and activity of HMG CoA (Shefer *et al.*, 1972) in the jejunum and ileum were detected. Other experiments revealed strong diurnal variations in activity of numerous digestive enzymes in jejunum, particularly maltase, sucrase, trehalase, lactase, leucine aminopeptidase, and alkaline phosphatase. The highest activity was observed in the middle of the active (dark in rat) phase in animals fed *ad libitum*, and with maximal peak of activity shifted to the light phase, under condition of daytime feeding (Saito *et al.*, 1975; Stevenson *et al.*, 1975). Similarly, polyamine synthesis enzyme, ornithine decarboxylase, displayed rhythmic changes in activity with highest rate after food intake (Fujimoto *et al.*, 1978).

3.2.2 Transport rhythms

3.2.2.1 Organic molecules transport

Studies on circadian intestinal transport were pivoted by experiments of Baril and Potter (Baril & Potter, 1968), who found diurnal variations in amino acid ^{14}C-cycloleucine uptake by small intestine. Similarly, daily fluctuations in intestinal transport of L-histidine with greater transport capacity during dark phase were observed in animals kept on conventional light-dark

conditions with *ad libitum* access to diet, whereas shift of maximal transport rate to light phase was detected in animals with daytime food access (Furuya & Yugari, 1971).

In addition, diurnal changes in sucrose, glucose and water transport were detected in small intestine with higher rate during the dark phase, while daytime feeding shifted the maximal rate to the light phase (Fisher & Gardner, 1976; Stevenson & Fierstein, 1976). Further experiments revealed that circadian changes in glucose absorption reflect rhythmic availability of carbohydrate transporters. mRNA and protein levels of apical fructose transporter *Glut5* and basolateral glucose transporter *Glut2* were found to exhibit diurnal oscillations and regulation of *Glut5* is influenced by food intake (Castelló *et al.*, 1995; Corpe & Burant, 1996; Houghton *et al.*, 2008).

Sglt1 (sodium glucose transporter 1), a gene coding protein responsible for active glucose absorption via antiport with sodium, was also found to be diurnally regulated at both mRNA and protein levels in particular segments of the small intestine (Corpe & Burant, 1996; Rhoads, 1998) and is considered as the main source of diurnal rhythmicity in intestinal absorptive capacity (Rhoads, 1998; Tavakkolizadeh *et al.*, 2001).

Food intake is a potent entrainment stimulus which is able to induce phase shift of *Sglt1* expression (Pan *et al.*, 2004). However, no direct local effect of luminal content on *Sglt1* rhythm was observed. Laparotomically formed isolated loops of jejunum enable to study jejunum epithelium without luminal content *in vivo*. Expressions of both mRNA, and protein were unaltered in such isolated loops comparing to the intact jejunum (Stearns *et al.*, 2009), which suggests that intrinsic mechanism of regulation is involved rather than direct effect of luminal content.

Various studies uncovered circadian pattern of mRNA, protein expression, and activity of PEPT1, the H^+/peptide cotransporter, in rat duodenum (Pan *et al.*, 2002), jejunum, but not ileum (Qandeel *et al.*, 2009) under standard and RF conditions. The phases of both mRNA and protein rhythmic expressions were shifted from the beginning of the dark phase to the light phase according to food availability (Pan *et al.*, 2004). Interestingly, after 4 days of food deprivation, the rhythmicity of protein expression as well as the absorption capacity were gradually abandoned, while the expression of mRNA remained rhythmic (Pan *et al.*, 2003, 2004).

Circadian patterns of transport capacity and expression were also observed in intestinal drug transporters, which play a substantial role in pharmacokinetics of many drugs, including chemicals used in cancer therapy, and participate in intestinal barrier as well as in tumour ability to dispose anticancer drugs (Chan *et al.*, 2004). Apparent rhythmic expression and activity of

P-glycoprotein and one of its gene, *Abcb1a*/*Mdr1a*, was observed in mouse liver and intestine (Ando *et al.*, 2005). Another member of ATP-binding cassette transporter (ABC) family, *Abcc2*, involved in the cellular efflux of cytotoxic drug irinotecan, exhibited circadian expression in mouse ileum (Okyar *et al.*, 2011). Similarly, in rat jejunum, diurnal rhythmicity was observed for *Abcb1*/*Mdr1* (multidrug-resistance like protein 1) and its expression was phase-shifted by restricted feeding (Hayashi *et al.*, 2010; Stearns *et al.*, 2008). In addition, *Mct1* (monocarboxylate transporter), *Abcc2*/*Mrp2* (multidrug resistance protein 2), and *Bcrp* (breast cancer resistance protein) exhibited diurnal variations, while no significant rhythm was detected in *Mdr3*, *Mrp1*, *Mrp3*, *Octn2* (organic cation transporter 2), and *Oatp-b* (organic anion transporter B) in rat jejunum (Stearns *et al.*, 2008). Importantly, direct effect of peripheral clock was demonstrated, as clock-controlled transcription factor HLF activated transcription of the *Mdr1a* gene, whereas molecular clock component E4BP4 was able to suppress transcription at the same DNA binding site (Murakami *et al.*, 2008). Moreover, in *Clock*-mutant mice intestinal *Mdr1a* rhythm was lost suggesting clock dependent circadian regulation (Murakami *et al.*, 2008).

3.2.2.2 Ion transport

Generally, electrolyte transport is a fundamental mechanism for sustaining cell excitability and volume. Furthermore, renal and intestinal ion transport pathways are essential processes of maintaining electrolyte balance as well as blood and interstitial volume homeostasis in whole organism. There are several transporters and channels mediating transmembrane and transepithelial ion transport.

In the intestine, the electroneutral sodium absorption (Fig. 3.2 A) is mediated by apically localized hydrogen/sodium exchangers NHE3 and NHE2 (coded by genes *Slc9a3* and *Slc9a2*, respectively) (Zachos *et al.*, 2005). Parallel to sodium absorption, the apically located transporters DRA (Down-regulated in adenoma, coded by *Slc26a3* gene), PAT1 (Putative anion exchanger 1, coded by *Slc26a6* gene) and AE1 (Anion exchanger 1, coded by *Slc4a1* gene) mediate the exchange of luminal chloride for intracellular bicarbonate resulting in absorption of NaCl into epithelial cells (Geibel, 2005; Kunzelmann & Mall, 2002). On the basolateral membrane operates the sodium-potassium pump (Na^+/K^+-ATPase assembled from α and β subunits, coded by *Atp1a* and *Atp1b* genes), which extrudes 3 sodium ions in exchange for 2 potassium ions. The pump activity is the main driving force determining rate of sodium luminal uptake. Basolaterally located KCC1 transporter (coded by gene *Slc12a4*) extrudes potassium and

chloride ions, thus enabling NaCl to be fully transported through epithelium, while potassium ions are recycled (Kunzelmann & Mall, 2002).

In the condition of low sodium intake, electrolyte homeostasis is essentially maintained by raised sodium retention. The mechanism of aldosterone-dependent activation of electrogenic sodium transport (Fig. 3.2 B) takes a part in both the aldosterone-sensitive distal nephron segments and distal colon epithelium, where sodium channel ENaC (heterotrimeric channel complex composed of one α subunit, one β subunit, and one γ subunit) is activated (Geibel, 2005; Kunzelmann & Mall, 2002). Lowered dietary sodium intake is the stimulus for adrenal aldosterone synthesis and release via renin-angiotensin signalling. Aldosterone has direct effect on epithelial cells and after binding to its mineralocorticoid receptor, it enhances expression of regulatory subunits of ENaC (α and β in the kidney; β and γ in the colon) (Epple *et al.*, 2000). Moreover, it stimulates trafficking of vesicles with pre-synthesized sodium channels to apical membrane (Butterworth *et al.*, 2009) and enhances ENaC deubiquitylation (Fakitsas *et al.*, 2007).

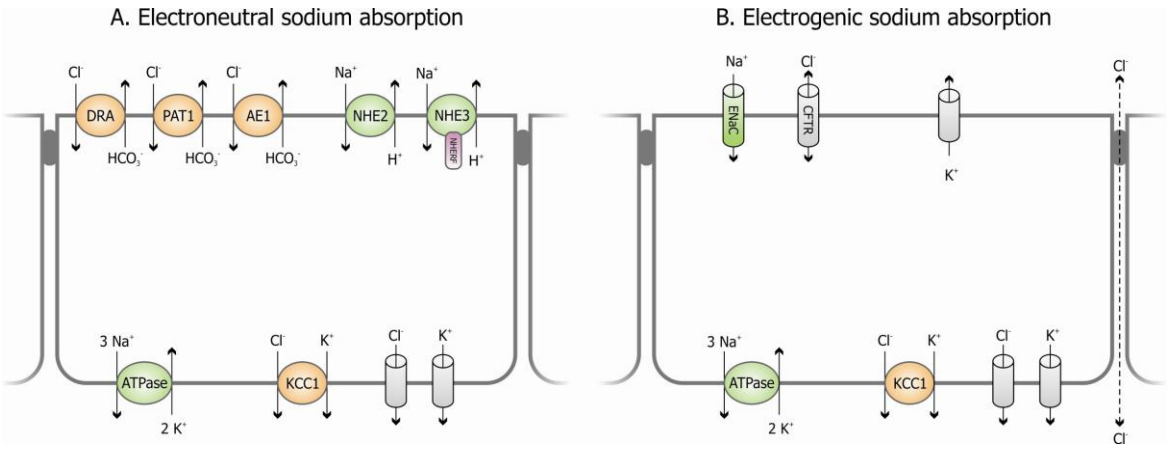

Figure 3.2 Transepithelial sodium absorption in the colon. A. Electroneutral transport dominates under standard diet condition. Participating transporters and channels are depicted. **B.** Electrogenic sodium transport operates in colon if the Na^+ dietary intake is decreased due to low-salt diet. Participating transporters and channels are depicted. DRA – Downregulated in adenoma, PAT1 – Putative anion exchanger 1, AE1 – anion exchanger 1, NHE2/3 – Na^+/H+ exchanger, NHERF – NHE3-regulatory factor, ATPase – Na^+/K^+ ATPase, KCC1 – K^+-Cl^- cotransporter 1. ENaC – epithelial Na^+ channel, CFTR – Cystic fibrosis transmembrane conductance regulator.

Maintenance of electrolyte/water homeostasis includes balance between ion and water absorption and secretion. The epithelial surface of the intestinal lumen is covered by secreted mucus which is maintained hydrated by accompanying KCl and NaCl secretion followed by water movement. Distal colon is the final segment where it is possible to regulate and adjust

absorption/secretion balance, so that stool is in an appropriate consistency avoiding diarrhoea or constipation. Primary source of intestinal chloride efflux is chloride channel CFTR (cystic fibrosis transmembrane conductance regulator) located in apical membrane (Fig. 3.3). Besides, potassium channels are present in the apical membrane allowing potassium efflux that results in KCl secretion. To maintain secretion through the epithelial cells, the basolateral membrane has to allow ion intake from the serosal site in sufficient rate. Na^+-K^+-$2Cl^-$ cotransporter type 1 (NKCC1, coded by *Slc12a2*) was identified to fulfil this function in basolateral membrane of colonic enterocytes (Barrett & Keely, 2000; Geibel, 2005). The imbalance between intestinal secretion and absorption may cause various pathologies such as cystic fibrosis or diarrhoea, which often accompany ulcerative colitis and Crohn's disease. Therefore studies of the intestinal ion transport regulatory mechanisms are of particular interest.

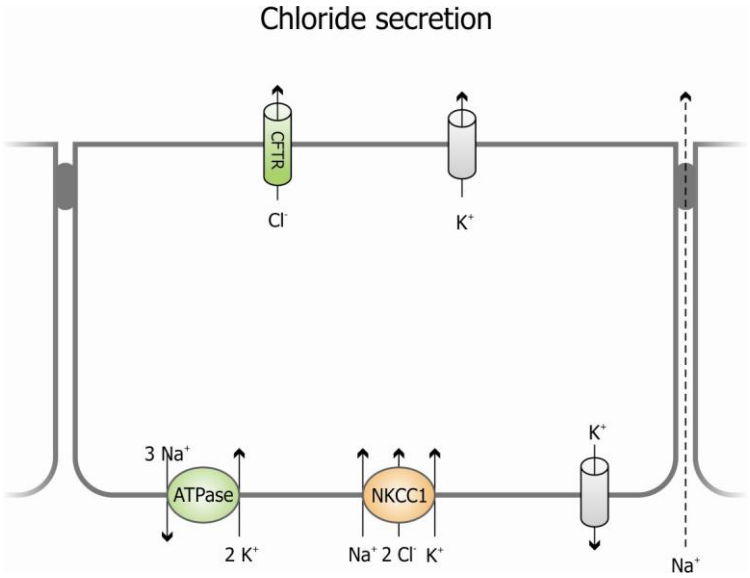

Figure 3.3 Transepithelial chloride secretion in the colon. CFTR – Cystic fibrosis transmembrane conductance regulator, ATPase – Na^+/K^+ ATPase, NKCC1 – Na^+-K^+-Cl^- cotransporter.

Various functions related to ion transport exhibit diurnal variations. For years it is known that excretion of urine follows circadian pattern with maximal peak in the first half of active phase (Mills & Stanbury, 1952). Similarly, renal excretion of electrolytes such as Na^+, K^+, Cl^- and PO_4^- was found to vary during 24 hours (Dossetor *et al.*, 1963; Wesson, 1980). Correspondingly glomerulal filtration rate (Cambar *et al.*, 1979) and blood pressure exhibit circadian oscillations (Lew, 1976; Millar-Craig *et al.*, 1978). This is in parallel with the rhythm of sodium extrusion. It was postulated that impairment in daytime renal sodium might be the major cause of high blood pressure in non-dipping patients (Bankir *et al.*, 2008; Burnier *et al.*, 2007). Likewise

concentration of plasma corticosterone or cortisol, also aldosterone which is the main regulator of sodium absorption, exhibits diurnal variations (Bligh *et al.*, 1993; Cugini *et al.*, 1984; Hilfenhaus, 1976; Hurwitz *et al.*, 2004). Moreover, it has been shown recently, that the gene coding 3 β-hydroxysteroid dehydrogenase VI (*Hsd3b6*), an enzyme involved in aldosterone synthesis, is modulated by circadian clock (Doi *et al.*, 2010). In addition, low aldosterone levels were observed in triple-knockout transgenic mice of clock-controlled transcriptional factors *Dbp/ Hlf/ Tef* and overproduction of aldosterone was detected in adrenal glands of *Cry*-null mice. These observations support the role of clock genes in aldosterone synthesis (Doi *et al.*, 2010; Wang *et al.*, 2010).

Diurnal changes of colonic transmural electrical potential difference were observed in rabbit, which is ceacotrophy animal model. Some small mammals, including rabbits are passing food through alimentary tract twice. They produce and eat soft faecal pellets directly from anus and store it in caecum, where it is to be mixed with ingested food. This process is time- and light-dependent and exhibit circadian rhythmicity (Jilge, 1982). In these animals, the transepithelial electrical potential measured in rectum varied during the day with maximal value of difference at the beginning of the night. Similarly, the plasma level of aldosterone exhibited circadian pattern peaking at the same daytime. Importantly, light/dark rhythm was determined as external synchronizer of observed rhythms, as light phase manipulation changed the transepithelial electrical potential differences regarding the part of the day (Clauss *et al.*, 1988). Moreover, diurnal fluctuations were observed in colonic short circuit current (I_{sc}) and sodium flux with greater extent during the late subjective day compared with the early subjective day (Clauss *et al.*, 1988).

Similar observations have been made in mice, where rectal amiloride-sensitive sodium transport exhibited diurnal variations with significantly higher activity during the late subjective day compared with the early subjective day. Moreover, the transport rate depended on salt intake and the potential difference in rectum exhibited greater negative values in condition of low-salt intake (Wang *et al.*, 2000). This is in concordance with previous findings in rats, where low-salt diet stimulates potential difference and amiloride sensitive short circuit current (Pácha & Pohlová, 1995). Very recently it has been demonstrated that both NHE3 transporter and α subunit of ENaC channel exhibit circadian expression in kidney and both genes might be under direct regulatory control of clock genes (Gumz *et al.*, 2009; Nishinaga *et al.*, 2009; Saifur Rohman *et al.*, 2005). Moreover, circadian expression of *Nhe3* mRNA was also detected in rat distal colon (Sládek *et al.*, 2007). Using whole transcriptome microarray gene expression assays, recent

studies identified rhythmic expression of several other genes coding transporters, channels and associated proteins in the colon, particularly the α and β subunits of the sodium pump, and the NHE regulatory factor scaffold protein NHERF1 coded by *Slc9a3r1* gene (Hoogerwerf *et al.*, 2008).

Implications for contribution of local peripheral clock to sodium transport have been documented in the kidney and suggestions have been made regarding similar regulation in the colon. However detailed functional study of possible circadian control of colonic electrolyte transport is still lacking.

3.2.3 Cell cycle rhythms

3.2.3.1 Cell cycle regulation

In order to maintain full-capacity absorptive, secretory, and barrier functions, the intestinal epithelium possesses huge renewal ability and is a site of enormous cell proliferation and exfoliation. The primary source of enterocytes are intestinal stem cells located in crypt base just few cells above Paneth cells, which provide innate immunity and are located at the very bottom of the crypts. Cells originated from stem cells continually differentiate and migrate along the crypt axis from the bottom to the crypt mouth or villus in the colon or small intestine, respectively, while being more differentiated and fully equipped to carry out their functions. Migrating cells differentiate into three functional types: most prominent enterocytes with absorptive/secretory functions, goblet cells producing and secreting mucus, and enteroendocrine cells producing paracrine/autocrine peptide hormones (Crosnier *et al.*, 2006; van der Flier & Clevers, 2009). The self-renewal process takes about 3 – 5 days (van der Flier & Clevers, 2009). The strict cell cycle regulation is essential to sustain homeostasis in the number of epithelial cells sufficient to maintain proper intestinal functions and at the same time to prevent tissue hyperplasia or malformation.

In general, epithelial cells share common principles of cell cycle regulatory pathways. Serine-threonine kinases, the so called cyclin dependent kinases (CDKs), their regulatory subunits called cyclins, and their inhibitors are taking part in regulatory pathways leading to either progression or regression of cell cycle. Cascades of subsequent varying levels of particular cyclins and their association with corresponding CDKs lead to activation of target substances by phosphorylation. It is followed by cell cycle progression and initiation of particular cellular responses finally directing cell to division. Opposite actions causing the withholding of cell cycle

are mediated by a class of regulators called CDK inhibitors (CKI). Most prominent are members of the Cip and Waf family, which universally inhibit CDK/cyclin complexes, particularly p21 (also known as $p21^{Waf1/Cip1}$), p27 ($p27^{Kip1}$) and p57 ($p57^{Kip2}$). p21 is directly activated by another important tumour suppressor p53 or p53-independently. p27 is able to mediate cell cycle arrest (Sherr & Roberts, 1999). Both p21 and p27 play significant role in intestinal epithelial cell differentiation (Quaroni *et al.*, 2000). Another family (INK4/ARF) of CKI, which includes p15 ($p15^{INK4B}$), p16 ($p16^{INK4A}$), p18 ($p18^{INK4C}$), p19 ($p19^{ARF}$), selectively binds and inhibits CDK4 and CDK6, thus stops cell cycle progression (Tian & Quaroni, 1999). Their members participate in regulation of cellular senescence and are associated with development of malignant diseases (Witkiewicz *et al.*, 2011).

Progression of cell cycle (Fig 3.4) involves relatively long G1 phase, during which association of cyclins D (D1, D2, D3) with CKD4 and CDK6 is required. G1/S transition is initiated by accumulation of cyclin E, which binds to CDK2. Additionally, important target of CDK4/6 are retinoblastoma protein pRb and pRb-related proteins p107 and p130. In their hypophosphorylated state, they can sequester and inactivate E2F family proteins, so that E2Fs cannot act as transcription factors responsible for activating genes participating in promoting of G1/S transition. CDK2/cyclin A complex is present during S phase and complexes of CDK1/cyclin A and CDK1/cyclin B start S/G2 transition. Finally, CDK1/cyclin B is characteristic for entry to mitosis (Arellano & Moreno, 1997; Ekholm & Reed, 2000). *Wee1/Myt1* kinases are potent negative regulators of that transition achieved by tyrosine phosphorylation and inactivation of CDK1/cyclin B kinase (McGowan & Russell, 1995). Their counterpart, family of CDC25 phospatases are able to dephosphorylate and activate CDK1/cyclin B resulting in mitosis progress (Karlsson-Rosenthal & Millar, 2006).

Although basic principles of cell cycle regulation are employed within colonic epithelium, there are other important regulatory pathways, which contribute to proper balance of number, density and differentiation state of epithelial cells. Major driving force of intestinal epithelium proliferation is Wnt/β-catenin pathway. In the absence of Wnt signal, β-catenin is targeted for degradation by proteolysis through phosphorylation signal mediated by complex of adenomatous polyposis coli (APC), casein kinase I (CKI), glycogen synthase kinase 3 (GSK3), and axin. After binding Wnt to its Frizzled membrane receptor, phosphorylatory complex is inhibited and β-catenin is no longer degraded, so it acts as transcription factor by binding T cell factor (TCF)and subsequently activating Wnt target genes (van der Flier & Clevers, 2009). One of the

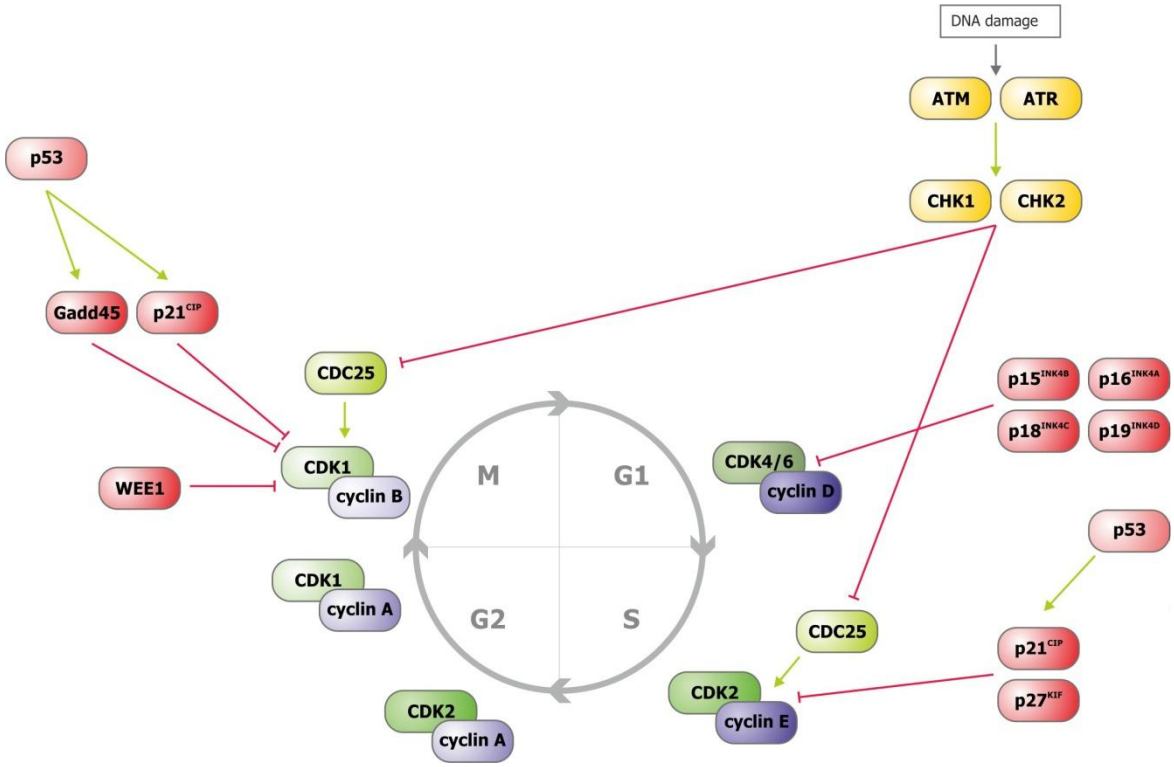

Figure 3.4 Scheme of cell cycle regulation. CDKs (cyclin dependent kinases, CDK1, CDK2, CDK4, CDK6) form complexes with particular cyclins (cyclin D, E, A, B) and mediate cell cycle progression. At different stages of cell cycle, CDK inhibitors (p15, p16, p18, p19, p21, p27, p53, Gadd45) are able to withhold cell cycle progression by interacting with CDK/cyclin complexes. WEE1 kinase inhibits G2/M progression by phosphorylation of CKD1/cyclin B complex, while the phosphatase CDC25 counteracts the process. DNA damage response proteins ATM and ATR activate Check kinases CHK1 and CHK2, which act as cell cycle repressors.

principal Wnt target genes is the oncogene *c-Myc*, which was shown to be repressed by APC and activated by β-catenin (He *et al.*, 1998).

In addition, there are some other pathways that play a role in a differentiation: BMP (bone morphogenetic factor) signalling associated with SMAD transcription factors operates in villi, but not in crypts; Notch signalling pathway is important in keeping crypt cells in non-differentiated proliferating state (Crosnier *et al.*, 2006; van der Flier & Clevers, 2009).

3.2.3.2 Involvement of circadian clock in cell cycle

As circadian clock is a strong pacemaker of different cellular and physiological processes, there were conducted several experiments to elucidate crosstalk between clock and cell cycle regulatory pathways. For long time it is known, that cell proliferation follows circadian pattern of rhythmic variations as detected by thymidine uptake (Burns *et al.*, 1972), mitotic figures (Scheving *et al.*, 1972) or DNA content (Ruby *et al.*, 1973) at different time of day. Also, parts of

mice alimentary tract, particularly oesophagus, stomach, jejunum and rectum exhibit strong daily variations in incorporation of labelled [^{3}H]-thymidine (Scheving, 2000).

Evidence for circadian expression of cell cycle associated proteins, as revealed by cosinor analysis, was documented by Bjarnason *et al.* (1999), when they detected in human oral mucosa circadian rhythm of protein expression of cyclins A, B1, and E, inhibitor of cell cycle progression p53, and marker of active proliferation Ki67. Similarly, other cell cycle regulators such as *c-Myc*, *p53*, *cyclin D1*, *Mdm2*, and *Gadd45α*, were demonstrated to exhibit circadian pattern of expression in mouse liver (Fu *et al.*, 2002). Recent reports showed that expression of important cell cycle inhibitor *p21* followed diurnal changes in wild type mice liver, while the expression pattern was substantially elevated in *Bmal1*-null mice (Gréchez-Cassiau *et al.*, 2008). Further analysis demonstrated that these circadian changes were p53-independent and that they were not regulated directly by CLOCK/BMAL1 as promoter region of *p21* lacked E-box sequences. Nonetheless, performed experiments suggested direct responsiveness of *p21* promoter to ROR/REVERB signalling (Gréchez-Cassiau *et al.*, 2008). Furthermore, PER1 overexpression was able to induce c-Myc and repressed p21 in response to ionizing radiation (Gery *et al.*, 2006). Additionally, the expression of many cell cycle regulatory genes was changed in *Clock* mutant mice cells. In particular, inhibitors *p21* and *p27* were up-regulated, while *Cdk2* and cyclins *D3* and *E1* were down-regulated as well as the expression of apoptosis-related genes such as *Akt1*, *Bcl2*, and *Pbef* (Miller *et al.*, 2007). These experiments indirectly suggest an interfering role of clock genes in cell cycle regulation.

Direct link of intestinal molecular clock and cell cycle regulation was proposed by Matsuo *et al.* (2003), who showed that regenerating liver (after 2/3 partial hepactomy; PH) is associated with intensive cell proliferation and entry to mitosis is dependent on the *Zeitgeber* rather than the time of hepactomy. This has been shown by experiment where massive entry to mitosis was observed 40 hours after PH performed at ZT8, whereas in animals with PH performed at ZT0 most prominent entry to mitosis was observed 48 hours after PH, suggesting a substantial role of circadian clock timing in proliferation. Further, the same authors have shown that regulation of complex CDK1/cyclin B activity is crucial for circadian time-dependent entry to mitosis. In addition, potential candidates for mediating circadian time to cell cycle associated proteins were explored and it was found, that the expression of *Wee1*, the regulator of CDK1/cyclin B complex, is rhythmic and also circadian time-dependent regardless of PH timing. Moreover, *Wee1* promoter was found to contain E-boxes, which were able to functionally bind CLOCK/BMAL1 heterodimer in circadian manner. Additionally, mitotic timing, and *Wee1*, *Cdc2*, and *cyclin B*

expression is impaired in *Cry*-deficient transgenic mice, while simultaneously WEE1 protein level is elevated, what is assigned to lack of CLOCK/BMAL1 inhibition by CRY (Matsuo *et al.*, 2003). This study is an evidence for direct participation of peripheral clock in cell cycle regulation.

Further, *c-Myc* gene is also supposed to be influenced by clock, as it was demonstrated *in vitro* that BMAL1/NPAS2 can suppress expression of *c-Myc* by direct binding to its promoter and inhibiting transcription (Fu *et al.*, 2002).

3.2.4 Clock in cancer

3.2.4.1 Chronodisruption

Recently, a lot of findings give an indication of the substantial role of repeated disruption of circadian rhythms, exposure to light at night, and impairment of circadian clock in pathological deregulation of proliferation, tissue malformation and carcinogenesis.

Epidemiological studies documented increased risk of cancer incidence in shift workers or people with abnormal working hours, like pilots or flight service. As meta-analysis from several studies proves, in-flight personnel have 70% higher risk for breast cancer incidence and excess of 40% risk assessment for prostate cancer occurrence (Erren *et al.*, 2008). Although it is not possible to exclude participation of higher gained radiation during flights, data from other studies suggest that chronodisruption in shift workers might be the main source of elevated cancer risk. Combined meta-analysis of large epidemiological studies of shift workers estimated 40-50% higher risk for breast cancer (Erren *et al.*, 2008). Furthermore, colorectal cancer risk was assessed in nurses working on night shifts with more profound risk when working more than 15 years (Schernhammer *et al.*, 2003). In addition, long-term sleeping duration less than 6 hours was found to be of 50% higher risk of colonic adenoma incidence (Thompson *et al.*, 2011).

In experimental studies, chronodisruption was shown to affect rate of tumour growth as observed in xenograft model with implanted HeLa cancer cells; animals kept on constant light had significantly greater volume of tumour and higher rate of microvessel angiogenesis (Yasuniwa *et al.*, 2010).

3.2.4.2 Molecular clock disruption

Disruption of clock at molecular level has been shown to be linked to process of tissue transformation and sensitizing cells to various harmful environmental cues (Khapre *et al.*, 2010). Fu *et al.* (2002) have shown that *Per2*-mutant mice are cancer prone (with salivary gland

hyperplasia and developed teratomas around the genitals) and show substantially higher susceptibility to γ radiation induced lymphoma tumour growth. Also, reduced apoptosis rate was detected in thymocytes of *Per2*-mutant mice after γ radiation. Furthermore, rhythmicity of several cell cycle regulatory genes like *c-Myc*, and *cyclin D1* is altered in *Per2*-mutant mice comparing wild type, suggesting significant role of *Per2* in cell cycle regulation and tumourigenesis (Fu *et al.*, 2002). Further research revealed that mice deficient in other clock genes, in particular *Bmal1* (*Bmal1*$^{+/-}$), *Cry1* and *Cry2* (*Cry1*$^{-/-}$;*Cry2*$^{-/-}$), *Per1* and *Per2* (*Per1*$^{-/-}$;*Per2*$^{m/m}$) or *Per2* alone (*Per2*$^{-/-}$) are also all cancer prone (Lee *et al.*, 2010).

Attenuation of *Per2* expression in colon cell line led to increased proliferation, presumably via up-regulation of *β-catenin* and *cyclin D*. Similarly, *Per2*$^{m/m}$ mice develop colonic polyps and show an increase of small intestinal mucosa β-catenin and cyclin D protein levels compared with the wild type mice (Wood *et al.*, 2008). Moreover, increased colonic and small intestinal polyp formation can be found in mice with combined genotype of *Per2* mutation with *Apc*$^{Min/+}$ mutation (Min - *multiple intestinal neoplasia*; mouse with a heterozygous mutation of the *Apc* gene - *adenomatous polyposis coli*) (Wood *et al.*, 2008). Rhythmic expression of *Per2* protein is substantially altered in the small intestine of *Apc*$^{Min/+}$ mice and similarly, attenuation or phase shift of rhythmic mRNA expression was observed in several other clock genes, and clock-controlled genes *Dbp* and *Wee1* (Yang *et al.*, 2009a).

In vitro overexpression of PER2 in lung and mammary cancer cell lines rapidly reduced proliferation and induced apoptosis, presumably via *c-Myc*, *Bcl-XL* and *Bcl-2* down-regulation together with *p53* and *Bax* up-regulation (Hua *et al.*, 2006). Intratumoural *mPer2* gene delivery in C57BL/6 mice was able to induce apoptosis *in vivo* (Hua *et al.*, 2007). In addition, mutation of *Per2* gene, which is known to be responsible for familial advanced sleep phase syndrome, led to enhanced resistance to X-ray-induced apoptosis and increased RAS-mediated oncogenic transformation in fibroblasts (Gu *et al.*, 2012).

Down-regulation of tumour *Per1* expression increased breast cancer cell growth *in vitro* and implanted tumour growth *in vivo*, by enhancing the circadian amplitude of the two daily tumour growth peaks (Yang *et al.*, 2009b). Conversely, overexpression of *Per* sensitized human colorectal cancer cell line to DNA damage-induced apoptosis, while in contrast, inhibition of *Per1* blunted apoptosis (Gery *et al.*, 2006). However, on the contrary, anti-apoptotic effect of *Per1* was observed in hepatocellular, pancreatic, and gingival cancer cell lines (Sato *et al.*, 2009, 2011) suggesting tissue specific and possibly multivariable role of *Per1*.

Although cryptochromes deficient mice (*Cry1*$^{-/-}$*Cry2*$^{-/-}$) do not show any difference in radiation-induced tumour incidence or DNA damage checkpoint response comparing with the wild type mice (Gauger & Sancar, 2005), triple gene deficient mice (*p53*$^{-/-}$*Cry1*$^{-/-}$*Cry2*$^{-/-}$) exhibit extended lifespan and reduced tumour incidence comparing with single *p53* deficient mice (*p53*$^{-/-}$), which are known to be strongly cancer prone (Ozturk *et al.*, 2009). Observed effect was likely caused by increased genotoxic stress-induced apoptosis sensitivity of triple knockout cells mediated probably by *p73* induction (Lee & Sancar, 2011).

Clock gene deficiency has been shown to result in significantly increased pro-apoptotic and simultaneously decreased pro-proliferative gene expression (Antoch *et al.*, 2008; Miller *et al.*, 2007), which correlated with a higher rate of radiation-induced apoptosis in both spleen and thymus of *Clock* deficient mice (Antoch *et al.*, 2008). Although no radiation-induced tumour development was observed in *Clock*-mutant mice, they manifested significant weight loss, higher mortality, severe eye pathologies, acceleration of a number of degenerative processes associated with aging, and exhibited the signs of premature aging phenotype comparing with the irradiated wild type mice (Antoch *et al.*, 2008).

Similarly, substantially reduced lifespan, and premature ageing marks, including eye, skin and hair pathologies appeared in *Bmal1* knockout mice (*Bmal1*$^{-/-}$). This manifestation was in correlation with elevated reactive oxygen species in several tissues, suggesting possible mechanism of CLOCK/BMAL1 involvement in physiological oxidative stress responses (Kondratov *et al.*, 2006).

Interestingly, γ-radiation was able to advance substantially the phase of mice locomotor activity, and this phase shift depended on irradiation timing (Oklejewicz *et al.*, 2008). Similarly, ultraviolet ionizing and oxidative stress phase-advanced *Per2* expression in fibroblasts by a mechanism involving ATM/ATR DNA damage response pathway as documented by attenuated phase advance response after the pathway inhibition (Oklejewicz *et al.*, 2008). Human TIMELESS protein (*Drosophila* orthologue), which interacts on one hand with circadian clock gene *Cry2* and on the other hand with cell cycle checkpoint proteins CHK1 and ATR, plays an important role in the DNA damage checkpoint response (Unsal-Kaçmaz *et al.*, 2005). Moreover, direct interaction of PER1 and DNA damage response proteins ATM and CHK2 (Gery *et al.*, 2006) implicate bidirectional signalling of clock and checkpoints regulatory pathways.

Above mentioned studies indicate possible linking between circadian clock, cell cycle regulation, and tumourigenesis. However, exact mechanism and potential involvement of circadian clock in intestinal tumourigenesis is still lacking.

4 AIMS

"I have no special talents. I am only passionately curious."

-Albert Einstein, 1952-

Although circadian rhythms in intestinal tract have been documented, the exact role of molecular circadian clock in intestinal physiology and pathology is not understood. Therefore the objective of the thesis was to characterize the circadian clock in distinct parts of the alimentary tract and to determine its role in epithelial transport as well as in the regulation of proliferation under physiological and pathological conditions.

The following particular aims were addressed:

1. To determine circadian expression of main clock genes in the epithelium of doudoenum, jejunum, ileum, and colon.

2. To examine circadian regulation of electrolyte transport in colon:

    a. To determine circadian expression of genes coding transporters and channels mediating electroneutral and electrogenic electrolyte transport.

    b. To determine circadian variations in functional electrogenic sodium transport.

3. To examine link between circadian clock and cell cycle regulation by determination of putative circadian rhythmicity of mRNA of genes coding cell cycle regulators in colonic epithelium.

4. To examine the rhythmicity of circadian clock in tumourigenesis using the analysis of circadian mRNA expression of clock and clock-controlled genes in healthy and neoplastic colonic tissue in a model of chemically induced colorectal cancer.

5 MATERIALS AND METHODS

"I did not think; I investigated."

-Wilhelm Röntgen, 1896-

5.1 Experimental animals

5.1.1 Rats

6-week-old male Wistar rats (Velaz, Únětice, Czech Republic) were housed at local facility for at least 2 weeks of acclimatization to local conditions at 21 ± 2 °C under standard light regime (12 hours of light following 12 hours of darkness; CT0 is referred to light onset) with standard laboratory chow (6.7 mmol Na^+/kg BW per day) and water access *ad libitum*. Animals were randomly divided in experimental and control groups. Control group animals (n = 28) had free access to standard chow *ad libitum*, whereas the animals subjected to "restricted feeding" (RF; n = 28) were given food for only 6 hours during light period (i.e., CT3 – CT9). Animals in another experimental group were fed a low-salt diet (mean dietary Na^+ intake 0.75 mmol Na^+/kg BW per day) for 7 days *ad libitum* in order to stimulate secondary hyperaldosteronism (Pácha & Pohlová, 1995).

5.1.2 Mice

20-week-old male CD-1 (ICR) mice (Charles River, Germany) were kept on standard light-dark conditions with 12 hours of light (light on at 6:00, defined as CT0) and 12 hours of dark (light off at 18:00, defined as CT12) with access to standard chow and water *ad libitum*. Animals were randomly split to intact control group and experimental group with induced colitis-associated colorectal cancer. Experimental animals were injected by a single i.p. injection of azoxymethane (AOM; Sigma-Aldrich, St. Louis, MO, U.S.) dissolved in saline at a dose of 10 mg/kg. One week later they were subjected to 6 repeated cycles of consumption of 2% DSS (MP Biomedicals, Irvine, CA, USA) dissolved in drinking water for 7 days followed by consumption of drinking water for the following 14 days (Bissahoyo *et al.*, 2005; Švec *et al.*, 2010; Tanaka *et al.*, 2003). 3 months after the last cycle (at the age of 52 weeks) tissue samples were collected from both treated and intact animals.

To compare young and aged animals, untreated intact 10-week-old mice kept under the same conditions were used in some experiments.

All experiments were conducted in accordance with the *European Union Law*, and *Principals of Laboratory Animal Care* and were approved by the institutional *Animal Care and Use Committee*.

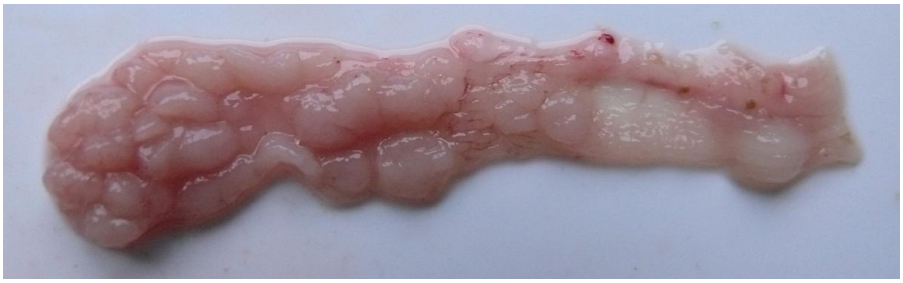

Figure 5.1 Representative colorectal tumours in CD-1 mouse model of AOM-induced colitis-associated colorectal cancer. Tumours are predominantly localized in rectum (left in the picture), and in distal colon of mice.

5.2 Tissue harvesting

5.2.1 Rat tissue harvesting

On the day of sample collection, light was not switch on at the beginning of subjective day and animals were released into constant darkness, in order to avoid direct effect of light and maintain endogenous rhythms during sample collection. Animals were euthanized after deep anaesthesia (thiopental i.p. at dose 50 mg/kg) every 4 hours and small intestine and colon were excised and flushed with cold saline solution (0.15 M NaCl). Epithelial layers of intestinal segments of duodenum (1 cm from stomach pylorus), jejunum (in 1/3 of small intestine length), and ileum (in 2/3 of small intestine length) were mechanically scrapped in length of 2 cm and immediately stored in RNA-protective *RNAlater* solution (Applied Biosystems). Similarly epithelial layer of distal colon (2 cm from rectum) in length of 3 cm was scrapped and stored in *RNAlater*. For low-salt diet experiments, samples of distal colon scrapped mucosa were collected in the same way at early light phase (CT4) and early dark phase (CT16), respectively.

5.2.2 Mice tissue harvesting

In order to reveal internal rhythms, on the day of the samples collection, the light was not turned on as usually and the CT0 referred to the time of previous day light on. Every 4 hours, 5 animals of each experimental group (intact control and AOM/DSS treated) were euthanized and colon was excised of its full length from caecum to rectum, rinsed with cold saline solution and longitudinally cut. From AOM/DSS treated mice approximately 20 mg of colorectal tumour tissue (hereafter "AOM/DSS tumour") and 1 cm of surrounding, macroscopically healthy, whole thickness distal colon tissue (hereafter "AOM/DSS surroundings") were collected. From control mice, 1 cm of whole thickness distal colon (hereafter "CTRL whole colon") and scrapped

mucosa layer enriched with epithelial cells (hereafter "CTRL mucosa") were collected. Moreover liver tissue from both experimental groups was collected. All tissues were immediately placed in *RNAlater* solution and stored according to manufacturer's recommendations until RNA isolation.

5.3 Laser capture microdissection (LMD)

In some experiment, microsamples from control rats were collected using LMD. Excised colon was rinsed, longitudinally cut and sample of 1 cm long distal colon (2 cm from rectum) was mounted in box with cryomold *OCT substance* (Sakura, Torrance, CA, USA) and frozen in liquid nitrogen. Sections of 8 μm were prepared on a cryostat (Leica Microsystems CM 3000, Wetzlar, Germany), subsequently placed on polyethylene naphthalate membrane coated slides (Leica) and immediately fixed in ice cold 96% ethanol. Water-free staining was performed in 2% cresyl violet acetate in 96% ethanol for 1 min followed by 3 ethanol washes, each of 1 min. After complete ethanol evaporation (5-10 minutes), samples were processed using LMD6000 laser capture microdissection system (Leica Microsystems, Wetzlar, Germany). Total area of approximately 0.2 mm^2 of crypt base (defined as lower 1/3 of crypt length) and crypt mouth (defined as upper 1/3 of crypt length and surface epithelium) were excised using laser beam (representative sample is displayed in Fig 5.2), collected in tube with 70 μl of lysis buffer (RNeasy Micro Kit, Qiagen, Hilden, Germany), homogenized by vortexing for 30 s and stored in -80°C until RNA isolation.

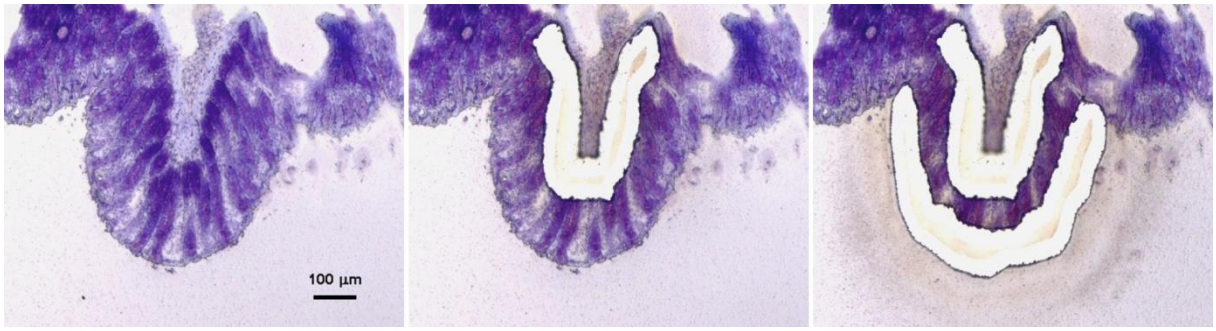

Figure 5.2 Laser capture microdissection procedure. Samples are excised from histological slices using laser beam and collected to the tube with lysis solution. Collection of rat colon crypt base and mouth are depicted.

5.4 RNA extraction and quantitative real time RT-PCR

For macrosamples obtained by mucosal scrapping, whole-thick colon or tumour samples, respectively, the tissue was homogenized using MagnaLyser Green Beads (Roche Diagnostics), total RNA was isolated using GeneElute Mammalian total RNA miniprep kit (Sigma-Aldrich.) and first strand cDNA synthesis was performed with ImProm II Reverse transcription system (Promega, Madison, WI, USA) using random primers in total volume of 15 μl according to manufacturer's recommendations.

For microdissected tissues, total RNA was isolated using the RNeasy Micro Kit (Qiagen) according to the manufacturer's recommendations including on-column DNase I digestion. First strand cDNA was subsequently prepared in a total volume of 20 μl using Sensiscript Reverse Transcriptase (Qiagen) according to the protocol.

The resulting cDNAs were used as templates for quantitative PCR. All reactions were performed according to manufacturer's instructions.

Measurements using pre-made TaqMan probe Assays (Table 5.1; Applied Biosystems, Foster City, CA, USA) were performed in total volume of 20 μl comprised of 8.25 μl of PCR grade water, 0.75 μl of FAM- or VIC-dyed TaqMan probe assay mix, 10 μl of GeneExpression Master Mix (Applied Biosystems) and 1 μl of cDNA sample (4 times diluted in the case of macrosamples) on the ABI PRISM 7000 Sequence Detection System (Applied Biosystems). PCR reaction was performed according to following program: 2 min at 50 °C, initial denaturation for 10 min at 95 °C, followed by 45 (or 55 respectively for microdissected samples) cycles of denaturation for 30 sec at 95 °C followed by annealing and elongation for 60 sec at 60 °C. Ct (cycle threshold) values were determined by method of fit points using SDS Software (Applied Biosystems).

Measurements using SYBR Green and specific primers (Table 5.2) were performed in total volume of 15 μl comprised of 5.75 μl of PCR grade water, 0.75 μl primers mix (20μM each), 7.5 μl of LightCycler480 SYBR Green I Mix (Roche) and 1 μl cDNA sample (4 times diluted in the case of macrosamples) on the LightCycler 480 instrument (Roche). PCR reaction was carried out according to following thermal profile: initial denaturation for 10 min at 95 °C, followed by 45 (or 55 respectively for microdissected samples) cycles of denaturation for 30 sec at 95 °C, annealing for 20 sec at 61 °C and elongation for 20 sec at 72 °C. Fluorescence acquiring

was performed at the end of each cycle. Ct values were determined using 2nd derivative maximum method performed by LightCycler480 software (ver. 1.50; Roche).

Standard curve method was applied to express relative concentrations and efficiency of PCR reactions. Data were normalized to normalization factor expressed as geometric mean of normalized concentrations of genes B2m, Gapd, and 18S rRNA for rat sample analysis, while Ywhaz, and Gapd were used for mouse sample analysis. Normalization genes were selected by particular housekeeping gene stability analysis (Andersen *et al.*, 2004; Vandesompele *et al.*, 2002).

Table 5.1. TaqMan Gene Expression Assays (Applied Biosystems) used for quantification of particular transcripts.

Gene	Alternative name	catalog no.	NCBI RefSeq
rSlc9a3	*Nhe3*	Rn00561944_m1	NM_012654.1
rScnn1a	*αENaC*	Rn00580652_m1	NM_031548.2
rSccn1b	*βENaC*	Rn00561892_m1	NM_012648.1
rSccn1g	*γENaC*	Rn00566891_m1	NM_017046.1
rSlc26a3	*Dra*	Rn00709709_m1	NM_053755.1
rSlc4a1	*Ae1*	Rn00561909_m1	NM_012651.2
rAtp1a1		Rn01533986_m1	NM_012504.1
rAtp1b1		Rn00565405_m1	NM_013113.2
rSlc9a3r1	*Nherf1*	Rn00572154_m1	NM_021594.1
rSlc12a4	*Kcc1*	Rn00570248_m1	NM_019229.1
rSlc12a2	*Nkcc1*	Rn00582505_m1	NM_031798.1
rCftr		Rn01455968_m1	NM_031506.1
rWee1		Rn01279391_m1	NM_001012742.1
rMyc	*c-Myc*	Rn00561507_m1	NM_012603.2
rCcnb1	*cyclin B*	Rn00596848_m1	NM_171991.2
rCcnd1	*cyclin D*	Rn00432359_m1	NM_171992.4
rCcna1	*cyclin A*	Rn01761351_m1	NM_001011949.1
rCdkn1a	*p21 (Cip1, Waf1)*	Rn00589996_m1	NM_080782.3
rCdkn1b	*p27 (Kip1)*	Rn00582195_m1	NM_031762.3
rCdkn2a	*p16 (Arf)*	Rn00580664_m1	NM_031550.1
rB2m		Rn00560865_m1	NM_012512.2
rGapdh		4352338E	NM_017008.3
18S rRNA		4319413E	X03205.1
mGapdh		4352339E	NM_008084.2
mWee1		Mm00494175_m1	NM_009516.3
mMyc	*c-Myc*	Mm00487804_m1	NM_001177352.1
mCdkn1a	*p21 (Cip1, Waf1)*	Mm00432448_m1	NM_007669.4

Table 5.2. Sequences of primers used for quantification of clock genes and some normalization genes in combination with SYBR Green I. *Sequence of primers for *Ywhaz* were selected from NCBI mouseprimerdepot (Cui *et al.*, 2007), database issue D805.

species	Gene	position	5' → 3' sequence	NCBI RefSeq
rat				
	rPer1	forward	CGCACTTCGGGAGCTCAAACTTC	NM_001034125.1
		reverse	GTCCATGGCACAGGGCTCACC	
	rPer2	forward	CACGCAACGGGGAGTACATCACAC	NM_031678.1
		reverse	CAAGGGGAGGCTGCGAACACAT	
	rBmal1	forward	CAATGCGATGTCCCGGAAGTTAGA	NM_024362.2
		reverse	AAATCCATCTGCTGCCCTGAGAAT	
	rRevErba	forward	GCTGTGCGGGAGGTGGTAGAAT	NM_145775.2
		reverse	TGTAGGTTGTGCGGCTCAGGAA	
mouse				
	mPer1	forward	TCTGGCCTGGGCTCTGGGTCTGGTTC	NM_011065.4
		reverse	GCTGCGGGTGATGCTGGCTGAGGT	
	mPer2	forward	CCTCTGGCCCCTGTGGATTG	NM_011066.3
		reverse	AGCTGGGCCCTTGGTGGATAG	
	mBmal1	forward	CAGAGCCGGAGCAGGAAAAATAGGT	NM_007489.3
		reverse	CAGGGGGAGGCGTACTTGTGATGT	
	mRevErba	forward	TTTTGGCGGCTCAGCGTCATAAT	NM_145434.3
		reverse	CCAGGTAGGCGGGTAGGAGGAAG	
	mDbp	forward	TTTTTGCGCCGCTGCTGTGGGAACG	NM_016974.3
		reverse	GGGGGAGGGCGCGGGAGTGC	
	mB2m	forward	TCTCACTGACCGGCGTGTATGCTATC	NM_009735.3
		reverse	AATGTGAGGCGGGTGGAACTGTG	
	*mYwhaz**	forward	TTGAGCAGAAGACGGAAGGT	NM_011740.3
		reverse	CTTTCTGGTTGCGAAGCATT	

5.5 Plasma collection and aldosterone measurements

Blood samples (volume 1 ml) were collected from hearts of anesthetized rats (n=28) every 4 hours during 24 hour-interval (starting from CT0). Animals were kept on standard light conditions with *ad libitum* access to standard diet and water. In another experiment, blood samples were collected from rats kept on low-salt diet and their control counterparts kept on standard diet at early subjective day (CT4) and early subjective night (CT16). Samples were incubated at room temperature for 5 minutes in order to allow erythrocytes to precipitate.

Subsequently samples were centrifuged (10 min at 3000g) and plasma aliquots were stored at -80 °C for further analysis. Level of aldosterone was determined using ^{125}I aldosterone radioimmunoassay (Immunotech, Czech Republic) by competitive binding of radioactively labelled aldosterone according to manufacturer's instructions. Plasma aldosterone is expressed in pg per ml of blood plasma.

5.6 Electrophysiological experiments

Rats kept on low-salt diet (n = 12) and corresponding controls (n = 11) were euthanized at early subjective day (CT4) and early subjective night (CT16). Subsequently, distal colon was excised, rinsed to get rid of content and cut longitudinally. The muscle layer was stripped and the mucosa was mounted in modified Ussing chambers containing Krebs-Ringer solution (composition in mM: 140.5 Na^+, 5.4 K^+, 1.2 Ca^{2+}, 1.2 Mg^{2+}, 119 Cl^-, 21 HCO_3^-, 0.6 $H_2PO_4^-$, 2.4 HPO_4^{2-}, 10 D-mannitol, 10 D-glucose, 2.5 L-glutamine, 0.5 β-hydroxybutyrate) gassed with carbogen (95% O_2 + 5% CO_2) and kept at 37 °C. After an equilibration period of 15 min (10 min in open-circuit mode and 5 min in voltage-clamp mode), amiloride (10^{-5} M) was added from mucosal side and the tissue response was recorded by a computer-controlled voltage clamp. Net active ion transport across the epithelium was measured as a short-circuit current (SCC, expressed in $\mu A.cm^{-2}$) in voltage-clamp mode in which the spontaneous potential difference was maintained at 0 mV. Amiloride sensitive current represents electrogenic net flux of Na^+ through ENaC channel (Benos, 1982). In addition to SCC, potential difference and tissue resistance were recorded at a sampling frequency of 1 Hz and the data were processed using Excel and Statistica 6.1 (StatSoft, Tulsa, OK) softwares.

5.7 Statistics

The data of expression are presented as mean ± SEM. To test the simple effect of time on expression of particular transporters, channels and cell cycle regulators, one-way analysis of variance (ANOVA) was used and values $P < 0.05$ were considered significant. Post-hoc Fisher's least-significance difference (LSD) test was used to assess differences between particular time points, where ANOVA test P values achieved significant level.

To evaluate rhythmicity, the cosinor analysis was applied using mathematical model of cosine curve expressed by formula $M = A*cos(2\pi t/T - \varphi/T)$, where t represents time, M is the estimate of the mean of the oscillation, A is the amplitude of the cosine wave, T is the fixed period 24 h, and φ is the time at which the peak of the rhythm occurs. Simple mathematical rearrangement using goniometric functions gave a linear model that was fitted using multiple linear regression, and the coefficient of determination (R^2) and the P value corresponding to the non-null amplitude of the rhythm were calculated. Rhythms were considered being present when the effect of time detected by ANOVA reached significance, and the cosinor analysis revealed significant fit to cosine curve.

In the case of determining the effect of feeding schedule, circadian time, and their interactions with gene expression, two-way ANOVA where time (CT0-CT24), and feeding schedule (normal feeding/restricted feeding) were the between-subject factors. The LSD test was used to evaluate differences between time points where appropriate.

Two-way ANOVA was also used to identify the differences in gene expression along the crypt axis (crypt mouth/crypt base x circadian time) and the effect of dietary salt intake (low-salt diet/standard diet x circadian time) followed by the LSD test. Student's t-test was used where the transcripts were detected only in the colonocytes of crypt mouth.

All calculations were performed using Statistica 6.1 software.

6 RESULTS

"What we observe is not nature itself, but nature exposed to our method of questioning."

-Werner Heisenberg, 1958-

6.1 Intestinal clocks

6.1.1 Clock gene expression along intestinal tract

Diurnal variations of various intestinal functions have been observed and documented in numerous studies. Recently, also rhythmic expression of clock genes in colon has been detected. However, detailed study of peripheral clock and its regulation along intestinal tract is still missing. Hence, our first goal was to find out whether functional clock system exists within particular parts of the intestinal tract. We employed quantitative "real time" RT-PCR to determine daily mRNA expression profiles of main genes constituting molecular clock in duodenum, jejunum, ileum and colon of rats.

Initial immunocytochemical analysis (data not shown) revealed dominant presence of core clock genes in epithelial cells compared to subepithelial and muscular layers (Sládek *et al.*, 2007), therefore, we focused on intestinal epithelium in following studies. Circadian expression profiles of clock genes detected in intestinal segments from duodenum to colon are presented in Fig. 6.1. As revealed by analysis of variance (ANOVA), we found significantly rhythmic mRNA expression of *Per1*, *Per2*, *RevErbα* and *Bmal1* in all studied segments, except *Per2* in ileum, where P value was slightly higher than criterion of significance (P=0.51). Rhythmic expression of *Per* genes reached peak of maximum at the beginning of subjective night (CT12 for *Per1*, CT12 – CT16 for *Per2*) in all studied segments. The expression of clock gene *RevErbα* began to rise at early subjective day with the peak of maximum reached during late subjective day (CT8 – CT12) in all segments. In contrast, expression of *Bmal1* reached maximum level at the end of subjective night, while minimal expression occurred at the end of subjective day. Furthermore, cosinor analysis confirmed rhythmic expression of all genes in all segments ($P<0.05$) except *Per1* and *Per2* in colon where profiles did not fit cosine curve significantly. Together, presented data demonstrate functional circadian clock in the epithelium along alimentary tract from duodenum to colon.

6.1.2 Spatial organization of colonic epithelial intestinal clock along colonic crypts

As we found rhythmic expression of clock genes within macrosamples of scraped mucosa, we extended focus on distinct region of colonic crypts. We harboured technique of laser capture microdissection to analyze circadian expression profiles in the region of crypt base, where

intestinal stem cells and proliferating colonocytes are located, and in the region of crypt mouth/surface cells, where fully differentiated intestinal epithelial cells are located.

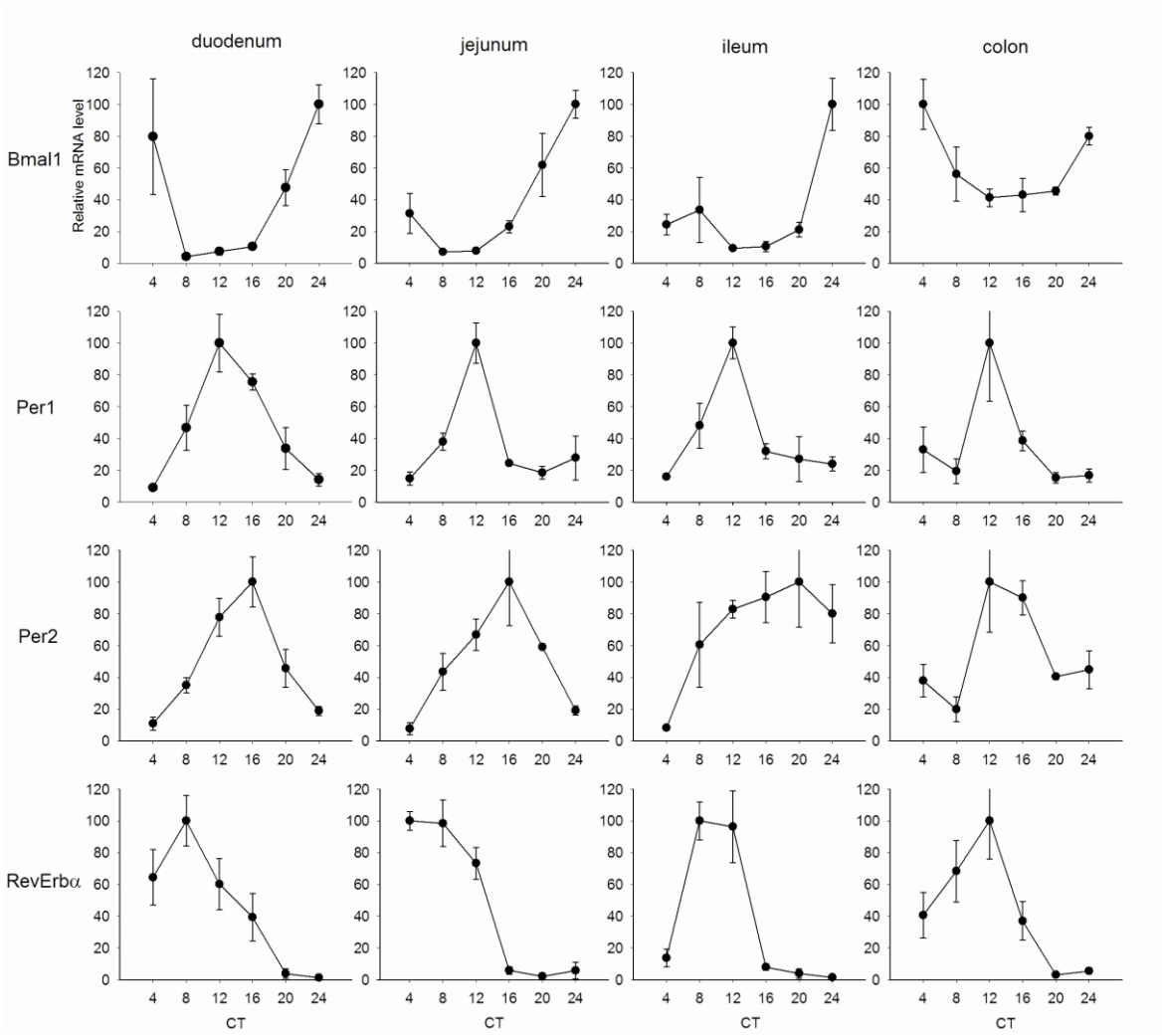

Figure 6.1 Relative mRNA expression of clock genes along the rat intestine. Mucosa samples of rats kept on standard light-dark regime with 12 hours of light and 12 hours of dark period (LD 12:12) with access to standard chow diet and water *ad libitum* were collected within 24 hours. On the day of sample collection, light was not switched on as usual and animals were maintained and euthanized in constant darkness. Time is expressed as circadian time (CT); CT12 corresponds to time of previous light-off, and CT24 corresponds to time of previous light-on. mRNA levels of genes *Bmal1*, *Per1*, *Per2* and *RevErbα* were determined in samples of duodenum, jejunum, ileum, and colon using quantitative RT-PCR. Each timepoint is represented by mean ± SEM and converted to a percentage of the maximum level for each transcript.

As revealed by one-way ANOVA, we detected significant diurnal rhythms in mRNA expression of core clock genes *Per1*, *Per2* and *Bmal1* (for all $P<0.001$) in both crypt base and crypt mouth/surface (Fig 6.2). In both histological compartments of colonic epithelium, *Per1* reached maximal expression at CT12 and minimal at CT4, and similarly, *Per2* reached maximum

at CT16 and minimum at CT4. In contrast, *Bmal1* expression peaked at CT24, being in opposite phase compared with *Per1*/*Per2*.

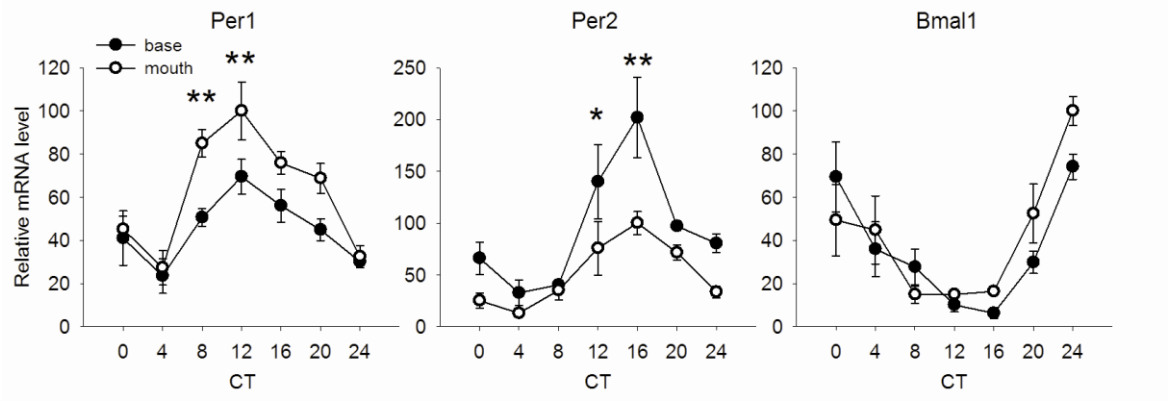

Figure 6.2 Spatio-temporal organization of core clock genes *Per1*, *Per2* and *Bmal1* mRNA expression in colonocytes of crypt base (●) and crypt mouth (○). The rats were kept under a 12:12-h light-dark schedule, and, on the day of sampling, they were released into constant darkness. Transcript levels were determined using quantitative RT-PCR of laser-microdissected samples. Normalized data are expressed as a percentage of the maximum values found in the crypt mouth/surface colonocytes for each transcript and represent means ± SEM (4 animals for each time point). The rhythmicity of the genes was analyzed by 2-way ANOVA, which showed significant effects of time for all three genes ($P<0.001$) and that of position along the crypt axis for *Per1* and *Per2* ($P<0.001$). Individual differences were calculated by the post hoc LSD test; significant differences between crypt base and crypt mouth are indicated by *$P<0.05$ or **$P<0.01$.

Moreover, the analysis proved the effect of position (crypt base vs. crypt mouth/surface cells) on expression of genes *Per1* ($P<0.001$) and *Per2* ($P<0.001$). Significantly higher expression of *Per1* was observed in crypt mouth/surface cells at CT8 and CT12 (both $P<0.01$). On the contrary, *Per2* exhibited significantly higher expression in crypt base at CT12 ($P<0.05$) and CT16 ($P<0.01$). In spite of distinct cellular physiology of crypt base and crypt mouth/surface cells, these data demonstrate functional clock with the same phase in both cell compartments in accordance with data obtained from mucosal macrosamples.

6.2 Circadian regulation of intestinal electrolyte transport

6.2.1 Diurnal variations of electrolyte transporters and channels mRNA expression in distal colon

To determine whether intestinal electrolyte transport, particularly NaCl absorption and secretion, is under circadian regulation, we studied daily expression of transporters and channels operating in colonic electrolyte transport. In samples of scraped colonic mucosa we studied the

expression profiles of chloride/bicarbonate transporters *Dra* and *Ae1*, sodium/hydrogen ion antiporter *Nhe3* and its regulatory factor 1 (*Nherf1*), α1 and β1 subunit of sodium-potassium pump (*Atp1a1*, *Atp1b1*), α, β and γ subunit of sodium channel *ENaC*, chloride-potassium cotransporter *Kcc1*, chloride channel cystic fibrosis transmembrane conductance regulator (*Cftr*), and sodium-potassium-chloride cotransporter *Nkcc1* in order to examine possible regulation of electrolyte transport by intestinal circadian clock.

As shown in Fig 6.3, we revealed diurnal variations in expression of genes *Dra* ($P<0.01$), *Ae1* ($P<0.001$) and *Nhe3* ($P<0.05$), which operate in the apical membrane of epithelium in the process of NaCl absorption and reached maximum peak at the beginning of subjective night (CT12). Catalytic α1 subunit of sodium-potassium pump (*Atp1a1*), which extrudes sodium ions across the basolateral membrane and thus is the rate limiting factor of NaCl absorption, displayed similar rhythmic expression ($P<0.001$) with peak also at CT12. Moreover, *Nherf1* exhibited circadian expression ($P<0.001$) with pattern resembling *Nhe3* diurnal variation. Surprisingly, we detected expression of regulatory γ subunit of sodium channel *ENaC*, the rate-limiting subunit of ENaC, which mediate electrogenic sodium absorption and is activated in state of salt deprivation (Pácha & Pohlová, 1995). Its expression exhibited rhythmic pattern ($P<0.01$) with elevated level throughout subjective night and declined level throughout subjective day.

Cosinor analyses confirmed a significant circadian rhythmicity for all of these genes (*Dra*: $R^2=0.43$, $P<0.001$; *Nhe3*: $R^2=0.28$, $P<0.001$; *Ae1*: $R^2=0.28$, $P<0.01$; *Atp1a1*: $R^2=0.49$, $P<0.001$; *Nherf1*: $R^2=0.28$, $P<0.01$; *γENaC*: $R^2=0.26$, $P<0.05$). Amplitudes of fitted curves were similar among studied genes (*Dra*: 24.65, 95 % CI = 14.71-34.59; *Nhe3*: 28.36, 95 % CI = 12.70-44.01; *Ae1*: 20.30, 95 % CI = 8.63-31.98; *Apt1a1*: 24.75, 95 % CI = 15.93-33.57; *Nherf1*: 26.07, 95 % CI = 18.66-33.48; *γENaC*: 27.62, 95 % CI = 7.91-47.33), as well as mesor (midway value between highest and lowest values of the fitted cosine function) (*Dra*: 77.95, 95 % CI = 71.01-84.88; *Nhe3*: 71.06, 95 % CI = 59.49-82.62; *Ae1*: 51.81, 95 % CI = 43.09-60.53; *Apt1a1*: 61.23, 95 % CI = 54.76-67.71; *Nherf1*: 64.93, 95 % CI = 59.63-70.23; *γENaC*: 60.78, 95 % CI = 47.26-74.31). Furthermore, we did not detect any significant differences in acrophase (timepoint of maximal value) of fitted cosine curves (*Dra*: 16.02, 95 % CI = 14.55-17.49; *Nhe3*: 13.58, 95 % CI = 11.29-15.88; *Ae1*: 13.39, 95 % CI = 10.94-15.85; *Apt1a1*: 13.96, 95 % CI = 12.50-15.43; *Nherf1*: 14.57, 95 % CI = 13.47-15.67; *γENaC*: 17.12, 95 % CI = 14.61-19.62).

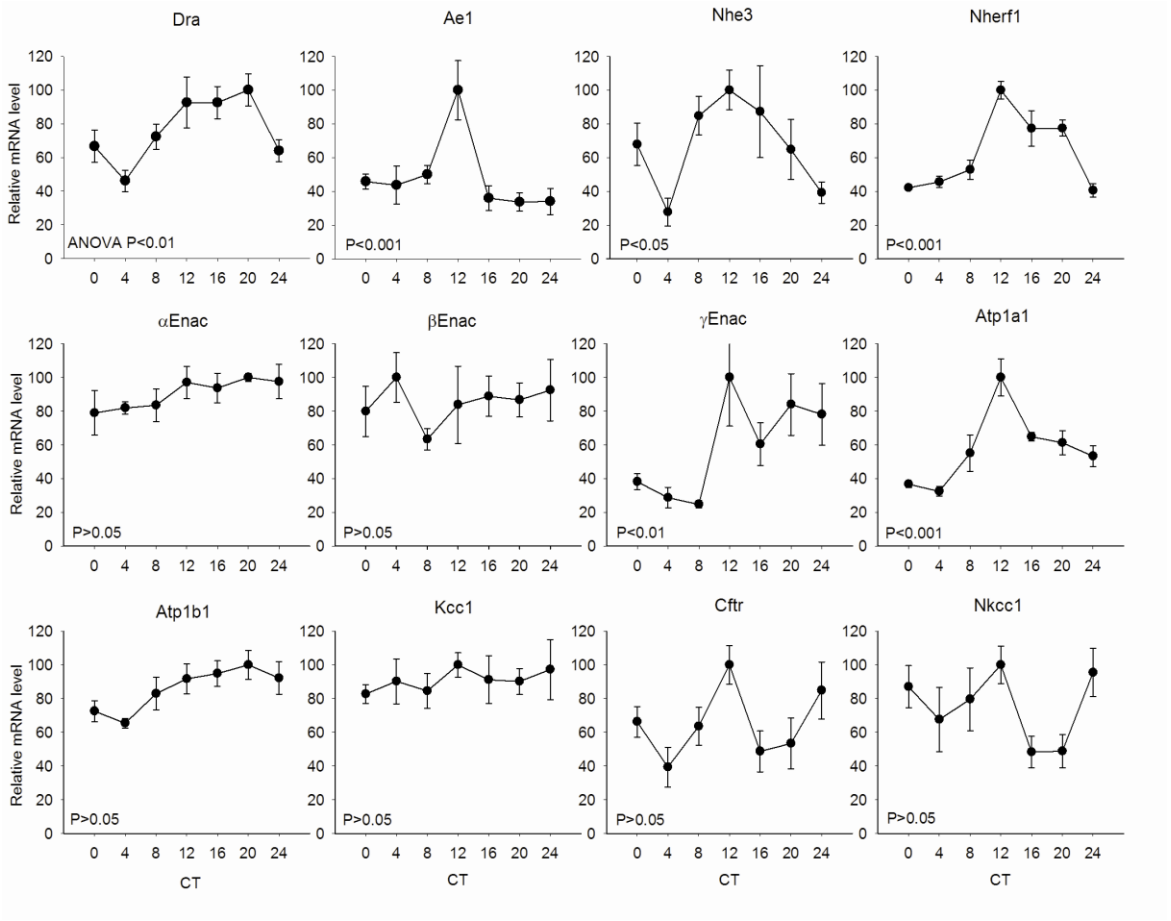

Figure 6.3 Daily profile of mRNA expression of transporters and channels participating in colonic NaCl and KCl absorption and NaCl secretion in distal colon mucosa. Rats were kept on standard light-dark regime with 12 hours of light and 12 hours of dark period (LD 12:12) with access to standard chow diet and water *ad libitum*. Transcription levels of Cl^-/HCO_3^- exchangers (*Dra*, *Ae1*), Na^+/H^+ exchanger (*Nhe3*) and its regulator (*Nherf1*), α, β, and γ subunits of the epithelial Na^+ channel (*αEnac*, *βEnac*, and *γEnac*), α and β subunits of the Na^+ pump (*Atp1a1*, *Atp1b1*), K^+-Cl^- cotransporter (*Kcc1*), Cl^- channel (*Cftr*), and Na^+-K^+-Cl^- cotransporter (*Nkcc1*) were determined using quantitative RT-PCR, and the normalized values were converted to a percentage of the maximum level for each transcript. Each timepoint is represented by mean ± SEM. The rhythmicity of the genes was analyzed by a one-way ANOVA (P values are displayed in figure), cosinor analysis, and post hoc least-significant difference (LSD) test (for further details, see the text). CT, circadian time.

In contrast to the above mentioned genes, we did not detect significant diurnal variation in basolaterally located β1 subunit of sodium-potassium pump or chloride-potassium cotransporter *Kcc1*. Furthermore, neither *Cftr*, nor *Nkcc1* displayed significant 24-hour rhythmic pattern and their expression resembled rather ultradian circadian variation with phase approximately 12 hours.

6.2.2 Effect of restricted feeding on expression of genes participating in NaCl absorption

Food intake is known to affect peripheral circadian clock (Sládek *et al.*, 2007; Stokkan *et al.*, 2001), hence we investigated the impact of feeding restricted to the light phase of the day (from CT3 to CT9) on expression of genes involved in colonic NaCl absorption.

As shown in Fig 6.4, two-way ANOVA revealed significant effect of feeding regime on expression of *γENaC* (P<0.05), *Atp1a1* (P<0.001) and *Nherf1* (P<0.001). Furthermore, the analysis uncovered significant interaction of circadian time and feeding schedule for genes *Ae1* (P<0.01), *Dra* (P<0.001), *γENaC* (P<0.05) and *Nherf1* (P<0.05) but not for *Atp1a1*. Restricted feeding induced increased expression of *Dra* at CT4 but decreased expression at CT20, increased expression of *Ae1* at CT4 but decreased expression at CT 12, and increased expression of *γENaC* and *Nherf1* at CT4 and CT8. In addition, restricted feeding significantly reduced the amplitude of *Atp1a1* expression.

Even if the effect of meal time was significant only in *γENaC*, *Atp1a1*, and *Nherf1*, the significant interaction between the variables suggests there are differences between the profiles of rats kept on *ad libitum* and restricted feeding conditions. Taken together the restricted feeding regime phase-advanced the rhythm of *Dra*, *Ae1*, and *Nherf1* expression, decreased the amplitude of *Atp1a1* expression, and abolished the rhythm of *γENaC* expression.

6.2.3 Spatial localization of day/night variations in expression of transporters and channels operating in NaCl absorption

To elucidate spatio-temporal distribution of expression along crypt axis, we determined mRNA level of particular transporters and channels in early subjective day (CT4) and early subjective night (CT16) in samples of crypt base, and crypt mouth/surface cells. Unlike the clock genes, the expression pattern of transporter, and channel genes differs along the crypt axis.

As presented in Fig 6.5, we detected expression of *Dra*, *Nhe3* and *γENaC* exclusively in crypt mouth/surface cells with no signal in crypt base. In contrast, expression of *Cftr* and *Atp1a1* was detected higher in crypt base. In crypt mouth/surface cells we observed diurnal variation in expression of *Dra* (P<0.01), *γENaC* (P<0.01), *Nhe3* (tendency) and *Nherf1* (P<0.01), with higher expression in dark phase (CT16). We did not observe any significant diurnal changes in expression of *Cftr* or *Atp1a*. The expression of *Ae1* could not be determined as the amount of this transcript is relatively low and was below the detection limit in microdissected samples. Taken

together, the data from microsamples mostly confirmed the results obtained from scraped mucosa and demonstrate diurnal regulation of genes involved in colonic NaCl absorption.

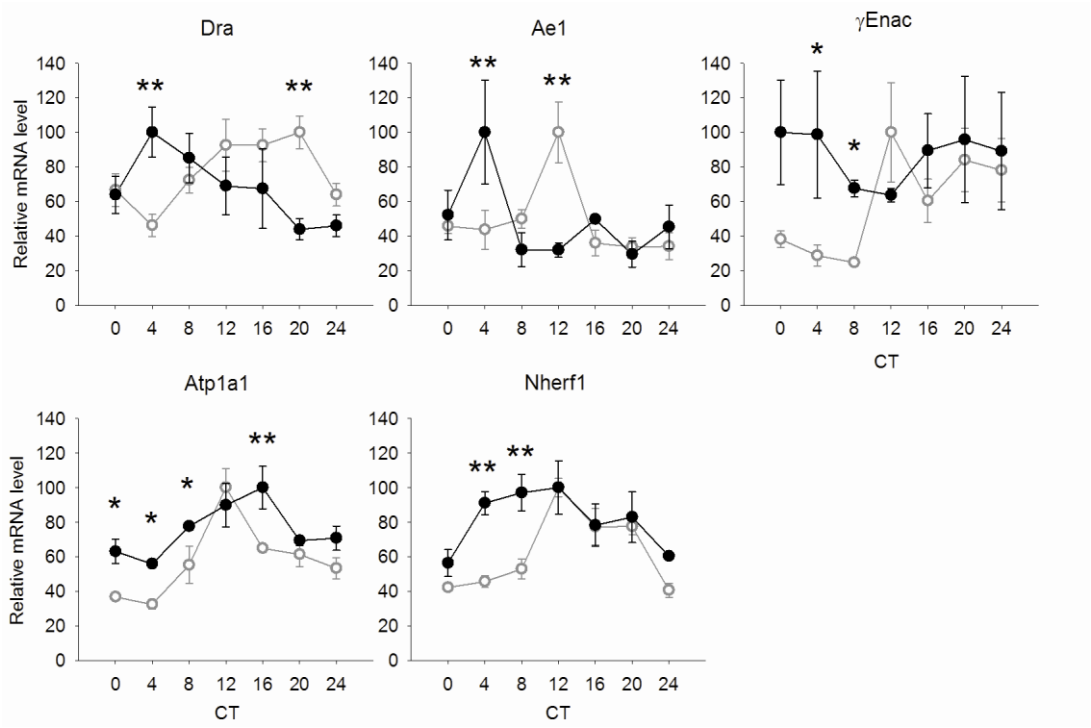

Figure 6.4 The effect of restricted feeding schedule on the 24-hour expression profiles of *Dra, Ae1,* γ*Enac,* A*tp1a1,* and *Nherf1*. Rats were kept on a 12:12-h light-dark schedule fed *ad libitum* (○), or subjected to restricted feeding regime (●) from CT3 to CT9 for 14 continual days. On the day of sampling, animals were released into constant darkness. Data are expressed as a percentage of the maximum mean value for each gene and represent means ± SEM (4 animals for each time point). Significant differences between normal and restricted feeding are indicated by **P<0.05* or ***P<0.01*.

6.2.4 Involvement of aldosterone in circadian regulation of electrogenic transport

As aldosterone is known to be a potent regulator of electrogenic sodium absorption by stimulation of regulatory subunits of ENaC channel (Epple *et al.*, 2000), we investigated its level during 24 hours in order to reveal a possible role of aldosterone in oscillation of *γENaC* subunit. As shown in Fig 6.6, we observed diurnal changes of plasma concentration of aldosterone ($P<0.05$) in rats kept on standard diet and standard light conditions with strong peak at the beginning of dark phase (CT12). This observation corresponds with the expression of *γENaC,* and thus it implicates that oscillation of aldosterone might be involved in circadian regulation of intestinal sodium absorption and might drive the circadian changes of target gene expression.

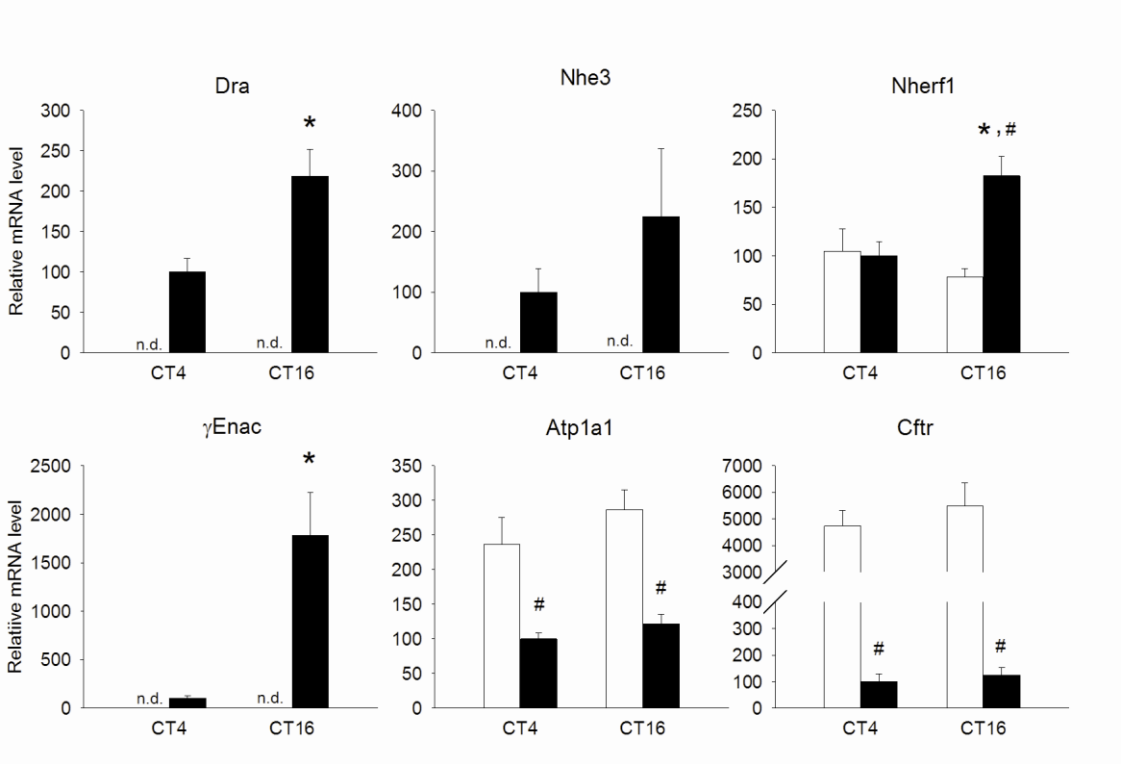

Figure 6.5 Expression of genes, participating in colonic ion transport along the crypt axis. The mRNA levels were determined at CT4 (early subjective day) and CT16 (early subjective night) in microsamples of colonic crypt base (open bars) and crypt mouth/surface colonocytes (closed bars) harvested by laser microdissection. The rats were maintained under a 12:12-h light-dark schedule, and, on the day of sampling, they were released into constant darkness. The mRNA values of crypt mouth/surface cells at CT4 were set to 100%, and other data are presented as a percentage of this value. No expression of Dra, Nhe3,or γEnac was found in the crypt base [not detected (n.d.)]. The values are means ± SEM (4–5 animals). *Significant difference between CT4 and CT16 at the same position along the axis ($P<0.01$); #significant difference between the crypt base and crypt mouth at the same time of day ($P<0.01$) as established by 2-way ANOVA and post hoc LSD test (*Nherf1*, *Atp1a1*, and *Cftr*) or Student's t-test (*Dra*, *Nhe3*, and *γEnac*).

6.2.5 Circadian regulation of Na^+ absorption in secondary hyperaldosteronism

Colonic electrogenic sodium absorption plays physiological role mainly during secondary hyperaldosteronism, which is induced by low sodium intake (Pácha & Pohlová, 1995). As only very little is known about diurnal changes of this process, our goal was to elucidate possible clock and aldosterone participation in the regulation of electrogenic sodium absorption under the condition of secondary hyperaldosteronism. Hence, we determined plasma aldosterone level and colonic expression of *ENaC* channel subunits as well as core clock genes in early subjective day (CT4) and early subjective night (CT16) of rats fed low-salt diet and kept under standard light conditions.

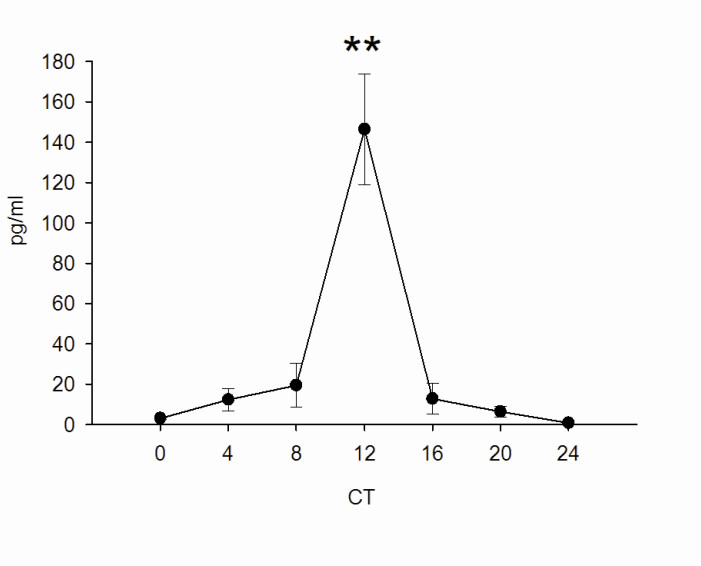

Figure 6.6 Daily profile of aldosterone plasma levels in rats kept on a standard diet. The rats were maintained under a 12:12-h light-dark schedule, and, on the day of sampling, they were released into constant darkness. CT0 corresponds to time of previous lights on. Each time point represents the mean ± SEM (4 animals for each time point). **Significantly different from the lowest value (P<0.01).

As expected, low-salt diet rapidly stimulated plasma aldosterone concentration (Fig. 6.7; P<0.001). While low-salt diet had no significant effect on expression of non-regulatory *αENaC*, it substantially stimulated expression of regulatory subunits *βENaC* and *γENaC* (both P<0.001). Furthermore, circadian time affected expression of both *βENaC* (P<0.001) and *γENaC* (P<0.01), and expression reached higher level at early subjective night (CT16).

As these results imply putative role of circadian clock, we analyzed core clock gene expression in the same conditions. The expression of clock genes *Per1* and *Bmal1* exhibited expected diurnal changes in standard diet fed animals, with higher expression at early night in case of *Per1* (P<0.01) and at early day in case of *Bmal1* (P<0.01). However, although *Bmal1* exhibited very similar expression pattern in low-salt diet group [i.e. higher expression during early day (P<0.01), with no significant changes between diet groups in either time point], *Per1* gene exhibited no significant difference between day and night in the low-salt diet group. The expression of *Per1* reached similar levels at both time points and this value was not significantly different from the night-time value of the standard diet group.

Taken together these data demonstrate circadian regulation of intestinal sodium absorption in secondary hyperaldosteronism. Not only diurnal variations in expression of genes involved in electrogenic NaCl absorption, but also changes in *Per1* expression and plasma aldosterone implicate considerable role of intestinal clock in regulation of NaCl absorption.

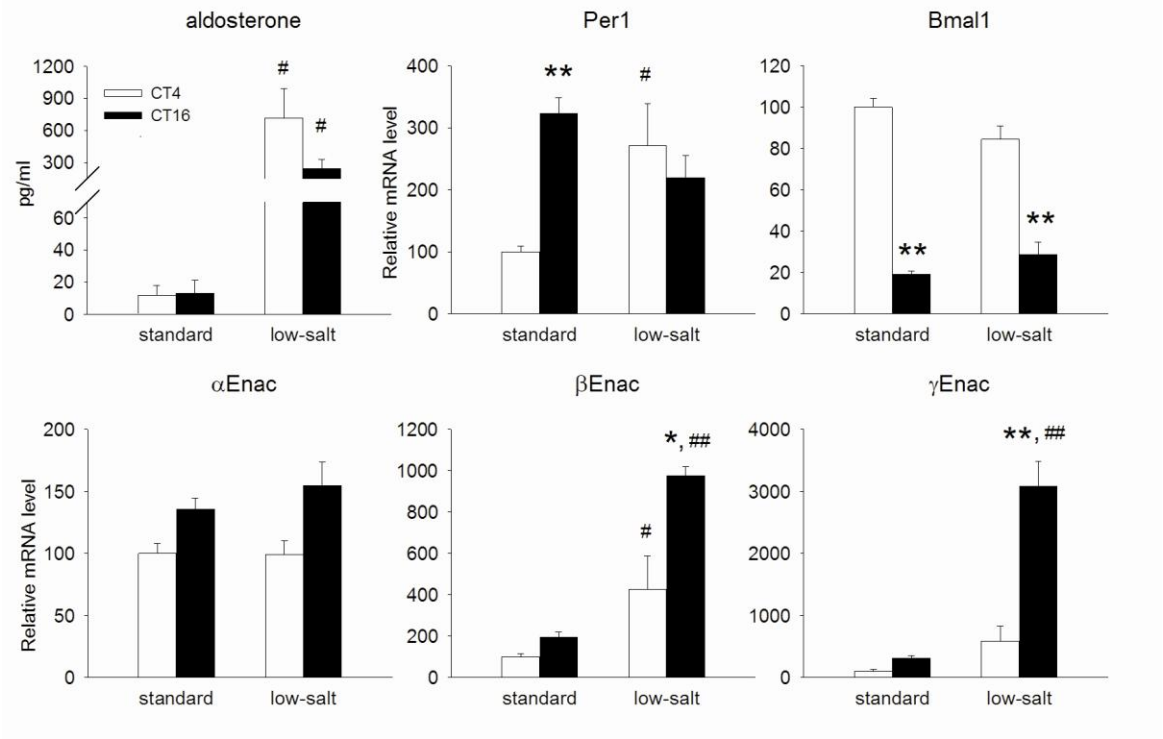

Figure 6.7 Effect of secondary hyperaldosteronism induced by low-salt diet on the diurnal changes in the plasma level of aldosterone and in expression of clock genes *Per1* and *Bmal1* and genes encoding subunits of epithelial Na^+ channel (αEnac, βEnac, and γEnac). Rats fed a low-salt diet were maintained under a 12:12-h light-dark schedule, and, on the day of sampling, they were released into constant darkness. CT4 corresponds to 4 h after the previous lights on and CT16 to 4 h after lights off. Blood plasma and colonic mucosa were collected at CT4 (open bars) and CT16 (closed bars) for subsequent radioimmunoassay of plasma aldosterone and quantitative RT-PCR analyses. The gene expression levels are presented as a percentage of the morning (CT4) levels reached on the standard diet. The values are means ± SEM (4–5 animals). *Significant difference between CT4 and CT16 in animals kept on the same diet; #significant difference between animals kept on standard and low-salt diets at the same time of day, as established by 2-way ANOVA and post hoc LSD test (*,#P< 0.05 and **,##P <0.01).

6.2.6 Diurnal variations of amiloride-sensitive short circuit current in distal colon of control and low-salt diet fed rats

Although, we have shown circadian regulation of Na^+ channel subunit expression, there is lack of functional evidence of such regulation. Hence, we utilized the method of short circuit current measurement in Ussing chamber to assess the values of electrogenic Na^+ transport. This transport was quantified as amiloride-sensitive SCC, which reflects the rate of electrogenic Na^+ absorption via ENaC channel. We measured amiloride-sensitive SCC in both, control and low-salt diet fed group, at two time points (early subjective day, CT4; early subjective night, CT16). In addition, we recorded potential difference (PD_0) and transepithelial electrical resistance (R_0), all parameters with frequency 1 Hz.

As expected, we detected no significant amiloride-sensitive SCC in control group (ΔSCC: -4.1 ± 5.4 at CT4; -7.9 ± 2.3 at CT16; P>0.05), showing the absence of electrogenic sodium absorption via ENaC in normal diet fed rats. On the contrary, we detected significant changes of SCC in colon of rats kept on low-salt diet (Fig. 6.8). ΔSCC at early subjective night (-78.4 ± 19.6 at CT16; P<0.05) reached significantly greater value comparing to early subjective day (-19.7 ± 6.9 at CT4). We detected also significant rise of PD_0 at CT16 (P<0.05), while no significant change in ΔR (Table 6.1).

Regarding the observed data, we demonstrated in animals with induced secondary hyperaldosteronism diurnal variation of electrogenic sodium absorption with higher transport activity during the dark phase. We suggest that these changes are due to circadian regulations of key regulatory subunits of channel ENaC, which mediate electrogenic sodium absorption in distal colon. In addition, circadian oscillation of aldosterone might play a considerable role in this regulation.

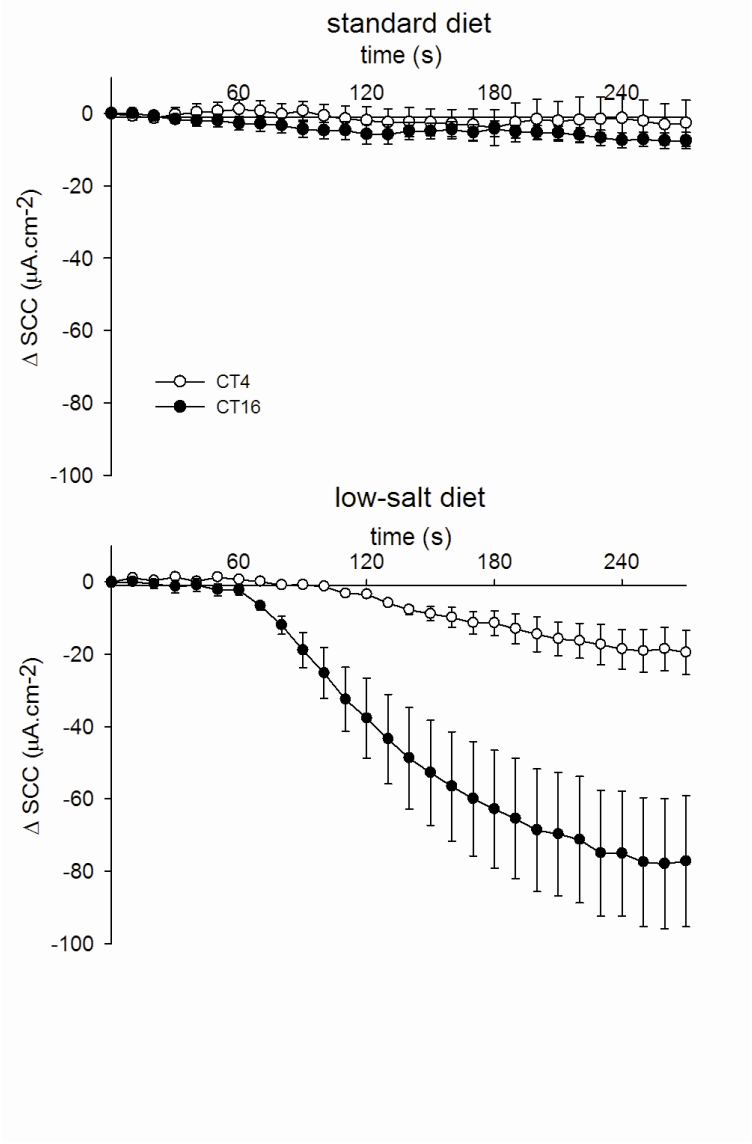

Figure 6.8 Differential effect of amiloride on short-circuit current (SCC) in rat colon during the light and dark period. The animals were kept on a 12:12-h light-dark schedule and fed a standard or low-salt diet. On the day of the experiment, the rats were released into darkness, and short-circuit currents were measured at 2 time points corresponding to CT4 (4 h after the previous lights on) and CT16 (4 h after the previous lights off). Each average curve represents the mean ± SEM of 4–6 original 270-s tracings, but, for simplicity, only every 10th point is displayed. Significant differences between diet and time of day were tested by repeated-measures ANOVA: low-salt diet, CT4 vs. low-salt diet CT16: P<0.05; standard diet, CT16 vs. low-salt diet, CT16: P<0.01.

Table 6.1. Summary of electrophysiological data in colon of rats kept on standard and low-salt diet

Time	PD_0, mV	SCC_0, μA/cm²	R_0, Ω . cm²	Δ SCC, μA/cm²	Δ R, Ω . cm²
			Standard diet		
CT4	2.9 ± 0.4	57.6 ± 11.7	44.1 ± 4.0	4.1 ± 5.4	0.5 ± 3.4
CT16	4.4 ± 1.2	71.6 ± 12.7	54.0 ± 6.5	7.9 ± 2.3	0.6 ± 3.1
			Low-salt diet		
CT4	3.9 ± 0.6	68.8 ± 8.0	43.3 ± 5.1	19.7 ± 6.9	3.8 ± 3.6
CT16	11.6 ± 2.4*	284.0 ± 40.9**	39.3 ± 4.3	78.4 ± 19.6*	22.7 ± 8.3

Data represent means ± SE; n = 5–6 rats in each group. PD_0, potential difference; SCC_0, short-circuit current; R_0, transepithelial electrical resistance at the beginning of the experiment; ΔSCC, amiloride-sensitive SCC; ΔR, the change of R in the presence of amiloride; CT, circadian time. Significantly different from CT4: *P<0.05 and **P<0.01.

6.3 Circadian regulation of cell cycle in the intestinal epithelium

6.3.1 Diurnal variations in expression of cell cycle regulator genes under standard conditions

Colonic epithelium undergoes very dynamic cell renewal process associated with intensive proliferation and rapid turnover of colonocytes. Regarding these facts, and the presence of functional clock in the intestinal epithelium, and the findings suggesting interconnection between peripheral clock and cell proliferation, we examined putative circadian clock role in cell cycle regulation of colon epithelial cells.

We determined daily mRNA expression profiles of key cell cycle regulators, transcription factor *c-Myc*, *cyclins A*, *B1*, and *D1*, inhibitory kinase *Wee1*, and cyclin-dependent kinase inhibitors *p21*, *p27*, and *p16* in scraped mucosa from distal colon of rats kept under standard light and feeding regime (LD12:12; food and water *ad libitum*). Circadian expression profiles are presented in Fig. 6.9.

As revealed by one-way ANOVA, we detected significant rhythm in expression of *cyclin A* (P<0.05) with maximum expression at CT8 and *cyclin B1* (P<0.05) with maximum peak at CT4, while no significant diurnal changes were found in expression of *cyclin D1* (P=0.77). Surprisingly, we did not observe any significant variation in expression of proto-oncogene *c-Myc* (P>0.05). However, the expression of inhibitory kinase *Wee1* exhibited circadian oscillations (P<0.05) with maximum peak reached at CT12 – CT16. Furthermore, *p27*, one of studied cyclin-dependent kinase inhibitors, showed significant diurnal variation (P<0.01) with maximum at CT24, while the others, *p21*, and *p16*, did not reach level of significance (P>0.05).

Additionally, we applied cosinor analyses to expression profiles of genes, which were determined as significant by ANOVA analyses. All analyzed genes exhibited significant rhythm fitted to cosine curve (*cyclin A*: R^2=0.56, P<0.05, mesor: 53.56, 95 % CI = 42.37-64.75, amplitude: 24.42, 95 % CI = 7.76-41.07, acrophase: 4.98, 95 % CI = 2.64-7.32; *cyclin B1*, R^2=0.40, P<0.01, mesor: 72.01, 95 % CI = 60.37-83.66, amplitude: 30.16, 95 % CI = 13.06-47.26, acrophase: 4.30, 95 % CI = 2.30-6.30; *Wee1*, R^2=0.55, P<0.001, mesor: 69.95, 95 % CI = 62.68-77.22, amplitude: 25.09, 95 % CI = 14.57-35.61, acrophase: 17.28, 95 % CI = 15.77-18.80; *p27*, R^2=0.44, P<0.01, mesor: 69.88, 95 % CI = 63.69-76.06, amplitude: 16.50, 95 % CI = 7.89-25.11, acrophase: 2.53, 95 % CI = 0.47-4.58).

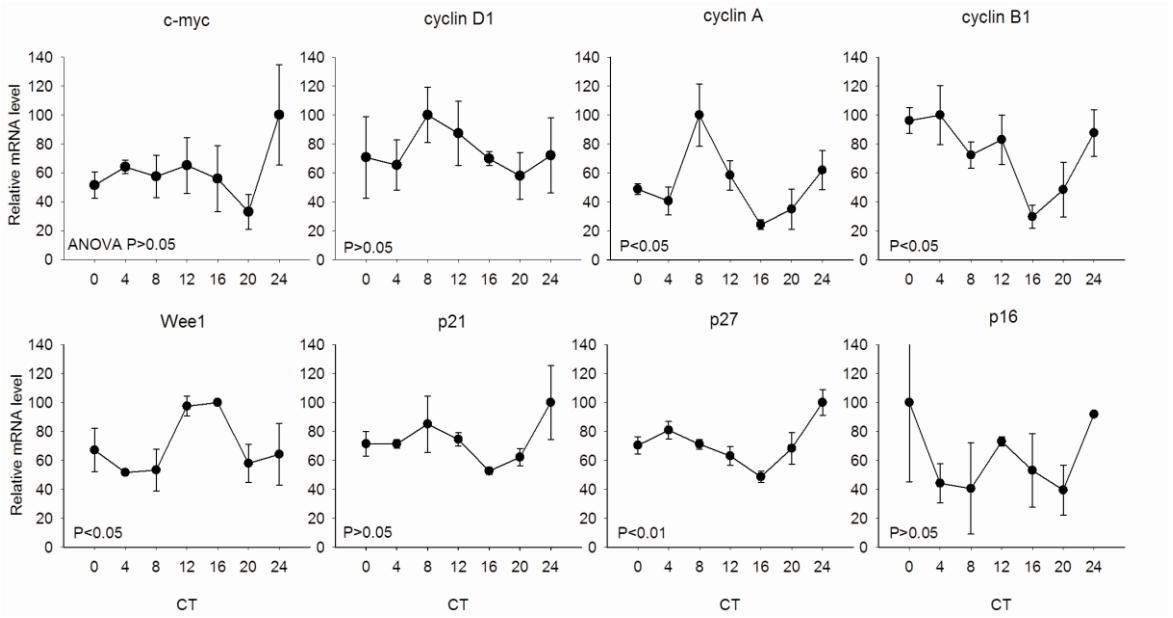

Figure 6.9 Daily profiles of mRNA expression of cell cycle regulatory genes in distal colon mucosa. Rats were maintained under standard light-dark regime schedule with 12-h light and 12-h dark phase, with access to standard chow diet and water *ad libitum*. mRNA abundance of genes *c-myc*, *cyclins D1*, *A*, and *B1*, G2/M inhibitory kinase *Wee1*, and cyclin-dependent kinase inhibitors *p21*, *p27*, and *p16* were determined by quantitative RT-PCR. Levels of transcript are expressed as percentage of the maximum mean level for each particular transcript, and presented as means ± SEM (3-5 animals per time point). One-way ANOVA was used to assess significance of variations according circadian time, and P values are presented. Further post-hoc LSD test was employed to reveal differences between particular time points, and cosinor analysis was applied (see text for details).

In conclusion, we found circadian rhythmicity in several cell cycle regulators, although there are others without circadian pattern of expression. These results suggest that regulation of colonocyte proliferation might be interconnected with intestinal circadian clock; however detailed analysis would be needed to fully elucidate this process.

6.3.2 Circadian expression of cell cycle regulator genes under restricted feeding regime

As it is known that feeding schedule is powerful modulator of peripheral clock phasing, particularly in the liver and intestine (Sládek *et al.*, 2007; Stokkan *et al.*, 2001), and its manipulation could reveal more deeply coupling of peripheral circadian clock and proliferation, we determined expression profiles of rhythmic genes from previous experiment in animals kept under restricted feeding schedule with food availability between CT3 – CT9.

As shown in Fig. 6.10, two-way ANOVA revealed significant effect of factors feeding regime, and time respectively, only in *cyclin B1* (both effects $P<0.05$). No interaction between the factors was observed. In contrast, neither significant differences of any factor nor interaction were detected in case of *cyclin A*, *Wee1*, and *p27* ($P>0.05$).

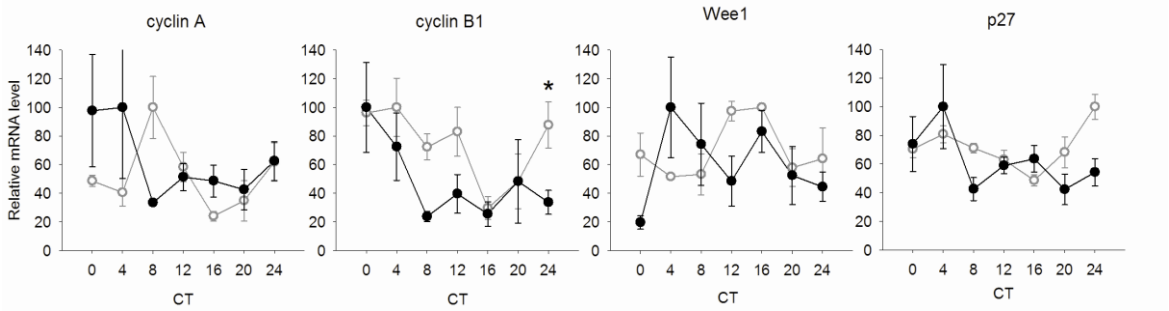

Figure 6.10 The effect of restricted feeding schedule on the daily expression profiles of cell cycle regulatory genes *cyclin A, B1*, kinase *Wee1*, and CDK-inhibitor *p27* in distal colon mucosa. Rats were kept on a 12:12-h light-dark schedule fed *ad libitum* (○), or subjected to restricted feeding regime (●) from CT3 to CT9 for 14 days. On the day of sampling, animals were released into constant darkness. Data are expressed as a percentage of the maximum mean level for each transcript and represent means ± SEM (4 animals for each time point). Significant differences between normal and restricted feeding are indicated by **P<0.05*.

These results suggest that though there is diurnal variation in expression of some cell cycle regulators in standard light conditions, coupling to intestinal circadian clock is not preserved during changes of feeding regime. It is likely that another regulatory pathway plays dominant role in control of cell cycle regulation.

6.3.3 Spatio-temporal organization of cell cycle regulators' circadian rhythms within colonic crypts

Cell division of stem cells occurs at the base of the crypt from where the cells move up to the mouth, while continuously progress in differentiation. As these differentiation processes

might impact clock and cell cycle regulation, therefore we have studied circadian expression of selected cell cycle regulators in the colonic crypt base and crypt mouth respectively, using laser capture microdissection and real time RT-PCR. Circadian expression profiles of particular genes are presented in Fig 6.11.

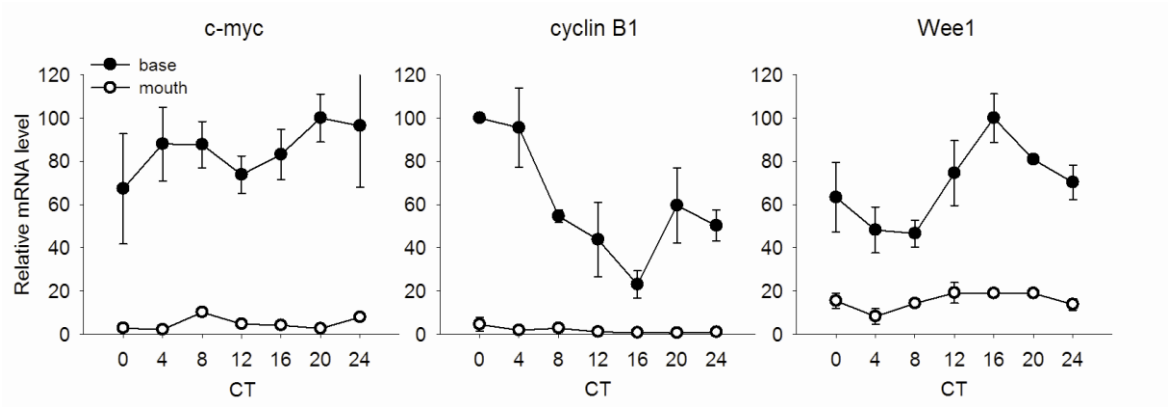

Figure 6.11 Spatio-temporal organization of mRNA expression of cell cycle regulatory genes in rat colonic crypt base, and crypt mouth. The mRNA levels of c-myc, cyclin B1, and Wee1 genes were determined by qunatitative RT-PCR in microsamples of colonic crypt base (●) and crypt mouth/surface (○) colonocytes harvested by laser microdissection. Data are expressed as percentage of maximum mean levels for each transcript, and presented as means ± SEM of 4 animals per each time point. 2-way ANOVA analysis revealed the effect of colonocyte position along crypt axis in all three genes, and the effect of circadian time in *Wee1* and *cyclin B1*. For details see the text.

Two-way ANOVA revealed significant effect of both factors (circadian time, $P<0.001$; tissue position, $P<0.01$) for *cyclin B1* expression profile. Cosinor analysis confirmed periodic expression in crypt base ($P<0.05$), while no significant fitting was achieved in crypt mouth ($P>0.05$). Post-hoc analysis showed differences in expression during 24 hours within crypt base with the highest expression level reaching maximum at CT0 – CT4, then declining to minimum at CT16 (with statistical values of $P<0.001$ for both CT0 vs. CT16 and CT4 vs. CT16). Moreover, interaction of the factors reached significant value ($P<0.01$). Similarly, the significant effect of both circadian time ($P<0.01$), and tissue localization ($P<0.001$) was identified for *Wee1* gene.

Cosinor analysis confirmed the cyclic expression in both compartments (crypt base, $P<0.05$; crypt mouth $P<0.01$). In the crypt base, where post-hoc test revealed significant differences, the expression of *Wee1* raised from minimal values at CT4 – CT8 to maximum reached at CT16. Both time comparisons of CT4 vs. CT16 and CT8 vs. CT16 respectively, reached significance ($P<0.001$). In case of *c-Myc*, no effect of circadian time was observed,

however colonocytes position displayed significant effect with greater level in the crypt base ($P<0.001$).

In summary, all studied genes showed substantially higher expression in crypt base, where intensive proliferation is going on. Circadian profiles of genes were in concordance to the results obtained from scrapped mucosa samples.

6.4 Role of circadian clock in tumourigenesis

6.4.1 Expression of clock genes in colorectal neoplastic tissue

Though we found some evidence of putative interplay between peripheral clock and a few cell cycle regulators in the intestinal epithelium, it remains unclear whether there is any contribution of circadian clock to disturbance of cell cycle regulation. Therefore, in the next experiments we examined the role and the contribution of clock and its alteration in process of neoplastic transformation and tumourigenesis. We have worked with the mouse model of azoxymethane (AOM) induced carcinogenesis associated with dextran sodium sulfate (DSS) induced colitis. AOM in combination with DSS induces multiple nodular and polypoid tumours in the distal part of colon that display characteristics of intraepithelial neoplasia ranging from low-grade dysplasia (usually located at the periphery of polyps, next to non-dysplastic colonic epithelium) to high-grade dysplasia/intramucosal carcinoma forming the central part of the tumour (Švec *et al.*, 2010). In this model we studied expression of core clock genes in tumour and surrounding healthy looking tissue in mice bearing induced colorectal tumour. Simultaneously, we analyzed scrapped colonic mucosa and whole-thick colon tissue from corresponding intact control animals of the same age.

As shown in Fig. 6.12, we have observed substantially suppressed oscillations of clock genes in tumour tissue. One-way ANOVA revealed diurnal variation in mRNA expression of *Bmal1*, *Per1*, *Per2* and *RevErbα* in all studied tissues (tumour and surroundings from induced tumour bearing mice, and mucosa and whole colon from intact mice). Highly significant effect of time was found in all tissues and genes. In most of the time points, the post-hoc analysis showed strong down-regulation of clock genes expression in tumour tissue in comparison with the surroundings of the same colon and in mucosa and colon of intact animals. For details see Fig. 6.12.

In addition, cosinor analysis was employed in order to characterize and compare circadian parameters of clock gene expression patterns. Expression of *Per1*, *Per2* and *RevErbα* significantly fitted the 24h sinusoidal curve in all studied tissues (P<0.001, except of Per1 in surrounding tissue, where P<0.01). For *Bmal1*, no rhythm was detected in tumour or surrounding tissue of tumour bearing mice (P>0.05), however, significant rhythm of this gene

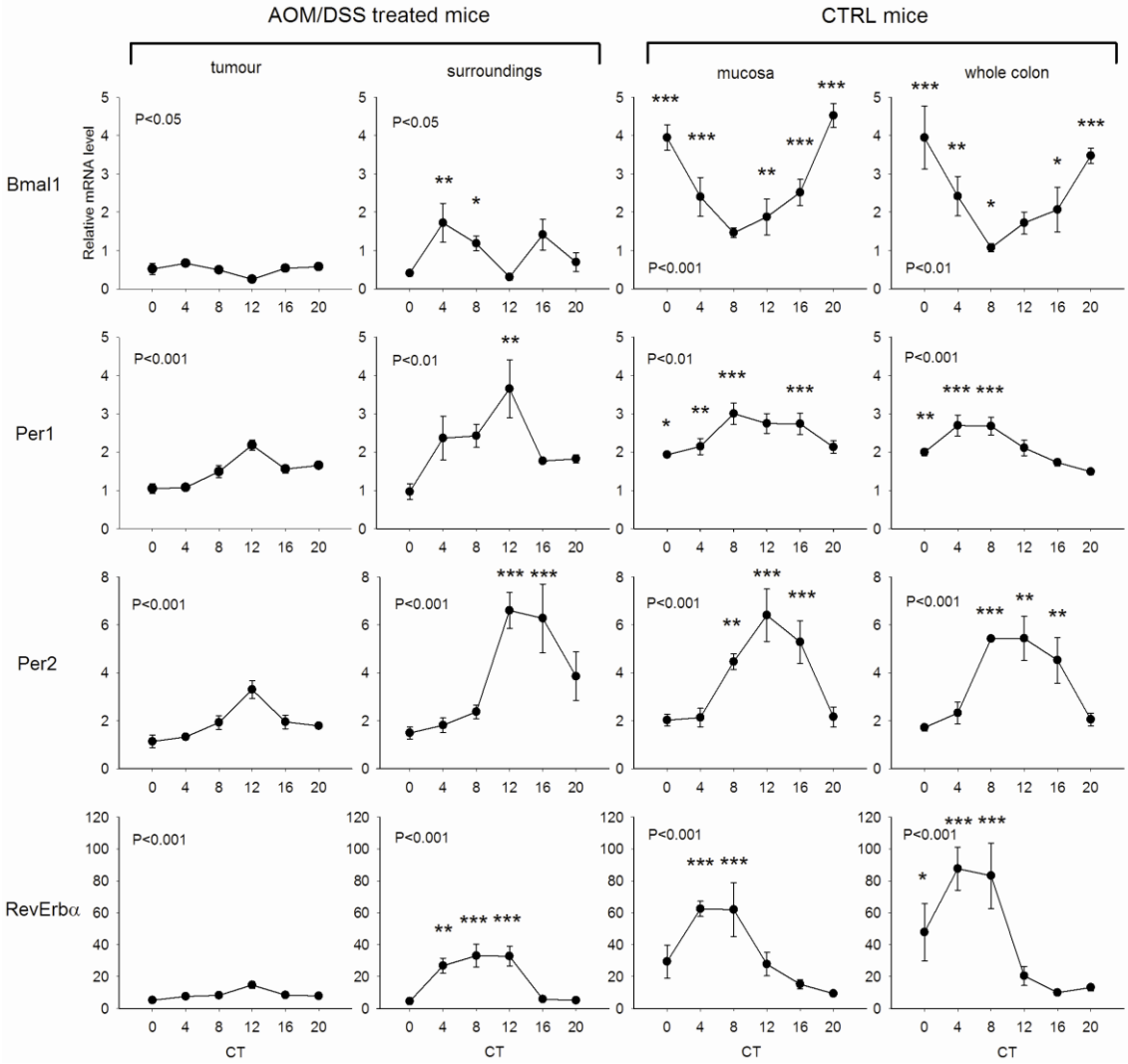

Figure 6.12 The daily profiles of mRNA expression levels of clock genes in mice bearing colorectal tumors and in healthy counterparts. The expression levels of the genes *Bmal1*, *Per1*, *Per2*, and *Rev-erbα*, were measured in intraepithelial neoplasia, and in the surrounding colonic tissue of mice with induced colorectal tumours (AOM/DSS) and in the whole colon and colonic mucosa of control animals of the same age (CTRL). The mice were maintained under a 12:12-h light-dark schedule; on the day of sampling, they were released into constant darkness. The points represent the mean ± SEM (5 animals for each time point). One-way ANOVA revealed significant effects of time for all four genes and tissues (the P value is given in each figure). Significant differences from tumours are indicated by **P<0.05*, ***P<0.01* or ****P<0.001*.

was observed in mucosa (P<0.001) and whole colon (P<0.01) of intact controls. Moreover, parameters of mesor, amplitude and acrophase were determined with confidence intervals. As shown in Fig. 6.13, we revealed in tumour substantially lowered value of mesor in all studied clock genes and reduced amplitude of *Per2* and *RevErbα*. In addition, the phase-delay of acrophase of *RevErbα* in tumour suggests that disturbances in circadian organization are associated with modulation of the phase of clock gene expression.

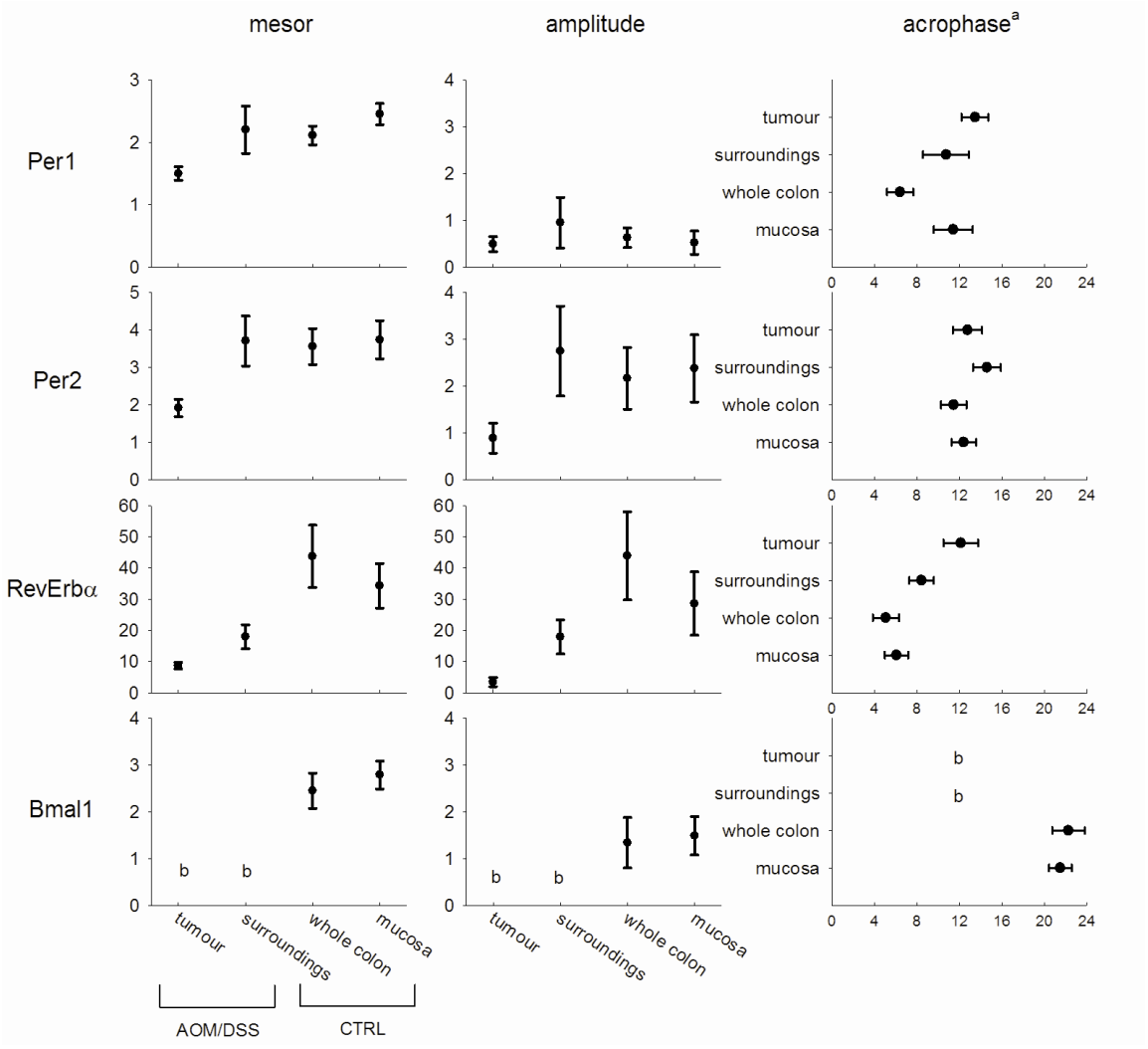

Figure 6.13 Cosinor analysis parameters of the circadian rhythmicity of the clock genes in tumours and surroundings of induced tumour bearing mice, and mucosa and whole-thick colon tissue of control mice of the same age. Data are given as the calculated values of mesor (rhythm-adjusted mean), amplitude of the rhythm, acrophase (time of day when the maximum values are reached), and the estimated 95 % confidence intervals. AOM/DSS – mice with colorectal tumor, CTRL – healthy controls of the same age. [a]Acrophase is given in hours after the subjective dark-to-light transition; [b], not given if *P>0.05*.

In summary, we found strong deregulation of circadian clock in tumour. The amplitude of circadian rhythmicity of clock gene expression was substantially damped and mRNA abundance was lowered. Likewise, in surrounding tissue of tumour bearing mice we found reduction of mesor and amplitude of *RevErbα* circadian rhythm, which together with absence of significant rhythm of *Bmal1* indicates clock alteration also in tumour adjacent healthy tissue.

6.4.2 Expression of clock-controlled genes in tumours and associated tissues

Although clock genes constitute the core clock system, the clock-controlled genes are outputs, which spread the information about timing to regulatory networks. Therefore, we analyzed transcription factor *Dbp*, which is directly regulated by CLOCK/BMAL1 heterodimer, and *Wee1*, which regulates cell cycle progression and exhibits rhythmic expression in rat intestinal epithelium (Fig. 6.14). Furthermore we measured expression of *c-Myc* and *p21* as representatives of cell cycle regulators, which might be rhythmically expressed in mouse intestine.

We compared expression profiles of particular mRNA levels in tumour of AOM/DSS mice and colonic mucosa of intact controls and determined robust rhythmic expression of *Dbp* in control mucosa (ANOVA P<0.001; cosinor analysis P<0.001), whereas strongly reduced rhythm in tumour (as defined by amplitude, P<0.001). Moreover, maximal daily level of expression was phase delayed approximately 4 hours in tumour (acrophase: 12.07; 95 % CI = 10.49-13.64) compared to mucosa of healthy animals (acrophase: 8.71; 95 % CI = 7.49-9.92).

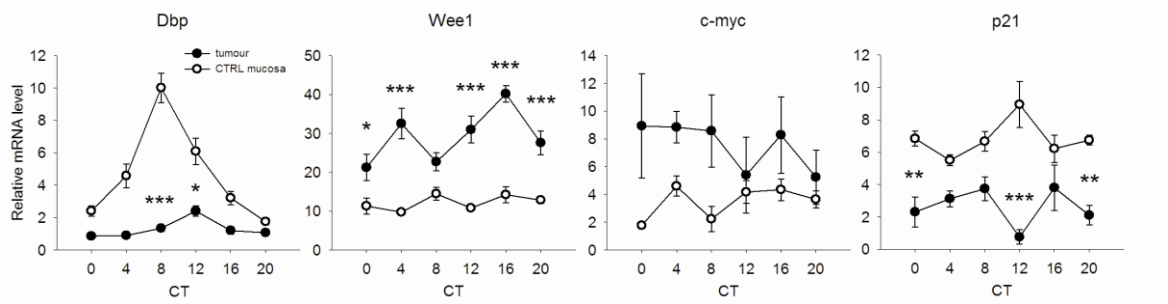

Figure 6.14 The daily profiles of clock-controlled genes in colorectal tumours of AOM/DSS mice (●) and in the colonic mucosa of healthy mice of the same age (○). One-way ANOVA revealed a significant effect of time on *Dbp* expression in both tissues (P<0.001) and *Wee1* expression in tumours (P<0.01), but no effects were observed in the cases of *c-myc*, and *p21*. Significant differences from healthy controls are indicated by **P<0.05*, ***P<0.01* or ****P<0.001*.

Although one-way ANOVA analysis revealed significant changes in *Wee1* expression in tumours as factor of time (P<0.05), cosinor analysis did not detect any significant rhythm

(cosinor analysis P>0.05), and so was considered as non-rhythmic. Furthermore, we failed to observe significant circadian rhythm of *Wee1* expression in control mucosa (both ANOVA and cosinor analysis P>0.05). Similarly, in case of genes *c-Myc* and *p21* there was no significant rhythm in expression either in healthy mucosa or tumour samples. However, analysis of overall average expression within 24 hours revealed significant differences between tumour an mucosa samples. In particular, there was increased expression of *Wee1* and *c-Myc* in tumour samples compared to healthy mucosa (28.8 ± 1.7 vs. 12.2 ± 0.6, P<0.001; 7.7 ± 1.0 vs. 3.6 ± 0.3, P<0.01) and reduced expression of p21 (2.7 ± 0.4 vs. 6.8 ± 0.3; P<0.001).

6.4.3 Expression of clock and clock-controlled genes in young animals

The results obtained for clock-controlled gene *Wee1* were in conflict with our previous findings, where we observed rhythmic expression of *Wee1* in rat colonic mucosa (Fig. 6.9). To rule out the possibility that the loss of rhythmicity is associated with ageing (aged mice had been used in experiments with induced tumours), we determined circadian expression of selected core clock and clock-controlled genes in 10 weeks old mice, which are typically used in experimental studies. All measured clock genes, particularly *Bmal1* and *Per1*, were rhythmically expressed in colonic mucosa of young animals as detected by both one-way ANOVA (P<0.001) and cosinor analysis (P<0.01). Expression profiles (Fig. 6.15) corresponded to that obtained from aged animals and cosinor analysis displayed similar parameters (acrophases: *Bmal1*: 22.2, 95 % CI = 21.6-22.8; *Per1*: 11.7, 95 % CI = 9.4-14.0).

Furthermore, the expression of clock-controlled gene *Wee1* fluctuated in a circadian manner (one-way ANOVA: P<0.001; cosinor analyses: P<0.01) in young animals with profile resembling our previous findings, and the rhythm of *p21* reached borderline significance (one-way ANOVA: P=0.048; cosinor analysis: P<0.01). The acrophases of *Wee1* and *p21* were reached at 14.9 (95 % CI = 13.0-16.8) and 4.4 (95 % CI = 1.7-7.1), respectively. In contrast to these genes, *c-Myc* did not display any diurnal rhythmicity. Moreover, two-way ANOVA revealed a significant effect of ageing on circadian rhythmicity for *Bmal1* (P<0.05), *Per1* (P<0.01), and *Wee1* (P<0.01).

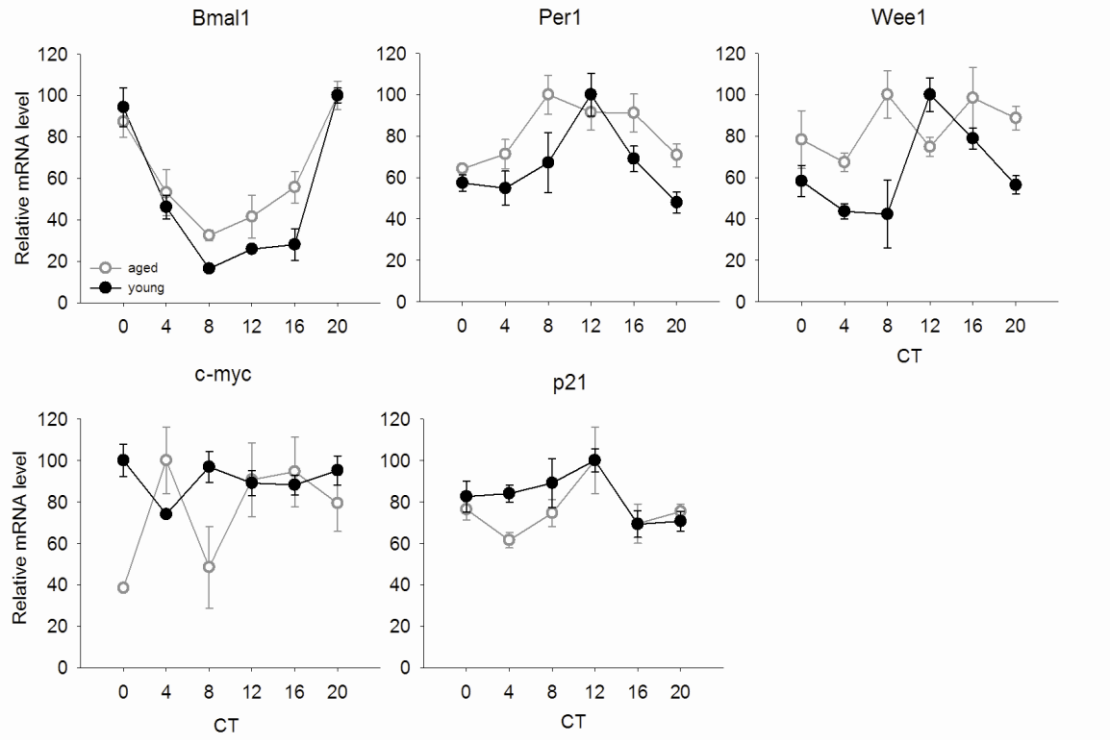

Figure 6.15 The effect of ageing on the daily expression profiles of Bmal1, Per1, Wee1, c-myc, and p21 in the colonic mucosa of healthy young (10-week-old; ●) and aged (52-week-old; ○) mice. The values are the mean ± SEM for 5 mice. One-way ANOVA revealed significant effects of time for Bmal1, Per1, Wee1, c-myc ($P<0.001$) and p21 ($P<0.05$) in young mice but only for Bmal1 ($P<0.001$) and Per1 ($P<0.01$) in aged animals.

6.4.4 Clock gene expression in the liver of colorectal tumour bearing mice

To test the possibility that other peripheral organs might be affected by colorectal tumourigenesis, we studied expression of clock genes in liver, where AOM is metabolized. As shown in Fig. 6.16, mRNA levels of clock genes *Bmal1*, *Per1*, *Per2*, *Rev-Erbα*, and clock-controlled genes *Dbp,* and *Wee1* exhibited circadian rhythms that were statistically validated with both one-way ANOVA ($P<0.001$; $P<0.01$ for *Dbp* and *Wee1* in control mice) and cosinor analysis ($P<0.0001$; $P<0.005$ for *Dbp* in control mice). Parameters of fitted cosine curves revealed increased amplitude of *Per1* and *Per2* rhythms in AOM/DSS mice (Per1: 0.27, 95 % CI = 0.17-0.37 vs. 0.73, 95 % CI = 0.53-0.94; Per2: 1.04, 95 % CI = 0.79-1.29 vs. 1.73, 95 % CI = 1.39-2.08) but the amplitudes of *Bmal1*, *Rev-Erbα*, *Dbp* and *Wee1* did not vary.

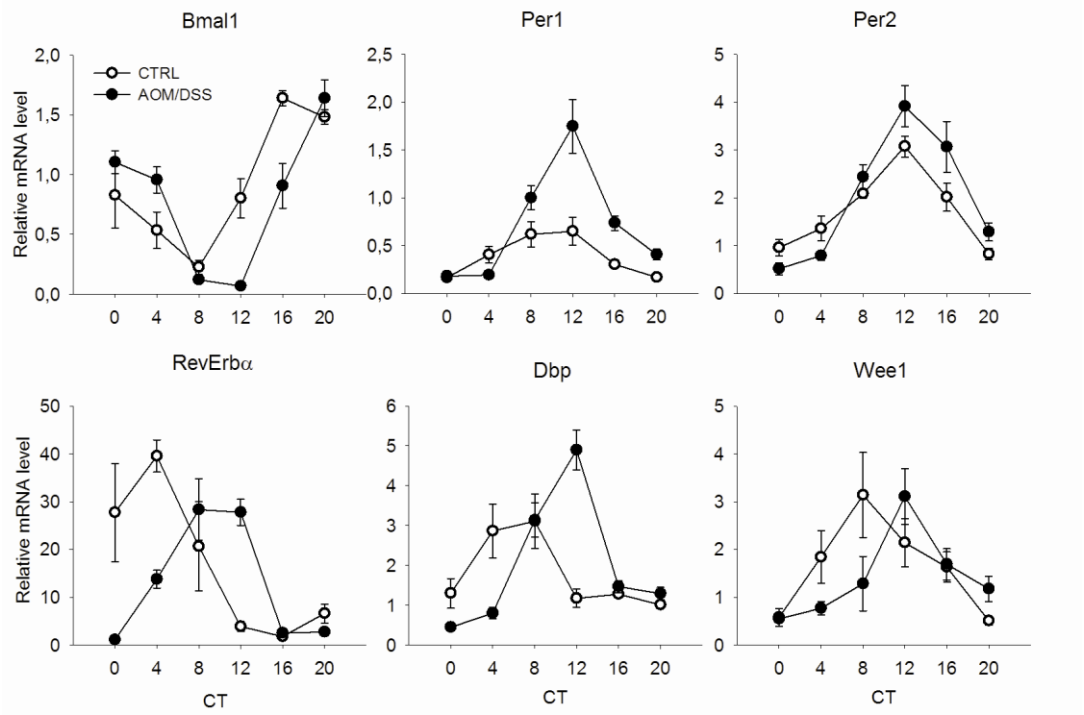

Figure 6.16 The expression levels of clock and clock-controlled genes in liver of tumour-bearing mice (AOM/DSS; ●) and in healthy mice of the same age (CTRL; ○). The mice were maintained under a 12:12-h light-dark schedule and on the day of sampling, they were released into constant darkness. The points represent the mean ± SEM (5 animals for each time point). For details see the text.

On the contrary, AOM treatment significantly affected the phase of expression of *Bmal1*, *Rev-Erbα*, *Dbp* and *Wee1* but not *Per1* and *Per2* (Fig 6.17). Two-way ANOVA showed significant differences in the expression of *Bmal1*, *Rev-Erbα*, *Per1*, *Dbp* and *Wee1* between the liver of healthy and tumour-bearing mice. The circadian expression of *Per2* did not vary between both groups (Fig 6.17).

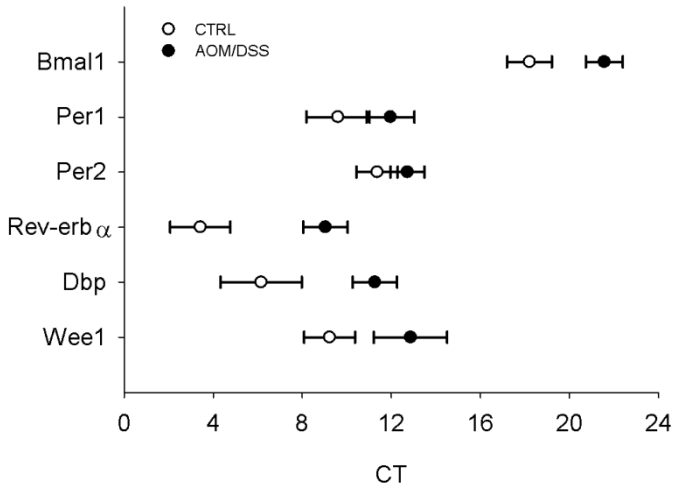

Figure 6.17 The shift of the acrophase of clock genes and clock-controlled genes in liver of healthy control mice (CTRL; ○) and in tumour-bearing mice (AOM/DSS; ●) mice. Data were obtained by cosinor analysis and the values are given in hours after dark/light transition. The horizontal bars represent 95% confidence interval. Differences were considered as significant in cases where confidence intervals did not overlap.

7 DISCUSSION

"Our love of being right is best understood as our fear of being wrong."

-Kathryn Schulz, 2010-

Our objective was to determine whether particular parts of the gastrointestinal tract possess functional peripheral clockwork system and to show whether it is involved in regulation of enterocyte proliferation and electrolyte transport.

7.1 Intestinal circadian clock

We documented molecular clock in epithelium of rat duodenum, jejunum, ileum and colon by detecting circadian oscillations in mRNA expression of core clock genes *Bmal1*, *Per1*, *Per2*, and *RevErbα*. The findings are in concordance with general basic clock transcriptional-translational feedback loop mechanism previously described (Ko & Takahashi, 2006; Reppert & Weaver, 2001). Further, the expression profile patterns resemble those observed previously in rat colon (Sládek *et al.*, 2007), and in mouse stomach, colon (Hoogerwerf *et al.*, 2007), and jejunum (Froy & Chapnik, 2007), with the phase of *Bmal1* opposite, and *RevErbα* phase-advanced comparing to *Per* genes. In addition, colonic epithelium is known to be the site of rhythmic clock gene expression as detected by quantitative PCR, hybridization technique, and immunohistochemistry (Sládek *et al.*, 2007). These findings implicate that oscillating mRNA represents functional clock in all segments of gastrointestinal tract along the cranio-caudal axis. Furthermore, the profiles of clock genes in gut were very similar to those observed in rat liver, which points to the importance of synchronization of physiologically related metabolic processes between liver and intestine, and to the substantial role of peripheral clock in governing this synchronization. Similar to our findings in rats, the expression of clock genes was shown also in human colonocytes (Pardini *et al.*, 2005) and the experiments of Yang *et al.* (2009a) confirmed also the existence of clock gene fluctuations in small intestine.

As intestinal epithelium is highly diversified in cellular and physiological functions, we investigated whether circadian clock differs in distinct parts of the epithelium. We focused on colonic crypts, where at least two different cell populations can be found. The area at the bottom of the crypts contains stem cells which are the source of undifferentiated sister cells. These cells move along the crypt-surface axis to the mouth of the crypt, while simultaneously differentiate into mature colonocytes and finally undergo apoptosis and exfoliation; i.e. the surface colonocytes represent the fully differentiated cells without any proliferation potential. To our

knowledge, we showed for the first time that the circadian clock is present in both functionally distinct crypt compartments. The phases of expression of all studied genes (*Per1*, *Per2*, and *Bmal1*) were identical in both compartments, which implicates full coordination of epithelial clock between different crypt regions. Moreover, the phases of expression were in concordance with our previous observations in scrapped colonic mucosa (Fig 6.1). However, we detected differences in amplitudes between crypt base and crypt mouth in *Per1* and *Per2* profiles what might be linked to distinct differentiation phases of colonocytes in both compartments. Whether these dissimilarities could have functional significance is not clear, and further experiments would be needed to clarify this hypothesis.

7.2 Role of the circadian clock in intestinal transport

Colonic epithelium is the site of intensive water and electrolyte transport mediated by transcellular secretion and absorption of main electrolytes Cl^-, Na^+ and K^+. Previously Sládek *et al.* (2007) demonstrated circadian expression of *Nhe3* mRNA in rat colon epithelia. Similarly, in this study, we confirmed rhythmic *Nhe3* mRNA and furthermore extended this observations for novel demonstration of circadian regulation of other genes (i.e. *Dra*, *Ae1*, *Nherf1*, *γENaC*, *Atp1a1*) coding proteins participating in intestinal NaCl and KCl transport. Our findings are in concordance with several microarray studies, which revealed that a lot of genes are rhythmically expressed in various tissues including brain, liver, muscle, and colon including various members of transporter, channel and associated protein families such as *Nhe3*, *Nherf1*, *Atp1a1* (Hughes *et al.*, 2009; Panda *et al.*, 2002; Ueda *et al.*, 2002; Zuber *et al.*, 2009).

The rhythmicity of transporters is likely due to circadian clock operating in the intestinal epithelia. This hypothesis is supported by experiments showing *Nhe3* rhythmicity at both mRNA and protein level in kidney, other important epithelial tissue. In this tissue, a direct binding of clock gene products CLOCK/BMAL1 heterodimer was observed in E-box sequences of promoter region of *Nhe3* gene (Nishinaga *et al.*, 2009; Saifur Rohman *et al.*, 2005). This observation strongly suggests similar mechanism of direct circadian regulation of colonic *Nhe3*. The same mechanism seems to control other transporters, although direct evidence is lacking and we cannot exclude the possibility of the regulation via other promoter regions like D-boxes or involvement of other rhythmically expressed transcription factors. However, the phases of the genes studied in our experiments are similar to the expression patterns of *Per* genes, which suggests regulation by CLOCK/BMAL1 heterodimer. Simultaneously synchronized regulation

of genes coding absorption would be of particular importance as it is known that DRA exchanger is functionally coupled to NHE3 (Walker *et al.*, 2008) and *Nherf1*-deficient mice exhibit defective sodium absorption in intestinal epithelium (Broere *et al.*, 2009).

Previous studies demonstrated circadian regulation of some intestinal nutrient transporters coupled with electrolyte transport. For example, the SGLT1 protein cotransporting Na^+ and glucose has been shown to exhibit diurnal rhythmicity at the level of mRNA, protein and activity (Pan *et al.*, 2004; Rhoads, 1998; Tavakkolizadeh *et al.*, 2001). Mice with mutated *Clock* gene lost rhythmic expression of *Sglt1* and feeding regime was unable to induce or entrain it. Similarly, diurnal changes in transport capacity were lost (Pan & Hussain, 2009). Furthermore, binding of BMAL1 to promoter region of *Sglt1* gene was demonstrated (Iwashina *et al.*, 2011). These findings support direct role of clock genes in circadian regulation of transporters.

Other small intestinal transporters, such H^+/peptide cotransporter PEPT1 and hexose transporters GLUT2, and GLUT5 were shown to be under similar circadian regulation associated with the regulatory role of CLOCK and BMAL1 proteins (Iwashina *et al.*, 2011; Pan & Hussain, 2009). Furthermore, several members of multidrug resistance protein family were demonstrated to exhibit rhythmicity in small intestine (Stearns *et al.*, 2008), while the source of the rhythm were determined to be the protein products of clock-regulated genes *Hlf*, and *E4bp4* (Murakami *et al.*, 2008). Thus, these experiments and our finding of rhythmic expression of transporters indicate that colonic electrolyte transporters are controlled by circadian clockwork system.

Whereas electroneutral sodium transport is active during standard sodium intake conditions, electrogenic sodium transport mediated by sodium channel ENaC is mostly active during sodium deprivation (Pácha & Pohlová, 1995). Surprisingly, we detected rhythmic expression of *γENaC* in animals kept on standard diet while we observed no rhythms in expression of α or β subunits. Although, this transport system does not significantly participate in overall sodium absorption during standard conditions, circadian regulation of γENaC, which is the main regulatory subunit of colonic ENaC, may be redundant due to described Per1 regulatory effect on expression of renal ENaC main regulatory subunit (Gumz *et al.*, 2009) or due to circadian oscillation of aldosterone (Fig 6.6). However, it is functionally relevant during sodium deprivation, when ENaC expression and activity are up-regulated (Asher *et al.*, 1996; Stokes & Sigmund, 1998). Our results confirm this fact – the expressions of β and γ subunits of ENaC in distal colon were up-regulated during low-salt diet. In concordance with the literature

(Kunzelmann & Mall, 2002), we identified simultaneously elevated plasma level of aldosterone, which stimulates channel activity.

Our analysis revealed alterations in colonic circadian clock during secondary hyperaldosteronism induced by low-salt diet. Whereas *Bmal1* expression remained unaltered with increased expression during subjective day independent of dietary salt intake, *Per1* varied according to the expectation only in the case of rats kept on standard conditions. Interestingly, the expression of *Per1* did not vary between day and night in colon of rats kept on low-salt diet and reached similar values in both day and night. The day-time value of *Per1* in rats kept on low-salt diet was elevated comparing to the day-time values in rats kept on standard diet and reached values similar to the night-time levels found in both groups of animals. These observations are in accordance with the study of Gumz *et al.* (2009), who found that αENaC, the main regulatory subunit of renal sodium channels, exhibits circadian pattern of expression and the rhythm diminishes in mice lacking functional *Per1* gene. Moreover, they showed involvement of PER1 protein in channel activation in response to aldosterone, and conversely, altered stimulation by aldosterone when *Per1* was silenced. Therefore, we suggest that the same mechanism might operate in colon and the elevated plasma aldosterone during secondary hyperaldosteronism might alter *Per1* gene expression.

Since we do not know the whole day expression profile of clock genes in animals with secondary hyperaldosteronism, we cannot exclude other changes in different time points. Nonetheless, we detected strong diurnal changes in βENaC and γENaC expression under conditions of low-salt diet – expression of both subunits was significantly higher during night. Secondly, our functional study of transepithelial electrogenic sodium transport via ENaC channel confirmed rhythmic activity of electrogenic transport during low-salt diet condition with increased transport capacity during the dark phase of the day. This increase was observed in the same period of the day as we detected up-regulation of *βENaC* and *γENaC* mRNA expression. The detected diurnal differences of electrogenic sodium transport was found only in the group fed low-salt diet, while no differences were detected in animals kept on standard diet. This finding demonstrates that circadian rhythmicity of electrogenic sodium absorption in colon is dominant in physiologically relevant condition, i.e. secondary hyperaldosteronism. Some other authors also confirm the role of aldosterone in control of rhythmic changes of electrogenic sodium transport. In rabbit colon, Clauss and co-workers showed rhythmic transmural electrical potential difference (PD) and diminished rhythm after treatment with spironolactone, an aldosterone antagonist (Clauss *et al.*, 1988). Experiments of Wang *et al.* (2000) showed diurnal changes in

amiloride-sensitive rectal PD in mice with more negative PD in the afternoon compared to the morning values. Afternoon values were even greater, when angiotensin II inhibitor was applied what indicates time dependent role of renin-angiotensin system. Similarly, Gumz with co-workers demonstrated aldosterone participation in circadian regulation of ENaC channel regulatory subunit in the rat kidney (Gumz *et al.*, 2009), where aldosterone was able to stimulate *Per1* expression and *αENaC* mRNA expression was PER1 dependent. Interestingly, plasma aldosterone did not vary significantly between day and night values in our experiment. As there are only two time points (CT4 and CT16) measurements, we cannot exclude the possibility that aldosterone concentration rose in earlier or later time points, especially, if we take into account the daily profile of aldosterone level in intact rats where sharp peak was detected only in CT12 (Fig. 6.6) and the same pattern of aldosterone oscillation was found in rats kept on low-salt diet (Hilfenhaus, 1976). Moreover, aldosterone effect is known to be mediated by additional proteins (Lee *et al.*, 2008) and the peak of maximal transmural potential difference in rabbit colon is delayed by 4 hours compared to the peak of plasma aldosterone (Clauss *et al.*, 1988). It is noteworthy, that the peaks of both rabbit and rat plasma aldosterone reach maximum in the late day and middle day, respectively, and plasma levels of aldosterone reach equal values in timepoints used in our experimental schedule (Clauss *et al.*, 1988; Hilfenhaus, 1976). Although, it is possible, that aldosterone fluctuation is not the primary force that drives oscillation of electrogenic sodium transport and ENaC expression, the observations in kidney and our results support hypothesis that aldosterone mediates oscillations of both ENaC expression and function.

Taking together, we found diurnal rhythmicity of genes participating in sodium absorption in rat distal colon. Furthermore, we showed that times phases of transporter and channel transcripts are in accordance with colonic clock gene expression under both normal and restricted feeding conditions, which implies significant role of colonic clock in anticipation of food load. In contrast, we did not find significant 24-hour rhythm of genes coding transporters and channels operating in chloride secretion.

7.3 Role of the circadian clock in epithelial cell cycle regulation

The intestinal epithelium as the dominant absorptive and secretory tissue requires regular renewal and thus total turnover of epithelial cells is enormously frequent. Strict balance between cell division and apoptosis is essential to maintain cellular replenishment. As there is some evidence for circadian timing of cell proliferation from a few experiments *in vitro* as well as *in vivo*

(Al-Nafussi & Wright, 1982; Buchi *et al.*, 1991; Neal & Potten, 1981) we examined possible role of clock and circadian timing in basic cell cycle regulation of colonic epithelial cells. Firstly, we examined circadian mRNA expression of genes involved in different phases of cell cycle with both activatory and inhibitory functions. Using ANOVA and cosinor analysis we found that some of the studied genes showed circadian changes within 24 hours. Interestingly, the oscillating genes were members of various families; cyclin A and B are activatory units of CDKs promoting cell cycle, while WEE1 kinase prevents G2/M progression and p27 is CDK inhibitor. Similarly, non-rhythmic genes were both activatory and inhibitory. From cell cycle progression point of view, dominant presence/activity of rhythmic genes was condensed to G2 and M phases, as cyclin A is present in complex with CDK2 in S/G2 progression and in complex with CDK1 during G2 phase. Further, cyclin B1 is in active complex with CDK1 in G2/M transition and this complex is inhibited by WEE1 kinase, a protein product of rhythmically expressed gene (Arellano & Moreno, 1997; Ekholm & Reed, 2000; McGowan & Russell, 1995). Moreover, mRNA expression phases of these genes were in accordance with cell cycle progression, as expression of the gene coding cyclin A reached maximum level at CT8, while the gene coding cyclin B exhibited increased expression between CT0 and CT12. *Wee1* reached maximal values at CT12 – CT16, which is the latest peak from rhythmic genes. These observations suggest that the phases of cell cycle progression correspond to the circadian clock rhythm. However, gating of cell cycle may be clock-independent under particular conditions, especially in tissues that do not undergo intensive proliferation (Johnson, 2010).

As the matter of fact, other regulatory factors enter to regulatory check-points of cell cycle progression, especially as response to specific ambient stimuli (DNA damage, apoptotic signals etc.). Furthermore, cell cycle is not completely altered in animals or cell lines bearing mutant or lacking any of clock genes. In these animals, embryogenesis and organogenesis occur normally and they live to adulthood, however, their cell cycles can be partially altered. For example, impaired cell cycle progression from S to M phase and the loss of mitotic wave during hepatic regeneration were detected in *Cry* deficient mice (Matsuo *et al.*, 2003). In addition, there is the evidence that clock genes are able to interact with cell cycle regulators (Borgs *et al.*, 2009).

Our findings of *Wee1* oscillations are in perfect concordance with the study of Matsuo *et al.* (2003), who found circadian regulation of *Wee1* expression in mouse liver, where regenerating hepatocytes were gating their cell division according to rhythmically expressed *Wee1*, a gene that is directly regulated by CLOCK/BMAL1 complex via E-box promoter sequence. Hence we propose that *Wee1* might represent direct link between molecular clock and cell cycle

progression in colonocytes. The hypothesis is supported also by findings of elevated WEE1 protein in spleen of *Cry1/Cry2* double knockouts (Hashiramoto *et al.*, 2010) and diminished rhythm of *Wee1* expression in the liver of *Bmal1* knockout mice (Gréchez-Cassiau *et al.*, 2008). It is of considerable importance, that one of the most important points of cell life, the entry to mitosis, is directly linked to circadian clock and timely synchronized with other processes within the organism and ambient environmental cues. This conclusion is supported by studies documenting that circadian gene expression persists in daughter cells after division, however, cell division can shift the phases of circadian cycle (Nagoshi *et al.*, 2004). Furthermore, gating of cytokinesis to specific time spans was described in individual fibroblasts and interestingly, mitosis was able to induce phase shift in these cells (Nagoshi *et al.*, 2004). Circadian rhythmic gating of the S phase, when DNA is doubled, was observed in several experiments in human oral and gastrointestinal mucosa, skin (Bjarnason & Jordan, 2002; Bjarnason *et al.*, 1999), and human and mouse bone marrow (Smaaland *et al.*, 2002; Sothern *et al.*, 1995).

In case of rhythmic cyclins, our results are also in concordance with described cyclin A and cyclin B rhythms of expression in human oral mucosa (Bjarnason *et al.*, 1999), which functionally resembles intestinal epithelium. Although the study of Bjarnason *et al.* (1999) and our experiments were conducted on distinct species and different part of alimentary tract, the phases of the rhythms are similar and maximal levels are reached during light phase in both cases. We can speculate about mechanism how cyclins are maintained in rhythmic expression, however indirect evidence implies the role of clock genes. Knockdown of *Csnk1e*, a gene coding CK1ε, was demonstrated to down-regulate substantially mRNA expression of *cyclin A* and *cyclin B* in human fibrosarcoma cell line (Yang & Stockwell, 2008). In contrast, investigation using luminescence reporters in Rat-1 fibroblasts revealed that while the period of *Bmal1* expression in free running synchronized cell line is temperature compensated (period length is the same regardless ambient temperature), the expression of *cyclin B1* is not temperature compensated and thus period length is temperature dependent (Yeom *et al.*, 2010). This inconsistence shows uncoupling of circadian cycle and cell cycle in particular mammalian cell lines, what could be due to fact that Rat-1 is an immortalized cell line and possibly differs from other cell lines or *in vivo* status.

Another member of cyclin family, *cyclin D*, did not reach the border of significance, although indication of the rhythm is noticeable. Previous studies documented cyclin D as rhythmically expressed in human intestinal mucosa (Griniatsos *et al.*, 2006). Furthermore, markedly decreased expression of mRNA was observed in liver of *Cry*-deficient mice (Matsuo *et*

al., 2003) and in hair follicle of *Bmal1* knockout mice (Lin *et al.*, 2009). However, conflicting results were also found. While other cell cycle regulators were affected by *Per2* mutation in mouse embryonic fibroblast cell line, the expression of *cyclin D* was not affected (Gu *et al.*, 2012). Similarly, knockdown of *Csnk1e*, whose protein product CKε is circadian clock regulator, did not affect *cyclin D* expression in human sarcoma cell line (Yang & Stockwell, 2008). Moreover, the role of microRNA in modulation of translational activity of cyclins D was documented in the rat intestine – only rhythmic expression of cyclin D1 protein but not mRNA was detected (Balakrishnan *et al.*, 2010). These findings suggest that other regulatory pathways might also play a substantial role in the regulation of *cyclin D* expression and that it can be tissue specific.

Surprisingly, *c-Myc* did not exhibit circadian variation in our experiments even if it is considered to be clock-controlled gene as its promoter contains E-box sequence and P1 site, which are known to mediate *c-Myc* repression by binding of CLOCK/BMAL1 (Wierstra & Alves, 2008). Previously there was demonstrated rhythmic expression of *c-Myc* in liver and cell lines (Filipski *et al.*, 2009; Hua *et al.*, 2006), and within whole transcriptome in circadian microarray studies of SCN and liver (Akhtar *et al.*, 2002; Duffield *et al.*, 2002; Panda *et al.*, 2002). However, the amplitude of the rhythm has never been extremely robust and moreover, absence of the rhythm in mouse liver was also reported (Wu *et al.*, 2004). In another study, strong rhythms of cell cycle associated genes including *c-Myc* were revealed in the liver, while no or weak rhythms appeared in kidney, suggesting tissue specific distribution of the rhythmicity (Kita *et al.*, 2002). Similarly, no circadian rhythm of *c-Myc* was detected in whole transcriptome microarray study in mouse colon but the gene *Mycbp* coding c-MYC binding protein exhibited circadian variation (Hoogerwerf *et al.*, 2008), i.e. circadian changes could alter c-MYC activity on functional level. Furthermore, c-MYC regulates cyclin D expression (Pelengaris *et al.*, 2002) and thus the absence of its rhythm in our experiments might contribute to insignificant rhythmicity of cyclin D transcription observed in our study.

p21, the cell cycle inhibitor induced by *p53* pathway and decelerating G1/S transition by repressing activity of CDK complexes, is induced in intestinal epithelium mainly by environmental insults like radiation (Wilson *et al.*, 1998). According to earlier experiments of other groups in mouse liver (Gréchez-Cassiau *et al.*, 2008; Gu *et al.*, 2012) or human intestinal mucosa (Griniatsos *et al.*, 2006) we expected rhythmic expression of *p21*. In contrast, we observed no significant rhythm in colonic epithelium. Direct connection of *p21* expression to molecular clock was proposed, as *Bmal1* knockouts exhibited altered cell proliferation and

diminished *p21* rhythm. Subsequently, functional RORE binding site in *p21* promoter region was identified and orphan receptors REVERBs and RORs were able to bind actively to this region (Gréchez-Cassiau *et al.*, 2008). In addition, *p21* phase shift was detected in mouse embryonic fibroblasts of *Per2* mutants comparing with wild type (Gu *et al.*, 2012). Nonetheless, multiple signals and factors are able to regulate transcription from the *p21* gene promoter (Abbas & Dutta, 2009) and thus putative clock regulation may only participate in complex regulatory network.

We detected rhythmic expression of the CDK inhibitor p27, which is another member of CIP family and functionally similar to p21. Though it partially substitutes function of p21, its role in cell cycle withholding and suppressing of malignancy is generally less extensive than p21. p27 plays an important role in homeostasis of intestinal epithelium. It binds to CDK2 and CDK4, suppresses their activities in proliferating zone of crypts and gates cell cycle progression (Smartt *et al.*, 2007). In the view of this fact, circadian rhythmicity of p27 could play a substantial regulatory role particularly in crypt proliferation zone and functionally participate in circadian orchestration of intestinal cell proliferation.

Although, oscillatory expression of CDK inhibitor p16 in human intestinal mucosa has been observed (Griniatsos *et al.*, 2006), the abundance of detected mRNA was extremely low in our experiments and standard deviations of particular measurements were too high to reach statistically significant level.

From the expression profiles of cell cycle regulators under standard conditions it seems that circadian orchestration has only partial impact on intestinal epithelial proliferation and that this influence might be species and tissue specific.

As the peripheral circadian clocks, including the intestinal one, are robustly affected by feeding regime (Sládek *et al.*, 2007; Stokkan *et al.*, 2001), and circadian variation in proliferation was documented in rodent, and human intestinal epithelium (Buchi *et al.*, 1991; Burholt *et al.*, 1985; Burns *et al.*, 1972; Marra *et al.*, 1994; Scheving, 2000), we studied the effect of reverse feeding regime on above mentioned genes of cell cycle.

In contrast to rhythmically expressed genes detected under standard condition, only the gene *cyclin B1* showed significant effect of both factors (feeding regime and circadian time) using two-way ANOVA. All other genes, in particular *Wee1*, *cyclin A*, and *p27* did not show any main effects or interaction between time and feeding regime, suggesting rather partial uncoupling or misbalancing of cell cycle regulator rhythm under time-restricted feeding. However, it is documented that kinetics of cell proliferation in mouse colon measured as [^{3}H]-thymidine incorporation into DNA exhibit circadian rhythm and is governed by feeding time rather than

light regime (Burholt *et al.*, 1985; Lakatua *et al.*, 1983). Inconsistency of these results with our data could be attributed to the use of different animal experimental model or to the fact that epithelium of colon crypts is comprised of functionally diverse cell types with particularly different cellular functions. These cell types might be unequally affected by feeding regime and potential changes in particular compartment might be difficult to see in the whole epithelium harvested as one sample. We confirmed such heterogeneity using histological laser microsdissection approach as we found substantially higher expression of cell cycle regulators in crypt base than in crypt mouth/surface cells. Furthermore, in spite of homogenous circadian clock distribution along the crypt axis, the rhythmicity of cell cycle regulators was distributed differentially. In addition, previous experiments showed that circadian variation in proliferation of rectal mucosa cells was detected in the lower parts of crypt, while no rhythm was observed in the upper parts (Marra *et al.*, 1994), which supports again the hypothesis of different rhythm distribution. In overall, although suggestions and some evidence for linking circadian clock and cell cycle have been claimed, our study documents, that colonic intestinal epithelium exhibits at least partially uncoupled clock and cell cycle. These results indicate additional strong stimuli and regulators, which can interfere and alter clock-cell cycle link.

Speculatively, an important role in the putative circadian orchestration of epithelial cells proliferation could be played also by Wnt pathway, which is very active in regulation of epithelial proliferation in the intestine. Interestingly, it was found that CKε acts on the β-catenin degradation complex and directly participates in canonical Wnt and noncanonical Wnt/JNK pathways (Schwarz-Romond *et al.*, 2002). In addition, β-catenin was able to regulate expression of *cyclin D1* (Tetsu & McCormick, 1999) and was increased in *Per2*-knockdown intestinal cell lines (Wood *et al.*, 2008). Moreover, increased β-catenin was able to destabilize PER2 by inducing β-TrCP, an F-box protein of SCF ubiquitin E3 ligase, in the intestinal mucosa of the $Apc^{(Min/)(+)}$ mouse and decrease of PER2 levels was associated with altered circadian rhythms of clock-controlled genes *Dbp* and *Wee1* (Yang *et al.*, 2009a). Thus Wnt pathway is other possible way how clock might affect intestinal epithelial proliferation.

7.4 Role of the clock in tumourigenesis

Supportive evidence for linking circadian clock and regulation of cell proliferation emerged from the experiments documenting significant role of clock disruption or its deficiency in carcinogenesis of various cancers in experimental models as well as in human epidemiological

studies (Greene, 2012). Numerous experimental investigations show that alteration or dysfunction of circadian clock at molecular lever may lead to cancer development or tumour growth acceleration (Fu *et al.*, 2002; Gu *et al.*, 2012; Yang *et al.*, 2009b; Zhang *et al.*, 2008). Similarly, increased polyp formation was detected in the small intestine of mice with Apc/Min phenotype combined with *Per2* mutation (Wood *et al.*, 2008) and the increased β-catenin was shown to modulate PER2 protein stability (Yang *et al.*, 2009a). In the present study, we show significantly altered circadian machinery during tumourigenesis in the model of colitis-associated colorectal cancer. This model was previously characterized as high-grade dysplasia or intramucosal carcinoma (Švec *et al.*, 2010) that accurately recapitulates the phases of initiation and progression of human tumour (De Robertis *et al.*, 2011).

To our knowledge, we show for the first time comprehensive examination of local circadian clock and its output (clock-controlled) in induced colorectal tumour and simultaneously in adjacent healthy-looking colonic tissue. Our results demonstrate disruption of colonic clock in tumour associated with strongly dampened expression of most clock genes. The impaired amplitude of clock gene rhythms in tumour tissue was not due to clock desynchronisation among tumour cells, as average expression represented by mesor was rapidly decreased in all studied genes. Moreover, rhythm of clock-controlled gene *Dbp* was depressed but other putative clock-controlled genes were either down-regulated (*p21*) or up-regulated (*Wee1*, *c-Myc*). Most interestingly, we detected clock gene expression alterations also in surrounding healthy-appearing tissue, and in liver of tumour-bearing mice. These tissues exhibited phase delay of clock gene or clock-controlled gene expression, respectively, which implicates that molecular circadian disruption might precede tissue malformation.

Putative involvement of circadian clock in tumourigenesis was reported in animal studies, where higher incidence of spontaneous and γ-radiation induced tumour incidence was observed in animals with clock gene deficiency (Fu *et al.*, 2002; Lee *et al.*, 2010; Wood *et al.*, 2008; Yang *et al.*, 2009b). Altered expression of clock genes was reported in malignant tissue of several human cancer types detected in patients at single time point (Chen *et al.*, 2005; Lin *et al.*, 2008; Yeh *et al.*, 2005; Zeman *et al.*, 2008). In addition, chronodisruption caused by jet-lag or shiftwork negatively affected tumour incidence, tumour growth progression or survival rate in both humans and animal models (Erren *et al.*, 2008; Filipski *et al.*, 2004, 2005; Schernhammer *et al.*, 2003; Yasuniwa *et al.*, 2010). Further experiments from cancer cell lines demonstrated more or less the involvement of clock genes in regulation of cell proliferation and/or apoptosis (Gery *et al.*, 2006; Hua *et al.*, 2006; Yang *et al.*, 2009b). However, to date only a few studies report detailed analysis

of mouse tumour molecular clock. The expression of clock genes was attenuated or completely diminished in implanted tumours (Li *et al.*, 2010; You *et al.*, 2005) and in intestinal polyps of $Apc^{Min/+}$ mice (Yang *et al.*, 2009a).

To our knowledge, the present study shows for the first time the evidence of endogenous rhythmicity in primary tumour compared with its adjacent surrounding tissue in colorectal tumourigenesis. Our results obtained from control animals are in concordance with our determination of rat intestinal clockwork system (Fig. 6.1) and with other observations (Hoogerwerf *et al.*, 2008, 2007; Sládek *et al.*, 2007). Comparison of colonic tissue in control animals and in tissue surrounding the tumour showed the damping of *Per1*, *Per2*, and *RevErbα* expression and complete loss of *Bmal1* rhythmicity in neoplastic tissue. Thus, we confirmed the hypothesis of disrupted clock in neoplastic tissue and extended it to mouse carcinogen-induced colorectal primary tumour. In support of this hypothesis, we also detected down-regulation of rhythmic expression of clock-controlled gene *Dbp*, whose altered expression pattern was found in intestinal polyps of $Apc^{Min/+}$ mice (Yang *et al.*, 2009a).

Interestingly, we observed that clock genes were partially affected also in surrounding tissue of tumour bearing mice. Whereas *Per1* and *Per2* expression did not differ compared to mucosa and whole colon of control mice, the expression profile of *RevErbα* was depressed and *Bmal1* rhythm was abolished. These findings suggest that in carcinogen-induced model of tumourigenesis, clock disruption might precede the malignant transformation. Such hypothesis is supported by observed changes of peripheral circadian clock in liver of tumour bearing mice, where precancerous lesions were found (Nishihara *et al.*, 2008; Nozaki *et al.*, 2003). Alternatively, clock in surrounding tissue could be affected by factors secreted by tumour cells. Treatment with AOM is associated with up-regulation of COX-2, a key enzyme of prostaglandin synthesis, and prostaglandins are able to shift the peripheral circadian rhythms (Tsuchiya *et al.*, 2005). Similarly, tumour cells secrete TGFβ that is able to modulate the circadian clock (Kon *et al.*, 2008).

Our results demonstrate altered regulation of key cell cycle regulators *Wee1*, *c-Myc*, and *p21*, which are putatively under circadian clock control. *Wee1*, a candidate for linking element between clock and cell cycle, was demonstrated to be directly regulated by CLOCK/BMAL1 binding to its E-box promoter sequence (Matsuo *et al.*, 2003). Its overexpression was detected in various cancer types (Vriend *et al.*, 2013). Furthermore, *c-Myc* is a well known proto-oncogene stimulating G0/G1 transition by enhancing expression of numerous cell cycle promoting factors. In opposite, *p21* is one of the main CDK inhibitors, which withhold cell cycle progression and

REVERBα/RORα are able to regulate expression of p21 protein (Gréchez-Cassiau *et al.*, 2008). Thus clock disruption in tumour tissue might be causally involved in the changes of *Wee1*, *c-Myc*, and *p21* expression. Although involvement of other regulatory pathways in these changes cannot be ruled out, the observed gene specific changes suggest that they might be related to clock disruption. Suppressed rhythms of *Per1* and *Per2* expression in tumour might up-regulate *Wee1* and *c-Myc* expression, because PER/CRY heterodimer is not able to suppress sufficiently the transcription of clock genes and other E-box regulated genes including *Wee1* and *c-Myc*. Similarly, disruption of REVERBα-mediated transcription due to suppression of *Rev-Erb*α might lead to impaired regulation of *p21* expression.

The absence of *Wee1* expression rhythm in murine colon samples was in contrary to our previous findings in rat colon as well as observations of other authors (Hoogerwerf *et al.*, 2007; Polidarová *et al.*, 2009; Sládek *et al.*, 2007). We demonstrate that this lack of rhythm was due to aging, as tumours bearing mice, as well as parallel controls, were 52-weeks-old. In contrast, we detected rhythmic expression of *Wee1* in younger 10-week-old animals. Beside *Wee1*, also the rhythm of *p21* was suppressed in aged animals. Importantly, all studied clock genes were permanently rhythmic in aged animals even if the amplitude of these rhythms was reduced. Others authors described increased proliferation rate of colonic epithelium (Holt & Yeh, 1988) and altered expression of cell cycle regulators like *p21*, *Cdk2,* and *cyclin E* (Xiao *et al.*, 1999) in aged animals. Together these findings indicate partial uncoupling and disruption of output genes downstream the basic circadian clock transcriptional loop in aged animals.

The exact mechanism of AOM-induced disruption of molecular circadian clock remains uncovered. However, several pathways might be theoretically involved. The gene mutations or increased activation of β-catenin, K-ras, and TGFβ signalling pathways were reported in AOM-induced colorectal cancer (De Robertis *et al.*, 2011; Takahashi & Wakabayashi, 2004). Furthermore, increased activity of iNOS, and COX-2 was detected in adenocarcinomas (Tanaka *et al.*, 2003, 2005) and prostaglandins as well as TGFβ have been previously demonstrated to shift and reset the phase of clock genes (Kon *et al.*, 2008; Tsuchiya *et al.*, 2005).

β-catenin is a key co-transcriptional activator in *Wnt* signalling, the essential pathway in intestinal proliferation homeostasis (Fevr *et al.*, 2007). Activation of Wnt inhibits GSK3β and leads to releasing of previously bound β-catenin. This protein is translocated to nucleus where it acts as a co-activator of target genes. Mutations of β-catenin prevents its binding to GSK3β and so β-catenin is not phosphorylated and degraded in proteasome and thus it activates

exaggeratedly the target genes, which results in extended proliferation. Knockdown of PER2 protein in colon cancer cell lines or *Per2* mutation in transgenic mice leads to increased β-catenin and enhanced cell proliferation (Wood *et al.*, 2008). Mutually, β-catenin is able to decrease PER2 protein stability (Yang *et al.*, 2009a), thus mutation can affect clock function. Alternatively, the mutation can also modulate phosphorylation of BMAL1 by GSK3β resulting in altered stability and circadian output of the clock gene (Sahar *et al.*, 2010). Furthermore, GSK3β exhibits rhythmic catalytic activity (Harada *et al.*, 2005) and is required for the entry of PER2 into nucleus, allowing the repression of CLOCK/BMAL1-mediated gene transcription (Iitaka *et al.*, 2005). In other experiments, NHERF1, rhythmically expressed adaptor protein interacting with β-catenin, was shown to undergo consecutive alterations during colorectal tumourigenesis (Hayashi *et al.*, 2010). These findings indicate another possible link between clock disruption and neoplastic state of colon epithelium. Furthermore, the elevated β-catenin may be involved in iNOS up-regulation resulting in increased synthesis of NO, which seems to play a role in clock function regulation (Menger *et al.*, 2007). Simultaneously, iNOS mRNA regulation is postulated to be clock regulatory target mediated by circadian deadenylase nocturnin (Niu *et al.*, 2011). In addition, histone deacetylase SIRT1, which is expressed in a circadian manner (Asher *et al.*, 2008), suppresses intestinal tumourigenesis and colon cancer growth in the β-catenin-driven mouse model of colon cancer as it deacetylates β-catenin and suppresses its ability to activate transcription of target genes and drive cell proliferation (Firestein *et al.*, 2008).

K-ras mutation leads to constitutive activation of its downstream targets in Raf/MEK/MAPK and PI3K/Akt/PKB signalling pathways. MAPK kinases cascade is known to be involved in inducing circadian expression in culture cells (de Paula *et al.*, 2008) and in phosphorylating of CRY1 and CRY2 (Sanada *et al.*, 2004). Moreover, some of these kinases, particularly *Mapk10* and *Map3k2*, are markedly reduced in scrapped colonic mucosa after AOM/DSS treated mice (Suzuki *et al.*, 2007), while other isoforms are rhythmically expressed as detected by microarray studies (Hughes *et al.*, 2009). Interconnected signalling pathway induced by IGF-1R and mediated by PI3K/PDK1/Akt has been shown to be substantially involved in tumour growth acceleration after light-at-night exposure (Wu *et al.*, 2011).

TGFβ pathway was found to be deregulated in AOM-treated mice (Chen & Huang, 2009; Takahashi & Wakabayashi, 2004) where the active form of TGFβ is decreased and TGFβ pathway is attenuated. TGFβ binds to its receptor, consequently, the protein product of rhythmically expressed *Smad3* gene (Sato *et al.*, 2012), the protein SMAD3, is phosphorylated

and forms dimer with SMAD4. Subsequently the dimer activates transcription of *Dec1*, whose protein product DEC1 is a potent clock regulator. Via this mechanism, TGFβ seems to play a role in clock resetting and in induction of phase-shifting of peripheral clock via *Dec1* induction (Kon *et al.*, 2008). In parallel, DEC2 is able to inhibit TGFβ-induced tumour progression in human pancreatic cancer cell line (Sato *et al.*, 2012). Moreover, TGFβ inhibits expression of the majority of clock genes as was shown in murine fibroblasts (Gast *et al.*, 2012).

Considerable role in clock-related tumourigenesis may play also melatonin, as its inhibitory actions have been demonstrated in various cancer cell lines and animal models (Reiter *et al.*, 2007). Melatonin was shown to inhibit dose-dependently the development of colonic adenocarcinoma in rat AOM/DSS model (Tanaka *et al.*, 2009) and its rhythm was depressed in patients with colorectal cancer (Kos-Kudla *et al.*, 2002). Exogenous administration of melatonin increased survival rate and decreased tumour growth in experimental mouse model (Otálora *et al.*, 2008). Furthermore, its ability to increase *Per2* and *Clock* expression and to decrease *Bmal1* expression was demonstrated in prostate cancer cells (Jung-Hynes *et al.*, 2010). These data suggest possible interaction between peripheral melatonin and clock even if the mechanism is not clear. The possible link could be via sirtuin signalization, as melatonin is able to inhibit SIRT1 expression and activity and thus to decrease cell proliferation (Jung-Hynes *et al.*, 2011).

Another possible candidate for mediating clock function disruption during neoplastic transformation is adiponectin, a hormone of adipose tissue, which enhances insulin sensitivity, suppresses inflammatory processes and affects metabolism (Kadowaki *et al.*, 2006). Adiponectin deficiency enhances colorectal carcinogenesis in murine AOM-induced cancer model, specifically the tumour size and incidence (Nishihara *et al.*, 2008). Compared to its deficiency, adiponectin is able to suppress carcinogenesis in mice under specific dietary condition (Fujisawa *et al.*, 2008) and has anti-proliferative effect on cancer cell lines (Jardé *et al.*, 2011). Diurnal variations of plasma concentration of adiponectin and its receptors (*AdipoR1* and *AdipoR2*) have been observed both in human and mice (Blüher *et al.*, 2005; Gavrila *et al.*, 2003; Gómez-Abellán *et al.*, 2010). Adiponectin has been show to modulate some clock-related pathways. It blocks phosphorylation of Akt and GSK3β thus suppressing intracellular accumulation of β-catenin. Furthermore, it modulates the expression of *c-Myc,* and *cyclin D1* and represses MAPK pathway (Jardé *et al.*, 2011). Adiponectin gene transcription is enhanced by PPARγ, C/EBPα, and SIRT1/FOXO1, all of which have been shown to exhibit circadian regulation and to be involved

in linkage between the clock and metabolism (Phillips & Kung, 2010). Together these findings imply a possible role of adiponectin in clock-related tumourigenesis.

In summary, all mentioned mechanism could play a partial role in the development of circadian clock-related tumourigenesis. However, we also cannot exclude the known mutagenic effect of AOM directly on DNA of clock genes. Significant number of mutations was detected in *Clock* gene in human colorectal cancer (Alhopuro *et al.*, 2010). In rats, AOM administered in morning hours caused twice as many foci aberrant crypts in comparison with administration in the afternoon (Pereira *et al.*, 1994). The demonstration of time-dependent cytotoxicity points to importance of local subjective clock setting in susceptibility of colon epithelium to carcinogenic cues. In addition, methylazoxymethanol (MAM) acetate, the downstream metabolite of AOM, showed circadian rhythmicity in the apoptotic incidence both in the small and large intestine (Ijiri, 1989).

8 CONCLUSION

In summary, we demonstrated functional peripheral clock in the epithelial cells of distinct intestinal segments and its involvement in the regulation of epithelial transport, cell cycle regulation and neoplastic transformation.

The epithelium exhibited homogenous expression pattern in colonic crypt cells independently of the cell position along the crypt-villus(surface) axis despite of distinct cellular function of enterocytes along the crypt axis, which documents universal role of the clock independent of cellular function. Furthermore, we documented circadian regulation of colonic sodium absorption. We showed that genes coding proteins which mediate sodium chloride absorption (*Nhe3*, *Dra*, *Atp1a1*, *Nherf1*, *γENaC*) exhibited rhythmic expression that was affected by feeding regime. Moreover, we demonstrated that rats kept under condition of low-salt diet exhibit diurnal variation in electrogenic sodium absorption mediating by ENaC channel. We suppose that this variation is mediated by plasma aldosterone and clock gene *Per1*.

We also demonstrated that cell cycle regulation of colonic epithelial cells was partially under circadian regulation. Some of the regulatory genes exhibited rhythmic expression (*Wee1*, *p27*, *cyclin A*, *cyclin B1*), while others did not vary significantly in time (*c-Myc*, *cyclin D1*, *p21*). The feeding regime influenced only *cyclin B1* expression. These data indicate a partial role of the clock in colonic epithelium proliferation, while other regulatory factors seem to be involved.

We showed substantial disruption of cellular clockwork during neoplastic transformation of colonic epithelium. This disruption was most prominent in tumour tissue, where main clock genes were severely dampened. However, the healthy-looking adjacent colonic tissue also exhibited marked changes in expression of clock genes comparing to control animals. These findings suggest that clock disruption might contribute to the tissue malformation and this conclusion is supported by findings of altered liver clock in colorectal tumour-bearing animals. Furthermore, we observed changes in expression of cell-cycle regulators in tumour tissue. However, their circadian rhythms were lost not only in tumour tissue, but also in the colonic epithelium of control animals. This loss of rhythm was associated with ageing, which suggests partial uncoupling of clock and cellular functions during ageing.

In overall, we have shown substantial participation of peripheral clock in regulation of key intestinal functions. The intestinal circadian clock governs colonic absorption of sodium chloride. Furthermore, it participates in cell cycle regulation of colonic enterocyte proliferation and its disruption contributes to tissue malformation and colorectal tumourigenesis.

9 REFERENCES

Abbas T, Dutta A: **p21 in cancer: intricate networks and multiple activities.** *Nature Reviews. Cancer* 2009, 9:400–14.

Abe M, Herzog ED, Yamazaki S, Straume M, Tei H, Sakaki Y, Menaker M, Block GD: **Circadian rhythms in isolated brain regions.** *Journal of Neuroscience* 2002, 22:350–6.

Akhtar RA, Reddy AB, Maywood ES, Clayton JD, King VM, Smith AG, Gant TW, Hastings MH, Kyriacou CP: **Circadian cycling of the mouse liver transcriptome, as revealed by cDNA microarray, is driven by the suprachiasmatic nucleus.** *Current Biology* 2002, 12:540–50.

Alhopuro P, Björklund M, Sammalkorpi H, Turunen M, Tuupanen S, Biström M, Niittymäki I, Lehtonen HJ, Kivioja T, Launonen V, Saharinen J, Nousiainen K, Hautaniemi S, Nuorva K, Mecklin J-P, Järvinen H, Orntoft T, Arango D, Lehtonen R, Karhu A, Taipale J, Aaltonen LA: **Mutations in the circadian gene CLOCK in colorectal cancer.** *Molecular Cancer Research* 2010, 8:952–60.

Al-Nafussi AI, Wright NA: **Circadian rhythm in the rate of cellular proliferation and in the size of the functional compartment of mouse jejunal epithelium.** *Virchows Archiv B. Cell Pathology Including Molecular Pathology* 1982, 40:71–9.

Andersen CL, Jensen JL, Ørntoft TF: **Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets.** *Cancer Research* 2004, 64:5245–50.

Ando H, Yanagihara H, Sugimoto K, Hayashi Y, Tsuruoka S, Takamura T, Kaneko S, Fujimura A: **Daily rhythms of P-glycoprotein expression in mice.** *Chronobiology International* 2005, 22:655–65.

Antoch MP, Gorbacheva VY, Vykhovanets O, Toshkov I a, Kondratov R V, Kondratova A a, Lee C, Nikitin AY: **Disruption of the circadian clock due to the Clock mutation has discrete effects on aging and carcinogenesis.** *Cell Cycle* 2008, 7:1197–204.

Arellano M, Moreno S: **Regulation of CDK/cyclin complexes during the cell cycle.** *International Journal of Biochemistry & Cell Biology* 1997, 29:559–73.

Asher C, Wald H, Rossier BC, Garty H: **Aldosterone-induced increase in the abundance of Na+ channel subunits**. *American Journal of Physiology. Cell Physiology* 1996, 271:C605–11.

Asher G, Gatfield D, Stratmann M, Reinke H, Dibner C, Kreppel F, Mostoslavsky R, Alt FW, Schibler U: **SIRT1 regulates circadian clock gene expression through PER2 deacetylation.** *Cell* 2008, 134:317–28.

Balakrishnan A, Stearns AT, Park PJ, Dreyfuss JM, Ashley SW, Rhoads DB, Tavakkolizadeh A: **MicroRNA mir-16 is anti-proliferative in enterocytes and exhibits diurnal rhythmicity in intestinal crypts.** *Experimental Cell Research* 2010, 316:3512–21.

Balsalobre A jou., Damiola F, Schibler U: **A serum shock induces circadian gene expression in mammalian tissue culture cells.** *Cell* 1998, 93:929–37.

Balsalobre A: **Resetting of Circadian Time in Peripheral Tissues by Glucocorticoid Signaling**. *Science* 2000, 289:2344–47.

Bankir L, Bochud M, Maillard M, Bovet P, Gabriel A, Burnier M: **Nighttime blood pressure and nocturnal dipping are associated with daytime urinary sodium excretion in African subjects.** *Hypertension* 2008, 51:891–8.

Baril EF, Potter VR: **Systematic Oscillations of Amino Acid Transport in Liver from Rats Adapted to Controlled Feeding Schedules**. *Journal of Nutrition* 1968, 95:228–37.

Barrett KE, Keely SJ: **Chloride secretion by the intestinal epithelium: molecular basis and regulatory aspects.** *Annual Review of Physiology* 2000, 62:535–72.

Benos DJ: **Amiloride: a molecular probe of sodium transport in tissues and cells.** *American Journal of Physiology* 1982, 242:C131–45.

Berson DM, Dunn FA, Takao M: **Phototransduction by retinal ganglion cells that set the circadian clock.** *Science* 2002, 295:1070–3.

Bissahoyo A, Pearsall RS, Hanlon K, Amann V, Hicks D, Godfrey VL, Threadgill DW: **Azoxymethane is a genetic background-dependent colorectal tumor initiator and promoter in mice: effects of dose, route, and diet.** *Toxicological Sciences* 2005, 88:340–5.

Bjarnason GA, Jordan R: **Rhythms in human gastrointestinal mucosa and skin.** *Chronobiology International* 2002, 19:129–40.

Bjarnason GA, Jordan RC, Sothern RB: **Circadian variation in the expression of cell-cycle proteins in human oral epithelium.** *American Journal of Pathology* 1999, 154:613–22.

Bligh ME, Bhagwat S a, Castonguay TW: **Aldosterone diurnal rhythm in the rat: a question of cross-reactivity?** *Physiology & Behavior* 1993, 53:845–8.

Blüher M, Fasshauer M, Kralisch S, Schön MR, Krohn K, Paschke R: **Regulation of adiponectin receptor R1 and R2 gene expression in adipocytes of C57BL/6 mice.** *Biochemical and Biophysical Research Communications* 2005, 329:1127–32.

Borgs L, Beukelaers P, Vandenbosch R, Belachew S, Nguyen L, Malgrange B: **Cell "circadian" cycle: new role for mammalian core clock genes.** *Cell Cycle* 2009, 8:832–7.

Boulos Z, Terman M: **Food availability and daily biological rhythms.** *Neuroscience and Biobehavioral Reviews* 1980, 4:119–31.

Bozek K, Relógio A, Kielbasa SM, Heine M, Dame C, Kramer A, Herzel H: **Regulation of clock-controlled genes in mammals.** *PloS One* 2009, 4:e4882.

Broere N, Chen M, Cinar A, Singh AK, Hillesheim J, Riederer B, Lünnemann M, Rottinghaus I, Krabbenhöft A, Engelhardt R, Rausch B, Weinman EJ, Donowitz M, Hubbard A, Kocher O, de Jonge HR, Hogema BM, Seidler U: **Defective jejunal and colonic salt absorption and alteredNa(+)/H (+) exchanger 3 (NHE3) activity in NHE regulatory factor 1 (NHERF1) adaptor protein-deficient mice.** *Pflügers Archiv. European Journal of Physiology* 2009, 457:1079–91.

Buchi KN, Moore JG, Hrushesky WJ, Sothern RB, Rubin NH: **Circadian rhythm of cellular proliferation in the human rectal mucosa.** *Gastroenterology* 1991, 101:410–5.

Burholt DR, Etzel SL, Schenken LL, Kovacs CJ: **Digestive tract cell proliferation and food consumption patterns of Ha/ICR mice.** *Cell and Tissue Kinetics* 1985, 18:369–86.

Burnier M, Coltamai L, Maillard M, Bochud M: **Renal sodium handling and nighttime blood pressure.** *Seminars in Nephrology* 2007, 27:565–71.

Burns ER, Scheving LE, Tsai TH: **Circadian rhythm in uptake of tritiated thymidine by kidney, parotid, and duodenum of isoproterenol-treated mice.** *Science* 1972, 175:71–3.

Busino L, Bassermann F, Maiolica A, Lee C, Nolan PM, Godinho SIH, Draetta GF, Pagano M: **SCFFbxl3 controls the oscillation of the circadian clock by directing the degradation of cryptochrome proteins.** *Science* 2007, 316:900–4.

Butterworth MB, Edinger RS, Frizzell R a, Johnson JP: **Regulation of the epithelial sodium channel by membrane trafficking.** *American Journal of Physiology. Renal Physiology* 2009, 296:F10–24.

Cailotto C, Lei J, van der Vliet J, van Heijningen C, van Eden CG, Kalsbeek A, Pévet P, Buijs RM: **Effects of nocturnal light on (clock) gene expression in peripheral organs: a role for the autonomic innervation of the liver.** *PloS One* 2009, 4:e5650.

Cambar J, Lemoigne F, Toussaint C: **Diurnal variations evidence of glomerular filtration in the rat**. *Experientia* 1979, 35:1607–9.

Castelló A, Gumá A, Sevilla L, Furriols M, Testar X, Palacín M, Zorzano A: **Regulation of GLUT5 gene expression in rat intestinal mucosa: regional distribution, circadian rhythm, perinatal development and effect of diabetes.** *Biochemical Journal* 1995, 309:271–7.

Clauss W, Dürr JE, Krattenmacher R, Hörnicke H, Van Driessche W: **Circadian rhythm of apical Na-channels and Na-transport in rabbit distal colon.** *Experientia* 1988, 44:608–10.

Corpe CP, Burant CF: **Hexose transporter expression in rat small intestine: effect of diet on diurnal variations.** *American Journal of Physiology* 1996, 271:G211–6.

Crosnier C, Stamataki D, Lewis J: **Organizing cell renewal in the intestine: stem cells, signals and combinatorial control.** *Nature Reviews. Genetics* 2006, 7:349–59.

Cugini P, Centanni M, Murano G, Letizia C, Lucia P, Scavo D, Halberg F, Sothern R, Cornelissen G: **Toward a chronophysiology of circulating aldosterone.** *Biochemical Medicine* 1984, 32:270–82.

Cui W, Taub DD, Gardner K: **qPrimerDepot: a primer database for quantitative real time PCR.** *Nucleic Acids Research* 2007, 35(Database issue):D805–9.

Damiola F: **Restricted feeding uncouples circadian oscillators in peripheral tissues from the central pacemaker in the suprachiasmatic nucleus**. *Genes & Development* 2000, 14:2950–61.

Davidson AJ, Cappendijk SL, Stephan FK: **Feeding-entrained circadian rhythms are attenuated by lesions of the parabrachial region in rats.** *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology* 2000, 278:R1296–304.

Dibner C, Schibler U, Albrecht U: **The Mammalian circadian timing system: organization and coordination of central and peripheral clocks.** *Annual Review of Physiology* 2010, 72:517–49.

Doi M, Takahashi Y, Komatsu R, Yamazaki F, Yamada H, Haraguchi S, Emoto N, Okuno Y, Tsujimoto G, Kanematsu A, Ogawa O, Todo T, Tsutsui K, van der Horst GTJ, Okamura H: **Salt-sensitive hypertension in circadian clock-deficient Cry-null mice involves dysregulated adrenal Hsd3b6.** *Nature Medicine* 2010, 16:67–74.

Dossetor JB, Gorman HM, Beck JC: **Diurnal Rhythm of Urinary Electrolyte Excretion. I. Observations in Normal Subjects**. *Metabolism* 1963, 12:1083–99.

Dudley CA, Erbel-Sieler C, Estill SJ, Reick M, Franken P, Pitts S, McKnight SL: **Altered patterns of sleep and behavioral adaptability in NPAS2-deficient mice.** *Science* 2003, 301:379–83.

Duffield GE, Best JD, Meurers BH, Bittner A, Loros JJ, Dunlap JC: **Circadian Programs of Transcriptional Activation, Signaling, and Protein Turnover Revealed by Microarray Analysis of Mammalian Cells**. *Current Biology* 2002, 12:551–57.

Dunlap JC: **Molecular bases for circadian clocks.** *Cell* 1999, 96:271–90.

Edwards PA, Muroya H, Gould RG: **In vivo demonstration of the circadian thythm of cholesterol biosynthesis in the liver and intestine of the rat.** *Journal of Lipid Research* 1972, 13:396–401.

Ekholm S V, Reed SI: **Regulation of G(1) cyclin-dependent kinases in the mammalian cell cycle.** *Current Opinion in Cell Biology* 2000, 12:676–84.

Epple HJ, Amasheh S, Mankertz J, Goltz M, Schulzke JD, Fromm M: **Early aldosterone effect in distal colon by transcriptional regulation of ENaC subunits**. *American Journal of Physiology. Gastrointestinal and Liver Physiology* 2000, 278:G718–24.

Erren TC, Pape HG, Reiter RJ, Piekarski C: **Chronodisruption and cancer.** *Naturwissenschaften* 2008, 95:367–82.

Fakitsas P, Adam G, Daidié D, van Bemmelen MX, Fouladkou F, Patrignani A, Wagner U, Warth R, Camargo SMR, Staub O, Verrey F: **Early aldosterone-induced gene product regulates the epithelial sodium channel by deubiquitylation.** *Journal of the American Society of Nephrology* 2007, 18:1084–92.

Feillet CA, Mendoza J, Albrecht U, Pévet P, Challet E: **Forebrain oscillators ticking with different clock hands.** *Molecular and Cellular Neurosciences* 2008, 37:209–21.

Feillet CA, Ripperger JA, Magnone MC, Dulloo A, Albrecht U, Challet E: **Lack of food anticipation in Per2 mutant mice.** *Current Biology* 2006, 16:2016–22.

Fevr T, Robine S, Louvard D, Huelsken J: **Wnt/beta-catenin is essential for intestinal homeostasis and maintenance of intestinal stem cells.** *Molecular and Cellular Biology* 2007, 27:7551–9.

Filipski E, Delaunay F, King VM, Wu M-W, Claustrat B, Gréchez-Cassiau A, Guettier C, Hastings MH, Francis L: **Effects of chronic jet lag on tumor progression in mice.** *Cancer Research* 2004, 64:7879–85.

Filipski E, Innominato PF, Wu M, Li X-M, Iacobelli S, Xian L-J, Lévi F: **Effects of light and food schedules on liver and tumor molecular clocks in mice.** *Journal of the National Cancer Institute* 2005, 97:507–17.

Filipski E, Subramanian P, Carrière J, Guettier C, Barbason H, Lévi F: **Circadian disruption accelerates liver carcinogenesis in mice.** *Mutation Research* 2009, 680:95–105.

Firestein R, Blander G, Michan S, Oberdoerffer P, Ogino S, Campbell J, Bhimavarapu A, Luikenhuis S, de Cabo R, Fuchs C, Hahn WC, Guarente LP, Sinclair DA: **The SIRT1 deacetylase suppresses intestinal tumorigenesis and colon cancer growth.** *PloS One* 2008, 3:e2020.

Fisher RB, Gardner ML: **A diurnal rhythm in the absorption of glucose and water by isolated rat small intestine.** *Journal of Physiology* 1976, 254:821–5.

Van der Flier LG, Clevers H: **Stem cells, self-renewal, and differentiation in the intestinal epithelium.** *Annual Review of Physiology* 2009, 71:241–60.

Froy O, Chapnik N: **Circadian oscillation of innate immunity components in mouse small intestine.** *Molecular Immunology* 2007, 44:1954–60.

Fu L, Pelicano H, Liu J, Huang P, Lee C: **The circadian gene Period2 plays an important role in tumor suppression and DNA damage response in vivo.** *Cell* 2002, 111:41–50.

Fujimoto M, Kanaya A, Nakabou Y, Hagihira H: **Circadian rhythm in the ornithine decarboxylase activity of rat small intestine.** *Journal of Biochemistry* 1978, 83:237–42.

Fujisawa T, Endo H, Tomimoto A, Sugiyama M, Takahashi H, Saito S, Inamori M, Nakajima N, Watanabe M, Kubota N, Yamauchi T, Kadowaki T, Wada K, Nakagama H, Nakajima A: **Adiponectin suppresses colorectal carcinogenesis under the high-fat diet condition.** *Gut* 2008, 57:1531–8.

Furuya S, Yugari Y: **Daily rhythmic change in the transport of histidine by everted sacs of rat small intestine.** *Biochimica et Biophysica Acta* 1971, 241:245–8.

Gast H, Gordic S, Petrzilka S, Lopez M, Müller A, Gietl A, Hock C, Birchler T, Fontana A: **Transforming growth factor-beta inhibits the expression of clock genes.** *Annals of the New York Academy of Sciences* 2012, 1261:79–87.

Gauger M a, Sancar A: **Cryptochrome, circadian cycle, cell cycle checkpoints, and cancer.** *Cancer Research* 2005, 65:6828–34.

Gavrila A, Peng C-K, Chan JL, Mietus JE, Goldberger AL, Mantzoros CS: **Diurnal and ultradian dynamics of serum adiponectin in healthy men: comparison with leptin, circulating soluble leptin receptor, and cortisol patterns.** *Journal of Clinical Endocrinology and Metabolism* 2003, 88:2838–43.

Geibel JP: **Secretion and absorption by colonic crypts.** *Annual Review of Physiology* 2005, 67:471–90.

Gery S, Komatsu N, Baldjyan L, Yu A, Koo D, Koeffler HP: **The circadian gene per1 plays an important role in cell growth and DNA damage control in human cancer cells.** *Molecular Cell* 2006, 22:375–82.

Godinho SIH, Maywood ES, Shaw L, Tucci V, Barnard AR, Busino L, Pagano M, Kendall R, Quwailid MM, Romero MR, O'neill J, Chesham JE, Brooker D, Lalanne Z, Hastings MH, Nolan PM: **The after-hours mutant reveals a role for Fbxl3 in determining mammalian circadian period.** *Science* 2007, 316:897–900.

Golombek DA, Rosenstein RE: **Physiology of circadian entrainment.** *Physiological Reviews* 2010, 90:1063–102.

Gómez-Abellán P, Gómez-Santos C, Madrid JA, Milagro FI, Campion J, Martínez JA, Ordovás JM, Garaulet M: **Circadian expression of adiponectin and its receptors in human adipose tissue.** *Endocrinology* 2010, 151:115–22.

Greene MW: **Circadian Rhythms and Tumor Growth.** *Cancer Letters* 2012, 318:115–23.

Gréchez-Cassiau A, Rayet B, Guillaumond F, Teboul M, Delaunay F: **The circadian clock component BMAL1 is a critical regulator of p21WAF1/CIP1 expression and hepatocyte proliferation.** *Journal of Biological Chemistry* 2008, 283:4535–42.

Griniatsos J, Michail OP, Theocharis S, Arvelakis A, Papaconstantinou I, Felekouras E, Pikoulis E, Karavokyros I, Bakoyiannis C, Marinos G, Bramis J, Michail PO: **Circadian variation in expression of G1 phase cyclins D1 and E and cyclin-dependent kinase inhibitors p16 and p21 in human bowel mucosa.** *World Journal of Gastroenterology* 2006, 12:2109–14.

Groos G, Hendriks J: **Circadian rhythms in electrical discharge of rat suprachiasmatic neurones recorded in vitro.** *Neuroscience Letters* 1982, 34:283–8.

Gu X, Xing L, Shi G, Liu Z, Wang X, Qu Z, Wu X, Dong Z, Gao X, Liu G, Yang L, Xu Y: **The circadian mutation PER2(S662G) is linked to cell cycle progression and tumorigenesis.** *Cell Death and Differentiation* 2012, 19:397–405.

Gumz ML, Stow LR, Lynch IJ, Greenlee MM, Rudin A, Cain BD, Weaver DR, Wingo CS: **The circadian clock protein Period 1 regulates expression of the renal epithelial sodium channel in mice.** *Journal of Clinical Investigation* 2009, 119:2423–34.

Hankins MW, Peirson SN, Foster RG: **Melanopsin: an exciting photopigment.** *Trends in Neurosciences* 2008, 31:27–36.

Harada Y, Sakai M, Kurabayashi N, Hirota T, Fukada Y: **Ser-557-phosphorylated mCRY2 is degraded upon synergistic phosphorylation by glycogen synthase kinase-3 beta.** *Journal of Biological Chemistry* 2005, 280:31714–21.

Hashiramoto A, Yamane T, Tsumiyama K, Yoshida K, Komai K, Yamada H, Yamazaki F, Doi M, Okamura H, Shiozawa S: **Mammalian clock gene Cryptochrome regulates arthritis via proinflammatory cytokine TNF-alpha.** *Journal of Immunology* 2010, 184:1560–5.

Hayashi Y, Molina JR, Hamilton SR, Georgescu M-M: **NHERF1/EBP50 is a new marker in colorectal cancer.** *Neoplasia* 2010, 12:1013–22.

Hayashi Y, Ushijima K, Ando H, Yanagihara H, Ishikawa E, Tsuruoka S-I, Sugimoto K-I, Fujimura A: **Influence of time-restricted feeding schedule on daily rhythm of abcb1a gene expression and its function in rat intestine.** *Journal of Pharmacology and Experimental Therapeutics* 2010, 335: 418-23.

He TC, Sparks AB, Rago C, Hermeking H, Zawel L, da Costa LT, Morin PJ, Vogelstein B, Kinzler KW: **Identification of c-MYC as a target of the APC pathway.** *Science* 1998, 281:1509–12.

He Y, Jones CR, Fujiki N, Xu Y, Guo B, Holder JL, Rossner MJ, Nishino S, Fu Y-H: **The transcriptional repressor DEC2 regulates sleep length in mammals.** *Science* 2009, 325:866–70.

Hilfenhaus M: **Circadian rhythm of the renin-angiotensin-aldosterone system in the rat.** *Archives of Toxicology* 1976, 36:305–16.

Holt PR, Yeh KY: **Colonic proliferation is increased in senescent rats.** *Gastroenterology* 1988, 95:1556–63.

Honma S, Kawamoto T, Takagi Y, Fujimoto K, Sato F, Noshiro M, Kato Y, Honma K: **Dec1 and Dec2 are regulators of the mammalian molecular clock.** *Nature* 2002, 419:841–4.

Hoogerwerf W a, Sinha M, Conesa A, Luxon B a, Shahinian VB, Cornélissen G, Halberg F, Bostwick J, Timm J, Cassone VM: **Transcriptional profiling of mRNA expression in the mouse distal colon.** *Gastroenterology* 2008, 135:2019–29.

Hoogerwerf WA, Hellmich HL, Cornélissen G, Halberg F, Shahinian VB, Bostwick J, Savidge TC, Cassone VM: **Clock gene expression in the murine gastrointestinal tract: endogenous rhythmicity and effects of a feeding regimen.** *Gastroenterology* 2007, 133:1250–60.

Houghton SG, Iqbal CW, Duenes J a, Fatima J, Kasparek MS, Sarr MG: **Coordinated, diurnal hexose transporter expression in rat small bowel: implications for small bowel resection.** *Surgery* 2008, 143:79–93.

Hua H, Wang Y, Wan C, Liu Y, Zhu B, Wang X, Wang Z, Ding JM: **Inhibition of tumorigenesis by intratumoral delivery of the circadian gene mPer2 in C57BL/6 mice.** *Cancer Gene Therapy* 2007, 14:815–8.

Hua H, Wang Y, Wan C, Liu Y, Zhu B, Yang C, Wang X, Wang Z, Cornelissen-Guillaume G, Halberg F: **Circadian gene mPer2 overexpression induces cancer cell apoptosis.** *Cancer Science* 2006, 97:589–96.

Hughes ME, DiTacchio L, Hayes KR, Vollmers C, Pulivarthy S, Baggs JE, Panda S, Hogenesch JB: **Harmonics of circadian gene transcription in mammals.** *PLoS Genetics* 2009, 5:e1000442.

Hurwitz S, Cohen RJ, Williams GH: **Diurnal variation of aldosterone and plasma renin activity: timing relation to melatonin and cortisol and consistency after prolonged bed rest.** *Journal of Applied Physiology* 2004, 96:1406–14.

Chan LMS, Lowes S, Hirst BH: **The ABCs of drug transport in intestine and liver: efflux proteins limiting drug absorption and bioavailability.** *European Journal of Pharmaceutical Sciences* 2004, 21:25–51.

Chen J, Huang X-F: **The signal pathways in azoxymethane-induced colon cancer and preventive implications.** *Cancer Biology & Therapy* 2009, 8:1313–7.

Chen S-T, Choo K-B, Hou M-F, Yeh K-T, Kuo S-J, Chang J-G: **Deregulated expression of the PER1, PER2 and PER3 genes in breast cancers.** *Carcinogenesis* 2005, 26:1241–6.

Iijima M, Yamaguchi S, van der Horst GTJ, Bonnefont X, Okamura H, Shibata S: **Altered food-anticipatory activity rhythm in Cryptochrome-deficient mice.** *Neuroscience Research* 2005, 52:166–73.

Iitaka C, Miyazaki K, Akaike T, Ishida N: **A role for glycogen synthase kinase-3beta in the mammalian circadian clock.** *Journal of Biological Chemistry* 2005, 280:29397–402.

Ijiri K: **Apoptosis (cell death) induced in mouse bowel by 1,2-dimethylhydrazine, methylazoxymethanol acetate, and gamma-rays.** *Cancer Research* 1989, 49:6342–6.

Iwashina I, Mochizuki K, Inamochi Y, Goda T: **Clock genes regulate the feeding schedule-dependent diurnal rhythm changes in hexose transporter gene expressions through the binding of BMAL1 to the promoter/enhancer and transcribed regions.** *Journal of Nutritional Biochemistry* 2011, 22:334–43.

Jardé T, Perrier S, Vasson M-P, Caldefie-Chézet F: **Molecular mechanisms of leptin and adiponectin in breast cancer.** *European Journal of Cancer* 2011, 47:33–43.

Jilge B: **Monophasic and diphasic patterns of the circadian caecotrophy rhythm of rabbits**. *Laboratory Animals* 1982, 16:1–6.

Jin X, Shearman LP, Weaver DR, Zylka MJ, de Vries GJ, Reppert SM: **A molecular mechanism regulating rhythmic output from the suprachiasmatic circadian clock.** *Cell* 1999, 96:57–68.

Johnson C: **Circadian clocks and cell division: What's the pacemaker?** *Cell Cycle* 2010, 9:3864–3873.

Jung-Hynes B, Huang W, Reiter RJ, Ahmad N: **Melatonin resynchronizes dysregulated circadian rhythm circuitry in human prostate cancer cells.** *Journal of Pineal Research* 2010, 49:60–8.

Jung-Hynes B, Schmit TL, Reagan-Shaw SR, Siddiqui I a, Mukhtar H, Ahmad N: **Melatonin, a novel Sirt1 inhibitor, imparts antiproliferative effects against prostate cancer in vitro in culture and in vivo in TRAMP model.** *Journal of Pineal Research* 2011, 50:140–9.

Kadowaki T, Yamauchi T, Kubota N, Hara K, Ueki K, Tobe K: **Adiponectin and adiponectin receptors in insulin resistance, diabetes, and the metabolic syndrome.** *Journal of Clinical Investigation* 2006, 116:1784–92.

Karlsson-Rosenthal C, Millar JB a: **Cdc25: mechanisms of checkpoint inhibition and recovery.** *Trends in Cell Biology* 2006, 16:285–92.

Khapre R V, Samsa WE, Kondratov R V: **Circadian regulation of cell cycle: Molecular connections between aging and the circadian clock.** *Annals of Medicine* 2010, 42:404–15.

Kita Y, Shiozawa M, Jin W, Majewski RR, Besharse JC, Greene AS, Jacob HJ: **Implications of circadian gene expression in kidney, liver and the effects of fasting on pharmacogenomic studies.** *Pharmacogenetics* 2002, 12:55–65.

Ko CH, Takahashi JS: **Molecular components of the mammalian circadian clock.** *Human Molecular Genetics* 2006, 15 Spec No 2:R271–7.

Kon N, Hirota T, Kawamoto T, Kato Y, Tsubota T, Fukada Y: **Activation of TGF-beta/activin signalling resets the circadian clock through rapid induction of Dec1 transcripts.** *Nature Cell Biology* 2008, 10:1463–9.

Kondratov R V, Kondratova A a, Gorbacheva VY, Vykhovanets O V, Antoch MP: **Early aging and age-related pathologies in mice deficient in BMAL1, the core componentof the circadian clock.** *Genes & Development* 2006, 20:1868–73.

Kos-Kudla B, Ostrowska Z, Kozlowski A, Marek B, Ciesielska-Kopacz N, Kudla M, Kajdaniuk D, Strzelczyk J, Staszewicz P: **Circadian rhythm of melatonin in patients with colorectal carcinoma.** *Neuroendocrinology Letters* 2002, 23:239–42.

Kunzelmann K, Mall M: **Electrolyte transport in the mammalian colon: mechanisms and implications for disease.** *Physiological Reviews* 2002, 82:245–89.

Lakatua DJ, White M, Sackett-Lundeen LL, Haus E: **Change in phase relations of circadian rhythms in cell proliferation induced by time-limited feeding in BALB/c X DBA/2F1 mice bearing a transplantable Harding-Passey tumor.** *Cancer Research* 1983, 43:4068–72.

Landry GJ, Simon MM, Webb IC, Mistlberger RE: **Persistence of a behavioral food-anticipatory circadian rhythm following dorsomedial hypothalamic ablation in rats.** *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology* 2006, 290:R1527–34.

Lee I-H, Campbell CR, Cook DI, Dinudom A: **Regulation of epithelial Na+ channels by aldosterone: role of Sgk1.** *Clinical and Experimental Pharmacology & Physiology* 2008, 35:235–41.

Lee JH, Sancar A: **Circadian clock disruption improves the efficacy of chemotherapy through p73-mediated apoptosis.** *Proceedings of the National Academy of Sciences of the United States of America* 2011, 108:10668–72.

Lee S, Donehower LA, Herron AJ, Moore DD, Fu L: **Disrupting circadian homeostasis of sympathetic signaling promotes tumor development in mice.** *PloS One* 2010, 5:e10995.

LeSauter J, Hoque N, Weintraub M, Pfaff DW, Silver R: **Stomach ghrelin-secreting cells as food-entrainable circadian clocks.** *Proceedings of the National Academy of Sciences of the United States of America* 2009, 106:13582–7.

Lew GM: **Circadian rhythms in blood pressure and norepinephrine in genetically hypertensive and normotensive rats.** *General Pharmacology* 1976, 7:35–40.

Li X-M, Delaunay F, Dulong S, Claustrat B, Zampera S, Fujii Y, Teboul M, Beau J, Lévi F: **Cancer inhibition through circadian reprogramming of tumor transcriptome with meal timing.** *Cancer Research* 2010, 70:3351–60.

Lin KK, Kumar V, Geyfman M, Chudova D, Ihler AT, Smyth P, Paus R, Takahashi JS, Andersen B: **Circadian clock genes contribute to the regulation of hair follicle cycling.** *PLoS Genetics* 2009, 5:e1000573.

Lin Y-M, Chang JH, Yeh K-T, Yang M-Y, Liu T-C, Lin S-F, Su W-W, Chang J-G: **Disturbance of circadian gene expression in hepatocellular carcinoma.** *Molecular Carcinogenesis* 2008, 47:925–33.

Logan RW, Arjona A, Sarkar DK: **Role of sympathetic nervous system in the entrainment of circadian natural-killer cell function.** *Brain, Behavior, and Immunity* 2011, 25:101–9.

Lowrey PL, Takahashi JS: **Mammalian circadian biology: elucidating genome-wide levels of temporal organization.** *Annual Review of Genomics and Human Genetics* 2004, 5:407–41.

Marra G, Anti M, Percesepe A, Armelao F, Ficarelli R, Coco C, Rinelli A, Vecchio FM, D'Arcangelo E: **Circadian variations of epithelial cell proliferation in human rectal crypts.** *Gastroenterology* 1994, 106:982–7.

Matsuo T, Yamaguchi S, Mitsui S, Emi A, Shimoda F, Okamura H: **Control mechanism of the circadian clock for timing of cell division in vivo.** *Science* 2003, 302:255–9.

McGowan CH, Russell P: **Cell cycle regulation of human WEE1.** *EMBO Journal* 1995, 14:2166–75.

Menger GJ, Allen GC, Neuendorff N, Nahm S-S, Thomas TL, Cassone VM, Earnest DJ: **Circadian profiling of the transcriptome in NIH/3T3 fibroblasts: comparison with rhythmic gene expression in SCN2.2 cells and the rat SCN.** *Physiological Genomics* 2007, 29:280–9.

Mieda M, Williams SC, Richardson JA, Tanaka K, Yanagisawa M: **The dorsomedial hypothalamic nucleus as a putative food-entrainable circadian pacemaker.** *Proceedings of the National Academy of Sciences of the United States of America* 2006, 103:12150–5.

Millar-Craig M, Bishop C, Raftery E.: **Circadian variation of blood-pressure**. *Lancet* 1978, 311:795–7.

Miller BH, McDearmon EL, Panda S, Hayes KR, Zhang J, Andrews JL, Antoch MP, Walker JR, Esser KA, Hogenesch JB, Takahashi JS: **Circadian and CLOCK-controlled regulation of the mouse transcriptome and cell proliferation.** *Proceedings of the National Academy of Sciences of the United States of America* 2007, 104:3342–7.

Mills JN, Stanbury SW: **Persistent 24-hour renal excretory rhythm on a 12-hour cycle of activity.** *Journal of Physiology* 1952, 117:22–37.

Mistlberger RE, Mumby DG: **The limbic system and food-anticipatory circadian rhythms in the rat: ablation and dopamine blocking studies.** *Behavioural Brain Research* 1992, 47:159–68.

Mistlberger RE: **Circadian food-anticipatory activity: formal models and physiological mechanisms.** *Neuroscience and Biobehavioral Reviews* 1994, 18:171–95.

Mitra A, Lenglos C, Martin J, Mbende N, Gagné A, Timofeeva E: **Sucrose modifies c-fos mRNA expression in the brain of rats maintained on feeding schedules.** *Neuroscience* 2011, 192: 459-74.

Mohawk JA, Baer ML, Menaker M: **The methamphetamine-sensitive circadian oscillator does not employ canonical clock genes.** *Proceedings of the National Academy of Sciences of the United States of America* 2009, 106:3519–24.

Moore RY, Eichler VB: **Loss of a circadian adrenal corticosterone rhythm following suprachiasmatic lesions in the rat.** *Brain Research* 1972, 42:201–6.

Moriya T, Aida R, Kudo T, Akiyama M, Doi M, Hayasaka N, Nakahata N, Mistlberger R, Okamura H, Shibata S: **The dorsomedial hypothalamic nucleus is not necessary for food-anticipatory circadian rhythms of behavior, temperature or clock gene expression in mice.** *European Journal of Neuroscience* 2009, 29:1447–60.

Murakami Y, Higashi Y, Matsunaga N, Koyanagi S, Ohdo S: **Circadian clock-controlled intestinal expression of the multidrug-resistance gene mdr1a in mice.** *Gastroenterology* 2008, 135:1636–1044.e3.

Nagoshi E, Saini C, Bauer C, Laroche T, Naef F, Schibler U: **Circadian gene expression in individual fibroblasts: cell-autonomous and self-sustained oscillators pass time to daughter cells.** *Cell* 2004, 119:693–705.

Nakahata Y, Yoshida M, Takano A, Soma H, Yamamoto T, Yasuda A, Nakatsu T, Takumi T: **A direct repeat of E-box-like elements is required for cell-autonomous circadian rhythm of clock genes.** *BMC Molecular Biology* 2008, 9:1.

Namihira M, Honma S, Abe H, Tanahashi Y, Ikeda M, Honma K: **Daily variation and light responsiveness of mammalian clock gene, Clock and BMAL1, transcripts in the pineal body and different areas of brain in rats.** *Neuroscience Letters* 1999, 267:69–72.

Neal J V, Potten CS: **Circadian rhythms in the epithelial cells and the pericryptal fibroblast sheath in three different sites in the murine intestinal tract.** *Cell and Tissue Kinetics* 1981, 14:581–7.

Nelson W, Scheving L, Halberg F: **Circadian rhythms in mice fed a single daily meal at different stages of lighting regimen.** *Journal of Nutrition* 1975, 105:171–84.

Nishihara T, Baba M, Matsuda M, Inoue M, Nishizawa Y, Fukuhara A, Araki H, Kihara S, Funahashi T, Tamura S, Hayashi N, Iishi H, Shimomura I: **Adiponectin deficiency enhances colorectal carcinogenesis and liver tumor formation induced by azoxymethane in mice.** *World Journal of Gastroenterology* 2008, 14:6473–80.

Nishinaga H, Komatsu R, Doi M, Fustin J-M, Yamada H, Okura R, Yamaguchi Y, Matsuo M, Emoto N, Okamura H: **Circadian expression of the Na+/H+ exchanger NHE3 in the mouse renal medulla.** *Biomedical Research* 2009, 30:87–93.

Niu S, Shingle DL, Garbarino-Pico E, Kojima S, Gilbert M, Green CB: **The circadian deadenylase Nocturnin is necessary for stabilization of the iNOS mRNA in mice.** *PloS One* 2011, 6:e26954.

Noshiro M, Furukawa M, Honma S, Kawamoto T, Hamada T, Honma K, Kato Y: **Tissue-specific disruption of rhythmic expression of Dec1 and Dec2 in clock mutant mice.** *Journal of Biological Rhythms* 2005, 20:404–18.

Nozaki T, Fujihara H, Watanabe M, Tsutsumi M, Nakamoto K, Kusuoka O, Kamada N, Suzuki H, Nakagama H, Sugimura T, Masutani M: **Parp-1 deficiency implicated in colon and liver tumorigenesis induced by azoxymethane.** *Cancer Science* 2003, 94:497–500.

Oklejewicz M, Destici E, Tamanini F, Hut R a, Janssens R, van der Horst GTJ: **Phase resetting of the mammalian circadian clock by DNA damage.** *Current Biology* 2008, 18:286–91.

Okyar A, Piccolo E, Ahowesso C, Filipski E, Hossard V, Guettier C, La Sorda R, Tinari N, Iacobelli S, Lévi F: **Strain- and sex-dependent circadian changes in abcc2 transporter expression: implications for irinotecan chronotolerance in mouse ileum.** *PloS One* 2011, 6:e20393.

Otálora BB, Madrid JA, Alvarez N, Vicente V, Rol MA: **Effects of exogenous melatonin and circadian synchronization on tumor progression in melanoma-bearing C57BL6 mice.** *Journal of Pineal Research* 2008, 44:307–15.

Ozturk N, Lee JH, Gaddameedhi S, Sancar A: **Loss of cryptochrome reduces cancer risk in p53 mutant mice.** *Proceedings of the National Academy of Sciences of the United States of America* 2009, 106:2841–6.

Pácha J, Pohlová I: **Relationship between dietary Na+ intake, aldosterone and colonic amiloride-sensitive Na+ transport.** *British Journal of Nutrition* 1995, 73:633–40.

Pan X, Hussain MM: **Clock is important for food and circadian regulation of macronutrient absorption in mice.** *Journal of Lipid Research* 2009, 50:1800–13.

Pan X, Terada T, Irie M, Saito H, Inui K-I: **Diurnal rhythm of H+-peptide cotransporter in rat small intestine.** *American Journal of Physiology. Gastrointestinal and Liver Physiology* 2002, 283:G57–64.

Pan X, Terada T, Okuda M, Inui K: **Altered diurnal rhythm of intestinal peptide transporter by fasting and its effects on the pharmacokinetics of ceftibuten.** *Journal of Pharmacology and Experimental Therapeutics* 2003, 307:626–32.

Pan X, Terada T, Okuda M, Inui K: **The diurnal rhythm of the intestinal transporters SGLT1 and PEPT1 is regulated by the feeding conditions in rats.** *Journal of Nutrition* 2004, 134:2211–5.

Panda S, Antoch MP, Miller BH, Su AI, Schook AB, Straume M, Schultz PG, Kay SA, Takahashi JS, Hogenesch JB: **Coordinated transcription of key pathways in the mouse by the circadian clock.** *Cell* 2002, 109:307–20.

Pardini L, Kaeffer B, Trubuil a, Bourreille a, Galmiche J-P: **Human intestinal circadian clock: expression of clock genes in colonocytes lining the crypt.** *Chronobiology International* 2005, 22:951–61.

De Paula RM, Lamb TM, Bennett L, Bell-Pedersen D: **A connection between MAPK pathways and circadian clocks.** *Cell Cycle* 2008, 7:2630–4.

Pelengaris S, Khan M, Evan G: **c-MYC: more than just a matter of life and death.** *Nature Reviews. Cancer* 2002, 2:764–76.

Pendergast JS, Nakamura W, Friday RC, Hatanaka F, Takumi T, Yamazaki S: **Robust food anticipatory activity in BMAL1-deficient mice.** *PloS One* 2009, 4:e4860.

Pereira MA, Barnes LH, Rassman VL, Kelloff G V, Steele VE: **Use of azoxymethane-induced foci of aberrant crypts in rat colon to identify potential cancer chemopreventive agents.** *Carcinogenesis* 1994, 15:1049–54.

Pezuk P, Mohawk J a, Yoshikawa T, Sellix MT, Menaker M: **Circadian organization is governed by extra-SCN pacemakers.** *Journal of Biological Rhythms* 2010, 25:432–41.

Phillips S a, Kung JT: **Mechanisms of adiponectin regulation and use as a pharmacological target.** *Current Opinion in Pharmacology* 2010, 10:676–83.

Pitts S, Perone E, Silver R: **Food-entrained circadian rhythms are sustained in arrhythmic Clk/Clk mutant mice.** *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology* 2003, 285:R57–67.

Polidarová L, Soták M, Sládek M, Pácha J, Sumová A: **Temporal gradient in the clock gene and cell-cycle checkpoint kinase Wee1 expression along the gut.** *Chronobiology International* 2009, 26:607–20.

Qandeel HG, Duenes JA, Zheng Y, Sarr MG: **Diurnal expression and function of peptide transporter 1 (PEPT1).** *Journal of Surgical Research* 2009, 156:123–8.

Quaroni A, Tian JQ, Seth P, Ap Rhys C: **p27(Kip1) is an inducer of intestinal epithelial cell differentiation.** *American journal of physiology. Cell Physiology* 2000, 279:C1045–57.

Reiter RJ, Tan D, Korkmaz A, Erren TC, Piekarski C, Tamura H, Manchester LC: **Light at night, chronodisruption, melatonin suppression, and cancer risk: a review.** *Critical Reviews in Oncogenesis* 2007, 13:303–28.

Reppert SM, Weaver DR: **Molecular analysis of mammalian circadian rhythms.** *Annual Review of Physiology* 2001, 63:647–76.

Rhoads DB: **Circadian Periodicity of Intestinal Na+/Glucose Cotransporter 1 mRNA Levels Is Transcriptionally Regulated**. *Journal of Biological Chemistry* 1998, 273:9510–6.

Ripperger J a, Schibler U: **Rhythmic CLOCK-BMAL1 binding to multiple E-box motifs drives circadian Dbp transcription and chromatin transitions.** *Nature Genetics* 2006, 38:369–74.

De Robertis M, Massi E, Poeta ML, Carotti S, Morini S, Cecchetelli L, Signori E, Fazio VM: **The AOM/DSS murine model for the study of colon carcinogenesis: From pathways to diagnosis and therapy studies.** *Journal of Carcinogenesis* 2011, 10:9.

Ruby JR, Scheving LE, Gray SB, White K: **Circadian rhythm of nuclear DNA in adult rat liver.** *Experimental Cell Research* 1973, 76:136–42.

Sahar S, Zocchi L, Kinoshita C, Borrelli E, Sassone-Corsi P: **Regulation of BMAL1 protein stability and circadian function by GSK3beta-mediated phosphorylation.** *PloS One* 2010, 5:e8561.

Saifur Rohman M, Emoto N, Nonaka H, Okura R, Nishimura M, Yagita K, van der Horst GTJ, Matsuo M, Okamura H, Yokoyama M: **Circadian clock genes directly regulate expression of the Na(+)/H(+) exchanger NHE3 in the kidney.** *Kidney International* 2005, 67:1410–9.

Saito M, Murakami E, Nishida T, Fujisawa Y, Suda M: **Circadian rhythms in digestive enzymes in the small intestine of rats. I. Patterns of the rhythms in various regions of the small intestine.** *Journal of Biochemistry* 1975, 78:475–80.

Sanada K, Harada Y, Sakai M, Todo T, Fukada Y: **Serine phosphorylation of mCRY1 and mCRY2 by mitogen-activated protein kinase.** *Genes to Cells* 2004, 9:697–708.

Sato F, Kawamura H, Wu Y, Sato H, Jin D, Bhawal UK, Kawamoto T, Fujimoto K, Noshiro M, Seino H, Morohashi S, Kato Y, Kijima H: **The basic helix-loop-helix transcription factor DEC2 inhibits TGF-β-induced tumor progression in human pancreatic cancer BxPC-3 cells.** *International Journal of Molecular Medicine* 2012, 30:495–501.

Sato F, Nagata C, Liu Y, Suzuki T, Kondo J, Morohashi S, Imaizumi T, Kato Y, Kijima H: **PERIOD1 is an anti-apoptotic factor in human pancreatic and hepatic cancer cells.** *Journal of Biochemistry* 2009, 146:833–8.

Sato F, Sato H, Jin D, Bhawal UK, Wu Y, Noshiro M, Kawamoto T, Fujimoto K, Seino H, Morohashi S, Kato Y, Kijima H: **Smad3 and Snail show circadian expression in human gingival fibroblasts, human mesenchymal stem cell, and in mouse liver.** *Biochemical and Biophysical Research Communications* 2012, 419:441–6.

Sato F, Wu Y, Bhawal UK, Liu Y, Imaizumi T, Morohashi S, Kato Y, Kijima H: **PERIOD1 (PER1) has anti-apoptotic effects, and PER3 has pro-apoptotic effects during cisplatin (CDDP) treatment in human gingival cancer CA9-22 cells.** *European Journal of Cancer* 2011, 47:1747–58.

Shefer S, Hauser S, Lapar V, Mosbach EH: **Diurnal variation of HMG CoA reductase activity in rat intestine.** *Journal of Lipid Research* 1972, 13:571–3.

Sherr CJ, Roberts JM: **CDK inhibitors: positive and negative regulators of G1-phase progression.** *Genes & Development* 1999, 13:1501–12.

Shibata S: **Neural regulation of the hepatic circadian rhythm.** *Anatomical Record Part A. Discoveries in Molecular, Cellular, and Evolutionary Biology* 2004, 280:901–9.

Schernhammer ES, Laden F, Speizer FE, Willett WC, Hunter DJ, Kawachi I, Fuchs CS, Colditz GA: **Night-shift work and risk of colorectal cancer in the nurses' health study.** *Journal of the National Cancer Institute* 2003, 95:825–8.

Scheving L a.: **Biological Clocks and the Digestive System**. *Gastroenterology* 2000, 119:536–49.

Scheving LE, Burns ER, Pauly JE: **Circadian rhythms in mitotic activity and 3 H-thymidine uptake in the duodenum: effect of isoproterenol on the mitotic rhythm.** *American Journal of Anatomy* 1972, 135:311–7.

Schwarz-Romond T, Asbrand C, Bakkers J, Kühl M, Schaeffer H-J, Huelsken J, Behrens J, Hammerschmidt M, Birchmeier W: **The ankyrin repeat protein Diversin recruits Casein kinase Iepsilon to the beta-catenin degradation complex and acts in both canonical Wnt and Wnt/JNK signaling.** *Genes & Development* 2002, 16:2073–84.

Silver R, LeSauter J, Tresco PA, Lehman MN: **A diffusible coupling signal from the transplanted suprachiasmatic nucleus controlling circadian locomotor rhythms.** *Nature* 1996, 382:810–3.

Sládek M, Rybová M, Jindráková Z, Zemanová Z, Polidarová L, Mrnka L, O'Neill J, Pácha J, Sumová A: **Insight into the circadian clock within rat colonic epithelial cells.** *Gastroenterology* 2007, 133:1240–9.

Smaaland R, Sothern RB, Laerum OD, Abrahamsen JF: **Rhythms in human bone marrow and blood cells.** *Chronobiology International* 2002, 19:101–27.

Smartt HJM, Guilmeau S, Nasser S V, Nicholas C, Bancroft L, Simpson SA, Yeh N, Yang W, Mariadason JM, Koff A, Augenlicht LH: **p27kip1 Regulates cdk2 activity in the proliferating zone of the mouse intestinal epithelium: potential role in neoplasia.** *Gastroenterology* 2007, 133:232–43.

Sothern RB, Smaaland R, Moore JG: **Circannual rhythm in DNA synthesis (S-phase) in healthy human bone marrow and rectal mucosa.** *FASEB Journal* 1995, 9:397–403.

Stearns AT, Balakrishnan A, Rhoads DB, Ashley SW, Tavakkolizadeh A: **Diurnal rhythmicity in the transcription of jejunal drug transporters.** *Journal of Pharmacological Sciences* 2008, 108:144–8.

Stearns AT, Balakrishnan A, Rhoads DB, Ashley SW, Tavakkolizadeh A: **Diurnal expression of the rat intestinal sodium-glucose cotransporter 1 (SGLT1) is independent of local luminal factors.** *Surgery* 2009, 145:294–302.

Stephan FK, Swann JM, Sisk CL: **Entrainment of circadian rhythms by feeding schedules in rats with suprachiasmatic lesions.** *Behavioral and Neural Biology* 1979, 25:545–54.

Stephan FK, Zucker I: **Circadian rhythms in drinking behavior and locomotor activity of rats are eliminated by hypothalamic lesions.** *Proceedings of the National Academy of Sciences of the United States of America* 1972, 69:1583–6.

Stephan FK: **The "other" circadian system: food as a Zeitgeber.** *Journal of Biological Rhythms* 2002, 17:284–92.

Stevenson NR, Ferrigni F, Parnicky K, Day S, Fierstein JS: **Effect of changes in feeding schedule on the diurnal rhythms and daily activity levels of intestinal brush border enzymes and transport systems.** *Biochimica et Biophysica Acta* 1975, 406:131–45.

Stevenson NR, Fierstein JS: **Circadian rhythms of intestinal sucrase and glucose transport: cued by time of feeding.** *American Journal of Physiology* 1976, 230:731–5.

Stokes JB, Sigmund RD: **Regulation of rENaC mRNA by dietary NaCl and steroids: organ, tissue, and steroid heterogeneity.** *American Journal of Physiology* 1998, 274:C1699–707.

Stokkan KA, Yamazaki S, Tei H, Sakaki Y, Menaker M: **Entrainment of the circadian clock in the liver by feeding.** *Science* 2001, 291:490–3.

Sujino M, Masumoto K, Yamaguchi S, van der Horst GTJ, Okamura H, Inouye S-IT: **Suprachiasmatic nucleus grafts restore circadian behavioral rhythms of genetically arrhythmic mice.** *Current Biology* 2003, 13:664–8.

Suzuki R, Miyamoto S, Yasui Y, Sugie S, Tanaka T: **Global gene expression analysis of the mouse colonic mucosa treated with azoxymethane and dextran sodium sulfate.** *BMC Cancer* 2007, 7:84.

Švec J, Ergang P, Mandys V, Kment M, Pácha J: **Expression profiles of proliferative and antiapoptotic genes in sporadic and colitis-related mouse colon cancer models.** *International Journal of Experimental Pathology* 2010, 91:44–53.

Takahashi JS, Hong H-K, Ko CH, McDearmon EL: **The genetics of mammalian circadian order and disorder: implications for physiology and disease.** *Nature Reviews. Genetics* 2008, 9:764–75.

Takahashi M, Wakabayashi K: **Gene mutations and altered gene expression in azoxymethane-induced colon carcinogenesis in rodents.** *Cancer Science* 2004, 95:475–80.

Tanaka T, Kohno H, Suzuki R, Yamada Y, Sugie S, Mori H: **A novel inflammation-related mouse colon carcinogenesis model induced by azoxymethane and dextran sodium sulfate**. *Cancer Science* 2003, 94:965–73.

Tanaka T, Suzuki R, Kohno H, Sugie S, Takahashi M, Wakabayashi K: **Colonic adenocarcinomas rapidly induced by the combined treatment with 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine and dextran sodium sulfate in male ICR mice possess beta-catenin gene mutations and increases immunoreactivity for beta-catenin, cycloox**. *Carcinogenesis* 2005, 26:229–38.

Tanaka T, Yasui Y, Tanaka M, Tanaka T, Oyama T, Rahman KMW: **Melatonin suppresses AOM/DSS-induced large bowel oncogenesis in rats.** *Chemico-Biological Interactions* 2009, 177:128–36.

Tataroglu O, Davidson AJ, Benvenuto LJ, Menaker M: **The methamphetamine-sensitive circadian oscillator (MASCO) in mice.** *Journal of Biological Rhythms* 2006, 21:185–94.

Tavakkolizadeh A, Berger U V., Shen KR, Levitsky LL, Zinner MJ, Hediger MA, Ashley SW, Whang EE, Rhoads DB: **Diurnal rhythmicity in intestinal SGLT-1 function, V(max), and mRNA expression topography.** *American Journal of Physiology. Gastrointestinal and Liver Physiology* 2001, 280:G209–15.

Tetsu O, McCormick F: **Beta-catenin regulates expression of cyclin D1 in colon carcinoma cells.** *Nature* 1999, 398:422–6.

Thompson CL, Larkin EK, Patel S, Berger NA, Redline S, Li L: **Short duration of sleep increases risk of colorectal adenoma.** *Cancer* 2011, 117:841–7.

Tian JQ, Quaroni A: **Involvement of p21(WAF1/Cip1) and p27(Kip1) in intestinal epithelial cell differentiation.** *American Journal of Physiology* 1999, 276(6 Pt 1):C1245–58.

Tosini G, Menaker M: **Circadian rhythms in cultured mammalian retina.** *Science* 1996, 272:419–21.

Tsuchiya Y, Minami I, Kadotani H, Nishida E: **Resetting of peripheral circadian clock by prostaglandin E2.** *EMBO Reports* 2005, 6:256–61.

Ueda HR, Chen W, Adachi A, Wakamatsu H, Hayashi S, Takasugi T, Nagano M, Nakahama K, Suzuki Y, Sugano S, Iino M, Shigeyoshi Y, Hashimoto S: **A transcription factor response element for gene expression during circadian night.** *Nature* 2002, 418:534–9.

Unsal-Kaçmaz K, Mullen TE, Kaufmann WK, Sancar A: **Coupling of human circadian and cell cycles by the timeless protein.** *Molecular and Cellular Biology* 2005, 25:3109–16.

Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F: **Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes.** *Genome Biology* 2002, 3:research0034.

Vriend LEM, De Witt Hamer PC, Van Noorden CJF, Würdinger T: **WEE1 inhibition and genomic instability in cancer.** *Biochimica et Biophysica Acta* 2013, 1836:227–35.

Wakamatsu H, Yoshinobu Y, Aida R, Moriya T, Akiyama M, Shibata S: **Restricted-feeding-induced anticipatory activity rhythm is associated with a phase-shift of the expression of mPer1 and mPer2 mRNA in the cerebral cortex and hippocampus but not in the suprachiasmatic nucleus of mice.** *European Journal of Neuroscience* 2001, 13:1190–6.

Walker NM, Simpson JE, Yen P-F, Gill RK, Rigsby E V, Brazill JM, Dudeja PK, Schweinfest CW, Clarke LL: **Down-regulated in adenoma Cl/HCO3 exchanger couples with Na/H exchanger 3 for NaCl absorption in murine small intestine.** *Gastroenterology* 2008, 135:1645–1653.e3.

Wang Q, Horisberger JD, Maillard M, Brunner HR, Rossier BC, Burnier M: **Salt- and angiotensin II-dependent variations in amiloride-sensitive rectal potential difference in mice.** *Clinical and Experimental Pharmacology & Physiology* 2000, 27:60–6.

Wang Q, Maillard M, Schibler U, Burnier M, Gachon F: **Cardiac hypertrophy, low blood pressure, and low aldosterone levels in mice devoid of the three circadian PAR bZip transcription factors DBP, HLF, and TEF.** *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology* 2010, 299:R1013–9.

Wesson LG: **Diurnal circadian rhythms of electrolyte excretion and filtration rate in end-stage renal disease.** *Nephron* 1980, 26:211–4.

Wierstra I, Alves J: **The c-myc promoter: still MysterY and challenge.** *Advances in Cancer Research* 2008, 99:113–333.

Wilson JW, Pritchard DM, Hickman JA, Potten CS: **Radiation-induced p53 and p21WAF-1/CIP1 expression in the murine intestinal epithelium: apoptosis and cell cycle arrest.** *American Journal of Pathology* 1998, 153:899–909.

Witkiewicz AK, Knudsen KE, Dicker AP, Knudsen ES: **The meaning of p16(ink4a) expression in tumors: functional significance, clinical associations and future developments.** *Cell Cycle* 2011, 10:2497–503.

Wood PA, Yang X, Taber A, Oh E-Y, Ansell C, Ayers SE, Al-Assaad Z, Carnevale K, Berger FG, Peña MMO, Hrushesky WJM: **Period 2 mutation accelerates ApcMin/+ tumorigenesis.** *Molecular Cancer Research* 2008, 6:1786–93.

Wu J, Dauchy RT, Tirrell PC, Wu SS, Lynch DT, Jitawatanarat P, Burrington CM, Dauchy EM, Blask DE, Greene MW: **Light at night activates IGF-1R/PDK1 signaling and accelerates tumor growth in human breast cancer xenografts.** *Cancer Research* 2011, 71:2622–31.

Wu M-W, Xian L-J, Li X-M, Pasquale I, Francis L: **Circadian expression of dihydropyrimidine dehydrogenase, thymidylate synthase, c-myc and p53 mRNA in mouse liver tissue.** *Chinese Journal of Cancer* 2004, 23:235–42.

Xiao ZQ, Yu Y, Khan A, Jaszewski R, Ehrinpreis MN, Majumdar AP: **Induction of G(1) checkpoint in the gastric mucosa of aged rats.** *American Journal of Physiology* 1999, 277:G929–34.

Yamazaki S, Numano R, Abe M, Hida A, Takahashi R, Ueda M, Block GD, Sakaki Y, Menaker M, Tei H: **Resetting central and peripheral circadian oscillators in transgenic rats.** *Science* 2000, 288:682–5.

Yang WS, Stockwell BR: **Inhibition of casein kinase 1-epsilon induces cancer-cell-selective, PERIOD2-dependent growth arrest.** *Genome Biology* 2008, 9:R92.

Yang X, Wood P a, Ansell CM, Ohmori M, Oh E-Y, Xiong Y, Berger FG, Peña MMO, Hrushesky WJM: **Beta-catenin induces beta-TrCP-mediated PER2 degradation altering circadian clock gene expression in intestinal mucosa of ApcMin/+ mice.** *Journal of Biochemistry* 2009a, 145:289–97.

Yang X, Wood PA, Ansell CM, Quiton DFT, Oh E-Y, Du-Quiton J, Hrushesky WJM: **The circadian clock gene Per1 suppresses cancer cell proliferation and tumor growth at specific times of day.** *Chronobiology International* 2009b, 26:1323–39.

Yasuniwa Y, Izumi H, Wang K-Y, Shimajiri S, Sasaguri Y, Kawai K, Kasai H, Shimada T, Miyake K, Kashiwagi E, Hirano G, Kidani A, Akiyama M, Han B, Wu Y, Ieiri I, Higuchi S, Kohno K: **Circadian disruption accelerates tumor growth and angio/stromagenesis through a Wnt signaling pathway.** *PloS One* 2010, 5:e15330.

Yeh K-T, Yang M-Y, Liu T-C, Chen J-C, Chan W-L, Lin S-F, Chang J-G: **Abnormal expression of period 1 (PER1) in endometrial carcinoma.** *Journal of Pathology* 2005, 206:111–20.

Yeom M, Pendergast JS, Ohmiya Y, Yamazaki S: **Circadian-independent cell mitosis in immortalized fibroblasts.** *Proceedings of the National Academy of Sciences of the United States of America* 2010, 107:9665–70.

You S, Wood P a, Xiong Y, Kobayashi M, Du-Quiton J, Hrushesky WJM: **Daily coordination of cancer growth and circadian clock gene expression.** *Breast Cancer research and Treatment* 2005, 91:47–60.

Zachos NC, Tse M, Donowitz M: **Molecular physiology of intestinal Na+/H+ exchange.** *Annual Review of Physiology* 2005, 67:411–43.

Zeman M, Vician M, Monosíková J, Reis R, Herichová I: **Deregulated expression of the per2 gene in human colorectal carcinoma.** *Molecular Medicine Reports* 2008, 1:599–603.

Zhang EE, Kay SA: **Clocks not winding down: unravelling circadian networks**. *Nature Reviews. Molecular Cell Biology* 2010, 11:764–76.

Zhang J, Zhu B, Liu Y, Jiang Z, Wang Y, Li Y, Hua H, Wang Z: **High expression of circadian gene mPer2 diminishes radiosensitivity of tumor cells.** *Cancer Biotherapy & Radiopharmaceuticals* 2008, 23:561–70.

Zuber AM, Centeno G, Pradervand S, Nikolaeva S, Maquelin L, Cardinaux L, Bonny O, Firsov D: **Molecular clock is involved in predictive circadian adjustment of renal function.** *Proceedings of the National Academy of Sciences of the United States of America* 2009, 106:16523–8.

10 LIST OF ABBREVIATIONS

ATP	adenosine-5'-triphosphate
ABC	ATP-binding cassette
Abcb1a	ATP-binding cassette, sub-family B, member 1a
AdipoR1/R2	adiponectin receptor 1/2
Ae1	anion exchanger 1
Akt	v-akt murine thymoma viral oncogene homolog; known as protein kinase B
ANOVA	analysis of variance
AOM	azoxymethane
APC	adenomatous polyposis coli
ATM	ataxia telangiectasia mutated
Atp1a	ATPase, Na+/K+ transporting, alpha 1 polypeptide
Atp1b	ATPase, Na+/K+ transporting, beta 1 polypeptide
ATR	ataxia telangiectasia and Rad3-related protein
Avp	arginine vasopressin
B2m	beta-2-microglobulin
Bcl2	B-cell CLL/lymphoma 2
Bcl-XL	B-cell lymphoma-extra large
beta-TrCP	beta-transducin repeat containing
Bmal	brain and muscle Arnt-like protein-1
Bmp	bone morphogenetic factor
BW	body weight
C/EBP a	CCAAT-enhancer-binding protein alpha
C57BL/6	C57 black 6
CDC	cell division cycle
CDK	cyclin dependent kinase
cDNA	copy DNA
Cftr	cystic fibrosis transmembrane conductance regulator
CI	confidence interval
Cip1	CDK-interaction protein 1
CK	casein kinase
Clock	circadian locomotor output cycles kaput
c-Myc	myelocytomatosis oncogene
Cox-2	cyclooxygenase-2
Cry1/2	cryptochrome 1/2
Csnk1e	casein kinase 1, epsilon
CT	ciradian time
CTRL	control
Dbp	D-box binding protein
Dec1/2	differentially expressed in chondrocytes 1/2
DHM	dorsomedial hypothalamus
DNA	deoxyribonucleic acid
Dra	downregulated in adenoma
DSS	dextran sodium sulfate
E4bp4	E4 promoter binding protein 4; known as Nfil3 (Nuclear Factor, Interleukin 3 Regulated)

ENaC	epithelial sodium channel
FAA	food-anticipatory activity
Fbxl3	F-box and leucine-rich repeat protein 3
FEO	food-entrainable oscillator
Foxo1	forkhead box protein O1
Gadd45a	growth arrest and DNA-damage-inducible, alpha
Gapd	glyceraldehyde-3-phosphate dehydrogenase
Glut2/5	glucose transporter 2/5
GSK3	glycogen synthase kinase 3
Hlf	hepatic leukemia factor
HMG CoA	3 hydroxy 3 methylglutaryl coenzyme A
Hsd3b6	3 b-hydroxysteroid dehydrogenase VI
Chk1/2	serine/threonine-protein kinase Chk1/2
IGF-1R	insulin-like growth factor 1 (IGF-1) receptor
IGL	intergeniculate leaflets
iNOS	nitric oxide synthase, inducible isoform
Isc	short-circuit current
JNK	c-Jun N-terminal kinase
Kcc1	potassium/chloride cotransporter 1
Kip1	cyclin-dependent kinase inhibitor 1
Klf10	Krüppel-like factor 10
LD	light/dark
LMD	laser capture microdissection
LSD	least-significance difference
MAM	methylazoxymethanol
Mapk	mitogen-activated protein kinase
Mct1	monocarboxylate transporter 1
Mdm2	double inute 2 protein
Mdr	multidrug resistance protein
MEK	mitogen-activated protein kinase kinase
mesor	midline estimating statistic of rhythm
Min	multiple intestinal neoplasia
mRNA	messenger ribonucleic acid
Mrp2	multidrug resistance protein 2
Myt1	protein kinase, membrane associated tyrosine/threonine 1
Nhe2/3	sodium/hydrogen exchanger, member 3
Nherf1	sodium-hydrogen exchanger regulatory factor 1
Nherf1	Nhe3 regulatory factor 1
Nkcc1	sodium/potassium/chloride transporter
Npas2	neuronal PAS domain-containing protein 2
Oatp-b	organic anion transporter B
OCT	optimum cutting temperature
Octn2	organic cation transporter 2
PAS	Period-Arnt-Single-minded
Pat1	putative anion transporter-1
Pbef	pre-B cell-enhancing factor; known as Nampt (nicotinamide phosphoribosyltransferase)

PD	potential difference
Pept1	peptide transporter 1
Per1/2/3	period gene 1/2/3
PH	partial hepactomy
PI3K	phosphatidylinositide 3-kinases
PKB	protein kinase B
Ppar a/g	peroxisome proliferator-activated receptor alpha/gama
Raf	Raf/MEK/MAPK and PI3K/Akt/PKB
RAR	retinoic acid receptor
RAS	rat sarcoma protein
RevErb	known as Nr1d1 (nuclear receptor subfamily 1, group D, member 1)
RF	restricted feeding
RHT	retino hypothalamic tract
RNA	ribonucleic acid
ROR	RAR-related orphan receptor
RRE	Rev response element
RT-PCR	reverse transcription polymerase chain reaction
SCC	short-circuit current
SCF	Skp, cullin, F-box containing complex
SCN	suprachiasmatic nuclei of hypothalamus
SE	standard error of the mean
Sglt	sodium-glucose linked transporter
Sirt1	sirtuin (silent mating type information regulation 2 homolog) 1
Slc	solute carrier family
Smad	mothers against decapentaplegic homolog
SREBP	sterol regulatory element-binding protein
Tcf	T cell factor
Tef	thyrotroph embryonic factor
Tgf-beta	Transforming growth factor beta
Waf1	wild-type p53-activated fragment 1
Wee1	Wee1 homolog (Schizosaccharomyces pombe)
Wif1	Wnt inhibitory factor 1
Wnt	wingless-type MMTV (mouse mammary tumor virus) integration site family
Ywhaz	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta (14-3-3-zeta)

11 LIST OF PUBLICATIONS

Publications related to the thesis results:

Soták M, Sumová A, Pácha J: **Cross-talk between the circadian clock and the cell cycle in cancer.** Annals of Medicine 2014, 46:221-32. **Review.** (IF, 2012 = 5.094)

Polidarová L, Olejníková L, Paušlyová L, Sládek M, Soták M, Pácha J, Sumová A: **Development and entrainment of the colonic circadian clock during ontogenesis.** *American Journal of Physiology. Gastrointestinal and Liver Physiology* 2014, 306:G346–56. (IF, 2012 = 3.649)

Soták M, Polidarová L, Ergang P, Sumová A, Pácha J: **An association between clock genes and clock-controlled cell cycle genes in murine colorectal tumors.** *International Journal of Cancer* 2013, 132:1032–41. (IF, 2012 = 6.198)

Soták M, Polidarová L, Musílková J, Hock M, Sumová A, Pácha J: **Circadian regulation of electrolyte absorption in the rat colon.** *American Journal of Physiology. Gastrointestinal and Liver Physiology* 2011, 301:G1066–74. (IF, 2012 = 3.649)

Polidarová L, Sládek M, Soták M, Pácha J, Sumová A: **Hepatic, Duodenal, and Colonic Circadian Clocks Differ in their Persistence under Conditions of Constant Light and in their Entrainment by Restricted Feeding.** *Chronobiology International* 2011, 28:204–15. (IF, 2012 = 4.350)

Polidarová L*, Soták M*, Sládek M, Pácha J, Sumová A: **Temporal gradient in the clock gene and cell-cycle checkpoint kinase Wee1 expression along the gut.** *Chronobiology International* 2009, 26:607–20. (*authors contributed equally) (IF, 2012 = 4.350)

Publications not related to the thesis results:

Vodička M, Ergang P, Mikulecká A, Řeháková L, Klusoňová P, Makal J, Soták M, Musílková J, Zach P, Pácha J: **Regulation of 11β-hydroxysteroid dehydrogenase type 1 and 7α-hydroxylase CYP7B1 during social stress.** *PLoS One* 2014, 9:e89421. (IF, 2012 = 3.730)

Ergang P, Kuželová A, Soták M, Klusoňová P, Makal J, Pácha J: **Distinct effect of stress on 11beta-hydroxysteroid dehydrogenase type 1 and corticosteroid receptors in dorsal and ventral hippocampus.** *Physiological Research* 2014, 63:255-61. (IF, 2012 = 1.531)

Hock M, Soták M, Kment M, Pácha J: **The early effect of dextran sodium sulfate administration on carbachol-induced short-circuit current in distal and proximal colon during colitis development.** *Physiological Research* 2011, 60:921–31. (IF, 2012 = 1.531)

Soták M, Mrnka L, Pácha J: **Heterogeneous expression of melatonin receptor MT1 mRNA in the rat intestine under control and fasting conditions.** *Journal of Pineal Research* 2006, 41:183–8. (IF, 2012 = 7.304)

Commentary/Letter:

Soták M: **NextGen speaks: Definition of Success** (edited by Jennfier Sills). *Science* 2012, 336:33.