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European Biological Rhythms Society

THESIS SUMMARY

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19th June 2020

# Molecular Mechanisms of Intercellular Coupling among Peripheral Circadian Oscillators

Circadian clocks can be found in almost all living organisms and cell types. They evolved as endogenous timekeepers in adaptation to periodically reoccurring environmental changes and allow for the optimal temporal coordination of intrinsic biological processes, physiology and behavior. Mammalian circadian systems are hierarchically organized with the suprachiasmatic nucleus (SCN), the so-called master clock, on top. The SCN is required for entrainment of circadian systems to the environmental light-dark cycle as it receives photic information through the retinohypothalamic tract (RHT). It then passes on entrainment information to non-SCN brain and peripheral clocks in order to align body clocks with each other and with the environmental light-dark cycle. On the cellular level, circadian clocks are self-sustained and autonomous, as well as driven by transcriptional-translational feedback loops between genes and their own protein products. At the very core of the mammalian rhythm generating machinery BMAL1/CLOCK heterodimers drive the E-box dependent expression of their target genes *Periods* (*Per1-3*) and *Cryptochromes* (*Cry1-2*), which, after a defined time delay important for generating ca. 24-hour rhythms, translocate back into the nucleus where they suppress their own activators.

As mentioned above cellular oscillators in mammals (but also other species) are self-sustained and autonomous and cycle with their own period and phases. Thus, over time, single cell oscillators within tissue networks would desynchronize due to progressive de-phasing. Therefore, intercellular coupling serves to keep individual oscillators in synchrony and maintain coherent ensemble rhythms. Theoretical models show that intercellular coupling promotes narrower period and phase distributions of single cell oscillators.

Moreover, intercellular coupling strength is positively correlated with network amplitude (due to resonance effects) and negatively correlated with network damping (due to increased synchrony). Additionally, intercellular coupling (by promoting high amplitudes and fast amplitude relaxation rates) renders oscillator networks more robust against perturbation by Zeitgeber pulses and governs the entrainment range of circadian clocks.

While it is known that the SCN constitutes a network of strongly coupled oscillators, coupling among cellular clocks in peripheral tissues is still highly debated. This is mainly because the SCN cycles with high amplitudes and almost no damping ex vivo, while rhythms of peripheral clocks have been found to dampen out quickly in vitro and ex vivo. However, more recent in vivo imaging approaches, allowing for the recording of peripheral clock rhythms in individual and freely-moving animals have suggested otherwise: bioluminescence rhythms of liver circadian clocks in SCN-lesioned mice housed under constant conditions (ad libitum feeding, constant darkness) persisted for at least seven days despite the absence of SCN-derived or external entrainment information. This suggested that individual oscillators within livers remain synchronized to maintain coherent tissue rhythms. Nevertheless, mechanisms of such peripheral coupling remained completely unknown.

Therefore, my thesis was aiming at answering three main questions: (i) do peripheral circadian oscillators couple intercellularly, (ii) what is the nature and identity of peripheral coupling factors, as well as (iii) what are molecular mechanisms of peripheral coupling? To answer these questions, we decided to use human osteosarcoma cells (U-2 OS) as simplified, yet well-characterized and -established, in vitro model of human peripheral clocks.

Initially, we showed that peripheral oscillators indeed couple intercellularly. We performed co-culture experiments of U-2 OS cells that differed in circadian period and phase. For coupled systems it would be expected that cellular populations display (period-/phase-)

pulling effects dependent on the co-culture ratios. Excitingly, we observed that the phase of U-2 OS reporter cells was shifted towards the phase of either 6-hour delayed or 6-hour advanced co-cultured non-reporter cells in a cell number dependent fashion, i.e. phases of reporter cells became later with delayed and earlier with advanced co-cultured cells. Moreover, the period of either U-2 OS *CRY2* KO (long period) or *TNPO1* KO (short period) reporter cells was pulled towards a more wildtype period phenotype upon co-culture with U-2 OS WT non-reporter cells, i.e. the period of long period cells was shortened and the period of short period cells was lengthened upon co-culture (as 3-D spheroids). In addition, we found that U-2 OS ensemble rhythms, with respect to amplitude, damping, and period, displayed strong dependency on culture density, suggesting that adjacent oscillators exchange coupling signals to maintain coherent, high amplitude network rhythms. To test whether this coupling depends on direct cell-cell contact or paracrine communication mechanisms, we again performed co-culture experiments with low density, low amplitude, highly damped U-2 OS reporter cells and increasing numbers of non-reporter cells. Interestingly, amplitude and damping parameters were rescued by this co-culture protocol even when reporter and non-reporter cell populations were physically separated using membrane inserts. This suggested that secreted and diffusible signals play an important role in intercellular coupling in the periphery.

Thus, using conditioned medium, we showed that secreted molecules act as Zeitgebers for peripheral circadian oscillators in an inter-species and inter-tissue manner. Moreover, time dependent phase responses to conditioned medium appeared to be associated with the time and cAMP response enhancer element (CRE) dependent immediate early expression of *PER2* (and subsequent repression of E-box driven clock genes). Interestingly, RNA sequencing (RNAseq) showed that the top-10 differentially regulated genes in response to conditioned medium stimulation were associated with growth factor signaling, including the TGF-β/SMAD signaling pathway. This strengthened the assumption that peripheral coupling factors are secreted molecules, likely proteins, acting on the molecular clock machinery and via growth factor dependent pathways.

We further confirmed the proteinergic nature of potential coupling factors by size fractionation, heat inactivation, and ammonium sulfate precipitation. Ultimately, together with our collaboration partners at the Max Planck Institute in Berlin, we were able to identify active conditioned medium factors using a two-step chromatography and mass spectrometry approach. Chromatography fractions with high specific activity (or low specific activity as background controls) were analyzed regarding their protein content using ESI-MS/MS. Thereby, we identified TGF-β as top candidate, contained in all active fractions analyzed and in agreement with RNAseq results. We validated the role of TGF-β as active conditioned medium factor by immunodepletion, pharmacological inhibition of TGF-β receptor, as well as by using recombinant TGF-β. Very much to our excitement, it had previously been reported that TGF-β acts as Zeitgeber for peripheral oscillators in vitro and in vivo using rat, mouse, and even zebrafish model systems (Kon et al. 2008; Sloin et al. 2018).

Lastly, we were able to show that TGF-β and its associated signaling pathway indeed promote intercellular coupling among peripheral circadian oscillators by performing genetic and pharmacological perturbation experiments. Not only did RNA interference (RNAi), targeting components of the TGF-β pathway, result in altered CRE induction upon conditioned medium stimulation, but it also decreased amplitudes and increased damping of circadian rhythms of U-2 OS reporter cells. Similarly, pharmacological inhibition of TGF-β receptor promoted higher damping in U-2 OS reporter cells and rendered U-2 OS networks more susceptible to perturbation by an 8-hour 20°C temperature stimulus. Overall, these results indicate that functional TGF-β signaling pathway plays an important role for intercellular coupling and that pathway disruption weakens intercellular coupling, leading to reduced network amplitudes, higher damping, and increased susceptibility to perturbation by external Zeitgeber pulses.

In summary, we were able to answer our initial questions. Using U-2 OS cells as model system, we were able to show that peripheral oscillators couple intercellularly. Moreover, we found that peripheral oscillators likely couple by exchange of paracrine signaling

molecules and identified TGF-β as candidate coupling factors. We suggest a molecular mechanism by which TGF-β is secreted by peripheral oscillators and subsequently, in adjacent oscillators, triggers its canonical SMAD signaling cascade resulting in the time and CRE dependent immediate early induction of PER2, as well as subsequent repression of E-box driven clock genes. Ultimately, such TGF-β mediated coupling will promote phase-synchronization among peripheral circadian oscillators, though with reduced potency compared to SCN neuronal networks.

Kon, Naohiro et al. 2008. "Activation of TGF-β/Activin Signalling Resets the Circadian Clock through Rapid Induction of Dec1 Transcripts." *Nature Cell Biology*.

Sloin, Hadas E. et al. 2018. "Interactions between the Circadian Clock and TGF-β Signaling Pathway in Zebrafish." *PLoS ONE*.