development, differentiation, division, survival, and death.[2] In the nucleus, lncRNAs have been shown to participate in critical epigenetic processes controlling the nuclear organization that influences gene expression.[4,5] In erythroid cells, an lncRNA called LincRNA-erythrocyte prosurvival participates in regulation of cellular differentiation and programed cell death.[6]

In the first part of the study by Alvarez-Dominguez et al, a well-defined set of bioinformatic criteria is used to identify and comprehensively catalog lncRNAs expressed during erythropoiesis. These data were further refined to identify erythroid-specific lncRNAs. Determined by examining patterns of expression in burst forming units-erythroid, colony forming units-erythriod, and erythroblasts, erythroid-specific lncRNAs were found to demonstrate dynamic patterns of expression throughout erythropoiesis. Integration of lncRNA expression data with genomewide data sets of chromatin state revealed that changes in lncRNA expression during erythropoiesis were reflected at the chromatin level, with patterns of gene expression correlated with patterns of histone architecture reflecting active and repressive chromatin.

Genomewide maps of the erythroid transcription factors GATA1, KLF1, and TAL1 were intersected with the promoter regions of differentially expressed erythroid lncRNAs. Individual and combinations of the transcription factors were found in the proximal promoters of most differentially expressed lncRNAs, with co-occupancy of GATA1 and TAL1 found at lncRNAs induced during erythropoiesis. These studies fit well with initial observations of lncRNAs that indicate that their transcription is tightly regulated by cellular developmental or differentiation stage, external environment, etc.[4]

The second half of this study is a series of functional analyses of 12 carefully chosen erythroid-specific lncRNAs. Knockdown studies of these 12 candidates using short hairpin RNA technology were performed in erythroid cells. Varying phenotypic effects on erythroid differentiation, including influence on TER119 expression, cell size, and enucleation, were observed in knockdown cells.

Detailed analyses of lncRNAs have revealed that there is poor conservation of lncRNA sequence across species.[7] Orthologs have been identified between species, leading to "syntelogs" with minimal or no sequence similarity, but with conserved positions relative to neighboring protein coding genes through evolution.[2,7] Based on these observations, the authors also examined the expression of neighboring genes of knocked down erythroid lncRNAs. Nine of the knocked down lncRNAs had no influence on neighboring gene expression, 1 knocked down lncRNA was associated with an increase in neighboring gene expression, and 2 knocked down lncRNAs were associated with decreased neighboring gene expression. Further analysis of one of the lncRNAs associated with decreased expression, alncRNA-EC7, revealed that it is an enhancer for *SLC4A1*, the gene encoding band 3, the primary anion exchanger of the erythrocyte. Knockdown of alncRNA-EC7, which is located 10 kb away from the band 3 gene locus, was associated with an 80% decrease in band 3 gene mRNA expression. Experimental data suggested a model for looping of the alncRNA-EC7 enhancer to the *SLC4A1* gene locus with subsequent activation of band 3 gene expression in erythroid cells.

The field of lncRNA biology is exploding. The list of lncRNA transcripts continues to expand across differing cell types, with functional understanding limited to a few well-characterized examples. Varying cell types express varying patterns and amounts of lncRNAs suggesting that complex patterns of lncRNA expression regulate cells in many different ways. Despite their apparent importance, to date, there has been no unifying structural, biochemical, or functional characteristic that define a transcript as a lncRNA.

There is much more to be learned about lncRNAs. What are their roles in regulating cellular gene expression? What are their roles in normal and perturbed hematopoiesis?[8] Based on studies of inherited neuromuscular diseases and select malignancies, we know they make important contributions to the pathogenesis of inherited and acquired disease.[4,9] What are the mechanisms of these contributions? Can lncRNAs be used as biomarkers of disease presence or progression? Can we exploit them as potential therapeutic targets in disease?[10] We are at the beginning of a long journey into the new field of ncRNA biology. Expect many surprises along the way.

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● ● ● THROMBOSIS & HEMOSTASIS

Comment on Scheer and Shea, page 590

PAI at breakfast (whether you like it or not)

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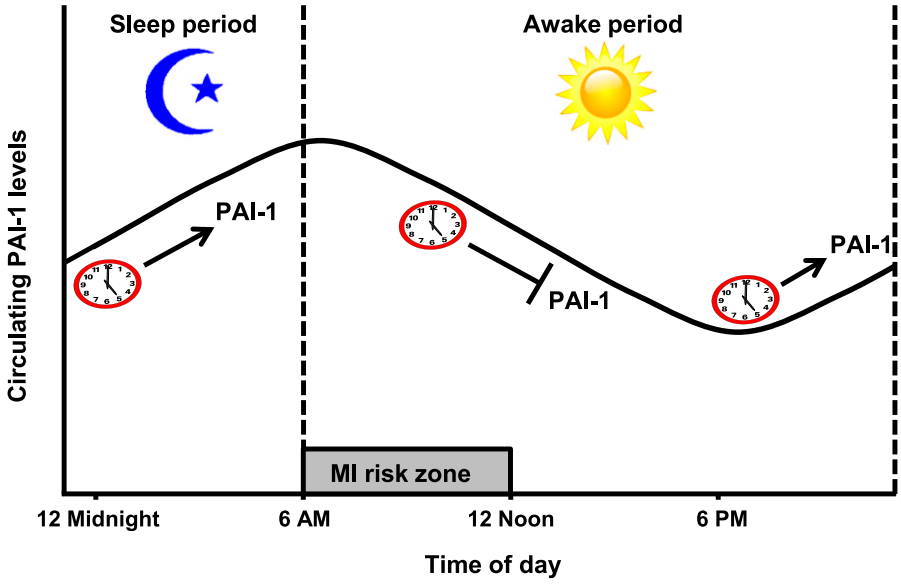
In this issue of *Blood*, Scheer and Shea report that the morning surge of the prothrombotic factor plasminogen activator inhibitor-1 (PAI-1) observed in

humans is driven by an endogenous mechanism (as opposed to behavioral/environmental influences).[1]

The waking hours are a dangerous time. On awakening, animals in the wild must venture out in search of food sources and/or mating opportunities, while at the same time fending off predators and rivals. Although humans living in Westernized civilizations are typically not confronted with these same hazards during their journey to the kitchen, the morning still remains a period of significant risk. This is exemplified by a disproportionately greater incidence of adverse cardiac events (eg, myocardial infarctions, sudden cardiac death) that occur between the hours of 6 AM and 12 noon.[2] These observations have led to speculation that a wide range of variables drive increased risk during the sleep-to-wake transition, including both behavioral (ie, physical and emotional stress) and biochemical (ie, autonomic stimulation, vessel shear stress, prothrombotic factors) influences.[2] Somewhat surprisingly, it appears that alone the act of awakening is not a sufficient "stress" to elicit these events; an 11-year retrospective study reported on investigations of vacationers in Hawaii and showed that the peak incidence of a myocardial infarction corresponded more closely to the early hours of the morning at the vacationers' point of origin (ie, time at their homes), as opposed to the local time in Hawaii.[3] This study raises the intriguing possibility that increased risk of adverse cardiac events in the early hours of the morning is mediated by intrinsically driven factors (as opposed to sleep-to-wake associated behavioral fluctuations). But what might these factors be, and what drives their intrinsic daily oscillations? One such candidate is PAI-1 oscillations driven by cell autonomous circadian clocks.

PAI-1 reduces the activity of tissue plasminogen activator (an enzyme known to cleave plasminogen to plasmin) thereby attenuating fibrinolysis and thrombi dissolution.[4] PAI-1 levels oscillate in a time-of-day–dependent fashion, peaking in the early hours of the morning.[4] An evolutionary advantage of the rise in PAI-1 levels at this time might be to promote clot formation in anticipation of injuries associated with increased physical activity, foraging for food, and avoidance of predation on awakening. However, what was once a selective advantage is now likely a detriment for humans in our modern-day setting, for which the PAI-1 surge would promote thrombi-induced ischemic events in susceptible individuals. Previous cell- and animal-based studies have established a molecular link between PAI-1 and the circadian clock.[5] Circadian clocks are cell-autonomous transcriptionally based mechanisms that modulate various biological functions in a time-of-day–dependent manner. At the heart of the clock mechanism are 2 transcription factors, CLOCK and BMAL1, which, on heterodimerization, directly bind to E-boxes within the promoter of various target genes, including PAI-1.[5] However, the extent to which the morning rise in circulating PAI-1 levels observed in humans is mediated by the intrinsic circadian mechanism (as opposed to behavioral influences) is unknown. This important question was the focus of the current study by Scheer and Shea.



Oscillations in circulating PAI-1 levels are driven by an intrinsic circadian system (depicted as a clock) in humans. The peak in circulating PAI-1 levels around 6:30 AM occurs during the period when the risk of a myocardial infarction (MI) is elevated.

Dissection of the contribution of intrinsic vs extrinsic influences on a biological process in humans presents significant methodological challenges. Through the use of a recently established desynchrony protocol (DP), the current study attempted to dissociate these influences on the morning rise in circulating PAI-1 levels. Indeed, the investigators have successfully used this DP to uncover the contribution of the intrinsic circadian system on daily rhythms of multiple cardiovascular relevant parameters, such as platelet activation, plasma epinephrine and norepinephrine, as well as heart rate and blood pressure in humans.[6-8] Briefly, during the DP, healthy subjects adhere to 12 contiguous 20-hour days, during which sleep/wake and feeding/fasting routines, as well as various environmental factors (eg, temperature, lighting), are strictly controlled. Previous studies have established that under such conditions, the intrinsic circadian (24-hour) system in humans is unable to reentrain successfully to the enforced 20-hour day. Any 24-hour oscillations that persist during this DP must be driven by an intrinsic circadian system. This was the case for circulating PAI-1 levels; the persistent 24-hour oscillations in PAI-1 levels peaked during the DP at a time corresponding to 6:30 AM. This study therefore showed that the morning surge in PAI-1 levels observed in humans is driven by an intrinsic circadian system.

These findings raise a number of important questions. For example, could the circadian system be pharmacologically targeted (eg, small molecule modulators) to reduce PAI-1 levels in patients at cardiovascular risk, or could disruption/misalignment of the intrinsic circadian system increase risk of adverse cardiac events? Evidence exists for the latter. Circadian misalignment occurs on a frequent basis, secondary to behavioral/lifestyle and environmental fluctuations. For example,

a break during the weekend from the normal daily "workweek" routine is associated with an increased risk of ischemic events on a Monday morning (relative to any other day of the week).[9] Similarly, the 1-hour time change during "Spring Forward" is associated with an approximate 10% increase in the incidence of myocardial infarctions on the subsequent Monday (whereas a reciprocal decrease in incidence is observed during the "Fall Back" time change).[10] These observations raise the intriguing possibility that even subtle circadian misalignment may result in greater susceptibility to adverse cardiac events in at-risk subjects.

In conclusion, the study by Scheer and Shea has successfully exposed that the morning rise in circulating PAI-1 levels in humans are driven by an intrinsic circadian mechanism (see figure). Future studies are required to determine whether prevention of the morning surge in PAI-1 levels (perhaps through manipulation of the circadian system) reduces risk of adverse cardiac events.

Conflict-of-interest disclosure: The author declares no competing financial interests.

DEDICATION

This commentary is dedicated to William C. Stanley, Professor of Physiology and Editor-in-Chief of the American Journal of Physiology: Heart and Circulatory Physiology, *who died recently from a myocardial infarction in the early hours of a Monday morning. You will be missed dearly.* ■

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