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Reproductive rhythms, longevity and cancer

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Reproduction is dominated by various rhythms: daily, cyclic and seasonal, together with those imposed by non-reproductive factors. Timing is essential for the control of daily, monthly, seasonal and other rhythms, as it is for very long-term cycles, including longevity. The reproductive period of life is expensive physiologically. Current concepts stress the high cost of reproduction may shorten life spans. It may have to be protected throughout infancy and puberty, fending off cancers and other disasters until well into reproductive life.

Long cycles and lifespans

Numerous primary factors determine lifespans including gene silencing, telomeric activity, nutrition, accumulation of mutations and inadequate body rhythms. Gene silencing is a significant longevity factor in yeast, operated via the Sir proteins (silent information regulator). This gene system is composed of the three genes *Sir2*, *Sir3* and *Sir4*. It is conserved in many organisms including humans (Guarente and Kenyon, 2000). One action of *Sir2* is to silence DNA encoding for ribosomal RNA, which enhances the lifespan. Deleting deacetylase genes also lengthens lifespans in yeast, which also silences ribosomal DNA sequences, although this system may be more variable in other species. Silencing may also act indirectly, e.g. the genes *HLM* and *HMR* extend lifespan because their simultaneous silencing induces sterility, which triggers longevity. Normal ageing may be due to the slow erosion of *Sir2* silencing by stress, although this remains to be shown in mammals.

Several processes influence lifespan in *C. elegans* including calorie restriction, signalling through insulin/IGF-1, germline activity, the *cdk* pathway and defective DNA and its repair. Each of these factors

can increase lifespans, sometimes by 30%. (Finkel and Holbrook, 2000). Destroying germ-cell precursors extends lifespans by 60%, prompting suggestions that reproductive cyclicality coordinates longevity. Factors such as defective DNA repair, or damage, may be oncogenic and impair longevity (Guarente and Kenyon, 2000). Defective DNA could be identified in a protein interaction map, and RNA interference measured on gene activity. Nutritional effects on lifespans have also been clarified in *C. elegans*. Metabolic sensors may be involved in genes such as *elk* in *C. elegans*, acting via pathways other than *daf*. They may 'sense' mitochondrial function, enabling genes to be set at correct times for behaviour and ageing. Feeding diets lacking coenzyme Q, a lipid with a significant role in aerobic respiration, added 60% to lifespans (Larsen and Clarke, 2002). Coenzyme Q might interact with the *daf-2* pathway in mitochondria, with the enhanced scavenging of reactive oxygen species then extending lifespans. Mice display similar responses, a restricted food intake extending lifespans by one-third perhaps by slowing metabolism and the production of toxic reactive oxygen species (Guarente and Kenyon, 2000).

Telomere clocks measure and may influence longevity. Telomeres have a 5–15 kbp length in human cells, varying between different cell types and between individual chromosomes. Between 50–150 bp of repeat sequences are lost during each cell division. Telomerase prevents this loss by adding repeat sequences, although other mechanisms operate. Shortened telomeres might signify limited lifespans, witnessed in Dolly's arthritis apparently due to early senescence, and positional effects may delay replication of nearby genes. Timing telomere expansion varies on different chromosomes or on the two strands of a single chromosome. They replicate before and during the S phase in human cell lines (Hultdin

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et al., 2001). Early replication is typical of household and tissue-specific genes, late replication involving heterochromatin and centromeres. Variations arise between patients, indicated by the varying telomere length in their cell samples, although its consequences remain to be clarified.

Longevity is influenced by the actions of some cancer genes, effects sometimes modified by reproductive systems. The p53 tumour suppressor protein plays a significant role, noted in early studies generating mice with modified forms of p53, which were hampered by the onset of early-forming tumours. It seemed that p53 was involved in ageing, a link confirmed by Tyner et al. (2002). They generated mice carrying a normal p53 gene and a homologue with deleted regions in exons 1–6 and some other sequences. The very high p53 activity of these mice led to a high efficiency in tumour suppression, but also to unexpected early signs of ageing. Their skin thinned prematurely, and they developed osteoporosis, organ atrophy and slow wound-healing. p53 had clearly modified various timing systems, possibly including stem cell death in some tissues, the fewer cell numbers then causing the loss of organ homeostasis. p53 also seemed to be involved with telomeres. Shortened telomeres enhance cell senescence and impair proliferation, an effect modified by lack of p53. Cancers might also be involved in gene silencing. Immense care will be needed in making drugs to counteract cancers, in view of the 'shocking possibility' that ageing is a side-effect of natural safeguards protecting us from cancer (Ferbeyre and Lowe, 2002).

Circadian and other central and peripheral rhythms

Immense amounts of data, and not a few surprises, have emerged in studies on other timing systems, especially circadian rhythms. Their importance in reproductive phenomena and many other systems cannot be overstressed. They are manifest in locomotion, feeding, sleep/wake cycles and many metabolic and physiological processes (McDonald and Rosbash, 2001). Four gene families are known. One unique aspect of these rhythms is their apparent control by a single central system, at least in *Drosophila*, while genetic analyses also reveal how a single gene knock-out can effectively subvert all circadian rhythms in mice (Bunger et al., 2000).

Circadian rhythms are established by translational and transcriptional feedback loops regulating a central circadian pacemaker. Their nature was summed up as positive and negative elements of autoregulating systems of translational and transcriptional control (Shearman et al., 2000). In *Drosophila*, the positive limb of this loop is driven by the transcription

factors *CLOCK* (*CLK*) and *CYCLE* (*CYC*). Orthologous mammalian genes with similar functions include the transcription factors *CLK* and *BMAL1*. Heterodimers of *CLK:CYC* bind to E boxes to activate two genes directly involved in rhythms: the three *Period* genes, *mPer-1*, 2 and 3, and two so-called *cryptochrome* genes, *Cry1* and 2. Their products then interrupt *CLK:CYC* function, to terminate transcription and close the circadian period. Regulation claimed by the mammalian gene *Timeless*, apparently homologous with the *Drosophila* gene, may be incorrect due to a false homology.

Mutations, natural or induced, in several genes reveal their roles in circadian rhythms and suprachiasmatic nucleus (SCN), the well known centre of such rhythmicity. Mutations in *Clock* lead to long circadian rhythms, because heterodimerization occurs but transcription does not, so *Per* and *Cry* genes become ineffective (Shearman et al., 2000). Another loop identified in mutants might regulate oscillatory rhythms. Mice lacking *Cry2* display reduced sensitivity to light induction in the SCN, and have a slightly extended circadian cycle. A raised transcription of *mPer1* and 2 and a slightly shortened circadian cycle characterize *Cry1^{-/-}* and *Cry2^{-/-}* deficient mice, which also display a blunted SCN response to the induction of *mPer1* in response to light (Vitaterna et al., 1999). The loss of *BMAL1* in mice invokes a total loss of circadian rhythms in constant darkness, with a loss of circadian rhythm at molecular level, and *Bmal1^{-/-}* mice do not entrain to light responses (Bunger et al., 2000).

Differential display analyses applied to circadian gene expression in *Drosophila* identified 134 cycling genes (McDonald and Rosbash, 2001). Other clock-controlled genes may have been excluded by strict selection criteria or by the choice of probe sequences. Many of the cycling genes located in gene clusters, suggesting a role for genomic organization. They influenced potassium channels, protein kinases, calcium oscillations, resting membrane potential, and other aspects of *Drosophila* physiology. Some *CLK*-controlled transcripts were involved in detoxification, olfaction, clearance, neuropeptide signals, nutritive status and immunity. Others regulated K^+ channels or Ca^{2+} oscillations. *CLK* was apparently among the master transcriptional circadian regulators, perhaps controlling the activity of a few key transcription factors.

These gene systems are involved in the classic regulatory system identified in the SCN of mammals, where circadian rhythms are well known to be regulated by retinal signals passing via the retinal hypothalamic tract to the SCN. A second circadian system involves 'masking', possibly via a retinal agent, which suppresses locomotor effects independently of the SCN. Light influences both clocks. The SCN produces 32 SCN peptides or proteins mostly with unknown functions, some probably secreted as

diffusible substances into the third ventricle, perhaps as major projections to the nearby hypothalamic subparaventricular zone (SPZ). Kramer et al. (2001) assayed all 32 factors for their control of circadian activities especially locomotor responses, by means of 3-week infusions into the brains of hamsters, near the SCN. Transforming growth factor- α (TGF- α) was exceptional, preventing circadian running activity but without any other effects. Its mRNA levels in SCN displayed a circadian rhythm, high in daytime and low at night, comparable to those regulated by *Cry1* and *Cry2*. Epidermal growth factor (EGF) receptor is a common receptor for EGF and TGF- α , and many of them were identified in the subparaventricular nuclear zone. They were capable of influencing diurnal locomotor behaviour (SPZ) (Kramer et al., 2001). Infusing TGF- α did not modify REM and non-REM sleep, although it changed the sleep-wake cycle to 5–6 ultradian rhythms daily. It had simultaneously revealed the significance of the TGF- α controlled circadian rhythm while exposing underlying sleep-wake and temperature rhythms resembling those formed by stimulating SPZ neurons. Mutant mice generated with hypomorphic receptors and an intact hypothalamic system also developed excessive daytime locomotor activity. Their EGF receptors had seemingly been stimulated by retinal EGF photoreceptors with a distribution typical of transmission to the SPZ via the retino-hypothalamic tract. EGF receptor in the SPZ was clearly stimulated by TGF- α from the SCN, and by retinal EGF, to provide the basis of a circadian rhythm and a masking process respectively (Kramer et al., 2001). Two circadian inputs to EGF receptor thus explained the nature of the two circadian rhythms.

Reproductive and other hormones exert major effects on lifespans and ageing. Insulin-like growth factor (IGF)-1 is one such regulator in *C. elegans*, mediated via the insulin/IGF-1 receptor encoded by *daf-2*. Lifespans are more than double in carriers of *daf-2* mutants (Guarente and Kenyon, 2000). Other genetic systems regulating extended lifespans include *DAF-16*, a transcription factor, and ageing may be curtailed if it is down-regulated. Similar insulin/IGF-1 systems also operate in mice. Reproductive signalling may coordinate reproduction with the rate of ageing as in *Drosophila* where selecting for short- or long-lived strains has implied that germ line has a role in ageing. Reproductive systems are entrained to several rhythms including circadian rhythms. One clear example is the onset of the LH surge. Linked with circadian cortisol rhythms, it begins at 3 a.m. in four-fifths of women attending IVF clinics in winter. In the remainder, it onsets at various times of day, again in concert with the cortisol rhythm. Its onset is highly predictable in patients assessed in successive cycles. After long-distance travel, it requires between one and two menstrual cycles to adjust to the light times

(Edwards, 1985). It also has a seasonal component, which weakens its circadian rhythm during long hours of daylight.

Equally significant are the regulators of timing systems in somatic cells and tissues. Such programmes are an essential aspect of all organ systems, perhaps shown most clearly in successive developmental stages from fertilization onwards. Some of these systems are regulated by hormones. For example, the LH surge initiates a time-regulated maturation of oocytes in mammals, yet itself displays circadian and seasonal rhythms, as discussed above. This circadian timing is then imposed on developmental events in the oocyte, such as germinal vesicle breakdown, and the successive series of closely-timed stages of meiosis in oocytes and ovulation which take 37h in women. Clearly, maturation timing in the oocyte especially is set, ready for the circadian LH stimulus, and ovulation can be forecast to occur in most women in mid-afternoon in winter. Perhaps a satisfactory circadian rhythm contributes to good quality oocytes. The overall duration of maturation is highly regular in all oocytes of a species, with considerable species variation, e.g. it requires 11.5h in mice compared with 37.0h in humans (Edwards, 1965). These timings remain unchanged if ovulation is induced by an HCG injection, or occurs *in vitro*. Similar rules no doubt apply in men, where stages of spermatogenesis and spermiogenesis are highly regular (Leblond and Clermont, 1952; Sirlin and Edwards, 1957) although circadian effects have not been investigated.

Reproductive tissues also respond to rhythmic systems. The LH surge imposes luteinization on the follicle, to introduce a luteal phase lasting for a fairly standard time, varying between species. In cattle, for example, the luteolytic signal is known to involve prostaglandins. This is apparently not the case in humans, where the nature of the luteolytic signal is unknown. Earlier suggestions of a luteal clock timing the lifespan of corpora lutea were not followed up, yet today it would not be surprising if luteal cells timed their own demise. Several genes involved in timing are expressed in human oviductal and uterine tissues, e.g. the *Per* genes, *Cry1*, *Bmall*, *Clock*, and *Timeless* (Johnson, 2002).

Long-term rhythms also regulate ovarian oocytes and follicles. Some such control must regulate the timely migration of specific numbers of oocytes from the pool in the fetal ovary until menopause. Henderson and Edwards (1968) postulated that first-formed oocytes and follicles migrated first, the rest following in the sequence of their formation. Labelling later-formed oocytes in the mouse ovary led Crolla and Polani (19) to conclude this production line existed. Oocytes and embryos also express *Timeless*, their levels varying partly due to the timing of major transcription (Johnson et al., 2002). Members of the

four canonical gene families are each expressed, with *Per1*, *Cry1* and *Bmal1* persisting to the blastocyst. The development and differentiation of blastocysts and beyond also display considerable regularity, the widest variations arising in human embryos with those cleaving early having the highest implantation potential (Edwards et al., 1984). Timers regulating cell cycles also regulate fine details of cell function and differentiation, e.g. the control of a K^+ and other channels in oocytes (Johnson, 2002). Genes such as *ped* can modify cleavage timings, and developmental clocks are well known to arrest blastocyst growth at diapause in some species, associated with long-range timing factors such as lactation and seasonal breeding.

Conclusions

Perhaps it is time to accept the idea that germinal and embryonic cells possess their own timers. The SCN can be cultured *in vitro*, independent of neural inputs, and displays circadian rhythms for up to 32 days. Somatic cells of liver, lung and skeletal muscle cells also display such rhythms *in vitro*, being damped after 2–7 cycles *in vitro*. Perhaps *in vivo* the SCN pacemaker entrains oscillations in peripheral cells, to maintain their normal rhythms in a hierarchical manner while self-sustaining its own SCN oscillations (Yamazaki et al., 2000). In turn, the SCN may in turn be responsive to humoral signals.

It is also fascinating how immortalised fibroblasts grown over 25 years *in vitro* retain their timing systems, with high precision (Balsalobre et al., 1998). Exposing them to serum from various sources activates the circadian expression of *mper-1* and other genes which persists for 3 days. Adding serum to rat fibroblasts or hepatic cells also invokes a rhythmic expression of several genes known to oscillate and have circadian expressions *in vivo*. Circadian pacemakers typical of the SCN also exist in cultured mammalian cell lines. Positive and negative feedback loops involve *mPer-1*, 2 and 3 and *mCry1* and 2 (Yagita et al., 2001).

It is time to investigate timers and clocks in embryos in greater detail. They must be significant from all stages in ovarian oocytes, fertilization and in the control of hundreds of genes switching on and off between the oocyte and blastocyst (Ko et al., 2000). Implantation must also be a testing time to synchronize embryonic and endometrial clocks, especially since embryos are outside any central systemic influences while in oviduct and uterine cavities. We look forward to better knowledge of how all these systems are integrated and phase-linked with

other regulatory systems such as programmed cell death, polarities and integrating genes.

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