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Review

Open-minded scepticism: inferring the causal mechanisms of human ageing from genetic perturbations

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Abstract

Given the myriad of age-related changes and the many proposed mechanistic theories of ageing, a major problem in gerontology is distinguishing causes from effects. This review aims to identify and evaluate those mechanisms which have gathered experimental support in favour of seeing them as a cause rather than an effect of ageing. Recent results related to energy metabolism and ageing, the free radical and the DNA damage theories of ageing are reviewed and their predictions evaluated through a systems biology rationale. Crucial in this approach are genetic manipulations in animal models that enable researchers to discriminate causes from effects of ageing and focus on the causal structure of human ageing. Based on a system-level interpretation, the GH/IGF-1 axis appears the most likely explanation for caloric restriction and a possible causal mechanism of human ageing. Although much work remains to fully understand the human ageing process, there is little evidence that free radicals are a causal factor in mammalian ageing, though they may be involved in signalling pathways related to ageing. On the other hand, studying how the DNA machinery affects ageing appears a promising avenue for disclosing the human ageing process.

Keywords: Causes of ageing; DNA damage; Free radicals; Genetics of ageing; Gerontology; Metabolism

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1. Introduction

The human ageing process affects multiple organs and tissues (reviewed in Finch, 1990; Hayflick, 1994) and involves the progressive deterioration of virtually every bodily function (Austad, 1997; Strehler, 1999). Given the multiple effects of ageing, one major problem in gerontology is distinguishing causes from effects. Although there are many age-related changes, just because two processes are parallel to each other we cannot imply a causal relation in any direction (Strehler, 1986). Others have claimed before how the goal of gerontology should be to discriminate causes from effects of ageing and find the one or few physiological processes that control ageing (Medawar, 1955). This review aims to identify and debate those mechanisms which have gathered experimental support in favour of seeing them as a cause rather than an effect of ageing. Namely, mechanistic theories of ageing must explain the two major observations in gerontology (Finch, 1990; Hayflick, 1994): (1) what changes occur in adult humans to increase the chances of dying by over 30-fold? (2) Why different species age at markedly different paces?

Genetic manipulations in animal models enable researchers to discriminate causes from effects of ageing and focus on the causal structure of human ageing rather than merely describe effects of the ageing process. Yet, care must be taken when analysing such results. Since human ageing can be defined as the changes that render human beings progressively more likely to die (Medawar, 1952), one outcome of ageing is the exponential increase in mortality associated with chronological age, which was first recognized by Benjamin Gompertz. Yet, death can be unrelated to ageing. Consequently, changes in parameters such as longevity, which measures how long an organism is expected to live, and lifespan, which represents maximum longevity, may or may not be related to ageing. Since ageing results in an increase in mortality, many interventions affecting longevity are often confused with interventions that affect the ageing process (Hayflick, 1994). A careful evaluation of the mortality acceleration rate and age-related changes is necessary to determine whether a given intervention changes the rate of ageing or merely affects overall health.

2. Mechanistic theories of ageing

Despite a myriad of age-related changes and many proposed mechanistic theories of ageing, only a few mechanisms have been directly shown to influence ageing. For example, some genes appear capable of shifting the rate of ageing and age-related debilitation in mammals (Table 1). These can be grouped into three broad pathways: DNA metabolism (*CKN1*, *Lamin A*, *WRN*, *XPD*, *Terc*, *PASG*, *ATM*, and *p53*), energy metabolism and the growth hormone/insulin-like growth factor-1 (GH/IGF-1) axis (*GHR/BP*, *GHRHR*, *IGF-R1*, *Pit1*, *Prop1*, and *UPA*), and oxidative stress (*MSRA*, $p66^{shc}$, and *Thdx1*). Only one gene, *klotho*, does not fit into these pathways, though its functions remain mostly unknown (Kuro-o et al., 1997). So far, these are the only known mechanisms that may directly influence mammalian ageing and so are the ones that we may presently assume as capable of being part of the causal structure of human ageing. Therefore, this review will focus only

Table 1
Genes that appear to modulate ageing in mammals

Gene name	Common name	Phenotype	Primary reference
Mouse (*Mus musculus*)			
GHR/BP	Growth hormone receptor	Increase in lifespan of 40–50% in homozygous knock-outs	Coschigano et al. (2000)
GHRHR	Growth hormone releasing hormone receptor	Lifespan increase of about 20% in homozygous knock-out mice	Flurkey et al. (2001)
IGF-R1	Insulin-like growth factor type 1 receptor	Heterozygous mice live 26% longer than wild-type	Holzenberger et al. (2003)
klotho	Klotho	Possible accelerated ageing phenotype of homozygous knock-outs	Kuro-o et al. (1997)
MSRA	Methionine sulfoxide reductase A	Decreased lifespan and possible accelerated ageing in homozygous knock-outs	Moskovitz et al. (2001)
p53	Tumour protector 53	Heterozygous mutant mice display signs of accelerated ageing	Tyner et al. (2002)
$p66^{shc}$	$p66^{shc}$ (SHC1)	Roughly 30% increase in lifespan in −/− mice	Migliaccio et al. (1999)
PASG	Proliferation associated SNF2-like gene	Possible accelerated ageing phenotype due to disruption	Sun et al. (2004)
Pit1	Snell dwarf mouse	Lifespan increase of 42% in homozygous mice	Flurkey et al. (2001)
Prop1	Ames dwarf mouse	Homozygous mice show over 50% increases in lifespan	Brown-Borg et al. (1996)
Terc + ATM	Telomerase RNA component + ataxia telangiectasia mutated	Possible accelerated ageing in double mutant mice	Wong et al. (2003)
Thdx1	Thioredoxin	35% increase in average longevity in transgenic mice	Mitsui et al. (2002)
UPA	Urokinase-type plasminogen activator	Roughly 20% increase in lifespan of transgenic mice	Miskin and Masos (1997)
XPD	Xeroderma pigmentosum, group D	Possible accelerated ageing phenotype due to homozygous mutation	de Boer et al. (2002)
Human (*Homo sapiens sapiens*)			
CKN1	Cockayne syndrome Type I	Possible premature ageing due to recessive mutation	Henning et al. (1995)
WRN	Werner syndrome	Possible accelerated ageing due to recessive mutation	Yu et al. (1996)
Lamin A	Hutchinson–Gilford syndrome	Possible premature ageing due to dominant mutation	Eriksson et al. (2003)

on these three pathways: I will first succinctly present these pathways and the theory behind them and then debate their impact on ageing through a systems biology approach.

2.1. From the energy consumption hypothesis to neuroendocrine signal transduction

In 1908, physiologist Max Rubner discovered a relationship between metabolic rate, body size, and longevity. In brief, long-lived animal species are on average bigger and spend fewer calories per gram of body mass than smaller, short-lived species. The energy consumption hypothesis states that animals are born with a limited amount of some substance, potential energy, or physiological capacity and the faster they use it, the faster they will die (Hayflick, 1994). Later, this hypothesis evolved into the rate of living theory: the faster the metabolic rate, the faster the biochemical activity, the faster an organism will age. In other words, ageing derives from the pace at which life is lived (Pearl, 1928). This theory is in accordance with the life history traits of mammals in which long lifespan is associated with delayed development and slow reproductive rates (reviewed in Austad, 1997).

Despite its intuitive concept, several experiments have cast doubts on the rate of living theory. For instance, mice kept at lower temperatures eat 44% more than control mice and yet do not age faster (Holloszy and Smith, 1986). Marsupials have lower body temperatures than eutherians but, on average, age faster. In contrast, birds have higher body temperatures and metabolic rates than mammals and yet, on average, age slower. Although a correlation between metabolic rates and rate of ageing can be found amongst some mammalian species, even correcting for metabolism, rate of ageing varies widely amongst mammals (Austad and Fischer, 1991; Austad, 1997). As such, the ideas proposed by Rubner and Pearl have been mostly discarded. Even so, their concepts paved the way for our current view of the impact of energy metabolism on ageing and age-related disease.

Probably the biggest discovery so far in the biology of ageing was made in 1935, following earlier findings, by veterinary nutritionist Clive McCay and colleagues: they discovered they could slow ageing in laboratory rats by making them eat less calories while maintaining normal levels of proteins, vitamins, and minerals (McCay et al., 1935). This process became known as caloric restriction (CR) and appears to work in many animals; it has been particularly well-studied in mice. From mouse studies we know CR not only increases longevity and lifespan but it also postpones age-related diseases, decreases the rate of ageing, and delays development (reviewed in Weindruch and Walford, 1988). Doubts have for long existed on whether CR results from some technical artefact. For example, perhaps life-extension due to CR derives from alterations in body fat (Bluher et al., 2003 for arguments). Nonetheless, CR remains the most impressive way to delay ageing in mammals, particularly since it derives from a very simple intervention.

Since CR involves a decrease in calories, one obvious hypothesis is that CR works by delaying metabolic rates. Yet, results are contradictory. On one side, since the rate of chemical reactions rises with temperature, the decrease in body temperature of animals under CR, such as mice, rats, and monkeys (Weindruch and Walford, 1988; Ramsey et al., 2000), is consistent with the view CR shifts metabolic pathways (Duffy et al., 1990). In contrast, some evidence indicates that mice under CR burn the same amount of energy per unit lean mass as controls (McCarter and Palmer, 1992). Similar results have been reported

in rhesus monkeys (Lane et al., 1995). These and similar studies, however, remain controversial in the way metabolic rate – in this case measured indirectly through O_2 consumption – is normalized to metabolic mass (Greenberg and Boozer, 2000) and so whether CR changes or not metabolic rate remains under debate.

CR affects the endocrine system and, for instance, decreases the plasma levels of IGF-1 and increases the levels of growth hormone (Sonntag et al., 1999). Interestingly, several genes have been identified in model organisms whose mutation results in a phenotype similar to CR (Table 2). At least four of these appear to mimic CR in generally affecting body size, growth hormone and IGF-1, and body temperature (reviewed in Bartke et al., 2001a): *Pit1* (Flurkey et al., 2001), *Prop1* (Brown-Borg et al., 1996), *GHR/BP* (Coschigano et al., 2000), and *GHRHR* (Flurkey et al., 2001). Therefore, one plausible hypothesis is that energy depletion leads to neuroendocrine adaptations that, probably indirectly, affect ageing. The GH/IGF-1 axis may thus be related to ageing. By combining CR and mutations of one of these genes – the *Prop1* gene – however, we witness an even greater increase in longevity (Bartke et al., 2001b), so even though CR may partly operate through the GH/IGF-1 pathway, other mechanisms might be involved (Shimokawa et al., 2003; Tsuchiya et al., 2004).

2.2. Free radical theory of ageing

The idea that free radicals are toxic agents was first suggested by Gerschman et al. (1954). In 1956, Denham Harman developed the free radical theory of ageing (Harman, 1956). Free radicals and oxidants – such as singlet oxygen that is not a free radical – are commonly called reactive oxygen species (ROS) and are such highly reactive molecules that can damage all sorts of cellular components. ROS can originate from exogenous sources such as ultraviolet (UV) and ionising radiations or from several intracellular sources. Since oxidative damage of many types accumulate with age, the free radical theory of ageing argues that ageing results from the damage generated by ROS (reviewed at length in Beckman and Ames, 1998).

To protect against oxidation there are many different types of antioxidants, from Vitamins C and E to enzymes such as superoxide dismutase (SOD), catalase, and glutathione peroxidase. Briefly, antioxidant enzymes are capable of degrading ROS into inert compounds through a series of chemical reactions (Halliwell, 2001). In addition to antioxidants, some enzymes catalyze the repair caused by ROS. One of such enzymes is methionine sulfoxide reductase A (MSRA), which catalyzes the repair of protein-bound methionine residues oxidized by ROS. The simple existence of enzymes to prevent and repair damage by ROS is a strong indicator that ROS are biologically important, dangerous molecules.

Most experimental evidence in favour of the free radical theory of ageing comes from invertebrates (Table 3). Transgenic fruit flies, *Drosophila melanogaster*, overexpressing the cytoplasmic form of SOD, called Cu/ZnSOD or SOD1, and catalase have a 34% increase in average longevity and a delayed ageing process (Orr and Sohal, 1994). Also in *Drosophila*, expression of SOD1 in motor neurons increases longevity by 40% (Parkes et al., 1998). Certain long-lived strains of *Drosophila* (Hari et al., 1998) and the nematode worm *Caenorhabditis elegans* (Larsen, 1993) have increased levels of antioxidant

Table 2
Genes involved in metabolism or the GH/IGF-1 axis and their impact on the ageing process

Gene name	Phenotype	Function	Organism	Main reference
atp-3	Perturbing mitochondrial function during development extended lifespan	Subunit of mitochondrial ATP synthase	*C. elegans*	Dillin et al. (2002)
Ceinsulin-1	Disruption extends lifespan	Homologue of mammalian insulin/insulin-like growth factor	*C. elegans*	Kawano et al. (2000)
chico	Mutation extends longevity	Insulin receptor substrate	*D. melanogaster*	Clancy et al. (2001)
daf-2	Mutation extends lifespan	Insulin pathway	*C. elegans*	Wolkow et al. (2000)
eat-2	Mutants show increased longevity	Nicotinic acetylcholine receptor subunit	*C. elegans*	Lakowski and Hekimi (1998)
F26E4.6	Impaired mitochondria was associated with longer lifespan	Component of the mitochondrial complex IV	*C. elegans*	Lee et al. (2003)
GH-1	Laron syndrome; decrease in longevity	Tissue proliferation	*H. sapiens*	Besson et al. (2003)
GHR/BP	Knock-outs have an increase in lifespan	GH transmembrane receptor	*M. musculus*	Coschigano et al. (2000)
GHRHR	Knock-outs have an increase in lifespan	Regulation of GH synthesis and secretion in the pituitary	*M. musculus*	Flurkey et al. (2001)
IGF-R1	Heterozygous mice have increased longevity	Proliferation and growth	*M. musculus*	Holzenberger et al. (2003)
IGF-R1	Intrauterine growth retardation, mental retardation, amongst other features	Proliferation and growth	*H. sapiens*	Roback et al. (1991)
Indy	Mutations lead to extended lifespan that could mimic CR	Unknown	*D. melanogaster*	Rogina et al. (2000)
InR	Mutation results in dwarf animals and extended longevity	Insulin-like receptor	*D. melanogaster*	Tatar et al. (2001)
Pit1	Lifespan increase in homozygous animals	Pituitary-specific transcription factor	*M. musculus*	Flurkey et al. (2001)
Pit1	Combined pituitary hormone deficiency	Pituitary-specific transcription factor	*H. sapiens*	Radovick et al. (1992)
Prop1	Lifespan increase in homozygous animals	Pituitary transcriptional activation	*M. musculus*	Brown-Borg et al. (1996)
Prop1	"Little People" of Krk; mutations might increase longevity	Pituitary transcriptional activation	*H. sapiens*	Krzisnik et al. (1999)
UPA	Transgenic animals showed an increase in lifespan	Appetite suppressor	*M. musculus*	Miskin and Masos (1997)

Table 3
Genes involved in oxidant levels and redox potential and their impact on the ageing process

Gene name	Phenotype	Function	Organism	Main reference
CAT	Use of catalase mimetics led to an increase in lifespan	Anti-oxidant enzyme	*C. elegans*	Melov et al. (2000)
CAT	Catalase and SOD1 overexpression led to a slower rate of ageing	Anti-oxidant enzyme	*D. melanogaster*	Orr and Sohal (1994)
CAT	No effects witnessed in nucleus-targeted overexpression	Anti-oxidant enzyme	*M. musculus*	Schriner et al. (2000)
CAT	Hereditary catalase deficiencies	Anti-oxidant enzyme	*H. sapiens*	Goth and Eaton (2000)
CLK1	Mutations extend lifespan	Mitochondrial inner membrane protein directly involved in ubiquinone biosynthesis	*C. elegans*	Ewbank et al. (1997)
GPX	Deficient animals are apparently healthy	Anti-oxidant enzyme	*M. musculus*	Ho et al. (1997)
GPX	Overexpression leads to insulin resistance and obesity	Anti-oxidant enzyme	*M. musculus*	McClung et al. (2004)
GSR	Overexpression increased longevity under hyperoxia but nor normoxia	Anti-oxidant enzyme	*D. melanogaster*	Mockett et al. (1999)
MSRA	Knock-out decreases lifespan and accelerates ageing	Oxidative-damage repair	*D. melanogaster*	Ruan et al. (2002)
MSRA	Knock-out decreases lifespan and may accelerate ageing	Oxidative-damage repair	*M. musculus*	Moskovitz et al. (2001)
$p66^{shc}$	Mutants feature an increase in lifespan	Apoptosis and redox potential	*M. musculus*	Migliaccio et al. (1999)
Prdx1	Mutants feature decreased lifespan but probably not premature ageing	Anti-oxidant	*M. musculus*	Neumann et al. (2003)
SOD	Use of SOD mimetics led to an increase in lifespan	Anti-oxidant enzyme	*C. elegans*	Melov et al. (2000)
SOD1	Overexpression led to an increase in longevity	Anti-oxidant enzyme	*D. melanogaster*	Parkes et al. (1998)
SOD1	Overexpression did not extend lifespan	Anti-oxidant enzyme	*M. musculus*	Huang et al. (2000)
SOD1	Inverse correlation between lifespan and SOD1 expression levels	Anti-oxidant enzyme	*Lasius niger*	Parker et al. (2004)
SOD2	The longevity and ageing process of heterozygous animals was not affected	Anti-oxidant enzyme	*M. musculus*	Van Remmen et al. (2003)
Thdx1	Transgenic animals had increased longevity	Redox-active protein	*M. musculus*	Mitsui et al. (2002)

enzymes. Recent results from ants, however, found a decreased SOD1 expression in long-lived animals (Parker et al., 2004). Lastly, overexpression of MSRA in the nervous system of *Drosophila* increases longevity and delays age-related decline, supporting the free radical theory of ageing (Ruan et al., 2002).

One intriguing discovery was that targeted mutation of $p66^{shc}$ in mice increases longevity by about 30%, inducing resistance to oxidative damage, and maybe delaying ageing (Migliaccio et al., 1999). Although the exact functions of $p66^{shc}$ remains unclear, some evidence suggests it may be related to intracellular oxidants and apoptosis (Trinei et al., 2002). In another intriguing experiment, transgenic mice overexpressing the human thioredoxin gene featured an increased resistance to oxidative stress and an extended longevity of 35% (Mitsui et al., 2002). Like $p66^{shc}$, thioredoxin regulates the redox content of cells but, contrary to $p66^{shc}$, is thought to have anti-apoptotic effects (Kwon et al., 2003). Moreover, injection of spin trapping agents, which quench the more reactive radicals to produce long-lived stable radical adducts, into animals of a strain called senescence-accelerated mice extended longevity (Edamatsu et al., 1995). Lastly, since ROS can originate in the cellular metabolism taking place in the mitochondrion, the cell's energy source, then perhaps the free radical theory can be combined with the energy consumption hypothesis. Indeed, one proposed mechanism for CR is that animals under CR produce less ROS and therefore age slower (Weindruch, 1996).

Although there is evidence to support the role of ROS in ageing, there is also experimental evidence against it. Overexpressing bovine SOD2, the mitochondrial form of SOD, also called MnSOD, in *Drosophila* slightly extends average longevity but does not delay ageing (Fleming et al., 1992). In fact, recent findings suggest that the influence of SOD1 and catalase in *Drosophila* ageing might have been overestimated because it only took into account short-lived strains (Orr et al., 2003). Experiments in feeding mice antioxidants – either a single compound or a combination of compounds – were able to decrease oxidative damage and increase average longevity of animals but none of them delayed ageing (Comfort et al., 1971; Heidrick et al., 1984; Saito et al., 1998; Holloszy, 1998). These results suggest that antioxidant proteins are already optimized in mammals. Indeed, correlations between rate of ageing and antioxidant levels in mammals are, if they exist, very weak (reviewed in Finch, 1990; Sohal and Weindruch, 1996). Some studies found correlations between the levels of certain antioxidants and longevity in mammals, but failed to find any consensus (Tolmasoff et al., 1980; Cutler, 1985; Sohal et al., 1990).

One hypothesis is that the rate of mitochondrial ROS generation rather than the antioxidant level may act as a longevity determinant (Sohal and Brunk, 1992; Barja, 2002b). Some results suggest that the rate of ROS generated in the mitochondria of post-mitotic tissues helps explain the differences in lifespan amongst some animals, particularly amongst mammals (Ku et al., 1993; Barja and Herrero, 2000) and between birds and mammals (reviewed in Barja, 2002a). One pitfall of these studies is that technical limitations exist in measuring ROS production in isolated mitochondria. For example, none of these studies measured the levels of hydroxyl radical, the most reactive and destructive of the ROS; often, hydrogen peroxide and superoxide anion are measured since they can react to give the hydroxyl radical and so such studies may not be representative of what actually occurs. In addition, one recent study in *Drosophila* found that lowering ROS leakage from the mitochondria through over-expression of the mitochondrial adenine

nucleotide translocase did not result in extended longevity. The same study failed to find differences in ROS production in CR flies despite these living longer (Miwa et al., 2004). Finally, an involvement in ageing of the mitochondrion has been described in invertebrates (Dillin et al., 2002), but it is not clear whether longer lifespan can be assigned to a lower free radical production and whether oxidative damage can be coupled to metabolism and longevity (Lee et al., 2003). Similarly, although accelerated ageing has been described in mice expressing a defective mitochondrial DNA polymerase, the authors found no evidence that this phenotype originates from increased susceptibility to oxidative damage (Trifunovic et al., 2004).

2.3. DNA damage as a causal mechanism of ageing

The DNA, due to its central role in life, is bound to be implicated in ageing. As arguably first proposed by physicist Leo Szilard, one hypothesis is that damage accumulation to the DNA causes ageing (Szilard, 1959a,b). DNA mutations and chromosomal abnormalities increase with age in mice (Martin et al., 1985; Vijg, 2000; Dolle and Vijg, 2002) and humans (reviewed in Morley, 1995). Yet, it is impossible to tell whether these changes are effects or causes of ageing. Correlations have been found between DNA repair mechanisms and rate of ageing in some mammalian species (Grube and Burkle, 1992; Cortopassi and Wang, 1996), though this may be an artefact of long-lived species being on average bigger (Promislow, 1994). While radiation appeared to accelerate ageing in some cases (Henshaw et al., 1947; see Vijg, 2000 for arguments), embryos of mice and flies irradiated with X-rays do not age faster (Cosgrove et al., 1993; see Strehler, 1999 for arguments), though one report argued that Chernobyl victims do (Polyukhov et al., 2000).

One of the most intriguing phenotypes in the biology of ageing is the accelerated ageing witnessed in humans and animals as a result of certain mutations. Progeroid syndromes, as they are called, are rare genetic diseases of which the two most impressive forms are Werner's (WS) and Hutchinson–Gilford's syndromes (Martin and Oshima, 2000). Both these diseases originate a phenotype that is remarkably similar to an accelerated ageing process, particularly WS (Goto, 1997). Though differences exist in terms of pathology, what most markedly distinguishes these syndromes is age of onset with Hutchinson–Gilford's syndrome almost exclusively affecting children while WS patients normally reach adulthood.

Werner's syndrome originates in a recessive mutation in a gene, *WRN*, encoding a RecQ helicase (Yu et al., 1996; Gray et al., 1997). Since WRN is unique amongst its family in also possessing exonuclease activity (Huang et al., 1998), it may be involved in DNA repair. Although the exact functions of WRN remain a mystery, it is undeniable that WRN plays a role in DNA biology, particularly on unusual DNA structures (reviewed in Shen and Loeb, 2000; Bohr et al., 2002). In fact, cells taken from patients with WS have increased genomic instability (Fukuchi et al., 1989). WS cells are hypersensitive to topoisomerase inhibitors (Pichierri et al., 2000a,b). Since topoisomerases are enzymes that regulate the supercoiling in duplex DNA, WS is an indicator that alterations in the DNA over time play a role in ageing.

As with WRN, the protein whose mutation causes Hutchinson–Gilford's syndrome is a nuclear protein: lamin A/C (Eriksson et al., 2003). Recent results also suggest that some atypical cases of WS may be derived from mutations in lamin A/C (Chen et al., 2003). The exact functions of lamin A/C remain unknown, but it appears to be involved in the biology

of the inner nuclear membrane. Further evidence suggests that the DNA machinery is impaired in Hutchinson–Gilford's syndrome (Wang et al., 1991; Sugita et al., 1995), again suggesting that changes in the DNA are important in normal ageing.

Other segmental progeroid syndromes exist, though the classification is subjective (Table 4). For example, Nijmegen breakage syndrome, which derives from a mutated DNA double-strand break repair protein (Varon et al., 1998), has been considered as progeroid (Martin and Oshima, 2000). Cockayne syndrome Type I may also be accelerated ageing and *CKN1* participates in transcription and DNA metabolism (Henning et al., 1995). Murine accelerated ageing syndromes have also been implicated in DNA repair such as the mouse homologues of xeroderma pigmentosum, group D (de Boer et al., 2002), ataxia telangiectasia mutated or ATM (Wong et al., 2003), *p53* (Donehower, 2002; Tyner et al., 2002), and *Ercc1* (Weeda et al., 1997). Accelerated chromosomal aberrations have also been reported in senescence-accelerated mice (Nisitani et al., 1990).

One possibility is that ROS damage DNA and some evidence exists showing an increase in oxidative damage to DNA with age (Hamilton et al., 2001). Since ROS originates in the mitochondria, and since mitochondria possess their own genome, many advocates of the free radical theory of ageing consider that ROS damage to mitochondrial DNA (mtDNA) is critical to ageing (Harman, 1972; Linnane et al., 1989; Barja, 2002a). For example, some evidence exists suggesting that under CR oxidative damage to mtDNA is more important than oxidative damage to nuclear DNA (reviewed in Barja, 2002a). On the other hand, there are contradictory results in terms of age-related declines in mitochondria (Khaidakov et al., 2003; Rasmussen et al., 2003) and current technology does not appear capable of assessing the true relevance of damage to mtDNA in ageing (Lightowlers et al., 1999). Moreover, little direct evidence exists to support the notion that mitochondria are a causal factor in human ageing. As aforementioned, even though some evidence suggests that mitochondria are involved in ageing of invertebrates (Dillin et al., 2002), it is not clear whether longer lifespan can be assigned to a lower free radical production (Lee et al., 2003). Although mice expressing a defective mitochondrial DNA polymerase appear to age faster, the mechanistic basis for this phenotype is not clear (Trifunovic et al., 2004). Therefore, and although the mtDNA may play a role in age-related diseases, its role in ageing remains unproven.

3. Genetic perturbations of ageing: a system-level approach

Given the large number of age-related changes, we should be open-minded to theories of ageing but also evaluate each one with scepticism. Herein, I will follow a system-level approach in which genetic manipulations in animal models are a crucial tool. Obviously, one important, and old, debate concerns the relevance of model organisms to study human ageing. Ideally, only data obtained from human studies should be used to infer the causal mechanisms of human ageing, but that is clearly impossible. As such, I will solely focus on mammalian models for they, particularly mouse studies, provide numerous clues about ageing while being evolutionary and biologically close to humans to minimize possible errors. Since the discussion of paradigms in aging research is not the subject of this review, the use of mammalian models appears a reasonable compromise. Therefore, the systems biology in this review consists of three steps: (1) define, to the best of knowledge, the

Table 4
Genes involved in DNA metabolism and their impact on the ageing process

Gene name	Phenotype	Function	Organism	Main reference
ATM	Mutations may accelerate ageing	DNA damage recognition	*M. musculus*	Wong et al. (2003)
ATM	Ataxia telangiectasia mutated	DNA damage recognition	*H. sapiens*	Gatti et al. (1991)
CKN1	Cockayne syndrome type I; possible premature ageing	DNA repair	*H. sapiens*	Henning et al. (1995)
clk-1	Mutations extend lifespan	Regulation of transcription	*C. elegans*	Ewbank et al. (1997)
DNA-PK	Severe combined immune-deficient mice	DNA repair	*M. musculus*	Araki et al. (1997)
Ercc1	Disruption may accelerate ageing phenotype	DNA repair	*M. musculus*	Weeda et al. (1997)
Histone Deacetylase 1	Heterozygous feature extended lifespan	Regulation of transcription	*D. melanogaster*	Rogina et al. (2002)
Lamin A	Hutchinson-Gilford syndrome; possible premature ageing	Unclear but may be involved in DNA metabolism	*H. sapiens*	Eriksson et al. (2003)
Nibrin	Nijmegen breakage syndrome	DNA double-strand repair	*H. sapiens*	Varon et al. (1998)
p53	Heterozygous mutants display signs of accelerated ageing	Tumour suppressor	*M. musculus*	Tyner et al. (2002)
p53	Germline mutations cause Li-Fraumeni syndrome	Tumour suppressor	*H. sapiens*	Varley (2003)
PARP	Correlation between PARP levels and lifespan	Chromatin-associated enzyme involved in DNA repair	Mammals	Grube and Burkle (1992)
PASG	Proliferation associated SNF2-like gene	DNA methylation and maybe DNA repair	*M. musculus*	Sun et al. (2004)
Sir-2	Increased dosage extends lifespan	Epigenetic gene silencing	*C. elegans*	Tissenbaum and Guarente (2001)
Sirtuin 1	Knock-out animals died shortly after birth	Sir-2 homologue	*M. musculus*	McBurney et al. (2003)
Terc	Mutations may accelerate ageing	Elongating the telomeres	*M. musculus*	Wong et al. (2003)
Terc	Mutations lead to Dyskeratosis congenita	Elongating the telomeres	*H. sapiens*	Vulliamy et al. (2001)
top3b	Disruption decreases lifespan though it may not affect ageing	DNA topoisomerase III beta; chromosomal structure	*M. musculus*	Kwan and Wang (2001)
WRN	Werner syndrome, possible accelerated ageing	Helicase and exonuclease	*H. sapiens*	Yu et al. (1996)
XPD	Mutations may accelerate ageing	DNA repair	*M. musculus*	de Boer et al. (2002)
XPD	Xeroderma pigmentosum, group D	DNA repair	*H. sapiens*	Stefanini et al. (1986)

pathway under study and its components; (2) perturb each pathway component by genetic manipulation; and (3) integrate the observed responses to formulate new hypothesis (Ideker et al., 2001). In other words, after previously showing the theoretical basis of the most important theories of ageing and defining the components of the pathways under study, i.e. step (1), I will now integrate the observations derived from genetic perturbations of each component of the pathways. A more detailed overview of the impact of these genes on ageing and their relevance to human biology is available online at the GenAge database, a curated database of genes related to human ageing (http://genomics.senescence.info/genes).

3.1. The causal structure of CR

As mentioned earlier, both the GH/IGF-1 axis and ROS production appear to change in CR. Hypotheses aiming to explain CR based on either mechanism are theoretically sound. Yet, while a clear cause–effect phenotype is witnessed when manipulating the components of the GH/IGF-1 axis (Table 2), such is not the case for ROS and antioxidants (Table 3). In other words, contrary to what is witnessed with pathways related to antioxidant defences, genetic manipulations of the components of the GH/IGF-1 pathway result not only in a phenotype similar to CR but also in a series of physiological and mechanistic changes that are similar to what occurs in CR (reviewed in Bartke et al., 2001a). A clear overlap between mechanisms of life-extension in CR and the GH/IGF-1 axis occurs, even if the possibility of other mechanisms playing a role in CR exists (Shimokawa et al., 2003; Tsuchiya et al., 2004). Therefore, the GH/IGF-1 axis, not oxidative damage, is the main candidate for explaining CR.

3.2. Energy metabolism and ageing

As mentioned earlier, the way animals with changes in the GH/IGF-1 axis develop a phenotype similar to CR clearly supports the view that CR derives, at least partly, from the GH/IGF-1 axis (Table 2). Even though the mechanisms by which energy restriction regulates the neuroendocrine systems remain obscure, CR appears to operate through a neuroendocrine signalling cascade of which the GH/IGF-1 axis is a pivotal component (reviewed in Berner and Stern, 2004). One possibility is that food shortage triggers a delay in development aiming to postpone reproduction to more favourable conditions, which as a side-effect, extends lifespan (Holliday, 1989). Energy metabolism, the GH/IGF-1 axis, and associated neuroendocrine mechanisms should thus influence mammalian ageing. Mutations in energy metabolism or components of the GH/IGF-1 pathway extend longevity and may delay ageing (Fig. 1; Table 2), clearly supporting the role of energy usage and GH/IGF-1 on ageing. Yet, even though the impact of the GH/IGF-1 axis on ageing appears well-conserved amongst animal models, it remains to be seen whether the influence of the GH/IGF-1 axis on ageing is preserved in humans (Krzisnik et al., 1999).

In conclusion, energy metabolism and the GH/IGF-1 axis appear as two of the few pieces of the ageing process's puzzle that we know of, even though their true relevance to human ageing remains unknown.

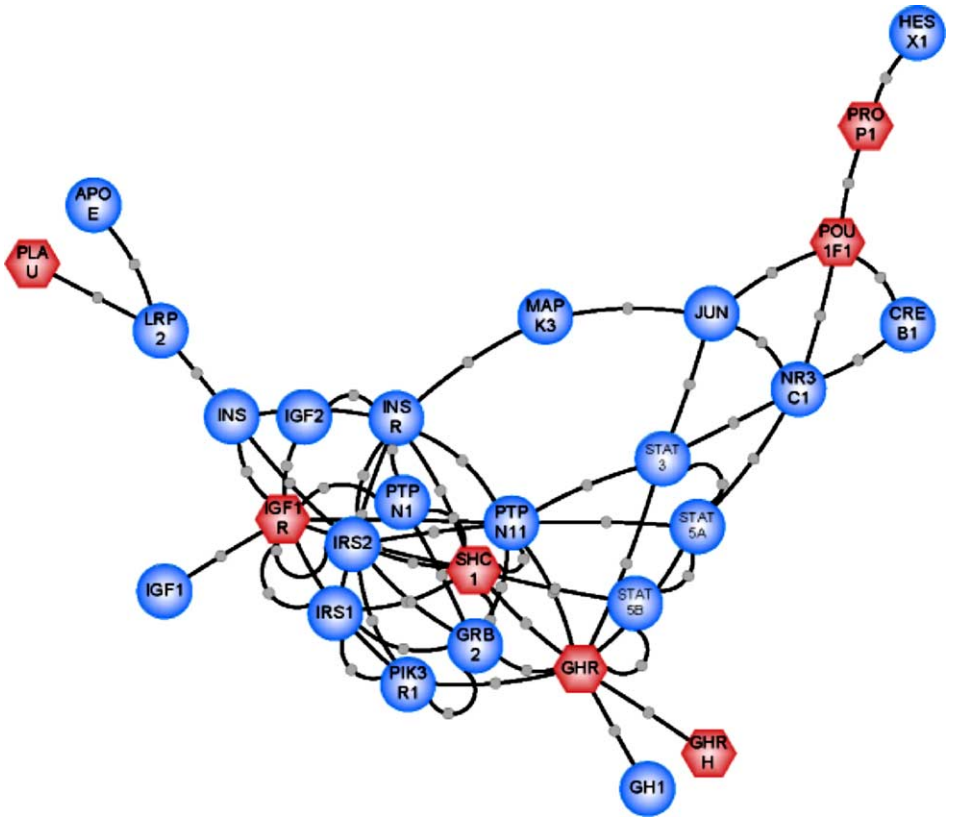

Fig. 1. Protein interaction map of the GH/IGF-1 pathway and associated proteins. Proteins directly linked to ageing in mice through genetic perturbations (red hexagons) as well as proteins associated with these (blue circles) are represented using the HUGO nomenclature. Lines represent published protein–protein interactions derived from the Human Protein Reference Database (http://www.hprd.org/). Calculated using the GenAge database (http://genomics.senescence.info/genes) and having as basis the GH/IGF-1 axis proteins influencing ageing: PLAU, IGF1R, GHR, GHRHR, PROP1, and POU1F1 (Pit1). Network layout computed using the PathwayAssist Software 2.5 (Stratagene, La Jolla, CA).

3.3. Oxidative damage and ageing

Deemed by many as the fundamental cause of ageing, the free radical theory of ageing has faced several hurdles, namely in its inability to modulate mammalian ageing. Several attempts have been made to overexpress or knock-out antioxidants in mice, but the results have been largely disappointing (Table 3). In several cases, animals do not show any differences in their ageing phenotype when compared to controls (Reaume et al., 1996; Ho et al., 1997; Schriner et al., 2000). For instance, ubiquitous overexpression of SOD1 in mice failed to increase longevity (Huang et al., 2000). Mice without MSRA have a decreased longevity of about 40% but whether their ageing process is affected remains to be seen (Moskovitz et al., 2001). Inactivation of peroxiredoxin 1, an antioxidant enzyme, shortens longevity in mice though these do not appear to age faster but rather develop more

tumours and often feature severe haemolytic anaemia probably derived from protein oxidation in erythrocytes (Neumann et al., 2003). Another enzyme that repairs oxidative damage is 8-oxo-dGTPase, which repairs 8-oxo-7,8-dihydroguanine, an abundant and mutagenic form of oxidative DNA damage. Again in disagreement with the free radical theory of ageing, knocking out the gene responsible for 8-oxo-dGTPase in mice, although resulting in an increased cancer incidence, did not alter the ageing phenotype (Tsuzuki et al., 2001). Recently, mice with extra catalase in their mitochondria lived 18% more than controls and were less likely to develop cataracts. Yet, it is not known whether these catalase-enhanced mice actually age slower (Martin, 2003). Moreover, mice heterozygous for SOD2 showed increased oxidative damage at a cellular and molecular level but did not show significant changes in longevity or rate of ageing (Van Remmen et al., 2003).

Since these are complex systems involving multiple components, investigating single enzymes might not guarantee an accurate holistic view of the biological processes involved. As previously mentioned, one hypothesis is that ROS production rather than antioxidant defence systems regulates the ageing process. Interestingly, several pathologies exist in mice and humans derived from mutations affecting the mitochondrion, which often involve an increase in ROS leakage (Wallace, 1999; Halliwell, 2001; DiMauro and Schon, 2003). Yet, these pathologies do not yield an accelerated ageing phenotype, but frequently result in diseases of the central nervous system. One example is Friedreich's ataxia, which appears to derive from increased oxidative stress in the mitochondria and does not resemble accelerated ageing (Wong et al., 1999). Deficiency of the mitochondrial complex I has been reported in a variety of pathologies such as neurodegenerative disorders (reviewed in Robinson, 1998). Cytochrome *c* deficiency has also been associated with neurodegenerative disorders (reviewed in DiMauro and Schon, 2003) as has selective Vitamin E deficiency (Burck et al., 1981). Perhaps ROS are involved in some pathologies involving post-mitotic cells, such as neurons; another alternative is that mitochondrial diseases affect mainly the central nervous system due to its high energy usage (Parker, 1990 for arguments). Intriguingly, both *Drosophila* and *C. elegans* are mostly composed of post-mitotic cells, which can explain why results from these invertebrates are much more supportive of the free radical theory of ageing than results from mice or observations in humans (Table 3).

Although it is undeniable that ROS play a role in several pathologies, including age-related pathologies such as neurodegenerative diseases, the exact influence of ROS in mammalian ageing is undetermined. In conclusion, there is very little evidence that ROS influence mammalian ageing and a sceptical mind cannot accept the free radical theory of ageing as a causal mechanism in human ageing.

3.3.1. Free radicals as messengers: a speculative hypothesis

Since redox status and ROS act as messengers in development and cellular growth (Berner and Stern, 2004), one hypothesis is that the decrease in oxidative damage observed in CR animals results from the delayed development and growth witnessed. For instance, SHC1 could play a role in the signal transduction derived from the GH/IGF-1 axis (Fig. 1) by mediating intracellular redox status, which then impacts on apoptosis and cellular proliferation (Trinei et al., 2002). It has been shown that GH suppresses components of the anti-oxidant defence systems and hence the suggestion that an increased oxidative damage contributes to ageing (Brown-Borg and Rakoczy, 2003). Yet, in view of the inability of

ROS to regulate mammalian ageing (Table 3), my suggestion is that redox and oxidative pathways should be seen as important signalling networks that impact on apoptosis, development and, indirectly, ageing. As such, oxidative damage should be seen mostly as a result rather than a cause of ageing. Assuming ROS as messengers explains why oxidative damage increases with age, why changes in antioxidants do not influence mammalian ageing, and why oxidative damage decreases in CR mice.

3.4. DNA alterations as a causal factor in ageing

Many mutations in DNA repair proteins have been associated with ageing in mammals. In fact, as previously mentioned, the most impressive human segmental progeroid syndromes originate in proteins that are part of the DNA machinery (Table 1). On the other hand, certain mutations in DNA repair proteins, such as *p53* in humans (Varley, 2003), despite affecting longevity and increasing cancer incidence, fail to accelerate ageing. Exemplifying, mice deficient in *Pms2* have elevated levels of mutations in multiple tissues and yet do not appear to age faster than controls (Narayanan et al., 1997). In addition, there are no cases of delayed ageing in mammalian models that could be related to increased DNA repair. For example, mice overexpressing p48, which is important in repairing DNA damage deriving from UV radiation, have improved DNA repair mechanisms and still do not live longer or age slower (Tang et al., 2000). Consequently, the DNA damage theory of ageing has so far failed to explain why different species age at different rates. The idea that DNA damage derived from ROS is involved in ageing is also debatable. As mentioned earlier, overexpression of catalase in the nucleus did not prevent oxidative damage to DNA (Schriner et al., 2000) and knocking out the gene responsible for 8-oxo-dGTPase failed to accelerate ageing (Tsuzuki et al., 2001). These results hint that the free radical and the DNA damage theories of ageing are not complementary.

As with antioxidant defence and ROS production, DNA repair mechanisms are multiple and so studying the effects of single proteins on the ageing process may not be representative of the impact the DNA machinery may have on ageing. If progeroid syndromes represent a phenotype of accelerated ageing then changes in DNA over time most likely play an important role in ageing. Nevertheless, the essence of those changes remains to be determined. Since many genetic perturbations affecting DNA repair do not influence ageing (Table 4), is doubtful overall DNA repair is related to ageing. Understanding which aspects, if any, of DNA biology play a role in ageing remains a great challenge in gerontology.

4. Conclusion

In a field such as gerontology, where probably all theories and hypothesis are unproven, it is crucial to keep an open-mind and we should accept all theories as possible. Yet, we must also judge every theory ruthlessly and doubt every assumption. The only way we can cut through the forest of theories of ageing is by being sceptic about every theory, as proposed before (Strehler, 1986). That is why only three mechanistic theories of ageing are reviewed herein. Clearly, the bulk of genes that appear to influence mammalian ageing can be related to the GH/IGF-1 axis, the free radical theory of ageing, or DNA metabolism (Table 1). Keeping

an open-mind, these mechanistic theories offer a preliminary theoretical framework, though a sceptic would rightfully argue that none truly addresses the major questions in gerontology. We still do not know what changes occur in adult humans to increase the chances of dying by over 30-fold or why different species age at markedly different paces.

Since ageing is a universal human feature, it is not surprising that from the dawn of civilization many have sought to avoid it. From the Babylonian epic of Gilgamesh, to Ponce de Leon seeking the ''fountain of youth'', countless men have dreamed of finding a way to avoid ageing. I suggest that studying development pathways, including neuroendocrine signals, and how the DNA machinery affects ageing appears the most promising avenue of research for disclosing the secrets of ageing.

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