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Review

Pleiotropic responses to methionine restriction

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ABSTRACT

Methionine restriction (MR) extends lifespan across different species. The main responses of rodent models to MR are well-documented in adipose tissue (AT) and liver, which have reduced mass and improved insulin sensitivity, respectively. Recently, molecular mechanisms that improve healthspan have been identified in both organs during MR. In fat, MR induced a futile lipid cycle concomitant with beige AT accumulation, producing elevated energy expenditure. In liver, MR upregulated fibroblast growth factor 21 and improved glucose metabolism in aged mice and in response to a high-fat diet. Furthermore, MR also reduces mitochondrial oxidative stress in various organs such as liver, heart, kidneys, and brain. Other effects of MR have also been reported in such areas as cardiac function in response to hyperhomocysteinemia (HHcy), identification of molecular mechanisms in bone development, and enhanced epithelial tight junction. In addition, rodent models of cancer responded positively to MR, as has been reported in colon, prostate, and breast cancer studies.

The beneficial effects of MR have also been documented in a number of invertebrate model organisms, including yeast, nematodes, and fruit flies. MR not only promotes extended longevity in these organisms, but in the case of yeast has also been shown to improve stress tolerance. In addition, expression analyses of yeast and *Drosophila* undergoing MR have identified multiple candidate mediators of the beneficial effects of MR in these models.

In this review, we emphasize other in vivo effects of MR such as in cardiovascular function, bone development, epithelial tight junction, and cancer. We also discuss the effects of MR in invertebrates.

Contents

1. Introduction . . . 84
2. Pleiotropic responses to MR . . . 84
   2.1. Cardiovascular effects of MR . . . 84
   2.2. MR affects bone development . . . 84
   2.3. MR improves epithelial tight junctions . . . 85
   2.4. MR alters cancer progression . . . 85
3. MR extends the lifespan of invertebrate organisms . . . 85
   3.1. Effects on *Drosophila* . . . 85
   3.2. Effects on *C. elegans* . . . 86
   3.3. Effects on yeast . . . 86
   3.4. MR confers stress tolerance to yeast . . . 86
   3.5. MR-dependent gene expression changes affect multiple pathways involved in determining longevity . . . 86
4. Perspectives and future directions . . . 87
Acknowledgments . . . 87
References . . . 87

Abbreviations: SAM, S-adenosylmethionine; Hcy, homocysteine; tHcy, total homocysteine; Bhmt, betaine-Hcy methyltansferase; Mthf, 5-methyltetrahydrofolate-Hcy methyltransferase; Cbs, cystathionine β-synthase; SAH, S-adenosylhomocysteine; DMG, dimethylglycine; HFD, high-fat diet; Ptp1B, protein tyrosine phosphatase; Gcn2, general control non-derepressible 2; CVD, cardiovascular disease; HHCy, hyperhomocysteinemia; CF, control-fed; Apo-E KO, apolipoprotein-E knockout; ECG, electrocardiogram; vBMD, volumetric bone mass density; SAAR, sulfur amino acid reduced.

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1. Introduction

The purpose of this review is to understand how MR extends lifespan and involves identifying its effects on various tissues in animal models as well as determining its potential mechanisms using invertebrates. Collating data from these different studies provides for a comprehensive explanation for the prolonged homeostasis in MR.

The metabolism of methionine has already been established. Briefly, methionine is converted to S-adenosylmethionine (SAM), the universal methyl donor, which eventually leads to the synthesis of homocysteine (Hcy). Hcy can then either be remethylated or undergo transsulfuration. Remethylation of Hcy will synthesize methionine in the liver by betaine-Hcy methyltansferase (Bhmt), or in other tissues via the folate cycle by 5-methyltetrahydrofolate-Hcy methyltransferase (Mthf) and vitamin B12. Transsulfuration occurs when Hcy generates the intermediate cystathionine, which is catalyzed cystathionine β-synthase (Cbs). Following this reaction, cysteine and glutathione are synthesized, which have significant functions in protein synthesis and antioxidant capabilities, respectively.

Under conditions of MR, hepatic levels of SAM, S-adenosylhomocysteine (SAH), cystathionine, and methylthioadenosine were decreased while methyl donors dimethyl glycine (DMG) and betaine were increased (Mentch et al., 2015). In plasma, MR decreased levels of methionine, cystathionine, and 2-keto-4-methylthiobutyrate, increased betaine and DMG levels, and left SAM and SAH unchanged (Mentch et al., 2015). Our group has shown that the transulfuration pathway is downregulated by MR (Elshorbagy et al., 2010; Perrone et al., 2012), although Gupta et al. reported that a deficiency in Cbs does not alter the MR phenotype (Gupta et al., 2014). In addition, supplementation of cysteine during MR reversed a majority of its effects (Elshorbagy et al., 2011; Perrone et al., 2012). Future studies should therefore examine the influence of MR on the folate cycle as well as identify the roles of other metabolites and enzymes that are involved in its metabolism.

Several reviews have discussed the effects of MR in the liver and adipose tissue, in which it improves insulin sensitivity and reduces mass, respectively (Ghosh et al., 2014; Orgeron et al., 2014; Zhou et al., 2016). Recently, molecular mechanisms in liver and adipose tissue during MR were identified to elicit an improved healthspan phenotype (Lees et al., 2015; Wanders et al., 2016). For example, upregulated hepatic gene expression and elevated circulating fibroblast growth factor 21 (FGF21) in MR appear to be the main factors that improve glucose metabolism in response to a high-fat diet (HFD) or in aged mice (Ables et al., 2012; Lees et al., 2014; Stone et al., 2014). In addition, increased uncoupling protein 1 (Ucp1) expression in adipose tissue could contribute to the elevated energy expenditure with concomitant adipose tissue remodeling in mice under MR (Plaisance et al., 2010; Wanders et al., 2015). Finally, the metabolic effects of MR appear to be independent of hepatic protein tyrosine phosphatase 1B (Ptp1B) enzyme and general control non-derepressible 2 (Gcn2) protein (Lees et al., 2015; Wanders et al., 2016).

Oxidative stress is one of the proposed mechanisms involved in accelerated aging. That MR reduced mitochondrial oxidative stress has been described previously in brain, heart, liver, and kidneys (Barja, 2014; Sanchez-Roman and Barja, 2013; Pamplona and Barja, 2006). These studies demonstrated that concentration of complexes I and III in the mitochondria of the liver and brain, and complex I of the rat heart, were decreased (Naudi et al., 2007; Sanchez-Roman et al., 2012; Sanz et al., 2006). Additionally, it was suggested that the reduced glutathione levels observed in animals experiencing MR may reflect intrinsically low levels of oxidative stress (Maddineni et al., 2013).

This review focuses on the other in vivo effects of MR, including effects on cardiovascular function, bone development, epithelial tight junction, and cancer. Additionally, we also discuss the effects of MR in invertebrates such as *Drosophila*, *C. elegans*, and yeast, wherein novel mechanisms and pathways have been characterized.

2. Pleiotropic responses to MR

2.1. Cardiovascular effects of MR

It may be that MR extends lifespan through its ability to delay the occurrence of cardiovascular disease (CVD), a primary cause for early mortality. Interestingly, it is paradoxical that MR extends lifespan in rodents despite high levels of Hcy, which is a known risk factor for CVD. The mechanisms by which Hcy causes deleterious toxic effects in cultured epithelial cell lines have been previously discussed (D'Angelo and Selhub, 1997; Mayer et al., 1996). However, studies in animal models are inconsistent, which indicate that Hcy could either induce cardiac injury or not (Ma et al., 2013; Dayal et al., 2012). Furthermore, clinical trials to lower Hcy levels did not induce beneficial CVD outcomes (Armitage et al., 2010; Ebbing et al., 2010). Finally, recent clinical studies add further confusion to its effects such that in one trial, hyperhomocysteinemia (HHCy) is strongly associated increased risk for CVD while another study does not support its association (Cioni et al., 2016; Lupton et al., 2016).

In our model, rodents on MR diet develop elevated plasma Hcy levels in comparison to control-fed (CF) animals (Ables et al., 2015; Elshorbagy et al., 2010). While MR induced HHCy, circulating plasma levels of other sulfur-containing amino acids—cystathionine, cysteine, and taurine—were reduced, suggesting an alteration to the transulfuration pathway (Ables et al., 2015; Elshorbagy et al., 2010). Our studies in young, aged, acute-fed, chronic-fed, and apolipoprotein-knockout (Apo-E KO) mice undergoing MR exhibited upregulation of cardiac hypertrophy markers *Nppa* and *Nppb* compared with CF mice (Ables et al., 2015). Non-invasive electrocardiogram (ECG) at basal conditions in young animals demonstrated an extended QRS segment in MR mice compared to CF mice (Ables et al., 2015). In addition, chronic feeding resulted in a reduced heart rate, which translated to an extended RR segment in MR mice compared to chronic-fed CF mice (Ables et al., 2015). Interestingly, under stress conditions using isoproterenol, mice on the MR diet exhibited an attenuated response to injection compared to CF counterparts, suggesting improved cardiac adaptability during MR (Ables et al., 2015). These data were supported by tests using retrograde isolated heart perfusion in aged mice, which showed similarities in cardiac contractility in both groups (Ables et al., 2015). Elevated levels of adiponectin and FGF21upon MR, both of which confer cardioprotection, could explain the improved cardiac adaptability of these animals despite HHcy (Liu et al., 2013; Shibata et al., 2012). Finally, signaling pathways and gene expression analyses revealed altered cardiac metabolic signaling in mice on the MR diet as a response to increased adiponectin and FGF21levels, which may support their improved cardiac adaptability (Ables et al., 2015).

Notably, a study by Troen et al. (2003) implicated methionine rather than tHcy as the main cause for inducing aortic plaque. In those experiments, ApoE-KO mice were fed excess methionine, which resulted in a moderate increase in levels of tHcy (tHcy = 86.7 ± 25.3 nmol/ml) compared with ApoE-KO mice fed a diet with normal methionine levels, but lacking vitamin B, which exhibited severe levels of tHcy (243.7 ± 82.0 nmol/ml). ApoE-KO mice with severe HHcy had a total lesion area of 23,986 ± 1877 μm^2, while mice fed high methionine showed a total lesion area nearly twice the size (45,923 ± 2804 μm^2) (Troen et al., 2003). This finding further implicates methionine, not Hcy, as causing increased plaque sizes. Furthermore, this study corroborates our findings that MR does not affect cardiac function despite HHCy.

2.2. MR affects bone development

Studies on the effects of MR on bone growth and development have only recently been reported. To our knowledge, our studies were the first to demonstrate that MR altered bone growth and osteoblast differentiation in rodents (Ables et al., 2012; Huang et al., 2014; Ouattara et al., 2016; Plummer et al., 2016). The effects of MR in mice were

influenced by age and gender, with changes being more robust in young and aged males but not in aged females (Ouattara et al., 2016). MR in young male and female mice, and aged male mice, had shorter body lengths compared to CF counterparts (Ouattara et al., 2016). Mice on MR indicated reduced absolute cortical, trabecular and total volumetric bone mass density (vBMD) and bone mineral content (BMC) compared to their CF counterparts (Ouattara et al., 2016). However, when adjusted for body weight, cortical, trabecular, and total vBMD were higher in both age groups of males on MR compared to their CF counterparts, while mild effects were observed in females (Ouattara et al., 2016). More stringent measurements on body weight-adjusted parameters by using microchromotomography indicated that bone surface to trabecular volume ratios were higher in young and aged male mice on MR than in CF counterparts, while all parameters in aged CF and females on MR were similar, suggesting that the small bones seen under MR are appropriate for its body size (Ouattara et al., 2016). More importantly, nanoindentation revealed that MR did not affect bone material properties as indicated by indentation depth increase, hardness, and modulus parameters (Ouattara et al., 2016). Furthermore, mechanical tests revealed that yield load energy was similar in rats under MR and CF conditions, while bones from rats on MR had lower extrinsic bending strength, energy resorption, and stiffness compared to CF animals (Huang et al., 2014). Interestingly, MR in rats induced a higher intrinsic yield stress, toughness, and elastic modulus (Huang et al., 2014).

The possible molecular mechanism of MR on bone development was described in time course studies using MC3T3-E1 preosteoblast cell lines fed media low in sulfur amino acids (sulfur amino acids reduced, SAAR). For those experiments, control media (CF) contained 100 mg/l cysteine, 31 mg/l cystine, and 15 mg/l methionine, while the SAAR media contained 20 mg/l cysteine, 6.2 mg/l cystine, and 3 mg/l methionine (Ouattara et al., 2016). Gene expression analysis for bone differentiation and collagen formation revealed that CF cells underwent differentiation after 6 days in culture when *Runx2*, *Bglap*, *Spp1*, *Alpl* and *Col1a1* were upregulated (Ouattara et al., 2016). In contrast, those genes from SAAR cells were unchanged or remained at lower levels than CF cells after 6 days in culture indicating delayed osteoblast differentiation (Ouattara et al., 2016). That MR targets osteoblast differentiation was supported by downregulation of osteocalcin (*Bglap*) in whole bone of aged MR male mice (Ouattara et al., 2016). Our group recently reported miRNAs that target *Runx2* expression which alters bone structure in young male mice (Plummer et al., 2016). Using bone marrow cells from young mice, the expression of miR-133a, miR335-5p and miR-204 was upregulated by MR, which subsequently downregulated *Runx2* gene expression (Plummer et al., 2016). Because *Runx2*, an essential marker for osteoblast differentiation, is downregulated in the bone marrow cells of young male mice on MR, and in the MC3T3-E1 cell line in SAAR medium, our data strongly suggest that MR delays osteoblast differentiation. It is notable that MR in mice also do not gain as much weight as their CF counterparts, indicating that small bones in rodents undergoing MR are appropriate for their body size.

2.3. MR improves epithelial tight junctions

Loss of integrity in tight junctions could lead to a myriad of complications in an organism and cause early mortality. Leaks in tight junctions are involved in inflammation, cancer, and aging (Mullin et al., 2015). In vitro studies using cell lines revealed that MR does improve epithelial tight junctions, which may protect against potential infection. When the porcine kidney epithelial cell line LLC-PK_1 was exposed to SAAR medium comprising 50% cysteine, 0% cystine and 10% methionine (as compared with normal medium), transepithelial electrical resistance was elevated in relation to control cells grown in medium having 100% of all sulfur amino acids, suggesting decreased permeability of tight junctions. (Skrovanek et al., 2007). Interestingly, the transepithelial diffusion rate by a smaller probe, mannitol, but not with a larger probe, polyethylene glycol, was also reduced in cells grown in SAAR medium (Skrovanek et al., 2007). While levels of proteins involved in tight junctions, such as occludin and claudins 1 and 2, remained similar between the two groups, claudins 3 and 7 were downregulated in cells grown in SAAR medium, which could explain the selective permeability of cells during SAAR conditions (Skrovanek et al., 2007). MR in rats reduced colonocyte methionine levels compared to CF counterparts (Ramalingam et al., 2010). Distal colon tissue sheets in rats undergoing MR exhibited increased transepithelial electrical resistance and reduced mannitol flux across the tissue compared to CF rats from 2 to 9 weeks of feeding (Ramalingam et al., 2010). Altered tight junction signaling of claudin 3 and occludin suggest a possible molecular mechanism for the effect of MR despite unchanged crypt architecture and cell proliferation index (Ramalingam et al., 2010). Collectively, these studies reveal a critical mechanism of how MR protects against infection and delays early mortality.

2.4. MR alters cancer progression

Along with CVD, cancer is one of the main causes of early mortality in the human population. The elevated metabolic demand for methionine in cancer cells provides an enticing target for cancer therapy. In colon and prostate cancer animal models, we examined the effects of MR on tumor progression (Komninou et al., 2006; Sinha et al., 2014). MR in rats had a significantly reduced formation of aberrant crypt foci in the colon, primarily occurring during the post-initiation phases of carcinogenesis. This may be due, in part, to an inhibition of colonic cell proliferation (Komninou et al., 2006). In addition, in the transgenic adenocarcinoma of the mouse prostate (TRAMP) mouse model, MR resulted in a decrease in prostatic intraepithelial neoplasia and a reduction in prostate-lobe specific cell proliferation compared to CF mice (Sinha et al., 2014). Finally, breast cancer tumors were suppressed by MR. In triple negative breast cancer cell lines, MR reduced the phosphorylation of focal adhesion kinase and downregulated expression and activities of matrix metalloproteinases MMP-2 and MMP-9, while upregulating TIMP1 and PAI-1 (Jeon et al., 2016). In mammary tumor-bearing athymic nude mice, MR resulted in smaller tumors compared to those of CF mice (Hens et al., 2016). MR in mice exhibited decreased proliferation and increased apoptosis in cells that constitute the mammary glands and tumors of mice (Hens et al., 2016). Elevated expression of P21 occurred in both MCF10AT1-derived tumor tissue and endogenously in mammary gland tissue of mice undergoing MR (Hens et al., 2016). Breast cancer cell lines subjected to MR also upregulated gene expression of the cell cycle inhibitors P21 and P27 (Hens et al., 2016).

Using an in vitro MR model system in prostate cancer cell lines, methionine-free media with 10% FBS supplemented with 100 μM Hcy arrested cells in the the G2/M and G1 phases of the cell cycle due to upregulated CDKN1A and CDKN1 inhibitors (Lu and Epner, 2000). Subsequent studies determined that JNK1 signals cancer cells to undergo apoptosis in response to MR (Lu et al., 2002), which involved cleavage and the activation of initiator and effector caspases (Lu et al., 2003). When a similar MR system was used to investigate telomere metabolism in fibroblasts, it altered the composition of the ALT-associated promyelocytic leukemia bodies (APBs) that are associated with a telomerase-independent telomere maintenance pathway (Jiang et al., 2007).

Taken together, these findings suggest that MR delays cancer progression by regulating the cell cycle and by modulating DNA repair mechanisms, both of which could support the benefits of MR to lifespan.

3. MR extends the lifespan of invertebrate organisms

3.1. Effects on Drosophila

While the beneficial effects of MR have been well-documented in vertebrate animals, a growing body of work has sought to explore the mechanisms underlying MR by studying this intervention using more

facile, genetically-tractable model organisms. In fact, the first exploration of MR-dependent prolongation of lifespan in *Drosophila melanogaster* revealed that flies fed an amino acid-limited diet (0.1×) received a lifespan benefit when dietary methionine levels were restricted from 0.405% to 0.135% (65 days vs. 72 days, ~10% extension) (Troen et al., 2007). This finding was confirmed by Lee et al., who reported that MR (0.15 mM methionine) extended the lifespan of mated flies (~13% longer-lived than control-fed animals, 1 mM methionine), but only under conditions of limiting amino acids (Lee et al., 2014). A more robust effect was observed in virgin flies, for which MR resulted in lifespan extensions of up to ~19%. Interestingly, the authors also found that inhibition of the TOR pathway, through overexpression of Tsc2 or a dominant negative form of the insulin receptor (dInRDN), abrogated the relative benefit of MR. In addition, levels of MR that resulted in lifespan extension also severely compromised egg production, confirming that, similar to other organisms, flies experience a trade-off between extended somatic cell maintenance and reproduction.

In a subsequent study, Obata and Miura presented data suggesting that the benefits of MR in *Drosophila* might be due not to reduced methionine levels per se, but to a decreased pool of its primary metabolite, SAM (Obata and Miura, 2015). The authors reported that overexpression of Glycine *N*-methyltransferase (Gnmt), which catabolizes SAM by using it as a methyl donor for sarcosine production, significantly extended the lifespan of both male and female flies. Notably, the authors also found that Gnmt is required for multiple lifespan-extending interventions in flies, including both TOR inhibition (via dInRDN expression) and dietary restriction (DR).

3.2. Effects on C. elegans

That SAM catabolism can have implications for longevity appears to be at least somewhat conserved across phylogeny. In a 2005 study, 23 genes were identified in *C. elegans* that significantly impacted lifespan, including the gene encoding the SAM synthetase, SAMS-1 (Hansen et al., 2005). Worms with reduced SAM synthetase activity were up to 55% longer-lived than control animals. Interestingly, epistasis analyses demonstrated that extension of lifespan by downregulation of *SAMS-1* is at least partially redundant with lifespan extension by DR, as *eat-2* mutant worms fed *SAMS-1* RNAi containing bacteria exhibited a complete loss of the extended lifespan observed for control *eat-2* mutant animals. Furthermore, downregulation of SAM synthetase impaired reproductive capability, as evidenced by both decreased brood size and a delay in reproductive timing. In a complementary study, another group explored the genetic requirements for worm lifespan extension by downregulation of *SAMS-1*, and found that inhibition of TOR signaling reduced the expression of *SAMS-1*, suggesting that *SAMS-1* might be involved in mediating the longevity benefits of DR (Ching et al., 2010). Taken together, such findings provide further evidence that the relevant benefit of MR to organismal longevity might be reduced flux through SAM and/or its metabolites, and further, that the pathways underlying MR might overlap with those supporting DR.

An additional study demonstrating the benefits of MR to *C. elegans* focused on the biguanide drug metformin, which is the standard treatment for type 2 diabetes and metabolic syndrome, and further, has been demonstrated to extend lifespan in a number of settings (Cabreiro et al., 2013). In their 2013 paper, Cabreiro et al. explored the mechanistic basis for the metformin-mediated lifespan extension of worms, and found that the drug alters folate and methionine metabolism in the *E. coli* used for feeding of worm cultures. Treatment of the co-culture with 25 mM and 50 mM metformin resulted in mean lifespan extensions of 18% and 36%, respectively. Given both that *C. elegans* live longer on *E. coli* with reduced folate levels (Virk et al., 2012) and that metformin is known to decrease folate pools in human patients (Sahin et al., 2007), the authors decided to characterize folate metabolism in metformin-treated *E. coli*. As expected, levels of various folate-containing compounds were altered (both up and down), and polyglutamylation was increased. In addition, metformin negatively affected the methionine cycle in bacteria, resulting in increases of 86% and 33% in SAM and SAH levels, respectively. Subsequent biochemical and genetic studies indicated that, despite the observed effects of metformin on both bacterial folate and methionine metabolism, ingestion of metformin-treated *E. coli* by worms altered only their methionine, and not folate levels. Moreover, with respect to longevity, feeding of metformin-treated *E. coli* to worms failed to produce a benefit when coupled with either methionine synthetase (*metr-1*) and SAM synthetase (*sams-1*) mutants. This intervention can therefore be considered yet another bona fide method of producing a state of MR in *C. elegans*.

3.3. Effects on yeast

To test whether lifespan extension by MR might also be modeled in *S. cerevisiae*, we and others made use of the yeast chronological lifespan (CLS) assay, which measures the length of time that yeast cells remain viable in a non-dividing state. In the resulting work, it was reproducibly demonstrated that dietary MR robustly extends yeast CLS (Johnson and Johnson, 2014; Lee et al., 2014; Ruckenstuhl et al., 2014; Wu et al., 2013). In our study, we also demonstrated that the state of MR could be produced by genetic means, through deletion of either of two genes involved in methionine production (*MET2* and *MET15*) (Johnson and Johnson, 2014). These manipulations, which we call “genetic MR”, perfectly recapitulate the lifespan extension observed for dietary MR. We also determined that extension of yeast CLS was due specifically to methionine limitation, rather than being the consequence of simply reducing amino acid levels. When exploring the genetic requirements for CLS extension by MR, several genes were identified as being required for the benefits of this intervention, including the retrograde stress response factor *RTG3*, the amino acid sensor *GCN2*, and the autophagy-related genes *ATG5*, *ATG7*, and *ATG8* (Johnson and Johnson, 2014; Ruckenstuhl et al., 2014; Wu et al., 2013). Notably, the retrograde stress response had previously been implicated in the control of yeast replicative lifespan (Jazwinski, 2000). In total, the above studies demonstrate that there are integral roles for retrograde stress signaling, amino acid sensing, and autophagy in the extension of yeast CLS by MR. Indeed, the involvement of these processes in lifespan prolongation by MR appears to be conserved from yeast to vertebrates.

3.4. MR confers stress tolerance to yeast

The lifespan extension conferred by MR (and other longevity-promoting interventions) is often associated with an improvement in stress tolerance. For example, *MET15*-deficient cells were found to be resistant to treatment with the heavy metals methyl mercury and cadmium, as well as the oxidizing agent diamide (Hwang et al., 2007; Singh and Sherman, 1974; Warringer and Blomberg, 2003). Yeast undergoing MR were also found to be resistant to heat shock, as well as incubation with the toxic compound 1,10-phenanthroline (Johnson and Johnson, 2014). In addition, as yeast cells undergoing both dietary and genetic MR have an extended chronological lifespan, they can therefore be considered to be resistant to the nutritional and metabolic stresses encountered during yeast chronological aging.

3.5. MR-dependent gene expression changes affect multiple pathways involved in determining longevity

In an attempt to further explore the mechanistic basis of lifespan extension by MR, we previously subjected yeast cells undergoing MR to gene expression profiling. 1625 probes were differentially regulated in *met15*Δ cells as compared with wild-type, while the altered expression of 313 (19%) was dependent on a functional retrograde response (Johnson and Johnson, 2014). Further, retrograde signaling is downregulated by TOR, and inhibition of TOR is known to extend both replicative and chronological lifespan in yeast, as well as organismal lifespan in

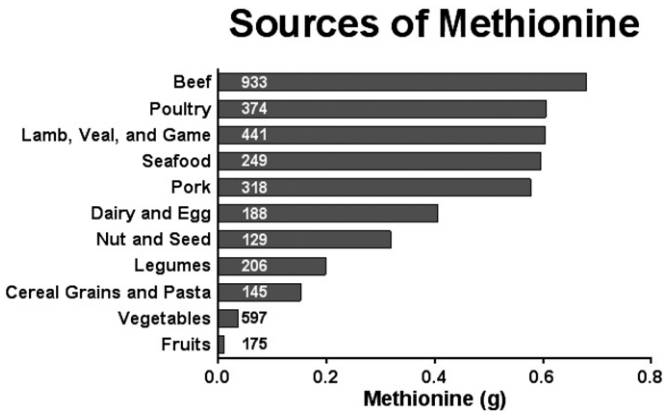

Fig. 1. Methionine content of various food sources. Bars represent average Met concentration. Numbers inside bars represent the number of samples of food sources available from the database.
Source: National Nutrient Database for Standard Reference Release 28 slightly revised May 2016. Software v.2.6.1. The National Agricultural Library.

vertebrates (Harrison et al., 2009; Jazwinski, 2013; Kaeberlein et al., 2005; Powers et al., 2006; Wei et al., 2008; Wilkinson et al., 2012). 222 functional categories were found to be enriched within MR-regulated probe sets, several of which (particularly those associated with up-regulated genes) corresponded to genes and pathways with functions in metabolism, stress response, and protein quality control. This finding is notable given that all three pathways have been implicated in the regulation of longevity (Lopez-Otin et al., 2013). Interestingly, Lee et al. (2014) performed gene expression analyses of methionine-restricted *Drosophila*, and found upregulation of factors involved in lipid and steroid metabolism, as well as the response to toxic stress. It is probable that some of the expression changes identified in yeast and flies underlie the extended lifespan conferred to these organisms by MR, and might provide clues as to which factors or pathways are key mediators of the MR phenotype in mammals.

4. Perspectives and future directions

The potential to translate MR to humans involves access to appropriate food sources. We compiled methionine content from various food sources based on the US National Nutrient Database for Standard Reference Release 28. By selecting food sources that have more than 100 samples within each food source, 11 groups are shown in Fig. 1. A complete list of food sources is included in Supplementary Table 1. Food sources for beef revealed the highest content of methionine at 0.680 g ± 0.14 g met/100 g. In addition, poultry, lamb, veal, game, finfish, shellfish, pork, dairy and egg all contained more than 0.4 g met/100 g. On the other hand, nuts, seeds, legumes, cereals, vegetables and fruits had less than 0.32 g met/100 g. These data suggest that in order to achieve MR, an individual has to eat less animal-based food source and more plant-based food source. This information supports the hypothesis that a vegan diet, naturally low in methionine, could be beneficial to healthspan (McCarty et al., 2009).

In conclusion, as the benefits of MR become more prominent, it is essential to direct future research on its effects on healthy aging. Studies on the role of MR on microbiota, cognition, behavior, and muscle function, coupled with mechanisms identified from invertebrate models will be relevant as the aging human population continues to expand.

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