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Short report

Rapamycin additively extends lifespan in short- and long-lived lines of the nematode *Caenorhabditis remanei*

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ABSTRACT

Despite tremendous progress in finding genes that, when manipulated, affects lifespan, little is known about the genetics underlying natural variation in lifespan. While segregating genetic variants for lifespan has been notoriously difficult to find in genome-wide association studies (GWAS), a complementary approach is to manipulate key genetic pathways in lines that differ in lifespan. If these candidate pathways are down regulated in long-lived lines, these lines can be predicted to respond less to pharmaceutical down-regulation of these pathways than short-lived lines. Experimental studies have identified the nutrient-sensing pathway TOR as a key regulator of lifespan in model organisms, and this pathway can effectively be down regulated using the drug rapamycin, which extends lifespan in all tested species. We expose short- and long-lived lines of the nematode *Caenorhabditis remanei* to rapamycin, and investigate if long-lived lines, which are hypothesized to already have down-regulated TOR signaling, respond less to rapamycin. We found no interaction between line and rapamycin treatment, since rapamycin extended lifespan independent of the intrinsic lifespan of the lines. This shows that rapamycin is equally effective on long and short-lived lines, and suggests that the evolution of long life may involve more factors that down-regulation of TOR.

1. Introduction

Lifespan has a strong genetic basis and differs greatly within species (Austad, 1993; Gems and Partridge, 2013; Ivanov et al., 2015). While theory on the evolution of ageing does not necessarily predicts that certain genetic or physiological pathways should be responsible for the evolution of long lifespan (Medawar, 1952), experimental studies have identified the nutrient-sensing target-of-rapamycin (TOR) pathway as a potential key regulator of lifespan across organisms (reviewed in Gems and Partridge, 2013). TOR exists in two protein complexes (mTORC1 and mTORC2) and mTORC1 forms a signaling network with the insulin/insulin-like growth factor 1 (IGF-1) pathway (IIS) and genetic manipulations of this pathway extends lifespan in model organisms (Kapahi et al., 2010; Apelo and Lamming, 2016) suggesting that it may be key pathway underlying natural variation in lifespan. Moreover, the TOR pathway can be pharmaceutically down-regulated by the drug rapamycin, which extends lifespan in yeast, flies, mice and nematodes (Kapahi et al., 2010; Gems and Partridge, 2013). Rapamycin thus allows TOR to be manipulated without the need of genetic manipulations, and can therefore be used at large scale on genetically heterogeneous populations (Miller et al., 2014) and non-model organisms (Lind et al., 2016), even though rapamycin is less specific than genetic manipulation, since it is also affecting mTORC2 (Apelo and Lamming, 2016).

While rapamycin extends lifespan of individuals by plastic nutrient-sensing responses, individuals can also differ in lifespan as a response to evolutionary factors (Austad, 1993; Shattuck and Williams, 2010). In many cases, these differences in lifespan can be interpreted as physiological trade-offs with other life-history traits such as reproduction (Flatt, 2011) or growth (Lee et al., 2013), trade-offs that are partly mirrored by rapamycin exposure (Lind et al., 2016). However, despite tremendous progress in identifying genes that, when manipulated, affects lifespan (Kenyon, 2010; Gems and Partridge, 2013), we know very little about the genetics underpinning natural variation in lifespan. One fruitful pathway is to search for genetic variants that are correlated to natural variation in lifespan using a genome-wide association study (GWAS) approach, a work that has been initiated for humans (reviewed in Murabito et al., 2012) and the *Drosophila* Genetic Reference Panel (DGRP) lines (Ivanov et al., 2015). However, a complementary approach is to manipulate candidate genetic pathways in short- and long-lived lines. If the candidate pathway is genetically down regulated in long-lived lines, further pharmaceutical down-regulation is expected to have a larger effect in short-lived than in long-lived lines, the latter expected to already have a down-regulated pathway. The question whether lifespan extending pharmaceuticals mostly benefit individuals

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with short intrinsic lifespan, or if the same positive effect on lifespan would be expected across all individuals is also of vital important for medical gerontology.

We investigated these questions using lines of the free-living outcrossing nematode *C. remanei* that differ in lifespan as a result of selection using different intensity of condition dependent or random extrinsic mortality (Chen and Maklakov, 2012). We expose these lines to either rapamycin or control conditions, to investigate whether rapamycin affects all lines independent of intrinsic lifespan, or if the short-lived lines have a stronger increase in lifespan by down-regulating the candidate TOR pathway. We found no interaction with rapamycin treatment, since rapamycin extended lifespan independent of the intrinsic lifespan of the lines. This shows that rapamycin is equally effective on long and short-lived lines, and although rapamycin is not as specific as a targeted mTORC1 mutation, the finding nevertheless suggests that the evolution of long lifespan may involve more factors that down-regulation of TOR.

2. Methods

We used females from experimental lines, created by artificial selection from the wild-type SP8 strain of *C. remanei*. For a full description of the selection procedure, see Chen and Maklakov (2012). Briefly, the lines were subjected to two crossed selection regimes: mortality source (random [R] or condition dependent heat-shock [C-d]) and mortality rate (high [H] or low [L]), resulting in four selection regimes (HR, LR, HC-d, LC-d). Four replicate lines were subjected to each selection regime, resulting in a total of 16 lines. The lines were evolving under these conditions for 12 generations, and were then kept for two generations under relaxing selection and frozen for future use. The lines in this study were originating from an expansion and re-freeze of the original lines, kept under relaxed selection for an additional 2 generations (6 generations in total).

Rapamycin (LC Laboratories, Woburn, MA) was dissolved in dimethyl sulfoxide (DMSO) at 50 mg/ml and added to agar at 100 μM following the protocol for *C. remanei* (Lind et al., 2016). An equal amount of DMSO was added to the control plates. Before each assay, worms were recovered from freezing and cultivated for 2 generations under standard laboratory conditions (Stiernagle, 2006) at 20 °C, with the addition of the antibiotics nystatin, streptomycin and kanamycin to agar and bacterial growth medium. For lifespan assays, kanamycin was added to the agar from day 4 of adult life. Worms were feeding on the antibiotic resistant *E. coli* OP50-1 (pUC4K) obtained from J. Ewbank at the Centre d'Immunologie de Marseille-Luminy, France. Lifespan assays were established using 20 age-synchronised worms (10 target females, 10 background males from the SP8 populations, to keep females mated) in the L4 stage (54 h old) per replicate plate, which had been developing on control plates since egg-laying. Worms were set up on either control or rapamycin plates and transferred to new plates every second day. Sex ratio was adjusted to 1:1 according to the focal sex (females) throughout the assay. Worms were scored as dead, dead of matricide (internal hatching of eggs) or censored (escaped from agar).

The experiment was run in four blocks, each block containing one of the four replicate lines of each selection treatment. Each line was replicated four times within each treatment (rapamycin/control) and block, resulting in 32 replicate plates per block, and 128 replicate plates with 1280 target females for the entire experiment.

2.1. Statistical analysis

Lifespan was analysed in Cox proportional hazard models with Gaussian random effects using the *coxme* package for *R* 3.2.2. Selection treatment and rapamycin (rapamycin/control) were fitted as crossed fixed factors, and block and plate as nested random factors. Female dying of matricide were censored. Significance of fixed factors were evaluated using likelihood ratio tests. Note that one replicate line of each selection treatment was run in each block, thus the block effect estimates both the block and line effect.

3. Results

We found that female lifespan was influenced by selection treatment ($\chi^2 = 47.31$, df = 3, $p < 0.001$), and extended by rapamycin ($\chi^2 = 18.22$, df = 1, $p < 0.001$, Fig. 1). However, we found no interaction between selection treatment and rapamycin ($\chi^2 = 0.54$, df = 3, $p = 0.910$), implying that the effect of rapamycin was not statistically different for the different treatments, thus rapamycin has an additive effect on lifespan.

4. Discussion

We found that the TOR signaling antagonist rapamycin additively extends lifespan of female *C. remanei* nematodes from selection lines differing in lifespan. Thus, rapamycin is as effective in short- and long-lived lines and is therefore an applicable method for lifespan extension independent of differences in intrinsic lifespan. This also suggests that evolved differences in lifespan are not necessarily caused by regulation of TOR signaling.

The nutrient sensing TOR pathway has emerged as a key regulator of lifespan in model organisms, and genetic or pharmaceutical manipulations of this pathway extends lifespan across taxa. These findings suggest that TOR signaling may underline natural variation in lifespan (Kapahi et al., 2010; Gems and Partridge, 2013). If this is the case, it is possible that further down-regulation of this pathway would have different effects in individuals differing in intrinsic lifespan, since long-lived individuals can be predicted to already have down-regulated TOR signaling. Such an effect would limit the effectiveness of pharmaceutical life-span extension. Therefore, females from selection treatments known to differ in lifespan (Chen and Maklakov, 2012) were exposed to the TOR signaling antagonist rapamycin.

We found that females from the different selection treatments differed in lifespan, and that lifespan was further increased by rapamycin exposure. While rapamycin prolongs lifespan in all organisms tested, it does so in a sex specific matter (Bjedov et al., 2010; Miller et al., 2014; Lind et al., 2016), and in *C. remanei* rapamycin has the strongest effect in males (Lind et al., 2016). However, due to substantially increased replication, we can here show that rapamycin also extends lifespan in female *C. remanei*, which we did not had statistical power to do in our previous study (Lind et al., 2016).

We predicted that rapamycin would have a larger effect on lifespan in short- than in long-lived lines, since we presumed long-lived population to already have down-regulated TOR-signaling. However, we found no interaction between selection treatment and rapamycin exposure, meaning that the effect of rapamycin was additive and independent of the intrinsic lifespan of the organism (see Fig. 1). This finding has important consequences for the use of TOR-signaling inhibitors as anti-ageing drugs, since our results suggests that the effect of the drug is independent of the intrinsic lifespan of the individual. However, this result is surprising given the importance of TOR signaling for lifespan in genetic studies (Kapahi et al., 2010; Gems and Partridge, 2013), and the suggestion that natural variation in lifespan is determined by different levels of TOR signaling. It is possible that the additive effect of rapamycin on lifespan is simply caused by the ability of long-lived lines to down-regulate TOR even further and to the same degree as short-lived populations. But it could also indicate that, while TOR signaling undoubtedly affects lifespan, it is not underlying natural variation in lifespan in genetically heterogeneous populations. Possible support for the latter explanation comes from the fact that genetic manipulations of TOR (Meissner et al., 2004) as well as exposure to rapamycin (Lind et al., 2016) reduces female fecundity, while the long-lived populations in this experiment did not pay a fecundity cost relative to the short lived

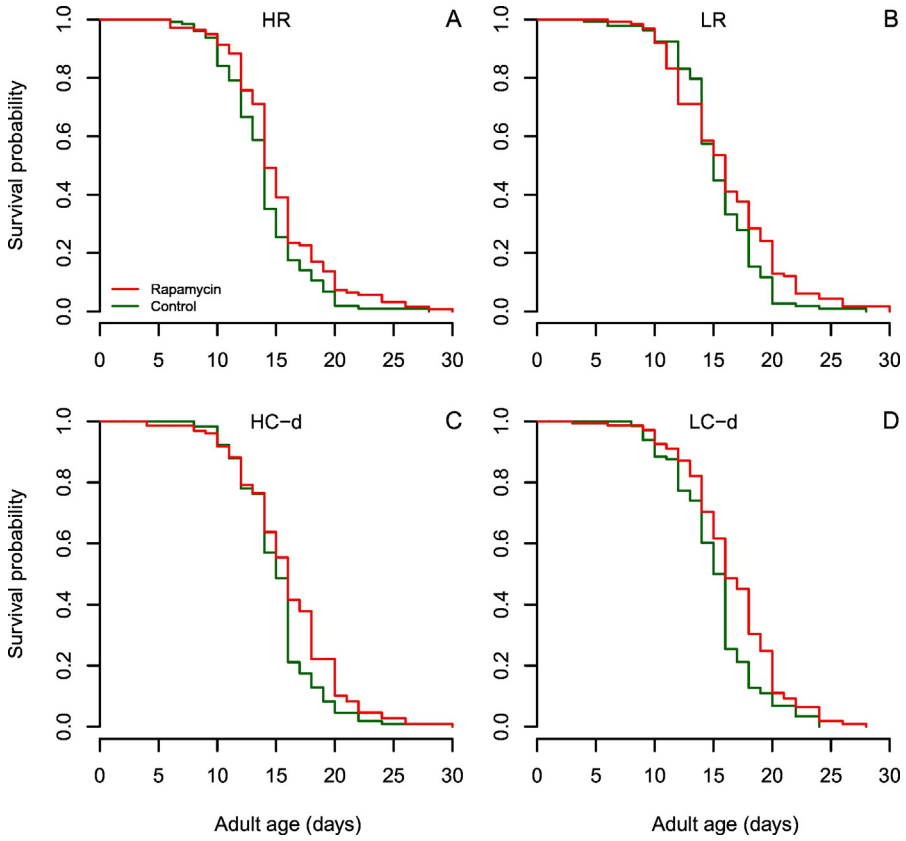

Fig. 1. The effect of rapamycin on survival probability of females from (A) High Random HR, (B) Low Random LR, (C) High Condition-dependent HC-d and (D) Low Condition-dependent LC-d selection lines. Each line represents pooled data from the four replicate selection lines of each selection regime. Green colour denotes control conditions and red rapamycin exposure. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

lines, and indeed had higher fecundity than the intermediate LR and LC-d populations (Chen and Maklakov, 2012). Moreover, although genes involved in the TOR pathway are enriched in the top ranked genes associated with longevity in the *Drosophila* DGRP lines, no gene variants passed the significant threshold which suggests that alleles with large to moderate effects on lifespan is not segregating in DGRP, despite a high heritability of lifespan (0.413) (Ivanov et al., 2015). This agrees with findings in humans, where GWAS for lifespan has been notoriously difficult to find (Murabito et al., 2012; Deelen et al., 2014), and often seems to be related to pathologies rather than longevity. In addition, using re-sequencing and transcriptome profiling of *D. melanogaster* lines selected for increased late-life performance, Remolina et al. (2012) found only weak support for the involvement of the TOR pathway in segregating genetic variation for lifespan. It should however be noted that rapamycin, as a drug, is not as specific as targeted gene mutation. In addition to targeting mTORC1, the kinase responsible for nutrient sensing, rapamycin also affect mTORC2, which may be related to the negative side effects of rapamycin treatment. The two kinases however differ in their sensitivity to rapamycin, while mTORC1 is acutely sensitive, chronic exposure is needed to activate mTORC2 (Apelo and Lamming, 2016). Perhaps lifespan should be seen as a quantitative trait with numerous underlying genes, and no naturally segregating genetic variants has large effects. Despite the obvious effects of TOR signaling on lifespan in experiments (Kapahi et al., 2010), extrapolating this to naturally segregating genetic variation for lifespan may be a too simplistic view.

The lines used in the study is a refreeze of the original lines from Chen and Maklakov (2012) that has experienced an additional 2 generations of relaxed selection (in total 6 generations of relaxed selection). We recover the previous lifespan difference between the short lived lines evolving under high rates of random extrinsic mortality (HR) and the longer lived lines from low levels of random (LR) or condition dependent (LC-d) mortality. However, we did not recover the most extended lifespan of the lines from the high condition-dependent mortality treatment (HC-d), but found that during the 4 additional generations of relaxed selection, their lifespan had returned to that of the LR and LC-d lines, while they still lived longer than the short-lived HR lines. Given that the HC-d lines evolved extended lifespan at the cost of extended development time (Lind et al., 2017) this trade-off may explain the loss of the extreme lifespan extension during prolonged relaxed selection, since delayed development has a direct negative effect on population growth rate (Stearns, 1992).

In summary, we found that short- and long-lived lines responded in the same way to pharmaceutical down-regulation of the candidate pathway TOR that is believed to be a major regulator of lifespan. Together with limited evidence for major genetic variants underlying genetic variation in lifespan from GWAS studies, this suggests that lifespan is a quantitative trait regulated by many underlying genetic pathways. As a consequence, it indicates that drugs like rapamycin effectively extends lifespan even in already long-lived individuals.

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