Chapter 6

The association of mTOR and familial longevity in the blood transcriptome is not reflected in primary skin fibroblasts

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Abstract

The Target of Rapamycin (TOR) pathway is an evolutionary conserved signaling pathway involved in lifespan regulation. In a gene expression study of whole blood, we recently reported that the mammalian TOR pathway was associated with familial longevity in humans. The aim of the present study was to investigate whether such an association can also be found at the cellular level. For this purpose, primary fibroblasts from a subset of our initial discovery cohort consisting of 40 middle aged members of long-lived families and 40 controls of similar age were analyzed. The protein expression, phosphorylation state, and gene expression of key molecules within the mTOR pathway as well as total protein content and markers for autophagy were measured. Although a significant effect on total protein content was observed, that might reflect differences in cell growth and proliferation, no differences were observed on the other parameters. The variation in gene expression levels of mTOR genes in cultured fibroblasts did not reflect at all the variation in these levels in whole blood from the same individuals. We conclude that contribution of the mTOR pathway to familial longevity cannot be studied in fibroblasts of donors which do express marks of such contribution in blood.

Introduction

After extensive studies in animal models, mammalian target of rapamycin (mTOR) signaling has recently also been implicated in human ageing and familial longevity (1;2). Gene expression studies in blood showed that expression changes of downstream targets of mTOR complex 1 (mTORC1) are downregulated with aging (3;4). It has been suggested that, in line with what is known in animal models, inhibition of mTOR signaling with advancing age might be a survivor effect, since decreased mTOR signaling is associated with increased survival (3). Our study investigating expression levels of genes belonging to the mTOR pathway showed that familial longevity in middle age associated with downregulation of the expression of genes in the mTOR pathway (4). These results correspond with previous studies showing that increased mTOR signaling is associated with increased prevalence and progression of disease (5;6), and that reduced mTOR signaling extends lifespan in model organisms (7-10) and is associated with better metabolic health (11). Thus, taken together, accumulating evidence suggests that mTOR signaling can play an important role in human metabolic health and longevity.

mTOR is an evolutionarily conserved nutrient-sensing protein kinase (6). The core kinase is present in two distinct multiprotein complexes (mTORC1 and mTORC2), each with different functions (12), although their exact respective roles remain to be elucidated. The main known functions of mTORC1 include translation initiation, protein synthesis and autophagy, while those of mTORC2 include cytoskeletal organization and stress resistance (13). In addition, the two complexes are connected in that mTORC2 regulates Akt phosphorylation, part of an upstream pathway that controls activation of mTORC1 (6). The mTOR pathway has been extensively studied in cell lines, including human and mouse fibroblasts (14-18).

The mTOR pathway was found to be involved in human familial longevity in the Leiden Longevity Study (LLS) in which nonagenarian siblings, their offspring and the partners thereof were studied. Expression levels of mTOR pathway related genes in blood differed between nonagenarian and middle aged individuals and between middle aged offspring and similarly aged partners as controls (4). The middle aged members of the long lived families (offspring) have a decreased prevalence of myocardial infarction and type 2 diabetes (19), and display a healthier metabolism reflected by beneficial lipid profiles (20;21) and preservation of insulin sensitivity (22;23) than the controls. Previously we demonstrated differences in the *in vitro* cellular responses to stress of the offspring's fibroblasts as compared to controls, including a lower increase in senescence, but an increase in apoptosis, which is seen as advantageous to the organism (24;25). To what extent mTOR signaling contributes to human familial longevity is not yet investigated at the protein and signaling level.

In this paper primary skin fibroblasts of members of long-lived families are investigated *in vitro* for differences in mTOR signaling as compared to similarly aged controls. The expression level of the most important proteins in the mTOR pathway were determined, as well as the phosphorylation of these proteins, total protein content and autophagy. We also inspected the correlation between the transcription levels of mTOR related genes in cultured skin fibroblasts and in blood from the same donors.

Results

The characteristics of the randomly selected fibroblast strains subset of subjects from the Leiden Longevity Study are shown in Table 1. Offspring of long-lived individuals and their partners, which will act as controls, were of similar age. Experiments were performed in fibroblasts obtained from 37

offspring and 36 controls, with a similar proportion of male and female donors.

Table 1 Analyzed samples in this study

Samples	Number	Mean Age	Age Range	Women (%)
Offspring	37	63.0	45-78	20 (54.1%)
Controls	36	62.5	44-78	19 (52.8%)

Protein expression and phosphorylation in skin fibroblasts

To investigate whether gene expression differences in blood represent lower mTOR signaling in peripheral tissues like the skin, we measured protein expression and phosphorylation of the most important proteins in this pathway (Figure 1) in skin fibroblasts using; mTOR, P70S6K, 4EBP1, Raptor, Rictor and Akt. mTOR itself is part of both mTORC1 and mTORC2 with complex-specific proteins Raptor and Rictor respectively. P70S6K and 4EBP1 are downstream targets of mTORC1 and Akt is a downstream target of mTORC2 as well as an upstream effector of mTORC1.

The comparison of offspring of long-lived individuals and controls for association with familial longevity showed no significant difference in expression or phosphorylation of any of the proteins after multiple testing correction (Bonferroni p=0.005, Table 2).

Protein synthesis and autophagy

Biological processes regulated by the mTOR pathway include autophagy and protein synthesis. Lower mTOR signaling, which is hypothesized to be the case in familial longevity, results in higher autophagy levels. An increase in autophagy levels could be indicated by a decrease in Light Chain3-I-on-Light Chain3-II isoform (LC3-I-on-LC3-II) ratio. However, the members of long-lived families show the same ratio as controls.

Although total protein content also reflects cell growth and differentiation, we could speculate that decreased total protein levels might indicate a decreased protein synthesis or an increased protein turnover to which a decreased mTORC1 activity may have contributed. Furthermore, since a lower mTOR signaling reduces protein synthesis, we speculate that total protein yield from 10^6 cells, as a surrogate marker for protein synthesis, might be decreased in members of long-lived families as compared to controls. The skin fibroblasts of members of long-lived families indeed had a lower protein content than controls (Table 2, p=0.007).

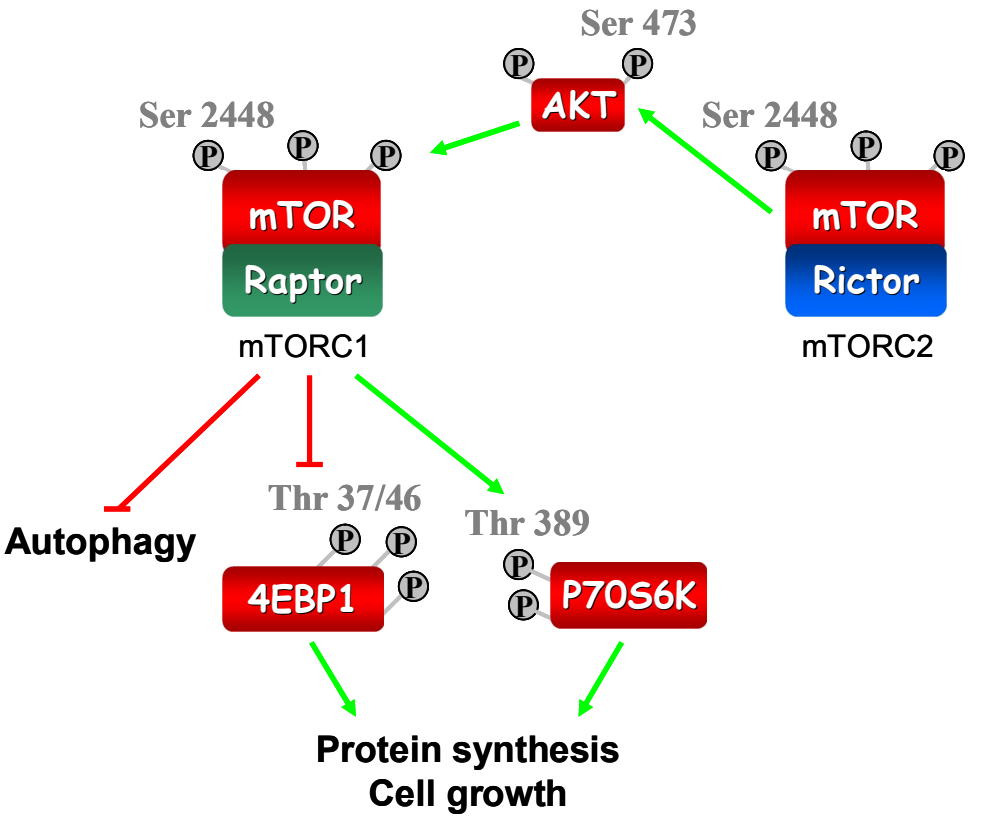

Figure 1. Core of mTOR signaling pathway of which protein expression and phosphorylation are investigated for their association with human longevity. Phosphorylation at Ser2448 activates mTOR in both complexes mTORC1 and mTORC2. mTORC1 inhibits 4EBP1 by phosphorylation at Thr37/46 and activates P70S6K by phosphorylating Thr389. mTORC2 activates Akt by phosphorylation at Ser473 and Akt indirectly activates mTORC1.

Gene expression and protein expression in skin fibroblasts

In blood we have previously established a difference between the offspring and controls in mTOR related gene expression levels (4). To investigate whether gene expression levels in skin fibroblasts correlate with protein levels we additionally measured transcription levels of *MTOR*, *RPTOR* and *RPS6KB1* in 66 fibroblast samples using RT-qPCR. Pearson pairwise correlation analysis of protein and gene expression levels in skin fibroblasts of the same individuals showed unexpectedly no significant correlation except for 4EBP1 ($r=0.324$, $p=0.008$ (Supplementary Table S1).

Gene expression in blood and gene expression in skin fibroblasts

The observed gene expression differences in blood between members of long-lived families and controls was apparently not reflected by any marked difference in mTOR signaling in the skin fibroblasts of these groups. The question raises therefore to what extent mTOR gene expression in blood and fibroblasts of the same donor correlates. We tested gene expression levels of the 40 mTOR genes in 72 individuals for such correlation by Pearson correlation analysis. We found no significant correlation of transcription levels for any of the genes in blood and skin fibroblasts of the same individuals (Supplementary Table S2).

Table 2. Linear regression results of protein expression in skin fibroblasts of offspring compared to controls

	Controls vs Offspring	
	Coef	p
Proteins		
mTOR	0.054	0.277
p-mTOR (S2448)	0.113	0.024
P70S6K	-0.003	0.938
p-P70S6K (T389)	0.003	0.967
p-4EBP1 (T37/46)	0.013	0.812
Raptor	0.039	0.504
Rictor	-0.038	0.606
p-Akt (S473)	0.117	0.270
Protein synthesis		
Total protein	-0.065	0.007
Autophagy		
LC3-I/LC3-II ratio	-0.005	0.962

Coef: coefficient from linear regression of protein expression (arbitrary units), positive indicated higher expression in offspring. p: raw p value from linear regression.

Discussion

The aim of this study was to further investigate our previous finding that expression levels of genes from the mTOR pathway in blood are associated with human familial longevity. In the skin fibroblasts of members of long-lived families and controls, however, we found no differences in the levels of proteins from the mTOR pathway nor their phosphorylation state. Hence we conclude that we are not able to detect an effect of mTOR signaling on human familial longevity in cultured skin fibroblasts. This is also reflected by the similarity between members of long-lived families and

controls in levels of autophagy as downstream effect of the mTOR pathway. The total protein content in members of long-lived families as compared to controls was slightly lower, potentially indicating downregulation of mTOR signaling. But since gene expression and protein levels of mTOR related genes in cultured skin fibroblasts and blood from the same donors did not correlate, we conclude that cultured skin fibroblasts may not be suitable to investigate the basis and consequences of natural variation of mTOR related gene expression in blood that marked familial longevity at a functional level.

Besides the biological explanation that mTOR signaling itself might differ between blood and skin of the same donor, our lack of clear validation might be due to the technical variations, of which the major one is that the skin fibroblasts were cultured several passages *in vitro* before RNA extraction, while blood was drawn and it's RNA immediately stabilized and frozen, representing the *in vivo* status. Thus, our current findings may stress that mTOR signaling in cultured primary skin fibroblasts is different from that in whole blood and therefore cannot provide sufficient insights about the role of mTOR signaling in human ageing and familial longevity.

Another explanation for the lack of clear validation might be that investigating natural occurring genetic variation in a primary cell system does not result in clearly measurable changes in protein expression and phosphorylation state. Most successful studies on mTOR signaling in skin fibroblasts investigate the effects of the knockdown of genes (14;18), the use of inhibiting compounds (17), mutant genes, overexpression of genes (14), amino acid deprivation or induction of oxidative stress (15). Because the effect of naturally occurring genetic variation is expected to be much smaller than the effect of such challenges, it may be that that the culturing of cells introduces too much background variation in mTOR signaling to detect the subtle differences between members of long-lived families and controls. Hence we may need to challenge the mTOR pathway to be able to detect the differences associated with human familial longevity in fibroblasts like was applied in the study of MTOR signaling in fibroblasts of long lived mouse strains (15).

In summary, the gene expression differences in mTOR related genes in blood associated with human familial longevity was not observed for primary skin fibroblast. Moreover, we were not able to detect differences in levels or phosphorylation status of mTOR proteins in skin fibroblast in relation to human familial longevity. We conclude that under unchallenged conditions cultured skin fibroblasts may not be suitable to investigate the

basis and consequences of natural variation in mTOR signaling in humans as determinant of human longevity. Potentially, cultured PBMCs better resemble the effects found in blood, or the exploration of differential response to a challenged mTOR pathway may provide functional insight in the association of mTOR related gene expression in blood with human familial longevity.

Materials and methods

Study design

The individuals investigated in this study are participants of the Leiden Longevity Study. The families participating in this study have at least two siblings with a minimum age for men of 89 years and for women of 91 years (28). The offspring of these long-lived individuals, who have an increased potential to become long-lived individuals (30% reduced standardized mortality rate), were also included. In addition, the partners of the offspring were included as population controls of similar age and environmental exposures as the offspring, and as a young control group for the nonagenarian siblings. Blood samples were taken from all the participants. During the period November 2006 and May 2008, a biobank was established from fibroblasts cultivated skin biopsies from 150 offspring-control pairs. Because it was expected that the difference in biological age between the offspring and control groups would be small, a relatively large sample size of 80 strains from 40 couples was chosen, consisting of 20 fibroblast strains from the 65 female offspring, 20 strains from the 86 male offspring, as well as 20 strains from the 86 female controls and 20 strains from the 63 male controls (Table 1).

Fibroblast cultures

Four mm skin biopsies were taken from the sun unexposed medial side of the upper arm. Fibroblasts were grown in D-MEM:F-12 (1:1) medium supplemented with 10% fetal calf serum (FCS), 1 mM MEM sodium pyruvate, 10 mM HEPES, 2 mM glutamax I and antibiotics (100 U mL^{-1} penicillin, 100 µg mL^{-1} streptomycin and 0.25-2.5 µg mL^{-1} amphotericin B), all obtained from Gibco (Breda, the Netherlands). One specific FCS batch was used during the whole experiment (Bodinco, Alkmaar, the Netherlands, batch no. 162229). Fibroblasts were incubated at 37 °C with 5% CO_2 and 100% humidity. All cultures that are used in the present study were grown under predefined, highly standardized conditions as published earlier (29) and frozen at low passage. Of the 73 individuals from which the fibroblasts

are tested, gene expression was measured in whole blood for 72 of them (4).

Experiments were set up in batches of maximally eight fibroblasts strains which were thawed from frozen stocks on day zero. On day one, the medium was changed and on day 4 fibroblasts were passaged 1:4 and passed further in equal numbers to have similar confluences for experiments. On day 25, fibroblasts were seeded in 9 cm dishes and grown for three days to similar confluences before harvesting cells for RNA or protein isolation.

Western Blot preparation and analysis

Fibroblast cells were washed with PBS, lysed in EBSB buffer (4 mM glycerol, 3% SDS, 100 mM TRIS-PO4 pH 6.8), boiled at 100 °C for 5 minutes and stored at -20 °C.

Whole cell lysates were prepared as described (30), and cleared by centrifugation. Protein content was determined using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL). Expression and phosphorylation of proteins were determined by SDS-PAGE Western Blotting. Antibodies against total mTOR (#2972), P70S6K (#9202), LC3-I-on-LC3-II (#4108), and phospho-specific mTOR-Ser2448 (#2971S), P70S6K-Thr389 (#9205S), 4EBP1-Thr37/46 (#9459), and Akt-Ser473 (#9271) were from Cell Signalling Technology (Danvers, MA, USA). Antibodies against total Raptor (sc-27744) and Rictor (sc-50676) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Bound antibodies were visualized by enhanced chemiluminescence and quantified by densitometry analysis of scanned films (Image J 1.34S; National Institutes of Health, Bethesda, MD, USA).

Statistical analysis was conducted with the software package STATA/SE 11.0 (DPC Software, StataCorp 2009). Differences in protein expression or phosphorylation (in arbitrary units) between the offspring and controls were determined with linear regression with age, gender and their interactions as covariates and adjusted for cell batch, cell confluence and where applicable western blot batch.

RNA sample preparation

From the 40 offspring and 40 controls RNA was extracted from both the cultured fibroblasts and whole blood. Harvesting of fibroblast cells included

washing with PBS and the cells were lysed in RLT buffer (Qiagen, Venlo, the Netherlands) and stored at -80 °C. RNA from fibroblasts was extracted using the RNeasy mini kit, RNA from whole blood (PAXgene tubes) was extracted using PAXgene Blood RNA kit (both Qiagen, Venlo, the Netherlands) following the manufacturer's recommended protocols. The quality and integrity of the total RNA was evaluated on the 2100 Bioanalyzer (Agilent Technologies, Amstelveen, The Netherlands) and the concentration was measured using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

RT-qPCR and correlation analysis of gene expression data

For all 40 mTOR signaling genes the suggested Taqman® assay (Applied Biosystems) was selected. Reverse transcription was performed with total RNA from fibroblasts and blood of in total 80 individuals, using the First Strand cDNA Synthesis Kit according to the manufacturer's protocol (Roche Applied Science). cDNA was amplified using the DNA Engine Tetrad® 2 Peltier Thermal Cycler (Bio-Rad). qPCR was then performed with the Taqman® method using the BiomarkTM 48.48 and 96.96 Dynamic Arrays (Fluidigm). Relative gene expression of the BioMark™ Array data were calculated by using the 2-ΔΔCt method, in which Ct indicates cycle threshold, the fractional cycle number where the fluorescent signal reaches the detection threshold (31). YKT6 was used as housekeeping gene and commercially available human total reference RNA (Clontech Laboratories, Mountain View, CA, USA) as reference RNA.

Gene expression and protein expression levels of 5 genes/proteins were correlated between the two tissues, skin fibroblasts and whole blood, within the same individuals using pairwise Pearson correlation in STATA adjusted for age, gender, their interaction, cell batch and cell confluence (Table 3). Relative gene expression levels of the 40 genes were also correlated between the two tissues within the same individuals using pairwise Pearson correlation in STATA adjusted for age, gender, their interaction, cell batch and cell confluence (Table 4).

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Supplementary information

Table S1 Pearson correlation between gene expression and protein expression in fibroblasts of the same donors (n = 66)

Gene	Protein	Correlation	P
MTOR	mTOR	-0.058	0.646
RPTOR	Raptor	-0.132	0.292
RPS6KB1	P70S6K	-0.198	0.112

p: raw p value from 2-tailed Pearson correlation.

Table S2 Pearson correlation between gene expression in fibroblasts and gene expression in whole blood of the same donors (n = 72)

Gene	Protein	Correlation	P
EIF4EBP1	4EBP1	0.060	0.639
AKT1	AKT	0.070	0.585
C12orf44	ATG101	0.067	0.575
ATG13	ATG13	-0.130	0.276
DEPTOR	Deptor	-0.139	0.245
EIF4B	eIF4B	0.074	0.538
EIF4E	eIF4E	0.011	0.930
RB1CC1	FIP200	0.031	0.793
FOXO1	FOXO1	-0.072	0.550
FOXO3	FOXO3	0.030	0.803
HIF1A	HIF1a	-0.056	0.638
MLST8	mLST8	0.127	0.289
MAPKSP1	MP1	-0.107	0.370
MAPKAP1	mSIN1	0.140	0.249
MTOR	mTOR	0.063	0.621
LAMTOR2	p14	0.075	0.530
CDKN2C	p18	0.218	0.066
PPARGC1A	PGC1a	-0.056	0.651
PRKCA	PKCa	0.105	0.381
PPARG	PPARg	-0.057	0.632
AKT1S1	PRAS40	0.046	0.701
PRR5	Protor1	-0.145	0.224
PRR5L	Protor2	0.117	0.327
RRAGA	RagA	-0.088	0.467
RRAGB	RagB	0.038	0.752
RRAGC	RagC	-0.110	0.356
RRAGD	RagD	-0.139	0.245
RPTOR	Raptor	-0.153	0.221
RHEB	Rheb	-0.057	0.632

RHOA	RhoA	0.032	0.787
RICTOR	Rictor	0.113	0.344
RPS6	S6	0.034	0.777
RPS6KB1	S6K1	-0.070	0.581
SGK1	SGK	-0.160	0.179
POLDIP3	SKAR	-0.131	0.315
SREBF1	SREBP	0.149	0.210
TSC1	TSC1	-0.109	0.361
TSC2	TSC2	0.107	0.372
ULK1	ULK1	-0.074	0.559
YY1	YY1	0.033	0.786

p: raw p value from 2-tailed Pearson correlation