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HEALTHY AGING RESEARCH

Age related changes in mTOR-related gene expression in two primary human cell lines

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Abstract

Background: Leukocyte transcriptomics studies suggest disrupted mTOR gene expression is a feature of human aging. We aimed to establish whether gene expression is also disrupted in other human tissues senesced in vitro.

Methods: We aged two primary cell types (fibroblasts and endothelial cells) in vitro, by repeated passage until growth arrest, and measured the expression of an mTOR-related transcript set by quantitative real-time PCR in low passage ('young') and high passage ('old') cells.

Results: We identified expression differences for 7/28 mTOR-related transcripts in senescent fibroblasts, which included up-regulation of mTORC1 inhibitory transcripts (*DEPTOR, TSC1, TSC2;* p=0.047, 0.007, and 0.024 respectively) with concurrent up-regulation of down-stream transcripts usually inhibited by mTOR activation (*NFKB1*, *PRKCA*, *EIF4G3* and *FOXO1*; p=0.047, 0.007 0.005 and 0.038 respectively). Endothelial cells demonstrated 2 significant expression changes between senescent and non-senescent cells (*PRCKA* and *SGK1*; p<0.001 and p=0.0031 respectively), transcripts involved in mTORC2 signaling.

Conclusions: Our preliminary study suggests that age-related gene expression changes noted in human population studies also occur with senescence in some primary human tissue types. Age-related expression changes in primary human fibroblasts involve mainly mTORC1 transcripts, whilst in human endothelial cells, expression changes are less apparent and involve mTORC2-related transcripts.

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Introduction

The mechanistic TOR (mTOR) signaling pathway is a highly conserved network which is present in eukaryotic cells and is one of the central modulators of cell growth and metabolism [1]. The pathway is mediated by two distinct complexes (mTORC1 and mTORC2), which have unique functions and respond to different stimuli [2]. The mTORC1 complex is activated via the AKT/ PI3K pathway and leads to the modulation of the cellular metabolic, inflammatory and stress response states by regulating protein and lipid synthetic processes, autophagy, and inflammatory and stress response pathways, whereas the mTORC2 complex is modulated by the RAS/RAF pathway and is involved with cell survival and cellular proliferation [3] (Fig. 1).

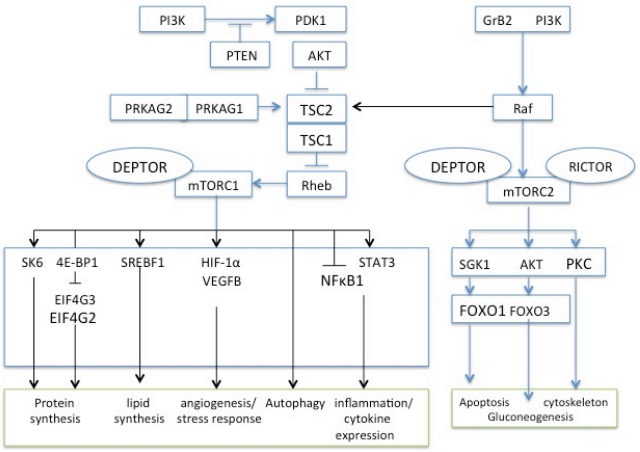

Figure 1. The mTOR signaling pathway

The conformation and regulatory relationships within the mTOR pathway are illustrated in the figure. Adapted from [38]

Modification of TOR signaling modulates longevity in yeast, nematodes and fruit flies laboratory models, as well as in mammals [4,5]. The importance of this pathway in determination of longevity and aging in these systems is demonstrated by the observation that the immunosuppressant, rapamycin, which targets the mTORC1 pathway, is the only pharmacological intervention demonstrated to increase lifespan in rodents [6,7]. Calorific restriction, known to regulate mTOR signaling, is also associated with increased lifespan in animal models [8,9]. Accordingly, many cellular processes influenced by the mTOR pathway are associated with advancing age in humans and animals; age-related changes in processes such as protein synthesis, autophagy, vascular plasticity, inflammation and lipid metabolism are all well characterized [10-13]. The pathway is also increasingly being implicated in many age-related human diseases such as cancer, type 2 diabetes, neurodegeneration and obesity [14].

We have recently demonstrated that altered mTOR signaling is a feature of aging in the human population, as assessed by molecular epidemiological studies in two distinct human populations with different characteristics (the InCHIANTI cohort and the San Antonio Family Heart Study; SAFHS). Microarray analysis using a bespoke transcript set of mTOR-related genes demonstrated that older individuals from both cohorts demonstrated expression changes consistent with an overall down-regulation of both mTORC1 and mTORC2 components of the mTOR pathway, consistent with findings from animal models [15]. These data reinforce the potential importance of mTOR signaling in aging, and demonstrate the relevance of studying the status of the mTOR pathway in the human population.

In the present study, we aimed to determine if the changes in mTOR-related transcript levels suggested by our in-vivo molecular epidemiology studies are also present in the more controlled environment of primary cell cultures that have undergone cellular senescence in vitro, and whether these responses are conserved between cell types. We have taken two unique primary cell types of different lineages (normal human dermal fibroblast cells and human aortic endothelial cells), which we have "aged" by continuous culture until growth arrest. Expression profiling of an expanded panel of mTOR-related genes was subsequently carried out in "young" and in "old" cells. The use of populations of cells that differ only in their "age", to assess senescence-related transcript changes thus offers the opportunity to differentiate transcript changes arising directly from the senescence process itself, from those arising from differences in the composition of tissue types with advancing age.

Methods

Senescence of primary cell lines in vitro

Two primary cell types commonly used for senescence studies, normal human dermal fibroblast cells (nHDF) and human aortic endothelial cells (HAOEC) were purchased commercially (Promocell, Heidelberg, Germany). HDFs were derived from human skin from the thigh and HAOEC were isolated from the human thoracic and abdominal aorta. We choose not to analyze primary leukocytes in vitro because the potential effects of the techniques necessary for separation of different blood cell subtypes, and the necessity for additional co-factors in culture may induce alterations in gene expression levels. Cells were tested for the presence of microbiological pathogens such as mycoplasma that could interfere with cell growth at source and also for cell type specific markers to confirm identity. Three independent cultures were set up to undergo in-vitro senescence for each cell line. The cells were grown in culture flasks with specialized medium (Promocell, Heidelberg, Germany) containing 1% penicillin and

streptomycin and supplement mixture (fibroblasts - fetal calf serum 0.03mL/mL, recombinant fibroblast growth factor 1ng/mL and recombinant human insulin 5µg/mL; and endothelial cells - fetal calf serum 0.05mL/mL, endothelial cell growth supplement 0.004mL/mL, epidermal growth factor 10ng/mL, hydrocortisone 1µg/mL, heparin 90µg/mL). Medium was replaced every 2 days and cells were rinsed twice with PBS. Cells were grown until 80% confluence and then transferred into new flasks. Cells were cultured in humidified chambers at 37˚C with 5% CO2 and cultured serially until late-passage cells had reach growth arrest. It is important to note that each passage represents several cell division events per cell.

Biochemical and morphological assessment of cell senescence

Cells were stained for senescence-associated β-galactosidase activity (Sigma Aldrich, Gillingham, UK) following the methods previously described by Dimri et al. [16]. Senescence was also assessed by morphological changes and the slowing of growth.

RNA extraction and reverse transcription

RNA was extracted from "young" and "old" fibroblasts and endothelial cells using the Qiagen extraction kit (Qiagen, Crawley, UK), following manufacturer's instructions. RNA was quality controlled using a Nanodrop spectrophotometer (Thermo Scientific Ltd, Wilmington, USA) and was not accepted for analysis if the concentration was < 30µg/ng. Total RNA (100ng) was reversed transcribed in 20µl reactions using the Superscript III VILO kit (Life Technologies, Foster City USA), according to the manufacturer's instructions.

Molecular definition of cell senescence

Cell senescence was assessed at the molecular level by measuring levels of CD248 transcripts, levels of which were demonstrated to be associated with age in our previous microarray study of age in humans [17]. Real-time PCR reactions to each target were carried out in triplicate from "young" and "old" fibroblasts and endothelial cells on the ABI Prism 7900HT platform (Life Technologies, Foster City USA). Reactions contained 5 ml TaqMan Fast Universal Mastermix (no AMPerase) (Life Technologies, Foster City USA), 0.9 mM each primer, 0.25 mM probe and 2 mL cDNA reverse transcribed as above in a total volume of 10 ml. PCR conditions were a single cycle of 95ºC for 20 seconds followed by 50 cycles of 95 ºC for 1 second and 60 ºC for 20 seconds. The probes and primers to CD248 comprised a pre-validated off-the-shelf assay provided by Life Technologies (Foster City USA); Assay identification number available on request). The relative expression level of CD248 was then determined relative to the GUSB endogenous control and normalized to the level of CD248 transcripts in the low passage cells.

Selection of transcripts for study

Transcripts were selected via their association to the mTOR signaling pathway according to Gene ontology (GO) (www.geneontology.org) and the Kyoto Encyclopedia of Gene and Genomes (KEGG) and had also shown importance in our previous microarray analysis [15]. The transcripts included AKT, AKT1S1, DEPTOR, EIF4EBP2, EIF4G2, EIF4G3, FOXO1, FOXO3, GRB2, HIF1A, MTOR, NFKB1, PDK1, PIK3CA, PRKAG1, PRKAG2, PRKCA, PTEN, RAF1, RHEB, RICTOR, RPS6KB1, SGK1, SREBF1, STAT3, TSC1, TSC2, and VEGFB. The endogenous controls were IDH3B, GUSB, PPIA and GAPDH and were selected based a complete lack of any association with age in microarray study of aging [17].

TaqMan low-density array (TLDA) analysis

Reaction mixes included 50µl 2x TaqMan universal master mix (no AMPerase) (Life technologies, Foster City, USA), 40µl dH2O and 10µl cDNA template. 100 ml reaction solution was aliquoted into the chamber in the TLDA card, and centrifuged twice for 2 minutes at 1500rpm to ensure distribution of solution to the each TLDA micro-well. PCR amplifications were performed on the ABI 7900HT platform (Life Technologies, Foster City, USA). Cycling conditions were 50°C for 2 minutes, 94.5°C for 10 minutes followed by 40 cycles of 97°C for 30 seconds and 57.9°C for 1 minute. The expression of each gene was measured in triplicate for each sample. Relative gene expression levels were calculated using the comparative Ct technique [18], relative to the geometric mean of the four endogenous controls IDH3B, GUSB, PPIA and GAPDH. The values for

each candidate gene were then normalized back to the median value for that transcript in the low passage cells.

Statistical analysis

The statistical significance of apparent differences in gene expression levels between "young" and "old" cells was investigated using Mann Whitney-U analysis. Non-parametric statistics were employed due to the relatively small sample numbers and the fact that the data obtained were not expected to be normally distributed. All statistical analyses were carried out using STATA v10.1.

Results

Cell senescence in vitro

Normal human dermal fibroblasts (nHDF) and human aortic endothelial cells (HAOEC) underwent cell senescence by continuous culture until growth arrest in vitro. The two cell types underwent senescence at different rates, and senescence was accompanied in both cell types by increased β-galactosidase activity, a marker of cellular senescence [16], and alterations in morphology and doubling time. Fibroblasts ceased growing at p22 whilst endothelial cells ceased growth at p15. Both cell types demonstrated increases in the time-to-confluence during the senescence process from 3 to 10 days in the endothelial cells and from 4 to 27 days in the fibroblasts. Cells were analyzed two passages prior to growth arrest, since terminally senescent cells are rare in living people; data suggest that although there is a relationship between the senescence of cells in culture and lifespan in vivo, it may not be clear cut [19]. B-galactosidase staining was apparent in 68% of the endothelial cells at p13 and 58% of the fibroblasts at p20 (Fig. 2).

Confirmation of cell senescence using a molecular marker

Levels of *CD248* transcripts were found to be significantly higher in the 'old' cells compared to the 'young' cells for both fibroblasts and endothelial cells. In fibroblasts, the expression level of *CD248* relative to the endogenous control in 'old' cells was 1.33 (IQR 0.21) compared with 0.96 (IQR 0.12) in young cells (p=0.001). In 'old' aortic endothelial cells, the median level of *CD248* expression relative to the endogenous control in 'old' cells was 2.13 (IQR 2.72) compared with 0.75 (IQR 0.31) in 'young' cells (p=0.004).

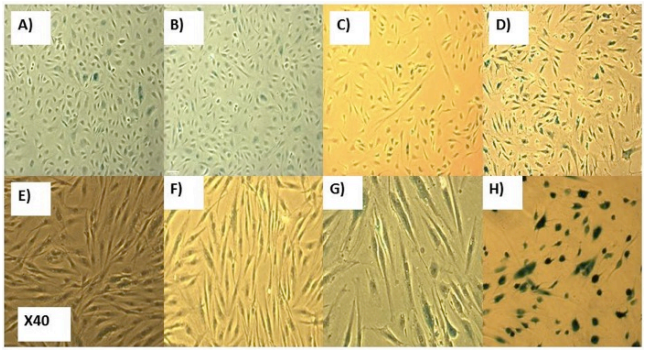

Figure 2. Biochemical markers of cell senescence

The figure demonstrates cell senescence as demonstrated by b-galactosidase staining in human aortic endothelial cells (HAOEC) and human normal dermal fibroblasts (nHDF). Panels A-D demonstrate staining patterns in human endothelial cells at passages p6; 7% staining (**A**), p8; 12% (**B**), p12; 38% staining (**C**) and p13; 68% staining (**D**). Panels E-G illustrates staining patterns for human fibroblasts at passages p13; 4% staining (**E**), p15; 39% staining (**F**), p20; 58% staining (**G**) and p22; 96% staining (**H**).

Senescence-induced gene expression changes in the levels of mTOR-related transcripts - fibroblasts.

Seven of the 28 genes tested for differential expression with cell senescence showed a significant change ($p<0.05$) between senescent and non-senescent fibroblasts in multiple independent analyses (Table 1).

Table 1. Gene expression changes in non senescent and senescent fibroblasts and endothelial cells*

	Median expression	IQR	Median expression	IQR	p-value
nHDF (fibroblasts)					
	p7		**p20**		
DEPTOR	0.960	0.490	1.840	1.350	0.047
EIF4G2	2.340	2.430	5.310	4.400	0.005
FOXO1	0.830	1.420	2.420	3.350	0.038
NFKB1	1.100	0.670	1.560	0.680	0.047
PRKCA	0.300	0.280	0.660	0.650	0.007
TSC1	0.900	0.530	2.940	2.990	0.007
TSC2	1.280	1.300	2.680	2.650	0.024
HAOEC (endothelial)					
	p7		**p13**		
SGK1	0.950	0.490	1.960	1.390	0.031
PRKCA	1.610	1.560	4.100	1.920	<0.001

* *The change in the expression of mTOR-related genes in fibroblasts and endothelial cells is given. nHDF = normal human dermal fibroblasts, HAOEC = human aortic endothelial cells. Fibroblasts were senescent at p20 and endothelial cells were senescent at p13. Differences in transcript expression levels were assessed by Mann-Whitney U analysis.*

The *TSC1* and *TSC2* genes which play a central inhibitory role in inhibition of mTORC1 signaling both demonstrated up-regulation in cells at p20 just prior to growth arrest (2.9 [IQR 3.0] v/s 0.9 [IQR 0.5]; p = 0.007 (*TSC1*) and 2.7 [IQR 2.7] v/s 1.3 [IQR 1.3]; p = 0.024 (*TSC2*). The mTORC1 and mTORC2 inhibitor *DEPTOR* also demonstrated increased expression in 'old' cells compared with 'young cells' (1.8 [IQR 1.4] v/s 1.0 [IQR 0.5]; p=0.047). Aged fibroblasts also demonstrated increased expression of transcripts such as *NFκB1,* a key regulator of inflammation, that are inhibited by mTOR signaling (1.6 [IQR 0.7] v/s 1.1 [IQR 0.7]; p=0.047). Other downstream targets such as the translation initiation factor *EIF4G2*, the cellular differentiation and proliferation factor *PRKCA* and the transcription factor *FOXO1* also demonstrated an increase in expression in aged cells (5.3 [IQR 4.4] v/s 2.3 [IQR 2.4]; p=0.005, 0.3 [IQR 0.3] v/s 0.7 [IQR 0.7]; p=0.007 and 2.4 [IQR 3.4] v/s 0.8 [IQR 1.4]; p=0.038 respectively).

Senescence-induced gene expression changes in the levels of mTOR-related transcripts – endothelial cells

Only two transcripts out of the 28 tested were differentially expressed in this tissue type (Table 1). The two genes were *SGK1* which plays a role in cell proliferation, migration, survival and apoptosis and the cellular differentiation and proliferation factor *PRKCA*, both of which demonstrated increased expression in aged cells, (relative expression 2.0 [IQR 1.4] v/s 1.0 [IQR 0.5]; p=0.031) and 4.1 [IQR 1.9] v/s 1.6 [IQR 1.6]; p<0.001 respectively). *PRKCA* and *SGK1* are components of the mTORC2 complex alone (Fig. 1). No other transcript demonstrated statistically significant changes in gene expression in aging endothelial cells.

Discussion

In vivo studies of the role of mTOR signaling in human senescence suggest that gene expression changes consistent with overall down-regulation of this pathway are evident in older humans in vivo [15]. However, the heterogeneous nature of the study tissue (whole blood) and the confounding factors that can influence gene expression in living human subjects pose difficulties for distinguishing between aging effects and other factors. To address this and to assess whether the expression of mTOR-related transcripts was also disturbed in human tissues other than blood, we carried out a series of in vitro cell senescence experiments on primary human dermal fibroblasts and aortic endothelial cells.

Here, we present data on senescence-associated changes in gene expression levels of 28 mTOR-related transcripts in two human cell lines of different lineages; human dermal fibroblasts and aortic endothelial cells. Human fibroblasts and endothelial cells became senescent after different numbers of passages, which is not unexpected, since different cell types are known to undergo senescence at different rates in vitro as they do in vivo [20]. Whilst cells of the fibroblast lineage demonstrate gene expression changes for transcripts located in the mTORC1 component of the mTOR signaling pathway (Fig. 1), the expression of mTORC1-related genes appears to be less apparent in senescent cells of the endothelial lineage. These cells instead demonstrate gene expression changes in transcripts involved in mTORC2-related processes, indicating that age-related mTOR-related gene expression changes may demonstrate some degree of tissue specificity.

Four of the seven transcripts we have found to be significantly up-regulated in senescent fibroblasts code for negative regulators of mTOR signaling (Table 1; Fig. 1). TSC1 and TSC2 are tumor suppressor genes which interpret cellular stress signals such as DNA damage or oxidative insult to repress mTORC1 signaling [21]. When TSC1 is activated it associates with the TSC2 gene product to inhibit RHEB and other down-stream processes [22]. DEPTOR is a key gene in the mTOR signaling pathway as it has the ability to inhibit both mTORC1 and mTORC2 complexes. Under stress conditions, DEPTOR accumulates leading to an increase in autophagy as a cellular protective mechanism [23]. Thus the balance between DEPTOR and other components of the mTOR pathway may play an essential role in maintenance of cellular homeostasis. Finally, the FOXO1 gene product maintains cellular homeostasis in mammals; once activated by stress conditions FOXO1 signaling can lead to inhibition of mTOR signaling, cell cycle arrest and an increase in autophagy [24]. These new findings are of particular importance because they suggest that the changes noted in our previous study were consistent with direct effects of advancing age, rather than merely representing changes in the composition of the blood

cell pool as has been documented to occur with age [25,26]. This provides reassurance that human leukocyte based samples can provide valid insights into in-vivo aging processes.

Two of the remaining transcripts demonstrating altered expression in senescent fibroblasts are also consistent with inhibition of mTORC1 signaling. The translation inhibitor EIF4G2 demonstrates increased expression in late-passage fibroblasts when compared to early passage cells. EIF4G2 is usually under the inhibitory control of the EIF4EBP1 repressor, the activity of which is itself known to be inhibited by the activity of TSC1/TSC2/DEPTOR (see Fig. 1). Although we see no concurrent decrease in EIF4EBP1 levels, it is important to note that most of the regulation of the mTOR pathway, like other signaling cascades, is coordinated by kinase and phosphatase activity at the level of the protein product [27]. Similarly, the NFKB1 gene, inhibited by up-stream mTORC1 signaling, demonstrates an increase in gene expression levels in our data. NFκB1 is one of the key mediators of inducible transcription in the innate immune system. It acts by controlling the expression of a network of inducers and effectors that dictate responses to pathogens and other classes of danger signals [28]. Increases in chronic innate immune activation are well documented in human aging [29] and the increase in NFKB1 expression we note in our data is entirely consistent with this observation. There are data in the literature to suggest that NFKB1 is involved in maintaining the aging phenotype; in a study between young and old tissues, it was found that there were significantly higher levels of genes associated with NFκB signaling. This may indicate that the aging phenotype might be associated with an enhanced need for active maintenance [30]. It is worth noting however that NFκB is regulated by other pathways and they may be other underlying reason for the increase in gene expression. The remaining gene that we find to be differentially expressed in senescent fibroblasts, PRKCA, is a component of the mTORC2 pathway. Increases in PRKCA expression have previously been reported in senescent rodent prostate, where it acts as an anti-apoptotic factor by activating the Bcl(2) protein [31]. The up-regulation of PRKCA expression we note in aged fibroblasts could also reflect an adaptive change in mTORC2 signaling to compensate for potential inhibition of the mTORC1 pathway. This is supported by our observation that FOXO1, another mTORC2 component is also up-regulated at the level of the mRNA transcript in senesced fibroblasts (Table 1).

Our data suggest that the expression of mTOR-related genes in human aging may be tissue specific. In contrast to fibroblasts, endothelial cells do not exhibit expression changes consistent with decreases in the expression of mTORC1-related genes, although the up-regulation of mTORC2-related genes we observed in senescent fibroblasts is conserved in endothelial cells as demonstrated by the elevated expression of the SGK1 and PRKCA genes (Table 1; Fig. 1). SGK1 is activated by insulin and nutrient signaling and mediates transport, hormone release, neuroexcitablity, cell proliferation, and apoptosis [32]. In endothelial cells, the increase in SGK1 expression is not accompanied by an increase in FOXO1 expression, but again, it is important to note that the regulation of FOXO1 by SGK1 is probably mediated by kinase activity at the level of the protein gene product. Differences in the specific pathways activated by the senescence processes have previous been reported between fibroblasts and endothelial cells. In a whole transcriptome microarray study, it was demonstrated that fibroblasts shared only a limited overlap in the pattern of genes altered by replicative senescence compared with endothelial cells and epithelial cells [33]. Fibroblasts demonstrated constitutive over-expression of inflammatory genes, consistent with the observation of elevated NFKB1 expression note in our data. It is possible that the differences in expression could have been due to differences in the culture media between the tissue types. However, no component of either medium has been linked with effecting aging to date. It remains a possibility that some of the differences in tissue-specific expression may arise from differences in the culture medium.

The strengths of this study include the use of primary cells of a single, homogeneous cell type that have undergone the aging process in vitro, with biochemically, morphologically and molecular definition of when senescence is reached. As such, our results are likely to represent genuine changes in the expression of genes linked with the senescence process, rather than reflecting alterations in the composition of different cell types with age. Our study is also on human cells, whereas the majority of work on the effects of mTOR signaling on aging and longevity has used data from animal models, which are much shorter lived than man. The use of in vitro cultures is a weakness of the study, in that it is unclear whether replicative senescence occurs in vivo [34].

However, recent studies by Sedivy et al. [35,36] have demonstrated the presence of senescent cells in aging mammals with >15% of cells displaying senescence markers [35,36]. With other groups also showing the presence of senescent cells in vivo [37], it is considered that in vitro senescence is a reasonable proxy for the aging process. In our study, we have elected not to assess the expression patterns of terminally senescent cells; our data derive from cells that are several passages away from growth arrest, which may be more representative of the situation in living humans. Finally, gene expression data of course do not inform on the post-translational regulation of the genes in question, and mTOR, like most other signaling pathways, is partly regulated by protein modifications [27]. However, identification of effects on downstream targets at the level of the RNA transcript can sometimes infer activity changes for their upstream regulators.

Overall our data suggests that reported human in-vivo down regulation of mTOR transcripts with age might be broadly consistent with in-vitro changes, at least in some primary human tissue types. Age-related transcript changes in primary human fibroblasts in-vitro involve mainly transcripts encoding factors involved in mTORC1 signaling. However, in human endothelial cells, expression changes are less apparent and appear to involve mTORC2-related transcripts.

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