Rapamycin Prevents Senescence and Cellular Aging of Mesenchymal Stem Cells in Culture

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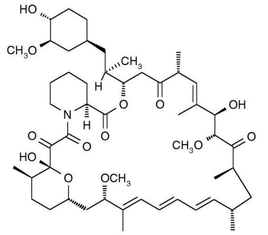


ABSTRACT

Objective: To confirm Rapamycin prolongs human placental mesenchymal stem cell (hPMSC)) survival in cultures and identify pathways through which Rapamycin slows progression of cellular aging and specify proteins acted upon to better define drug mechanism.

Results: Rapamycin was shown to decrease IL-6 levels while consistently increasing IL-8's. Significantly higher autophagy activity and reduced SA-B Gal staining were revealed in the rapamycin-treated group, Microarray analysis indicated a total of 396 genes significantly impacted in expression, with many downregulated in cell cycle promotion.

Conclusion: Rapamycin has proven effective in enhancing hPMSC longevity through regulation of autophagy activity, cell cycle progression, and metabolism. Cellular senescence-promoting pathways and proteins were significantly decreased, while pathways implicated in longevity, such as p53 and FOXO, were significantly increased following drug treatment.

INTRODUCTION

Aging is a common risk factor for chronic and severe disease, including neurodegeneration, tissue injury, and cancer. On a cellular level, senescence is characterized by many dysregulated processes. A reduction in cellular repair and maintenance mechanisms, such as protein ubiquitination, autophagy, and anti-inflammatory pathways, leads to toxin accumulation, inflammation, reduced immunity, and cancer-inducing mutation potential.

Stem cell therapy is a promising prevention and remedy to congenital, acquired, and age-related injury or disease. However, the difficulty in maintaining MSC viability and aptitude in sheet culture has limited their scope of clinical usage and regenerative potential.

Rapamycin, a natural mechanistic target of rapamycin (mTOR) inhibitor currently used in tumor-based cancer treatment and transplant rejection prevention, has exhibited anti-aging capabilities by increasing the lifespan of model organisms tested. Abundant evidence suggests enhanced mTOR signaling negatively influences cellular lifespan and plays an important role in autophagy, inflammation, and apoptosis pathway regulation, therefore, it is hypothesized mTOR inhibition can promote viability. However, there is insufficient research regarding the exact mechanism by which rapamycin prolongs cellular longevity. Characterizing rapamycin's mechanism of action would support manufacturing a new clinical function, age prevention, for this drug. This discovery would also enable prolonged MSC cultural growth and reduce therapeutic limitation.

This study aims to characterize rapamycin's efficacy in prolonging hPMSC lifespan in sheet culture and provide insight into which pathways are altered by this drug.

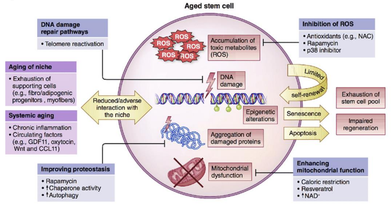


Figure 1. Mechanisms of Stem Cell Aging.

APPROACH

hPMSC Preparation: hPMSCs were isolated from human placenta delivered at normal term. Stemness was analyzed by colony formation and flow cytometry previously. The following experiments were performed on hPMSCs of passage 5-10.

High Density Culture and Treatment: hPMSCs were seeded at 70,000 cell/cm^2 into appropriately sized wells. After 24 hours, culture media was exchanged for culture media + rapamycin (10 nM) or DMSO (control) of equivalent volume. Treatment media was changed every other day.

SA-B Gal Staining: After 4 and 7 days of culture in 6-well plates, high-density monolayers were stained for SA B-Gal activity (Cell Signaling). Percent of SA B-Gal positive area was measured using ImageJ threshold method. Forward and side scatter were used to measure cell size and complexity, respectively.

Cytokine Measurements: 1 ml of culture media was extracted at day 1, 2, and 4 (each extraction 24 hours after re-treatment). IL-6 and IL-8 levels were quantified using ELISA.

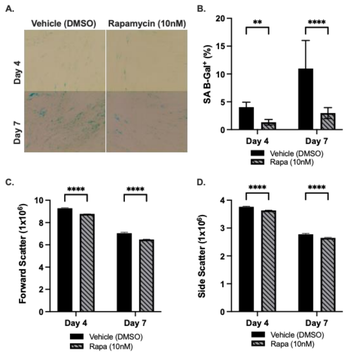
Autophagy Assessment: DAPI nuclear stain and green detection reagent were used in staining via autophagy assay kit. ImageJ was used to quantify fluorescence and the mean percent area of autophagy-related (green) stain was compared with DAPI (blue).

Microarray Gene analysis: Extracted RNA was hybridized through Clariom S Human arrays and quality and quantity were assessed with Agilent Tape Station and Qbit Broad Range RNA Assays, respectively. Pixel intensity measurement, feature extraction, data summarization, normalization, and differential gene analysis were performed in Transcriptome Analysis Console (TAC). Arrays were normalized using the SST-RMA (Signal Space Transformation Robust Multi-Chip Analysis) algorithm, which consists of background adjustment, quantile normalization and summarization. A gene map was constructed using STRING network.

Statistics: All experiments were carried out in technical triplicate, and treatment group (rapamycin) was compared to vehicle (DMSO) by 1-tailed Student's t-test. ** denotes p< 0.01 and * denotes p < 0.05

RESULTS

Figure 1. (**A**) High-density PMSC monolayers were stained for senescence-associated Beta-galactosidase activity on day 4 and 7. (**B**) At day 4, the rapamycin group had 67.7% less SA Beta-Gal positive area (0.0067, n=15). At day 7, the rapamycin group had 72.9% less SA Beta-Gal positive area (p < 0.001, n=15). (**C**) Forward scatter and side scatter (**D**) were used as measures of relative cell size and complexity. The rapamycin group had a 5.52% and 8% decrease in forward scatter at day 4 and 7, respectively (**C**). Side scatter decreased by 3.4% and 4.7% at day 4 and 7, respectively (**D**).



RESULTS (Con't)

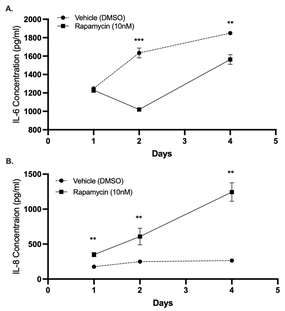
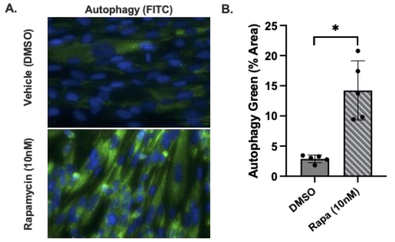


Figure 2. Rapamycin increases the expression of IL-8 and decreases IL-6 levels when compared to control following 24 hr, 48 hr, and 4-day treatment. (**A**) IL-6 concentration in rapamycin-treated culture was significantly reduced at 48 hours (614.3 pg/ml decrease, p=0.0004, n=3), and day 4 of treatment (286.6 pg/ml decreased difference, p=0.0008, n=3) compared to control (DMSO). (**B**) IL-8 concentration in rapamycin-treated culture was significantly increased in all time points, following 24 hours (173.1 pg/ml difference, p=0.00006, n=3), 48 hours (359.3 pg/ml increase, p=0.006, n=3), and 4 days (979.9 pg/ml increase, p=0.0002, n=3) of treatment.

Figure 3. (**A**) PMSCs in high-density monolayer were stained for autophagy associated vacuoles and DAPI. At day 4, the PMSCs with 10nM rapamycin showed a marked increase in autophagy. The percent positive area of autophagy stain was normalized to DAPI to quantify the relative change of autophagy level. (**B**) The rapamycin treated cells had a 3.94-fold increase in autophagy-associated stain compared to the vehicle control (p= 0.004, n=5). p<0.05.



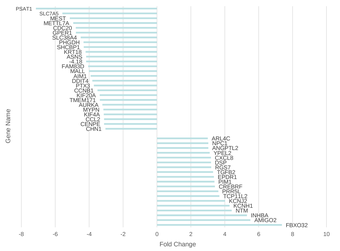
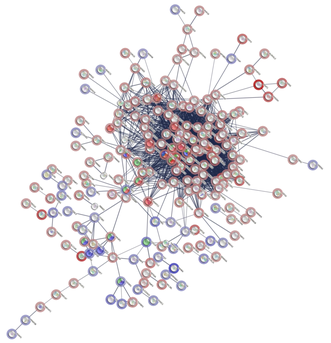


Figure 4. Bar graph of Microarray analysis genes with a 3-fold change or greater following rapamycin treatment. Upregulation of CXCL8 confirms rapamycin induces IL-8 production. FBXO32, implicated in FoxO signaling pathway, was most upregulated (fold-change 7.37), indicating potential contribution to cell longevity. PSAT1, involved in serine biosynthesis and cancer proliferation, was the highest downregulated gene (-7.14).

RESULTS (Con't)



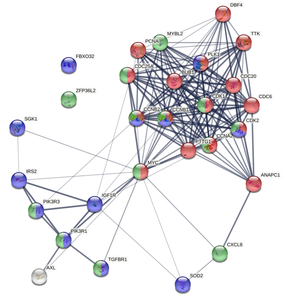


Figure 5a. A gene enrichment map was constructed from genes with a 2-fold or greater change in expression between rapamycin and DMSO groups. Nodes represent proteins encoded by their labelled gene locus. Edges represent protein-protein associations of high confidence (0.7). Halo color and intensity signifies gene down-regulation (red) or up-regulation (blue) and fold-change value, respectively. A total of 429 genes identified, with colored nodes indicating involvement in KEGG FoxO (blue; 16 genes), cellular senescence (red; 16 genes), and p53 pathways (green; 7 genes).

Figure 5b. FoxO (blue), cellular senescence (green), and cell cycle (red) KEGG pathway genes selected from whole enrichment gene map (above). 28 total genes with high confidence (0.7) protein interaction shown. Upregulated genes: SGK1 (oxidative stress resistance), FOXO32 (tumor suppressor), IRS2 (glucose homeostasis), CXCL8 (IL-8 pro-inflammatory cytokine). Down-regulated genes: CDC20, CDK1, CDC6, CCNB1, PLK1, BUB1 (cell cycle initiators or promoters).

CONCLUSIONS

Rapamycin has proven effective in prolonging placental mesenchymal stem cell lifespan, evident through decreased SA-B Gal staining and cellular size.
Interleukin-6, a pro-inflammatory cytokine, was significantly reduced in rapamycin-treated hPMSCs . Interleukin-8, shown to maintain cell stemness, had consistently increased production levels under drug treatment. Further promoting cell survival.
An increase in autophagy activity in rapamycin-treated cells supports a potential mechanism this drug acts on to prevent cell functional decline through direct mTOR signaling inhibition.
Gene analysis revealed complex interactions between rapamycin and genes associated with age-related diseases, specifically FoxO, p53, and cellular senescence signaling pathways.
Future experiments will be focused on how rapamycin regulates these differentially expressed proteins. Western blots will be performed to confirm protein expression alteration and significance.

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