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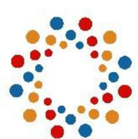

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RESEARCH ARTICLE

Metadichol®-Induced Expression of Sirtuin 1-7 In Somatic and Cancer Cells

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ABSTRACT

The Sirtuins 1-7 family and Klotho (KL), Forkhead box protein O1 (FOXO1), telomerase reverse transcriptase (TERT), tumor suppressor p53 (TP53) and growth differentiation factor 11 (GDF11) regulate aging, metabolism, and DNA repair and are involved in age-related diseases such as cancer, cardiovascular disease, and diabetes.
Seven sirtuin genes in humans encode seven sirtuin enzymes (SIRT1–7), each of which has unique functions and subcellular locations. Sirtuins are nicotinamide adenine dinucleotide (NAD+)-dependent protein deacetylases that play a significant role in physiological processes such as energy metabolism, stress responses, DNA repair, and gene expression and are potential targets for age-related diseases such as type 2 diabetes, inflammatory diseases, and neurodegenerative disorders. They also play a role in cancer by regulating critical cellular processes such as DNA repair and energy metabolism. Other genes, such as Klotho (KL), Forkhead box protein O1 (FOXO1), telomerase reverse transcriptase (TERT), tumor suppressor p53 (TP53) and growth differentiation factor 11 (GDF11), also regulate aging, metabolism, and DNA repair and are involved in age-related diseases such as cancer, cardiovascular disease, and diabetes.

In addition, these proteins are closely related to sirtuins. A single molecule that can activate these five genes and sirtuin genes is challenging because each isoform has a unique structure, substrate, and regulatory mechanism. Most known sirtuin activators are specific for Sirtuin 1, the most studied isoform of the sirtuin family. This study was initiated based on previous work in which we showed that metadichol can express all nuclear receptors if it is possible to express all seven sirtuin families 1-7 using metadichol as a small molecule inducer. Herein, we report that at concentrations ranging from 1 pg/mL to 100 ng/mL, Metadichol®, a nanoemulsion of long-chain alcohols, induced the expression of the human Sirtuin 1-7 gene in dermal fibroblasts and a variety of cancer cells in a concentration-dependent manner and that KL, GDF11, telomerase, Foxo1 and P53 could have significant beneficial effects on mitigating age-related diseases. The results were quantified by using qRT–PCR, and proteins were characterized using western blot techniques. The experimental procedure used is unique in that it did not involve the use of viruses or other gene insertion technique.

Introduction

The sirtuin family is a family of signaling proteins involved in metabolic regulation and cellular stress responses. Nicotinamide adenine dinucleotide (NAD+)-dependent deacetylases or ADP ribosyltransferases can modulate the acylation state of various histone and nonhistone proteins.[1,2] These proteins are highly conserved throughout all kingdoms of life but are diverse in eukaryotes. Mammals possess seven sirtuins (SIRT1–7) that occupy different subcellular compartments: SIRT1, SIRT6, and SIRT7 are predominantly found in the nucleus; SIRT2 is found in the cytoplasm; and SIRT3, SIRT4, and SIRT5 are found in mitochondria.[3,4]

Sirtuins have been implicated in several diseases, including cancer, metabolic disorders, neurodegenerative diseases, and cardiovascular diseases (Figures 1-3 and Table 1). For example, SIRT1 has been shown to play a role in tumor suppression,[5] whereas SIRT2 has a role in neurodegenerative diseases such as Parkinson's disease and Alzheimer's disease.[6] Moreover, SIRT3 has been found to regulate the oxidative stress response in cells and may play a protective role in cardiovascular diseases.[7] SIRT6 has also been shown to regulate glucose homeostasis, and its deficiency has been linked to aging-related diseases such as diabetes and osteoporosis.[8] There is evidence suggesting that sirtuin family members interact with each other to regulate their activity and function.[9] For example, SIRT1 has been shown to regulate the activity of SIRT3 and SIRT6, and SIRT1 increases SIRT3 activity through deacetylation, which enhances mitochondrial function and increases energy production. SIRT1, together with SIRT6, regulates glucose homeostasis and DNA repair.[10] Similarly, SIRT3 interacts with SIRT4 and SIRT5 to regulate cellular metabolism.[11] SIRT4 inhibits glutamine metabolism by suppressing glutamate dehydrogenase,[12] and SIRT5 regulates the urea cycle by desuccinylating and demalonylating carbamoyl phosphate synthase1.[13]

By interacting with TP53, telomerase reverse transcriptase (TERT), Klotho (KL), FOXO1, and the growth differentiation factor 11 (GDF11) sirtuin regulate vital cellular processes[14-17] that can maintain cellular homeostasis.

TP53 is a tumor suppressor gene. SIRT1 has been shown to interact with p53 in response to DNA damage and to regulate TP53-dependent transcriptional activity. SIRT1 deacetylates p53, leading to decreased activity and expression of p53, which impacts cell apoptosis and cell cycle arrest.[18]

KL regulates aging and metabolism. SIRT1 upregulates KL gene expression in various tissues, including the brain, kidney, and liver. KL overexpression has been associated with increased lifespan and improved glucose homeostasis, and SIRT1 has a role in mediating these effects.[19,20]

Figure 1. Enzymatic functions of sirtuins

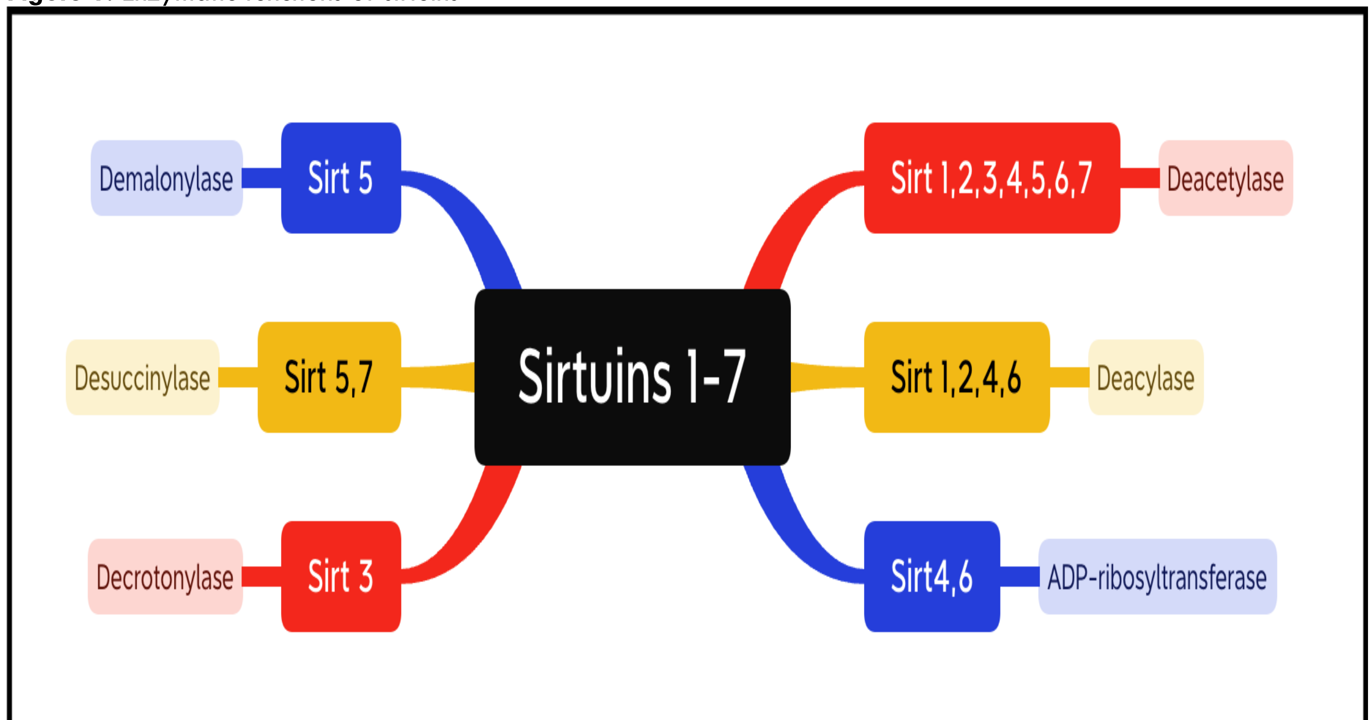
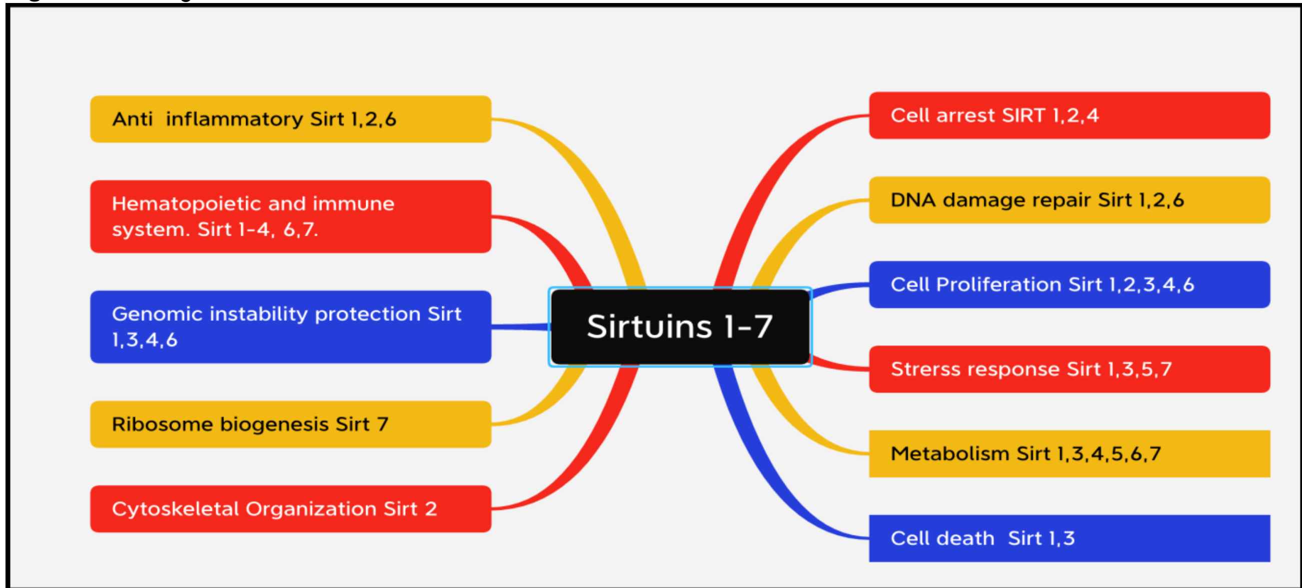


Figure 2. Biological functions of sirtuins



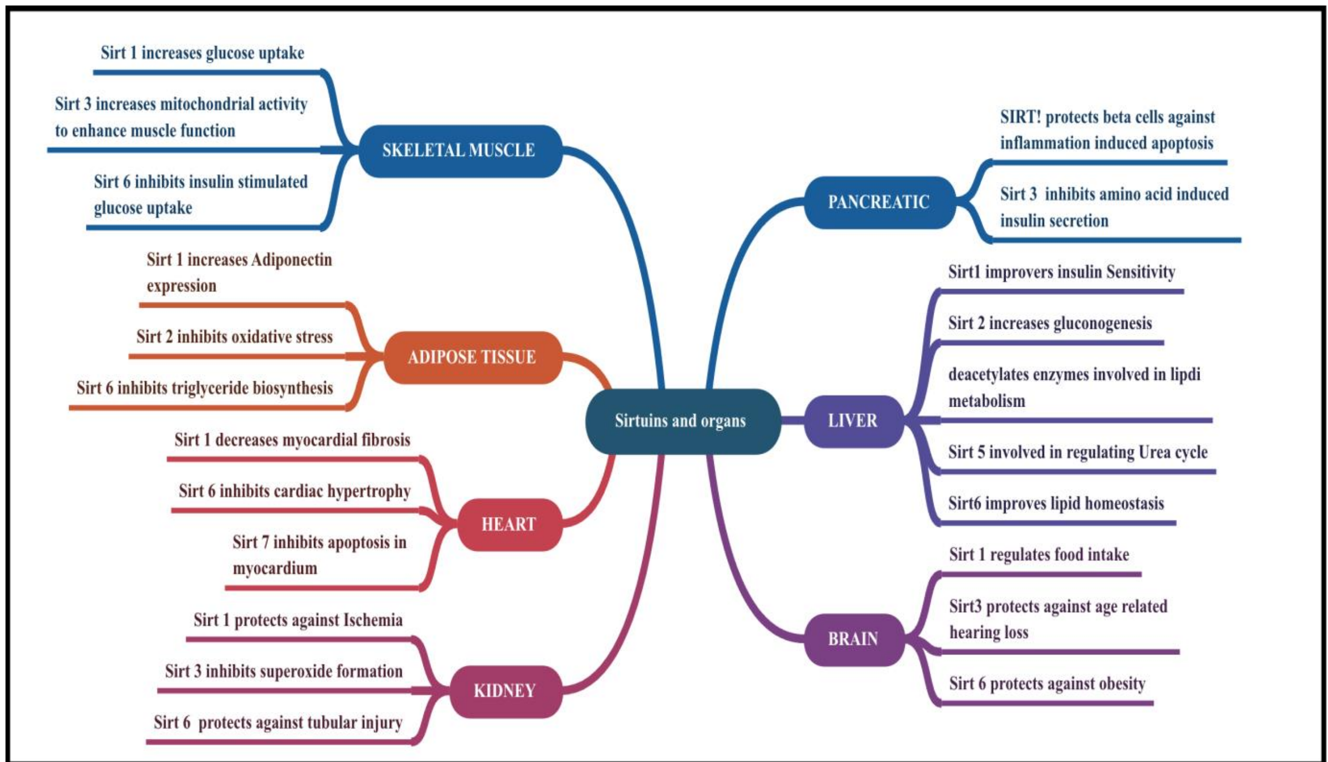


Figure 3. Biological effects of sirtuins on organs and tissues

Table 1. Summary of sirtuin functions and related pathways

SIRT1	Involved in gene expression, DNA repair, metabolism, and aging
SIRT2	Involved in cell division, DNA repair, and metabolism
SIRT3	Involved in energy metabolism, mitochondrial function, and oxidative stress
SIRT4	Involved in insulin secretion, amino acid metabolism, and mitochondrial function
SIRT5	Involved in amino acid metabolism, mitochondrial function, and DNA repair
SIRT6	Involved in DNA repair, telomere maintenance, and metabolism

SIRT7	Involved RNA polymerase activity, ribosome biogenesis, and DNA repair
TP53	Encodes tumor protein p53; critical in regulating cell growth, DNA repair, and apoptosis; is commonly called the "guardian of the genome" because it helps prevent mutations that can lead to cancer
TERT	Encodes telomerase reverse transcriptase, which maintains the length of telomeres, the protective caps at the end of chromosomes
KL	Encodes Klotho, which regulates aging, mineral metabolism, and cardiovascular function
FOXO1	Encodes forkhead box protein O1, which regulates metabolism, cell cycle, and apoptosis; involved in the development of insulin resistance and diabetes
GDF11	A member of the transforming growth factor β superfamily; has important roles in processes other than differentiation and embryonic development, such as age-related muscle dysfunction, skin function, metabolism, and notably, cancer

FOXO1 is a forkhead box transcription factor that regulates glucose metabolism, apoptosis, and the stress response.[21] SIRT1 deacetylates and activates FOXO1, which leads to increased expression of genes involved in gluconeogenesis and stress resistance.[22] SIRT1 regulates the activity of other FOXO family members, including FOXO3 and FOXO4.[23,24]

The TERT gene and sirtuins modulate each other's activity and function. SIRT1 deacylates the TERT gene and increases its expression. The TERT gene activates SIRT1 by inhibiting p53 signaling.[15] Both SIRT1 and the TERT gene improve cellular senescence, stem cell function, and tumorigenesis. SIRT3 also deacetylates the TERT gene and increases its expression and activity.[25] SIRT3 is activated by the TERT gene via the inhibition of ROS production. SIRT3 and the TERT gene interact to protect against mitochondrial dysfunction, oxidative stress, and apoptosis. SIRT6 deacetylates the TERT gene and decreases its expression and activity.[26,27] The TERT gene can also inhibit SIRT6 through the activation of NF-κB signaling.[28] Moreover, SIRT6 and the TERT gene can antagonize each other in the regulation of cellular senescence, the DNA damage response, and tumorigenesis.

Growth differentiation factor 11 (GDF11), a member of the transforming growth factor β superfamily, plays an important role in processes other than differentiation and embryonic development, such as age-related muscle dysfunction, skin function, metabolism, and, notably, cancer.[29] GDF11 is expressed in various tissues, and its expression can be influenced by pathological conditions or age. The functions of GDF11 in cancer are complex and somewhat controversial. GDF11 has shown tumor-suppressor functions in certain types of cancer, such as triple-negative breast cancer, where its overexpression can inhibit tumor progression.[30] Conversely, GDF11 is also related to carcinogenesis, and its pattern of expression in tumors is intricate. It can promote tumor growth in some cancers, such as colon cancer, and inhibit cell proliferation in others, such as breast cancer.[31] GDF11 can influence metabolism in pancreatic cells, which may support its role in cancer metabolism.[32]

Most known sirtuin activators are specific for SIRT1, the most studied isoform of the sirtuin family. Some examples of SIRT1 activators are resveratrol[33] and benzimidazoles.[34] Resveratrol is found in red wine, grapes, berries, and peanuts. Resveratrol decreases the Michaelis constant of SIRT1 for acetylated substrates and NAD+ and increases cell survival by stimulating the SIRT1-dependent deacetylation of p53. Resveratrol also activates other sirtuin isoforms, such as SIRT3 and SIRT5, albeit with lower potency. Benzimidazoles are synthetic compounds developed by Sirtris Pharmaceuticals based on the structure of resveratrol. They include SRT1720, SRT1460, and SRT2183. These compounds are indirect activators of Sirt1.[35]

There are a few reports of small-molecule activators specific for other sirtuin isoforms.[36,37] For example, quercetin is a natural compound found in apples, onions, tea, and red wine that can activate SIRT2 and SIRT6. Tenovin-6 is a synthetic compound that inhibits sirtuins at high concentrations but paradoxically activates SIRT3 at low concentrations. Honokiol is a natural compound derived from magnolia bark that activates SIRT7.

In summary, there is no known single molecule that can activate all seven sirtuin genes, and finding a molecule that can do so is challenging because each sirtuin isoform has a unique structure, substrate, and regulatory mechanism. Herein, we report that metadichol, a nanoformulation of long-chain alcohols[38], induces the expression of the Sirtuin 1-7 gene, as well as the KL,[39] TERT,[40] TP53, FOXO1 and

GDF11 genes, in normal human dermal fibroblasts (NHDFs) at concentrations ranging from 1 pg to 1 ng/mL.

Methods

EXPERIMENTAL PROCEDURES

Quantitative RT–PCR, western blotting, and cell culture were performed by Skanda Life Sciences, Bangalore, India, a commercial service provider. Gene network analysis using Pathway Studio was performed by Elsevier R&D Solutions, Inc.[41] The raw qRT–PCR data are provided in the Supplemental files.

CHEMICALS AND REAGENTS

Normal human dermal fibroblasts (NHDFs) and cancer cells were obtained from ATCC (USA). Primary antibodies were purchased from ABclonal (Woburn, Massachusetts, USA) and E-lab Science (Maryland, USA). The primers used were obtained from SahaGene (Hyderabad, India) (Table 2). Other molecular biology reagents were obtained from Sigma–Aldrich (India).

Table 2 Target-specific forward and reverse primers

Primer		Sequence	Annealing Temperature (°C)
GAPDH	Forward	GTCTCCTCTGACTTCAACAGCG	60
	Reverse	ACCACCCTGTTGCTGTAGCCAA	
SIRT-1	Forward	TAGACACGCTGGAACAGGTTGC	54
	Reverse	CTCCTCGTACAGCTTCACAGTC	
SIRT-2	Forward	CTGCGGAACTTATTCTCCCAGAC	60
	Reverse	CCACCAAACAGATGACTCTGCG	
SIRT-3	Forward	CCCTGGAAACTACAAGCCCAAC	52
	Reverse	GCAGAGGCAAAGGTTCCATGAG	
SIRT-4	Forward	GTGGATGCTTTGCACACCAAGG	64
	Reverse	GGTTCAGGACTTGGAAACGCTC	
SIRT-5	Forward	GTCCACACGAAACCAGATTTGCC	56
	Reverse	TCCTCTGAAGGTCGGAACACCA	
SIRT-6	Forward	TGGCAGTCTTCCAGTGTGGTGT	64
	Reverse	CGCTCTCAAAGGTGGTGTCGAA	
SIRT-7	Forward	TGGAGTGTGGACACTGCTTCAG	64
	Reverse	CCGTCACAGTTCTGAGACACCA	
FOXO-1	Forward	CTACGAGTGGATGGTCAAGAGC	54
	Reverse	CCAGTTCCTTCATTCTGCACACG	
P53	Forward	CCTCAGCATCTTATCCGAGTGG	58
	Reverse	TGGATGGTGGTACAGTCAGAGC	
TERT	Forward	GCCGATTGTGAACATGGACTACG	56
	Reverse	GCTCGTAGTTGAGCACGCTGAA	
KLOTHO	Forward	GTGCGTCCATCTGGGATACG	60
	Reverse	TGTCGCGGAAGACGTTGTT	
GDF 11	Forward	CCACCGAGACCGTCATTAGC	60
	Reverse	CAGGCCGTAGGTACACCCA	

MAINTENANCE AND SEEDING

The cells were maintained in the appropriate medium with or without the required supplements and 1% antibiotics in a humidified atmosphere of 5% CO_2 at 37 °C. The medium was changed every other day until the cells reached confluency. The viability of the cells was assessed using a hemocytometer.

When the cells reached 70–80% confluence, single-cell suspensions containing 106 cells/mL were prepared and seeded in 6-well plates at a density of 1 million cells/well. The cells were incubated for 24 h at 37 °C in 5% CO_2. After 24 h, the cell monolayer was rinsed with serum-free medium and treated with metadichol at predetermined concentrations.

CELL TREATMENTS

Different concentrations of metadichol (1 pg/mL, 100 pg/mL, 1 ng/mL, and 100 ng/mL) were prepared in serum-free medium. Subsequently, Metadichol-containing medium was added to the predesignated wells. The control cells received medium without the drug. The cells were incubated for 24 h. After treatment, the cells were gently rinsed with sterile PBS. Whole-cell RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's instructions and reverse transcribed into cDNA. The samples were used for

qPCR and western blot analysis of various biomarkers.

QUANTITATIVE REAL-TIME PCR (qRT–PCR)

RNA isolation

Total RNA (Table 2) was isolated from each treatment group using TRIzol. Approximately 1×106 cells were collected in 1.5 mL microcentrifuge tubes. The cells were centrifuged at 5000 rpm for 5 min at 4 °C, after which the supernatant was discarded. Then, 650 µL of TRIzol was added to the pellet, and the contents were mixed well and incubated on ice for 20 min. Subsequently, 300 µL of chloroform was added to the mixture, and the samples were mixed well by gentle inversion for 1–2 min and incubated on ice for 10 min. The samples were centrifuged at 12000 rpm for 15 min at 4 °C.

Table 3. Total RNA yield

Treatment group	Treatment	RNA yield (ng/µL)
Control	NA	434.08
Sample	1 pg	288.32
	100 pg	593.44
	1 ng	301.6
	100 ng	400.64

The upper aqueous layer was transferred to a new, sterile 1.5 mL centrifuge tube, and an equal amount of prechilled isopropanol was added to the tube. The samples were incubated at -20 °C for 60 min. After incubation, the mixture was centrifuged at 12000 rpm for 15 min at 4 °C. The supernatant was discarded carefully, and the RNA pellet was retained. The pellet was washed with 1.0 mL of 100% ethanol followed by 700 µL of 70% ethanol via centrifugation, as described above, after each step. The RNA pellet was air-dried at RT for approximately 15–20 min and then resuspended in 30 µL of DEPC-treated water. The RNA concentration was quantified using a Spectradrop (SpectraMax i3x, USA) spectrophotometer (Molecular Devices), and cDNA was synthesized using reverse-transcription PCR (RT–PCR).

cDNA SYNTHESIS

cDNA was synthesized from 2 µg of RNA using a PrimeScript cDNA synthesis kit (Takara) and oligo dT primers per the manufacturer's instructions. The reaction volume was 20 µL, and cDNA synthesis was performed on an Applied Biosystems instrument (Veritii). cDNA was used for qPCR (50 °C for 30 min followed by 85 °C for 5 min).

PRIMERS AND qPCR

The PCR mixture (final volume of 20 µL) contained 1 µL of cDNA, 10 µL of SYBR Green Master Mix, and one µM complementary forward and reverse primers specific for the respective target genes. The reaction conditions were as follows: initial denaturation at 95 °C for 5 min, followed by 30 cycles of secondary denaturation at 95 °C for 30 s, annealing at the optimized temperature for 30 s, and extension at 72 °C for 1 min. The number of cycles that allowed amplification in the exponential range without reaching a plateau was selected as the optimal number of cycles. The obtained results were analyzed using CFX Maestro software.

The fold change was calculated using the ΔΔCT method.

The comparative CT method was used to determine the relative expression of target genes to that of the housekeeping gene (β-actin) in untreated control cells.

The delta CT for each treatment was calculated using the following formula: Delta Ct = Ct (target gene) – Ct (reference gene).

To compare the delta Ct of individually treated samples with that of the untreated control samples, the Ct of each group was subtracted from that of the control to obtain the delta CT.

Delta delta Ct = delta Ct (treatment group) – delta Ct (control group).

The fold change in target gene expression for each treatment group was calculated using the following formula: Fold change = 2^ (−delta delta Ct).

PROTEIN ISOLATION

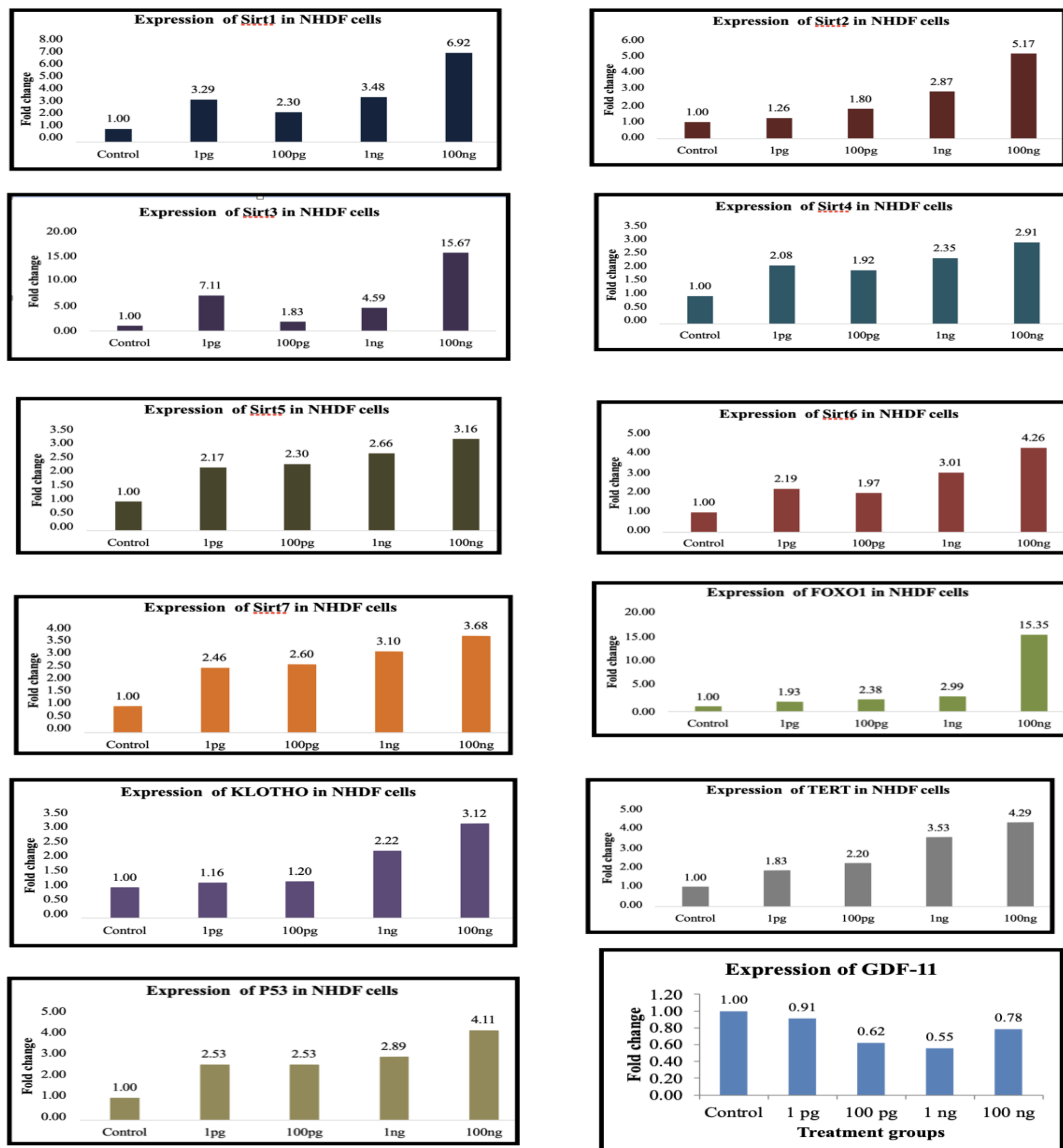
Total protein was isolated from 106 cells using RIPA buffer supplemented with the protease inhibitor PMSF. The cells were lysed for 30 min at 4 °C with gentle inversion. The cells were centrifuged at 10,000 rpm for 15 min, and the supernatant was transferred to a fresh tube. The Bradford method was used to determine the protein concentration, and 25 µg of protein was mixed with 1× sample loading dye containing SDS and loaded onto a gel. The proteins were separated under denaturing conditions in Tris-glycine buffer. The proteins were

transferred to methanol-activated PVDF membranes (Invitrogen) using a Turbo transblot system (Bio-Rad, USA). Nonspecific binding was blocked by incubating the membranes in 5% BSA for 1 h. The membranes were incubated overnight with the respective primary antibodies at 4 °C, followed by incubation with a species-specific secondary antibody for 1 h at RT. The blots were washed and incubated with an enhanced chemiluminescence (ECL) substrate (Merck) for 1 min in the dark. Images were captured at appropriate exposure settings using a ChemiDoc XRS system (Bio-Rad, USA).

Results

To determine whether Metadichol can activate all seven sirtuin isoforms, we first aimed to determine measure the expression of sirtuins and related genes by qPCR treatment of NHDFs with Metadichol® increased the expression of all sirtuin family genes and the closely related genes KL, GDF11, TERT, FOXO1, and TP53 (Figure 4). The fold increase in expression ranged from 2- to 15-fold after treatment with various concentrations of Metadichol®, ranging from 1 pg to 100 ng. The expression of SIRT3 and FOXO1 increased 15-fold, whereas that of SIRT1 increased 7-fold.

Figure 4. qRT–PCR analysis of SIRT1-7 gene expression in human dermal fibroblasts after treatment with Metadichol®



Discussion

Unlike existing methodology for gene expression studies this work did not involve the use of viruses or other gene insertion techniques. The results indicate that treatment of HDFCs with Metadichol® can induce the gene expression of all sirtuin family members (SIRT1–7), despite the differences between sirtuin isoforms.[10] Given their different structures, it is improbable that Metadichol® binds directly to DNA elements of the gene to activate them. It is far more likely that Metadichol® activates upstream regulatory genes, leading to the cascade of genes being activated downstream. Sirtuins are NAD^+-dependent enzymes.[42] Nuclear receptors do not directly regulate NAD+, they can influence the expression of genes involved in metabolic pathways that impact NAD+ levels. For instance, peroxisome proliferator-activated receptors (PPARs) are a group of nuclear receptor proteins that function as transcription factors regulating the expression of genes involved in fatty acid storage and glucose metabolism. These metabolic processes can indirectly affect NAD+ levels within cells.[43,44] We have recently shown that Metadichol® treatment increases nuclear receptor expression[45] in stem cells and fibroblasts (Table 4).

Table 4. Nuclear receptor expression induced in HMSCs by metadichol treatment; the bold text indicates the greatest increase in the expression of each of the nuclear receptors

Nuclear receptor	Common name	Control	1 picogram	100 picogram	1 nanogram	100 nanogram
NR1D1	Rev-ErbAα	1	1.5	0.64	0.07	0.75
NR1D2	Rev-ErbAβ	1	2.19	1.18	1.8	1.82
NR1H2	LXRa	1	4.95	1.03	0.03	1.71
NR1H3	LXRB	1	1.63	1.79	3.84	0.71
NR2E1	TLX	1	3.38	1.49	1.69	0.88
NR5A1	SF1	1	3.2	2.56	1.92	5.09
NR3B1	Err alpha	1	10.31	8.3	6.58	11.1
NR1C1	PPAR alpha	1	1.9	1.24	0.72	1.8

Sirt3 was overexpressed fifteenfold. Sirt3 overexpression protects cochlear hair cells against noise-induced hearing loss by reducing oxidative stress and apoptosis, disrupting mitochondrial proteostasis, and influencing cell cycle progression by altering the acetylation levels of mitochondrial proteins and inducing DNA damage.[46,47] SIRT3 also inhibits high glucose-induced cellular senescence by deacetylating and activating FOXO1, which influences the expression of antioxidant enzymes and reduces oxidative stress. This process can protect against thoracic aortic dissection by attenuating oxidative stress, apoptosis, and inflammation in vascular smooth muscle cells.[48]

FOXO1 is overexpressed fifteenfold and regulates many substrates,[49] including IRS2, GLUT4, PGC-1α, and SOD2. Previously, we reported that both IRS proteins and GLUT4 exhibit increased expression after treatment of stem cells with Metadichol®.[50] These substrates are involved in various cellular processes, such as glucose metabolism, lipid metabolism, oxidative stress, cell cycle arrest, and apoptosis. FOXO1 has been implicated [43,51-53] in the pathogenesis and prevention of various diseases, such as diabetes, obesity, cardiovascular diseases, neurodegenerative diseases, and cancers. For example, FOXO1 can improve insulin sensitivity and glucose homeostasis by regulating gluconeogenic enzymes, and GLUT4 expression can inhibit adipogenesis and lipogenesis by suppressing PPARγ and SREBP-1c expression. FOXO1 can protect against cardiac hypertrophy.

There are nuclear receptors activate transcription factors regulate the expression of genes involved in NAD^+ biosynthesis or consumption.[54] NAD^+ is a cofactor for various enzymatic reactions and a substrate for NAD^+-dependent enzymes such as sirtuins, poly-ADP-ribose polymerases, and cyclic ADP-ribose (cADPR) synthases. NAD^+ levels are influenced by circadian rhythms, nutrient availability, and cellular stress. The nuclear receptors that activate NAD^+ are shown in Table 4. REV-ERBα and REV-ERBβ are repressive components of the molecular circadian clock and regulate metabolism and inflammation.[55] REV-ERBs repress the expression of E4BP4, a transcriptional repressor of NAMPT, the rate-limiting enzyme in the NAD^+ salvage pathway. By repressing E4BP4, REV-ERBs activate NAMPT expression and NAD^+ production in a circadian manner.

TLX is a transcriptional repressor that promotes neural precursor cell proliferation and self-renewal. TLX directly binds to the promoter of SIRT1 and activates its transcription. LXR regulates cholesterol and lipid metabolism, inflammation, and immunity. LXR upregulates the expression of SIRT1, which can deacetylate and activate LXR,[56] increasing its

transcriptional activity. LXR activation also induces the expression of SIRT6, which modulates lipid metabolism and inflammation.[57]

NR5A1 encodes the steroidogenic factor 1 (SF-1) protein, a nuclear receptor and transcription factor that regulates the expression of genes involved in the development and function of the adrenal glands, gonads, and hypothalamus. There is some evidence that SF-1 and SIRTs interact with each other in different ways.[58] For example, SF-1 can regulate the expression of SIRT1 in the adrenal glands and gonads. SF-1 binds to the promoter region of the SIRT1 gene and activates its transcription. In addition, SIRT1 and SF-1 can cooperate to regulate fetal and adult Leydig cell development. Leydig cells are the primary source of testosterone in males.[59]

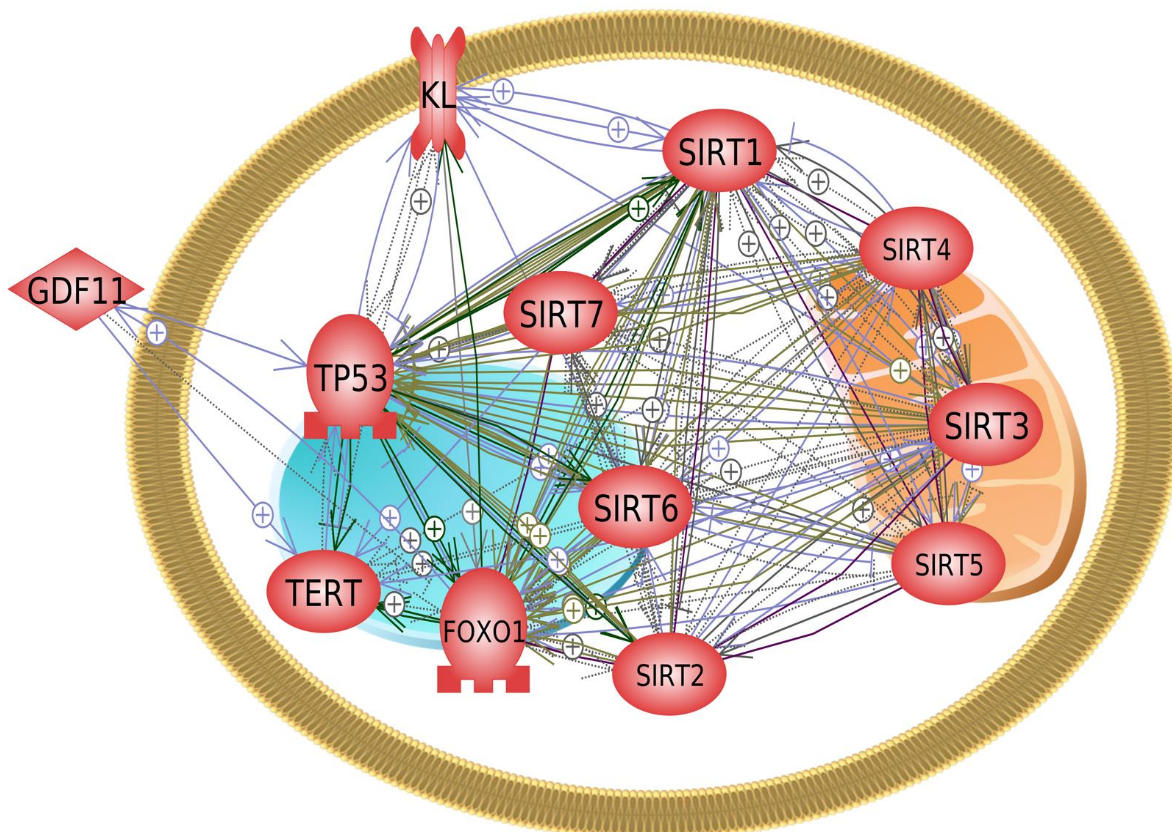
Other nuclear receptors, such as estrogen-related receptor α (ERRα), peroxisome proliferator-activated receptor α (PPARα), and nuclear respiratory factor 2 (NRF2), can control the expression of NAD^+ biosynthetic enzymes.[56,60-63] These nuclear receptors can induce the expression of nicotinamide mononucleotide adenylyltransferase 1 (NMNAT1), which is a nuclear enzyme that catalyzes the conversion of nicotinamide mononucleotide (NMN) to NAD^+. ERRα, PPARα, and NRF2 can induce the expression of NMNAT1.

In addition, thyroid receptors, such as THRA and THRB, interact with SIRTs.[57,64-65] For example, the thyroid can regulate the expression and activity of SIRT6. SIRT1 can affect TH signaling by deacetylating some target genes or coregulators. Metadichol® binds to and interacts with both THRA and THR.[66]

It is likely that all genes of the sirtuin family are activated directly by upstream genes involved in transcription, i.e., nuclear receptors. Given the many nuclear receptors involved, sirtuin gene regulation is likely a complex process. These nuclear receptors produce NAD^+, which is needed for the function of sirtuins. NAD+ is involved in the activation of nuclear receptors by Metadichol. [67]

We utilized Pathway Studio software [68,69] to perform gene set enrichment analysis (GSEA) with Elsevier's proprietary pathway collection. More genes were expressed than expected for a gene set of the same size and distribution randomly selected from the genome. This enrichment indicates that this set of genes shares a significant biological connection [70] Figure 5 and Tables 5-10.

Figure 5. Interactions between gene sets.



All the relationships between the 12 genes are available in the Elsevier Biology Knowledge Graph.

- SIRT1 interacts with p53 to regulate DNA repair and apoptosis. SIRT1 can deacetylate TP53, enhancing p53 protein-mediated transcriptional activity and increasing apoptosis.
- SIRT6 interacts with TP53 to regulate DNA repair and apoptosis. SIRT6 can deacetylate TP53, reducing p53 protein-mediated transcriptional activity and decreasing apoptosis.
- SIRT3 and SIRT5 are both involved in regulating cellular metabolism and mitochondrial function. SIRT3 has been shown to deacetylate and deactivate SIRT5, and the SIRT5 protein can deacetylate and activate enzymes involved in fatty acid metabolism.
- TP53 has been shown to transcriptionally repress the expression of TERT, which is involved in maintaining the length of telomeres. In addition, TERT can interact with p53 to regulate apoptosis and the DNA damage response.
- FOXO1 can be deacetylated and activated by SIRT1, leading to increased expression of genes involved in metabolism and the cellular stress response.

These interactions suggest that the different sirtuin family members work together to regulate various cellular processes and maintain cellular homeostasis. In a previous study, a detailed analysis of the protein–protein network of sirtuin family members was performed.[9] Researchers have shown that the network covers 25% of all protein interactions in humans. Their analyses support many of the known metabolic functions of sirtuins in humans and provide insight into the complex molecular mechanisms of action of sirtuins, which are still poorly understood.

The expressed genes play a role in regulating aging, metabolism, and DNA repair.[71-72] In addition, all of these genes have been linked to age-related diseases such as cancer, cardiovascular disease, and diabetes (Table 1). These genes are essential for maintaining the health and proper functioning of cells and tissues, and their dysregulation has been associated with various age-related pathologies. These results were further confirmed by pathway studio analysis and are shown in Tables 5-8. We can make a few observations from these results.

The key biological process involved is the regulation of the mTOR pathway. The overexpression of Sirt6 has been shown to inhibit tumor growth in non-small cell lung cancer and promote cell apoptosis and cell cycle arrest in diffuse large B-cell lymphoma (DLBCL) cells and in non small cell cancers.[73] Additionally, Sirt6 overexpression or activation has beneficial effects on cellular metabolic regulation and aging,[74] and it has been found to inhibit the mTOR signaling pathway, which is associated with various cellular processes, such as the cell cycle, DNA damage, and cell apoptosis. The table shows pathways where that Sirt1, Sirt2, Sirt3, Sirt6, and Sirt7 play important roles in anti-aging pathways.

Table 5. List of the top 10 significantly enriched pathways (Fisher's exact test, p value < 0.05)

Biological Process	Parent Folder	# of Entities	Expanded # of Entities	Overlap	Percent Overlap	Overlapping Entities	p Value
Regulation of cell metabolism by MTOR/TP53	Eating behavior and metabolism regulation (biological process); Hallmarks of deregulated metabolism in cancer	47	90	3	3	SIRT6, TP53, FOXO1	3.53E-05
SIRT7 signaling in aging	Genomic instability associated with aging (aging biology)	27	98	3	3	SIRT7, TP53, FOXO1	4.55E-05
SIRT1 signaling in aging	Genomic instability associated with aging (aging biology)	64	170	3	1	TP53, FOXO1, SIRT1	2.34E-04
Longevity-related drugs	Aging biology (biological process)	43	173	3	1	SIRT7, SIRT3, SIRT1	2.46E-04
SIRT2 signaling in aging	Genomic instability associated with aging	38	238	3	1	TP53, FOXO1, SIRT2	6.29E-04
SIRT3 signaling in aging	Genomic instability associated with aging	47	75	2	2	TP53, SIRT3	1.40E-03
Inhibition of mTOR signaling by persistent DNA repair	DNA repair (cell process); fundamental signaling pathways changed during aging	55	95	2	2	TP53, SIRT1	2.24E-03
Adipokine production by adipocytes	Lipogenesis and lipolysis (biological process); adipose tissue	58	104	2	1	FOXO1, SIRT1	2.68E-03
SIRT6 signaling in aging	Genomic instability associated with aging (aging biology)	32	170	2	1	SIRT6, TP53	7.00E-03
ADCY-PKA-|myofibroblasts in the corneal stroma	Corneal stroma remodeling	126	171	2	1	TP53, SIRT1	7.08E-03

Table 6. Cellular processes

Cellular process	Parent Folder	# of Entities	Expanded # of Entities	Overlap	Percent Overlap	Overlapping Entities	p value
Telomere maintenance	DNA machinery	27	36	2	5	TERT, TP53	6.14227E-05
Inhibition of mTOR signaling by persistent DNA repair	DNA repair; fundamental signaling pathways changed during aging	55	95	2	2	TP53, SIRT1	4.32228E-04
SCF/SKP2 complexation	Chromatin remodeling	35	277	2	0	TP53, FOXO1	3.61940E-03
G2/M damage checkpoint	Cell cycle regulation	22	44	1	2	TP53	1.58036E-02
Histone sumoylation	Histone modification	25	65	1	1	TP53	2.32798E-02
Histone acetylation	Histone modification	33	139	1	0	SIRT1	4.92856E-02

The key cellular processes of telomere maintenance and the inhibition of mTOR are key players in aging and cancer

Table 7. Gene sets and disease

Associated disease or condition	# of Entities	Expanded # of Entities	Overlap	Percent Overlap	overlapping entities	p Value
Huntington's disease	111	192	4	2	TP53, SIRT2, SIRT3, SIRT1	3.3414E-06
Werner syndrome (adult progeria)	28	238	4	1	TP53,TERT, SIRT4, SIRT1	7.8282E-06
Alveolar epithelial cell dysfunction in COPD	91	179	3	1	SIRT6 , TP53, SIRT1	1.3394E-04
Cell Cycle dysregulation in mantle cell lymphoma	30	61	2	3	TP53, Sirt1	5.6333E-04
Adiponectin synthesis reduction in insulin resistance	53	78	2	2	SIRT1,Foxo1	9.1939E-04
Ca2+ overload in Huntington's disease	94	361	3	0	TP53, FoxO1, Sirt1	1.0449E-03
Impaired adipokine production by adipocytes in Obesity	56	98	2	2	FOXO1, Sirt1	1.4458E-03
B-Cell acute lymphoblastic leukemia	103	548	3	0	TP53,FoxO1, Tert	3.4505E-03
NSP14 interaction	8	8	1	12	SIRT5	4.69057E-03

Table 8. Cell processes regulated by the input gene set

Regulated Process	Total # of Neighbors	Overlap	Percent Overlap	Overlapping Entities	p Value
Apoptosis	13743	12	0	GDF11, SIRT2, SIRT7, SIRT6, TP53, SIRT5, FOXO1, SIRT4, SIRT3, SIRT1, TERT, KL	0E+00
Cell proliferation	15770	12	0	GDF11, SIRT2, SIRT7, SIRT6, TP53, SIRT5, FOXO1, SIRT4, SIRT3, SIRT1, TERT, KL	0E+00
Protein acetylation	127	8	6	SIRT5, SIRT4, SIRT3, SIRT1, SIRT2, SIRT7, SIRT6, TP53	2.62195E-14
Mitochondrial organization and biogenesis	1139	12	1	GDF11, SIRT2, SIRT7, SIRT6, TP53, SIRT5, FOXO1, SIRT4, SIRT3, SIRT1, TERT, KL	1.45053E-13
Cell lifespan	168	8	4	SIRT5, SIRT4, SIRT1, TERT, SIRT2, SIRT6, TP53, KL	2.57085E-13
Chromatin silencing	100	7	6	SIRT5, SIRT4, SIRT3, SIRT1, SIRT2, SIRT7, SIRT6	8.34739E-13
Endothelial cell aging	200	8	3	FOXO1, SIRT3, SIRT1, TERT, SIRT7, SIRT6, TP53, KL	1.05681E-12
Tumor metabolism	356	9	2	SIRT5, FOXO1, SIRT4, SIRT3, SIRT1, SIRT2, SIRT7, SIRT6, TP53	1.29182E-12
Recombinational repair	212	8	3	SIRT5, SIRT4, SIRT3, SIRT1, SIRT2, SIRT7, SIRT6, TP53	1.69262E-12
Cell redox homeostasis	624	10	1	SIRT5, FOXO1, SIRT4, SIRT3, SIRT1, TERT, SIRT2, SIRT6, TP53, KL	2.8804E-12

The key processes involved in apoptotic cancer and aging are apoptosis, cell proliferation, and cell life span.

Table 9. Diseases regulated by the gene set

Regulated Disease	Total # of Neighbors	Overlap	Percent Overlap	Overlapping Entities	p value
Geriatric disorders	257	12	4	GDF11, SIRT2, SIRT7, SIRT6, TP53, SIRT5, FOXO1, SIRT4, SIRT3, SIRT1, TERT, KL	1.49528E-24
Posteoarthritis	1615	12	0	GDF11, SIRT2, SIRT7, SIRT6, TP53, SIRT5, FOXO1, SIRT4, SIRT3, SIRT1, TERT, KL	7.0638E-15
Neurodegenerative diseases	1626	12	0	GDF11, SIRT2, SIRT7, SIRT6, TP53, SIRT5, FOXO1, SIRT4, SIRT3, SIRT1, TERT, KL	7.66541E-15
Involutional osteoporosis	102	7	6	GDF11, FOXO1, SIRT3, SIRT1, SIRT2, SIRT6, KL	1.43961E-14
Heart disease	1816	12	0	GDF11, SIRT2, SIRT7, SIRT6, TP53, SIRT5, FOXO1, SIRT4, SIRT3, SIRT1, TERT, KL	2.89956E-14
Metabolic stress	443	9	2	SIRT5, FOXO1, SIRT4, SIRT3, SIRT1, SIRT2, SIRT7, SIRT6, TP53	4.2635E-14
Cardiomyopathy	1230	11	0	GDF11, SIRT5, FOXO1, SIRT4, SIRT3, SIRT1, TERT, SIRT7, SIRT6, TP53, KL	6.06494E-14
Hypertrophy	2122	12	0	GDF11, SIRT2, SIRT7, SIRT6, TP53, SIRT5, FOXO1, SIRT4, SIRT3, SIRT1, TERT, KL	1.88873E-13
Cardiac hypertrophy	2144	12	0	GDF11, SIRT2, SIRT7, SIRT6, TP53, SIRT5, FOXO1, SIRT4, SIRT3, SIRT1, TERT, KL	2.13827E-13

The key diseases impacted are geriatric disorders and other diseases, such as arthritis and neurodegenerative diseases, and others, which are aging-related diseases.

Table 10. Protein entities related to different organs

Related Organ/Cells	Total # of Neighbors	Overlap	Percent Overlap	Overlapping Entities	p Value
Testis	10388	12	0	GDF11, SIRT2, SIRT7, SIRT6, TP53, SIRT5, FOXO1, SIRT4, SIRT3, SIRT1, TERT, KL	0.00000E+00
Cerebral cortex	8885	12	0	GDF11, SIRT2, SIRT7, SIRT6, TP53, SIRT5, FOXO1, SIRT4, SIRT3, SIRT1, TERT, KL	0.00000E+00
Brain	11791	12	0	GDF11, SIRT2, SIRT7, SIRT6, TP53, SIRT5, FOXO1, SIRT4, SIRT3, SIRT1, TERT, KL	0.00000E+00
Neurons	9349	12	0	GDF11, SIRT2, SIRT7, SIRT6, TP53, SIRT5, FOXO1, SIRT4, SIRT3, SIRT1, TERT, KL	0.00000E+00
Lung	10058	12	0	GDF11, SIRT2, SIRT7, SIRT6, TP53, SIRT5, FOXO1, SIRT4, SIRT3, SIRT1, TERT, KL	0.00000E+00
Kidney	10916	11	0	GDF11, SIRT5, FOXO1, SIRT4, SIRT3, SIRT1, TERT, SIRT2, SIRT7, SIRT6, TP53	0.00000E+00
Liver	10017	12	0	GDF11, SIRT2, SIRT7, SIRT6, TP53, SIRT5, FOXO1, SIRT4, SIRT3, SIRT1, TERT, KL	0.00000E+00
H9c2(2-1) cells (rat cardiac cells that exhibit many of the properties of skeletal muscle cells; can be used in cardiovascular disease research)	1228	12	0	GDF11, SIRT2, SIRT7, SIRT6, TP53, SIRT5, FOXO1, SIRT4, SIRT3, SIRT1, TERT, KL	6.98566E-11
Adipose derived stem cell	1012	11	1	GDF11, SIRT5, FOXO1, SIRT4, SIRT3, SIRT1, TERT, SIRT2, SIRT6, TP53, KL	6.22104E-10
JVM-3 cells (chronic B-cell leukemia)	8	3	33	SIRT1, SIRT2, TP53	1.16327E-07

Gene sets expressed in key organs are shown. Interestingly, all 12 genes are present in the testis,[77] brain, cerebral cortex,[78] neurons,[79] lung,[80] and liver.[81] The expression of 12 genes in the testis, brain, liver, lung and neurons showed that these genes are absolutely necessary for human reproduction and survival.

We also extended the study of the expression of sirtuins in cancer cell lines treated with metadichol. The results seen in Figures 6-13. we observed dose-dependent changes in the expression of 12 genes (SIRT1-7, KL, TERT, TP53, FOXO1, and GDF11). The sirtuin family (Figure 14) plays crucial roles in cancer biology and has diverse implications.[82]

Figure 6. Gene set expression in Jurkat cells (leukemia cells)

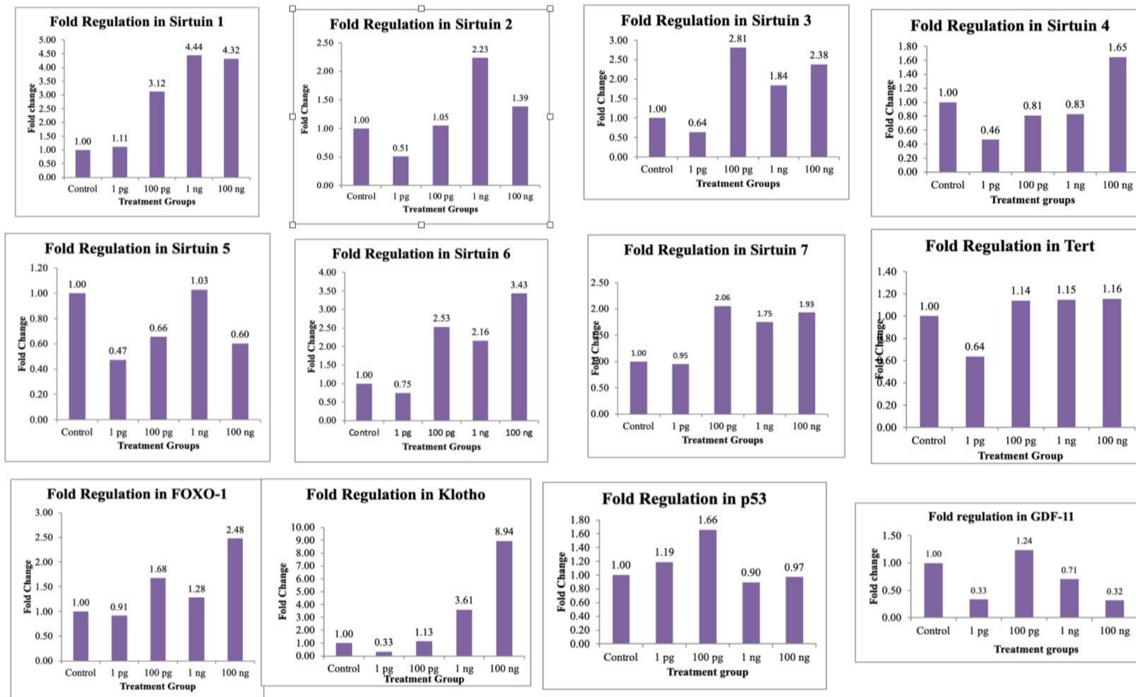


Figure 7: Gene set expression in U87MF (Glibosoma cells)

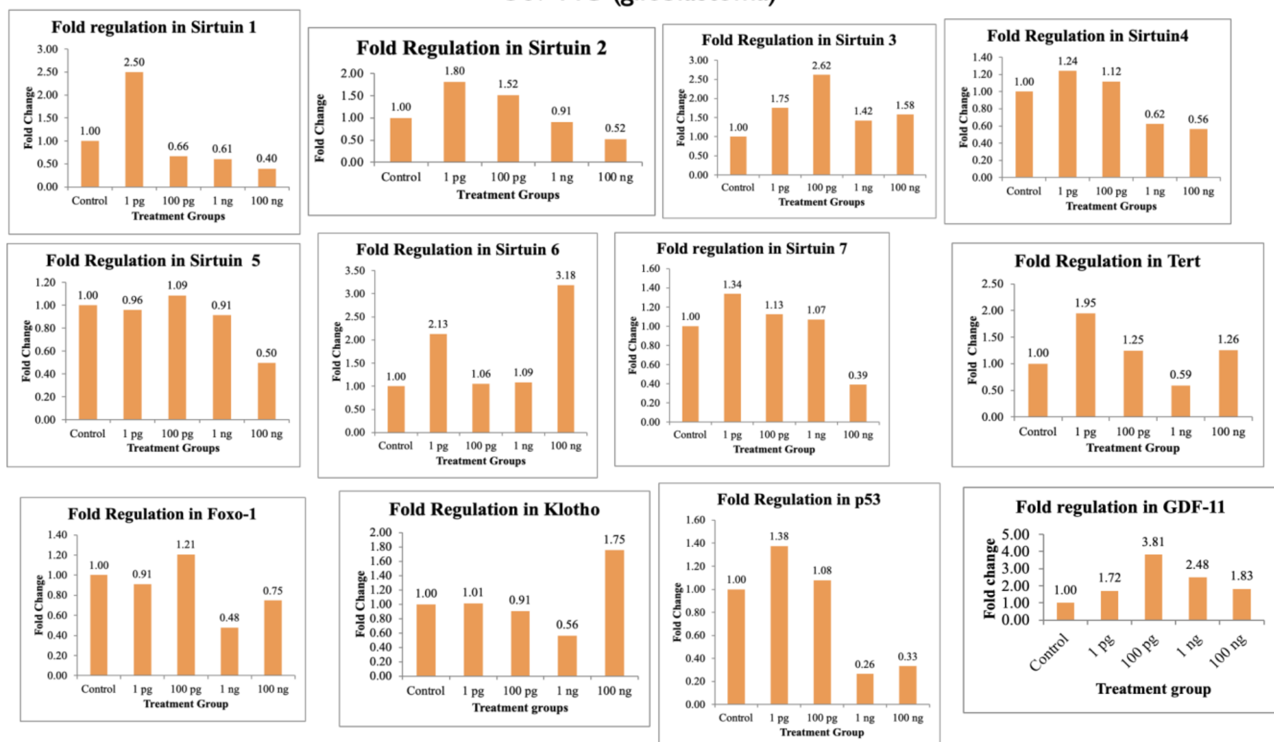


Figure 8. Gene set expression in lung cancer cell lines.

A-549(lung cancer)

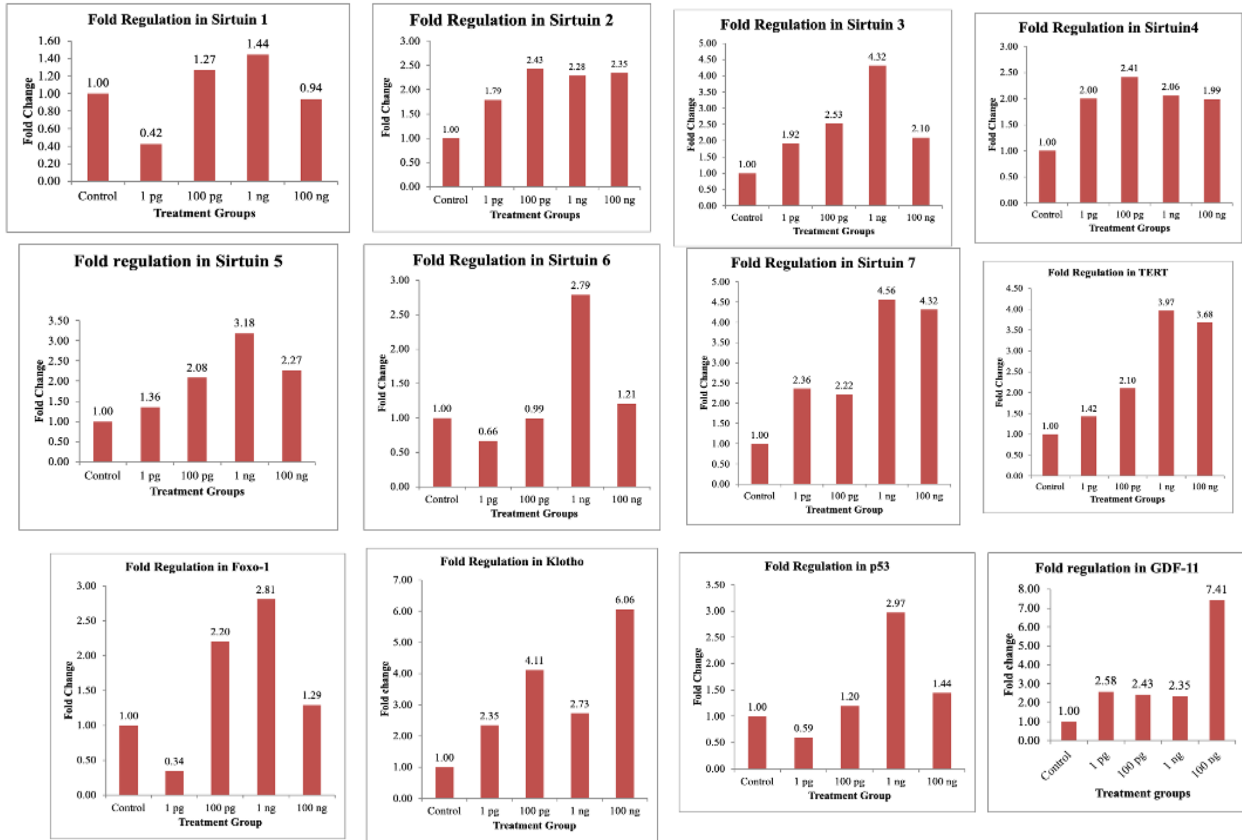


Figure 9. Gene set expression in the ovarian cancer cell line

SKOV3.1 (ovarian cancer)

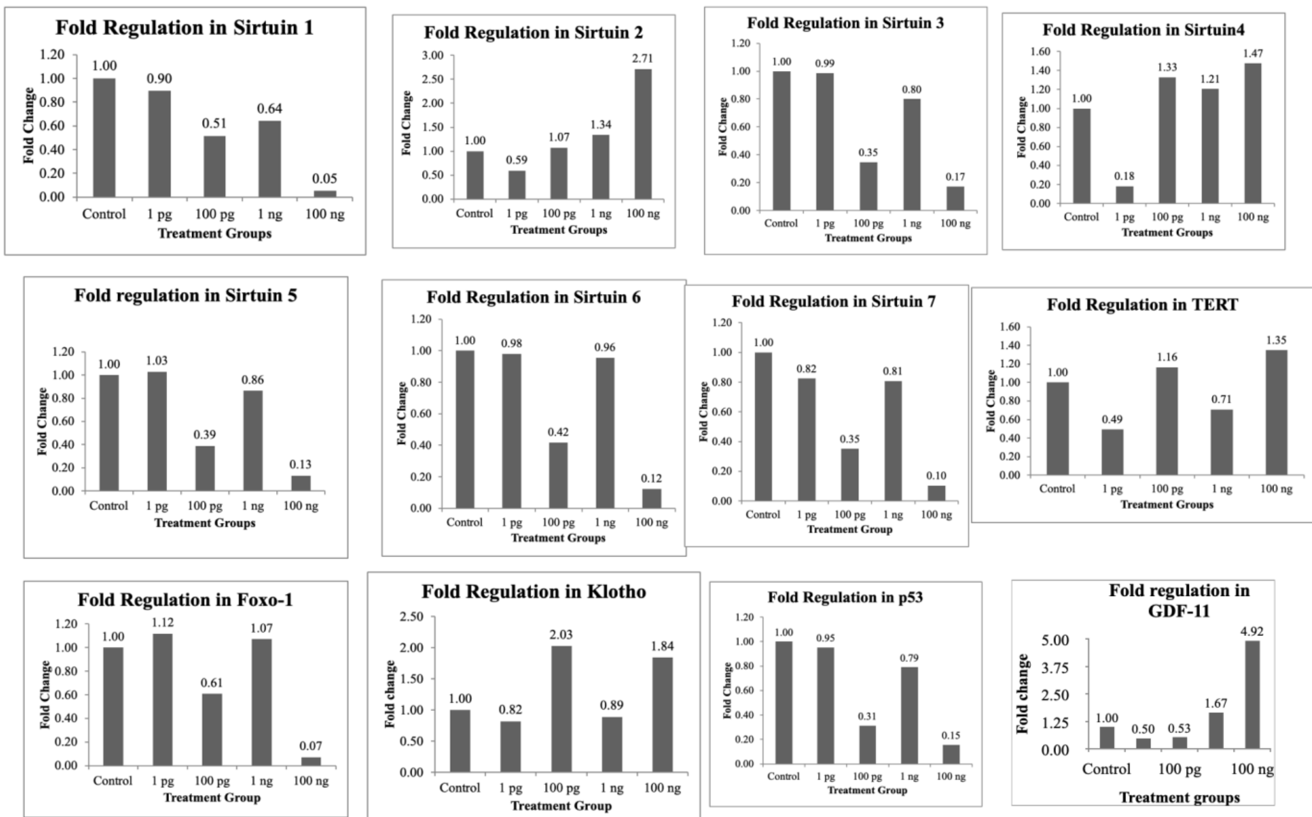


Figure 10. Gene set expression in pancreatic cancer cell lines

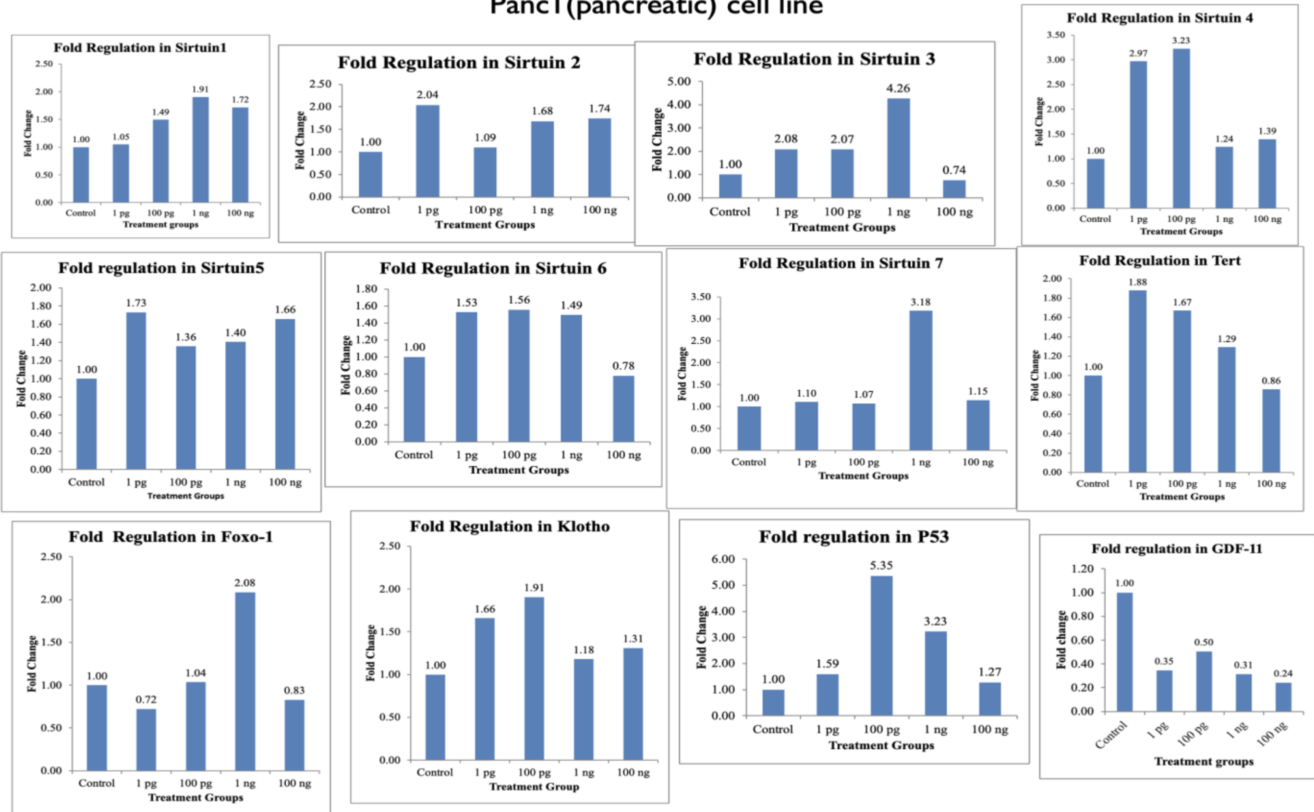


Figure 11. Gene set expression in colorectal cancer cell lines.

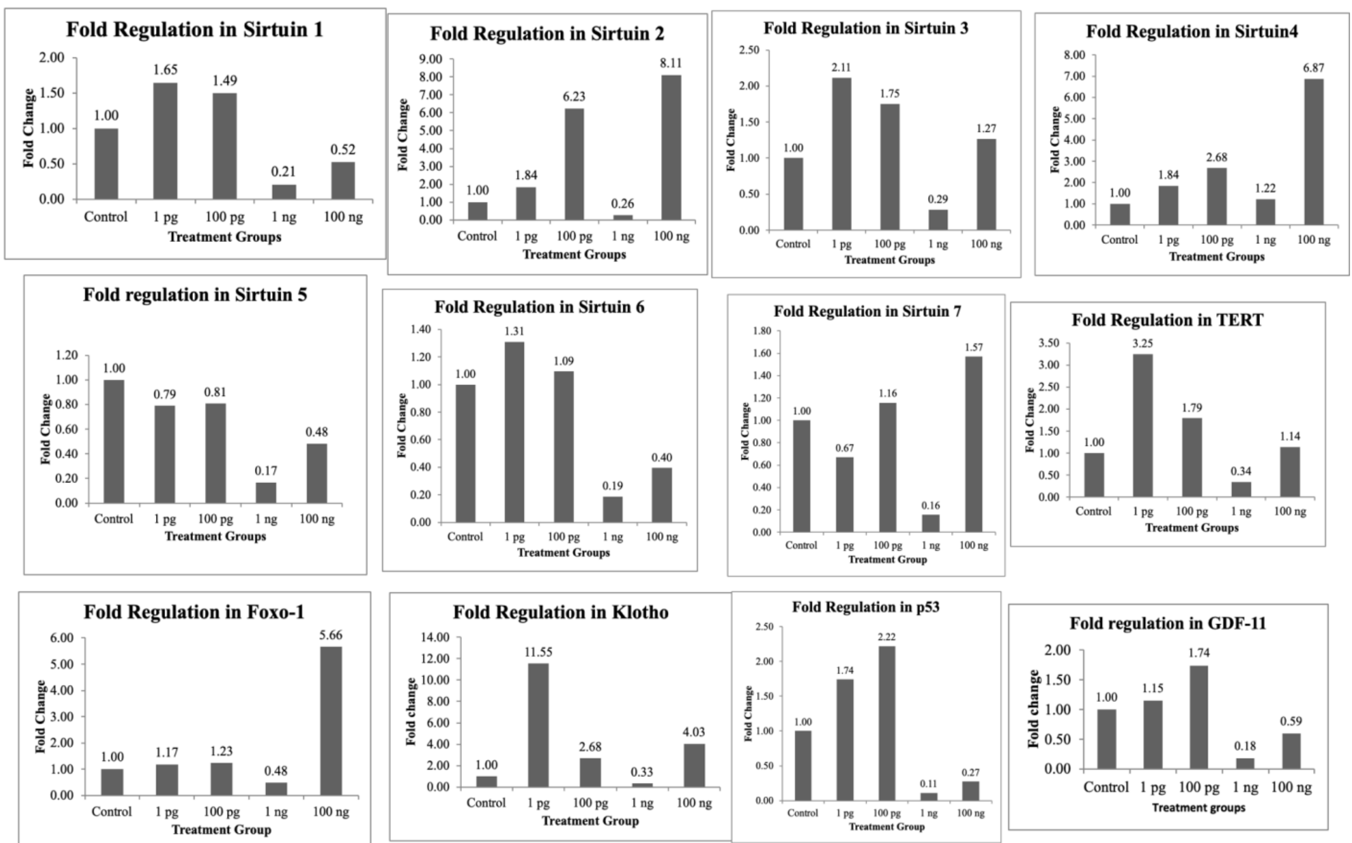


Figure 12. Gene set expression in the primary breast cancer cell line.

BCFA (primary breast cancer)

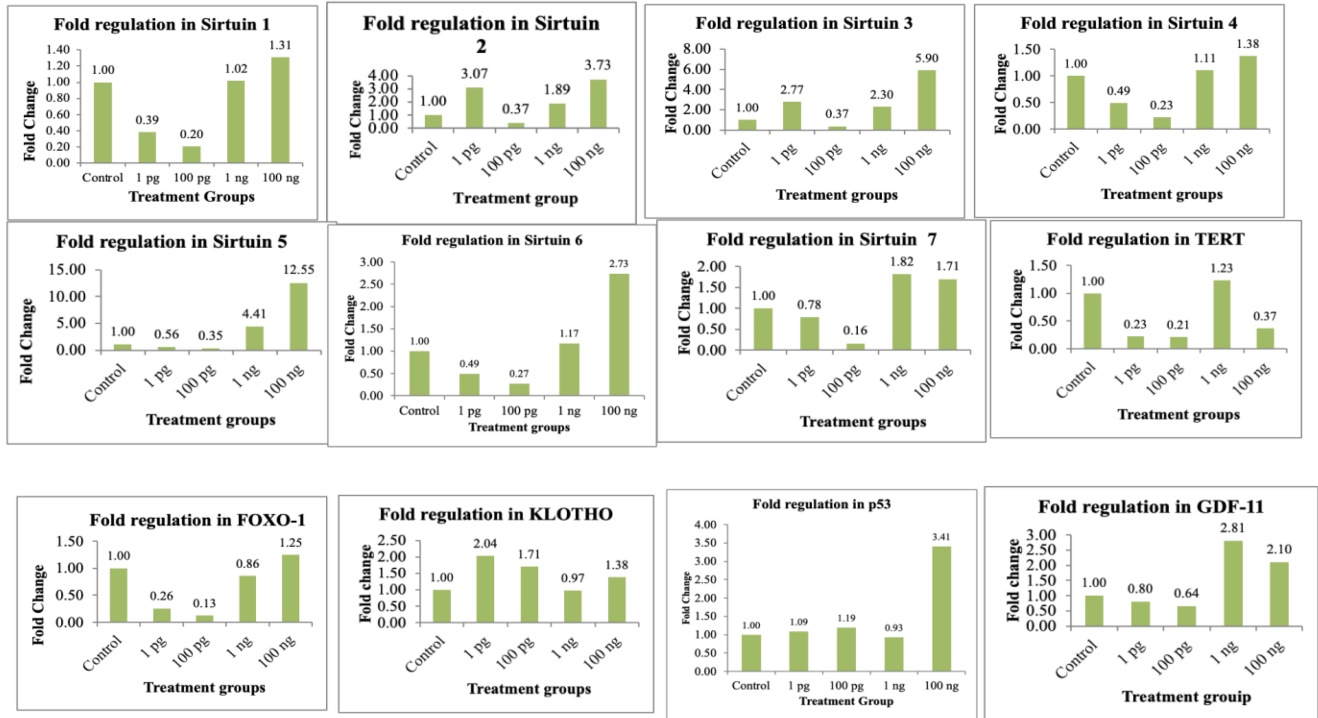
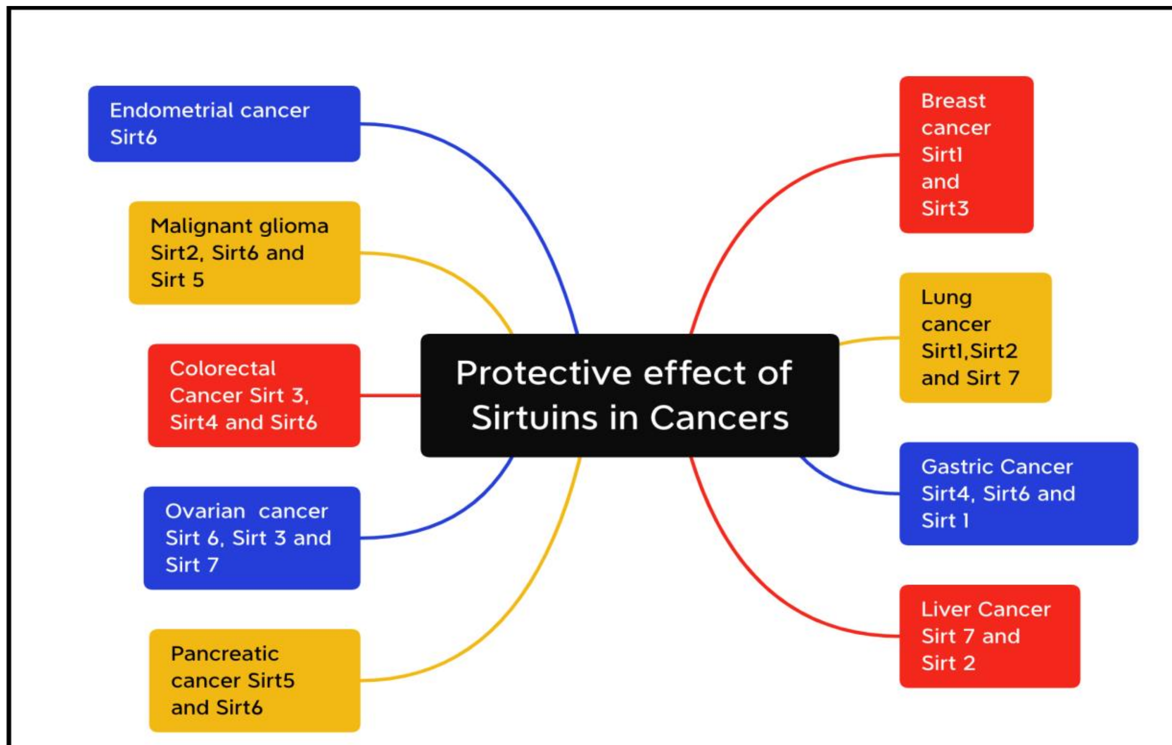


Figure 13. Gene set expression in an epithelial human breast cancer cell line.

MDAMB-231 ((Epithelial, human breast cancer)

Fold Regulation in Sirtuin 1
Control 1.00, 1 pg 1.18, 100 pg 6.77, 1 ng 0.29, 100 ng 1.13
Fold regulation in Sirtuin 2
Control 1.00, 1 pg 0.37, 100 pg 0.81, 1 ng 1.83, 100 ng 1.90
Fold regulation in Sirtuin 3
Control 1.00, 1 pg 1.06, 100 pg 3.94, 1 ng 4.56, 100 ng 3.75
Fold regulation in Sirtuin 4
Control 1.00, 1 pg 1.10, 100 pg 2.75, 1 ng 2.91, 100 ng 1.72
Fold regulation in Sirtuin 5
Control 1.00, 1 pg 0.67, 100 pg 1.66, 1 ng 1.93, 100 ng 1.02
Fold regulation in Sirtuin 6
Control 1.00, 1 pg 1.13, 100 pg 2.62, 1 ng 3.58, 100 ng 1.62
Fold regulation in Sirtuin 7
Control 1.00, 1 pg 0.75, 100 pg 1.61, 1 ng 1.83, 100 ng 0.90
Fold regulation in TERT
Control 1.00, 1 pg 1.08, 100 pg 3.27, 1 ng 3.76, 100 ng 3.82
Fold regulation in FOXO-1
Control 1.00, 1 pg 0.69, 100 pg 1.62, 1 ng 2.58, 100 ng 1.59
Fold regulation in KLOTHO
Control 1.00, 1 pg 3.00, 100 pg 5.08, 1 ng 12.74, 100 ng 1.93
Fold regulation in p53
Control 1.00, 1 pg 1.47, 100 pg 3.73, 1 ng 5.13, 100 ng 1.80
Fold regulation in GDF-11
Control 1.00, 1 pg 2.94, 100 pg 4.17, 1 ng 21.71, 100 ng 3.99

Figure 14. Sirtuins and their protective roles in cancer



Depending on the cell line at least one of the sirtuin isoforms was upregulated to play a protective role. For example, in lung cancer cells (Figure 8), the levels of the sirtuins 2 and Sirt7 (Figure 14) were markedly increased. In primary breast cancer cells (Figure 12),

Sirt 5 was highly upregulated, which is consistent with the information provided in Figure 14. Similarly in colorectal cancer cells (Figure 11), Sirt3, Sirt4 and Sirt6 were upregulated.

Surprisingly, GDF11 was expressed in all cancer cell lines. There are no known reports of the use of small molecule compounds that induce GDF11 expression specifically in cancer cell lines. Many studies have demonstrated the context-dependent tumor suppressive or tumor-promoting roles of GDF11 in different cancers, 30 they have focused on modulating GDF11 levels through recombinant protein treatment, overexpression or knockdown techniques. This is the first study of small molecule induced expression of GDF11 in cancer cells in the annals of the medical literature.

NR5A1 plays a crucial role in mediating the effects of GDFs, which are members of the BMP family, by transducing their signals and influencing the expression of genes related to growth and differentiation processes.83 GDF11 has shown tumor-suppressor functions in certain types of cancer,30 such as triple-negative breast cancer, where its overexpression can inhibit tumor progression. GDF expression was nearly 3-fold greater in BCAF breast cancer cells than in MDAMB cells (Figures 12 and 13).

SIRTUIN EXPRESSION IN CANCER CELL LINES AND ITS ROLE IN DISEASES.

Metadichol, induces the expression of all sirtuins, nuclear receptors, KL, GDF11, telomerase, Foxo1 and P53, could have significant beneficial effects in mitigating age-related diseases.[84]

CANCER

Telomerase, which maintains telomere length, might have both positive and negative effects on cancer.[85] While elongated telomeres are associated with longevity, they can also contribute to the uncontrolled cell division observed in many cancers. A careful balance is required to harness the benefits of telomerase without promoting cancer growth. KL, P53 and Sirt6 are also expressed in cancer, and they have a role in regulating the effects of telomerase in cancer cells.

NEURODEGENERATIVE DISEASES

KL has been associated with neuroprotection and cognitive function. It could contribute to reducing the risk or severity of neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease.[86]

CARDIOVASCULAR AND METABOLIC DISORDERS

Sirtuins and Foxo1 have implications for cardiovascular health. They can regulate factors such as inflammation, oxidative stress, and cell survival, which are relevant to heart disease and stroke.87 The combination of sirtuins, nuclear

receptors, and Foxo1 could impact metabolic pathways, potentially leading to insulin resistance and diabetes, as well as obesity-related issues.

OSTEOPOROSIS
KL[88] and Foxo1[89] play roles in bone health. These factors might contribute to increased bone density and a reduced risk of fractures associated with osteoporosis.

INFLAMMATION
Sirtuins and Foxo1 are involved in dampening chronic inflammation, which is a common factor in many age-related diseases.[90] These proteins could mitigate inflammatory responses.

CELLULAR SENESCENCE
Sirtuins and Foxo1 have been implicated in controlling cellular senescence,[91,92] a state in which cells lose their ability to divide and function properly. Targeting cellular senescence could impact various age-related conditions.

STEM CELL FUNCTION
Telomerase and Foxo1 are associated with stem cell maintenance and regeneration. These molecules can enhance tissue repair and regeneration.[93-95]

HORMONE REGULATION
Nuclear receptors are key regulators of hormone signaling and might contribute to maintaining hormonal balance and alleviating age-related endocrine disorders.[96]

TISSUE REGENERATION
The combination of proteins such as KL, telomerase and FOXO1,[97-99] promotes tissue regeneration and repair, potentially leading to age-related tissue degeneration.

LONGEVITY
Since Sirtuin levels decrease with age,[100] increased expression of transcription factors of the sirtuin family could increase lifespan, potentially by reducing the incidence of various age-related diseases, as shown in Figures 2 and 3.

Conclusions

Like sirtuins, there are other genes that regulate the expression of multiple downstream genes. Metadichol also expresses gene [101], which regulates hundreds of other genes. Metadichol targets the Yamanaka factors Sox2, KLF2, KLF4, Oct3/4, and Nanog, which have been shown to regulate mammalian embryogenesis, embryonic stem cell self-renewal, and pluripotency.[102] Based on our previous work, metadichol induces the expression of key regulatory genes that have a role in cancer and reversing age-related diseases.[103-106]

The development of omics technologies (genomics, proteomics, metabolomics, metagenomics, phenomics, and transcriptomics) has increased interest in regulatory genes because they play a crucial role in organism development and are potential targets for gene therapies and drugs. The application of regulatory genes for gene therapy and targeted drugs remains challenging, and the clinical translation of these therapies requires further research. Understanding the functions of these genes and their interactions could help identify new avenues for treating and preventing age-related diseases.

Metadichol® is among the first safe therapeutics[107-109] that target regulatory genes affecting multiple genes, pathways, and diseases.

Data availability

Supplementary materials

- Raw data: qRT–PCR data
- Western blot analysis
- Sirtuin gene network analysis
- Sirtuin family interactions
- References

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