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RESEARCH ARTICLE

Metadichol, a Natural Ligand for the Expression of Yamanaka Reprogramming Factors in Human Cardiac, Fibroblast, and Cancer Cell Lines

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ABSTRACT

The reprogramming of somatic cells into embryonic-like stem cells requires the activation of four essential transcription factors, referred to as the Yamanaka factors: Sox2, Oct4, Klf4, and c-Myc. However, the utilization of these genes and viral vectors for their delivery presents a risk of carcinogenesis, which renders induced pluripotent stem cell (iPSC) lines unsuitable for clinical applications. Typically, direct reprogramming involves using viral vectors to induce the expression of these factors. In contrast, metadichol, a novel approach, enhances the expression of Yamanaka factors in cells using a dose ranging from 1 pg to 100 ng, thereby preventing the need for viral vectors. This non-viral method renders cellular reprogramming safer and more clinically viable. Treatment with metadichol within the specific dose range resulted in a substantial augmentation of the fibroblast expression of OCT4, SOX2, KLF4, KLF2, and Nanog, which was confirmed through qRT-PCR and western blot analyses. The expression levels of OCT4, KLF4, Nanog, and Sox2, exhibited an increase of 4.01-, 3.51-, 1.26-, and 2.5-fold, respectively, compared to the controls. Notably, A549 and Colo-205 cancer cells demonstrated a marked elevation in expression levels. The reprogramming of primary human cancer cells presents considerable challenges. However, in triple-negative primary breast cancer cells, metadichol treatment led to the substantial upregulation of the expression levels of OCT4, KLF4, Nanog, and Sox2 by 19.6-, 8.07-, 2.45-, and 6.91-fold, respectively, across the dose range of 1 pg to 100 ng. Klotho, an antiaging gene modulated by metadichol, downregulates TP53, a crucial factor for producing somatic cell iPSCs. Additionally, metadichol enhances the availability of vitamin C, which is essential for generating iPSCs from somatic cells. Increased alkaline phosphatase (ALP) levels, indicative of cellular differentiation, were observed in the treated group compared to the controls in fibroblasts and cancer cells. ALP is highly expressed for cell maturation in juvenile cells, including early preimplantation mouse embryos, pluripotent stem cells, primordial germ cells, and certain somatic stem cells, such as neuronal stem cells. This indicates that ALP may contribute to the cell maturation process and differentiation. The emergence of intermediate cells expressing tissue-nonspecific ALP is crucial for reprogramming into iPSCs.

Introduction

In 2006, Yamanaka et al.[1] demonstrated that somatic cells could be reprogrammed using only four genes, namely Oct4, Sox2, Klf4, and c-Myc (OSKM, also called the Yamanaka factors). These genes facilitate the reprogramming of adult mouse fibroblasts (connective tissue cells) into a pluripotent state that resembles embryonic stem cells, which are capable of differentiating into any cell type in the body. In 2011, Wang et al.[2] introduced two additional factors (Nanog and LIN28) to the Yamanaka factors. Their effects were investigated in aged fibroblasts from healthy older adults and individuals over 100 years. These factors were found to promote cellular rejuvenation. The combination of these six factors enabled the reprogramming of these cells into induced pluripotent stem cells (iPSCs), which can then differentiate into various cell types within the body[3]. López-Otín et al.[4] demonstrated that aged cells, such as fibroblasts, exhibited shorter telomeres and dysfunctional mitochondria.

While isolating and reprogramming cells in a Petri dish using Yamanaka factors is relatively straightforward, this approach is not feasible in living animals because of the inability to erase cellular memory and revert cells to a pluripotent state. Moreover, the expression of Yamanaka factors may induce the development of cancer in animals[5]. Belmonte et al. reported the successful rejuvenation of cells and organs in living animals[6]. They utilized a specially engineered mouse strain that was designed to age rapidly and a mouse strain that aged normally. Upon exposure to the antibiotic doxycycline in the drinking water, these mice expressed Yamanaka factors transiently for two days, followed by the silencing of these factors when doxycycline was discontinued. In 2020, Sinclair restored vision in aged mice and those with damaged retinal nerves through partial cellular reprogramming[7], utilizing only three of the Yamanaka factors while eliminating MYC to mitigate the cancer risk. This approach also ameliorated age-related vision impairment and increased eye pressure, which is indicative of glaucoma, in the treated mice. In 2021, Gill et al.[8] performed research focusing on the use of a doxycycline-inducible lentiviral system to expose cells to OSKM, which is similar to previous animal studies. This strategy reversed fibroblast aging by approximately 30 years, thereby enabling aged cells to function similarly to those of individuals approximately 25 years old.

For the translation of partial cellular reprogramming strategies into humans, methods for inducing the expression of the Yamanaka factors in cells without relying on drugs such as doxycycline must be developed. The critical challenges for the clinical application of these factors in cells include ensuring their safety and addressing their tumorigenic potential upon transplantation back into patients. The generation of iPSCs via viral integration and the use of viral vectors for the delivery of transcription factors are associated with tumorigenesis[9]. Therefore, there is a pressing need for new reprogramming protocols that circumvent the permanent genomic integration of or reliance on these viral vectors, which represents the future of regenerative medicine. Deng et al.[10] reported that a combination of specific small molecules could reprogram mouse fibroblasts into iPSCs via a

single transcription factor, Oct4, thereby eliminating the need for the expression of Sox2, Klf4, and c-Myc. This discovery enabled the generation of iPSCs using small molecules without requiring genetic modification. Further research is required to identify additional small molecules that are capable of preventing the necessity for exogenous Oct4 expression.

The presence of alkaline phosphatase (ALP) in somatic cells such as fibroblasts and cancer cells treated with small molecule inducers like MS023[11] has various implications. For cell maturation, ALP is highly expressed in juvenile cells, including early preimplantation mouse embryos, pluripotent stem cells, primordial germ cells, and certain somatic stem cells, such as neuronal stem cells[12]. This indicates that ALP may contribute to the cell maturation process and differentiation. The emergence of intermediate cells that express tissue-nonspecific ALP (TNSALP) is crucial for their reprogramming into iPSCs. ALP-positive cells are considered "intermediate cells" and may undergo reprogramming into undifferentiated cells like iPSCs. ALP also plays a physiological role where it is widely distributed in various tissues and is a marker of differentiation in undifferentiated embryonic stem cells and hard tissue-forming cells such as osteoblasts and odontoblasts[13]. Its enzymatic activity serves as an indicator of cellular differentiation[13].

Our findings demonstrate that metadichol[14], a small non-toxic molecule, naturally induces the expression of Yamanaka factors in fibroblasts[15], A549 and Colo-205 cancer cells, and triple-negative primary breast cancer (HCAF-TNPBC) cells, while also increasing the ALP activity in cancer cells. Treatment with metadichol regulates the key cardiac progenitors, such as ISL1, NKX2-5, GATA4, WT-1, and c-Kit, in fibroblasts and embryonic stem cells, which are crucial for heart development[16]. This finding indicates the potential role of ALP in influencing the regulation of cardiac progenitors.

Methods

All work was planned, supervised, and outsourced to the service provider, Skanda Life Sciences (Bangalore, India).

Chemicals and reagents

A549 cells, Colo-205 cells, and human cardiac fibroblasts (HCF) were procured from ATCC (USA). Primary breast cancer cells were obtained from BIOIVT (Detroit, Michigan, USA). Primary antibodies were purchased from ABclonal (Woburn, Massachusetts, USA) and E-lab Science (Maryland, USA). The primers were obtained from SahaGene (Hyderabad, India) (Table 1). All other molecular biology reagents were sourced from Sigma Aldrich, Bangalore, India .

Table 1: Primers used in this study Base pairs 5′ to 3′

OCT4 (POU5F1)	F	GTTGATCCTCGGACCTGGCTA	134
	R	GGTTGGCTCACTCGGTTCT	
KLF4	F	CCCACACAGGTGAGAAACCT	199
	R	ATGTGTAAGGCGAGGTGGTC	
SOX2	F	AACCCCAAGATGCACAACTC	234
	R	CGGGGCCGGTATTTATAATC	
c-MYC	F	CCATCCAGGTGAACCACCTA	241
	R	ATCTCCGAACACATCACTTC	
NANOG	F	ATCTGCTGGAGGCTGAGGTA	180
	R	GTGGTTTCAAGGCCAGATGT	
KLF 2	F	CTTCTCTCGACGCCATCTCC	160
	R	AGCCATCCAAAAGCCCCATT	
KL	F	AGGGTCCTAGGCTGGAATGT	158
	R	CCTCAGGGACACAGGGTTTA	

Maintenance and seeding

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

Upon reaching 70%–80% confluency, single-cell suspensions containing 1 × 10^6 cells/mL were prepared and seeded into 6-well plates at a density of 1 × 10^6 cells/well. The plates were then incubated for 24 h at 37°C in 5% CO_2. After 24 h, the cell monolayer was washed 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 a serum-free medium. The metadichol-containing medium was subsequently added to the predetermined wells, while the control cells received the medium without metadichol. The cells were incubated for 24 h, after which they were gently rinsed with sterile phosphate-buffered saline. Following the manufacturer's instructions, whole-cell RNA was isolated using TRIzol reagent (Invitrogen), and cDNA was prepared. The samples were analyzed by qPCR and western blotting for the various biomarkers.

Quantitative real-time PCR (qRT–PCR)

RNA ISOLATION

Total RNA was extracted from each treatment group using TRIzol. Approximately 1 × 10^6 cells were collected in 1.5 mL microcentrifuge tubes and centrifuged at 5000 rpm for 5 min at 4°C, after which the cell supernatant was discarded. Then, 650 µL of TRIzol was added to the pellet, and the contents were thoroughly mixed and incubated on ice for 20 min. Subsequently, 300 µL of chloroform was added to the mixture, and the samples were

gently inverted for 1–2 min and incubated on ice for a further 10 min. The samples were centrifuged at 12000 rpm for 15 min at 4°C, and the upper aqueous layer was carefully transferred to a new, sterile 1.5 mL centrifuge tube. An equal amount of prechilled isopropanol was added to the tube, and 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 carefully discarded, 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 room temperature (RT) for approximately 15–20 min and then resuspended in 30 µL of diethylpyrocarbonate (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 AND qPCR

cDNA was synthesized using a PrimeScript cDNA synthesis kit (Takara) with oligo dT primers, according to the manufacturer's instructions. A total of 2 µg of RNA was used for each reaction in a 20 µL reaction volume. The cDNA synthesis process was performed on an Applied Biosystems instrument (Veritii). Subsequently, the synthesized cDNA was used for qPCR, incubating at 50°C for 30 min and 85°C for 5 min.

PCR

The PCR mixture, with a final volume of 20 µL, comprised 1 µL of cDNA, 10 µL of SYBR Green Master Mix, and 1 µM each of the forward and reverse primers specific to the target genes. The PCR mixture then underwent the following PCR protocol: an initial denaturation step 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 optimal number of cycles was determined and selected as the amplification point in the exponential range without reaching a plateau. Analysis of the obtained results was performed using CFX Maestro software.

FOLD CHANGE (FC) WAS CALCULATED USING THE Δ Δ CT METHOD

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

The ΔCt for each treatment was determined using the formula:

ΔCt = Ct (target gene) - Ct (reference gene)

To compare the ΔCt of individually treated samples with the untreated control sample, the Ct was subtracted from the control to obtain the ΔΔCt.

ΔCt = ΔCt (treatment group) - ΔCt (control group)

The FC in the target gene expression for each treatment was calculated using the formula: $FC = 2^{-\Delta\Delta Ct}$

PROTEIN ISOLATION

Total protein was extracted from 1 × 10^6 cells using RIPA buffer supplemented with the protease inhibitor phenylmethylsulfonyl fluoride (PMSF). The cells were lysed for 30 min at 4°C with occasional gentle inversion. Subsequently, the lysed cells were centrifuged at 10,000 rpm for 15 min, and the resulting supernatant was transferred to a fresh tube. Protein concentration was determined using

the Bradford method, and 25 µg of protein was mixed with 1× sample loading dye containing sodium dodecyl-sulfate before being loaded onto a gel. Protein separation was performed under denaturing conditions in a Tris-glycine buffer.

The separated proteins were transferred to methanol-activated polyvinylidene fluoride (PVDF) membranes (Invitrogen) using a Turbo transblot system (Biorad, USA). They were blocked by incubation in 5% bovine serum albumin for 1 h to prevent non-specific binding to the membranes. Following this, the membranes were incubated overnight with their respective primary antibodies at 4°C, followed by incubation with a species-specific secondary antibody for 1 h at RT. Subsequently, the blots were washed and incubated with an enhanced chemiluminescence (ECL) substrate (Merck) for 1 min in the dark. Finally, the images were captured at the appropriate exposure settings using a ChemiDoc XRS system (BioRad, USA).

ALP activity

CELL CULTURE AND MAINTENANCE

All tested cell lines (including HCFs and cancer cells) were cultured in the appropriate complete media supplemented with 10% fetal bovine serum and 1% Pen-Strep. Culturing was performed in a humidified atmosphere containing 5% CO_2 at 37°C, with media changes performed every other day until confluence was reached. Cell viability was assessed by staining with trypan blue using a hemocytometer. Cells were seeded at an appropriate density to expand the cell lines for further studies.

CELL SEEDING

After adjusting the media to a density of 5 × 10^5 cells/mL, 100 µL of the media was seeded into 96-well plates and incubated for 24 h at 37°C with 5% CO_2 to allow the cells to acclimatize and form a monolayer.

TREATMENT CONDITIONS

After 24 h of seeding, the media was carefully removed, and the cells were then incubated with or without metadichol at various concentrations (1 pg/mL, 100 pg/mL, 1 ng/mL, or 100 ng/mL) at 37°C with 5% CO_2 for an additional 24 h. The cells were harvested after treatment, and alkaline phosphatase activity was determined.

ALP ASSAY

Total protein was harvested from the cells by suspending them in 200 µL of lysis buffer containing PMSF. Subsequently, an ALP assay master stock solution containing AMP and the pNPP substrate (2.5 mM) was prepared. Ten microliters of protein sample and 100 µL of the master stock solution were combined and incubated at 37°C for 3 min. Kinetic measurements were recorded at a wavelength of 405 nm, and ALP activity was determined using the following formula:

$$\text{ALP activity (U/L)} = \Delta A/\text{min} \times 2720.$$

Results

Table 2: Q-Rt-PCR results for human cardiac fibroblast cells

Metadichol concentration	NANOG	OCT 04 (POU5F1)	SOX2	KLF2	Klotho
	Fold Increase				
Control	1.00	1.00	1.00	1	1
1 pg	2.39	0.28	0.85	1.265	2.82
100 pg	5.86	10.36	10.21	2.468	10.13
1 ng	1.40	1.67	1.04	6.052	0.26
100 ng	0.22	0.96	2.55	14.81	0.19

Table 3: Q-RT-PCR results for A-549 cancer cells

Metadichol concentration	Oct4 (POU5F1)	Klf4	Nanog	SOX2	Klotho
	Fold increase				
Control	1	1	1	1	1
1 pg	0.27	1.05	0.82	0.47	0.37
100 pg	0.92	0.44	0.82	0.80	0.60
1 ng	4.01	3.51	1.26	2.51	1.99
100 ng	1.25	1.58	0.81	0.73	0.83

Table 4: RT-PCR results in COLO205 cancer cells

Metadichol concentration	Oct4 (POU5F1)	Klf4	Nanog	SOX2	Klotho
	Fold increase				
Control	1	1	1	1	1
1 pg	0.41	2.55	1.74	1	1
100 pg	0.88	2.98	0.68	2.16	1.89
1 ng	1.79	13.17	0.41	2.21	1.22
100 ng	0.81	6.77	1.19	2.25	2.58

Table 5: RT-PCR results following metadichol treatment in primary human cancer-associated fibroblasts (HCAF) triple-negative primary breast cancer (TNPBC)

Metadichol concentration	c-MYC	Oct4 (Pou5F1)	Klf4	SOX2	Klotho
	Fold increase				
Control	1	1	1	1	1
1 pg	3.36	4.56	3.36	0.57	2.13
100 pg	4.39	4.94	4.39	0.62	1.10
1 ng	4.04	19.63	8.07	2.45	0.64
100 ng	6.91	9.32	6.91	1.16	1.02

Figure 1 ; Alkaline phosphatase (ALP) activity in human cardiac fibroblasts (HCFs)

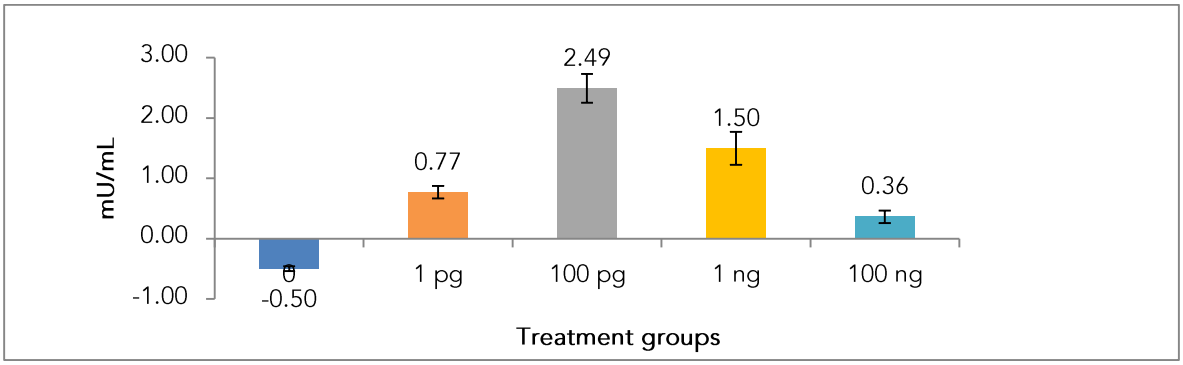


Figure 2: ALP activity in A549 Cells

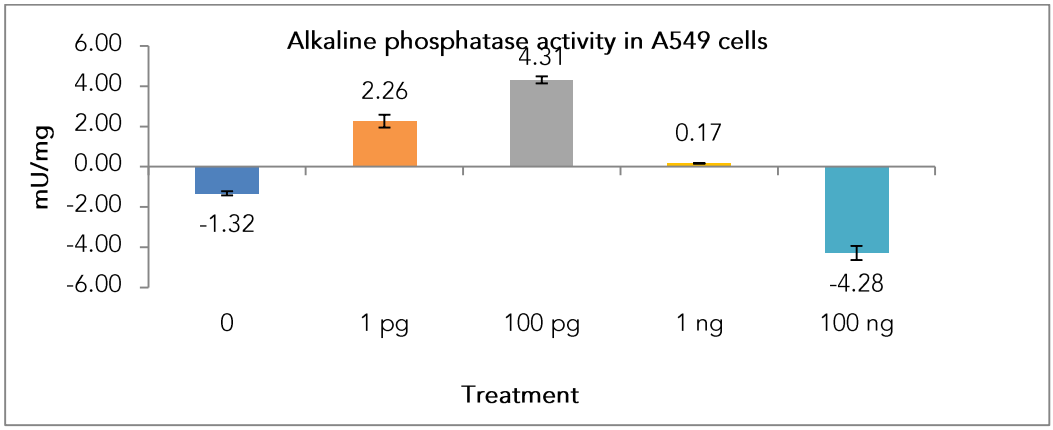
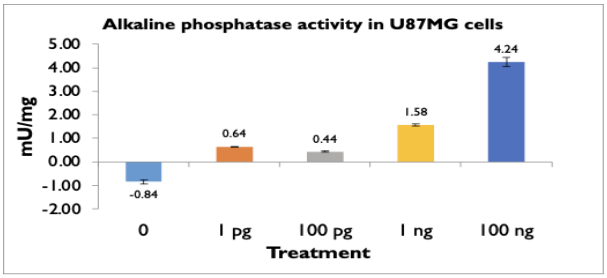
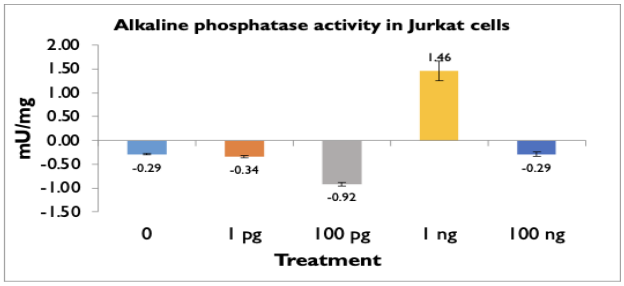
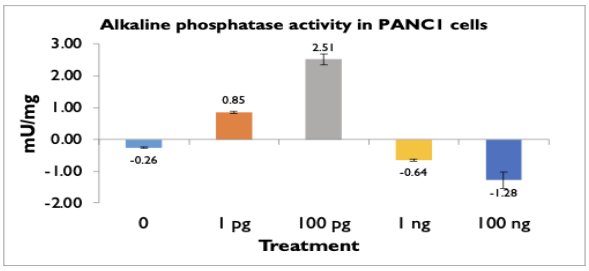
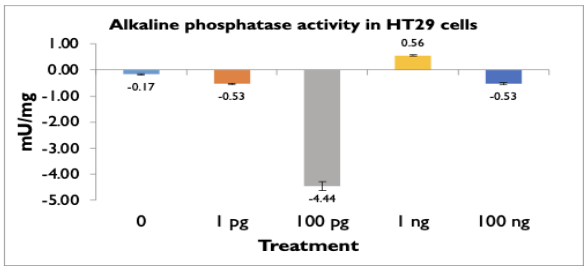


Figure 3: Alkaline phosphatase (ALP) activity induced by metadichol in U87MG, Jurkat, Panc1, and HT 29 cancer cell lines









Discussion

Metadichol is a nanoemulsion composed of long-chain alcohols that primarily consist of C26 and C28 (>80%) and C30. Our findings indicated that low doses of metadichol ranging from 1 pg to 1 ng augmented the expression of Yamanaka factors in fibroblasts and cancer cell lines. Notably, we observed that the optimal expression of Yamanaka factors in cancer cell lines required a dose of 1 ng. However, reprogramming primary human cancer cells remains a challenge. Hu et al.[17] were the first to successfully reprogram primary human lymphoblasts from a patient using trans-free gene iPSC technology to express OKSM factors ectopically.

The effects of metadichol on HCFs are presented in Table 2, while its impact on the cancer cell line A-549 and pancreatic cancer cell line COLO205 are presented in Table 3. Additionally, the effects of metadichol on HCAF-TNPBCs are detailed in Table 4. In all these cell types, a notable increase in the OKSM factors was observed. Furthermore, the heightened expression of the KLF2 transcription factor was revealed. KLF2 plays a substantial role in stem cell reprogramming and the maintenance of pluripotency. Notably, KLF2 is crucial for sustaining base-state pluripotency, thus rendering it more efficient in somatic cell reprogramming than other factors, such as KLF1, KLF4, or KLF5[18]. KLF2, along with KLF4 and KLF5, can maintain the undifferentiated state of mouse embryonic stem cells (ESCs) and can also reprogram epiblast-derived stem cells into iPSCs when overexpressed[19]. In addition, studies have indicated that the induced expression of KLF2, in combination with Nanog, is adequate to induce a naive state in ESCs, thereby highlighting its pivotal role in maintaining pluripotency[20].

Recent research has demonstrated that Klotho is expressed in fibroblasts and numerous cancer

cells when treated with metadichol[21]. The expression of these factors is essential for maintaining the pluripotency of stem cells. Klotho is recognized as a longevity hormone and exerts a dual function by shielding cells from oxidative stress and serving as a tumor suppressor in cancers[22]. Notably, a mutation in the Klotho gene (KL (−/−)) substantially diminishes the proliferation of adipose-derived stem cells, consequently reducing the expression of pluripotent transcription factors (Nanog, Sox-2, and Oct-4) in mice[23,24]. Our recent findings[25] indicate that metadichol induces the expression of all 48 nuclear receptors (NRs) in stem cells and fibroblasts. Among these, nuclear receptor NR5A1 (SF1) can reprogram somatic cells, which leads to the generation of iPSCs and potentially replacing Oct4, thereby enhancing the yield of iPSCs[26]. NR5A1 lies upstream of OCT4 in the pluripotency network, so its ectopic expression can directly activate endogenous OCT4 expression, resulting in iPSC generation[27]. Treatment with metadichol induced a remarkable 27-fold increase in the expression of NR5A1 in stem cells, which has profound importance for the reproductive implications and survival of the human species.

Another critical factor required for cell reprogramming is vitamin C. Numerous studies have demonstrated that supplementing the culture medium of somatic cells with vitamin C during reprogramming enhances the efficiency and quality of iPSC formation[28,29]. Metadichol, as evidenced by previous studies, elevates patients' vitamin C levels in vivo[30,31,32]. The generation of iPSC through the induced expression of OSKM leads to an increased production of reactive oxygen species (ROS), which can induce DNA damage and senescence[33,34]. Given its antioxidant properties, vitamin C was initially incorporated into the culture medium of mouse and human cells during reprogramming to counteract the effects of ROS, thus potentially improving the efficiency and quality of reprogramming[36]. Compared with other antioxidants, such as glutathione, N-acetylcysteine, vitamin E, and lipoic acid, vitamin C substantially enhances the generation of mouse ESCs and iPSCs from mouse or human fibroblasts. Vitamin C facilitates reprogramming[37] by promoting histone demethylation, a process that is crucial for expressing the master transcription factor Nanog in ESCs. Additionally, studies have indicated that vitamin C increases the population of ESCs in humans and induces DNA demethylation at genomic loci known to undergo an extensive loss of methylation during the reprogramming of somatic cells into iPSCs[37].

Recently, we have demonstrated that metadichol is expressed across all 48 NRs in fibroblasts and stem cells[25]. Notably, NRSA1 (SF1) and NRSA2 are among these NRs, and NR5A1 guides the different[38,39,40]. Sertoli cells, located in the testis, are essential nurse cells that regulate spermatogenesis, the process of male germ cell production. Additionally, Sertoli cells secrete cytokines and growth factors, thereby modulating immune processes to safeguard the germ cells from immunological attacks[41]. Furthermore, they suppress T, B, and NK cell proliferation. These findings indicate a potential reduction in immune responses following cell transplantation in patients with diabetes, neurogenerative diseases, skin implants, and other illnesses[42]. However, abnormalities in

Sertoli cells can lead to infertility[43,44]. Conversely, Oct4, a pluripotent transcription factor, is essential for maintaining self-renewal capabilities in stem and somatic cells[45,46]. In this context, SF1 serves a critical function in regulating the transcription of Oct4[47]. However, NR5A2 (LRH-1) can reprogram somatic cells, leading to the generation of iPSCs, and may act as an Oct4 substitute, thus resulting in an enhanced iPSC yield[40].

The detection of ALP in somatic cells like fibroblasts and cancer cell lines after treatment with metadichol implies its involvement in various cellular processes such as maturation, iPSC reprogramming, regulation of cardiac progenitors, and differentiation. Based on these observations, ALP expression in cancer cell lines induced by small-molecule activators like MS023 and Entinostat has considerable implications[13]. MS023 and Entinostat have been observed to elevate ALP activity, notably in colon cancer cell differentiation. This increase promoted cell differentiation and retarded cell growth, in both 2D and 3D cultures. This suggests a promising avenue for the use of these small-molecule activators to induce differentiation in colon cancer cells. Regarding the prevention of metastatic progression, the inhibition of PRMT1[48], an arginine methyltransferase protein, resulted in a substantial upsurge (~20-fold) in ALP expression in colon cancer cells. This increase emphasizes the effectiveness of ALP as a marker for differentiation and hints at its potential role in impeding metastatic advancement. Within metastatic prostate cancer, tumor-derived ALPL (tissue-nonspecific ALP) was identified to govern tumor growth, epithelial plasticity, and disease-free survival. Notably, the inhibition of ALPL activity augmented cell death, promoted mesenchymal-to-epithelial transition, and reduced migration of prostate cancer cells[49]. In addition, the small-molecule DIPQUO rapidly stimulated ALP expression in C2C12 cells, an immortalized mouse myoblast cell line, thereby fostering osteoblast differentiation through p38 MAPK activation[50]. This highlights the pivotal role of ALP in regulating the cellular differentiation process. Therefore, the induction of ALP expression by metadichol in cancer cell lines bears implications for inducing cell differentiation, impeding metastatic progression, regulating tumor growth in prostate cancer, and influencing osteoblast differentiation[48].

The nuclear receptor that regulates the expression of ALP (alkaline phosphatase) in cancer cells appears to be PPARγ(peroxisome proliferator-activated receptor gamma)[51]. Expression of PPARγ is seen in human osteosarcoma cells. It has been reported that RXR is ubiquitously expressed, whereas expression of PPARγ is more restrictive to certain cell types, including adipocytes and osteoblasts[52]. This suggests that PPARγ is expressed in osteosarcoma cells, a type of cancer cell. Since ALP is a marker of osteoblast differentiation, it implies that PPAR gamma regulates ALP expression in cancer cells[53].

Nuclear receptors Nuclear Receptor Subfamily 5 Group A Member 2 (Nr5a2) could replace Oct4 in the reprogramming of human fibroblasts[41] to induced pluripotent stem cells (iPSCs). Rarg-induced iPSCs express alkaline phosphatase, a pluripotency marker[54]. Metadichol expresses all the 48 Nuclear receptors[25] in fibroblast cells. PPARγ RARG, and RXR's, NR5A1 and NR5A2 are also expressed from the mentioned study, as shown in Table 6.

Table 6 ;Expression of Nuclear receptors in Fibroblast cells

Common Name	Nomenclature name	1 pg	100 pg	1 ng	100 ng	Control
PPARG	NR1C3	3.78	6.11	7.31	3.07	1
RXRA	NR2B1	2.5	0.86	1.32	0.98	1
RXRB	NR2B2	4.21	1.65	1.03	2.7	1
RXRG	NR2B3	2.84	2.95	3.9	1.09	1
RARG	NR1B3	2.84	2.95	3.9	1.09	1
SF1	NR5A1	27.27	13.78	13.28	2.69	1
LRH1	NR5A2	3.32	1.79	1.74	2.02	1

Thus, it is likely that PPAR gamma expression controls ALP expression in cancer cells, and in fibroblasts, RAR gamma controls ALP expression. These insights reveal the multifaceted functions of ALP in cellular mechanisms and its potential therapeutic utility in cancer research and regenerative medicine[55].

We carried out a theoretical bioinformatics study to analyze the expression of transcription factors by metadichol, utilizing Pathway Studio[56,57]. The analysis revealed protein-protein interaction maps, which illustrated the formation of a feedback loop network as shown in Figure 4. Additionally, we identified the top 25 most substantially regulated cell processes by the gene set, with $p < e^{-9}$, as outlined in Table 7. The biological processes are what one can expect form the expression of the Yamanaka factors and ALP expression like cell renewal and cell trans differentiation etc. The supplementary material provides a comprehensive list of the processes regulated by the gene subset. Notably, metadichol acts as an inverse agonist, possibly a protean agonist[58], of the vitamin D receptor (VDR)[14]. Through this analysis (figure 4) , we observed that TP53 controls the expression and inhibits Oct4 (POU5F1), Nanog, MYC, KLF4/KLF2, and SOX2. Moreover, the expression of Klotho inhibits the actions of TP53[59], thereby facilitating the expression of Yamanaka factors Oct 4 and Sox2 and facilitating iPSC conversion[60,61]. Additionally, it is worth noting that VDR regulates both MYC and Klotho[62,63]. Previously we have shown that Metadichol also expresses Telomerase[64]. Thus all the transcription factors in Figure 4 are accounted for by the actions of Metadichol.

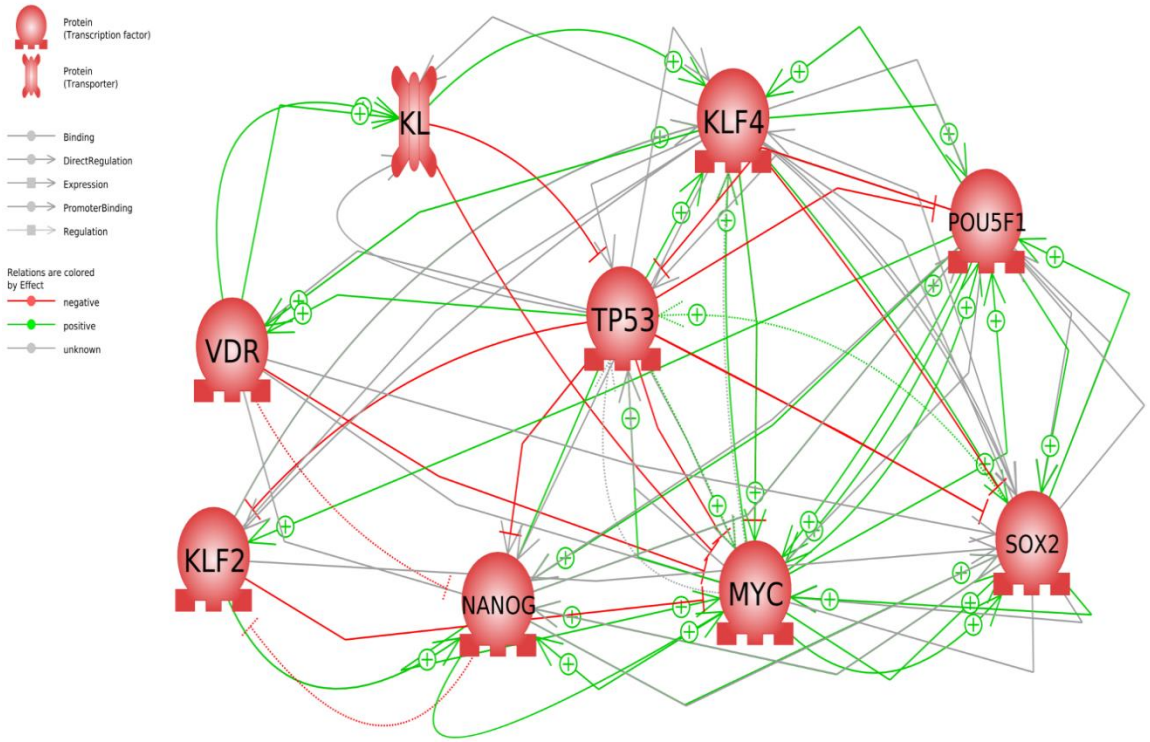
The processes outlined in Table 5 encompass various essential cellular functions, including cell differentiation, pluripotency maintenance, cellular senescence, regulation of DNA methylation, fibroblast differentiation, and the development of stem cells. Importantly, these genes exhibit a higher degree of interaction than expected for a random set of genes of similar size and degree distribution drawn from the genome, indicating a substantial biological connection in the cell pluripotency process[65]. Our results demonstrate that the forced programming of external Yamanaka factors and transfection using viral vectors are not necessary for reprogramming. Metadichol is a safe compound, with an LD50 of >5000 mg/kg in rats[66-68], and it is readily available commercially as a supplement. Moreover, vitamin C plays a crucial role in cell reprogramming by counteracting the ROS

effects, thereby increasing the expression of Yamanaka factors. Our work suggests Klotho, can6effectively reactivate silenced tumor suppressor genes. The impact of metadichol on the expression of critical genes involved in the reprogramming of somatic cells prompts further evaluation in patients to ascertain whether these promising in vitro results translate into in vivo effects. Previous studies utilizing metadichol[69,70] to treat various skin diseases have demonstrated its effect on skin renewal, including wound healing and rejuvenation. Leveraging this strategy to harness the body's cells not only circumvents the incompatibility issues associated with external donor cell sources but also has the potential for effectively mitigating numerous human diseases, including cardiovascular and neurological disorders, where stem cell therapy can play an important role.

Using metadichol to express Yamanaka factors and the presence of ALP has considerable implications for basic and applied research. Researchers can manipulate and understand the reprogramming and aging-related biological pathways with metadichol, which can generate iPSCs from diverse cell or tissue types, produce specific cell types for disease modeling or therapy, or rejuvenate aging or damaged cells and tissues. Its impact on cancer cell lines indicates its potential to hinder metastasis and influence cell differentiation, thereby exhibiting its potential effectiveness against various cancer types, and selectively targeting specific cancer cells. These findings highlight the various implications of metadichol in cancer research and its potential role in developing novel therapeutic approaches for combating cancer. Metadichol-induced ALP expression by cancer cells and pluripotency represents a complex and multifaceted phenomenon that may have diagnostic, prognostic, and therapeutic implications for various cancer types. Elucidating the molecular mechanisms and interactions of ALP and pluripotency factors in cancer cells could offer novel insights and therapeutic targets for cancer prevention and treatment. These findings enhance our understanding of cellular reprogramming mechanisms and offer potential new applications in regenerative medicine and disease modeling.

Figure 4 Gene network of reprogramming factors

POU5F1 is another alias for Oct4



Conclusion

The research presented highlights Metadichol's ability to induce Yamanaka factor expression in somatic and cancer cells without the need for viral transduction or genetic modification is a significant finding, as it opens new possibilities for non-viral iPSC generation and regenerative medicine applications where till today viral vectors had posed safety concerns.

Table7: Biological cell processes regulated by the input genes from Figure 4

Protein regulators of	Total # of neighbors	Overlap	Percent Overlap	Overlapping Entities	p-value
Messenger RNA synthesis	615	9	1	POU5F1, KLF4, NANOG, MYC, KL, KLF2, VDR, SOX2, TP53	4.2643E-13
The cancer stem cell population	203	7	3	POU5F1, KLF4, NANOG, MYC, VDR, SOX2, TP53	3.383E-12
Cancer stem cell differentiation	100	6	5	POU5F1, KLF4, NANOG, MYC, SOX2, TP53	7.7156E-12

Protein regulators of	Total # of neighbors	Overlap	Percent Overlap	Overlapping Entities	p-value
Cell renewal	938	9	0	POU5F1, KLF4, NANOG, MYC, KL, KLF2, VDR, SOX2, TP53	1.9437E-11
Dedifferentiation	969	9	0	POU5F1, KLF4, NANOG, MYC, KL, KLF2, VDR, SOX2, TP53	2.6076E-11
Stem cell development	529	8	1	POU5F1, KLF4, NANOG, MYC, KL, KLF2, SOX2, TP53	2.6336E-11
Cell trans-differentiation	976	9	0	POU5F1, KLF4, NANOG, MYC, KL, KLF2, VDR, SOX2, TP53	2.7829E-11
Cancer stem cell development	48	5	10	POU5F1, NANOG, MYC, SOX2,TP53	4.0368E-11
Cancer stem cell renewal	144	6	4	POU5F1, KLF4, NANOG, MYC, SOX2,TP53	7.156E-11
Cancer stem cell proliferation	147	6	4	POU5F, KLF4, NANOG, MYC, SOX2,TP53	8.1118E-11
Cell maturation	1156	9	0	POU5F1, KLF4, NANOG, MYC, KL, KLF2, VDR, SOX2, TP53	1.284E-10
Endothelial cell homeostasis	64	5	7	KLF4, NANOG, MYC, KL,KLF2	1.7908E-10
Trophoblast differentiation	375	7	1	POU5F1, KLF4, NANOG, MYC, VDR, SOX2, TP53	2.5516E-10
Cancer stem cell phenotype	383	7	1	POU5F1, KLF4, NANOG, MYC, KLF2, SOX2, TP53	2.9585E-10
Cell plasticity	385	7	1	POU5F1, KLF4, NANOG, MYC, KL, SOX2, TP53	3.0685E-10
Cell dedifferentiation	389	7	1	POU5F1, KLF4, NANOG, MYC, KL, SOX2, TP53	3.2989E-10
Gene repression	737	8	1	POU5F1, KLF4, NANOG, MYC, KLF2, VDR, SOX2, TP53	3.7447E-10
Stem cell function	764	8	1	POU5F1, KLF4, NANOG, MYC, KL, VDR, SOX2, TP53	4.9918E-10

Protein regulators of	Total # of neighbors	Overlap	Percent Overlap	Overlapping Entities	p-value
Stem cell maintenance	779	8	1	POU5F1, KLF4, NANOG, MYC, KL, KLF2, SOX2, TP53	5.8304E-10
Stem cell fate determination	81	5	6	POU5F1, KLF4, NANOG, MYC, SOX2	5.9944E-10
Blastocyst formation	208	6	2	POU5F1, KLF4, NANOG, KL, SOX2, TP53	6.6392E-10
Stem cell fate	473	7	1	POU5F1, KLF4, NANOG, MYC, VDR, SOX2, TP53	1.2955E-09
Nuclear reprogramming	497	7	1	POU5F1, KLF4, NANOG, MYC, KLF2, SOX2, TP53	1.8304E-09
Cellular senescence	1563	9	0	POU5F1, KLF4, NANOG, MYC, KL, KLF2, VDR, SOX2, TP53	1.9549E-09
Stem cell fate commitment	262	6	2	POU5F1, KLF4, NANOG, MYC, SOX2, TP53	2.6661E-09

Abbreviations: KL, Klotho; KLF2, Krüppel like factor 2; KLF4, Krüppel like factor 4; MYC, MYC proto-oncogene bHLH transcription factor; NANOG, Nanog homeobox; POU5F1 (OCT 4), POU class 5 homeobox 1; SOX2, SRY-box transcription factor 2; TP53, tumor protein p53; VDR, vitamin D receptor; IPSC, induced pluripotent stem cell

Conflicts of Interest Statement:

The author is the founder and CEO of Nanorx Inc. NY, USA, and is a majority shareholder.

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Availability of data and materials

All raw data are presented in the supplementary materials, including qRT-PCR and western blot raw data.cell processes regulated by entities enriched in the input genes; and raw ALP activity data.

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