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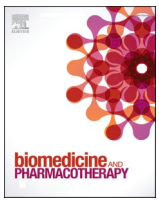

Dietary marine-derived ingredients for stimulating hair cell cycle

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ABSTRACT

In normal condition human hair growth occurs through three phases, anagen (growth phase included about 85 % of hairs, last from 2 to 6 years), catagen (transitional phase lasting up to 2 weeks) and telogen (resting phase which last from 1 to 4 months). Natural dynamics of the hair growth process can be impaired by several factors, such as genetic predisposition, hormonal disorders, aging, poor nutrition or stress, which can lead to the slowdown in the growth of hair or even hair loss. The aim of the study was to assess the hair growth promotion effect of marine-derived ingredients, hair supplement Viviscal® and its raw components (marine protein complex AminoMarC®, shark and oyster extract). Cytotoxicity, production of alkaline phosphatase and glycosaminoglycans, as well as expression of genes involved in hair cycle-related pathways were investigated using dermal papilla cells, both immortalized and primary cell lines. Tested marine compounds showed no evidence of cytotoxicity under in vitro conditions. Viviscal® significantly increased proliferation of dermal papilla cells. Moreover, tested samples stimulated cells to produce alkaline phosphatase and glycosaminoglycans. Increased expression of hair cell cycle-related genes was also observed. The obtained results indicate that marine-derived ingredients stimulate hair growth through anagen activation.

1. Introduction

Natural dynamics of hair growth occurs through three phases: anagen (active growth phase lasting 2–7 years and determining hair length), catagen (transition phase usually lasting between 10 and 20 days during which hair growth ends) and telogen (resting phase when hair is released and falls out) [1–3]. Genetic predisposition, hormonal disorders, unbalanced diet or stress – an integral element of modern life, may contribute to disturbance to the hair cycle [4–7]. It can result in deterioration of hair condition, the slowdown of hair growth as well as increased hair loss and, consequently, telogen effluvium [8–10]. This problem concerns both men and women and has a negative influence on many important aspects of their life. For people affected by the loss of hair, this is a problem not only aesthetic, but also often social or psychological. People affected by the problem of excessive hair loss are exposed to mental disorders like depression, anxiety or social anxiety disorder [11,12].

Modern medicine does not have the answers to all hair loss problems. The only treatments for androgenic alopecia approved by the Food and Drug Administration (FDA) are minoxidil and finasteride [13]. Minoxidil, 2,4-diamino-6-piperidinipyrimidine-3-oxyde, is a vasodilator and was primarily used for hypertension treatment. The first mention of the potential use of this drug as a hair loss therapeutic agent appeared in 1972 when hair growth was observed as a side effect of treatment of patients with high blood pressure [14]. Finasteride is used for male pattern hair loss treatment as it inhibits 5-alpha-reductase (5-AR), the enzyme converts testosterone to dihydrotestosterone (DHT) [15,16]. The effects of using this drug for treating female hair loss were not as positive as in the case of men [17]. Both therapeutics are characterized by the occurrence of side effects, including allergic reactions, headaches, heart arrhythmia, weight gain, cold sweats, confusion as well as impotence and decreased libido [18–21].

In some cases of hair loss, taking medications like minoxidil or finasteride and even surgical procedures seem to be the only form of therapy. However, when the loss of hair relates to the lifestyle (poor diet, smoking), side effects associated with drug treatment may be inadequate to the scale of the problem. It is commonly known that balanced diet is a very important factor for maintaining a health and can reduce the risk of many diseases. The hair follicle, as the fastest growing organ in the human body, is exceptionally exposed to deficiencies resulting from an inadequate diet. Lack of vitamins, minerals or other nutrients is very quickly visible in the hair condition and may lead to acute and chronic telogen effluvium, diffuse alopecia, androgenetic alopecia, female pattern hair loss or alopecia areata [22–26].

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In such cases it is worth thinking about a more natural way to fight with the hair loss problem. Various types of herbal extracts, vitamins, and micronutrients, as well as marine-derived extracts, are successfully used to support the return to the balance of the body and natural hair growth cycle [27–31].

Oceans, covering 70 % of our planet and representing over 95 % of the biosphere, are a great source of bioactive ingredients with the most diverse properties to support human health. Seawater is the living environment for many kinds of living organisms belonging to all five kingdoms of life - Animalia, Plantae, Fungi, Protista and Monera. The positive effects of substances obtained from aquatic organisms has already been described by Hippocrates [32]. Development of science and the exploration of the marine environment meant that today we know more than 30,000 marine-derived compounds, and each year more than 1000 are added to this pool [33,34]. Ingredients with marine origin, due to the presence of peptides, minerals, polyunsaturated fatty acids, vitamins, polysaccharides, or polyphenols are commonly used by nutraceutical and supplements industry [35]. These types of supplements are becoming increasingly popular also due to the increase in consumer awareness. More and more people prefer to replace medical treatments with natural ones that do not promise immediate results, but also do not cause so many side effects [36]. Marine-derived compounds fit perfectly into this trend. The biological activity of substances obtained from marine organisms makes them useful in prevention and treatment of many diseases and disorders. Ingredients with aquatic origin are known to be antioxidants [37], immunomodulators [38] or antimicrobial agents [39]. Studies have shown that marine-derived substances can be used as natural hair growth promoters [27,40–43]. It was found that polyphenols extracted from marine algae *Eclonia cava* increase proliferation rate of dermal papilla cells and the expression of hair growth-related factors, IGF-1 and VEGF. The antioxidant property of tested extract, effect on the increase of hair follicle length and inhibition of the 5 α-reductase was also observed [27,40,41]. Two others marine macroalgae, *Laminaria japonica* and *Cistanche tubulosa*, have been investigated for their potential hair growth promotion effect. The results from clinical trial, performed with healthy females and males diagnosed with mild to moderate patterned hair loss, indicate that tested extracts positively affect hair growth and scalp inflammation [43]. Another marine-derived ingredient, fermented mackerel oil, promotes dermal papilla cells proliferation, increases hair-fibers length and stimulates anagen phase by nuclear translocation of β-catenin [42].

One of the marine-derived supplements with proved hair growth promoting properties, supported by clinical data, is Viviscal®. This hair restoral product was originally developed in the late 1980 s in Scandinavia and the first clinical trial proving its efficacy was published in 1992 [44]. Since then, 12 trials have been conducted to prove the hair growth properties and safety of Viviscal® supplements in nourishing thinning hair and promoting existing hair growth (Table 1).

Viviscal® supplements contain the proprietary AminoMar C® marine protein complex together with the vitamins and minerals, i.a. zinc oxide, D-biotin, niacin, iron (Table 2), that are essential to promote healthy hair and were proven to work in promoting hair growth [23,55–59]. The AminoMar C™ is an active ingredient and comprises of a proprietary blend of shark cartilage and oyster extract powder derived from sustainable marine sources.

Hair growth promoting properties of the Viviscal® supplements have been proven in vivo on a group of 659 participants (both female and male), however, the mode of action of the products at a cellular level was not determined. In this study, we examined the effects and mechanisms of Viviscal® supplements and raw ingredients on dermal papilla cells, both immortalized and primary.

Table 1
Summary of Viviscal® clinical studies.

Year	Double blinded	Placebo control	Test group	Results	Ref.
2015	Yes	Yes	60 females	32 % statistically significant increase in terminal hairs after 3 months and 39 % decrease in hair shedding After 3 months there was a significant self-perceived improvements in overall hair volume, scalp coverage and hair strength.	[45]
2014	Yes	Yes	36 females	Statistically significant 57 % increase in terminal hairs after 3 months, 80 % increase in terminal hairs after 6 months and 12 % increase in hair diameter after 6 months After 3 and 6 months there was a significant self-perceived improvements in overall hair volume, scalp coverage, hair and nail strength	[46]
2014	Yes	Yes	72 females	Statistically significant 7.4 % increase in hair diameter after 6 months and 18 % reduction in hair shedding after 3 months.	[47]
2012	Yes	Yes	15 females	111 % increase in terminal hairs after 3 months, 125 % increase in terminal hairs after 6 months. After 3 and 6 months there was a significant self-perceived improvements in overall hair volume, thickness and scalp coverage.	[48]
2011	No	No	16 females	the greatest change in hair growth and hair quality occurred during the initial 2 months of treatment. Increased changes continued to occur after that time except there was a very slight decrease in the number of hairs lost on an average day.	[49]
2010	No	No	16 females	a directional reduction in hair shedding was observed. After a 10-week period, there was an average 46 % reduction in hair loss reported. 75 % saw an increased thickness in the body of their hair and 75 % saw an increase in overall hair volume.	[50]
1997	No	No	178 males	75.3 % of patients observed a significant decrease in hair loss; 14.6 % of patients showed partial regrowth	[51]

(*continued on next page*)

Table 1 (*continued*)

Year	Double blinded	Placebo control	Test group	Results	Ref.
1996	No	No	55 females, 23 males	After 6 months of treatment 92 % of areata group showed regrowth of permanent hair. After 4 months of treatment 83.3 % of totalis group showed regrowth of permanent hair and 31.8 % of universalis group showed regrowth of permanent hair after 5 months. Complete cure was observed in 14 % of areata, 25 % of totalis and 5 % of universalis.	[52]
1994	No	No	30 males	Hair loss stopped for 100 % of subjects after 2 months treatment. 43 % showed total regrowth, 23 % showed three quarter regrowth, 13 % showed half regrowth and 13 % showed 30–50 % regrowth.	[53]
1992	No	No	20 females, 20 males	85 % of subjects with Alopecia Areata were completely cured and 10 % showed significant improvement. 25 % of subjects with Alopecia Totalis were completely cured and 20 % showed significant improvement.	[54]
1992	Yes	Yes	37 males	100 % of participants reported hair loss had stopped after 2 months of treatment. Mean increase in non-vellus hair of 38 % was recorded in patients after 6 months treatment. 95 % of subjects showed both clinical and histological cure.	[44]

Table 2
The list of ingredients of Viviscal® hair growth supplements.

Ingredient [mg/daily dose]	Viviscal Max EU	Viviscal Max US	Viviscal Professional
AminoMar C®	600	900	950
Ascorbic acid	77	118	40
Calcium	0	0	120
Niacin	16	24	0
Biotin	0.156	0.24	0.2
Iron	13.6	20	0
Zinc	14.9	22	0
Horsetail extract	49	73.4	0
Millet seed	10	15	0
Apple extract (Procyanidin B-2)	0	0	80
L-cysteine	0	0	25
L-methionine	0	0	25

2. Materials and methods

2.1. Sample preparation

Since Viviscal® is oral nutritional supplements, Viviscal® samples and its raw ingredients were artificially digested to mimic the natural processes occurring in the human digestive system. Simulated Salivary Fluid (SSF), Simulated Gastric Fluid (SGF) and Simulated Intestinal Fluid (SIF) were prepared according to the publication of Minekus et al. [60]. As the recommended daily dose of the Viviscal® supplement is two tablets and it was also the amount taken by the participants of clinical studies, the same dose was used to prepare samples for the in vitro study. Oral digestion was achieved by first mixing weighed amounts of the products with simulated salivary fluid, then adding calcium chloride and distilled water to produce a liquid sample. Gastric digestion was achieved by adding a set volume of gastric fluid to the liquid sample, followed by porcine pepsin, calcium chloride and then bringing the pH to 3 with hydrochloric acid. More distilled water was added, and the samples were placed in a shaking incubator at 37 °C for two hours. Intestinal digestion was performed by adding simulated intestinal fluid, pancreatin, freshly made bile and calcium chloride. The pH was increased to pH 7 with sodium hydroxide. The samples were thoroughly mixed and incubated at 37 °C for two hours in the shaking incubator. The samples were then heated to 60 °C for 20 min to inactivate the enzymes. The samples were centrifuged at 4000 rpm for 5 min. The pH was checked to ensure that it was pH 7. The supernatants were removed, filter-sterilised and stored at −20 °C in the freezer for the further analysis. The additional control, digestive fluids without Viviscal®, was also prepared to assess the impact of solvent on dermal papilla cell behaviour.

2.2. Cell culture

The immortalized human dermal papilla cells, iDPC, were purchased from Kyungpook National University (South Korea). Cells were cultured in complete Dulbecco's modified Eagle's medium (DMEM; GibcoTM) with GlutaMAX™, 10 % FBS and penicillin/streptomycin (100 unit/ml and 100 μg/ml, respectively; GibcoTM). Primary human dermal papilla cells, isolated from human scalp follicles, were purchased from NBS Biologicals (Lot 1614424 and 1614431, for male and female cell lines, respectively). Cells were cultured in complete media recommended by the supplier (Human Dermal Papilla Primary Cell Culture Medium with Serum, NBS03809S). Immortalized and primary cell lines were maintained at 37 °C in a humidified 5 % CO_2 incubator.

2.3. Viability assay

Cell viability after treatment with tested samples was evaluated using PrestoBlue™ Cell Viability Reagent (Invitrogen). Dermal Papilla Cells were seeded at a density of 10,000 cells per well in 96-well plates in 100 μl of complete media. Cells were allowed to adhere for 24 h. Treatment media was prepared to contain an appropriate concentration of Viviscal® supplements and its raw ingredients in complete media. Treatments were performed in triplicate wells. Cells were cultured for a further 24, 48 and 72 h. No media changes were performed between initial treatment and cell assays. Media only was used to treat controls cells. An additional control was used to assess the impact of solvent (digestion fluids without Viviscal®). After exposure for the desired period of time 11 μl PrestoBlue™ reagent was added to each well of the 96-well plate. Plates were incubated in the dark for 2 h at 37 °C. Fluorescence was read using a 560 nm excitation/590 nm emission filter set (10 nm bandwidth). Fluorescence data in wells containing cells were corrected for background fluorescence using cell-free media control replicates.

2.4. Alkaline phosphatase production assay

The SensoLyte® Alkaline Phosphatase Assay Kit (AnaSpec) was used for quantitative evaluation of enzyme production by Dermal Papilla Cells. Cells were seeded in transparent 12-well plates at density 30,000 cells per well in 2 ml of complete media. Cells were allowed to adhere overnight. Treatment media was prepared to contain an appropriate concentration of Viviscal® supplements and its raw ingredients in

complete media. Alkaline phosphatase expression was assessed at 72, 120 and 168 h. Cells were gently washed twice with 1X assay buffer. Lysis buffer (1X assay buffer with TritonX-100) was added to each well. Cells were transferred into Eppendorf tubes and subjected to 3 freeze thaws to ensure cells lysis. Cell suspensions were centrifuged at 2500 g for 10 min at 4 °C. Supernatants were used to alkaline phosphatase assay according to the manufacturer's protocol.

2.5. RNA extraction and quantitative RT-PCR

Total RNA was extracted from the male dermal papilla cells using High Pure RNA Isolation kit (Roche) according to the manufacturer's protocol. A total of 500 ng of DNA-free RNA was subjected to reverse transcription using qScript cDNA Synthesis Kit (Quanta bio, USA). Relative cDNA levels were measured using FastStart Essential DNA Green Master (Roche) in a LightCycler® 96 System (Roche). Two housekeeping genes, *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) and *ACTB* (actin beta) were used as reference genes in the calculations for relative expression. The relative mRNA expression level was calculated using the $2^{-\Delta\Delta CT}$ analysis method. All measurements were repeated for three biological and three technical replicates. The primers used for the quantitative PCR are shown in Table 3.

2.6. Statistical analysis

All results were expressed as the means ± SEM from three independent experiments. GraphPad Prism 9 (GraphPad Software, CA, USA) and Dunnets's multiple comparison tests were used to analyze significant differences ($p < 0.05$) between the mean values of the individual group.

3. Results

3.1. Viability assay

PrestoBlue® Cell Viability Reagent was used to assess cell viability after treatment with Viviscal® and its key ingredients. The results are presented in Fig. 1.

There were no statistical differences in cellular viability level of dermal papilla cells, both immortalized and primary, treated with digestive fluids only and untreated ones (negative control; data not shown). It was proven that the solvent used during the artificial digestion process, mixture of digestive fluids, do not affect proliferation of tested dermal papilla cells. None of the tested hair growth Viviscal® supplements show cytotoxic activity against used cell lines and observed cell viability in relation to the control cells (not treated with tested samples) was not lower than 85.5 %. In addition, the tested hair growth supplements caused statistically significant increase in dermal papilla cell proliferation. For the immortalized cell line, the best results were observed after treatment with 2 mg/ml Viviscal® Professional. Hair supplement caused significant increase in iDPC viability. There were not statistically significant differences between activity of this sample and positive control, 10 μM minoxidil. Results obtained for Viviscal® Professional used at concentration 2 mg/ml, for all three timepoints, were significantly higher in comparison to the raw ingredient, Shark Extract. Immortalized DPC viability after treatment with all key marine-derived components of hair growth supplement, AminoMar C®, Shark Extract and Oyster Extract, was significantly lower in relation to the positive control, minoxidil. In the case of male primary cell line, Viviscal supplements and raw ingredients were not cytotoxic against the cells. Viviscal® Max EU at the concentration 2 mg/ml, as the only tested sample, significantly increased cell proliferation rate after all three timepoints. Moreover, results obtained for this sample after 48 h incubation were significantly higher in comparison to all three raw ingredients. It was found that marine-derived component of Viviscal® supplements, Shark Extract, were slightly cytotoxic against tested female dermal papilla cells (cell viability 62.5 % after 24 h treatment). It was not observed after longer incubation period, 48 and 72 h. Viviscal® Max EU used at the highest concentration, 5 mg/ml, statistically significant increase female cells viability after 24, 48 and 72 h.

Table 3
Sequences of primer pairs used in RT-PCR.

Gene name	Forward primer sequence (5′ → 3′)	Reverse primer sequence (5′ → 3′)
GAPDH	TCGACAGTCAGCCGCATCTT	GCCCAATACGACCAAATCCGT
ACTB	GAGCACAGAGCCTCGCCTTT	CATCACGCCCTGGTGCCT
CAMK2G	CCCGTCTCCTCCTCTTGCTC	ACAGAGAAAGCACCCTTGCC
CSNK1A1	CTCTTCCCAGAGGTGTCGGG	GCTTCACTGCCACTTCCTCG
CXCL2	AGGGGTTCGCCGTTCTCGG	CGAGGAGGAGAGCTGGCAAGG
DUSP1	CACTCTACGATCAGGGTGGC	TCCTTGCGGGAAGCGTGATA
EGR1	TGACCGCAGAGTCTTTTCCTG	CCAGGGAAAAGCGGCCAGTA
EGR2	GCGAGGAGCAAATGATGACCG	TTGATCATGCCATCTCCGGC
FOS	CACTCCAAGCGGAGACAGACC	AGGCCCCCAGTCAGATCAAG
JUNB	CGCATCAAAGTGGAGCGCAA	TTCTCGGCCTTGAGCGTCTT
MEF2C	AGTGCAGGGAACGGGTATGG	GCAGGTCGACATCCTCAGACA
PORCN	CTTCGCAAGTGGCTGCGAG	TCCACCATTGACCGAGGCAG
PPM1A	GAGGCGCGAAAGCGATGAG	CAGATCATCCGGGCGTTGGA
PRICKLE1	TTCTGGGCTCTGGATGGTTCG	TCAAACAATGGCTGCTCGC

3.2. Alkaline phosphatase production assay

The influence of hair supplements and marine-derived key ingredients on alkaline production by dermal papilla cells was assessed using the SensoLyte® Alkaline Phosphatase Assay Kit.

The results shown in Fig. 2 demonstrate that Viviscal® supplements significantly promote the production of the enzyme in tested cell lines. When immortalized dermal papilla cells were treated with Viviscal Max EU, statistically significant increase in alkaline phosphatase production was observed after 72 h (at concentrations 2 and 0.1 mg/ml) and 168 h (0.1 mg/ml). Viviscal® Max US at both tested doses, as well as AminoMarC® and Shark Extract, significantly inhibited production of enzyme by iDPC. In the case of primary male dermal papilla cells, Viviscal® Professional used at the lowest concentration, 0.1 mg/ml significantly promoted enzyme production after all three incubation periods. When the same supplement was used at a concentration 2 mg/ml, significant decline in obtained alkaline phosphatase was observed after 168 h. Similar pattern was seen for Viviscal® Max US. Viviscal® Max EU significantly increase enzyme expression after 72 h of incubation only. Among marine-derived raw ingredients, Oyster Extract significantly stimulated alkaline phosphatase production after 120 h. For female cells, treatment with 2 μg/ml Viviscal® Max EU resulted in statistically significant increase in alkaline phosphatase production in all three tested timepoints. Level of enzyme was significantly higher for Viviscal® Max EU 0.1 μg/ml and Viviscal® Professional 0.1 μg/ml (120 and 168 h). Moreover, alkaline phosphatase production was strongly stimulated by Oyster Extract after 72 h incubation.

3.3. Quantitative RT-PCR

Changes in expression of genes of interest after male human follicle dermal papilla cells treatment with tested samples was assessed using quantitative RT-PCR.

As can be seen in Fig. 3, all tested samples, both Viviscal supplement and key ingredients, affect the expression of tested genes in a similar way, but not all obtained differences in comparison to the untreated cells were statistically significant. In general, treatment of male dermal papilla cells with tested samples caused an up-regulation of 6 genes (CAMK2G; CXCL2; DUSP1; EGR1; EGR2 and PPM1A) and down-regulation of 3 (FOS; JUNB and PRICKLE1). For 3 genes of interest, CSNK1A1, MEF2C and PORCN significant difference in expression level were not observed.

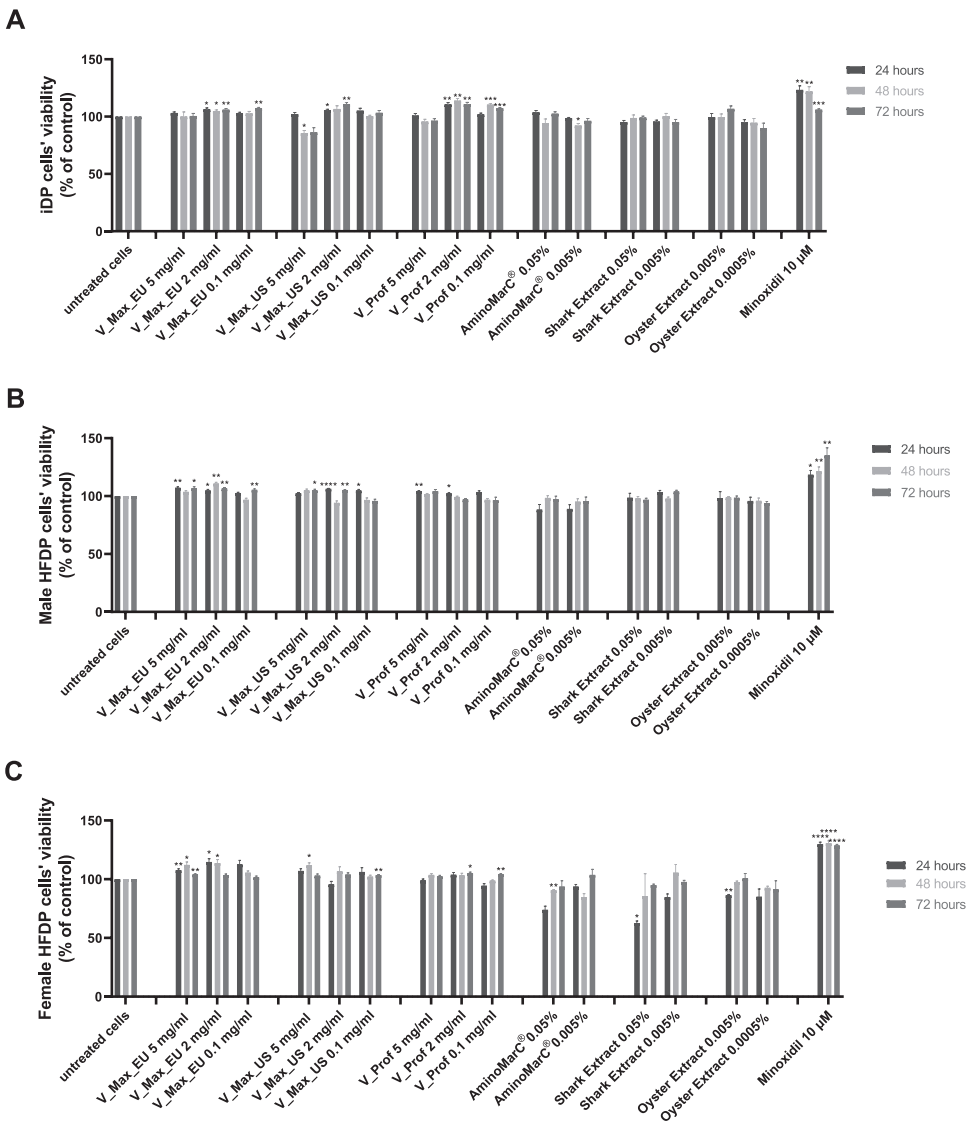

Fig. 1. Effect of Viviscal® supplements and its key ingredients on cell proliferation of dermal papilla cells: immortalized (A), primary male (B) and primary female (C). Cells were treated with different concentrations of tested samples for 24, 48 and 72 h, and proliferation was assessed using PrestoBlue® Cell Viability Reagent. The data are presented as the mean ± SEM. Statistical significance in comparison to the negative control (untreated cells) was assessed using one-way ANOVA followed by Dunnett's multiple comparison test; *$p < 0.05$; * * $p < 0.01$; * **$p < 0.001$; * ** *$p < 0.0001$.

4. Discussion

Dermal papilla cells (DPC), derived from the dermis mesenchyme and located at the base of the hair follicle, play a key role in hair growth process [61,62]. These specialized mesenchymal cells are capable of inducing follicle development from the epidermis and the production of hair fibre. The important role played by these cells in the process of hair growth makes them an excellent model for in vitro study of hair growth supplements effectiveness. Independently of the type of remedy to hair loss, all strategies are based on manipulating the natural course of hair growth cycle. It can occur through prolonging of anagen phase, induction of anagen in telogen phase follicles, inhibition of catagen as well as inhibition of exogen (shedding phase of hair growth cycle). Especially important from the patient point of view seems to be a first strategy. Duration of the anagen directly affects hair length and rate of their growth. During this phase of hair cycle, very intensive growth processes take place – number of dermal papilla cells is increased, and some specific markers and enzymes are expressed [63].

The results obtained in this study revealed that treatment of dermal papilla cells with Viviscal® supplements and raw ingredients cause an increase in proliferation rate of the cells (Fig. 1). Since duration of the anagen depends on the proliferation and differentiation of dermal papilla cells, based on this parameter, we can conclude that positive effects of Viviscal® therapy observed in the clinical studies are connected to proliferative properties of supplements on dermal papilla cells. The ability of the supplement to increase a number of dermal papilla cells is a very important parameter evaluated its hair growth promoting properties [31,64]. Improving hair cells proliferation rate is one of the mechanisms of action of minoxidil, compound used as a positive control in the present study. Han et al. [65] investigated minoxidil effect on the dermal papilla cells of human hair follicle proliferation and apoptosis. It was found that tested sample had proliferative and anti-apoptotic action, which translated to the anagen prolongation. Increased proliferation of dermal papilla cells was also observed by Yoo et al. [66] and Oh et al. [67]. Both research groups used MTT assay to assess dermal papilla cells proliferation after treatment with minoxidil and combination of minoxidil with retinoic acid or retinol. The obtained results confirm findings of Han et al. [65] about the proliferative activity of minoxidil. Outcome of the present study is in agreement with the previous research. Minoxidil increases proliferation rate of all three types of tested dermal papilla cells, both immortalized and primary. Proliferated effect of tested Viviscal® was not as strong as in the case of positive control, but the hair growth supplements caused statistically significant increase in dermal papilla cell proliferation. Significantly higher level of cell proliferation after treatment with raw ingredients, AminoMar C®, shark and oyster extracts was not observed, but the samples are also

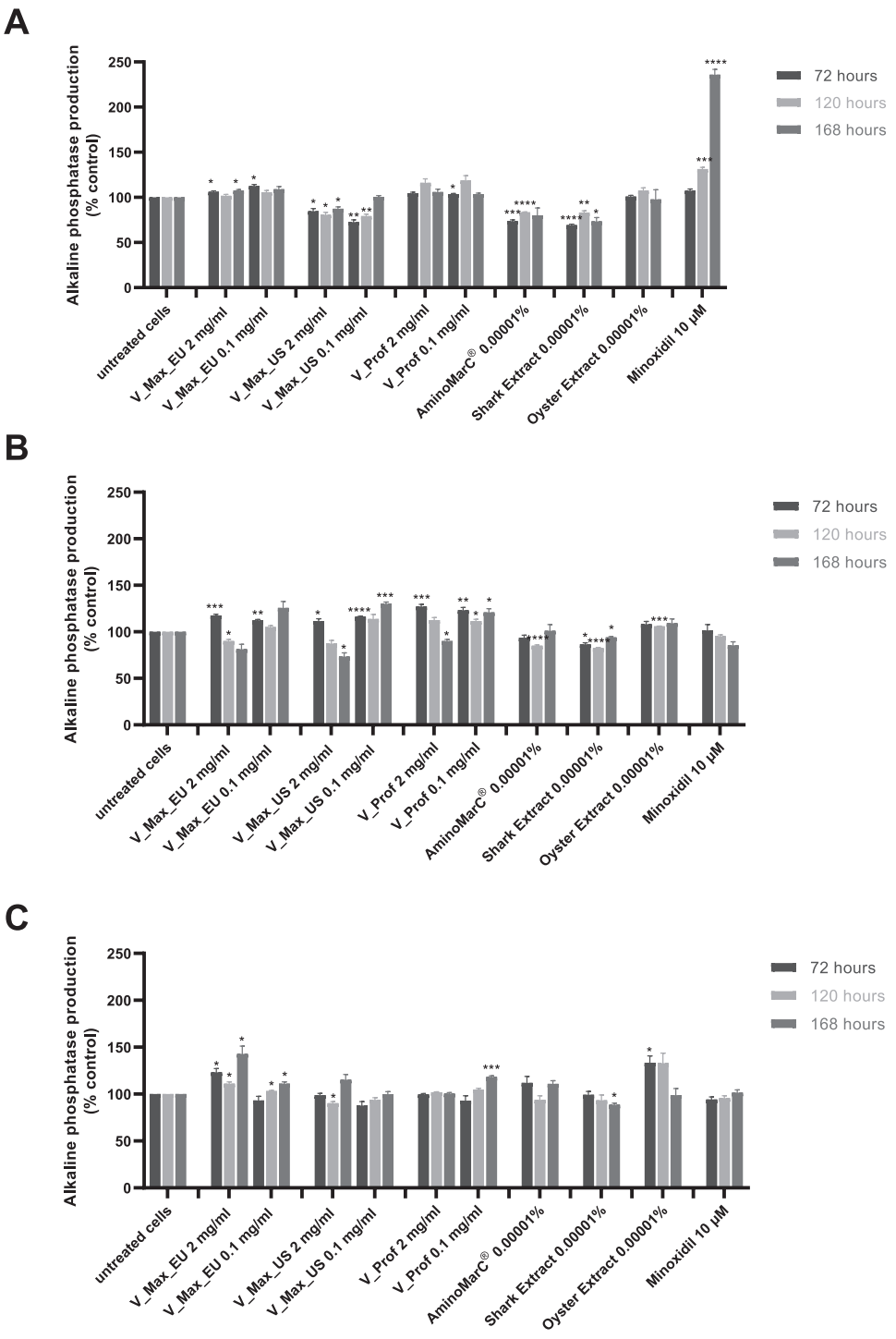

Fig. 2. Effect of Viviscal® supplements and raw ingredients on alkaline phosphatase production by immortalized (A), primary male (B) and primary female (C) dermal papilla cells. Cells were treated with different concentrations of tested samples for 72, 120 and 168 h, and enzyme concentration was assessed using the SensoLyte® Alkaline Phosphatase Assay Kit. The data are presented as the mean ± SEM. Statistical significance in comparison to the negative control (untreated cells) was assessed using one-way ANOVA followed by Dunnett's multiple comparison test; *$p < 0.05$; * *$p < 0.01$; * **$p < 0.001$; * ** *$p < 0.0001$.

noncytotoxic against tested cell lines. Although increase in cell proliferation is a commonly results observed when testing potential hair growth supporting agents, positive in vivo effects are not always associated with the proliferative activity [68–70].

Extract from *Sophora flavescens* root induced a conversion form telogen to anagen in C57BL/6 mice, but significant changes in proliferation level of human dermal papilla cells measured by [3H]thymidine incorporation were not observed [68]. Similar results were obtained by Park [69]. Tested *Origanum vulgare* extract stimulated hair growth in C57BL/6 mice, but this was not accompanied by an increase in proliferation of cultured human hair dermal papilla cells. Enhanced hair growth in telogenic C57BL/6 mice with no effect on human cell proliferation in vitro was also identified by Park et al. [70].

Among different type of cells forming hair follicle, dermal papilla cells can be characteristic by the expression of unique proteins, like laminin, fibronectin or alkaline phosphatase [71]. Alkaline phosphatase is a zinc-metallo enzyme expressed in highly proliferating tissues [72, 73]. Activity of this enzyme was observed in hair follicles as well [71]. Alkaline phosphatase is a mechanistic marker of dermal papilla cells in vivo and its activity is associated with hair regeneration. According to the research of Iida et al. [74] the activity of alkaline phosphatase changed during the hair growth cycle – a maximal level was found to be

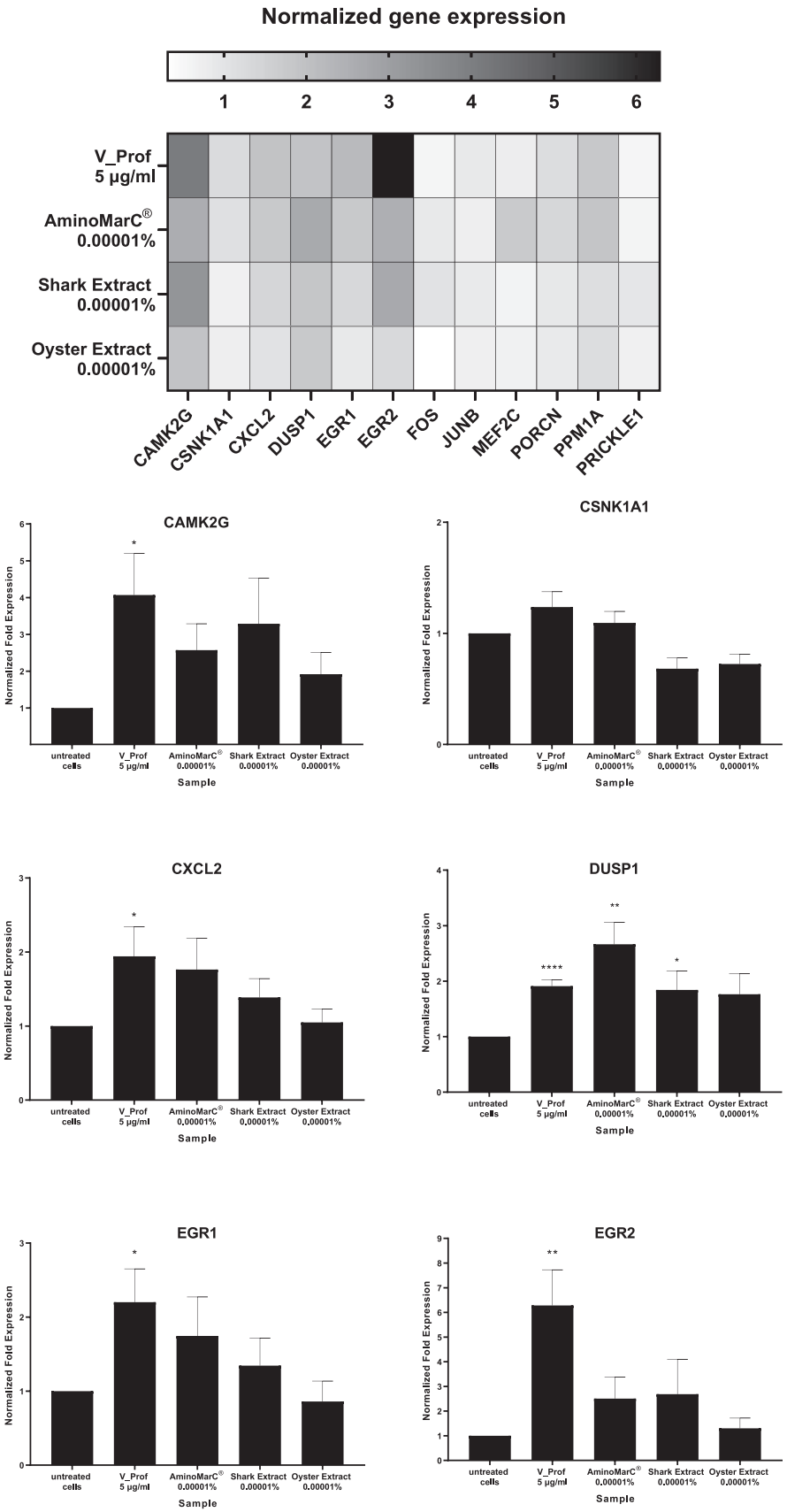

Fig. 3. Gene expression in male human follicle dermal papilla cells treatment with Viviscal® Professional and raw ingredients. The data are presented as the mean ± SEM. Statistical significance in comparison to the negative control (untreated cells) was assessed using one-way ANOVA followed by Dunnett's multiple comparison test; *$p < 0.05$; * * $p < 0.01$; * **$p < 0.001$; * ** *$p < 0.0001$.

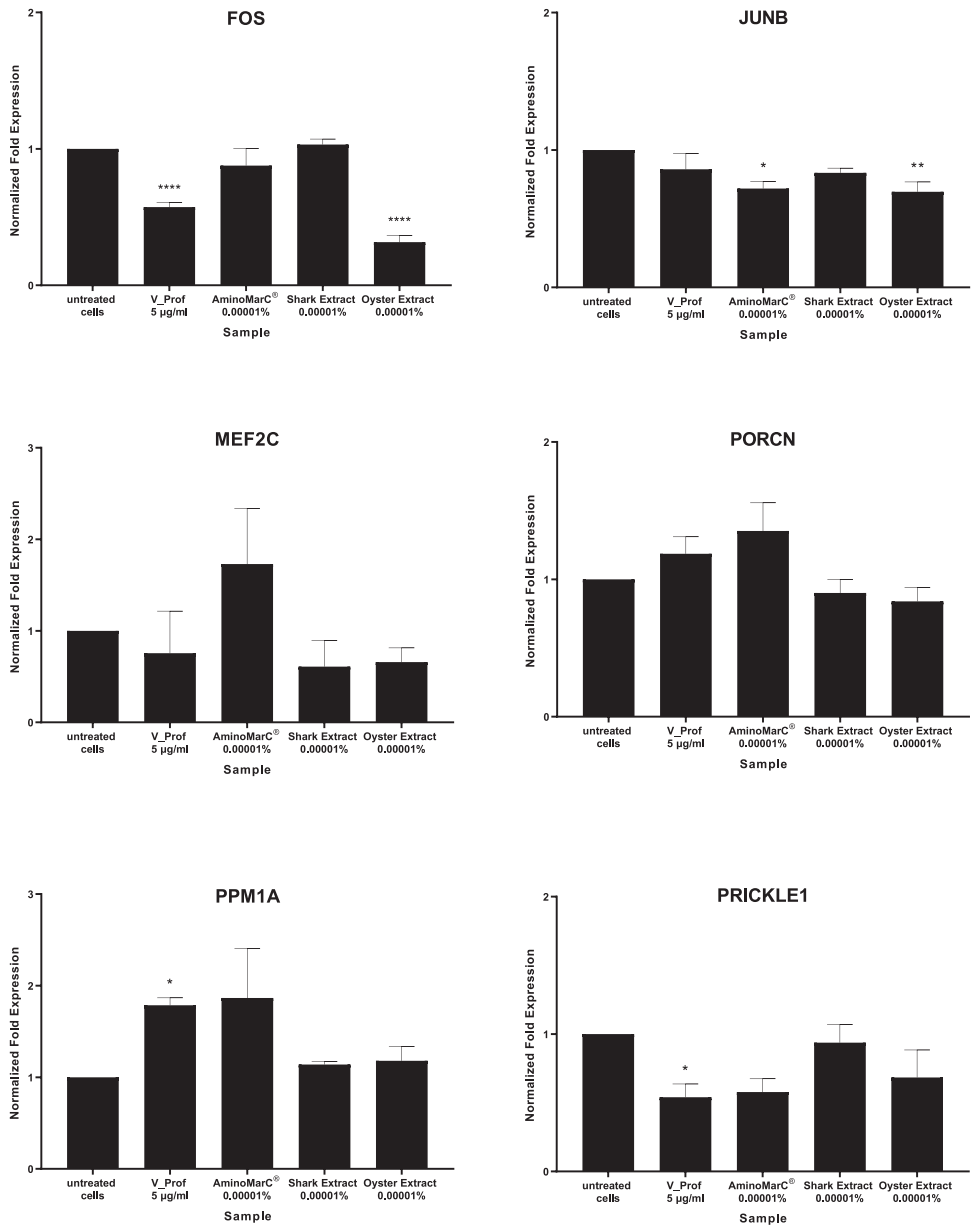

Fig. 3. (*continued*).

characteristic for early anagen. The data from in vitro experiments, performed in the present study, indicate that tested hair growth promoting supplements have ability to promote expression of alkaline phosphatase in dermal papilla cells (Fig. 2). As the high level of the enzyme can be used as an indicator of the anagen, increasing in alkaline phosphatase activity in DPC may reveal that Viviscal® stimulates the recovery of growth phase in hair cycle [75]. Similar results can be found in the previously published studies. Kim et al. [76] observed 1.5-fold increase in alkaline production by human dermal papilla cells after treatment with rice bran mineral extract (by western blotting). Analysis of tested sample effect on genetic level revealed that the β-catenin/Wnt signalling pathway was activated. Moreover, expression of TGF-β2 was reduced due to the activity of rice bran mineral extract.

Wnt signalling pathways are considered to be one of the most important for growth and development of hair follicles [77]. Highly conserved Wnt proteins activate distinct intracellular cascades, known as canonical (the Wnt-β-catenin) and noncanonical (the Wnt/Ca^{2+}, the Wnt/planar cell polarity (PCP) and Wnt/Ror2) pathways through regulation of transcriptional and posttranscriptional processes [78,79]. Both canonical and noncanonical Wnt pathways plays role during development of hair follicle [80–84]. In the present study, increased expression of *CAMK2G*, gene involves in Wnt/Ca^{2+} pathway was observed after male primary dermal papilla cells treatment with Viviscal® Professional (Fig. 3). Product of the gene, calmodulin-dependent kinase II (CamKII), in noncanonical Wnt pathway is stimulated by calcium released from the endoplasmic reticulum and activates transcription factor NFAT [85]. Another gene from the Wnt noncanonical signalling pathway, *PRICKLE1*, was found to be significantly downregulated by the tested hair growth supplements. The gene encodes prickle planar cell polarity protein 1, a nuclear receptor involve in the Wnt/PCP pathway. Several studies indicate that PRICKLE1 may function as a negative regulator of canonical Wnt/β-catenin pathway [86–89]. It has been proven that canonical Wnt signalling pathway plays a crucial role in promoting hair growth, but the knowledge about the involvement of noncanonical pathway still remain not sufficiently explored. The changes observed on a genetic level and resulted from dermal papilla cell exposure to tested Viviscal® may indicate that supplements action is mediated through noncanonical Wnt pathways.

Another hair growth-related signalling cascade affects by Viviscal® Professional treatment is TGF-β signalling. It was proven that this pathway acts as a negative regulator of hair growth, reduces the anagen phase and promotes early enter the catagen [90,91]. In the present study upregulation of *PPM1A* gene was observed. Product of the gene expression, protein phosphatase, $Mg^{2+/}Mn^{2+}$ dependent 1A, is a negative regulator of TGF-β signalling. Protein dephosphorylates SMAD2 and SMAD3 what results in their dissociation from SMAD4, nuclear export of the SMADs and termination of the TGF-β-mediated signalling [92]. Reduced expression of TGF-β was observed in the previous studies of hair growth promoting ingredients. According to Shin et al. [93] expression of TGF-β after human dermal papilla cells treatment with 1 μM ginsenoside F2 was 72 % lower in comparison to the control group. Moreover, expression of phosphorylated SMAD2 and SMAD3 decreased by 58 % and 72 %, respectively. In the work of Kang et al. [94] anagen stage vibrissae follicles were treated with ethanol extract of *Schisandra nigra*. After 7 days of incubation, expression of TGF-β was lower when compared to the control follicles.

As can be seen in Fig. 3, Viviscal® Professional significantly stimulates expression of two genes from EGR family of transcription-regulatory factors, *EGR1* and *EGR2*. Early growth response factor 1, product of *EGR1* gene expression, was found to promote proliferation of dermal papilla cells [95]. Cellular expression of EGR1 was related to the proliferation rate – cells with up-regulated EGR1 have higher proliferation rate when compared to the control, down-regulation of the transcription factor was associated with lower proliferative effect. Moreover, the markers of proliferation (PCNA, CDK2) were upregulated when *EGR1* gene was overexpressed. Study of Yang et al. [96] showed that topical application of conditioned media from adipose-derived stromal/stem cells treated with LL-37, naturally occurring antimicrobial peptide, and EGR-1 overexpressing cells promotes hair growth C57BL/6 mice with hair loss. EGR2 (KROX20) is a zinc finger transcription factor expressed in subpopulations of hair follicle cells [97]. Liao et al. [98] investigated the role of protein during the hair follicle development. It has been reported that KROX20-expressing cells produce stem cell factors, necessary element of maintaining differentiated melanocytes and hair pigmentation. Moreover, after deletion of *KROX20* lineage cells complete arrest of new hair growth was observed in vivo. Analysis of hair cycle-related genes performed by Lin et al. [99] by using microarray and replicate variance revealed that *EGR2* gene belongs to the cluster 7 displaying hair growth pattern that peak at late anagen.

Two genes of interest, *FOS* and *JUNB*, were significantly downregulated as a result of treatment with tested samples. Products of the genes expression are the members of activator protein 1 (AP-1) family of transcription factors regulated gene expression in response to different stimuli and controlled a large number of cellular processes, like proliferation, differentiation or apoptosis [100–103]. One of the signalling pathway AP-1 is involved in is TGF-β-Smad. Study from 2020 [104] revealed that knockdown of two subunits of AP-1, JUN and FOS or blockage of activator protein 1 signalling by using two inhibitors (50 μM T-5224 and SR11302) caused the downregulation of *TGF-β1*. Han et al. [105] investigated the role of TGF-β downregulation in death of cancer cell. They found that increased production of reactive oxygen species (ROS) was associated with TGF-β downregulation. Expression of two proteins protected cells from oxidative stress, thioredoxine (Trx) and glutathione S-transferase Mu1 (GSTM1) was found to be suppressed as a result of TGF-β downregulation. As the promoters for both Trx and GSTM1 contains several AP-1 binding sides, the relation of activator protein 1 and TGF-β was further investigated. The obtained results indicate that TGF-β downregulation decreased Trx and GSTM1 promoter binding by AP-1 proteins, most likely due to decrease in AP-1 protein levels. Moreover, Western blot analysis showed that the expression levels of phospho-Smad2 and phospho-Smad3 were decreased after silencing *TGF-β1* or *TGF-β2* genes by infection with adenoviruses expressing shRNAs. The results may suggest that Viviscal® hair growth properties are connected with the inhibition of TGF-β pathway, involved in the anagen reduction and catagen progression [106,107].

Results from in vitro experiments performed in this study reveal differences between activity of Viviscal® supplements and their raw ingredients. Although AminoMar C® complex, consisted of shark cartilage extract and oyster powder, is the key Viviscal® ingredient, there are more compounds with proven hair growth promoting properties within the hair growth supplements. The positive effect of Viviscal® is the sum of the hair growth potential of each of its components. In addition to the marine-derived ingredients, vitamins and minerals, in Viviscal® supplements we can find horsetail (*Equisetum sp.)* extract, millet seed or procyanidin B-2 which effects on hair cells and hair growth were reported [108–112]. The results observed in the study suggest that dermal papilla cells exposure to Viviscal® affected TGF-β signalling pathway. Similar mechanism of action is attributed to procyanidin B-2 [113,114].

In summary, our study for the first time provides results from in vitro evaluation of activity of marine-derived hair growth supplements with proven clinical efficacy. To the knowledge of the authors, the comparison of data obtained from treatment of three different dermal papilla cell lines, primary female, male and immortalized ones, with the tested samples is the first of its kind. Tested samples significantly influences anagen markers of DPCs, cellular proliferation rate and the production of alkaline phosphatase. Viviscal effect on the growth phase of hair was also confirmed by the results from gene analysis.

CRediT authorship contribution statement

Helena Mc Mahon: Conceptualization, Funding acquisition, Project administration, Methodology, Resources, Supervision, Validation, Writing- review and editing. **Aleksandra Augustyniak**: Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing-Original draft.

Declaration of Competing Interest

The authors declare that there is no conflict of interest.

Data Availability

All data generated or used during the study appear in the submitted article.

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