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REGULAR PAPER

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A designer mixture of six amino acids promotes the extracellular matrix gene expression in cultured human fibroblasts

Laura Tedesco,[1] Fabio Rossi,[1] Chiara Ruocco,[1] Maurizio Ragni,[1] Michele O. Carruba,[1] Alessandra Valerio,[2] and Enzo Nisoli [1,*]

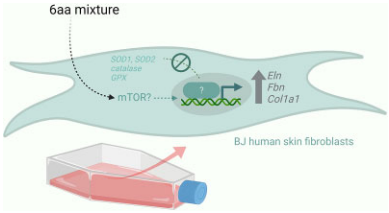
[1]Center for Study and Research on Obesity, Department of Medical Biotechnology and Translational Medicine, University of Milan, via Vanvitelli, 32–Milan, Italy; and [2]Department of Molecular and Translational Medicine, Brescia University, viale Europa, 11– Brescia, Italy

*Correspondence: Enzo Nisoli, enzo.nisoli@unimi.it

ABSTRACT

The deterioration of the skin is caused by dermatological disorders, environmental conditions, and aging processes. One incisive strategy for supervising the skin aging process is implementing healthy nutrition, preserving a balanced diet, and a good supply of food supplements. Here, we compared H-Pro-Hyp-OH peptide, hydrolyzed collagen, and an original mixture of six amino acids (we named 6aa)—including glycine, L-alanine, L-proline, L-valine, L-leucine, and L-lysine—effects on the production of extracellular matrix (ECM) components, particularly the elastin, fibronectin, collagen 1, and collagen 4. Treatment of BJ human skin fibroblasts with the 6aa mixture upregulated elastin, fibronectin, and collagen 1 gene expression, without affecting the expression of anti-reactive oxygen species enzymes. Moreover, the mammalian target of rapamycin (mTOR) signaling pathway seems to be involved, at least in part. Collectively, these results suggest that the six amino acid mixture exerts beneficial effects in human skin fibroblasts.

Graphical Abstract



An original mixture of six amino acids promotes extracellular matrix component production in BJ human skin fibroblasts through mTOR and independently from ROS defense enzymes.

Keywords: amino acids, extracellular matrix, fibroblasts, hydrolyzed collagen, peptide

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The impact of aging on the appearance and function of the skin has received notable interest in recent decades. The passage of time caused chronological skin aging which takes place in the entire body skin, including facial skin. To keep self-esteem and social relations, it's become essential to obtain a young-looking appearance (Dobos *et al*. 2015). Therefore, there is an increasing demand for anti-aging interventions to delay or even reverse signs of skin aging. Dietary supplements have received growing attention for improving the appearance and role of aged skin. Indeed, many dietary components, such as polyphenols (Chen *et al*. 2017), vitamins (Tran *et al*. 2014), fatty acids (Latreille *et al*. 2013), trace minerals (Fanian *et al*. 2013), and proteins (Murata *et al*. 2014), have been described to exert beneficial effects on aged skin and have been used as nutraceuticals or functional foods in many countries. Recently, researchers have examined protein hydrolysates as potential dietary supplements. As a matter of fact, collagen, the main structural protein of different connective tissues, such as skin, bones, and tendons, has been broadly used in the medicine and food industries.

The wound healing process is one of the most complicated processes described regarding the skin and implicates different phases, such as inflammation, proliferation, migration of keratinocytes, new tissue formation, and tissue remodeling: to achieve wound healing, all these steps are required (Colella *et al*. 2009). Moreover, biochemical, molecular, and morphological changes and the identification of biomarkers can be used to monitor these phases. During the remodeling phase, extracellular matrix (ECM) proteins, which are synthesized, assembled, and reorganized by fibroblasts to reconstruct tissues, play an essential role (Gosain and Pietro 2004). Multiple collagen types and fibronectin belong to these proteins and the local bioavailability of constituent amino acids (AA) is required for their biosynthesis. Collagen triple helix consists of glycine, L-proline, and L-lysine in well-determined positions. Its viscoelastic properties are due to the hydroxylation of glycine and proline residues which produces inter- and intra- molecular bonds (Trackman 2005). Elastin is a polymeric protein that originated from the crosslinking of monomers of tropoelastin precursors. Collagen and elastin together form the ECM, which gives the skin its structure, elasticity, and firmness (Krieg and Aumailley 2011).

Since previous work showed that topical application of an AA mixture (ie glycine, proline, leucine, and lysine) and sodium hyaluronate accelerated wound closure and prevented fibrosis in a rat model of excisional wound (Corsetti *et al*. 2010), and another study demonstrated the relevance of specific AA mixtures to extend the average life span of mice (D'Antona *et al*. 2010), the present screening study aimed to compare H-Pro-Hyp-OH peptide, hydrolyzed collagen and a designer combination of six AA (we named 6aa) in stimulating the production of ECM components, particularly the synthesis of elastin (Eln) and fibronectin 1 (Fbn1), while at the same time maintaining the stimulation of collagen 1 (Col1a1) and collagen 4 (Col4a1), in cultured human skin fibroblasts. Furthermore, we examined the possible involvement of mammalian target of rapamycin (mTOR) pathway.

Materials and methods

Cell cultures and treatment

Human skin fibroblast BJ (ATCC® CRL-2522™) was used in the study. BJ cells were cultured in standard conditions in EMEM medium (Sigma-Aldrich Milan, Italy), supplemented with 10% fetal bovine serum (Sigma-Aldrich) and antibiotics (Sigma-Aldrich). Cell cultures were incubated at 37 °C, under 95% humidity and 5% CO_2. Cells were treated with 0.1%-1% of the 6aa mixture (composition is reported in Table 1), or H-Pro-Hyp-OH peptide (Bachem #1 072 927), or hydrolyzed collagen (GELITA Deutschland GMBH, Eberbach, Germany) for 3 days. Pro-Hyp is a dipeptide composed of L-proline and L-hydroxyproline residues. For mTOR pathway analysis, BJ cells were treated for 30 min with 50 nM rapamycin and then for 48 h with 1% 6aa mixture. Untreated cells were plated as controls. Every 24 h, media were replaced in both control and treatment flasks, with fresh media or with the different products. At the end of the experimental treatments, cells were used for mRNA extraction.

Table 1. 6aa mixture composition

Mixture	6aa (%)
Glycine	30.2
l-Alanine	22.8
l-Proline	22.7
l-Valine	16.8
l-Leucine	4.2
l-Lysine (chlorhydrate)	3.3

Total RNA extraction and gene expression analysis

RNA was isolated from BJ cells using the RNeasy Mini Kit (Qiagen). The iScript cDNA Synthesis Kit (Bio-Rad Laboratories Segrate, Milan, Italy) was used to synthesize cDNA. Quantitative reverse transcription polymerase chain reactions (RT-PCRs) were performed with the iQ SybrGreenI SuperMix (Bio-Rad Laboratories) on an iCycler iQ real-time PCR detection system (Bio-Rad Laboratories) as previously described (Vettor *et al*. 2014). Human primers were designed with Beacon Designer 2.6 software from Premier Biosoft International (Palo Alto, CA, USA) and are shown in Table 2. The cycle number at which the various transcripts were detectable (threshold cycle, CT) was compared with that of TATA binding protein (TBP), referred to as ΔCT. The relative gene level was expressed as 2-(ΔΔCT), in which ΔΔCT equals the difference between the ΔCT of either treated cells and the ΔCT of the untreated cells.

Western blot analysis

Protein extracts were obtained from BJ cells in mammalian protein extraction reagent (M-PER; Pierce, ThermoScientific, Rockford, USA), as indicated by the manufacturer, in 1 mM $NaVO_4$, 10 mM NaF, and a cocktail of protease inhibitors (Sigma-Aldrich). The bicinchoninic acid protein assay (BCA, Pierce, Euroclone, Milan, Italy) was used to determine the protein content. A total of 30 µg of proteins were separated under reducing conditions (SDS-PAGE). The separated proteins were then electrophoretically transferred to a nitrocellulose membrane (Bio-Rad Laboratories) (Vettor *et al*. 2014). Proteins of interest were detected with specific antibodies: anti-Fbn1 (1:1000, GeneTex, Cat# GTX112794), and anti-GAPDH (1:1000, Cell Signaling Technology Cat# 2118). Immunostaining was performed using horseradish peroxidase-conjugated anti-rabbit or anti-mouse immunoglobulin for 1 h at room temperature. The protein was detected using SuperSignal substrate (Pierce, Euroclone, Milan, Italy) and quantified by densitometry with ImageJ image analysis software.

Table 2. Human primers for qRT-PCR

Gene	Primer Sense (5'-3')	Primer Antisense (5'-3')	PCR Product (bp)	T_a (°C)
Cat	ATACCTGTGAACTGTCCCTACC	GTTGAATCTCCGCACTTCTCC	183	60
Col1a1	TGATGGTGCTACTGGTGCTG	CCTCGCTTTCCTTCCTCTCC	437	60
Col4a1	CTACGTGCAAGGCAATGAACG	GCAGAACAGGAAGGGCATTGT	93	60
Eln	TTCCCCGCAGTTACCTTTCC	ACGTTCCCAGGCTTCACTCC	175	60
Fbn1	GTGGTGTGGTCTACTCTGTGG	TCTGGTCGGGCATCATAGTTCTG	434	60
GPX1	CGCTTCCAGACCATTGAC	GGTGTTCCTCCCTCGTAG	172	60
TBP	AGGCACCACAGCTCTTCCAC	CCCAGAACTCTCCGAAGCTG	130	60
SOD1	GAGACGGGGTGCTGGTTTGC	ACGCCGAGGTCCTGGTTCC	82	60
SOD2	TAACGGTGGTGGAGAACCCAAA	TTGAAACCAAGCCAACCCCAAC	138	60

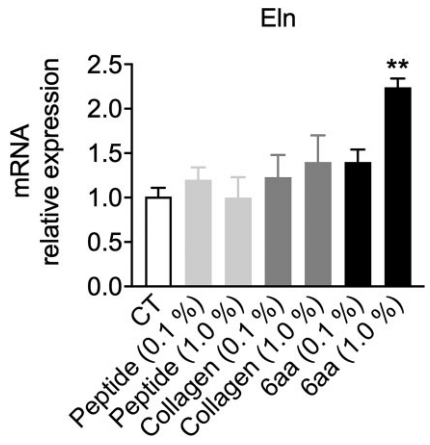

Figure 1. Eln mRNA expression in BJ cells treated with 0.1% or 1% of H-Pro-Hyp-OH peptide or hydrolyzed collagen or 6aa mixture for 72 h. Quantitative PCR, performed in triplicate and normalized to TBP of Eln ($n = 3$, mean ± SD). ** $P < .01$ *vs.* untreated cells, expressed as 1.0.

Statistical analysis and data presentation

Statistical analysis was performed with a one-way ANOVA followed by Student-Newman-Keuls' test or Student's *t*-test. Data are presented as the means ± standard deviations (SD). A statistically significant difference was accepted at $P < .05$.

Results

6aa mixture is more effective than hydrolyzed collagen or H-Pro-Hyp-OH peptide in inducing Eln and Fbn1 expression

As reported in Table 1, the 6aa mixture was created especially for the conservation and nourishment of fibroblasts and skin. The optimal ratio of the 6aa mixture between the AA for collagen and elastin production was previously identified (De Servi *et al.* 2018). In particular, it was demonstrated that while only glycine, proline, leucine, and lysine are essential for the synthesis of collagen, alanine and valine were also essential for the production of elastin (De Servi *et al.* 2018). To compare the efficacy of the new amino acid formula 6aa with H-Pro-Hyp-OH peptide and hydrolyzed collagen, we first analyzed the *Eln* and *Fbn1* gene expression in human fibroblast BJ cells. As shown in Figures 1 and 2, 1% 6aa mixture significantly increased both *Eln* and *Fbn1* mRNA levels by 124% and 60%, respectively, compared with untreated cells after 72 h of treatment. H-Pro-Hyp-OH peptide and hydrolyzed collagen do not significantly affect *Eln* and *Fbn1*'s gene expression.

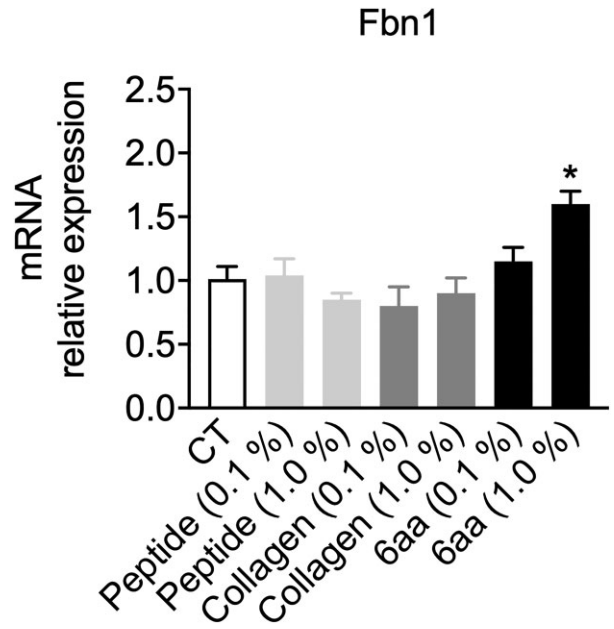

Figure 2. Fbn1 mRNA expression in BJ cells treated with 0.1% or 1% of H-Pro-Hyp-OH peptide or hydrolyzed collagen or 6aa mixture for 72 h. Quantitative PCR, performed in triplicate and normalized to TBP of Eln ($n = 3$, mean ± SD). * $P < .05$ *vs.* untreated cells, expressed as 1.0.

Only 6aa mixture activates Col1a1 and Col4a1 expression

We then investigated the effects of 6aa mixture, H-Pro-Hyp-OH peptide and hydrolyzed collagen on *Col1a1* and *Col4a1* gene expression in human fibroblast BJ cells. After 72 h of treatment, only 1% 6aa mixture significantly increased mRNA levels of *Col1a1* by 40% compared with untreated cells (Figure 3).

However, we observed only a trend to higher *Col4a1* mRNA levels than those in untreated BJ cells after 6aa treatment (Figure 4). Moreover, in line with *Eln* and *Fbn1* data, H-Pro-Hyp-OH peptide and hydrolyzed collagen were ineffective. The effects observed with the H-Pro-Hyp-OH peptide are in line with those obtained by other researchers who did not find any significant changes in *Eln*, *Col1a1*, and *Col4a1* in cultured fibroblasts (Ohara *et al.* 2010). Furthermore, hydrolyzed collagen seemed to down-regulate *Col1a1* and *Col4a1*.

Altogether, these results suggest that the 6aa mixture is a more potent inducer of ECM components production, particularly *Eln*, *Fbn1*, and *Col1a1*, than H-Pro-Hyp-OH peptide and hydrolyzed collagen.

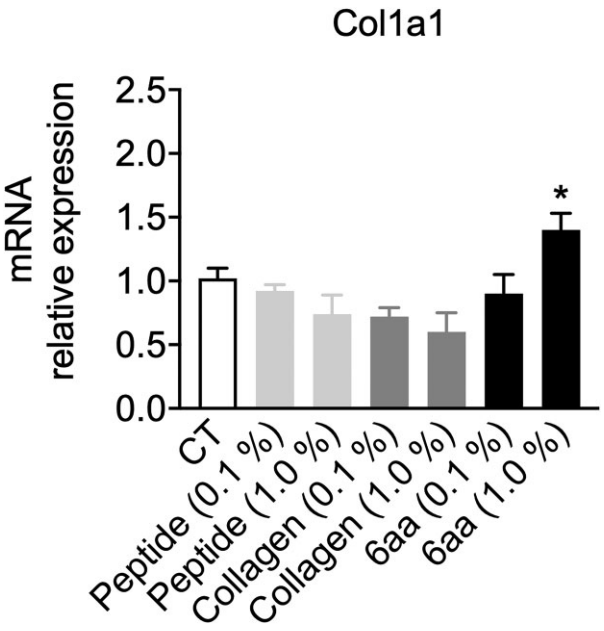

Figure 3. Col1a1 mRNA expression in BJ cells treated with 0.1% or 1% of H-Pro-Hyp-OH peptide or hydrolyzed collagen or 6aa mixture for 72 h. Quantitative PCR, performed in triplicate and normalized to TBP of Eln ($n = 3$, mean ± SD). * $P < .05$ *vs.* untreated cells, expressed as 1.0.

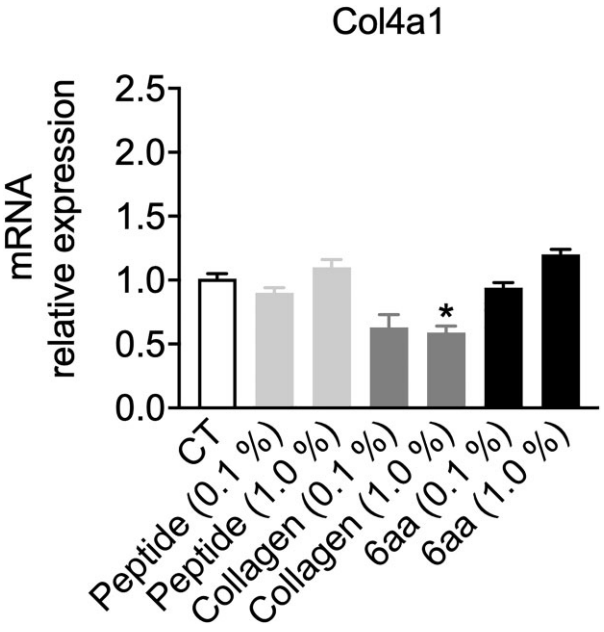

Figure 4. Col4a1 mRNA expression in BJ cells treated with 0.1% or 1% of H-Pro-Hyp-OH peptide or hydrolyzed collagen or 6aa mixture for 72 h. Quantitative PCR, performed in triplicate and normalized to TBP of Eln ($n = 3$, mean ± SD).

Mixture does not impair oxidative stress

To verify the effects of the 6aa mixture on oxidative stress, we investigated the expression of anti-ROS enzymes in BJ cells treated with 0.1% or 1% of H-Pro-Hyp-OH peptide or hydrolyzed collagen or 6aa mixture for 72 h. We observed that the 6aa mixture did not affect superoxide dismutase 1 and 2 (*SOD1* and *SOD2*), *catalase* and glutathione peroxidase (*GPX*) mRNA levels, whereas hydrolyzed collagen increased their levels compared with untreated cells (Figure 5). These findings suggested that the designer 6aa mixture does not interfere with the ROS defense system in cultured human fibroblasts, suggesting that it does not promote oxidative stress.

mTOR signaling pathway seems to be implicated in the effects of 6aa mixture

Since branched-chain AAs are known to activate the mTOR complex 1 (mTORC1) (Avruch *et al.* 2009), to test the possible involvement of the mTOR pathway in the effects of 6aa mixture, we treated BJ fibroblasts with 50 nM mTOR inhibitor rapamycin (Rapa) 30 min before the treatment of 1% 6aa mixture for 48 h. While 1% 6aa mixture increased *Fbn1*, *Eln*, and *Col1a1* mRNA levels, as reported before, the addition of 50 nM Rapa inhibited their expression respectively by 50%, 135%, and 114% compared with 6aa treatment alone. Moreover, the Fbn1 protein levels confirmed this data: the 6aa treatment alone upregulated the Fbn1 protein level by 60%, whereas 6aa plus Rapa downregulated it by 80% compared with untreated cells and by 87.5% compared with 6aa-treated cells (Figure 6). Our results suggested that the 6aa mixture affects the ECM components through the mTOR signaling pathway at least in part.

Discussion

The present work demonstrates that a novel, designer 6aa mixture is more effective than hydrolyzed collagen or H-Pro-Hyp-OH peptide in inducing ECM components, including Eln and Fbn1, beyond collagen 1 and 4. The low molecular weight of hydrolyzed collagen has been demonstrated to be efficiently absorbed and distributed to the dermis to stimulate fibroblasts' proliferation and motility. Moreover, collagen peptides increase collagen fibers and hyaluronic acid production and protect against ultraviolet A (UVA) radiation. In *in vitro* studies, the Pro-Hyp peptide has been found to increase hyaluronan synthase 2 mRNA levels and chemotactic activity for fibroblasts and peripheral blood neutrophils (Postlethwaite, Seyer and Kang 1978). Furthermore, the primary component of the food-derived collagen peptides that stayed in the blood was Pro-Hyp (Ohara *et al.* 2007). Based on these facts it has been speculated that food-derived collagen peptides such as Pro-Hyp might improve wound healing through the stimulation of the fibroblasts migration and other cells to the wounded tissue (Iwai *et al.* 2005). It's to be noted that the millimolar concentrations of collagen peptides needed for *in vitro* chemotactic activity were higher than the micromolar concentrations of food-derived collagen peptides in human peripheral blood.

It is well-known that glycine is a principal constituent of every collagen (the primary ECM protein) (Devlin 2006) and elastin in animals (Chow *et al.* 1989); proline is another critical AA in collagen (Devlin 2006). For this reason, glycine and proline must be supplied sufficiently for all types of collagen synthesis, maximal growth performance, and good health. Thus, in the present study, we compared the effects of hydrolyzed collagen, Pro-Hyp peptide, and a new formula named 6aa, an AA mixture (alanine, leucine, lysine, and valine) added with glycine and proline. The precise stoichiometric ratio among the diverse AAs of this formula adequately promotes the contemporary expression of collagen and elastin proteins (De Servi *et al.* 2018), essential for skin consistency and elasticity. Additionally, the 6aa mixture modulates the ECM protein production slowly and lengthy, avoiding the elastin synthesis peak and break—with toxic oligomers production, generally seen during the acute UVA exposure (Meloni, Farina and de Servi 2010).

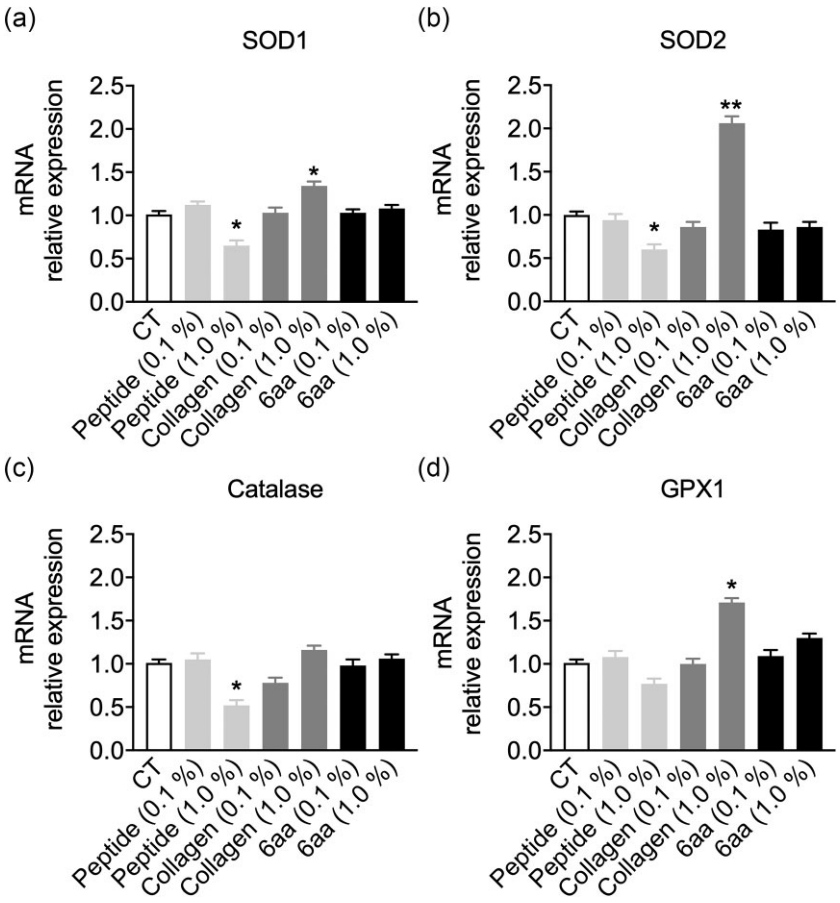

Figure 5. SOD1, SOD2, Catalase and GPX1 mRNA expression in BJ cells treated with 0.1% or 1% of H-Pro-Hyp-OH peptide or hydrolyzed collagen or 6aa mixture for 72 h. Quantitative PCR, performed in triplicate and normalized to TBP of Eln ($n = 3$, mean ± SD).

We observed an upregulation of different components of the ECM by the 6aa mixture. Of note, this effect of the 6aa mixture seems to be mediated through the mTOR signaling pathway.

The 6aa mixture was effective at 1% concentration in the culture medium. Since the culture medium contains many AAs at high concentrations compared with the physiological range, we should discuss the effective concentrations of the single AAs present in our 6aa mixture compared with their physiological range. We focus on two diverse AAs in our formula as paradigmatic examples, the most (glycine) and the less (leucine) concentrated. In particular, our 1% w/v 6aa solution contains ~40 mM concentration of glycine, which is not present in the culture medium we used, and only 0.4 mM in blood—in other words, 100-times physiological levels. When added to the culture medium for cell growth, the glycine concentration is only 0.1 mM; however, when used to investigate its actions *in vitro* or *in vivo*, glycine concentrations are higher. It was shown that 2-10 mM glycine is protective against hypoxic injury of rat hepatocytes (Carini *et al.* 1997; Frank, Rauen and de Groot 2000), 4 mM glycine inhibits Toll-like receptor 4 upregulation after LPS exposure in Kupffer cells (Xu *et al.* 2008), and 3 mM glycine increases the cell viability of isolated cardiomyocytes and isolated rat hearts after ischemia/reperfusion (Ruiz-Meana *et al.* 2004). *In vivo*, 2.2% glycine preserves function and decreases necrosis in skeletal muscle undergoing ischemia and reperfusion injury (Ascher *et al.* 2001); similarly, local perfusion with 20% glycine can diminish warm ischemia-reperfusion damage to the rat small intestine in an *in vivo* model (Lee *et al.* 2002). These reports confirm that high glycine concentrations are effective under multiple experimental conditions and strengthen our findings in human fibroblasts.

Also, the 1% w/v 6aa solution contains ~3 mM concentration for leucine, which is 0.3 mM in the culture medium and 0.15–0.30 mM in blood—in other words, 10-times physiological levels were used. The *in vitro* experiments with leucine are usually reported at 0.5-15 mM concentrations (Fox *et al.* 1998; Areta *et al.* 2014; Mobley *et al.* 2016; Zhang *et al.* 2016), confirming that our experimental conditions are in line with other reports.

Although an exciting advance, the discovery of the elusive intracellular AA sensors and transporters (eg leucine, arginine, and glycine) as regulators of the mTORC1 pathway (Goberdhan, Wilson and Harris 2016; Wolfson and Sabatini 2017) is questioned by the lack of clear understanding of the affinities of these sensors for their respective AAs correlating with concentrations of those AAs in cells or *in vivo*. While it is clear that sensors like sestrin1 and 2, for an example, bind leucine with affinities that are consistent with those in the media (Wolfson *et al.* 2016), it is not entirely known how concentrations of AAs in the media correlate with intracellular, specifically cytosolic, or extracellular matrix AA concentrations. Wolfson and Sabatini (2017) have recently stated that "AA levels within different subcellular compartments, specifically within various organelles, could easily vary with respect to the cytosol, as organelles are enclosed by membranes and contain a distinct set of amino acid transporters compared to those that are on the plasma membrane." In addition, concentrations of AAs may exist in gradients across the in-

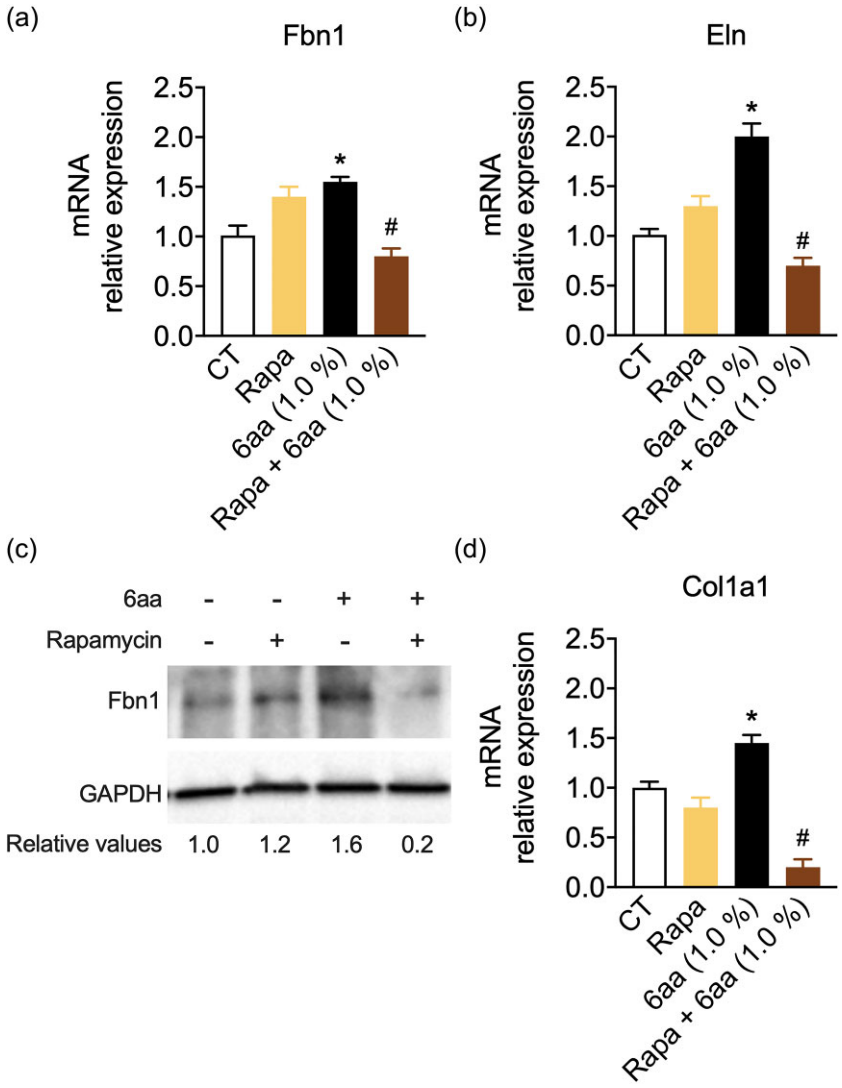

Figure 6. Fbn1, Eln and Col1a1 mRNA levels and Fbn1 protein levels in BJ cells treated with 50 nM Rapa for 30 min and 1% 6aa mixture for 48 h ($n = 3$, mean ± SD).

tracellular and extracellular space, thus further underlining the difficulty to define a concrete "physiological AA concentrations" meaning.

Finally, because high concentrations of certain AAs, such as branched-chain AAs, promote oxidative stress and ROS production (Zhenyukh *et al.* 2017), we analyzed the effects of our formula on these parameters. Of note, we found that the 6aa mixture did not affect the anti-ROS machinery, including SOD1, SOD2, catalase, and GPX1 enzymes, suggesting that it does not interfere with the ROS defense system in cultured human fibroblasts.

In conclusion, our findings suggest that a designer AA mixture may be a practical approach to promoting ECM component production and ameliorating dermatological disorders.

Data availability

The authors confirm that the data supporting the findings of this study are available from the corresponding author on reasonable request.

Author contribution

L.T. and F.R. performed experiments; L.T., C.R., M.R., A.V., and E.N. analyzed data; L.T. and C.R. prepared the figures; L.T., A.V., and E.N. wrote the manuscript with suggestions from all authors, all of whom read and approved the final version.

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Disclosure statement

The authors declare that they have no conflict of interest.

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