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Mimicking key stages of skin aging to propose innovative and targeted solutions

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1. Introduction

Aging is psychosocially and biologically defined as being older. It is a complex and progressive deterioration of physiological functions. Aging biology research focuses on understanding both the molecular and cellular mechanisms that underlie these changes [1]. The quest for longevity aims to delay the aging process to maintain homeostasis and functionalities of the organism. In this context, scientific publications identify and categorize the hallmarks of aging [2, 3]. The skin, as the largest organ of the body at the interface with the environment, is submitted to intrinsic and extrinsic factors inducing its aging. Understanding the cell mechanisms underlying skin aging is essential to develop model mimicking skin aging and demonstrate the action mechanism of active ingredients. Among the hallmarks of aging, altered intercellular communication and cell senescence are very important mechanisms to investigate in the context of skin physiology. Moreover, the family of sirtuins (NAD^+-dependent protein deacylases) is a key actor involved in various biological pathways supporting longevity.

The dermis organization is involved in the firmness of the skin. With age it has been proven that it is altered, thus leading to skin slackening. The epidermis and its properties are not as directly associated with the phenomenon of aging. Indeed, this skin compartment is above all considered as the envelope of the body exposed to external aggression. Characterized by a reduction of its thickness and an alteration of its self-renewal capacity, the aging of the epidermis results in a loss of complexion radiance and a deterioration of cutaneous microrelief. In a tissue as heterogeneous and complex as the skin, dialogue between the different cell types composing it is indispensable for guaranteeing its development and maintaining its biological functions [4–6].The compartments of the dermis and the epidermis cannot be reduced to two separate and autonomous entities. For several years, new studies have been emerging to analyze the influence of the dermis on aging of the epidermis, in particular by studying mechanisms of intercellular communication [7, 8].

In addition, cell senescence is now described as a central factor of tissue aging. Indeed, all cells possess an intrinsic capital of divisions that when reached, causes the onset of cell senescence. This is a state in which the senescent fibroblast can no longer divide but nevertheless remains metabolically active and therefore capable of affecting the fate of the tissue. It involves the secretion of a large quantity of inflammatory molecules clustered under the term of SASP (Senescence-associated secretory phenotype). This SASP alters the functioning of surrounding cells in the dermis and the epidermis, thus leading to progressive skin aging [9, 10]. In young skin, immune cells are attracted by senescent cells and after specific recognition, they eliminate them. During aging, some senescent fibroblasts however install clever strategies dedicated to break communication with immune cells and thereby escaping their fatal destiny. This is called the immunoevasion mechanism. Even though they account for only a very small fraction of senescent cells in the dermis, immunoevasive senescent fibroblasts are by far the most harmful cells for the skin. Since they in fact are no longer recognized by the immune system and so are not eliminated, this elusive population can easily propagate harmful signals in the form of the prolonged secretion of inflammatory molecules composing the SASP [11].
To improve skin longevity, three examples of development will be detailed below (Figure 1).

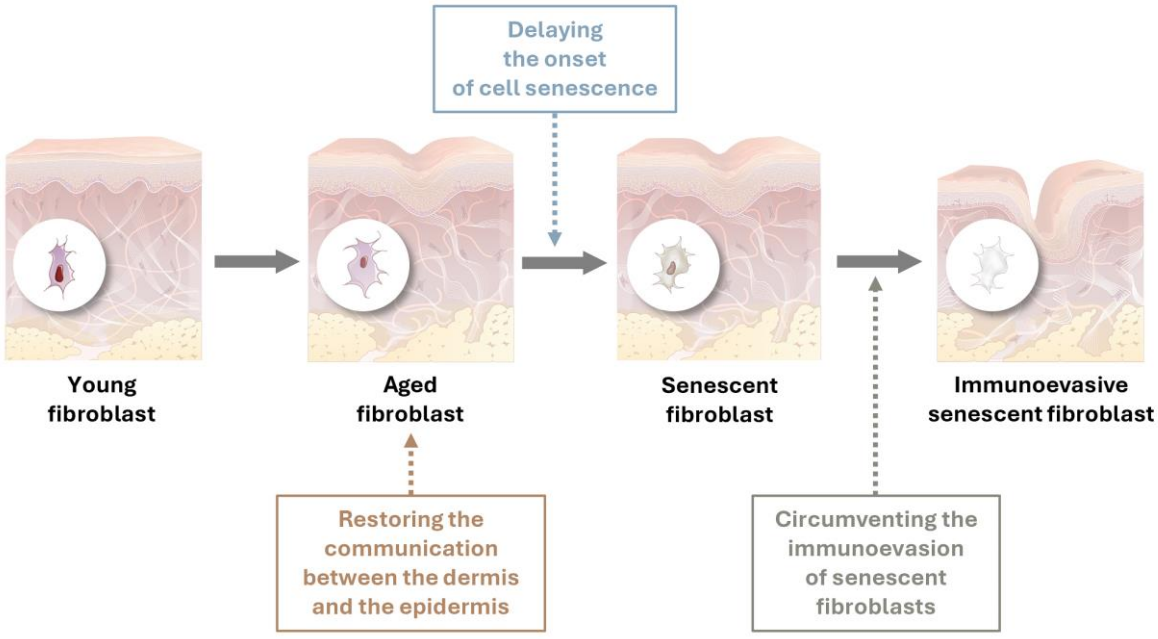

Figure 1. Schematic representation of three strategies aiming to improve skin longevity.

In this context, it was of interest to develop *in vitro* study models to substantiate and demonstrate the mechanism of action of natural active ingredients.

2. Materials and Methods

1. Restoring the communication between the dermis and the epidermis

This model was developed to investigate communication from the dermis to the epidermis during aging and its impacts on epidermal biological functions.

It involved the secretome of human fibroblasts young and aged by successive passages (> P20). Moreover, human keratinocytes from young (< 30 years old) and old donors (> 60 years old) were treated or not with a secretome of aged fibroblasts. The fibroblasts' secretomes were sent to the "Bordeaux Proteome" scientific platform for proteomic analysis. The proteome was determined by LC-MS/MS (nano-LC coupled to an Orbitrap Fusion Lumos mass spectrometer (Thermo-Fisher)) using a quantitative Shotgun label-free approach. Proteins that were modulated between young and aged secretomes were analyzed using databases (Reactome, Wikipathways, etc.) to detect biological pathways of interest. Moreover, extracellular vesicles secreted by fibroblasts were isolated from the secretome using the exoEasy Maxi Kit (Qiagen). They were characterized with a NanoSight LM14 (Malvern). The miRNAs in extracellular vesicles were extracted, reverse-transcripted and the complementary DNAs obtained were analyzed by quantitative PCR. Analysis of Ct (relative quantification) was done with LC480 software (Roche). Finally, the effects of aged fibroblasts' secretome on keratinocytes functionalities were investigated thanks to the synthesis of the proliferation marker Ki-67, determined by immunocytofluorescence and the expression of epidermal markers filaggrin, desmoglein-1, aquaporin-3 and laminin-332 by quantitative PCR.

The efficacy of a natural active ingredient (INCI: *Pichia* ferment lysate extract) was then evaluated on this model.

2. Delaying the onset of cell senescence

A model has been used to investigate the impact of sirtuins, described as strategic targets to improve longevity, on the dermis during aging.

To this end, human fibroblasts were treated or not with a solution of hydrogen peroxide (H_2O_2), a senescence inducer. Then, two sirtuins, described for their capacity to limit glycation and delay the onset of cell senescence, as well as their coactivators (NAD^+ and phosphorylated AMPK) were investigated [17, 18]. Cells were recovered for the luminescence assay of intracellular NAD^+. The levels of phosphorylated AMPK, of SIRT1 and SIRT7 were quantified by capillary Western blot. The ratio of the quantity of AMPK-P over the total quantity of AMPK was calculated and is expressed as arbitrary units (AU). A cell staining kit was used to visualize SA-β-galactosidase activity, a senescence marker. DAPI labeling was used to obtain cell counts and the ratio of the number of positive cells showing β-galactosidase staining near the nucleus. The results are expressed as percentage of SA-β-galactosidase-positive cells.

The efficacy of a natural active ingredient (INCI: *Myrtus communis* leaf extract) was then evaluated on this model.

3. Circumventing the immunoevasion process

A novel *in vitro* model of immunoevasive senescent fibroblasts was developed to assess their impact on the skin. Senescent fibroblasts were obtained by subjecting normal human fibroblasts to successive replications (P30). The immunoevasive phenotype was then activated by the application of repeated stress to this senescent model. The SASP composition was determined by the study of the secretions of MMP-1, MMP-3 and IL-6 by ELISA assay. The immunoevasion mechanism was assessed by analyzing the cleavage of the immunogenic “eat me” signal MICA by ELISA assay and the lysis of immunoevasive senescent fibroblasts by Natural Killer (NK) cells by a morphological analysis using a fluorescent probe (Figure 4a).

The efficacy of a natural active ingredient (INCI: *Ginkgo biloba* leaf extract) was then evaluated on this model.

3. Results

1. Restoring the communication between the dermis and the epidermis

The proteomic analysis of the secretome of young fibroblasts showed that almost 40% of secreted proteins are related to intercellular communication function. The investigation of these proteins revealed four major biological pathways involved: IGF-1 pathway regulation, inflammation, growth factors and cell interactions. Compared to young fibroblasts, the secretome of aged fibroblasts is characterized by a modification of the synthesis of more than half the proteins involved in intercellular communication (Figure 2A), translating a general degradation of these mechanisms. Tested at 0.4% on old fibroblasts, the *Pichia* ferment lysate extract regulates the synthesis of proteins involved in different dermis-epidermis communication pathways (Figure 2a).

In addition, compared to young fibroblasts, the secretome of aged fibroblasts is characterized by a significant increase in the expression of 9 miRNAs, epigenetic actors transported by extracellular vesicles (among them exosomes) and described in publications as being involved in regulation of epidermal physiology [12–16]. Tested at 0.4% in aged fibroblasts, the *Pichia* ferment lysate extract significantly limits the expression of miRNAs that are involved in aging of the epidermis (Figure 2a).

Finally, it was proven that the secretome of aged fibroblasts has a significant and negative effect on the biological pathways of old human keratinocytes. Applied to old human keratinocytes, the secretome of aged human fibroblasts treated with this natural active ingredient at 0.4% significantly improves the synthesis or the expression of Ki-67 by 21%, filaggrin by 41%, desmoglein-1 by 25%, aquaporin-3 by 36% and laminin-332 by 93% (Figure 2c).

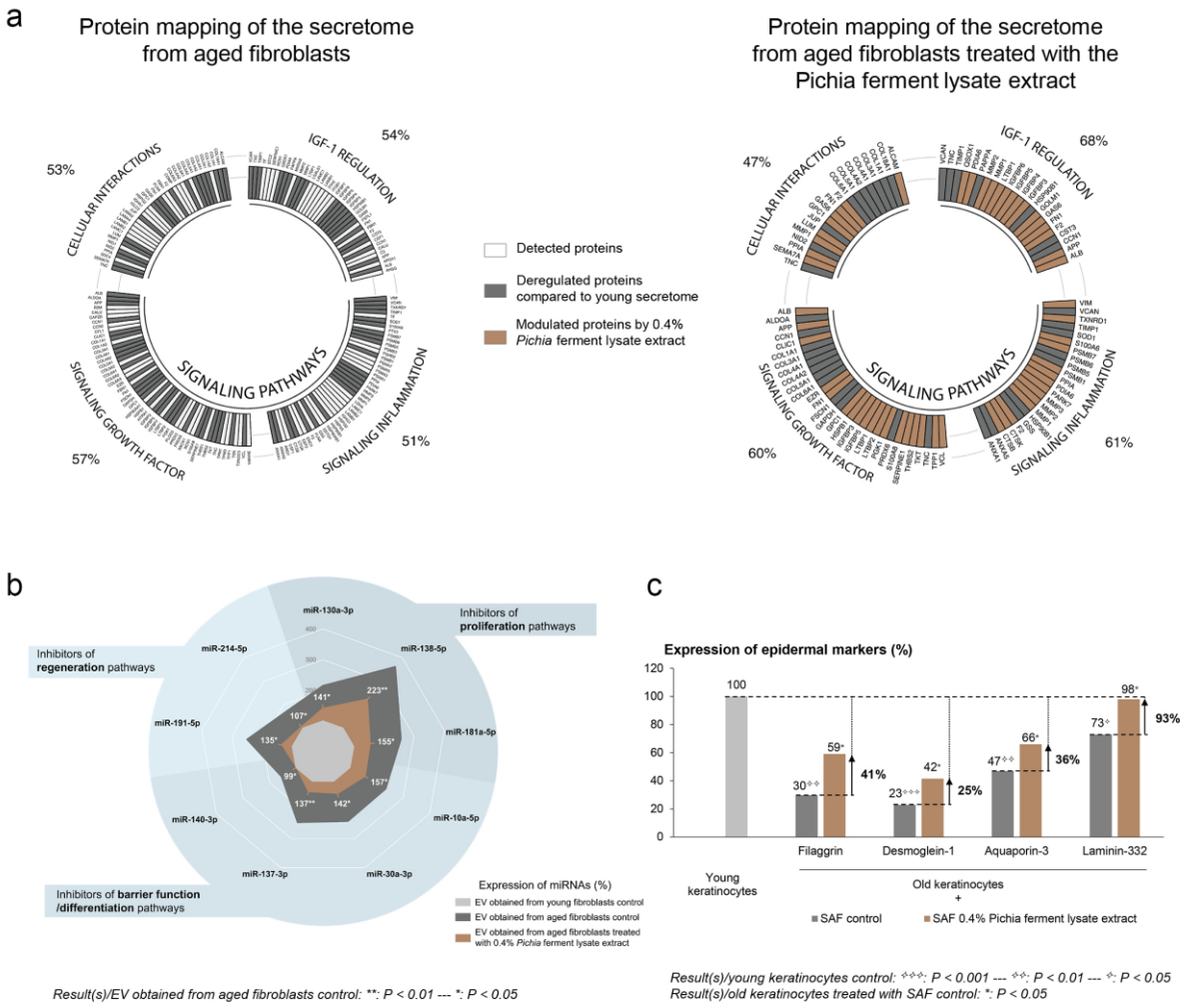

Figure 2. Investigation of communication from the dermis to the epidermis. (a) Proteomic investigation of secretomes from aged fibroblasts treated or not with the *Pichia* ferment lysate extract. (b) Analysis of the expression of miRNAs isolated from EVs from young and aged fibroblasts, treated or not with the active ingredient. (c) Study of the effect of the secretome of aged fibroblasts (SAF) treated or not with the active ingredient on the expression of epidermal biological markers by old keratinocytes.

Hence, the *Pichia* ferment lysate extract restores communication from the dermis to the epidermis to compensate the communication shortfall appearing in the course of aging, thus favoring epidermal functions. These effects are seen *in vivo* in a mature Caucasian panel by an improved renewal and thickness of the epidermis. After 28 days of twice daily use at 1.7%, microrelief is smoother and the skin's hydration and radiance are restored.

2. Delaying the onset of cell senescence

The syntheses of SIRT1 and SIRT7 are significantly reduced with aging. Tested at 0.10% on fibroblasts subjected to an H_2O_2 stress, the *Myrtus communis* leaf extract significantly increases the phosphorylation of AMPK and the production of NAD^+ by 42% and 35%, respectively (Figure 3a). It also significantly restores the syntheses of SIRT1 and SIRT7 by 95% and 115%, respectively (Figure 3b).

In response to senescence-inducing stress, the number of SA-β-galactosidase-positive cells increases significantly. When the *Myrtus communis* leaf extract at 0.10% is applied at the same time as the senescence-inducing stress, it significantly limits the number of SA-β-galactosidase-positive cells by 50%, revealing a delay of the onset of cell senescence (Figure 3c). Moreover, by limiting glycation, this natural active ingredient preserves the networks of collagen I by 39% and of elastin by 94% (data not shown). After 14 days of treatment *in vivo*, these effects are shown by a firming effect of the active ingredient tested at 1% in a body care formula. After 28 days of use at 2% in a facial care formula by Caucasian and Asian volunteers, it attenuates wrinkles and significantly improves the quality of the matrix and of the biomechanical properties of the skin.

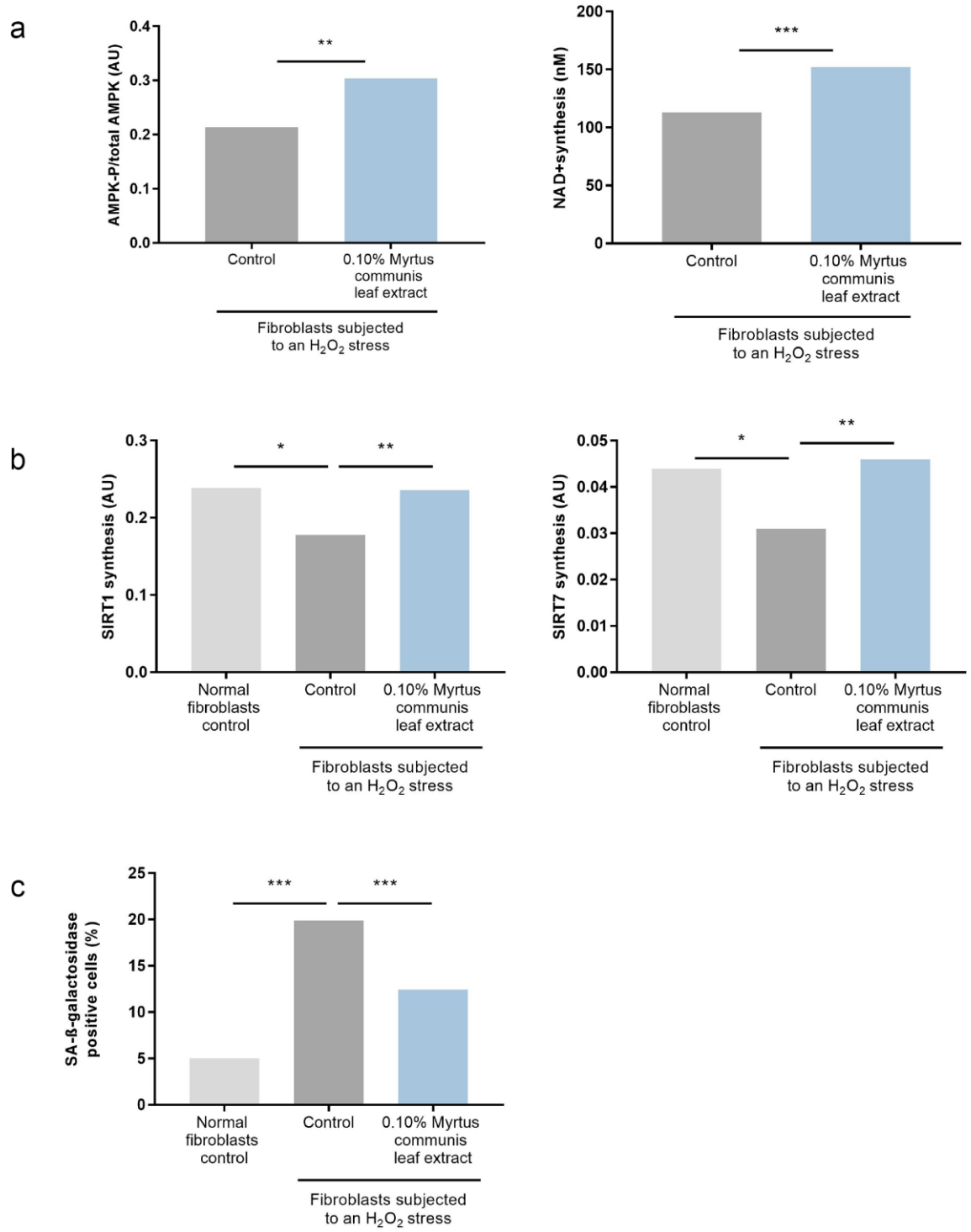

Figure 3. Study of the onset of cell senescence. (a) Study of the phosphorylation of AMPK and synthesis of NAD^+ by fibroblasts subjected to an H_2O_2 stress and treated or not with the *Myrtus communis* leaf extract. (b) Analysis of the SIRT1 and SIRT7 syntheses by fibroblasts subjected to an H_2O_2 stress and treated or not with the active ingredient. (c) Study of the SA-β-galactosidase activity by fibroblasts subjected to an H_2O_2 stress and treated or not with the active ingredient. Statistical analyses with: ***: $P < 0.001$ --- **: $P < 0.01$ --- *: $P < 0.05$.

3. Circumventing the immunoevasion process

Compared to senescent fibroblasts, immunoevasive senescent fibroblasts secrete significantly more MMP-1, MMP-3, and IL-6 (data not shown). Tested at 0.5% on immunoevasive senescent fibroblasts, the *Ginkgo biloba* leaf extract significantly limits the secretion of deregulated proteins of the SASP by 46%, 28% and 87%, respectively (Figure 4b). In addition, the quantity of MICA cleaved in immunoevasive senescent fibroblasts is significantly higher than that in senescent fibroblasts. Tested at 0.5% on immunoevasive senescent fibroblasts, the active ingredient significantly limits the release of MICA by 31% (Figure 4c). It therefore enables senescent cells to retain their ligand for recognition by the immune system. Compared to senescent fibroblasts, the lysis of immunoevasive senescent fibroblasts by NK cells is significantly lower. Tested at 0.5% on immunoevasive senescent fibroblasts, the natural active ingredient significantly restores their lysis by NK cells by 101% (Figure 4d). It therefore re-establishes the elimination of these resistant senescent cells by NK cells. Applied to old human fibroblasts, the secretome of immunoevasive senescent fibroblasts pretreated with the *Ginkgo biloba* leaf extract at 0.5% significantly preserves the collagen I network synthesis by 64%. The *Ginkgo biloba* leaf extract therefore preserves the matrix environment by regulating the SASP composition secreted by immunoevasive senescent fibroblasts. After 28 days of treatment in mature Caucasian skin, this natural active ingredient improves the density of the matrix and elasticity, attenuates wrinkles and enhances the complexion radiance.

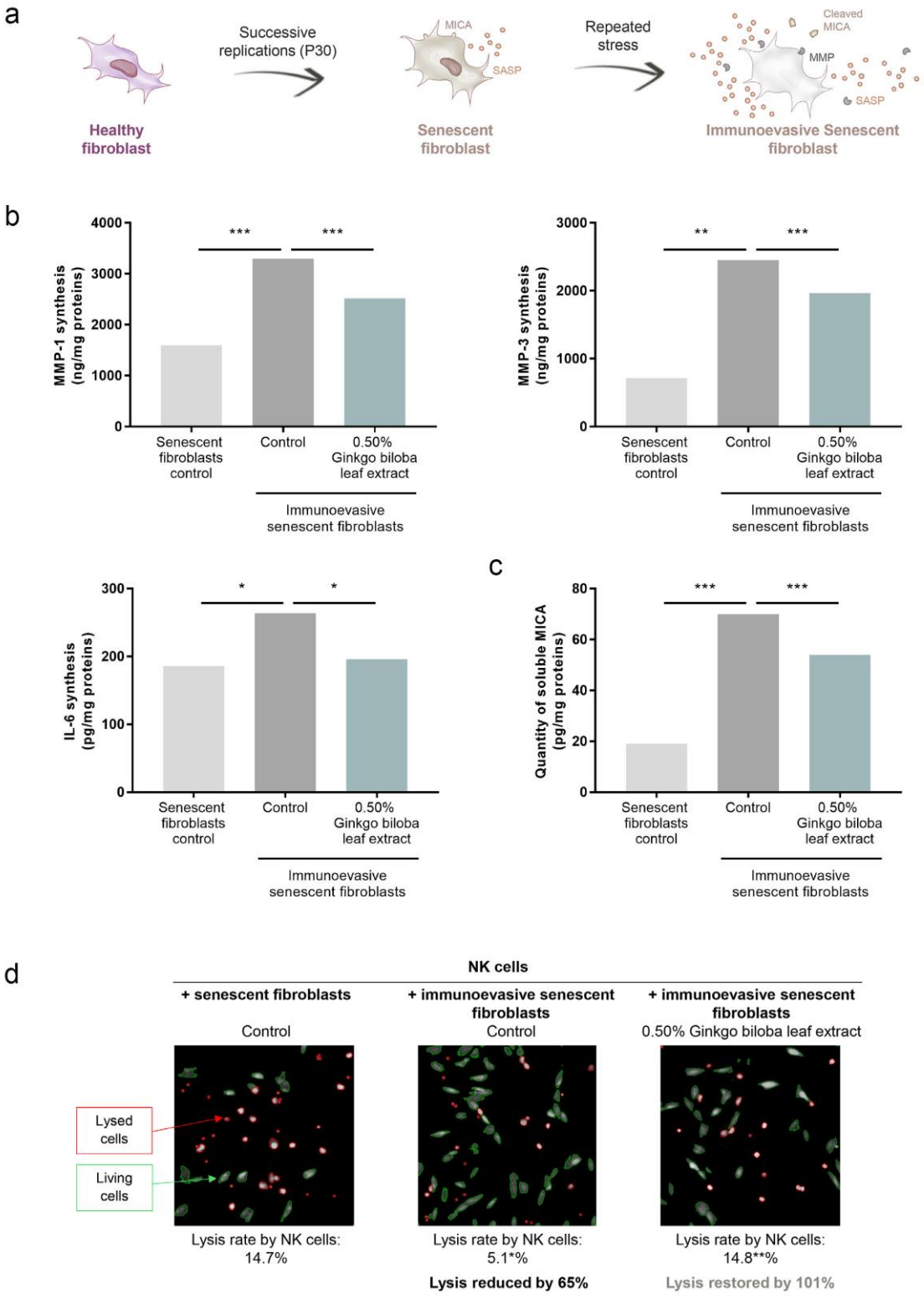

Figure 4. Study of the immunoevasion of senescent fibroblasts. (a) Schematic representation of the development of an immunoevasive senescent fibroblast model. (b) Analysis of MMP-1, MMP-3 and IL-6 syntheses. (c) Study of MICA release. (d) Study of the lysis of immunoevasive senescent fibroblasts. Statistical analyses with: ***: $P < 0.001$ --- **: $P < 0.01$ --- *: $P < 0.05$

4. Discussion

Thanks to its 40 years of expertise in cutaneous biology and in the development of natural active ingredients, SILAB is able to develop *in vitro* models mimicking skin aging. These ones are used to substantiate the efficacy of natural active ingredients targeting the hallmarks of aging. Hence, restoring the communication between the dermis and the epidermis with cutaneous exosomes modulation, delaying the onset of cell senescence and reactivating the immune system's natural elimination of senescent fibroblasts are three innovative strategies to improve skin longevity.

5. Conclusion

There are many ways to approach longevity in the field of cosmetics, among them the hallmarks of aging, aiming to define the biological pathways of longevity. Consumers pay attention to the long-term health of their skin and so are increasingly attracted by preventive approaches at the same time as realizing that aging is neither a disease nor an abnormal phenomenon.
Science is constantly advancing and so enables this scope of action to be continually broadened. SILAB's expertise in skin biology has enabled it to demonstrate the role of certain pathways and key markers, belonging to the hallmarks of aging, and involved in the process of biological aging. Natural active ingredients have been developed and are capable of acting on these essential players to sustain longevity of the skin.

6. References

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