Isolated Botanical Polyphenol Actin-Binding Compounds Impact Human Dermal Fibroblast Morphology: Potential Anti-Aging Effects & Insight Into YAP/TAZ Mechanical Cytoskeletal Modulation

Marina Elia

University of Michigan

April 23rd, 2024

This thesis has been read and approved by Dr. Gary Fisher.

Signed:  Date: April 23rd, 2024

Faculty Advisor Email: gjfisher@med.umich.edu Phone: (734) 585-6415

Table of Contents

Abstract……..……...……..2

Introduction……...……..3

I. Purpose…….3

II. The Actin Project..……...…6

III. Compounds of Interest……...……13

Materials and Methods…….…….……...…21

I. Sourcing & Management of Human Dermal Fibroblast Cultures……...……..21

II. Phalloidin Actin/YAP Staining & Visualization Techniques……...……..22

III. Rigidity Plate Preparation & Management……23

IV. Treatment of HDFs with Compounds of Interest……...……...24

V. Final Image Measurements & Analysis……25

Results…...……...……...…..27

I. Effects of CytoSoft Stiffnesses & ECM Substrates on HDF Morphology……28

II. Compounds of Interest Impact HDF Phenotype……34

III. Actin Cytoskeleton Organization & YAP/TAZ Localization…….……...38

Discussion……...……..41

Conclusions……...……...……44

Acknowledgments…….……..46

References……...……47

Abstract

Aging happens to everyone. Coupled with a multi-billion dollar industry, it's no secret that aging is something that each and every one of us wishes to delay or avoid entirely. The skin is the largest human organ. The extracellular matrix (ECM), which is localized below the outer surface of the skin, provides strength and resiliency and is a major determinant of skin health. The ECM is produced by cells called fibroblasts. Fibroblasts attach to the ECM and this attachment allows fibroblasts to stretch. During aging, structural proteins that make up the ECM, such as type 1 collagen and fibronectin, are degraded through various cell signaling mechanisms. This degradation alters the mechanical properties of the ECM and impairs fibroblast attachment. With loss of attachment, fibroblasts become smaller (collapse) and acquire an aged phenotype. These processes lead to the most common markers of aging, including wrinkles, laxity, and carcinomas. The research described in this thesis focuses on how ECM mechanical properties (i.e. stiffness) and treatment with botanical polyphenol compounds impact fibroblast morphology. The data demonstrate that some of the compounds alter fibroblast morphology through modulation of actin polymerization. Additional data are presented regarding the impact of the botanical compounds on fibroblast function using the mechano-sensitive transcriptional coactivator Yes-associated protein (YAP) as a marker.

Introduction

I. Purpose

Aging is a universal experience for all, no matter the amount of effort applied to circumvent it. Research appears to be expanding on the topic, yet a firm conclusion on the true remedy for aging, to either prevent or reverse it, has not been announced within the scientific community. Yet, aging has become a strong social construct that negatively impacts body image and overall mental well-being, especially in Western cultures where youth is valued above all else; it's as if there actually is an all-encompassing solution to aging that the rest of the world looks down on you for not taking advantage of. With chronological age comes chronological signs of aging, something most of us act extremely fearful of. This is blatantly apparent in our current choices of skin-care products, their ingredients, and the emergence of increasingly more extreme measures people take to retain a Goffmanianly managed impression.[1] A solution to the multifaceted and increasingly socially polarizing topic of aging would require an equally diverse approach consisting of not three, not four, but maybe even hundreds of components intricately balanced with one another to produce the desired pharmacological effect of so-called "anti-aging".

Despite the lack of fully "science-backed" products, the global market is dramatically overflowing with an endless stream of those advertised to the general public to be exactly that, "anti-aging". I use "*dramatically*" here to refer to the projected 93 billion dollar valuation the anti-aging market is estimated to hold in 2027.[2] We send our bare skin cells out to the battlefield day after day only to try and salvage the outermost layer with acids, creams, and emulsions containing ingredients and fragrances of all chemical kinds. In fact, a new survey conducted by

the Centers for Disease Control and Prevention (CDC) reveals that only about 30% of women and 15% of men use sunscreen regularly.[3]

Wrinkles, then, for the average person, are almost inevitable. This could possibly be either by default of what's written in the code of what we call "human nature" or due to a severe lack of public exposure to research articles that discuss such problems or caution against using products with certain ingredients that cause poor downstream side effects. Beyond prevailing issues of a widespread scarcity of sunscreen use, the CDC has also found significant data linking chronic low-level exposure to phthalates, a class of developmental and reproductive toxins and a major component of plastics, to several widely used skin-care cosmetics.[4] Interestingly enough, most products included in the list of cosmetics-to-be-wary-of are largely endorsed by celebrities, if not in direct collaboration with them.[5] But, these are separate stories on the ethics of open-access publishing and proper package labeling—both of which would allow for individuals to make more well-informed skin-care decisions—that are probably best saved for another time. It is too often that people succumb to the pressures of carefully tailored advertising, leading most to a negative-sum game of using overpriced products in all the wrong combinations for their skin.

It logically follows that the skincare industry would want to concoct a product that reverses aging rather than just preventing aging. This opens their market up to a much larger target audience and thus to more potentially empty-promise money. Saving those who have already aged as well as those who have yet to age is a much heftier bet than simply leaving behind those who have missed the supposed new youthful era. Perhaps, many already know the solution to delay aging: a healthy diet, frequent light exercise, and a calm and balanced mind. How about a routine that costs upwards of 2 million dollars per year? This is exactly what Bryan

Johnson, 46-year-old American billionaire and tech mogul, claims to have done for himself through what he calls "*Project Blueprint*".[6] His overarching goal is to closely measure biological DNA markers and improve the functioning of all of his organs via 100-plus daily supplements, exercise, heavily restricted caloric intake from strictly vegan sources, and dystopian-like procedures (i.e. blood transfusions from his own 17-year-old son). With a particular interest in the skin, Johnson has stated that he has biologically reversed to that of a 28-year-old, almost half of his true age. This routine certainly takes a strong will and mind, with the former overpowering the latter for most. Still, don't fret! Tightly controlled studies have found that dietary supplements have little to no positive effects on overall health or reversal of skin aging compared to placebos.[7] The notion that the body's biological clock can be reversed with pharmaceutical intervention has led to a sense of laziness when it comes to taking care of oneself. Medicalization of every natural symptom stresses reversal over prevention. This is particularly the case with aging since it is inevitable.

Why, then, study the mechanisms of aging? Within the laws of entropy, there lies a relevant point: systems naturally tend to progress towards states of higher entropy (or disorder) with time. Low entropy systems are associated with states of youth. The skin inexorably becomes progressively more disordered, wrinkled, and the underlying dermis environment more jumbled with age. However, it does not cost 2 million dollars to keep our skin in good health well into our old age. But then, is this only applicable to occupants of societies who have long been suffering from poor nutrition? There is notable evidence of elderly Asian populations exhibiting remarkably lower amounts of aging markers in the skin compared to samples from non-Asians, presumably due to better nutrition.[8] Asian diets are generally abundant in berries, beans, cruciferous and fermented vegetables, whole grains, and seafood, and are easily

characterized by a generous usage of a diverse range of herbals and spices.[9] Those living in countries with provisions embracing these elements consume larger concentrations of compounds called polyphenols and isoflavones, without necessarily being aware of the fact. These compounds have been found to have anti-aging and anti-cancer properties, though human studies are still lacking to make stronger assertions.

Many studies investigating the effects of plant extracts on cancer, for example, use cultured cells. Scientists add certain amounts of plant extract onto cancerous cells seeded into culture dishes. Then, they incubate and watch to see if the cancerous tumor cells commit programmed suicide, or apoptosis.[10] Results from these types of studies and nutritional studies conducted in rodents or humans suggest that compounds that are present in plants, or botanicals, may provide health benefits. Alternatively, observational studies may reveal that certain foods in the diet are associated with healthier skin with age. Now, we test the compounds contained within these foods—with particular interest in anti-aging potential—*in vitro* to determine whether these effects can be isolated to then be effectively added into novel cosmetics. My hope is to switch course from the bleak future that mindless consumption of empty-promise (and potentially toxic) products holds for our general population.

II. The Actin Project

Actin filaments are a key component of the cytoskeleton in eukaryotic cells that mediate cell shape, movement, cell division, signal transduction, and intracellular transport. They begin as actin monomers called G-actin, nucleating then polymerizing over time with the help of ATP hydrolysis to form a twisted, double-stranded, polarized helix structure termed F-actin. This structure is not permanent, however. The actin cytoskeleton is a fluid dynamic system, a

sensitive machinery that is responsive to all kinds of mechanical triggers from the environment when in complex with myosin. The polymerization, subsequent depolymerization, and re-polymerization that must happen in order for movement to occur are exothermic processes with branching being facilitated by the Arp2/3 complex, a 7-protein actin-binding assembly.[11]

As a major component of human skin fibroblast cells, actin directly modifies much of the overall phenotype of the skin. Actin filaments congregate close to the plasma membrane and, when healthy, can be seen both as straight, long, parallel stretches and as small patches along a cell's elongation axis, clueing us back into their main role of cell morphology determination.[12] This proximity to the plasma membrane aids in their easy (and beautiful) recognition when fibroblast cells are stained and visualized under a microscope (Fig. 1).

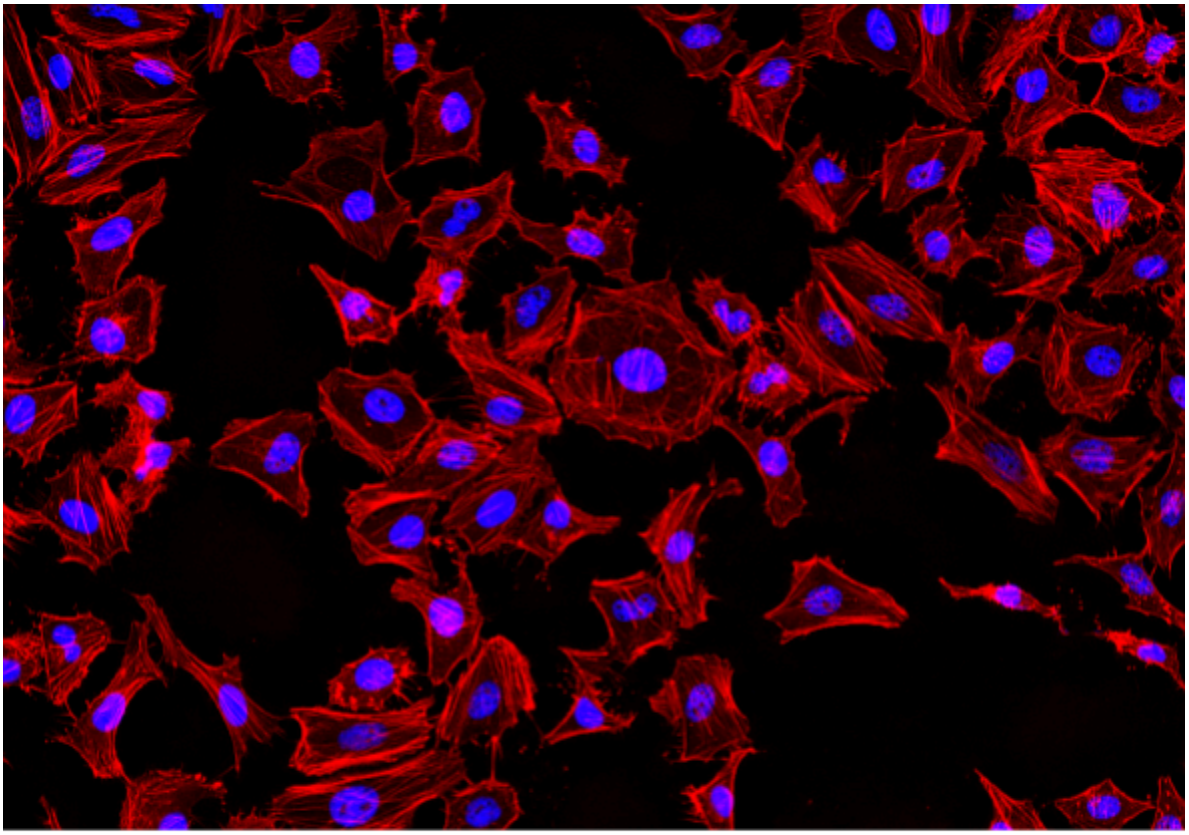

Figure 1. Human dermal fibroblasts. Actin filaments are stained with phalloidin, a red fluorescent marker. Blue dots indicate nuclear staining. Adopted from AAT Bioquest.[13]

Fibroblasts are predominantly responsible for synthesizing the extracellular matrix (ECM) that comprises the bulk of the skin dermis (Fig. 2).

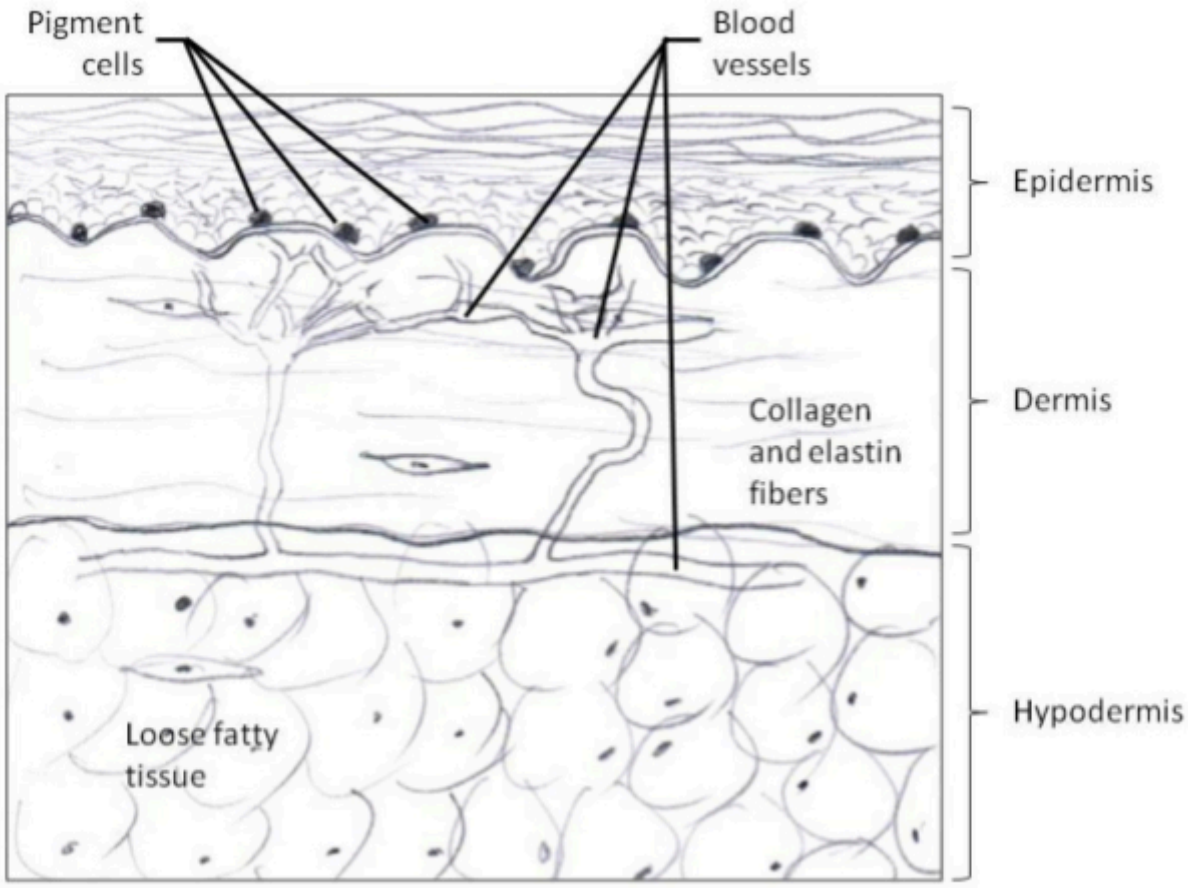

Figure 2. Illustration of the organized layers of the skin dermis.[14]

The ECM is a large network of proteins that surrounds cells in the dermis, providing structural and functional support through regulating cell-matrix interactions. Further, the dermal ECM is able to impact fibroblast cell migration, gene expression, and morphology.[15] The ECM is made up of proteoglycans, glycosaminoglycans, collagens, fibrin, fibronectin, fibrillin, elastin, and vitronectin. Collagen is the most abundant structural protein in the dermis. In addition to providing structural/mechanical support the dermal ECM acts as a vital bioactive molecule

stockpile.[16] A concerto of balance exists within the ECM as its components have complementary charges and consequent secretions that moderate the upkeep of the skin.

Unfortunately, our environment is sometimes hostile and as time passes, enough harmful stimuli have damaged the ECM that visible signs of age begin to appear on the outermost surface of the skin in the form of wrinkles, sagging, hyperpigmentation, age spots, dryness/roughness, and, in some cases, carcinomas (photocarcinogenesis).[17] The skin is not only a protective barrier, but also maintains internal body temperature and hydration conditions, relays essential sensory information, and monitors the immune system via the release of cytokines. As extrinsic (and intrinsic) factors like ultraviolet rays (UV) and air pollution harm the skin continuously over time, the accumulated damage attenuates these functions, causing a more rapid rate of aging in the process. It is known that our sun emits harmful UV rays that induce oxidative stress, and thus "photo-aging" occurs. Exposed methionine and cysteine residues make up actin filaments, at the primary level, and are particularly susceptible to oxidation by reactive oxygen species (ROS) such as singlet oxygen, superoxide anions, hydrogen peroxide, and hydroxyl radicals, that form as a result of UV-induced oxidative stress.[18] With the use of sunscreen, these ROS are kept at a balanced physiological range and are quenched by antioxidant molecules and superoxide dismutase (SOD), protecting the skin.[19]

When the skin is not protected, however, UV exposure generates high levels of ROS that overwhelm the cells' natural quenching mechanisms (depleting crucial stores of Vitamins E and C) and activate mitogen-activated protein kinases (MAPKs). This family of enzymes works together to activate a transcription factor called AP-1.[20] AP-1 and NF-κB (another transcription factor important in the expression of cytokines, cell adhesion molecules, and growth factors) then lead to upregulated expression of matrix metalloproteinases (MMPs) which are

endopeptidases, enzymes capable of degrading the ECM proteins.[20] There are various classifications of MMPs based on their structures and substrates, though what most have in common is the connection to certain skin-catabolizing proteases—either being small proteases in and of themselves or recruiting, or activating, those nearby—that ultimately alter, the basal lamina, or the extracellular surface of the skin.[21] Botanical polyphenols like EGCG's ((–)-epigallocatechin-3-gallat, containing epicatechin) have been previously found to specifically downregulate MMPs in the skin, presenting hopeful mechanistic explanations for EGCG's observed anti-aging properties.[22]

+1 on superscripts, insert as new 20

Additionally, YAP and TAZ are transcriptional coactivators of the Hippo signaling pathway that are found localized in the nuclei of fibroblasts when in stiff environments, regulated by filamentous actin tension, structure, and resistance. When the ECM is degraded, fibroblasts do not adhere well and thus YAP/TAZ will be absent from the nuclei.[23] The list of characters involved in photoaging continues to grow longer still, now adding in the effects imparted by the TGF-β pathway. This cell-surface receptor, second messenger signaling pathway regulates the production of ECM proteins by human dermal fibroblasts through activation of even more downstream transcription factors, Smad2-4, that directly affect their gene expression, reorganizing the actin cytoskeleton.[24]

Altogether, aging and UV exposure generate ROS that cause overexpression of MMPs, thus degrading the proteins making up the structural scaffolding of the skin. This impairs the attachment of fibroblasts to the dermal ECM, thereby reducing actin polymerization, which then decreases fibroblast size/length and mechanotransduction. (Fig. 3). Thankfully, this information points us to a starting direction when considering pharmaceutical targets of aging: actin. The

processes aforementioned are reversible and, with the right actin-binding compounds, we may be able to stretch the fibroblasts and thereby rebuild the ECM and reduce signs of aging.

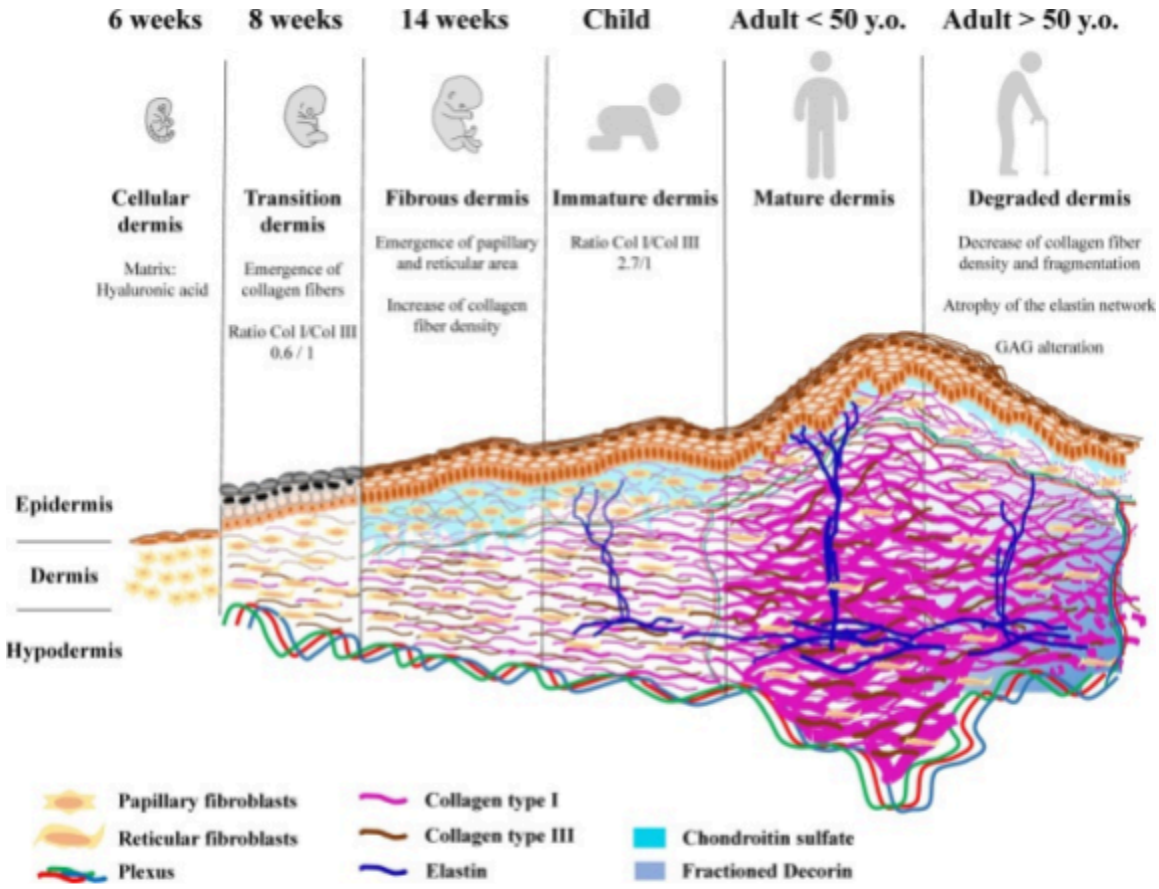

Figure 3. Schematic of the development of the dermis with age. Collagen and elastin fiber bundles become thinner, fibroblasts become rounder and sparser with deformed actin filaments leading to outward signs of aging.[25]

As mentioned in *I. Purpose*, there are many naturally occurring compounds that are of interest to the scientific community when it comes to the skin and the most notable are plant-derived polyphenols, which come in the forms of flavones, flavanones, flavanols, flavonols, and isoflavones. They harness anti-inflammatory, anti-oxidant, and DNA repair powers that can be photoprotective and potentially counter signs of aging through modulation of enzymatic activity (more specifically, inhibition of oxidizing enzymes), cell cycle progression, and cell signaling.[26] The specific mechanisms of action of polyphenols in the skin are largely

unknown but there is significant evidence of their anti-tumoral, anti-aging, and anti-inflammatory effects in the literature (Fig. 4).[27]

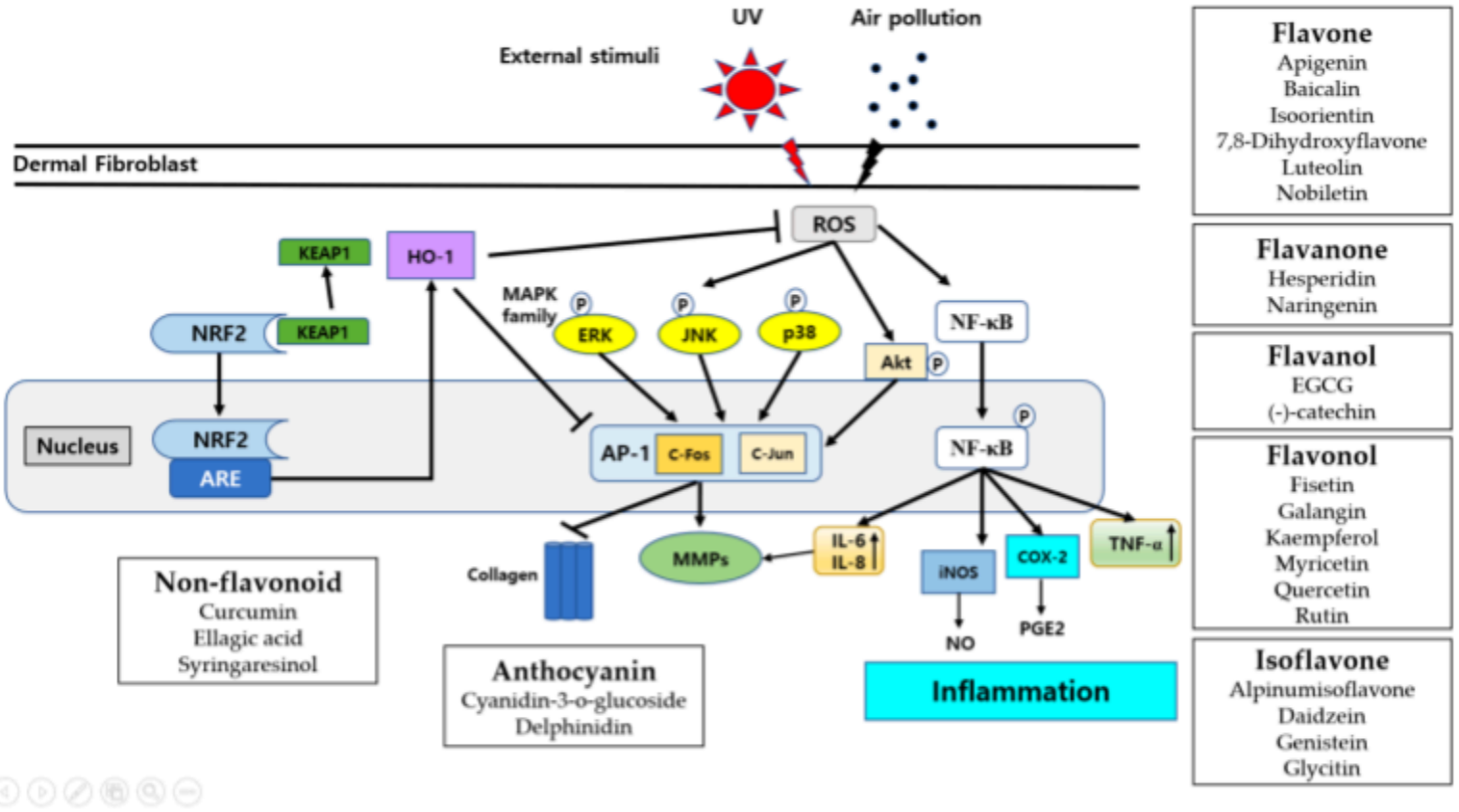

Figure 4. Diagram depicting numerous pathways cited to be involved in polyphenol mediated anti-aging. Notably, UV-induced ROS causes MAPK family to form AP-1 and upregulate MMPs that degrade collagen in the ECM, NF-kB upregulation leading to inflammation, and common polyphenols (flavones, flavanones, flavanols, isoflavones) that potentially protect dermal fibroblasts from these processes.[30]

Polyphenolic compounds have been found to directly increase estrogen production through binding to estrogen receptors α (ERα) and β (ERβ) and may have an anti-aging effect in this regard as estrogen deficiencies can also lead to accelerated aging.[28] Around age 60, our immune systems experience substantial alterations and progressively lose the ability to protect against cancer and infections and mediate proper wound healing.[29] It intuitively follows that

immune system regulating compounds such as the polyphenols commonly found in healthy diets would suppress the aging-related deconstruction of the ECM. In the next section, I cover each of the botanical polyphenol compounds we found to be of greatest interest for further study and isolated characterization in individual detail.

III. Compounds of Interest

In alphabetical order…..

Allantoin:

Allantoin's history in the biochemistry field is seen as being a molecule first identified as the product of uric acid oxidation found in animal (and human) urine, getting its name from its discovery in the allantoic fluid of cows.[31] As research expanded into investigating oxidative stress in the body, allantoin resurfaced in popularity. It neutralizes hydrogen peroxide to reduce its mutagenic effects and conveniently serves as a sensitive marker of oxidative stress.[31] Allantoin is naturally found in some plants including comfrey, aloe vera, chamomile, wheat germ, and black salsify (a root vegetable) with $C_4H_6N_4O_3$ being its chemical formula.[32] Allantoin can be purchased as a component in several types of plant extracts but has since been artificially synthesized in the lab (Fig. 5). It is most widely known for its role in promoting quicker wound healing times with a lower risk of bacterial infection.[33]

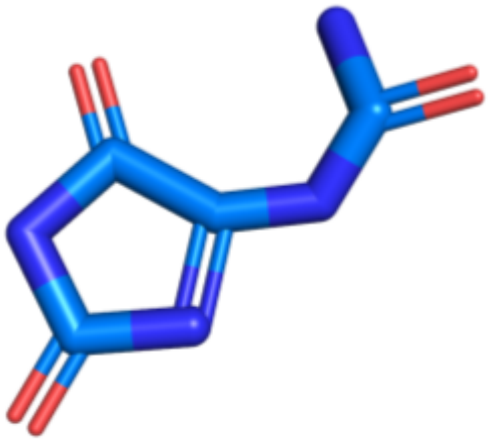

Figure 5. PyMOL generated chemical structure of allantoin. Extracted from PDB 2FXL.[34]

Apigenin:

The top naturally-occurring sources of apigenin (with chemical formula $C_{15}H_{10}O_5$) are parsley, celery, chamomile, artichokes, oregano, and spinach with concentrations increasing as the sources are dried down (Fig. 6).[35]Apigenin is in the flavone class of polyphenols and has extensive credentials in the anti-cancer, antioxidant, and anti-inflammatory scientific communities. In previous mouse model studies, apigenin was shown to downregulate AP-1, MAPK, and NF-κB pathways, induce synthesis of mouse skin barrier factors, and increase the hydration level of the skin's outermost layer, the stratum corneum.[36] In a study more closely related to human dermal fibroblasts, apigenin directly promoted type-I and type-III collagen production in the dermis through activation of the Smad2/3 signaling pathway, thus maintaining the texture and appearance of the skin.[37] This would be especially applicable if normal fibroblast functioning of synthesizing collagen for the ECM was impaired by excess UV-incited damage. Its implications in anti-aging research are most promising.

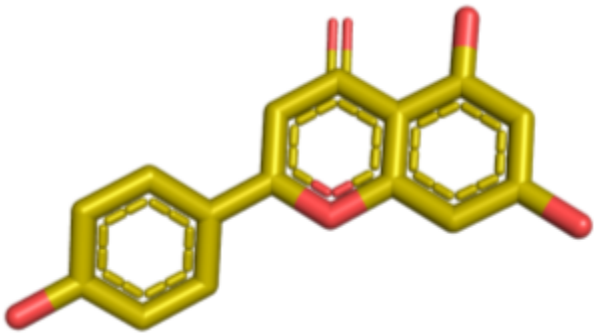

Figure 6. PyMOL generated chemical structure of apigenin. Extracted from PDB 3CF9.[38]

Catechin:

(+)-catechin is a stereospecific notation for "catechin", as I will later show the distinction with epicatechin. This compound is in the flavanol class of polyphenols that are commonly found in green tea, red wine, cocoa extract, and fruits like apricots, black grapes, and

strawberries.[38] A previous study has shown that when UV-irradiated human dermal fibroblasts are treated with (R)-DHPV (or (+)-catechin), MMP-1 levels are slightly decreased compared to no treatment at all (Fig. 7).[39] MMP-1 expression levels were used as a marker for collagen degradation and thus also of dermis collapse. (+)-catechin has also been found to protect fibroblasts from oxidative stress-induced apoptosis, or cell death.[40]

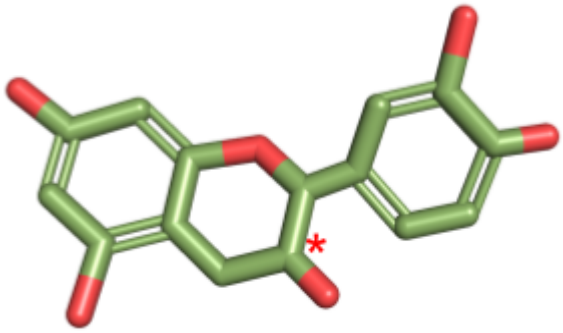

Figure 7. PyMOL generated chemical structure of (+)-catechin. A red star indicates the stereochemically relevant carbon. Extracted from PDB 4C94.[41]

Chlorogenic Acid:

Chlorogenic acid (in the flavanoid class) is found mainly in black coffee, apples, grapefruit, and strawberries, as well as in some Chinese herbal medicines (Fig. 8).[42] As with the other compounds, chlorogenic acid is a powerful antioxidant that has been found to reduce UV-induced collagen degradation by preventing the expression of MMP-1 in mice when applied from an extract mixture.[43] It also increased the rate of fibroblast remodeling for wound healing at concentrations below a maximal 20 µg/mL.[44]

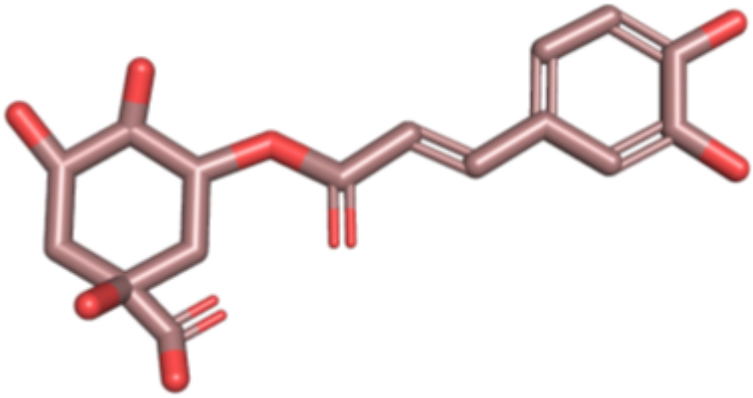

Figure 8. PyMOL generated chemical structure of chlorogenic acid. Extracted from PDB 6LK2.[45]

DMSO:

DMSO is a solvent with no antibacterial properties. This compound is often used as a control, though the chemical structure is still relevant enough to keep in mind (Fig. 9). Curiously, when DMSO is applied at too high a concentration, fibroblast growth, and proliferation are greatly reduced.[46] DMSO was used as a vehicle control in the treatment stage of the study and can be removed from the alphabetically ordered list for the sake of the image notation in Figure 26.

Figure 9. PyMOL generated chemical structure of DMSO. Extracted from PDB 1D7H.[47]

Epicatechin:

Epicatechin, or (-)-catechin/(S)-DHPV, is also a flavanol compound found in the same sources as (+)-catechin, listed above. It is the enantiomer of (+)-catechin that is also described above (Fig. 10). It was determined that the stereochemistry affects outcomes in human dermal fibroblasts blasted with UV, and is more potent of a MMP-1 inhibitor than its enantiomer.[39] They have also been shown to reduce activation of platelets (and thus of the endothelium), impacting transendothelial migration and permeability, and preventing immune responses, as well as decreasing signs of oxidative stress markers.[48,50]

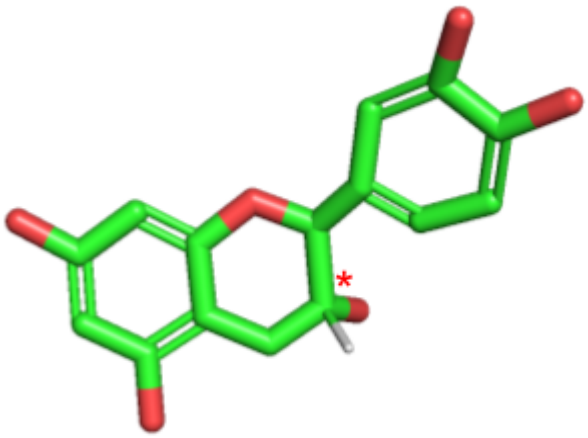

Figure 10. PyMOL generated chemical structure of epicatechin ((-)-catechin). A red star indicates the stereochemically relevant carbon. Extracted from PDB 4MA6.[49]

Gallic Acid:

In nature, gallic acid (from the flavanoid class) is sourced from blueberries, strawberries, cashews, tea, and wines, among many other common fruits (Fig. 11).[51] Studies show gallic acid as a formidable inhibitor of AP-1 mediated transcription of MMP-1 and of migration/invasiveness of several cancers through direct inhibition of the MAPK pathway.[52] Its antioxidant, antimicrobial, and anti-inflammatory properties make it suitable for wound dressings and treating dryness caused by atopic dermatitis.[53]

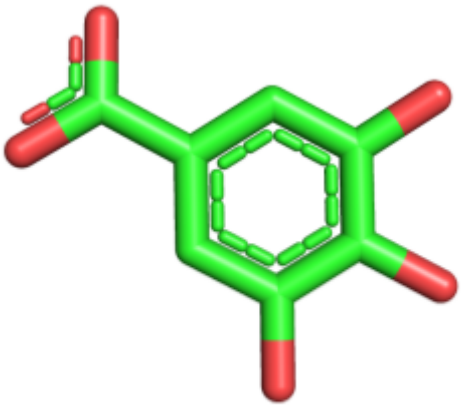

Figure 11. PyMOL generated chemical structure of gallic acid. Extracted from PDB 4Z5X.[54]

Jasplakinolide:

Jasplakinolide is a peptide derived from marine sponges (Fig. 12).[55] It is the only compound listed in this introduction that is not consumed through our ideal diet. Jasplakinolide is known to stabilize F-actin directly, increase the rate of actin filament nucleation, and competitively inhibit the binding of phalloidin (making visualization more difficult than with the other compounds mentioned), a widely used fluorescent F-actin stain.[56] Similarly to the aforementioned compounds, jasplakinolide shows potential as an anti-cancer drug.[58]

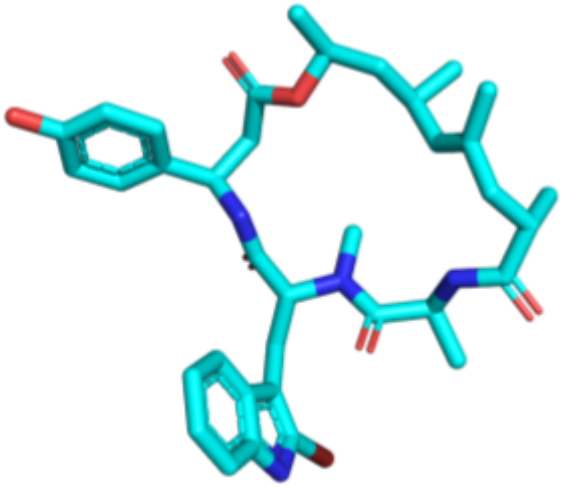

Figure 12. PyMOL generated chemical structure of jasplakinolide. PDB 9UE.[57]

Kaempferol 3-O-glucoside:

This compound is the 3-O glycosylated kaempferol flavenoid (Fig. 13). Foods high in kaempferol include most dark, leafy greens, tomatoes, beans, hibiscus tea, strawberries, apples, and many other fruits and vegetables.[59] Clincal human skin studies have shown its potential in reverting cellular senescence pathways, increasing collagen fibers through collagenase inhibition, and improving overall skin phenotype (anti-wrinkle), though studies in other organ tissues have also shown significant anti-cancer properties through downregulation of the AKT pathway.[60,61]

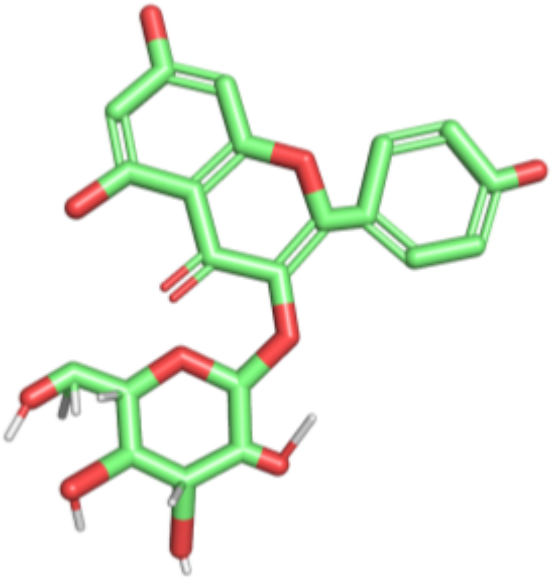

Figure 13. PyMOL self-generated chemical structure of kaempferol 3-O-glucoside.

Luteolin 7-O-glucoside:

Luteolin 7-O-glucoside is a derivative of the polyphenol luteolin and is found in oregano, anise, wild celery and carrots, lentils, and corn.[68] Its chemical structural elements such as a C2-C3 double bond with an oxo group at C4 allow it to bind transition metal ions, boosting its antioxidant activities (Fig. 14). When tested as a component of plant

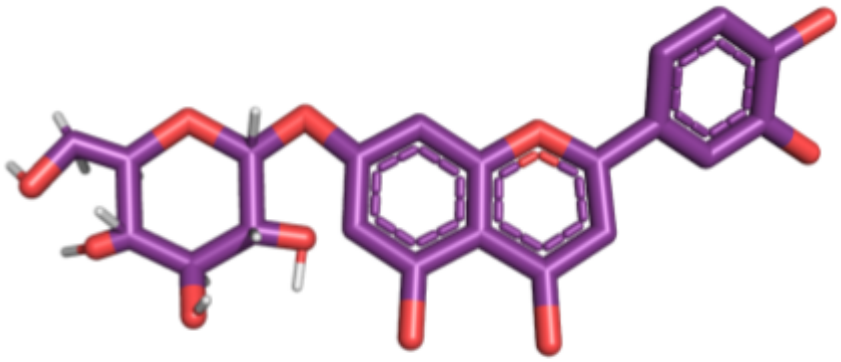

Figure 14. PyMOL generated chemical structure of luteolin 7-O-glucoside. Extracted from PDB 4QYA.[69]

extracts on human dermal fibroblasts, anti-aging effects were observed as reduced UV-induced oxidative stress via significant inhibition of MMP-1 secretion.[70]

Myricetin:

Myricetin is a flavonoid found in garlic, blue, black, and cranberries, spicy peppers, and beans (Fig. 15).[62] In mice, it has been shown to exert anti-photoaging effects by extinguishing MMP-9 (related to MMP-1) expression and activity by suppressing Raf kinase, an enzyme that activates downstream effectors involved in cancerous proliferation prompted by UV rays.[63] In combination, these effects lead to inhibition of photo-induced wrinkle formation and degradation of type I collagen. In regards to UV-related oxidative stress, myricetin enhances mitochondrial function and has anti-cancer/inflammatory potential, even ameliorating the effects of diabetes on fibroblasts.[65] This compound was not used in the final treatment stage of the study and can be removed from the alphabetically ordered list for the sake of the image notation in Figure 26.

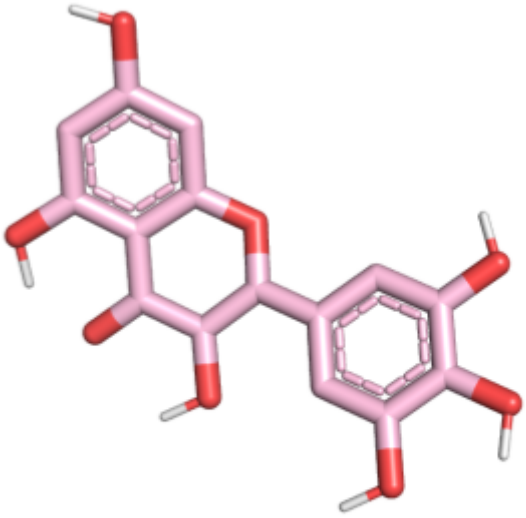

Figure 15. PyMOL generated chemical structure of myricetin. Extracted from PDB 6TTC.[64]

Quercetin 3-β-D-glucoside:

Quercetin 3-β-D-glucoside (isoquercetin) is a naturally occurring form of quercetin and is found in the peels of apples, mangoes, pears, and darkly colored vegetables.[66] When used as a component of plant extracts, isoquercetin has anti-inflammatory and antioxidant capabilities via

the prevention of NF-κB formation.[66] Antioxidant effects are provided by the compound's hydroxyphenolic groups and the 4-oxo function of its C ring, enabling it to quench ROS, enhance wound healing, and reduce damage to fibroblasts (Fig. 16).

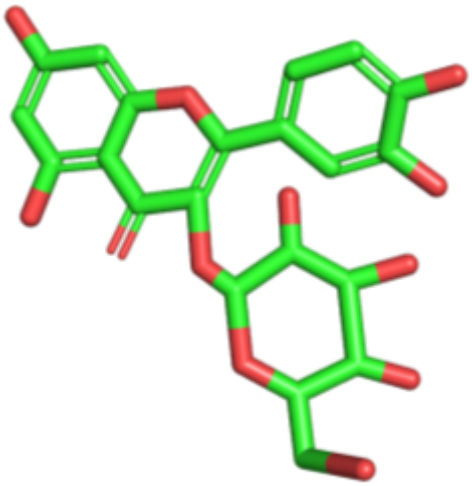

Figure 16. PyMOL generated chemical structure of quercetin 3-β-D-glucoside. Extracted from PDB 6QCE.[67]

The compounds listed above have several key property commonalities among them: anti-inflammatory, analgesic, antimicrobial, antioxidant, naturally derived, and potentially anticancer depending on the tissue to which they are administered. Of course, purity is always an issue as most of the knowledge about their effects stems from studies that focus on plant extracts rather than in isolation. Other substances—those besides those of interest to this study—present in the extracts used in previous studies could be responsible for the anti-aging effects reported. Indeed, most of the background regarding the compounds chosen for this more surface-level study is derived from plants and leaves. Though it could quite possibly be that these compounds will have greater effects when naturally consumed in a modified version of the average Western diet, the inclusion of these compounds in cosmetics is a much more promising method for implementing them into the lives of many. The mechanisms by which these compounds exert their effects are not fully understood and few studies are mentioning their effects when in isolation. This study aimed to investigate the potential phenotypical anti-aging effects of the compounds contained in this curated list using cultured primary human dermal fibroblasts as a model. This model allows screening of the effects of bioactive compounds in living human cells quickly, (relatively) inexpensively, and reproducibly.

Materials and Methods

I. Sourcing & Management of Human Dermal Fibroblast Cultures:

All cells collected for the purpose of this study were sourced from the University of Michigan, Department of Dermatology. Spanning several years of biopsy, both old/young and

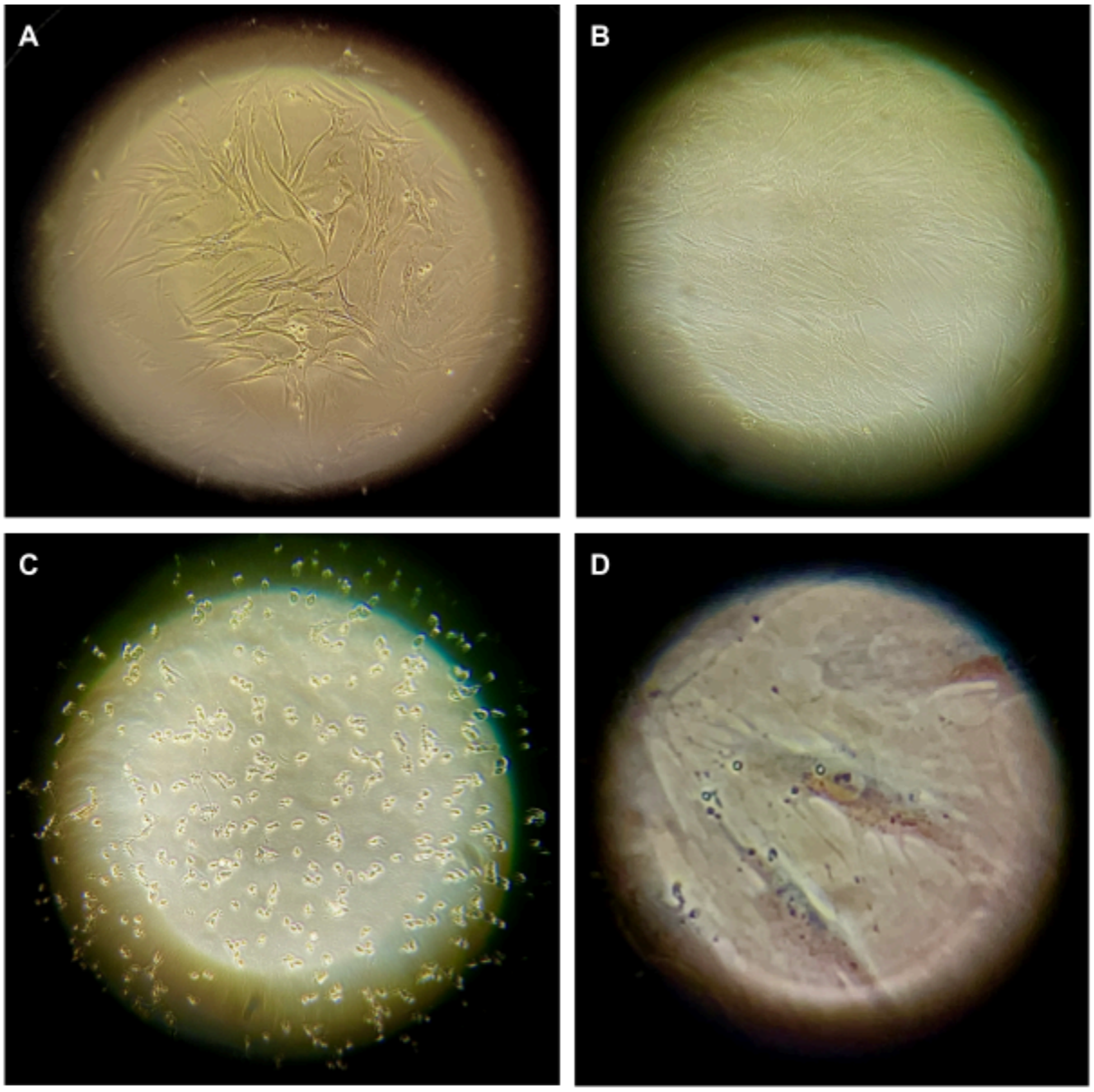

Figure 17. (A) Young male derived human dermal fibroblasts (HDFs) cultured for 24hr in complete media, under 10X brightfield microscope lens. **(B)** Young male derived HDFs cultured for 48hr in complete media, crowding under 10X. **(C)** Young male derived HDFs undergoing trypsinization process for removal from plate, 10X. **(D)** Young male derived HDF phenotype, 20X.

male/female samples were collected and stored in a liquid nitrogen chamber that was timely managed and replenished, preserving all cells until needed in the laboratory. Cell medium was produced by dilution of 500mL alpha-modified minimum essential medium (10X MEM-α Glutamax) with fetal bovine serum (FBS, 10% dilution) and penicillin-streptomycin (pen-strep, 5% dilution). This cell medium was consistent across all cultures managed. Cells were extracted from their liquid nitrogen chamber, allowed to thaw, centrifugated for 2 minutes at 2000 RPM, and resuspended in 10mL of medium before being pipetted into culture-treated Corning® plates for incubation and growth (Fig. 17A, B, D). Cells were placed in an incubator set at 37˚C and 5% CO_2 atmosphere. Removal of cells from plates to expand the number of cells began with draining plates, followed by washing with Dulbecco's Phosphate Buffered Saline (DPBS), treatment with 0.25% trypsin-EDTA (1X) enzyme for 1-3 minutes to allow detachment of cells, and final centrifugation, resuspension, and incubation in duplicate plates in the incubator (Fig. 17C). For counting purposes, 0.4% trypan blue was added to the cell suspension to verify cell viability (dead cells stain blue)and placed onto a glass grid under the 10X objective of an inverted microscope. Cells were passed up to a maximum of twelve times before growth weaknesses and greater susceptibility to air-borne cross-contamination were observed.

II. Phalloidin and DAPI Actin/YAP Staining & Visualization:

Phalloidin conjugated with a fluorescent dye was used to stain F-actin. First, a paraformaldehyde (PF)/phosphate-buffered saline (1X PBS) solution was prepared with 2g PF per 100mL PBS; several aliquots of this mixture were needed per experiment, so large batches were created at one time, measured out, and stored in a laboratory refrigerator. This mixture was heated with a stir bar and then allowed to cool after full dissolution. Cells were washed with PBS

4-5 times, fixed with appropriate amounts of PF/PBS (relative to dish size) for 15 minutes, and followed with another PBS wash cycle. 0.5-1mL of 0.25% Triton X-100/PBS solution was added to each dish and let sit for 20 minutes to allow cell membrane permeabilization for maximal F-actin staining. 2mL of 3% bovine serum albumin (BSA) was added to each cell plate and let sit for 60 minutes, minimizing non-specific Phalloidin binding in the subsequent staining. Phalloidin was prepared per recommended package instructions (avoiding as much exposure to direct light as possible) by dilution with PBS and methanol (MeOH). 0.5mL phalloidin/PBS/MeOH solution was pipetted into each cell plate and incubated for 30 minutes in a dark space.

In experiments, this phalloidin staining protocol was combined with antibody-based staining through the addition of 10% donkey serum in PBS and allowed blocking for 1 hour, followed by YAP/TAZ antibody solution in PBS (1:20 ratio), which was added to cultures of interest and allowed to incubate overnight. Plates were washed with PBS and a secondary antibody (5% donkey serum in PBS) was incorporated and left for an hour waiting time, followed by a final PBS wash. For nuclear staining, 1mL 4′,6-diamidino-2-phenylindole (DAPI, in water) was additionally added to phalloidin-stained cells and incubated for another 5 minutes before subsequent visualization. Zeiss Zen software was used for computerized microscope visualization and imaging of all stained cells.

III. Rigidity Plate Preparation & Management:

Rigidity tests were performed using Advanced BioMatrix CytoSoft® rigidity plates and prepared per package instructions. In a sterile hood, ECM protein solutions of fibronectin or type I collagen were diluted to 1:10 or 1:30 with 1X DPBS, respectively, and then warmed to room

temperature. Before reseeding cells, the 6-well rigidity plates and corresponding duplicates (for each stiffness plate, 0.2kPa, 0.5kPa, 2kPa, etc.) were prepared with 3mL each of either ECM solution, fibronectin or collagen, incubated for 1 hour at 37 ˚C, and then rinsed with PBS twice, making sure to leave behind sufficient PBS in the wells to avoid drying of the coated plate ECM substrate. Cells were reseeded at low numbers (~50k per well, to obtain maximal accuracy when taking measurements of individual cells; overcrowding is not ideal) and incubated for 48 hours to allow maximal attachment and growth on the stiffness plates, ranging from 0.2kPa to 64kPa. For reference, brain tissue stiffnesses range from 0.2-0.5kPa, followed by higher stiffness ranges corresponding to lung, liver, muscle, eye, and cartilage/bone tissue (Fig. 18).[71]

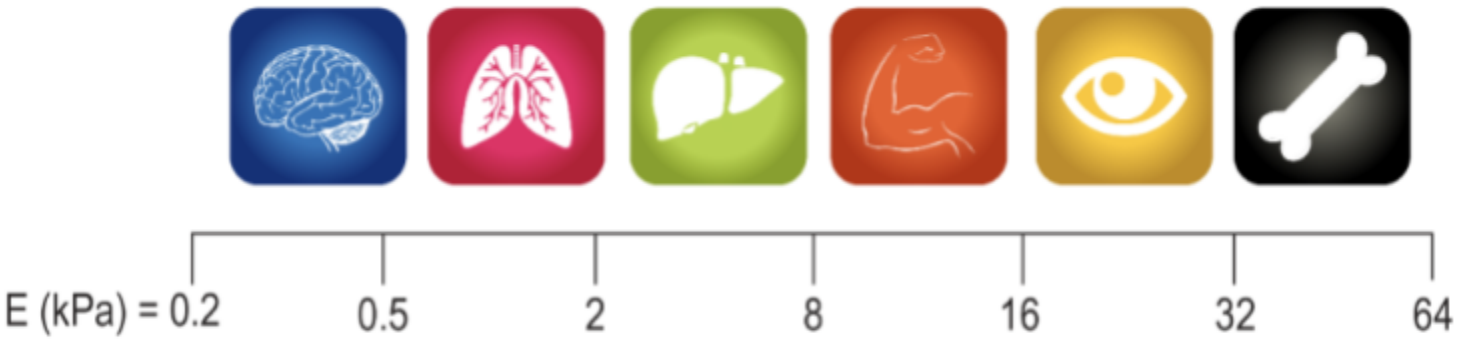

Figure 18. Chart depicting the wide ranges of plate stiffnesses (0.2 - 64kPa) provided in Advanced BioMatrix CytoSoft Rigidity plate line and their corresponding tissue equivalencies in the human body. Adapted from Advanced BioMatrix.[71]

IV. Treatment of Cells with Compounds of Interest:

After allowing cells to grow to an ideal confulency, they were removed from their original growth plates and reseeded at low numbers (~30-50k per well, same reasoning as stated in part III above) in 6-well 0.2kPa rigidity plates. These cells were allowed to incubate and multiply for 24-48 hours at 37 ˚C. Our treatments began with obtaining our compounds of interest (see *Introduction*), which were provided in solid form (with the exception of DMSO,

which is a liquid). Myrecetin was not used in the studies due to time constraints. These solid compounds were first dissolved in DMSO, making 10mM stocks which were then diluted 100-fold. Final treatment concentrations were 100μM compound of interest and 1% DMSO.

V. Final Image Measurements & Analysis:

As previously stated, all cell images were taken and processed with Zeiss Zen software. Hundreds of pictures were taken of resulting cells and stained actin fibers for maximal internal validity, based on both brightfield and red flourescence channel visualizations. After numerous, meticulously labeled images were produced, singular cell masks were isolated in the field of view (FOV) for variable analyses (Fig. 19).

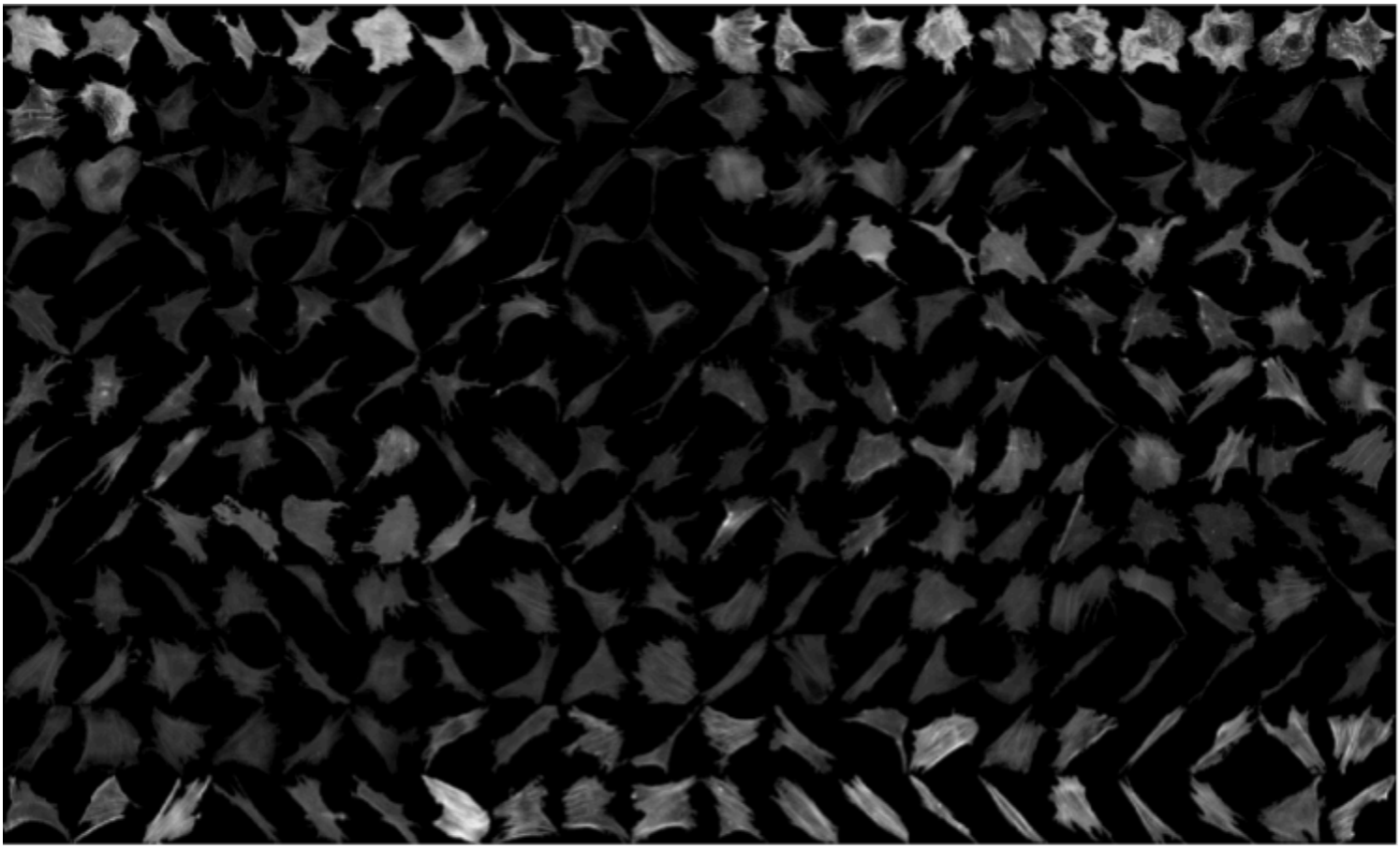

Figure 19. FOVs for a collection of individual HDFs in grayscale, compiled from a bank of images used to make formal measurements.

Measurements were made for cell area, cell aspect ratio, percentage of actin fibers aligned with the cell longitudinal axis (major cell axis), and S-parameter of observed fibers (concerning fiber angle deviance from a cell's longitudinal axis). From the best-fit-ellipse of each cell, the aspect ratio was calculated as $\frac{major\ cell\ axis}{minor\ cell\ axis}$, with the major axis defined as the longest vertical center line and the minor axis as the longest horizontal center line (perpendicular to the major axis).[72] Larger aspect ratio indicates a more stretched cell phenotype, while a smaller aspect ratio indicates a rounder cell phenotype. All cell fiber orientation vectors were summed and averaged for a mean fiber orientation value to calculate S-parameters. S-parameters of cells were calculated with $\overline{cos(2\theta)}$, where $\theta = |\theta_{\text{major cell axis} - \theta\text{mean fiber orientation}|}$ (Fig. 20).

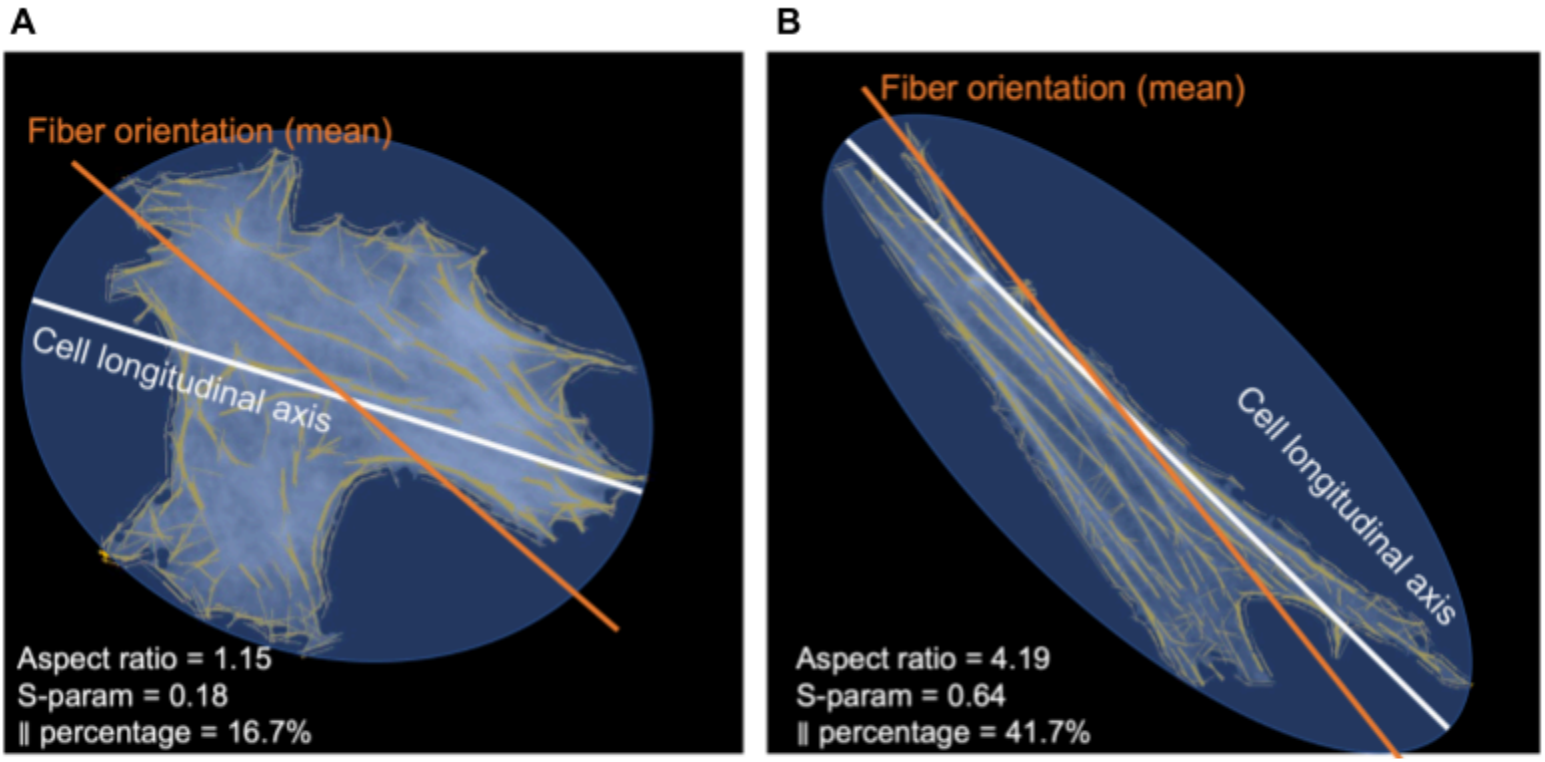

Figure 20. Illustrations of calculations made from cellular measurements: mean fiber orientation, aspect ratio, S-parameter, percentage alignment of actin fibers with cell's major axis. Comparison between example values for an "aged" cell with less overall F-actin cytoskeleton organization **(A)** and a more "youthful" cell that displays a more stretched morphology and thus more organized F-actin cytoskeleton **(B)**.

Results

Human dermal fibroblasts (HDFs) are known to display different phenotypes and morphologies in response to different tissue states.[72] With environmental damage to the skin over time, biochemical processes that are harmful to the skin's mechanotransduction abilities are induced. The consequent, gradual degradation of the underlying dermal ECM deleteriously impacts the skin's surface, where signs of aging manifest as wrinkles, laxity, and dyspigmentation. An efficient model is required to easily manipulate the cell's environment in order to begin to understand the skin cell's (specifically HDFs, in our case) structural response to varying extracellular conditions and extrapolate from there to consider treatments that may reverse or prevent these signs of aging *in vivo* in the future.

This study first served as a series of morphological assays of HDFs cultured in plates of varying stiffnesses in order to mimic the varying ECM conditions that may arise with aging (Fig. 21). Degradation of the ECM directly translates to the stiffness of a cell's surroundings, thus dictating whether a cell remains stretched—to an ideal state proportional to the cell's functions and needs—and attachment to the ECM or detaches, becoming more rounded and and acquiring a detrimental aged phenotype. Advancing our knowledge of the interplay between the surrounding extracellular environment and the regulation of actin fiber assembly in HDF's provides a critical foundation for later determining the impact of the chosen botanical compounds on actin cytoskeleton assembly. We set out to initially characterize a model for studying cell stretch and actin assembly under conditions of various ECM stiffness.

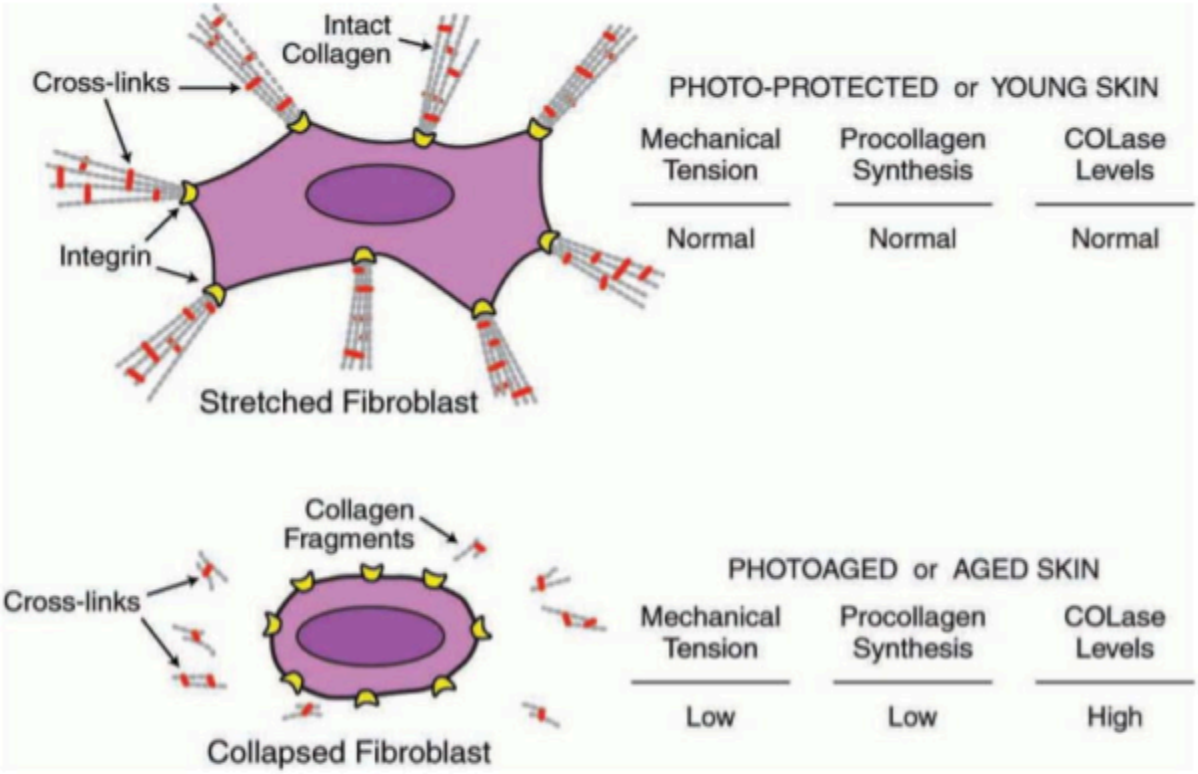

Figure 21. Illustrations comparing photo-protected/young and photoaged/aged skin fibroblast morphology. Low mechanical tension and a lack of cross-linking to the surrounding ECM through integrin proteins bring about a collapsed fibroblast phenotype.[73]

I. Effects of Varying CytoSoft Stiffnesses & ECM Substrates on HDF Morphology

Fibroblasts are essential in generating the ECM in the skin dermis. Our primary interests lie in the potential metamorphosis of fibroblast morphology and phenotype in varying stiffness conditions to mimic the mechanical environment in aged skin ECM *in vivo*. However, the first and foremost problem to tackle when conducting observational analysis studies on fibroblasts—or any kind of cells is the repetitive process of trial and error to decide how many cells to seed per plate. Cell overlap greatly interferes with isolating individual cells for accurate and precise measurements. Further, cell overcrowding in plates may also have confounding effects on ECM composition, gene regulation, consequent protein expression, and eventual

phenotypes of fibroblasts in vitro.[77] Figure 22 illustrates our matter of cell overcrowding versus seeding plates at lower cell numbers to allow for more precise measurements for our studies.

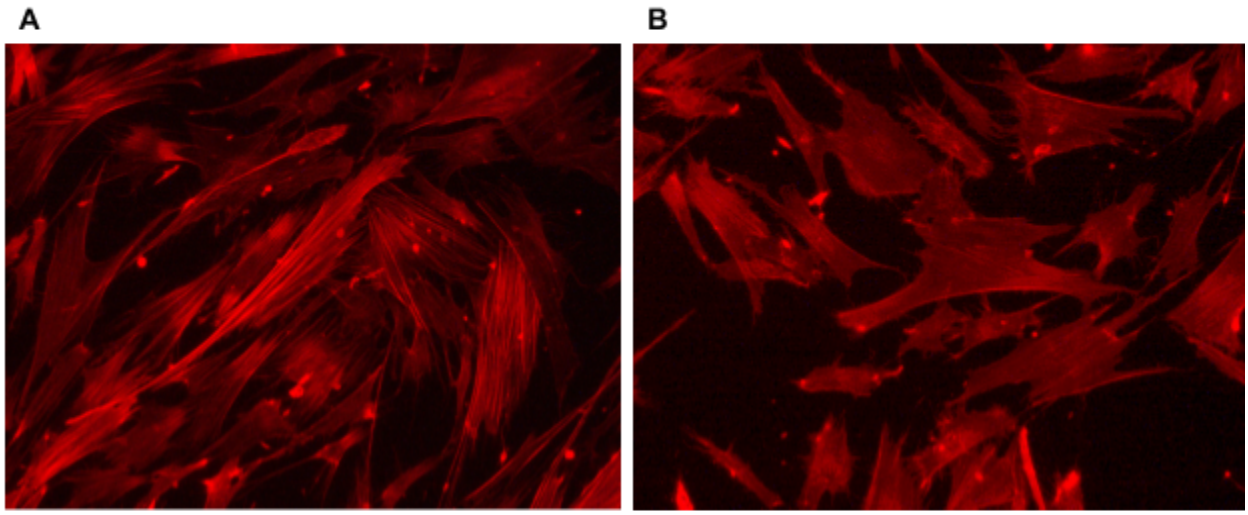

Figure 22. (A) Fluorescent staining of actin in 16kPa collagen loaded plates seeded with a high number of fibroblast cells under 20X, making individual cell measurements difficult due to excessive overlap. **(B)** Cells are seeded at lower numbers in the same conditions, reducing cell overlap for visualization and allowing ease of individual cell measurement and analysis.

After refining our methods to optimize cell viability and visualization on basic plastic culture plates, we moved on to investigate the direct effects of cell morphology on plates with varying surface stiffnesses. Plates with surface stiffness ranging from 0.2kPa to 64kPa were acquired and prepared according to *Materials and Methods, III*, coated with either collagen or fibronectin ECM protein for adhesion. Collagen type I is a major fibrilar protein in connective tissue that self-assembles as fibrils to form ECM scaffolding *in vivo*. Fibroblasts bind to type I collagen primarily through $\alpha_{11}\beta_1$ integrin receptors.[74, 75] Fibronectin, on the other hand, is a matrix protein that forms a thinner fibrillar gel than collagen, though it also participates in regulating fibroblast adhesion through $\alpha_5\beta_1$, $\alpha_v\beta_1$, and $\alpha_v\beta_5$ transmembrane integrin protein complex receptors.[75] These are the fibroblast receptors that interact with type I collagen and fibronectin

that are currently known, though there may be more involved that have not been definitively identified yet.[76]

As a reminder, with aging, the ECM proteins—most notably collagen and fibronectin—are degraded. This degradation impairs attachment of fibroblast to the drmal ECM, negatively affecting overall mechanotransduction abilities and actin polymerization. Thus, we expected that the cells grown on the lowest stiffness substrate (0.2kPa) would show a collapsed morphology, similar to that observed in aged skin in vivo. This finding would be indicated by reduced stretching of cells, decreased percentage of actin fibers aligned with the cells' major (or longitudinal) axes, lower cell aspect ratio, and lower S-parameter (see *Materials and Methods, V*). Our previous 2D matrix (no collagen or fibronectin), plastic plate setup serves as a positive control that allows the exhibition of maximal cell stretching for comparison (Fig. 26). Additionally, to achieve clear visualization of single cell morphologies, HDFs were fixed and stained with phalloidin, a highly selective F-actin-binding peptide that is commonly used for staining F-actin in a large variety of animal and plant species, it has been found to bind polymerized actin with much higher affinity than actin monomers.[77]

Collagen and fibronectin ECM substrates were compared in rigidity plates of 8, 16, 32, and 64kPa stiffnesses. There were no substantial differences detected between using collagen versus fibronectin for fibroblast adhesion on the rigidity plates (Fig. 23). Fibroblasts in human skin preferentially bind collagen.[75,76] Therefore, we decided to employ collagen as the ECM substrate protein for our studies. Looking at Figure 23, we can see that HDFs grown in 0.2kPa stiffness plates coated with collagen show the lowest overall stretching compared to those grown in higher rigidity conditions. Cells are more rounded and less stretched, and the red fluorescently stained actin fibers (corresponding to the straight, red lines seen in Fig. 23) are not all parallel to

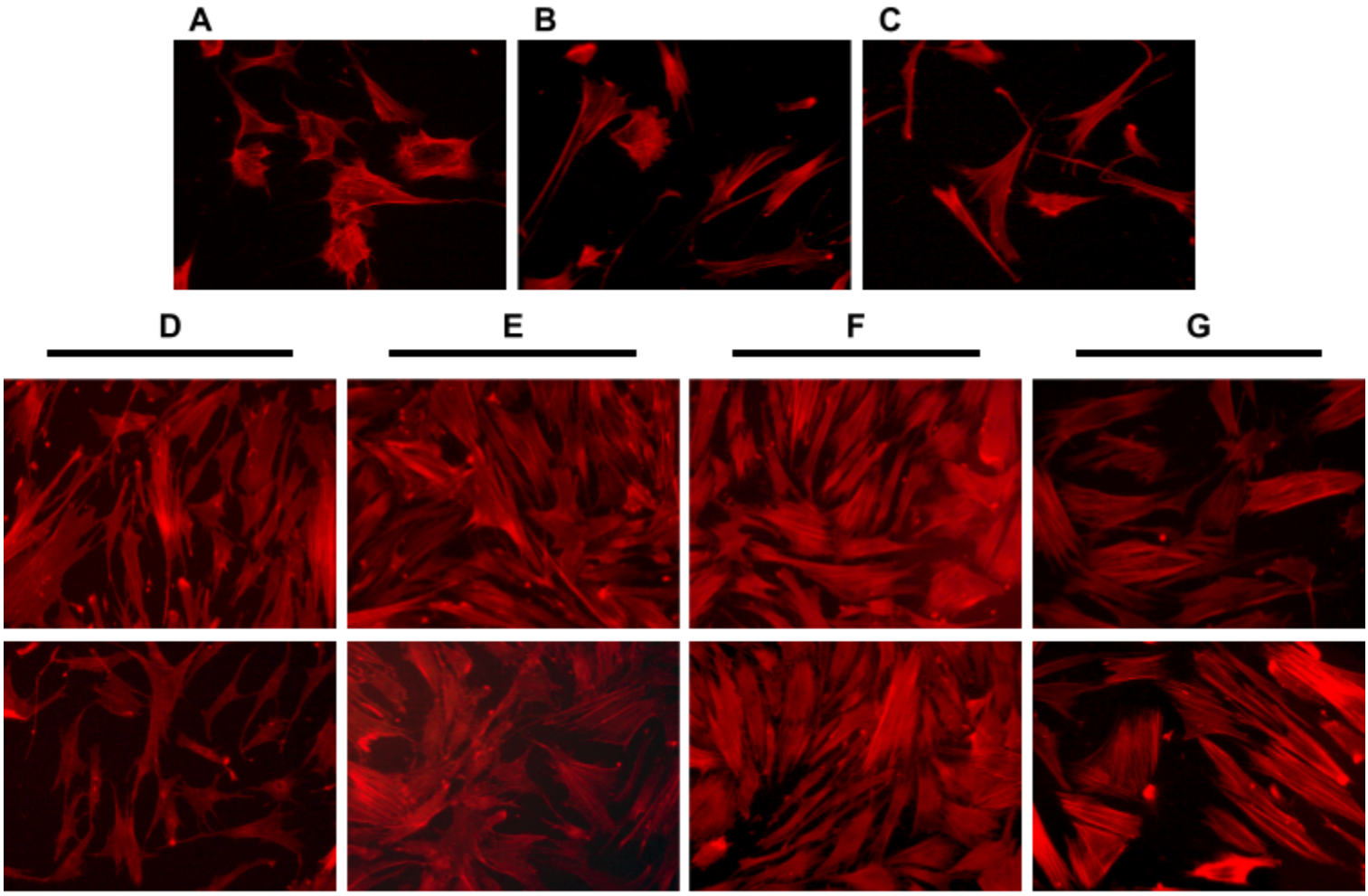

Figure 23. Varying HDF morphology across rigidity plate stiffnesses prepared with either collagen or fibronectin ECM proteins. **A - C** represent 0.2, 0.5, and 2 kPa in collagen only, respectively. **D - G** represent 8, 16, 32, and 64 kPa in collagen (top row) and fibronectin (bottom row), respectively.

the cells' major axes and are seen oriented in inconsistent directions from the centers of the cells. Moving from left to right across Fig. 23A - C, the cells' actin cytoskeletons are gradually more organized, with the cells becoming much more stretched out, with F-actin fibers orienting themselves with the cells' major axes. The effects of increasingly stiff environments on cell morphology are more pronounced in stiffnesses of 8kPa through 64kPa (Fig. 23D - G), on both fibronectin and collagen ECM matrices. The visual morphological results seen in Fig. 23 were

then grouped into the following categories for quantification and further analysis: cell area, cell aspect ratio, cell fiber percentage along the major axis, and fiber S-parameter. These values serve as indicators of how varying stiffness conditions affect fibroblast morphology in vitro, laying a foundation for understanding the mechanics underlying F-actin polymerization in different stiffness states of the ECM as it changes with age.

Cells were isolated in the field of view, counted, measured, and averaged per *Materials and Methods, V* for quantification. Since there were no significant differences found between cells grown in either collagen or fibronectin-coated culture plate surfaces in higher stiffness rigidity plates, the values calculated for each category were eventually combined across both substrates for comparison to the lower stiffness rigidity plates. For example, cells were grown in 8kPa in both collagen and fibronectin, measured, summed, and later averaged together to combine into one 8kPa condition. After quantification within all of these categories, several key findings came to light. Measurements of cell area and cell aspect ratio, on average, were highly variable and thus not helpful measures in and of themselves in determining any definitive effects on cell morphology across plate stiffnesses. Cell area tended to increase with increasing stiffnesses above 0.2kPa. Cell aspect ratio tended to remain in the same range across stiffnesses, on average, though making a more stark increase when moving from 0.2kPa to the next highest stiffness plates.

Fibroblast actin fiber percentage along individual cell major axes (Fig. 24) and fiber S-parameter (Fig. 25) increased with increasing substrate stiffnesses, actin fibers tended to become more closely oriented with the major spindle axis of each cell. This result indicates that stiffer substrates increased fibroblast stretch. It logically follows that the average actin fiber S-parameter values also tended to increase with stiffness, meaning that not only did actin fibers

become more oriented along the main body of the cell but also showed an overall corresponding decrease in angle deviation from the mean fiber orientation (recall, back to Fig. 20).

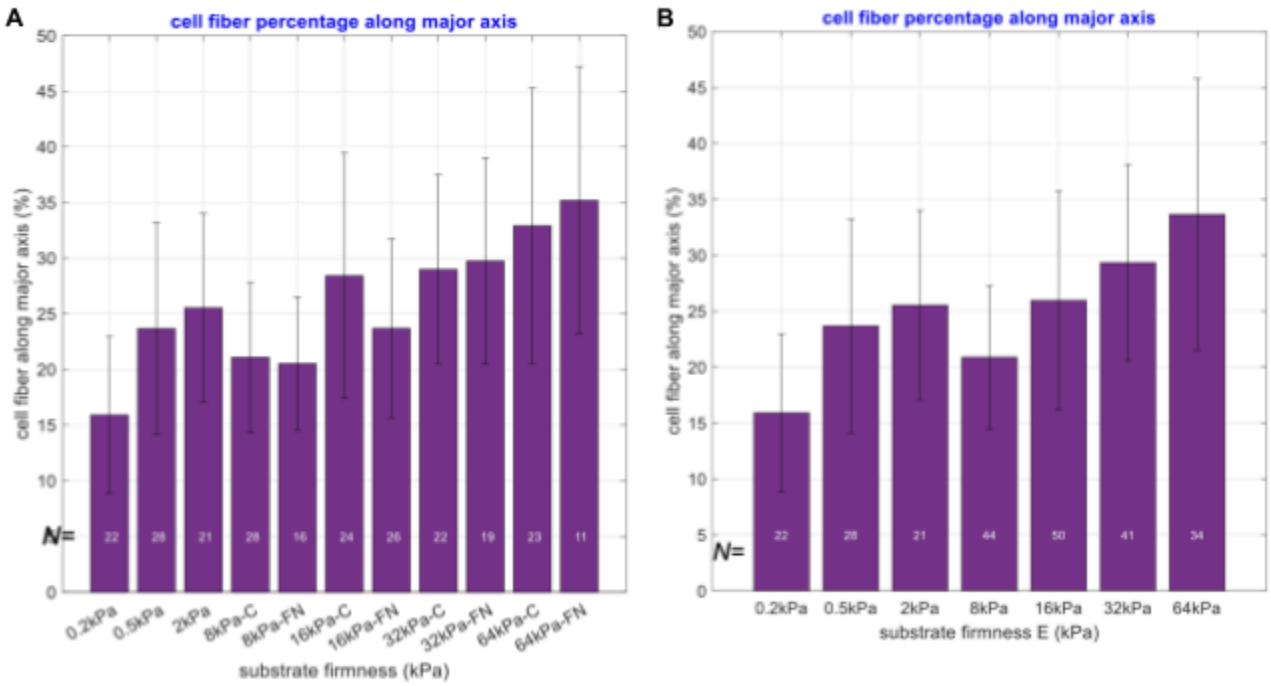

Figure 24. (A) Substrate firmness (kPa) vs cell fiber percentage along the cell's major axis. As stiffness increases, percentage of cell fibers aligned with the major axis tends to increase. **(B)** C and FN conditions combined for 8kPa-64kPa substrate stiffnesses. "N" indicates the cell count for each group.

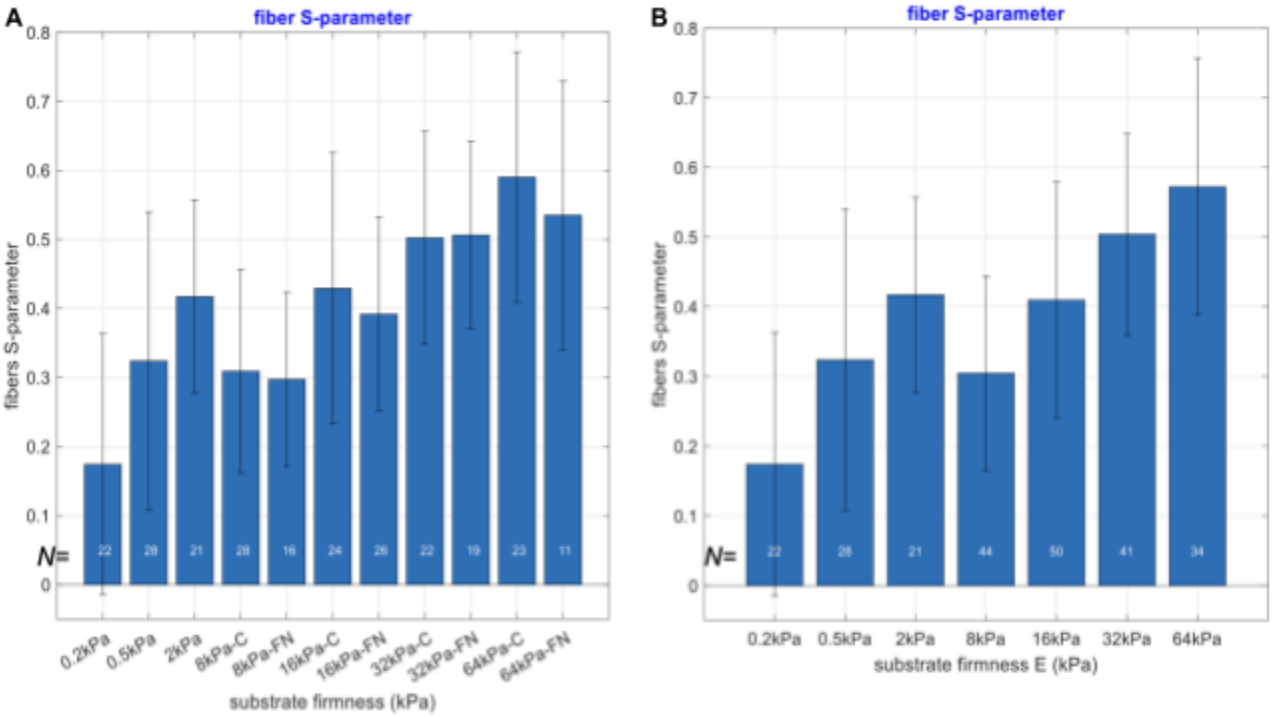

Figure 25. (A) Substrate firmness (kPa) vs fiber S-parameter. Similar trend to cell fiber orientation along major axis, S-parameter tends to increase with substrate stiffness. **(B)** C and FN conditions combined for 8kPa-64kPa substrate stiffnesses.

II. Compounds of Interest Impact HDF Phenotype

The actin cytoskeleton is a very dynamic and sensitive mechanotransducing complex that allows for several essential processes in the skin to take place. Fibroblasts, which are largely regulated by the actin cytoskeleton, are the principal cells of the skin that produce and maintain the dermal ECM. ECM stiffness is known to be critical for fibroblast function in the skin, and since the ECM's stiffness is significantly altered with age as a result of numerous environmental and endogenous factors, normal fibroblast functioning is disrupted. With aging, this disruption increases in a positive feedback loop like fashion.[78] Stiffness-mediated mechanotransduction upregulates fibroblast activation—F-actin polymerization and corresponding morphological effects—-to increase collagen production, deposition, and cross-linking, cell proliferation, and significant changes in gene expression.[79]

We next cultured human dermal fibroblasts on low stiffness plates to model aged skin dermal ECM environment and investigated whether treatment of the cells with isolated botanical polyphenols could stimulate assembly of the actin cytoskeleton and stretch the cells, to induce a more youthful phenotype. The treatments were chosen based on previously conducted studies demonstrating their potential anti-inflammation, anti-cancer, antimicrobial, and antioxidant activities that we hypothesize may have positive effects on aging markers in our experimental model. To simulate the fragmented ECM in aged skin, HDFs were grown in 0.2kPa stiffness rigidity plates that were that were coated with type I collagen following our findings from *Results, I*. Treatment condition groups were organized and equal concentrations of each previously described botanical polyphenol actin-binding compound (see *Introduction, III*) were administered accordingly. After incubation for several hours, cells were stained with phalloidin for F-actin visualization per *Materials and Methods, II*. Results are shown in Figure 26. For

comparison, HDFs were also plated on plastic plates as a positive control of maximum cell stretching (Fig. 26L) and on collagen-loaded 0.2kPa stiffness rigidity plates with DMSO treatment only as a vehicle (negative) control of minimal stretching in a low-stiffness ECM

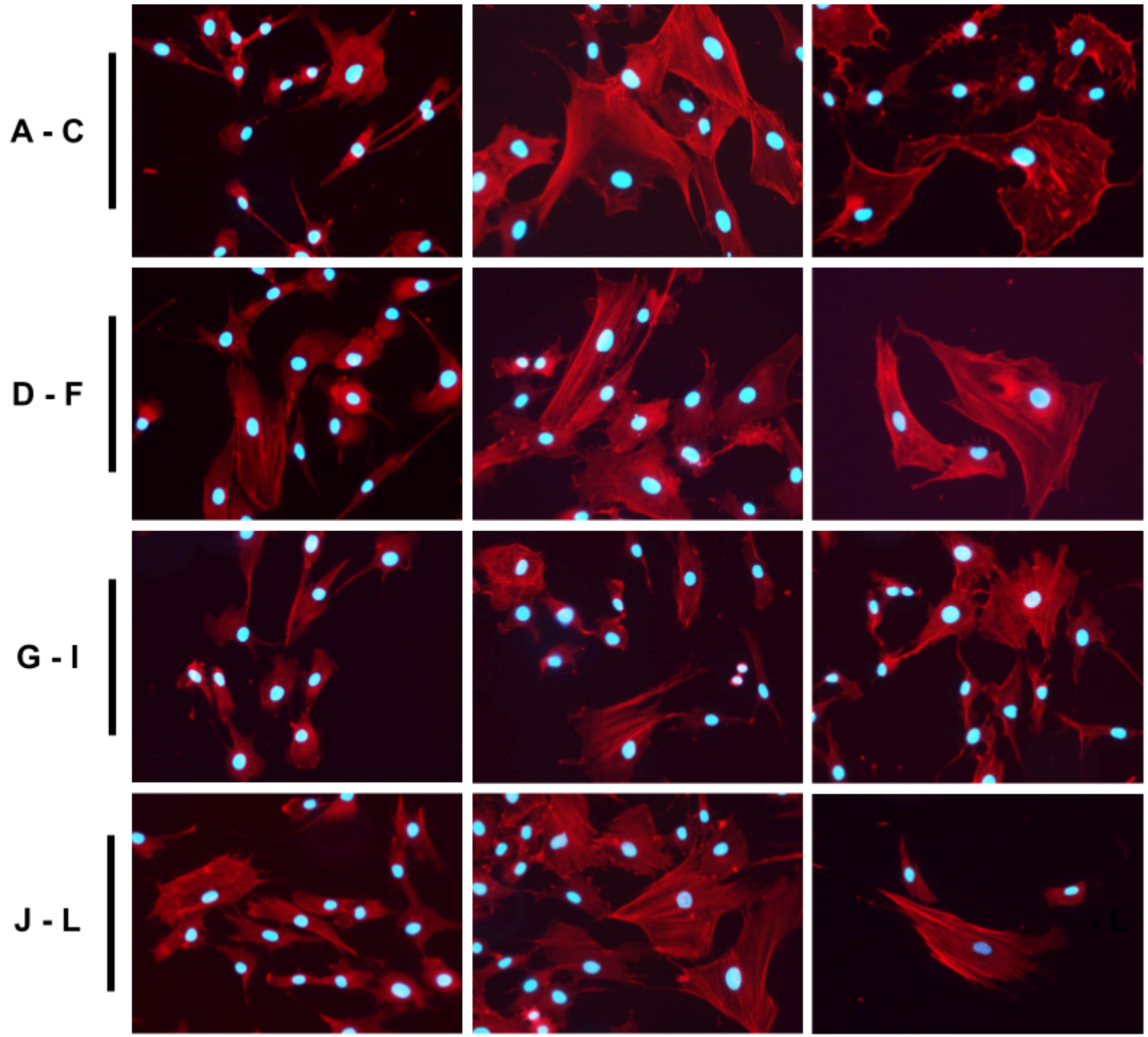

Figure 26. Images of HDFs grown on 0.2kPa plates prepared with collagen, post-100μM-treatment. From **A-J**, corresponding treatments are in alphabetical order (see *Introduction, III*). **K** and **L** represent DMSO and the plastic control plates, respectively.

environment (Fig. 26K). Cell nuclei are seen in bright blue and cell bodies can be seen in red with individual F-actin glowing as fluorescent red fibers; their orientations are clearly visible with respect to the whole cell body.

Hundreds of images were captured from each treatment group and compared to controls and each other to determine their individual effects on HDF's as well as to conclude which of them provides the most optimal cell morphologies in terms of cell area, overall stretching, and actin fiber orientation. Allantoin (Fig. 26A), for example, seems to decrease cell area though stretches HDF cell bodies to be more elongated and condensed compared to DMSO vehicle control cells.

Treatment with apigenin (Fig. 26B), on the other hand, results in larger, more plump yet also more stretched cell bodies with centrally located nuclei compared to DMSO treated controls. Interestingly, (+)-catechin (Fig. 26C) resulted in cells that grew dendrite-like extensions from their outer borders and individual fibers were much harder to observe compared to other treatments. Chlorogenic acid (Fig. 26D) and gallic acid (Fig. 26F) had somewhat similar effects on stretching of cells with average area increased slightly more in cells treated with the former. Unlike the previous two, cell area was decreased in cell groups treated with luteolin 7-O-glucoside (Fig. 26I) and jasplakinolide (Fig. 26G). Despite earlier mention, myricetin was not obtained for this study however it remains in this paper as a possible directive for future relevant studies for its potential effects in altering fibroblast morphology in low-stiffness environments.

Cell area values were averaged among all treatment groups after field-of-view isolation as described in Materials and Methods, V, then normalized to DMSO values for comparison. The quantified results of all data relevant to Figure 26—with cell treatment groups ranging from

69-237 cells being considered in the final values—are displayed in Figure 27. The compounds are listed in the graph in order of decreasing percent effects on average cell area with respect to DMSO, with average cell area of cell groups grown on plastic plate 2D matrices (no loading with either 3D ECM matrix substrate prior to cell seeding) labeled as the "Control" to the far-right side of Figure 27. Apigenin had the greatest positive effect on cell area, with a staggering 66% increase with respect to DMSO vehicle controls. Chlorogenic acid is up next on the list with cells exhibiting an average 56% increase in cell area, followed by gallic acid with 49%, allantoin (39%), quercetin-3-β-D-glucoside (38%), epicatechin (or (-)-catechin, 30%), kaempferol-3-O-glucoside (20%), catechin (or (+)-catechin, 4%), luteolin-7-O-glucoside (-16%), jasplakinolide (-21%), and finally plastic plate controls (83%).

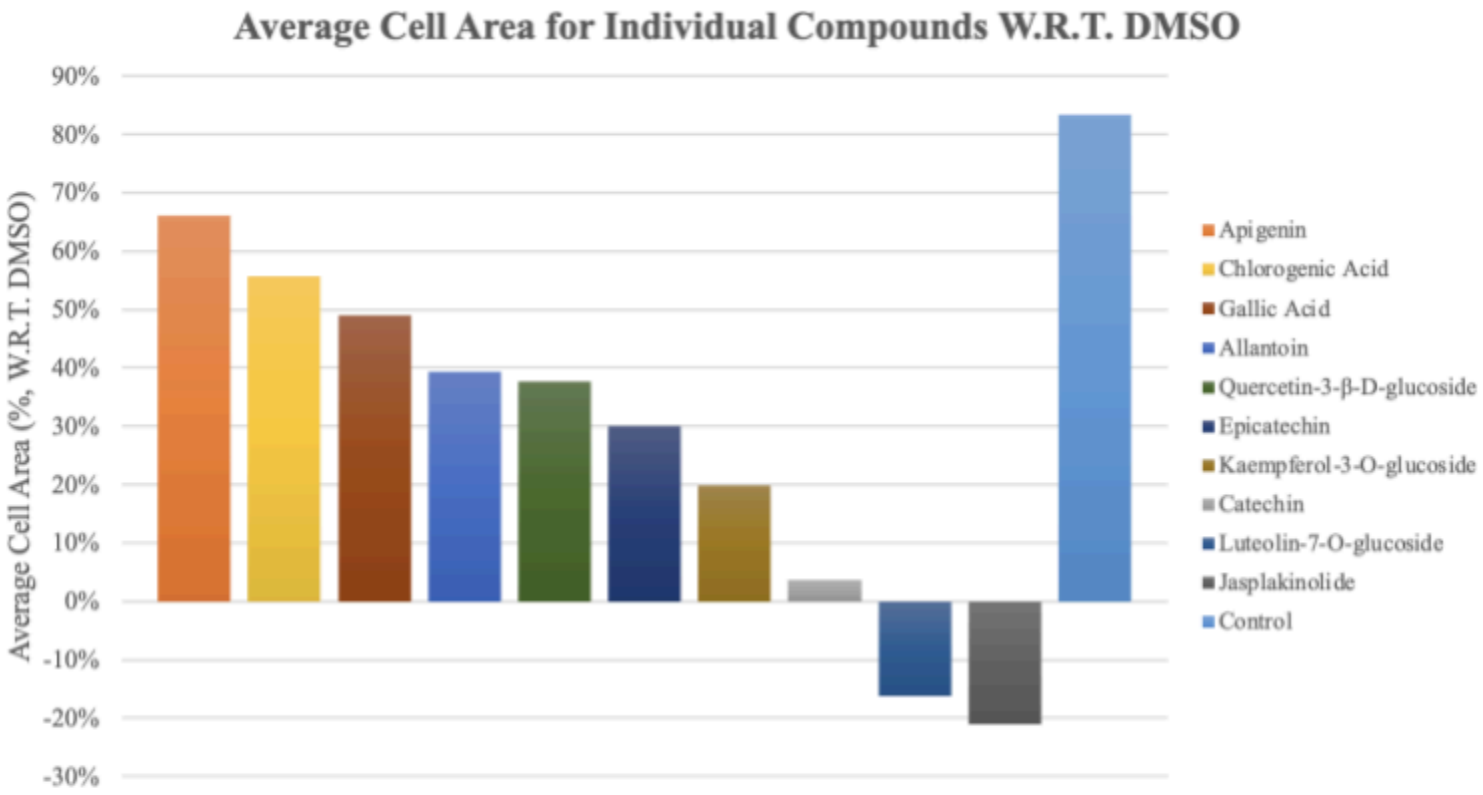

Figure 27. Final data for cells grown in 0.2kPa rigidity plates with collagen as the chosen ECM stiffness substrate. Average cell area (total cell area in pixels when visualized in 10X/number of nuclei) as a percentage with respect to (W.R.T) DMSO. DMSO is set as the zero point so, for example, cells treated with apigenin displayed a 66% larger cell area than those treated with DMSO. "Control" refers to cell groups grown on 2D plastic plate matrices.

III. Actin Cytoskeleton Organization & YAP/TAZ Localization

As described above, the stiffness of the dermal ECM greatly affects many processes in the skin. Fibrosis, or the development of collagen-rich, dense filamentous connective tissue in response to injury, is positively regulated by active differentiation of fibroblasts to pro-fibrotic myofibroblasts that then promote the restructuring of the ECM, resulting in scar formation.[80] With age, these processes are greatly diminished. In research done on normal fibroblast functioning in healthy skin, Yes-Associated Protein (YAP) and a transcriptional coactivating PDZ-binding motif (TAZ, also known as WWTR1) are shown to play pivotal roles in fibrosis.[81] YAP and TAZ are both downstream effectors of the Hippo pathway, which controls organ size through the regulation of cell proliferation and apoptosis. YAP and TAZ are also regulated by mechanical forces in the cell and respond to ECM stiffness, cell stretch, and actin assembly.[83] YAP/TAZ act as sensitive sensors of mechanical cues induced by changing ECM rigidity independent of the Hippo pathway.[84]

YAP/TAZ are thought to prevent senescence in cells by localizing to the nucleus and upregulating target genes to maintain the integrity of the nuclear envelope, keeping cyclic GMP-AMP synthase (cGAS) and the STING pathway (the cell's sensors for the presence of cytosolic double stranded DNA, or dsDNA) at bay.[85] YAP/TAZ are sequestered in the cytosol in cells with reduced mechanical force, such as fibroblasts in aged skin (Fig. 28). Thus, the cellular localization of YAP and TAZ, cytosol versus nucleus serves as a reliable marker of the state of activation of mechanotransduction pathways in human dermal fibroblasts.

Therefore, additional experiments were performed to determine YAP/TAZ cellular localization in fibroblasts treated with botanical polyphenol compounds described above (see *Results, II*). The most potent compounds, showing a 50%+ increase in average cell area with

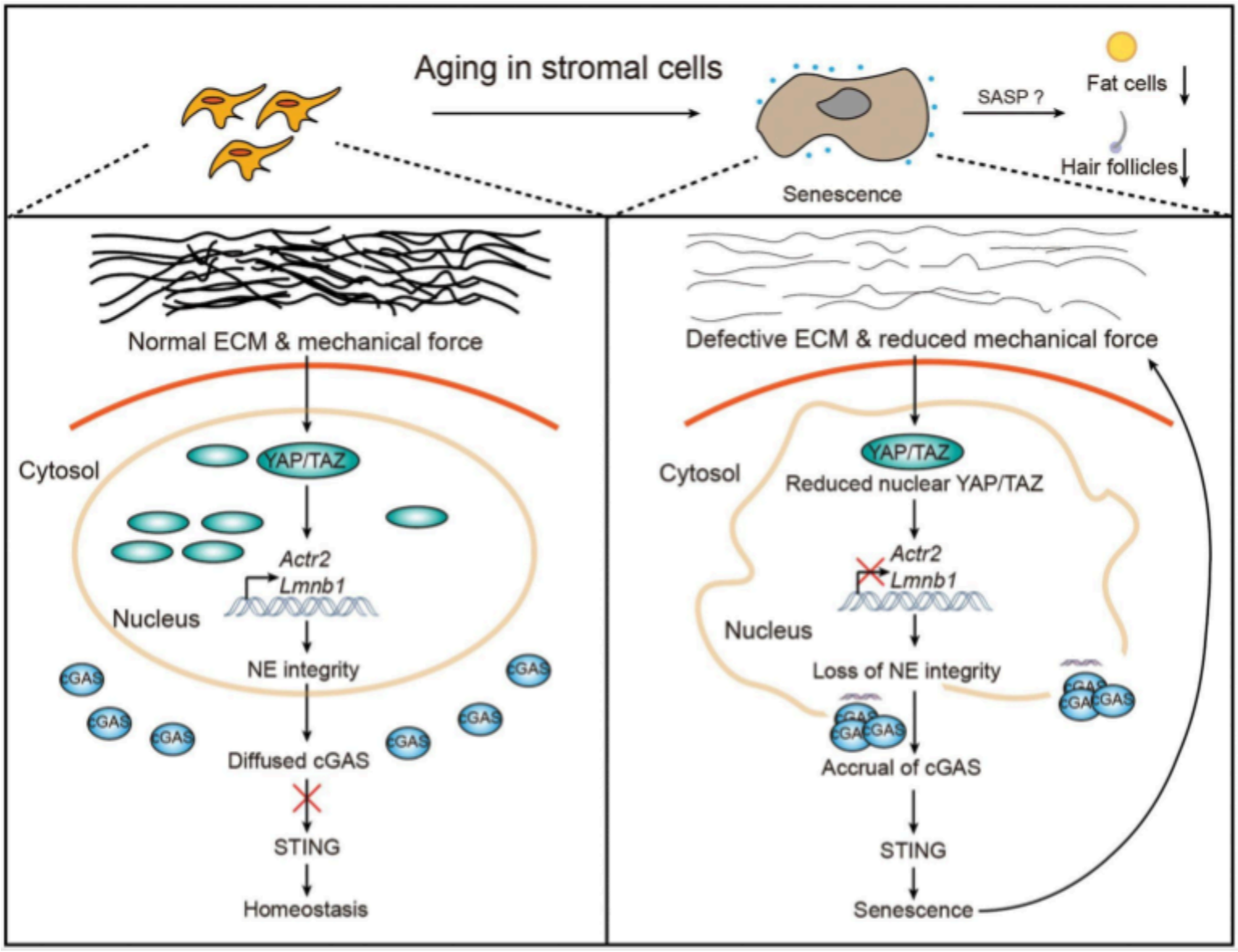

Figure 28. Illustration of the contribution of the YAP/TAZ modulation of aging in healthy vs. aged senescent stromal cells (i.e. fibroblasts) through cGAS-STING.[82]

respect to DMSO (Fig. 27), are apigenin, chlorogenic acid, and gallic acid. It then follows that these three compounds are expected to show higher nuclear YAP/TAZ localization compared to vehicle controls. Figure 29 outlines the results of the YAP/TAZ immunostaining experiments.

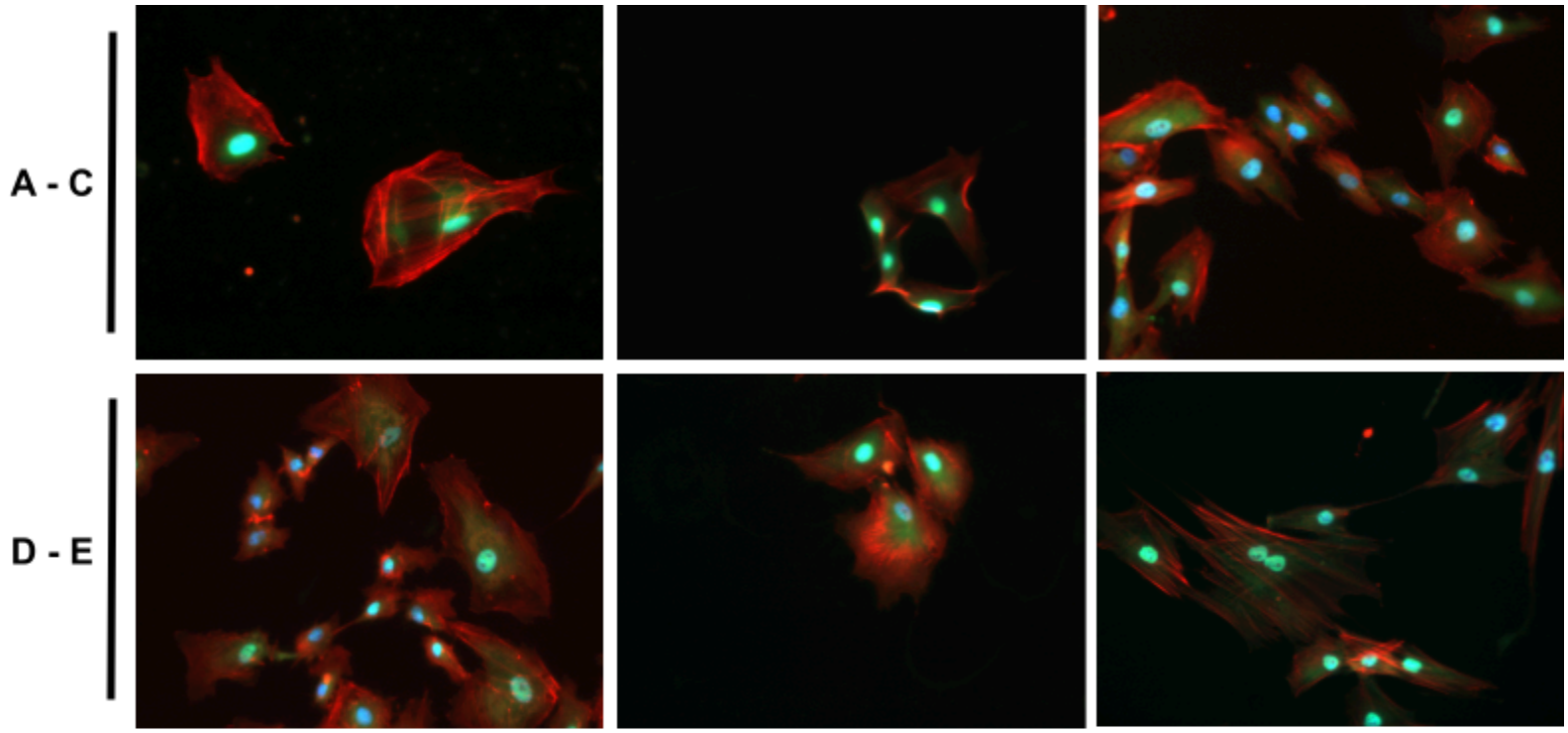

Figure 29. (A - C) 20X images of cells grown in 0.2kPa stiffness plates stained with DAPI (blue, nuclear), phalloidin (red), and YAP/TAZ antibody (green) and treated for 24hrs with apigenin, chlorogenic acid, and gallic acid, respectively. **(D - E)** 20X images of cells grown in same conditions treated for 24hrs with jasplakinolide, DMSO vehicle control, and no treatment (plastic plate controls), respectively.

These results are preliminary to ongoing research and deeper analysis of YAP/TAZ and in response to treatment with botanical polyphenol compounds. High ECM stiffness, as communicated to the cell through integrin receptors, leads to actin polymerization and YAP/TAZ localization to the nucleus. In the nucleus, YAP/TAZ form complexes with TEAD (final effectors of the Hippo pathway) and numerous other transcription factors that bind gene enhancers to regulate transcription of target genes.[86] These results indicate that the treatments that stimulate actin assembly and increase fibroblast size induce YAP/TAZ nuclear localization, while those that negatively affect cell area sequester YAP/TAZ in the cytosol.

Discussion

Targeting human dermal fibroblast morphology through botanical polyphenol actin-binding compounds is a promising venture for the anti-aging scientific research community. Further research can take profit from the diets of cultures exhibiting a lower abundance of aging markers in the skin (as well as in overall health and disease). These non-Western diets avoid processed and refined sugars and dairy fats and are instead heavily composed of whole fruits, vegetables, grains, and select tea leaves with major antioxidant, anti-inflammatory, and potential anti-cancerous activities. Healthy skin truly begins from within as applying expensive, improperly used, and potentially toxic chemicals to the bare skin without a second thought about why the skin exhibits signs of aging or malnourishment in the first place is more like a hamster running in place on a wheel in its cage rather than a marathoner completing a 5k.

The compounds tested within the scope of this study were isolated and not present as their naturally derived extracts from the commonly eaten foods in these ideal diets. Indeed, we aimed to isolate those most often mentioned in previously reported credibly significant study results to pinpoint the exact components with the anti-aging potential that was observed. It is known that fibroblasts undergo morphological changes when put under the pressures of different ECM stiffnesses, as also observed through the use of our specialized rigidity plates and actin staining methods. The data on measurements of cell area and cell aspect ratio were not optimal in regard to the rigidity experiments due to excessive overlapping cell-to-cell contacts in the FOV that prevented taking more accurate measurements, leading to large variability although the overall trends aforementioned (see *Results, I*) followed what was expected.

Although *in vitro* cultures are a simple model system, I expect that our results can be reasonably extrapolated to the skin *in vivo* since the fundamental mechanisms by which fibroblasts perceive and respond to mechanical forces are likely to be similar *in vitro* and *in vivo*. For the sake of future research, extra care and time should be taken to optimize cell seeding numbers and growth conditions to avoid overcrowding to obtain the most reliable data. Staining protocols were mostly successful through our methods and provided clear visualization of individual actin fibers, advancing the insight into the state of fibrillar polymerization when cells are grown in different stiffness conditions, mimicking those of different stages of ECM degradation obsrved in human skin.

In *Introduction, I*, it was mentioned that isolated compounds were chosen over the extracts they are found to be components of in relevant studies previously published. This was decided as extracts are composed of several different compounds and causation cannot be determined over correlation when the exact compound is not known. Although our results are promising on some compounds in isolation (i.e. apigenin, chlorogenic acid, and gallic acid), those that performed more poorly in terms of increasing average cell area should not be entirely thrown out in their potential in different contexts. These compounds may fare better when used in a naturally derived extract rather than alone, with positive pathway crosstalk and productive interactions with other compounds present in extracts being a possibility.

YAP and TAZ are two effectors from the Hippo pathway that act as markers of mechanical force imposed on HDFs. YAP/TAZ analyses are still ongoing within the Fisher Photoaging & Aging Lab at the University of Michigan. However, the experiments performed at least helped to confirm the morphological changes detected in HDFs treated with our chosen botanical polyphenol actin-binding compounds in *Results, II*. The objective was to investigate

whether induction of actin assembly resulted in increased YAP/TAZ nuclear localization, as visualized by immunostaining (*Results, III*). More YAP/TAZ should be seen around the nuclei of larger, more stretched cells with more organized F-actin cytoskeletons as this nuclear shuttling of these transcription cofactors is associated with YAP/TAZ activation and target gene regulation to induce the production of more ECM proteins by fibroblasts. As YAP/TAZ is reduced with aging, a therapeutic approach that is worth considering is to target regulation of YAP/TAZ to induce ECM production and thereby regain proper cell functioning, potentially reducing the outward signs of aging. The reciprocities between the several effectors of the Hippo pathway (as well as those that engage in crosstalk with Hippo) are not well understood and should be further researched in detail in order to determine whether YAP or TAZ (or both, or neither) are causative markers that can be targeted in isolation for these purposes.

Conclusion

In aged human skin, fibroblasts acquire an aged phenotype when the surrounding ECM stiffness is low, and this phenotypic response can be modeled *in vitro* by the culture of fibroblasts under low stiffness conditions. Cell area and cell aspect ratio should be helpful measures when it comes to overall fibroblast structure, but we found there was great variability that held back definitive conclusions. The general trends still followed as cells in greater stiffness plates were somewhat correlated with greater cell area, though cell aspect ratio was relatively similar among all groups. Results were comparatively significant for the percentage of cell fibers oriented with the major cell axis and fiber S-parameter. Results for this analysis would likely have been significant across the board had more time and resources been available to extend the initial portion of the study during which we were calculating cell growth rates and determining optimal cell seeding patterns for each dish type. Overall, our results reflect observations made in previous studies and can be made clearer through additional research, as well as some expansion on ancillary ECM proteins not described here.

Our botanical compound treatment results demonstrated that measurements of cell area were reliable and significant. For these studies, cell seeding was kept to a minimum, using our earlier studies as reference for reducing cell overlap in the FOV. The botanical polyphenol actin-binding compounds chosen for this study are naturally derived, isolated compounds from extracts frequently mentioned in published research studies on their potential anti-aging effects. The studies presented here were conducted with pure compounds, rather than extracts, in order to compare the bioactivities of known compounds. In order of least to most effective in increasing average fibriblast cell area, jasplakinolide and luteolin-7-O-glucoside came last with a negative impact, followed by catechin, kaempferol-3-O-glucoside, epicatechin,

quercetin-3-β-D-glucoside, allantoin, gallic acid, chlorogenic acid, and finally apigenin with the most promising effects on average cell area. These results should be expanded upon in further research with direct comparisons between their isolated effects and when in extracts from different plants, as well as additional work regarding the pathways that they affect to understand the deeper mechanisms behind their actions.

YAP/TAZ are undergoing further research within the lab through which the research regarding this paper was conducted. With the information collected, however, it is clear that YAP/TAZ are major mechanical markers for cell activity and the corresponding orientations and polymerized states of their actin fibers. With a more organized actin cytoskeleton that came as a result of treatment with either apigenin, chlorogenic acid, or gallic acid, more YAP/TAZ was found localized to the nucleus, thus indicating the higher levels of mechanical force experienced by HDFs. In contrast, jasplakinolide treatment resulted in more diffused green staining in the cytosol rather than concentrated at the nucleus as with control cells grown on plastic plates.

The results presented here provide a foundation for further studies on the underlying mechanisms of skin aging and the development of novel approaches to prevent or reverse its consequences. Additional research on these matters will benefit the cosmetics industry and the people who use it for dermal therapies.

Acknowledgments

For my family and friends who believed in me from the beginning, I thank them for their endless, unconditional support while I worked on this research these past two years and on this thesis throughout my beyond-memorable final semester as a University of Michigan undergraduate student. Without them, these opportunities would not have even been within my reach to begin to achieve. I owe them more than these words could ever offer.

Further, I would like to express my special thanks of gratitude to the virtuoso scientists so committed to contributing to novel discoveries within the field of dermatology for providing me with the foundations of how fine research is conducted by sharing their time, expertise, and advice with me. These thanks specifically go out to the members of the Fisher Photoaging & Aging Lab with the University of Michigan's Department of Dermatology: Dr. Gary Fisher, the pioneer of this project and my first mentor in research science who took me in with no prior experience, training me from the ground up and who provided the space, materials, and team of talented professionals for this project to transpire; Dr. Yilei Cui, my kindhearted rock in the laboratory throughout the lengthy process, dedicating her invaluable time to teaching new methods, providing me with solidarity, appreciation, and encouragement each step of the way despite having several simultaneous scientific commitments outside of this particular project; Dr. Yunfeng Ma, for spearheading the biochemical treatments of cells; Ziqi Wu, PhD, for their meticulous work in extracting useful data from the images taken for this study and creating beautiful figures for our use; Sarina Sadeghzadeh for all of her assistance with cell maintenance. I am thankful for all members of the University of Michigan's Dermatology Department with whom I worked throughout my time there, whether through simply advising me on how to best use a microscope or working through complicated texts, data, and experiments alongside me.

References

(1) Hareven, Tamara K. "Changing images of aging and the social construction of the life course." *Images of aging*. Routledge, 2003. 129-144.

(2) Petruzzi, D. *Anti-aging market size worldwide 2021-2027*. Statista. https://www.statista.com/statistics/509679/value-of-the-global-anti-aging-market

(3) Holman, D. M.; Berkowitz, Z.; Guy, G. P.; Hawkins, N. A.; Saraiya, M.; Watson, M. *Patterns of Sunscreen Use on the Face and Other Exposed Skin among US Adults*. Journal of the American Academy of Dermatology 2015, 73 (1), 83-92.e1. https://doi.org/10.1016/j.jaad.2015.02.1112.

(4) Barrett, J. R. Chemical Exposures: The Ugly Side of Beauty Products. *Environmental Health Perspectives* **2005**, *113* (1). https://doi.org/10.1289/ehp.113-a24.

(5) Nutrition, C. for F. S. and A. Phthalates in Cosmetics. *FDA* **2022**.

(6) Landsverk, G. *A 45 year-old tech executive's extreme longevity routine to age backwards involves taking over 100 daily supplements and waking up at 5 a.m.* Business Insider. https://www.businessinsider.com/tech-executive-shared-two-million-daily-routine-age-reverse-2023-7.

(7) Maughan, R. J.; King, D. S.; Lea, T. Dietary Supplements. *Journal of Sports Sciences* **2004**, *22* (1), 95–113. https://doi.org/10.1080/0264041031000140581.

(8) Draelos, Z. D. Nutrition and Enhancing Youthful-Appearing Skin. *Clinics in Dermatology* **2010**, *28* (4), 400–408. https://doi.org/10.1016/j.clindermatol.2010.03.019.

(9) Ooraikul, B.; Sirichote, A.; Siripongvutikorn, S. Southeast Asian Diets and Health Promotion. *Wild-Type Food in Health Promotion and Disease Prevention* **2008**, 515–533. https://doi.org/10.1007/978-1-59745-330-1_36.

(10) Qazi, A.; Pal, J.; Maitah, M. Y.; Fulciniti, M.; Dheeraj Pelluru; Puru Nanjappa; Lee, S.; Batchu, R. B.; Prasad, M.; Bryant, C.; Rajput, S.; Gryaznov, S. M.; Beer, D. G.; Weaver, D. F.; Munshi, N. C.; Goyal, R. K.; Shammas, M. A. Anticancer Activity of a Broccoli Derivative, Sulforaphane, in Barrett Adenocarcinoma: Potential Use in Chemoprevention and as Adjuvant in Chemotherapy. Translational Oncology 2010, 3 (6), 389–399. https://doi.org/10.1593/tlo.10235.

(11) Carlsson, A. E. Actin Dynamics: From Nanoscale to Microscale. *Annual review of biophysics* **2010**, *39*, 91–110. https://doi.org/10.1146/annurev.biophys.093008.131207.

(12) Geary, L. E.; Lazarides, E.; Goetz, I.; Roberts, E. Actin Filaments in Human Skin Fibroblasts Are Similar in Normal Persons and Patients with Huntington's Disease. *Annals of Neurology* **1978**, *4* (6), 554–556. https://doi.org/10.1002/ana.410040614.

(13) AAT Bioquest. *Human Dermal Fibroblasts Stained with Phalloidin-IFluor® 514.* (Image).

(14) *Skincare science: Skin anatomy*. Nagarkar Plastic Surgery. https://drnagarkar.com/skin-anatomy/. (Image).

(15) Tracy, L. E.; Minasian, R. A.; Caterson, E. J. Extracellular Matrix and Dermal Fibroblast Function in the Healing Wound. Advances in Wound Care 2016, 5 (3), 119–136. https://doi.org/10.1089/wound.2014.0561.

(16) Yue, B. Biology of the Extracellular Matrix. *Journal of Glaucoma* **2014**, *23* (8), S20–S23. https://doi.org/10.1097/ijg.0000000000000108.

(17) Farage, M. A.; Miller, K. W.; Elsner, P.; Maibach, H. I. Characteristics of the Aging Skin. *Advances in Wound Care* **2013**, *2* (1), 5–10. https://doi.org/10.1089/wound.2011.0356.

(18) Wilson, C.; González-Billault, C. Regulation of Cytoskeletal Dynamics by Redox Signaling and Oxidative Stress: Implications for Neuronal Development and Trafficking. *Frontiers in Cellular Neuroscience* **2015**, *9*. https://doi.org/10.3389/fncel.2015.00381.

(19) Papaccio, F.; D′Arino, A.; Caputo, S.; Bellei, B. Focus on the Contribution of Oxidative Stress in Skin Aging. *Antioxidants* **2022**, *11* (6), 1121. https://doi.org/10.3390/antiox11061121.

(20) Pittayapruek, P.; Meephansan, J.; Prapapan, O.; Komine, M.; Ohtsuki, M. Role of Matrix Metalloproteinases in Photoaging and Photocarcinogenesis. *International Journal of Molecular Sciences* **2016**, *17* (6), 868. https://doi.org/10.3390/ijms17060868.

(21) Cabral-Pacheco, G. A.; Garza-Veloz, I.; Castruita-De la Rosa, C.; Ramirez-Acuña, J. M.; Perez-Romero, B. A.; Guerrero-Rodriguez, J. F.; Martinez-Avila, N.; Martinez-Fierro, M. L. The Roles of Matrix Metalloproteinases and Their Inhibitors in Human Diseases. *International Journal of Molecular Sciences* **2020**, *21* (24), 9739. https://doi.org/10.3390/ijms21249739.

(22) Syed, D. N.; Mukhtar, H. Botanicals for the Prevention and Treatment of Cutaneous Melanoma. *Pigment Cell & Melanoma Research* **2011**, *24* (4), 688–702. https://doi.org/10.1111/j.1755-148x.2011.00851.x.

(23) Melchionna, R.; Trono, P.; Tocci, A.; Nisticò, P. Actin Cytoskeleton and Regulation of TGFβ Signaling: Exploring Their Links. *Biomolecules* **2021**, *11* (2), 336. https://doi.org/10.3390/biom11020336.

(24) Qin, Z.; Fisher, G. J.; Voorhees, J. J.; Quan, T. Actin Cytoskeleton Assembly Regulates Collagen Production via TGF‐β Type II Receptor in Human Skin Fibroblasts. *Journal of Cellular and Molecular Medicine* **2018**, *22* (9), 4085–4096. https://doi.org/10.1111/jcmm.13685.

(25) Haydont, V.; Bernard, B. A.; Fortunel, N. O. Age-Related Evolutions of the Dermis: Clinical Signs, Fibroblast and Extracellular Matrix Dynamics. *Mechanisms of Ageing and Development* **2019**, *177*, 150–156. https://doi.org/10.1016/j.mad.2018.03.006. (Image).

(26) Vauzour, D.; Rodriguez-Mateos, A.; Corona, G.; Oruna-Concha, M. J.; Spencer, J. P. E. Polyphenols and Human Health: Prevention of Disease and Mechanisms of Action. *Nutrients* **2010**, *2* (11), 1106–1131. https://doi.org/10.3390/nu2111106.

(27) Afaq, F.; Katiyar, S. K. Polyphenols: Skin Photoprotection and Inhibition of Photocarcinogenesis. *Mini reviews in medicinal chemistry* **2011**, *11* (14), 1200–1215. https://doi.org/10.2174/13895575111091200.

(28) Cipolletti, M.; Solar Fernandez, V.; Montalesi, E.; Marino, M.; Fiocchetti, M. Beyond the Antioxidant Activity of Dietary Polyphenols in Cancer: The Modulation of Estrogen Receptors (ERs) Signaling. *International Journal of Molecular Sciences* **2018**, *19* (9), 2624. https://doi.org/10.3390/ijms19092624.

(29) Weyand, C. M.; Goronzy, J. J. Aging of the Immune System. Mechanisms and Therapeutic Targets. *Annals of the American Thoracic Society* **2016**, *13* (Supplement_5), S422–S428. https://doi.org/10.1513/annalsats.201602-095aw.

(30) Lee, J. H.; Park, J.; Shin, D. W. The Molecular Mechanism of Polyphenols with Anti-Aging Activity in Aged Human Dermal Fibroblasts. *Molecules* **2022**, *27* (14), 4351. https://doi.org/10.3390/molecules27144351. (Image).

(31) Шестопалов, А. В.; Shkurat, T. P.; Микашинович, З. И.; Kryzhanovskaia, I. O.; Bogacheva, M. A.; S.V. Lomteva; Prokof'ev, V. P.; Gus'kov, E. P. Biological Functions of Allantoin. **2006**, *33* (5), 437–440. https://doi.org/10.1134/s1062359006050037.

(32) Caldwell, A. *Allantoin in skin care: Benefits, safety & side effects*. www.medicalnewstoday.com. https://www.medicalnewstoday.com/articles/allantoin.

(33) Zulema, K.; Rosa Alicia Saucedo-Acuña; Rios-Arana, J. V.; Lobo, N.; Rodriguez, C. J.; Juan Carlos Cuevas-González; Karla Lizette Tovar-Carrillo. Natural Film Based on

Pectin and Allantoin for Wound Healing: Obtaining, Characterization, and Rat Model. *BioMed Research International* **2020**, *2020*, 1–7. https://doi.org/10.1155/2020/6897497.

(34) Gabison, L.; Chiadmi, M.; Colloc'h, N.; Castro, B.; El Hajji, M.; Prangé, T. Recapture of [S]-Allantoin, the Product of the Two-Step Degradation of Uric Acid, by Urate Oxidase. *FEBS Letters* **2006**, *580* (8), 2087–2091. https://doi.org/10.1016/j.febslet.2006.03.007.

(35) Shankar, E.; Goel, A.; Gupta, K.; Gupta, S. Plant Flavone Apigenin: An Emerging Anticancer Agent. *Current Pharmacology Reports* **2017**, *3* (6), 423–446. https://doi.org/10.1007/s40495-017-0113-2.

(36) Yoon, J. H.; Kim, M.-Y.; Cho, J. Y. Apigenin: A Therapeutic Agent for Treatment of Skin Inflammatory Diseases and Cancer. *International Journal of Molecular Sciences* **2023**, *24* (2), 1498. https://doi.org/10.3390/ijms24021498.

(37) Zhang, Y.; Wang, J.; Cheng, X.; Yi, B.; Zhang, X.; Li, Q. Apigenin Induces Dermal Collagen Synthesis via Smad2/3 Signaling Pathway. *European Journal of Histochemistry* **2015**, *59* (2). https://doi.org/10.4081/ejh.2015.2467.

(38) Vo, J. *Nutrition & Health Info Sheets for Health Professionals - Catechins and Epicatechins*. UC Davis Nutrition Department. https://nutrition.ucdavis.edu/outreach/nutr-health-info-sheets/pro-catechins.

(39) Hur, J.; Kim, A-Ram.; Hyun Su Kim; Lim, C.; Kim, T.; Kim, T.-A.; Sim, J.; Suh, Y.-G. Concise Synthesis of Catechin Metabolites 5-(3′,4′-Dihydroxyphenyl)-γ-Valerolactones (DHPV) in Optically Pure Form and Their Stereochemical Effects on Skin Wrinkle-Reducing Activities. *Molecules* **2020**, *25* (8), 1970–1970. https://doi.org/10.3390/molecules25081970.

(40) Tanigawa, T.; Kanazawa, S.; Ichibori, R.; Fujiwara, T.; Magome, T.; Shingaki, K.; Miyata, S.; Hata, Y.; Tomita, K.; Matsuda, K.; Kubo, T.; Tohyama, M.; Yano, K.; Hosokawa, K. (+)-Catechin Protects Dermal Fibroblasts against Oxidative Stress-Induced Apoptosis. *BMC Complementary and Alternative Medicine* **2014**, *14* (1). https://doi.org/10.1186/1472-6882-14-133.

(41) A. Casanal; Zander, U.; V. Valpuesta; Marquez, J. A. Crystal Structure of the Strawberry Pathogenesis-Related 10 (PR-10) Fra a 3 Protein in Complex with Catechin. **2013**. https://doi.org/10.2210/pdb4c94/pdb.

(42) Lu, H.; Tian, Z.; Cui, Y.; Liu, Z.; Ma, X. Chlorogenic Acid: A Comprehensive Review of the Dietary Sources, Processing Effects, Bioavailability, Beneficial Properties, Mechanisms of Action, and Future Directions. *Comprehensive Reviews in Food Science and Food Safety* **2020**, *19* (6), 3130–3158. https://doi.org/10.1111/1541-4337.12620.

(43) Alves, G. de A. D.; Oliveira de Souza, R.; Ghislain Rogez, H. L.; Masaki, H.; Fonseca, M. J. V. Cecropia Obtusa Extract and Chlorogenic Acid Exhibit Anti Aging Effect in Human Fibroblasts and Keratinocytes Cells Exposed to UV Radiation. *PloS One* **2019**, *14* (5), e0216501. https://doi.org/10.1371/journal.pone.0216501.

(44) Moghadam, S.; Ebrahimi, S.; Salehi, P.; Moridi Farimani, M.; Hamburger, M.; Jabbarzadeh, E. Wound Healing Potential of Chlorogenic Acid and Myricetin-3-O-β-Rhamnoside Isolated from Parrotia Persica. *Molecules* **2017**, *22* (9), 1501. https://doi.org/10.3390/molecules22091501.

(45) N. Neetu; M. Katiki; Kumar, P. Crystal Structure of Providencia Alcalifaciens 3-Dehydroquinate Synthase (DHQS) in Complex with Mg2+, NAD and Chlorogenic Acid. **2020**. https://doi.org/10.2210/pdb6lk2/pdb.

(46) Moskot, M.; Jakóbkiewicz-Banecka, J.; Kloska, A.; Piotrowska, E.; Narajczyk, M.; Gabig-Cimińska, M. The Role of Dimethyl Sulfoxide (DMSO) in Gene Expression Modulation and Glycosaminoglycan Metabolism in Lysosomal Storage Disorders on an Example of Mucopolysaccharidosis. *International Journal of Molecular Sciences* **2019**, *20* (2), 304. https://doi.org/10.3390/ijms20020304.

(47) Burkhard, P.; Taylor, P.; Walkinshaw, M. D. FKBP COMPLEXED with DMSO. **1999**. https://doi.org/10.2210/pdb1d7h/pdb.

(48) Milenkovic, D.; Wim Vanden Berghe; Morand, C.; Claude, S.; Annette; Gorressen, S.; Laurent-Emmanuel Monfoulet; Chandra Sekhar Chirumamilla; Declerck, K.; Katarzyna Szarc vel Szic; Maija Lahtela-Kakkonen; Gerhäuser, C.; Merx, M. W.; Kelm, M. A Systems Biology Network Analysis of Nutri(Epi)Genomic Changes in Endothelial Cells Exposed to Epicatechin Metabolites. *Scientific Reports* **2018**, *8* (1). https://doi.org/10.1038/s41598-018-33959-x.

(49) Offermann, L. R.; Hurlburt, B. K.; K.A. Majorek; McBride, J. K.; Maleki, S. J.; M. Chruszcz. Crystal Structure of Ara H 8 with Epicatechin Bound. **2013**. https://doi.org/10.2210/pdb4ma6/pdb.

(50) Carnevale, R.; Loffredo, L.; Nocella, C.; Bartimoccia, S.; Bucci, T.; De Falco, E.; Peruzzi, M.; Chimenti, I.; Biondi-Zoccai, G.; Pignatelli, P.; Violi, F.; Frati, G. Epicatechin and Catechin Modulate Endothelial Activation Induced by Platelets of Patients with Peripheral Artery Disease. *Oxidative Medicine and Cellular Longevity* **2014**, *2014*. https://doi.org/10.1155/2014/691015.

(51) Daglia, M.; Di Lorenzo, A.; Nabavi, S. F.; Talas, Z. S.; Nabavi, S. M. Polyphenols: Well beyond the Antioxidant Capacity: Gallic Acid and Related Compounds as Neuroprotective Agents: You Are What You Eat! *Current Pharmaceutical Biotechnology* **2014**, *15* (4), 362–372. https://doi.org/10.2174/1389201015041408251207 37.

(52) Pang, J.-H. S.; Yen, J.-H.; Wu, H.-T.; Huang, S.-T. Gallic Acid Inhibited Matrix Invasion and AP-1/ETS-1-Mediated MMP-1 Transcription in Human Nasopharyngeal Carcinoma Cells. *International Journal of Molecular Sciences* **2017**, *18* (7), 1354–1354. https://doi.org/10.3390/ijms18071354.

(53) Tsang, M.; Jiao, D.; Chan, B.; Hon, K.-L.; Leung, P.; Lau, C.; Wong, E.; Cheng, L.; Chan, C.; Lam, C.; Wong, C. Anti-Inflammatory Activities of Pentaherbs Formula, Berberine, Gallic Acid and Chlorogenic Acid in Atopic Dermatitis-like Skin Inflammation. *Molecules* **2016**, *21* (4), 519. https://doi.org/10.3390/molecules21040519.

(54) E. Kyriakis; G.A. Stravodimos; A.L. Kantsadi; D.S.M. Chatzileontiadou; Skamnaki, V. T.; Leonidas, D. D. Natural Flavonoids as Antidiabetic Agents. The Binding of Gallic and Ellagic Acids to Glycogen Phosphorylase B. *FEBS Letters* **2015**, *589* (15), 1787–1794. https://doi.org/10.1016/j.febslet.2015.05.013.

(55) Bubb, M. R.; Senderowicz, A. M.; Sausville, E. A.; Duncan, K. L.; Korn, E. D. Jasplakinolide, a Cytotoxic Natural Product, Induces Actin Polymerization and Competitively Inhibits the Binding of Phalloidin to F-Actin. *The Journal of Biological Chemistry* **1994**, *269* (21), 14869–14871.

(56) Hellewell, A. L.; Gong, X.; Karsten Schärich; Christofidou, E. D.; Adams, J. C. Modulation of the Extracellular Matrix Patterning of Thrombospondins by Actin Dynamics and Thrombospondin Oligomer State. *Bioscience Reports* **2015**, *35* (3). https://doi.org/10.1042/bsr20140168.

(57) Bank, R. P. D. *RCSB PDB - 9UE Ligand Summary Page*. www.rcsb.org. https://www.rcsb.org/ligand/9UE (accessed 2024-02-16).

(58) Holzinger, A. Jasplakinolide: An Actin-Specific Reagent That Promotes Actin Polymerization. *Cytoskeleton Methods and Protocols* **2009**, 71–87. https://doi.org/10.1007/978-1-60761-376-3_4.

(59) Kashyap, D.; Sharma, A.; Tuli, H. S.; Sak, K.; Punia, S.; Mukherjee, T. K. Kaempferol – a Dietary Anticancer Molecule with Multiple Mechanisms of Action: Recent Trends and Advancements. *Journal of Functional Foods* **2017**, *30*, 203–219. https://doi.org/10.1016/j.jff.2017.01.022.

(60) Kim, J.; Kim, H.-S.; Choi, D.-H.; Choi, J.; Cho, S. Y.; Kim, S.-H.; Baek, H.-S.; Yoon, K. D.; Son, S. W.; Son, E. D.; Hong, Y.-D.; Ko, J.; Cho, S.-Y.; Park, W.-S. Kaempferol Tetrasaccharides Restore Skin Atrophy via PDK1 Inhibition in Human Skin Cells and Tissues: Bench and Clinical Studies. *Biomedicine & Pharmacotherapy* **2022**, *156*, 113864. https://doi.org/10.1016/j.biopha.2022.113864.

(61) Imran, M.; Salehi, B.; Sharifi-Rad, J.; Aslam Gondal, T.; Saeed, F.; Imran, A.; Shahbaz, M.; Tsouh Fokou, P. V.; Umair Arshad, M.; Khan, H.; Guerreiro, S. G.; Martins, N.; Estevinho, L. M. Kaempferol: A Key Emphasis to Its Anticancer Potential. *Molecules* **2019**, *24* (12), 2277. https://doi.org/10.3390/molecules24122277.

(62) Imran, M.; Saeed, F.; Hussain, G.; Imran, A.; Mehmood, Z.; Gondal, T. A.; El‐Ghorab, A.; Ahmad, I.; Pezzani, R.; Arshad, M. U.; Bacha, U.; Shariarti, M. A.; Rauf, A.; Muhammad, N.; Shah, Z. A.; Zengin, G.; Islam, S. Myricetin: A Comprehensive Review on Its Biological Potentials. *Food Science & Nutrition* **2021**, *9* (10), 5854–5868. https://doi.org/10.1002/fsn3.2513.

(63) Jung, S. K.; Lee, K. W.; Kim, H. Y.; Oh, M. H.; Byun, S.; Lim, S. H.; Heo, Y.-S.; Kang, N. J.; Bode, A. M.; Dong, Z.; Lee, H. J. Myricetin Suppresses UVB-Induced Wrinkle Formation and MMP-9 Expression by Inhibiting Raf. *Biochemical Pharmacology* **2010**, *79* (10), 1455–1461. https://doi.org/10.1016/j.bcp.2010.01.004.

(64) Rivière, G.; Oueslati, S.; Gayral, M.; Créchet, J.-B.; Nhiri, N.; Jacquet, E.; Cintrat, J.-C.; Giraud, F.; van Heijenoort, C.; Lescop, E.; Pethe, S.; Iorga, B. I.; Naas, T.; Guittet, E.; Morellet, N. NMR Characterization of the Influence of Zinc(II) Ions on the Structural and Dynamic Behavior of the New Delhi Metallo-β-Lactamase-1 and on the Binding with Flavonols as Inhibitors. *ACS Omega* **2020**, *5* (18), 10466–10480. https://doi.org/10.1021/acsomega.0c00590.

(65) Wu, Z.; Zheng, X.; Gong, M.; Li, Y. Myricetin, a Potent Natural Agent for Treatment of Diabetic Skin Damage by Modulating TIMP/MMPs Balance and Oxidative Stress. *Oncotarget* **2016**, *7* (44). https://doi.org/10.18632/oncotarget.12330.

(66) Marefati, N.; Ghorani, V.; Shakeri, F.; Boskabady, M.; Kianian, F.; Rezaee, R.; Boskabady, M. H. A Review of Anti-Inflammatory, Antioxidant, and Immunomodulatory Effects of Allium Cepa and Its Main Constituents. *Pharmaceutical Biology* **2021**, *59* (1), 287–302. https://doi.org/10.1080/13880209.2021.1874028.

(67) You, W.; Zheng, W.; Weiss, S.; Chua, K. F.; Steegborn, C. Structural Basis for the Activation and Inhibition of Sirtuin 6 by Quercetin and Its Derivatives. *Scientific Reports* **2019**, *9* (1). https://doi.org/10.1038/s41598-019-55654-1.

(68) Park, C. M.; Song, Y.-S. Luteolin and Luteolin-7-O-Glucoside Inhibit Lipopolysaccharide-Induced Inflammatory Responses through Modulation of NF-KB/AP-1/PI3K-Akt Signaling Cascades in RAW 264.7 Cells. *Nutrition Research and Practice* **2013**, *7* (6), 423. https://doi.org/10.4162/nrp.2013.7.6.423.

(69) Begum, A.; Olofsson, A.; A.E. Sauer-Eriksson. Crystal Structure of Human Transthyretin Variant V30M in Complex with Luteolin. **2015**. https://doi.org/10.2210/pdb4qya/pdb.

(70) Eui Jeong Nam; Yoo, G.; Joo Young Lee; Kim, M.; Changho Jhin; Son, Y.-J.; Sun Young Kim; Sang Hoon Jung; Chu Won Nho. Glycosyl Flavones from *Humulus Japonicus* Suppress MMP-1 Production via Decreasing Oxidative Stress in UVB Irradiated Human Dermal Fibroblasts. *Journal of Biochemistry and Molecular Biology* **2020**, *53* (7), 379–384. https://doi.org/10.5483/bmbrep.2020.53.7.253.

(71) *Advanced BioMatrix - CytoSoft® Rigidity Plates*. advancedbiomatrix.com. https://advancedbiomatrix.com/cytosoft/.

(72) Norzain, N. A.; Yu, Z.-W.; Lin, W.-C.; Su, H.-H. Micropatterned Fibrous Scaffold Produced by Using Template-Assisted Electrospinning Technique for Wound Healing Application. *Polymers* 2021, 13 (16), 2821. https://doi.org/10.3390/polym13162821.

(73) Harvey, M. H.; Wedgwood, K. R.; Reber, H. A. Treatment of Acute Pancreatitis with Beta-Adrenergic Agonist Drugs. *Surgery* **1987**, *102* (2), 229–234. (Image).

(74) Ramalingam, R.; Jiang, G.; Larjava, H.; Häkkinen, L. Macromolecular Crowding Regulates Matrix Composition and Gene Expression in Human Gingival Fibroblast Cultures. *Scientific Reports* 2023, *13* (1). https://doi.org/10.1038/s41598-023-29252-1.

(75) Hakkinen, K. M.; Harunaga, J. S.; Doyle, A. D.; Yamada, K. M. Direct Comparisons of the Morphology, Migration, Cell Adhesions, and Actin Cytoskeleton of Fibroblasts in

Four Different Three-Dimensional Extracellular Matrices. *Tissue Engineering Part A* 2011, *17* (5-6), 713–724. https://doi.org/10.1089/ten.tea.2010.0273.

(76) Lacouture, M. E.; Schaffer, J. L.; Klickstein, L. B. A Comparison of Type I Collagen, Fibronectin, and Vitronectin in Supporting Adhesion of Mechanically Strained Osteoblasts. *Journal of Bone and Mineral Research* 2002, *17* (3), 481–492. https://doi.org/10.1359/jbmr.2002.17.3.481.

(77) Cooper, J. A. Effects of Cytochalasin and Phalloidin on Actin. *The Journal of Cell Biology* **1987**, *105* (4), 1473–1478. https://doi.org/10.1083/jcb.105.4.1473.

(78) Solon, J.; Levental, I.; Sengupta, K.; Georges, P. C.; Janmey, P. A. Fibroblast Adaptation and Stiffness Matching to Soft Elastic Substrates. *Biophysical Journal* 2007, *93* (12), 4453–4461. https://doi.org/10.1529/biophysj.106.101386.

(79) Shutova, M. S.; Boehncke, W.-H. Mechanotransduction in Skin Inflammation. *Cells* 2022, *11* (13), 2026. https://doi.org/10.3390/cells11132026.

(80) Kendall, R. T.; Feghali-Bostwick, C. A. Fibroblasts in Fibrosis: Novel Roles and Mediators. *Frontiers in Pharmacology* 2014, *5* (123). https://doi.org/10.3389/fphar.2014.00123.

(81) Haak, A. J.; Enis Kostallari; Sicard, D.; Ligresti, G.; Kyoung Moo Choi; Nunzia Caporarello; Jones, D. L.; Tan, Q.; Meridew, J. A.; Diaz, A. M.; Aravamudhan, A.; Maiers, J. L.; Britt, R. D.; Roden, A. C.; Pabelick, C. M.; Prakash, Y. S.; Mehdi Nouraie; Li, X.; Zhang, Y.; Kass, D. J. Selective YAP/TAZ Inhibition in Fibroblasts via Dopamine Receptor D1 Agonism Reverses Fibrosis. *Science Translational Medicine* 2019, *11* (516). https://doi.org/10.1126/scitranslmed.aau6296.

(82) Cao, X.; Wang, W.; Zhao, B. YAP/TAZ Links Mechanosensing to Aging. *Life Medicine* 2022. https://doi.org/10.1093/lifemedi/lnac039. (Image).

(83) Lee, M.-J.; Byun, M. R.; Furutani-Seiki, M.; Hong, J.-H.; Jung, H.-S. YAP and TAZ Regulate Skin Wound Healing. *Journal of Investigative Dermatology* 2014, *134* (2), 518–525. https://doi.org/10.1038/jid.2013.339.

(84) Dupont, S.; Morsut, L.; Aragona, M.; Enzo, E.; Giulitti, S.; Cordenonsi, M.; Zanconato, F.; Le Digabel, J.; Forcato, M.; Bicciato, S.; Elvassore, N.; Piccolo, S. Role of YAP/TAZ in Mechanotransduction. *Nature* 2011, *474* (7350), 179–183. https://doi.org/10.1038/nature10137.

(85) Hanna Lucie Sladitschek-Martens; Guarnieri, A.; Giulia Brumana; Zanconato, F.; Battilana, G.; Romy Lucon Xiccato; Panciera, T.; Mattia Forcato; Silvio Bicciato; Guzzardo, V.; Matteo Fassan; Ulliana, L.; Alessandro Gandin; Tripodo, C.; Foiani, M.; Brusatin, G.; Michelangelo Cordenonsi; Piccolo, S. YAP/TAZ Activity in Stromal Cells Prevents Ageing by Controlling CGAS–STING. *Nature* **2022**, *607* (7920), 790–798. https://doi.org/10.1038/s41586-022-04924-6.

(86) Rognoni, E.; Walko, G. The Roles of YAP/TAZ and the Hippo Pathway in Healthy and Diseased Skin. *Cells* 2019, *8* (5), 411. https://doi.org/10.3390/cells8050411.