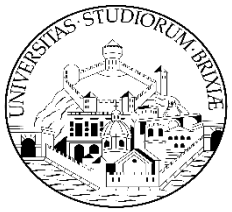

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DI BRESCIA

DOTTORATO DI RICERCA IN

INTELLIGENZA ARTIFICIALE IN MEDICINA E INNOVAZIONE

NELLA RICERCA CLINICA E METODOLOGICA

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DEVELOPMENT OF A 3D SKIN MODEL BASED ON BIOCOMPATIBLE POLYMERIC HYDROGEL SCAFFOLD ENGINEERED WITH HUMAN MESENCHYMAL STROMAL CELLS FOR HEALING OF SKIN WOUNDS

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RIASSUNTO

La medicina rigenerativa è una branca della medicina estremamente interdisciplinare focalizzata sulla riparazione, rigenerazione e sostituzione di cellule, tessuti e organi al fine di ripristinare funzionalità fisiologiche compromesse da malattie, difetti congeniti, traumi o invecchiamento.

Gli approcci ricostruttivi tradizionali non sempre hanno portato alla risoluzione del danno. La medicina rigenerativa è nata con l'idea di proporre applicazioni innovative e alternative ai metodi tradizionali. L'obiettivo principale è la rigenerazione dei tessuti tramite l'utilizzo di cellule stromali mesenchimali umane (hMSCs) da sole o in combinazione con scaffolds tridimensionali (3D).[1]

La medicina rigenerativa ha avuto delle buone applicazioni nel trattamento di lesioni cutanee di grado severo quali ulcere, ustioni, ferite profonde, difficili da trattare con metodi convenzionali. In clinica sono già presenti dei sostituti dermici, quali Integra® e MatriDerm® (che è attualmente il gold standard), che permettono di rigenerare il tessuto cutaneo promuovendo una cicatrizzazione stabile del tessuto, ma che presentano dei limiti dovuti all'alta incidenza di infezioni e agli alti costi di produzione.[2,3]

L'associazione di scaffold ingegnerizzati con le cellule stromali mesenchimali rappresenta una valida alternativa come trattamento delle lesioni cutanee.

Lo sviluppo di scaffolds ottimali per la crescita e il differenziamento cellulari è fondamentale per le tecniche di ingegneria tissutale. Gli scaffolds sono substrati 3D porosi progettati al fine di promuovere le interazioni cellula-scaffold e il trasporto di gas, sostanze nutritive e fattori regolatori per la sopravvivenza, proliferazione e differenziazione cellulari. Inoltre, gli scaffolds devono essere biocompatibili, bioriassorbibili, biodegradabili, bioattivi, non tossici e non immunogenici.[4-6]

In ambito dermatologico sta aumentando l'interesse per gli idrogeli, biomateriali polimerici 3D che possono presentare proprietà simili a quelle della matrice extracellulare (ECM). Gli idrogeli di derivazione naturale sono biocompatibili, bioattivi, biodegradabili e promuovono molte funzioni cellulari, mentre gli idrogeli sintetici sono riproducibili e caratterizzati da elevate proprietà meccaniche. Percìò gli idrogeli ibridi, che combinano i benefici sia dei polimeri naturali che di quelli sintetici, sono di notevole interesse per diverse applicazioni biologiche e biomediche.[7]

Sostanze naturali come i polimeri (acido ialuronico, collagene, fibrina) sono ampiamente utilizzati negli idrogeli. Tra questi, il chitosano (CH) ha suscitato notevole interesse poiché ha diverse proprietà che lo rendono idoneo alle applicazioni biomediche, tra le quali la biocompatibilità, la biodegradabilità, la non antigenicità, l'attività emostatica, antiinfiammatoria e antimicrobica. Inoltre, il CH ha una struttura simile ai componenti della ECM e presenta una superficie idrofilica che promuove l'adesione, la proliferazione e la differenziazione cellulare.[8] Gli idrogeli a base di gelatina (G) e CH sono stati ampiamente utilizzati nell'ambito della medicina rigenerativa per le loro proprietà di biocompatibilità e biodegradabilità, trovando una potenziale applicazione anche nella rigenerazione cutanea.[9,10]

Le hMSCs sono cellule ideali per la medicina rigenerativa in quanto possono essere espanse in grandi quantità, hanno capacità di autorinnovamento, migrano verso i tessuti danneggiati e hanno effetti immunomodulatori e trofici. Le hMSCs possono essere isolate da vari tessuti, principalmente da midollo osseo (BM), da cordone ombelicale (UC) e da tessuto adiposo (AT) e hanno un potenziale differenziativo multilineare. [11,12]

In diversi studi le hMSCs sono state coltivate in vitro sia in presenza di siero fetale bovino (FBS) che di lisato piastrinico umano (hPL) in quanto contengono fattori di crescita in grado di aumentare il grado di proliferazione delle hMSCs. hPL è un prodotto "clinical grade" e soddisfa la certificazione "Good Manufacturing Practices" (GMP) e può essere impiegato nei pazienti, mentre FBS non può essere somministrato nei pazienti a causa dell'elevato rischio di trasmissione di infezioni xenogeniche e di induzione di potenziali risposte immunitarie agli antigeni animali. I derivati piastrinici sono ampiamente usati in ambito clinico, in quanto contengono fattori di crescita responsabili della proliferazione cellulare, dell'angiogenesi e della migrazione cellulare che favoriscono la rigenerazione dei tessuti.[13]

In questo studio, abbiamo cercato di creare in vitro un dispositivo medicale "clinical grade" per un potenziale utilizzo nella medicina rigenerativa della cute. In particolare, sono stati sintetizzati, con una metodica innovativa, due tipologie di scaffold porosi a partire da G, polietilene glicole (PEG), CH e glicerolo (Gly): G-PEG-CH-Gly e G-PEG-CH.

È stata messa a punto una coltura monolayer (2D) per settare le condizioni di coltura per il differenziamento delle BM-hMSCS e delle UC-hMSCs a fibroblasti e cheratinociti. È seguita la coltura 3D in cui le BM-hMSCs e le UC-hMSCs sono state seminate negli idrogeli ibridi G-PEG-CH-Gly e G-PEG-CH in presenza di FBS o di hPL ed è stata valutata la loro vitalità, la proliferazione e la differenziazione verso la linea fibroblastica e cheratinocitica. È stata fatto un confronto tra il potenziale proliferativo e differenziativo degli idrogeli ibridi e quello del MatriDerm® (MD).

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INTRODUCTION

Tissue engineering and regenerative medicine

Tissue engineering is an interdisciplinary field that enforces the principle of biology and engineering to the development of substitutes for damaged tissue. The loss or failure of an organ or tissue is one of the most frequent and injurious problems in human health care.[14]

In the early 1970's W.T. Green, M.D. at the Children's Hospital performed some experiments to generate new cartilage using chondrocytes seeded onto spicules of bone and implanted in nude mice. Even if experiments were unsuccessful, he rightly concluded that the use of biocompatible scaffolds on which cells are seeded will allow new tissue to be generated.[15]

In the late 1970's and early 1980's the first successful tissue-engineered skin products were made.[16]

Bioengineering pioneer Fung originally introduced the term "tissue engineering" in 1985.[17]

The key event in the advent of tissue engineering was in the mid-1980's when Dr. Joseph Vacanti of the Children's Hospital and Dr. Robert Langer of MIT undertaken studies with the idea of being able to create tissue equivalents that promote cell transport instead of seeding cells in scaffolds with chemical and physical properties that cannot be controlled, thus resulting in unpredictable outcomes.[15] In 1993, Langer and Vacanti defined tissue engineering as an "interdisciplinary field that applies the principles of engineering and the life science toward the development of biological substitutes that restore, maintain, or improve tissue function". The following years, several centres have been organized in developed country of the world with the aim of exploring and defining the potential of tissue engineering.

Three pillars have supported tissue engineering:

-human stem cells capable of proliferating and differentiating.

-biocompatible scaffolds that constitute a three-dimensional support that promotes tissue regeneration.

-growth factors that activate relevant process such as cell differentiation, vascularization, and others, which are crucial for the process of tissue regeneration.

Regenerative medicine has been defined as "the process of replacing or regenerating human cells, tissues or organs to restore or establish normal function".[18] Regenerative medicine uses other strategies to induce organ regeneration including cell-based therapies, immunomodulation, gene therapy, nanomedicine and tissue engineering itself. Because of the similar goals, tissue engineering and regenerative medicine have been merging in recent years, originating the broad field of TERM.

In the human body there are tissues with a limited repair/regenerative capacity, which represents an arduous challenge to overcome. Therefore, to achieve the proposed objectives, TERM was based on different strategies, such as the combined use of scaffolds, cells and growth factors.[19]

Cells are important in tissue regeneration as they contribute to the development of new tissue through the production of extracellular matrix during the maturation process (endogenous regeneration). Scaffolds can be made of both natural and synthetic materials. The scaffolds are subject to degradation over time, and this allows their replacement with regenerated and native tissue. The goal is to isolate a specific cell line from a patient o donor biopsy, grow the cells on the scaffolds for a certain period and subsequently implant the scaffold-cell construct in vivo. The support, being bioabsorbable, will be reabsorbed by the body and replaced by new tissue regenerated from the healthy implanted cells.[20] The nature and chemical-physical properties of the biomaterial are therefore fundamental to create the conditions favourable to the formation of the desired tissue.[20] Currently, there are various commercial products, consisting of autologous or allogeneic somatic cells combined with biomaterials.[21,22]

Skin regenerative medicine

Wounds of the skin tissue are classified as epidermic, dermic or dermo-epidermal, depending on the degree of these wounds.[23] The molecular mechanism underlying the healing process of skin wounds mainly involves the production of various growth factors, such as epidermal growth factor (EGF) and alpha and beta transforming growth factors (TGF-α e TGF-β). The conventional approach used for instant healing of skin wounds includes the use of natural products, which have anti-inflammatory, antimicrobial and antioxidant properties.[24,25] In recent years, the field of TERM has established itself in the development of artificial tissues and organ regeneration, with the aim of solving serious human health problems[26]. The skin plays an important role as an organ that acts as the body's first defence system and is involved in protection against the external environment and thermoregulation.[27] It is composed of an outer layer of epidermis and an inner layer of dermis, and each has specific functions. The epidermis is mainly composed of keratinocytes (about 90%), that proliferate from the basal layer and differentiate in the terminal layer. The epidermis can regenerate itself thanks to stem cells that enable skin maintenance and wound healing. Wound healing and thus regeneration of skin tissue is a well-coordinated process dependent on several factors such as the type of wound, tissue damage caused by burns or physical trauma, inflammation, secondary infections and many others.[24] The skin healing process involves the formation of new cells through the secretion of ECM by fibroblasts, followed by the formation and proliferation of keratinocytes and finally the differentiation of keratinocytes to form the outer layers of the epidermis. Minor wounds are easily resolved by the growth of cells within the wound, while severe skin wounds take longer to heal and are subject to risks such as inflammation, infection, leading to chronic wound formation.[28] In addition, comorbidities, such as diabetes, negatively affect the healing process and hence tissue repair. Therefore, it is relevant to consider these factors when engineering the scaffolds.[24,27,28] In recent years, various dermal substitutes (autologous, allogenic, heterologous) of dermal, epidermal or dermo-epidermal origin have been used in skin regeneration.[23,28] Currently, the use of scaffolds with cell populations such as keratinocytes and fibroblast is particularly widespread.[28]

Physiology and skin structure

The skin is a continuous tissue belonging to the integumentary apparatus. After the skeletal musculature, it is one of the largest organs in terms of size and weight (about 15% of body weight). It is made up of a series of tissues of ectodermal and mesodermal origin; it has two fundamental properties, which are self-repair (it regenerates following injury) and extensibility (it adapts to changes in body size).[29]

The human skin is the largest vital organ in the body that provides an essential protective barrier against environmental factors such as exogenous chemical, physical, immune and pathogens insults, free radical and UC radiation.[30] In addition the skin performs numerous indispensable physiological functions: processing and metabolism of structural biomolecules such as lipids, proteins and glycans and in the production and secretion of hormones (vitamin D synthesis, peripheral conversion of pro-hormones).[31] The skin exerts further functions: a sensory function due to the presence of receptors that allow the reception of various external stimuli (tactile, thermal and painful), in particular the more superficial layer registers and transmits pressure (tactile), painful and thermal stimuli while the deeper layer transmits thermal and vibratory signals; an excretory function therefore allows the excretion of salts, water and catabolites through sweating; an absorption function that allows the absorption of active ingredients that pass from the external environment to the cells and into the bloodstream. Finally, a thermoregulatory function, i.e. the regulation of body temperature through sweating and the presence of an adipose tissue capable of regulating blood flow, increasing (vasodilation) or decreasing (vasoconstriction) heat dispersion.[32]

Its thickness varies from 0.05 to 2 mm and is formed of three main layers: epidermis, dermis and hypodermis (**Figure 1**).

The epidermis is the outermost layer, which originates from the ectoderm during embryonic development.[33,34] The epidermis plays a major defensive role.[35] This layer limits the flow of water and chemical absorption while safeguarding the skin from harm and stress.[36] Around 95% of the layer is made up mostly of keratinocytes, which are found in the epidermis in various maturation phases.[37] Numerous keratins, important structural proteins that give the skin its strength, are produced by these cells.[38] It is (~100–150 µm thick) and is divided into five distinct strata: stratum corneum, stratum lucidium, stratum granulosum, stratum spinosum, and stratum germinatevum (basal). The basal layer is home to keratinocyte stem cells, which gradually develop through the layers until they reach the stratum corneum, when they become terminally differentiated and are eventually replaced by keratinocytes preventive from the lower levels.[39,40] With 95% of the epidermis' cells being keratinocytes, this cell type predominates. Some people use the terms "basal cells" or "basal keratinocytes" to describe the keratinocytes that make up the stratum germinatevum, or base layer, of the epidermis. The epidermis contains keratinocytes as well as dendritic T cells, adipose cells, melanocytes, Merkel cells, Langerhans cells, and epidermotropic lymphocytes, among many other cell types.[30]

Langerhans cells in the stratum spinosum participate in an immune response and shield the skin from microorganisms.[41] Merkel cells also serve as sensory receptors for inputs including touch, warmth, and pain.[42] Additionally, there are melanocytes, which generate melanin and deliver it to keratinocytes. Melanin gives the skin and hair colour and shields the skin from ultraviolet (UV) radiation damage.[43,44] The intercellular space contains a variety of active catabolic enzymes, including but not limited to nucleotidases, lipases, phosphatases, esterases, and proteases[33] As mesenchymal cells proliferate across the skin, they provide signals that direct epidermis stratification throughout development and direct the placement of downgrowths that indicate the beginning of hair follicle (HF) morphogenesis. During maturation, the stratum basal cells undergo mitotic proliferation, during which the daughter cells migrate upward from basal to suprabasal to spinosum, subjecting the epidermis to homeostatic control. Due to the use of certain transcriptional programs, migratory cells undergo various phases of cell differentiation and alter their shape. The transcriptional programs of the cellular genome are altered during migration and entrance into the spinosum layer. For instance, the transcriptional upregulation of the genes encoding cytokeratin 1 (KRT1) and cytokeratin 10 (KRT10) is turned on while the expression of the genes encoding keratins 5 and 14 (KRT5 and KRT14) is turned off. This change in keratin synthesis is thought to be crucial for creating a strong network of intermediate filament (IF) proteins that are when cross-linked, create structural 3D support for lipid bilayers that are extruded from intracellular lamellar granules into the extracellular space between squamous (dead stratum corneum cells) at the late stage of terminal differentiation. You may think of this procedure as "waterproofing" or "sealing" the skin's surface. Desquamation is the eventual shedding of terminally developed cells from the stratum corneum surface.[30]

The cutaneous basement membrane zone, which connects basal keratinocytes with collagen fibers on the surface of the dermis, lies between the epidermis and the dermis.[45] This structure's primary purpose is to promote adhesion between the two layers, the basement membrane zone permits oxygen and nutrient exchange from the vascular dermis to the epidermis since the epidermis is an avascular layer.[46]

The dermis is essential for the body's structure and cushioning. A mesh-like network of connective tissue, blood vessels, lymphatic vessels, and mast cells make up this layer.[47] Fibroblasts, which produce the proteins elastin and collagen, are primarily responsible for the formation of connective tissue.[79] Elastin proteins help to maintain the skin's elasticity and resilience. The structural proteins known as collagen fibers are crucial for stretching and giving the skin tensile strength.[48] Mast cells are in charge of the skin's inflammatory response to pathogens, allergies, and physical injuries.[49]

The innermost layer of skin is called the hypodermis, and it is mostly made up of adipocytes. From this layer, a rich vascular plexus connects to the dermis and supplies it with blood. Adipose cells, hMSCs, blood, and lymphatic veins are present in the subcutaneous or hypodermis, the last layer. Numerous studies have shown that adipose tissue secretes a large variety of bioactive substances with a wide range of activities, which have

a remarkable impact on the microenvironment. This includes insulin sensitivity, energy balance, and lipid metabolism, all of which have been shown to be crucial in controlling angiogenesis, immunomodulation, and inflammatory response. Adipose tissue's secretory repertoire has the potential to affect gene expression, autocrine, paracrine, and/or endocrine systems, as well as tissue and organ homeostasis.[50] Furthermore, the hypodermis makes the connection of the skin to muscles.

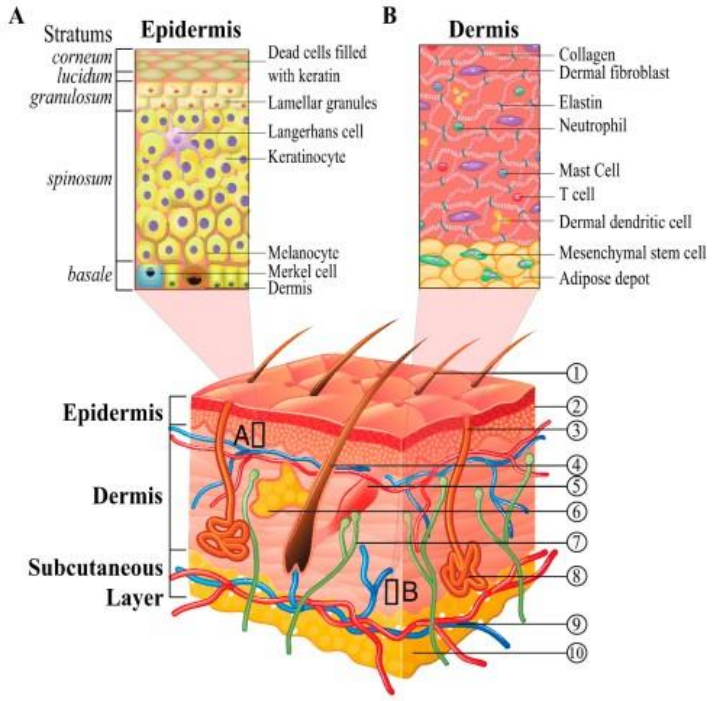


Figure 1. Skin Structure. The skin is composed of 3 main layers-Epidermis, Dermis and Subcutaneous layers containing (1) hair shaft; (2) stratum corneum; (3) sweat-pore; (4) hair follicle; (5) arrector pili muscle; (6) sebaceous gland; (7) nerve; (8) eccrine sweat gland; (9) cutaneous vascular plexes; (10) adipose depot. Detailed structure of (**A**) epidermis is shown with the stratum layers and (**B**) cellular composition and dermis is shown.[30]

Skin healing processes

The skin is a self-repairing organ and maintains homeostasis through organised and complex mechanisms. The regenerative property is essential for the maintenance of the skin barrier during the normal development process and for wound healing. This capacity is maintained by stem cells (SCs) in the skin, through the secretion of cytokines, chemotactic factors, ECM remodels, growth factors and their inhibitors.[51-53]

The healing of skin wounds is a complex process involving a series of cellular and molecular events. It is divided into four overlapping phases: haemostasis, inflammation, proliferation and migration of mesenchymal cells to the wound site and re-epithelialisation (**Figure 2**).[54] After a wound, damaged vessels are closed by blood clots (haemostasis phase), macrophages and neutrophils are recruited at the wound site to eliminate dead cells and secrete growth factors necessary for cell proliferation (inflammation phase). Cells, including keratinocytes, fibroblasts, stem cells and epithelial progenitors, continue their proliferation, migration and differentiation to repair the wound, restore vascular networks and form granulation tissue. Fibroblasts produce collagen to restore ECM, while matrix metalloproteinases remodel the matrix itself by replacing collagen 3 with collagen 1, which gives the skin greater strength. Keratinocytes begin to proliferate, preferably at the wound edges and secreting various growth factors and cytokines such as EGF, keratinocyte growth factor (KGF), vascular endothelial growth factor (VEGF), TGF-β and others that mediate the healing process.[55,56] During the healing process, cells no longer needed are removed from the wound site and the ECM is remodelled to restore the natural structure of the skin.

The process of wound healing, differentiation and regeneration of the skin, under both physiological and pathological conditions, is regulated by various signalling pathways spatially and temporally coordinated. The following pathways are included: WNT, NOTCH, MAPK, NF-kB and ILK. In addition to these pathways there are also components of the cell membrane and cytoskeleton (e.g. actin and RhoA) ion channels, catenin complexes, cell adhesion molecules (e.g. focal adhesions and integrins), know to act as mechanosensors that transmit mechanical signals to the cells which, in turn, trigger further cascades of responses.[57] Numerous transcriptional factors that guide development processes and cell transcriptional programs are involved in the communication between these signalling pathways and target genes. These factors include the transcriptional regulators protein 63 (p63), the AP2 family, binding protein CCAAT/enhancer-binding protein (C/EBP), kruppel-like factor-4 (KLF4), TGF-β and many others.[58]

The role of interleukins and their receptors in the skin has been demonstrated by various studies. These factors appear to be essential to regulate the inflammatory phase of the wound healing process and skin regeneration.[58,59]

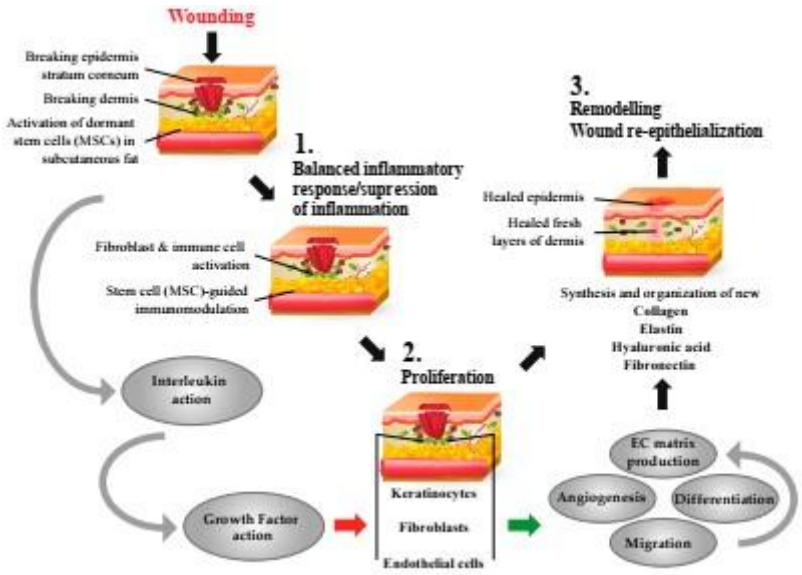


Figure 2. Stages of Wound Healing: Wound healing is a 3-step process. (1) Inflammation stage initiated by migration of inflammatory and stem cells to the wound-site; (2) Proliferation and Granularization stage involving secretion of growth factors and migration of fibroblasts, keratinocytes and endothelial cells to initiate ECM production and repair and; (3) Re-epithelialization stage is the final stage of wound healing process marked by the synthesis and organization of new collagen and elastin fibers resulting in healed epidermis.[30]

Cells for tissue engineering: human mesenchymal stromal cells (hMSCs)

Stem cells are non-specialized cells able to develop into many different cell types in the body during early life and growth, through a process named cell differentiation. The process of self-renewal involves stem cells dividing to produce additional stem cells, preserving the stem pool throughout life and keeping it in an undifferentiated form. Potency is the ability to differentiate into different specialized cell lineages.[60]

The ability to self-renewal is fundamental for stem cells to expand their numbers during development, to be maintained within adult tissues and restore the stem cell pool after injury. Developmental abnormalities, phenotypes associated with early aging, and cancer can result from errors in the self-renewal pathway.

One differentiated cell and one stem cell can be produced by asymmetrical division of stem cells, whereas two differentiated cells or two stem cells can be produced by symmetrical division of stem cells.[61] These characteristics allow stem cells to maintain tissue homeostasis, providing effective replacement of injured cells and proliferation in accordance with tissue needs while preventing over-proliferation.[62]

There are several degrees of potency divided into:

- **totipotent stem cells**: stem cells that can form both embryonic and extra-embryonic tissue. The totipotent stem cells are the individual cells of the early embryo, which are called blastomeres;
- **pluripotent stem cells**: stem cells that can differentiate into all three germ layers; endoderm, mesoderm or ectoderm. Embryonic stem cells are pluripotent stem cells derived from the inner cell mass of the developing embryo;
- **multipotent stem cells**: stem cells that are able to differentiate into a number of cell or tissue types;
- **unipotent stem cells**: stem cells that can only divide into a single cell or tissue type.

It is possible to divide stem cells into two distinct groups based on their origin: embryonic stem cells (ESCs) and adult stem cells (ASCs). The interest in ESCs in the regenerative field is quite limited due firstly to the risk of teratoma formation and then due to the ethical and political controversies that their use entails.[63] ASCs are found in terminally differentiated tissues or organs from which they can be isolated.[64,65] ASCs are postnatal multipotent stem cells that are present in the body for the duration of a person's life. They are able to self-renew and give rise to a small number of mature cell types that develop into the tissue in which they are found in response to traumatic events or regular cell turnover, ensuring the preservation of tissue homeostasis. ASCs are typically kept in a quiescent condition, where they multiply when triggered to repair injured tissues. Numerous organs and tissues, such as the brain, BM, peripheral blood, blood arteries, skeletal muscle, skin, teeth, heart, gut, liver, ovarian epithelium, and testis, have been shown to contain ASCs. They are thought to reside in a specific area of each tissue, called “stem cell niche”. ASCs are different from each other in terms of localization, abundance and specialization since they must generate different tissue types. Among many types of ASCs there are: hematopoietic stem cells that give rise to all the types of blood cells and hMSCs that have been reported to be present in many tissues including BM (BM-hMSCs).[66]

ASCs have great interest in basic research, and they have the potential to be one of the most significant aspects of medicine for drug testing, rejuvenation through cell programming, cell-based therapies, treatment of incurable neurodegenerative diseases, and therapeutic potential of extracellular vesicle-based therapies.[67]

Definition and main features

hMSCs are multipotent adult stem cells that have the ability of self-renewal and multipotency, which could differentiate into cells of the mesodermal lineages and other embryonic lineages, including adipocytes, osteocytes, chondrocytes, hepatocytes, neurons, muscle cells, epithelial cells. Although hMSCs do not differentiate to the various cell lineages as effectively as other cell types, there is a more potent tissue-specific differentiation when it comes to the tissues from whence it originated.[68]

Since these cells are connective tissue cells that make up the supporting framework of an organ, the name "mesenchymal stem cell" is controversially debated, and some researchers in the area prefer to use the word "mesenchymal stromal cells" instead. Most scientists simply refer to these cells as "MSCs" even though there is disagreement about their exact description. hMSCs were first described in 1967 by Friedenstein et al., who isolated adherent, fibroblast-like clonogenic cells from BM called colony-forming unit-fibroblasts (CFU-F). These cells showed a strong capacity for replication in vitro, could differentiate into osteoblasts, chondrocytes, adipocytes and supported hematopoietic stroma when a single CFU-F was retransplanted in vivo.[69]

Later, the International Society for Cellular Therapy (ISCT) established the following criteria for identifying unique populations of hMSCs:

- hMSCs must be purified from the BM stromal population based on plastic adherence and fibroblastic like morphology under standard culture conditions;
- hMSCs must be positive for CD105, CD90 and CD73 (CD= cluster of differentiation);
- hMSCs must be negative for CD45, CD34, CD14 o CD11b, CD79alpha or CD19 and HLA-DR;
- hMSCs must differentiate in vitro into osteocytes, chondrocytes and adipocytes.[12]

Main properties and advantages of hMSCs are:

- easy availability from adult tissues;
- few ethical concerns;
- extensive proliferation in vitro;
- maintenance of their phenotype and undifferentiated multi-potent status;
- low immunogenicity due to the absence of MHC class II molecules (MHCII):
- immunomodulation;
- tissue regeneration.

Currently, there is a lack of a definitive positive marker for hMSCs. It is generally agreed that hMSCs lack the expression of the following markers: CD11b (an immune cell marker), CD45 (a marker of all hematopoietic cells), CD34 (a primitive marker of hematopoietic cells), CD31 (expressed on endothelial and hematopoietic cells), and CD117 (a marker of hematopoietic stem/progenitor cells). Stro-1, CD13, CD29, CD44, CD73 (lymphocyte-vascular adhesion protein 2), CD105 (SH2, endoglin), and CD106 (VCAM-1, vascular cell adhesion molecule-1) are some of the most well-known and optimistic positive markers for hMSCs.[70]

hMSCs are used in many clinical applications due to their therapeutic potential. These biological properties are summarised in: differentiation potential, paracrine effects, immunomodulation and homing mechanism (**Figure 3**).[12]

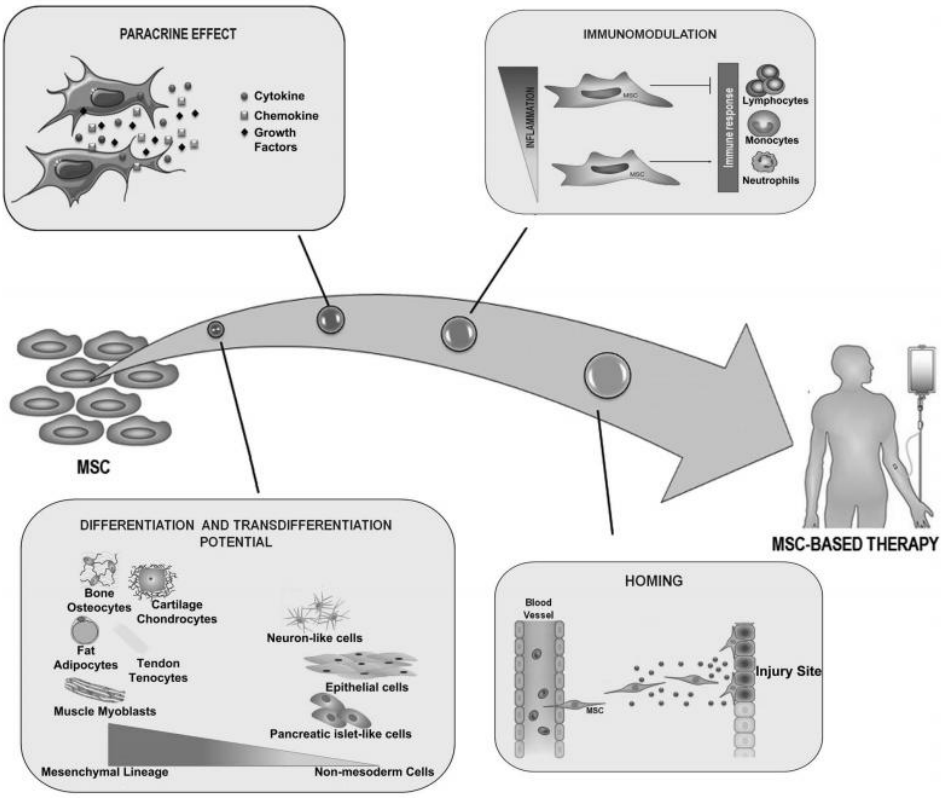


Figure 3. Biological properties supporting MSCs clinical use. The therapeutic potential of MSCs relies on their unique properties as i) the capacity to differentiate into various cell lineage (bottom, left), ii) the ability to secrete soluble factors that are crucial for cell survival and proliferation (top, left), iii) the ability to modulate immune response (top, right), iv) the ability to migrate to the exact site of injury (bottom, right).[12]

Differentiation potential

They may develop into a variety of mesenchymal tissues, including those of bones, cartilage, fat, muscles, tendons, and BM, both in vitro and in vivo since they are multipotent stem cells. hMSCs are also very flexible due to their capacity to transdifferentiate, or experience a sudden change in phenotype, which results in the development of cells with traits from several lineages. It is known that hMSCs may transdifferentiate in vitro into cells other than mesoderm-like cells, such as neurons, hepatocytes, and pancreatic islet-like cells. The development of keratinocytes and other types of skin cells by these cells has been shown by Sasaki et al. to aid in wound healing processes.[71-73]

Paracrine effects

It has been discovered that the ability of hMSCs to release a wide range of cytokines, chemokines, and growth factors is responsible for the advantages of hMSC transplantation.[74,75] In particular, hMSCs secrete a number of growth factors including VEGF, NGF, EGF, HGF and TGF-β as well as cytokines such as IL, G-CSF and GM-CSF. These facts coordinate local tissue regeneration, promoting angiogenesis, cell chemotaxis, anti-apoptotic processes, proliferation, and immunomodulation.[76]

Immunomodulation

hMSCs are able to modulate the immune system, by negatively regulating the immune response in the case of severe inflammation, both by positively stimulating the immune system through the release of proinflammatory cytokines in case the level of cytokines is too low. hMSCs are excellent candidates in the regenerative field as they are poorly immunogenic and immunomodulatory. The poor immunogenicity is due to the fact that they express low levels of HLA class I histocompatibility antigens and do not express class II one and co-stimulatory molecules such as CD80, CD86 and CD40.[78] In vitro e in vivo studies have shown that hMSCs interact with a wide range of immune cells and have the ability to suppress the excessive response of T lymphocytes, B lymphocytes, dendritic cells, macrophages and natural killer cells. Therefore, hMSCs could be considered as suitable candidates for the treatment of autoimmune diseases and GVHD. Furthermore, they could be used to treat tissue and organ damage associated with intense inflammatory activity, such as rheumatoid arthritis, kidney failure or cardiac lesions, given their positive role in promoting tissue repair.[64,79]

Homing mechanism

The homing mechanism of MSCs is based on their capacity to bind to signalling molecules from wounded tissue and equivalent receptors on the MSCs themselves to go to damaged tissue.

Inflammatory chemicals and chemokines are released by the cells around damaged tissues, creating a gradient of ligands that innate immune cells are drawn to through the chemotactic process.

This chemical signal draws hMSCs, which then go right to the injured location to start the regeneration process. The homing receptors found in the hMSCs and endothelial co-receptors, as well as the adhesive junctions between the cells and the vascular endothelium in the target tissue, enable this process. hMSCs express the most prevalent immune cell-attracting chemokine receptors, such as CXCR4 and CCR, which interact with chemoattractants overexpressed in the damaged region, such as Stromal-cell-derived factor 1 (SDF-1) and Monocyte-chemotactic protein 3 (MCP3), to assist this shift.[76]

hMSCs in different adult tissues

hMSCs were initially identified and isolated from BM, but can also be isolated from other tissues, such as AT, umbilical cord blood, peripheral blood, lungs, medullary spaces of long bones, synovial fluids, muscle, placenta and dental pulp.[80]

Bone marrow hMSCs (BM-hMSCs) are the most widely used in the clinical setting for tissue repair and regeneration. Another cell type used in regeneration are hMSCs isolated from UC.[80] Umbilical cord hMSCs (UC-hMSCs) can be isolated from different sources that include umbilical cord blood, umbilical vein sub-endothelium and Wharton's jelly.[213] High proliferative potential and distinctive morphological traits of differentiation and trophicity set umbilical cord hMSCs apart from other hMSCs.[81] UC-hMSCs are also appropriate for allogeneic usage due to their low immunogenicity and the fact that non-invasive procedures may be used to separate them, unlike BM-hMSCs, which needs a bone marrow needle aspiration.[82] The amount of BM-hMSCs extracted from the aspirate is negligible, and as they get older, their capacity for differentiation and development tends to decrease.[83] It's important to note that BM-hMSCs and UC-hMSCs have similar cytokine expression patterns, leading to similar biological potential, despite these discrepancies.[76]

hMSCs from adipose tissue (AT-hMSCs), in addition to BM-hMSCs and UC-hMSCs, are frequently employed in the field of regeneration. They are easily accessible, and a minimally invasive method can only gather a little amount of them. Additionally, they share with BM-hMSCs biological traits, immunogenicity, and differentiation potential.[84,85]

Accordingly, recent research provides no evidence that one tissue origin from which MSCs are produced gives a benefit in the wound healing process over another. hMSCs are thought to exert their therapeutic effects through a variety of interactions with various cell types at every step of the healing process.[84]

hMSCs are therefore very successful in the TERM. Recent research has revealed that secretion factors produced by cells themselves can be employed in place of cell therapy to regenerate tissues.[76,86]

hMSCs in skin regeneration

hMSCs are useful in all phases of the wound healing process and the use of these cells can improve healing and reduce scar formation. hMSCs target the damaged skin area, inhibit inflammation and increase the proliferation and differentiation capacity of fibroblast, epidermal and endothelial cells.[85,87] Furthermore, hMSCs have antimicrobial activity, essential for eliminating infections from wounds.[88,89] hMSCs have the ability to move in vivo in response to chemotactic signals that control inflammation, mending harmed tissues, and promoting regeneration.[90] Both hMSCs differentiation and paracrine signalling are involved in tissue healing; differentiation helps to regenerate damaged tissues, while paracrine signalling controls cellular responses to inflammation at the local level.[91]

hMSCs are known to secrete numerous mediators for tissue repair, including growth factors, cytokines and chemokines such as VEGF, Platelet-Derived Growth Factor (PDGF), basic Fibroblast Growth Factor (bFGF), Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF), EGF, KGF, Insulin-like Growth Factor-1 (IGF-1) and TGF-β.[90,92]

EGF is thought to have a role in keratinocyte migration, fibroblast proliferation and differentiation into granulation tissue, and the improvement of wound healing. In the initial phases of healing, bFGF and aFGF are found in the wound fluid where they promote neovascularization. While the receptor is expressed on epithelial cells of the epidermis, suggesting a potential paracrine control of epithelial cell development, KGF is mostly concentrated in the dermis and hypodermis beneath the wound. Due to its capacity to promote endothelial cell migration and proliferation, basement membrane breakdown, increased vascular permeability, and the development of new blood vessels, VEGF is the key agent inducing skin regeneration.

Overexpression of IGF-1 accelerates epidemic wound healing and inhibits apoptotic pathways, whereas reduced expression is associated with delayed cutaneous wound healing. PDGF is implicated in all phases of the healing process and is responsible for the differentiation of fibroblasts into myofibroblasts to allow general tissue contraction. Finally, TGF-β is highly expressed by many different cell types and can act both autocrinally, stimulating its own synthesis within target cells like keratinocytes, monocytes, macrophages, and fibroblasts, and paracrinely, inducing the surrounding cells to synthesize and release other growth factors involved in skin healing and regeneration.[52,92-98]

Several studies demonstrate that the different cell types that include epithelial cells, endothelial cells, keratinocytes and fibroblast respond to the paracrine signal of MSCs that regulates cell survival, proliferation, migration and gene expression.[98] hMSCs also secrete mitogens that stimulate the proliferation of keratinocytes, dermal fibroblasts and endothelial cells in vitro.[99]

hMSCs can be used for multiple applications such as skin regeneration which includes wound healing and burn treatment, but also skin rejuvenation.[100]

hMSCs in skin repair: differentiation to fibroblasts and keratinocytes

hMSCs, regardless of their origin, are cells capable of differentiating into different cell lines, generating cells specialized in carrying out a certain function. The fibroblast and keratinocyte differentiation of hMSCs is characterized by the action of specific markers that induce the acquisition of a fibroblast and keratinocyte phenotype.[101,102] It has been demonstrated that hMSCs derived from different sources, including BM and UC, are able to differentiate thanks to specific induction factors in both two-dimensional (2D) and three-dimensional (3D) cultures in fibroblasts and keratinocytes through the evaluation of morphological changes, the expression of specific epidermal and dermal markers and finally, the expression of specific proteins.[103,104]

The main molecular markers of fibroblasts and keratinocytes are respectively: collagen 1 (COLL1) and collagen 4 (COLL4), protein 63 (p63) and cytokeratin 10 (KRT10).[105-111]

Collagen has a triple helix structure that initiates and maintains the interaction between cells and matrix. To date, 28 different types of collagens have been identified. COLL1 is the most common type of protein and is present in 90% of the human body. It is commonly found in the skin, bones, organ capsules, tendons and cornea. Human COLL1 is encoded by the COL1A1 gene located in the long arm of chromosome 17q21.3-q22 and by the COL1A2 gene located in the long arm of the chromosome 7q21.3-q22, which encode respectively the two alpha1 chains and the alpha2 chain that constitute the triple helix.[112-115] COLL1 and other ECM compounds are primarily produced by fibroblasts that have differentiated from hMSCs. While collagen gene expression is kept low under normal circumstances, fibroblasts upregulate COLL1 transcription during the healing phase to start the process of repairing injured tissue. TGF-β is one of the soluble substances that modulates the activation of the genes that code for COLL1. It is a potent factor capable of promoting the synthesis of ECM in the processes of wound healing, scarring, and fibrosis.[105] The type I receptor kinase-activin is phosphorylated when TGF-β binds to its type II receptor (ALKs). ALKs then attract and activate Smad protein complexes, which go into the nucleus and, together with other transcription factors, stimulate the transcription of COLL1.[106,116]

COLL4 is only present in the basement membrane of the epidermal junction, as opposed to COLL1, which is localized in all dermal layers. COLL4 promotes cell adhesion, migration, and differentiation through intricate intramolecular and intermolecular interactions. Six distinct genes - COL4A1 and COL4A2 on chromosome 13, COL4A3 and COL4A4 on chromosome 2, COL4A5 and COL4A6 on the x chromosome - encode human COLL4. In specifically, these genes are all descended from a single ancestral gene, which gave rise to these genes through three successive gene duplications. COLL4's six alpha chains come together to form distinct heterodimers that can identify one another.[107]

The transcription factor p63, initially described as a keratinocyte transcription factor (KET) in 1997, is encoded by the TP63 gene and belongs to the p53 gene family. It is the key regulator of epidermal development and keratinocyte proliferation, and differentiation as reported in the literature.[109,117] Furthermore, high

expression of p63 has been detected in epidermal stem cells, underlining the importance of this factor in keratinocyte proliferation. This has been demonstrated by several studies in which p63 knock-down approaches have been used to investigate its role in cell proliferation and differentiation processes. In particular, it was noted that knock-down of p63 in primary keratinocytes results in hypo-proliferation, cell cycle arrest and abnormalities in cell adhesion. Furthermore, down-regulation prevents cells from differentiating and stratifying in both 2D and 3D patterns of keratinocyte differentiation.[95] Although the processes of proliferation and differentiation are related, p63 appears to play an independent role in both processes. At the molecular level, silencing p63 causes the activation of genes involved in cell cycle arrest such as p21 and genes that suppress cell proliferation such as JunB. Simultaneously, such silencing results in the repression of genes that can positively promote cell proliferation such as Fos and c-Jun and genes essential for epidermal differentiation such as Perp and cytokeratin 14 (KRT14).[109] Because it guarantees the activation of epidermal genes during differentiation and represses the genes of other cell lines, it is hypothesized that p63 is the "guardian" of the epithelial lineage.[118] As it is expressed in the nuclei of basal layer keratinocytes, p63 is regarded as an early marker in keratinocyte development. To ensure the expression of all other epidermal markers, including cytokeratin 5 (KRT5) and KRT14, which are later replaced by cytokeratin (KRT1) and KRT10, its early expression is essential. This was confirmed by an in vitro study conducted on mouse embryonic stem cells (mESCs) in which it was seen that the expression of p63 was detected before the expression of the marker KRT14. Furthermore, the absence of p63 in mESCs hinders the progression towards stratified epithelial fate through the overexpression of mesodermal genes.[109] In the metabolism of human keratinocytes, p63 acts instead as a direct regulator of genes involved in the process of glycolysis. Among these genes are hexokinase 2 (HK2) which phosphorylates glucose to obtain glucose-6-phosphate and 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKKB3) which represents the key regulator of the glycolytic process.[119] It has been shown that a reduction in p63 causes a reduction in the expression of HK2 and PFKFB3, which in turn reduces glycolysis and, as a result, cell proliferation while boosting the rate of differentiation.[119] This demonstrates that p63 plays a crucial role in the metabolism of keratinocytes, thus representing an indispensable factor in modulating the levels of proliferation and differentiation in these cells.[120]

In epithelia, keratins perform a variety of homeostatic and stress-enhanced mechanical and nonmechanical tasks, including as preserving cellular integrity, controlling cell growth and migration, and preventing apoptosis. Both keratin-associated proteins and post-translational modifications strictly control these actions. A significant subclass of the extensive family of IF is represented by keratins. proteins that self-assemble into filaments 10 nm in width.[121] The basal layer's keratinocytes are extremely proliferative and express KRT5, KRT14 and cytokeratin15 (KRT15) in tiny levels. Once the differentiation process is triggered, the basal keratinocytes stop dividing and begin to express KTR1 and KRT10 as they migrate in the suprabasal

layers (spinous, granular and corneous). the early events of keratinocyte differentiation involving cell growth arrest and the expression of the molecular markers KRT1 and KRT10 are modulated by the transcription factor CCAAT/enhancer binding protein (C/EBP). It appears to have a major role in the control of the genes expressed throughout the differentiation process of the squamous layer, in particular, being located at the level of the suprabasal keratinocytes' nuclei. Although it appears to have little impact on the differentiation markers that define the suprabasal layers.[122]

After the expression of the characteristic molecular markers for fibroblasts and keratinocytes, the expression of protein markers will follow. By secreting soluble molecules including IL-1, IL-10, keratinocyte growth, and other substances, fibroblasts that express COLL1 and COLL4, fibronectin (FN), and fibroblast-specific protein 1 (FSP1) are able to promote the proliferation and differentiation of keratinocytes in the epidermis.[101,102] Reticular and papillary fibroblasts are two types of dermal fibroblasts that are present in various parts of the dermis and have unique properties. The papillary dermis contains proteoglycans and non-fibrillar collagen bundles, whereas the reticular dermis mostly consists of fibrillar collagen bundles. The production of fibrillar collagen gradually increases as dermal fibroblasts migrate toward the site of the injury until the collagenic matrix no longer completely replaces the fibrin matrix. In the early stages of the process of cutaneous regeneration, the COLL3 and the FN are deposited and gradually replaced. As an illustration, the COLL3 is replaced by the COLL1. Keratinocytes begin to move when activated by the COLL1 Integrine.[123] The differentiation process is therefore a dynamic process that involves the induction and repression of various genes and is substantially regulated by calcium which is the main regulator both in vitro and in vivo.[124] For example, the transition of the basal keratinocyte from the basal to the spinous layer is accompanied by the repression of basal keratinocyte transcripts such as KRT5, KRT14 and integrin α4β6, as well as the activation of later-stage differentiation markers such as KRT1 and KRT10. The transition from the spinous to the granular layer is instead characterized by the suppression of KRT1 and KRT10 transcripts and by the overexpression of the transcripts of keratinized envelope precursor proteins such as involucrin, loricrin and filaggrin (**Figure 4**).[125]

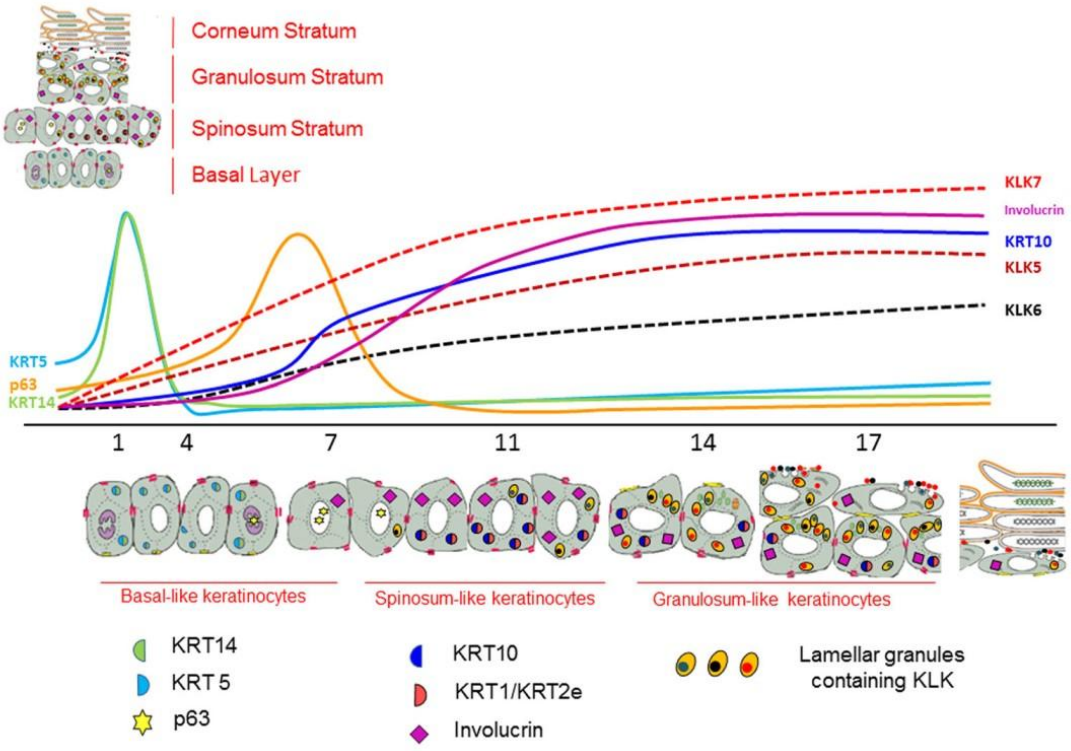


Figure 4. Representation of the temporal expression of proteins involved in the differentiation of hMSCs into keratinocytes. The expression of p63 was observed in the basal layer while the expression of 10, present in the spinous and granulosa layers, was detected in the more advanced stages of differentiation.[73]

Fibroblast differentiation appears to be influenced by the ascorbic acid (AA) molecule and connective tissue growth factor (CTGF) also known as CCN2, which act as enhancers of COLL1 and COLL4 gene expression. As connective tissues are unable to maintain the triple helix structure of collagen, a lack of AA is linked to a fault in them.[126] CTGF plays a central role in fibrotic processes and wound healing, as it is believed to interact with TGF to promote the production of ECM. In addition to promoting the proliferation of fibroblasts, it seems to induce hMSCs to differentiate towards the fibroblastic lineage by increasing the synthesis of COLL1 and tenascin -C (TN-C), thus acting as a true differentiation stimulus.[127-129] In research by Chang, it was quantitatively shown that hMSCs treated with CTGF produced more COLL1 and Tn-C than hMSCs not treated with CTGF. Therefore, CTGF may be crucial in causing hMSCs to differentiate into fibroblasts.[127]

The terminal differentiation of keratinocytes is instead promoted by the epidermal growth factor EGF. Both keratinocytes and fibroblasts in culture release this substance, although in the case of keratinocytes, the production of EGF by them promotes migration.[130] In general, it is a mitogen capable of promoting the in vitro expansion of hMSCs as well as the proliferation and migration of fibroblasts and keratinocytes, at least in a short-term culture scenario since the additive effects of EGF on hMSCs proliferation appear to be related to down-regulation in long-term culture.[131,132]

In summary, fibroblasts are resident cells of the connective tissue responsible for the production of the ECM, whose main function is that of active tissue support, i.e. capable of controlling physiological phenomena such as morphogenesis, pathophysiological phenomena such as wound healing. Fibroblasts express certain dermal markers during differentiation, including collagens like COLL1 and COLL4, as well as to a lesser extent fibronectin, fibrillins, and tenascins.[105]

Keratinocytes, during the differentiation process, express epidermal markers such as p63, involucrin and filaggrin in the different layers of the epidermis.[104] p63 is a transcriptional factor that is involved in the regulation of epidermal development. is a transcriptional factor that controls genes for cell adhesion mechanisms, signalling, and cytoskeletal elements like KRT.[133] p63 is expressed in the nuclei of proliferating keratinocytes present in the basal layer, so it is considered an early marker in differentiation.[134] KRT5 and KRT14 are considered late markers since they are expressed in the basal layer while KRT1 and KRT10 are found in the spinous layer. Last but not least, the stratum granulosum and spinosum express filaggrin, involucrin, and loricin, which promotes the latter stages of development towards the keratinocyte lineage.[104]

Clinical applications of hMSCs for skin repair

The first clinical trial using MSCs grown in vitro was conducted in 1995, involving 15 patients who received autologous cells. Since that time, a large number of clinical experiments have been carried out to examine the viability and efficacy of hMSCs therapy.[65] In 1014 clinical trials employing hMSCs for a variety of therapeutic purposes, according to the public clinical trials database http://clinicaltrials.gov (July 2021).[135] The majority of these studies are in stages I or II, which examine patient safety and effectiveness, respectively, or they combine phases I and II. Sierra-Sanchez et al. report that a total of 13 types of hMSCs products have been used as advanced therapies in dermatology over the past 5 years (2015-2020). The most studied types of hMSCs were UC-hMSCs, AT-hMSCs and BM-hMSCs. The most studied diseases were skin wounds, burns, ulcers and psoriasis.[118] Montero-Vilchez et al. report in their work that the conditioned medium in which hMSCs were grown improves wound healing, hair restoration, skin rejuvenation, atopic dermatitis and psoriasis, both in animal models and in humans.[136]

From 2015 to 2020, several studies have been conducted on the use of hMSCs as a cell therapy for the treatment of wounds and ulcers. Among these, two cases reported significant results regarding the reduction in size of the lesions. Other phase I and phase II trials that used autologous MSCs have looked at a variety of wounds, including chronic varicose leg ulcers, traumatic heel lesions, and diabetic foot ulcers.[137] Patients with diabetic foot ulcers have benefited greatly from the use of MSCs in combination with three-dimensional structures for wound closure.[137-139]

Human platelet lysate (hPL) in hMSCs expansion for clinical translation

hPL for regenerative medicine purposes

Human platelet lysate (hPL) is a hemoderivative, obtained from a preparation of platelets isolated from the platelet-rich plasma fraction (PRP) containing numerous growth factors such as PDGF, TGF-β1, bFGF, EGF, VEGF, IGF, HGF, soluble CD40L, VCAM-1, ligand 5 (CCL5, RANTES) and intercellular adhesion molecule-1 (ICAM-1). These factors play a role in wound healing processes, including proliferation, cell chemoattraction, extracellular matrix synthesis, induction of angiogenesis and cell recruitment.[12,140]

Fetal bovine serum (FBS) has been the traditional media supplement and source of growth factor for cell expansion and tissue engineering thus far.[12] Cellular treatments cannot yet be used in clinical settings due to the requirement for employing products generated from animals in cell culture. In reality, there are strict restrictions on using FBS due to safety and moral issues.[141] It has been demonstrated that hPL is a reliable alternative to FBS for hMSCs growth protocols, eliminating the inherent risk of spreading unknown animal illnesses, the potential immunological reactions to animal antigens, and getting around the stringent regulations of regulatory agencies in charge of approving experimental protocols for somatic cell therapies.[142] Numerous studies have demonstrated that allogenic and autologous hPL are more effective than FBS at promoting cell proliferation. Furthermore, the effectiveness of using hPL for hMSCs expansion has also been described.[13]

In one single culture period without additional passages, the highly effective stimulation of cell proliferation allowed for the large-scale expansion of hMSCs for clinical use while meeting all the requirements of the International Society for Cellular Therapy (ISCT), preserving a normal karyotype and the immunosuppressive ability.[143]

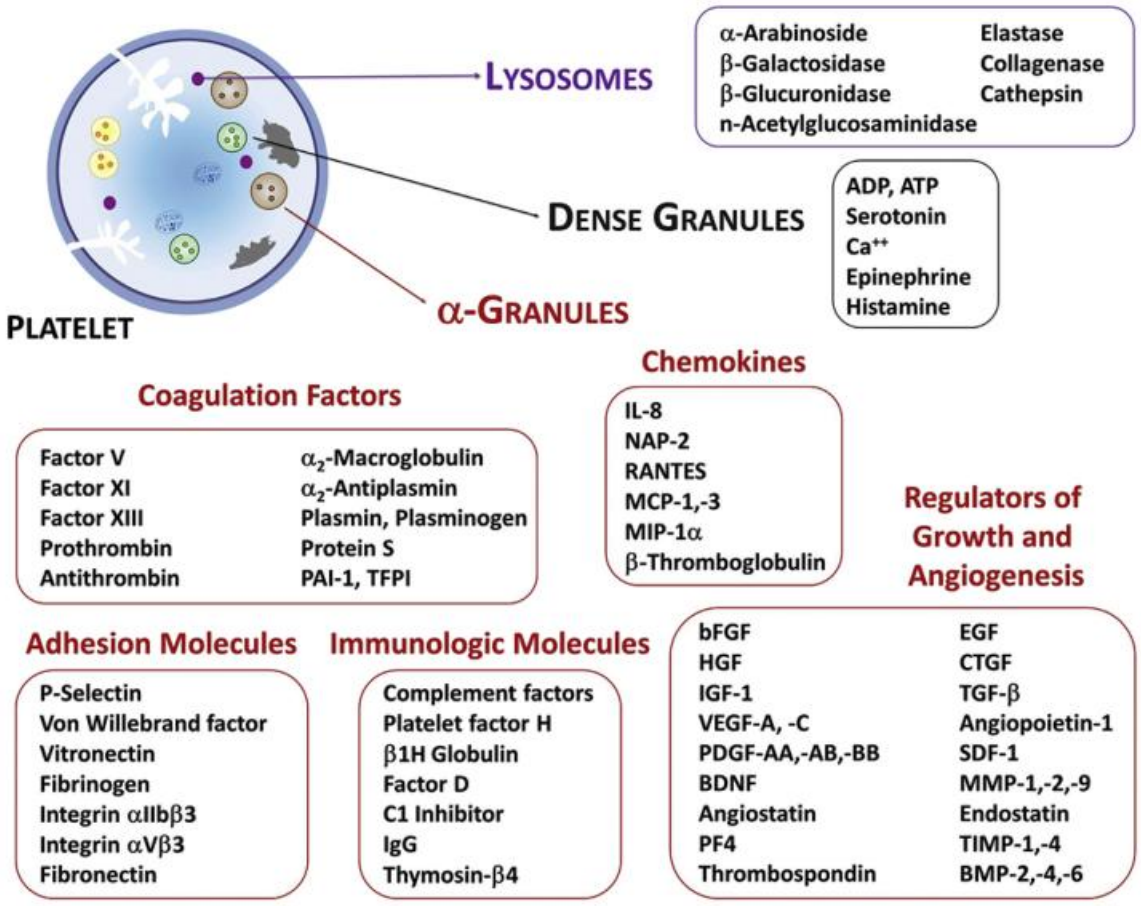


Figure 5. Platelet granule cargo. The various types of platelet granules store a plethora of potent substances including lysosomal enzymes, coagulation factors, immunologic and adhesion molecules, chemokines and growth factors for hemostasis, host defense, angiogenesis and tissue repair. Abbreviations: ADP, adenosine diphosphate; ATP, adenosine triphosphate; BDNF, brain derived neurotrophic factor; bFGF, basic fibroblast growth factor; BMP, bone morphogenetic protein; C, complement; CTGF, connective tissue growth factor; EGF, epidermal growth factor; HGF, hepatocyte growth factor; IGF-1, insulin-like growth factor-1; IL-8, interleukin 8 (CXCL8); MCP-1, monocyte chemotactic protein-1 (CCL2); MCP-3, monocyte chemotactic protein-3 (CCL7); MIP-1a, macrophage inflammatory protein 1a (CCL3); MMP, matrix metalloproteinase; NAP-2, neutrophil-activating protein-2 (CXCL7); PAI-1, plasminogen activator inhibitor-1; PDGF, platelet derived growth factor; PF4, platelet factor 4 (CXCL4); RANTES, regulated on activation, normal T cell expressed and secreted (CCL5); SDF-1, stromal cell derived factor-1; TFPI, tissue factor pathway inhibitor; TGF-b, transforming growth factor-b; TIMP, tissue inhibitor of metalloproteinases; VEGF, vascular endothelial growth factor.[144]

Clinical use of platelet-derived products and their derivates

Platelet-derived products has been of clinical interest in some indications in regenerative medicine. Numerous clinical trials investigating platelet-derived products, and their derivatives are now registered for use in maxillofacial, orthopaedic, and plastic surgery as well as the treatment of burns, scars, chronic ulcers and wounds, osteoarthritis, and the restoration of tendons and ligaments.[144]

For the treatment of severe radiation burns, the use of hPL represents a novel therapeutic approach, and it may bring fresh opportunities for radiotherapy problems. An effective method for treating irradiation burns in conjunction with skin or muscle grafts was found when single donor hPL was used to enhance blood flow.[145]

hPL represents a great opportunity for bone defect repair as a cell culture supplement for hMSCs expansion in 2D or in 3D culture.[146]

These data suggest that hPL has the potential to become the gold standard supplement for the expansion of human cells for tissue engineering and regenerative applications.[144]

The use of platelets derivates in cell therapy and in regenerative medicine procedures is still emerging and for ex vivo expansion of therapeutic cells for transplantation in compliance with Good Manufacturing Practice (GMP), platelet-derived products need to be standardized and subject to quality control. An example is the study of Bianchetti et al. that describes a method of standardising the platelet lysate preparation process.[147]

Biomaterials for skin tissue engineering

Biomaterials for tissue engineering

Scaffolds are three-dimensional (3D) porous structures that play a key role in modern strategies of regenerative medicine and tissue engineering. They represent a biophysical and biochemical support capable of directing and controlling function and behaviour cellular.[148-150] The scaffolds were designed to promote cell-biomaterial interaction, allowing adequate transport of gases, nutrients and survival regulatory factors cell, proliferation and differentiation, while respecting the specific mechanical characteristics of the tissue to be regenerated. They can also "biodegrade" with one controlled rate, inducing minimal inflammation or toxicity in vivo.[151] Various technologies and materials come together to construct porous scaffolds to generate the tissues/organs o to control and target the release of bioactive agents in tissue engineering applications.[152]

Scaffolds restore the structure and function of damaged or dysfunctional tissues in cell-based therapies, in which they induce the morphogenesis of transplanted cells or matrices in ex vivo bioengineered tissues, and in acellular therapies, in which they promote growth and the differentiation of healthy residual tissue cells in situ. The basic goal is to create a structure that closely resembles the extracellular matrix in nature. Using bioactive materials for TE has advanced significantly in recent years.

Many scaffolds that are currently using for regeneration applications are:

-**polymers**: naturals (chitosan, fibrin, hyaluronic acid and collagen) or synthetics (polyanhydride, polypropylene fumarate - PPF, PCL, polyphosphazene, PLA, polyether ether ketone - PEEK, and PGA). An important class of polymers utilized is represented by hydrogels both naturals (agarose, alginate and gelatins) and synthetics (poly(vinylalcohol)-based);

-**bioactive ceramics**: natural or synthetic origin (coralline, hydroxyapatite, tricalciumphosphate, sulphate, bioactive glass and calcium silicate);

-**hybrids**: combination of two or more materials with different properties in the form of co-polymers, polymer-polymer blends or polymer-ceramic composites.

Polymeric scaffolds in skin regeneration: hydrogels

The development of scaffolds with optimal characteristics is more readily achievable in polymeric scaffolds, in particular a great interest has been given to the hydrogels since they offer the possibility to generate well-defined 3D biofabricted tissue analogue to the native extracellular environment.[150,153]

Hydrogels are three-dimensional polymeric biomaterials, having the ability to retain large quantities of water like that of biological tissues (70%). They have a liquid phase and a solid phase: the liquid phase is that which makes them suitable and compatible with living tissue, while the solid phase is the one that maintains the three-dimensional structure. Hydrogels are capable of mimicking properties structural, biochemical, immunomodulatory and interaction of the cells with natural ECM.[154]

Hydrogels can have various compositions: they can be made of synthetic polymers, from naturally derived polymers or the combination of both.

Hydrogels consisting mainly of natural polymers are biocompatible, bioactive, biodegradable and capable to promote many cellular functions as cell viability, differentiation and proliferation.[150,151] However, their limitations include poor mechanical properties, inability to control their degradation and structure, difficulty in sterilization and purification. On the other hand, synthetic hydrogels are highly reproducible, characterized by high properties mechanical, possess controllable degradation rate and microstructure, but they lack biological cues to interact with cells and protein.[151] Therefore, hybrid hydrogels, that combine the benefits of natural and synthetic polymers, have attracted significant attention for biological and biomedical applications. To tailor the structural, chemical, and mechanical properties of the hydrogels for a particular tissue engineering application, many types of natural and synthetic polymers have been combined.[7]

Chitosan (CH)-based scaffolds in skin healing

In recent years, CH-based scaffolds and their applications in the field of tissue engineering have attracted the interest of the scientific community. CH is a linear polysaccharide with β-1,4 bonds between the polymers of glucosamine and N-acetylglucosamine. It is produced by the partial deacetylation of chitin, which can be achieved through enzymatic hydrolysis promoted by chitin deacetylase or by chemical hydrolysis under alkaline conditions. It is used in production of various scaffolds due to the high affinity to anionic proteins and polysaccharides.[155]

CH is therefore a carbohydrate biopolymer and is known to mimic a biological material capable of promoting the healing process of connective tissue. It's a material biocompatible, biodegradable, bioactive, biologically renewable, non-toxic, non-antigenic, non-immunogenic, capable of stimulating the activity of growth factors and with antibacterial property.

CH has a hydrophilic surface that promotes cell adhesion, proliferation, differentiation and has a large hydrophilic capacity, therefore it is able to maintain and attract fluids and cells at an injured site. Furthermore, at a structural level it presents positively charged amino groups, which make it a mucoadhesive, haemostatic material with the ability to bind cell membranes.[156] It also has anti-inflammatory activity and can act as a healing accelerator of the wound.

The already anticipated antibacterial activity is guaranteed by the fact that CH can bind to the DNA of a microorganism and inhibit the synthesis of mRNA and proteins and the proliferation of microorganism. The antibacterial activity is related to the interaction between the charged amino groups positively and the anionic groups of lipoteichoic acids of Gram-positive bacteria and of Gram-negative lipopolysaccharide; this interaction leads to the destruction of the membrane microbial.

CH can be used alone or in combination with other polymers. Gels, sponges, fibers, or porous mixtures of CH with ceramic or other polymeric materials as collagen or gelatin are examples of potential matrix preparations for cell cultures. These can be used to modify the mechanical behavior of cell transplants and the seeding characteristics of cells for the intended therapeutic uses.[157]

CH offers a wide range of potential applications for cell-based tissue engineering (**Figure 6**). Since a few decades ago, chitosan-based bioactive polymers have been extensively employed in tissue engineering. The most well-known applications of tissue engineering that make use of chitosan for the production of biomaterials are cartilage, bone, intervertebral disc, blood vessel, corneal regeneration, and skin.

CH-based scaffold for skin tissue engineering should be characterized by mechanical strength and the ability to promote adhesion, proliferation and differentiation. It has been combined with various material such as polycaprolactone, collagen, gelatin and fibrinogen for potential applications in skin regeneration.[10]

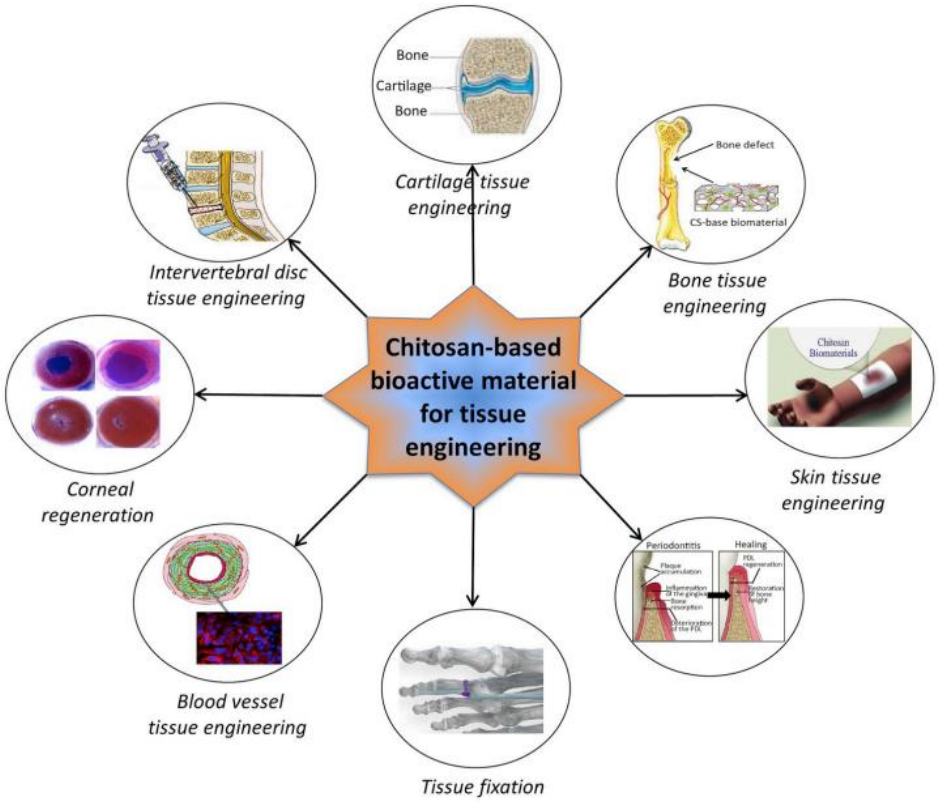


Figure 6. Schematic representation of applications of chitosan-based bioactive materials[10]

Scaffolds applied in dermatology clinic

Clinical dermatology currently offers a wide range of dermal substitutes, including synthetic collagen or nylon-based acellular skin substitutes, allografts made from cadaver skin's acellular matrix, allografts made from sheets of neonatal foreskin cells, xenografts made from animals like pigs or cows, and natural skin substitutes made from the patient's own cells.

One of the most widely used dermal substitutes is AlloDermTM acellular dermal matrix (LifeCell Corp., Branchburg, NJ, USA). This extracellular matrix, which has been lyophilized and was taken from cadaver skin, promotes wound healing and lessens scar shrinkage, allowing for dermal regeneration. However, there are restrictions on using human-derived skin products because they are made from donated human tissue and may have quantity restrictions in addition to their high price.[158]

The most widely used synthetic skin substitute in burn patients at present is Integra® (Integra Life Sciences Corp, Plainsboro, NJ, USA), an artificial skin. Integra®, first described by Yannas et al., has a bilaminar, therefore bilayer structure made up of shark-derived chondroitin-6-sulfate and bovine flexor tendon type 1 collagen that have been cross-linked with glutaraldehyde. Integra® is also coated on one side with a silicone membrane (polysiloxane film) that serves as the epidermis and regulates wound moisture loss. The pores of

this media are between 70 and 200 µm in size so that endothelial cells and fibroblasts from patients can migrate through for adequate media colonization and tissue regeneration. Larger pores don't offer enough surface area for invasive host cells to connect to, whereas smaller pores either slow down or prohibit biointegration. After application to a wound, the collagen layer integrates with the wound to form new skin, a process that takes about 3-6 weeks. The silicone layer can be removed at this point so that a thin skin graft can be placed in its place. Integra® is used in cases of severe burns in which skin tissue autotransplantation is not feasible.[159] Integra® is relatively expensive compared with cadaveric allograft skin from skin banks, and it is believed that there is a high probability of initial failure. On the other hand, it offers better elasticity and aesthetics than a split-skin transplant, with reduced donor-site morbidity as it heals more quickly with less scarring. Additionally, it does away with the typical infection risk that comes with allografts. The second stage can be carried out whenever is convenient, and donor sites can be used again and again.[158]

Alternatively, another graft widely used in dermatology for the treatment of burns and ulcers turns out to be MatriDerm® (MedSkin Solution Dr. Suwelack AG, Billerbeck, Germany), which, today, is among the gold standards. It is a highly porous single laminar matrix composed of native (non-cross-linked) collagen and elastin, the two main components of ECM. While elastin is produced by hydrolysis of bovine nuchal ligament, the collagen that makes up this carrier is taken from bovine dermis and contains collagen types I, III, and V. After freeze-drying, the media is gamma irradiated and stored at room temperature. It is packaged in sterile double packaging that can only be opened in a sterile environment. It must be rehydrated in saline before use, and it must be positioned on the water's surface rather than immersed to prevent the development of trapped air pockets that might impede diffusion and compromise transplanting. The matrix is ready for grafting when the surface changes from whitish to translucent.[160] MatriDerm® controls the formation of scars and functions as a skeleton for tissue regeneration. In addition, it has excellent hemostatic properties and thus reduces the risk of subtransplant hematoma, such as between the matrix and the wound bed. It supports orderly healing and acts as a temporary adhesive barrier and exhibits limited mechanical stability when wetted but fits the wound bed very well. It is a substitute for acellular tissue in which native, structurally unaltered collagen functions as a part of the new extracellular matrix for the fusion of blood vessels and cells. The low thickness of MatriDerm® allows the graft to be supplied with nutrients through diffusion; subsequently, rapid vascularization occurs and as the healing process progresses, fibroblasts begin to produce their own collagen matrix. MatriDerm®, in contrast to other synthetic dermal matrices, has good integration, enabling one-step skin grafting in both the animal model and in people.[161] It provides, in fact, an adequate structure to restore the skin and modulate the formation of scar tissue at the skin level. it reduces the risk of the appearance of hematoma that occurs after skin grafting due to its haemostatic properties. MatriDerm® encourages the regeneration of the dermis in this way, decreasing the production of scars and restoring the treated area's suppleness and skin barrier to those of healthy skin. MatriDerm® is therefore thought to be a useful alternative for the healing of full-thickness skin defects.[162]

The ideal skin substitute should have precise characteristics such as sterility, the ability to provide a barrier against infection, maintain optimal moisture, and promote skin healing. In addition, It should have a low inflammatory reaction, be nontoxic, permit water vapor passage, stick easily to the wound surface, be able to dissolve over time, be simple to handle, and be affordable.[163-165]

In conclusion, obtaining an ideal dermal substitute with the suitable requirements is a complex and time-consuming and costly process. This justifies the fact that there is no universal TE product in the literature that meets all the requirements for an ideal skin graft.[163]

The use of dermal substitutes such as MatriDerm® and Integra® in the treatment of deep burns offers simpler and less disabling reconstructive possibilities for the patient as surgeries are less time-consuming, there is no morbidity of donor areas, and the postoperative course is easier. By encouraging stable tissue recovery and preventing minor excoriations and injuries from the lightest trauma, as can happen on scars that are not sufficiently maintained, such replacements enable the best possible regeneration of skin tissue. However, the main limitations of these substitutes are the high incidence of infection and high production costs. The two "neoderms" made from the application of MatriDerm® and Integra® replacements showed some thickness variation. In particular, the dermis regenerated through the use of MatriDerm® appears to be thinner as this substrate degrades more rapidly because its components are not cross-linked unlike Integra®. Thus, it is known that crosslinking reinforces matrix stability and makes it less susceptible to degradation, increasing strength. Nevertheless, crosslinking also has a negative effect on cell survival, proliferation, and adhesion because of breakdown products. This could explain the reduced cell density observed in regenerated dermis through the use of Integra®.[166,167] Another study has demonstrated, for the time being only in vitro, that MadriDerm® can easily transport and transfer growing epithelial cells (keratinocytes and melanocytes) on substance loss and can regenerate and produce a well-differentiated epidermis. In addition, the presence of melanocytes indicates that MatriDerm® has the ability to regenerate a pigmented epidermis.[168]

The ability to create in vitro dermal or epidermal substitutes on which proliferating epithelial cells (fibroblasts and keratinocytes) are cultured and then be able to transplant them onto the injured portion of the skin opens up great reconstructive possibilities on severe posttraumatic skin injuries, chronic injuries, and large burns.

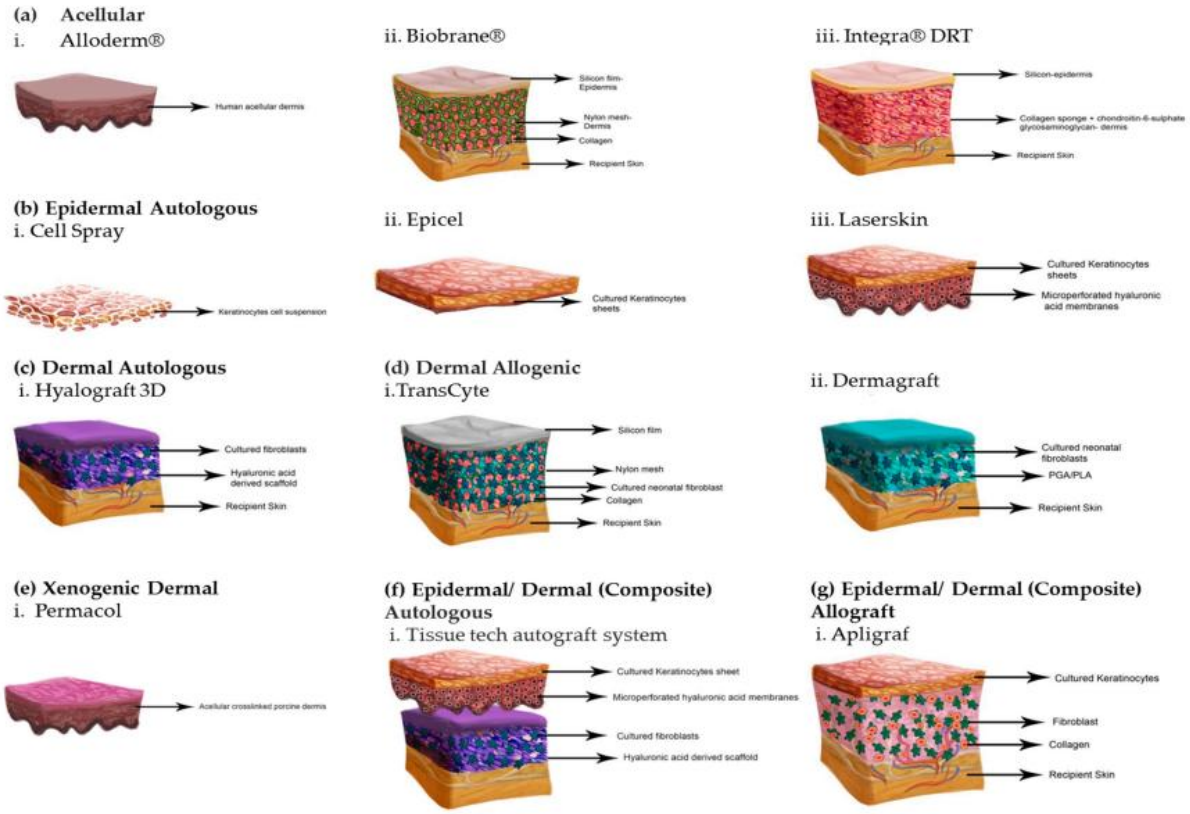


Figure 7. Examples of skin substitutes applied in the dermatology clinic [212]

AIM OF THE THESIS

The aim of this thesis project is to analyse in vitro the molecular expression of BM-hMSCs and UC-hMSCs induced to differentiate towards the fibroblast and keratinocyte lineage both in monolayer culture (2D) and on scaffolds (3D).

In detail, the main objectives were:

-develop new chitosan-based hydrogels.
-test biocompatibility: analysis of the viability and proliferation of BM-hMSCs and UC-hMSCs in G-PEG-CH-Gly and G-PEG-CH in the presence of FBS and hPL.
-design and apply the protocol for differentiation of BM-hMSCs and UC-hMSCs towards the fibroblast and keratinocyte lineage in the presence of FBS and hPL in monolayer culture (2D culture).
-design and apply the protocol for differentiation of BM-hMSCs and UC-hMSCs towards the fibroblast and keratinocyte lineage in G-PEG-CH-Gly, G-PEG-CH and MatriDerm® in the presence of FBS and hPL (3D culture).
-analyse the molecular expression of markers for differentiation in the fibroblast (COLL1 and COLL4) and keratinocyte (p63 and KRT) direction in both 2D and 3D cultures using the Digital PCR (dPCR) method.
-perform a preliminary histomorphological evaluation of the 3D model.
-assessing in immunohistochemistry (IHC) the markers of differentiation of BM-hMSCs and UC-hMSCs in a fibroblast and keratinocyte sense.

MATERIALS AND METHODS

Hybrid hydrogels synthesis

Hydrogels were prepared in aqueous solution and the synthetic procedure involved the reaction between the amine groups of gelatin and chitosan with the epoxy groups of functionalised polyethylene glycol (PEGDGE). PEGDGE can also react with other functional groups such as the hydroxyl groups of glycerol.

1. Preparation of the G-PEG-CH hydrogel

Briefly, gelatin (6g) was dissolved in 60g of distilled water at 40°C under gentle magnetic stirring, followed by the slow addition of PEGDGE (1.4g). A 2% solution of chitosan in 1% acetic acid (32.5g) was added to the reaction mixture and, again under slow stirring at 40°C after 15 minutes, a few drops of Ethylenediamine EDA were added until pH 7 - 7.5 was reached. The reaction mixture was kept under magnetic stirring at 40°C for 30 minutes and then poured into Petri dishes for gel formation. The hydrogels were allowed to air dry to a volume reduction of approximately 40%, then cut with a 1 cm diameter circular die, frozen and then freeze-dried using an Edwards Modulyo freeze-dryer operating under vacuum at -60°C, for sublimation of the ice crystals. To further increase the degree of grafting, the dried samples were subjected to a post-reaction process in an oven at 45°C for 2 hours under vacuum. Finally, the samples were washed by immersion in distilled water at 40°C for 24 hours and again frozen and freeze-dried. The final products obtained after the freeze-drying and post-curing process maintained their size and shape well. The hydrogels were then closed and sealed in small polypropylene bags and sterilised by gamma irradiation at 25 kGy according to UNI EN ISO 11137 (Sterilisation of Health Care Products).

2. Preparation of the G-PEG-CH-Gly hydrogel

A second hydrogel was prepared as described in step 1 with the only difference being that after the addition of chitosan, glycerol (2.5g) was added.

Hydrogel	Composition (%)			
	G	**PEG**	**CH**	**Gly**
G-PEG-CH-Gly	56.93	13.28	6.07	23.72
G-PEG-CH	74.5	17.4	8.1	/

Table 1. Composition of hybrid hydrogels. G: gelatin; PEG: polyethyleneglycol; CH: chitosan; Gly: glycerol

Morphological characterization of hybrid hydrogels

The morphology of G-PEG-CH and G-PEG-CH-Gly hydrogels was carried out using an optical microscope (Leica DMS 300, Leica Microsystems, Wetzlar, Germany). Specimens were observed both in dry and wet conditions (i.e. after immersion in distilled water for 24h) without any treatment.

Physicochemical characterization of hybrid hydrogels

Fourier-transform-infrared (FTIR) analysis was carried out on dry samples by means of a Thermo Scientific, Nicolet iS50 FTIR spectrophotometer (Thermo Fisher Scientific, Madison, WI, USA) equipped with a PIKE MIRacle attenuated total reflectance attachment. The spectra were recorded over a range of 400–4000 cm^{-1} at a resolution of 4 cm^{-1}. In order to evaluate the qualitative chemical composition of the materials before and after the hydrogel grafting, the test was carried out on G-PEG-CH hydrogel and G-PEG-CH-Gly hydrogel and on starting PEG, G, CH and Gly.

Hydrolytic mass loss evaluation and swelling ratio of hybrid hydrogels

Pre-weighed samples (Wi) were incubated in 30 mL distilled water at 37°C for a period of 25 days. At regular intervals of 1, 4, 7, 12, 19 and 25 days, the samples were removed, weighed (Ww), rinsed with fresh water, air-dried followed by vacuum drying at 45°C for 4 hours, and finally the dry mass (Wf) was measured. Water absorption (%) and mass loss (%) were calculated using the following equations:

$$\text{Swelling capacity} = \frac{Ww-Wi}{Wi}100$$

$$\text{Weight loss} = \frac{Wi-Wf}{Wi}100$$

The synthesis of hydrogels and their morphological characterisation were carried out by the group of Prof. Luciana Sartore, Department of Mechanical and Industrial Engineering, University of Brescia.

hPL production

Human Platelet Lysate (hPL) for MSCs expansion was obtained from blood donations belonging to the Blood Bank of ASST Spedali Civili of Brescia, Italy and produced according to standardized clinical grade procedures in closed systems. For the technical procedure's details, please refer to F. Re et al.

Human bone marrow and umbilical cord mesenchymal stromal cells (BM-UC-hMSCs) culture

BM-hMSCs and UC-hMSCs were purchased from Promocell, Germany. BM-h-MSCs and UC-hMSCs were expanded in the presence of a growth medium (GM) consisting of Dulbecco's modified Eagle's medium (DMEM), a high glucose -based medium with 1% penicillin-streptomycin, L-glutamine, sodium pyruvate, MEM Non Essential Amino Acids Solution solution, 0.05% amphotericin B and 10% fetal bovine serum (FBS) (referred as complete medium FBS) or 5% hPL (referred as complete medium hPL) at 37°C and 5% CO_2 in an incubator. BM-hMSCs and UC-hMSCs were detached from the flask using trypsin ethylenediaminetetraacetic acid after reaching 80% of confluence, neutralized with complete medium FBS, centrifugated at 1100 rpm for 5 min, resuspended in the GM, and counted with Burker chamber. Cells could be seeded.

BM-UC-hMSCs fibroblast differentiation in 2D and 3D culture

In 2D culture a cell suspension at a cellular density of 2000 cells/cm^2 for BM-hMSCs and 3000 cells/cm^2 for UC-hMSCs were added to the wells in 24-coated well plates for fibroblast differentiation in static conditions. The cells were cultured for 21 and 28 days with fibroblast differentiation medium (FI), consisting of medium FBS or medium hPL with only 1% penicillin-streptomycin and 0.05% amphotericin B and supplemented with 100 ng/mL connective tissue growth factor (CTGF) and 50 µg/mL L-ascorbic acid. All samples analysed in triplicates. As controls, samples were also cultured in the complete medium FBS or complete medium hPL without additional fibroblastic factors for 21 and 28 days to evaluate the cells in basal conditions.
In 3D culture a cell suspension at a cellular density of 3000 cells/mm^3 (471 x 10^3 cells/scaffold) for BM-hMSCs and UC-hMSCs were added to the scaffolds G-PEG-CH-Gly, G-PEG-CH and to the MatriDerm® for fibroblastic differentiation in static conditions. Scaffolds and MatriDerm® with the volume of approximately 157 mm^3 (approximately scaffolds dimensions: d=10 mm; h=2 mm; MatriDerm® dimensions: 8.8 x 8.8 x 2 mm) were used. The cells were added to each scaffold by direct cell seeding in 24-uncotated well plates, then incubated at 37 °C for 2h. After this time, 500 µL of differentiation medium (+FBS/hPL) or complete medium (+FBS/hPL) were added to each well. To induce fibroblastic differentiation cells-scaffolds constructs were cultured for 21 days under the same conditions as the 2D culture. The control samples were used to evaluate the fibroblastic potential of the scaffolds alone.

BM-UC-hMSCs keratinocyte differentiation in 2D and 3D culture

In 2D culture a cell suspension at a cellular density of 2000 cells/cm^2 for BM-hMSCs and 3000 cells/cm^2 for UC-hMSCs were added to the wells in 24-coated well plates for keratinocyte differentiation in static conditions. The cells were cultured for 21 and 28 days with keratinocyte differentiation medium (CH), consisting of Keratinocyte basal medium (KBM) FBS or KBM medium hPL with only 1% penicillin-streptomycin and 0.05% amphotericin B and supplemented with 5 ng/mL epidermal growth factor (EGF) and 1.8 mM $CaCl_2$. All samples analysed in triplicates. As controls, samples were also cultured in the complete medium FBS or complete medium hPL without additional keratinocytes factors for 21 and 28 days to evaluate the cells in basal conditions.

In 3D culture a cell suspension at a cellular density of 3000 cells/mm^3 for BM-hMSCs and UC-hMSCs were added to the scaffolds G-PEG-CH-Gly, G-PEG-CH and to the MatriDerm® for keratinocyte differentiation in static conditions. The dimensions of the scaffolds and seeding methods were like those reported in the previous paragraph. To induce keratinocyte differentiation cells-scaffolds constructs were cultured for 21 days under the same conditions as the 2D culture. The control samples were used to evaluate the keratinocytic potential of the scaffolds alone.

BM-UC-hMSCs cell viability and cell proliferation assay in 3D culture

In 3D culture a cell suspension at a cellular density of 3000 cells/mm^3 for BM-hMSCs and UC-hMSCs were added to the scaffolds G-PEG-CH-Gly, G-PEG-CH and to the MatriDerm® for cell viability and cell proliferation assay in static conditions. The cells were cultured for 21 days with complete medium FBS or complete medium hPL and cell viability analysed after 21 days, and cell proliferation analysed after 2, 10, 14, 21 days.

Cell viability was evaluated using a Live/Dead kit for mammalian cells (Thermofisher, USA). The samples were washed with DPBS and incubated for 30-45 min at RT in DPBS wth 2 µM of calcein AM and 4 µM of ethidium homodimer-1 (EthD-1). NucBlue® Live reagent (2 drops/mL) for nuclei staining was added to the cultures. An analysis of live (stained in green with calcein AM) and dead (stained in red with EthD-1) cells was then performed using a Zeiss Observer Z1 fluorescence microscope.

Cell proliferation was determined using the Cell Counting Kit-8 (CCK-8, Sigma-Aldrich, USA) on days 2, 10, 14, 21 of cell culture, following manufacturer instructions. The formazan produced by the CCK-8 assay is shown to be proportional to the number of viable cells giving rise to a colored compound that can be measured spectrophotometrically at 450 nm. Briefly, the cell-cultured samples (three replicates) were moved to a new cell culture plate at each time point and incubated with a conditioned culture medium containing the CCK-8 reagent (ratio 1:10) at 37 ° C for 2h. Then, the absorbance of 100 µL of supernatant transferred to a new cell culture plate was measured at 450 nm with an Infinite 200 PRO plate reader (Tecan, Switzerland).

Molecular analysis

RNA Extraction

To perform the RNA extraction, the complete and ready-to-use TRIzol™ Reagent (Life Technologies) was used, which allows the isolation of high-quality total RNA or enables the simultaneous isolation of RNA, DNA and proteins from a variety of biological samples. This monophasic solution of phenol and guanidine isothiocyanate is designed to isolate separate fractions of RNA, DNA and proteins from cell and tissue samples of human, animal, plant, yeast or bacterial origin.

The protocol included the following steps:

-Add 1 mL of TRIzol Reagent for at least 100.000 cells to each eppendorf containing growth medium only in the case of 2D culture and containing growth medium + scaffolds (previously homogenised with pestles) in the case of 3D culture.

- Lysate well and add 200 µL of chloroform, vortex and centrifuge at 12000 rpm for 15 min at 4°C.

-Take the aqueous phase (approx. 500 µL) and transfer it to a new eppendorf. Then add 500 µL of isopropanol (1:1 ratio), vortex and incubate at 4° C for at least 30 min or overnight at -20°C to allow the RNA to precipitate.

-Centrifuge at 12000 rpm for 15 min at 4°C.

-Check that the precipitated RNA pellet is visible and remove the supernatant.

-Add 1 mL of 75% ethanol to perform washing, shake to mobilise the pellets and centrifuge at 7000 rpm for 10 min at 4°C.

-Discard the supernatant and repeat the previous step.

-Discard the supernatant and allow the pellets to air dry by leaving the Eppendorf open for up to 5 min.

-Resuspend the RNA pellets in 26 µL of Nuclease Free Water.

-Quantify to NanoDrop 2000c (ThermoFisher Scientific) by adding 1 µL of each sample and store the RNA sample at -80°C until further use.

Retro-Trascription

The RnaUsScript Reverse Transcriptase (LeGene Biosciences-TwinHelix) kit was used to perform RNA-to-cDNA retrotranscription. The protocol provided for two stages.

1st Phase

For the first denaturation step, a reaction mixture was prepared containing 1 µL of NTPs 10 mM, 1 µL of Random Primer 50 ng/µL and 1 µg of RNA. It was made up to volume with water to a final volume of 13 µL. The sample was loaded onto the Thermal Cycler Veriti (Applied Biosystems) at 65°C for 5 min. Once the incubation was completed, the sample was immediately placed on ice.

2nd Phase

The following reaction mixture was added to each sample:

Buffer 5x cDNA Synthesis	4 µL
H_2O	2,60 µL
RNAUsScript Reverse Transcriptase	0.40 µL
Final volume	7 µL

During the second phase, the sample was loaded onto the Thermal Cycler Veriti (Applied Biosystems) with a temperature profile of 25° C for 5 min, 50° C for 45 min and 70° C for 15 min. At the end of the run 1 µL of sample was quantified at NanoDrop 2000c. The cDNA sample was stored at -20° C until further use.

Digital PCR analysis

The dPCR system QuantStudio™ Absolute Q (Applied Biosystems) is a plate-based dPCR platform based on microfluidic properties (Microfluidic Array Plate, MAP) that allow all the steps involved in the technique to be performed, i.e. partitioning, thermocycling and data acquisition using a single instrument. The MAP technology allows for uniformity in the analysis of the microchambers, while the dead-ended reagent loading allows for the analysis of 95% of the incoming sample, compared to 25-60% in other dPCR platforms. Reagent digitization, thermal cycling and data collection take an average of 90 minutes. The QuantStudio Absolute Q system has the ability to read 4 different optical channels at the same time, which allows multiplex analysis, i.e. to measure more targets per sample in a single reaction.

dPCR was used for the absolute quantification of COLL1 and COLL4 (fibroblast markers), p63 and KRT10 (keratinocyte markers) transcripts. The GAPDH transcript was used as the housekeeper gene. Commercial assays from Life Technologies ThermoFisher were used for all transcripts. All the transcript probes analyzed were labelled with the fluorochrome FAM, except for GAPDH, which was labelled with the fluorochrome VIC.

The following reaction mixtures were prepared for fibroblast markers (for each sample):

Mastermix	1,8 µL	Mastermix	1,8 µL
$GAPDH_{VIC}$ (20x)	0,45 µL	$COLL4_{FAM}$ (20x)	0,45 µL
$COLL1_{FAM}$ (20x)	0,45 µL	H_2O	5,75 µL
H_2O	5,3 µL		

COLL1 was analysed in multiplex with GAPDH.

The following reaction mixtures were prepared for the keratinocyte markers (for each sample):

Mastermix	1,8 µL	Mastermix	1,8 µL
$GAPDH_{VIC}$ (20x)	0,45 µL	$KRT10_{FAM}$ (20x)	0,45 µL
$p63_{FAM}$ (20x)	0,45 µL	H_2O	5,75 µL
H_2O	5,3 µL		

p63 was analysed in multiplex with GAPDH.

A fixed volume of cDNA of 50 ng/µL was added to each reaction.

Each sample was then loaded onto the bottom of the various wells of the MAP plate for QuantStudio™ Absolute Q™ Digital PCR Instrument, taking care to tilt the tip 45° and not to insert bubbles. Then 15 µL of Absolute Q™ Isolation Buffer was added to each well, which placed over the reagent to prevent

contamination and evaporation of the sample. Finally, the wells of the various columns were covered using 5 MAP-specific closure strips provided by the kit. The samples were run at 60°C for 35 cycles for the analysis of the COLL1, p63 and KRT10 transcripts, while for the analysis of the COLL4 transcript they were run at 60°C for 45 cycles. At the end of the run, the instrument generated primary analysis files containing various qualitative and quantitative dates of the detected fluorescence signals. The results were then analysed and processed with the software QuantStudio™ Absolute Q™ Digital PCR Software 6.

Hystomorphological analysis at optical microscope

The analysis was performed on cells/scaffolds constructs in which fibroblast and keratinocyte differentiation was induced and on control samples.

Scaffolds were embedded in paraffin using an automatic processor Donatello series 2 (Diapath S.p.A., Bergamo, Italy). Serial paraffin sections (5µm thick) of each sample were cut with a semiautomatic microtome Galileo semi-series 2 (Diapath S.p.A., Bergamo, Italy). Alternate sections were deparaffinized, and rehydrated, according to standard procedures and stained with hematoxylin-eosin stain (automatic stainer Giotto; Diapath S.p.A., Bergamo, Italy) to appreciate the general morphology and Masson-Goldner Trichrome stain (automatic stainer Giotto; Diapath S.p.A., Bergamo, Italy) to identify collagen fibers of the scaffolds on day 21. The micrographs were recorded using a camera equipped with an image analysis system (Image-Pro Premier 9.1; 2018, Immagini e Computer, Milan, Italy).

Immunohistochemistry analysis

The analysis was performed on cells/scaffolds constructs in which fibroblast and keratinocyte differentiation was induced and on control samples.

Briefly, the sections were subjected to antigen retrieval in 0.05 M sodium citrate buffer (pH 6.0) in a hot water bath (98°C for 20 min) and then incubated first in adequate serum (10% in TBS plus 0,,1% Triton X-100) for 60 min and then in primary antiserum directed against collagen type I (rabbit anti-goat polyclonal antibody, 1:250, Santa Cruz Biotechnology) and cytokeratin 10 (mouse monoclonal antibody, 1:250, Santa Cruz Biotechnology) in TBS containing 3% normal serum and 0,1% Triton X-100, for 24h at 4° C. After incubation in the primary antiserum, the sections were sequentially incubated with appropriated biotinylated secondary antibodies and avidin-biotin peroxidase complex (Vector Laboratories, Burlingame, CA, USA).

The reaction product was visualized using hydrogen peroxide and diaminobenzidine (Sigma, St. Louis, MO, USA) as chromogen; the immunopositivity was identified as a brown color. To better visualize the positive reaction, the sections were counterstained with hematoxylin (blue/violet color), dehydrated, and mounted

with DPX for light microscopy detection. The immunohistochemical control was performed by omitting the primary antibody and incubating the sections with non-immune rabbit serum and with isotype-matched irrelevant rat IgGs as negative control.

Both histomorphological and immunohistochemical analysis were carried out at the Anatomy and Pathophysiology section of the University of Brescia in collaboration with Prof. Elisa Borsani.

Statistical analysis

For the statistical studies of the in vitro assessment of cells proliferation and differentiation on the scaffolds, GraphPad Prism was used. Two-way ANOVA with Bonferroni post-hoc test was performed as specified in the captions in the figures. Three replicates of each sample were used. Statistical significance was accepted at the probability level $p < 0.05$. Data are expressed as mean ± standard deviation.

RESULTS

Hybrid hydrogels

Two different scaffolds named G-PEG-CH and G-PEG-CH-Gly were synthesised, differing in the percentage of all components (**Table 1**). In order to create hydrogels, amine groups from gelatin and chitosan were reacted with epoxy groups from functionalized polyethylene glycol (PEGDGE) in an aqueous solution. As a crosslinking agent, functionalized PEG was utilized, with the primary players being the PEG epoxide groups and the amino groups of the G and CH chains. PEGDGE can also react with other functional groups such as the hydroxyl groups of glycerol. The hydrogel was frozen, then freeze-dried to produce the macroporous scaffold components. The final products obtained after the freeze-drying and post-curing process maintained their size and shape well.

The morphology of the hydrogels was analysed using an optical microscope. The **Figures 8 and 9** showed the morphology of hydrated (**Figures 8A and 9A**) and dry (**Figures 8B and 9B**) of G-PEG-CH and G-PEG-CH-Gly hydrogels respectively. As shown in **Figures 8B and 9B**, both the dry hydrogels showed a highly interconnected irregular pore morphology and micro-macro spherical pore and channels are homogeneously distributed into the network.

The chemical properties of the hydrogels were analysed by FTIR in order to confirm the qualitative composition. FTIR analysis was carried out on G-PEG-CH and G-PEG-CH-Gly and on samples of their components. The infrared spectra showed the signals of the presence of all the materials constituents for both scaffolds (**Figures 10A and 10B**). In the assembled hydrogels, the characteristic peaks of its constituents are present. In G-PEG-CH-Gly, there were two absorbance peaks relating to G, one at 1549.11 cm^{-1} and the other at 1632.20 cm^{-1}, and one peak relating to PEG at 1080.43 cm^{-1}. At 1032.78 cm^{-1} there were two peaks corresponding to the other two components, i.e. CH and Gly. On G-PEG-CH, two peaks at 1032.65 cm^{-1} and 1080.73 cm^{-1} for G, a peak at 1080.73 cm^{-1} for PEG and two peaks at 1548.80 cm^{-1} and 1632.74 cm^{-1} for CH were evident.

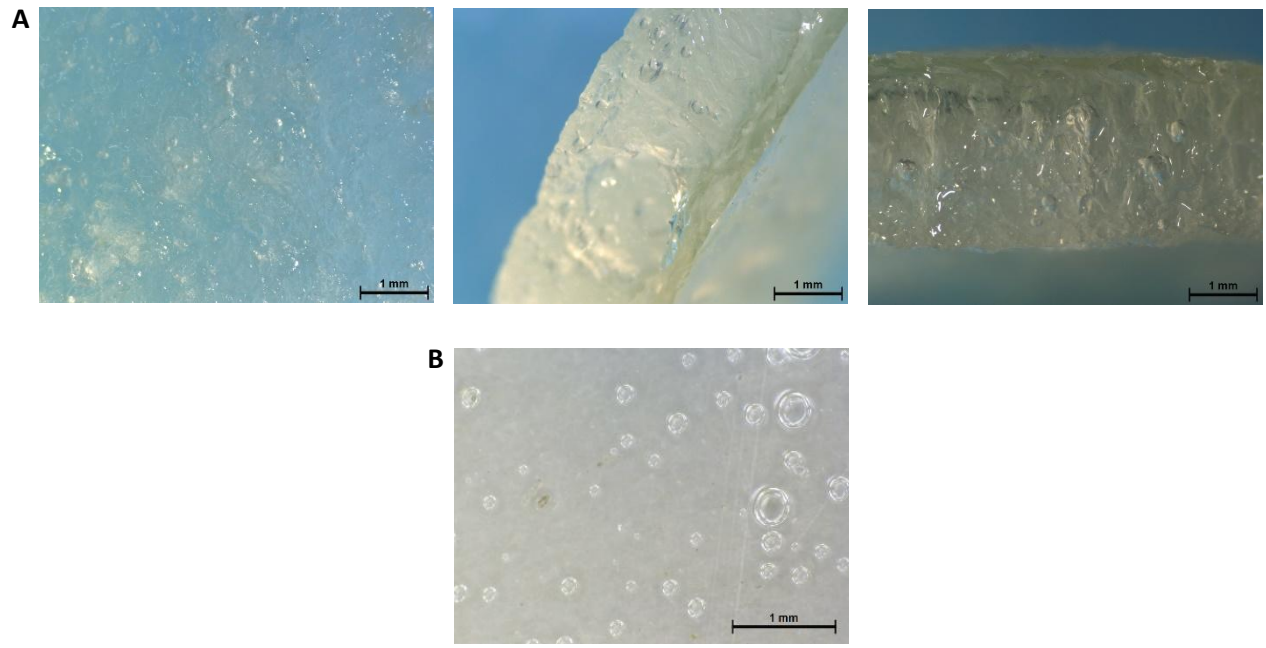


Figure 8. Macroscopical morphology of G-PEG-CH hybrid hydrogels. Optical visualization of the porous structure of hydrated (**A**) and dry (**B**) hydrogels. Scale bar: 1 mm.

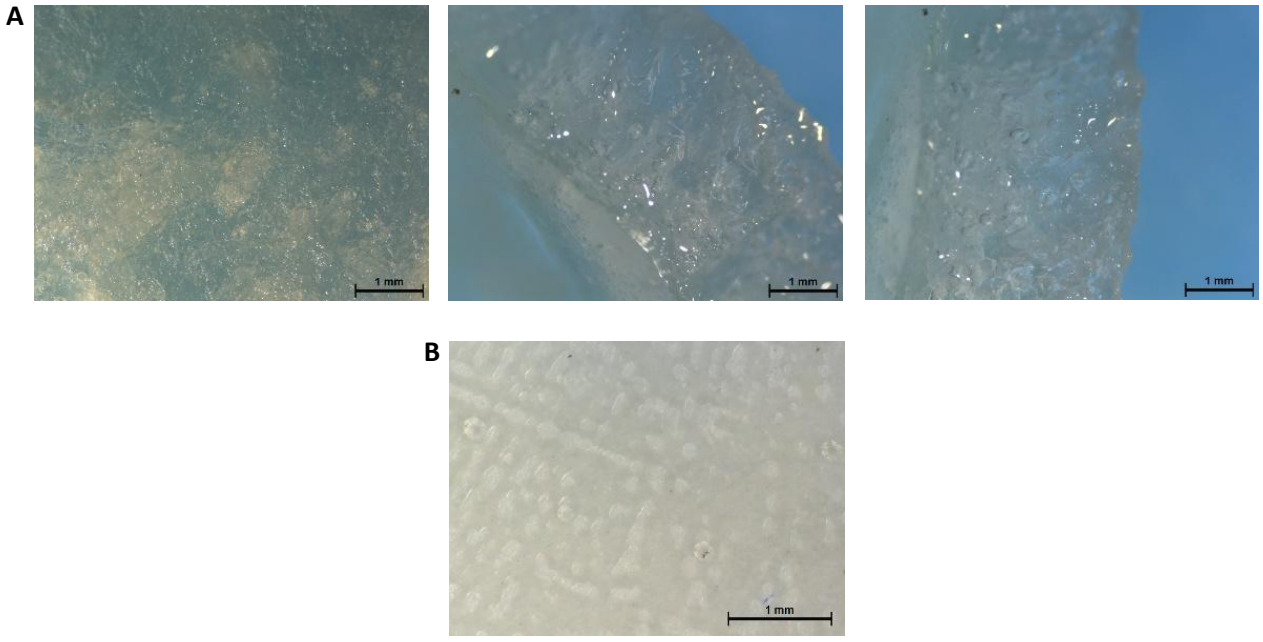
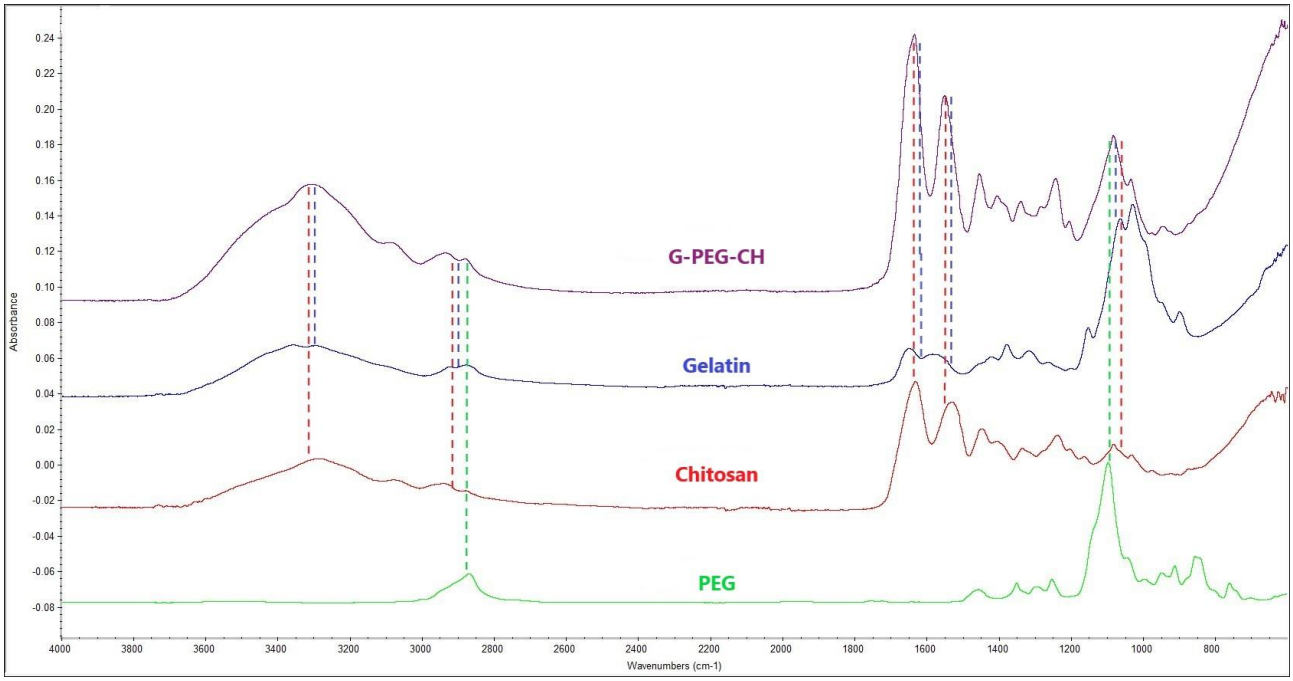


Figure 9. Macroscopical morphology of G-PEG-CH-Gly hybrid hydrogels. Optical visualization of the porous structure of hydrated (**A**) and dry (**B**) hydrogels. Scale bar: 1 mm.

A



B

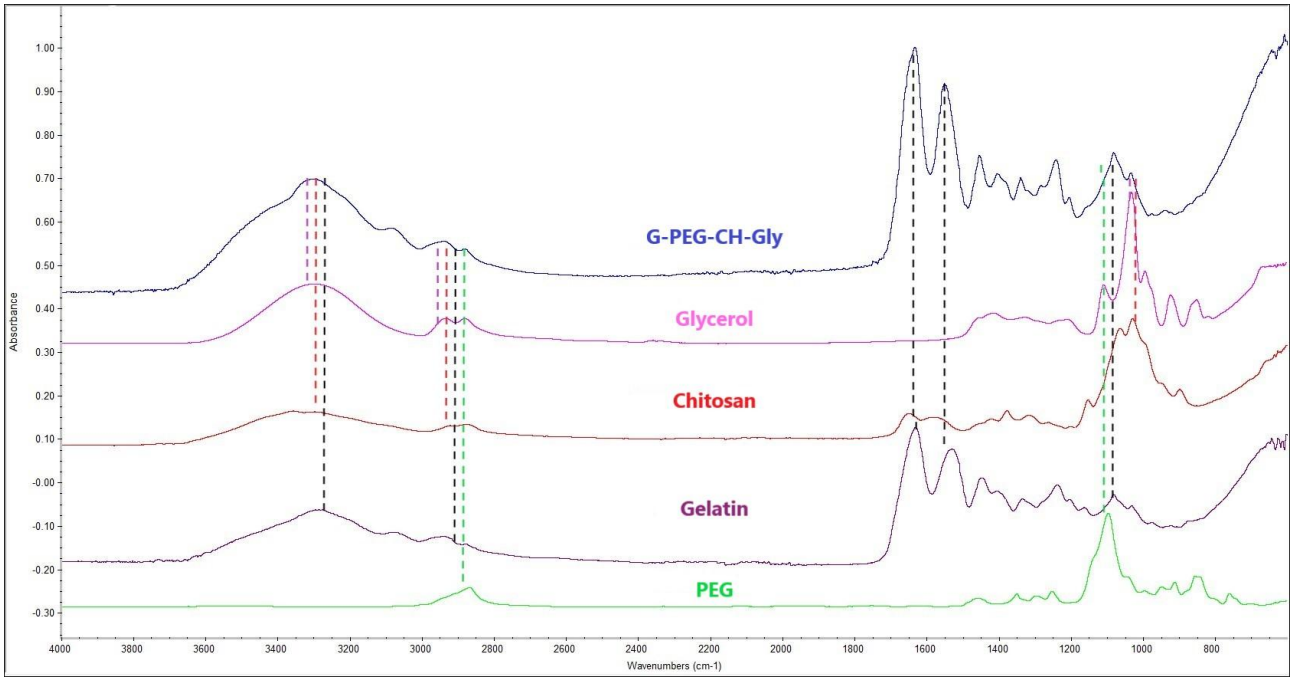


Figure 10. (**A**) FTIR spectra of starting materials (G,PEG,CH) and of the assembled scaffold G-PEG-CH. (**B**) FTIR spectra Of starting materials (G,PEG,CH,Gly) and of the assembled scaffold G-PEG-CH-Gly.

For long-term use of hydrogels, materials must be controlled in their degradation without losing their structural integrity. Structural stability and swelling ratio are impacted by degradation.

Therefore, we investigated hydrogel hydrolytic degradation by measuring mass loss and swelling ratio at predetermined time of 1, 4, 7, 12, 19 and 25 days.

It is noteworthy that the hydrolytic bonds in G, PEG, CH, and Gly, as well as hydrogels derived from their grafting and crosslinking, cause hydrolysis, which destroys the network and dissolves/leaches away the fragmented soluble fractions. These events may result in mass loss, which opens spaces for water and raises the swelling ratio.

Both hydrogels had an increase in swelling ratio and mass loss. Regarding the original values for G-PEG-CH-Gly and G-PEG-CH, the swelling ratio rose by almost 60% and 25%, respectively (**Figures 11A and 11B**). Compared to the original masses, mass losses of 40% for G-PEG-CH-Gly and 45% for G-PEG-CH were seen (**Figures 11C and 11D**).

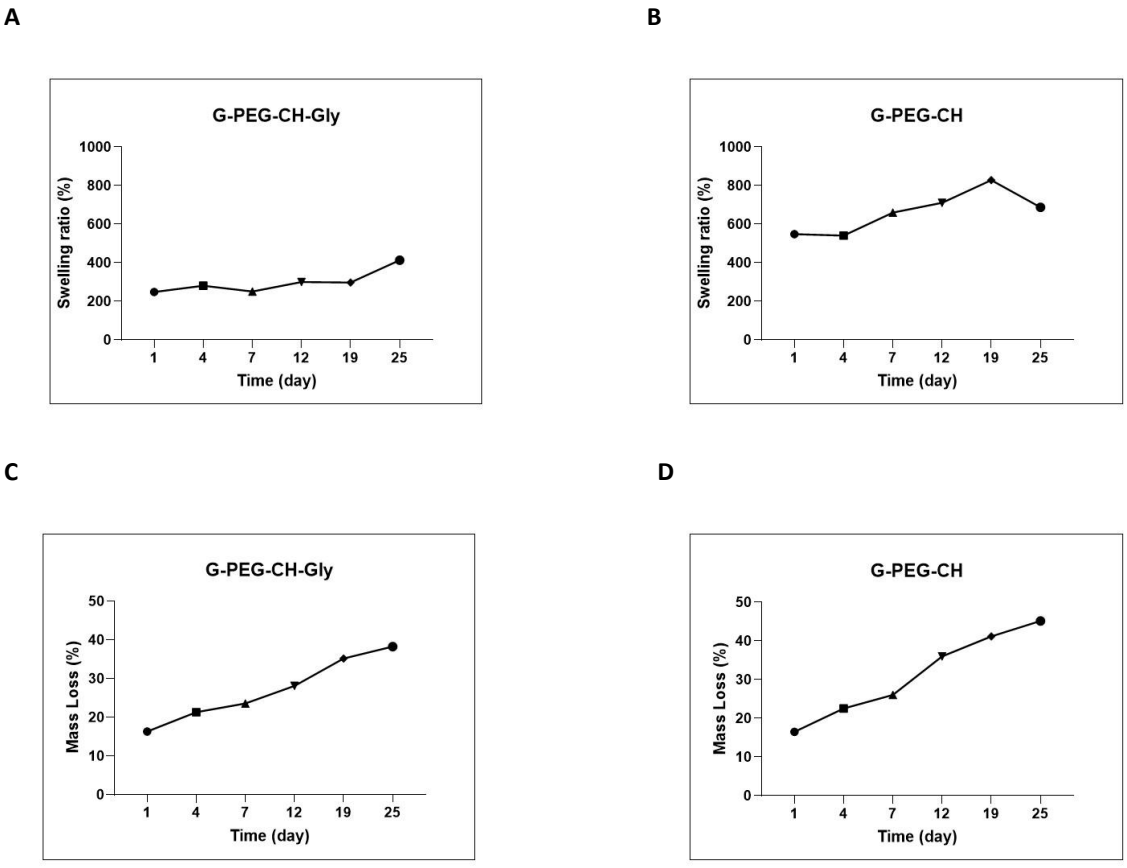


Figure 11. (**A-D**) Swelling ratio and mass loss trends during degradation period of G-PEG-CH-Gly (**A,C**) and G-PEG-CH (**B,D**).

Evaluation of cell viability in hybrid hydrogels

BM-hMSCs and UC-hMSCs were seeded in the hydrogels G-PEG-CH-Gly, G-PEG-CH and MatriDerm® and after 21 days in complete medium FBS or complete medium HPL, cell viability analysis was performed using a Live/Dead kit for mammalian cells (ThermoFisher, USA). The analysis was carried out using the Zeiss Observer Z1 fluorescence microscope, which made it possible to visualize the viable cells, stained in green with calcein-AM and the dead cells, stained in red with ethidium (EthD-1). Staining with Hoechst showed that there is no homogeneous distribution of cells in the scaffolds, in particular in G-PEG-CH-Gly and G-PEG-CH. In detail, both BM-hMSCs and UC-hMSCs tend to accumulate along the edges of the scaffolds and not in the centre of them. Both cell lines were found to be viable, and no significant differences were found between the conditions considered. This was supported by the fact that ethidium-labelled (EthD-1), and therefore non-viable, cells were small in number. In addition, a morphological difference was observed between BM-hMSCs (**Figure 12**) and UC-hMSCs (**Figure 13**) stained with calcein-AM. In particular, the BM-hMSCs showed a fibroblast-like morphology with a rounded appearance, whereas the UC-hMSCs showed a fibroblast-like morphology with a fusiform appearance. Through observation in the Bright Field, it was possible to visualize the structure of the various scaffolds (**Figures 12 and 13**). Observing the images acquired in bright field, in the **figures 12A and 13A,** it was possible to appreciate the morphology of the MatriDerm®, which appeared structurally uniform in all its parts with pores of homogeneous size, ensuring an even distribution of cells throughout the scaffold. The structure of G-PEG-CH-Gly had a heterogeneous porosity with larger, interconnected pores (**Figures 12B and 13B**). The structure of G-PEG-CH was found to be porous, although the degree of porosity was found to be lower than G-PEG-CH-Gly (**Figures 12C and 12C**). G-PEG-CH-Gly had some larger pores than G-PEG-CH. In addition, G-PEG-CH-Gly was characterized by a slight concavity of the central core of the scaffold, in which there was an accumulation of cells. In general, the porosity of increased from the outside to the inside of the scaffold to ensure a different distribution of cells in the different layers, emulating the native tissue. The MatriDerm® had a structure like the extracellular matrix, with dense texture and reduced porosity compared to G-PEG-CH-Gly and G-PEG-CH but uniform in its distribution.

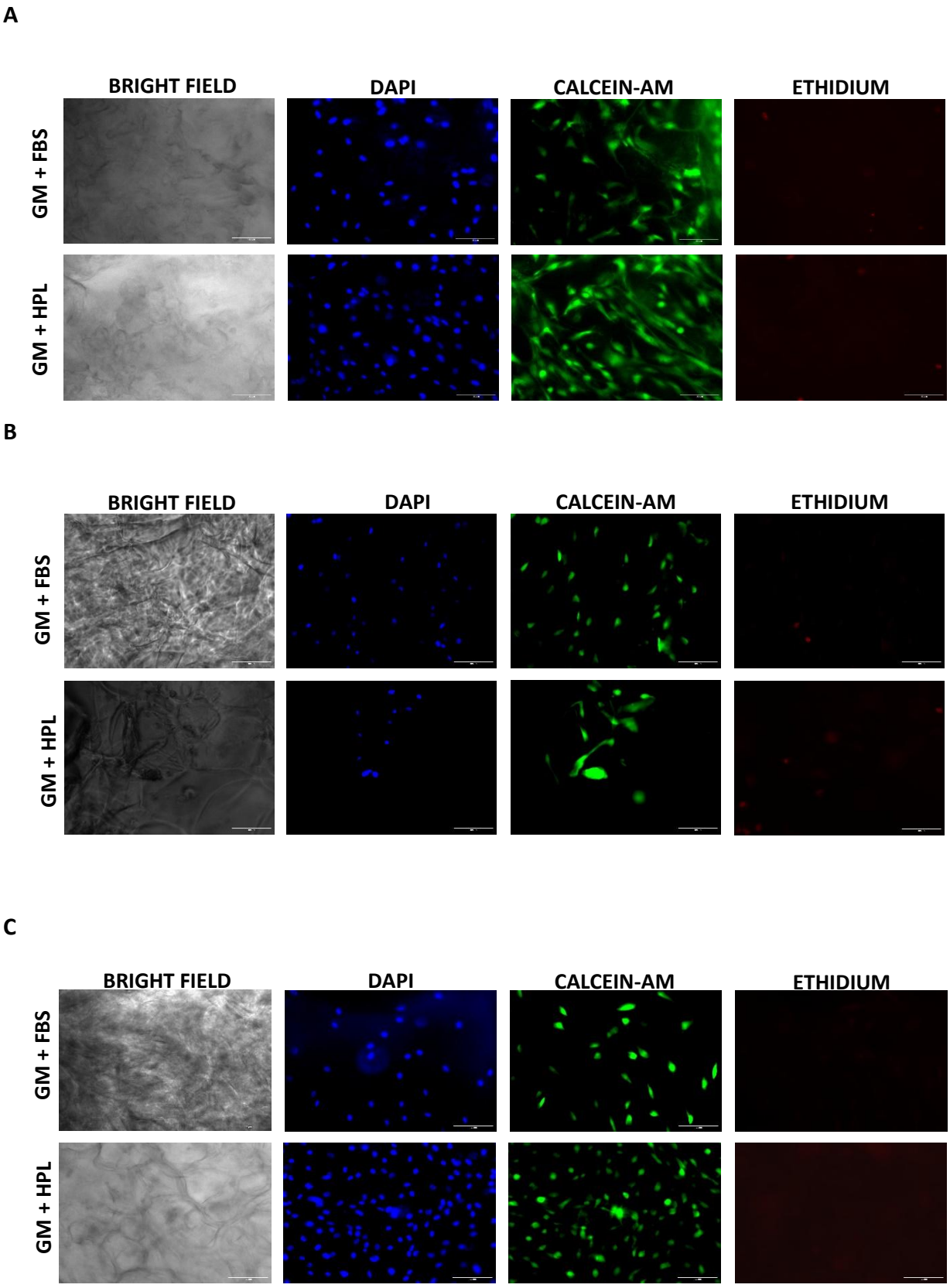


Figure 12. Live/Dead staining of BM-hMSCs cultivated for 21 days in MatriDerm® (**A**), G-PEG-CH-Gly (**B**) and G-PEG-CH (**C**) hydrogels in the complete medium FBS o complete medium hPL. Scale bar: 100 µm.

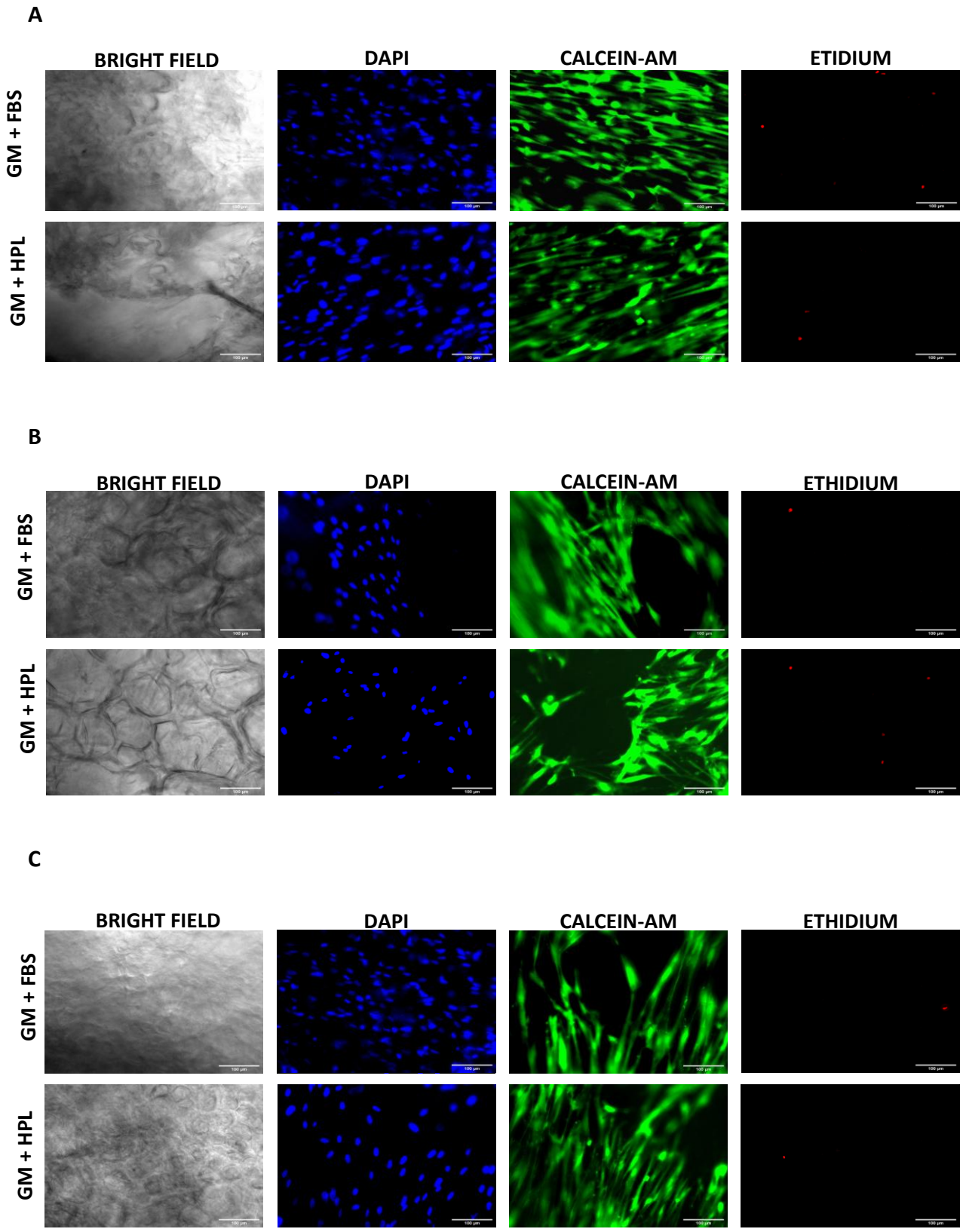


Figure 13. Live/Dead staining of UC-hMSCs cultivated for 21 days in MatriDerm® (**A**), G-PEG-CH-Gly (**B**) and G-PEG-CH (**C**) hydrogels in the complete medium FBS o complete medium hPL. Scale bar: 100 µm.

Evaluation of cell proliferation in hybrid hydrogels

Cell proliferation was determined by quantifying the number of viable cells in the scaffolds using the Cell Counting Kit-8 (CCK-8, Sigma-Aldrich, USA) on days 2, 10, 14, 21 of cell culture. Cells were cultured for 21 days with complete medium FBS or complete medium hPL on hydrogels G-PEG-CH-Gly, G-PEG-CH and MatriDerm®. The BM-hMSCs and UC-hMSCs grew differently in the various scaffolds during the 21 days of culture.

The BM-hMSCs in MatriDerm® grew exponentially reaching the plateau at the day 21 of culture as well as in G-PEG-CH-Gly despite the slight decrease at the day 14 of culture. In G-PEG-CH they had a proliferative peak at the day 2 of culture and then reached the plateau as early as day 10, remaining stable over the following days of the culture (**Figure 14**). Statistically significant differences were found after 10 and 14 days of culture between the various conditions.

In general, in the presence of the GM+FBS medium, bm grew exponentially in the G-PEG-CH-Gly scaffold while in the G-PEG-CH scaffold they maintained a steady-state growth during 21 days of culture. In the presence of the GM+HPL medium, BM-hMSCs in G-PEG-CH-Gly proliferated gradually up to day 10 of culture, stopping around day 14, and then resumed proliferation on day 21. In G-PEG-CH they grew exponentially on day 2 of culture and then maintained a stationary growth over the following days. In the MatriDerm®, on the other hand, they grew exponentially in the presence of both GM+FBS and GM+HPL medium, showing statistically significant values in both cases and reaching a plateau at 21 days.

Considering the growth kinetics of BM-hMSCs in relation to the condition in which they were grown, i.e. in the presence of GM+FBS and GM+HPL, it was possible to find statistically significant differences when considering the same scaffold on different culture days. In the images below are only the graphs for the G-PEG-CH-Gly and MatriDerm® scaffolds in which statistically significant differences were found between the conditions GM+FBS and GM+HPL (**Figure 15**).

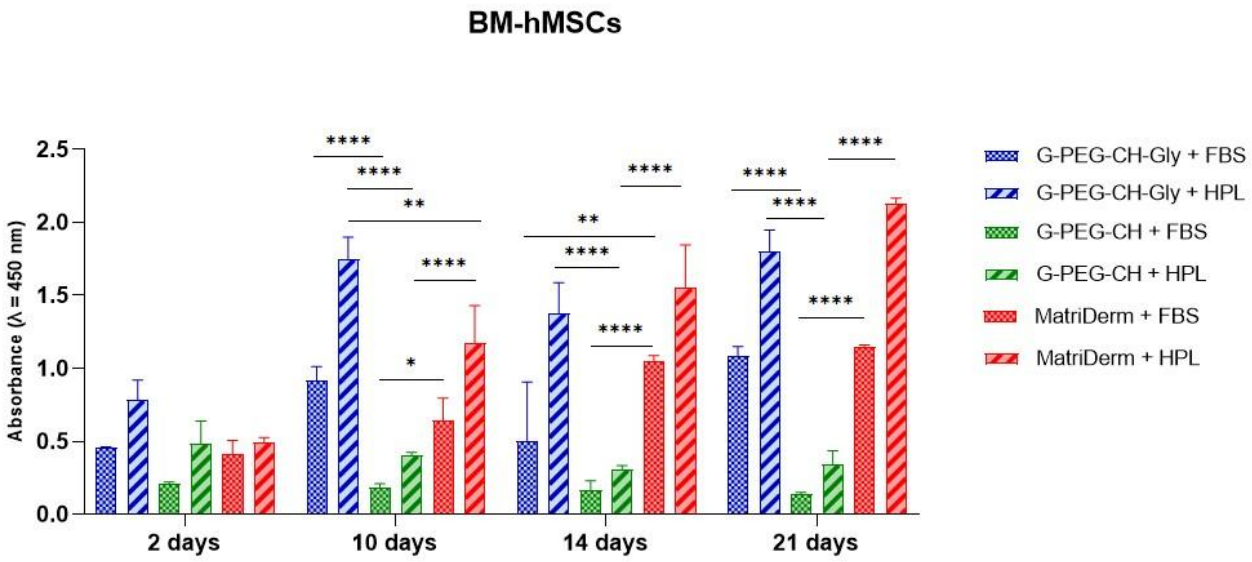


Figure 14. Proliferation of BM-hMSCs grown in G-PEG-CH-Gly, G-PEG-CH and MatriDerm® scaffolds in GM+FBS and GM+HPL complete media at different time points (2, 10, 14 and 21 days). Two-way ANOVA with test post-hoc Bonferroni was used for statistical analysis by GraphPad Prism (version 8). Data show the average values and their significant differences (*p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001).

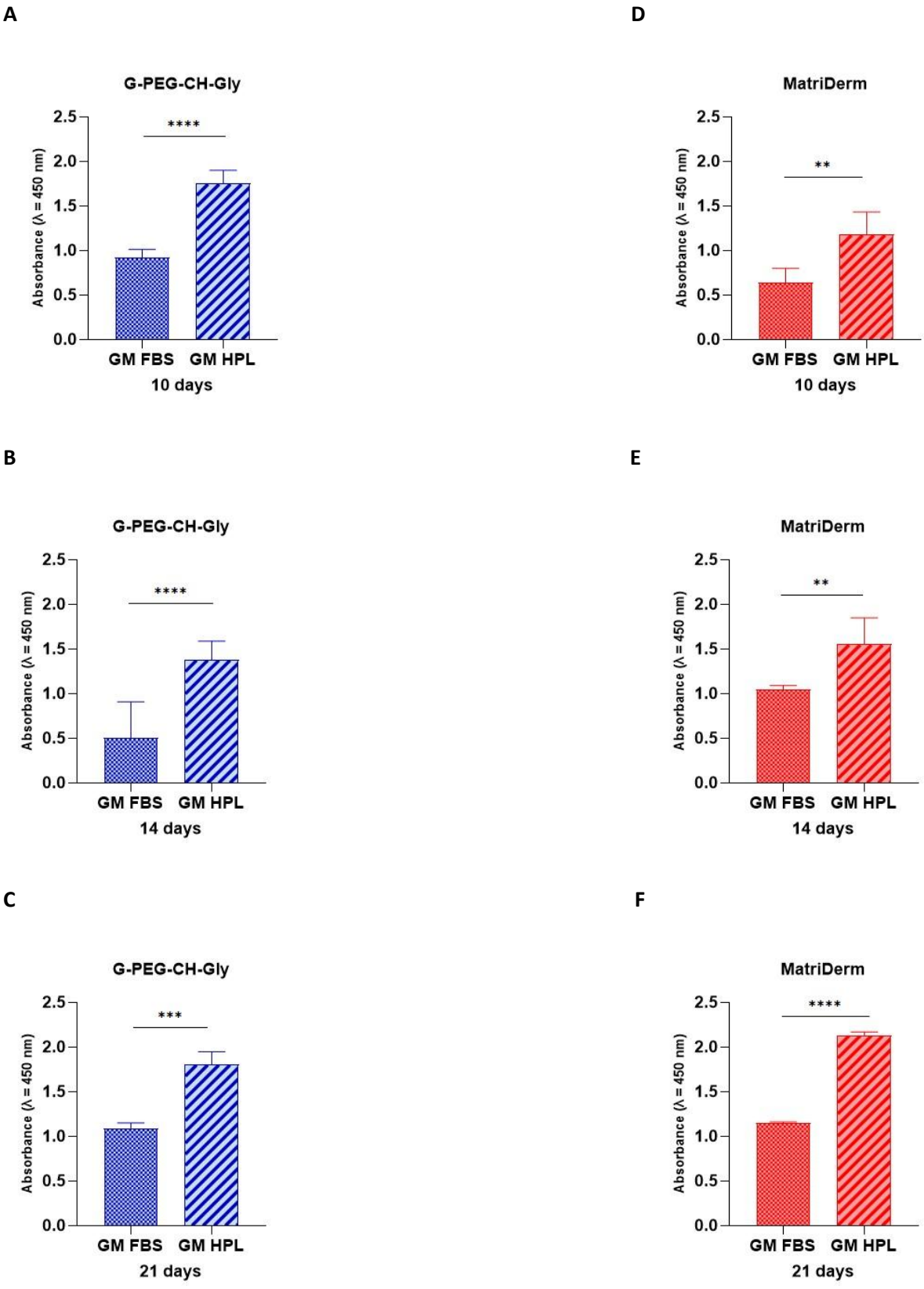


Figure 15. Proliferation of BM-hMSCs grown in G-PEG-CH-Gly and MatriDerm® scaffolds in GM+FBS and GM+HPL complete media at different time points (10, 14 and 21 days). (**A**) Comparison of the two conditions GM+FBS and GM+HPL in the G-PEG-CH-Gly scaffold at day 10 of culture. (**B)** Comparison of the two conditions GM+FBS and GM+HPL in the G-PEG-CH-Gly scaffold at day 14 of culture. (**C**) Comparison of the two conditions GM+FBS and GM+HPL in the G-

PEG-CH-Gly scaffold at day 21 of culture. (**D**) Comparison of the two conditions GM+FBS and GM+HPL in the MatriDerm® at day 10 of culture. (**E**) Comparison of the two conditions GM+FBS and GM+HPL in the MatriDerm® at day 14 of culture. (**F**) Comparison of the two conditions GM+FBS and GM+HPL in the MatriDerm® at day 21 of culture. Two-way ANOVA with test post-hoc Bonferroni was used for statistical analysis by GraphPad Prism (version 8). Data show the average values and their significant differences (*p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001).

The UC-hMSCs in MatriDerm® grew exponentially reaching the plateau at the day 21 of culture. In G-PEG-CH-Gly grew moderately to peak proliferation on day 21. In G-PEG-CH, growth reached a plateau on day 10 of culture while remaining stable on subsequent days (**Figure 16**).

In detail, already after 2 days of culture, statistically significant differences in UC-hMSCs cell growth were found between the various conditions at each time point.

In general, in the presence of the GM+FBS medium, the UC-hMSCs showed steady-state growth kinetics in G-PEG-CH-Gly up to day 14 with a statistically significant growth peak on day 21 of culture, while in G-PEG-CH they showed steady-state growth kinetics on all days of culture. In the presence of the GM+HPL medium, the UC-hMSCs in G-PEG-CH-Gly reached plateau between days 14 and 21, while in G-PEG-CH they grew exponentially on day 2 and then remained in a stationary phase until day 21. In the MatriDerm®, on the other hand, they grew exponentially in the presence of both GM+FBS and GM+HPL medium, in both cases reporting statistically significant values and reaching a plateau at 21 days. (**Figure 17**).

Considering the growth kinetics of UC-hMSCs in relation to the condition in which they were grown, i.e. in the presence of GM+FBS and GM+HPL, it was possible to find statistically significant differences when considering the same scaffold on different culture days. In the images below are only the graphs for the G-PEG-CH and MatriDerm® scaffolds at the time points where statistically significant differences were found between the conditions GM+FBS and GM+HPL (**Figure 17**).

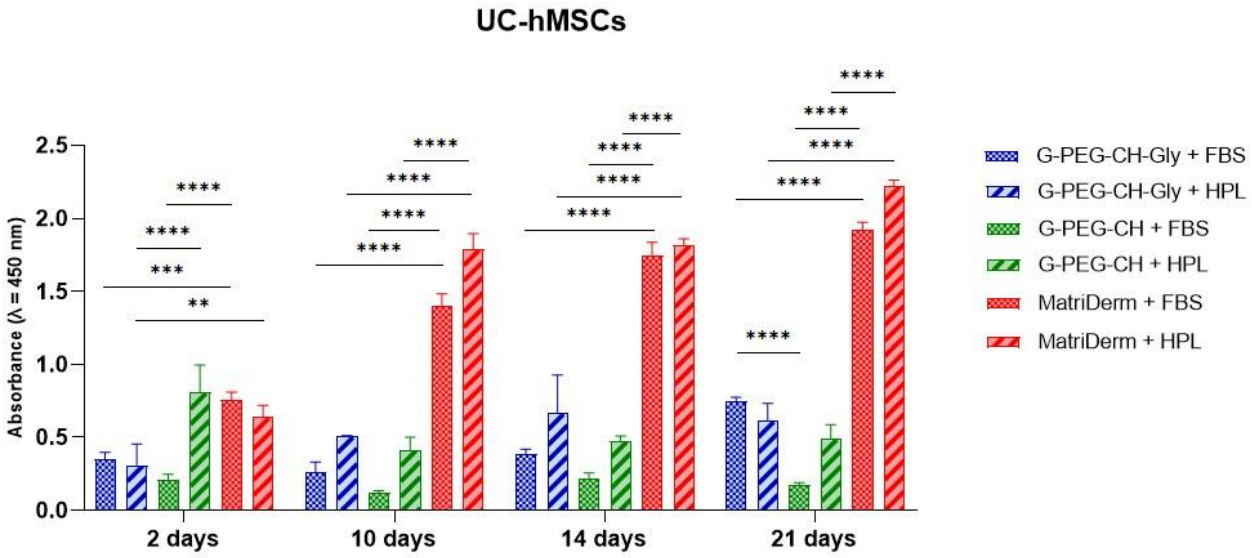


Figure 16. Proliferation of UC-hMSCs grown in G-PEG-CH-Gly, G-PEG-CH and MatriDerm® scaffolds in GM+FBS and GM+HPL complete media at different time points (2, 10, 14 and 21 days). Two-way ANOVA with test post-hoc Bonferroni was used for statistical analysis by GraphPad Prism (version 8). Data show the average values and their significant differences (*p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001).

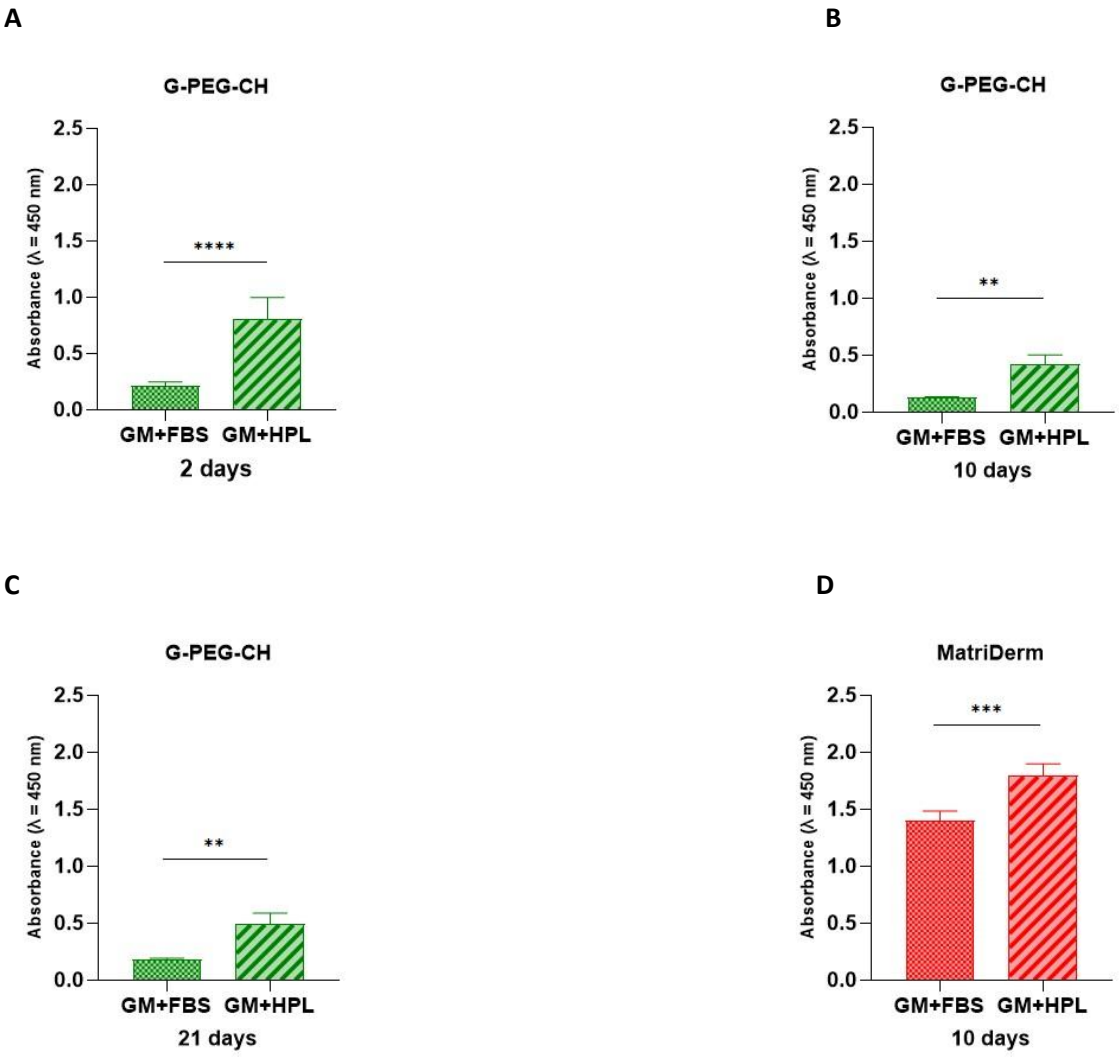


Figure 17. Proliferation of UC-hMSCs grown in G-PEG-CH and MatriDerm® scaffolds in GM+FBS and GM+HPL complete media at different time points (2, 10 and 21 days). (**A**) Comparison of the two conditions GM+FBS and GM+HPL in the G-PEG-CH scaffold at day 2 of culture. (**B)** Comparison of the two conditions GM+FBS and GM+HPL in the G-PEG-CH scaffold at day 10 of culture. (**C**) Comparison of the two conditions GM+FBS and GM+HPL in the G-PEG-CH scaffold at day 21 of culture. (**D**) Comparison of the two conditions GM+FBS and GM+HPL in the MatriDerm® at day 10 of culture. Two-way ANOVA with test post-hoc Bonferroni was used for statistical analysis by GraphPad Prism (version 8). Data show the average values and their significant differences (*p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001).

Analysis of fibroblast and keratinocyte differentiation in 2D culture

Differentiation towards the fibroblast and keratinocyte lines of BM-hMSCs and UC-hMSCs in two-dimensional culture (2D) was assessed by quantifying collagen 1 (COLL1) and collagen 4 (COLL4) transcripts for fibroblasts, protein 63 (p63) and cytokeratin 10 (KRT10) for keratinocytes at day 21 and 28.

The dPCR analysis of the different molecular markers was performed by evaluating the absolute number of copies/µL of the COLL1, COLL4, p63 and KRT10 transcripts, obtained by processing the data with the software QuantStudio™ Absolute Q™ Digital PCR Software 6 (ThermoFisher). The results considered are based on the absolute transcript quantification of each gene considered (copies/µL of the COLL1, COLL4, p63 and KRT10), normalised considering the absolute transcript quantification of the reference gene GAPDH (copies/ml of the housekeeper gene). The results obtained from the ratio of each marker and GAPDH were expressed as copies/ml for each sample (marker/GAPDH [copies/µL]).

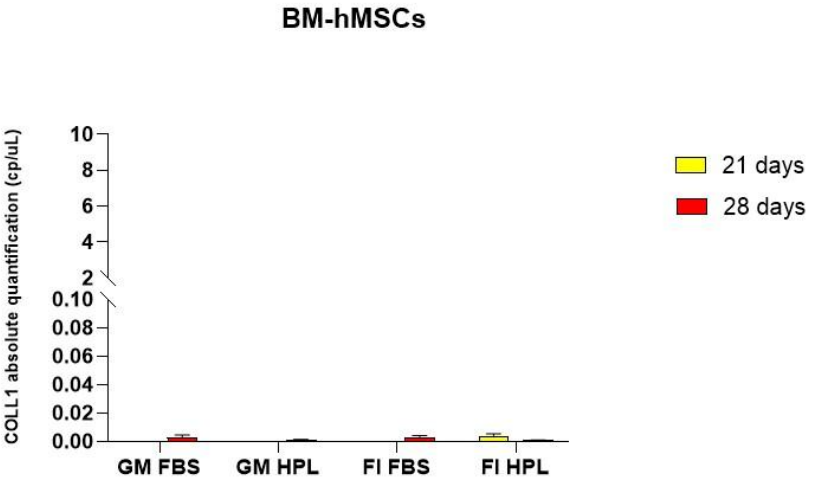
The expression of the fibroblast marker COLL1 by the BM-hMSCs, as the **Figure 18.A** shows, is evident both in the presence of the complete growth medium alone, i.e. without additional differentiating factors (GM+FBS and GM+HPL), and in the presence of the differentiating medium (FI+FBS and FI+HPL) at both 21 and 28 days (mainly at 28 days except in the FI+HPL condition), without statistically significant values between the different conditions and time points.

The fibroblast marker COLL4 is expressed by BM-hMSCs both in the presence of complete growth medium alone, i.e. without additional differentiating factors (GM+FBS and GM+HPL), and in the presence of differentiating medium (FI+FBS and FI+HPL). In particular, in the FI+FBS and FI+HPL conditions there is greater expression of the COLL4 marker at 21 days than at 28 days confirmed by statistically significant values as the **Figure 18.B** shows.

Regarding the expression of the keratinocyte marker p63 by BM-hMSCs emerged both in the presence of complete growth medium, i.e. without differentiation factors (GM+FBS and GM+HPL), and in the presence of differentiating medium (CH+FBS and CH+HPL) at both 21 and 28 days, without statistically significant values between the different conditions and time points (**Figure 19.A**).

While **Figure 19.B** shows the expression of the keratinocyte marker KRT10 by BM-hMSCs both in the presence of complete growth medium, i.e. without differentiation factors (GM+FBS and GM+HPL), and in the presence of differentiating medium (CH+FBS and CH+HPL) at both 21 and 28 days with statistically significant values between CH+FBS and CH+HPL conditions. In the first case there is a higher expression of the marker at 28 days while in the second case the expression is higher at 21 days.

A



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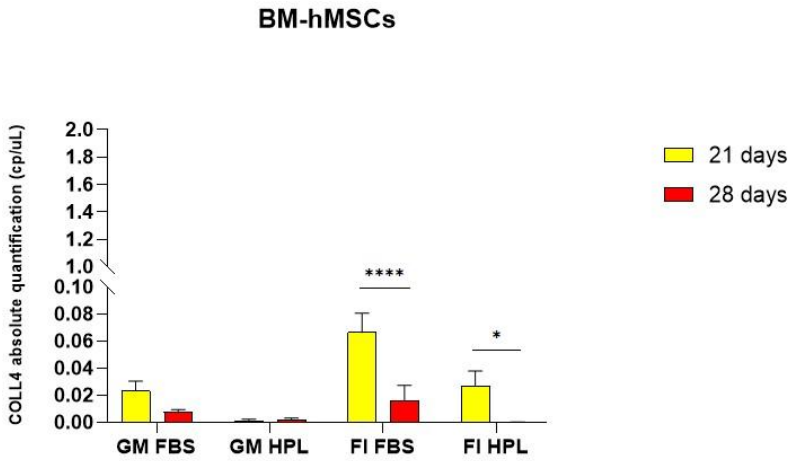
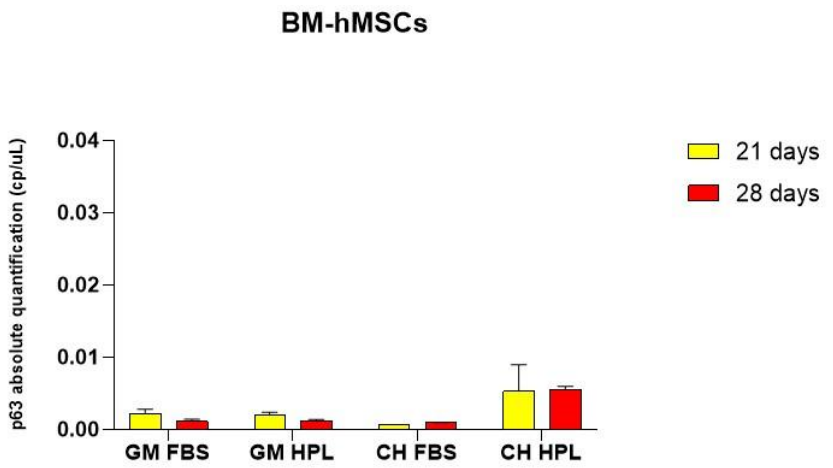


Figure 18. Analysis of the expression of COLL1 **(A)** and COLL4 **(B**), performed by dPCR in BM-hMSCs grown for 21 and 28 days in 2D in complete medium supplemented with FBS (GM+GBS), complete medium supplemented with HPL (GM+HPL), differentiating medium supplemented with FBS (FI+FBS) and differentiating medium supplemented with HPL (FI+HPL). Two-way ANOVA with test post-hoc Bonferroni was used for statistical analysis by GraphPad Prism (version 8). Data show the average values and their significant differences (*p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001).

A



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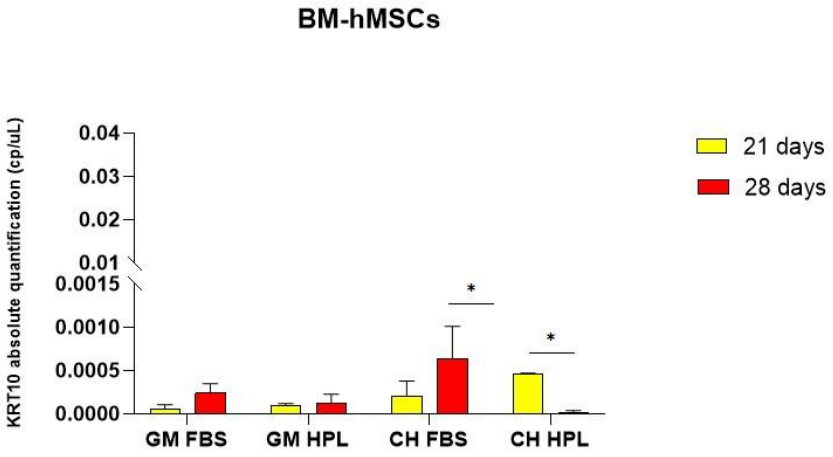


Figure 19. Analysis of the expression of p63 **(A)** and KRT10 **(B**), performed by dPCR in BM-hMSCs grown for 21 and 28 days in 2D in complete medium supplemented with FBS (GM+GBS), complete medium supplemented with HPL (GM+HPL), differentiating medium supplemented with FBS (CH+FBS) and differentiating medium supplemented with HPL (CH+HPL). Two-way ANOVA with test post-hoc Bonferroni was used for statistical analysis by GraphPad Prism (version 8). Data show the average values and their significant differences (*p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001).

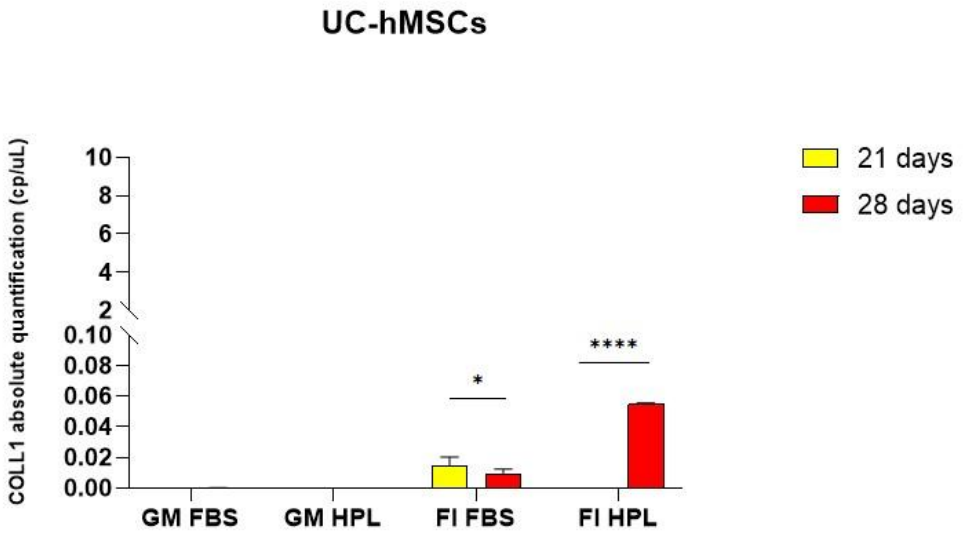
Regarding UC-hMSCs, **Figure 20.A** shows the expression of marker fibroblast COLL1 in both in the presence of differentiating medium (FI+FBS and FI+HPL). In particular, there is an expression of transcript in the FI+FBS condition at both 21 and 28 days: 21 days vs 28 days (*p = 0.0454); while the expression of COLL1 in the FI+HPL condition is present only at 28 days: 21 days vs 28 days (****p ≤ 0.0001).

While **Figure 20.B** shows the expression of marker fibroblast COLL4 by UC-hMSCs both in the presence of complete growth medium alone, i.e. without additional differentiating factors (GM+FBS and GM+HPL), and in the presence of differentiating medium (FI+FBS and FI+HPL). In the GM+HPL and FI+FBS conditions there is greater expression of the COLL4 marker at 21 days than at 28 days confirmed by statistically significant values: GM+HPL 21days vs 28 days (***p = 0.0005); FI+FBS 21 days vs 28 days (****p ≤ 0.0001).

The marker keratinocyte p63 is expressed by UC-hMSCs both in the presence of complete growth medium alone, i.e. without additional differentiating factors (GM+FBS and GM+HPL), and in the presence of differentiating medium (CH+FBS and CH+HPL). In detail, in the CH+FBS condition there is a higher expression of marker at 21 days than at 28 days confirmed by statistically significant values as the **Figure 21.A** shows.

The expression of marker keratinocyte KRT10 by UC-hMSCs, as the **Figure 21.B,** both in the presence of complete growth medium alone, i.e. without additional differentiating factors (GM+FBS and GM+HPL), and in the presence of differentiating medium (CH+FBS and CH+HPL). In detail, in the GM+HPL condition there is statistically significant expression of the marker KRT10 at 28 days compared to 21 days.

A



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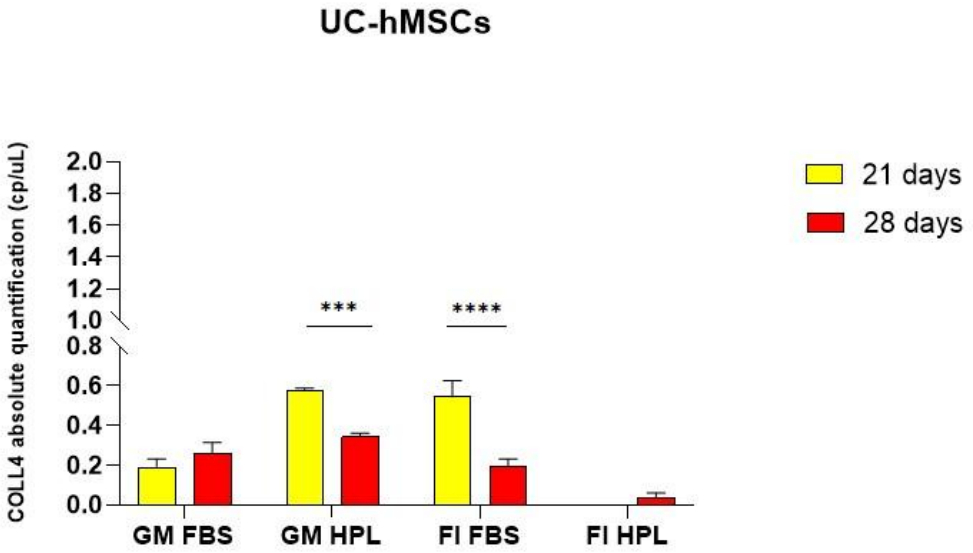

Figure 20. Analysis of the expression of COLL1 **(A)** and COLL4 **(B**), performed by dPCR in UC-hMSCs grown for 21 and 28 days in 2D in complete medium supplemented with FBS (GM+GBS), complete medium supplemented with HPL (GM+HPL), differentiating medium supplemented with FBS (FI+FBS) and differentiating medium supplemented with HPL (FI+HPL). Two-way ANOVA with test post-hoc Bonferroni was used for statistical analysis by GraphPad Prism (version 8). Data show the average values and their significant differences (*p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001).

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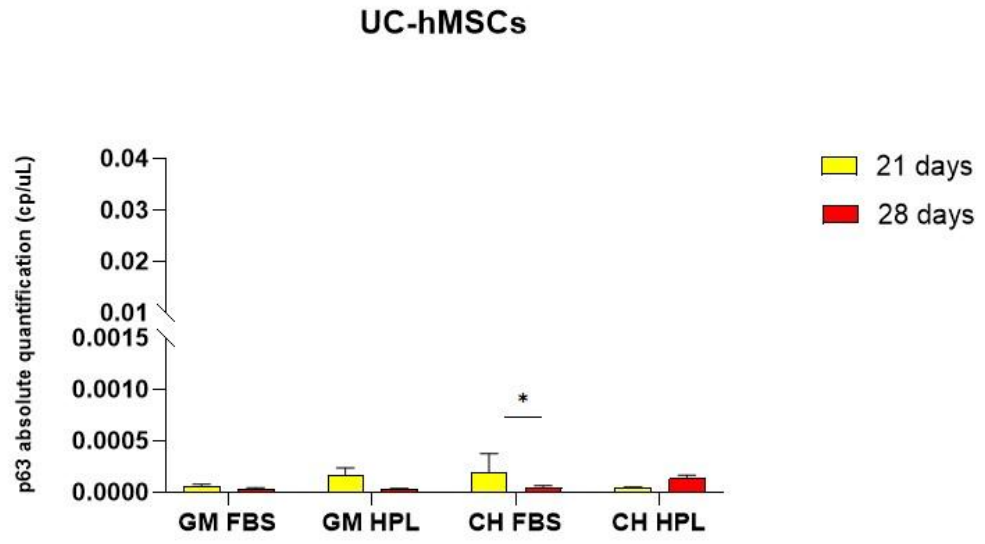

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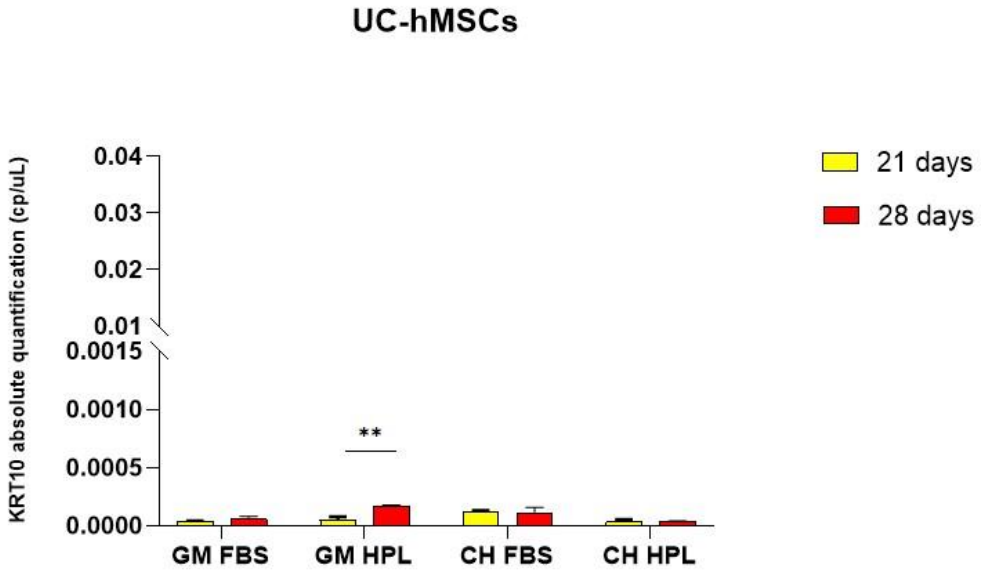


Figure 21. Analysis of the expression of p63 **(A)** and KTR10 **(B**), performed by dPCR in UC-hMSCs grown for 21 and 28 days in 2D in complete medium supplemented with FBS (GM+GBS), complete medium supplemented with HPL (GM+HPL), differentiating medium supplemented with FBS (CH+FBS) and differentiating medium supplemented with HPL (CH+HPL). Two-way ANOVA with test post-hoc Bonferroni was used for statistical analysis by GraphPad Prism (version 8). Data show the average values and their significant differences (*p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001).

Analysis of fibroblast and keratinocyte differentiation in 3D culture

The results obtained from the molecular analysis of differentiation in 2D culture confirmed that the 21-day time point, compared to the 28-day time point, is ideal for the analysis of gene expression of BM-hMSCs and UC-hMSCs towards the fibroblast and keratinocyte lines. Therefore, differentiation in 3D culture was assessed by quantifying the molecular markers COLL1, COLL4 for fibroblasts and p63 and KRT10 for keratinocytes in the G-PEG-CH-Gly, G-PEG-CH and MatriDerm® (MD) scaffolds at day 21.

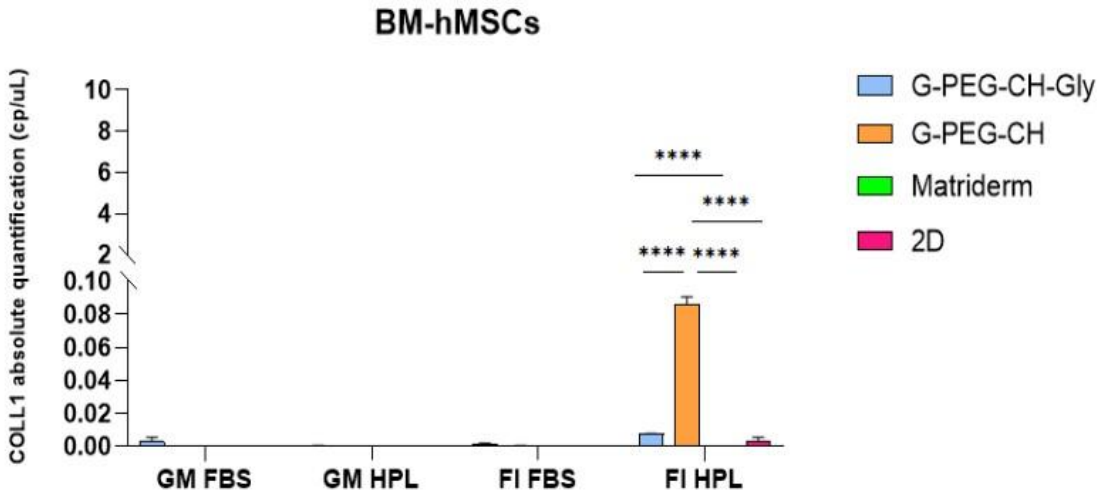
G-PEG-CH-Gly and G-PEG-CH scaffolds can promote the differentiation of BM-hMSCs towards the fibroblast line mainly in the presence of the differentiation medium supplemented with HPL (FI+HPL) by evaluating the expression of the marker COLL1, confirmed by statistically significant values as the **Figure 22.A** shows.

G-PEG-CH-Gly, G-PEG-CH and MatriDerm® scaffolds have the ability to induce the differentiation of BM-hMSCs towards the fibroblast line by evaluating the expression of the marker COLL4 , both in the presence of complete growth medium added with FBS (GM+FBS) and HPL (GM+HPL), and in the presence of differentiating medium added FBS (FI+FBS) and HPL (FI+HPL). Statistically significant values can be found in different culture conditions as shown in **Figure 22.B**.

G-PEG-CH-Gly, G-PEG-CH and MatriDerm® scaffolds have the ability to induce the differentiation of BM-hMSCs towards the keratinocyte line, by evaluating the expression of the marker p63, mainly in presence of differentiating medium added FBS (CH+FBS), while there is an expression of p63 by BM-hMSCs in 2D culture in presence of differentiating medium added HPL (CH+HPL), as reported by statistically significant values (**Figure 23.A**).

Figure 23.B shows that only G-PEG-CH-Gly and G-PEG-CH scaffolds have the ability to promote the differentiation of BM-hMSCs towards the keratinocyte line, by evaluating the expression of the marker KRT10, only in presence of differentiating medium added HPL (CH+HPL) confirmed by statistically significant values.

A



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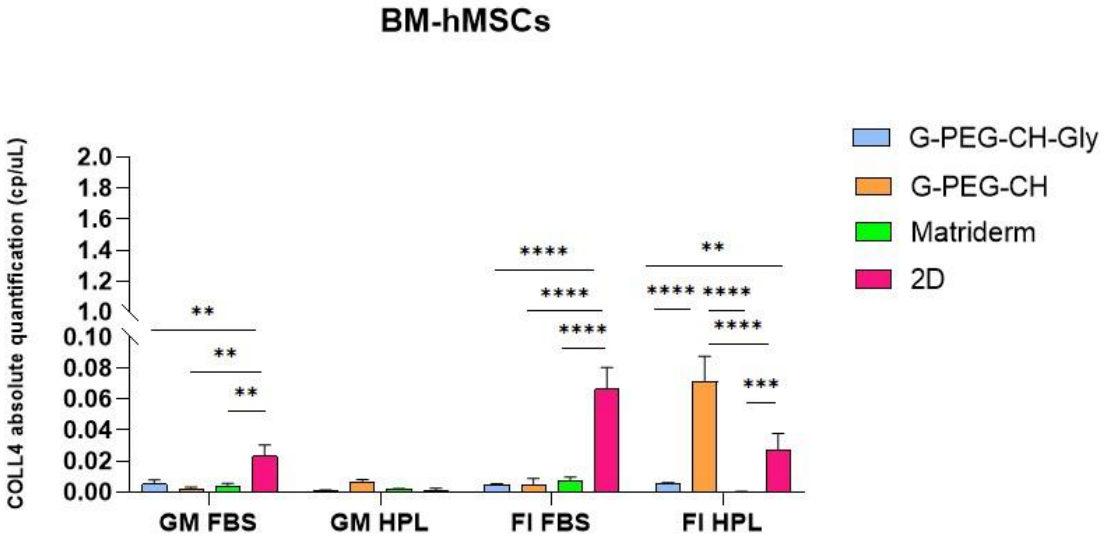
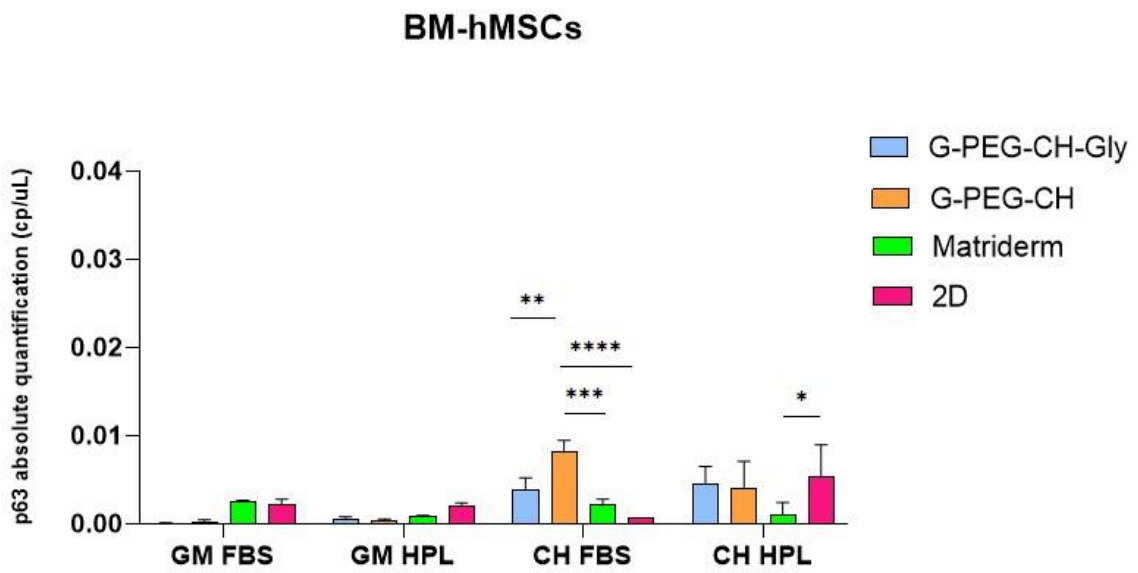


Figure 22. Analysis of the expression of COLL1 **(A)** and COLL4 **(B**), performed by dPCR in BM-hMSCs grown for 21 days in G-PEG-CH-Gly, G-PEG-CH and MatriDerm® in complete medium supplemented with FBS (GM+GBS), complete medium supplemented with HPL (GM+HPL), differentiating medium supplemented with FBS (FI+FBS) and differentiating medium supplemented with HPL (FI+HPL). Two-way ANOVA with test post-hoc Bonferroni was used for statistical analysis by GraphPad Prism (version 8). Data show the average values and their significant differences (*p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001).

A



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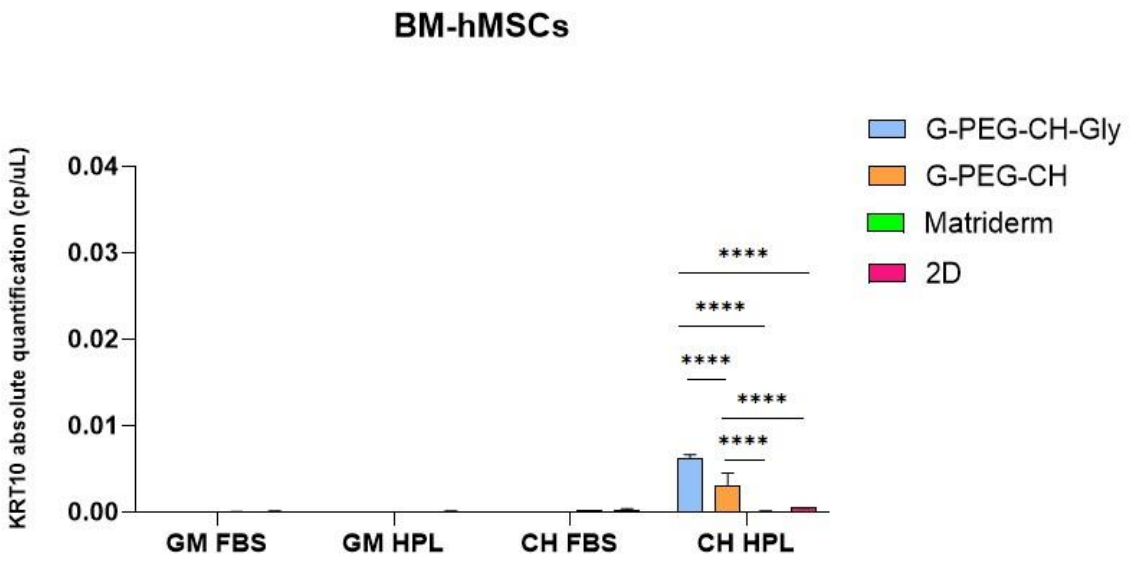


Figure 23. Analysis of the expression of p63 **(A)** and KRT10 **(B**), performed by dPCR in BM-hMSCs grown for 21 days in G-PEG-CH-Gly, G-PEG-CH and MatriDerm® in complete medium supplemented with FBS (GM+GBS), complete medium supplemented with HPL (GM+HPL), differentiating medium supplemented with FBS (CH+FBS) and differentiating medium supplemented with HPL (CH+HPL). Two-way ANOVA with test post-hoc Bonferroni was used for statistical analysis by GraphPad Prism (version 8). Data show the average values and their significant differences (*p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001).

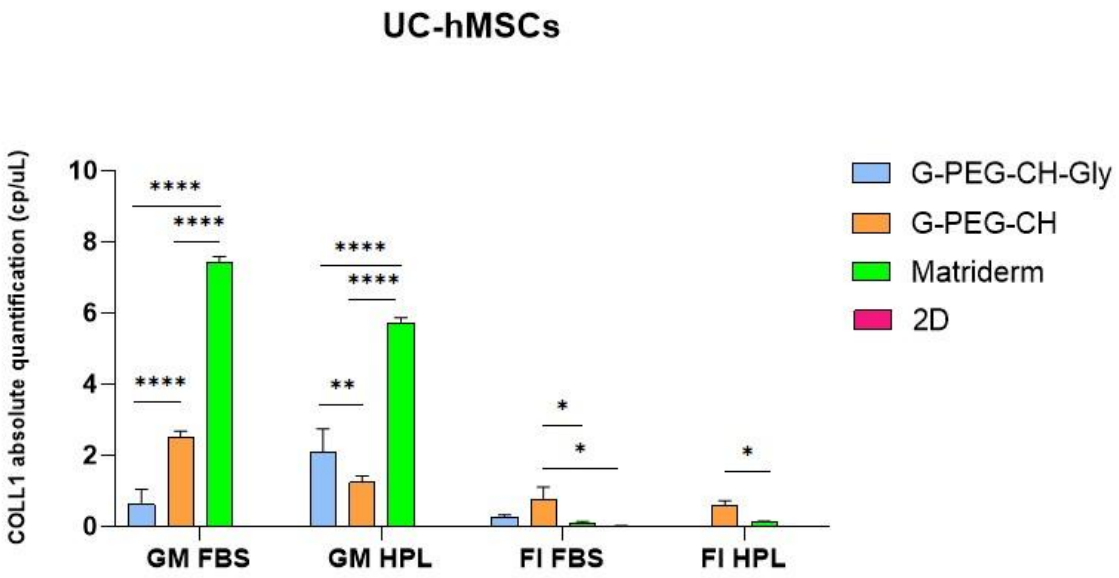
Regarding UC-hMSCs, **figure 24.A** shows that G-PEG-CH-Gly, G-PEG-CH and MatriDerm® scaffolds have the ability to induce the differentiation of UC-hMSCs towards the fibroblast line, by evaluating the expression of the marker COLL1, both in the presence of complete growth medium added with FBS (GM+FBS) and HPL (GM+HPL) and lower absolute value, in the presence of differentiating medium added FBS (FI+FBS) and HPL (FI+HPL) as reported by statistically significant values.

Figure 24.B shows that G-PEG-CH-Gly, G-PEG-CH and MatriDerm® scaffolds have the ability to promote the differentiation of UC-hMSCs towards the fibroblast line, by evaluating the expression of the marker COLL4, both in the presence of complete growth medium added with FBS (GM+FBS) and HPL (GM+HPL) and in the presence of differentiating medium added FBS (FI+FBS) and HPL (FI+HPL) as reported by statistically significant values.

G-PEG-CH-Gly scaffold has an increased capacity to induce the differentiation of UC-hMSCs towards the keratinocyte line, by evaluating the expression of the marker p63, in presence of complete growth medium added with HPL (GM+HPL), compared to G-PEG-CH scaffolds, which induces slight expression, and MatriDerm®, confirmed by statistically significant values as the **Figure 25.A** shows.

G-PEG-CH-Gly, G-PEG-CH scaffolds are the only ones able to induce UC-hMSCs differentiation towards the keratinocyte line, by evaluating the expression of the marker KRT10, both in the presence of complete growth medium added with FBS (GM+FBS) and HPL (GM+HPL) and in the presence of differentiating medium added FBS (CH+FBS) and HPL (CH+HPL). Specifically, scaffold G-PEG-CH-Gly induces higher expression of KRT10 in the GM+HPL condition and lower expression in the CH+HPL condition, while scaffold G-PEG-CH induces high expression of KRT10 in the CH+HPL differentiation condition, confirmed by statistically significant values (**Figure 25.B**).

A



B

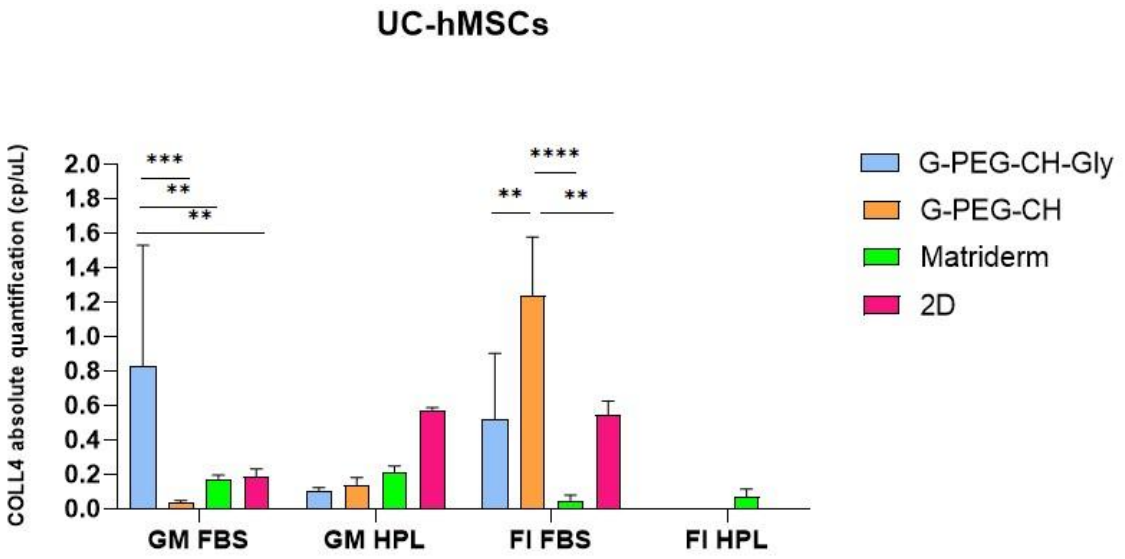
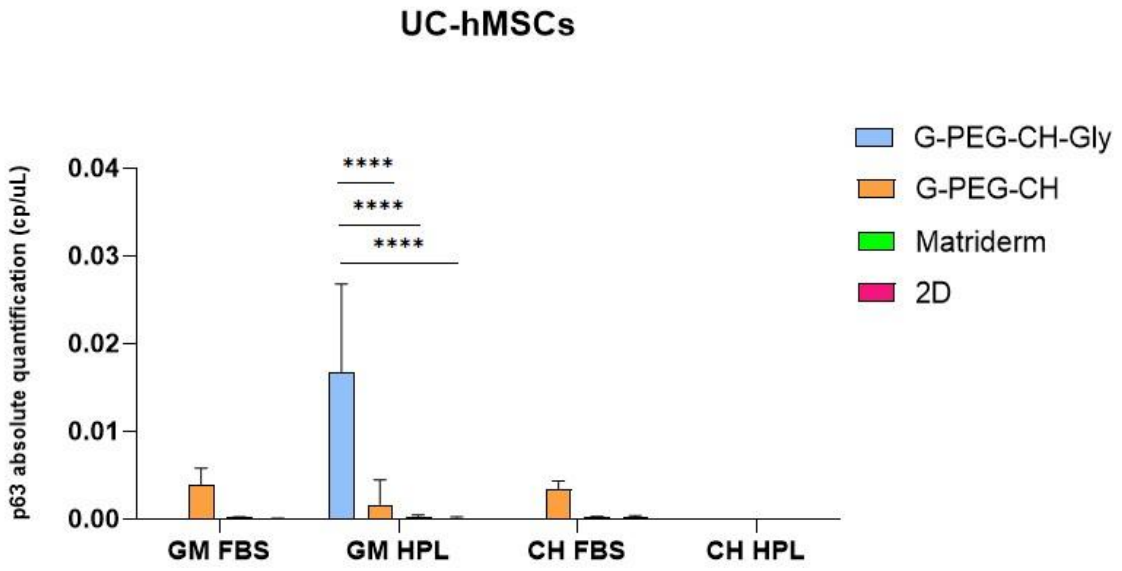


Figure 24. Analysis of the expression of COLL1 **(A)** and COLL4 **(B**), performed by dPCR in UC-hMSCs grown for 21 days in G-PEG-CH-Gly, G-PEG-CH and MatriDerm® in complete medium supplemented with FBS (GM+GBS), complete medium supplemented with HPL (GM+HPL), differentiating medium supplemented with FBS (FI+FBS) and differentiating medium supplemented with HPL (FI+HPL). Two-way ANOVA with test post-hoc Bonferroni was used for statistical analysis by GraphPad Prism (version 8). Data show the average values and their significant differences (*p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001).

A



B

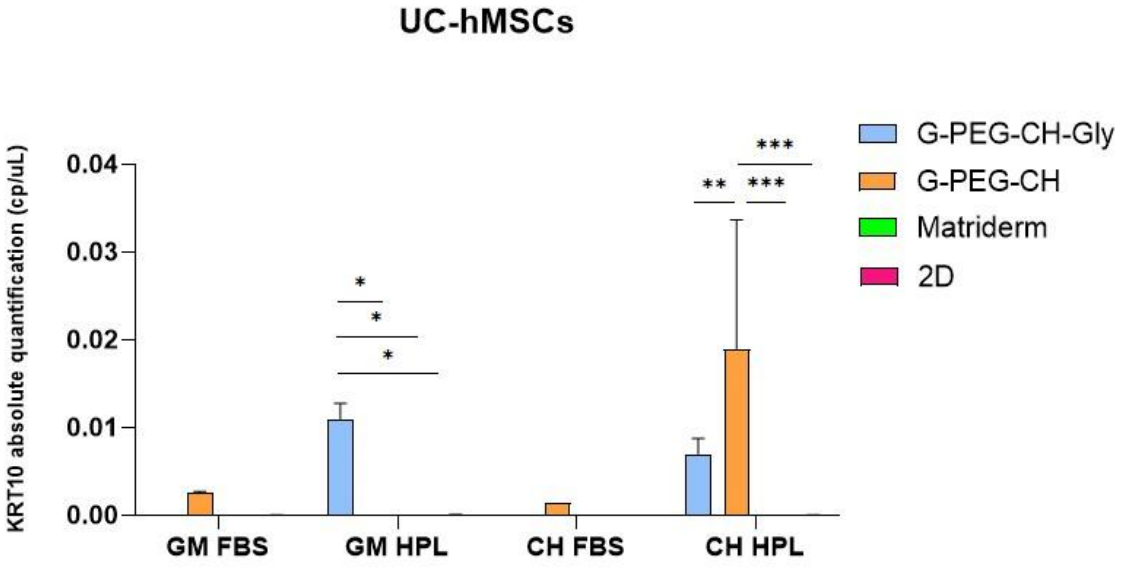


Figure 25. Analysis of the expression of p63 **(A)** and KRT10 **(B**), performed by dPCR in UC-hMSCs grown for 21 days in G-PEG-CH-Gly, G-PEG-CH and MatriDerm® in complete medium supplemented with FBS (GM+GBS), complete medium supplemented with HPL (GM+HPL), differentiating medium supplemented with FBS (CH+FBS) and differentiating medium supplemented with HPL (CH+HPL). Two-way ANOVA with test post-hoc Bonferroni was used for statistical analysis by GraphPad Prism (version 8). Data show the average values and their significant differences (*p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001).

Furthermore, the differentiation of hMSCs towards other cell lines was assessed by evaluating the expression of mesodermal markers such as osteopontin (OPN) (**Figures 26A – 27A – 28A**) aggrecan (ACAN) (**Figures 26B – 27B – 28B**) and fatty acid binding protein (FABP4) (**Figures 26C – 27C – 28C**) compared with the expression of each fibroblast and keratinocyte markers. The values obtained were not statistically significant.

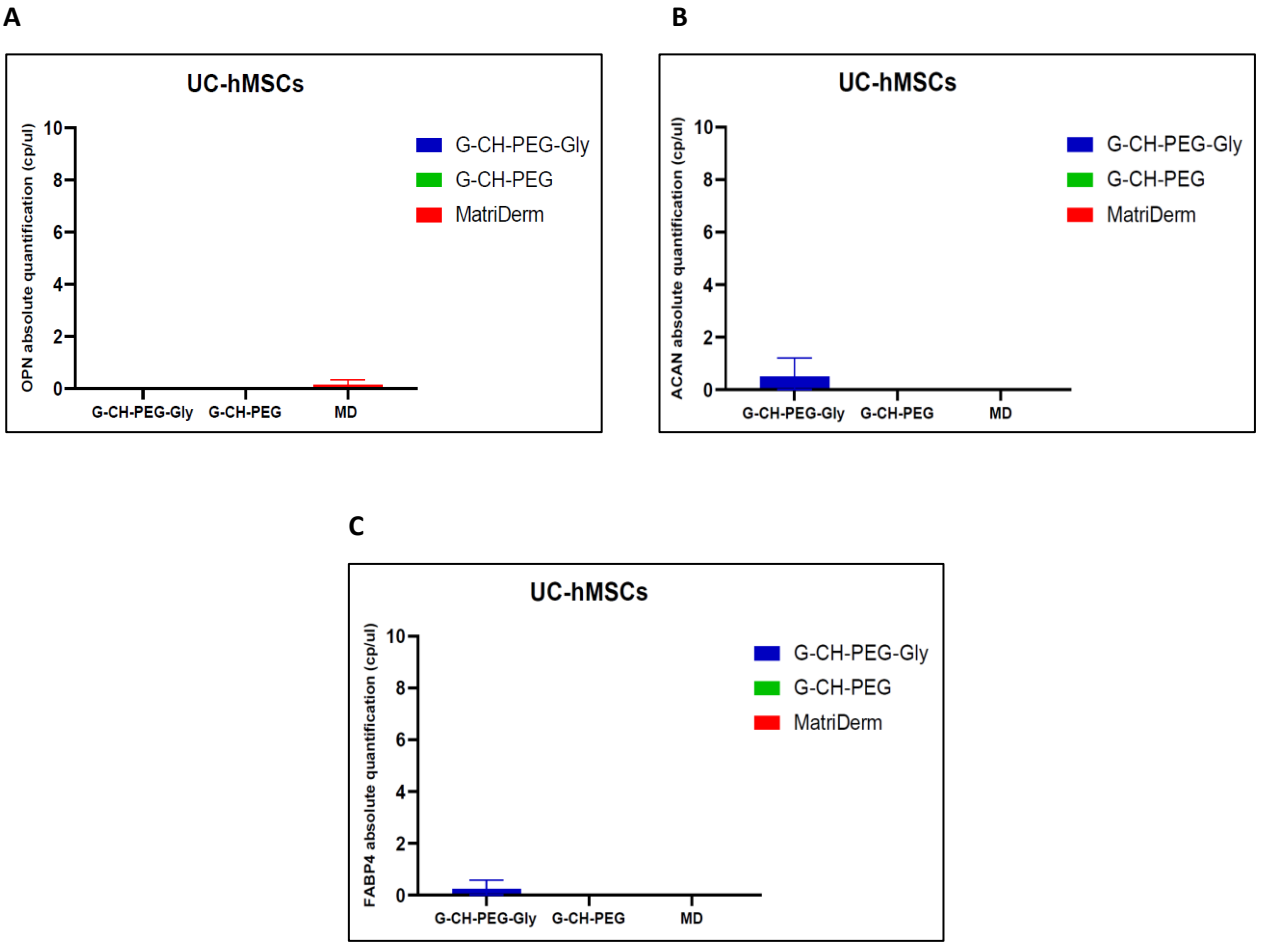


Figure 26. Analysis and absolute quantification by dPCR of OPN (**A**), ACAN (**B**) and FABP4 (**C**) expression, normalised in relation to GAPDH in G-PEG-CH-Gly, G-PEG-CH and MatriDerm® scaffolds and compared with expression of fibroblast marker COLL1. One-way ANOVA with test post-hoc Tukey was used for statistical analysis by GraphPad Prism (version 8).

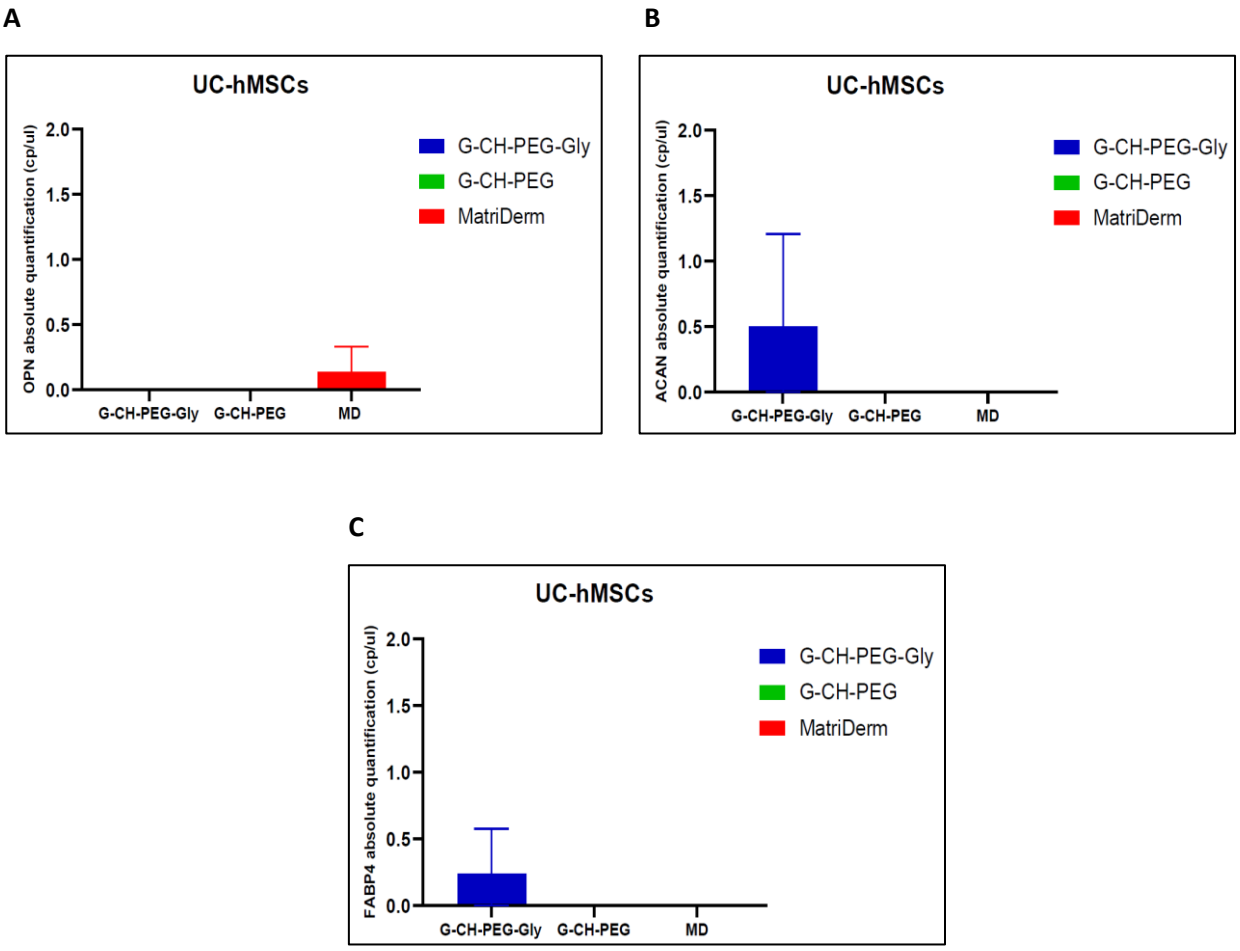


Figure 27. Analysis and absolute quantification by dPCR of OPN (**A**), ACAN (**B**) and FABP4 (**C**) expression, normalised in relation to GAPDH in G-PEG-CH-Gly, G-PEG-CH and MatriDerm® scaffolds and compared with expression of fibroblast marker COLL4. One-way ANOVA with test post-hoc Tukey was used for statistical analysis by GraphPad Prism (version 8).

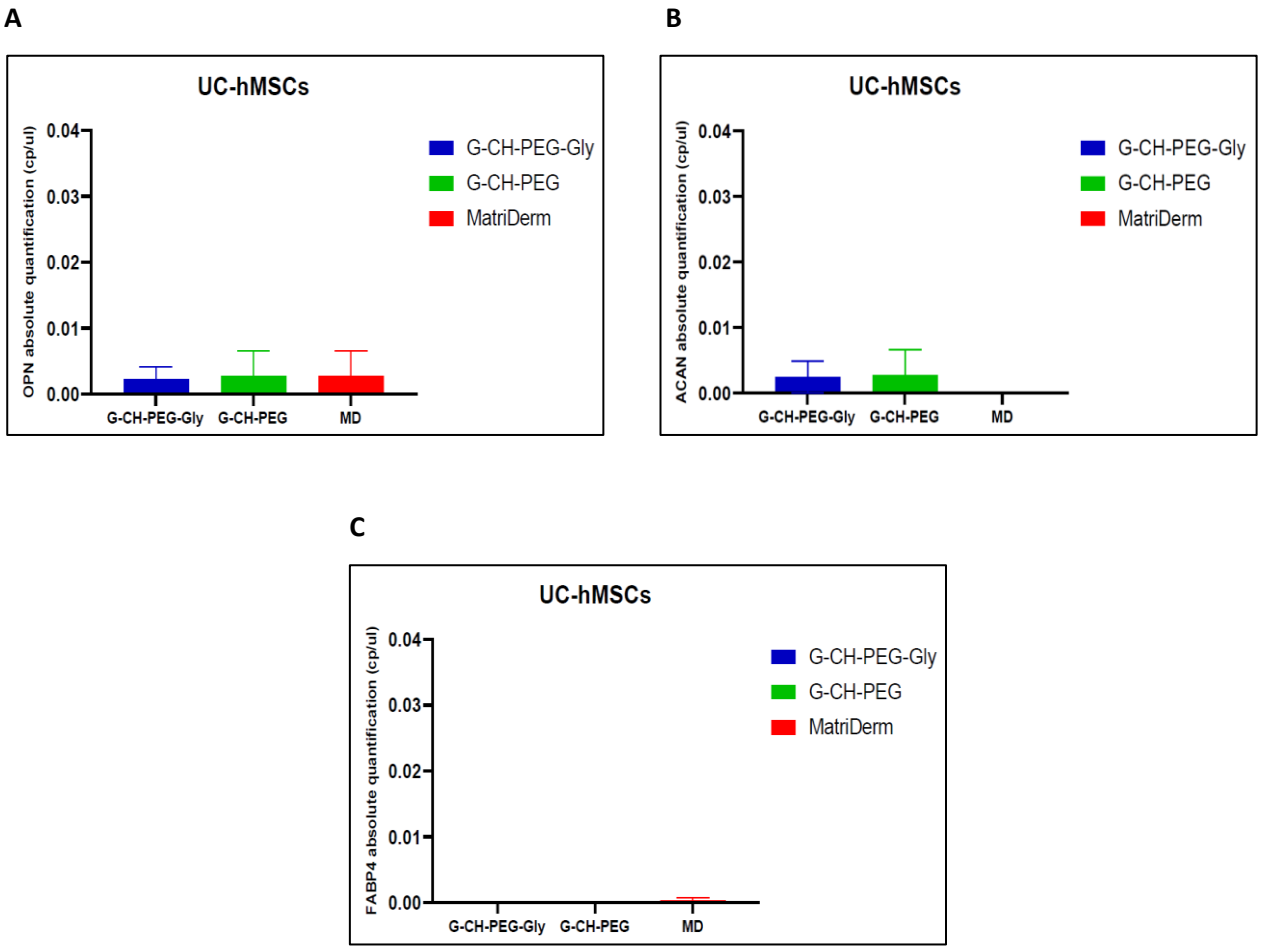


Figure 28. Analysis and absolute quantification by dPCR of OPN (**A**), ACAN (**B**) and FABP4 (**C**) expression, normalised in relation to GAPDH in G-PEG-CH-Gly, G-PEG-CH and MatriDerm® scaffolds and compared with expression of keratinocyte markers p63 and KRT10. One-way ANOVA with test post-hoc Tukey was used for statistical analysis by GraphPad Prism (version 8).

Histomorphological and immunohistochemical analysis of the 3D system

Histomorphology analysis, performed by H&E stain, allowed to appreciate the general morphology of the scaffolds at day 21. It was possible to observe the porous structure of the G-PEG-CH scaffold with pores of different sizes partly interconnected with each other (**Figure 29**). In particular, in the **Figure 29C** is shown the inner part of the scaffold, where it is possible to observe the presence of poorly interconnected pores that limit the passage and the adhesion of cells to the scaffold. The figures only show the conditions in GM HPL for UC-hMSCs and in GM FBS for BM-hMSCs (seeded on G-PEG-CH) due to technical problems that occurred in the other conditions and for which it was necessary to repeat the experiment.

The analysis performed by Masson-Goldner's Trichrome stain, allowed to observe extracellular matrix (ECM) deposition in G-PEG-CH-Gly, G-PEG-CH and MatriDerm® in different condition (**Figure 30**).

Immunohistochemical analysis identified the distribution and localization of the biomarkers COLL1 and KTR10. Both BM-hMSCs and UC-hMSCs were positive for differentiation markers in the presence of differentiation stimuli (FI FBS, FI HPL, CH FBS, CH HPL) in both G-PEG-CH-Gly and G-PEG-CH hydrogels and MatriDerm® (**Figure 31**).

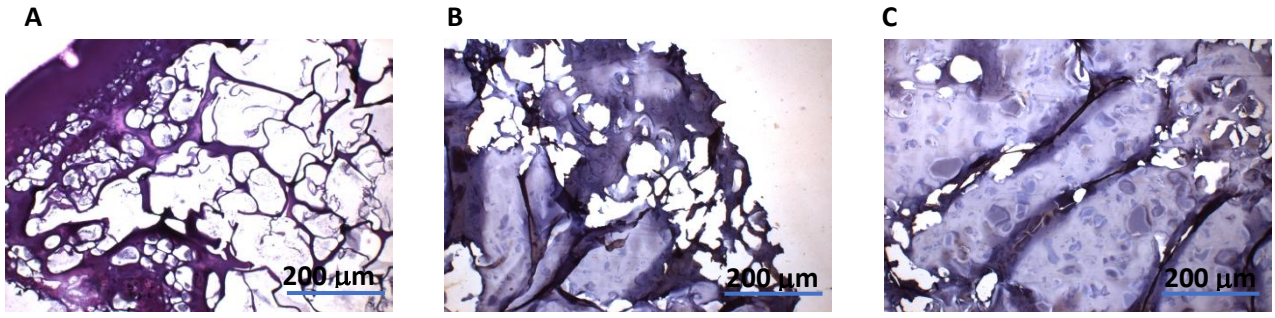


Figure 29. Microphotographs of H&E stain for morphology of G-PEG-CH seeded with UC-hMSCs (**A**) and BM-hMSCs (**B, C**) under the different culture conditions: GM+HPL (**A**), GM+FBS (**B, C**). The micrographs were recorded using a camera equipped with an image analysis system (Image-Pro Premier 9.1; 2018, Immagini e Computer, Milan, Italy). Scale bar: 200 µm.

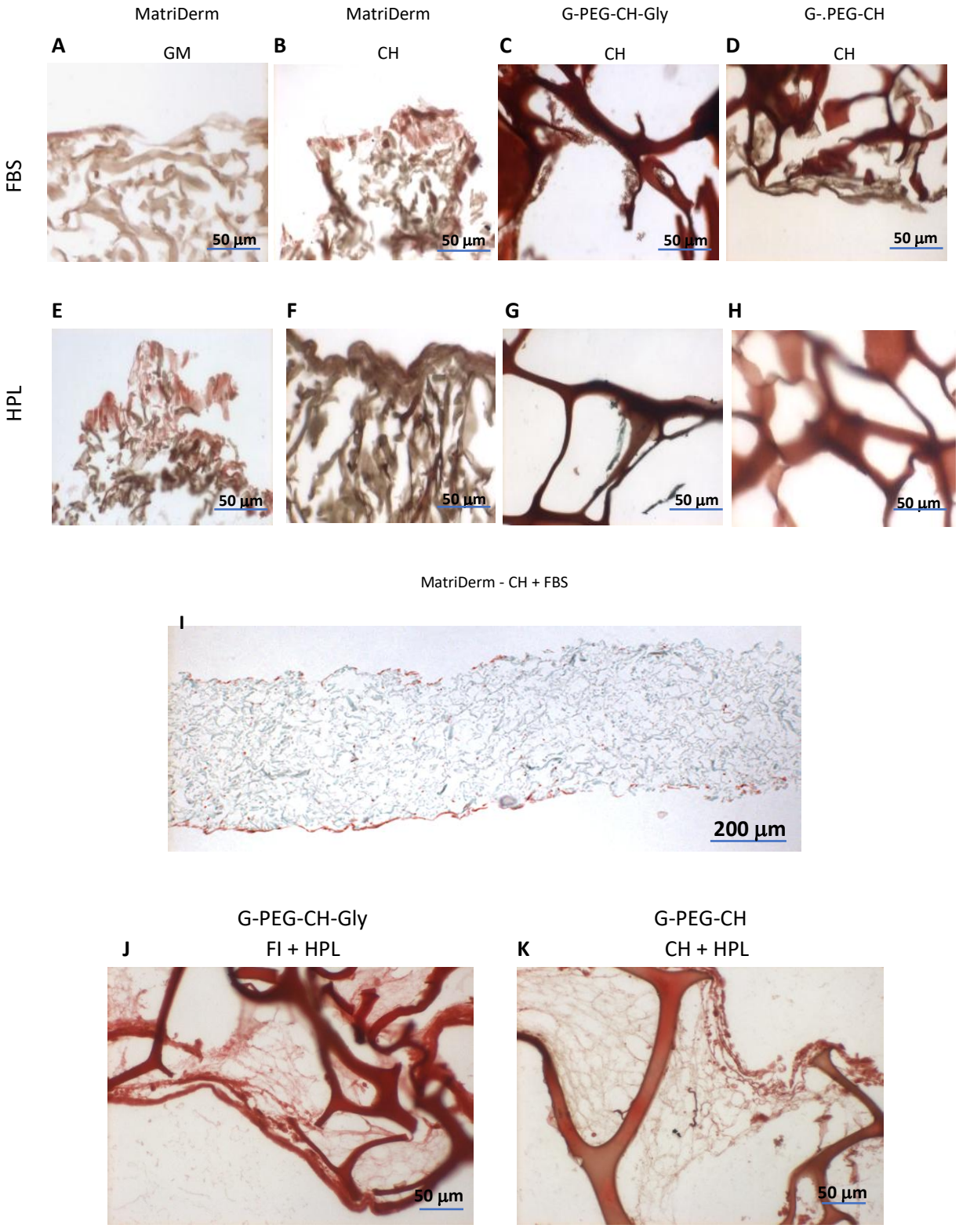
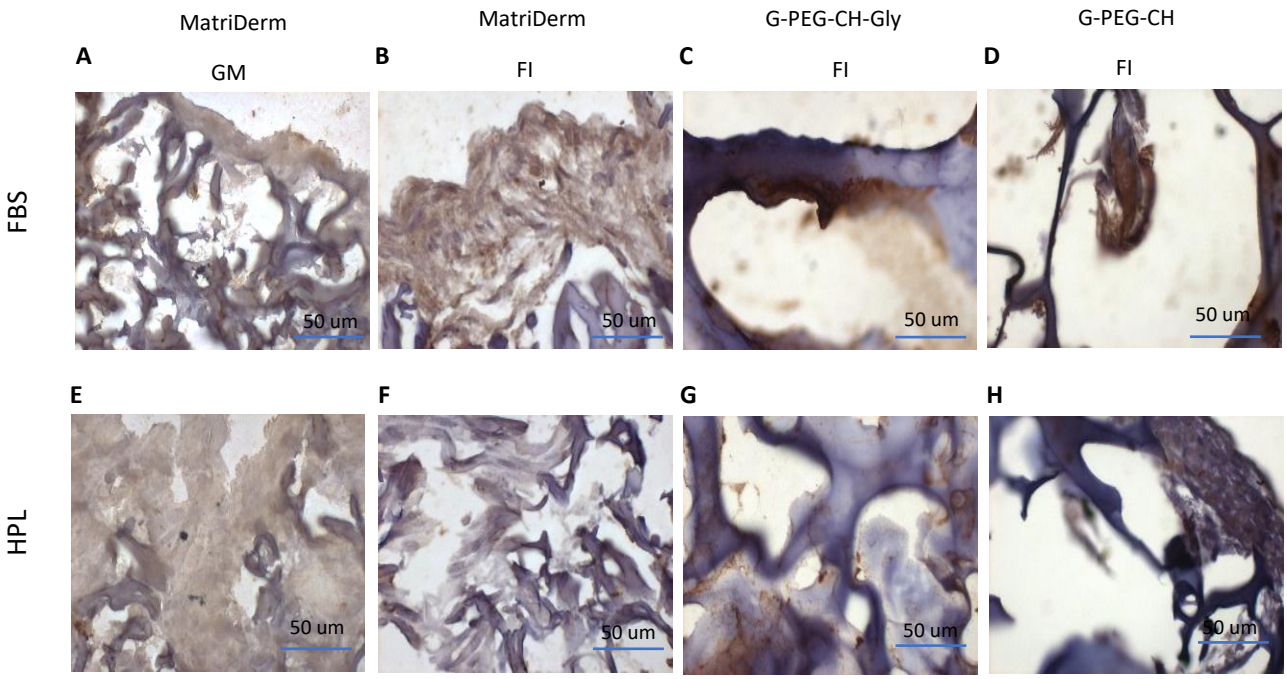
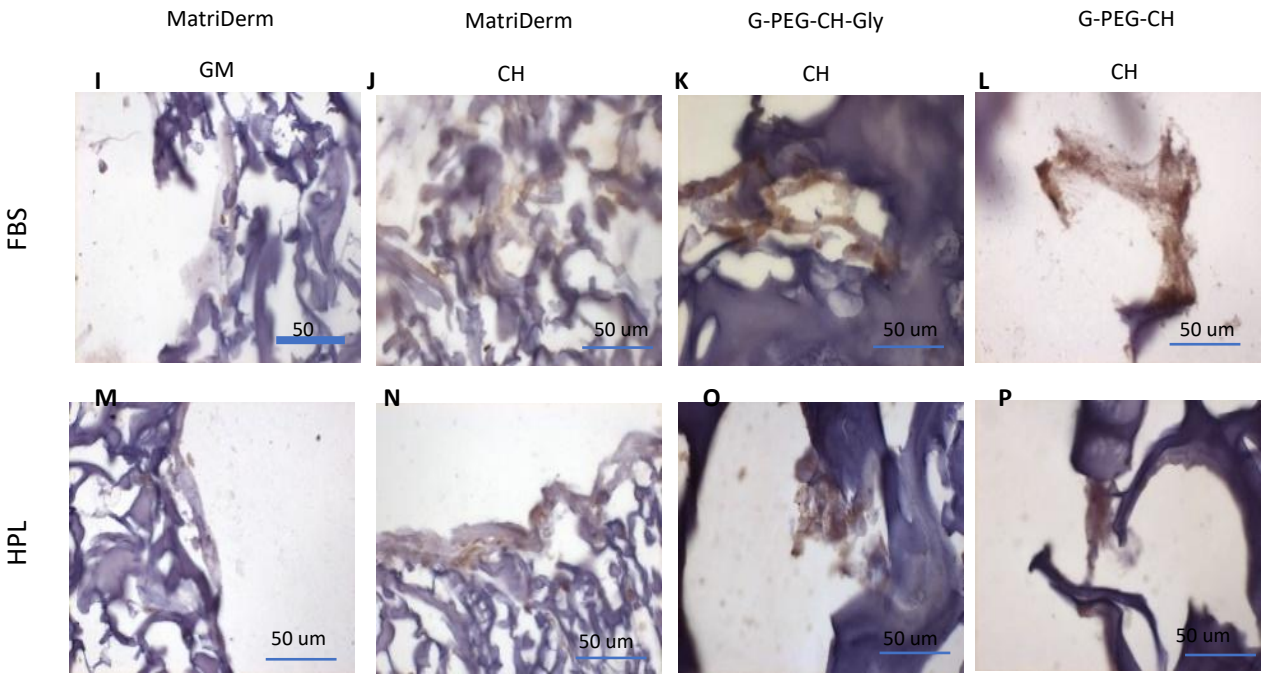


Figure 30. Microphotographs of Masson–Goldner's Trichrome stain of hydrogels (**A**–**J**) for extracellular matrix deposition. Microphotographs of MatriDerm, G-PEG-CH-Gly and G-PEG-CH seeded with BM-hMSCs (**A**-**I**) under the different culture conditions: GM+FBS (**A**), GM+HPL (**E**), CH+FBS (**B, C, D, I**) and CH+HPL (**F, G, H**). Microphotographs G-PEG-CH-Gly and G-PEG-CH seeded with UC-hMSCs (**I, J**) under the different culture conditions: FI+HPL (**I**) and CH+HPL (**J**). The micrographs were recorded using a camera equipped with an image analysis system (Image-Pro Premier 9.1; 2018, Immagini e Computer, Milan, Italy). Scale bar: 50 µm for (**A-H, J, K**); scale bar: 200 µm for (**I**).


MatriDerm
GM
MatriDerm
FI
G-PEG-CH-Gly
FI
G-PEG-CH
FI
A
B
C
D
FBS
50 um
E
F
G
H
HPL
50 um


MatriDerm
GM
MatriDerm
CH
G-PEG-CH-Gly
CH
G-PEG-CH
CH
I
J
K
L
FBS
50
50 um
M
N
O
P
HPL

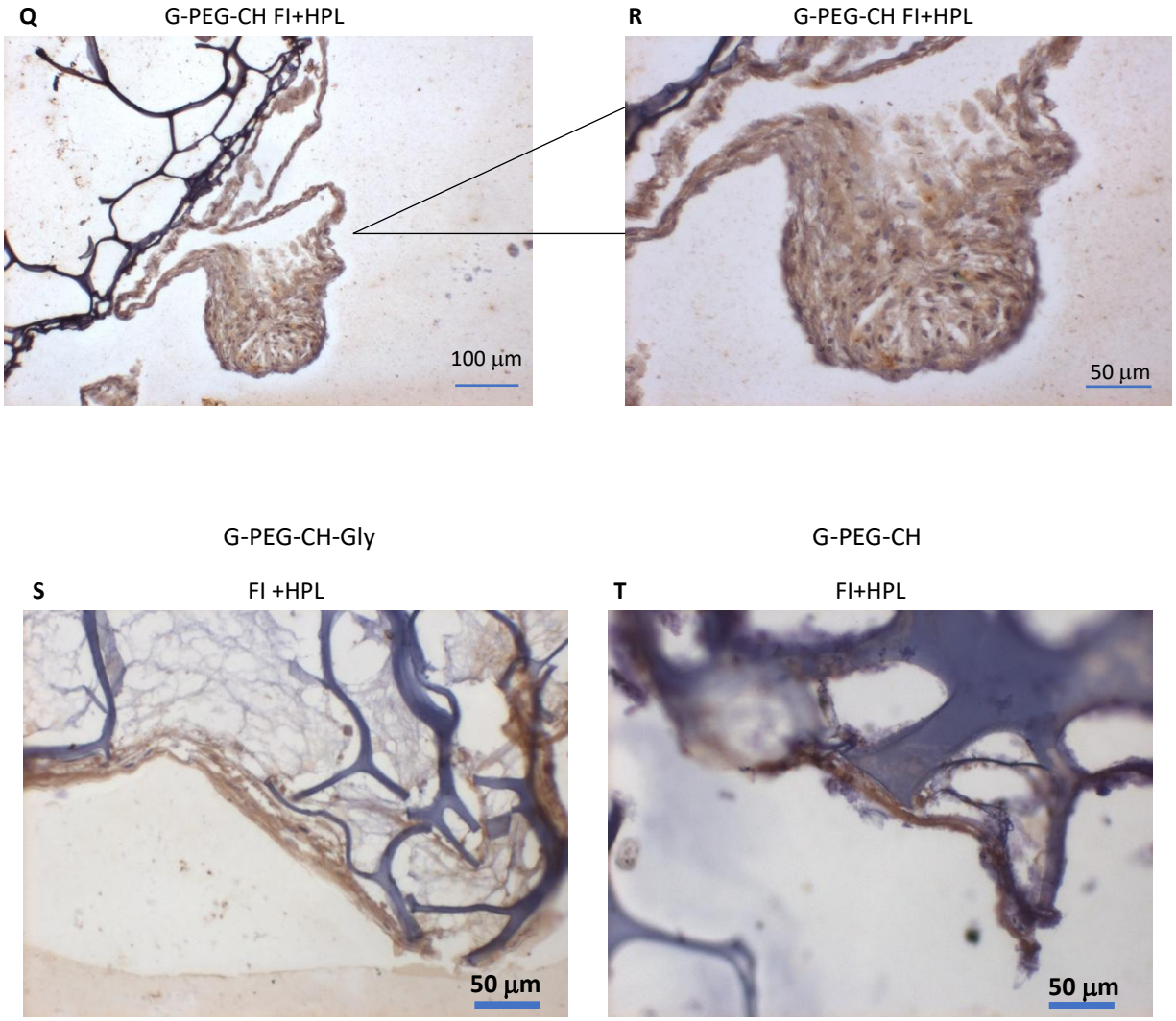


Figure 31. Microphotographs of immunohistochemical of hydrogels (**A**–**T**) for the distribution and localization of the biomarkers COLL1 and KTR10. Microphotographs of MatriDerm, G-PEG-CH-Gly and G-PEG-CH seeded with BM-hMSCs (**A**-**H, Q, R**) under the different culture conditions: GM+FBS (**A**), GM+HPL (**E**), FI+FBS (**B, C, D**) and FI+HPL (**F, G, H, Q, R**) for COLL1 marker. Microphotographs MatriDerm, G-PEG-CH-Gly and G-PEG-CH seeded with BM-hMSCs (**I-P**) under the different culture conditions: GM+FBS (**I**), GM+HPL (**M**), CH+FBS (**J,K,L**) and CH+HPL (**N,O,P**) for KRT10 marker. Microphotographs of G-PEG-CH-Gly and G-PEG-CH seeded with UC-hMSCs (**S,T**) under FI+HPL condition for COLL1 marker. The micrographs were recorded using a camera equipped with an image analysis system (Image-Pro Premier 9.1; 2018, Immagini e Computer, Milan, Italy). Scale bar: 50 µm for (**A-P, R-T**); scale bar: 100 µm for (**Q**).

DISCUSSION

Regenerative skin medicine is a field of research and treatment that focuses on the repair and recovery of skin tissue damaged or lost due to disease, trauma or ageing, restoring its functionality and normal appearance.[169-171]

No study has ever shown a clear advantage of a specific combination of biomaterials in promoting tissue repair of the skin. Skin tissue engineering is still in its early stages due to a number of limitations, such as the lack of experimental investigations, variable manufacturing technologies and the application of different materials.[172] To date, MatriDerm® is among the gold standards in clinical applications in dermatology. It is a porous dermal substitute composed of non-crosslinked collagen and bovine-derived elastin.

In this project, a 3D model consisting of new hydrogels bioengineered with hMSCs, from bone marrow and umbilical cord, is presented and analysed to test their biocompatibility. The protocol for differentiating hMSCs into the cell lineages that make up the skin tissue: fibroblasts and keratinocytes was developed and applied. Finally, hMSCs differentiated towards the fibroblast and keratinocyte lineages were cultured in the new hydrogels: G-PEG-CH-Gly and G-PEG-CH. Then the molecular and protein expression of BM-hMSCs and UC-hMSCs induced to differentiate towards the fibroblast and keratinocyte lines was analysed both in two-dimensional (2D) and on scaffolds (3D).

The hydrogels studied in this project were preliminarily developed following specifications for skin regeneration. The hydrogels were based on chitosan (CH) in combination with polyethylene glycol (PEG), gelatin (G) and glycerol (Gly). Specifically, the G-PEG-CH-Gly scaffold consisted of 56.93% G, 13.28% PEG, 6.07% CH and 23.72% Gly, while the G-PEG-CH scaffold consisted of 74.5% G, 17.4% PEG and 8.1% CH.

Chitosan-based hydrogels have previously been tested for bone regeneration.[173,174-182] In recent years, considerable attention has been paid to hydrogels, polymeric scaffolds that offer the possibility to generate well-defined 3D biofabric tissue analogue to the native extracellular environment. Hydrogels have the ability to retain large quantities of water similar to that of biological tissues (70%). They have a liquid phase and a solid phase: the liquid phase is that which makes them suitable and compatible with living tissue, while the solid phase is the one that maintains the three-dimensional structure. Hydrogels are capable of mimicking properties structural, biochemical, immunomodulatory and interaction of the cells with natural ECM. They can have various compositions: they can be made of synthetic polymers, from naturally-derived polymers or the combination of both.[150-154] They often include extracellular matrix elements that promote cell adhesion, such as collagen, fibrin, or polypeptides, and polysaccharides that promote cell encapsulation, such as agarose, hyaluronic acid, and chitosan.[183,184] An essential component of scaffolds is CH, which is a carbohydrate biopolymer, produced by the partial deacetylation of chitin, with a structure similar to the glycosaminoglycans (GAGs) present in the ECM whose role is to promote cell adhesion. It has a hydrophilic surface, which makes it suitable for promoting cell adhesion, proliferation and differentiation. It is able to

retain large amounts of water, which makes it suitable for trapping fluids and cells at the site of the wound. CH is known to mimic a biological material capable of promoting the healing process of connective tissue. It's a material biocompatible, biodegradable, bioactive, biologically renewable, non-toxic, non-antigenic, non-immunogenic, capable of stimulating the activity of growth factors, with antibacterial and anti-inflammatory property. CH offers a wide range of potential applications for cell-based tissue engineering, including skin regeneration. CH-based scaffold for skin tissue engineering should be characterized by mechanical strength and the ability to promote adhesion, proliferation and differentiation. It has been combined with various material such as polycaprolactone, collagen, gelatin and fibrinogen for potential applications in skin regeneration.[10,163,185-187]

Biocompatible hybrid hydrogels G-PEG-CH and G-PEG-CH-Gly were obtained after the freeze-drying and post-curing process maintained their size and shape well with the aim to produce scaffolds with functional properties for skin repair. For biological and medicinal applications, hybrid hydrogels that combine the advantages of natural and synthetic polymers have garnered considerable interest and a variety of crosslinking techniques have been used to obtain structurally stable hydrogels in physiological conditions. In order to tailor the structural, chemical, and mechanical properties of the hydrogels for a particular tissue engineering application, many types of natural and synthetic polymers have been combined.

In this study, we developed a crosslinking strategy to produce hydrogels based on G and PEG as a cross-linker.[188] In addition, natural polysaccharides CH and Gly were added. G has been widely used in the field of tissue engineering due to its various advantageous features, in including biocompatibility, biodegradability, low cost, and ease of manipulation. CH exhibited excellent properties that made it a good candidate for biomedical applications: it was biocompatible, biodegradable, non-antigenic, non-toxic and antimicrobial.[9] Moreover, its structural similarity to natural ECM and cationic surface charges promoted cell attachment and growth. No study has ever shown a clear advantage of the specific combination of biomaterials and a defined concentration of CH in these systems.

These hydrogel systems allowed to develop the possibility of epoxide groups of PEG to react not only with amino groups of gelatin and chitosan, but also with different groups such as the hydroxyl groups of glycerol. This allowed to increase the crosslinking density by post curing. The use of PEG as crosslinking agent, can provide a certain distance between the linked functional group and the gelatin or chitosan polymer chains, decreasing steric hindrance, facilitating adhesion of cells and triggering biological responses.[190]

The post-curing procedure, which is a step necessary for the crosslinking reaction to be finished, is very crucial for the hydrogels' increased mechanical qualities as well as their long-term stability. Hybrid hydrogels with anisotropic morphology and mechanical properties are very interesting in tissue engineering applications. The majority of biological tissue, including the heart, articular cartilage, skin, tendon, and muscles, is made up of well-defined anisotropic hierarchical designs.[191]

Anisotropy plays an essential role in mass transport, lubrication surface and force generation in biological systems. Moreover, it is also widely recognized that anisotropic structures have a significant impact on cell migration, proliferation, and differentiation. In this study we adopted a freezing technique followed by freeze-drying.[192]

The morphology of the hydrogels, analysed using an optical microscope, showed an interconnected porous network with macro and micro-porosity and channels homogeneously distributed. Porosity and pore size are believed to determine the final mechanical property of the scaffold and to influence cell behavior such as promote and regulate tissue regeneration and vascularization, transport of nutrients and removal of waste. From the results obtained, the scaffolds were found to be biocompatible in that they allow the survival and growth of the cells present. The margins of the G-PEG-CH-Gly and G-PEG-CH scaffolds are covered with cells. In G-PEG-CH-Gly, a significant fraction of the cells is dispersed across the scaffold's center core and outside margins. Histomorphological analysis shows that in some regions of the scaffold, the pores are poorly interconnected, preventing cell penetration and adhesion. In addition, pores oriented parallel to the direction of cell seeding appear to promote cell escape from the scaffold. Therefore, creating scaffolds with better interconnected pores and oriented perpendicular to the cell seeding plane could be a solution to trap more cells inside the scaffold while preventing them from escaping and adhering to the bottom of the well. The orientation of the pores can therefore modulate cell organisation and interaction with the scaffold, favouring cell adhesion.[193]

FTIR analysis served to confirm the qualitative and chemical composition of the hydrogels and was performed on the scaffolds and samples of their components. In both assembled hydrogels, the characteristic peaks of its constituents were present.

Swelling ratio and mass loss tests were performed to assess the degree of swelling following incubation with distilled water at different time points. The long-term use of hydrogels presupposes that the materials are monitored in their degradation in order to maintain their structural and mechanical integrity. These analyses revealed that both hydrogels showed a degree of swelling and loss of mass, peculiarities that a scaffold should possess for clinical use.[194-196]

The biocompatibility of G-PEG-CH-Gly and G-PEG-CH scaffolds, bioengineered with BM-hMSCs and UC-hMSCs, grown in GM complete medium supplemented with FBS and HPL, was analysed by means of viability and proliferation tests. The cell viability of the hydrogels was found to be comparable under all conditions regardless of cell type, hydrogel type and the presence of FBS or HPL. A more even distribution of viable cells was found in the MatriDerm® than in the tested hydrogels. This could be attributable to the fact that the collagen and elastin matrix has a more uniform architecture than the hydrogels, hence a more homogeneous distribution of cells. The low thickness, the reduced porosity but uniform in its distribution compared to hydrogels, and the dense texture, derived from a close interconnection of pores, allow for better cell colonisation. These results confirm that the ability not to interfere with the vital functions of cells is a

fundamental and necessary feature for scaffolds to be used in the field of regenerative medicine.[197-200] The Live/Dead experiment further revealed that UC-hMSCs have a fusiform fibroblast-like shape, whereas BM-hMSCs exhibit a more rounded fibroblast-like morphology. This was seen when the cells were stained with calcein-AM, which brings out the cytoplasm and makes it possible to see the cell morphology. In both of the settings taken into consideration (GM+FBS and GM+HPL), both cell lines seem to be firmly attached to the surface of the pores inside the scaffolds. This is supported by the fact that the cells labeled with ethidium, and therefore non-viable, are small in number. Additionally, it appears that the viable cells are not evenly distributed across the surface; rather, they prefer to gather toward the borders of the scaffolds G-PEG-CH-Gly and G-PEG-CH. There is also an accumulation in the central core of G-PEG-CH-Gly.

Cell proliferation was determined by quantifying the number of proliferating cells in the scaffolds using the Cell Counting Kit-8 (CCK-8) on days 2, 10, 14, 21 of cell culture. The BM-hMSCs and UC-hMSCs have been shown to have different growth kinetics in different scaffolds. In G-PEG-CH-Gly BM-hMSCs proliferated faster than UC-hMSCs although there was an exponential growth trend for both cell types over the 21 days of culture. The BM-hMSCs showed exponential growth despite the slight decrease on the 14th day of culture in G-PEG-CH-Gly. In G-PEG-CH both cell lines showed a stable growth trend, the growth kinetics of UC-hMSCs reaches the plateau on the 10th day of culture, remaining stable in the following days, while the BM-hMSCs have a growth peak on the 2nd day with reaching the plateau phase on the 10th day. Finally, in MatriDerm® both BM-hMSCs and UC-hMSCs grew exponentially reaching a plateau at day 21.

The UC-hMSCs, in the presence of FBS, present stationary growth kinetics until 14 days with a proliferative peak on the 21st day of culture in G-PEG-CH-Gly. On the contrary, BM-hMSCs present exponential growth kinetics under the same conditions. In G-PEG-CH, both cell lines showed steady-state growth until day 21. In the presence of GM+HPL medium, the UC-hMSCs grew exponentially in G-PEG-CH-Gly reaching the plateau between the 14th and 21st day of culture. Instead, BM-hMSCs proliferated gradually until day 10 with a subsequent decrease on day 14 and a proliferative recovery on day 21. In the same condition, both BM-hMSCs and UC-hMSCs grew exponentially in G-PEG-CH with a growth peak at day 2, then maintaining stationary growth until day 21. In the MatriDerm®, both cell types had exponential growth both in the presence of FBS and HPL.

It was evident that both cell lines exhibited increased growth kinetics in all three scaffolds when grown in the presence of complete medium supplemented with HPL (GM+HPL) compared to complete medium supplemented with FBS (GM+FBS). The cells were probably affected by a dual proliferative stimulus: one induced by the scaffolds and the other mediated by HPL, known to be a modulator of various physiological processes including proliferation, in different populations.[173,202]

It has been demonstrated that hPL is a reliable alternative to FBS for hMSCs growth protocols, eliminating the inherent risk of spreading unknown animal illnesses, the potential immunological reactions to animal antigens.[142] Despite that there is a need to reach a standardization and quality control of platelet-derived

products, especially in terms of time and temperature of freezing, storage conditions, the number of pooled units, characterization of donators (age, sex) and platelet count influenced growth factor content in hPL as well as capacity to support hMSCs expansion.[203] Standardization of hPL quality should be confirmed by validated measurements and associated controls of parameters such as pH, osmolality and proteomic analysis.[204]

In addition to enhancing cell expansion in vitro, hPL can enhance hMSCs differentiation towards the fibroblast and keratinocyte lineage as it contains specific growth factors capable of stimulating such differentiation. hPL is known to contain the following growth factors Alpha-1-antitrypsin, Hemoglobulin subunit delta, Hemoglobulin subunit alfa, 14-3-3 Protein zeta/delta, von Willebrand factor, Kininogen-1, capable of stimulating differentiation into fibroblasts, while Hemopexin, 14-3-3 Protein zeta/delta, Cofilin-1, Hepatocyte Growth Factor-like protein, Kininogen-1, capable of stimulating differentiation into keratinocytes. hPL has proven to be a good proliferative, as well as differentiating, stimulus in both BM-hMSCs and UC-hMSCs as previously described.

The results showed that scaffolds had an intrinsic ability to promote cell proliferation in BM-hMSCs and UC-hMSCs. The scaffold alone appears to be an active component in inducing cell proliferation as demonstrated by various studies in the literature.[182,205,206]

In parallel, the protocol for differentiating hMSCs towards the fibroblast and keratinocyte lineage in both 2D and 3D culture was optimised and standardised. It has been evaluated the molecular expression of markers for differentiation in the fibroblast (COLL1 and COLL4) and keratinocyte (p63 and KRT) direction using the Digital PCR (dPCR) method. This molecular biology technology allowed an absolute quantification of the number of copies of the transcripts considered to be present in the cDNA samples obtained from cells cultured in different conditions in both 2D and 3D. The idea of using the dPCR technique derives from the fact that this method is particularly sensitive and accurate compared to other molecular biology methods such as Real Time PCR. The basic principle of this technique involves the fragmentation of the sample into multiple independent PCR microreactions such that each individual reaction contains few or no target sequences. Following amplification, a statistically specified precision is employed to measure the concentration of the target sequence using the proportion of positive microreactions, which is based on the Poisson statistic. Furthermore, this technique requires a limited quantity of material on which to perform the analysis, making it particularly advantageous.[207-209]

Cell growth in monolayers revealed a high expression of COLL1 in the FI HPL condition compared to FI FBS in the UC-hMSCs, thus induced by both HPL and differentiation factors. In the same condition, low expression of COLL1 was observed in the BM-hMSCs at 21 days. The expression of the transcript by the UC-hMSCs was higher as they basally express this marker.[204] Expression of COLL4 was increased in the presence of both HPL and differentiation factors in both UC-hMSCs (GM+HPL, FI+FBS) and BM-hMSCs (FI+FBS, FI+HPL). Expression of COLL4 in BM-hMSCs in FI HPL medium could be affected by the association between CTGF and heparin,

components of the differentiation medium containing hPL. The heparin-binding kDa fragment of CTGF containing module 4 alone stimulates adhesion of fibroblast, myofibroblasts, endothelial cells and epithelial cells.[211]

Expression of p63 was low in both cell lines. This is related to the fact that p63 is an early marker of differentiation in the keratinocyte direction, so the time point 21/28 days is too high to detect its expression.[104]

KRT10 expression was found to be higher in BM-hMSCs in the presence of differentiating factors and hPL, whereas in UC-hMSCs there was a slight increase in expression in GM HPL, induced by hPL itself.

Compared to cells grown in monolayer, which were characterised by a relatively low expression of the markers COLL1, p63 and KTR10 at 21 days, a significant increase in the expression of the transcripts COLL1, COLL4, p63 and KRT10 was detected in the 3D culture.

In the 3D culture, the growth medium alone (GM+FBS and GM+HPL) together with the scaffold resulted in increased expression of the COLL1 transcript in the UC-hMSCs under all conditions tested. It is therefore confirmed that in UC-hMSCs, the combination of HPL and differentiation factors fails to induce an increase in COLL1 expression. Only scaffold stimulation in combination with growth supplements can induce increased expression of the marker. The hypothesis is that, under FI FBS and FI HPL conditions, COLL1 expression could be assessed at time points below day 21. The molecular marker COLL1 could in fact be expressed early, before day 21. To confirm this, IHC under the same conditions showed positivity for the maker COLL1. Therefore, although molecular expression was low in the FI FBS and FI HPL conditions, there was positivity for the marker COLL1 in IHC thus indicating that the UC-hMSCs were differentiating towards the fibroblastic lineage. The low molecular expression and positive protein expression of the marker could be attributed to the fact that there was a down-regulation of the gene in conjunction with an up-regulation of the protein.[104]

The expression of COLL4 in UC-hMSCs in hydrogels increased in the presence of differentiation stimuli with FBS, but not hPL, similar to the result obtained in 2D culture both UC-hMSCs and BM-hMSCs. This could confirm a possible interaction between differentiation factors and hPL itself, or an early expression of COLL4 at time points below day 21. Indeed, it was found that the combination of hPL with conditioned medium does not exert the synergistic effect in terms of expected gene expression.[211]

Concerning the expression of p63, only in G-PEG-CH-Gly was an increase in the GM HPL condition, induced by the scaffold and hPL itself, observed. The low expression of p63 could be attributable to the fact that, being an early marker of differentiation in the keratinocyte direction, the time point at 21 days is too late to detect transcript expression. This was confirmed by a study in the literature in which p63 expression was detected on day 7 of culture.[104] Therefore, future experiments will investigate the expression of p63 at lower time points.

Expression of the marker KRT10 was found in both the GM HPL condition in G-PEG-CH-Gly, but also in the CH HPL condition in G-PEG-CH. The expression of the marker in the latter condition is higher as it is induced by

the combination of the differentiation factors, scaffold and HPL. Expression was higher in the treated conditions than in the basal conditions, supporting the previous hypothesis. In this case, the 21-day time point allowed the detection of the marker KRT10, this being a late marker of keratinocytes differentiation.[104] Regarding BM-hMSCs in association with scaffolds, an increased expression of COLL1 and COLL4 was again detected in the FI HPL condition, particularly in G-PEG-CH. Expression of p63 was higher in the presence of differentiation stimuli in G-PEG-CH than in the other conditions, supporting the hypothesis of early molecular expression of this marker. The expression of KRT10 was found to be stimulated by the synergy of differentiation factors and HPL in both G-PEG-CH-Gly and G-PEG-CH.

To confirm that cellular differentiation is directed towards the fibroblast and keratinocyte lineages, osteogenic (OPN), chondrogenic (ACAN) and adipogenic (FABP4) molecular markers were evaluated using dPCR. This control to exclude the possibility that our cells have expressed the osteoblastic, chondrogenic and adipogenic markers, given that the MSCs differentiate spontaneously towards the cell lineages of the mesoderm. The data obtained were not statistically significant for these transcripts, which confirms that the differentiation occurred specifically in a fibroblastic and keratinocytic direction and therefore towards the epithelial lineage.

The analysis performed by Masson-Goldner's Trichrome stain, allowed to observe extracellular matrix (ECM) deposition in G-PEG-CH-Gly, G-PEG-CH and MatriDerm® in different condition both BM-hMSCs and UC-hMSCs. Both in basal conditions (GM+FBS, GM+HPL) and in the presence of differentiation medium (FI+HPL, CH+FBS, CH+HPL), the deposition of the extracellular matrix occurs with the presence of collagen fibers.

To confirm the molecular expression of COLL1 and KRT10, an evaluation of the expression of the protein counterpart was carried out for both the fibroblast and keratinocyte lineage. Immunohistochemical analysis confirmed protein expression of the markers COLL1 and KRT10 by hMSCs seeded on both scaffold G-PEG-CH-Gly, G-PEG-CH and MatriDerm®. In particular, a positivity for both markers was noted in the conditions with the differentiation media supplemented with HPL for UC-hMSCs, while for BM-hMSCs the expression of both COLL1 and KRT10 was noted in the conditions with the differentiation media supplemented with FBS and HPL. This confirmed that the BM-hMSCs and UC-hMSCs expressed the fibroblast marker COLL1 and keratinocyte marker KRT10, evidence of difference towards the fibroblast and keratinocyte lineage respectively. The analysis of protein expression is preliminary and will have to be completed by analysing the missing conditions.

The protein expression of the markers COLL1 and KRT10 in both cell types in G-PEG-CH-Gly and G-PEG-CH is an indication that the 3D model developed in this thesis project can promote hMSCs differentiation towards fibroblast and keratinocyte lines.

In conclusion, the generated 3D model is shown to have the ability to promote differentiation both towards the fibroblast lineage, a property necessary to promote dermal regeneration to support the epidermis in the case of wounds and severe burns, and towards the keratinocyte lineage.

It was found that G-PEG-CH-Gly and G-PEG-CH hybrid hydrogels show a greater ability to promote differentiation, confirmed both molecularly and protein-wise, of BM-hMSCs and UC-hMSCs towards the fibroblast and keratinocyte lineage than MatriDerm®. On the contrary, MatriDerm® proves to be more efficient in promoting proliferation, compared to scaffolds, of both BM-hMSCs and UC-hMSCs and in particular in the presence of hPL from the earliest time points. In the literature, there are no in vitro studies in which MatriDerm® is tested in combination with platelet lysate, whereas in this project, this additional analysis was introduced.

CONCLUSIONS

The data obtained from this work demonstrate the ability of G-PEG-CH-Gly and G-PEG-CH scaffolds to promote growth and differentiation in a fibroblast and keratinocyte sense, either independently or with the addition of proliferative or differentiative stimuli such as hPL and the factors contained therein, in two different cell populations, namely UC-hMSCs and BM-hMSCs. This demonstrates that the scaffolds in this study, chitosan-based hydrogels, not only serve as an inert support for cell growth and differentiation but also actively promote these two physiological processes by delivering both proliferative and differentiative stimuli. The hydrogels tested in this work were compared to MatriDerm®, among the current skin substitutes used in dermatology and considered the gold standard. According to the data, MatriDerm® appears to be more effective than the scaffolds evaluated at assuring and encouraging the proliferation and colonization of UC-hMSCs and BM-hMSCs, but it does not equally increase the expression of cell differentiation markers in the epithelial direction. CH-based hydrogels were better able to induce differentiation of UC-hMSCs and BM-hMSCs in a fibroblast and keratinocyte direction. UC-hMSCs showed better differentiation in the fibroblastic direction when cultured with the G-PEG-CH scaffold in the presence of FBS, whereas differentiation in the keratinocyte direction was greater with HPL. BM-hMSCs showed high levels of COLL1 and COLL4 expression when cultured with the G-PEG-CH scaffold in the presence of HPL, showing differentiation in a fibroblast direction. They expressed more of the p63 transcript in the same scaffold when FBS was present, while they expressed KRT10 in the G-PEG-CH-Gly scaffold when HPL was present, emphasizing differentiation in a keratinocyte sense.

In general, both cell lines showed a higher expression of fibroblast transcripts than keratinocyte transcripts. UC-hMSCs showed higher expression of COLL1 and COLL4 than BM-hMSCs, due to their higher stemness derived from the umbilical cord. In contrast to BM-hMSCs, UC-hMSCs normally express both markers.[210]

The protein expression of the markers COLL1 and KRT10 in both cell types in G-PEG-CH-Gly and G-PEG-CH and in the presence of the differentiation media indicates that the 3D model developed in this thesis project is able to promote the differentiation of hMSCs to fibroblast and keratinocyte lines.

Protein analysis of the differentiation into fibroblasts and keratinocytes of BM-hMSCs and UC-hMSCs in 2D culture are still ongoing. Protein analysis is a Western Blot in which COLL1 and KRT10 are analysed.

Future perspectives include optimizing the 3D model both by refining the structure and composition of the scaffold and by optimizing the cell differentiation protocol towards fibroblast and keratinocyte lines at different time points. This will be followed using patient-derived cells, and it would be very interesting to translate this model using iPSCs derived from fibroblasts. The last step to be considered for pre-clinical analysis would be the transition to the in vivo model, in the literature there are studies of skin regeneration mainly in the mouse model. The regeneration mechanism could be investigated by considering the process of neo-angiogenesis and how pro-angiogenic factors present in hPL can influence the system.

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